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Toll-like receptors (TLRs) and T cell effector function

By: PhD student

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Abstract

The aim of the project was to identify whether cattle T cell subsets express toll-like receptors (TLRs), and if so whether there was any functional consequence of this when they were stimulated with TLR-ligands. CD4+ T cells, CD8+ T cells, and γδ T cells were studied in the context of whole PBMC or as isolated cells. To develop rabbit Tregs as a source of cells to study expression of toll-like receptors (TLRs), putative rabbit regulatory T cells (Tregs) were developed as cultured cells from MLN as part of an ongoing project and to inform generation of cattle Treg development for this study. Culture of rabbit MLN cells resulted in detection of CD4+CD25hiFOXP3+ (putative nTregs) in about one fifth of the total cells. The cultured rabbit Tregs did not exhibit any immunosuppressive effect on autologous MLN (Mesenteric lymph node) cells in a suppression assy. This and the complexity of this method meant that the cattle Treg work was not initiated for this study.

The main part of the project involved screening of 10 bovine toll-like receptors (TLRs 1-10) on cattle blood samples (PBMC fraction and T cell subsets) plus investigation of their expression using RT-qPCR to detect transcripts in the T cell subsets. TLRs were variably expressed in the T cell subsets from the samples. With regard to function measured by proliferation in response to stimulation with TLR agonist (ligand), CFSE (Carboxy-fluorescein Succinimidyl Ester)-labelled PBMCs were stimulated with TLR1-9 agonists and each CFSE-labelled T cell subset identified by flow cytometry and proliferation based on the CFSE profile for each subset within the whole PBMC sample. No significant proliferation was recorded in activated PBMCs while T cell subsets showed variation in responses with

CD4 T cells generally most effective followed by CD8+ T cells and least proliferation was seen with γδ T cells.

The functional role of expressed TLRs stimulated with ligands and Con-A or antiCD3 were also assessed by expression or not of 7 cytokines (CXCL-8, IFNα, IFNγ, TNFα, TGFβ, IL-4, and IL-10) by specific ELISA. However, the results showed variation between different cattle blood samples. T cells generally activated PBMCs producing CXCL-8 while the isolated T cell subsets showed a more selective secretion. No IFN α expression was found. IFNγ was secreted in a limited amount in one PBMC animal and some γδ T cell samples rather than CD4 or CD8 T cells. TNF-α was produced by one PBMC sample stimulated with TLR2 agonist. The T cell subsets did not record any TNF-α secretion. There was no real functional link between the CFSE proliferation assay and the cytokine expression results.

In conclusion, TLRs are expressed on cattle T cell subsets but with variable activation outcomes after stimulation of the cells with TLR ligands.

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Majid

Dedication

To:

My parents, family members and all relatives … With love

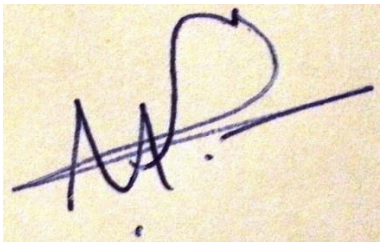
My real friends with a special respect … A friend in need is a friend indeed

Humanity … All human beings

Majid

DECLARATION

I declare that the information in this thesis was carried out in accordance with the Regulations of University of Nottingham. The work is original, except where indicated by special reference in the text, and no part of the thesis has been submitted for any other academic award. Any views expressed in the thesis are those of the author.

Signature: 

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Abs	Antibodies
ALRs	Absent in Melanoma 2 (AIM2)-Like Receptors
Amp	Ampere
ANOVA	Analysis Of Variance
APC	Allophycocyanin
BCR	B Cell Receptor
B-ME	Beta-Mercaptoethanol
bp	Base Pair
BSA	Bovine Serum Albumin
CD	Cluster of Differentiation
cDNA	Complementary DNA
CFSE	Carboxyfluorescein Succinimidyl Ester
CLRs	Transmembrane C-Type Lectin Receptors
CMI	Cell-Mediated Immunity
Con A	Concanavalin A
Conc	Concentration
Cq	Quantification Cycle
CTLs	Cytotoxic T Cells
CTV	CellTrace Violet
DAMPs	Damage-Associated Molecular Patterns
DCs	Dendritic Cells
ddH_2O or (DDI H_2O)	Double Distilled Water / or Double Ionised Distilled Water
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DPBS	Dulbecco's Phosphate Buffered Saline
dsRNA	Double Stranded RNA
DTH	Delayed Type Hypersensitivity
EDTA	Ethylenediamintetraacetic Acid
ELB	Erythrocyte Lysis Buffer
ELISA	Enzyme-Linked Immunosorbent Assay
FACS	Fluorescent Antibody Cell Sorting
FAM	6 Carboxyfluorescein
FCS / FBS	Foetal Calf Serum / Foetal Bovine Serum
FITC	Fluorescein Isothiocyanate
FOXP3	Forkhead Box P3
FSC	Forward Scatter
Fwd Primer	Forward Primer
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
gd or γδ T cells	Gamma-Delta T Cells
gDNA	Genomic DNA
HEK293	Human Embryonic Kidney 239
HI	Humoral Immunity
hr	Hour
IFN-γ	Interferon Gamma
IgA, IgG, IgM	Immunoglobulin A, G, M
IKK-β	I-Kappa B Kinase Complex
IL-	Interleukin-

IMDM	Iscove's Modified Dulbecco's Medium
IRAK	Interleukin-1 Receptor-Associated Kinase
IU	International Unit
kb	Kilobase
L	Litre
L-glu	L-glutamin
LN2	Liquid Nitrogen
LRR	Leucine-Rich Repeat
MACS	Magnetic Antibody Cell Sorting
MAPK	Mitogen-Activated Protein Kinase
MHC	Major Histocompatibility Complex
min	Minute
MKK6	Mitogen-Activated Protein Kinase (MAPK) Kinase-6
ml	Millilitre
MLN	Mesenteric Lymph Nodes
mM	Millimolar
MØs	Macrophages
MS	Microsoft Office
MyD88	Myeloid Differentiation Primary Response Gene 88
NCBI	National Centre for Biotechnology Information
NEB / UK	New England Biolabs / United Kingdom
NFκB	Nuclear Factor Kappa B
ng	Nanogram
NK cells	Natural Killer Cells
NK T cells	Natural Killer T Cells
NLRs	Nucleotide-Binding Oligomerization Domain (Nod)-Like Receptors
nm	Nanometre
nM	Nanomolar
NS	Non-Stained
nsSNP	Non-Synonymous SNP
NTC	No Template Control
PAMPs	Pathogen-Associated Molecular Patterns
PBMCs	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PE / or R-PE	Phycoerythrin / Or Red-Phycoerythrin
Penstrep	Penicillin/Streptomycin
PFA	Paraformaldehyde
PHA	Phytohaemagglutinin
PMA	Phorbol 12-Myristate 13-Acetate
PMN cells	Polymorphnuclear Cells
Pmol	Picomolar
PRRs	Pattern Recognition Receptors
RBCs	Red Blood Cells
Rev Primer	Reverse Primer
RLRs	Retinoic Acid-Inducible Gene (RIG)-I-Like Receptors
RNA	Ribonucleic Acid
RNS	Reactive Nitrogen Species
RO water	Reverse Osmosis Water
ROS	Reactive Oxygen Species

RPLP0	Ribosomal Protein, Large P0
RPLP2	Ribosomal Protein, Large P2
RPMI	Roswell Park Memorial Institute
RT-qPCR	Quantitative Real-Time PCR
SARM	Sterile-Alpha and Armadillo-Motif-Containing Protein
SDHA	Succinate Dehydrogenase
sec	Seconds
SNP	Single Nucleotide Polymorphism
SRs	Scavenger Receptors
SSC	Side Scatter
ssRNA	Single Stranded RNA
TAE buffer	Tris-Acetate-Edta Buffer
TAMARA	N,N,N,N'-Tetramethyl-6-Carboxyrhodamine
TCR	T Cell Receptor
Th1, Th2, Th17	T Helper 1, T Helper 2, T Helper 17
TIR	Toll/IL-1 Receptor
TIRAP	Toll-Interleukin 1 Receptor (TIR) Domain Containing Adapter Protein
TLRs	Toll-Like Receptors
TNF-α	Tumour Necrosis Factor alpha
TRAF6	Tumour Necrosis Factor (TNF) Receptor Associated Factor-6
TRAM	TRIF-Related Adaptor Molecule
Tregs	Regulatory T Cells
TRIF	TIR-Domain-Containing-Adapter-Inducing Interferon-beta
u/ul	Unit/Microliter
ug	Microgram
ul or uL	Microliter
uM	Micromolar
UPL probes	Universal Probe Library Probe
UV light	Ultraviolet Light
V	Volt
WEASEL	Walter & Eliza Analysis Software: Eclectic & Lucid

Chapter One

Introduction and

Literature Review

1.1 The immune response

The immune response is essential for the protection of animals (including humans) against pathogenic microbial attacks and other disease processes such as cancer (Chaplin, 2010). It is grossly divided into two components; innate immunity which is non-antigen specific, and involves cells, such as macrophages (MØs), dendritic cells (DCs), Natural killer (NK) cells, mast cells, and epithelial cells at tissues exposed to the exterior (the gut, lung and skin) that respond rapidly to infection or changes in tissues. It is usually of short duration (Galli *et al*., 2011, Kelly and O'neill, 2015, Whitsett and Alenghat, 2015, Man and Kanneganti, 2016). The second main component of immunity is the adaptive immune response (also called acquired immunity) involving T and B cells as well as their subsets, which are highly specific for antigens, are generated in lymph nodes and show immunological memory. This means they can be recalled after several years or decades to respond rapidly and specifically when, for example, an infection occurs that the host has experienced before (Mantovani *et al*., 2011, Lanier, 2013).

1.2 The innate immune response

Innate immunity is the starting point for foreign microbe recognition by the host. It is the front-line defence against invading pathogens or disease-related tissue changes. The innate immune cells at the site of microbial entry recognise relatively conserved molecules of the microbes not shared by the host (described in detail below). These pattern recognition receptors (PRR) on innate immune cells activate the cells (for example epithelial cells, monocytes / MØs, DCs) to produce a variety of mediators such as

pro-inflammatory cytokines, chemokines, and antimicrobial peptides (Medzhitov, 2001, Beutler, 2004). The cells involved in innate immune responses are listed in table 1.1.

Innate mechanisms of defence against pathogen infection or disease-induced change also include: apoptosis (controlled programmed cell death) (Roos *et al*., 2004); opsonisation and phagocytosis (Greenberg and Grinstein, 2002); the complement system (Rus *et al*., 2005) and clotting process (Engelmann and Massberg, 2012). Other components of innate immunity include the antimicrobial activity of normal gut flora (Boman, 2000); non-specific production of immunoglobulin A (IgA) from epithelial cell lining mucosal sites that access the exterior (Bidgood *et al*., 2014); secretion of antimicrobial peptides such as defensins and lysozymes (Ouellette, 2011, Leysen *et al*., 2013); cytolysis of target pathogen-infected cells by natural killer cells (NK cells) (Vivier *et al*., 2011).

The proinflammatory cytokines released by innate immune cells include: interleukin-1 (IL-1); IL-6; the chemokine CXCL8 (also known as IL-8); tumour necrosis factor alpha (TNF-α) and interferons including interferon gamma (IFN-γ), which is produced in abundance by NK cells. These are defined as low molecular weight proteins (usually glycoproteins) that attract, activate or differentiate leucocytes to stimulate inflammation and fever to create unsuitable environments for pathogen survival (Xu *et al*., 2010, Abbas *et al*., 2014).

Although multiple strategies for microbial elimination are involved in innate immunity, phagocytosis is an important element of innate immune responses. Neutrophils and mononuclear cells (for example MØs and some

DCs) have the ability to engulf pathogens that exceed 1 μm in size (Medzhitov, 2001, Singh and Singh, 2013).

Table 1.1: Major cells involved in the innate immune response.

Type of Cell	Description and function	Citation
Monocytes	Phagocytosis of circulating pathogens in blood, antigen presentation and chemotaxis of other immune cells via releasing cytokines/chemokines. Differentiation into MØs in tissues.	(Faist *et al*., 1988, Gordon and Taylor, 2005, Domínguez and Ardavín, 2010).
Macrophages (MØ)	Pathogen engulfment and presentation of antigens, releasing cytokines, healing process and scavenging after tissue damage, apoptosis.	(Morrissette *et al*., 1999, Nadella *et al*., 2012).
Kupffer cells	Liver MØs: see macrophage function.	(Movita *et al*., 2012).
Microglial cells	Brain MØs: see macrophage function.	(Faustino *et al*., 2011).
Dendritic cells (DC)	Efficient pinocytosis of pathogens (immature form) and presenting them (mature form), to T cells as antigens with MHC molecules. Cytokine release. Initiating and maintaining immune responses. Immune tolerance.	(Banchereau and Steinman, 1998, Geissmann *et al*., 2010).

Mast cells	Allergic reactions (Type 1 hypersensitivity mediated by IgE in particular); source of histamine, vasoactive amines, prostaglandins and leukotrienes. Tissue modulation.	(Kalesnikoff and Galli, 2008, Silver and Curley, 2013).
Natural Killer (NK) cells	Cytolysis of target, early sensors for viral and/or tumour attack. Inflammation, cytokine release (particularly IFN-gamma.	(Shi *et al*., 2011, Vivier *et al*., 2011).
Neutrophils	Found in profuse amounts in blood, early response to some microbes, mainly bacteria. Sepsis formation (dead and alive); acute inflammation phagocytosis; opsonisation; short acting, release of cytokines, secretion of antimicrobial peptides (e.g. lysozymes that degrade cell walls of bacteria).	(Wright *et al*., 2010, Amulic *et al*., 2012).
Eosinophils	Increased in blood and tissues in parasitic infestation; allergic reactions; inflammation; antigen presentation; interaction with mast cells; tissue modulation; involved in asthma.	(Akuthota *et al*., 2010, Lloyd and Saglani, 2013, Fajt *et al*., 2014),

Basophils	Less than 1% of total blood cells; allergic reactions associated with IgE; inflammation; production of cytokines; enhances T helper 2 progression.	(Ishida *et al*., 2011, Siracusa *et al*., 2011, Voehringer, 2013).
Epithelial cells	Work as natural filters due to their existence on surfaces of lining layers of canals or tracts of many body systems exposed to exterior such as respiratory, digestive, urinary may allow expulsion and elimination of microbes by ciliary movements; non-specific Ab-Ag binding via IgA secretion.	(Peterson and Artis, 2014, Artis and Spits, 2015, Whitsett and Alenghat, 2015)

1.2.1 Innate immune cell pattern recognition receptors

An important aspect of innate immune cells is their ability to recognise relatively-conserved components of microorganisms and initiate a rapid inflammatory response invasion or infection. Epithelial cells and dendritic cells amongst others express PRRs (pattern recognition receptors) that perform this function. They recognise pathogen-associated molecular patterns (PAMPs) on microbes and damage-associated molecular patterns (DAMPs) which are host related (Hayashi *et al*., 2010, Takeuchi and Akira, 2010).

Six classes of pattern recognition receptors (PRRs) have been listed which recognise different PAMPs (see table 1.2). These are: cytoplasmic retinoic acid-inducible gene (RIG)-I-like receptors (RLRs); absent in melanoma 2 (AIM2)-like receptors (ALRs); transmembrane C-type lectin receptors (CLRs); scavenger receptors (SRs); nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs); and Toll-like receptors (TLRs) (Areschoug and Gordon, 2009, Sattler *et al*., 2012, Singh and Singh, 2013). Viruses, bacteria, parasites and other microbes express PAMPs that can be sensed and distinguished by PRRs in immune cells as non-self antigens and thus initiating immune response to infection or tissue damage/ change (Kumar and Bot, 2013).

These PAMPs can be detected by different recognition patterns initiating multiple signalling pathways, i.e. RIG-I-like receptors (RLRs) are responsible for sensing of RNA viruses in humans resulting in triggering innate immune response as well as inflammation throughout IFN gamma (Loo and Gale Jr, 2011). Another group of PRRs, AIM2-like receptors plays a fundamental role in sensing bacterial and viral DNA (Rathinam *et al*.,

2010). The next group of PRRS is the C-type lectin receptors (CLRs)– expressed by DCs and MØs – which are responsible for recognition of bacterial and fungal carbohydrates (Yabe *et al*., 2014). In addition, scavenger receptors (SRs) expressed by mononuclear phagocytes (MØs and DCs) have the capability to detect or eliminate modified lipoproteins (the major constituent in both Gram negative and Gram-positive bacteria) thus enhancing innate immunity (Areschoug and Gordon, 2009, Canton *et al*., 2013). Moreover, NOD-like receptors (NLRs) play a key role in detecting intracellular microbes via induction of inflammation and apoptosis (Kersse *et al*., 2011). Finally, Toll-like receptors (TLRs), which will be the group of interest in this study, have been involved in a very wide range of sensing the variety of bacterial, viral, fungal, and protozoal PAMPs triggering inflammation and then innate immune responses (Kawai and Akira, 2010, Tartey and Takeuchi, 2014). The TLRs will be discussed in more detail below starting from section 1.5.

However, innate immunity is not always effective against pathogens; some fastidious microbes can cross over these barriers, and this is where the more specialised type of immune response named as adaptive immunity comes in (Beutler, 2004, Hoebe *et al*., 2004). Innate immunity effector function can inform the type of adaptive immune response that is generated to infection or disease.

Table 1.2: Non TLRs pathogen recognition pattern classes.

PRRs	Target(s)	Function(s)	References
RIG-I-like receptors (RLRs)	RNA viruses	Stimulation of innate immunity and inflammation via IFN-γ secretion	(Loo and Gale Jr, 2011)
AIM2-like receptors (ALRs)	Bacterial and viral DNA	Activation of inflammasome and cell death by induction of proinflammatory cytokines (IL-1β and IL-18).	(Rathinam *et al*., 2010)
C-type lectin receptors (CLRs)	Bacterial and fungal carbohydrates	Enhance APCs (mainly DCs & MØs) functions as well as regulation of DCs migration and their interaction with T cells	(Figdor *et al*., 2002)
Scavenger receptors (SRs)	Varied, but mainly bacterial lipoproteins	Phagocytosis of varieties of foreign bodies especially bacterial.	(Areschoug and Gordon, 2009)
NOD-like receptors (NLRs)	peptidoglycan of intracellular bacteria	Higher NLRs levels could indicate stress. Stimulation of inflammation and apoptosis.	(Kersse *et al*., 2011)

1.3 The adaptive immune response

Adaptive (acquired) immunity develops after an initial innate immune response, where T cells (involved in cell-mediated immunity) and B cells (involved in humoral immunity) are generated in lymph nodes (Bonizzi and Karin, 2004, Ferrand and Ferrero, 2013). The adaptive immune response confers specificity and memory on immune responses. To generate adaptive immune responses to infection, the following sequence of events typically takes place. The pathogen infects and is recognised by the innate immune system that generates inflammation, activation and recruitment of cells, some of which are involved in generating the adaptive immune response to infection. Pathogens are processed by innate immune antigen-presenting cells such as MØs and immature DCs. DCs are very important for generating and maintaining immune responses. As they mature after taking up pathogen or pathogen fragments, they migrate to local lymph nodes and become very good at presenting antigen fragments coupled to major histocompatibility complex molecules (MHC class I and MHC class II, see below) to T and B cells (Palm and Medzhitov, 2009).

The MHC molecules are highly variable (polymorphic) and differ between individuals in outbred animal populations. MHC class I molecules are present on all nucleated cells and act as restriction elements for antigen presentation for intracellular pathogens (for example all viruses, some bacteria) to cytotoxic T cells in particular (CTLs, see below). The CTLs will then destroy the infected cell. The MHC class II molecules are mainly on antigen presenting cells such as monocyte / MØs, DCs and B cells, and are restriction elements for antigen from extracellular invading pathogens to helper T cells in particular (see below). The MHC molecules are involved in

the thymus in making sure that self-reactive T cells do not develop. Their role in antigen presentation is to ensure that only pathogen-specific responses are generated (Comber and Philip, 2014, Fooksman, 2014).

As stated above, acquired immunity consists of two major compartments; humoral immunity and cell-mediated immunity (CMI). Humoral immunity (HI) is mainly dependent on stimulated / activated B cells (the name was derived from Bone marrow in mammals and Bursa of fabricious in avian as they are synthesised there) which have a unique receptor called the B cell receptor (BCR) expressed on the outer membrane (Michael, 1995).

B cells have detailed antigen specificity and develop into either effector plasma cells that secrete antibody, or into memory cells that circulate, ready to respond rapidly to infection with the same pathogen antigens that stimulated their development. Antibodies in mammals belong to five immunoglobulin classes: IgM, IgG, IgA, IgD and IgE. IgG is further divided into several subclasses, depending on the host species (Schroeder Jr and Cavacini, 2010).

Immature B cells express IgM, while IgM and IgD are displayed on more mature B cells to enable their infiltration and access to lymphoid tissues (Yuan and Witte, 1988, Brezski and Monroe, 2008). Mature B cells undergo differentiation into plasma cells or memory cells under stimulation by expressed antigen and regulatory cytokines (IL-4, IL-5, IL-10 and IL-13, IFN-gamma) secreted by Ag presenting and T helper cells (tables 1.3 and 1.4).

Plasma cells are responsible for maintaining secretion of immunoglobulins at about 10,000 Ig/second (Abbas *et al*., 2014). On the other hand, memory B cells are responsible for re-stimulation of the immune system

when a recurrent infection with the same antigen is encountered (Weill *et al*., 2013).

The second compartment of adaptive immunity is cell-mediated immunity (CMI). CMI depends on T lymphocytes (named so because they develop and mature in the Thymus). The T cell receptor (TCR) recognises antigen fragments (epitopes) and is made up of constant and variable gene segments – the alpha and beta gene products. Cluster of differentiation 3 (CD3) is the common marker for T cells which binds with TCR forming the TCR complex (Abbas *et al*., 2014). The two originally-described principal types of T cells are helper T cells (that express the CD4 molecule and use the MHC-II pathway) that are involved in helping B cells and other effector T cells, like Cytotoxic T cells (CTLs), to develop and function in immune responses, and CTLs (that express the CD8 molecule), which kill infected cells by the MHC-I restriction pathway. A third type of T cell uses a different configuration of the T cell receptor that uses gamma and delta TCR chains rather than alpha and beta ones. More recently the CD4+ T helper cell subset has been divided into several subtypes – Th1, Th2, TH17 and regulatory T cells (that can also express CD8) (Delves *et al*., 2011).

T helper 1 (Th1) cells are involved in cell-mediated immune responses to many pathogens, particularly intracellular ones. They are responsible for delayed type hypersensitivity (DTH) reactions to these. Th1 develop in the presence of the cytokine s IL-12 and IFN-gamma. Cytokines released from activated Th1 include IL-2, IFN-γ and TNF-β (table 1.4) amongst others that activate MØs for pathogen clearance and generate antibody responses with certain IgG subclasses (that differ between the different species of animal). Th2 cells are involved in allergic reactions and immune responses

to nematode parasites. They also help B cells make antibodies of the IgE class and some IgG subsets (depending on species, e.g. IgG1). Th2 release IL-2 in common with Th1 cells but are the principal T cell source of IL4, IL-5, IL-10 and IL13 (table 1.4) that stimulate the recruitment and activation of mast cells, eosinophils amongst others at the site of infection (Beckmann *et al*., 1992, Viallard *et al*., 1999).

T helper 17 (Th17) cells are a further subset of T cells developed from CD4 T cell differentiation that secretes IL-17 and IL-22 involved in neutrophil accumulation amongst other activities. This pathway is important in response to inflammation caused by intracellular bacteria (Delves *et al*., 2011) (Table 1.4).

CTLs are involved in the killing (by apoptosis induction mainly) of cells infected by intracellular pathogens. They require antigen presentation restricted by MHC class I molecules, but their activity can be enhanced by the activation of Th1 cells as helper cells. There is a principal mechanism where CTLS kill target cells. The perforin-granzyme pathway involves pore-forming molecules called perforin to puncturing the infected cell allowing the access of granzyme enzymes that induce apoptosis in the cell (Podack and Dennert, 1983, Tschopp and Nabholz, 1990).

Although naïve T cells develop and differentiate into either CD4+ or CD8+ effector T cells which, in turn, proliferate to increase their numbers to fight specific pathogens, they subsequently undergo death and reduction of their numbers to establish homeostasis inside the body (Abbas *et al*., 2014). Nonetheless, a small number of these cells persist and differentiate as memory T cells, which are responsible for rapid recall responses to

encountering the same pathogen antigens again (Sallusto *et al*., 1999, Masopust *et al*., 2001).

A further subset of T cells is the gamma-delta T cells that use the gamma and delta chains of the T cell receptor rather than the alpha and beta ones (Holtmeier and Kabelitz, 2005). This subset of T cells represents the least common T cells (up to 5% in blood), whereas they are more abundant in epithelial surfaces (25-60% in the gut). Although these cells had been described as of unknown function, the distinct γδ TCR can be used as a pathogen recognition receptor, thus it links innate and adaptive immunity. These cells are part of the CD4-CD8- T lymphocytes in peripheral blood; they recognise pathogens via non-classical MHC class I and MHC class II initiating cell cytotoxicity. Furthermore, these cells can neutralise intestinal, cutaneous and genital epithelium pathogens (Li *et al*., 1998, Carding and Egan, 2002, Dar *et al*., 2014). Termination of the immune response process is regulated by specific subsets of T cells called regulatory T cells (Tregs) formerly called T suppressor cells (Beissert *et al*., 2006).

Table 1.3: Major cells involved in the adaptive immune response.

Type of Cell	Function	Surface marker	Cytokine secretion	Citation
B lymphocytes (B cells)	Responsible for adaptive humoral immunity involving antibody production (IgM, IgG, IgA, IgE, IgD) through differentiation to plasma cells; antigen presentation; production of memory cells.	CD19, CD20, CD34, CD38, and CD45R	IL2, IL4, TNFα, IL6, IFNγ, IL12, and TNFα	(Lund and Randall, 2010, Yang *et al*., 2010, Mauri and Bosma, 2012)
Plasma cells	Generated from B cells. They are the antibody-secreting cells.	CD138, CD78	IL21, IgG3	(Calame, 2001)
T lymphocytes (T cells)	Responsible for adaptive cellular immunity; three major classes – helper T cells (CD4+, MHC class –II restricted), cytotoxic T cells (CD8+, MHC class I restricted), and γδ T cells (non-classical MHC class I and class II restricted). Regulatory T cells (CD4, many are FOXP3+), and memory T cell subsets (see below)	CD3, CD4, CD8	Many Interleukins, e.g. IL-2, IFN-γ, IL-4, TGF -beta, IL-10.	(Abbas *et al*., 2014)
T helper 1 (Th1)	Mainly direct adaptive immunity to drive cell-mediated immunity and provide B cell help for antibody production,	CD4+CCR5	E.g. IL1, IL2, and IFNγ	(Viallard *et al*., 1999)

	release cytokines (IL-2, IFN-γ and TNF-β), induction of phagocytosis via activation of MØs, DCs and help for CTL cells.			
Th2	Chiefly guide adaptive immunity against extracellular pathogens, and involved in anti-nematode parasite immune responses; release of IL-4, IL-5, IL-10 and IL13 driving humoral immunity pathway by stimulation of B cells, IgG1 and IgE production, class switching, stimulation of hematopoiesis	CD4+CCR4	IL4, IL5, IL10, IL13	(Delves *et al*., 2011)
Th17	Induction of inflammation via IL-17 secretion; recruitment and activation of neutrophils; antimicrobial activity via IL-22 released at mucosal surfaces	CD4+CCR6	IL17	(Ouyang *et al*., 2008, Lee *et al*., 2009)
Regulatory T cells (Tregs)	Mainly CD4+ CD25+ FOXP3+ T cells, but other subsets exist. Control (supress) immune responses and autoimmunity, tissue grafts and some cancers by termination of immune responses and establishing equilibrium and homeostasis. Secretion of inhibitory cytokines such as IL-10 and TGF-β	CD4, CD25, CD127, and FOXP3	TGF, IL-10	(Sakaguchi *et al*., 2008)

Memory T cells	Re-stimulation to generate T effector cells and B cell help on exposure to previously encountered pathogen antigens.	CD4, CD8	IL7, IFNγ, TNF-α	(Farber *et al*., 2014)
Cytotoxic T lymphocytes (CTLs)	Antiviral and antitumor activities. Recognition of intracellular pathogen antigens in association with MHC I.	CD8	IL1, TNF-α and IFN-γ	(Barry and Bleackley, 2002)
γδ T cells	Non-classical MHC class I and class II restricted. Act directly when exposed to antigen by secretion of either IL-17 causing inflammation or IFN gamma release when exposed to viruses or cancer (amongst other responses). Immunosuppression (thought to be a regulatory T cell subset in cattle).	γδ TCR	IFN-γ, IL4, IL10, and IL17	(Chien *et al*., 2014, Guzman *et al*., 2014, Hovav, 2017)

Table 1.4: Main functional cytokines released from immune cells.

Cytokine	Description	References
Interleukin-1 alpha and beta: (IL-1α) and (IL-1β)	Released by monocytes, MØs, DCs, T cells, B cells, neutrophils, glial cells, endothelium, myocytes, fibroblasts, NK cells and keratinocytes. Acts as T helper 2 (Th2) stimulation; B and T cell maturation; NK cell activator; induces pyrexia (endogenous pyrogen); takes part in acute inflammation, endothelial cell activation.	(Dinarello, 2009, Dinarello, 2011).
IL-2	Released by activated Th cells. Acts as activator and proliferator for T, B and NK cells.	(Gaffen and Liu, 2004).
IL-3	Produced by T helper cells and promotes stem cells progression and diversification along with SCF; induction of mucosal mast cell development.	(Llop-Guevara *et al*., 2011).
IL-4	Secreted by Th2 cells, MØs, mast cells, basophils. Stimulates B cells for IgG1 and IgE production, propagation and differentiation of stimulated B cell (class switching); stimulate Th2 cell development.	(Beckmann *et al*., 1992, Van Dyken and Locksley, 2013).

IL-5	Produced by Th2 cells, eosinophils and mast cells. Proliferation and differentiation of eosinophils and activated B cells; IgG1 and IgE production;	(Mainou-Fowler *et al*., 1994, Molfino *et al*., 2012).
IL-6	Produced by monocytes, MØs, fibroblasts, endothelial cells, Th2 cells, mast cells and hepatocytes. Also acts as a pro-inflammatory cytokine; maturation of B cells into plasma cells; stimulates plasma cells to produce antibodies; differentiation of stem cells; stimulates platelet synthesis (clotting formation); triggers liver to produce acute phase proteins;	(Scheller *et al*., 2011, Garbers *et al*., 2012).
IL-7	Released by thymocytes and bone marrow cells. Acts on stem cell differentiation into B and T progenitor cells; enhances monocyte and MØ killing mechanisms; activates T cytotoxic cells (CTLs).	(Fry and Mackall, 2002, Huang and Luther, 2012).
CXCL-8 (formerly IL-8)	Chemokine; Produced by monocytes, MØs, endothelial and epithelial cells, T cells, neutrophils, hepatocytes, keratinocytes, chondrocytes, fibroblasts. Acts as a neutrophil chemotactic factor, which activates them to produce lysozymes; supports phagocytic activity of mononuclear cells; angiogenesis.	(Meade *et al*., 2012).

IL-10	Produced by mononuclear cells, regulatory T cells, T helper 2 cells, B cells, (and γδ T cells in cattle). Immunosuppression (termination of immune response), known to work together with TGFβ	(Sabat *et al*., 2010, Guzman *et al*., 2014).
IL-17	Synthesised by Th17 CD4+ T cells and γδ T cells in humans and cattle. Multifunctional cytokine such as induction of inflammation, allergy-associated cytokine, stimulation of other immune cells to produce a wide range of cytokines during TB infection in combination with IL-22.	(Peckham *et al*., 2014, Gaffen, 2016, Steinbach *et al*., 2016)
Tumour necrosis factor alpha & beta (TNF-α) & (TNF-β)	Produced by MØs, NK and mast cells, T and B cells. Acts as antitumor cytokine; proinflammatory; apoptosis; hematopoietic cell proliferation; angiogenesis; B cell maturation and Ab synthesis; neutrophil activation; phagocytosis.	(Aggarwal *et al*., 2012).
Interferon gamma (IFN-γ)	Secreted by majority of lymphocytes, particularly Th1 cells and NK cells. Antiviral and antitumor activities; activates class switching in B cells (IgG); enhances MHC I and MHC II expression and antigen presentation enhances cell cytotoxicity.	(Young and Hardy, 1995).

IFN-α & IFN-β	Type I interferons. Various leukocytes release IFN-α as an antiviral and antitumor mediator, while IFN-β is released by fibroblasts with the same function as IFN-α.	(Taniguchi and Takaoka, 2001, Belardelli *et al*., 2002).
Transforming growth factor beta (TGFβ)	Approximately shares similar secretion cites and functions with those mentioned in IL-10 plus a few extra functional roles in reaction to cancer cells and initiation of fibrosis in chronic diseases.	(Calon *et al*., 2014, Meng *et al*., 2016)

1.4 Regulatory T cells (Tregs)

Peripheral immune tolerance (unresponsiveness status of the immune system represented by lymph nodes to a certain antigen or immunogen) is controlled by regulatory T cells (Tregs), which are an important category of T cells involved in down-regulating immune responses at the end of their effector phase in order to prevent tissue damage. As unregulated immune responses are seen in autoimmune reactions, Tregs are important in controlling these diseases (Sakaguchi *et al*., 1995, Takahashi *et al*., 2000). They were originally identified as CD4+CD25+FOXP3+ cells, where CD25 is the α-chain of the IL-2 receptor which is expressed on most activated T cells. FOXP3 is a transcription factor involved in Treg development and function (Sakaguchi *et al*., 2009, Kassiotis and Liston, 2011).

Two types of Tregs have been described; natural CD4+CD25+ Tregs (nTregs) and induced (iTregs) Tregs (or sometimes called adaptive Tregs) both of which express the transcriptional factor forkhead box P3 (FOXP3) expressed on the inner nuclear membrane (Hori *et al*., 2003, Rudra *et al*., 2012). nTregs originate in the thymus and iTregs develop out of it (Curotto de Lafaille and Lafaille, 2009). nTregs form 5-10% of a typical overall T cell population in humans and mice as they develop from naïve CD4+ T lymphocytes (Shevach, 2002). They are characterised by their anergic properties *in vitro* (lack of response to TCR of T cells), while they are able to suppress CD4+ and CD8+ T cell subset growth and cytokine release (Takahashi *et al*., 1998, Piccirillo and Shevach, 2001).

iTregs (CD4+CD25+FOXP3+) are induced during the later stages of immune responses and are the principal Tregs which take part in different immuno-suppressive mechanisms on effector immune cells. Their activities

include inhibition of target cell proliferation and cytokine production. They are active against a range of immune cells including CD4+ effector T cells and CD8+ effector T lymphocytes, NK cells as well as phagocytic cells and APCs, such as monocyte / MØ lineage cells (Sakaguchi *et al*., 2008). These suppressive activities are mediated mainly by two suppressive cytokines, TGF-beta and IL-10.

Induced Tregs require IL-2 for their growth, maintenance and function (Chen *et al*., 2011, Visan, 2014). In addition, TGF-β is required to generate FOXP3 gene expression which changes CD4+CD25− naïve Tregs into active CD4+CD25+ Tregs and full function (Chen *et al*., 2003).

Vignali *et al*. (2008) suggested the division of Tregs into four functional groups depending on the cytokines they produce; the first group releases IL-10, IL-35 (a potent immunosuppressive cytokine secreted by Tregs that inhibits T cell proliferation) and TGF-β, as immuno-suppressive cytokines; the second group uses cytolysis by releasing granzyme A or B as well as pore forming protein (perforin) to induce apoptosis in the target cells; the third group cause metabolic disruption which influences mainly effector T cells by interference with cyclic AMP (cAMP) pathway or deprivation of cytokines leading to apoptosis; and the final group modifies DC maturation and/or function by CD223-MHCII engagement which inhibits DC maturation.

1.5 Toll-like receptors (TLRs): A bridge between innate and adaptive immunity

Pathogen recognition receptors (PRRs) consist of several families of receptors of which the TLR group is the interesting group for this (my) study. The Toll receptor was discovered firstly in *Drosophila* insects more than 25 years ago as an immune defence factor (Hashimoto *et al*., 1988). Later, it was found that a homologue toll-like receptor in humans existed that also had similarity in terms of structure with the human IL-1 receptor which provided a link to a role in innate immunity in vertebrates (Rock *et al*., 1998, Aderem and Ulevitch, 2000). In mammals, it has been shown that TLRs play an important role in innate immunity and also informing adaptive immune responses (Akira, 2003). 10 types of TLR have been recognised in humans and 13 in mice (Pasare and Medzhitov, 2005), while there are up to 23 TLRs in fish (Palti, 2011).

Many immune cells and 'barrier' cells express TLRs such as DCs, MØs, T and B cells, polymorphonuclear cells, microglial cells, endothelium, and epithelium (Bsibsi *et al*., 2002, Armstrong *et al*., 2004, Miller *et al*., 2005). These cells recognise microbial PAMPs via TLRs and bind them, leading to the release of reactive oxygen species (ROS) metabolites, reactive nitrogen species (RNS) metabolites, pro-inflammatory cytokines like IL-1, IL-6, IL-8, and TNF-α, as well as co-stimulatory molecules, which include CD28, CD40, CD80 (B7.1) and CD86 (B7.2), which can activate T cells, B cells and DCs, thus triggering adaptive immunity (Werling and Jungi, 2003).

1.5.1 TLR structure and function

Figure 1.1 shows the structure typical of TLRs. They are type I transmembrane receptors which are glycoproteins consisting of 3 portions; first, a unique conserved intracytoplasmic Toll/IL-1 receptor (TIR) as a C-terminal signalling domain common to all TLRs; secondly, a transmembrane portion as a single helical connecting part; and third, an extracellular N-terminal leucine-rich repeat (LRR) domain that differs morphologically amongst TLRs (Werling *et al*., 2009, Reuven *et al*., 2014). The intracellular (TIR) domain is responsible for cascades of events leading toward triggering the signalling system which is regulated by one of 5 important adapter proteins: myeloid differentiation primary response gene 88 (MyD88), MyD88-adapter like (MAL) named as Toll-interleukin 1 receptor (TIR) domain containing adapter protein (TIRAP) as well; TIR-domain-containing-adapter-inducing interferon-β (TRIF); TRIF-related adaptor molecule (TRAM) and Sterile-alpha and Armadillo-motif-containing protein (SARM) (O'Neill and Bowie, 2007). TLRs can work individually or as heterodimers as in TLR1/2, TLR2/6 interaction by binding to each other to exert potential immunological effect where these proteins traffic to induce activation of transcription factors (Triantafilou *et al*., 2006).

The transmembrane domains of TLRs play an important role in recognition of RNA and/or DNA of PAMPs (mostly viruses) by binding to them in the endoplasmic reticulum via UNC93B (a transmembrane protein important for signalling of human and murine TLR3, 7 and 9) (Brinkmann *et al*., 2007, Kim *et al*., 2008).

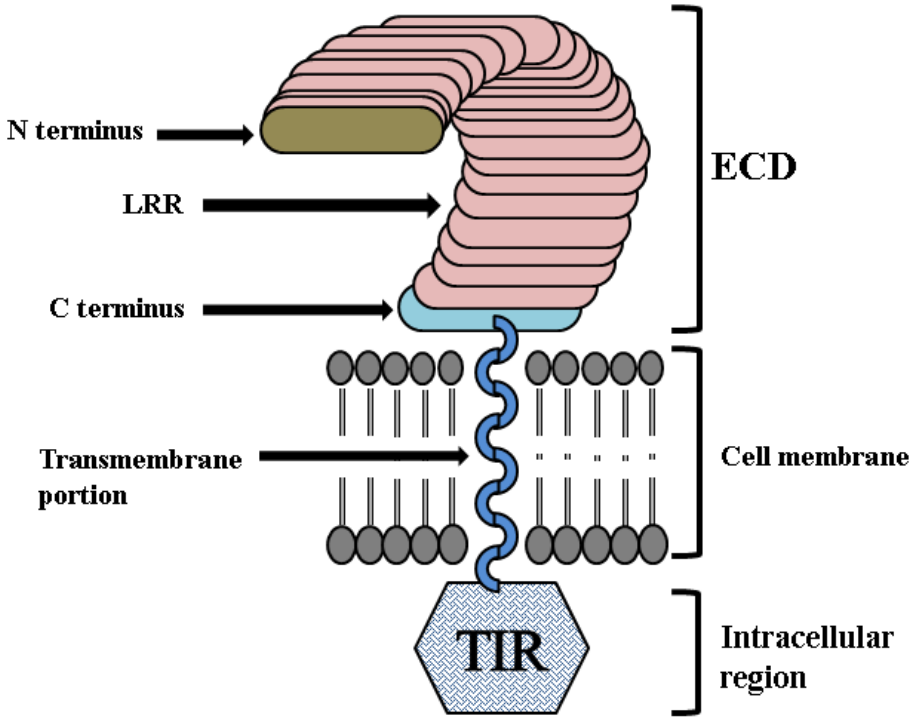


Figure 1.1: Schematic diagram showing a typical TLR structure. ECD= extracellular domain, LRR= leucine rich repeat, TIR= Toll/IL-1 receptor.

The glycoprotein part consists of LRRs of 20–30 amino acids which is the immunologically active component of TLRs responsible for capturing PAMPs. The LRR proteins give TLRs a horseshoe shape as a curved solenoid structure; therefore, its outline resembles two surface coils (convex and concave faces) forming an "m-shape" ending with two loops (Bella *et al*., 2008, Botos *et al*., 2011). These LRR proteins can be further divided into 7 subfamilies in accordance to their sequence and structural basis, six of them have been identified in vertebrates and one in plants (Matsushima *et al*., 2007). The differences in these LRR proteins result in capturing varieties of pathogens by their PAMPs as listed in table (1.5).

Functionally, TLRs have multiple signalling functions, i.e. the major specific TLRs for sensing viral antigens are TLR3, TLR7, TLR8 and TLR9 which are responsible for recognition of dsRNA viruses, ssRNA viruses and DNA

viruses respectively (Alexopoulou *et al*., 2001, Takeshita *et al*., 2001, Heil *et al*., 2004). Recently, expression of TLR10 has been reported in human monocytes *in vitro* after infecting them with Influenza virus resulting in proinflammatory cytokines secretion (Lee *et al*., 2014). TLR2/6 (heterodimer) on host cells can recognise PAMPs of *Saccharomyces cerevisiae*; and TLR2 and TLR4 recognise PAMPs in *Cryptococcus neoformans* and *Candida albicans* (Akira *et al*., 2006). Moreover, some large-size parasites (helminths) have PAMPs that could also be detected by TLRs (Venugopal *et al*., 2009) e.g. *Filaria* and *Schistosoma* species could be recognised by TLR2 and TLR4. Added to that, small size parasites (protozoa) can be recognised by TLR2, TLR4 and TLR9 for example in the response to malaria (*Plasmodium falciparum*); as well as specific findings illustrated in TLR11 and TLR12 which play an important role in detection of profilin antigen (actin-binding protein) expressed by *Toxoplasma gondii* in mice (Shi *et al*., 2012, Koblansky *et al*., 2013).

On the other hand, a wide spectrum of TLRs have been described in terms of bacterial PAMPs recognition, which could be subdivided into groups of TLRs, e.g. TLR1/2 heterodimer, TLR2 and TLR2/6 on mammalian cells can sense for bacterial lipoproteins; TLR2 peptidoglycan and lipoteichoic acid; TLR4 lipopolysaccharide and TLR5 flagellated bacteria (Philpott and Girardin, 2004, Akira *et al*., 2006). Moreover, protozoa can be sensed by certain types of TLRs such as *Toxoplasma gondii* which is recognised by TLR11 and TLR12 (Shi *et al*., 2012, Koblansky *et al*., 2013); *Trypanosoma cruzi*, *T. brucei*, *Leishmania major* and *Plasmodium falciparum* (Malaria) by TLR2 and TLR4 (Gazzinelli and Denkers, 2006).

Table 1.5: Ligands of different TLRs and their targets.

TLRs	Dimerization	Cells expressing TLRs	Adaptor molecule	Target recognition and ligands	References
TLR1	Heterodimer with TLR2	MØs and PMNs	MyD88/ TIRAP	Triacyl lipopeptides and peptidoglycans in majority of bacteria and *Mycobacteria*; other bacterial soluble components.	(Wyllie *et al*., 2000, Takeuchi *et al*., 2002)
TLR2	Heterodimer with TLR1 or with TLR6	Microglia, PMNs, MØs, monocytes, B cells, CD4+ T cells including Tregs, DCs and respiratory epithelia	MyD88/ TIRAP	Lipoprotein in different bacteria and other pathogens; Peptidoglycan (PGN) and/or Lipoteichoic acid (LPA) of many Gram +ve bacteria; Lipoarabinomannan in *Mycobacteria*; Zymosan in fungi; Heat shock protein 70 (Hsp70) in host cells; Hemagglutinin in *Paramyxovirus* and Cytomegalovirus (CMV).	(Aliprantis *et al*., 1999, Means *et al*., 1999, Asea *et al*., 2002, Bieback *et al*., 2002, Compton *et al*., 2003, Bellocchio *et al*., 2004)

TLR3	Homodimer	Intracellular in T cells, DCs and placental epithelia	TRIF	Double stranded RNA (dsRNA) viruses	(Alexopoulou *et al*., 2001)
TLR4	Homodimer	Placental epithelia, monocytes	MyD88/ TIRAP, SARM	Lipopolysaccharide in many Gram –ve bacteria; Fusion protein in Respiratory syncytial virus (RSV); Hsp60, Hsp70 and Fibrinogen in host cells.	(Chow *et al*., 1999, Ohashi *et al*., 2000, Smiley *et al*., 2001, Asea *et al*., 2002)
TLR5	Homodimer	Monocytes, NKT cells, intestinal epithelia and MØs	MyD88	Flagellin in motile bacteria	(Hayashi *et al*., 2001)
TLR6	Heterodimer with TLR2	Monocytes, spleenocytes and thymocytes	MyD88	Diacyl lipopeptides in *Mycoplasma*	(Takeuchi *et al*., 2001)

TLR7 & TLR8	Homodimer	Intracellular in DCs, T cells MØs and spleenocytes	MyD88	Single stranded RNA (ssRNA) and Single stranded DNA viruses	(Heil *et al*., 2004)
TLR9	Homodimer	Intracellular in DCs, T and B cells, NK cells and monocytes	MyD88	DNA viruses; Unmethylated CpG DNA in bacteria, viruses, fungi; hemozoin in Malaria	(Takeshita *et al*., 2001, Coban *et al*., 2005)
TLR10	Not confirmed	Spleenocytes, lymph nodes, thymocytes, and respiratory epithelia	MyD88	Influenza viruses (*Orthomyxoviruses*) / recently discovered	(Lee *et al*., 2014)
TLR11	No data	DCs, MØs, epithelial cells of liver, gut kidney, and bladder	MyD88	Blockage of *Salmonella* adhesion in murine gut; profilin in *Toxoplasma gondii*	(Shi *et al*., 2012, Koblansky *et al*., 2013)
TLR12	No data	DCs	MyD88	Profilin in Toxoplasma gondii	(Koblansky *et al*., 2013)

TLR13	No data	DCs and MØs	MyD88	Receptor for RNA Bacteria	(Hidmark *et al*., 2012)
TLR14-TLR23	No data	Unknown	Mainly MyD88	Fish TLRs	(Palti, 2011, Pietretti and Wiegertjes, 2014)

1.5.2 T and B lymphocytes expressing TLRs

Although previous studies showed the prominent role of TLRs in initiation of innate immune responses, recent studies have described that TLRs are expressed on adaptive immune cells (T and B cells) (Reynolds and Dong, 2013, Buchta and Bishop, 2014a and b). Both B cells (naïve and activated) and memory B cells express TLR1, 4, 6, 7, 9 and 10 in humans (Hornung *et al*., 2002, Mita *et al*., 2002, Bernasconi *et al*., 2003). In addition, TLR2 can be expressed on human B cells in response to protein A (a virulence factor in *Staphylococcus aureus*) resulting in B cell receptor (BCR) activation (Bekeredjian-Ding *et al*., 2007). The BCR consists of immunoglobulin molecules located on the outer surface of B cells. Further investigations showed that human plasma cells express TLR1-9 which enhance antibody production leading to a more powerful humoral immune response (Dorner *et al*., 2009).

T cells also have TLR expression in mice and humans. CD4+ T cells express TLRs 2,3,4 and 5 and TLR7, 8, 9 (Gelman *et al*., 2004, Komai-Koma *et al*., 2004, Caron *et al*., 2005, Zanin-Zhorov *et al*., 2007); while Th1, Th2 and Th17 express TLR2 and TLR4 (Matsuguchi *et al*., 2000, Imanishi *et al*., 2007, Reynolds *et al*., 2010, Reynolds *et al*., 2012). CD8+ T cells express TLR2, 3 and 9 (Komai-Koma *et al*., 2004, Babu *et al*., 2006, Tabiasco *et al*., 2006). Moreover, Gamma delta T cells have the capability to express TLR1, 2 and 3 and TLR6, 7 and 8 (Pietschmann *et al*., 2009), whereas natural killer T cells (NKT cells) could express TLR2, 3, 4 and 5, TLR7 and TLR9 (Shimizu *et al*., 2002, Saikh *et al*., 2003, Kulkarni *et al*., 2012) and finally Tregs are through to express TLRs 1-8 (Sutmuller *et al*., 2006).

1.5.3 TLR signalling pathways

1.5.3.1 Extracellular TLR signalling pathway

Extracellular TLR homodimers include TLR1, TLR2, TLR4, TLR5 and TLR6, plus heterodimers (TLR1/2, TLR2/6). They share approximately the same signalling pathway. As an example of extracellular TLRs, the TLR5 signalling pathway (see figure 1.2) shows the classical signalling route. The recognition of flagellin by TLR5 leads to the recruitment and activation of MyD88, which leads to activation of transcription factor NFκB resulting in secretion of pro-inflammatory cytokines (Barton and Medzhitov, 2003). MyD88 is an adaptor protein composed of a TIR domain that binds to the N-terminal protein in interleukin-1 receptor-associated kinase (IRAK) and tumour necrosis factor (TNF) receptor associated factor-6 (TRAF6) (Medzhitov *et al*., 1998). This involves the kinases IRAK1 then IRAK4. responsible for indirect activation of TRAF6 via a phosphorylation process (Kawai and Akira, 2007). The activated TRAF6 attracts TGF-β-activated kinase-1 (TAK1), which is an important enzyme for upregulation of I-kappa B kinase complex (IKK-β) and mitogen-activated protein kinase (MAPK) kinase-6 (MKK6) This series of events activates NFκB transcription factor (Wang *et al*., 2001, Adhikari *et al*., 2007). This in turn induces several events, most notably pro-inflammatory cytokine production that includes: IL-1β, IL-6, IL-8, and TNFα (figure 1.2) (Brasier, 2010, Tornatore *et al*., 2012). Also, Meade *et al*. (2012) added that TLR5 stimulated cells lead to CXCL-8 production by monocytes, MØs, endothelial and epithelial cells, T cells, and neutrophils, which enhance attraction of phagocytic cells and maintain the activity of mononuclear cells and angiogenesis.

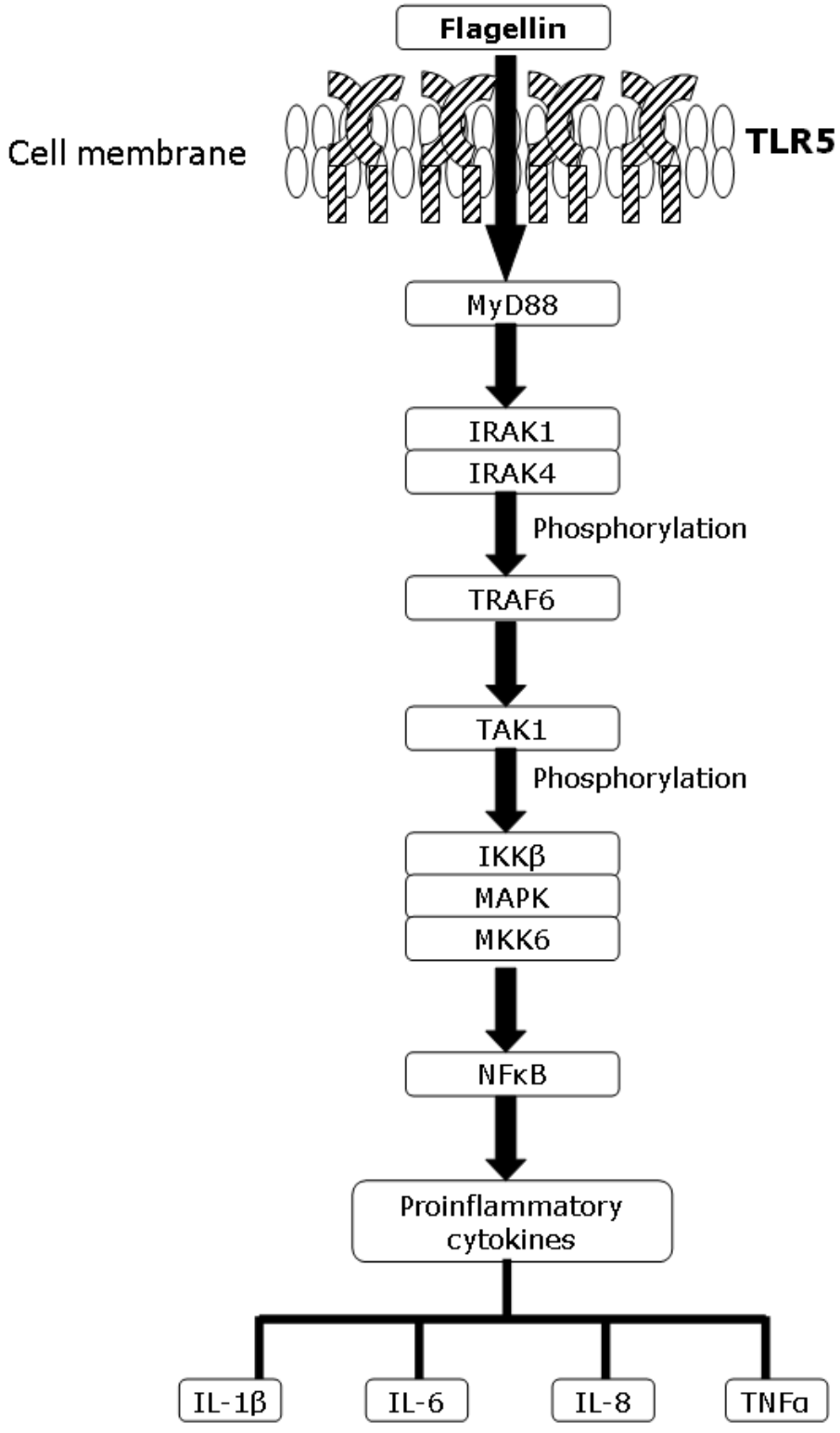


Figure 1.2: TLR5 signalling pathway as an example of extracellular TLR signalling pathway.

1.5.3.2 Intracellular TLRs signalling pathways

Intracellular TLRs include TLR3, 7, 8 and 9 (mostly antiviral sensors). These intracellular TLRs recognise the nucleic acids of viruses in a mechanism that terminates with type 1 Interferon (IFNα and β) production. This happens through interferon-regulatory factors (IRF3, IRF5, and IRF7 known as effective transcription factors), these are important for T lymphocyte activation (mainly cytotoxic T cells) to trigger antiviral immune responses (Moynagh, 2005, Kawai and Akira, 2007).

However, Gay *et al*. (2014) proposed that these intracellular TLRs possibly could share the same pathway with the extracellular TLRs through activation of the MyD88-dependent pathway (see above section 1.5.3.1) to end up with induction of pro-inflammatory cytokines. This is not necessarily only NFκB transcription factor activation, but some other transcription factors might be involved such as cAMP-responsive element-binding protein (CREB) and activator protein-1 (AP-1) in case of intracellular TLR7, 8, and 9 signalling pathways (Hemmi *et al*., 2002, Latz *et al*., 2007, Tanji *et al*., 2013).

In contrast, TLR3 uniquely reveals a different signalling route from all other TLRs after engagement with dsRNA viruses. Experimental infection with orthomyxoviruses (that contain the causative agents of influenza, also called influenza viruses) *ex vivo* resulted in activation of TIR domain-containing adaptor protein inducing IFNβ (TRIF), which (TRIF-driven route) leads to production of the TRAF3 effector proteins necessary for binding of IKK epsilon (IKKε) protein with either IRAK1 or TBK1 to produce IRF3 or IRF7 transcription factors respectively that penetrate the nuclear

membrane to stimulate transcription for type I IFN secretion (Liu *et al*., 2008, Luo *et al*., 2012, Teijaro *et al*., 2014).

1.6 Bovine TLRs – in depth highlights and specifications

After discovery of the PRR molecules (including TLRs) in humans and some laboratory animals (mice, rats, and guinea pigs), attempts commenced to study the presence and function of these sensory molecules in domestic animals and ruminants (Turin and Riva, 2008, Smith *et al*., 2012). Initial attempts (Menzies and Ingham, 2006) to investigate expression of TLR1-10 by real time PCR (RT-qPCR) in ruminants (a focus on cattle and sheep), revealed the presence of 10 TLRs (TLR1 to TLR10) that shared 83-90% amino acid similarity to human TLRs 1-10 [confirmed when McGuire *et al*. (2006) mapped them]. Menzies and Ingham also observed that all bovine TLR1-10 genes except TLR6 were expressed in the skin, Peyer's patches, and mesenteric lymph nodes (gut associated lymphoid organs) of cattle but at low levels, except for bovine TLR2, and 7 which were strongly expressed in the skin.

The genome of *Bos taurus* has also been mapped revealing 10 TLRs (TLR1-10) genes (McGuire *et al*., 2006, Seabury *et al*., 2010). These bovine TLR sequences are currently available on the website of the National Centre for Biotechnology Information (NCBI) (see table 1.6).

Table 1.6: Gene accession numbers of up to date TLR1-10 genes in cattle (species *Bos taurus*). Cited from National Centre for Biotechnology Information (NCBI). Accessed 30-08-2017.

Gene name	mRNA size / base pair (bp)	Location (chromosome No.)	Accession No. / GenBank (NCBI)
TLR1	2319 bp	6	NM_001046504
TLR2	3513 bp	17	NM_174197
TLR3	3025 bp	27	NM_001008664
TLR4	3739 bp	8	NM_174198
TLR5	2800 bp	16	NM_001040501
TLR6	3095 bp	6	NM_001001159
TLR7	3177 bp	X	NM_001033761
TLR8	3075 bp	X	NM_001033937
TLR9	3265 bp	22	NM_183081
TLR10	3163 bp	6	NM_001076918

1.6.1 Bovine TLR1

This is an extracellular protein expressed in response to exposure of host cells to the lipid compartments of bacteria (such as lipoteichoic acid, a lipoprotein in most Gram-positive bacteria). Farhat *et al*. (2010) sequenced the full length bovine TLR1 gene and showed the protein bound synthetic diacyl and triacyl lipoproteins. Later on, Ruiz-Larranaga *et al*. (2011) described the genetic diversity of this gene by describing 24 SNPS in the TLR1 of Holstein-Friesian cattle infected by *Mycobacterium avium paratuberculosis*. Further studies by Russell *et al*. (2012) pointed out the importance of bovine TLR1 and its variants, synonymous and non-

synonymous SNPs (sSNPs and nsSNPs), in detection of bacterial lipoproteins during clinical mastitis.

Upon stimulation with bacterial lipoproteins, studies in cattle showed expression of TLR1, 2, and 6 RNA in the epithelium of endometrial and stromal cells but functionally, only TLR1 and TLR2 resulted in induction of an inflammatory reaction represented by detection of high levels of IL-6 and IL-8 secreted cytokines (Turner *et al*., 2014).

1.6.2 Bovine TLR2

The first report to identify bovine TLR2 was in 2003 when White *et al*. (2003) studied the sequence similarity in this gene between human and bovine TLR2. In cattle, studies showed that bovine TLR2 and TLR4 were detected in mammary epithelial cells in *Staphylococcal* mastitis cases (Goldammer *et al*., 2004).

In the intestine, TLR2 and TLR4 were detected in the epithelial layer infected with *Cryptosporidium parvum* (a protozoan parasite that causes diarrheal enteritis in calves) through secretion of CXCL-8 (Yang *et al*., 2015). One of the most important respiratory diseases in livestock is *Mycoplasma*. Bovine TLR2 is thought to play an important role in detection of *Mycoplasma bovis* lipid associated particles through the MyD88-driven pathway resulting in pro-inflammatory IL-1β secretion (Wang *et al*., 2016). TLR2 has been detected in the cattle reproductive organs (mainly corpus luteum in the ovaries of Chinese Holstein cattle) which may play a role (along with TLR4) in early sensing of virulent Gram-negative bacteria by triggering inflammation (Lüttgenau *et al*., 2016). More recent observations (Zhao *et al*., 2017) revealed that some TLR2 SNPs might be associated

with recognition of *Mycobacterium* infected cattle (Chinese Holstein species).

1.6.3 Bovine TLR3

Bovine intracellular TLR3 was first confirmed by McGuire *et al*. (2006). TLR3 in cattle contains many SNPs and presents with numerous variants (Cargill and Womack, 2007, Zimin *et al*., 2009). A new cell line model for studying the immune functions of TLR3 (known as dsRNA sensor) was developed by Chiba *et al*., (2012) who used cells derived from bovine intestinal epithelial (BIE cells) on which TLR3 was highly expressed, which upon stimulation with polyI:C (TLR3 agonist) resulted in detectable levels of pro-inflammatory cytokines.

Rensetti *et al*. (2016) stated that TLR3 is involved in recognition of BoHV-1 and BoHV-5 in the infected neurons and olfactory tract. This notion has been recently further supported by Oliveira *et al*., (2017) who found a strong correlation between TLR3 signalling in the CNS with cows infected by BoHV-5 viruses.

1.6.4 Bovine TLR4

This particular receptor (TLR4) is a sensory receptor for LPS in most Gram-negative bacteria. In cattle, TLR4 structure and function does not differ much from those in other mammals. Bovine TLR4 often functions along with TLR2 to recognise infection, for example *C. parvum* in the intestine (see section 1.6.2) and trigger inflammation (Yang *et al*., 2015). Both TLR4 and TLR2 undergo biological changes for up to 7 days after birth. Their expression dropped in the endometrium from cows diagnosed with dystocia

due to retained placenta compared to healthy cows (Martins *et al*., 2016). Shimizu *et al*., (2017) identified numerous numbers of TLR4 SNPs in exon 3 of the gene in Holstein cows which might have functional consequences. TLR4 may be involved in susceptibility / resistance of Holstein cows to digital dermatitis infection (El-Shafaey *et al*., 2017). Moreover, properties of milk production in Vrindavani cattle (Indian breed) were thought to be linked to TLR4 gene expression and SNP variants (Mishra *et al*., 2016). In contrast, *Sahiwal* and *Hariana* cattle (Indian breeds) demonstrated marked down regulation of TLR4 gene expression in PMNs during winter and summer seasons (Swain *et al*., 2017).

Interestingly, bovine TLR4 can be negatively affected (downregulated) by exposure to certain antibiotics such as sodium houttuyfonate (SH). This compound is known to have anti-inflammatory properties in treatment of bovine mastitis, which also suppresses TLR4 expression in the mammary gland epithelium in response to LPS stimulation by Gram-negative bacteria (mainly *E. coli*). The suppression could be attributed to blockage of transcription factor NFκB activation (Wang *et al*., 2017).

1.6.5 Bovine TLR5

Flagellated motile bacteria can be sensed by TLR5 in mammals. This was investigated by Tahoun *et al*., (2015) who identified structural and functional similarity between human TLR5 (hTLR5) and bovine TLR5 (boTLR5). They transfected human embryonic kidney (HEK293) and embryonic bovine lung cell lines with boTLR5 and stimulated them with a flagellin derived from *E. coli* strain O157:H7. Both cell lines responded positively by production of CXCL-8 in the supernatant.

However, previous studies showed that bovine TLR5 was less responsive to flagellins than its human counterpart (Metcalfe *et al*., 2014; Lankester et al., 2016). In a flagellin TLR5 adjuvant study in Zebu cattle, the effect of flagellin was not effective but interestingly was associated with antibody immunosuppression (Lankester *et al*., 2016). Based on the above information, boTLR5 may play an additional role or even alternative to that known in humans.

1.6.6 Bovine TLR6

Bovine TLR6 was discovered in the same region of about 50 kB in chromosome 6 that contains TLR6, TLR1, and TLR10 (Opsal *et al*., 2006). Peptidoglycan binding and SNP variants have described (Seabury and Womack, 2008). In addition, bovine TLR6, 4, and 2 were screened for polymorphisms in European cattle breeds and 16 SNPs were identified (Mariotti *et al*., 2009)

As described in section 1.6.1, bovine TLR6 was expressed (along with either TLR1 or TLR2) in the endometrial epithelium but reported as non functional (Turner *et al*., 2014). However, Song *et al*. (2014) identified 4 SNPs of bovine TLR6 that played an important role against TB in Chinese Holstein cows and lack of them might expand the risk factor of TB. Finally, heterodimerisation of TLR6 with TLR2 was formerly described in humans and some animals (Triantafilou *et al*., 2006, Ren *et al*., 2016), but this is still untested in cattle.

1.6.7 Bovine TLR7 and TLR8

Both TLR7 and TLR8 are known in mammals as antiviral intracellular PRRs which recognise ssRNA viruses and to a lesser extent some DNA viruses and this leads to secretion of type I interferons (IFNα and β) through activation of IRF transcription factor (Hart *et al*., 2005, Stary *et al*., 2007, Wei *et al*., 2009, van Haren *et al*., 2016, Gidon *et al*., 2017). Bovine TLR7 and TLR8 (TLR7/8 hereafter) were mapped and confirmed by RT-qPCR (McGuire *et al*., 2006, Menzies and Ingham, 2006). More than 100 SNPs have been documented in TLR3, 7, and 8 by (Cargill and Womack, 2007) who attributed this to possible multiple functions of these TLRs. Also, Indian crossbred cows expressed TLR7 on PBMCs at variable rates according to geographical distribution of the livestock (Singh *et al*., 2014). Bovine TLR7/8 have been studied by Rensetti *et al*. (2016) who confirmed the expression of TLR7/8 in the central nervous system of calves infected with bovine alpha herpes virus (BoHV-1 and BoHV-5 strains). These TLRs participated (although TLR7 was much more prominently expressed) in initiation of inflammation as detected in the histopathological sections as part of their sensory role upon recognition of herpes viruses by comparison with calves infected with mock viral suspension.

Interestingly, Schaut *et al*. (2016) stated that monocyte-derived MΦs infected with bovine viral diarrheal virus-2 (BVDV-2) expressed higher levels of TLR7 determined by RT-qPCR and prompted large quantities of secreted IL-6. With regards to bovine TLR8 (boTLR8) protein, Zhu *et al*. (2009) noticed that it is located inside cells infected with BHV-1.

1.6.8 Bovine TLR9

TLR9 recognises non-methylated CpG motifs existing in the DNA of various viruses and bacteria. Bovine TLR9 (boTLR9) shares many of the features of other mammalian TLR9s (Griebel *et al*., 2005).

Schneberger *et al*. (2011) developed a mouse anti-bovine TLR9 antibody which detected TLR-9 by western blot, immunohistochemistry and electron microscopy for its expression in the bovine respiratory system (airway epithelium, endothelium, lung tissue including cellular compartments such as alveolar MΦs as well as intravascular monocytes and MΦs).

Molecular studies in cattle highlighted SNP variants of TLR-9, some of which were thought to participate in host susceptibility to TB infection in Chinese Holstein cows (Sun *et al*., 2012). In addition, the structure of three forms of *Bos taurus* TLR9 was further functionally identified by Ohto *et al*., (2015) as a sensory molecule for unmethylated CpG DNA viruses. All three forms of boTLR9 bound to CpG unmethylated DNA and elicited inflammation.

Parameswaran *et al*., (2014) used unmethylated CpG oligonucleotides (CpGODN) as an adjuvant to target TLR9 in immunisation studies against malignant catarrhal fever. They found that the inclusion of CpGODN did not have an enhancing effect on protective immunity over Emulsigen adjuvant when included together and little effect on its own.

1.6.9 Bovine TLR10

Less is known about TLR10 and its ligands in mammals. boTLR10 was first identified in the same region of the genome as TLR1 and TLR6 and is about 50 kB in chromosome 6 (Opsal *et al*., 2006). The biological topography of

boTLR10 was drawn by Werling *et al*. (2006) who noticed diversified presence of boTLR10 mRNA transcripts in some APC cells detected by RT-qPCR in monocytes, monocyte-derived MΦs, monocyte- derived DCs, and afferent lymph DCs (both $CD172a^{+}$ and $CD172a^{-}$), whereas alveolar MΦs, DCs derived from bone marrow, and B lymphocytes consistently expressed boTLR10.

This bovine PRR molecule, along with the rest of the bovine TLRs, has been sequenced (McGuire *et al*., 2006, Seabury *et al*., 2007, Zimin *et al*., 2009, Seabury *et al*., 2010).

1.7 TLR agonists

In mammals, conserved regions of microbial organisms (PAMPs) induce expression of cognate TLRs on immune cells. This stimulation results in an inflammatory immune response that results in pro-inflammatory cytokine secretion as described in previous sections. These microbial molecules (PAMPs) have been synthesised and are available from companies for research (e.g. InvivoGen and AdipoGen), where 10 ligands for human TLR1-9 are made by InvivoGen and 16 ligands by AdipoGen (see links):

1. (http://www.invivogen.com/human-tlr1-9-agonist-kit)
2. (http://www.adipogen.com/media/Catalogs/PDFs/Innate_Immunity_Flyer_2015_Adipogen.pdf).

These companies manufacture human TLR1-9 and TLR11/12 ligands for academic and commercial purposes. To date there are no available TLR10 agonists with the exception that TLR2/6 agonist might also be considered as a putative TLR10 agonist (Tocris-Bioscience, 2017).

1.8 TLR agonists /ligands as new generation adjuvants

The expression of TLRs on immune cells such as DCs, MØs, T cells and PMNs can control the direction and magnitude of immune responses affecting innate and adaptive immunity. Thus, TLR ligands (PAMPS etc) are likely to be useful new generation adjuvants that can affect the magnitude and type of immune response to vaccination (Hayashi *et al*., 2001, Kaisho and Akira, 2002, Hedayat *et al*., 2011).

Traditional vaccines, using well tested adjuvants such as alum and Quil-A amongst others still provide efficacies against existing pathogens and some newly emerging ones. However, the ability to direct the type of immune response to a protective one involving new adjuvants is particularly timely and important (Tomljenovic and Shaw, 2011, Tomljenovic and Shaw, 2012). In addition, the overuse of antibiotics is leading to antibiotic resistance in bacteria and new vaccines are very important to develop (Davies and Davies, 2010). For all those purposes, the use of TLR agonists as a new promising generation of adjuvants will be useful to deliver vaccines against pathogens in both human and veterinary medicine. In the case of TLR5, the starting point of this interest was in 2002 when Liaudet *et al*. (2002) showed that the flagellin-TLR5 pathway induced inflammation and that this could be utilised to help cure endotoxic shock, purulent infection attributed by pyogenic bacteria, pulmonary affections and airway epithelial disturbances. This idea was exploited by Blohmke *et al*. (2008) who stated that TLR5 agonists could be used effectively against harmful bacteria such as *Burkholderia cepacia* and *Pseudomonas aeruginosa* as a model for cystic fibrosis patients who developed severe chronic pulmonary

signs. They recorded increased pro-inflammatory mediators as well as higher levels of antibody in those patients compared with controls suggesting that TLR5 agonists could be used as part of a treatment to protect against cystic fibrosis.

A patent was lodged by Rhee *et al*. (2012) who created TLR5 agonist mutants by genetic manipulation of flagellins to produce a powerful TLR5-flagellin signalling cascade. Furthermore, Minton (2014) described the importance of adding TLR5 agonists to influenza vaccine in mice. They suggested that gut microbes stimulate the murine host to produce an antibody response to influenza vaccine in the presence of flagellin which leads to higher TLR5 expression on B cells resulting in induction of cytokine release. Finally, a patent was successfully obtained for a novel discovery of an immunomodulatory flagellin peptide that possesses TLR5 binding properties (Aderem *et al*., 2014).

1.9 Hypotheses, objectives, and questions

1. I hypothesise that cattle T cells express TLRs, and they are differentially expressed between the T cell subsets – CD4+, CD8+, γδ - TCR T cells, and in addition that they are functional.
2. Function of T cell TLRs will be measured by stimulation with TLR ligands and by comparison with Con-A (T cell mitogen) or anti-CD3 (a known stimulus for T cells) as controls.

It is possible that engagement of TLRs on DC/ monocyte antigen-presenting cells will have a different function than those on effector T cells (CD4 T cells, γδ T cells or CD8 T cells) or Tregs and understanding these interactions will inform the choice of vaccine adjuvant for protective immune responses to pathogens of veterinary importance. On this basis, we formed and developed our research objectives /questions:

1) To what extent do bovine PBMCs, T cells and/or their subsets express TLRs?

We proposed in the first instance to undertake a broad screen of bovine PBMCs and purified major T cell subsets (CD4, CD8 and γδ T cells) for reactivity to TLR ligands.

Chapter Two

General Materials & Methods

2.1 Animals

Cattle 18-24 months of age (table 2.7) were used. Two sources were used for the blood of these animals: Sera Laboratories, West Sussex, UK and Elliot Abattoir, Chesterfield, UK. Animals were kept off food for 24 hours and off water for 12 hours before slaughter. All animals were overtly clinically free of disease. Ethical approval was obtained from the SVMS Clinical Ethical Review Committee. All material (blood) was taken immediately post-mortem.

Table 2.7: Animals used for blood collection. Age, gender, and breed with the source are given.

Lot number (Seralab)/ blood animal	Animals description	Source
BOV4098	18 months female Limousine breed calf	Sera Laboratories, West Sussex, UK
Bov A (EA)*	22 months female Limousine breed calf	R B Elliott & Sons Abattoir, Chesterfield, UK
Bov B (EA)	18 months female Limousine breed calf	R B Elliott & Sons Abattoir, Chesterfield, UK
Bov 1 (EA)	24 months male Limousine breed calf	R B Elliott & Sons Abattoir, Chesterfield, UK
Bov 2 (EA)	18 months male Friesian calf	R B Elliott & Sons Abattoir, Chesterfield, UK
Bov 3 (EA)	20 months male Limousine calf	R B Elliott & Sons Abattoir, Chesterfield, UK
Bov 4 (EA)	20 months male Limousine calf	R B Elliott & Sons Abattoir, Chesterfield, UK
Bov 5 (EA)	20 months female Friesian calf	R B Elliott & Sons Abattoir, Chesterfield, UK
Bov 6 (EA)	20 months male Limousine calf	R B Elliott & Sons Abattoir, Chesterfield, UK
Bov 7 (EA)	20 months male Limousine calf	R B Elliott & Sons Abattoir, Chesterfield, UK
Bov 8 (EA)	20 months male Limousine calf	R B Elliott & Sons Abattoir, Chesterfield, UK

* EA = Elliot Abattoir, Chesterfield, UK

2.2 Blood and tissue collection

2.2.1 Rabbit MLN cells

These cells were already prepared by a previous Postdoctoral scientist (Nevi Parameswaran) from 6 New Zealand white healthy rabbits' (of either sex), 2-4 months of age (Charles River, UK). Mesenteric lymph nodes (MLN) were harvested and chopped into small slices, prior to single cell preparation in sterile bags by using a stomacher machine (10 seconds). Cells and detritus were filtered with PBS through two layers of Whatman sterile lens tissue then adjusted to $1X10^7$ cells/ml of cryopreservation medium (!0% v/v DMSO in DMEM containing 50% FCS) and stored in cryotubes in liquid nitrogen. These were harvested by thawing in a water bath prior to use.

2.2.2 Bovine blood

Sera Laboratories West Sussex, UK: whole bovine blood (for PBMC isolation), taken from healthy animals (vessel held under the throat during exsanguination) by vacutainer (containing lithium heparin, H0878, Sigma-Aldrich, Missouri, USA) and shipped immediately after collection at ambient temperature.

R B Elliott & Sons Abattoir (Chesterfield, Derbyshire, UK) was the other source of blood, post mortem from slaughtered cattle. In this instance, 20 mg Lithium heparin as an anticoagulant was dissolved in sterile 50 ml PBS and transferred into a sterile 1 L screw-capped glass bottle inside a class

II microbiology safety cabinet. The bottles were transported in a special tightly-sealed clinical transfer box.

2.3 Isolation of PBMCs from bovine blood

Blood was processed inside the class II microbiology safety cabinet under aseptic conditions. The blood was diluted 1:1 with sterile Dulbecco's Phosphate Buffered Saline (D8537 - Sigma-Aldrich, Haverhill, UK), which is abbreviated as DPBS hereafter, then distributed into 50 ml falcon tubes. Buffy coat was collected by Pasteur pipettes and diluted again with PBS (1:1), then centrifuged at 300g for 30 minutes at 22℃, brakes off. After that, the cell suspension was layered on Histopaque-1077 (10771 Sigma-Aldrich, Haverhill, UK). This was achieved by adding 30 ml cell suspension to 15 ml Histopaque solution then centrifuged at 300g for 30 min at 22 ℃, brakes off. The mononuclear cell layer (PBMCs) was aspirated and collected by Pasteur pipette into 50 ml falcon tubes (see figure 2.3), diluted with DPBS and centrifuged at 300g for 30 minutes at 22℃, brakes off. Cells were washed with DPBS twice and centrifuged at 300g for 10 minutes at 22 ℃, brakes on. Cell pellets were covered with 5ml Erythrocyte Lysis Buffer (ELB*), incubated at 37℃ for 5 minutes to lyse all the remaining RBCs and centrifuged at 300g for 10 minutes at 22 ℃, brakes on (repeated twice until seeing a clear translucent solution/ disappearance of the red colour). To remove the ELB solution, the cell suspension was washed again twice with RPMI 1640 complete medium. RPMI 1640 complete medium (see appendix 8.1.1) consists of the following: 500 ml RPMI 1640 medium (11544526 – Fisher Scientific, Loughborough, UK) plus 10% FCS (10073772 – Fisher Scientific, Loughborough, UK) plus 1% (10,000 U/ml)

penicillin and 1 mg/mL streptomycin (15140122 – Fisher Scientific, Loughborough, UK) plus 1% L-glutamine (G7513 - SIGMA-ALDRICH, Dorset, UK) and finally 1 ml Gibco Amphotericin B (previously named as Fungizone®) as an antimycotic (15290-018 – Fisher Scientific, Loughborough, UK) at 0.25 µg/ml as a final working concentration.

Cells were counted by haemocytometer then mixed with the freeze mix (see appendix 8.1.2) solution: 90% FCS + 10% DMSO (Dimethyl sulfoxide). The final cell count was adjusted to be 1 X 10^7 cells/ml and 1ml poured into cryotubes, which were stored in a -80 ℃ freezer overnight, and finally transferred to liquid nitrogen next day to store until use.

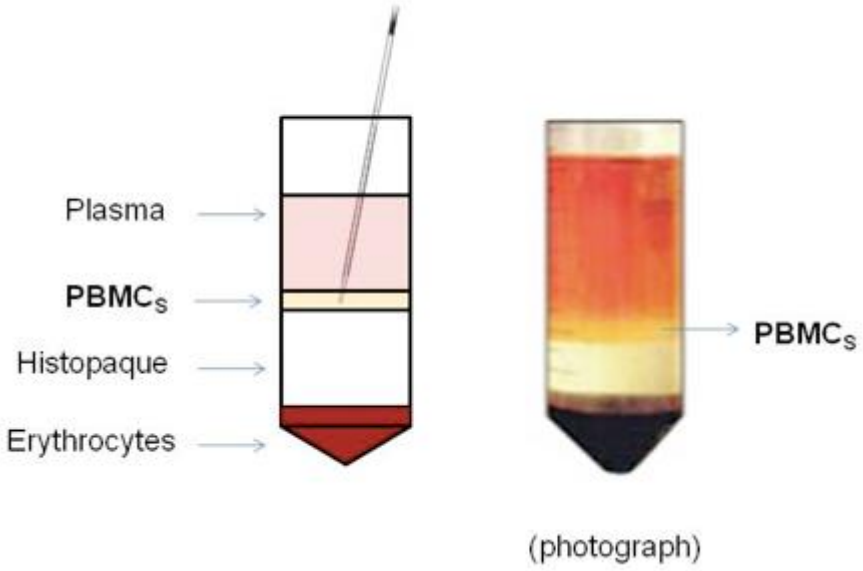


Figure 2.3: Layering buffy coat cell suspension on Histopaque 1077.

2.4 Reagents

2.4.1 IMDM Complete Medium

It was prepared as follows; Iscove's Modified Dulbecco's Medium (IMDM) (10474202 - Thermofisher Scientific, Rugby, UK). 1% 100U/mL penicillin and 50 (µg/mL) streptomycin (penstrep) made up from Pen/Strep stock (10,000 IU/ml penicillin and 10 mg/mL streptomycin at final working concentration 100 IU/mL penicillin and 100 (µg/mL) streptomycin (15140122 - Thermofisher Scientific, Loughborough, UK) plus 10% fetal calf serum (FCS) (10082139 - Thermofisher Scientific, Loughborough, UK) were added.

2.4.2 Reagents and cytokines

Concanavalin A (Con A) (C5275), phytohaemagglutinin (PHA) (L1668) and phorbol 12-myristate 13-acetate (PMA) (P1585) were all purchased from Sigma-Aldrich, Haverhill, UK. Transforming Growth Factor β1 (TGF-β1) (100-21 – Peprotech, USA), Interleukin 2 (IL-2) (202-IL-010 - R&D Systems, Abingdon, UK) and CellTrace™ Violet Cell Proliferation Kit (C34557 - Thermofisher Scientific, Rugby, UK).

2.4.3 Titration of reagents

Several titrations were done to choose the best cytokine concentration for putative rabbit Tregs and Treg target cell development. These include:

2.4.3.1 Interleukin-2 (IL-2)

Four concentrations of IL-2 were added to 1 X 10^6 MLN cells/ml in IMDM complete medium (0.1ng/ml, 1ng/ml, 10ng/ml, and 50 ng/ml) and

incubated for 4 days, when viable cells (trypan blue exclusion) were counted by haemocytometer.

2.4.3.2 Con A, PHA and PMA titration

These were titrated for optimal stimulation of MLN cells to develop putative rabbit Tregs or Treg assay T cells. For this purpose, 1 X 10^6 MLN cells/ml in IMDM medium were divided into 13 groups and treated as follows:

Group	Contents
G1	Con A 1 µg/ml
G2	Con A 5 µg/ml
G3	Con A 10 µg/ml
G4	Con A 1 µg/ml + 10 ng/ml PMA
G5	Con A 5 µg/ml + 10 ng/ml PMA
G6	Con A 10 µg/ml + 10 ng/ml PMA
G7	PHA 2 µg/ml
G8	PHA 5 µg/ml
G9	PHA 10 µg/ml
G10	PHA 2 µg/ml + 10 ng/ml PMA
G11	PHA 5 µg/ml + 10 ng/ml PMA
G12	PHA 10 µg/ml + 10 ng/ml PMA
Control	Only MLN cells without any additives

2.4.4 TLR Ligands (Agonists)

Human TLR1-9 agonist Kit (tlrl-kit1hw, InvivoGen, San Diego, USA), a commercial kit, was used to stimulate bovine PBMCs and fractionated T cell

subsets (CD4, CD8 and γδ T cells) seeded in RPMI complete medium. The kit components contained a wide range of TLR agonists synthesised from microbial components (mainly bacterial, viral and *Mycoplasma spp*) tested to stimulate human PBMCs and approved as specific stimulants (table 2.8). Instructions from the supplier were followed to inform dose response experiments to select optimally active concentrations for this study.

The concentrations of these TLR ligands used to stimulate bovine PBMCs and/or fractionated T cell subsets along with positive controls are illustrated in table 2.9.

Table 2.8: Human TLR1-9 Agonist Kit components

Agonists	Product	Working conc	Stock solution conc
TLR1/2	**Pam3CSK4**	0.1-1 µg/ml	100 µg/ml
TLR2	**HKLM**	10^8 cells/ml	10^{10} cells/ml
TLR3	**Poly(I:C)**	10 ng-10 µg/ml	1 mg/ml
TLR3	**Poly(I:C) LMW**	30ng-10 µg/ml	1 mg/ml
TLR4	**LPS**	10 ng-10 µg/ml	100 µg/ml
TLR5	**Flagellin**	10 ng-10 µg/ml	100 µg/ml
TLR6/2	**FSL-1**	1 ng-1 µg/ml	100 µg/ml
TLR7	**Imiquimod**	0.25-10 µg/ml	100 µg/ml
TLR8	**ssRNA40**	0.25-10 µg/ml	100 µg/ml
TLR9	**ODN2006**	5 µM	500 µM

Pam3CSK4 = synthetic tripalmitoylated lipopeptide, HKLM = heat-killed preparation of *Listeria monocytogenes*, Poly(I:C) = synthetic analogue of double-stranded RNA, Poly(I:C) LMW = the same but with low molecular weight, LPS = Lipopolysaccharide, FSL-1 = synthetic lipoprotein, Imiquimod = imidazoquinoline amine, ssRNA40 = 20-mer phosphothioate protected single-stranded RNA oligonucleotide, ODN2006 = synthetic oligonucleotides containing unmethylated CpG dinucleotides.

Table 2.9: TLR ligand concentrations used for stimulation of PBMCs and/or T cell subsets.

Product	Agonist	Working Conc 1 (Low) L	Working Conc 2 (Moderate) M	Working Conc 3 (High) H
Pam3CSK4	TLR1/2 Agonist	0.2 µg/ml	0.5 µg/ml	1 µg/ml
HKLM	TLR2 Agonist	1 X 10^7 cells/ml	5 X 107 cells/ml	1 X 10^8 cells/ml
Poly(I:C)	TLR3 Agonist	2.5 µg/ml	5 µg/ml	10 µg/ml
Poly(I:C) LMW	TLR3 Agonist	1 µg/ml	5 µg/ml	10 µg/ml
LPS	TLR4 Agonist	0.1 µg/ml	0.5 µg/ml	1 µg/ml
Flagellin	TLR5 Agonist	0.1 µg/ml	0.5 µg/ml	1 µg/ml
FSL-1	TLR6/2 Agonist	0.1 µg/ml	0.5 µg/ml	1 µg/ml
Imiquimod	TLR7 Agonist	0.5 µg/ml	1.5 µg/ml	2.5 µg/ml
ssRNA40	TLR8 Agonist	0.5 µg/ml	1 µg/ml	3 µg/ml
ODN2006	TLR9 Agonist	0.3 µM	1 µM	3 µM
Controls used				
Con A	Concanavalin A	Control +ve (For PBMCs & γδ T cells).		5 ug/ml
anti-bovine CD3 monoclonal antibody	CD3 *	Control +ve (for CD4 & CD8 T cells).		1 ug/ml

* CD3 = anti-bovine CD3 monoclonal antibody (WS0561B-100, Kingfisher Biotech, St. Paul, USA)

2.5 Methods specific to the putative rabbit Treg study

2.5.1 Generation of rabbit regulatory T cells

Putative rabbit Tregs were generated *in vitro*. Cryotubes of rabbit MLN cells were thawed gently in the water bath at 37°C and transferred dropwise into a 15ml falcon tubes loaded with pre-warmed IMDM complete medium (containing glutamax, 1% penstrep and 10% FCS) then spun at 300 g for 5 minutes to wash out any remaining DMSO (repeated twice). Cells were cultured in 24 well plates by seeding 2 X 10^5 MLN cells/ml in 1 ml IMDM complete culture medium for 6 days incubation at 37°C in a humidified atmosphere of 5% CO_2 in air. IL-2 (50 ng/ml), TGF-β1 (2 ng/ml) and Con A (1 µg/ml) were added to the culture as required according to the protocol. After 6 days incubation flow cytometry was done.

2.5.2 Flow cytometry detection of putative rabbit Tregs

After 6 days incubation, RPMI 1640 (61870010 - Thermofisher Scientific, Rugby, UK) was used with 2% FCS supplement to suspend cells for labelling with fluorescent-labelled antibodies: anti-CD4, anti-CD25 and anti-FOXP3 conjugated with three types of fluorochromes, Fluorescein isothiocyanate (FITC, for anti-CD4), Phycoerythrin (PE, for anti-CD25) and Allophycocyanin (APC, for anti-FOXP3) respectively (see tables 2.10 and 2.11 for antibodies used and their dilutions / working concentrations), also see appendix 8.2.

For this purpose, cells were spun at 250 x g (benchtop centrifuge) for 7 minutes and pelleted, then RPMI culture medium was added. Cells were

counted to 5 X 10^5 cells/45ul and placed in 1.5 ml eppendorf tubes. Antibodies (5ul, 1:20 diluted stock) mouse IgG2b anti-rabbit CD25 (unconjugated), 5ul (1:20 diluted) mouse IgG2b purified isotype control for CD25, 5ul mouse IgG2a anti-rabbit CD4 FITC, and 5ul mouse IgG2a CD4 isotype control-FITC were added to labelled eppendorf tubes (table 2.11) and incubated in the dark in the fridge (4 ℃) for 30 minutes. After that, cells were centrifuged at 2000 rpm for 2 minutes and washed twice with RPMI then 5ul of (1:20 diluted) goat anti-mouse IgG2b (R-PE-labelled) was added to the labelled wells and again incubated in the dark in the fridge for 30 minutes. Cells were washed twice by spinning at 2000 rpm for 2 minutes leaving 100ul supernatant.

FOXP3/Transcription Factor Staining Buffer kit (00-5523 – Thermofisher Scientific, Rugby, UK) containing cell permeabilization Buffer (00-8333 – Thermofisher Scientific, Rugby, UK) was used to make pores in putative rabbit Tregs to allow anti FOXP3 antibody penetration. The active components are formaldehyde (for fixation) and saponin (for permeabilisation). To all wells, 200 µl of eBioscience fixation/ permeabilisation working solution (supplied in the kit), was added. It was prepared by dilution of the fixation/ permeabilization concentrate in fixation/ permeabilization diluent (1 in 4), which was then added to wells and incubated for 30 minutes in the fridge, centrifuged at 2000 rpm for 2 min and supernatants discarded. 45 µl of eBioscience permeabilization buffer prepared by dilution of 10X eBioscience permeabilization buffer (supplied in kit) 1 in 10 distilled water) was added to all tubes. 5µl of rat IgG2a isotype control (FOXP3 isotype control) was added to control cell

wells and 5 µl of anti-rat/mouse FOXP3 to experimental cell wells were added then plates were incubated overnight in a fridge.

Next day, all cells were washed twice with eBioscience permeabilization buffer by centrifugation at 2000 rpm for 2 min. Cells were fixed with 2% PFA and finally suspended in 0.5 ml of PBS and shipped to the Queens Medical Centre (QMC, University of Nottingham) to be analysed in a Beckman Coulter MoFlo XDPflow cytometer for cell counting with the help of David Onion. Data were analysed by Walter & Eliza Analysis Software: Eclectic & Lucid abbreviated as WEASEL software.

Table 2.10: Antibodies used for labelling putative rabbit Tregs.

Ab	Isotype/ Clone	Primary/ secondary	Stock conc	Working conc	Cat #, supplier
Mouse IgG2a isotype control:FITC	IgG2a	Primary	0.1 mg/ml	10 µg/ml	MCA929F- BIO-RAD, Watford, UK
Mouse anti rabbit CD4:FITC	IgG2a / KEN-4	Primary	0.1 mg/ml	10 µg/ml	MCA799F - BIO-RAD, Watford, UK
Mouse IgG2b purified CD25 isotype control	IgG2a	Primary	0.1 mg/ml	2 µg/ml	MG2b00 - Thermofisher Scientific, Rugby, UK
Mouse anti rabbit CD25 (unconjugated)	IgG2b / KEI-alpha1	Primary	1 mg/ml	20 µg/ml	MCA1119GA - BIO-RAD, Watford, UK
Goat anti−Mouse IgG2b Human Adsorbed R−PE	IgG2b	Secondary	0.1 mg/ml	2 µg/ml	M32404 – Life technologies, Carlsbad, USA
Anti-Mouse/Rat FOXP3 APC	IgG2a, kappa / FJK-16s	Primary	0.2 mg/ml	20 µg/ml	17-5773-82 - Thermofisher Scientific, Rugby, UK
Rat IgG2a K Isotype Control APC	IgG2a, kappa / eBR2a	Primary	0.2 mg/ml	20 µg/ml	17-4321-41 - Thermofisher Scientific, Rugby, UK

2.5.3 Sorting of putative rabbit Tregs

Putative rabbit Tregs were generated as described above (2.5.1) and identified as being CD4, CD25, FOXP3+ cells. For cell sorting, CD4+ and CD25 high+ T cells were selected as these contained the FOXP3+ cells (as determined by flow cytometry analysis – see results). A fraction of labelled cells were not fixed with 2% PFA but kept alive and suspended in 1 ml complete RPMI medium then labelled with anti-CD4FITC and anti-CD25PE. Samples were subjected to cell sorting using the Beckman Coulter MoFlo XDP Cell Sorter equipped with 488nm and 405nm lasers to obtain forward and side scatter. Emitted fluorescence light was collected using 450/465nm band pass filter. Sorted putative rabbit Tregs (CD4+CD25hi) were gated on FITC and PE quadrants then collected into fresh RPMI complete medium to be used later in the assay.

2.5.4 Putative rabbit Treg functional assay: target cell proliferation and labelling

Cell trace violet is a DNA dye that binds to DNA as cells divide the daughter cells will have half the signal of the parent cells and so on through subsequent divisions, leading to a progressive diminution of CFSE signal.

Target cells for the putative rabbit Treg assay were autologous MLN cells that were thawed from liquid nitrogen to room temperature inside a class II microbiology safety cabinet under aseptic conditions, then cultured in IMDM complete medium (containing glutamax, 1% penstrep and 10% FCS) for 4 days in a CO_2 incubator with Con A (1 µg/ml) + PMA (10 ng/ml). The culture of these cells was co-ordinated such that the cells were ready for use as Treg assay target cells at the same time as the end of the culture

period of the generation of putative rabbit Tregs (2.5.1). For labelling with CFSE: $5X10^5$ target MLN cells/ml were labelled with 1µL of 5 mM CellTrace Violet using CellTrace™ Violet Cell Proliferation Kit (C34557 - Thermofisher Scientific, Rugby, UK) for each ml of cell suspension. Cells were incubated for 4 days at 37°C and wrapped with foil (to be protected from light) in the CO_2 incubator. A labelled sample was taken at day 0 to be a control for CFSE labelling efficiency of the cells.

2.5.5 Flow cytometry measurement of putative rabbit Treg functional assay T cells (inhibition/stimulation of proliferation by Treg dilutions).

Three concentrations of putative rabbit Tregs cells were used to suppress the proliferating CellTrace violet labelled Treg target cells (figure 2.4). These were 10^6, 5 X 10^5 and 10^5 Tregs cells/ml mixed each with 5 X 10^5 cell/ml CellTrace violet labelled target cells in 3 separate groups, while the fourth group was considered as a negative control containing only CellTrace violet labelled cells. The positive control for target cells were those treated with 5 µg/ml ConA. All groups of cells were incubated for 4 days (wrapped with foil) in a CO_2 incubator at 37°C. Samples were fixed with 2% PFA at day zero, 2 and 4 days of incubation then analysed by flow cytometry.

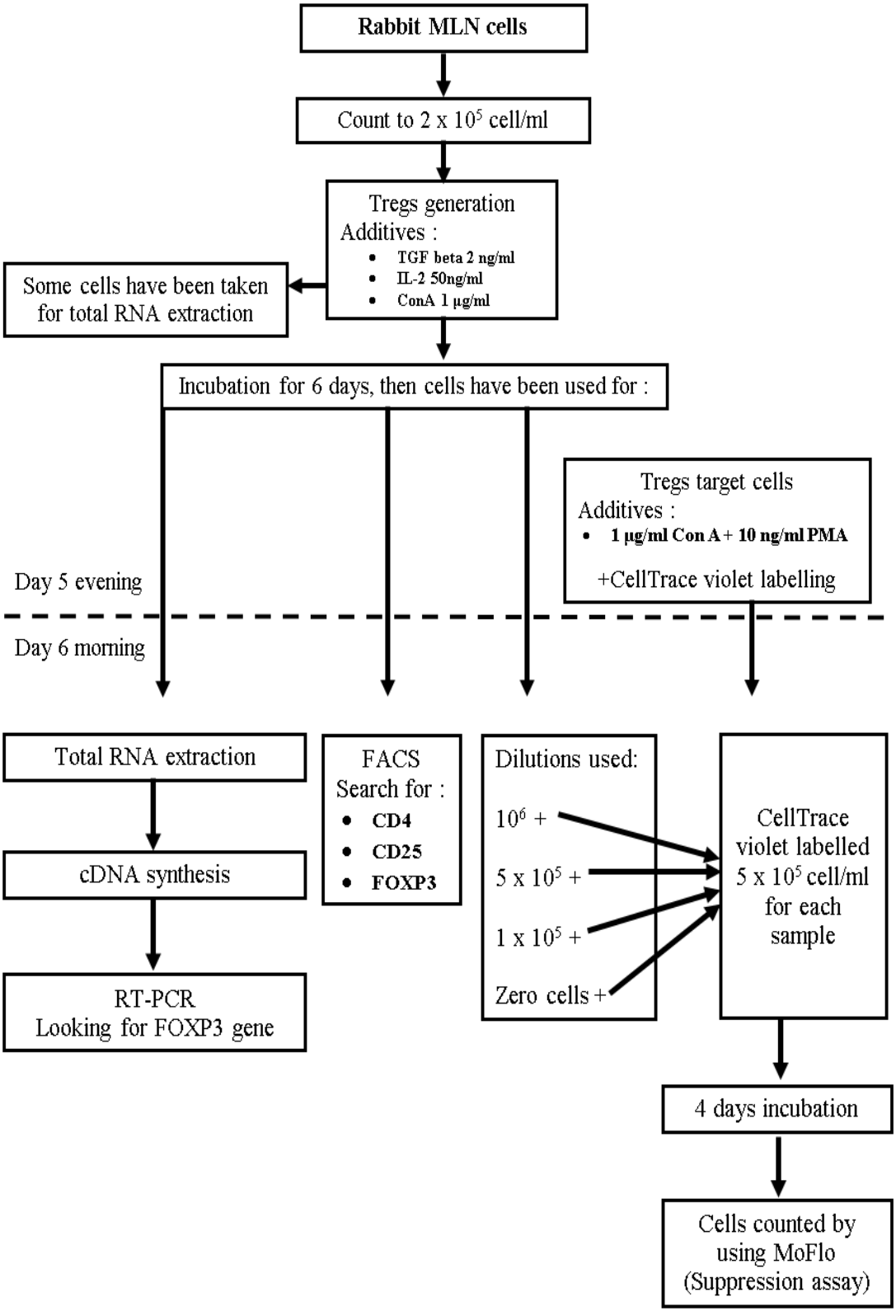


Figure 2.4: Study protocol for the generation of putative rabbit Tregs and the suppression assay.

2.6 Molecular techniques specific to the putative rabbit Treg study

2.6.1 Total RNA extraction for MLN and putative Treg cells

A portion of the putative rabbit Treg cells were kept for total RNA extraction at days 0 and 6 post-incubation. Cells were centrifuged and pelleted then counted and adjusted to $1X10^7$ cell/ml. A syringe with 21g needle was used for disruption and homogenisation in medium containing 10% β-mercaptoethanol (β-ME) to remove the activity of RNases. The RNeasy Plus Mini Kit (74136 - Qiagen, Manchester, UK) was used to extract RNA. Also, RNase-Free DNase Set (79254 - Qiagen, Manchester, UK) was used to digest any DNases. Eventually, the final concentration was measured by the Nanodrop machine (Thermofisher Scientific, UK).

2.6.2 cDNA synthesis

The Transcriptor First Strand cDNA Synthesis Kit (04897030001 – Roche, Burgess Hill, UK) was followed for 1 µg/ml cDNA preparation. Random hexamer primers were used in the protocol. The samples were loaded in a thermal block cycler machine using a program set up for 25 ℃ for 10 min, 55 ℃ for 30 min and 85 ℃ for 5 min, then held at 4 ℃.

2.6.3 SDHA (Housekeeping) gene expression

Succinate dehydrogenase (SDHA) was considered as a reference gene. Rabbit DNA from MLN cells was used as a template for DNA amplification by using a Taq PCR Kit (E5000S - New England Biolabs, Hitchin, UK) (Table

2.12). Conventional (normal) PCR on thermal block cycler machine was set to a program listed in table 2.11 for DNA amplification. The PCR product was run on a 1.5% agarose gel for 70 minutes. The band was excised out by using a sterile clean scalpel then the band was purified by using QIAquick® Gel Extraction Kit (28704 - Qiagen, Manchester, UK). The amount of DNA extracted from the gel was 16.63 ng/ul as measured by Nanodrop.

Table 2.11: PCR program for rabbit MLN DNA amplification.

PCR Phase	Cycles	Temperature & Timing
Initial Denaturation	1 x	95°C 5 mins
Denaturation	35x	94°C 30sec
Annealing	35x	60°C 30sec
Extension	35x	72°C 30 sec
Final extension	1 x	72°C 10mins
Hold	1 x	4°C hold

2.6.4 Real Time - Polymerase chain reaction (RT-qPCR)

Gene expression assays were performed by running quantitative real-time PCR (RT-qPCR). LightCycler® 480 System (Roche, Burgess Hill, UK) was used to run cDNA samples.

Quantitative real time PCR (RT-qPCR) was performed to quantify FOXP3 gene expression. SDHA (reference gene) cDNA samples were diluted (10-fold serial dilutions) and loaded into qPCR plate. Primer and hydrolysis probe sequences (TaqMan) for both target genes (FOXP3) and reference gene (SDHA) were optimised by Nevi Parameswaran. SDHA was used along

with three other reference genes: glceraldehyde-3-phosphate dehydrogenase (GAPDH), β-actin and 18SRNA. Nevi Parameswaran noted that SDHA was expressed most consistently in all samples in rabbit lymphoid samples studied. Hydrolysis probes were dual labelled with fluorescent reporter dye 6 carboxyfluorescein (FAM) at the 5' end and quencher N,N,N,N'-tetramethyl-6-carboxyrhodamine (TAMRA) at the 3' end.

The RT-qPCR 96 well white colour plate was loaded (as duplicates) with 20 ul for each well containing the final working concentration as follows; 10ul of 1x Probes Master Kit (04707494001 – Roche, Burgess Hill, UK), 3.6 ul of 900 nM primers (for each forward and reverse) 0.1 ul of 175 nM Universal Probe Library (UPL probes) and finally 0.7 ul PCR grade water (table 2.12). No Template Control (NTC, everything except the cDNA) was included. 10-fold serial dilutions of samples were applied to give a standard curve(s) for all reference and target genes which was/were measured by LightCycler® 480 software (Roche). The RT-qPCR 96 well plate was covered by a heat-resistant seal (applied manually). The typical value for a standard efficiency curve should range between 1.95 – 2.05 (Roche) and this was achieved by following absolute quantification analysis for the reference gene SDHA, while the results of target gene expression were evaluated by applying relative quantification analysis (Goni *et al*., 2009, D'haene *et al*., 2010, Liu *et al*., 2010, Mayo *et al*., 2010, Weaver *et al*., 2010, Hindson *et al*., 2011, Pinheiro *et al*., 2011, Bolha *et al*., 2012, Ji *et al*., 2012, Long *et al*., 2013a, Hartshorne *et al*., 2014, Ma and Chung, 2014). Primers and hydrolysis probe sequences used in the experiments are listed in table 2.13.

Table 2.12: RT-qPCR universal program followed for running samples (Roche).

Program name	Cycles	Temp	Duration	Analysis mode
1) Pre-incubation	1	95 ℃	10 min	None
2) Amplification	45	Varied	90 min	Quantification
a) Denaturation	45	95 ℃	10 sec	None
b) Annealing	45	60 ℃	30 sec	Data collection
c) Extension (Optional)*	45	72 ℃	1 sec	None
3) Cooling (Optional)*		4 ℃	1 min	None

* Optional = RT-qPCR program can be run without including this step which is not affecting the quality or quantity of the amplification.

Table 2.13: RT-qPCR primers and hydrolysis probes used for rabbit target and reference gene during the study*

Gene name	Forward primer (5′ – 3′)	Reverse primer (5′ – 3′)	Hydrolysis Probe (UPL) (5′ – 3′)
Target gene			
FOXP3	CTCTGCACCTTCCCAAGC	CACTTGCACACGCCATTT	UPL number 56
Reference gene			
SDHA	ACCGTGAAGGGCTCTGACT	TTTCTAGCTCGACCACAGAGG	UPL number 158

* Primers and probes were designated and normalised by Nevi Parameswaran (unpublished data).

The data obtained from running samples in a 96 RT-qPCR well plate were analysed according to Pfaffl method (formula) (Pfaffl, 2001, Pfaffl *et al*., 2002, Bustin *et al*., 2009, Taylor *et al*., 2010). Below is the Pfaffl equation used for relative quantification:

$$\text{Ratio} = \frac{(E_{target})^{\Delta C_T,\ target\ (calibrator - test)}}{(E_{ref})^{\Delta C_T,\ ref\ (calibrator - test)}}$$

The ratio of the target gene is expressed for a sample compared to a control in comparison to a reference gene. E_{target} is the real-time PCR efficiency of the target gene transcript; E_{ref} is the real-time PCR efficiency of a reference gene transcript; ΔCT_{target} is the CT deviation of control (calibrator) – sample of the target gene transcript; ΔCT_{ref} = CT deviation of control (calibrator) – sample of the reference gene transcript.

2.7 Bovine lymphocyte study

2.7.1 Cytospin and Diff Kwick staining

The frozen bovine PBMCs were thawed from liquid nitrogen with pre-warmed RPMI 1640 complete medium added dropwise, centrifuged at 300g for 10 min, supernatant was disposed of and cells re-suspended in 10 ml medium. 200ul of cell suspension was taken (containing cells that ranged between 1 X 10^5 to 2 X 10^5 cells) for cytocentrifugation at 500 x rpm for 5 minutes in the Shandon Cytospin®4 Cytocentrifuge (WD7020 - Thermofisher Scientific, Rugby, UK). The slides were dried overnight (left at room temperature) and stained with Diff Kwick stain (9990700 – Thermofisher Scientific, Rugby, UK).

Cover slips were applied, and then cells inspected under the light microscope: CTR500 Leica microscope (Leica Microsystems / Switzerland). This was used for imaging the PBMCs. LAS V3.8 software was utilised to obtain photos from the bright field (BF) area.

2.7.2 PBMC fractionation

The main components of PBMCs are monocytes and lymphocytes plus their subsets. To isolate each cell subset, Magnetic Antibody Cell Sorting (MACS) was used. The whole process involves binding a specific antibody to magnetic microbeads to add to the PBMCs so that detected cells can be attracted to the sides of the tube by a magnet.

2.7.3 MACS protocol for fractionation of CD4, CD8, and γδ T cells

The following protocol was designated by Miltenyi Biotec company to separate cells from a total of 1 X 10^7 cells, thus the numbers, volumes and concentrations could be doubled or tripled when the starting count is doubled or tripled accordingly.

The primary step involved labelling cells with purified Abs (primary Abs) (Table 2.14). For the secondary step, secondary Abs specific for the primary antibody isotypes conjugated with microbeads were used.

Table 2.14: Purified Abs used in the primary stage labelling.

Mouse anti-bovine Ab (mAb†)	**Isotype/ Clone**	**Working conc**	**Cat # (BIO-RAD, Watford, UK)**
CD4	IgG1/ CC30	20 µg/ml	MCA834GA
CD8	IgG2a/ CC63	20 µg/ml	MCA837GA
Gamma delta	IgG2a/ CC15	20 µg/ml	MCA838G

† mAb = Monoclonal Antibody

PBMCs (1 X 10^7 cells) were thawed from liquid nitrogen in pre-warmed RPMI 1640 complete medium (washed twice by spinning at 300 g for 10 min, supernatant discarded). A cell strainer was used to filter the cells to avoid having any clumps (clumps could block the columns). Cells were suspended in 95uL MACS running buffer (see appendix 8.1.3) which is made up by adding 4 ml ethylenediamintetraacetic acid (EDTA 500mM) + 5 gm bovine serum albumin (BSA) dissolved in 1 L PBS (commenced with

800 ml then finalised to 1 L). pH adjusted to 7.2 then sterile filtered and finally stored in the fridge (MACS running buffer). The cells were then mixed with 5uL purified mouse anti-bovine CD4 Ab (MCA834GA / BIO-RAD, Watford, UK) (dilution factor 1:20), and incubated in the fridge for 15 min. After that, cell suspension was spun at 300xg for 10 min, and the supernatant discarded. For the secondary step, 20uL goat anti-mouse IgG microbeads stock concentration (130-048-401 - Miltenyi Biotec, Woking, UK) + 80 uL MACS running buffer was added to the tubes and incubated in the fridge for 15 min. Cells were centrifuged at 300xg for 10 min, then the supernatant discarded and cell pellet re-suspended in 500uL Running buffer.

Magnetic separation of antibody-bound cells from others was achieved using the MidiMACS™ Separator (130-042-302 - Miltenyi Biotec, Woking, UK) inside a class II microbiology safety cabinet. LS columns (130-042-401 - Miltenyi Biotec, Woking, UK) were rinsed with 3 ml of rinsing buffer (2 ml EDTA (500mM) dissolved in 1 L PBS (started with 800 ml then topped up to 1 L and labelled as MACS rinsing buffer, see appendix 8.1.4). pH adjusted to 7.2, then sterile filtered was loaded inside the column to make sure that the column was washed. A 15-ml falcon tube was put underneath the magnet (for collection of the negative selection). The 500uL cell suspension was transferred onto the column and left dripping. 3 ml of rinsing buffer was added into the column (repeat 3 times). The column was removed from the magnet then loaded with 5 ml rinsing buffer, the plunger was applied and pressed quickly to collect the positive selected (bound) cells in a new sterile 15 ml falcon tube (positive selection = CD4+ T cells). Fractionated (CD4) cells were counted by haemocytometer.

Similar steps were followed by re-using the negative selection collected from above method to separate the other cell subsets, using (MCA837GA-BIO-RAD, Watford, UK) and (MCA838G - BIO-RAD, Watford, UK) as primary antibodies to enrich for CD8 T cells and γδ T cells respectively (see table 2.14). The purity of each of the above fractionated subsets was tested by flow cytometry which is described in the next section.

2.7.4 Flow cytometry

PBMCs or fractionated T cell subsets in RPMI 1640 (61870-010 Thermofisher Scientific, Rugby, UK) supplemented with 2% FCS were counted to 1 X 10^6 cell/45ul and placed in eppendorf tubes. A group of cells were left as non-stained controls (NS), while 5 ul of antibodies conjugated with fluorochromes was added to each tube on ice (~0℃) containing 45uL cell suspension. Cells were labelled with the Abs listed in table 2.17, also see appendix 8.3. Generally, those Abs were added at 1:100 dilutions of stock according to manufacturer's instructions. The negative controls (Mouse IgG2a negative control and Mouse IgG2b negative control) were used at 1:100 as recommended by the supplier as well as titrated and tested before use.

Table 2.15: Antibodies conjugated with fluorophores used to label bovine PBMCs.

Cell Target	Mouse anti-bovine (Ab) specific antigen	Isotype/ clone	Working Conc	Cat # / BIO-RAD, Watford, UK
Monocytes	CD11b FITC	IgG2b/ CC126	1 µg/ml	MCA1425F
B cells	CD21 FITC	IgG1/ CC21	1 µg/ml	MCA1424F
CD4 T cells	CD4 FITC	IgG2a/ CC8	1 µg/ml	MCA1653F
CD8 T cells	CD8 FITC	IgG2a/ 38.65	1 µg/ml	MCA2216F
γδ T cells*	WC1 (CC15 clone) FITC	IgG2a/ CC15	1 µg/ml	MCA838F
Mouse IgG2a Negative Control	FITC (negative control for CD4, CD8, γδ T cells & B cells)	IgG2a/ OX-34	1 µg/ml	MCA929F
Mouse IgG2b Negative Control	FITC (negative control for monocytes)	IgG2b	1 µg/ml	MCA691F

* γδ T cells = gamma delta T cells.

After mixing different antibodies (fluorochromes conjugated) with the cell suspension, the mixtures were incubated in the fridge for 30 min, wrapped with foil to avoid light exposure. After incubation, cell suspensions were centrifuged at 300g for 5 min, supernatant removed, and the pellet was topped up with 500 ul PBS and analysed by a BD FACS CANTO II flow cytometer (Becton Dickinson, Bioscience, USA).

The FACS Diva software (BD Bioscience, USA) analysis tool was used to calibrate and run the samples. Non-stained cells (NS) were used as a guide to set up the proper relevant gate(s) for the group(s) of cells of interest. Forward scatter (FSC), and side scatter (SSC) were determined according to the non-stained cell distribution and density. In general, 10,000 events were selected for cell acquisition. CellQuest pro-software (BD Bioscience, USA) was used for data analysis.

Kaluza software, an advanced software for FACS analysis, version 1.5 from Beckman Coulter / Life Sciences was utilised to analyse samples labelled with multicolour panel of fluorophores conjugated antibodies.

2.7.5 Major T cell subsets multicolour fluorophores panel

The major bovine T cell subsets were labelled within the pool of bovine PBMCs at the end of 3 days incubation and CD4, CD8, and γδ T cells were labelled (table 2.17) and also (see appendix 8.3) as follows; mouse anti bovine CD4:RPE (MCA1653PE - BIO-RAD, Watford, UK), mouse anti bovine CD8:Alexa Fluor® 647 (MCA837A647 - BIO-RAD, Watford, UK) and mouse anti bovine WC1:FITC (MCA929A647 - BIO-RAD, Watford, UK) respectively along with their isotype negative controls; mouse IgG2a negative control:RPE (MCA929PE- BIO-RAD, Watford, UK), mouse IgG2a negative control:

Alexa Fluor® 647 (MCA929A647 - BIO-RAD, Watford, UK), and mouse IgG2a negative control:FITC (MCA929F - BIO-RAD, Watford, UK) for CD4, CD8 and γδ T cells respectively.

Further to these 3 fluorophores, labelling with CellTrace™ Violet Cell Proliferation Kit (C34557 - Thermofisher Scientific, Rugby, UK) or so called CFSE stain was done by mixing PBMCs at day 0 at $1X10^6$ cells/ml with 1µL of 5 mM CellTrace Violet inside a class II microbiology safety cabinet and moved outside the hood and placed for 20 minutes in cold (4 ℃) dark environment. 4x RPMI complete medium was added to wash out excess stain (to remove the toxic effect) and incubated in a dark cold environment for 5 minutes, then spun at 300 g for 5 minutes and supernatant discarded. Cells were re-suspended in fresh RPMI complete medium and incubated for 3 days at 37°C wrapped with foil (protected from light) in a 5% CO_2 incubator. A fraction of cells was left non-stained as a control for the staining protocol. Labelled samples were harvested at the end of the incubation, washed and fixed with 2% PFA then washed and resuspended in 500ul PBS and analysed by flow cytometry.

Table 2.16: Fluorochrome-conjugated antibodies used for labelling major T cell subsets (CD4, CD8 and γδ T cells) within the pool of bovine PBMCs

Monoclonal antibody (mAb)	Stock conc	Working conc	Dilution factor	Isotype / Clone	Cat # / (BIO-RAD, Watford, UK)
MOUSE ANTI BOVINE CD4:RPE	0.1 mg/ml	1 µg/ml	1:100	IgG2a / CC8	MCA1653PE
MOUSE IgG2a NEGATIVE CONTROL:RPE	0.1 mg/ml	1 µg/ml	1:100	IgG2a / MRC OX-34	MCA929PE
MOUSE ANTI BOVINE CD8:Alexa Fluor® 647	0.05 mg/ml	0.5 µg/ml	1:100	IgG2a / CC63	MCA837A647
MOUSE IgG2a NEGATIVE CONTROL: Alexa Fluor® 647	0.05 mg/ml	0.5 µg/ml	1:100	IgG2a / MRC OX-34	MCA929A647
MOUSE ANTI BOVINE WC1:FITC	0.1 mg/ml	1 µg/ml	1:100	IgG2a / CC15	MCA838F
MOUSE IgG2a NEGATIVE CONTROL:FITC	0.1 mg/ml	1 µg/ml	1:100	IgG2a / MRC OX-34	MCA929F

2.8 Molecular techniques specific to Bovine lymphocyte study

2.8.1 Total RNA extraction

RNeasy plus mini kit (74134 – Qiagen, Manchester, UK) was used for total RNA extraction. This is a highly efficient, sensitive protocol for low amounts, and phenol-free method for RNA extraction with which up to 100 µg total RNA can be obtained for sensitive applications such as quantitative real-time PCR (Hanoux *et al*., 2007). Generally, 1 X 10^7 PBMCs (or any other cell type) were initially mixed with 600 ul of RLT buffer (cell lysis stage) and gently vortexed for 30 seconds (medium speed vortexing until clear translucent solution formed). A syringe with 21g needle was used for disruption and homogenisation in RLT buffer containing 10% β-mercaptoethanol (β-ME) to remove the activity of RNases. The lysates were transferred into a gDNA eliminator spin column (for genomic DNA removal) put on a 2-ml collection tube and spun at 8000 g for 30 seconds. The column was disposed of and the flow-through was collected and topped up by an equal volume of 70% ethanol, then pipetted up and down gently. 700 ul of the mixture was added to a RNeasy spin column which was spun at 8000 g for 15 seconds. The flow-through was discarded and this step was repeated with the rest volume of RNA- alcohol mixture. After that, 350 ul of RWT solution (washing step) was added to the spin column and spun for 15 seconds at 8000 g. To digest the genomic DNA, 10 ul DNase I stock was mixed thoroughly and carefully with 70 ul Buffer RDD (both supplied in RNase-Free DNase Set 79254 – Qiagen, Manchester, UK) and loaded to the spin column then incubated for 30 minutes in the fridge then spun at 8000 g for 15 seconds. 350 ul of RWT was loaded to the spin column (washing step) and spun at 8000 g for 15 seconds. 500 ul

of RPE buffer was added to the spin column (to remove any undesired chemical or protein precipitates) then was spun at 8000 g for 15 seconds. The RPE washing step was repeated again but for 2 minutes. The spin column was put over 1.5 eppendorf tube and loaded with 40 ul RNase-free water for final elution which was spun at 8000 g for 60 seconds. All centrifugations were performed by using benchtop centrifuge.

2.8.2 RNA quantification (Nanodrop®8000)

The Nanodrop®8000 machine (Thermofisher Scientific, UK) was used to measure the quality and quantity of RNA. It is basically a spectrophotometer that uses a UV light then absorbs the refracted beam light (from 220 to 750 nanometre wavelength). DNA (single and double stranded) as well as RNA samples can be measured at 260 nm wavelength. The machine is blanked with 1 ul PCR grade water, then the sample was loaded and measured accordingly. Up to 8 samples can be loaded at once. After measuring the concentration, RNA samples were stored in a -80 ℃ freezer.

2.8.3 Complementary DNA synthesis

Complementary DNA (cDNA) was synthesised by following the Roche kit instructions: Transcriptor First Strand cDNA Synthesis Kit (04897030001 - Roche, Burgess Hill, UK) was followed to make cDNA at 1 ug/ul. Random hexamer primers were used in the protocol. Ice was used in this protocol to ensure cold environment. The components added are listed in table 2.17.

Table 2.17: cDNA synthesis kit reagents (www.roche-applied-science.com).

Component	Final conc.	Volume
Random hexamer primer	60 uM	2 µl
Transcriptor Reverse Transcriptase Reaction Buffer	1x (8mM $MgCl_2$)	4 ul
Protector RNase Inhibitor	20 U	0.5 ul
Deoxynucleotide Mix	1 mM	2 ul
Transcriptor Reverse Transcriptase	10 U	0.5 ul
Total volume	-	9 ul
RNA volume (with or without water)	Varied	11 ul
Final working volume	-	20 ul

Sterile 0.2 ml nuclease-free, thin-walled PCR tubes were used which were loaded into a thermal block cycler machine (Alpha laboratories) using a program set up for 25 ℃ for 10 min, 55 ℃ for 30 min and 85 ℃ for 5 min, then held at 4 ℃. cDNA samples were stored in the freezer at -20 ℃.

2.8.4 Polymerase chain reaction (PCR)

2.8.4.1 Conventional PCR

Bovine PBMCs were subjected to RNA extraction and cDNA synthesis according to the above protocols. cDNA was used as a template to amplify DNA by using the Taq PCR Kit (E5000S - New England Biolabs, Hitchin, UK). Company instructions were followed as recommended. All kit components (except the enzyme "Taq") were thawed and placed on ice. Sterile 0.2 ml nuclease-free, thin-walled PCR tubes were used for this purpose. All the components were added inside PCR cabinets except the DNA which was added

outside the cabinet. A thermal block cycler (Alpha laboratories) was used to run the program. The components for each reaction and the cycling conditions are described in tables 2.18 and 2.19.

Table 2.18: PCR components for bovine cDNA amplification.

PCR materials	For 1x reaction
Nuclease free water	19.85 ul
10x buffer	2.5 ul
dNTPs (10mM)	0.5 ul
10mM Forward Primer (10 pmol/ul)	0.5 ul
10mM Reverse Primer (10 pmol/ul)	0.5 ul
DNA	1 ul
Taq (5u/ul)	0.15 ul
Total volume	25 ul

Table 2.19: PCR program for bovine cDNA amplification.

PCR Phase	Cycles	Temperature & Timing
Initial Denaturation	1 x	95°C 2 mins
Denaturation	30x	94°C 30sec
Annealing	30x	60°C 30sec
Extension	30x	72°C 1 min
Final extension	1 x	72°C 5 mins
Hold	1 x	4°C hold

2.8.4.2 Gel electrophoresis

The amplicon resulting from PCR amplification was run on 2% gel electrophoresis (small size amplicon products require higher gel concentration), 5 ul of Nancy-520 solution (01494 - Sigma-Aldrich, Haverhill, UK) was added as a visualising agent and as a safer alternative DNA stain to ethidium bromide. The comb from the set gel was removed to load the samples. This included 1 ul of 6X gel loading dye (N3233S - New England Biolabs, Hitchin, UK) mixed with 5 ul DNA product (amplicon) on parafilm then 5 ul loaded into the well. 5 ul from Low molecular weight DNA ladder (N3233S - New England Biolabs, Hitchin, UK) was mixed with 1 ul 6X gel loading dye, then 5 ul was loaded to each terminal well (ladder). Tris-acetate-EDTA buffer (TAE Buffer) working solution of 40 mM Tris acetate (pH 8.5) which was (1X TAE) simply made of as a 50X TAE stock solution (see appendix 8.1.5) by dissolving 242gm Tris free base and 18.61 gm Disodium EDTA in 57.1 ml Glacial acetic acid all mixed with 700 ml double ionised distilled water then stirred gently and topped up to 1 L. To make up 1 L from 1X TAE buffer (see appendix 8.1.6), 20 ml 50X TAE was aspirated and topped up with double ionised distilled water to 1 L. The 1x TAE solution is 40mM Tris, 20mM Acetate and 1mM EDTA, pH approximately 8.5. The settings for the electrophoresis apparatus were 90V, 400 Amp for 90 min. The bands were viewed by the UV illuminator (63005650 - ImageQuant 300 Imager (Amersham Bioscience, Part of GE Healthcare, UK).

2.8.4.3 Quantitative real-time PCR (RT-qPCR)

Gene expression assays were performed by running quantitative real-time PCR (RT-qPCR). The apparatus LightCycler® 480 System (Roche Applied Science, UK) was used to run the samples (cDNA samples) (Table 2.20).

Primers and hydrolysis probe sequences (TaqMan) for both target genes (TLRs 1-10) and three reference genes; Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), Ribosomal protein, large, P0 (RPLP0) and Ribosomal protein, large, P2 (RPLP2) were taken from previously published work which were used by other researchers (Werling *et al*., 2006, Gibson *et al*., 2012, Russell, 2012). Primers and probes were blasted online (Zhang *et al*., 1997). Both primers and hydrolysis probes were ordered from Sigma Aldrich. Hydrolysis probes were labelled (dual labelled) with fluorescent reporter dye 6 carboxyfluorescein (FAM) at the 5’ end and quencher N,N,N,N’-tetramethyl-6-carboxyrhodamine (TAMRA) at the 3’ end.

The RT-qPCR 96 well white colour plate was loaded (as duplicates) with 20 ul for each well containing the final working concentration as follows; 10ul of 1x Probes Master Kit (04707494001 - Roche Life Science, UK), 3.6 ul of 900 nM primers (for each forward and reverse) 0.7 ul of 175 nM non-UPL probes and finally 0.7 ul PCR grade water (table 2.22). No Template Control (NTC) was included. 10-fold serial dilutions of samples were applied to give a standard curve(s) for all reference and target genes which were measured by LightCycler® 480 software (Roche). The RT-qPCR 96 well plate was covered by a heat-resistant seal (applied manually). The typical value for a standard efficiency curve should be ranged between 1.95 – 2.05 (Roche) and this was achieved by following absolute quantification analysis while the results of target gene expression were evaluated by applying relative quantification

analysis (Goni *et al*., 2009, D'haene *et al*., 2010, Liu *et al*., 2010, Mayo *et al*., 2010, Weaver *et al*., 2010, Hindson *et al*., 2011, Pinheiro *et al*., 2011, Bolha *et al*., 2012, Ji *et al*., 2012, Long *et al*., 2013b, Hartshorne *et al*., 2014, Ma and Chung, 2014). Primers and hydrolysis probe sequences used in the experiments are listed in table 2.21

Table 2.20 RT-qPCR universal program followed for running samples (Roche).

Program name	Cycles	Temp	Duration	Analysis mode
1) Pre-incubation	1	95 ℃	10 min	None
2) Amplification	45	Varied	90 min	Quantification
a) Denaturation	45	95 ℃	10 sec	None
b) Annealing	45	60 ℃	30 sec	Data collection
c) Extension (Optional)*	45	72 ℃	1 sec	None
3) Cooling (Optional)*		4 ℃	1 min	None

* Optional = RT-qPCR program can be run without including this step which is not affecting the quality or quantity of the amplification.

Table 2.21: RT-qPCR primers and hydrolysis probes used for bovine target and reference genes during the study.

Gene name	Forward primer (5′ – 3′)	Reverse primer (5′ – 3′)	Hydrolysis Probe (non-UPL) (5′ – 3′)
Target genes			
BoTLR1	GCACCACAGTGAGTCTGGAA	GTACGCCAAACCAACTGGAG	TGTGTGCTTGATGATAATGGGTGTCCT
BoTLR2	ACGACGCCTTCGTGTCCTAC	GCTCCTGGACCATGAGGTTC	CGAGCGGGATTCCTACTGGGTGG
BoTLR3	AAAGAGTTCTCTCCTGGGTGTT	TGCTCAGGGACAGATTCTCA	CAATGCCAAGCTGAGCCCCA
BoTLR4	TGGAGGACATGCCAGTGCT	CACCGACACACTGATGATCGT	AGTTTCAGGAACGCCACTTGTCAGCTG
BoTLR5	CTAGACCTGGGTGGAAGTCAG	AGGGATGAAGGTAAAGACTCTGAA	TTCCTGTGGTCTCTCCGATGCTG
BoTLR6	CCTGCCCATCTGTAAGGAAT	TAGGTGCAAGTGAGCAATGG	TTGGCAACTTGACCCAACTGAATTTC
BoTLR7	GCTGAAGACTGTCCCTGAGA	TTTGAGCTGAGGTCCAGATG	TCCAACTGTTCCCGCAGCCTC
BoTLR8	TCCACATTTGAAACGAAGACC	ACATCGGTCAGTCTGGGAAC	CCTGACGTTCAGATTTCTGTCCATC
BoTLR9	CACCATCTTCAACGACCTGA	CTTCTCCAGGGACACCAGAC	TCCTTCGCCCACCTGCACCT
BoTLR10	TGGTTGGATGGTCAGATTCA	CAGGGCAAATCAAAGTGGA	CCATTGTTGTCATGCTCGTTCT
Reference genes			
BoGAPDH	CATGTTCCAGTATGATGATTC	GAGCTTCCCGTTCTCTGC	CGGCAAGTTCAACGGCAC
BoRPLP0	CTGATTACACCTTCCCACTTGCT	AGCCACAAATGCAGATGGATCA	AAGGCCTTGACCTTTTC
BoRPLP2	TCAACAAGGTCATCAGTGAGC	CCGATACCCTGAGCAATGA	CGTCCTCGATGTTCTTTCCGTGG

2.8.4.4 RT-qPCR method for TLR quantification

RNA was extracted then reverse transcribed into cDNA which was synthesised accordingly then analysed using ($2^{-\Delta\Delta CT}$) equation.

2.8.4.5 Normalisation of bovine reference genes

Although published primers and hydrolysis probes were used, it was worth confirming that they were able to amplify the genes of interest producing a product represented by a band of nucleic acid on a gel. Thus, bovine reference genes (primers only were used but not the hydrolysis probes) were validated by running PBMC cDNA samples in the Thermal block cycler (Alpha laboratories) following the conventional PCR protocol mentioned above. Annealing temperatures were manipulated starting from 60 ℃ downward to 58°C to find out the best conditions for the selected primers (both forward and reverse). The Taq PCR Kit (E5000S / New England Biolabs, Hitchin, UK) was used for this purpose following the instructions in "Taq DNA Polymerase Guidelines for PCR Optimization" published by New England Biolabs Inc., UK on their website. Bands on the gels were of the expected size for the reference genes. These were not checked by sequencing though.

2.9 Cytokine measurements (ELISA)

Cytokines were measured by collecting the supernatants which were subjected to Enzyme Linked Immuno-Sorbent Assay (ELISA). All manufacturers' instructions for ELISA protocols were followed. In total, 7 cytokines were assayed by purchasing commercial ELISA kits. These were Bovine Interleukin 8 ELISA kit (DIY1028B-003, Kingfisher Biotech Inc, St. Paul, USA), Bovine IFN-αA ELISA kit (DIY0663B-003, Kingfisher Biotech Inc,

St. Paul, USA), Bovine TNF-α ELISA kit (DIY0675B-003, Kingfisher Biotech Inc, St. Paul, USA) and Bovine IFN-γ ELISA development kit (3119-1H-6 / Mabtech, Cincinnati, USA), (see appendix 8.4).

Both IL-4 and IL-10 ELISA kits were purchased as "Matched Antibody Pairs" from BIO-RAD supplier. For IL-4, mouse anti bovine interleukin-4 (MCA2371, BIO-RAD, Watford, UK) was used as a capture Ab, while mouse anti bovine interleukin-4:Biotin (MCA2372B, BIO-RAD, Watford, UK) was used as a detection Ab. For IL-10, mouse anti bovine interleukin-10 (MCA2110, BIO-RAD, Watford, UK) was used as a capture Ab, while mouse anti bovine interleukin-10:Biotin (MCA2111B, BIO-RAD, Watford, UK) was used as a detection Ab. Finally, a TGFβ ELISA kit (ABIN996501, Antibodies-online, Aachen, Germany) was used to assay bovine TGF-beta.

2.9.1 Generic materials and common steps

- ELISA washing buffer and washing procedure: the whole plate was washed 3 times with washing buffer which was made of 0.05% Tween™20 in PBS (pH 7.2). Fourth wash was done with PBS only. After each wash, the plate was dried off by gentle blotting against tissue paper.
- ELISA Blocking buffer and procedure: all the wells were blocked by adding 300 ul of blocking buffer which was made of 1% bovine serum albumin (BSA) in PBS (pH 7.2). Blocking buffer was prepared by weighing out 1 gm BSA dissolved in 800 ml sterile fresh PBS, then the volume was topped up to 1 L and sterile filtered with 0.22 µm millipore filter. Blocking duration was set for 1 hour.

- ELISA coating buffer consisted of 1.5 g Sodium carbonate (Na_2CO_3) plus 2.93 g Sodium hydrogen carbonate ($NaHCO_3$) in 800 ml DW, then topped up to I L and pH adjusted to 9.6.
- ELISA 96 micro-well plate (475094 - Thermofisher Scientific, Rugby, UK).

2.9.2 IL-8 (CXCL-8)

To detect IL-8 in the supernatants, Bovine IL-8 ELISA kit (DIY1028B-003, Kingfisher Biotech Inc, St. Paul, USA) includes capture Ab, standard protein and detection Ab. Sandwich ELISA method was used.

Briefly, samples and reagents were warmed up to room temperature before use. All wells in ELISA plate were loaded with 100 ul of capture Ab (Anti-Swine IL-8 Polyclonal Antibody, PB0143S-100, Kingfisher Biotech Inc, St. Paul, USA) in fresh sterile PBS at 2ug/ml, covered with plate sealer and incubated overnight at 25 ℃.

The plate was washed by ELISA washing buffer (see section 2.9.1) and blocked by ELISA Blocking buffer (see section 2.9.1). 50 ul of samples were added in duplicates along with 50 ul of standard protein (Recombinant Bovine IL-8, RP0023B-005, Kingfisher Biotech Inc, St. Paul, USA), the standard protein (STD), dissolved in complete RPMI medium diluted as two-fold serial dilutions from 25 ng/ml down to 97.65 pg/ml whereas 2 wells were not loaded with STD where only complete RPMI medium was added.

Two more wells were loaded with PBS only (blank). The plate was sealed and incubated at 25 ℃ for 2 hours. The plate was washed with ELISA washing buffer (see section 2.9.1). Detection Ab (Biotinylated Anti-Swine IL-8 Polyclonal Antibody, PBB0266S-050, Kingfisher Biotech Inc, St. Paul, USA) was added at 0.3 ug/ml in blocking buffer (1%BSA) and loaded at 100 ul per well each then the plate was sealed and incubated at 25 ℃ for 1 hour. The plate was washed with ELISA washing buffer (see section 2.9.1).

50 ul of streptavidin-horseradish peroxidase (SA-HRP), the enzyme (AR0068-001, Kingfisher Biotech Inc, St. Paul, USA) was loaded as 1:25 diluted in blocking buffer (1%BSA) then sealed and wrapped with foil (protected from

light) and incubated at 25 ℃ for 30 minutes. The plate was washed with ELISA washing buffer (see section 2.9.1).

100 ul of ELISA Substrate 3,3',5,5'-tetramethylbenzidine (TMB) (DY999, R & D Systems, Minneapolis, USA) was added to each well by mixing equal volumes from pre-warmed vials A and B. These are stabilized hydrogen peroxide (895000, R & D Systems, Minneapolis, USA) for colour reagent A and stabilized tetramethylbenzidine, (895001, R & D Systems, Minneapolis, USA) for colour Reagent B. The plate was not sealed and incubated in the dark at 25 ℃ for up to 20 minutes. Once blue colour developed, all the wells were topped up with 100 ul of 0.18M sulphuric acid (H_2SO_4) as a stop solution changing the colour from blue to yellow.

Labtech spectrophotometer, a micro-plate reader, (LT-4000, Labtech, UK) was used to measure the absorbance (Optical Density which is abbreviated as "OD" values) at 450nm and 540nm wavelengths. OD values of all the samples were subtracted to omit background signal (corrected) as follows (Mean OD samples at 450nm –mean OD at 540nm). A standard curve line was plotted using recombinant bovine IL-8 (Figure 2.5). GraphPad Prism version 7 was used to interpolate the OD values into concentrations illustrated as ng/ml.

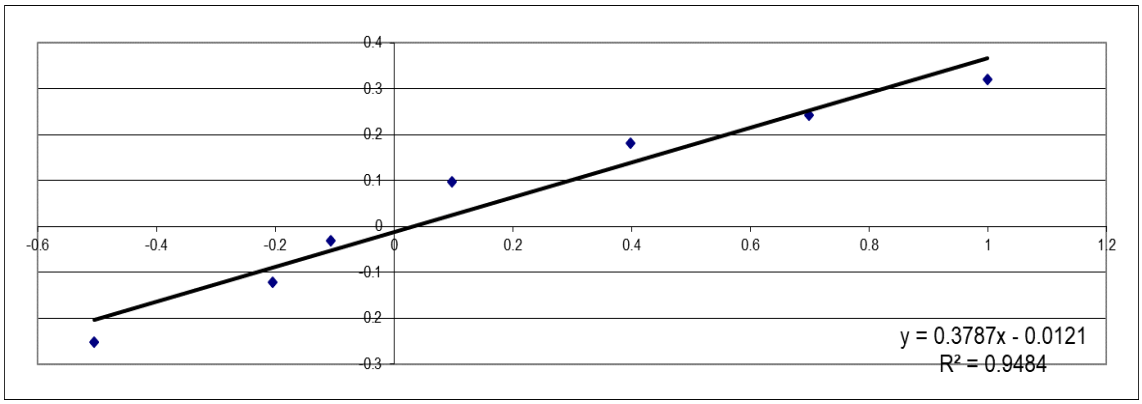


Figure 2.5: ELISA standard curve for Bovine IL-8. Mean OD values were determined at 2-fold serial dilutions of Recombinant Bovine IL-8 from 25 ng/ml down to 97.65 pg/ml concentrations. To generate the straight line, The X axis represents the log10 transformation of the standard concentrations expressed as a geometric mean and the Y axis the log10 of the O.D. expressed as a geometric mean. To get the sample concentrations the values are calculated from the standard curve (in Excel) and reverse transformed (backtransformed) to give a value in ng/ml or pg/ml.

2.9.3 IFNα

Bovine IFN-αA ELISA kit (DIY0663B-003, Kingfisher Biotech Inc, St. Paul, USA) was used to measure IFN-αA in the supernatants. Sandwich ELISA method was used. Briefly, samples and reagents were warmed up to room temperature before use. All wells in ELISA plate were loaded with 100 ul of capture Ab (Anti-Bovine IFN-αA Polyclonal Antibody, PB0474B-100, Kingfisher Biotech Inc, St. Paul, USA) in fresh sterile PBS at 1.6ug/ml, covered with plate sealer and incubated overnight at 25 ℃. The plate was washed by ELISA washing buffer (see section 2.9.1) and blocked by ELISA Blocking buffer (see section 2.9.1). 50 ul of samples were added in duplicates along with 50 ul of standard protein (Recombinant Bovine IFN-αA, RP0008B-005, Kingfisher Biotech Inc, St. Paul, USA), the standard protein (STD), dissolved in complete RPMI medium diluted as two-fold serial dilutions from 10 ng/ml down to 78.12 pg/ml whereas 2 wells were not loaded with STD

where only complete RPMI medium added. Two more wells were loaded with PBS only (blank). The plate was sealed and incubated at 25 ℃ for 2 hours. The plate was washed with ELISA washing buffer (see section 2.9.1). Detection Ab (Biotinylated Anti-Bovine IFN-αA Polyclonal Antibody, PBB0483B-050, Kingfisher Biotech Inc, St. Paul, USA) was added at 0.3 ug/ml in blocking buffer (1%BSA) and loaded at 100 ul per well each then the plate was sealed and incubated at 25 ℃ for 1 hour. The plate was washed with ELISA washing buffer (see section 2.9.1). 50 ul of SA-HRP, the enzyme (AR0068-001, Kingfisher Biotech Inc, St. Paul, USA), was loaded as 1:25 diluted in blocking buffer (1%BSA) then sealed and wrapped with foil (protected from light) and incubated at 25 ℃ for 30 minutes. The plate was washed with ELISA washing buffer (see section 2.9.1). 100 ul of ELISA Substrate 3,3',5,5'-tetramethylbenzidine (TMB) (DY999, R & D Systems, Minneapolis, USA) was added to each well by mixing equal volumes from pre-warmed vials A and B. The plate was not sealed and incubated in the dark at 25 ℃ for up to 15 minutes. Once blue colour appropriately developed, all the wells were topped up with 100 ul of 0.18M sulphuric acid (H_2SO_4) as a stop solution changing the colour from blue to yellow. Labtech spectrophotometer, a micro-plate reader, (LT-4000, Labtech, UK) was used to measure the absorbance (Optical Density which is abbreviated as "OD" values) at 450nm and 540nm wavelengths. OD values of the all samples were subtracted to omit background signal (corrected) as follows (Mean OD samples at 450nm– mean OD at 540nm). A standard curve line was plotted using recombinant bovine IFN-αA (Figure 2.6). GraphPad Prism version 7 was used to interpolate the OD values into concentrations illustrated as ng/ml.

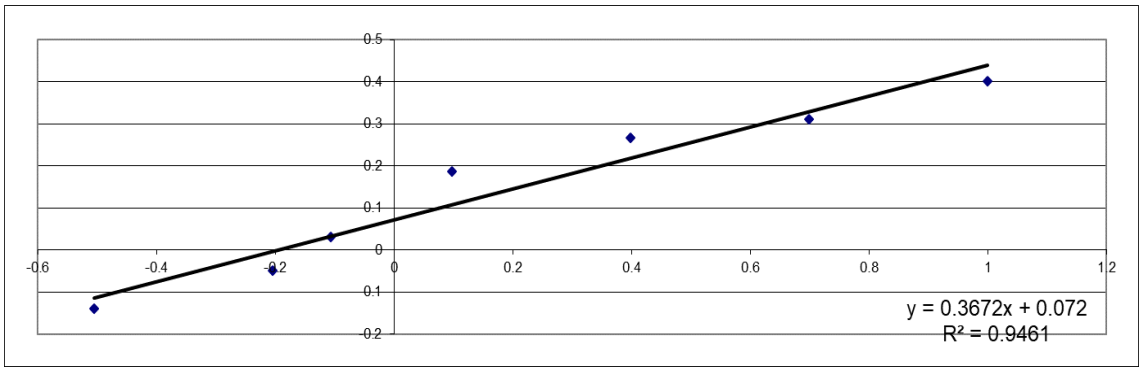


Figure 2.6: ELISA standard curve for Bovine IFNα. Mean OD values were determined at 2-fold serial dilutions of Recombinant Bovine IFN-αA from 10 ng/ml down to 78.12 pg/ml concentrations. To generate the straight line, The X axis represents the log10 transformation of the standard concentrations expressed as a geometric mean and the Y axis the log10 of the O.D. expressed as a geometric mean. To get the sample concentrations the values are calculated from the standard curve (in Excel) and reverse transformed (backtransformed) to give a value in ng/ml or pg/ml.

2.9.4 TNF-α

Bovine TNF-α ELISA kit (DIY0675B-003, Kingfisher Biotech Inc, St. Paul, USA) was used to measure TNF-α in the supernatants. Sandwich ELISA method was used. Briefly, samples and reagents were warmed up to room temperature before use. All wells in ELISA plate were loaded with 100 ul of capture Ab (Anti-Bovine TNF-α Polyclonal Antibody, PB0275B-100, Kingfisher Biotech Inc, St. Paul, USA) in fresh sterile PBS at 1.8ug/ml, covered with plate sealer and incubated overnight at 25 ℃. The plate was washed by ELISA washing buffer (see section 2.9.1) and blocked by ELISA Blocking buffer (see section 2.9.1). 50 ul of samples were added in duplicates along with 50 ul of standard protein (Recombinant Bovine TNF-α, RP0055B-005, Kingfisher Biotech Inc, St. Paul, USA), the standard protein (STD), dissolved in complete RPMI medium diluted as two-fold serial dilutions from 10 ng/ml down to 78.12 pg/ml whereas 2 wells were not loaded with STD where only complete RPMI

medium added. Two more wells were loaded with PBS only (blank). The plate was sealed and incubated at 25 ℃ for 2 hours. The plate was washed with ELISA washing buffer (see section 2.9.1). Detection Ab (Biotinylated Anti-Bovine TNF-α Polyclonal Antibody, PBB0278B-050, Kingfisher Biotech Inc, St. Paul, USA) was added at 0.3 ug/ml in blocking buffer (1%BSA) and loaded at 100 ul per well each then the plate was sealed and incubated at 25 ℃ for 1 hour. The plate was washed with ELISA washing buffer (see section 2.9.1). 50 ul of SA-HRP, the enzyme (AR0068-001, Kingfisher Biotech Inc, St. Paul, USA) was loaded as 1:25 diluted in blocking buffer (1%BSA) then sealed and wrapped with foil (protected from light) and incubated at 25 ℃ for 30 minutes. The plate was washed with ELISA washing buffer (see section 2.9.1). 100 ul of ELISA Substrate TMB Core+ (BUF062, BIO-RAD, Watford, UK). The plate was not sealed and incubated in the dark at 25 ℃ for up to 15 minutes. Once blue colour appropriately developed, all the wells were topped up with 100 ul of 0.18M sulphuric acid as a stop solution changing the colour from blue to yellow. Labtech spectrophotometer, a micro-plate reader, (LT-4000, Labtech, UK) was used to measure the absorbance (Optical Density which is abbreviated as "OD" values) at 450nm and 540nm wavelengths. OD values of the all samples were subtracted to omit background signal (corrected) as follows (mean OD samples at 450nm –mean OD at 540nm). A standard curve line was plotted using recombinant bovine TNF-α (Figure 2.7). GraphPad Prism version 7 was used to interpolate the OD values into concentrations illustrated as ng/ml.

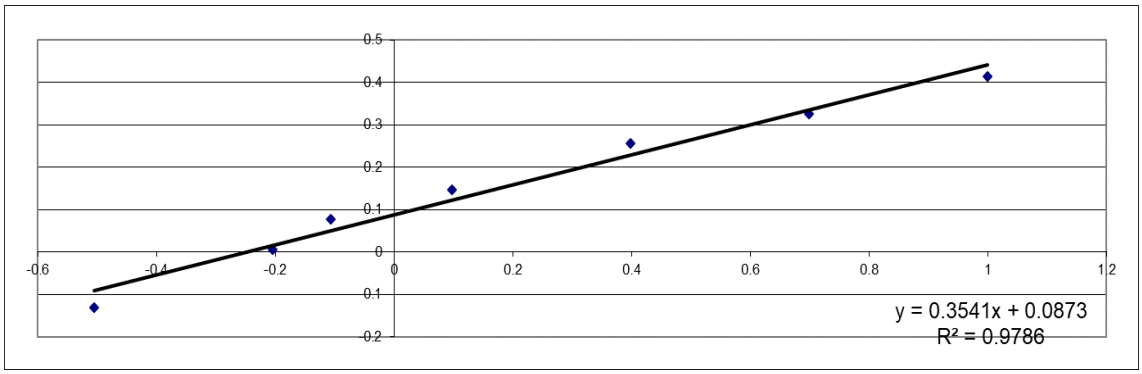


Figure 2.7: ELISA standard curve for Bovine TNFα. Mean OD values were determined at 2-fold serial dilutions of Recombinant Bovine IFN-αA from 10 ng/ml down to 78.12 pg/ml concentrations. To generate the straight line, The X axis represents the log10 transformation of the standard concentrations expressed as a geometric mean and the Y axis the log10 of the O.D. expressed as a geometric mean. To get the sample concentrations the values are calculated from the standard curve (in Excel) and reverse transformed (backtransformed) to give a value in ng/ml or pg/ml.

2.9.5 IL-4

In order to measure bovine IL-4 cytokine in the supernatant, Matched Antibody Pairs from BIO-RAD was used. Sandwich ELISA method was used. ELISA plate was loaded with 100 ul of ELISA coating buffer (see section 2.9.1) containing 3ug/ml mouse anti bovine interleukin-4 (MCA2371, BIO-RAD, Watford, UK) which was used as a capture Ab. The plate was sealed and incubated in the fridge overnight. The plate was washed by ELISA washing buffer (see section 2.9.1) and blocked by ELISA Blocking buffer (see section 2.9.1). 50 ul of samples were added in duplicates along with 50 ul of standard protein (recombinant bovine interleukin-4, PBP010, BIO-RAD, Watford, UK), the standard protein (STD), dissolved in ELISA washing buffer diluted as two-fold serial dilutions from 10 ng/ml down to 78.12 pg/ml whereas 2 wells were loaded with zero conc of STD. Two more wells were loaded with PBS only (blank). The plate was sealed and incubated at 37 ℃ for 2 hours. The plate was washed with ELISA washing buffer (see section 2.9.1). 100 ul of 1 ug/ml

mouse anti bovine interleukin-4:Biotin (MCA2372B, BIO-RAD, Watford, UK) was used as a detection Ab in ELISA washing buffer then the plate was sealed and incubated at 37 ℃ for 1 hour. The plate was washed with ELISA washing buffer (see section 2.9.1). 50 ul of SA-HRP, the enzyme (AR0068-001, Kingfisher Biotech Inc, St. Paul, USA) was loaded as 1:25 diluted in ELISA washing buffer then sealed and wrapped with foil (protected from light) and incubated at 37 ℃ for 60 minutes. The plate was washed with ELISA washing buffer (see section 2.9.1). 100 ul of ELISA Substrate TMB Core+ (BUF062, BIO-RAD, Watford, UK) was added. The plate was not sealed and incubated in the dark at 25 ℃ for up to 25 minutes. Once blue colour appropriately developed, all the wells were topped up with 100 ul of 0.2M sulphuric acid as a stop solution. Labtech spectrophotometer, a micro-plate reader, (LT-4000, Labtech, UK) was used to measure the absorbance at 450nm wavelength. OD values of all the samples were blank subtracted to omit background signal (corrected) as follows (mean OD samples at 450nm –mean OD blank). A standard curve line was plotted using recombinant bovine interleukin-4 (Figure 2.8). GraphPad Prism version 7 was used to interpolate the OD values into concentrations illustrated as ng/ml.

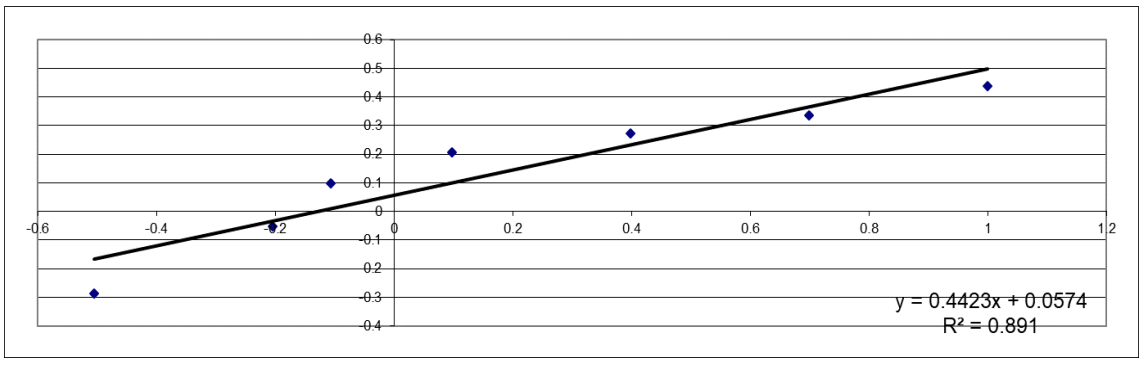


Figure 2.8: ELISA standard curve for Bovine IL-4. Mean OD values were determined at 2-fold serial dilutions of Recombinant Bovine IL-4 from 10 ng/ml down to 78.12 pg/ml concentrations. To generate the straight line, The X axis represents the log10 transformation of the standard concentrations expressed as a geometric mean and the Y axis the log10 of the O.D. expressed as a geometric mean. To get the sample concentrations

the values are calculated from the standard curve (in Excel) and reverse transformed (backtransformed) to give a value in ng/ml or pg/ml.

2.9.6 IL-10

Bovine IL-10 cytokine was measured in the supernatant by purchasing Matched Antibody Pairs from BIO-RAD. A Sandwich ELISA method was used. ELISA plate was loaded with 100 ul of ELISA coating buffer (see section 2.9.1) containing 3ug/ml mouse anti bovine interleukin-10 (MCA2110, BIO-RAD, Watford, UK) which was used as a capture Ab. The plate was sealed and incubated in the fridge overnight. The plate was washed by ELISA washing buffer (see section 2.9.1) and blocked by ELISA Blocking buffer (see section 2.9.1). 50 ul of samples were added in duplicates along with 50 ul of standard protein (recombinant bovine interleukin-10, PBP016A, BIO-RAD, Watford, UK). The standard protein (STD), dissolved in ELISA washing buffer diluted as two-fold serial dilutions from 10 ng/ml down to 78.12 pg/ml whereas 2 wells were loaded with zero conc of STD. Two more wells were loaded with PBS only (blank). The plate was sealed and incubated at 37 ℃ for 2 hours. The plate was washed with ELISA washing buffer (see section 2.9.1). 100 ul of 1 ug/ml mouse anti bovine interleukin-10:Biotin (MCA2111B, BIO-RAD, Watford, UK) was used as a detection Ab in ELISA washing buffer then the plate was sealed and incubated at 37 ℃ for 1 hour. The plate was washed with ELISA washing buffer (see section 2.9.1). 50 ul of SA-HRP, the enzyme (AR0068-001, Kingfisher Biotech Inc, St. Paul, USA), was loaded as 1:25 diluted in ELISA washing buffer then sealed and wrapped with foil (protected from light) and incubated at 37 ℃ for 60 minutes. The plate was washed with ELISA washing buffer (see section 2.9.1). 100 ul of ELISA Substrate TMB Core+ (BUF062, BIO-RAD, Watford, UK) was added. The plate was not sealed

and incubated in the dark at 25 ℃ for up to 25 minutes. Once blue colour developed, all the wells were topped up with 100 ul of 0.2M sulphuric acid as a stop solution. Labtech spectrophotometer, a micro-plate reader, (LT-4000, Labtech, UK) was used to measure the absorbance (Optical Density which is abbreviated as "OD" values) at 450nm wavelength. OD values of the all samples were blank subtracted to omit background signal (corrected) as follows (mean OD samples at 450nm –mean OD blank). A standard curve line was plotted using recombinant bovine interleukin-4 (Figure 2.9). GraphPad Prism version 7 was used to interpolate the OD values into concentrations illustrated as ng/ml.

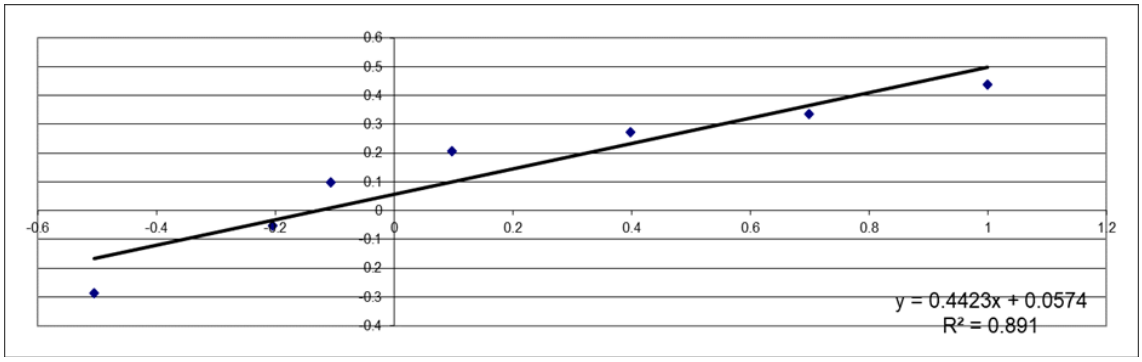


Figure 2.9: ELISA standard curve for Bovine IL-10. Mean OD values were determined at 2-fold serial dilutions of Recombinant Bovine IL-10 from 10 ng/ml down to 78.12 pg/ml concentrations. To generate the straight line, The X axis represents the log10 transformation of the standard concentrations expressed as a geometric mean and the Y axis the log10 of the O.D. expressed as a geometric mean. To get the sample concentrations the values are calculated from the standard curve (in Excel) and reverse transformed (backtransformed) to give a value in ng/ml or pg/ml.

2.9.7 TGFβ

To detect bovine TGF-beta in the supernatants, Bovine TGFβ ELISA kit (E11T0058, Blue Gene) which was purchased as Cow TGF-beta (TGFb) ELISA Kit (ABIN996501, Antibodies-online.com, Aachen, Germany). This commercial kit applied competitive ELISA method. Instructions were literally followed. A pre-coated 96 well micro-titre plate (supplied in the kit as 8 X 12

strips) was used. 100 ul of standards A, B, C, D, E, and F vials (supplied) was loaded in duplicates containing (0, 50, 100, 250, 500 and 1000) pg/ml bovine TGF-β standard protein. Two more wells (blank controls) were loaded with 100 ul PBS (Ph 7.2). 100 ul of samples were dispensed with 10 ul of Balance Solution (supplied) and gently mixed well. 50 ul of enzyme conjugate (supplied) was added to all wells (except the blank control) and gently mixed. The plate was covered with cling film and incubated for 60 minutes at 37 ℃. The plate was washed with was washed 5 times with 1x washing solution (supplied as 10x and diluted to 1x with deionised DW then warmed up to room temperature). After each wash, the plate was dried off by gentle tapping against tissue paper. All residual fluid was drained properly. Substrate was added as 50 ul Substrate A (supplied) along with 50 ul Substrate B (supplied) to all wells including the blank control. The plate was covered with foil and incubated for 30 minutes at 37 ℃ in the dark. Labtech spectrophotometer, a micro-plate reader, (LT-4000, Labtech, UK) was used to measure the absorbance at 450nm wavelength. A mean OD value for each sample was obtained and subtracted by the mean value of blank control before data interpolation (blank correction). A standard curve line was plotted to determine TGFβ in the samples (Figure 2.10). GraphPad Prism version 7 was used to interpolate the OD values into concentrations illustrated as pg/ml.

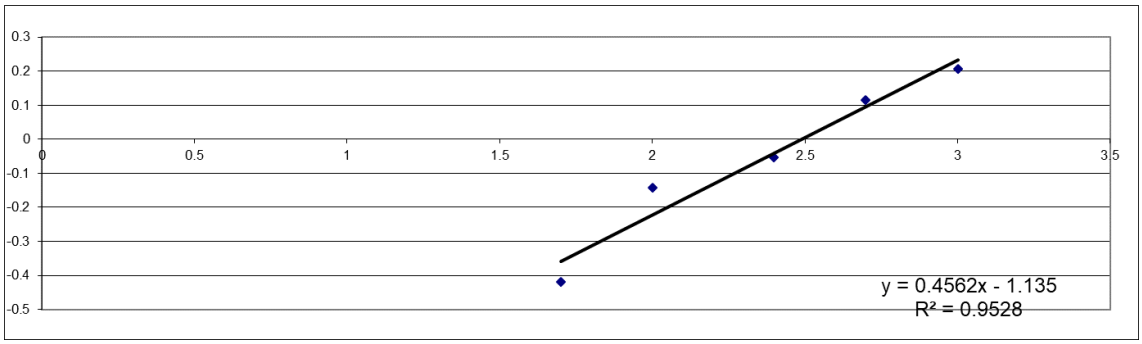


Figure 2.10: ELISA standard curve for Bovine TGFβ. Mean OD values were determined at (0, 50, 100, 250, 500 and 1000) pg/ml concentrations of bovine TGFβ standard protein. To generate the straight line, The X axis represents the log10 transformation of the standard concentrations expressed as a geometric mean and the Y axis the log10 of the O.D. expressed as a geometric mean. To get the sample concentrations the values are calculated from the standard curve (in Excel) and reverse transformed (backtransformed) to give a value in ng/ml or pg/ml.

2.9.8 IFNγ

Bovine IFN-γ ELISA development kit (3119-1H-6 / Mabtech, Cincinnati, USA) was used to determine IFN-γ. A sandwich ELISA method was used. ELISA plate was coated with 100 ul of PBS containing 2ug/ml monoclonal antibody MT17.1 which was used as a capture Ab. The plate was sealed and incubated in the fridge overnight. The plate was washed twice by PBS and blocked by adding 200 ul to each well blocking buffer (PBS with 0.05% Tween 20 containing 0.1% BSA) for 1 hour then washed 5 times with PBS containing Tween 20. 50 ul of samples were added in duplicates diluted 1:1 in blocking buffer along with 100 ul of standard protein (recombinant bovine IFN-γ standard). The standard protein (STD) dissolved in blocking buffer diluted as two-fold serial dilutions from 1000 pg/ml down to 7.81 pg/ml and 2 wells were loaded with PBS (blank). The plate was sealed and incubated at room temperature for 2 hours, then washed 5 times with washing buffer (PBS in 0.05% Tween 20). 100 ul of 0.25 ug/ml Biotinylated monoclonal antibody MT307 was loaded as a detection Ab in blocking buffer then the plate was

sealed and incubated at 25 ℃ for 1 hour then washed 5 times with washing buffer. 100 ul of SA-HRP, the enzyme, was loaded as 1:1000 dilution in blocking buffer then sealed and wrapped with foil (protected from light) and incubated at 25 ℃ for 60 minutes then washed with washing buffer. 100 ul of ELISA Substrate TMB Core+ (BUF062, BIO-RAD, Watford, UK) was added. The plate was not sealed and incubated in the dark at 25 ℃ for up to 25 minutes. Once blue colour appropriately developed, all the wells were topped up with 100 ul of 0.2M hydrochloric acid (HCl) as a stop solution. Varioskan™ Flash spectral scanning multimode reader (3001-1623 - Thermofisher Scientific, Rugby, UK), a micro-plate reader, was used to measure the absorbance (Optical Density which is abbreviated as "OD" values) at 450nm and 540 nm wavelengths. OD values of the all samples were subtracted to omit background signal (corrected) as follows (mean OD samples at 450nm– mean OD samples at 540nm). A standard curve line was plotted using recombinant bovine IFN-γ standard (Figure 2.11). GraphPad Prism version 7 was used to interpolate the OD values into concentrations illustrated as pg/ml.

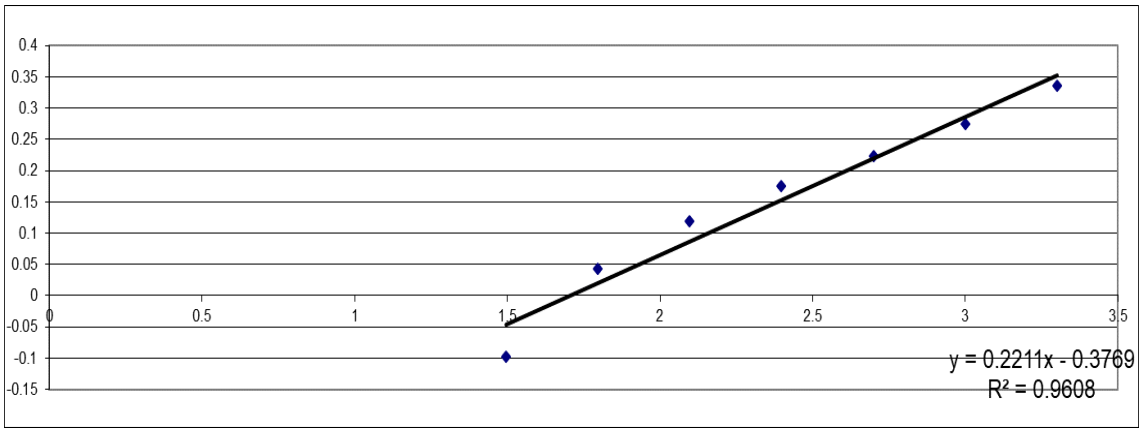


Figure 2.11: ELISA standard curve for Bovine IFN gamma. Mean OD values were determined at 2-fold serial dilutions of Recombinant Bovine IFN-γ from 1000 pg/ml down to 7.8125 pg/ml concentrations. To generate the straight line, The X axis represents the log10 transformation of the standard concentrations expressed as a geometric mean and the Y axis the log10 of the O.D. expressed as a geometric mean. To get the sample concentrations the values are calculated from the standard curve (in Excel) and reverse transformed (backtransformed) to give a value in ng/ml or pg/ml.

2.10 Statistical analysis

Data were analysed by using GraphPad Prism version 7. One-way analysis of variance (ANOVA) was used to compare the groups at a level of $P<0.05$ or less to show significance. Microsoft Office (MS) Excel 2016 and MS Word 2016 was used to input the raw data in groups or subgroups.

Chapter Three

Generation of putative rabbit regulatory T cells (Tregs) in culture

3.1 Abstract

The initial task in this chapter was to refine techniques for the generation of putative rabbit Tregs in culture for the cattle study. Some work had been done in D Haig laboratory to generate putative rabbit Tregs for another study, therefore the objective was to complete this work with a view to publication. Attempts were made to generate rabbit putative nTregs (natural regulatory T cells) *in vitro* by adding ConA to stimulate T cell activation, IL-2, and TGFβ in mesenteric lymph node (MLN) cells seeded in IMDM culture medium. At the end of incubation, cells were labelled with fluorochromes (FACS antibodies) to highlight the phenotypic changes of the cells. CD4+CD25hiFOXP3+ cells were gated by FACS software and considered as putative rabbit nTregs. To further confirm and identify the cells of interest (nTregs), a molecular detection of transcription factor FOXP3, a Treg marker, by RT-qPCR was performed. Putative Tregs were sorted from the cultures as CD4+CD25hi cells (all FOXP3+) and tested for activity in CFSE-labelled autologous MLN cells stimulated with ConA plus PMA. The results indicated that the putative rabbit nTregs did not inhibit autologous MLN cell proliferation.

3.2 Introduction

Rabbits are a good laboratory model animal for immunological research studies. Rabbits have been utilised in many research fields (Mapara *et al*., 2012), with a specific focus on infectious diseases (Peng *et al*., 2015) and immunity (Schnupf and Sansonetti, 2012, Guo *et al*., 2017) (Rittershaus *et al*., 2000, Tsenova *et al*., 2006, Cheng *et al*., 2012, El Sayed *et al*., 2016). From the point of view of my main thesis work, I hoped to gain experience in generating putative rabbit Tregs in the rabbit to complete a study and transfer the knowledge to generating cattle Tregs.

From an immunological point of view, the majority of regulatory T cells (in particular nTregs) are those cells phenotypically characterised by CD4+CD25hiFOXP3+ which are presented naturally at less than 1% *in vivo* but can be induced *in vitro* at a higher purity. Both natural and induced Tregs have been extensively studied in human and mice species (D'alessio *et al*., 2009) but there are a lack of studies in rabbits.

Phenotypically, nTregs are a subset of T cells carrying both external markers CD4 and CD25 (IL-2α receptor) and transcription factor FOXP3, which is the essential intracellular key marker for Tregs (Mottet and Golshayan, 2007, Kelley and Parker, 2010). Functionally, the role of these cells could be very important in autoimmunity and cancer through their suppressive activity (Curiel, 2007, Von Boehmer and Daniel, 2013).

TGFβ, ConA, and IL-2 were titrated and added as a combination to mesenteric lymph node (MLN) cells seeded in IMDM culture medium in an attempt to generate putative rabbit Tregs. Preliminary work on this had been done by Nevi Parameswaran. Autologous MLN cells were stimulated

with a combination of ConA and PMA and used as target cells in a putative Treg functional assay.

3.3 Materials and methods

All reagents were titrated as described in sections 2.4.2 and 2.4.3 in the Chapter 2. Putative rabbit Tregs were generated *in vitro* (section 2.5.1) from the MLNs (collected from 5 White New Zealand breed both sexes - two male and three female - rabbits around 12 weeks of age) and FACS labelling (section 2.5.2) was performed after 6 days of incubation, then CD4+CD25hi cells were sorted by flow cytometry (section 2.5.3) to test function in the CFSE assay (section 2.5.4 and 2.5.5). At the end of 6 days incubation, RNA was extracted from a proportion of the cultured cells (section 2.6.1), and cDNA was made (section 2.6.2) for PCR (section 2.6.4) detection of FOXP3 gene expression (nTreg marker)

Results

3.4 Preliminary results

These included titrations of reagents and generation of putative rabbit Tregs and target T cells for putative Treg functional assay.

3.4.1 TGF-β1

The concentration had been already titrated by Nevi Parameswaran (Haig lab postdoc) to 2 ng/ml for putative rabbit Treg generation.

3.4.2 IL-2

This reagent was titrated as mentioned in the previous chapter, section 2.4.3.1. The results showed significant proliferation (5.225 X 10^6 MLN cells/ml) when 10^6 cells were treated with 50 ng/ml IL-2 for 4 days (figure 3.12) by comparison with the control group (0.75 X 10^6 cell/ml) at a level $P<0.05$. This concentration was considered for putative rabbit Treg generated from MLN cells experiments.

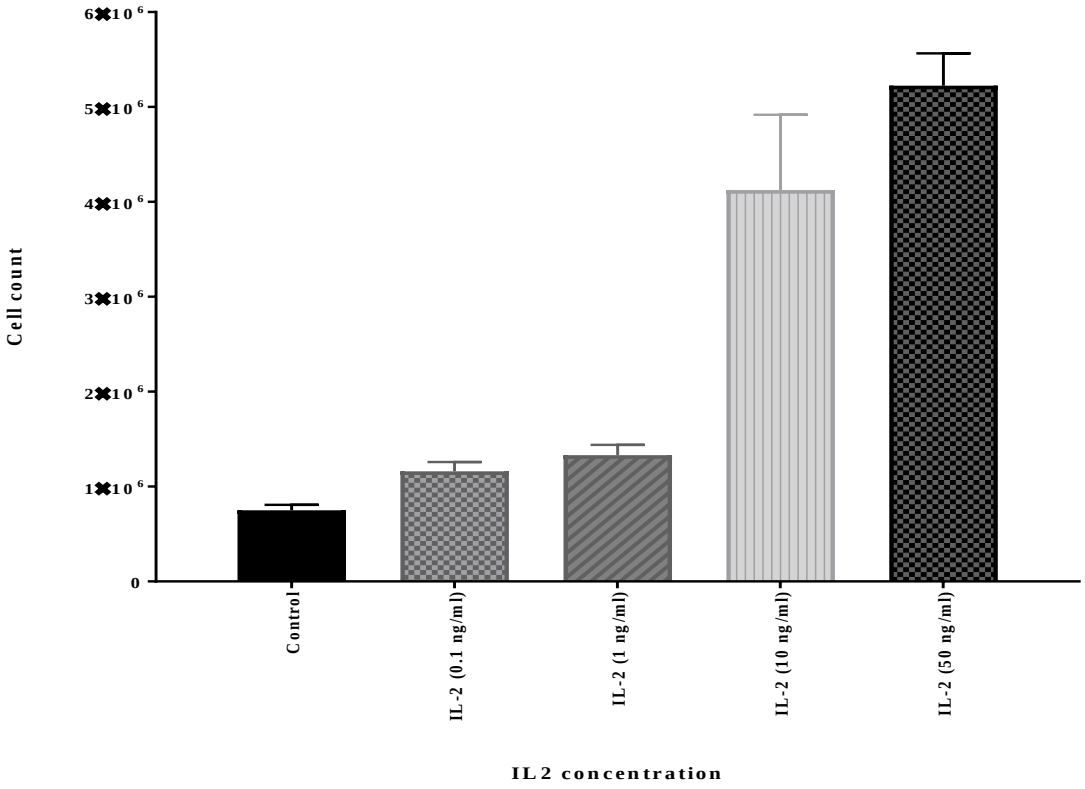


Figure 3.12: IL-2 titration for MLN cell proliferation. Proliferation was maximum when 50 ng/ml of IL-2 was used ($P<0.05$ by comparison with negative control untreated group). Cell count = cells/ml. Mean values ± SEM for each group.

3.5 Con A, PHA and PMA titrations for stimulating T cells

Thirteen groups (G1-13) of 1 X 10^6 MLN cells/ml in IMDM medium were used for optimal stimulation of MLN cells to develop putative rabbit Tregs for Treg assay (see section 2.4.3.2). G1, G2 and G3 were treated with Con A (1 µg/ml, 5 µg/ml and 10 µg/ml), while G4, G5 and G6 were treated with the same above concentrations of Con A plus 10 ng/ml PMA respectively. G7, G8 and G9 were treated with PHA (2 µg/ml, 5 µg/ml and 10 µg/ml) an alternative T cell mitogen to Con-A (Barabas *et al*., 2002), whereas G10, G11 and G12 were treated with the same concentrations of PHA plus 10 ng/ml PMA respectively. The control group contained only MLN cells without any stimulus.

The results illustrated that the fourth group (G4, 1 µg/ml Con A + 10 ng/ml PMA) showed significant stimulation (3.62 X 10^7 cell/ml) after 4 days incubation (figure 3.13) ($P<0.05$ by comparison with the unstimulated cell control group (1.595 X 10^7 cell/ml) when cells were counted by haemocytometer. This combination of ConA/PMA concentrations was used in Treg target cells experiments.

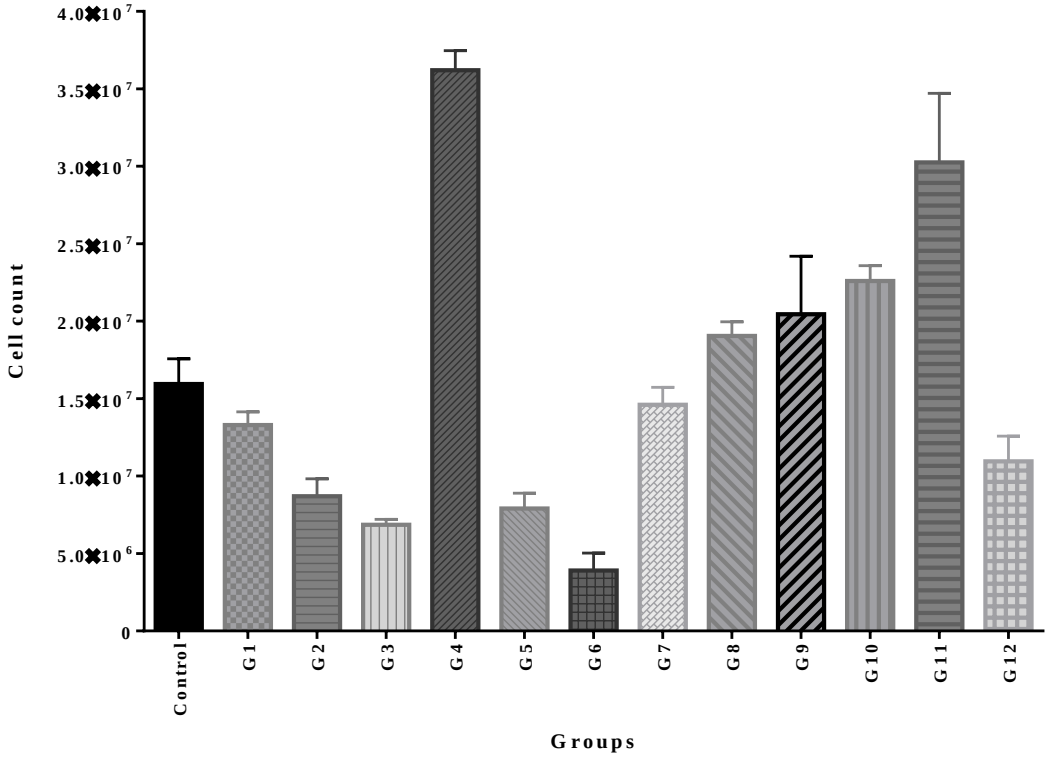


Figure 3.13: Con A, PHA, and PMA titration (Mean ± SEM) for Treg assay target cell proliferation, using MLN cells. Cell count = cells/ml. G1, G2 and G3 were treated with Con A (1 µg/ml, 5 µg/ml and 10 µg/ml), while G4, G5 and G6 were treated with the same concentrations of Con A plus 10 ng/ml PMA respectively. G7, G8 and G9 were treated with PHA (2 µg/ml, 5 µg/ml and 10 µg/ml) an alternative T cell mitogen to Con-A (Barabas *et al*., 2002), whereas G10, G11 and G12 were treated with the same concentrations of PHA plus 10 ng/ml PMA respectively. The control group contained only MLN cells without any stimulus.
The fourth group (1 µg/ml Con A + 10 ng/ml PMA) revealed significant stimulation (3.62 X 10^7 cell/ml) at a significant level ($P<0.05$ after 4 days incubation by comparison with negative control group).

3.6 Phenotype analysis of MLN and cultured MLN cells

Assessment of the generated putative rabbit Tregs was by flow cytometry as described in section 2.5.2. In general, putative rabbit Tregs were generated from MLN cells between 5% and 25.7% in 5 different animal MLN samples (Tr1, Tr2, Tr3, Tr4, and Tr5) on separate occasions. FACScan analysis was after 6 days in culture, as this was a consistent time to detect

FOXP3 in positive samples (N Parameswaran, unpublished data). As an example, the MLN cell culture of Tr2 sample shown in figure 3.14 showed gated cells (A) that contained 87.3% CD4+ T cells (B), and 23.6% of the total cells were FOXP3 positive (C), which indicates that these latter cells might be putative rabbit Tregs. Then, 25% of the CD4+ T cells were FOXP3+ (D), and this accounts for all the FOXP3+ cells in the total MLN population. In addition, the majority of CD4+ T cells appeared to be positive for CD25 (83.8%) (E). Whereas 19.5% of CD25+ cells were FOXP3+ (F) which indicate that the overall CD4+CD25+FOXP3+ cells were about 20% of the population after 6 days of culture. All MLN cells showed less than 0.5% CD4+CD25hi FOXP3+ (nTregs) at day 0 (prior to setting up the experimental cultures). There was a total of five rabbit putative Treg cultures that have generated between 18 and 48% CD4+ CD25+ FOXP3+ cells after 6 days in culture.

After 6 days of culture in Con-A + IL-2 + TGF-beta (n=3) results showed a range of 5%-25.7% FOXP3 positive cells (single labelled) detected by specific antibody (figure 3.15).

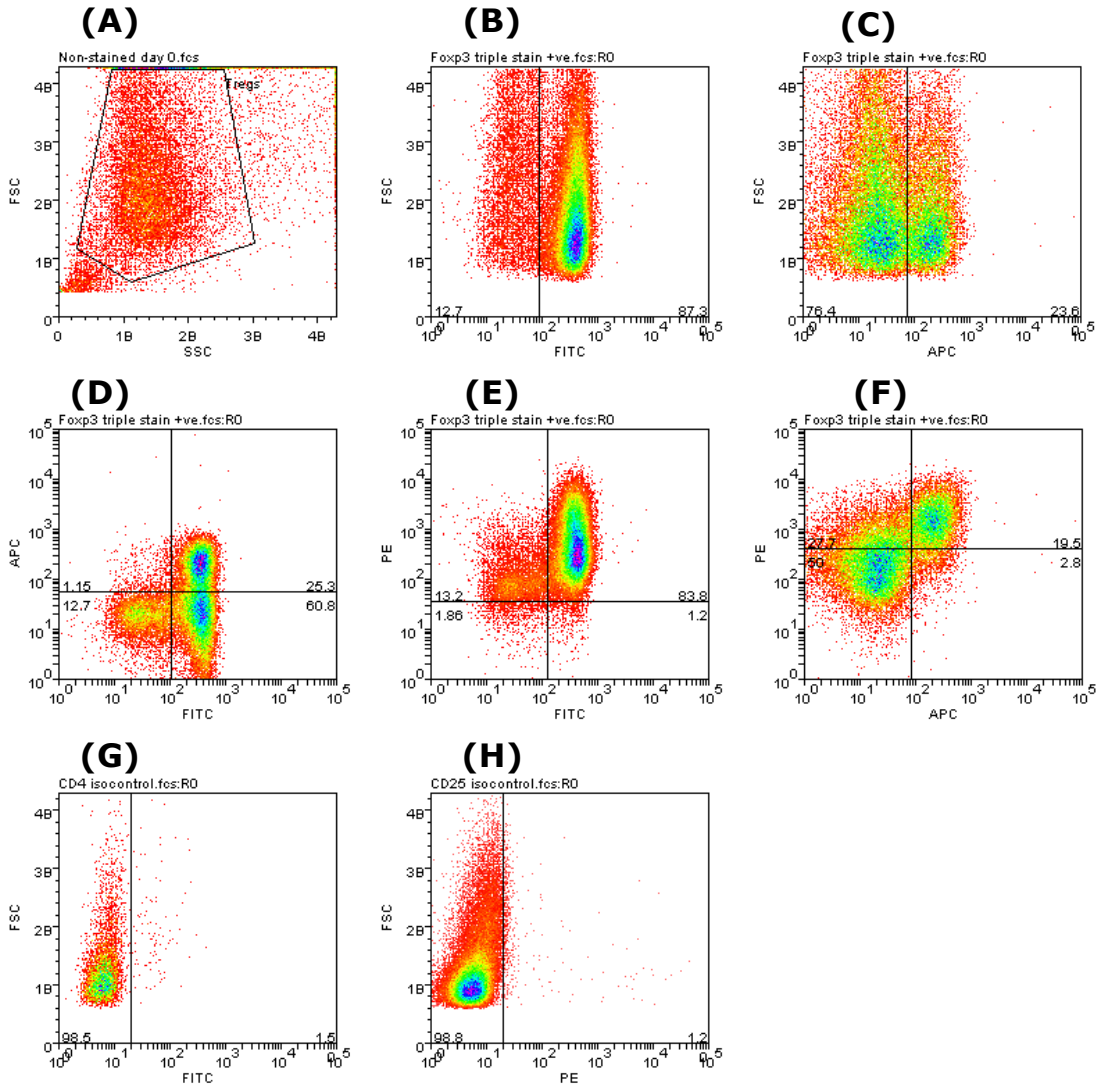


Figure 3.14: Triple stained generated putative rabbit Treg cells in Tr2 sample at day 6. (A): gated cells, (B): CD4+ T cells, (C): FOXP3+ cells, (D): CD4+(x axis) FOXP3+ T cells, (E): CD4+(x axis) CD25+ T cells, (F): CD25+FOXP3+ T cells, (G): CD4 isotype control and (H): CD25 isotype control.

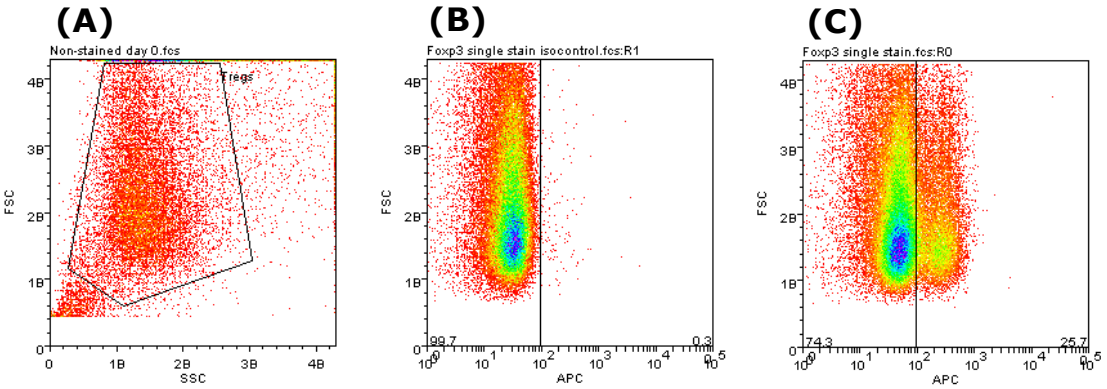


Figure 3.15: Validation FACscan for FOXP3 antibody-labelled cells in an MLN sample after 6 days incubation (Tr2 sample). (A): FSC /SSC gated cells (B): APC-labelled isotype control antibody labelled cells. (C): anti-FOXP3:APC labelled cells (25.7%).

3.7 Target cells for putative Treg functional assay

In this assay, MLN cells autologous with respect to the cultured putative Treg cells were stimulated by ConA and PMA and measured by CFSE as described in section 2.5.4. The Treg target MLN cells proliferated when $5X10^5$ MLN cells/ml labelled with 1 µL of 5 mM CellTrace Violet (CFSE) for each mL of cell suspension were stimulated with Con A (1 µg/ml) + PMA (10 ng/ml). This was to develop stimulation and culture conditions for these cells prior to their use as target cells for the cultured Treg cells in a functional assay.

The results indicated proliferation of the 5 animal MLN samples ranged between 6.2% to 35.9% after 4 days of incubation. Using Tr2 sample as an example, the analysis of Treg target cells showed proliferation in 13.1% and 35.9% of cells at 2 and 4 days post incubation respectively for the gated cells (figure 3.16). Note that we are looking for a decrease in the starting level of fluorescence (a shoulder or peak(s) of fluorescence to the left of the Day 0 peak) to indicate that daughter cells after each division contain a diminishing amount of fluorescent signal).

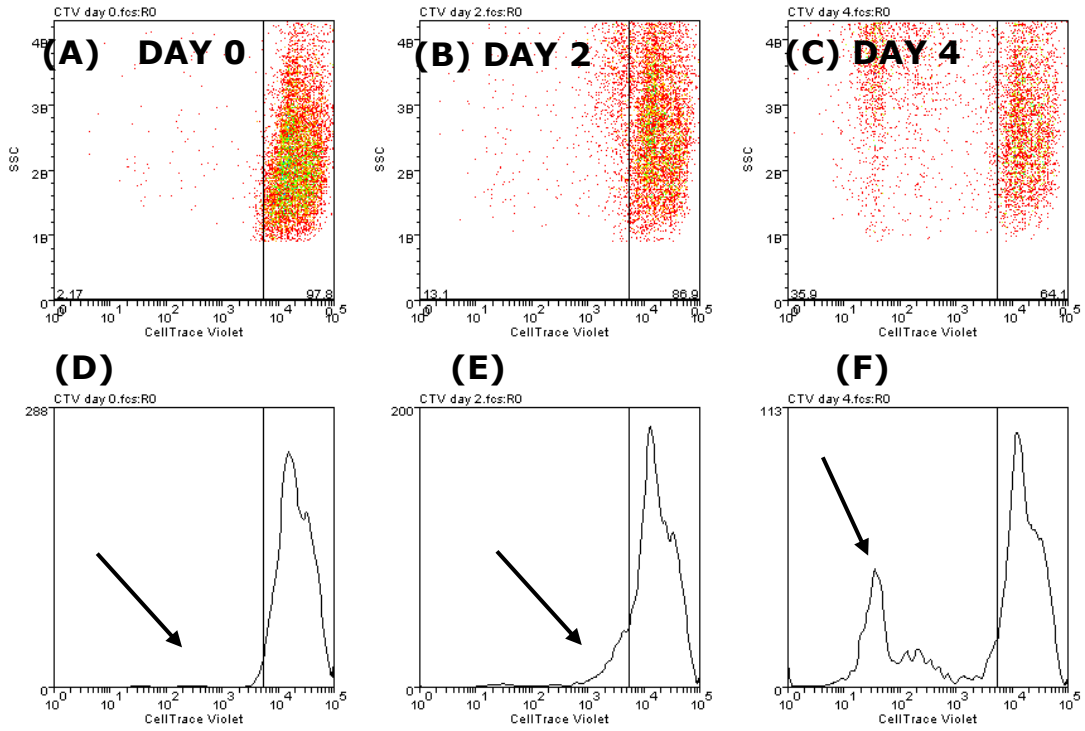


Figure 3.16: Treg target cell proliferation (labelled with CellTrace violet - CFSE, abbreviated as CTV). (A): Treg target cells dot plot at day 0. (B): Treg target cells dot plot at day 2. (C): Treg target cells dot plot at day 4. (D), (E) and (F): histograms derived from items (A), (B) and (C) respectively.

3.8 Putative rabbit Treg functional assay

This was done by sorting CD4+CD25hi cells from the cultured putative rabbit Tregs after 6 days incubation as described in section 2.5.3. These sorted cells (see figure 3.17) were mixed with Treg target cells (normal autologous MLN cells labelled with CFSE).

Unexpectedly, the results demonstrated no suppression of proliferating MLN cells. (figure 3.18). This was repeated with the same result 5 times.

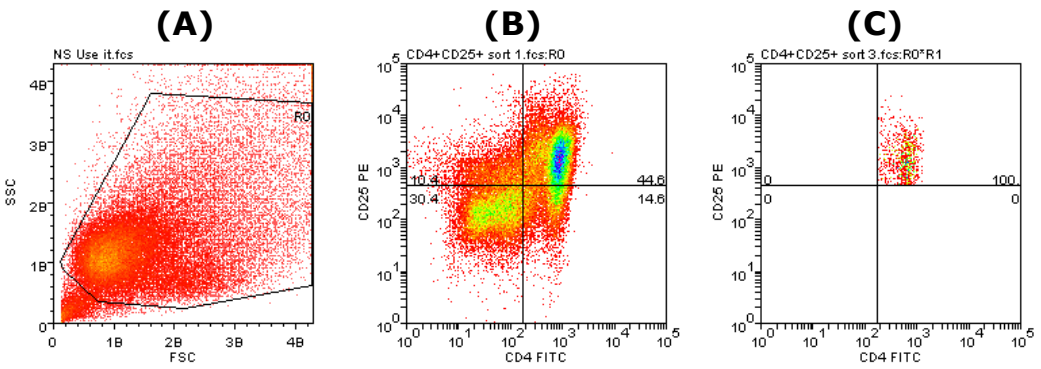


Figure 3.17: CD4+CD25hi cell sorting from putative rabbit Tregs generated in culture after 6 days incubation. (A): gated cells at day 6 post incubation (B): a quadrate gate to highlight CD4:FITC and CD25:PE double positives showing 44.6% frequency (the view before cell sorting). (C): purified sorted cells (CD4+CD25hi).

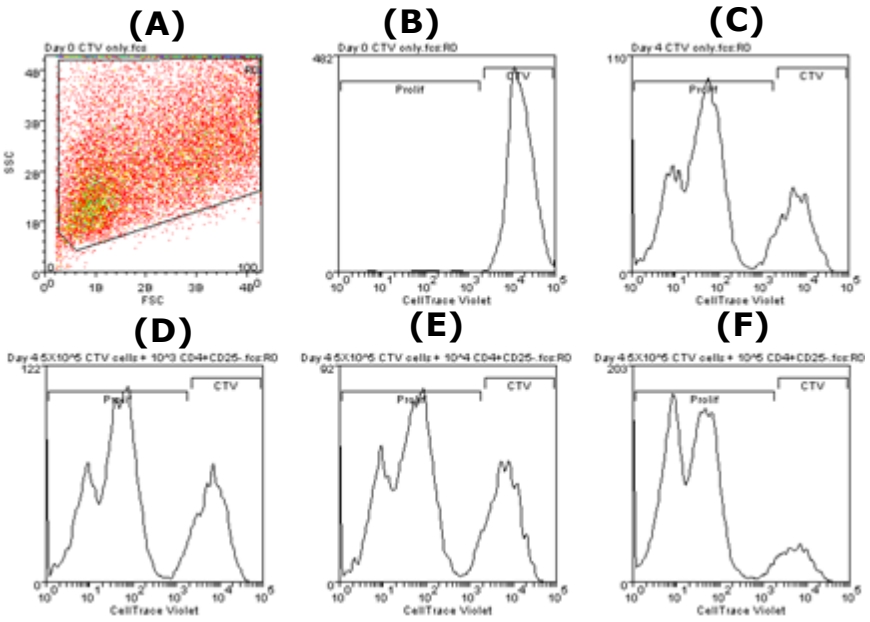


Figure 3.18: 5X10^5 cells/ml of Treg target cells (autologous MLN cells) labelled with CFSE. (A): Treg target cells dot plot at day 0. (B): histogram of A. (C): Treg target cells histogram at day 4. (D), (E), (F) Treg target cells mixed with 10^3, 10^4, and 10^5 cells/ml CD4+CD25hi sorted cells at day 4 respectively.

3.9 RT-qPCR for FOXP3 in the MLN cells

Total RNA was extracted from the five different animal MLN cells at day 0 (in duplicate) and putative rabbit Tregs generated from these at day 6 post incubation (see section 2.6.1). cDNA was made – reverse transcribed –as per Roche instructions (see section 2.6.2). All cDNA samples of generated putative rabbit Tregs were amplified in the Lightcycler 480® Roche (RT-qPCR machine) as explained in section 2.6.4, which showed significant expression of FOXP3 gene (the key marker for Tregs) at a level $P<0.05$ when compared with control group (figure 3.19).

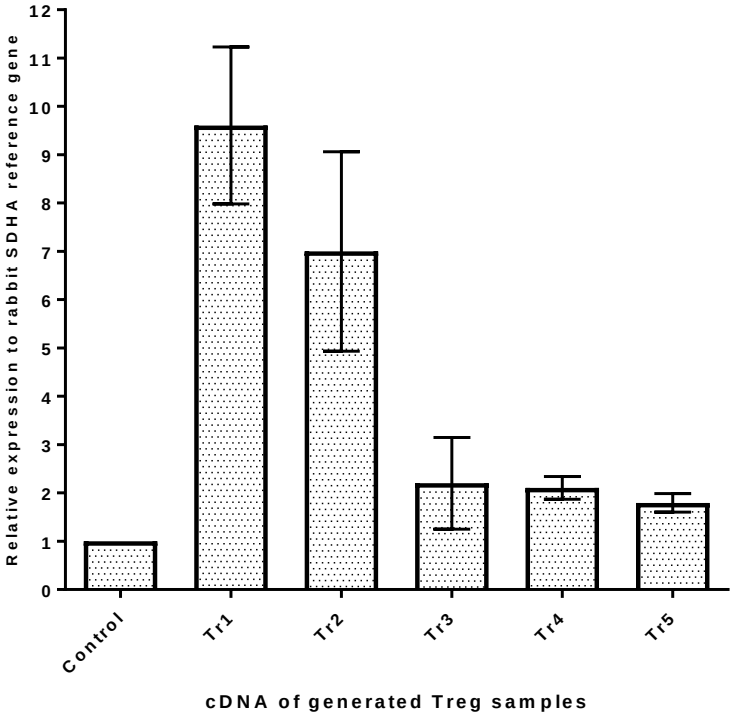


Figure 3.19: Average ± range of FOXP3 gene expression levels in duplicate samples of each of the MLN from 5 animals on day 6 of Treg culture (Tr1-Tr5) compared to day 0 levels (control) These fold change values were determined against SDHA reference gene (optimised by Nevi Parameswaran).

3.10 Discussion

In this study, rabbit mesenteric lymph node (MLN) cells were used to generate CD4+ CD25+, FOXP3+ putative rabbit nTreg in culture. The combination of Con-A (T cell mitogen), IL-2 and TGF-beta used to stimulate MLN cells was successful in developing a proportion of cultured cells (5%-27.5%) with a CD4+, CD25+ FOXP3+ phenotype representative of nTregs (Yi et al., 2006). This finding, regarding expression of FOXP3 in the presence of these cytokines is in line with previous studies in mice and man (De La Rosa *et al*., 2004, Fontenot *et al*., 2005a, Cesana *et al*., 2006, Koreth *et al*., 2011). In these species, TGFβ is required for developing and upregulating the intracellular nTreg marker FOXP3 (Nishioka *et al*., 2006, Bonelli *et al*., 2009, Kelley and Parker, 2010). Due to time constraints and the need to focus on the bovine T cell TLR expression work, this study was stopped at this stage so further characterisation of the cells was not done. The phenotype of Tregs has been recently extended in humans. CD127, which is the interleukin-7 receptor-α, was one of the external markers thought to correlate with an inhibitory role of Tregs in autoimmunity (Moradi *et al*., 2014, Wu *et al*., 2015a, Walter *et al*., 2016), whereas depletion of this molecule resulted in less functional Tregs in certain diseases such as diabetes and Guillain-Barré syndrome (a neuro-immunological disorder) (Marek-Trzonkowska *et al*., 2014, Wang *et al*., 2015). CD161 is expressed by natural Treg cells as well as induced Tregs (Duurland *et al*., 2017). CD161+Treg cells are thought to facilitate production of pro-inflammatory cytokines (Pesenacker *et al*., 2013).

In addition to time constraints, the challenges and limitations in this work for further phenotypic characterisation of putative rabbit Tregs were due to the scarcity of rabbit alloantigen-specific antibodies.

Several studies have confirmed the cross-reactivity of anti-FOXP3 mAb (clone FJK-16s) with many mammals (Schoenbrunn *et al*., 2012, Groen *et al*., 2013, Kronsteiner *et al*., 2013). The results in this study demonstrated significant amplification of FOXP3 gene ($P<0.05$) by comparison with control negative groups in putative rabbit Tregs generated *in vitro*.

In this study, the putative rabbit Tregs did not show any suppressive activity on proliferating autologous MLN cells. The reasons for this are not known. In mouse and the human, the immuno-inhibitory role of Treg cells in regulating peripheral immune responses, autoimmune diseases and cancer have been studied extensively (Ducloux, 2014, Whelan *et al*., 2014). FOXP3 is the transcription factor expressed internally by Tregs which defines many Tregs including nTregs (Sakaguchi, 2005). Treg immunosuppressive function is mediated by several mechanisms such as: production of the immunosuppressive cytokines IL-10 and TGF-β; DC growth and functional impairment; apoptosis or cytolysis of target cells by releasing granzyme A, granzyme B and pore forming protein (perforin) (Vignali *et al*., 2008). Because of the scarcity of nTregs *in vivo* (5% of overall T cells), it is important to grow them in culture so they can be characterised and studied further (Shevach, 2002). We found that putative rabbit Tregs can be successfully generated in culture (~25% of MLN cells after 6 days in the present experiments) as measured by flow cytometry, focussing on the putative rabbit Treg phenotype: CD4, CD25 and FOXP3 expression. This ratio is low compared to findings in human and murine

Tregs isolated from peripheral mononuclear blood cells (Wing *et al*., 2002, Gołąb *et al*., 2013), where frequencies of 40%- 66% could be seen.

We found that 19% of CD4+ T cells express CD25 (the alpha chain of the IL-2 receptor), but this is moderately higher than the human and murine findings where normally 10% (in humans) and 2-3% (in mice) of CD25+ can be noticed (Roncador *et al*., 2005, Wang *et al*., 2013). However, detection of FOXP3+ in the rabbit CD4+ T cells (representing about a quarter of MLN cells) is in line with some findings in human Tregs (Earle *et al*., 2005, Godfrey *et al*., 2005).

Chapter Four

Bovine TLR expression on T cells

4.1 Abstract

TLR expression has been detected by RT-qPCR in bovine macrophages, dendritic cells, epithelial cells, and neutrophils (Werling *et al*., 2006, Russell, 2012, Russell *et al*., 2012). The objective of this piece of work was to carry out in-depth detection of TLRs 1-10 gene expression in bovine PBMCs and the major bovine T cell subsets (CD4, CD8, and γδ T cells). PBMCs were fractionated by MACS into CD4, CD8, and γδ T cell subsets and purity determined by flow cytometry.

Results showed successful fractionation of γδ T cells, CD4+ T cells and CD8+ T cells from bovine PBMCs. Purity of these fractionated cells exceeded 90% for CD4 and γδ T cells but ranged between 83.4% - 87.1 in CD8 T cells. Reference and target genes were expressed in the majority of the PBMCs. All of TLRs 1-10 were expressed in CD4, CD8, and γδ T cell subsets to varying levels.

4.2 Introduction

TLRs have been described in most mammalian species including cattle (Kumar *et al*., 2009, Seabury *et al*., 2010, Pandey *et al*., 2015).

In the next three chapters (4, 5, 6), expression of TLRs on PBMC and T cell subsets will be analysed by RT-qPCR (chapter 4); PBMC and T cell subsets will be stimulated with TLR agonists (PAMPs) and activation measured by CFSE proliferation functional assays (chapter 5) and cytokine production (chapter 6).

In this chapter, PBMC isolation and fractionation of T cell subsets is described and levels of TLR1-10 expression measured by RT-qPCR.

There has been no previous reports of measurement of TLR1-10 gene expression in bovine T cell subsets. PBMC cells were MACS fractionated into CD4, CD8, and γδ T cells. Purified cells were validated by flow cytometry. TLR-1-10 gene expression was then determined.

4.3 Materials and methods

All methods and procedures related to this chapter have been described in the general materials and methods (chapter 2). Bovine PBMCs were prepared from the blood of individual animals as described in section 2.3. CD4, CD8 and γδ T cells subsets were MACS fractionated (section 2.7.3). The purity of each subset was tested by flow cytometry (section 2.7.4). Molecular techniques involved in gene expression were described in section 2.8 and associated sub-sections.

Results

4.4 PBMC separation

The bovine total PBMC counts and the mean PBMC count per 1 ml blood from each of the animals are shown in table 4.24. The total bovine PBMC count, (see general materials and methods section 2.3) ranged between 1.429×10^8 – 10.125×10^8 cells. A PBMC index was used to demonstrate the mean of isolated PBMCs per 1 ml blood collected from each adult cow blood sample (figure 4.20). This was done by dividing the total PBMC counts by the blood volume (ml).

Table 4.13: Volumes of bovine blood showing variable PBMC counts.

Animal identifier	**Blood volume (ml)**	**Total PBMC count**	**PBMC mean count/ 1 ml blood**
BOV4098	1000	10.125×10^8	10.125×10^5
Bov A (EA)*	500	1.781×10^8	3.562×10^5
Bov B (EA)	400	1.429×10^8	3.572×10^5
Bov 1 (EA)	1150	6.810×10^8	5.921×10^5
Bov 2 (EA)	1100	6.235×10^8	5.668×10^5
Bov 3 (EA)	900	5.901×10^8	6.557×10^5
Bov 4 (EA)	700	4.613×10^8	6.590×10^5
Bov 5 (EA)	700	2.968×10^8	4.240×10^5
Bov 6 (EA)	500	2.24×10^8	4.480×10^5
Bov 7 (EA)	500	4.08×10^8	8.160×10^5
Bov 8 (EA)	500	3.74×10^8	7.480×10^5
Mean			6.032×10^5

* EA = Elliot Abattoir, Chesterfield, UK

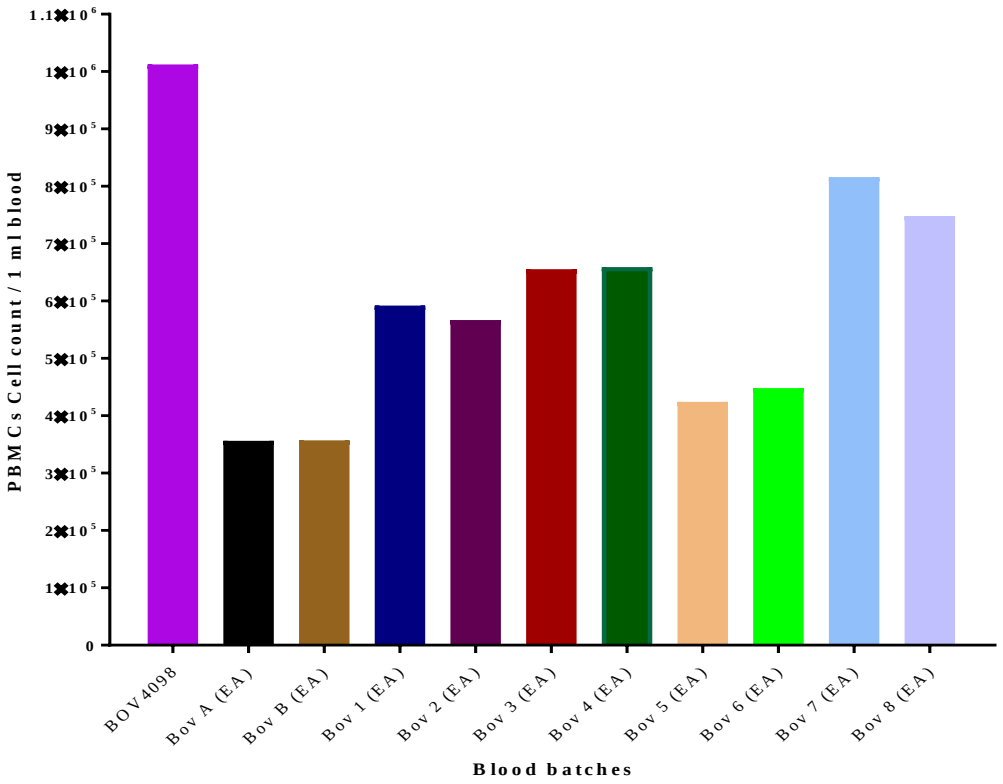


Figure 4.20: PBMC mean cell count per 1 ml blood (PBMC index). * EA= Elliot Abattoir

4.5 PBMCs stained with Diff Kwick

Stained PBMC on slides (see general materials and methods section 2.7.1) were inspected under the light microscope showing predominantly lymphocytes and fewer monocytes (figure 4.21). Monocytes seemed quite scattered over the slides but recognised because of their large size and U-shape, bean-shape or occasionally irregular blue-stained nuclei that filled half the space of the cell.

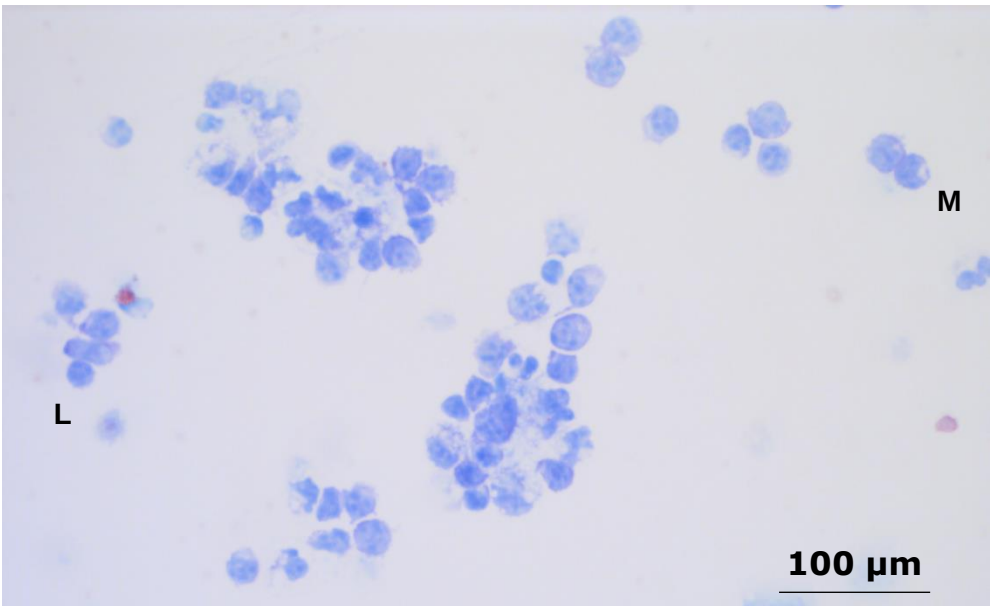


Figure 4.21: PBMCs stained with Diff Kwick. Scale bar 100 µm (20x lens/ Leica microscope). L = lymphocyte, M = monocyte.

4.6 Flow cytometry

4.6.1 Major PBMC components

Bovine PBMCs were labelled with specific Abs conjugated with fluorochromes (section 2.7.4) to detect specific lymphocyte subsets. Mouse anti-bovine CD11b FITC (which labels monocytes, but also granulocytes, and a proportion of activated CD8+ T cells in PBMC), CD21 FITC (that labels B cells in PBMC), CD4 FITC (that labels CD4+ T cells), CD8 FITC (that labels CD8+ T cells) and WC1 (CC15 clone) FITC antibodies (that labels the predominant γδ receptor+ T cell population in PBMC) and isotype controls were used. The results of flow cytometry per 1 million PBMCs is shown in figures 4.22, 4.23. shows representative FACs profiles for a few PBMC samples.

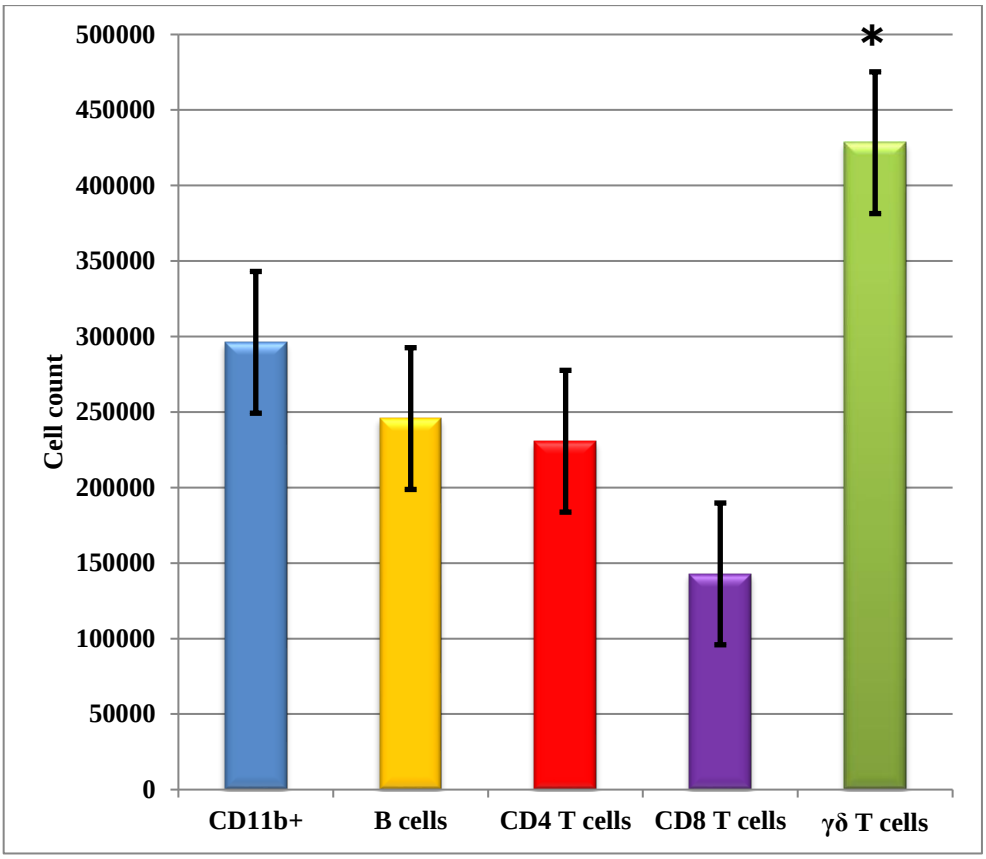
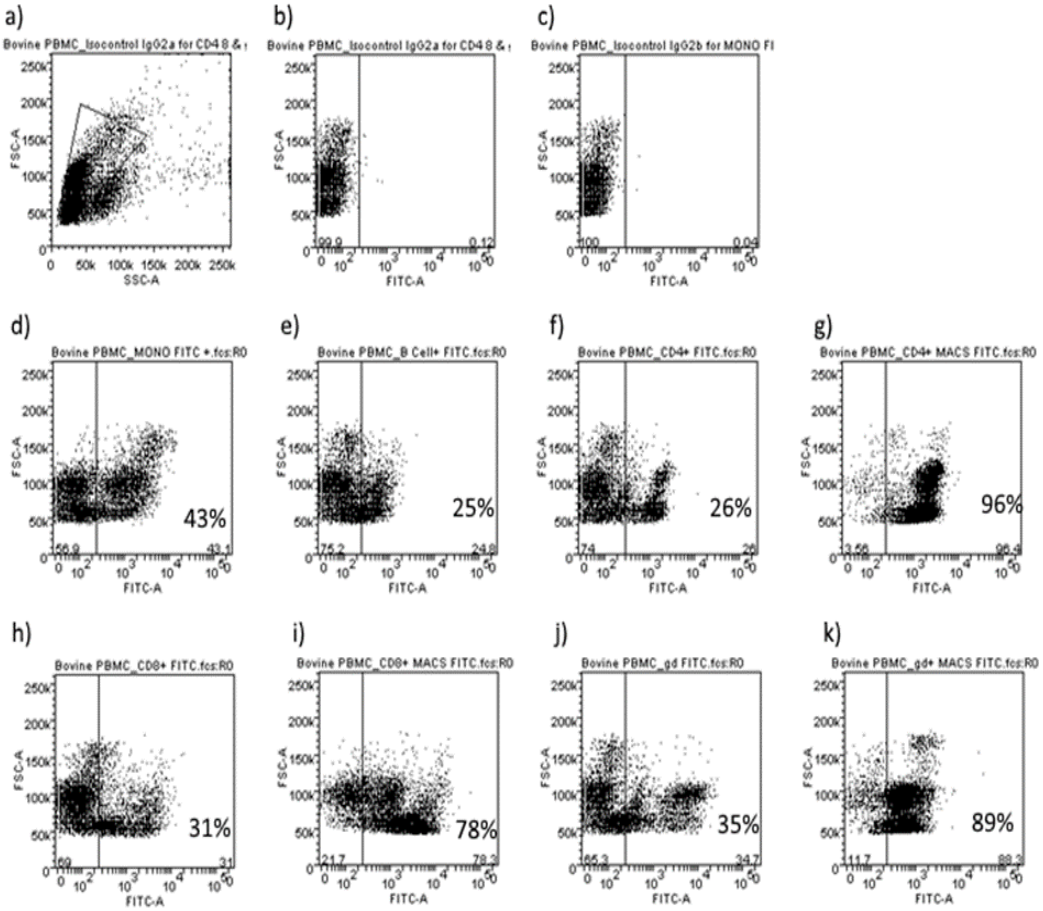


Figure 4.22: FACS results for bovine PBMCs (Mean of 7 animal PBMC samples and range). * = presence of significant differences at P<0.05 (multiple comparison between the means of each group with the mean of every other group). CD11b + monocytes only detected. CD21+ B cells only detected.

A) PBMCs (Bov A)



+

B) PBMCs (Bov B)

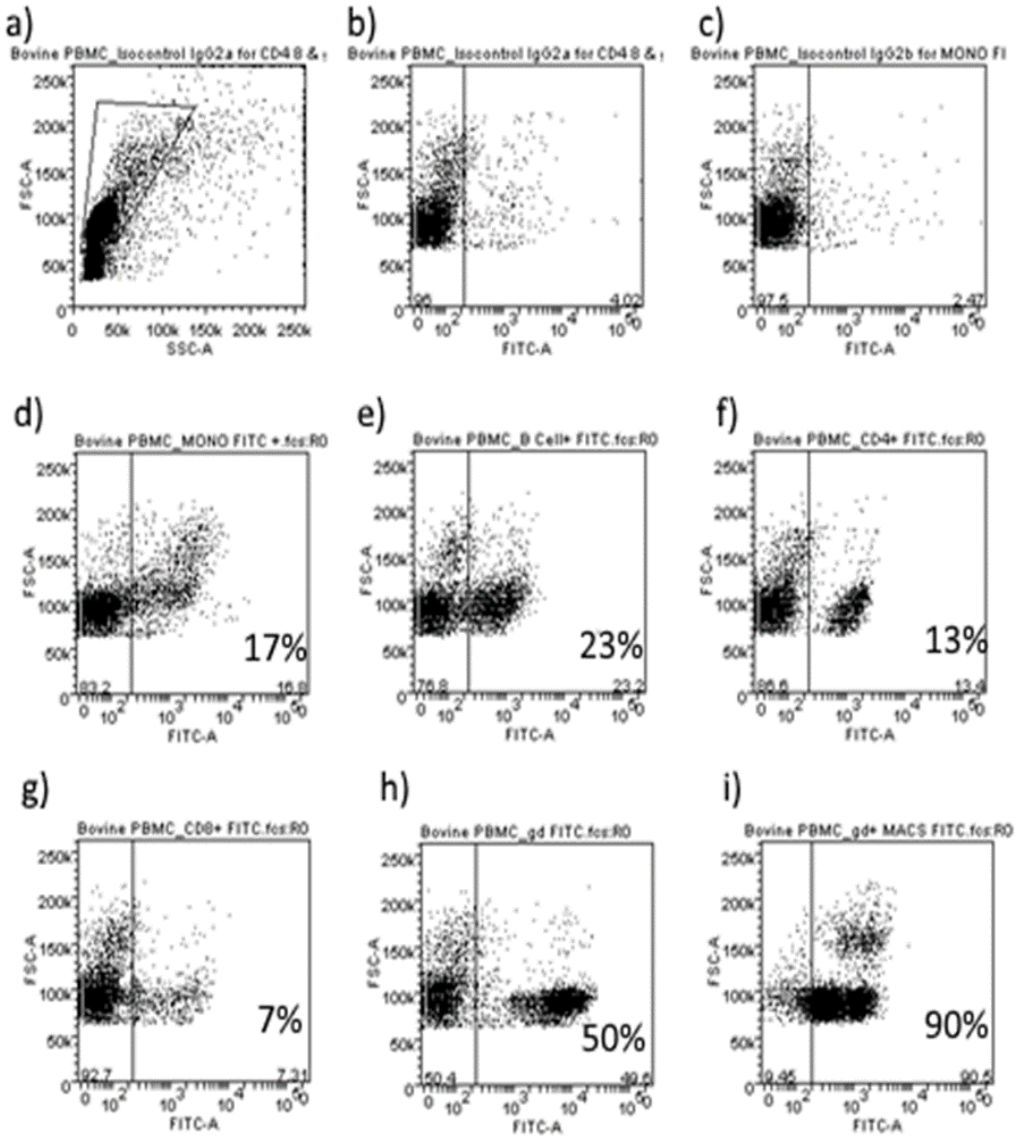


Figure 4.23. FACs dot plot analysis of PBMC samples from Bov A (A) and Bov B (B). (a) a gate was applied around the mononuclear fraction (low SSC) of bov A and B animal PBMC samples. (b) and (c) for (A) and (B) show the profiles for the isotype control (IgG2a and IgG2b respectively) FITC-labelled antibodies in Bov A and B respectively. A and B (d) show the proportion of cells of Bov A and Bov B respectively expressing CD11b. A and B (e) show the proportion of CD21+ B cells in Bov A and Bov B respectively. A and B (f) show the proportion of CD4+ T cells in Bov A and Bov B respectively. Figure A (g) shows the frequency of CD4+ T cells after MACS purification of Bov A cells. Figure A (h) and B (g) show the frequency of CD8+ T cells in Bov A and Bov B animal samples. Figure A (i) shows the frequency of CD8+ T cells in Bov A cells after MACS purification. Figure A (j) and (k) shows the frequency of WC1 γδ T cells before and after MACS purification respectively. Figure B (h) and (i) show the frequency of WC1 γδ T cells in Bov A sample before and after MACS purification respectively.

4.7 PBMCs fractionated by MACS

CD4, CD8, and γδ T cells were fractionated from PBMCs by MACs (section 2.7.3). The results obtained from this method for each subset are shown in figure 4.24. The results of fractionation (out of 1 X 10^8 cells suspended in 10 ml counted by haemocytometer method), indicated that total fractionated γδ T cells was the dominant phenotype amongst the others (mean 4.236X10^7, ranged between 3.716X10^7 – 4.160X10^7 cells/10 ml), followed by CD4 T cells (mean 3.443X10^7, ranged between 3.280X10^7 – 3.740X10^7 cells/10 ml). Finally, the lowest value was with the CD8 T cell subset (mean 1.469X10^7, ranged between 0.860X10^5 – 1.938X10^7 cells/10 ml).

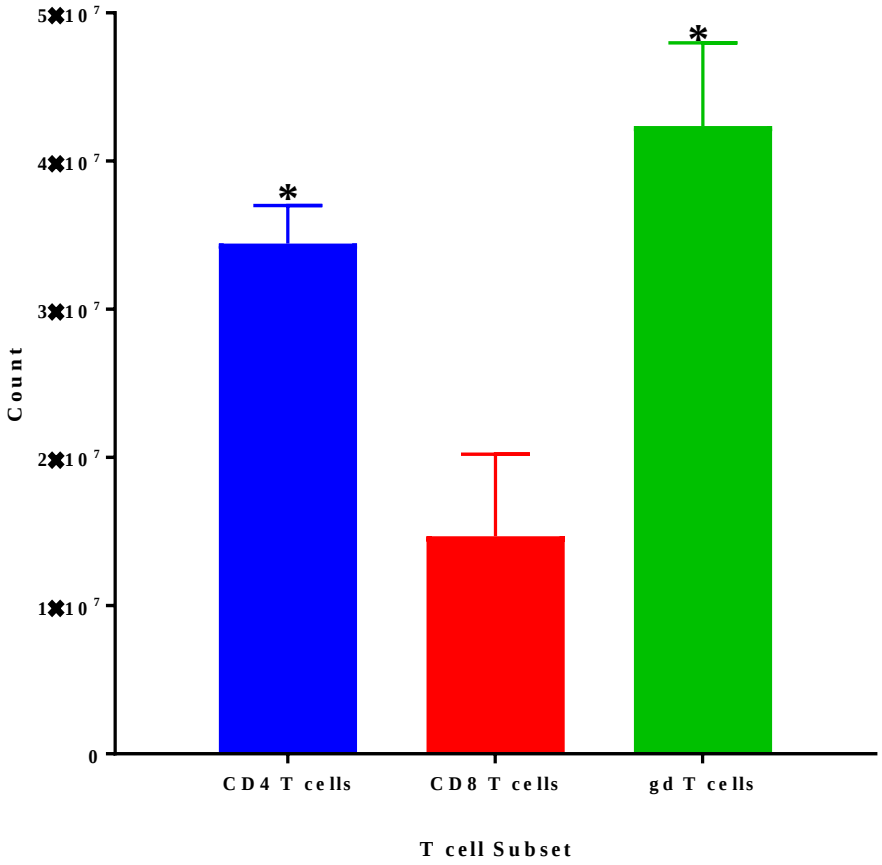


Figure 4.234: CD4, CD8 and γδ T mean cell counts +/- sem (counted by haemocytometer) fractionated by MACS from 10^8 PBMC cells from 5 animals .
*** = presence of significant differences at P<0.05.**

4.7.1 Flow cytometry analysis of T cell subsets fractionated by MACS (purity test)

The purity of fractioned CD4, CD8 and γδ T cells subsets separated by MACS method was checked by flow cytometry (see general materials and methods sections 2.7.3 and 2.7.4). The results illustrated high purity for the fractionated CD4 T cells and slightly lower purities for the CD8 and γδ T cells. The purity ranged between 90% - 96.9% in fractionated CD4 T cells, 82% - 87.1% in CD8 and 82% - 91.4% in γδ T cells (see figure 4.25).

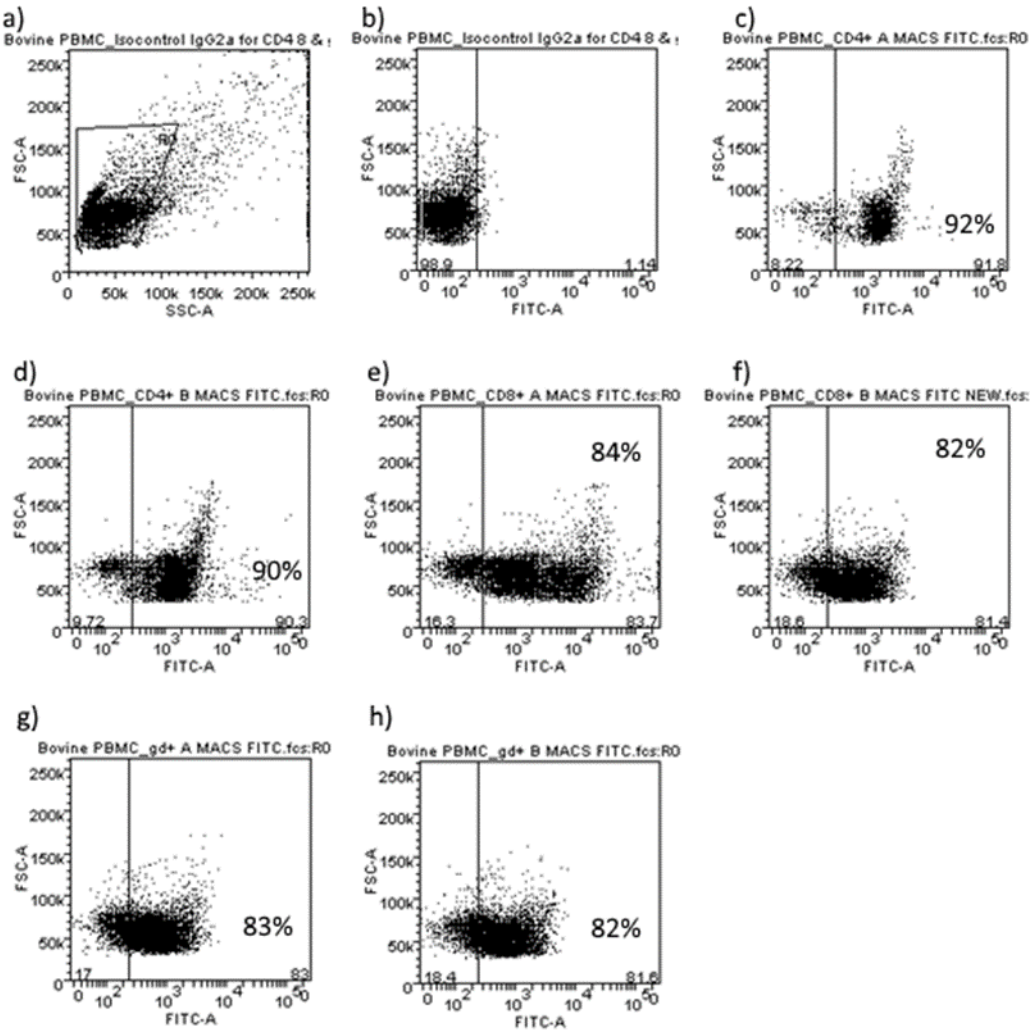


Figure 4.245: MACS enrichment of CD4+ T cells, CD8+ T cells and WC1+ γδ T cells in PBMC samples 'Bov A' and 'Bov B'. (a) gated mononuclear cell population, bov A. (b) isotype antibody-FITC control, bov A. (c) MACS enriched CD4+ T cell population, sample Bov A. (d) MACS enriched CD4+ T cell population, sample B. (e) and (f) MACS enriched CD8+ T cell population, sample A and sample B respectively. (g) and (h) MACS enriched WC1+ γδ T cells in PBMC samples from Bov A' and Bov B' respectively.

4.8 Bovine TLR gene expression

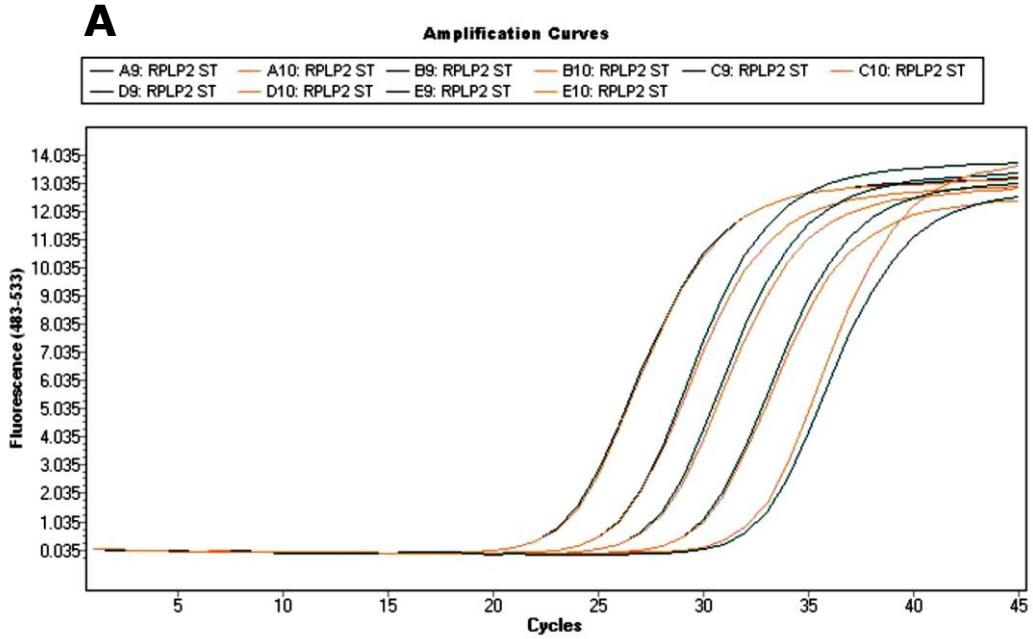
4.8.1 Choice of reference genes for TLR expression analysis by RT-qPCR

Gene expression by RT-qPCR for target genes must be done by comparing target genes together with reference genes (Pfaffl, 2001, Pfaffl and

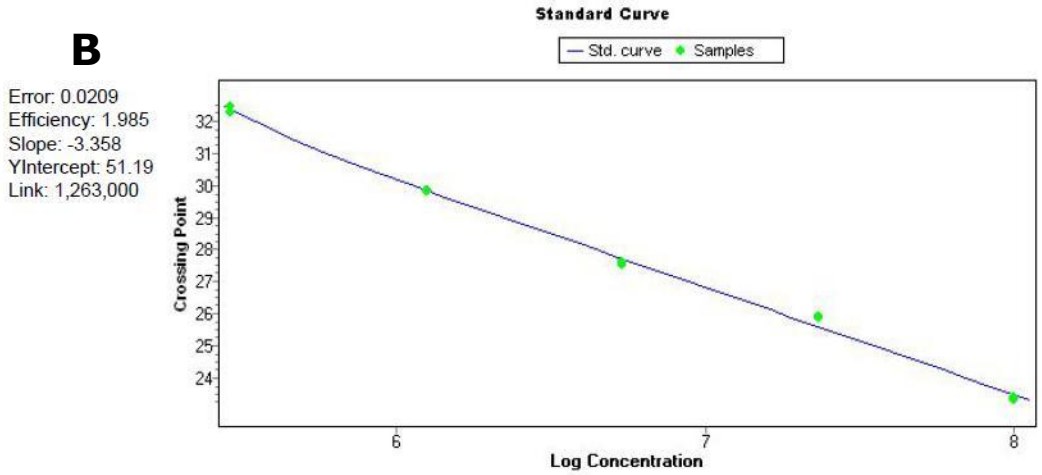
Hageleit, 2001, Bustin, 2002). Selecting the best reference gene depends on the consistency of its expression in cells with different treatments (Schmittgen and Livak, 2008, D'haene, 2013, Emam *et al*., 2015, Svingen *et al*., 2015).

GAPDH, RPLP0 and RPLP2 were selected as reference genes from the literature and from colleagues working with bovine cells (section 2.8.4.3). The RNA used in this experiment was extracted from $2X10^7$ PBMCs and cDNA was synthesised accordingly (2.8.1, 2.8.2 and 2.8.3). The results showed that the best reference gene was RPLP2 which gave an excellent standard curve efficiency of 1.985 where amplification efficiency was 98.52% and the Cq value= 23.33 (figure 4.26). In addition, it gave consistent expression in all the samples analysed, whereas the other reference genes did not. In contrast, GAPDH and RPLP0 were not as efficient as RPLP2, thus they were not used in further experiments. RPLP0 gave excellent standard curve efficiency of 1.987 (amplification efficiency 98.68%), but it showed only a fair Cq value= 26.98 (figure 4.27). In addition, it was not expressed consistently. Finally, GAPDH was the worst and excluded because of irregular standard curve efficiency of 1.904 (amplification efficiency 90.42%) and a very high Cq value = 33.21 as well as expression missing in some experiments (figure 4.28).

A


Amplification Curves
A9: RPLP2 ST
A10: RPLP2 ST
B9: RPLP2 ST
B10: RPLP2 ST
C9: RPLP2 ST
C10: RPLP2 ST
D9: RPLP2 ST
D10: RPLP2 ST
E9: RPLP2 ST
E10: RPLP2 ST
Fluorescence (483-533)
14.035
13.035
12.035
11.035
10.035
9.035
8.035
7.035
6.035
5.035
4.035
3.035
2.035
1.035
0.035
5
10
15
20
25
30
35
40
45
Cycles

B


Standard Curve
Std. curve
Samples
Error: 0.0209
Efficiency: 1.985
Slope: -3.358
YIntercept: 51.19
Link: 1,263,000
Crossing Point
32
31
30
29
28
27
26
25
24
6
7
8
Log Concentration

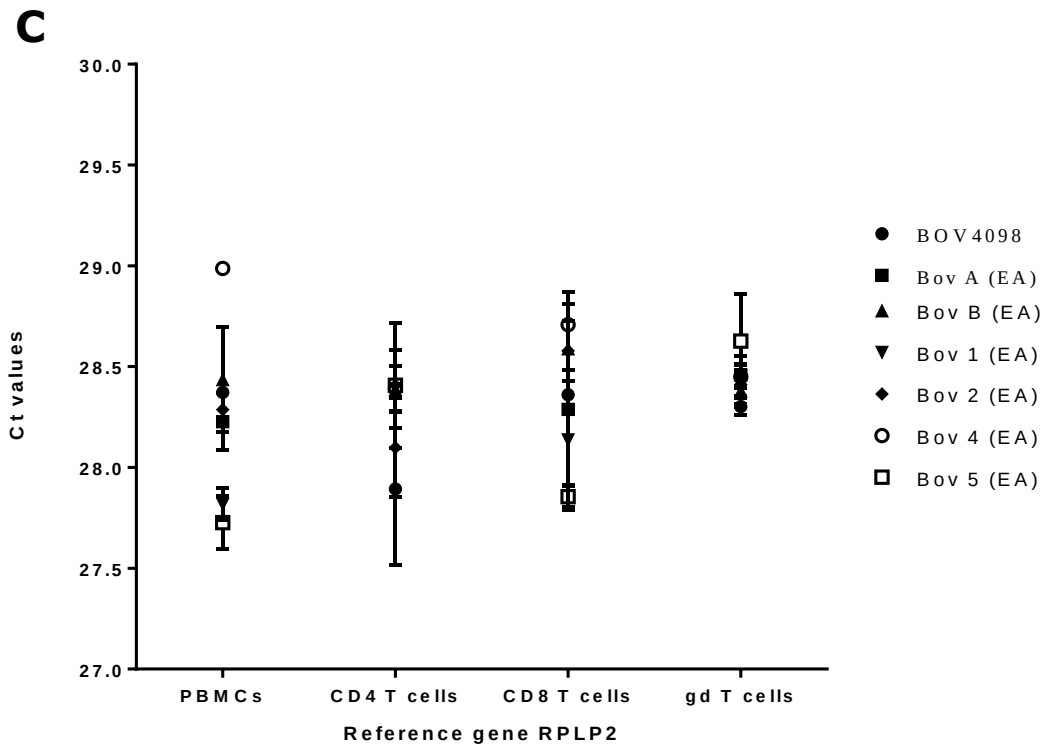


Figure 4.256: RPLP2 amplification and standard curves. (A) Amplification curve showing the Cq values starting from cycle 23 of the 10-fold serial diluted reference gene. (B) Standard curve efficiency showing the consistency of amplification through the straight line that is linking each diluted sample. Amplification efficiency =98.52%. (C) Mean Ct values (n=3) ± SD for reference gene RPLP2 in the assayed animals.

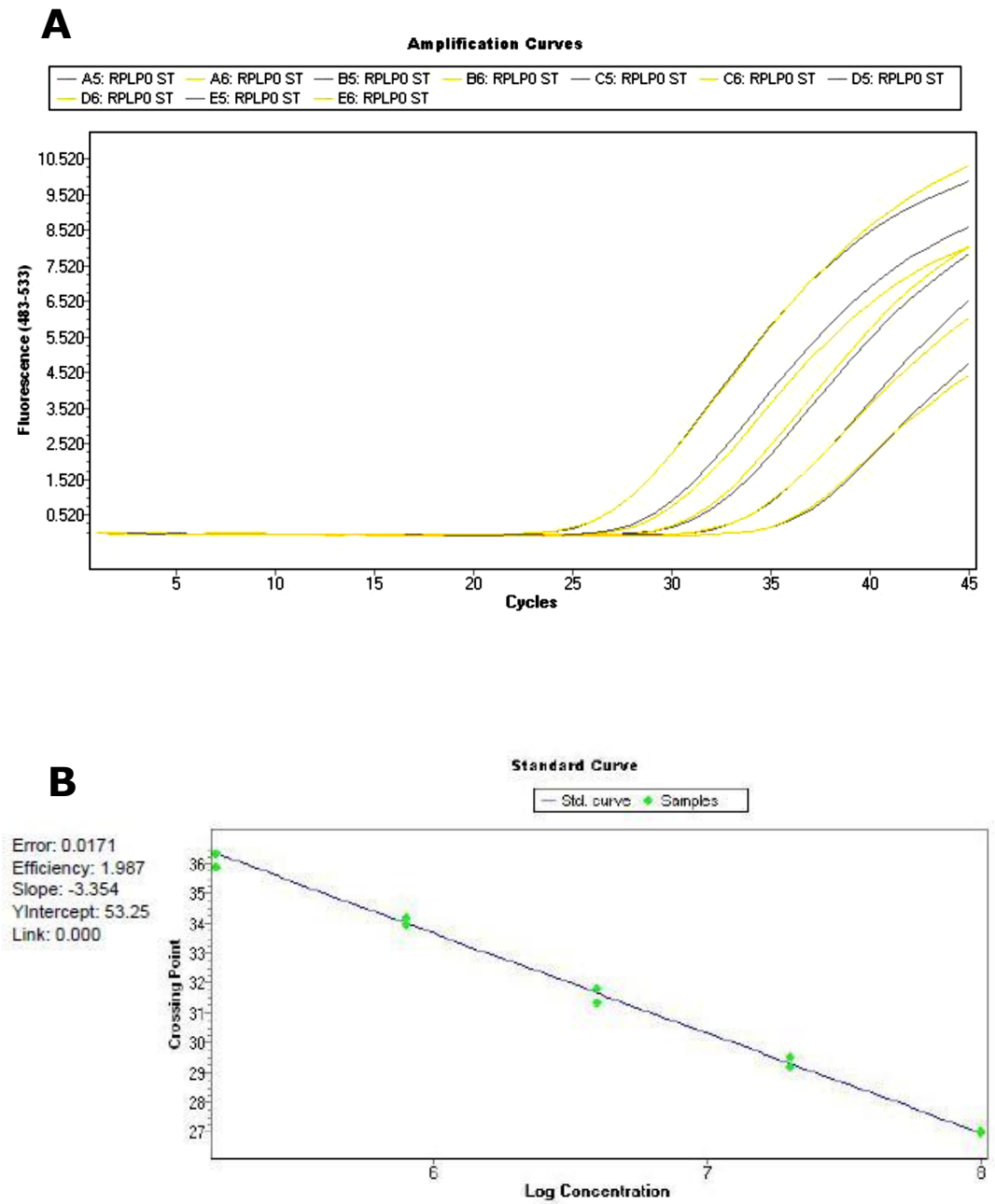


Figure 4.267: RPLP0 amplification and standard curves. (A) Amplification curve showing the Cq values starting from cycle 26 of the 10-fold serial diluted reference gene. (B) Standard curve efficiency is showing the consistency of amplification through the straight line that is linking each diluted sample. Amplification efficiency =98.68%.

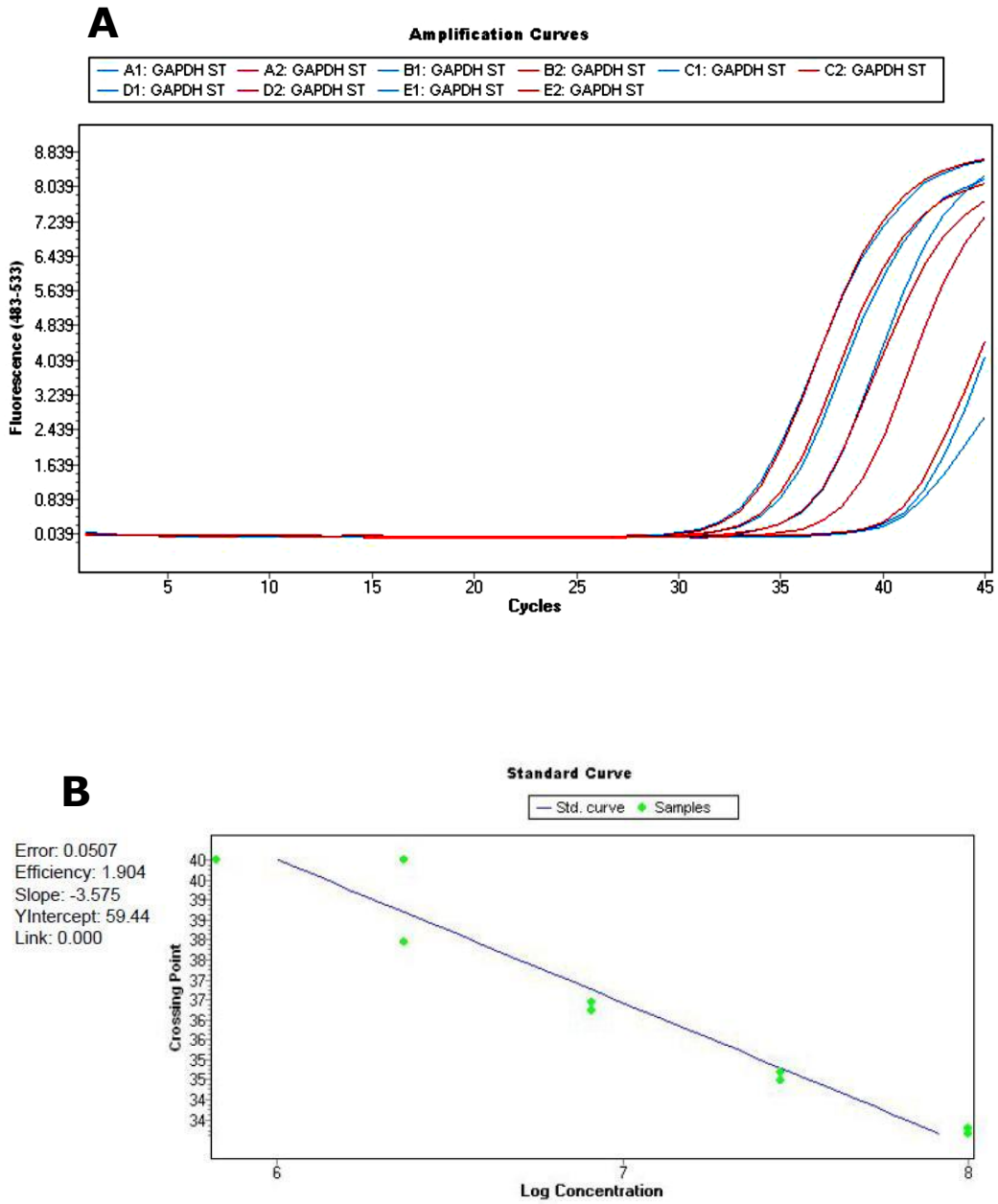


Figure 4.278: GAPDH amplification and standard curves. (A) Amplification curve showing the Cq values started from cycle 32 of the 10-fold serial diluted reference gene. (B) Standard curve efficiency showing the inconsistency of amplification through the straight line that is linking each diluted sample. Amplification efficiency =90.42%.

4.8.2 Gel analysis of TLRs and reference gene amplicons after expression measurement by RT-qPCR

RNA used in this experiment was extracted from $2X10^7$ PBMCs and cDNA was synthesised accordingly (sections 2.8.1, 2.8.2 and 2.8.3). One microgram µg of cDNA was prepared from each RNA sample. After that, cDNA samples were used as templates for RT-qPCR as described in section 2.8.4.3. Three reference genes (GAPDH, RPLP0 and RPLP2) were selected to allow a comparison of the expression of 10 TLR target genes. Gel imaging (figure 4.29) was done (section 2.8.4.2) to show the molecular size of each band (ranged from ~70 bp to ~260 bp). The two terminal wells were loaded with low molecular weight DNA ladder reagent. All genes gave a clear band at the expected size, however amplification of GAPDH relatively poor.

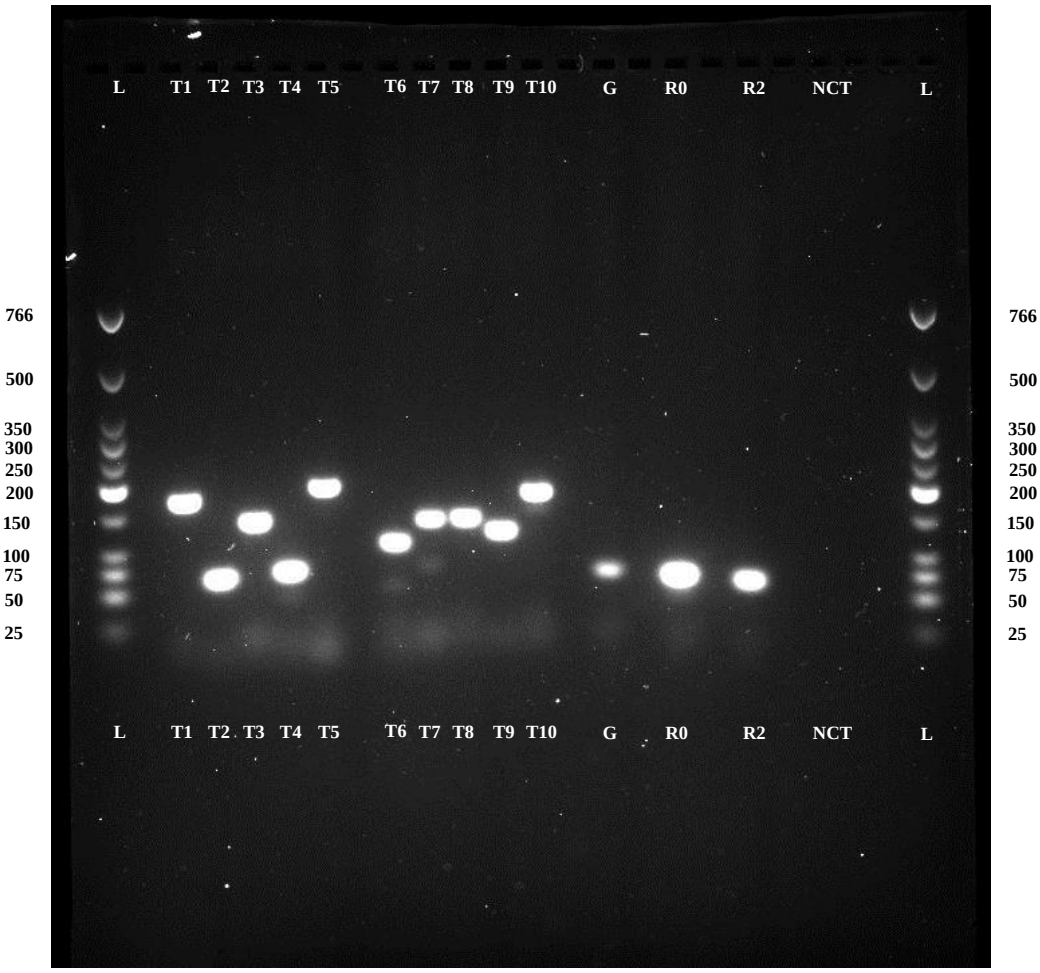


Figure 4.289: DNA products of reference and target genes expressed in PBMCs, amplified by RT-qPCR and analysed on a 2% agarose gel. Amplicon size unit is bp (base pair); L= Low molecular weight Ladder; T1-T10= TLR1-TLR10; G= GAPDH; R0= RPLP0; R2= RPLP2 and NCT= Negative control.

4.8.3 RT-qPCR results of TLR1-10 gene expression in PBMCs.

4.8.3.1 RT-qPCR results of TLR1-10 gene expression on PBMCs

RNA was extracted from PBMCs then cDNA was synthesised and run in the Lightcycler 480 (Real time PCR) machine and data were analysed as described in section 2.8 and its subsections.

The results of TLRs expressed on PBMCs were presented as log cDNA copy number per normalised reference gene (RPLP2) as described in general materials and methods (sections 2.8.4.3 and 2.8.4.4). Bovine PBMCs from seven animals showed different levels of expression of some target genes (TLR genes) without statistical significant differences between them at $P \geq 0.05$ (figure 4.30). However, a few genes were not amplified in some blood samples. These were TLR5, TLR6 and TLR10 as illustrated in table 4.25.

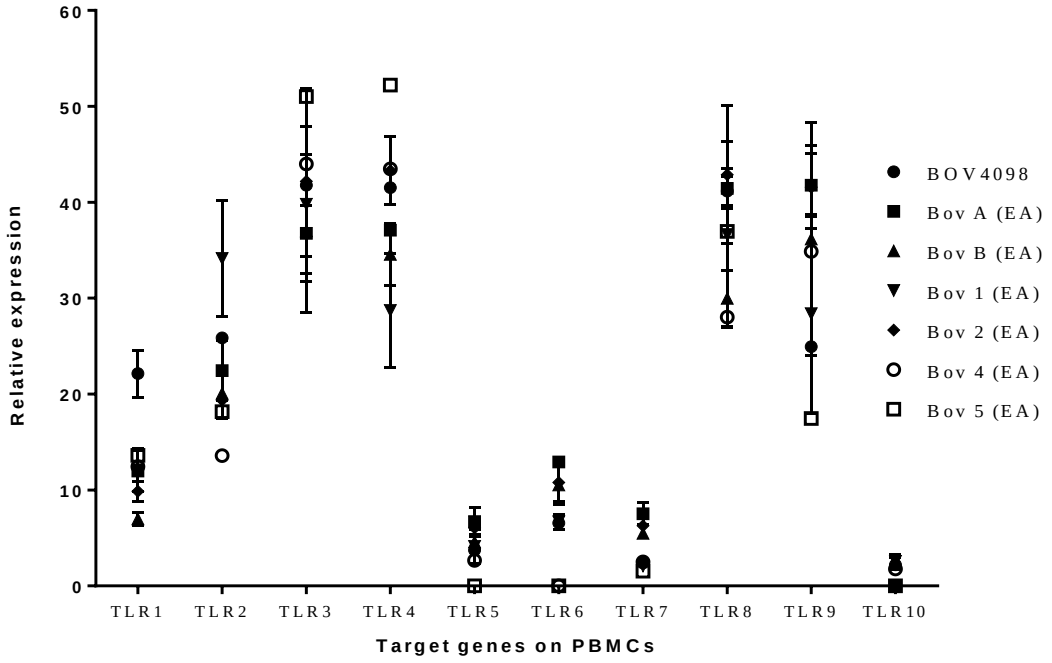


Figure 4.30: TLR 1-10 gene expression by RT-qPCR from 7 animals of bovine blood presented as the Mean ± standard error. The PBMCs expressed these TLR genes with no statistical significant differences between them, P>0.05.

Table 4.14: Presence/absence of TLR target genes on PBMCs measured by RT-qPCR from7 animals.

TLR	BOV4098	Bov A (EA)	Bov B (EA)	Bov 1 (EA)	Bov 2 (EA)	Bov 4 (EA)	Bov 5 (EA)
TLR1	+	+	+	+	+	+	+
TLR2	+	+	+	+	+	+	+
TLR3	+	+	+	+	+	+	+
TLR4	+	+	+	+	+	+	+
TLR5	+	+	+	+	+	+	-
TLR6	+	+	+	+	+	-	-
TLR7	+	+	+	+	+	+	+
TLR8	+	+	+	+	+	+	+
TLR9	+	+	+	+	+	+	+
TLR10	+	+	+	+	-	+	-

+ = Gene amplified (presence),
- = No amplification (absence).

4.8.3.2 RT-qPCR results of TLR1-10 gene expression in the fractionated T cell subsets

RNA was extracted from MACS fractionated T cell subsets (CD4, CD8 and γδ T cells) then cDNA was synthesised and run in the Lightcycler 480 (Real time PCR) machine. Data were analysed as described in the previous section.

CD4 T cells revealed TLR 1-10 gene expression with no significant differences at $P \geq 0.05$ compared to each other (figure 4.31). A few genes were not expressed in some animals. These were TLR5 and TLR6 (table 4.26).

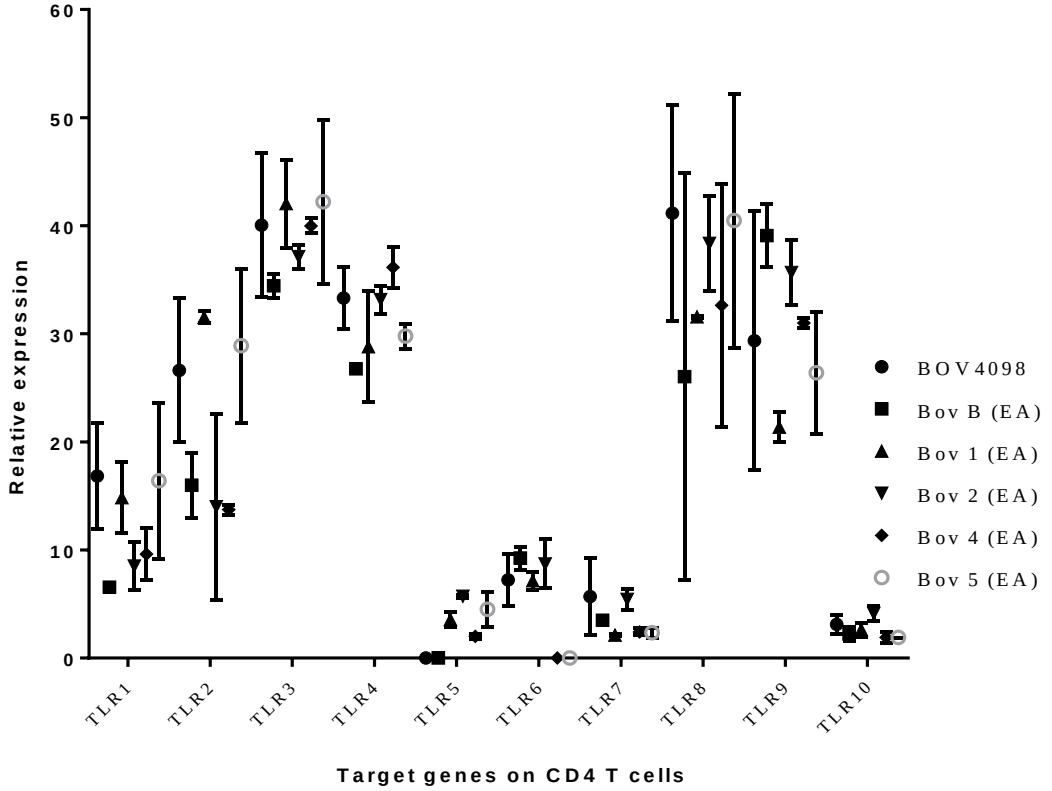


Figure 4.291: TLR 1-10 gene expression by RT-qPCR from 6 animals of bovine blood PBMC shown as the Mean ± standard error. Most CD4 T cells expressed genes with no statistical significant differences between them at P≥0.05.

Table 4.15: Presence/absence of TLR target genes on CD4 T cells measured by RT-qPCR from 7 animals.

TLR	BOV4098	Bov B (EA)	Bov 1 (EA)	Bov 2 (EA)	Bov 4 (EA)	Bov 5 (EA)
TLR1	+	+	+	+	+	+
TLR2	+	+	+	+	+	+
TLR3	+	+	+	+	+	+
TLR4	+	+	+	+	+	+
TLR5	-	-	+	+	+	+
TLR6	+	+	+	+	-	-
TLR7	+	+	+	+	+	+
TLR8	+	+	+	+	+	+
TLR9	+	+	+	+	+	+
TLR10	+	+	+	+	+	+

+ = Gene amplified (presence),
- = No amplification (absence).

CD8 T cells demonstrated TLR gene expression without significant differences at P≥0.05 compared to each other (figure 4.32). A few genes were not expressed in some blood animals. These are TLR5, TLR6 and TLR10 (table 4.27).

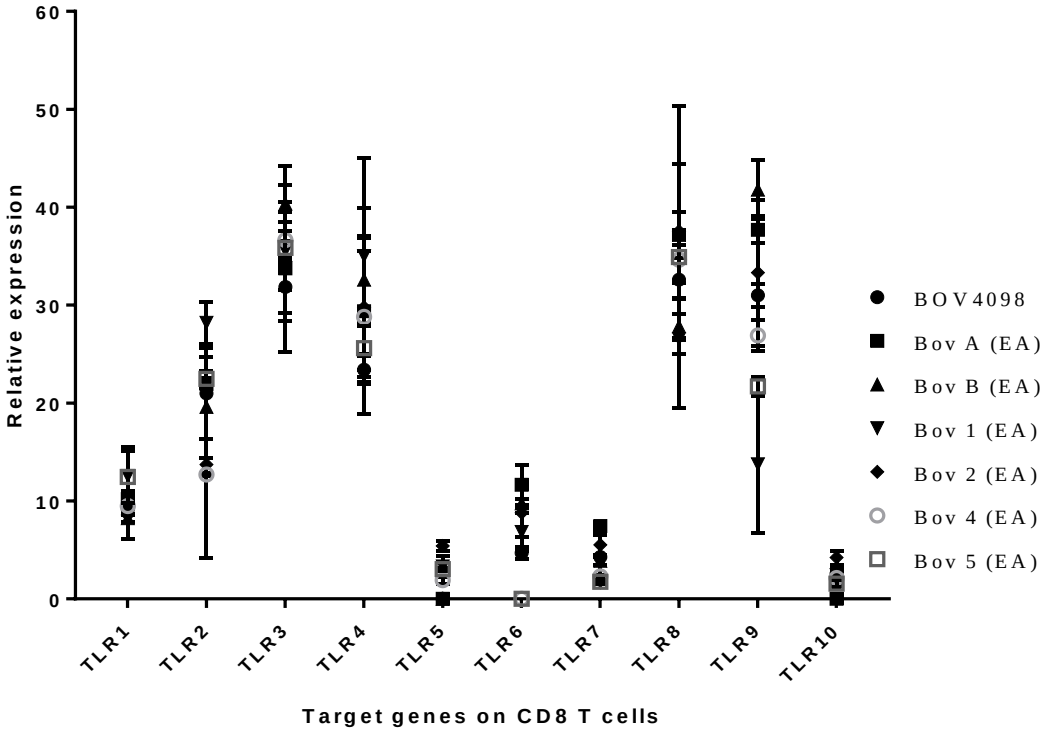


Figure 4.302: TLR 1-10 gene expression by RT-qPCR from 7 animals of bovine blood shown as the Mean ± standard error. Most CD8 T cells expressed TLR genes with no statistical significant differences between them at P≥0.05.

Table 4.16: Presence/absence of TLR target genes on CD8 T cells measured by RT-qPCR from 7 animals.

TLR	BOV4098	Bov A (EA)	Bov B (EA)	Bov 1 (EA)	Bov 2 (EA)	Bov 4 (EA)	Bov 5 (EA)
TLR1	+	+	+	+	+	+	+
TLR2	+	+	+	+	+	+	+
TLR3	+	+	+	+	+	+	+
TLR4	+	+	+	+	+	+	+
TLR5	-	-	-	+	+	+	+
TLR6	+	+	+	+	+	-	-
TLR7	+	+	+	+	+	+	+
TLR8	+	+	+	+	+	+	+
TLR9	+	+	+	+	+	+	+
TLR10	+	-	+	+	+	+	+

+ = Gene amplified (presence),
- = No amplification (absence).

γδ T cells demonstrated higher TLR gene expression without significant differences at P≥0.05 compared to each other (figure 4.33). Only TLR6 gene was not expressed in two blood animals of PBMC sample (table 4.28).

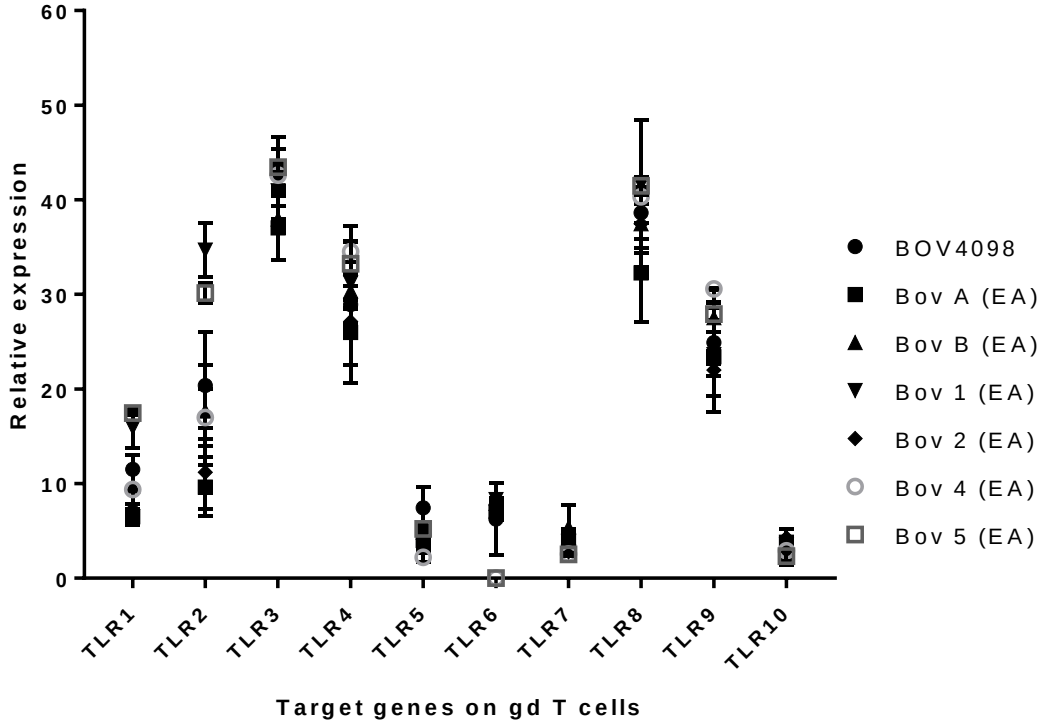


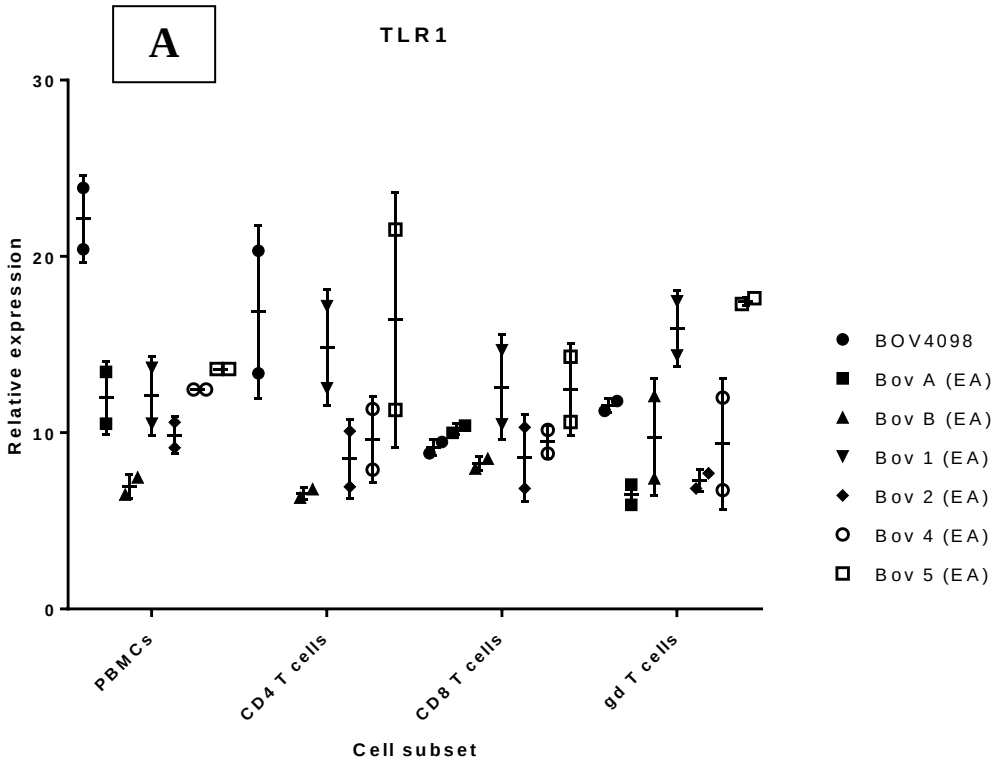
Figure 4.313: TLR 1-10 gene expression by RT-qPCR from 7 animals of bovine blood shown as the Mean ± standard error. Most γδ T cells expressed TLR genes with no statistical significant differences between them at P≥0.05.

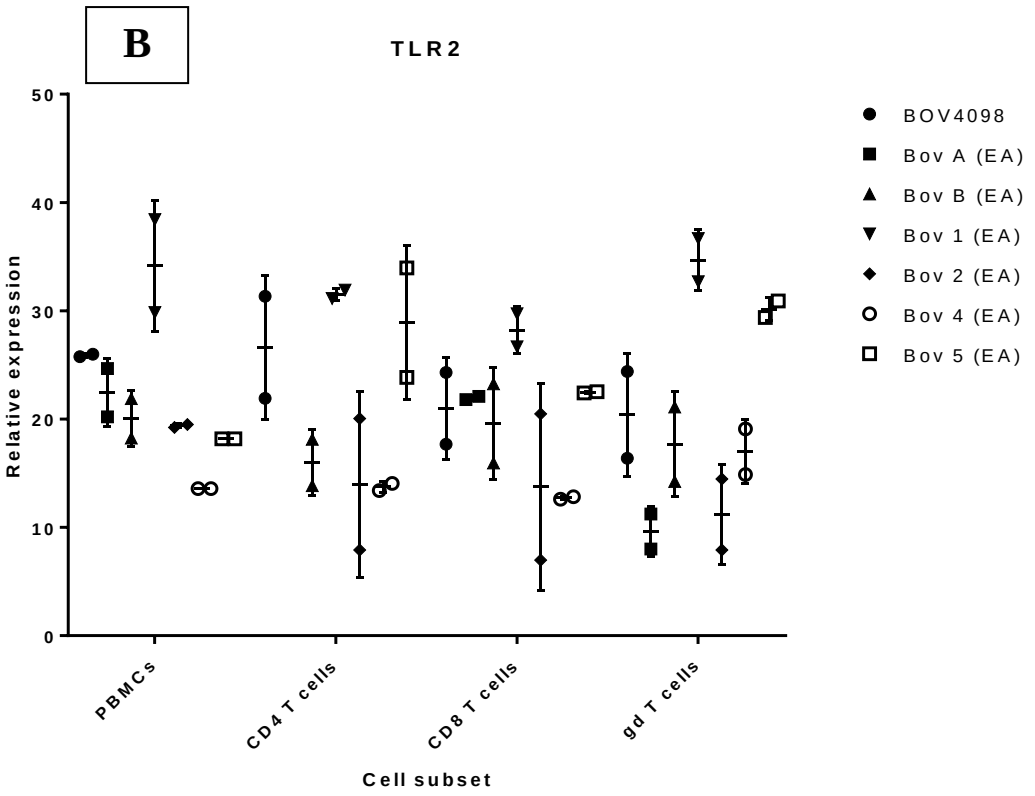
Table 4.17: Presence/absence of TLR target genes on γδ T cells measured by RT-qPCR from7 animals.

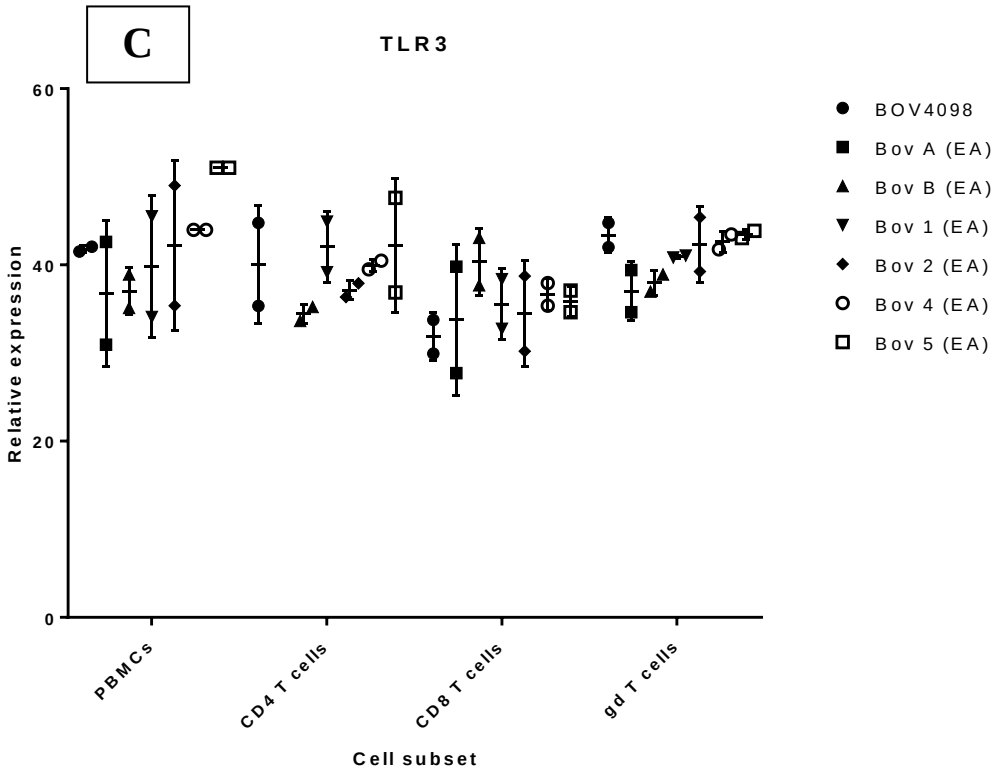
TLR	Sera Lab	Elliot abattoir					
	BOV4098	Bov A (EA)	Bov B (EA)	Bov 1 (EA)	Bov 2 (EA)	Bov 4 (EA)	Bov 5 (EA)
TLR1	+	+	+	+	+	+	+
TLR2	+	+	+	+	+	+	+
TLR3	+	+	+	+	+	+	+
TLR4	+	+	+	+	+	+	+
TLR5	+	+	+	+	+	+	+
TLR6	+	+	+	+	+	-	-
TLR7	+	+	+	+	+	+	+
TLR8	+	+	+	+	+	+	+
TLR9	+	+	+	+	+	+	+
TLR10	+	+	+	+	+	+	+

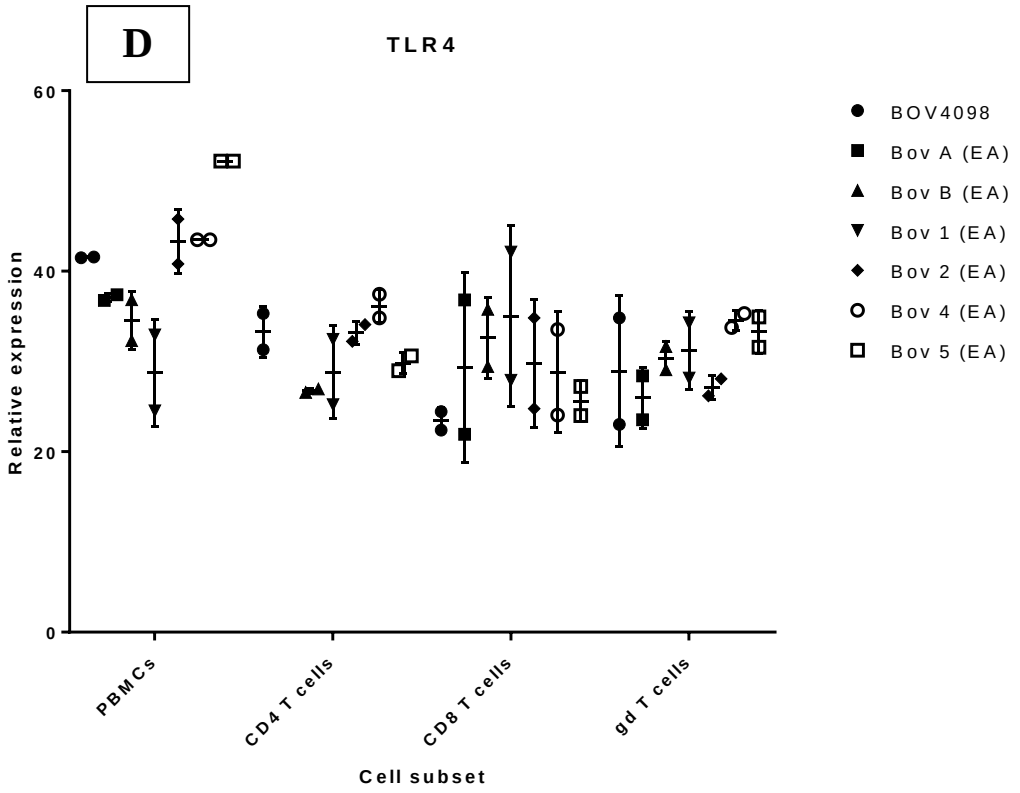
+ = Gene amplified (presence),
- = No amplification (absence).

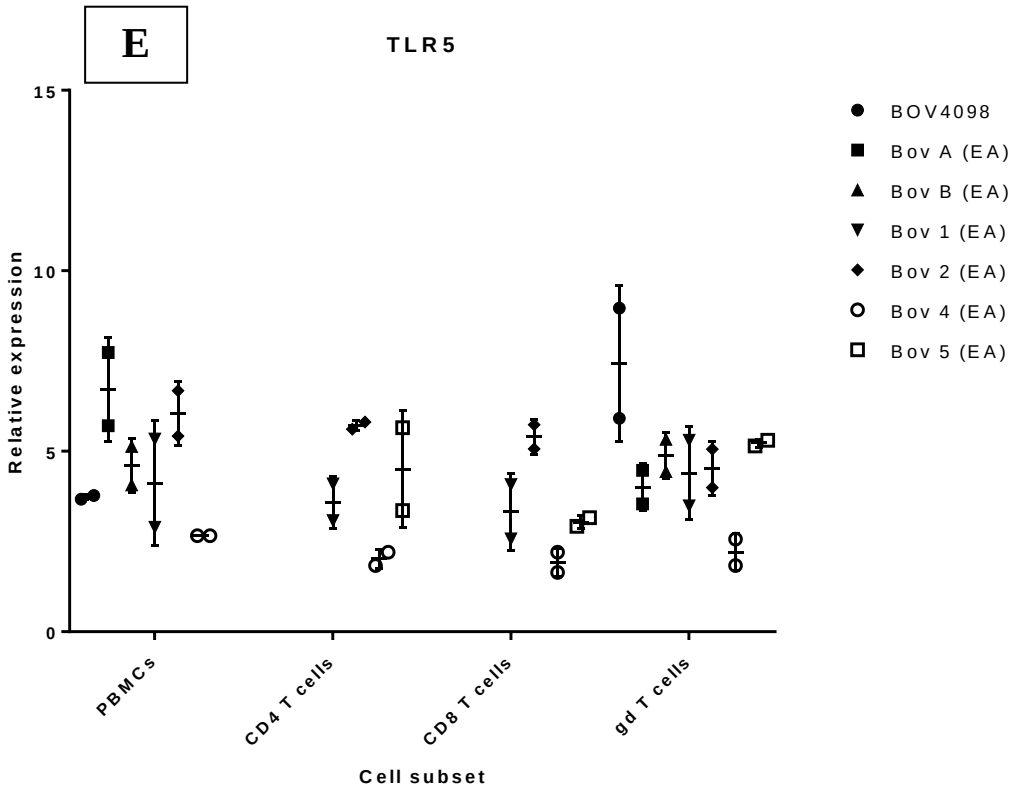
Graphs shown below (figure 4.34) are the same results of RT-qPCR but presented in the other way to show the difference between individual animals on the same graph. There are no significant differences.

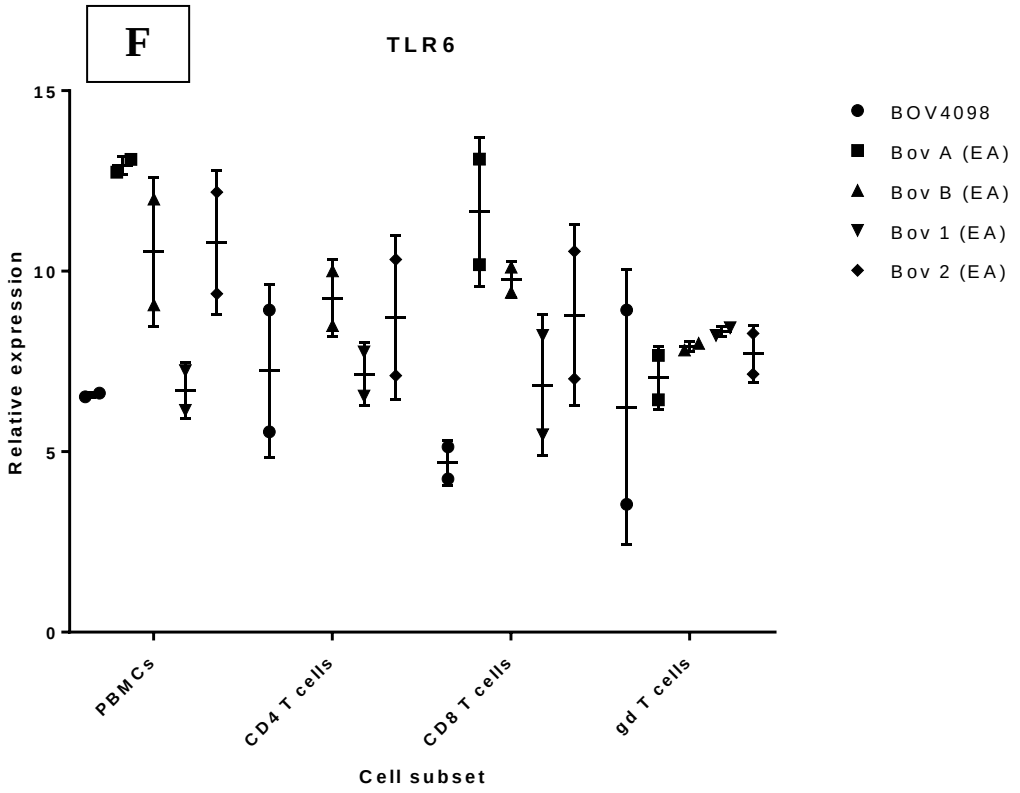


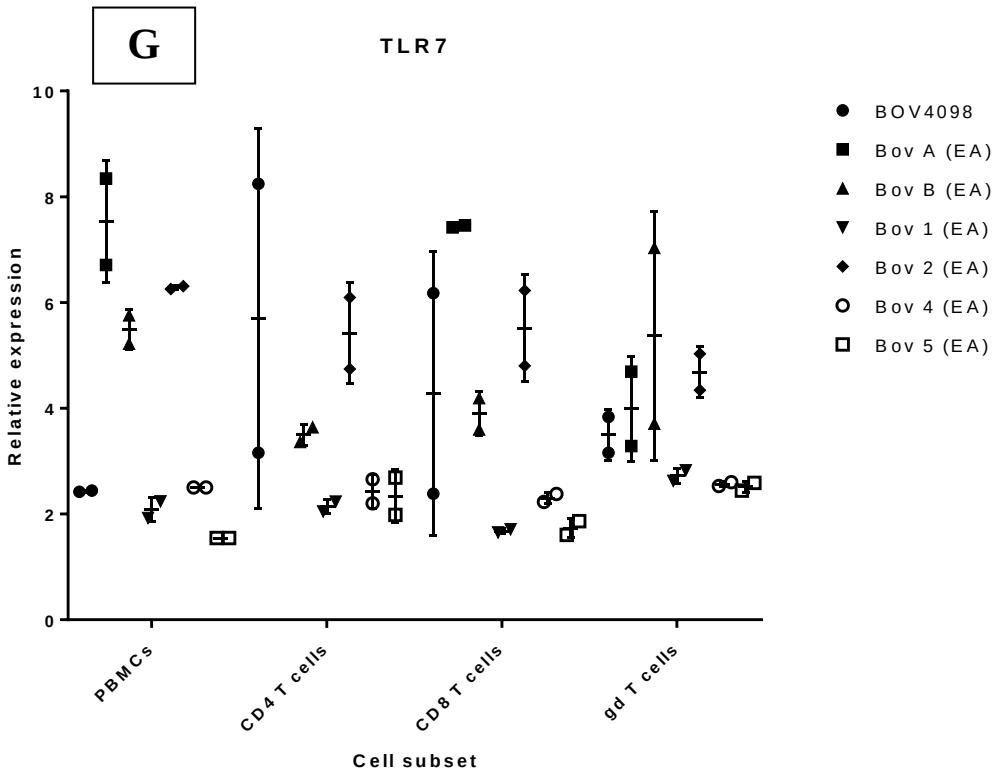

B
TLR2
Relative expression
0
10
20
30
40
50
PBMCs
CD4 T cells
CD8 T cells
gd T cells
Cell subset
BOV4098
Bov A (EA)
Bov B (EA)
Bov 1 (EA)
Bov 2 (EA)
Bov 4 (EA)
Bov 5 (EA)

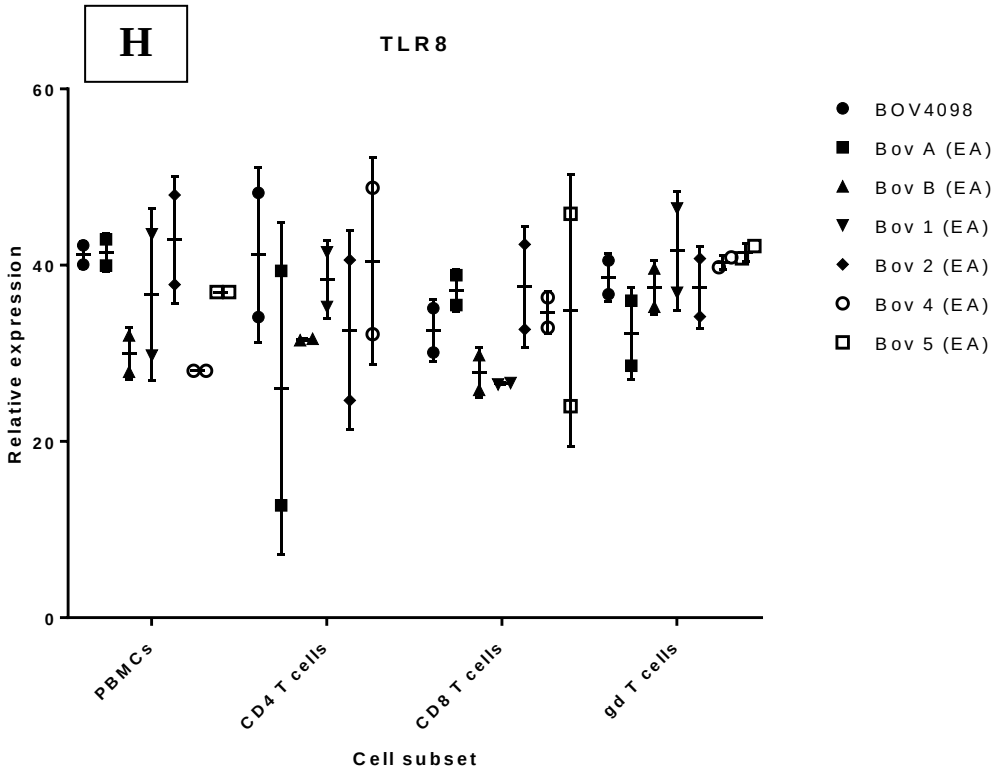

C
TLR3
Relative expression
0
20
40
60
PBMCs
CD4 T cells
CD8 T cells
gd T cells
Cell subset
BOV4098
Bov A (EA)
Bov B (EA)
Bov 1 (EA)
Bov 2 (EA)
Bov 4 (EA)
Bov 5 (EA)

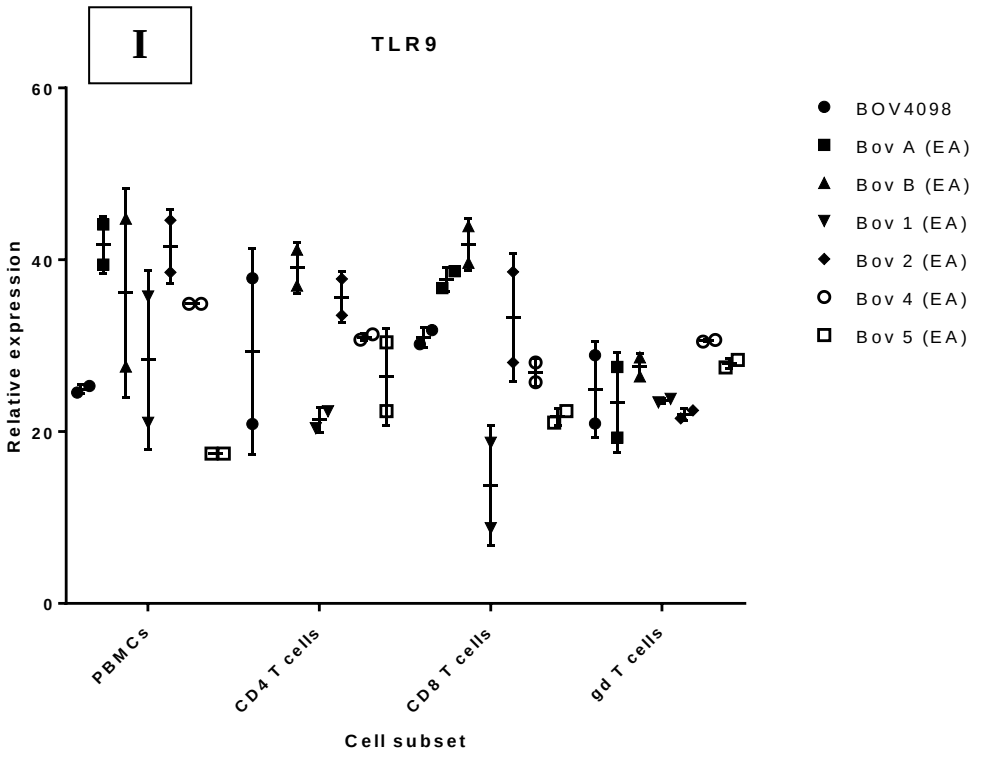

D
TLR4
60
40
20
0
Relative expression
PBMCs
CD4 T cells
CD8 T cells
gd T cells
Cell subset
BOV4098
Bov A (EA)
Bov B (EA)
Bov 1 (EA)
Bov 2 (EA)
Bov 4 (EA)
Bov 5 (EA)


E
TLR5
15
10
5
0
Relative expression
PBMCs
CD4 T cells
CD8 T cells
gd T cells
Cell subset
BOV4098
Bov A (EA)
Bov B (EA)
Bov 1 (EA)
Bov 2 (EA)
Bov 4 (EA)
Bov 5 (EA)


F
TLR6
Relative expression
15
10
5
0
PBMCs
CD4 T cells
CD8 T cells
gd T cells
Cell subset
BOV4098
Bov A (EA)
Bov B (EA)
Bov 1 (EA)
Bov 2 (EA)


G
TLR7
Relative expression
10
8
6
4
2
0
PBMCs
CD4 T cells
CD8 T cells
gd T cells
Cell subset
BOV4098
Bov A (EA)
Bov B (EA)
Bov 1 (EA)
Bov 2 (EA)
Bov 4 (EA)
Bov 5 (EA)


H
TLR8
Relative expression
60
40
20
0
PBMCs
CD4 T cells
CD8 T cells
gd T cells
Cell subset
BOV4098
Bov A (EA)
Bov B (EA)
Bov 1 (EA)
Bov 2 (EA)
Bov 4 (EA)
Bov 5 (EA)


I
TLR9
Relative expression
60
40
20
0
PBMCs
CD4 T cells
CD8 T cells
gd T cells
Cell subset
BOV4098
Bov A (EA)
Bov B (EA)
Bov 1 (EA)
Bov 2 (EA)
Bov 4 (EA)
Bov 5 (EA)

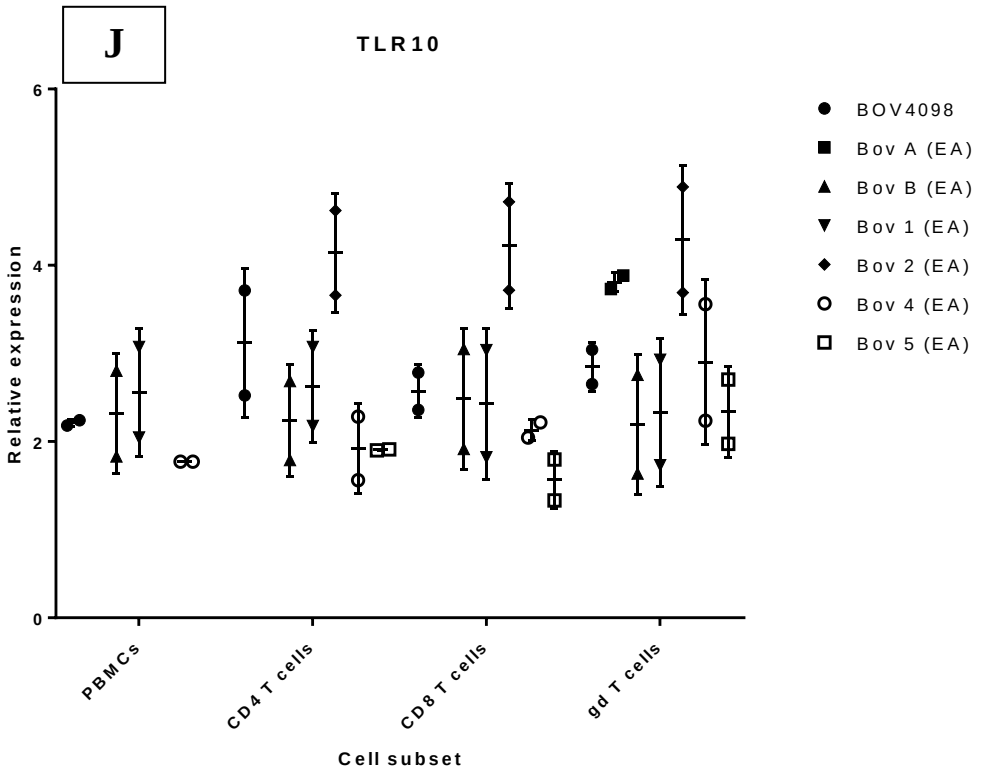


Figure 4.34. TLR expression in the studied cells (PBMCs, CD4 T cells, CD8 cells and gamma delta T cells) presented in groups to show the comparison. ANOVA was used for statistical analysis without any significant differences between cells at P>0.05 level. A: TLR1, B: TLR2, C: TLR3, D: TLR4, E:TLR5, F: TLR6, G: TLR7, H: TLR8, I: TLR9 and J: TLR10.

4.9 Discussion

Successful isolation of PBMCs from bovine blood and fractionation of T cell subsets CD4+, CD8+ and gd T cells was achieved in this study. Furthermore, expression of TLRs 1-10 was seen in the PBMC from the different animals. PBMC would include cells other than CD4+ T cells, CD8+ T cells and gd T cells, - principally monocytes that would have contributed to the TLR expression data. The PBMC were then fractionated into T cell subsets for TLR expression analysis. Fractionation of bovine PBMCs generally is characterised by higher numbers of gamma delta T cells (a feature of ruminants) which is compatible with what we found in this study which was around 30% (Jutila *et al*., 1994, Herzig and Baldwin, 2009, Baldwin and Telfer, 2015). CD8 T cells are less frequent in bovine PBMCs (approximately 10-15%) compared to CD4 T cells (range roughly 20% - 35%). Immunological status, age, gender and species could possibly affect these levels (Ayoub and Yang, 1996, Anderson *et al*., 1999, Hope *et al*., 2000, Sopp and Howard, 2001, Denholm *et al*., 2014, Kim *et al*., 2016). The T cell purities are adequate for CD4, gd T cells and CD8+ T cells to demonstrate TLR expression on these cells, although it would be preferable to have purities >98% in all cases to be confident that TLR expression is specific for the T cell subset under analysis. The purity of CD8 T cells was generally lower than for CD4+ T cells and γδ T cells.

PBMCs could be reduced to a very low count (less than 10%) in late pregnancy and during calving (Kimura *et al*., 1999). However, administration of recombinant bovine granulocyte colony stimulating factor to the pregnant cows may lead to significant increase in the total count of PBMCs after birth but not during pregnancy (Harp *et al*., 1991). PBMC

stimulated with concanavalin A isolated from Holstein and Brown Swiss cows and subjected to temperature from 39 to 43 ℃ showed decline in DNA synthesis of these PBMCs which reached to 22% in Brown Swiss cows and 40% Holstein cows less than normal in the two breeds (Lacetera *et al*., 2006). Adding food supplement such as Selenium to the diet of heifers did not show any effect on the total number of PBMCs in calves (Brennan *et al*., 2010). The effect of zinc supplementation (*in vitro*) on the cultured PBMC viability was ranged between 69% to 86% (Sheikh *et al*., 2016).

TLR expression on innate immune cells has been extensively studied in humans and mice as sensory molecules for the detection of infection leading to induction of inflammatory responses (Skevaki *et al*., 2015). Other studies, mainly in the human and mouse have underlined the importance of TLRs in innate immune recognition. Two subcategories of TLRs have been identified. Those that are expressed on the surface (e.g. TLR1, TLR2, TLR4, TLR5 and TLR6) and those that are intracellular (TLR3, TLR7, TLR8 and TLR9). Bacterial PAMPs can be recognised by TLR1, TLR2, TLR4, TLR5 and TLR6, whereas viral antigens are usually sensed by TLR3, TLR7, TLR8 and TLR9 (Filippi, 2015, Skevaki *et al*., 2015), although there are overlaps.

Expression of TLRs in ruminants, and particularly bovine immune cells has been studied (Jungi *et al*., 2011, Werling, 2012, Baldwin and Telfer, 2015). Werling and colleagues studying bovine TLRs indicated that these were very similar reagarding amino acid sequence homology and overall predicted structure to those of mice and humans (McGuire *et al*., 2006). Werling *et al*. (2009) and Jungi *et al*., (2011) highlighted the importance of TLRs in the bovine compared to those in other species regarding their

possible role as adjuvants for vaccines. The importance of bovine TLR in dendritic cells, and TLR comparisons in bovine species has been described (Bilgen *et al*., 2016, Gibson *et al*., 2016, Tombácz *et al*., 2017, Werling *et al*., 2017).

Peptidoglycan, double stranded RNA viruses, and LPS stimulated bovine PBMCs *in vitro* mediated by TLR2, TLR3, and TLR4 expression in the cells - mainly monocytes and lymphocytes (Doherty *et al*., 2013, Feldman *et al*., 2013, Singh *et al*., 2014, Karthikeyan *et al*., 2016, Martins *et al*., 2016).

Molecular detection of TLR1-10 genes in two ruminant species, ovine (sheep) and bovine (cattle), PBMCs commenced in 2006 when Menzies and Ingham used RT-qPCR to scan for these TLRs in a trial to compare their structural sequences with human TLRs (Menzies and Ingham, 2006). We used the advanced model of Menzies and Ingham's method for TLR1-10 gene detection. It is a TaqMan method where primers and probes were used to ensure specific and accurate quantification.

Our findings in this study reveal TLR expression on bovine CD4 T cells. This has also been recorded under pathological conditions in cells expressed in parasitic infestation of cattle with the protozoan parasite *Neospora caninum* (Mineo *et al*., 2010). Also, interestingly, in humans, TLR2 and TLR4 were expressed only under pathological conditions but not on cells from healthy individuals (Zastepa *et al*., 2014, Zhang *et al*., 2014, Borges da Silva *et al*., 2015). The activation state of the cells in our cattle used in this study is not known, although all animals presented disease-free and healthy.

Possibly related to activation state /healthy status of our cattle, it is interesting to note that in humans and mice CD8 T cells did not express TLRs unless stimulated. Specifically, T cytotoxic cells (CD8+ T cells) responded to viral particles and tumours by expressing intracellular TLR3 and TLR7, 8 and 9 genes (Marcondes *et al*., 2012, Melief, 2013, Mandraju *et al*., 2014, Schölch *et al*., 2015, Vanders *et al*., 2015, Wu *et al*., 2015b). In addition to intracellular TLRs, cytotoxic T cells (CD8+ T cells) have also been shown to express TLR4, which is involved in recognition of LPS in Gram negative bacteria (Komai-Koma *et al*., 2009). Moreover, stimulation of human neonatal cytotoxic T cells with TLR agonists (Pam3Cys and flagellin) encouraged them to express TLR2 and TLR5 genes (responsible for detection of peptidoglycan and flagellins, both found in bacteria (McCarron and Reen, 2009). In summary, to date, human CD8+ T cells have the capability to synthesise extracellular TLR1, TLR2, TLR4, 5 and 6 plus intracellular TLR3 and TLR9 (Freeman *et al*., 2013).

Finally, the MACS-separated γδ T cells showed expression of all TLR1-10 genes. This particular subset of T cells in cattle has not been studied in terms of TLR expression until now. However, human γδ T cells express intracellular TLR3 (Beetz *et al*., 2008). In addition, human γδ T cells activated with lipid A (part of endotoxin of gram negative bacterial cell membranes) expressed TLR2 and TLR4 (Cui *et al*., 2009). Pietschmann *et al*. (2009) observed expression of TLRs 1-9 in human γδ T cells at variable frequencies ranging from high expression of TLR1, TLR2, TLR6, and TLR7 genes, to lower expression of TLR3 and very low expression of TLR5 and TLR8.

This subset of T cells (γδ) is of particular interest to us as it is thought to be an important regulatory T cell subset in cattle with a role in terminating immune responses (suppressive function) either instead of or in addition to CD4+CD25+FOXP3+ T cells (nTregs) (Dar *et al*., 2014, Guzman *et al*., 2014, Baldwin and Telfer, 2015). In conclusion, the expression of TLR mRNAs in T cell subsets in cattle has been demonstrated in this study.

Chapter Five

Stimulation of PBMC and T cells with TLR agonists

5.1 Abstract

In this chapter the functional status of TLR receptors on T cells was tested by stimulating them with their ligands (TLR agonists) and assessing cell proliferation (by the CFSE method) as a read-out. Two approaches were taken: firstly analysis of purified T cells (CD4+, CD8+ and γδ T cells) from each of the bovine PBMC samles and secondly to examine bovine CD4, CD8 and γδ T cell function within the unfractionated PBMC population upon stimulation with TLR agonists (i.e. analysis of each T cell subset proliferation within PBMC without prior fractionation). This was to determine if T cell TLR activation could be detected within the context of whole PBMC. This aim was achieved by using a panel of T cell subset-specific antibodies conjugated to non-overlapping (signal) fluorophores: CD4:RPE, CD8:Alexa Fluor® 647, and WC1gd T cell:FITC.

TLR1-9 agonists (ligands) were used at 3 concentrations to stimulate bovine CFSE-labelled PBMCs and the major T cell subsets (CD4 T cells, CD8 T cells, and γδ T cells). There was then a focus on TLR3, TLR8, and TLR9 ligands to stimulate intracellular TLR3, TLR8, and TLR9 in CD4, CD8, and γδ T cells (MACS fractionated) separately. This selection prioritisation was because of the limited number of fractionated T cell subset cells (MACS method) obtained, and that intracellular TLR3, TLR8, and TLR9 were prioritised as play a role in detecting viral particles, an interest of our research group.

Finally, the third objective was to further study whether γδ T cells had immunosuppressive activity on autologous T cells as has been described previously (Guzman *et al*., 2014).

Regarding the two approaches to determining T cell TLR activation by measuring CFSE cell division, it was clear after several attempts with a selection of the different animal samples that the measurement of proliferation (cell division by CFSE) was giving inconsistent results. Therefore, some examples of this are given but a complete analysis of all animal samples was terminated in favour of measuring cytokine production as an indicator of T cell TLR activation by TLR agonists (Chapter 6).

In this limited study, there was a lack of CFSE-labelled PBMC cell division (proliferation) in any of the samples upon stimulation with TLR1-9 agonists compared to good proliferation in response to the T cell mitogen Con-A. Although generally inconclusive, purified CFSE-labelled CD4+ T cells revealed some proliferation when stimulated with TLR3, TLR8 and TLR9 agonists in Bov 1 sample, TLR3 and TLR8 agonists in Bov 5 sample and TLR8 agonist in Bov4 PBMCs ($P<0.05$). CD8+ T cells were activated /proliferated (CFSE assay) when stimulated with TLR3 and TLR9 ligands in Bov 1; TLR8 and TLR9 agonists in Bov 5 ; and TLR9 agonist in Bov 4 blood animals ($P<0.05$). Purified CFSE-labelled γδ T cells stimulated with TLR8 agonist in Bov 1 and TLR3 agonist in Bov A and Bov B showed $P<0.05$.

For the T cell subset analysis within unfractionated PBMC, the CFSE-labelled PBMCs stimulated with TLR1-9 agonists gave inconclusive results for CD4, CD8, and γδ T cell proliferation.

Finally, and unexpectedly, MACS fractionated γδ T cells (both unstimulated and ConA stimulated) showed a stimulatory effect on CFSE-labelled PBMCs (both unstimulated and ConA stimulated).

5.2 Introduction

To provide a better understanding for TLR function in bovine T cell subsets, PBMC and T cells were stimulated by TLR agonists (Bhardwaj *et al*., 2010). TLR agonists, or ligands, are molecules derived from certain microbes (infectious or non-infectious) which are responsible specifically for activation of TLR (upon exposure in the host cells), represented by TLR signalling pathways that lead to activation of the cells and the release of pro-inflammatory cytokines (e.g. IL-1β, IL-6, CXCL-8 and IFNγ) (Kawai and Akira, 2007).

One method for assessing the function of stimulated cells is the CFSE assay. Carboxyfluorescein succinimidyl ester is a DNA-binding fluorescent dye which is used to monitor the division of the cells (measured as peaks of fluorochrome signal of diminishing signal and amplitude as the cells divide) (Lyons *et al*., 2001). This assay is commonly used and has been successfully used to monitor T cell division (Hawkins *et al*., 2007, Quah and Parish, 2010).

The purpose of this study was to determine the proliferation (by CFSE measurement) of PBMC and purified T cell subsets upon stimulation with TLR agonists / ligands. Bovine CD4, CD8 and γδ T cell function was also to be studied within the pool of PBMCs in response to stimulation with TLR ligands, i.e. analysis of cell subset proliferation of T cell subsets in PBMC without prior fractionation. Finally, because bovine PBMCs showed an abundance of γδ T cells and based on previous publications (Guzman *et al*., 2014, Baldwin and Telfer, 2015) who proposed that γδ T cells possessed suppressive activity and considered them regulatory T cells we

re-investigated the role of these γδ T cells (both resting and ConA activated) on CFSE-labelled PBMCs (both resting and ConA activated).

5.3 Materials and methods

5.3.1 Stimulation of bovine CFSE-labelled PBMCs with TLR ligands

Bovine PBMCs were labelled with CellTrace™ Violet Cell Proliferation Kit (C34557 - Thermofisher Scientific, Rugby, UK) as described in section 2.7.5, seeded in RPMI complete medium at 5 X 10^5 cells per ml in 24 well plates. CFSE-labelled cells were stimulated with 3 concentrations of TLR ligands; low (L), moderate (M) and high (H) as described in section 2.4.4, along with two control positive groups treated with 5 ug/ml Con A and 1 ug/ml anti-bovine CD3 monoclonal antibody (these has previously been tested in dose-response experiments using bovine PBMC and purified T cells by Nevi Parameswaran, a postdoctoral researcher in the Haig lab), while a fraction of cells was left untreated as a negative control. The plates were incubated for 3 days at 37℃ in a humidified atmosphere of 5% CO2 in air and wrapped with foil to protect from light. After incubation, cells were fixed, stored and shipped for analysis as described in section 2.7.5.

5.3.2 Stimulation of bovine CFSE-labelled T cell subsets with TLR ligands

CD4, CD8 and γδ T cells were fractionated by MACS method (section 2.7.3) and labelled separately with CellTrace™ Violet Cell Proliferation Kit (C34557 - Thermofisher Scientific, Rugby, UK) as described in section 2.7.4 and seeded in RPMI complete medium at 2 X 10^5 cells/ml for CD4 T

cells, 1 X 10^5 cells/ml for CD8 T cells and 3 X 10^5 cells/ml for γδ T cells in 24 well plates. CFSE-labelled cells were stimulated with 3 concentrations of 3 TLR ligands (3, 8, and 9), low (L), moderate (M) and high (H) as described in section 2.4.4 (with actual concentrations shown). 5 ug/ml Con A was added to the control positive group of the γδ T cells and 1 ug/ml anti-bovine CD3 monoclonal antibody was added to each of the CD4 and CD8 T cell control positive groups, while a fraction of cells was left untreated as a negative control. The plate was incubated for 3 days at 37℃ in a humidified atmosphere of 5% CO2 in air and wrapped with foil to protect from light. After incubation, cells were fixed, stored and shipped for analysis as described in section 2.7.5.

5.3.3 Stimulation of CFSE-labelled PBMCs with TLR ligands and analysis of T cell activation to TLR ligands within the whole PBMC population.

Bovine PBMCs were labelled with CellTrace™ Violet Cell Proliferation Kit (C34557 - Thermofisher Scientific, Rugby, UK) as described in section 2.7.5, seeded in RPMI complete medium at 5 X 10^5 cells per ml in 24 well plate. CFSE-labelled cells were stimulated with one concentration, high (H), of nine TLR ligands (1-9) as described in section 2.4.4, along with two control positive groups stimulated with 5 ug/ml Con A and 1 ug/ml anti-bovine CD3 monoclonal antibody, while a fraction of cells was left unstimulated as a negative control. The cells were incubated for 3 days at 37℃ in a humidified atmosphere of 5% CO2 in air and wrapped with foil to protect from light. After incubation, cells were pelleted by spinning at 300 g for 5 minutes and the supernatant was stored at -20 ℃ for cytokine

assessment (in chapter 6). After incubation, cells were fixed, stored and shipped for analysis as described in section 2.7.5.

5.3.4 The effect of fractionated γδ T cells on CFSE-labelled bovine PBMC proliferation.

To attempt to highlight the functional role of bovine γδ T cells on suppression or not of autologous PBMC proliferation, this experiment planned to test both stimulated (Con-A) and unstimulated γδ T cells (MACS fractionated).

3.5 X 10^4 MACS fractionated (purified) γδ T cells were washed then mixed with 2 X 10^5 CFSE-labelled autologous PBMCs on day 0 in complete RPMI medium in a 24 well plate. Another 3.5 X 10^4 MACS fractionated γδ T cells were washed then stimulated with 5 ug/ml ConA, incubated for 2 days then washed and mixed with 2 X 10^5 CFSE-labelled autologous PBMCs and the mixture was harvested after 24 hours.

An additional 2 wells of CFSE-labelled PBMCs at day 0 were treated with 5 ug/ml and 25 ug/ml ConA to induce proliferation of the target cells, whereas a fraction of CFSE-labelled PBMCs was left as a control. After incubation, cells were fixed, stored and shipped for analysis as described in section 2.7.5.

5.3.4.2 Role of unstimulated purified γδ T cells on activated bovine autologous PBMCs (stimulated by Con A)

PBMCs from the blood of two animals (Bov 6 and Bov 7) were seeded in RPMI complete medium in two separate 25 ml flasks and stimulated with 5 ug/ml Con A each then incubated for 3 days. After that, activated PBMCs

for each sample was washed to remove any remaining Con A and labelled with CFSE.

Various cell counts of MACS-fractionated γδ T cells (1 X 10^5, 2 X 10^5, 3 X 10^5 and 4 X 10^5 cells/ml) were mixed separately with a constant number of CFSE-labelled autologous PBMCs (5 X 10^5 cells /ml) at day 0 in a 24 well plate then incubated for 48 hours. A fraction of Con A activated PBMCs, which was washed, was again re-stimulated with 5 ug/ml Con A (control positive group) while a fraction of these PBMCs was left as a control negative group. The 24 well plate was wrapped with foil to protect from light and incubated for 48 hours. After incubation, cells were fixed, stored and shipped for analysis as described in section 2.7.5.

These analyses were performed at the end of the project and for some of these there was only a limited amount of TLR-ligand for the tested TLRs left in the kits. Furthermore fractionated T cell subsets were also limited in quantity for any given animal, which limited the analyses that could be done.

Results

5.4 PBMCs stimulated with TLR ligands and labelled with CFSE (proliferation assay)

Results demonstrated no dose-dependent responses of the PBMCs from bov 1, 2, 4,5, and 4098 when stimulated with a selection of the TLR1-9 ligands in comparison with control negative groups. Figure 5.35 shows the lack of PBMC response to TLRs 1-9 in bov 5 sample, using the high concentration of each of the agonists to show the CFSE plots. Figure 5.36 shows a lack of a significant response in PBMCs (bov 1 and bov 2) to a selection of TLR agonists (to TLRs 2,3, 8 and 9, which is to be the focus of the study, as explained in the introduction) used in a dose response of low, medium and high concentrations of each of the agonists. A final attempt to get a response in two other animal PBMC samples (bov 4 and 4098) plus a repeat of bovine 5 using only the high concentration of each of the agonists to TLRs 1-9 is shown in Figure 5.37. Proliferation was low and not significant ($P \geq 0.05$) when PBMCs were stimulated with the TLR agonists compared to unstimulated cells, whereas increased (by comparison) proliferation was noticed with the Con A treated PBMC group (control positive). The anti-bovine CD3 monoclonal antibody did not stimulate the cells in this or any of the other experiments in this aspect of the study and the reason for this is not known. In the light of these results, further analyses of PBMC proliferation to TLR agonists was not undertaken.

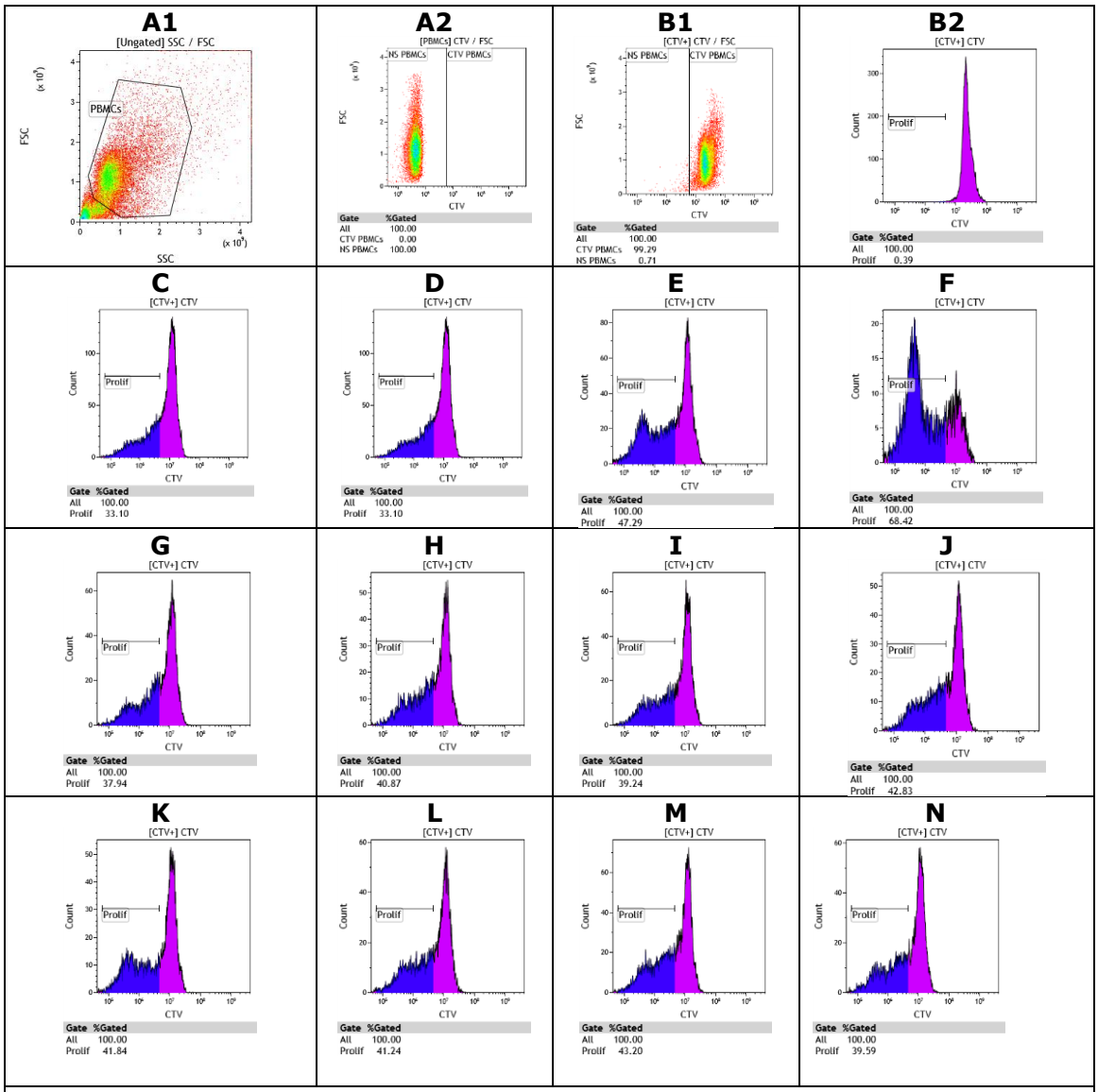


Figure 5.325: CFSE-labelled PBMCs stimulated with TLR ligands showing gated proliferative cells upon stimulation (blood animal Bov 5). A1 & A2= dot plots showing non-stained cells gated at day 0. B1= a dot plot of CellTrace violet (CFSE) stained cells (abbreviated as CTV) at day 0 showing 99% of cells were labelled. B2= a histogram of B1. C= CFSE-stained cells at day 3 - control negative group (37%). D= anti-bovine CD3 monoclonal antibody treated group (control positive) at day 3 showing 47.29% proliferation. E= ConA treated group (control positive) at day 3 showing 68.42% proliferation. F, G, H, I, J, K, L, M, and N= PBMCs stimulated with TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, and TLR9 agonists at day 3 showing 37.94%, 40.87%, 39.24%, 42.83%, 41.84%, 41.24%, 43.20%, 39.59%, and 35.17% proliferation rates respectively. The red colour indicates the peak of cells labelled at day 0 and the blue colour indicates putative PBMC division as indicated by the lower intensity of CFSE labelling. None of the TLR agonist stimulated cells showed any evidence of cell division over that of the negative control (P>0.05).

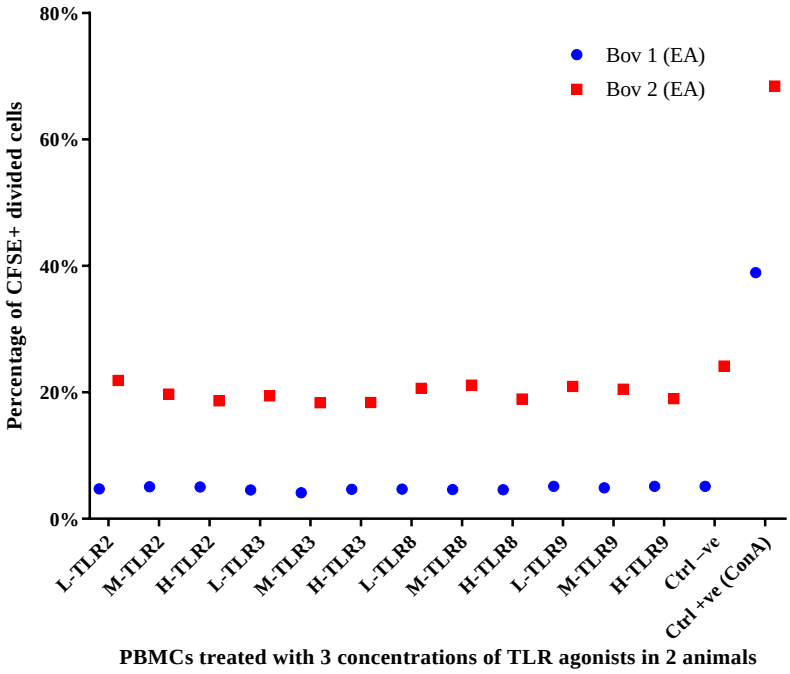


Figure 5.336: Proliferation (as percentages CFSE+) of 2 animal CFSE-labelled PBMCs stimulated with low (L), medium (M) and high (H) concentrations of TLR1-9 agonists as measured by flow cytometry. Each point represents the average of duplicates run in the assay. The reason for the limited analysis is due to the fact that these were performed at the end of the project and there was only a limited amount of TLR-ligand for the tested TLRs left in the kits

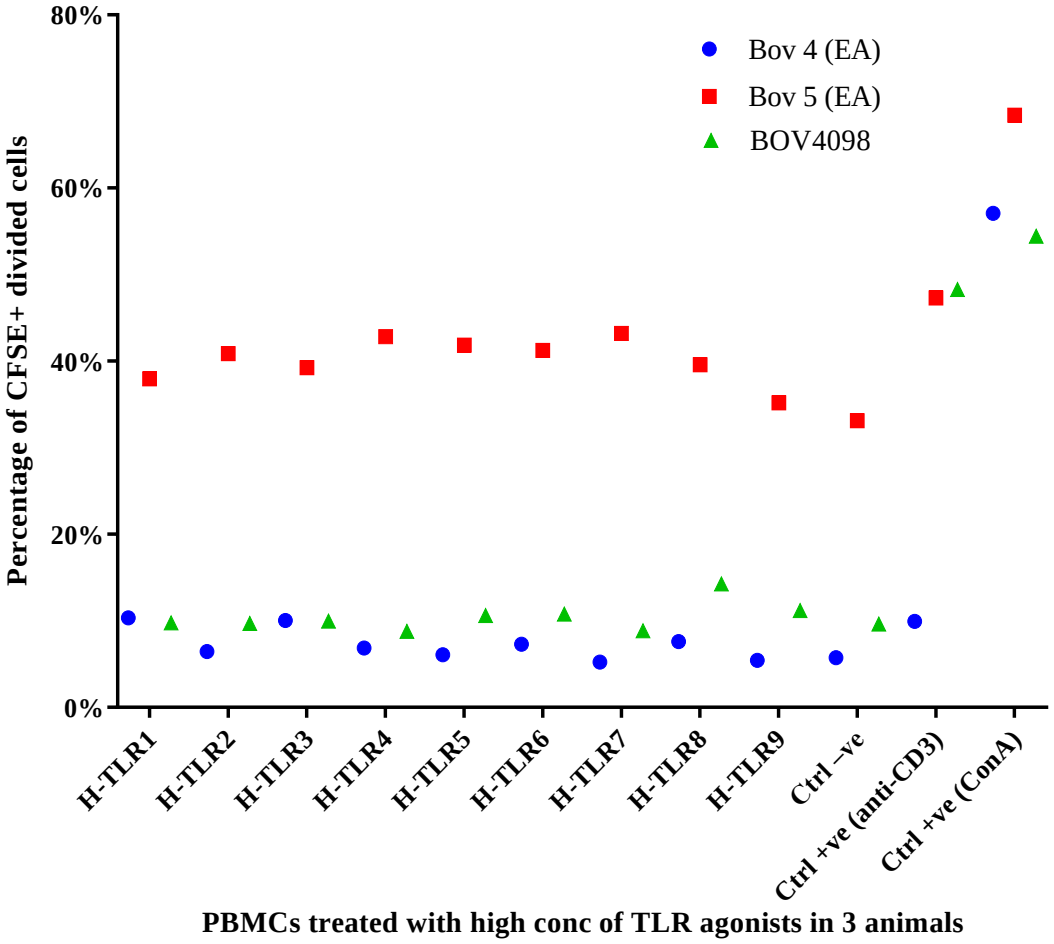


Figure 5.347: Proliferation (percentages CFSE+) of 3 bovine CFSE-labelled PBMCs stimulated with high concentrations only of TLR1-9 agonists as measured by flow cytometry. Each point represents the average of duplicates run in the assay. The reason for the limited analysis is due to the fact that these were performed at the end of the project and there was only a limited amount of TLR-ligand for the tested TLRs left in the kits

5.5 Proliferation of T cell subsets of the PBMC stimulated with TLR ligands

Bovine PBMCs were fractionated into the major T cell subsets (CD4, CD8 and γδ T cells) (section 2.7.3). Each subset was seeded in RPMI complete medium and stimulated with TLR ligands as described in section 5.3.2. Because of the limited number of fractionated T cell subset cells (MACS method), the intracellular TLR3, TLR8, and TLR9 were prioritised, which play a role in detecting viral particles, an interest of our research group.

5.5.1 Results of CFSE-labelled CD4 T cells stimulated with TLR ligands

A total of 5 bovine CD4+ T cell populations was analysed. The results showed dose-dependent proliferation of TLR3-agonist-stimulated Bov1 and Bov5 CD4 T cells compared with the control unstimulated group (figure 5.38, 5.39, 5.40). Bov 5 CD4 T cells also showed a dose-dependent response to TLR8 agonist compared to the control (Figure 5.40). None of the other bovine CD4 T cells responded to any of TLR 3, 8 or 9 (Figures 5.38, 5.39 and 5.40). Proliferation with the control positive group treated with anti-bovine CD3 monoclonal antibody was poor in comparison with the control negative group (figures 5.38, 5.39, 5.40).

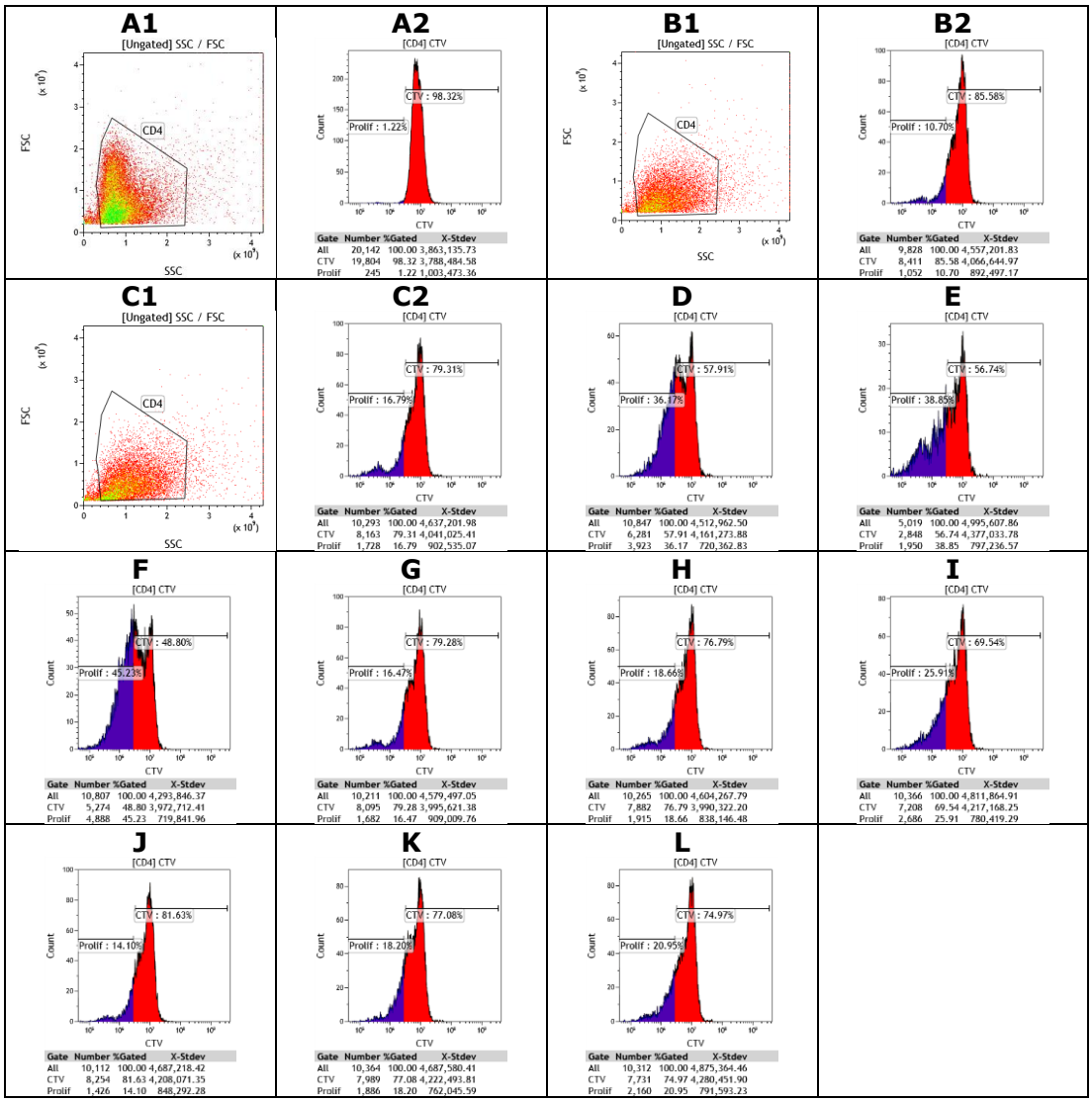

A1
[Ungated] SSC / FSC
CD4
FSC
SSC
A2
[CD4] CTV
CTV : 98.32%
Prolif : 1.22%
Count
CTV
Gate Number %Gated X-Stdev
All 20,142 100.00 3,863,135.73
CTV 19,804 98.32 3,788,484.58
Prolif 245 1.22 1,003,473.36
B1
[Ungated] SSC / FSC
CD4
FSC
SSC
B2
[CD4] CTV
CTV : 85.58%
Prolif : 10.70%
Count
CTV
Gate Number %Gated X-Stdev
All 9,828 100.00 4,557,201.83
CTV 8,411 85.58 4,066,644.97
Prolif 1,052 10.70 892,497.17
C1
[Ungated] SSC / FSC
CD4
FSC
SSC
C2
[CD4] CTV
CTV : 79.31%
Prolif : 16.79%
Count
CTV
Gate Number %Gated X-Stdev
All 10,293 100.00 4,637,201.98
CTV 8,163 79.31 4,041,025.41
Prolif 1,728 16.79 902,535.07
D
[CD4] CTV
CTV : 57.91%
Prolif : 36.17%
Count
CTV
Gate Number %Gated X-Stdev
All 10,847 100.00 4,512,962.50
CTV 6,281 57.91 4,161,273.88
Prolif 3,923 36.17 720,362.83
E
[CD4] CTV
CTV : 56.74%
Prolif : 38.85%
Count
CTV
Gate Number %Gated X-Stdev
All 5,019 100.00 4,995,607.86
CTV 2,848 56.74 4,377,033.78
Prolif 1,950 38.85 797,236.57
F
[CD4] CTV
CTV : 48.80%
Prolif : 45.23%
Count
CTV
Gate Number %Gated X-Stdev
All 10,807 100.00 4,293,846.37
CTV 5,274 48.80 3,972,712.41
Prolif 4,888 45.23 719,841.96
G
[CD4] CTV
CTV : 79.28%
Prolif : 16.47%
Count
CTV
Gate Number %Gated X-Stdev
All 10,211 100.00 4,579,497.05
CTV 8,095 79.28 3,995,621.38
Prolif 1,682 16.47 909,009.76
H
[CD4] CTV
CTV : 76.79%
Prolif : 18.66%
Count
CTV
Gate Number %Gated X-Stdev
All 10,265 100.00 4,604,267.79
CTV 7,882 76.79 3,990,322.20
Prolif 1,915 18.66 838,146.48
I
[CD4] CTV
CTV : 69.54%
Prolif : 25.91%
Count
CTV
Gate Number %Gated X-Stdev
All 10,366 100.00 4,811,864.91
CTV 7,208 69.54 4,217,168.25
Prolif 2,686 25.91 780,419.29
J
[CD4] CTV
CTV : 81.63%
Prolif : 14.10%
Count
CTV
Gate Number %Gated X-Stdev
All 10,112 100.00 4,687,218.42
CTV 8,254 81.63 4,208,071.35
Prolif 1,426 14.10 848,292.28
K
[CD4] CTV
CTV : 77.08%
Prolif : 18.20%
Count
CTV
Gate Number %Gated X-Stdev
All 10,364 100.00 4,687,580.41
CTV 7,989 77.08 4,222,493.81
Prolif 1,886 18.20 762,045.59
L
[CD4] CTV
CTV : 74.97%
Prolif : 20.95%
Count
CTV
Gate Number %Gated X-Stdev
All 10,312 100.00 4,875,364.46
CTV 7,731 74.97 4,280,451.90
Prolif 2,160 20.95 791,593.23

Figure 5.358: CFSE-labelled CD4 T cells stimulated with TLR ligands showing gated proliferative cells upon stimulation (animal Bov1). A1= a dot plot showing CFSE-labelled cells gated at day 0. A2= a histogram of A1. B1= a dot plot of CFSE-stained cells at day 3 of control negative group. B2= a histogram of B1 showing 10.7% proliferation (gated as Prolif). C1= a dot plot of anti-bovine CD3 monoclonal antibody treated group (control positive) at day 3. C2= a histogram of C1 showing 16.79% proliferation. D, E, and F= CD4 T cells stimulated with low, moderate, and high conc of TLR3 agonist at day 3 showing 36.17%, 38.85% and 45.23% proliferation respectively. G, H, and I= CD4 T cells stimulated with low, moderate, and high conc of TLR8 agonist at day 3 showing 16.47%, 18.66% and 25.91% proliferation frequencies respectively. J, K, and L= CD4 T cells stimulated with low, moderate, and high conc of TLR9 agonist at day 3 showing 14.10%, 18.20% and 20.95% proliferation respectively. The red colour indicates the peak of cells labelled at day 0 and the blue colour indicates putative PBMC division as indicated by the lower intensity of CFSE labelling.

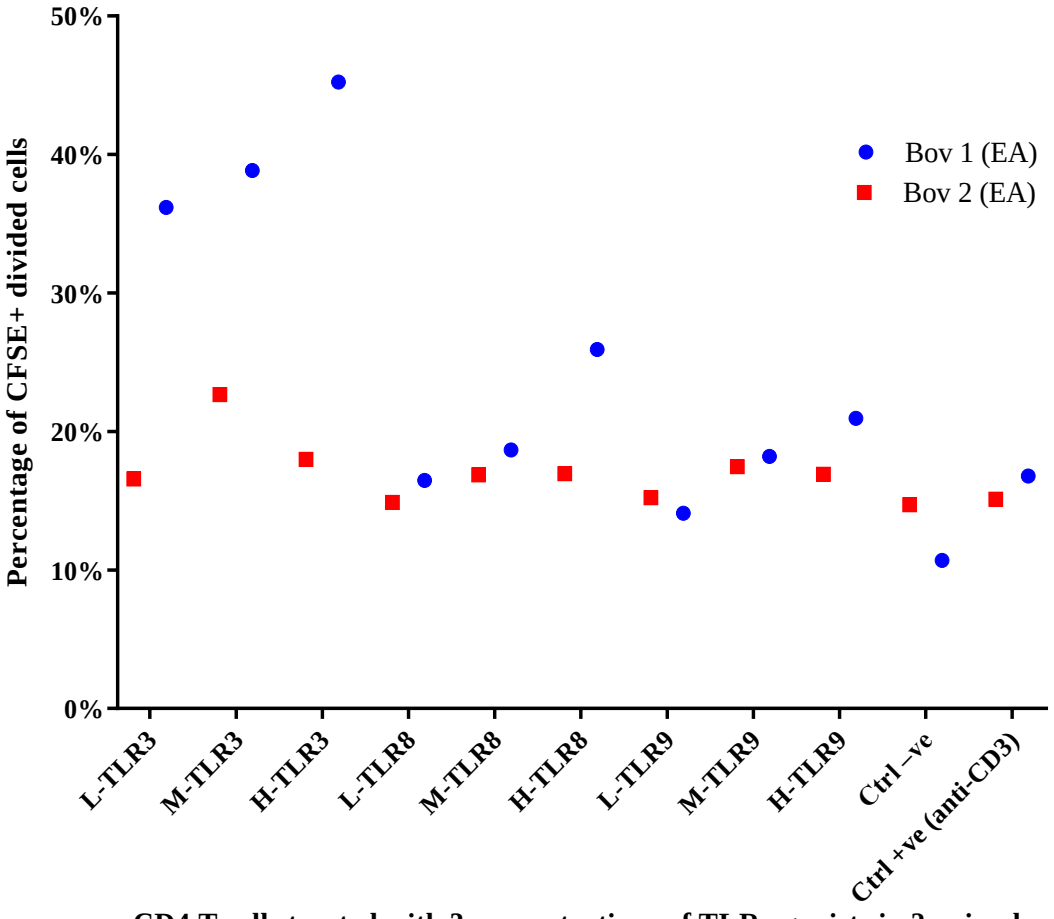


Figure 5.369: Proliferation (percentages) of bovine 1 and 2 CFSE-labelled CD4 T cells stimulated with 3 concentrations of TLR3, TLR8, and TLR9 agonists as measured by flow cytometry. Each point represents the average of duplicates run in the assay. The reason for the limited analysis is due to the fact that these were performed at the end of the project and

there was only a limited amount of TLR-ligand for the tested TLRs left in the kits

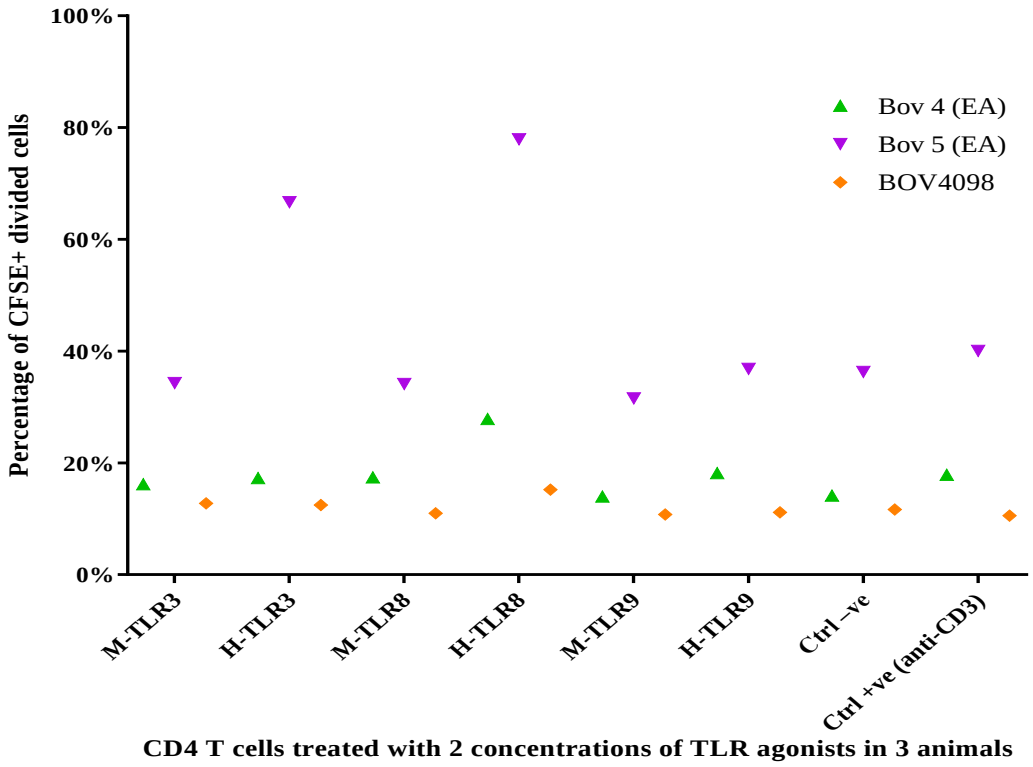


Figure 5.40: Proliferation (percentages) of bovine 4,5 and 4098 CFSE-labelled CD4 T cells stimulated with 2 concentrations of TLR3, TLR8, and TLR9 agonists as measured by flow cytometry. Each point represents the average of duplicates run in the assay. The reason for the limited analysis is due to the fact that these were performed at the end of the project and there was only a limited amount of TLR-ligand for the tested TLRs left in the kits

5.5.2 Results of CFSE-labelled CD8 T cells stimulated with TLR ligands

Results revealed in general no proliferation (CFSE assay) above unstimulated controls of TLR 3, 8 or 9 agonist-stimulated CD8 T cells from most of seven bovine animals (figures 5.41, 5.42, 5.43 and 5.44). However, bov1 CD8 T cells responded to high concentrations of TLR3 agonist and TLR9 agonist (Figure 5.41 F, L and Figure 5.42). Bov 5 CD8+ T cells responded to high concentrations of TLR8 agonist and TLR9 agonist (Figure 5.43). Once again, the anti-CD3 stimulation of the cells as a

positive control was variable. In the analyses shown in figures 5.44 A and B, high and medium and high only concentrations of TLR-agonist only were used as the the high concentration of agonist in particular gave positive results in responders.

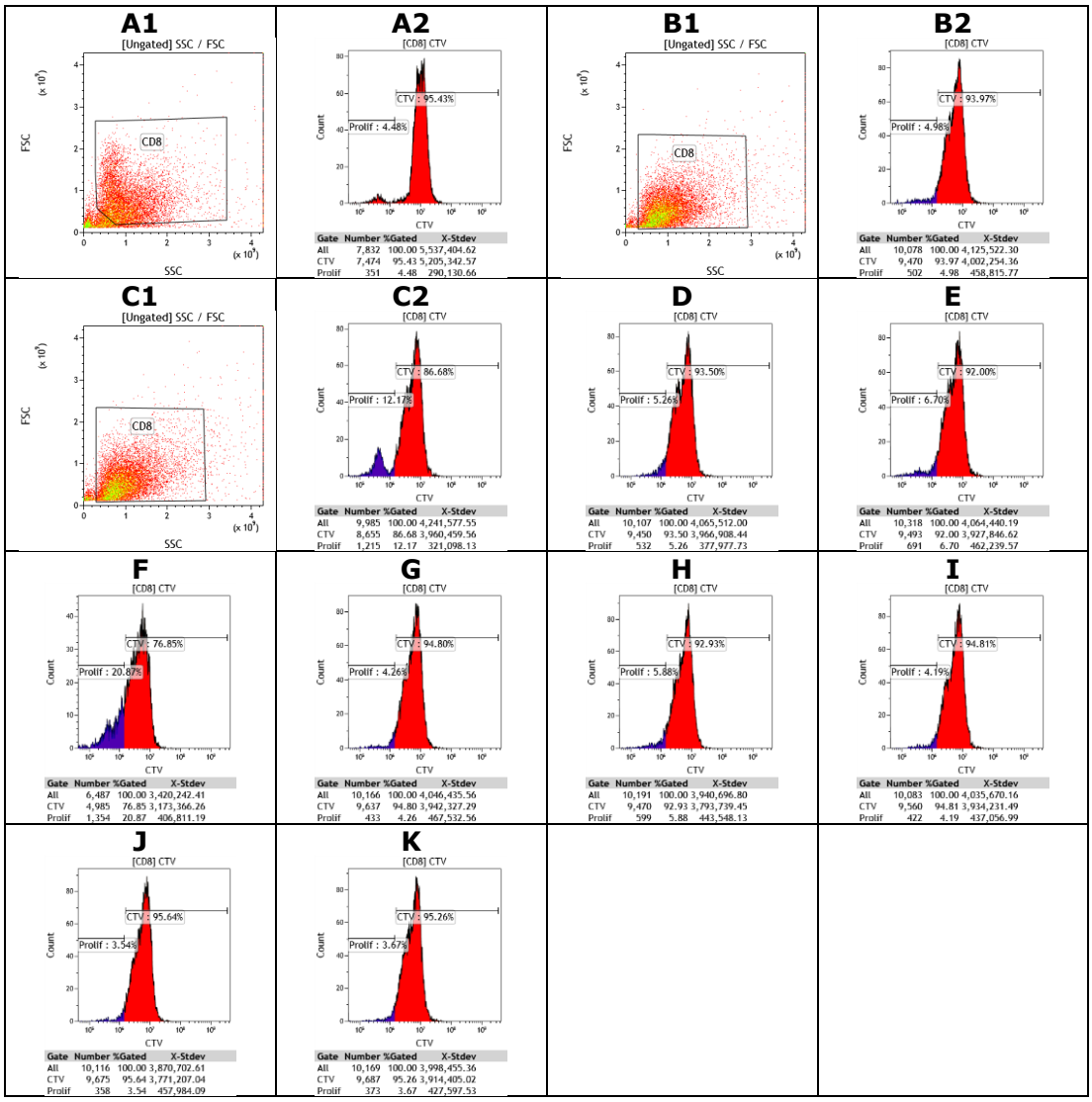


Figure 5.41: CFSE-labelled CD8 T cells stimulated with TLR ligands showing gated proliferative cells upon stimulation (blood animal Bov1). A1= a dot plot showing CFSE-labelled cells gated at day 0. A2= a histogram of A1. B1= a dot plot of CFSE-stained cells at day 3 of control negative group. B2= a histogram of B1 showing 4.98% proliferation (gated as Prolif). C1= a dot plot of anti-bovine CD3 monoclonal antibody treated group (control positive) at day 3. C2= a histogram of C1 showing 12.17% proliferation. D, E, and F= CD8 T cells stimulated with low, moderate, and high conc of TLR3 agonist at day 3 showing 5.26%, 6.70%, and 20.87% proliferation rates respectively. G, H, and I= CD8 T cells stimulated with low, moderate, and high conc of TLR8 agonist at day 3 showing 4.26%, 5.88%, and 4.19% proliferation rates respectively. J, K, and L= CD8 T cells stimulated with low, moderate, and high conc of TLR9 agonist at day 3 showing 3.54%, 3.67%, and 21.35% proliferation (CFSE+ cells) respectively. The red colour indicates the peak of cells labelled at day 0 and the blue colour indicates putative PBMC division as indicated by the lower intensity of CFSE labelling.

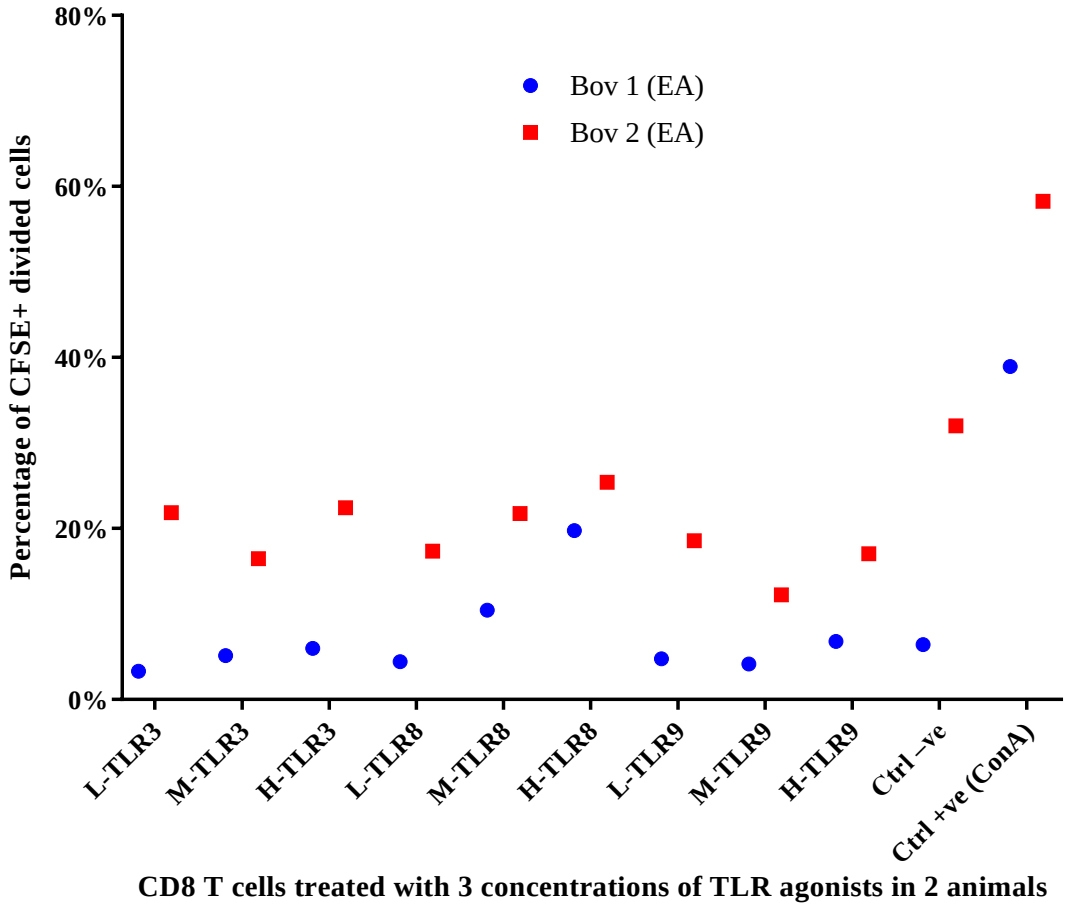


Figure 5.42: Proliferation (percentages) of bovine CFSE-labelled CD8 T cells stimulated with high, medium and low concentrations of TLR3, TLR8, and TLR9 agonists as measured by flow cytometry. The samples were loaded in duplicates. The kit was not enough to run more samples (and that was a limitation), therefore only 2 animals were selected. It was not

possible to present the data results as Mean ± Standard error because the results were not repeated twice (only once) and that was also due to the shortness of kit materials.

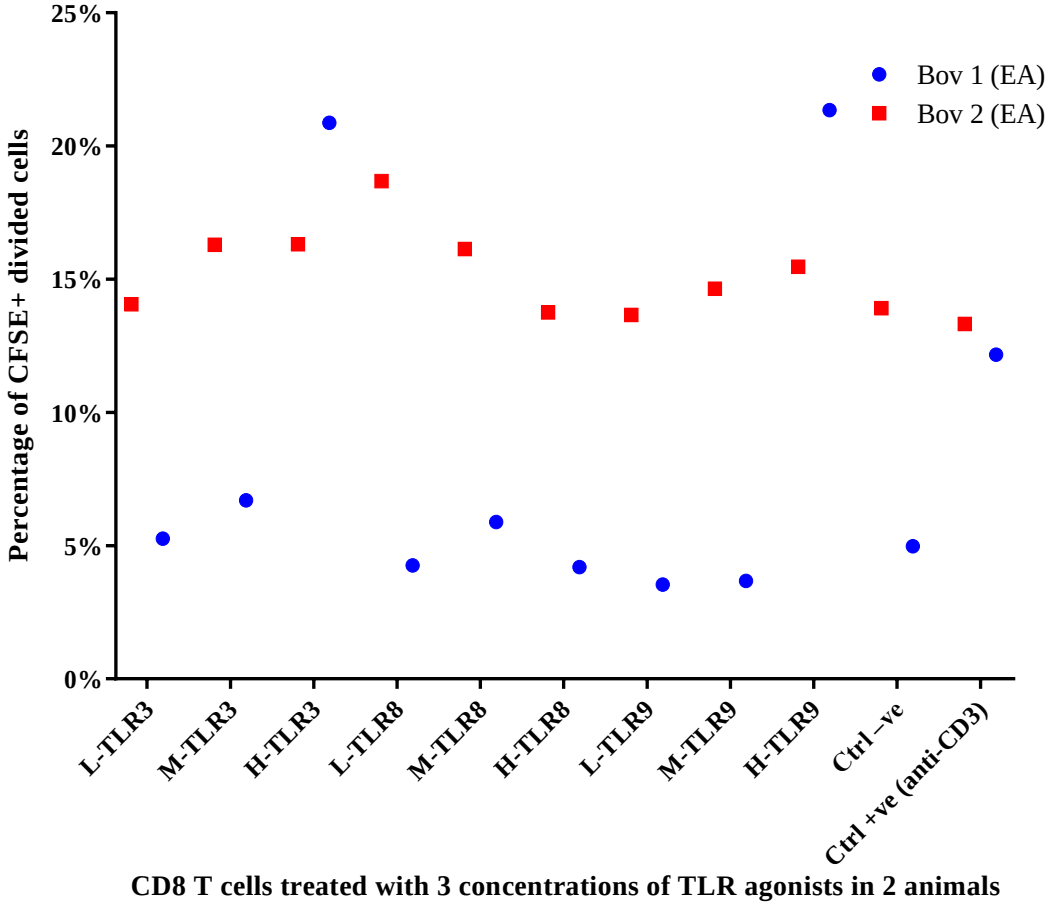
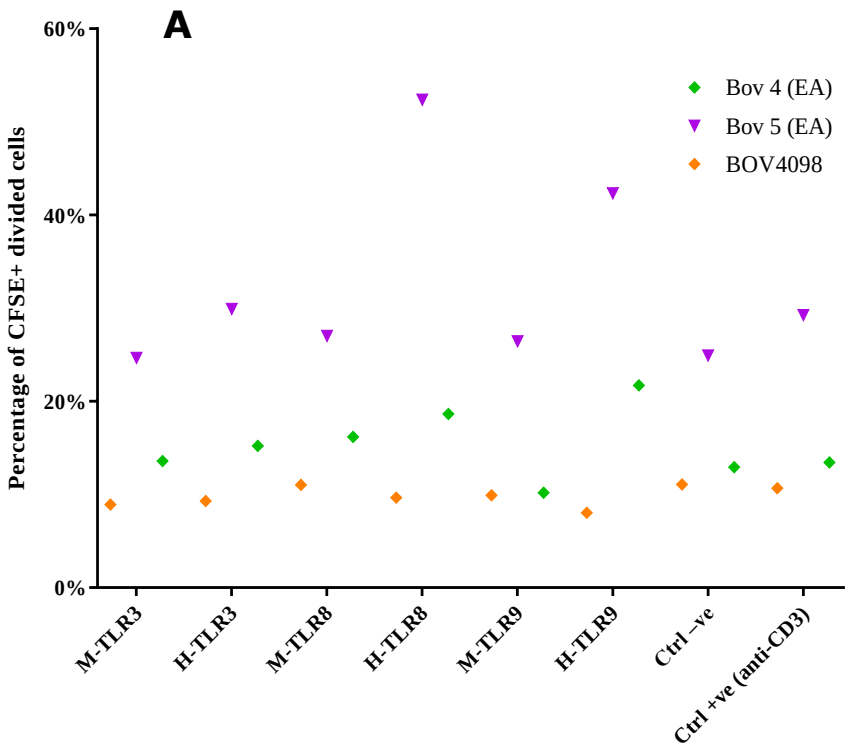
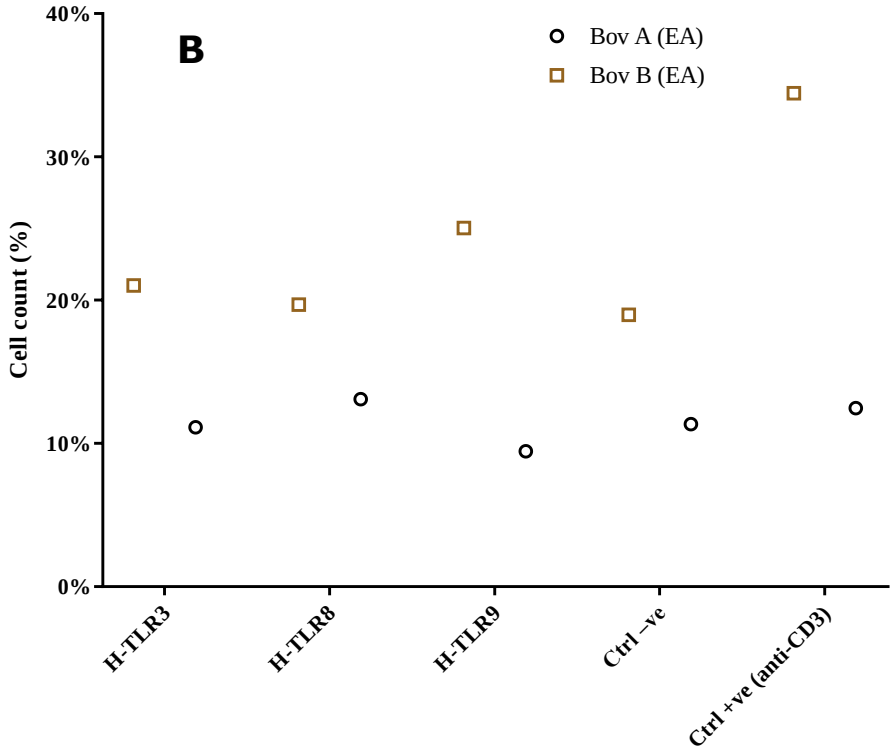


Figure 5.43: Proliferation (percentages) of bovine CFSE-labelled CD8 T cells stimulated with high, medium and low concentrations of TLR3, TLR8, and TLR9 agonists as measured by flow cytometry.



CD8 T cells treated with 2 concentrations of TLR agonists in 3 animals



CD8 T cells treated with high conc of TLR agonists in 2 animals

Figure 5.44. A) Proliferation (percentages) of bovine CFSE-labelled CD8 T cells stimulated with medium and high concentrations of TLR3, TLR8, and TLR9 agonists as measured by flow cytometry. B) CD8 T cell proliferation of the TLR agonists in 2 animals stimulated with high concentrations of TLR3, 8, and 9 agonists. There was only a limited amount of TLR-ligand

for the tested TLRs left in the kits. Furthermore, fractionated T cell subsets were also limited in quantity for any given animal, which limited the analyses that could be done.

5.5.3 Results of CFSE-labelled γδ T cells stimulated with TLR ligands

Results of the TLR agonist-stimulated γδ T cells from a selection of bovines showed no proliferation above that of the control negative groups (Figures 5.45 and 5.46) with the exception of γδ T cells from bov 1 stimulated with the high concentration of TLR8 agonist (figures 5.45I and 5.46). The Con A treated group (control positive) showed the most proliferative response of the cells (figures 5.45 and 5.46).

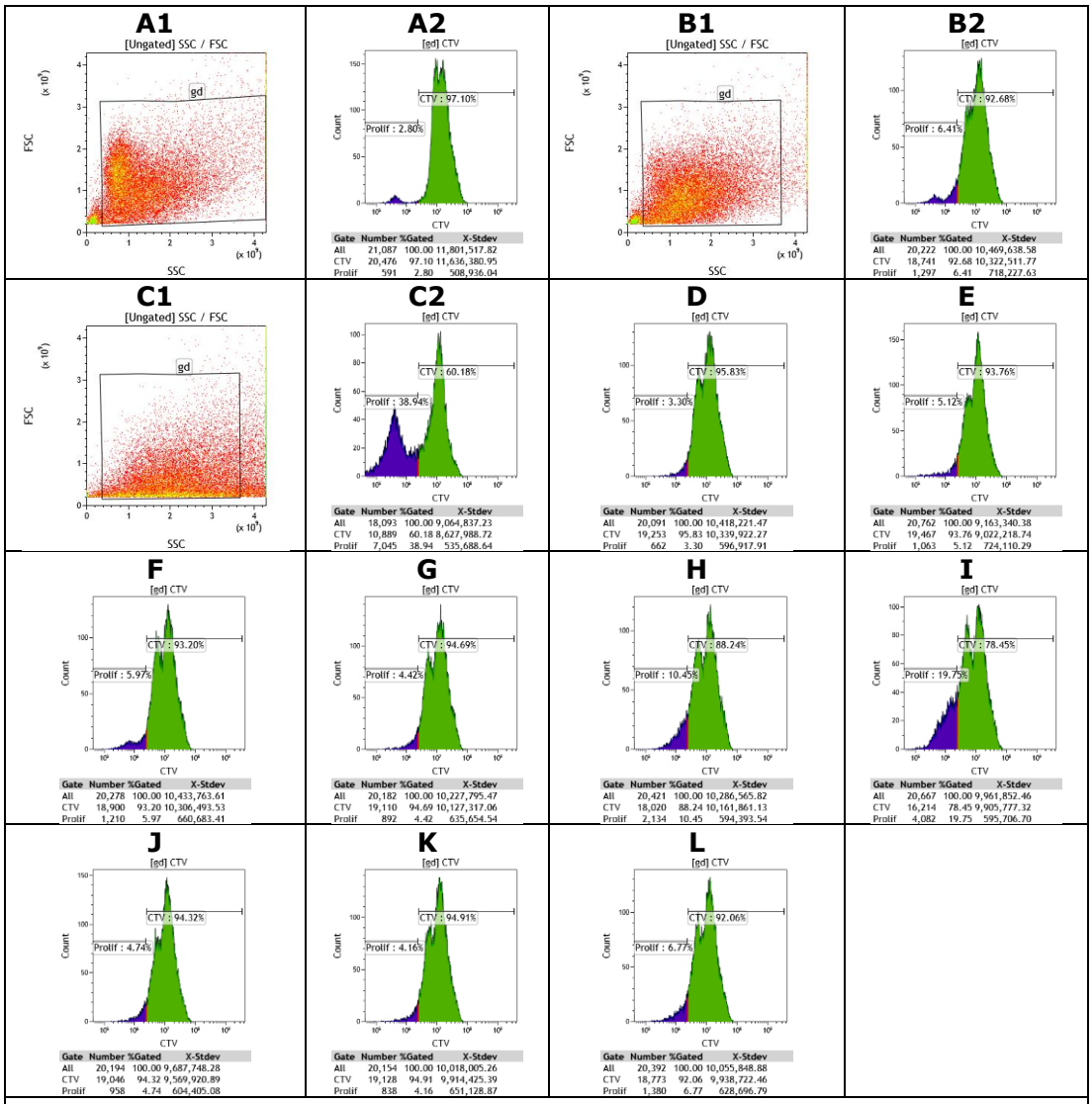
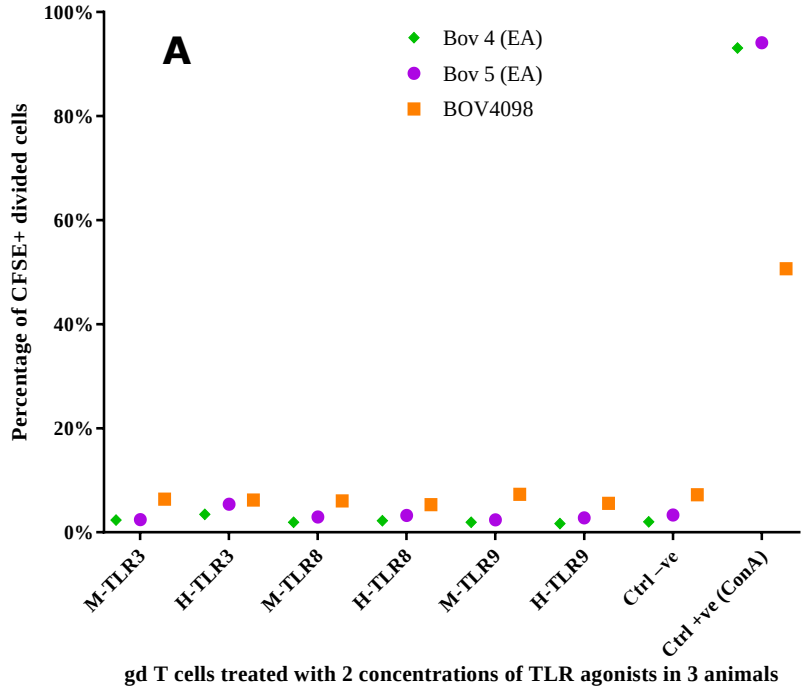


Figure 5.375: CFSE-labelled γδ T cells stimulated with TLR ligands showing gated proliferative cells upon stimulation (animal Bov1).
A1= a dot plot showing CFSE-labelled cells gated at day 0. A2= a histogram of A1. B1= a dot plot of CFSE-stained cells at day 3 of control negative group. B2= a histogram of B1 showing 6.41% proliferation (gated as Prolif). C1= a dot plot of Con A treated group (control positive) at day 3. C2= a histogram of C1 showing 38.94% proliferation. D, E, and F= γδ T cells stimulated with low, moderate, and high conc of TLR3 agonist at day 3 showing 3.30%, 5.12%, and 5.97% proliferation rates respectively. G, H, and I= γδ T cells stimulated with low, moderate, and high conc of TLR8 agonist at day 3 showing 4.42%, 10.45%, and 19.75% proliferation rates respectively. J, K, and L= γδ T cells stimulated with low, moderate, and high conc of TLR9 agonist at day 3 showing 4.74%, 4.16%, and 6.77% proliferation rates respectively. The green colour indicates the peak of cells labelled at day 0 and the blue/purple colour indicates putative PBMC division as indicated by the lower intensity of CFSE labelling.



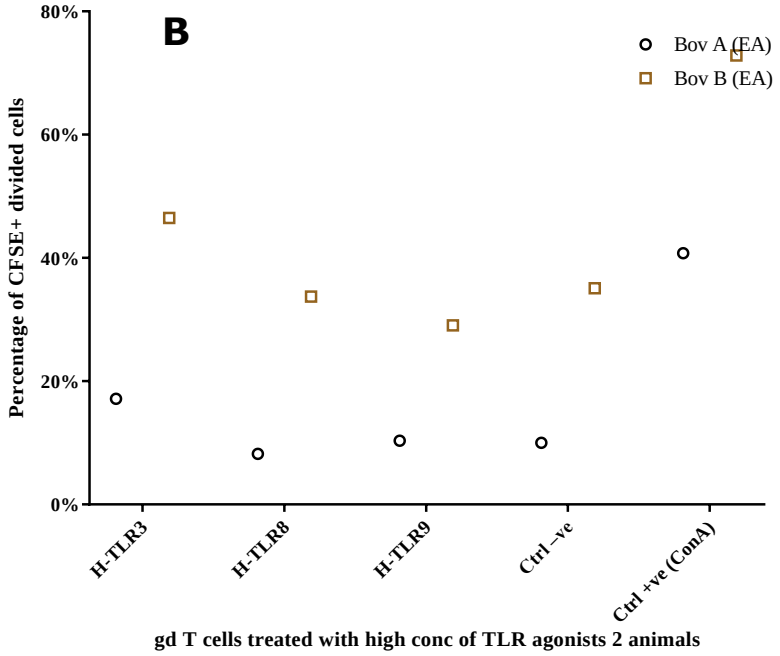


Figure 5.386: A) Proliferation (percentages) of bovine CFSE-labelled γδ T cells stimulated with medium and high concentrations of TLR3, TLR8, and TLR9 agonists as measured by flow cytometry. B) Proliferation of bovine CFSE-labelled γδ T cells stimulated with high concentrations of TLR3, TLR8, and TLR9 agonists. The samples were loaded in duplicates. The kit was not enough to run more samples (and that was a limitation), therefore only 3 animals were selected. It was not possible to present the data results as Mean ± Standard error because the results were not

repeated twice (only once) and that was also due to the shortness of kit materials.

5.6 Results of CFSE-labelled PBMCs stimulated with TLR ligands: T cell subset responses within the whole PBMC population

This experiment was designed to study responses to TLR ligands of subsets of T cells (CD4, CD8 and γδ T cells) within the context of unfractionated PBMC.

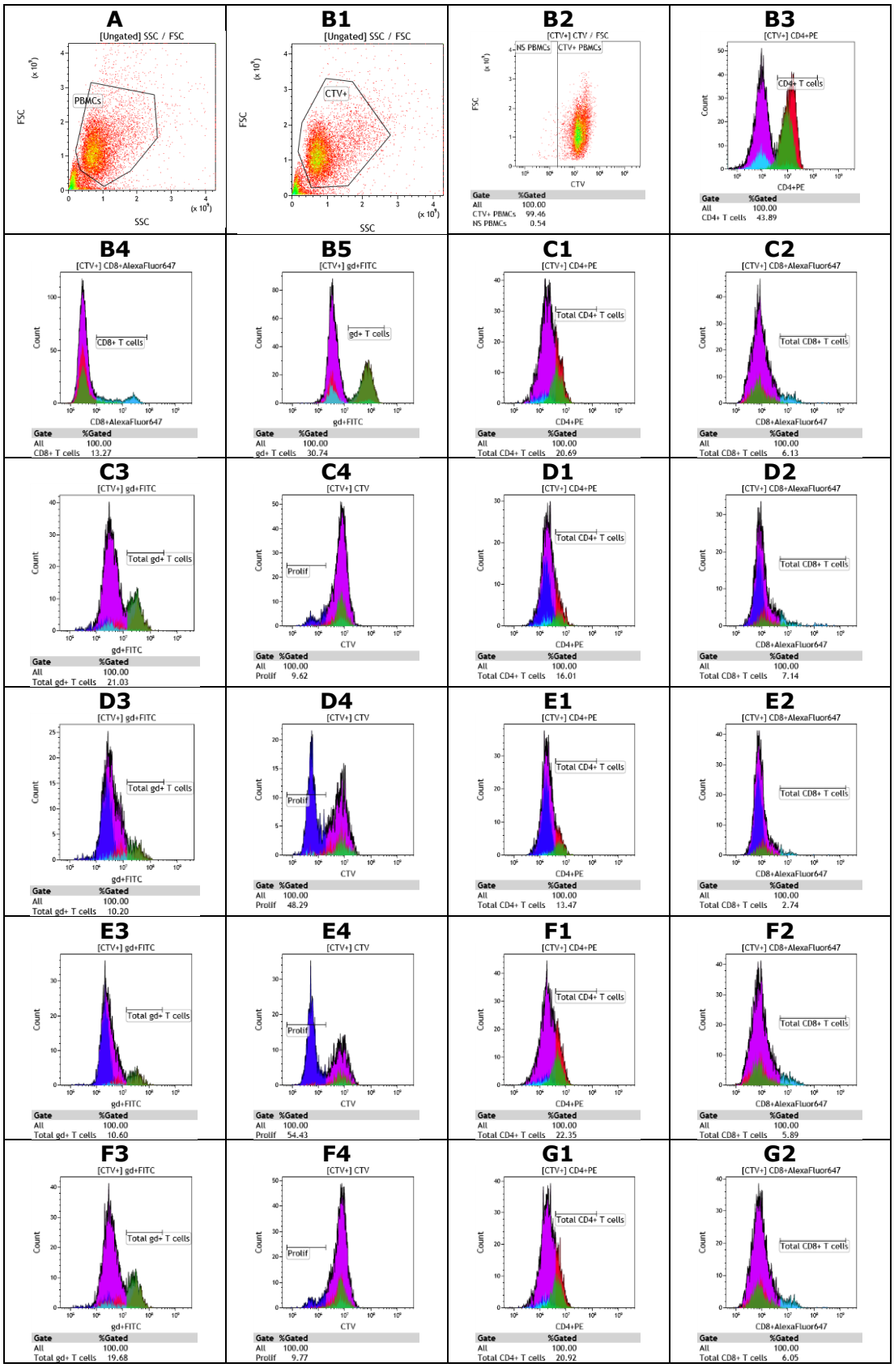
Bovine PBMCs were stimulated with TLR1-9 ligands and labelled with 4 fluorophores: CFSE at day 0 plus labelling major T cell subsets (CD4, CD8 and WC1 γδ T cells) with mouse anti bovine CD4:RPE, mouse anti bovine CD8:Alexa Fluor® 647, mouse anti bovine WC1:FITC, at days 0 and 3 post-stimulation. Isotype controls were used as negative controls to calibrate the FACscan gates applied (mouse IgG2a negative control; RPE-mouse IgG2a negative control; Alexa Fluor® 647-mouse IgG2a negative control; FITC-IgG2a (as a control for FITC-CD4 antibody); RPE-CD8 ab, Alexa Fluor® 647 γδ T cells labelled cells respectively) (sections 2.4.4 and 2.7.5). PBMCs stimulated with TLR ligands were seeded in 1 ml RPMI complete medium in 24 well plates along with two control positive groups treated with 5 ug/ml Con A and 1 ug/ml anti-bovine CD3 monoclonal antibody respectiveky, while a fraction of cells was left untreated as a negative control (see section 5.3.3).

Results for total PBMC proliferation were obtained by subtracting CFSE-labelled cells at day 0 from day 3 gated cells. A gate named "Prolif", dark blue colour in the histograms, showed the total proliferation rate as a percentage (%). For major T cell subsets (CD4, CD8 and γδ T cells), CD4:PE (red colour in the histograms) was gated at day 0 and 3 then 0

subtracted from 3 to show the proliferation as a percentage (%). The same method for determining proliferative CD4 T cells was repeated with CD8:AlexaFluor® 647 (for CD8 T cells) and WC1:FITC (for γδ T cells).

PBMCs did not proliferate noticeably to TLR1-9 ligands at the high concentration in comparison with the control negative groups in any of the animal PBMCs analysed (figures 5.47 and 5.48). ConA treated cells showed the highest proliferation, while anti-CD3 mAb treated groups responded well in only Bov 5 and BOV4098 animal cells but not in the bov 4 animal (see figures 5.47 and 5.48).

For CD4:PE labelled cells; CD8:AlexaFluor® 647 labelled cells and γδ T cells (FITC labelled) no convincing CFSE responses were seen to any of the the TLR agonists (Figures 5.49, 5.50 and 5.51). Indeed, none responded convincingly to the Con A positive control group, so the assay did not work.


A
[Ungated] SSC / FSC
PBMCs
FSC
SSC
B1
[Ungated] SSC / FSC
CTV+
FSC
SSC
B2
[CTV+] CTV / FSC
NS PBMCs
CTV+ PBMCs
FSC
CTV
Gate %Gated
All 100.00
CTV+ PBMCs 99.46
NS PBMCs 0.54
B3
[CTV+] CD4+PE
CD4+ T cells
Count
CD4+PE
Gate %Gated
All 100.00
CD4+ T cells 43.89
B4
[CTV+] CD8+AlexaFluor647
CD8+ T cells
Count
CD8+AlexaFluor647
Gate %Gated
All 100.00
CD8+ T cells 13.27
B5
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gd+ T cells
Count
gd+FITC
Gate %Gated
All 100.00
gd+ T cells 30.74
C1
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Total CD4+ T cells
Count
CD4+PE
Gate %Gated
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Total gd+ T cells
Count
gd+FITC
Gate %Gated
All 100.00
Total gd+ T cells 21.03
C4
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Prolif
Count
CTV
Gate %Gated
All 100.00
Prolif 9.62
D1
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Total CD4+ T cells
Count
CD4+PE
Gate %Gated
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Total CD8+ T cells
Count
CD8+AlexaFluor647
Gate %Gated
All 100.00
Total CD8+ T cells 7.14
D3
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Count
gd+FITC
Gate %Gated
All 100.00
Total gd+ T cells 10.20
D4
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Prolif
Count
CTV
Gate %Gated
All 100.00
Prolif 48.29
E1
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CTV
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CD4+PE
Gate %Gated
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Prolif 9.77
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Count
CD4+PE
Gate %Gated
All 100.00
Total CD4+ T cells 20.92
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Total CD8+ T cells
Count
CD8+AlexaFluor647
Gate %Gated
All 100.00
Total CD8+ T cells 6.05

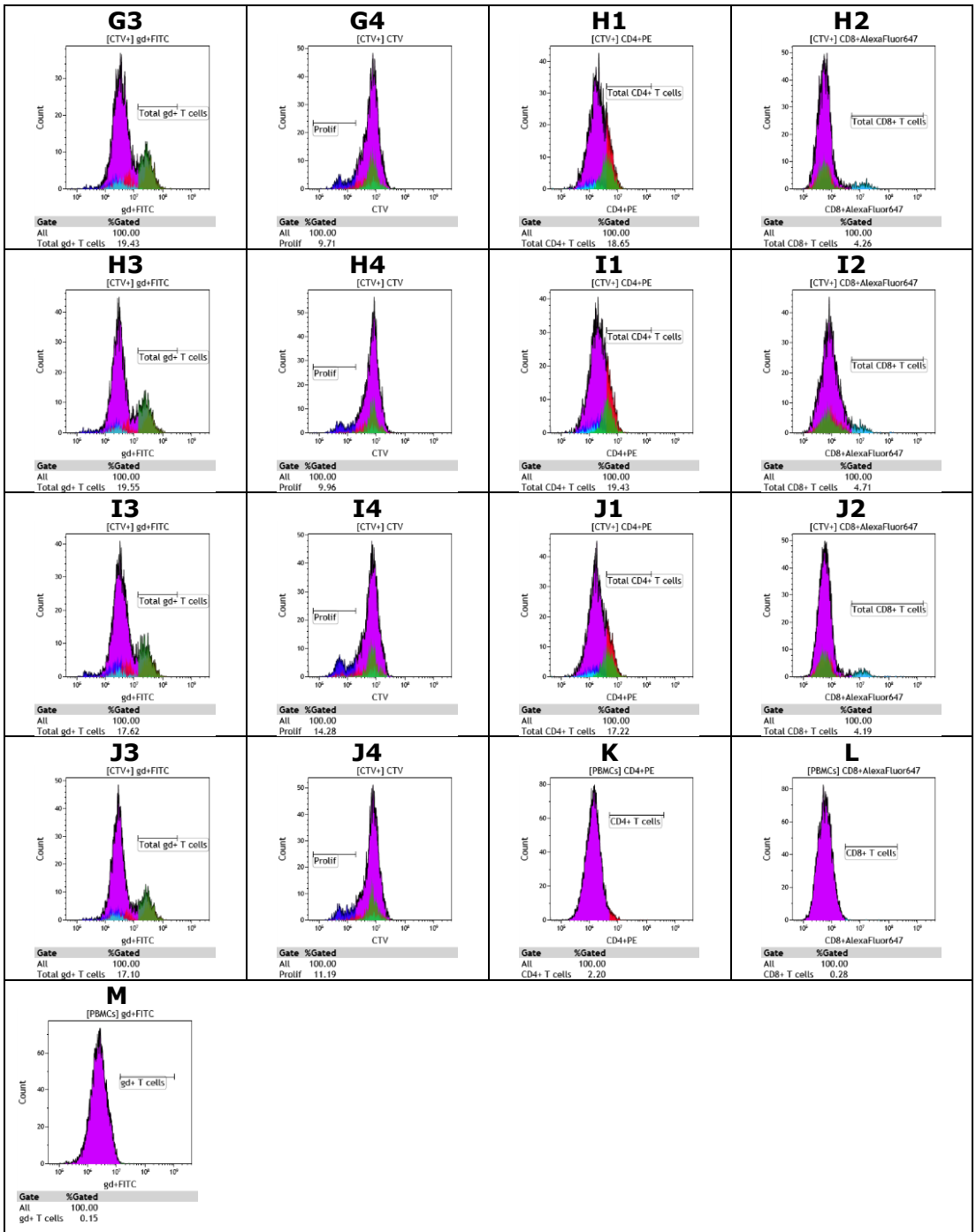


Figure 5.397: CFSE-labelled PBMCs (BOV4098 animal) stimulated with TLR1-9 ligands plus control positive groups (ConA and anti-CD3 mAb). A= dot plot of non-stained PBMCs at day 0. B1, B2= dot plots of CFSE-labelled PBMCs (CFSE-stained) at day 0. B3, B4, B5= CD4, CD8 and γδ T cells labelled PBMCs with PE, AF* and FITC respectively at day 0. C1, C2, C3= CD4, CD8 and γδ T cells labelled within the PBMCs with PE, AF and FITC respectively at day 3 in the control negative group. C4= total proliferation of PBMCs at day 3 in control negative group. D1, D2, D3= CD4, CD8 and γδ T cells labelled within PBMCs with PE, AF and FITC respectively at day 3 in anti-CD3 mAb treated group. D4= total proliferation of whole PBMCs at day 3 in anti-CD3 mAb treated group. E1, E2, E3= CD4, CD8 and γδ T cells labelled within PBMCs with PE, AF and FITC respectively at day 3 in ConA treated group. E4= total proliferation of whole PBMCs at day 3 in ConA treated group. F1, F2,

F3= CD4, CD8 and γδ T cells labelled within PBMCs with PE, AF and FITC respectively at day 3 in high conc TLR1 agonist treated group. F4= total proliferation of whole PBMCs in high conc TLR1 agonist treated group. G1, G2, G3= CD4, CD8 and γδ T cells labelled within PBMCs pool with PE, AF and FITC respectively at day 3 in high conc TLR2 agonist treated group. G4= total proliferation of whole PBMCs in high conc TLR1 agonist treated group. H1, H2, H3= CD4, CD8 and γδ T cells labelled within PBMCs with PE, AF and FITC respectively at day 3 in high conc TLR3 agonist treated group. H4= total proliferation of whole PBMCs in high conc TLR3 agonist treated group. I1, I2, I3= CD4, CD8 and γδ T cells labelled within PBMCs pool with PE, AF and FITC respectively at day 3 in high conc TLR8 agonist treated group. I4= total proliferation of whole PBMCs in high conc TLR8 agonist treated group. J1, J2, J3= CD4, CD8 and γδ T cells labelled within PBMCs with PE, AF and FITC respectively at day 3 in high conc TLR9 agonist treated group. J4= total proliferation of PBMCs in high conc TLR9 agonist treated group. K= CD4:PE isotype control labelled cells at day 3. L= CD8:AF isotype control labelled cells at day 3. M= γδ:FITC isotype control labelled cells at day 3.
***AF= Alexa Fluor® 647, conc= concentration, mAb= monoclonal antibody. Purple=CellTrace violet; blue=CD8 T cells; Green= Gamma delta T cells; Red=CD4 T cells.**

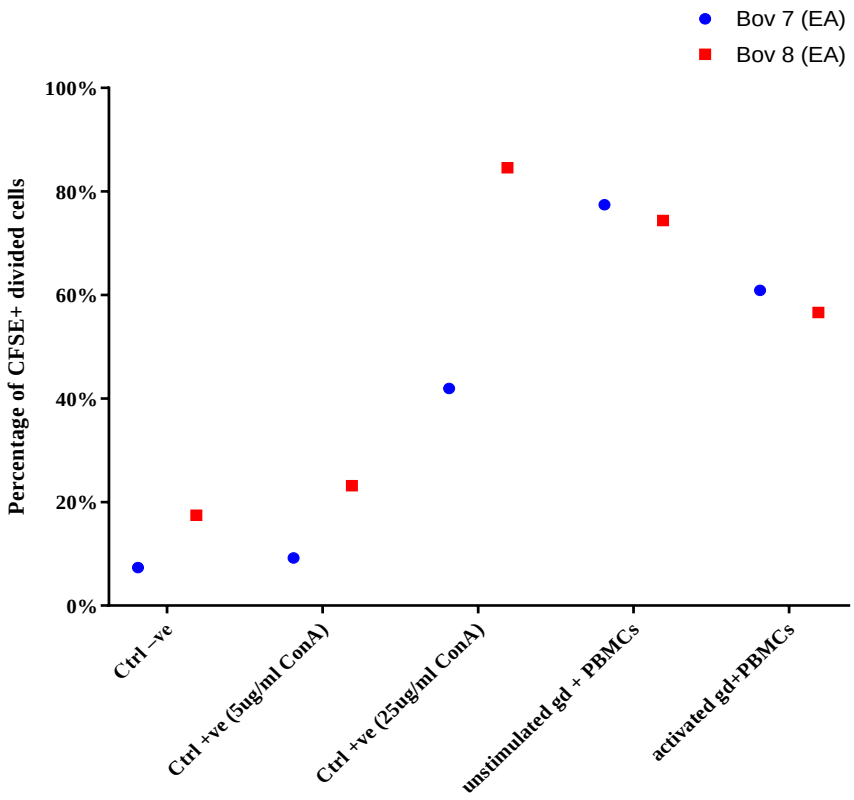


Figure 5.408: Total proliferation (%) of CFSE-labelled PBMCs stimulated with ConA in Bov 7 and Bov 8 blood animals. The samples were loaded in duplicates. The samples left were very limited because I consumed most of them in above tests (and that was a limitation), therefore only 2 animals were selected. It was not possible to present the data results as

Mean ± Standard error because the results were not repeated twice (only once) and that was also due to the shortness of kit materials.

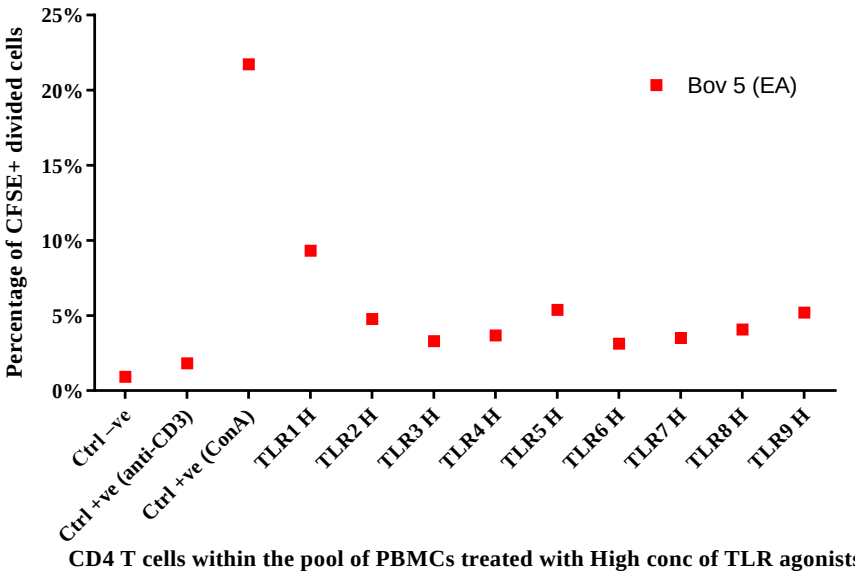


Figure 5.419: Proliferation (%) of CD4 T cells (labelled with PE) within the pool of CFSE-labelled PBMCs stimulated with TLR1-9 ligands, anti-CD3 mAb and ConA in Bov 5 blood animal. The samples were loaded in duplicates. The samples left were very limited because I consumed most of them in above tests (and that was a limitation), therefore only 1 animal was selected. It was not possible to present the data results as Mean ± Standard error because the results were not repeated twice (only once) and that was also due to the shortness of kit materials.

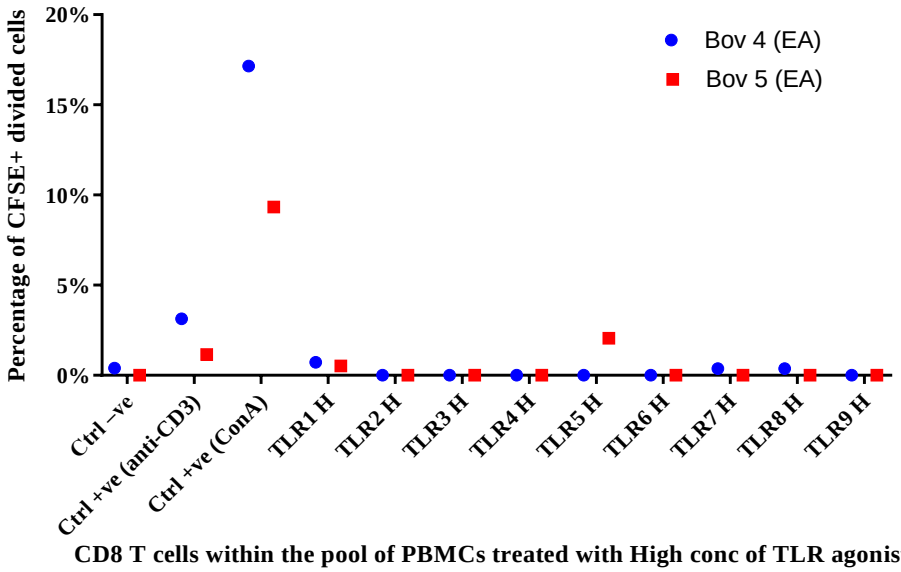


Figure 5.50: Proliferation (%) of CD8 T cells (labelled with Alexa Fluor® 647) within the pool of CFSE-labelled PBMCs stimulated with TLR1-9 ligands, anti-CD3 mAb and ConA in Bov 4 and Bov 5 blood animals. The

samples were loaded in duplicates. The samples left were very limited because I consumed most of them in above tests (and that was a limitation), therefore only 2 animals were selected. It was not possible to present the data results as Mean ± Standard error because the results were not repeated twice (only once) and that was also due to the shortness of kit materials.

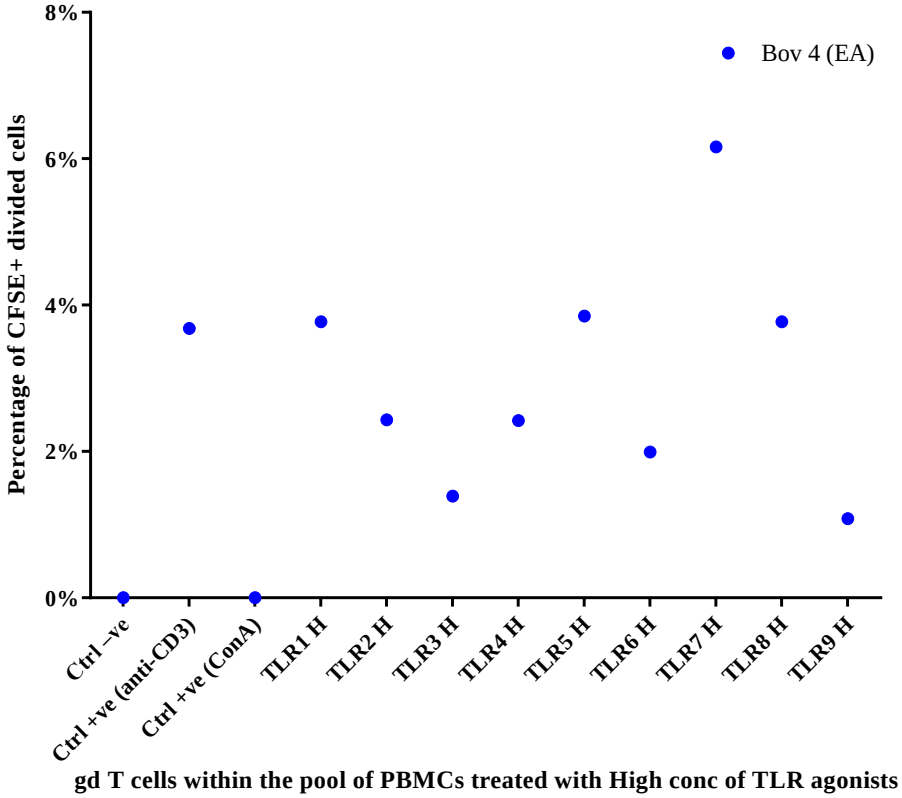


Figure 5.51: Proliferation (%) of γδ T cells (labelled with FITC) within the pool of CFSE-labelled PBMCs stimulated with TLR1-9 ligands, anti-CD3 mAb and ConA in Bov 4 blood animal. The samples were loaded in duplicates. The samples left were very limited because I consumed most of them in above tests (and that was a limitation), therefore only 1 animal was selected. It was not possible to present the data results as Mean ± Standard error because the results were not repeated twice (only once) and that was also due to the shortness of kit materials.

5.7 Functional role of bovine γδ T cells without TLR-agonist stimulation

To summarise: regardless whether γδ T cells were unstimulated or activated (by Con-A), autologous target PBMCs, either unstimulated or stimulated with Con-A showed enhanced proliferation (by CFSE assay) compared to control cells not incubated with γδ T cells.

5.7.1 Unstimulated and/or activated purified γδ T cells have a stimulatory effect on resting bovine autologous PBMCs (unstimulated)

As described in sections 2.7.3 and 5.3.4.1, 3.5 X 10^4 MACS fractionated γδ T cells were mixed with 2 X 10^5 CSFE-labelled autologous PBMCs from Bov 7 animal, while 6 X 10^4 γδ T cells were mixed with 3 X 10^5 CFSE-labelled autologous PBMCs from Bov 8 animal in complete RPMI medium. The γδ T cells were left unactivated or activated with 5 ug/ml ConA incubated for 2 days then washed and mixed with CFSE-labelled autologous PBMCs (unstimulated) in replicate wells of a 24 well plate. Control positive groups were both CFSE- labelled PBMCs treated with 5 ug/ml and 25 ug/ml ConA separately (the higher dose of Con-A to ensure maximal stimulation).

Results revealed a stimulatory activity of the γδ T cells on PBMCs in both animals Bov 7 and Bov 8 after 3 days of incubation. Unstimulated purified γδ T cells induced 77.42% (Bov 7) and 74.37% (Bov 8) CFSE shift representative of cell division compared with the control negative groups 7.36% and 17.43% respectively (figures 5.52, 5.53 and 5.54). Whereas, ConA activated γδ T cells also showed a stimulatory effect on PBMCs

60.89% and 56.63% in Bov7 and Bov 8 animals respectively, but it was lower than the effect of unstimulated γδ T cells (figure 5.54).

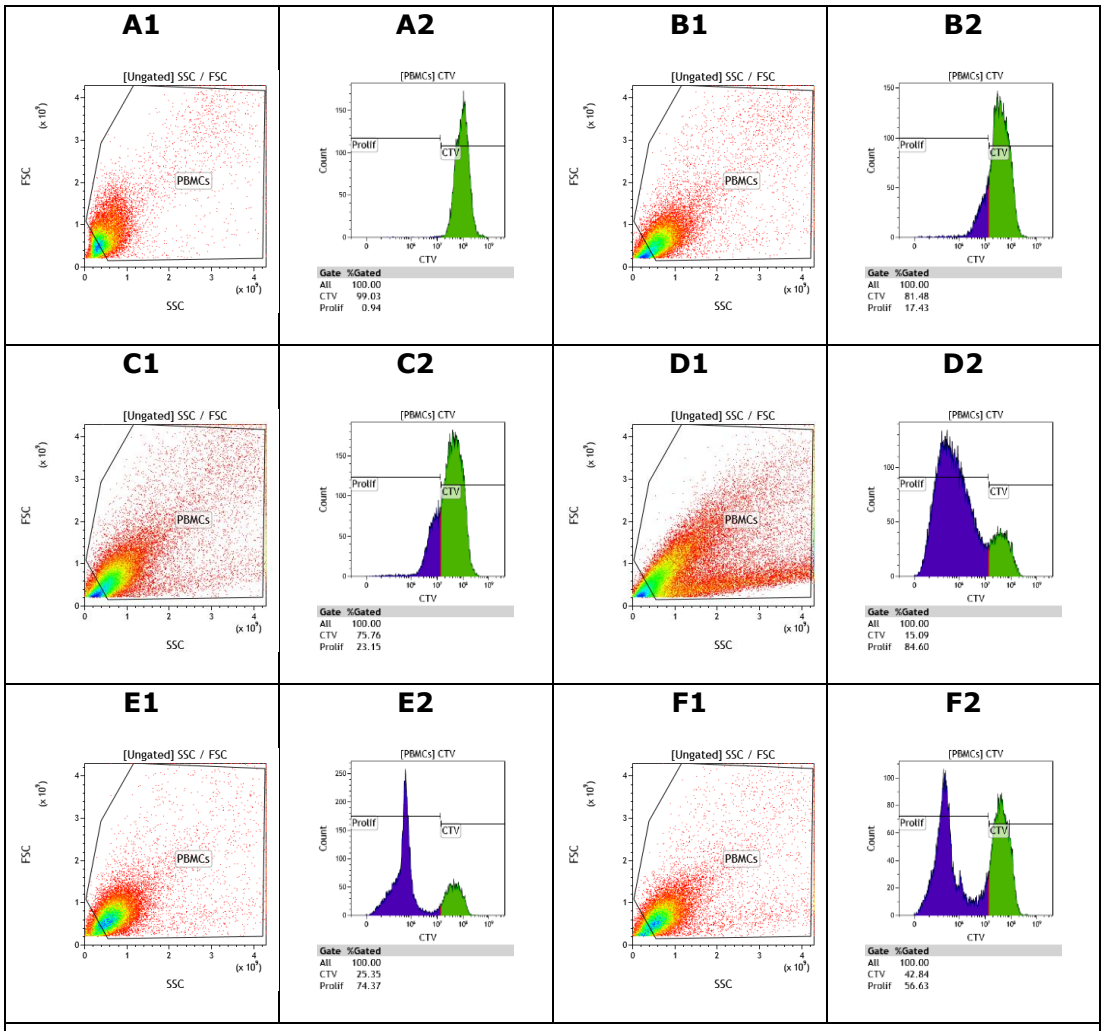


Figure 5.52: CFSE-labelled PBMCs from Bov 7 mixed with both unstimulated and activated γδ T cells separately. A1= dot plot of CFSE-stained PBMCs at day 0. B1= dot plots of CFSE-labelled PBMCs in the control negative group at day 3. C1= dot plot of control positive group treated with 5 ug/ml ConA at day 3. D1= dot plot of control positive group treated with 25 ug/ml ConA at day 3. E1= 3.5 X 10^4 unstimulated purified γδ T cells mixed with 2 X 10^5 CFSE-labelled PBMCs at day 3. F1= 6 X 10^4 purified activated γδ T cells (stimulated with 5 ug/ml ConA for 2 days) mixed with 3 X 10^5 CFSE-labelled PBMCs at day 3. A2, B2, C2, D2, E2, and F2= histograms of A1, B1, C1, D1, E1 and F1. Green indicates the peak of cells initially labelled on day blue/purple, the CFSE expression of progeny cells having divided by day 3.

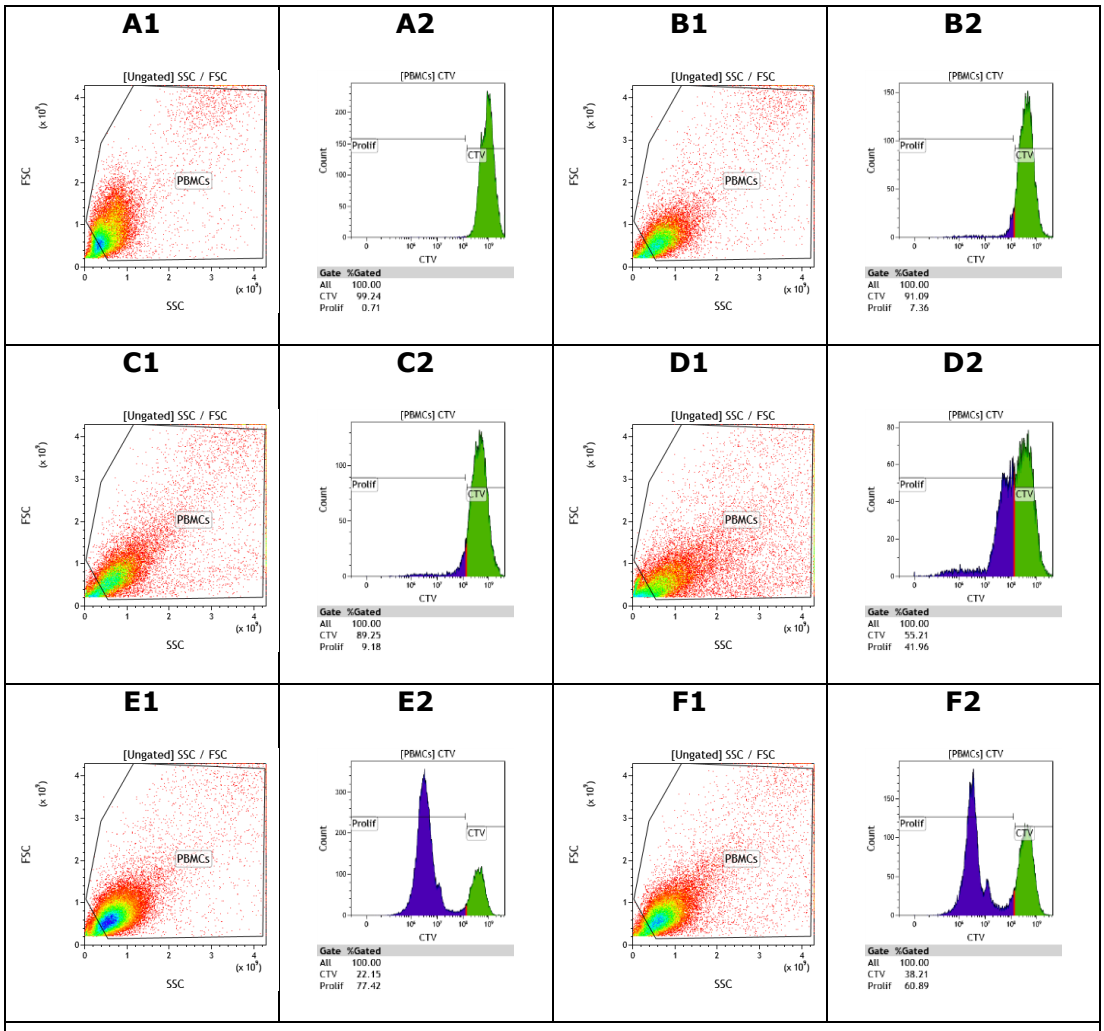


Figure 5.53: CFSE-labelled PBMCs from Bov 8 mixed with both unstimulated and activated γδ T cells separately. A1= dot plot of CFSE-stained PBMCs at day 0. B1= dot plots of CFSE-labelled PBMCs in the control negative group at day 3. C1= dot plot of control positive group treated with 5 ug/ml ConA at day 3. D1= dot plot of control positive group treated with 25 ug/ml ConA at day 3. E1= 3.5 X 10^4 unstimulated purified γδ T cells mixed with 2 X 10^5 CFSE-labelled PBMCs at day 3. F1= 6 X 10^4 purified activated γδ T cells (stimulated with 5 ug/ml ConA for 2 days) mixed with 3 X 10^5 CFSE-labelled PBMCs at day 3. A2, B2, C2, D2, E2, and F2= histograms of A1, B1, C1, D1, E1 and F1. Green indicates the peak of cells initially labelled on day blue/purple, the CFSE expression of progeny cells having divided by day 3.

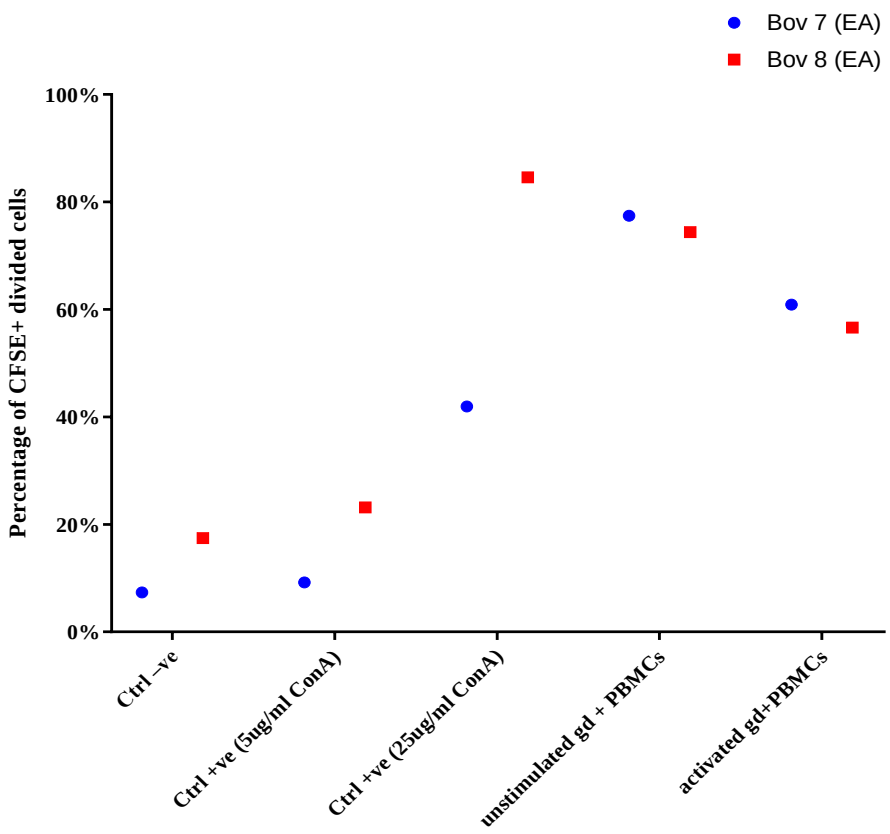


Figure 5.54: Proliferation (CFSE diminuition peak percentages) of CFSE-labelled PBMCs mixed with both unstimulated and activated γδ T cells separately in Bov 7 and Bov 8 showing the stimulatory role of γδ T cells. Unstimulated γδ T cells are potent stimulants for PBMCs (77.42% and 74.37% for bov 7 and bov 8 respectively) in comparison with control negative groups: (7.36% and 17.43%). The samples were loaded in duplicates. The samples left were very limited because I consumed most of them in above tests (and that was a limitation), therefore only 2 animals were selected. It was not possible to present the data results as Mean ± Standard error because the results were not repeated twice (only once) and that was also due to the shortness of kit materials.

5.7.2 Purified unstimulated γδ T cells have stimulatory effect on activated bovine autologous PBMCs (stimulated)

As described in section 5.3.4.2 in this chapter, a total of 1 X 10^5, 2 X 10^5, 3 X 10^5 and 4 X 10^5 cells/ml γδ T cells were mixed separately with 5 X 10^5 cells /ml CFSE-labelled autologous PBMCs from 2 animals (Bov 6 and Bov 7, which were previously activated with 5 ug/ml ConA in 25 ml flasks for 3 days and washed) in wells of a 24 well plate then incubated for 48 hours. Control positive group was re-stimulated with 5 ug/ml Con A.

Because this experiment was done in 2 stages and lasted for 5 days (PBMCs were stimulated for 3 days with ConA then washed and mixed with γδ T cells for 2 days), day 3 and day 5 were used to describe the duration of stimulation after the initial step of stimulation.

Results demonstrated a dose-dependent stimulatory role of γδ T cells on Con A-activated PBMCs. This was represented by elevated CFSE peaks of proliferation (lesser fluorescence than initially labelled cells) that ranged between 58.47% to 83.35% and 68.63% to 80.88% in the mixtures of (1X10^5 γδ T cells + 5X10^5 PBMCs) and (4X10^5 γδ T cells + 5X10^5 PBMCs) compared with control negative groups (13.86%) and (56.62%) in Bov 6 and Bov 7 animals respectively (see figures 5.55, 5.56, and 5.57).

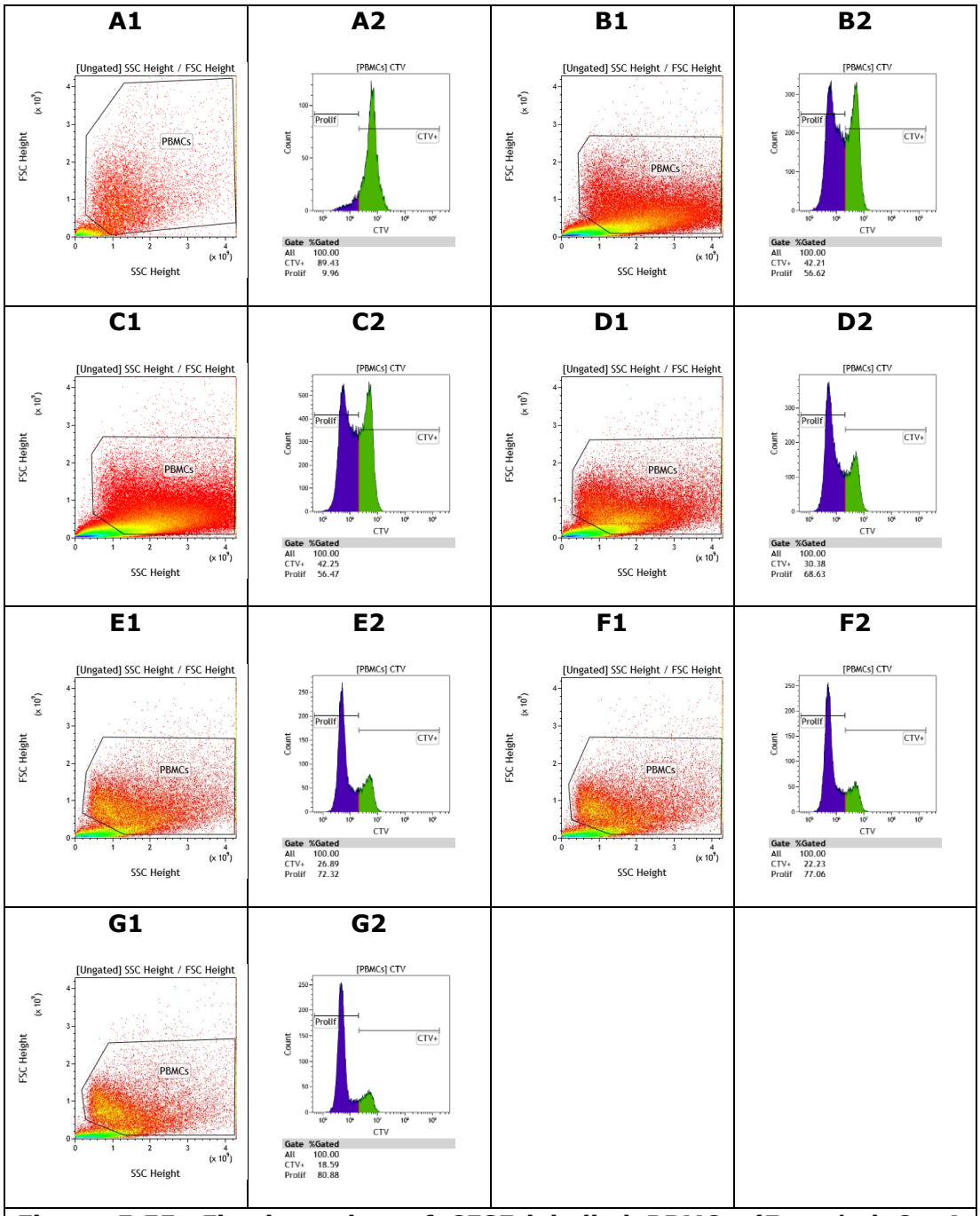


Figure 5.55: Fixed number of CFSE-labelled PBMCs (5 ug/ml ConA activated for 3 days) at days 3 (after activation) and 5 (after two days of incubation in the assay) from Bov 7 mixed with different numbers of unstimulated purified γδ T cells separately. A1= dot plot of CFSE-stained PBMCs at day 3. B1= dot plots of CFSE-labelled PBMCs in the control negative group at day 5. C1= dot plot of control positive group (5 ug/ml ConA) at day 5. D1= dot plot of a mixture of $1X10^5$ γδ T cells and $5X10^5$ PBMCs at day 5. E1= dot plot of a mixture of $2X10^5$ γδ T cells and $5X10^5$ PBMCs at day 5. F1= dot plot of a mixture of $3X10^5$ γδ T cells and $5X10^5$ PBMCs at day 5. G1= dot plot of a mixture of $4X10^5$ γδ T cells and $5X10^5$ PBMCs at day 5. A2, B2, C2, D2, E2, F2, and G2 = histograms of A1, B1, C1, D1, E1, F1, and G1. Green indicates the peak of cells initially labelled on day; blue/purple, the CFSE expression of progeny cells having divided by day 3.

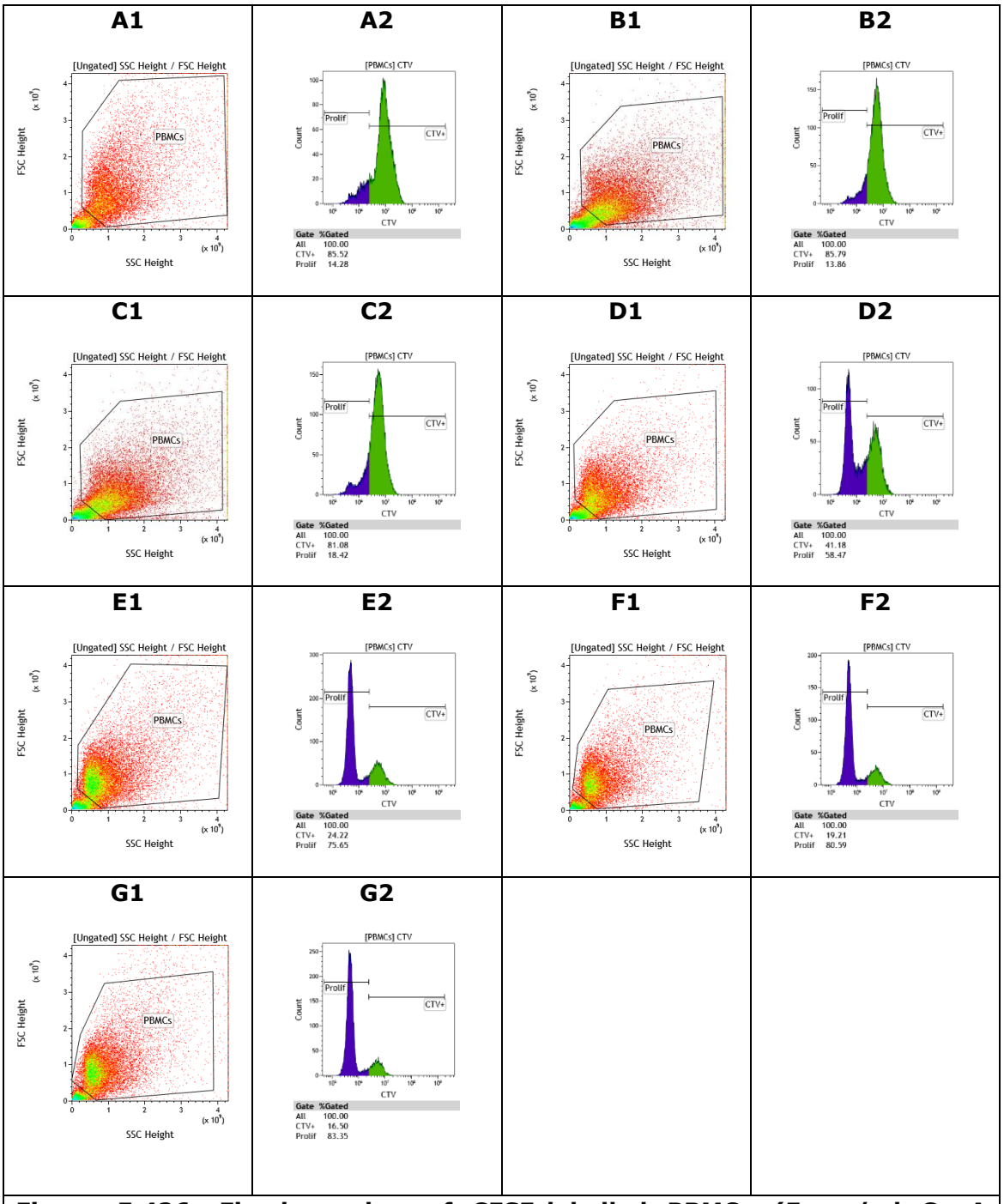


Figure 5.426: Fixed number of CFSE-labelled PBMCs (5 ug/ml ConA activated for 3 days) at days 3 and 5 (after two days of the co-culture assay) from Bov 6 mixed with diverse numbers of unstimulated purified γδ T cells separately. A1= dot plot of CFSE-stained PBMCs at day 3. B1= dot plots of CFSE-labelled PBMCs in the control negative group at day 5. C1= dot plot of control positive group (5 ug/ml ConA) at day 5. D1= dot plot of a mixture of $1X10^5$ γδ T cells and $5X10^5$ PBMCs at day 5. E1= dot plot of a mixture of $2X10^5$ γδ T cells and $5X10^5$ PBMCs at day 5. F1= dot plot of a mixture of $3X10^5$ γδ T cells and $5X10^5$ PBMCs at day 5. G1= dot plot of a mixture of $4X10^5$ γδ T cells and $5X10^5$ PBMCs at day 5. A2, B2, C2, D2, E2, F2, and G2 = histograms of A1, B1, C1, D1, E1, F1, and G1. Green indicates the peak of cells initially labelled on day; blue/purple, the CFSE expression of progeny cells having divided by day 3.

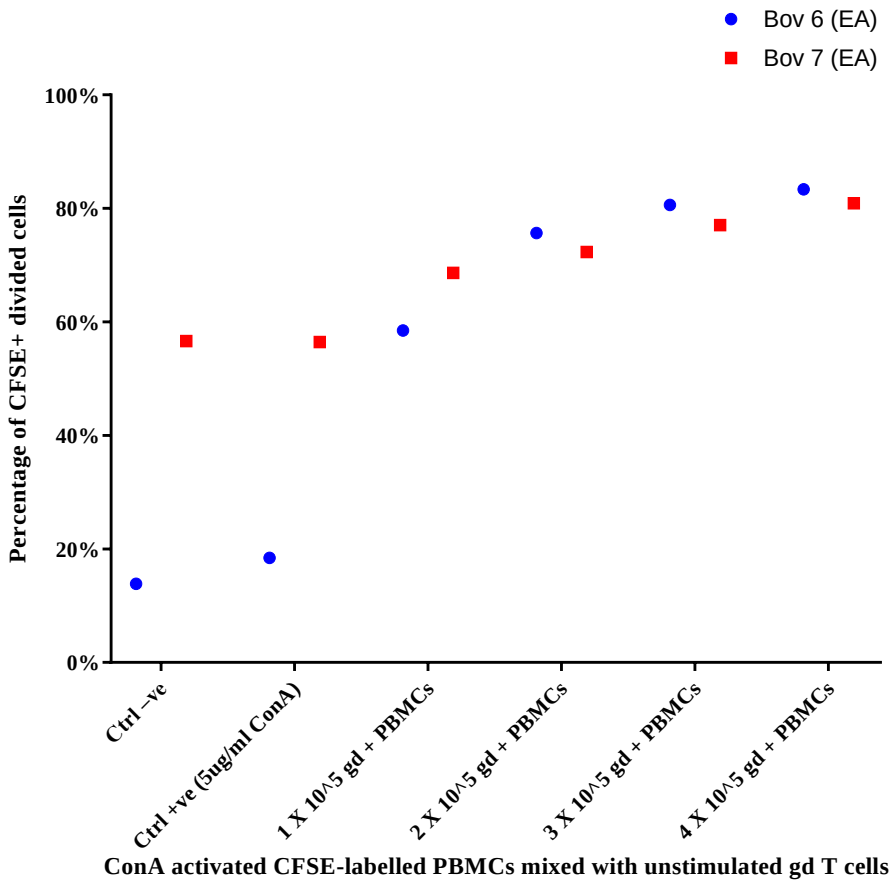


Figure 5.437: Proliferation (percentages) of CFSE-labelled PBMCs (5 ug/ml ConA activated for 3 days) mixed with unstimulated purified γδ T cells (for two days of the assay) separately in Bov 6 and Bov 7 blood animals showing the stimulatory role of γδ T cells. Proliferative PBMCs were 83.35% and 80.88% in the mixture of $4X10^5$ γδ T cells and $5X10^5$ PBMCs in bov 6 and bov 7 respectively in comparison with control negative groups (13.86% and 56.62% respectively. The samples were loaded in duplicates. The samples left were very limited because I consumed most of them in above tests (and that was a limitation), therefore only 2 animals were selected. It was not possible to present the data results as Mean ± Standard error because the results were not repeated twice (only once) and that was also due to the shortness of kit materials.

5.8 Discussion

The functional assessment of PBMCs, MACS fractionated CD4, CD8, and γδ T cells upon stimulation with TLR 1-9 agonists by CFSE assay (a cell proliferation read-out) was overall unsatisfactory. PBMCs did not proliferate significantly in response to TLR-agonist stimulation above unstimulated control levels. This was in spite of the Con-A positive control of stimulated cells that gave a good response by comparison with the control negative group. ConA provides a potent mitogenic activity for T cells through its binding to TCR receptor glycoproteins (Balch *et al*., 1984, Luzyanina *et al*., 2007, Quah and Parish, 2012). On the other hand, the anti-CD3 positive stimulation control gave mixed results. Both Con-A and anti-CD3 antibody had been previously tested by dose response on cattle PBMC and T cells and the concentrations used were optimal for 3-7 day cultures.

Bovine CD4+ T cells (5 animals studied) generally did not divide/ proliferate (CFSE assay) in reponse to any of the TLR-agonists, with the exception of bov 1 CD4 T cells that responded to the TLR3 agonist and bov 5 cells that responded to both the TLR-3 agonist and the TLR-8 agonist. Similarly for CD8+ T cells, where only those (seven animal cell preparations were studied) from bov1 responded to TLR3-agonist and TLR-9 agonist, and from bov 5 that responded to TLR8-agonist and TLR9 agonist. For γδ T cells, only those from bov1 responded to TLR8 agonist. As some stimulation was seen in a few bovine purified T cells, it was decided to try another assay where T cell subsets within PBMCs from the different animals were labelled differentially with fluorescinated antibodies and then looking for the effect of PBMC proliferation of the different cell

types. This avoided isolating purified populations of T cell subsets. This was unsuccessful. The difficulties encountered with the proliferation assays for measuring cell responses to TLR-agonists led us to the conclusion that troubleshooting further would take up limited time with no guarantee of success, and it was decided to proceed to measuring cytokine expression as a readout for TLR-agonist activation of the PBMC and T cells within (Chapter 6). This means that the results of this chapter are preliminary in nature and further work is required.

The reasons for the lack of success are not known, but could include: (1) the assays were not optimised. The TLR-ligands have been shown to stimulate bovine cells at the concentrations used (Werling *et al*., 2004, Mookherjee *et al*., 2006, Lee *et al*., 2008, Arsenault *et al*., 2009, Koets *et al*., 2010, Larsson, 2010, Nelson, 2010, Chu *et al*., 2011, Olivia, 2012, Porcherie *et al*., 2012, Feldman *et al*., 2013, Price et al., 2013, Berghuis *et al*., 2014, Dhanasekaran *et al*., 2014, Sei *et al*., 2014, van der Vlugt *et al*., 2014) but the concentrations used may not have been appropriate for stimulating T cells. It is interesting that where TLR-ligands stimulated a response it was usually the medium and high concentrations that were active. Extending the concentration up may reveal better reactivity.

(2) Different animals may have been immunologically compromised such that their cells were refractile to TLR-agonist stimulation. The animals used were healthy and clinically-disease-free when they presented prior to euthanasia, but as these are outbred animals in a microbial rich environment there may have been sublimal effects. Short of performing these experiments in specific pathogen free animals, it is difficult to get round this. This is discussed further in the general discussion. Interestingly

bov 1 and bov 5 animal T cells responded to some TLR-agonists differentially (TLR 3 agonist, TLR-8 agonist and TLR 9 agonist), whereas other animal T cells did not respond to the TLR-agonists. This is discussed further in the general discussion.

Finally, γδ T cells (both unstimulated and ConA activated) showed stimulatory but not inhibitory activity for autologous bovine CFSE-labelled PBMCs (unstimulated or ConA stimulated). This is different from other studies, for example (Hoek *et al*., 2009), the first authors who claimed an immunosuppressive role of γδ T cells, and evidenced *in vitro* functionally-immunosuppressive bovine IL-10 secreting γδ T cells but not CD4+CD25hi FOXP3+ T cells (conventionally known as nTregs). They speculated the presence of biological variation between bovine and non-ruminant species with regards to this particular subpopulation of T cells (γδ T cells) where at least a proportion of which expressed IL-10 (the immunosuppressive cytokine). Guzman *et al*., (2014) has also demonstrated that bovine γδ T cells were an immunosuppressive population, more dominant than CD4+CD25hi FOXP3+ cells (nTreg) and also detected IL-10 and TGFβ cytokines (known to have immune-inhibitory role on other immune cells) in the supernatant of co-culture of γδ and autologous CFSE-labelled mononuclear cells which showed significant regression in their number upon incubation *ex vivo* in culture. The reasons why γδ T cells were stimulatory for autologous PBMC is not known and requires further study.

γδ T cells in cattle consist of several subtypes.

The clone WC1 gamma delta T cells is most likely existed not only in bovine family, but it also discovered in pigs, goats, sheep, mice, horses as well as humans (Wijngaard *et al*., 1992). WC1 γδ T cells are subdivided in cattle

into 3 further clones which are WC1.1, WC1.2, and WC1.3 (Rogers *et al*., 2005, Rogers *et al*., 2006). In general, bovine WC1 γδ T cells are phenotypically divided into 2 main categories which are: WC1−CD2+CD3+ γδ T cells that located in the red pulp of the spleen and the intestinal canal, whilst the second phenotype WC1+CD2−CD3+ γδ T cells that existed mainly in the peripheral blood (Baldwin *et al*., 2019).

Additional markers have been noted on human, murine and bovine γδ T cells which included CD2, CD4, CD5, CD6, and CD8 but functionally these markers are not known to play a role in these cells, or these markers are less beneficial to these γδ T cells (Machugh *et al*., 1997, Hayday, 2000).

The function of γδ T cells can be basically explained by their lack to CD4 or CD8 (CD4-CD8- T cells), therefore these cells counterpart the invasive microbes without conjugated with either MHC I or MHC II as described in Chapter one. The second function is that γδ T cells can recognise lipid antigens and phosphorylated microbial metabolites (Baldwin *et al*., 2019). Third function is that these cells produce mediators (perforin and granzyme) involved in cellular cytotoxicity such as in TB infection (Dieli *et al*., 2000, Dieli *et al*., 2001). The last function is that cytokine production where γδ T cells yield IFN-*γ* and TNF-α, macrophage-colony stimulating factor, IL-17, and IL-21 , IL-4, IL-10, IL-11, keratinocyte growth factor or connective tissue growth factor (Wesch *et al*., 2001, O'Brien *et al*., 2007, Beetz *et al*., 2008).

In this study we used the WC1 γδ T cell antibody that recognises the majority of bovine γδ T cells and is excellent in methods to bind to and hence purify γδ T cells from the tissues.

Although human γδ T cells may not necessarily be the same as that of bovine counterparts (Baldwin and Telfer, 2015, Silva-Santos *et al*., 2015) (neither react with MHC class I or II on APC cells), human γδ T cells have been described in the literature to possess immunosuppressive effects in host responses to cancer (Dar *et al*., 2014) upon TLR agonist activation. In the same context, Peng *et al*. (2007) investigated the functional role of γδ T cells which demonstrated immunosuppression of T cells and dendritic cells in breast cancer *in vitro* and *in vivo,* during a response to TLR8 ligands. Finally, recent studies on human γδ T cells confirmed their defensive role against cancer and autoimmune diseases as well as in fighting some infectious diseases and play a role in clinical and medical future immunotherapy (Lawand *et al*., 2017).

Chapter Six

TLR-ligand activated PBMC and T cell subsets: Cytokine production

6.1 Abstract

The objective was to measure cytokine responses from TLR-ligand-stimulated PBMC and T cell subsets. ELISA techniques were used to measure 7 cytokines, known to be produced, differentially, in various cell-types after TLR-ligand stimulation. These were: CXCL-8, IFNα, IFNγ, TNFα, TGFβ, IL-4, and IL-10. There was no significant cytokine response at the entire group level for any cytokine, but individual samples did give a significant response for some analyses. In general, the results showed variable responses that were PBMC and T cell subset dependent. TLR1-9 agonists stimulated PBMC production of CXCL-8 in most samples. Purified CD4, CD8, and γδ T cells produced CXCL-8 in response to TLR3L, TLR8L and TLR9 ligand. Type I interferon (IFNα) was not reliably detected. IFNγ was produced in some PBMC samples to a range of TLRLs. However, purified CD4+ T cells, CD8+ T cells and γδ T cells did not produce any significant quantities of IFNγ in any sample to the restricted range of TLR3L, TLR8L and TLR9Ls.

TNF-α production was not significantly produced (compared to controls) in PBMC or T cell subsets (except one PBMC sample stimulated with a low concentration of TLR3 agonist). TGFβ also was not significantly produced after TLR-ligand stimulation of PBMC or associated T cell subsets.

IL-10 was increased in some TLR-ligand stimulated PBMCs and some CD4+ T cells and γδT cells (in response to TLR3, TLR8, and TLR9 ligands) IL-4 was produced in some PBMC samples and in CD4 T cells predominantly within the T cell subsets (to TLR3L, TLR8L and TLR9L).

6.2 Introduction

Bovine TLR expression was detected in PBMCs and T cells (Chapter 4) and a CFSE proliferation assay used as a readout for activation of the receptors by TLR-ligand (Chapter 5). As not all activated cells necessarily divide or proliferate, it is important to measure some other indicators of cell activation. For TLR activation, there are well-described signalling pathways described (Kabelitz, 2007). The outcome from this leads to production of pro-inflammatory cytokines in antigen-presenting cells, epithelial and endothelial cells and lymphocytes, but with the likelihood of some cell-specific cytokine production (e.g. IL-4 and IFNγ in T cells) (Reynolds and Dong, 2013). The cytokines produced include IL-1β, IL-6, IL-12, CXCL-8, TNFα, type I interferons (IFNα and β), IFNγ, G-CSF and GM-CSF (Takeda and Akira, 2004, Kawai and Akira, 2006, Kawai and Akira, 2007, Kawasaki and Kawai, 2014). Upon stimulation with TLR agonists, T lymphocytes responded to external stimuli and as a result some further sets of cytokines have been noted such as IL-4 (van Panhuys *et al*., 2008), TGFβ (Takebayashi *et al*., 2009), and IL-10 (Saraiva and O'Gara, 2010). Detailed information about each cytokine has been mentioned in chapter one (introduction), and this information included the cells that produce each cytokine, its function and the target cells.

Previous studies on bovine monocytes and macrophages stimulated with TLR ligands *in vitro* revealed secretion of abundant amounts of CXCL-8 (Russell, 2012, Russell *et al*., 2012, Smith, 2012). We will use ELISA to measure bovine CXCL-8, IFNα, IFNγ, TNFα, TGFβ, IL-4, and IL-10 in the supernatants of PBMCs, CD4, CD8, and γδ T cells co-cultured with TLR agonists.

6.3 Materials and methods

BMCs and major T cell fractions (CD4, CD8, and γδ T cells) were stimulated with TLR ligands in the presence/absence of CFSE stain as described in sections 2.4.4 and 2.7.5 (for FACS protocols). Seven cytokines were measured in response to stimulation with TLR ligands by ELISA (see section 2.9 and its branches 2.9.1 to 2.9.8), which were specific for:IL-8 (CXCL-8), IFNα, TNF-α, IL-4, IL-10, TGFβ, and IFNγ.

The pro-inflammatory chemokine (CXCL-8) secretion from the stimulated cells was considered the principal initial readout for TLR signalling pathways because many of the TLR+ cells have been shown to produce this (Kabelitz, 2007, Brasier, 2010, Tornatore *et al*., 2012). Other above listed cytokines also have been selected based on the recommendations collected from the literature.

Because the T cell subsets were shown to express TLR3, TLR8, and TLR9 and there is a limited supply of T cell subsets from any PBMC for testing, the focus was on these.

Results

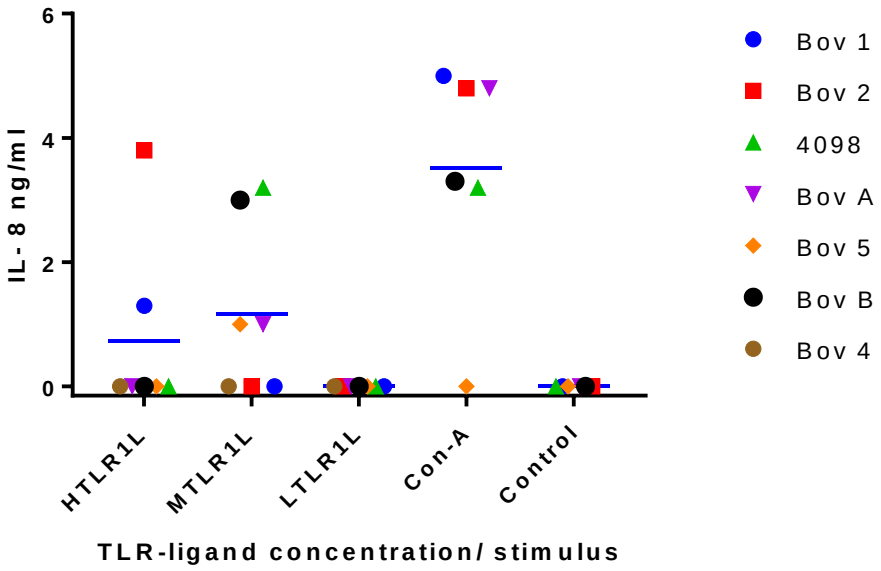
6.4 CXCL-8 production from TLR-ligand stimulated PBMCs and T cell subsets.

6.4.1 CXCL-8 results for PBMCs stimulated with TLR ligands

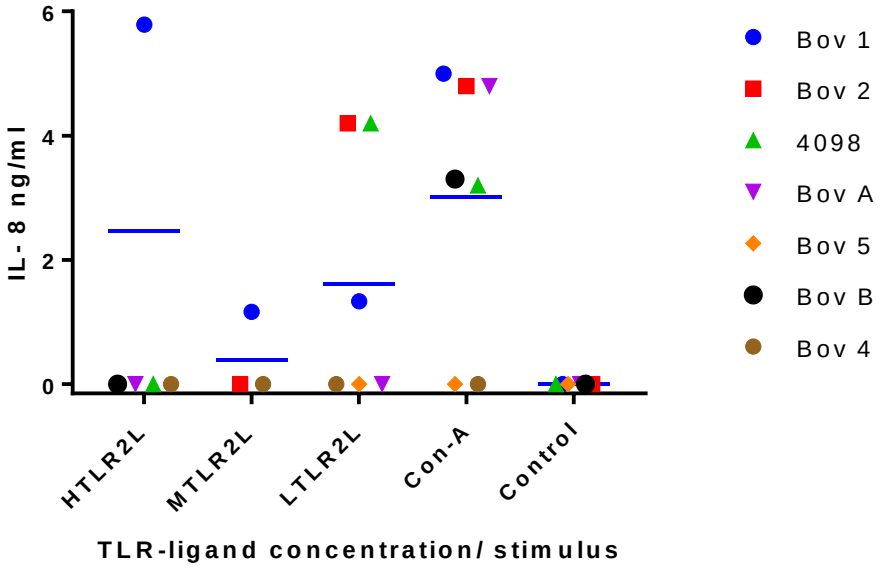
Figure 6.58 shows the mean of CXCL-8 (IL-8) responses in the supernatants of the seven PBMC samples studied and stimulated with TLR-ligands in a dose response. The comparison between PBMC TLR ligand-stimulated groups and unstimulated controls (Mean ± Standard error) showed no significant differences at the group level, but there were individual animal samples that exhibited a significant response and these are highlighted below. The PBMCs secreted IL-8 in response to high concentrations of TLR1 ligand in Bov 2 animal and also medium concentrations in Bov B and Bov4098 animals by comparison with the control negative group (P=0.041, P=0.0477 and P= 0.0473 respectively Figure 6.58a). In Figure 6.58b, responses to TLR 2 agonists at high concentration in Bov 1 and to low concentrations in Bov 2 and Bov 4098 animals were observed (P=0.03755, P= 0.0398 and P= 0.0406). Figure 6.58c showed a significant increase in CXCL-8 concentrations in response to high concentrations of TLR3 agonist in Bov 2 and Bov 4098 animals (P=0.00238 and P=0.00287) plus another significant increase in CXCL-8 in response to medium concentrations of TLR3 agonist in Bov 1 and Bov 4098 (P=0.0312 and P=0.0348). A significant increase in CXCL-8 was recorded in response to TLR4 ligand at high concentration in 3 animals

(Bov 1, Bov2, Bov 4098) by comparison with control negative group (figure 6.58d, P=0.0363, P=0.0329 and P=0.0373).In Figure 6.58e, high concentrations of TLR5 ligands gave significant increases of CXCL-8 in 3 animals (Bov 1, Bov 2 and Bov4098 animals) compared with the control group (P=0.0421, P=0.0407 and P=0.0441). Figure 6.58f shows a marked secretion of CXCL-8 in response to TLR6 ligands at high and low concentrations in 3 animals (Bov1, Bov2, and Bov 4098) by comparison with the control group (P=0.0414, P=0.0411 and P=0.0416). PBMCs in Figure 6.58g responded significantly by comparison with the control group to TLR7 agonists at high concentrations in 5 animals (Bov1, Bov2, Bov4, Bov5 and Bov4098) (P=0.0333, P=0.0302, P=0.0275, P=0.0228 and P=0.0341 respectively) and also when stimulated with low concentrations in 3 animals (Bov1, Bov2 and Bov4098) (P=0.0401, P=0.0442 and P=0.0403*)*. In figure 6.58h, 3 animals (Bov1, Bov2, and Bov4098) produced CXCL-8 at significant levels upon stimulation with high concentrations of TLR8 agonists (P=0.0418, P=0.0433 and P=0.0439 respectively) when compared to control unstimulated group*)*. Finally, high concentrations of TLR9 agonists stimulated a significant increase in CXCL-8 in 5 animals (Bov1, Bov2, Bov4, Bov5 and Bov4098) as shown in figure 6.58i (P=0.0407, P=0.0442, P=0.0488, P=0.0492 and P=0.0405 respectively) compared to unstimulated control group*)*.

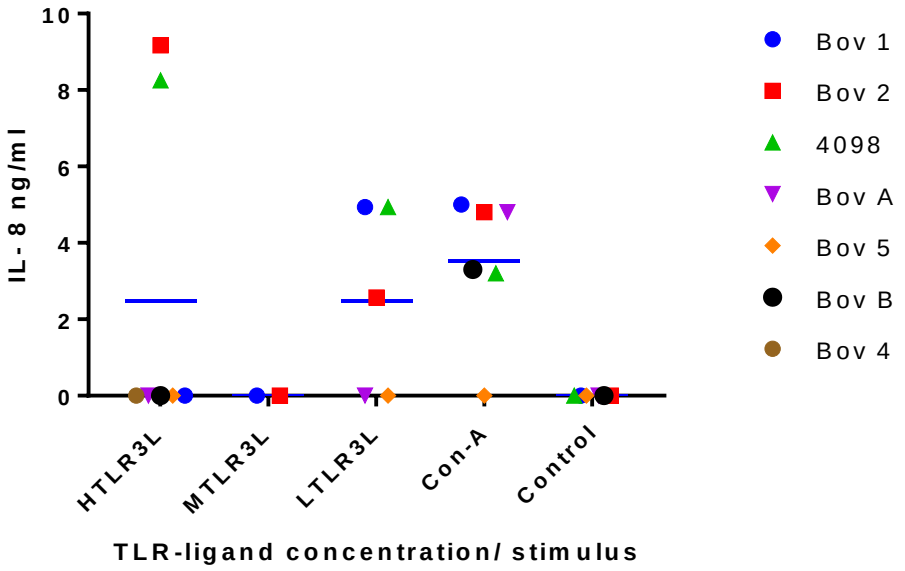
a) PBMCs: CXCL8 (IL-8) TLR1-ligand



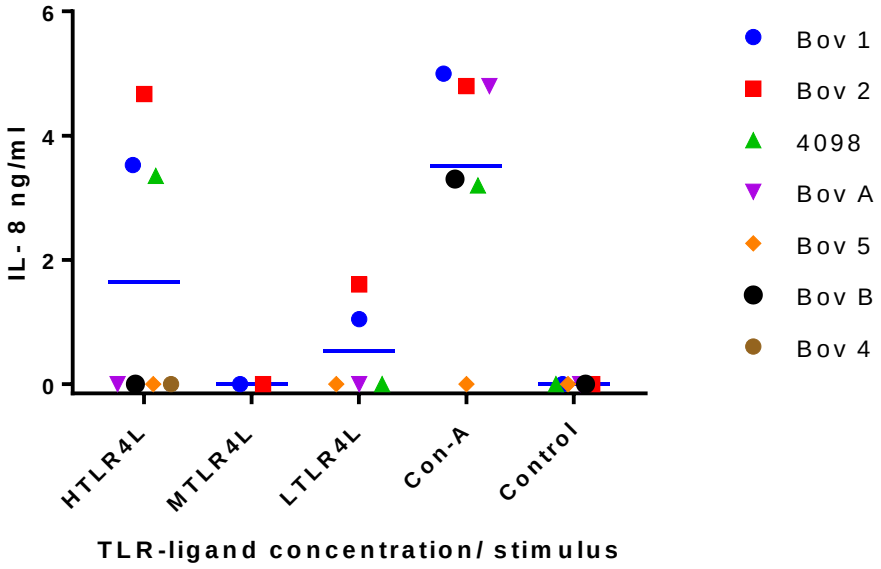
b) PBMCs: CXCL8 (IL-8) TLR2-ligand



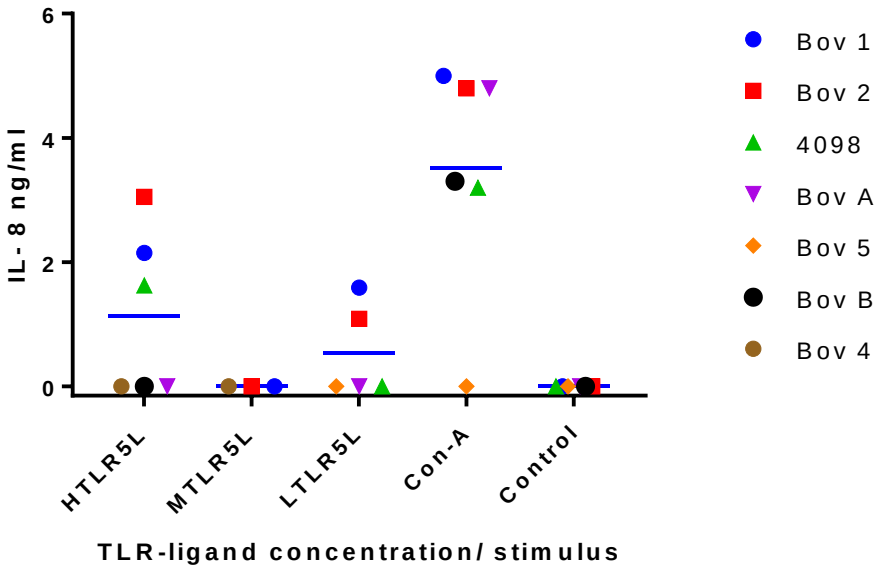
c) PBMCs: CXCL8 (IL-8) TLR3-ligand



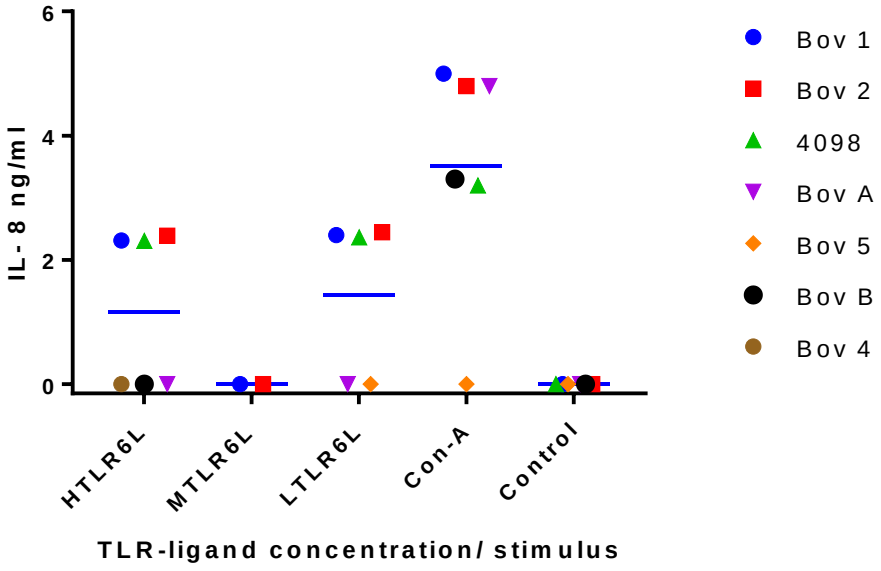
d) PBMCs: CXCL8 (IL-8) TLR4-ligand



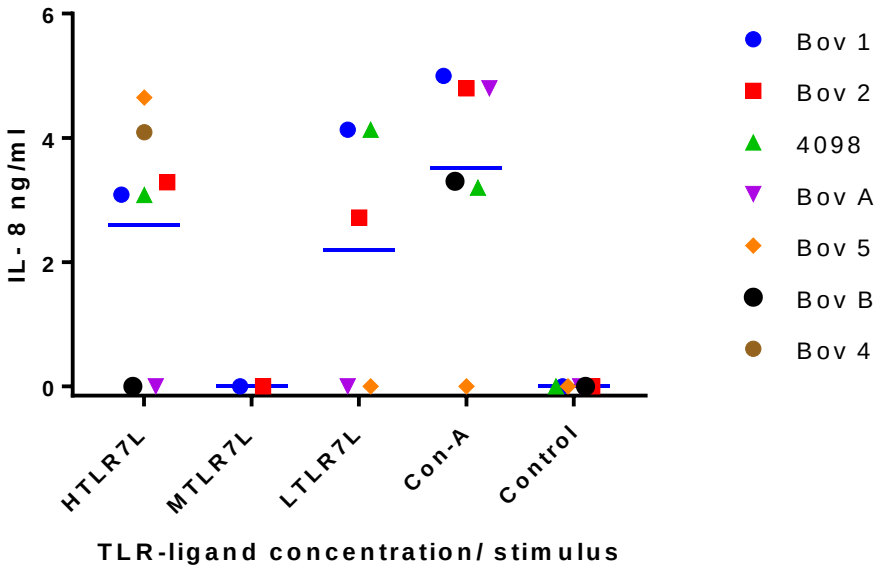
e) PBMCs: CXCL8 (IL-8) TLR5-ligand



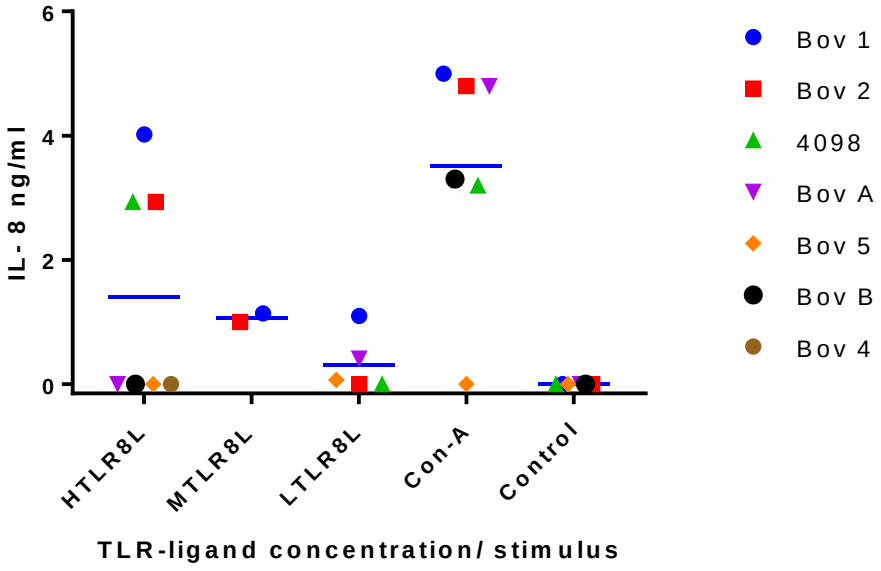
f) PBMCs: CXCL8 (IL-8) TLR6-ligand



g) PBMCs: CXCL8 (IL-8) TLR7-ligand



h) PBMCs: CXCL8 (IL-8) TLR8-ligand



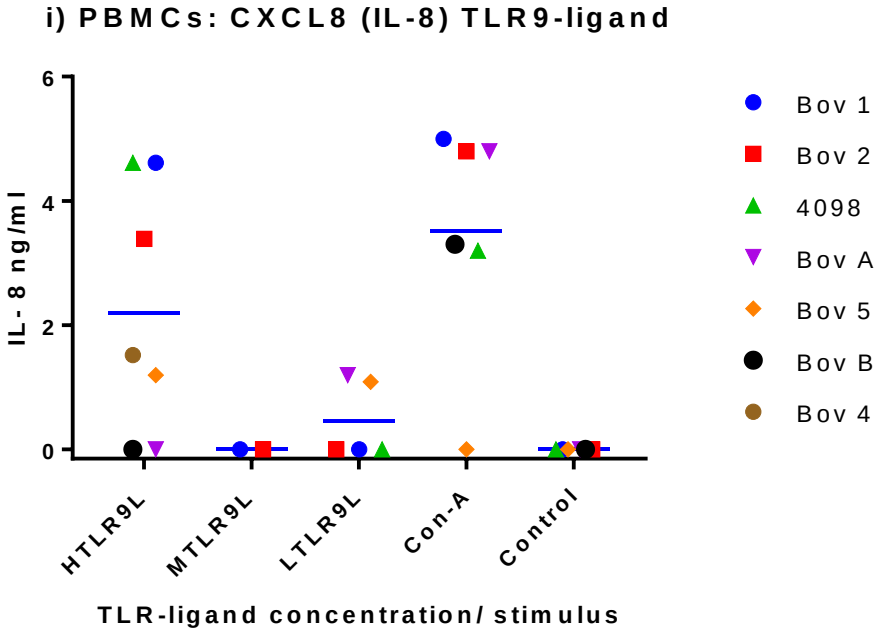
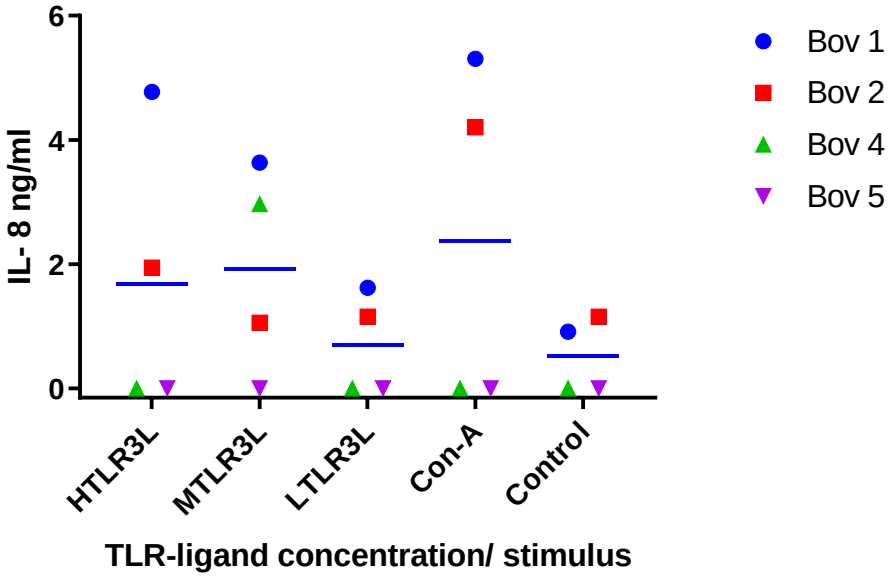


Figure 6.448: CXCL-8 concentrations measured in the supernatant of PBMCs cultured in response to TLR1-9 agonists in 7 animals. a) TLR1 ligand b) TLR2 ligand c) TLR3 ligand d) TLR4 ligand e) TLR5 ligand f) TLR6 ligand g) TLR7 ligand h) TLR8 ligand i) TLR9 ligand TLR ligand concentrations are shown as high (H), medium (M) and low (L), where individual TLR ligand concentration values have been listed previously (chapter 2 paragraph 2.4.4).

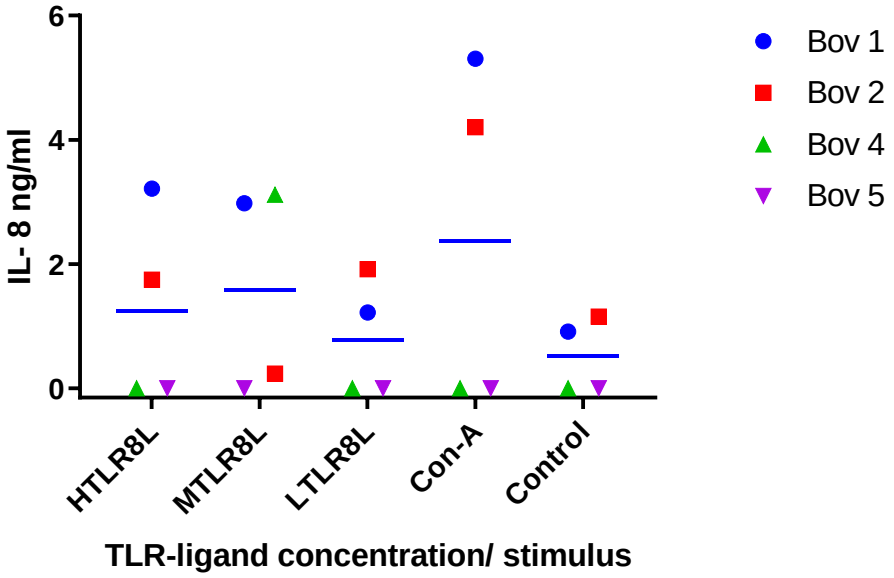
6.4.2 CXCL-8 results of T cell subsets: CD4 T cells stimulated with TLR ligands

The comparison between CD4 T cell TLR ligand-stimulated groups and unstimulated controls (Mean ± Standard error) showed no significant differences at the group level, but there were individual animal samples that exhibited a significant response and these are highlighted below. Samples of CD4 T cells from 4 animals in total were stimulated with TLR3-, TLR8-, and TLR9 agonists. It was not possible to analyse all blood sample T cell subsets and the full inventory of TLR-ligands, so these were chosen (see introduction section 6.2 for reasons).The results are shown in Figure 6.59a. CD4+ T cells of Bov 1 sample showed significant quantities (P=0.0409 compared to unstimulated control) of CXCL8 (IL-8) when medium and high concentrations of TLR3 agonist were used. Also, CXCL8 was secreted significantly (P=0.0483 compared to unstimulated control) when CD4 T cells were stimulated with a medium concentration of TLR3 ligand in Bov 4 animal. In figure 6.59b, high and medium concentrations of TLR 8 agonists induced a significant increase of CXCL8 in Bov 1 while only the medium concentration of TLR8 ligand stimulated a significant increase of CXCL8 in animal Bov 4 (P=0.0246 compared to unstimulated control). Finally, figure 6.59c shows a significant increase in the levels of CXCL8 in response to stimulation with high concentrations of TLR9 ligands in 2 animals (Bov1 and Bov2, P=0.0452 and P=0.0487 compared to unstimulated control), whereas 3 animals (Bov1 , Bov2 and Bov5) yielded significant amounts of CXCL8 when stimulated with medium concentrations of TLR9 ligands (P=0.044, P=0.0461 and P=0.0455 respectively).

a) CD4 T cells: CXCL8 (IL-8) TLR3-ligand



b) CD4 T cells: CXCL8 (IL-8) TLR8-ligand



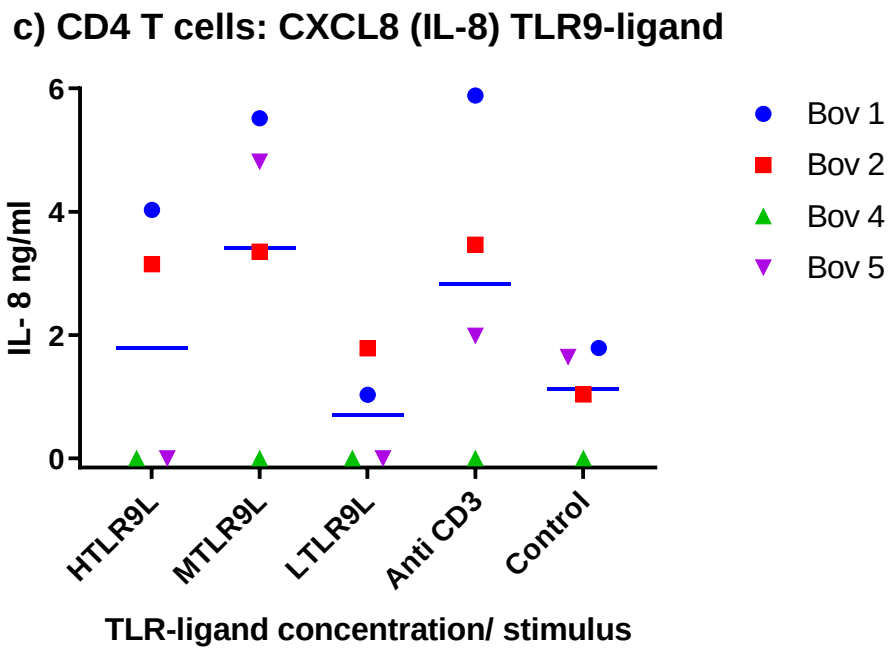


Figure 6.459: CXCL-8 concentrations measured in the supernatant of CD4 T cells cultured in response to TLR3, TLR8, and TLR9 agonists in 4 animals. a) 2 animals yielded significant amounts of IL-8 in response to 3 concentrations of TLR3 ligands. b) 2 animals yielded significant amounts of IL-8 in response to 3 concentrations of TLR8 ligands. c) 3 animals yielded significant amounts of IL-8 in response to 3 concentrations of TLR9 ligands. H, M, L explained as before (chapter 2, paragraph 2.4.4).

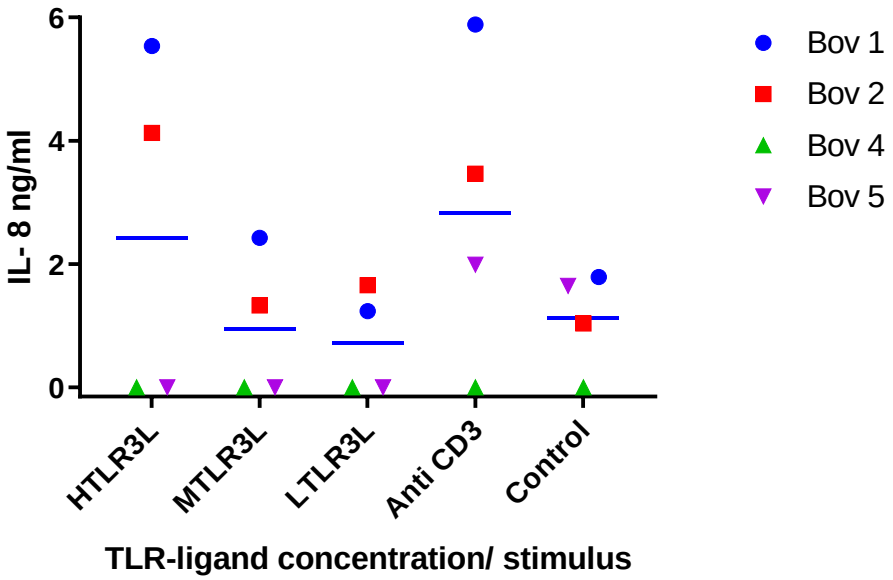
6.4.3 CXCL8 results of CD8 T cells stimulated with TLR ligands

CD8 T cells from the PBMC of 4 animals were stimulated with TLR3, TLR8, and TLR9 agonists.

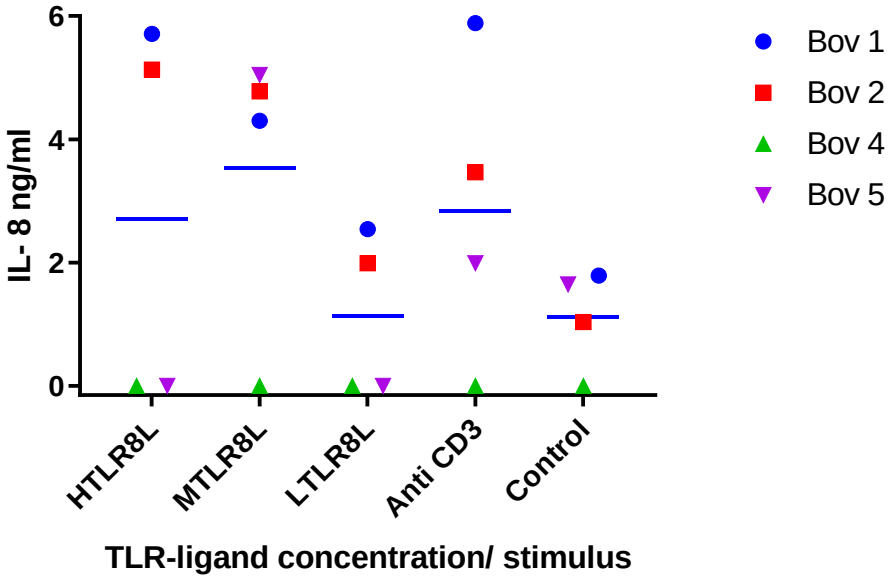
Figure 6.60a shows significant responses (P=0.0316 and P=0.0384) of CXCL8 in the CD8 T cells of Bov 1 and Bov 2 stimulated with high concentrations of TLR3 ligands by comparison with the unstimulated control. Figure 6.60b shows significant responses (P=0.0251 and P=0.0272) of CXCL8 in the CD8 T cells of Bov 1 and Bov 2 stimulated with high concentrations of TLR8 ligands by comparison with the unstimulated

control. Also, significant responses were seen in the Bov1, Bov2, and Bov5 T cells when stimulated with medium concentrations of TLR8 ligands (P=0.0372, P=0.0353 and P=0.0333 respectively). Finally, figure 6.60c shows significant responses (P=0.0386 and P=0.0403) of CXCL8 in the CD8 T cells of Bov 1 and Bov 2 stimulated with high concentrations of TLR9 ligands by comparison with the unstimulated control. Also, significant responses were detected in the T cells from animals Bov1, Bov2, and Bov4 when stimulated with medium concentrations of TLR9 ligands (P=0.0433, P=0.0442 and P=0.0399 respectively).

a) CD8 T cells: CXCL8 (IL-8) TLR3-ligand



b) CD8 T cells: CXCL8 (IL-8) TLR8-ligand



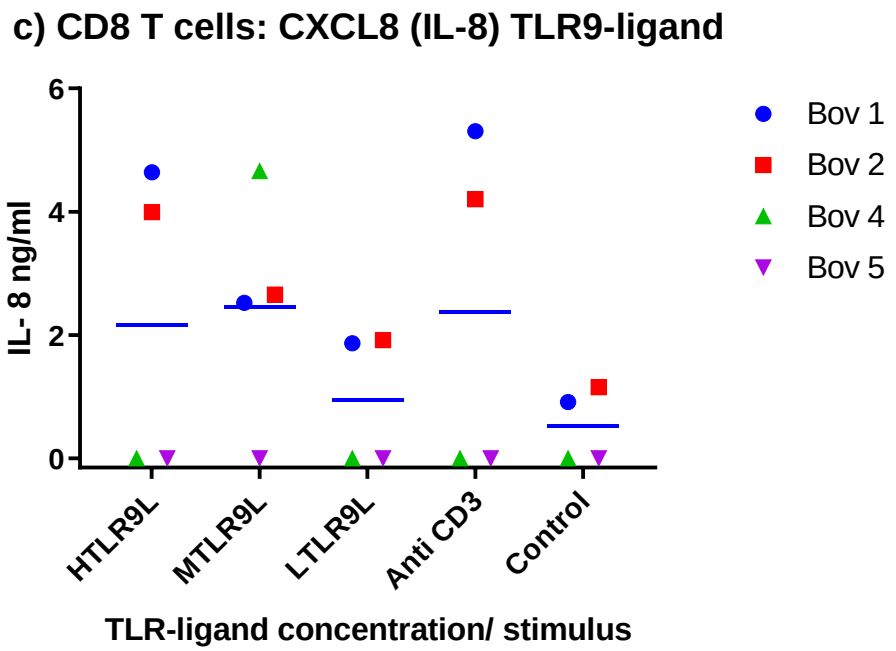


Figure 6.60: CXCL-8 concentrations measured in the supernatant of CD8 T cells cultured in response to TLR3, TLR8, and TLR9 agonists in 4 animals. a) TLR3 agonist. b) TLR8 agonist. c) TLR9 agonist.

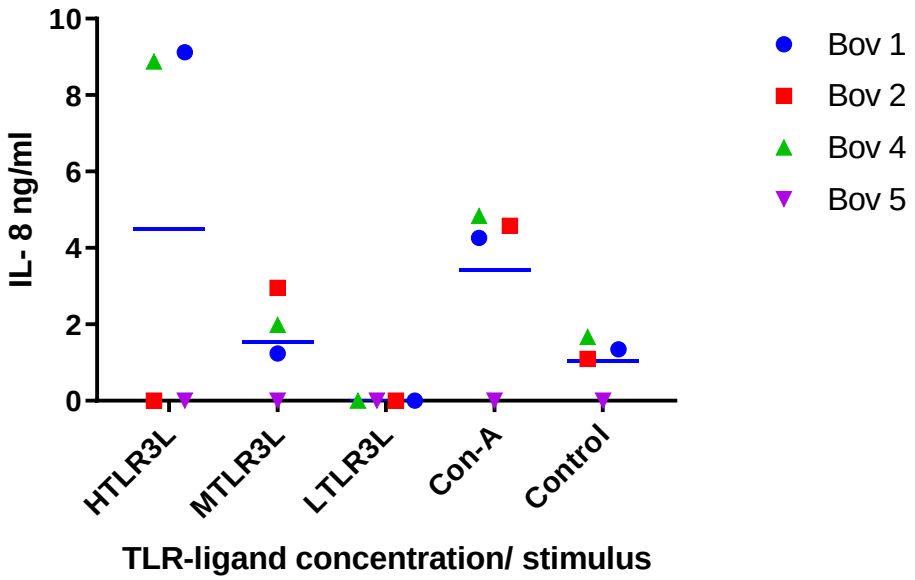
6.4.4 CXCL8 results of γδ T cells stimulated with TLR ligands

There was no significant difference between M±SEM of γδ T cells of the treated animal samples over unstimulated controls at the group level, but there were individual samples that were significantly different from controls. Stimulation with TLR3, TLR8, and TLR9 agonists of γδ T cells isolated from the PBMC of 4 animals were analysed (Bov 1, Bov 2, Bov 4, and Bov 5).

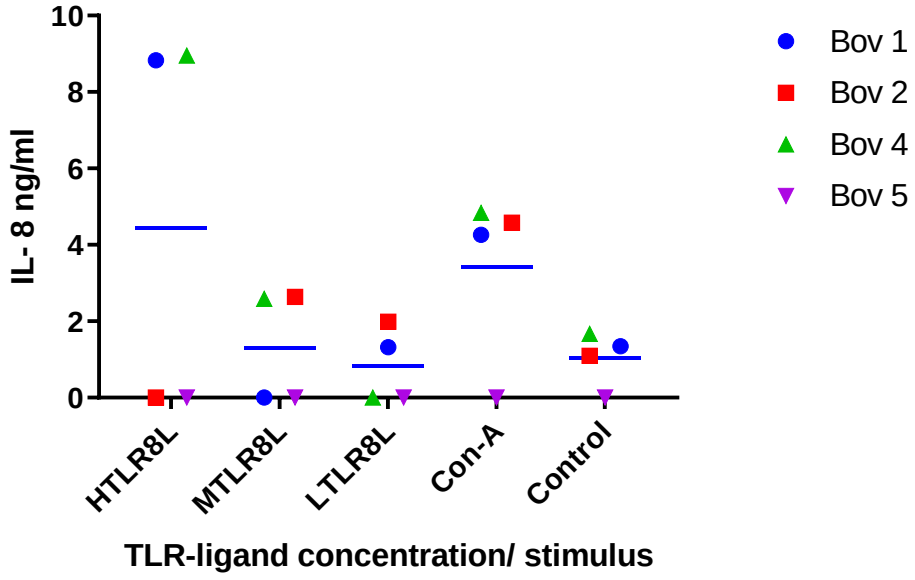
Figures 6.61a and b show significant production (P=0.0147 and P=0.0158) of CXCL8 in the γδ T cell supernatants of Bov1 and Bov4 stimulated with high concentrations of TLR3 and TLR8 agonists. Figure 6.61c shows a significant increase of CXCL8 in the γδ T cell supernatants of Bov 1 and Bov4 stimulated with high concentrations of TLR9 ligands (P=0.0126 and

P=0.0136). In addition, CXCL8 in the γδ T cell supernatants of Bov 1 Bov 2 and Bov 4 in response to medium concentrations of TLR9 ligands were significant (P=0.0176, P=0.0159 and P=0.0211 respectively) by comparison with the unstimulated control.

a) Gamma delta T cells: CXCL8 (IL-8) TLR3-ligand



b) Gamma delta T cells: CXCL8 (IL-8) TLR8-ligand



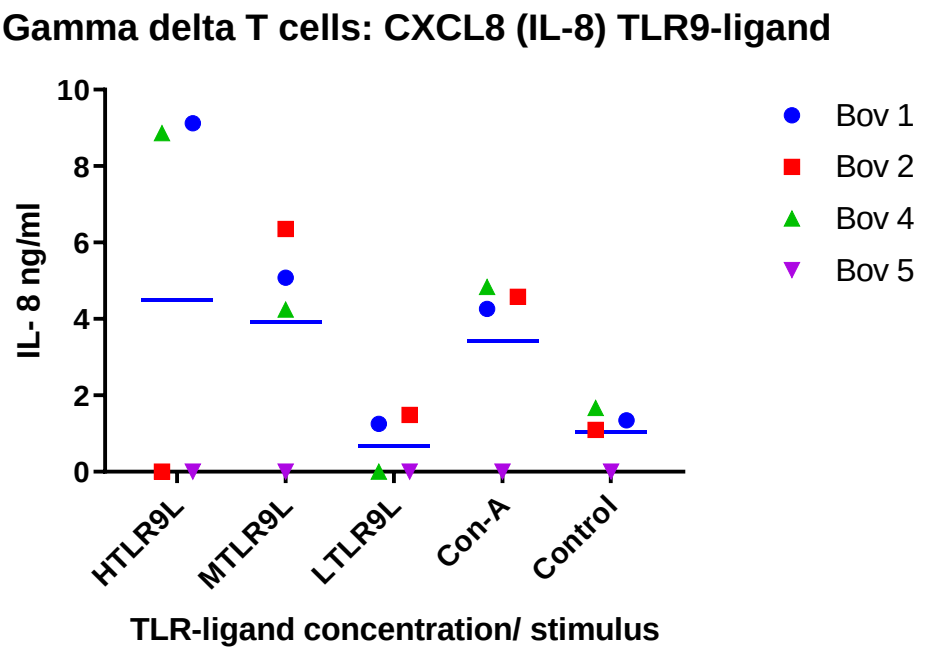


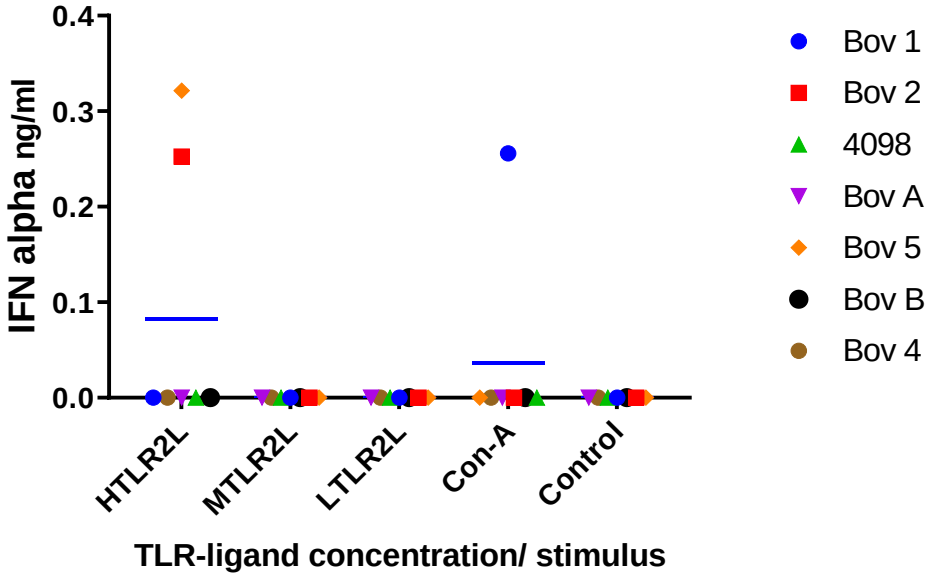
Figure 6.61: CXCL-8 concentrations measured in the supernatant of γδ T cells cultured in response to TLR3, TLR8, and TLR9 agonists in 4 animals. a) TLR3 agonist. b) TLR8 agonist. c) TLR9 agonists.

6.5 IFN-α, TNF-α, and TGF-beta in TLRL-stimulated PBMCs and T cell subsets

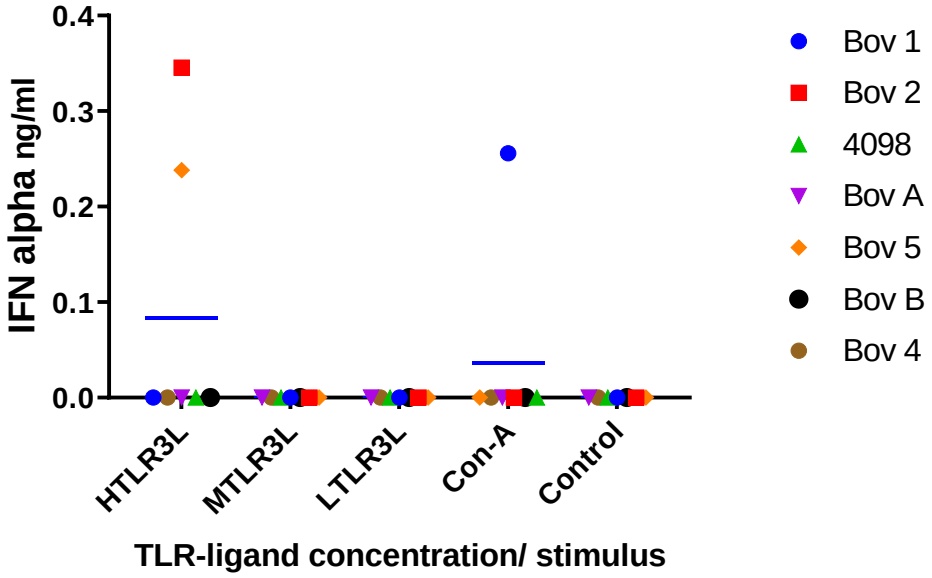
There was no significant difference between M±SE of TGF-β in PBMCs or T cells of the TLRL -treated groups over unstimulated controls. However, only cells from two animals were analysed (due to the capacity of the kit and a lack of time at the end of the project to complete the analysis) (Figure 6.65). There was no significant response in PBMCs or T cells to any TLRL stimulation for IFN-alpha, or TNF-alpha at the group level compared to unstimulated controls, however individual responses were significant compared to controls. Bov2 and bov5 PBMCs stimulated with the highest concentration of TLR2L and TLR3L showed significantly enhanced IFN-

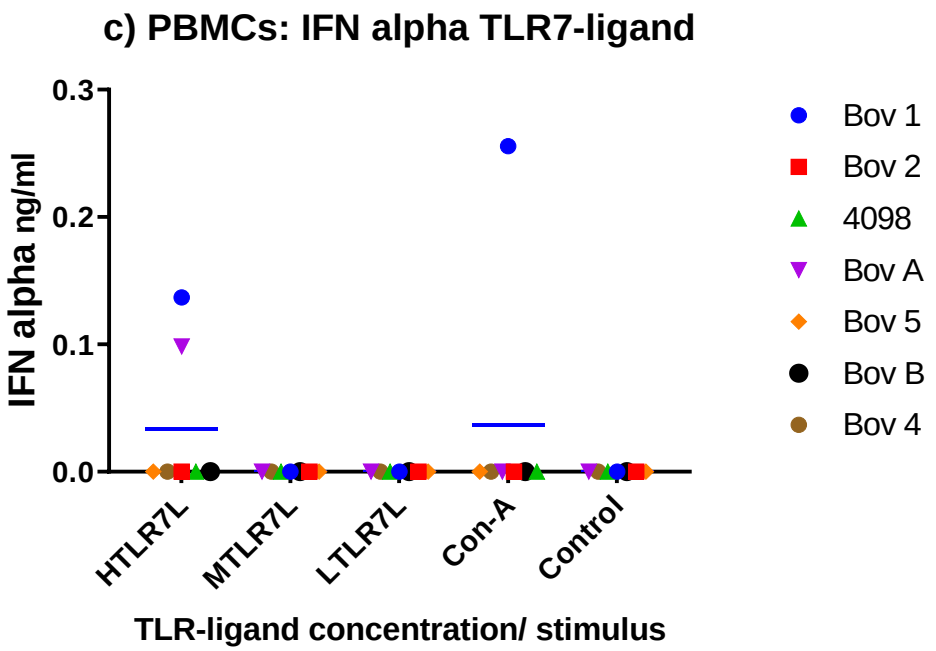
alpha compared to controls. PBMCs stimulated with TLR7L and TLR8L did not produce significant amounts of IFN-alpha compared to controls. CD8 T cells stimulated with high concentrations of TLR3 ligands yielded significant amount of IFNα in 2 animals Bov1 and Bov 2 (P=0.0425 and P= 0.0464 respectively) (Figure 6.63a), but other TLRLs were not tested. Gamma-delta T cells of bovs 4 and 5 stimulated with TLR9-L also showed a significant response compared to controls. On the other hand, individual PBMCs yielded significant amounts of TNFα when stimulated with high concentrations of TLR3 ligands in 5 animals Bov1, Bov2, Bov5, Bov A and Bov4098 (P=0.0482, P=0.0488, P=0.0482, P=0.0466, and P=0.0471) (Figure 6.64c). Finally, individually, PBMCs of Bov5 stimulated with the high concentration of TLR9 ligand gave a significant increase in TNFα (P=0.0468) compared to controls (Figure 6.64i).

a) PBMCs: IFN alpha TLR2-ligand



b) PBMCs: IFN alpha TLR3-ligand





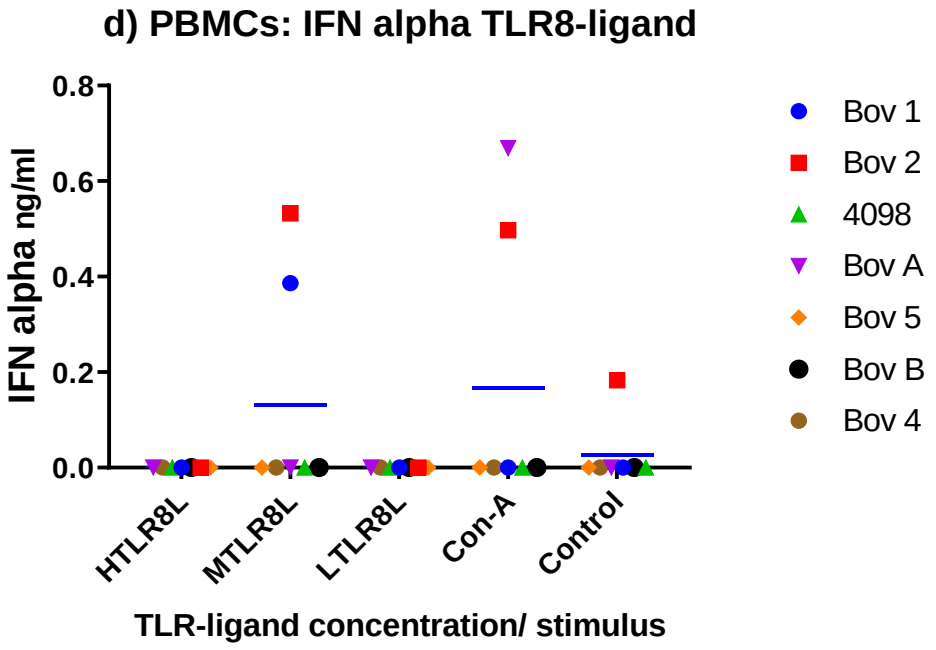
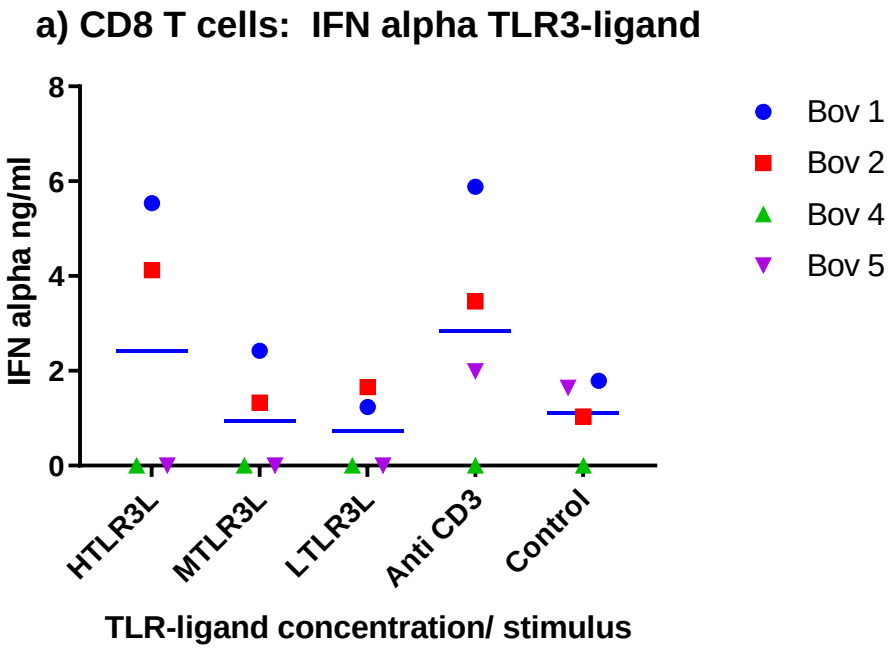
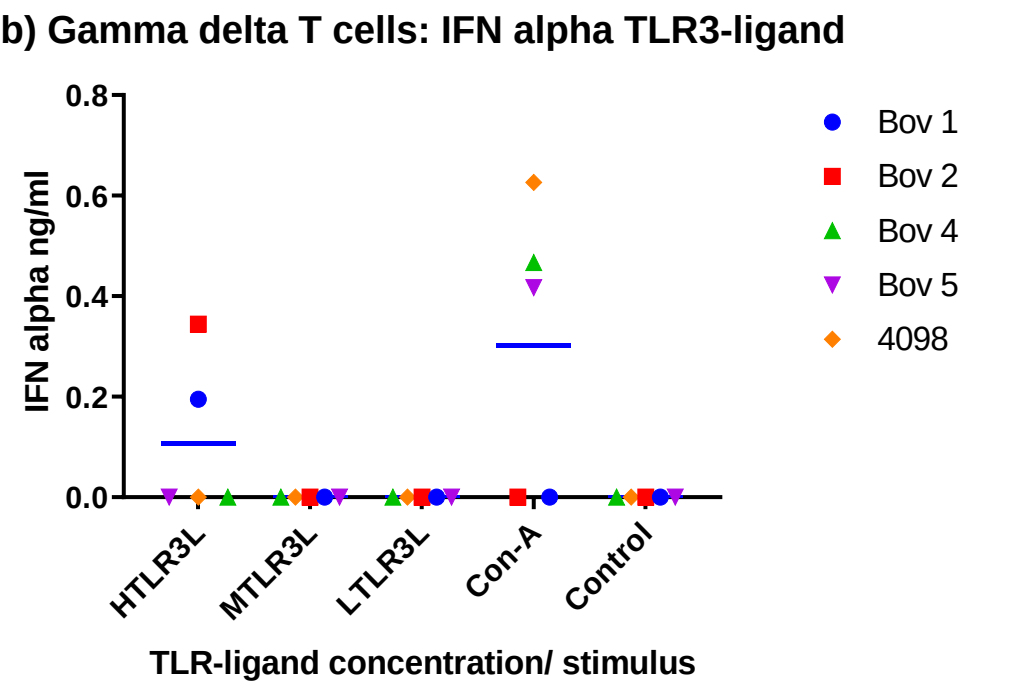


Figure 6.62(a-d): IFN-α concentrations measured in the supernatant of PBMCs cultured in response to TLR1-9 agonists in 7 animals.


a) CD8 T cells: IFN alpha TLR3-ligand
IFN alpha ng/ml
8
6
4
2
0
HTLR3L
MTLR3L
LTLR3L
Anti CD3
Control
TLR-ligand concentration/ stimulus
Bov 1
Bov 2
Bov 4
Bov 5



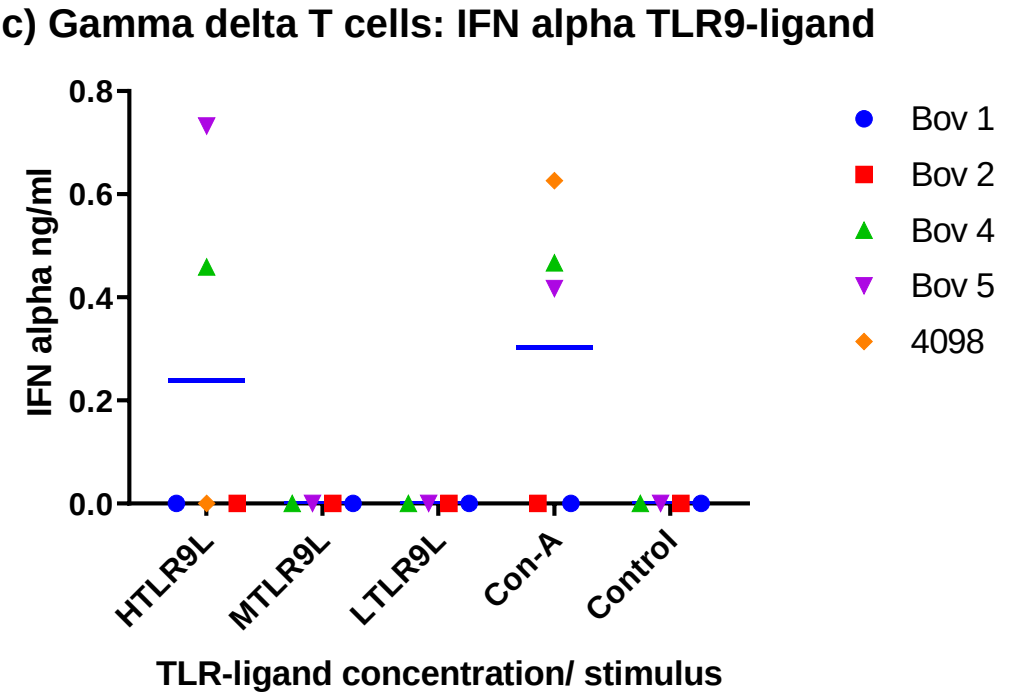
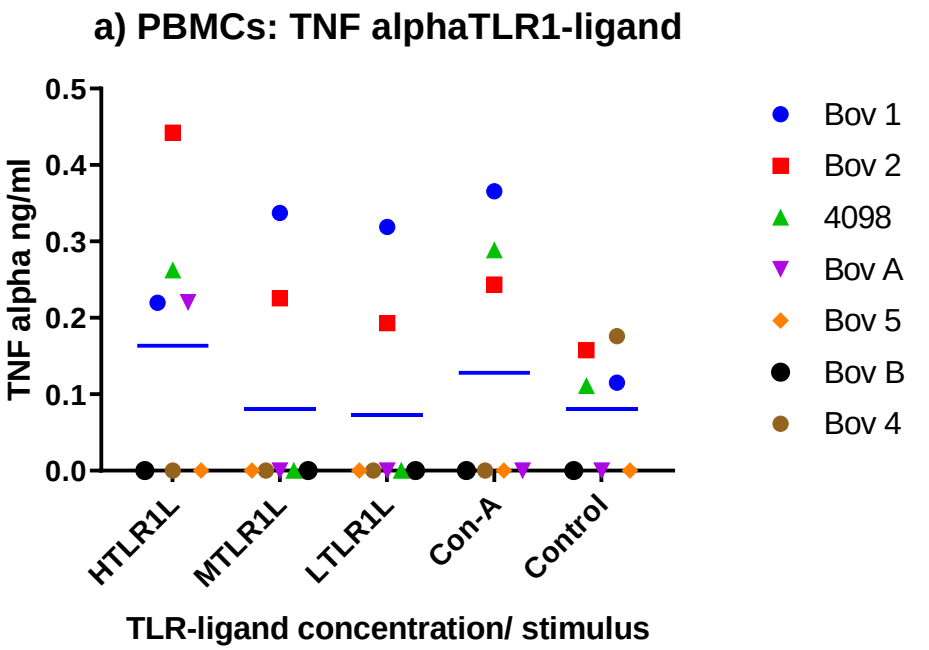
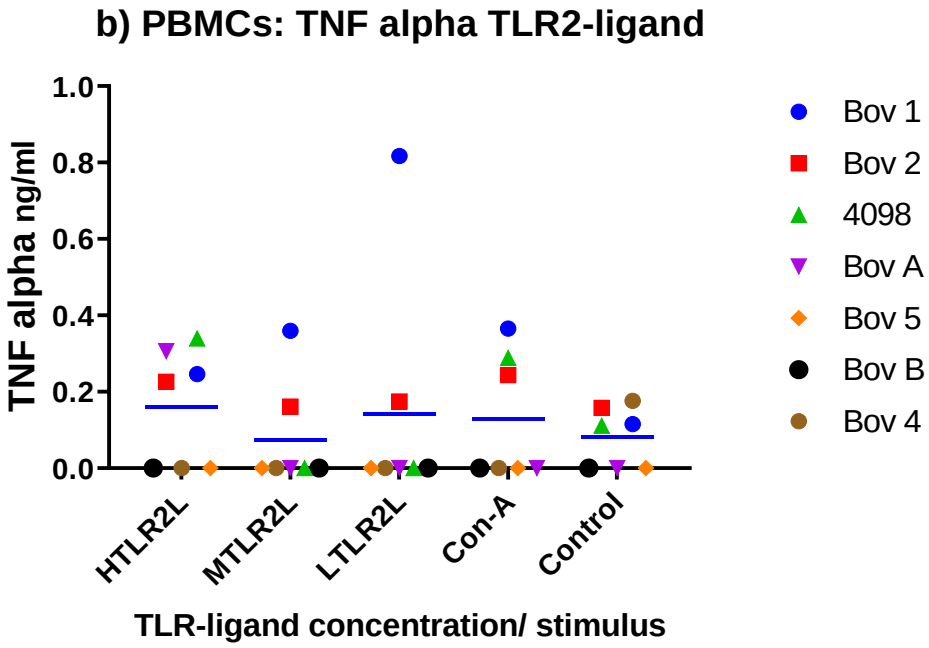
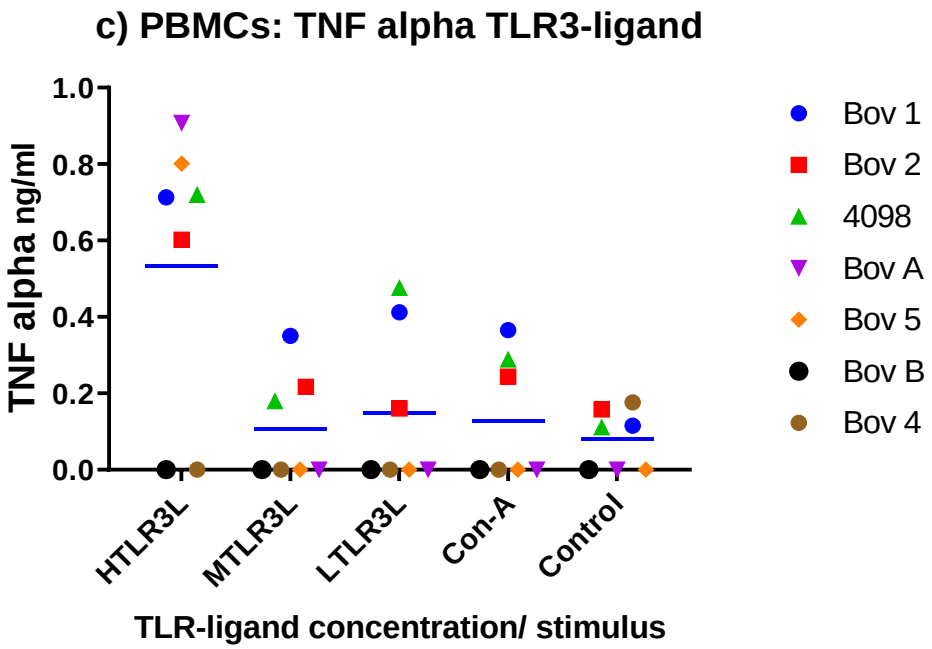


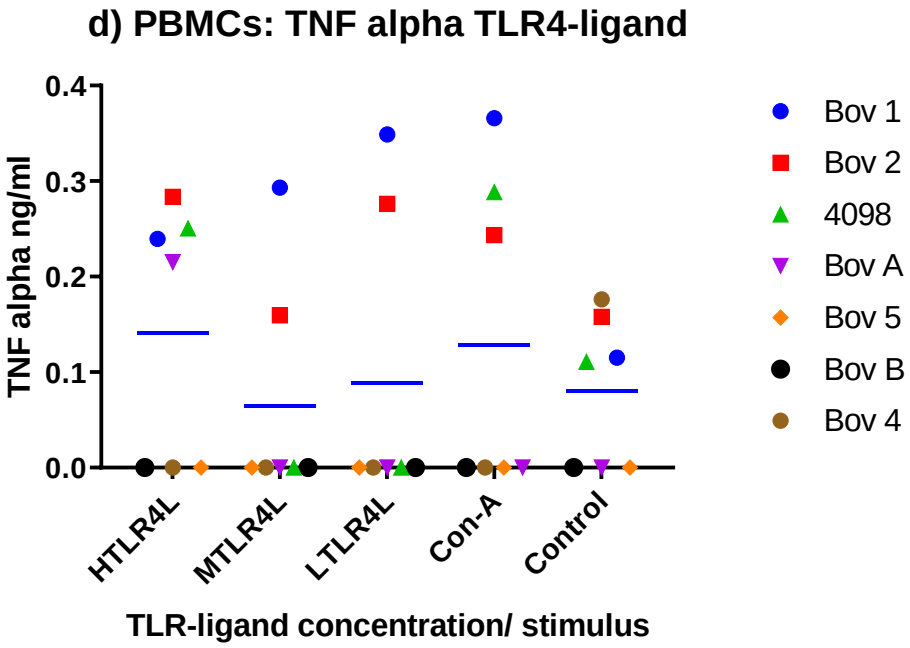
Figure 6.63 (a-c): IFN-α concentrations measured in the supernatant of T cell subsets cultured in response to TLR3 and TLR9 agonists from CD8 T

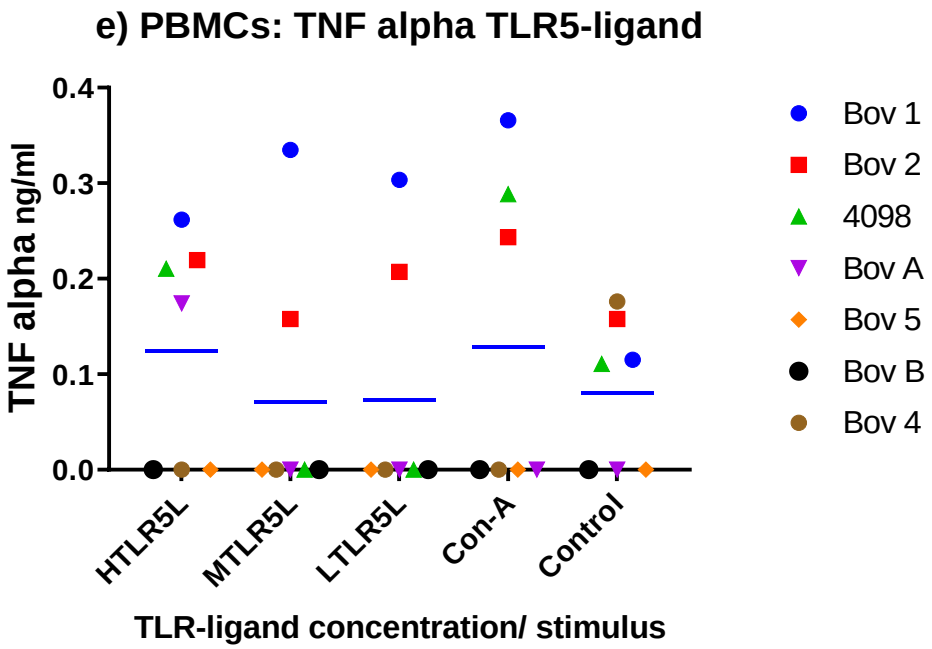
cells of 4 animals and γδ T cells of 5 animals. CD4 T cells produced nil concentrations.

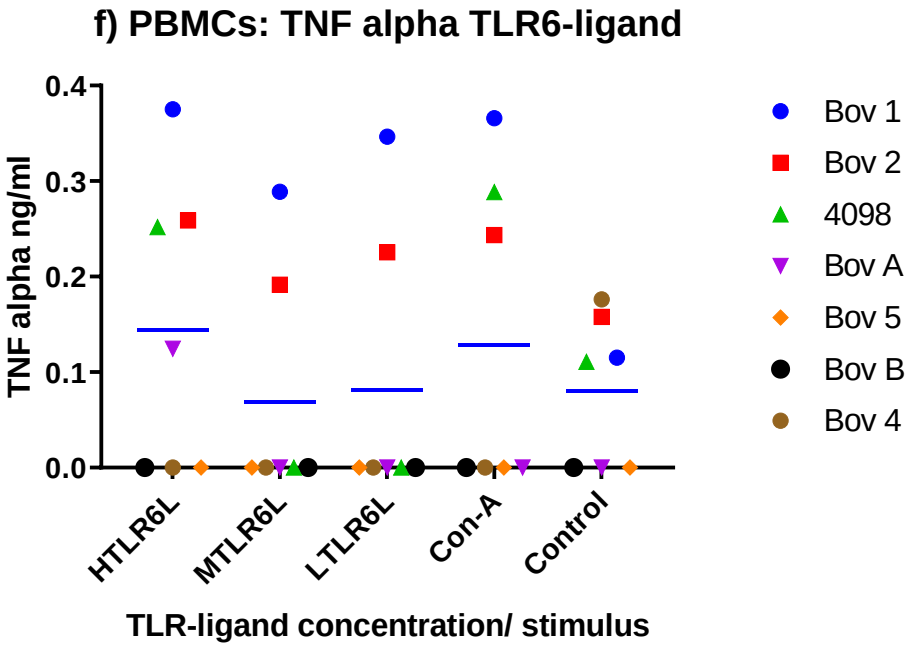




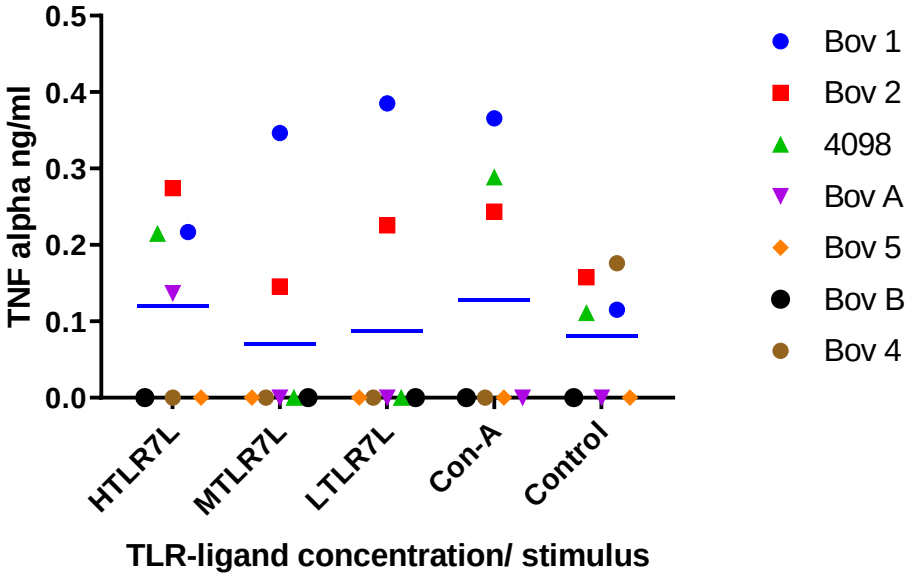

c) PBMCs: TNF alpha TLR3-ligand
TNF alpha ng/ml
1.0
0.8
0.6
0.4
0.2
0.0
HTLR3L
MTLR3L
LTLR3L
Con-A
Control
TLR-ligand concentration/ stimulus
Bov 1
Bov 2
4098
Bov A
Bov 5
Bov B
Bov 4


d) PBMCs: TNF alpha TLR4-ligand
TNF alpha ng/ml
0.4
0.3
0.2
0.1
0.0
HTLR4L
MTLR4L
LTLR4L
Con-A
Control
TLR-ligand concentration/ stimulus
Bov 1
Bov 2
4098
Bov A
Bov 5
Bov B
Bov 4

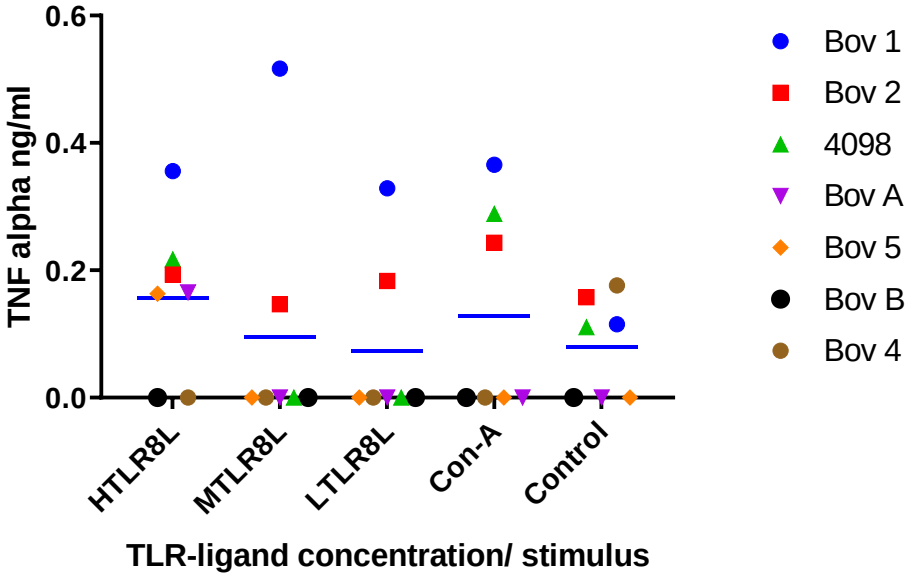

e) PBMCs: TNF alpha TLR5-ligand
TNF alpha ng/ml
0.4
0.3
0.2
0.1
0.0
HTLR5L
MTLR5L
LTLR5L
Con-A
Control
TLR-ligand concentration/ stimulus
Bov 1
Bov 2
4098
Bov A
Bov 5
Bov B
Bov 4


f) PBMCs: TNF alpha TLR6-ligand
TNF alpha ng/ml
0.4
0.3
0.2
0.1
0.0
HTLR6L
MTLR6L
LTLR6L
Con-A
Control
TLR-ligand concentration/ stimulus
Bov 1
Bov 2
4098
Bov A
Bov 5
Bov B
Bov 4

g) PBMCs: TNF alpha TLR7-ligand



h) PBMCs: TNF alpha TLR8-ligand



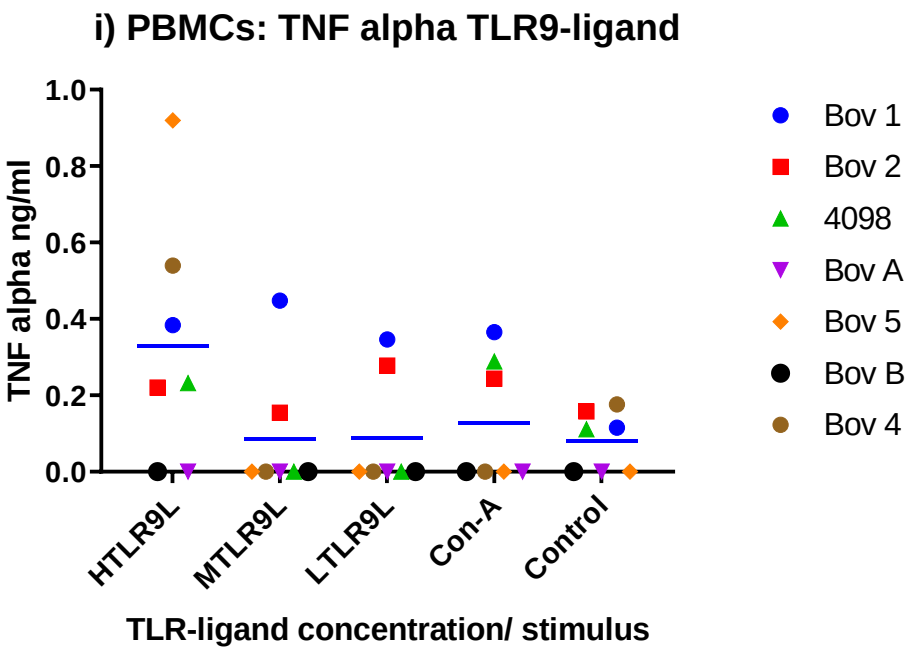
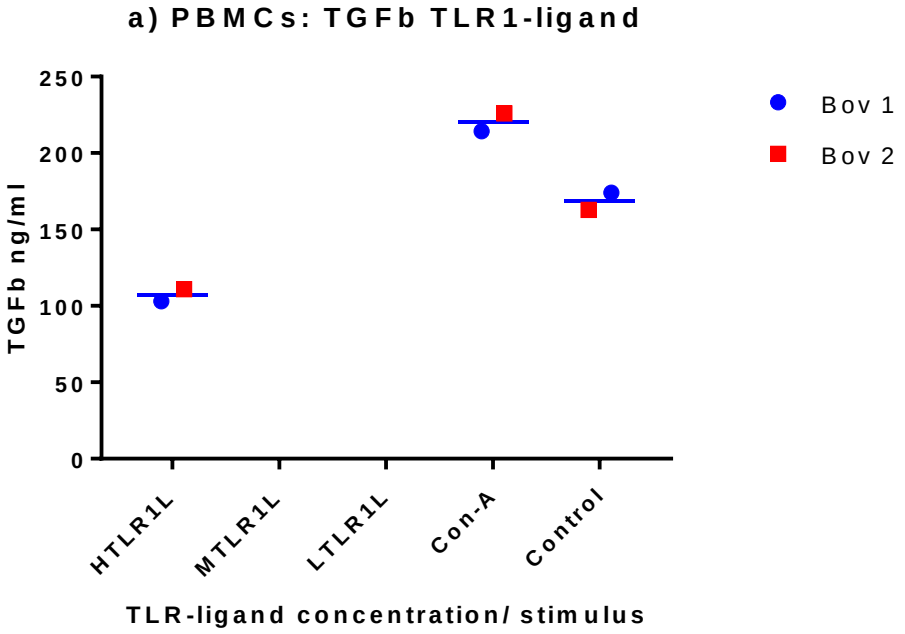
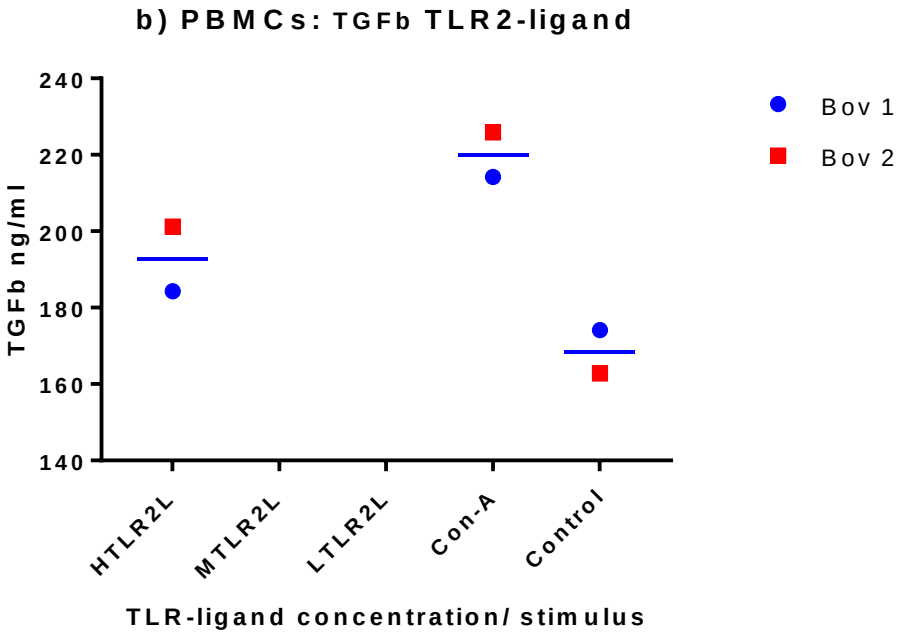
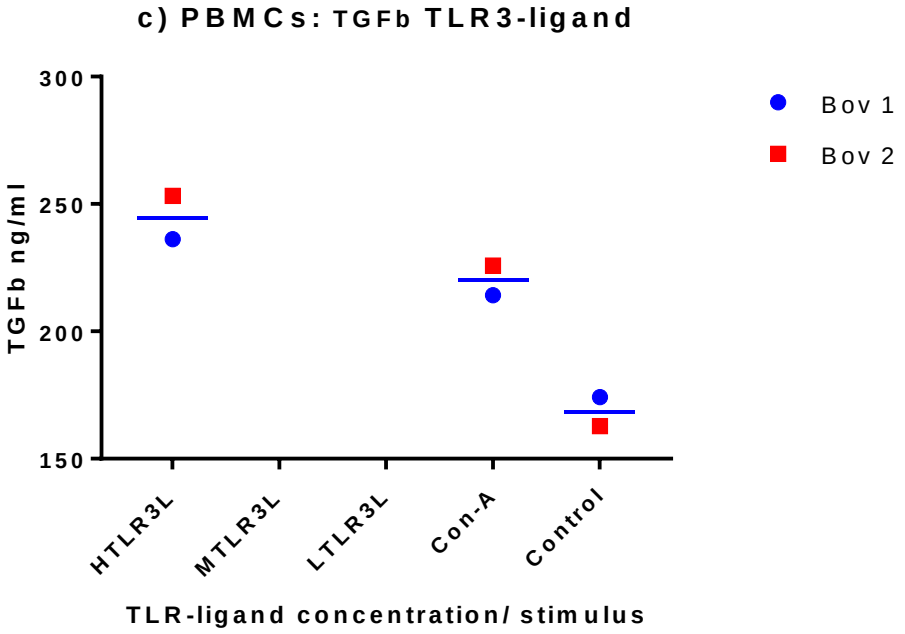
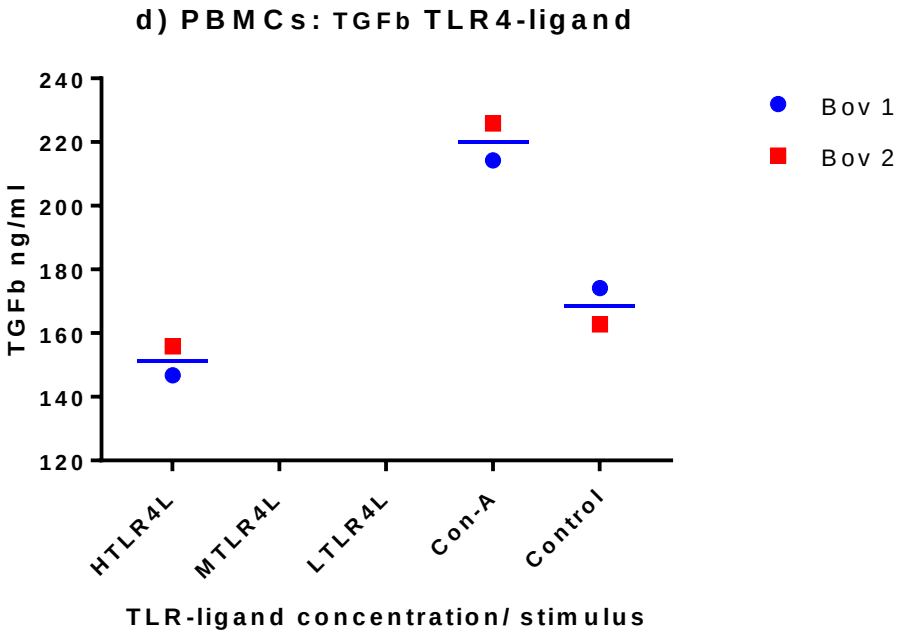


Figure 6.64 (a-i): TNF-α concentrations measured in the supernatant of PBMCs cultured in response to TLR1-9 agonists. CD4, CD8 and γδ T cells did not produce any TNF-α in the supernatant.

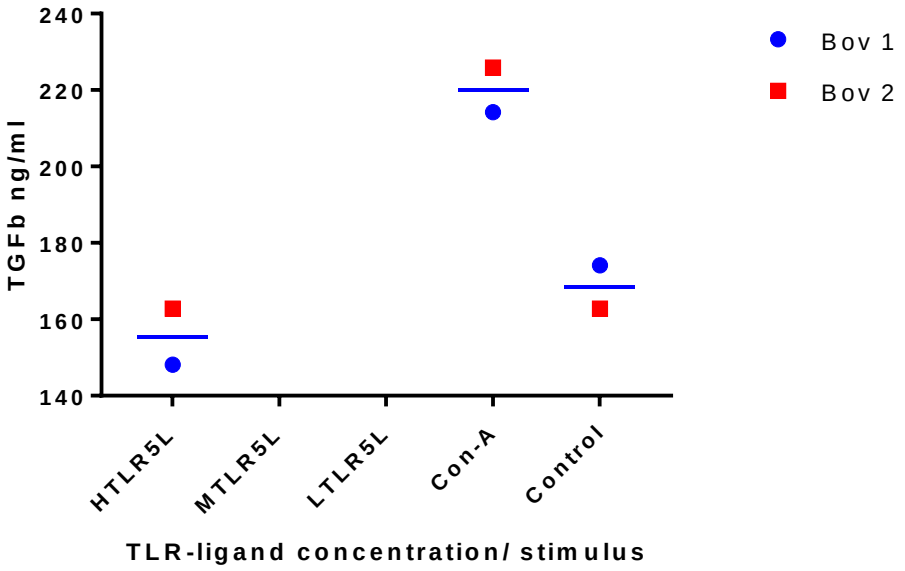

a) PBMCs: TGFb TLR1-ligand
TGFb ng/ml
250
200
150
100
50
0
HTLR1L
MTLR1L
LTLR1L
Con-A
Control
TLR-ligand concentration/ stimulus
Bov 1
Bov 2


b) PBMCs: TGFb TLR2-ligand
TGFb ng/ml
240
220
200
180
160
140
HTLR2L
MTLR2L
LTLR2L
Con-A
Control
TLR-ligand concentration/ stimulus
Bov 1
Bov 2

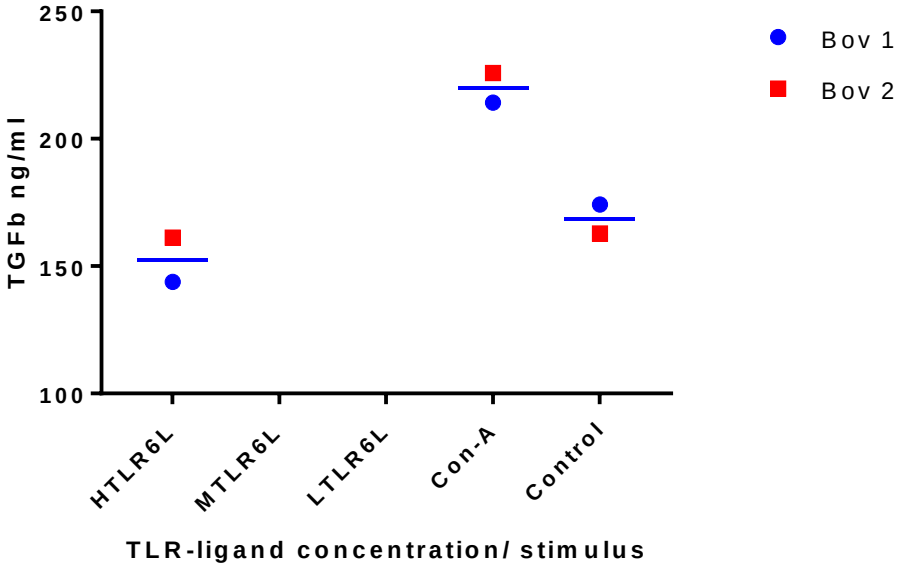

c) PBMCs: TGFb TLR3-ligand
300
250
200
150
TGFb ng/ml
Bov 1
Bov 2
HTLR3L
MTLR3L
LTLR3L
Con-A
Control
TLR-ligand concentration/ stimulus


d) PBMCs: TGFb TLR4-ligand
240
220
200
180
160
140
120
TGFb ng/ml
Bov 1
Bov 2
HTLR4L
MTLR4L
LTLR4L
Con-A
Control
TLR-ligand concentration/ stimulus

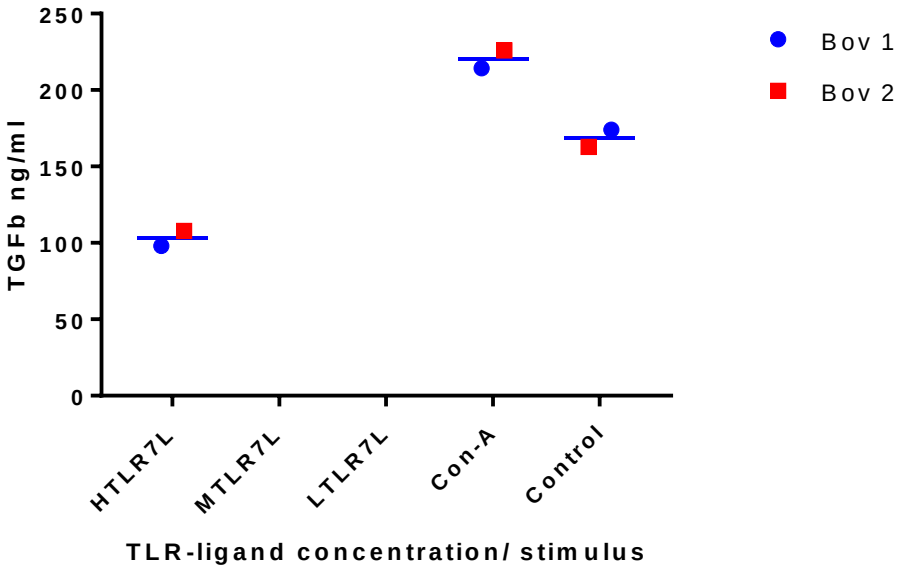
e) PBMCs: TGFb TLR5-ligand



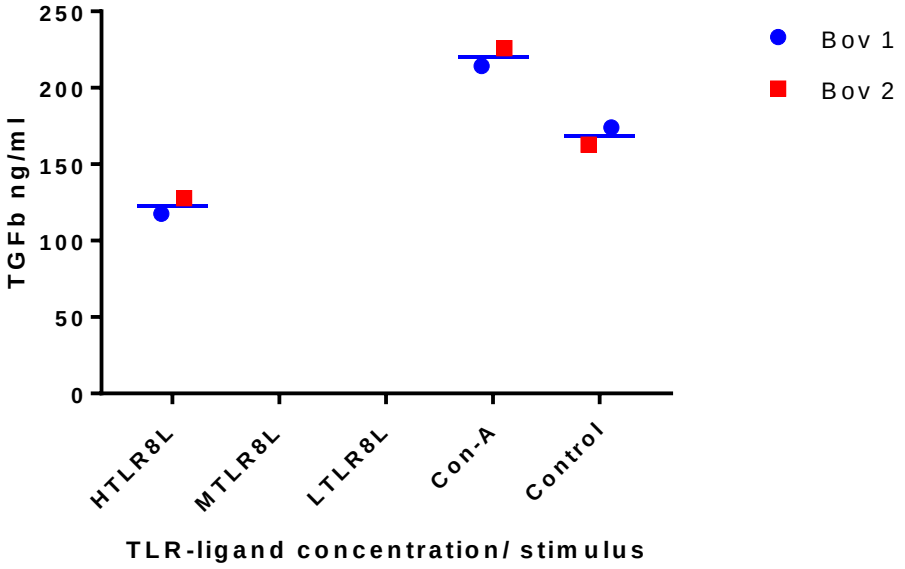
f) PBMCs: TGFb TLR6-ligand

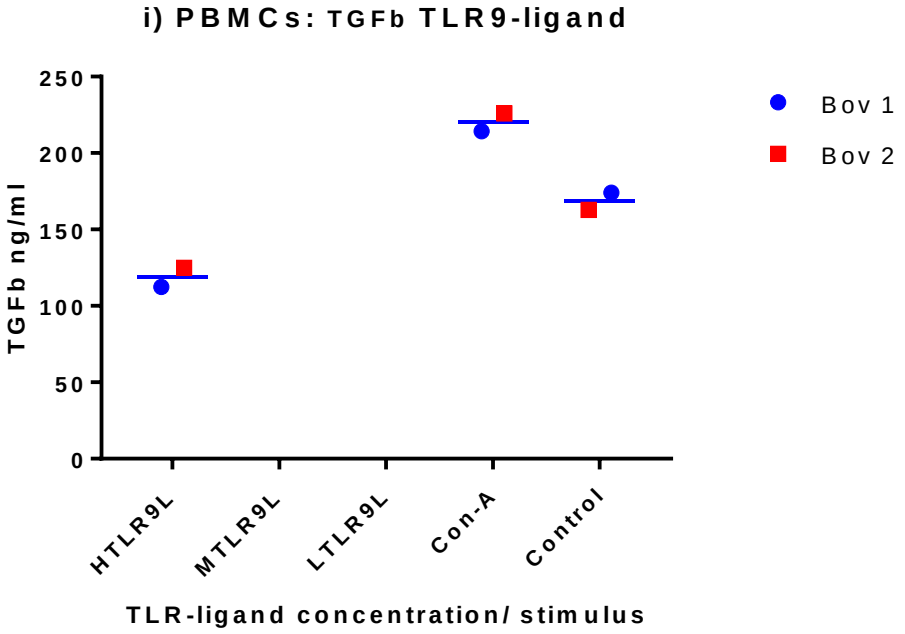


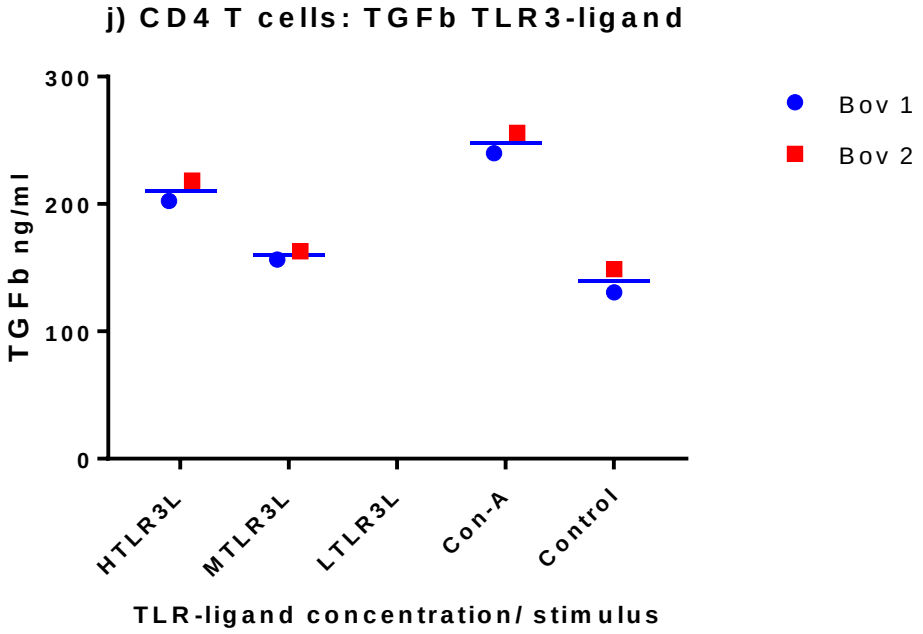
g) PBMCs: TGFb TLR7-ligand

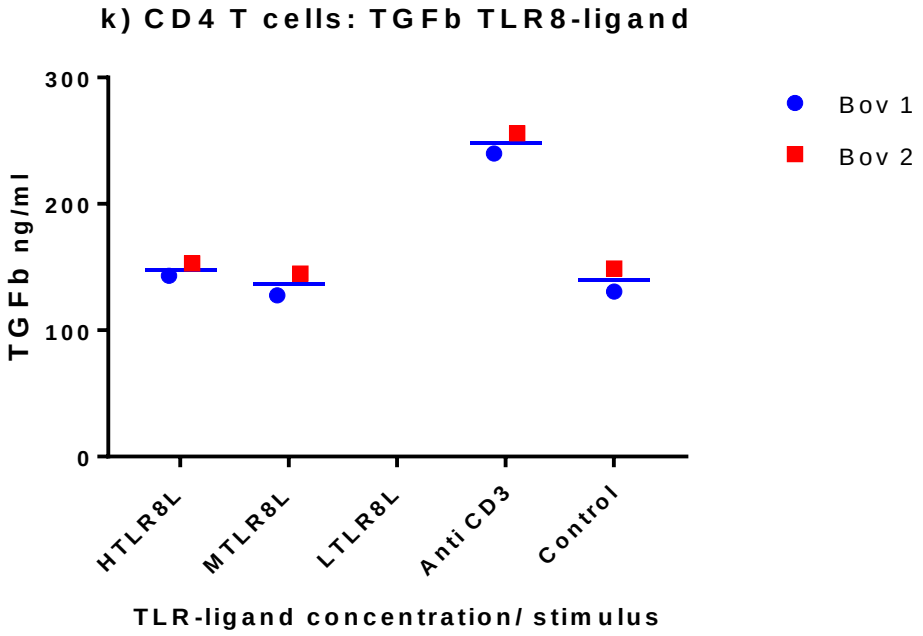


h) PBMCs: TGFb TLR8-ligand

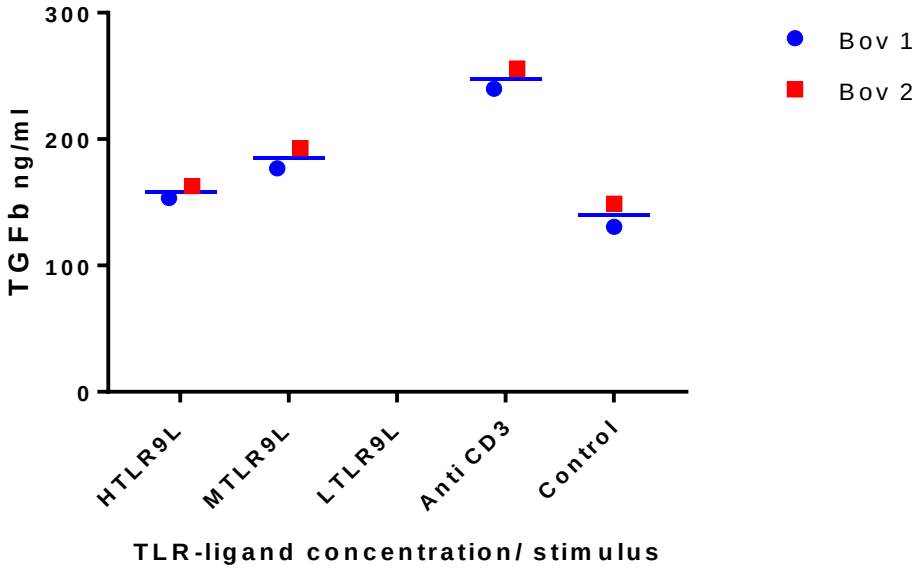



i) PBMCs: TGFb TLR9-ligand
TGFb ng/ml
250
200
150
100
50
0
HTLR9L
MTLR9L
LTLR9L
Con-A
Control
TLR-ligand concentration/ stimulus
Bov 1
Bov 2

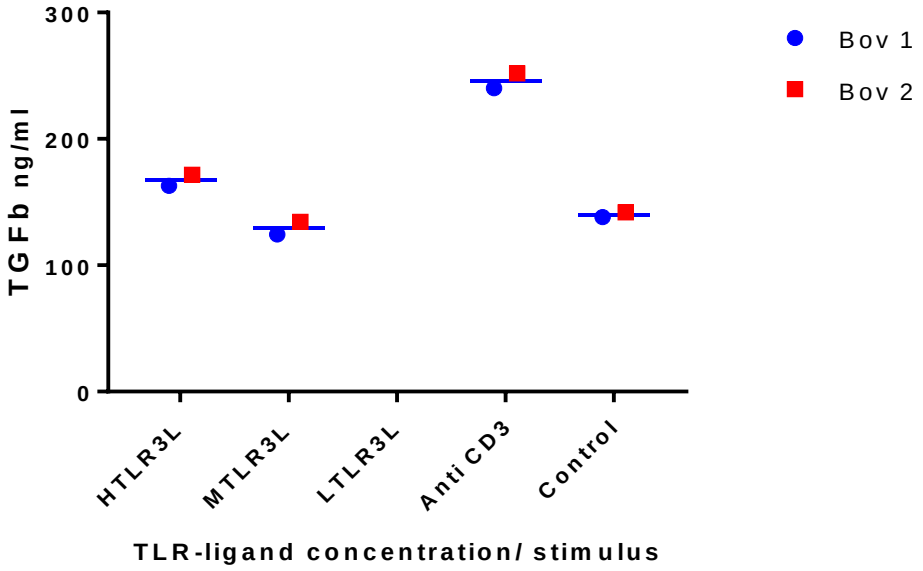

j) CD4 T cells: TGFb TLR3-ligand
TGFb ng/ml
300
200
100
0
HTLR3L
MTLR3L
LTLR3L
Con-A
Control
TLR-ligand concentration/ stimulus
Bov 1
Bov 2

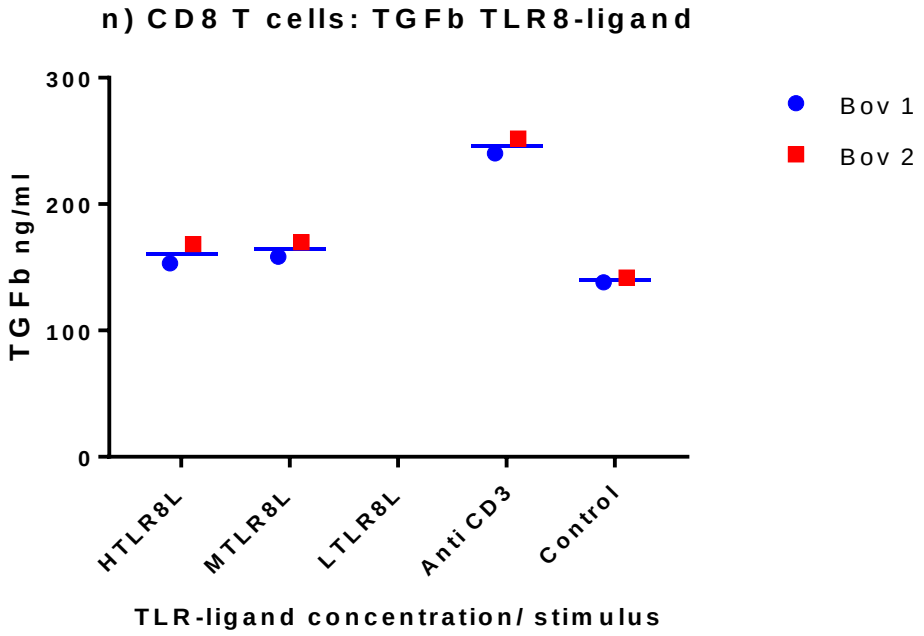

k) CD4 T cells: TGFb TLR8-ligand
TGFb ng/ml
300
200
100
0
HTLR8L
MTLR8L
LTLR8L
Anti CD3
Control
TLR-ligand concentration/ stimulus
Bov 1
Bov 2

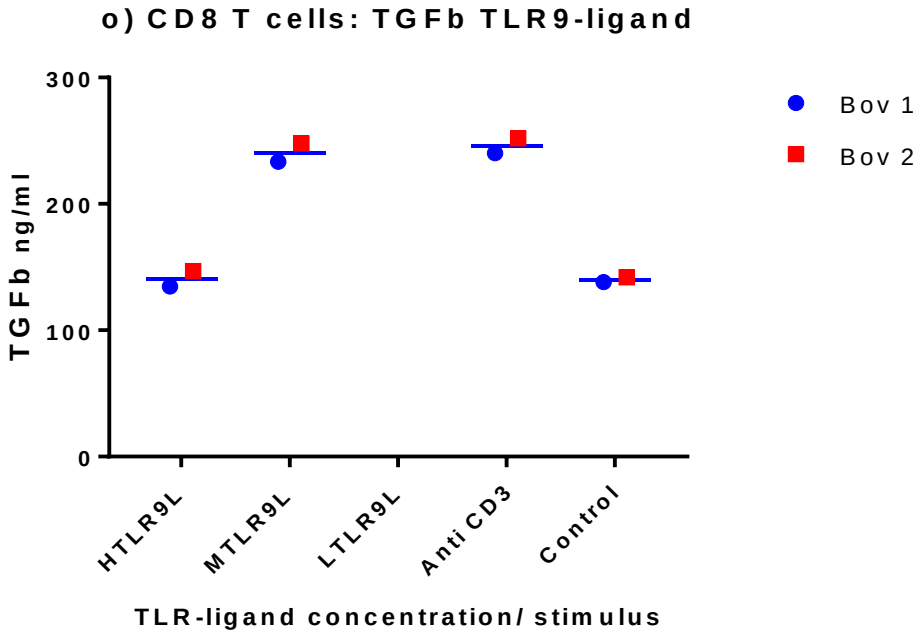
l) CD4 T cells: TGFb TLR9-ligand



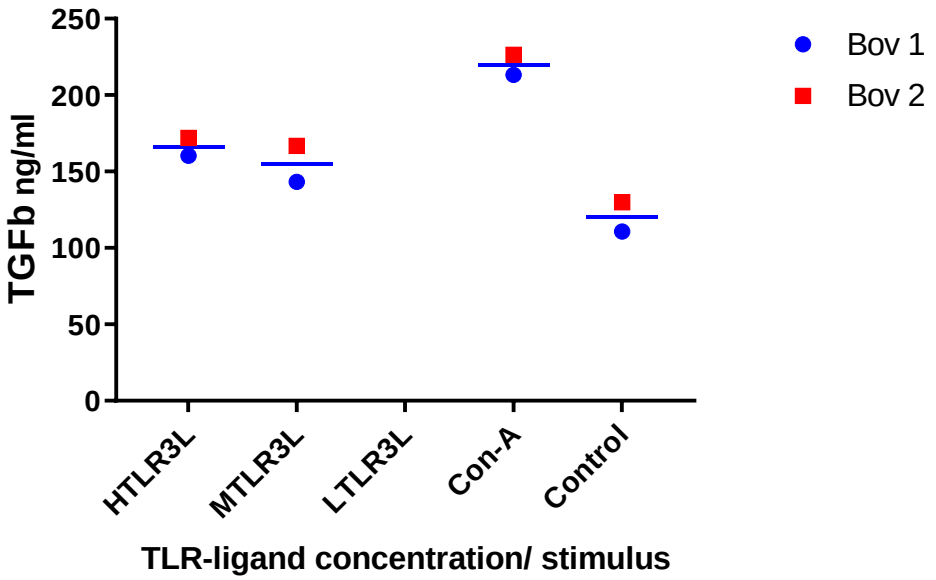
m) CD8 T cells: TGFb TLR3-ligand



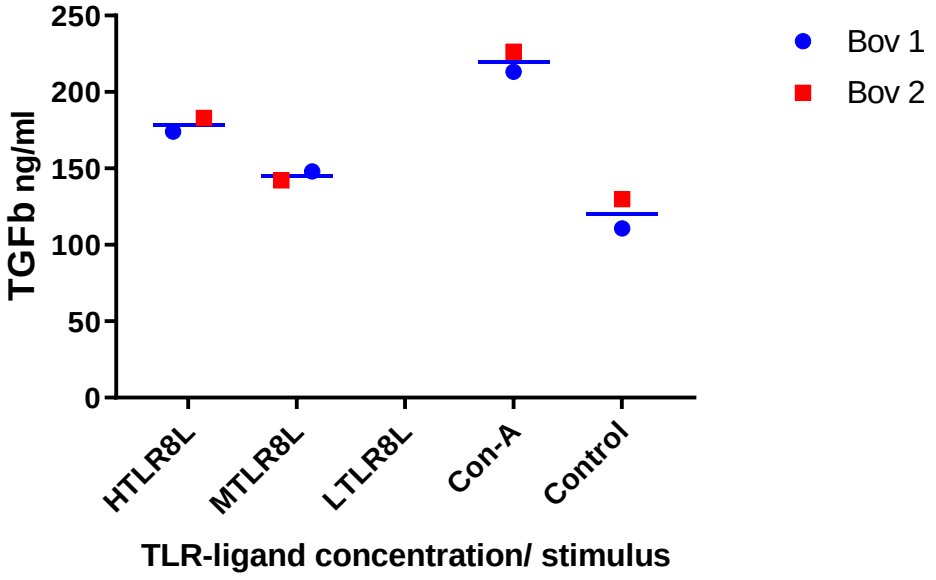

n) CD8 T cells: TGFb TLR8-ligand
TGFb ng/ml
300
200
100
0
HTLR8L
MTLR8L
LTLR8L
Anti CD3
Control
TLR-ligand concentration/ stimulus
Bov 1
Bov 2


o) CD8 T cells: TGFb TLR9-ligand
TGFb ng/ml
300
200
100
0
HTLR9L
MTLR9L
LTLR9L
Anti CD3
Control
TLR-ligand concentration/ stimulus
Bov 1
Bov 2

p) Gamma delta T cells: TGFb TLR3-ligand



q) Gamma delta T cells: TGFb TLR8-ligand



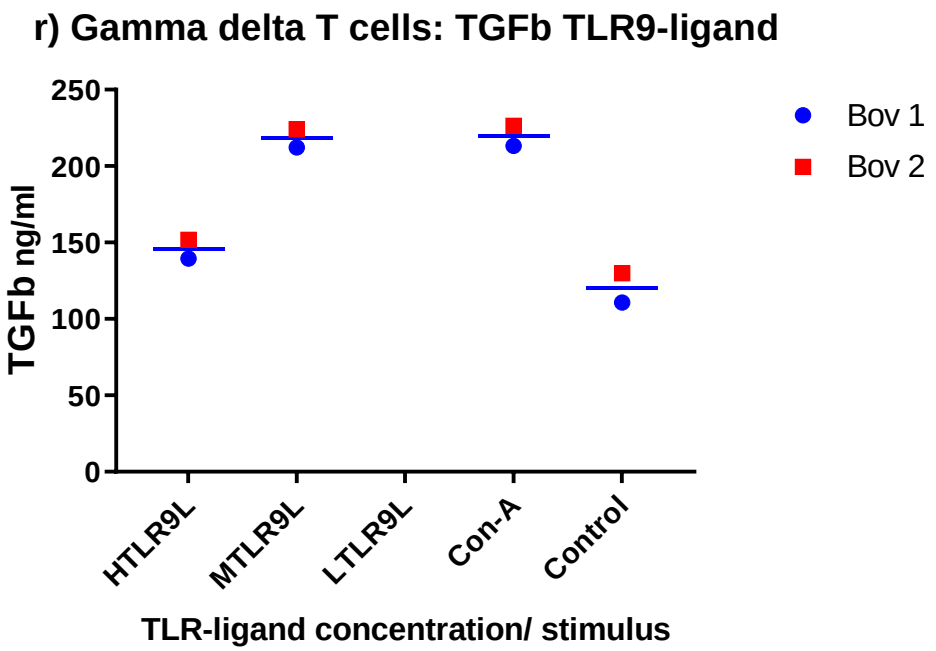


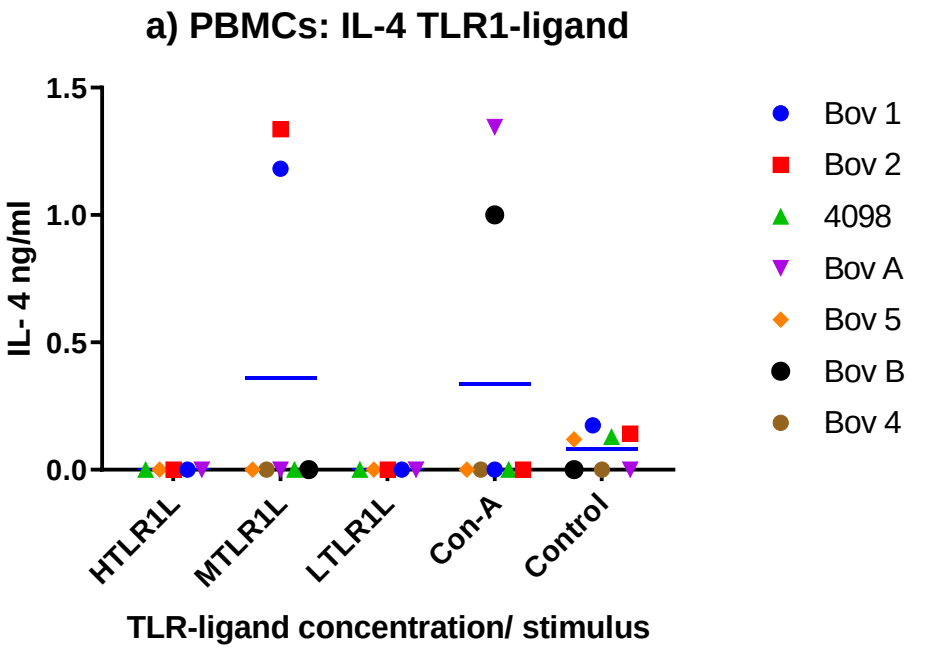
Figure 6.65: TGFβ concentrations measured in the supernatant of PBMCs (figures a-i) and T cell subsets (figures j-r) cultured in response to TLR agonists.

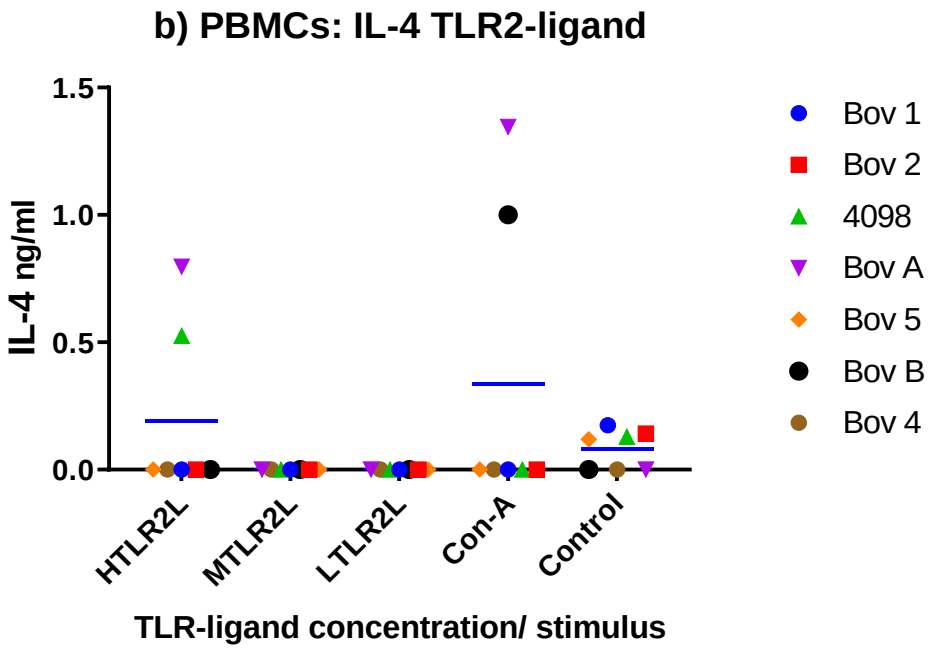
6.6 IL-4

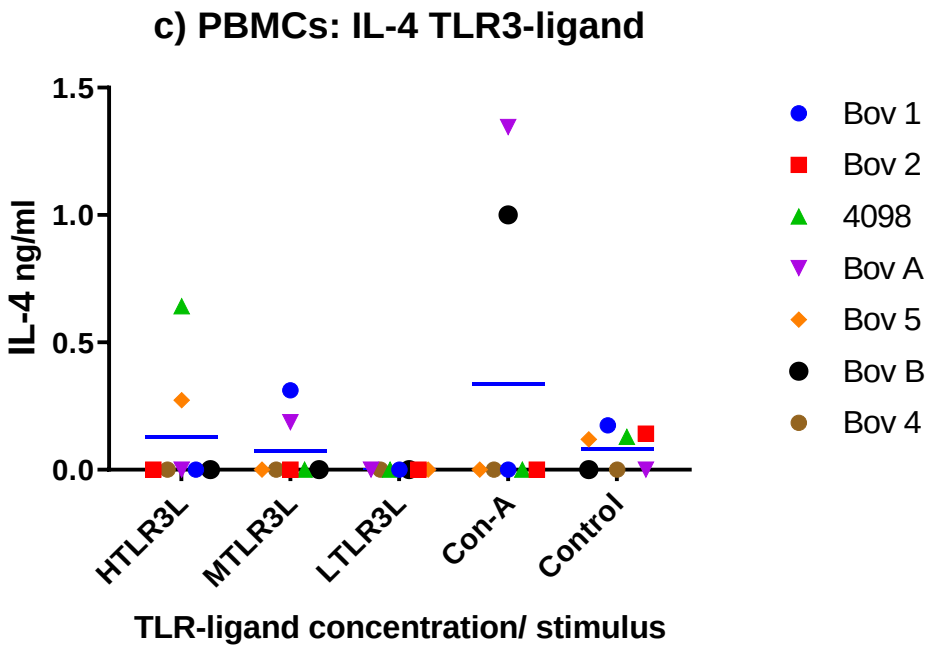
6.6.1 IL-4 results of PBMCs and T cell subsets stimulated with TLR ligands

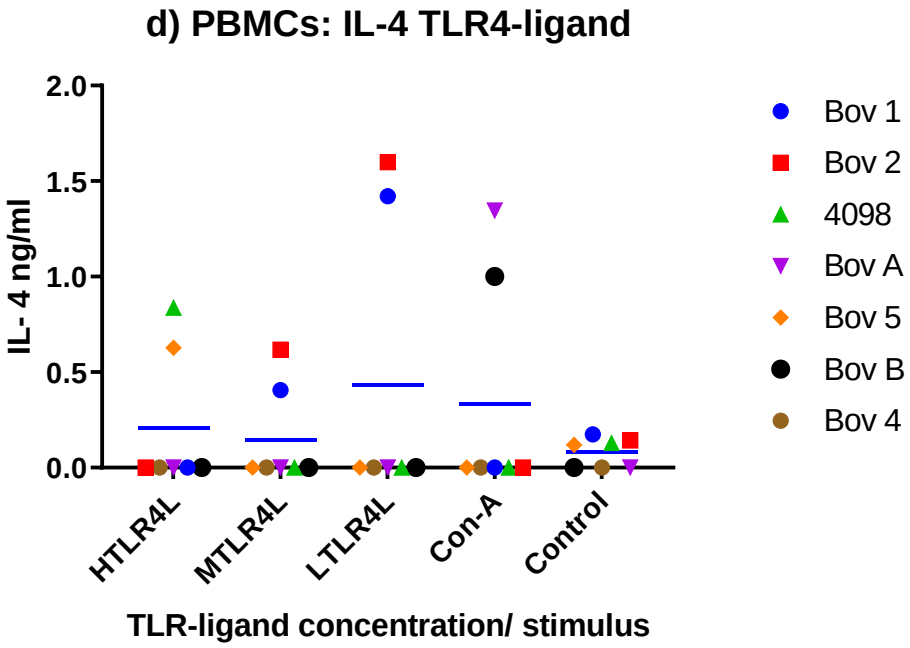
The comparison between PBMC TLR ligand-stimulated groups and unstimulated controls (Mean ± Standard error) showed no significant differences at the group level, but there were individual animal samples that exhibited a significant response and these are highlighted below. Figure 6.66a shows that the PBMC from 7 animals stimulated with TLR1 agonists, gave significant increases in IL-4 in 2 animals only (Bov1 and Bov2) at P=0.0458 and P=0.0441 by comparison with unstimulated controls. PBMCs treated with TLR2 and TLR3 agonists did not show significant increases of IL-4 (figures 6.66b and c). Low concentrations of

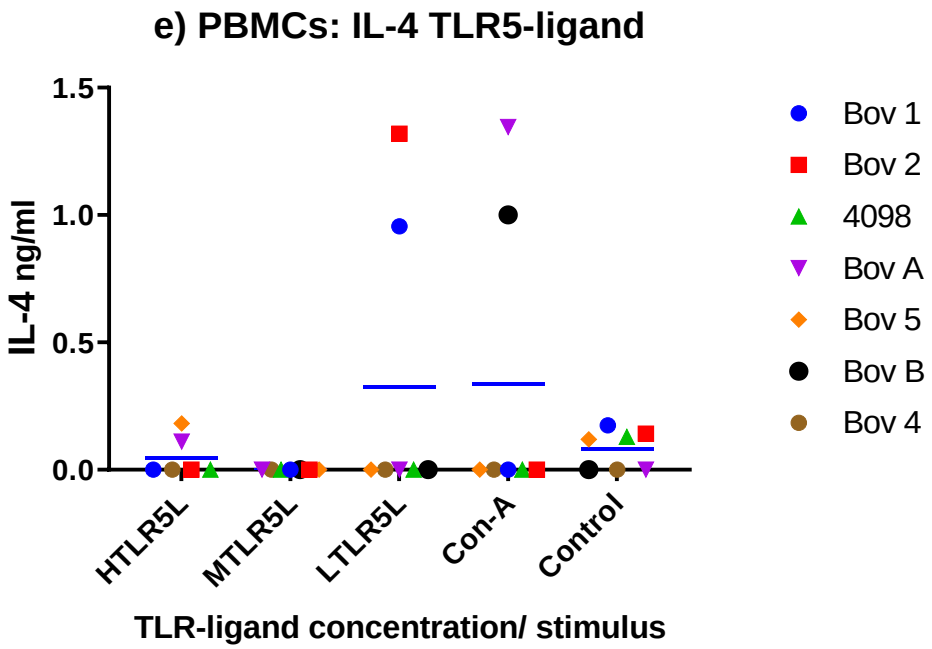
TLR4 and TLR5 agonists stimulated a significant increase in IL-4 in 2 animals (Bov1 and Bov2) as shown in figure (6.66d and e P=0.0471 and P=0.0458). There was no enhanced production of IL-4 from cells stimulated with 3 concentrations of TLR6 agonists (figure 6.64f). Stimulation of PBMCs with high concentrations of TLR7 ligands caused a significant increase in IL-4 in 2 animals (Bov A and Bov 5, P=0.0438 and P=0.0447 figure 6.66g) by comparison with the unstimulated control. Also stimulation with low concentrations of TLR7 agonists induced a significant increase in IL-4 in 2 animals (Bov 1 and Bov2, P=0.0488 and P=0.0496 compared to unstimulated control). In figure 6.66h, stimulation with high concentrations of TLR 8 agonists induced a significant increase in IL-4 in 2 animals (Bov 5 and Bov A, P=0.0467 and P=0.0453) compared to unstimulated control) while stimulation with medium concentrations of TLR8 agonists caused a significant increase in IL-4 in 2 animals (Bov1 and Bov2 P=0.0489 and P=0.0471 vs control). Finally, figure 6.66i shows that stimulation with the high concentration of TLR9 agonists caused a significant increase in IL-4 in 3 animals (Bov 2, Bov 4 and Bov 5, P=0.0472, P=0.0459 and P=0.0483 respectively vs control).

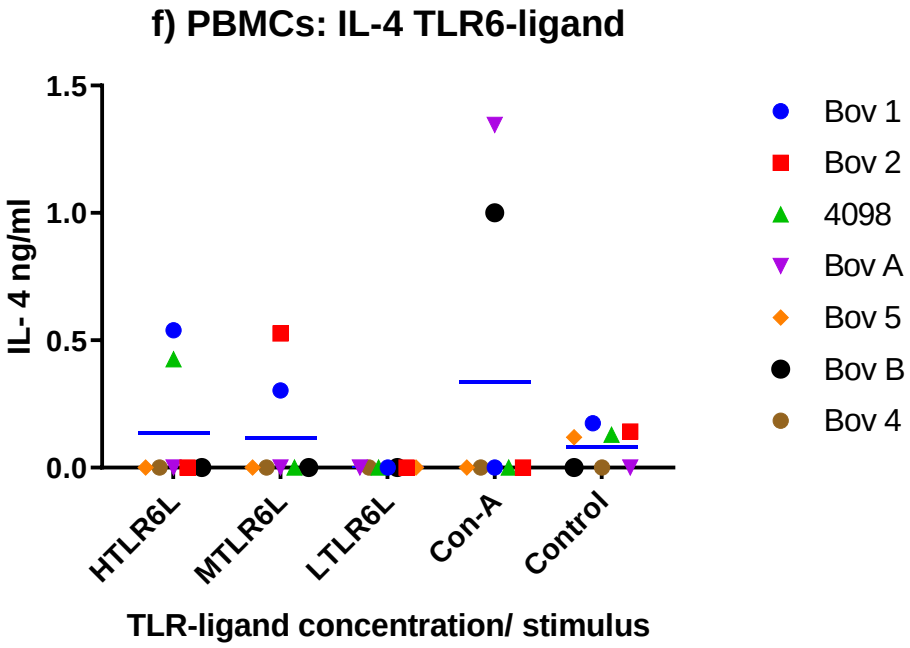

a) PBMCs: IL-4 TLR1-ligand
IL- 4 ng/ml
1.5
1.0
0.5
0.0
HTLR1L
MTLR1L
LTLR1L
Con-A
Control
TLR-ligand concentration/ stimulus
Bov 1
Bov 2
4098
Bov A
Bov 5
Bov B
Bov 4

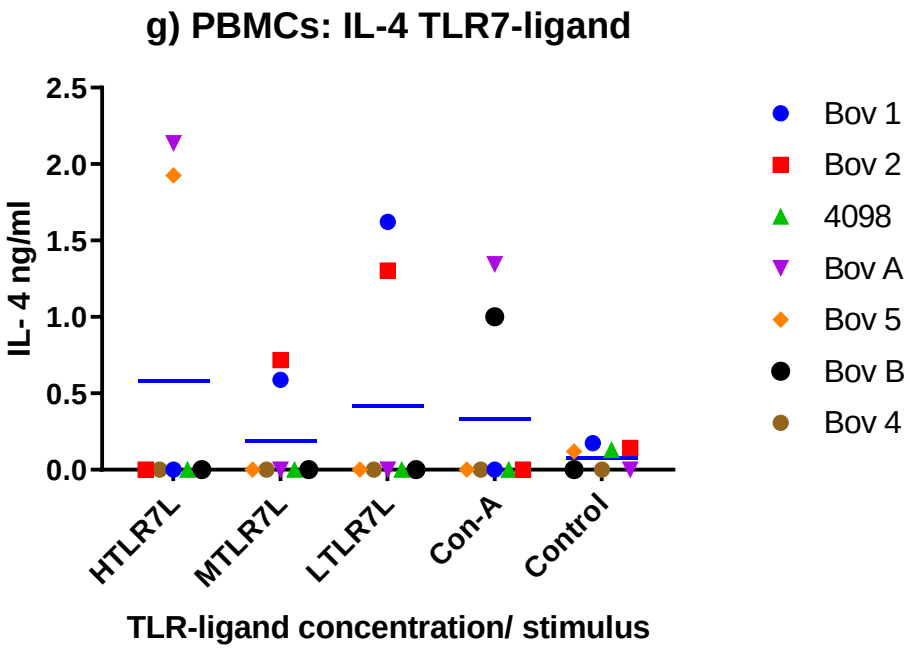

b) PBMCs: IL-4 TLR2-ligand
IL-4 ng/ml
1.5
1.0
0.5
0.0
HTLR2L
MTLR2L
LTLR2L
Con-A
Control
TLR-ligand concentration/ stimulus
Bov 1
Bov 2
4098
Bov A
Bov 5
Bov B
Bov 4

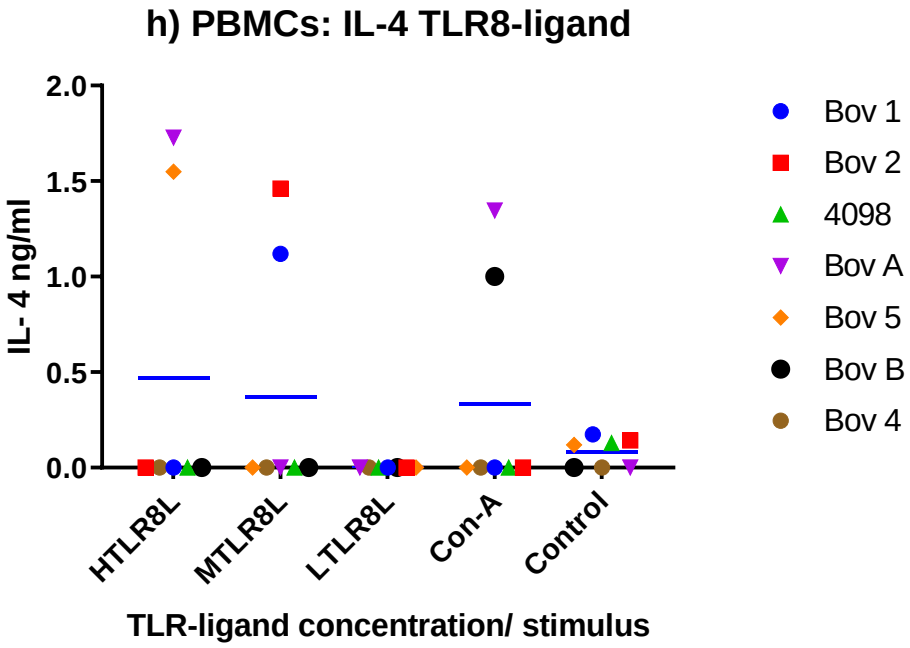

c) PBMCs: IL-4 TLR3-ligand
IL-4 ng/ml
1.5
1.0
0.5
0.0
HTLR3L
MTLR3L
LTLR3L
Con-A
Control
TLR-ligand concentration/ stimulus
Bov 1
Bov 2
4098
Bov A
Bov 5
Bov B
Bov 4


d) PBMCs: IL-4 TLR4-ligand
IL- 4 ng/ml
2.0
1.5
1.0
0.5
0.0
HTLR4L
MTLR4L
LTLR4L
Con-A
Control
TLR-ligand concentration/ stimulus
Bov 1
Bov 2
4098
Bov A
Bov 5
Bov B
Bov 4


e) PBMCs: IL-4 TLR5-ligand
IL-4 ng/ml
1.5
1.0
0.5
0.0
HTLR5L
MTLR5L
LTLR5L
Con-A
Control
TLR-ligand concentration/ stimulus
Bov 1
Bov 2
4098
Bov A
Bov 5
Bov B
Bov 4


f) PBMCs: IL-4 TLR6-ligand
IL- 4 ng/ml
1.5
1.0
0.5
0.0
HTLR6L
MTLR6L
LTLR6L
Con-A
Control
TLR-ligand concentration/ stimulus
Bov 1
Bov 2
4098
Bov A
Bov 5
Bov B
Bov 4


g) PBMCs: IL-4 TLR7-ligand
IL- 4 ng/ml
2.5
2.0
1.5
1.0
0.5
0.0
HTLR7L
MTLR7L
LTLR7L
Con-A
Control
TLR-ligand concentration/ stimulus
Bov 1
Bov 2
4098
Bov A
Bov 5
Bov B
Bov 4


h) PBMCs: IL-4 TLR8-ligand
IL- 4 ng/ml
2.0
1.5
1.0
0.5
0.0
HTLR8L
MTLR8L
LTLR8L
Con-A
Control
TLR-ligand concentration/ stimulus
Bov 1
Bov 2
4098
Bov A
Bov 5
Bov B
Bov 4

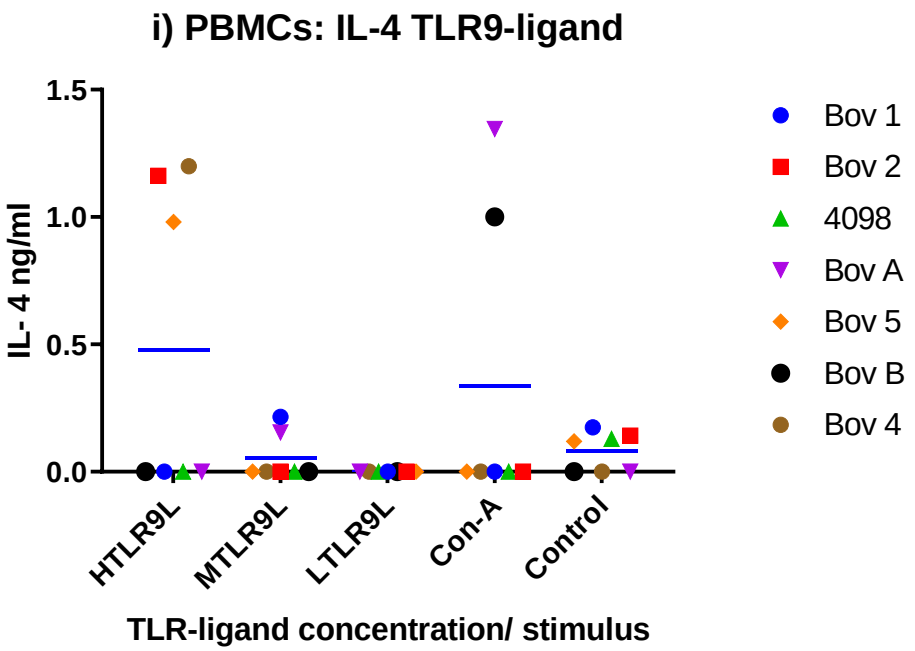
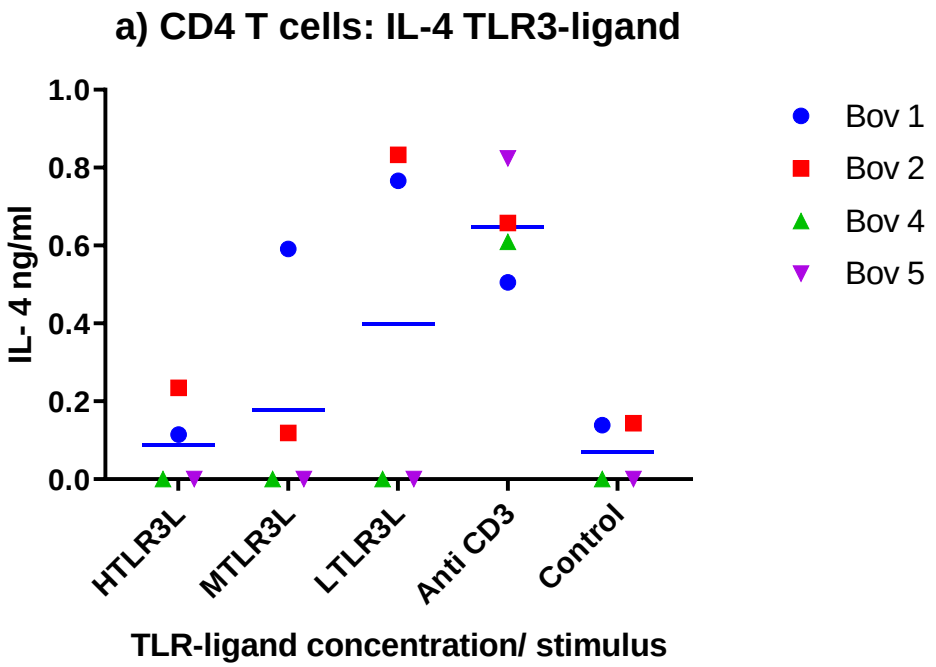
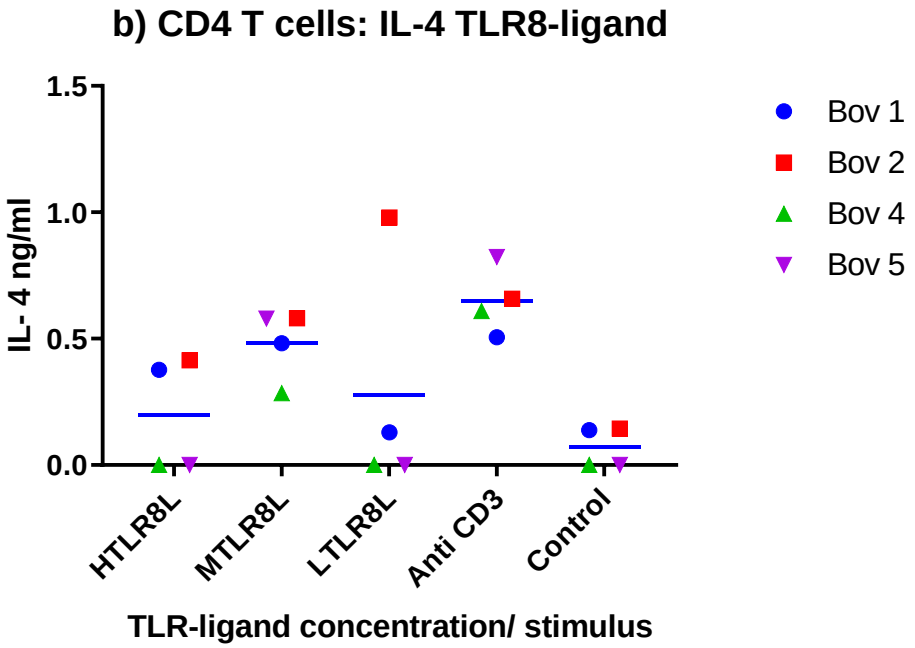


Figure 6.66: IL-4 concentrations measured in the supernatant of PBMCs cultured in response to TLR1-9 agonists in 7 animals. (a) TLR1 ligand. (b) TLR2 ligand. (c) TLR3 ligand. (d) TLR4 ligand. (e) TLR5 ligand. (f) TLR6 ligand. (g) TLR7 ligand. (h) TLR8 ligand. (i) TLR9 ligand.

6.6.2 IL-4 results of CD4 T cells stimulated with TLR ligands

The comparison between CD4 T cell TLR ligand-stimulated groups and unstimulated controls (Mean ± Standard error) showed no significant differences at the group level, but there were individual animal samples that exhibited a significant response and these are highlighted below. An analysis of the T cell subsets (figure 6.67) showed that an increase of IL-4 secreted from CD4 T cells was seen when stimulated with TLR3-, TLR8- and TLR9 agonists. Stimulation of CD4 T cells with low concentrations of TLR3 ligands caused significant production of IL-4 in 2 animals (Bov1 and Bov2) over control negative group (P=0.0493 and P=0.0484, Fig 6.67a). In figure 6.67b stimulation with low concentrations of TLR8 ligand induced significant production of IL-4 in 1 animal only (Bov2) over the unstimulated control (P=0.0491). In figure 6.67c stimulation with medium and high concentrations of TLR9 ligand induced significant production of IL-4 in 1 animal only (Bov1) compared to the unstimulated control (P=0.0488).


a) CD4 T cells: IL-4 TLR3-ligand
IL- 4 ng/ml
1.0
0.8
0.6
0.4
0.2
0.0
HTLR3L
MTLR3L
LTLR3L
Anti CD3
Control
TLR-ligand concentration/ stimulus
Bov 1
Bov 2
Bov 4
Bov 5


b) CD4 T cells: IL-4 TLR8-ligand
IL- 4 ng/ml
1.5
1.0
0.5
0.0
HTLR8L
MTLR8L
LTLR8L
Anti CD3
Control
TLR-ligand concentration/ stimulus
Bov 1
Bov 2
Bov 4
Bov 5

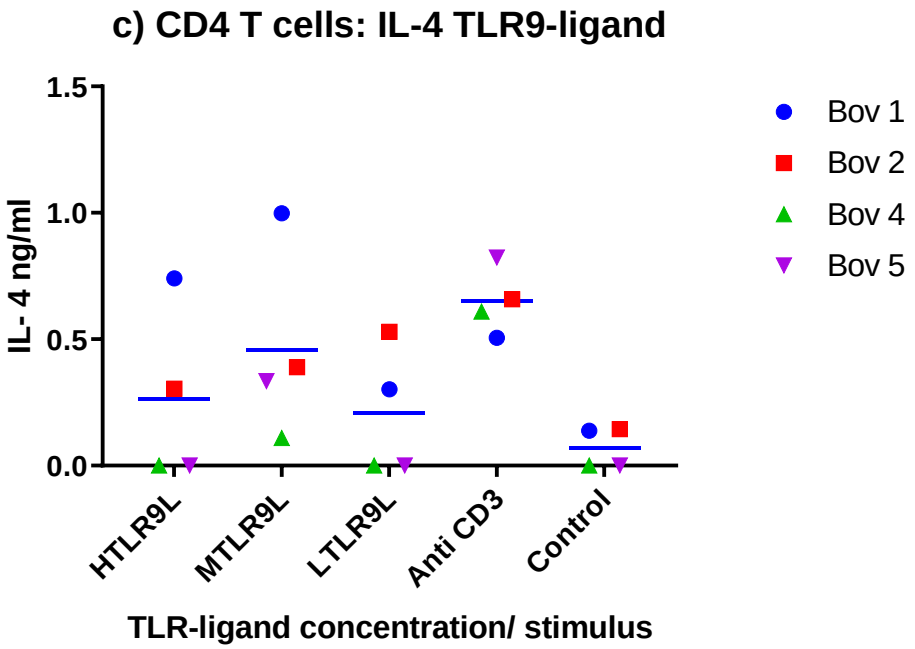
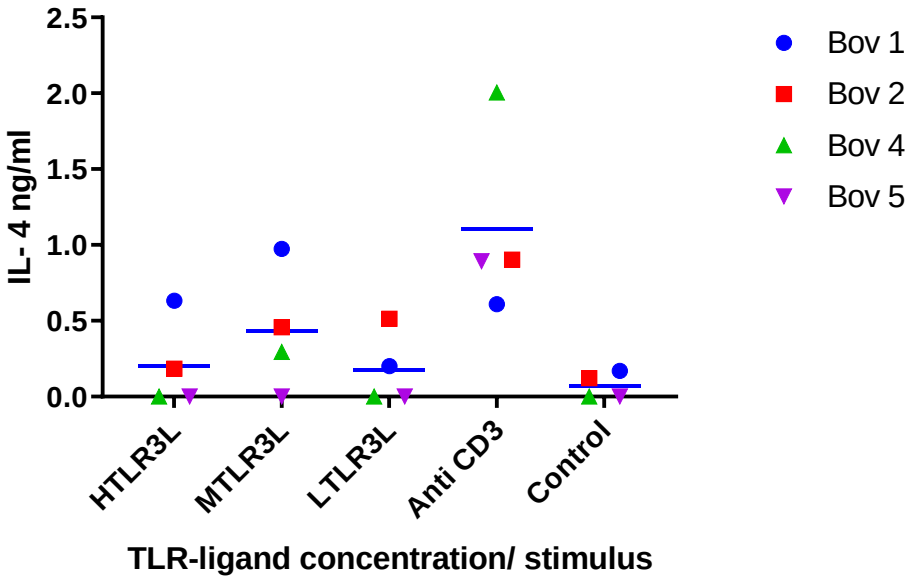


Figure 6.67: IL-4 concentrations measured in the supernatant of CD4 T cells cultured in response to TLR3, TLR8, and TLR9 agonists in 4 animals. (a) TLR3 ligand. (b) TLR8 ligand. (c) TLR9 ligand.

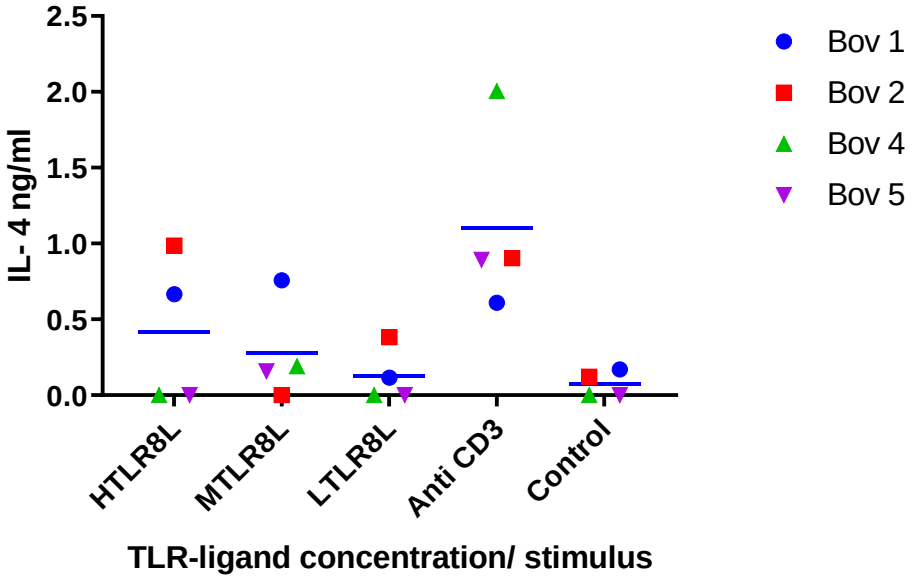
6.6.3 IL-4 results of CD8 T cells stimulated with TLR ligands

The comparison between CD8 T cell TLR ligand-stimulated groups and unstimulated controls (Mean ± Standard error) showed no significant differences at the group level, but there were individual animal samples that exhibited a significant response and these are highlighted below. Figure 6.68 shows that for the CD8 T cell subset, no significant increases over controls of IL-4 production ($P>0.05$) was seen in samples stimulated with TLR3, TLR8 and TLR9 agonists by comparison with control unstimulated groups (Figures 6.68a, b and c).

a) CD8 T cells: IL-4 TLR3-ligand



b) CD8 T cells: IL-4 TLR8-ligand



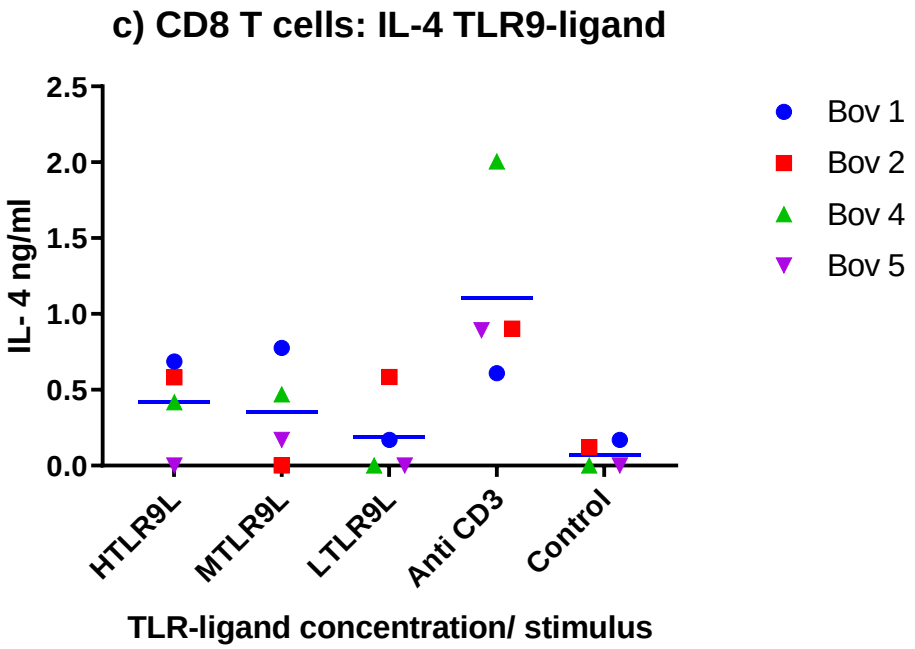
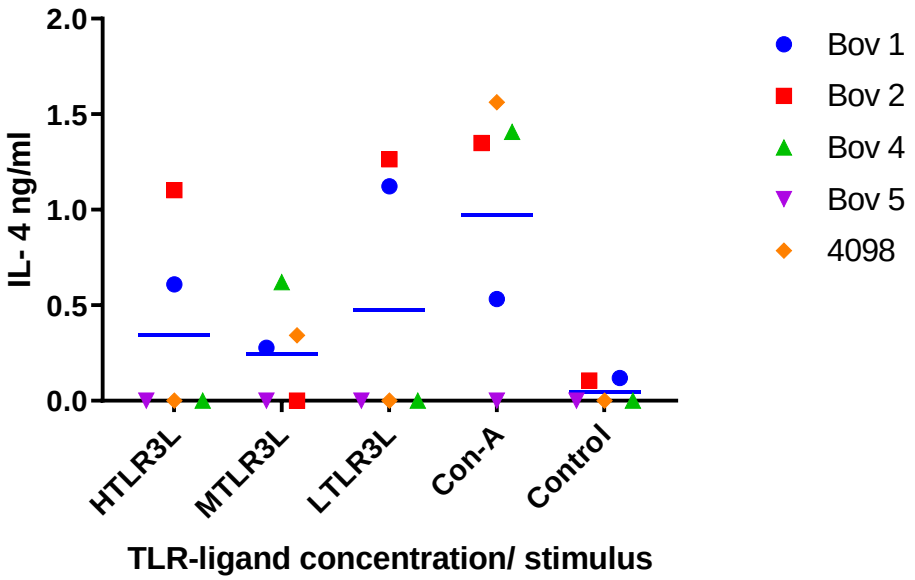


Figure 6.468: IL-4 concentrations measured in the supernatant of CD8 T cells cultured in response to TLR3, TLR8, and TLR9 agonists in 4 animals. (Figures a, b and c respectively).

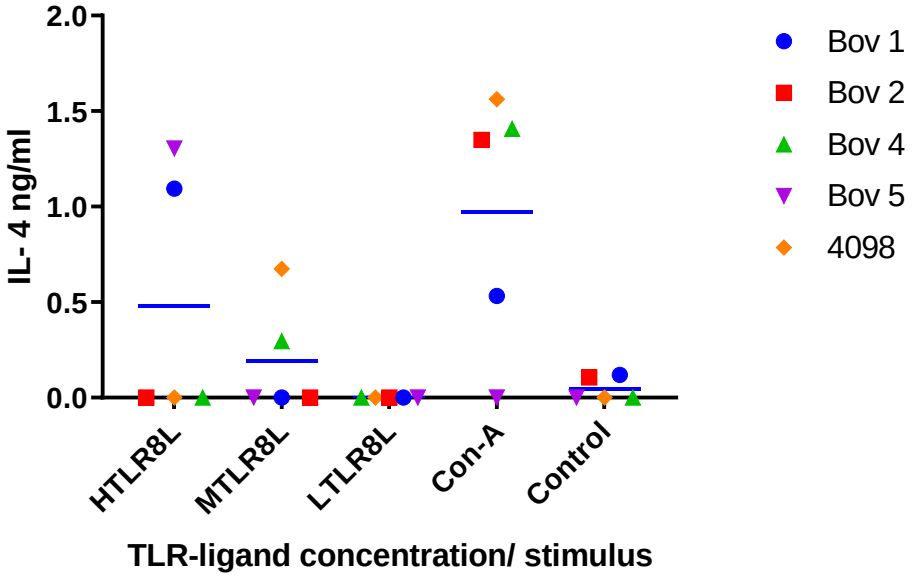
6.6.4 IL-4 results of γδ T cells stimulated with TLR ligands

The comparison between γδ T cells TLR ligand-stimulated groups and unstimulated controls (Mean ± Standard error) showed no significant differences at the group level, but there were individual animal samples that exhibited a significant response and these are highlighted below. Figure 6.69a shows that γδ T cells produced IL-4 at significant levels over controls ($P<0.04$) in 2 samples, to high and low TLR3 agonist (bov 2) and low concentration (bov 1) of by comparison with control negative groups. In figure 6.69b, high concentrations of TLR8 ligands induced a significant increase of IL-4 production in 2 animals (Bov 1 and Bov 5) over controls ($P=0.0471$ and $P=0.0462$). Finally figure 6.69c shows no significant production of IL-4 from the stimulated cells with TLR9 agonist over controls.

a) Gamma delta T cells: IL-4 TLR3-ligand



b) Gamma delta T cells: IL-4 TLR8-ligand



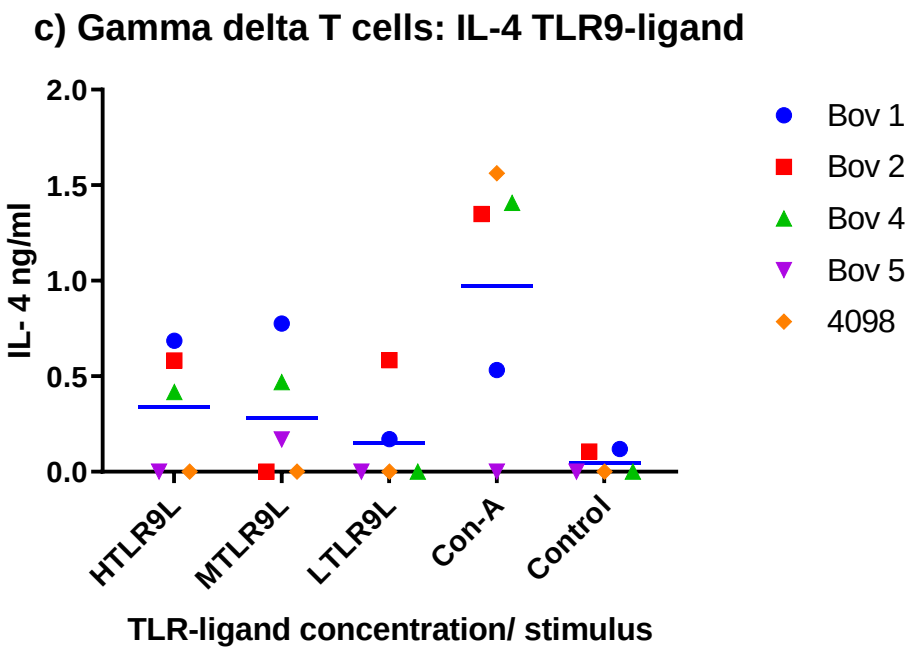
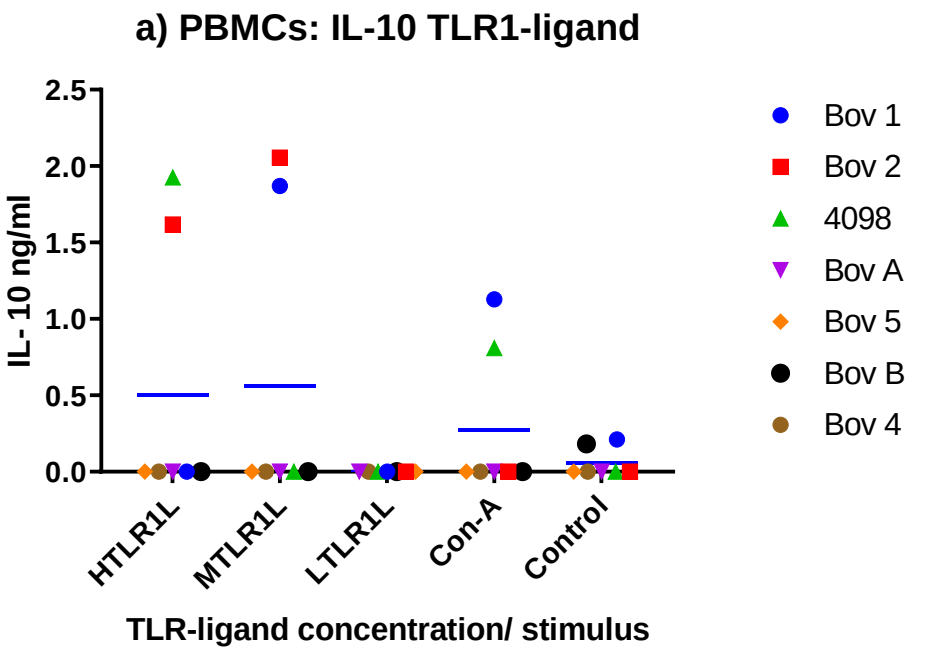


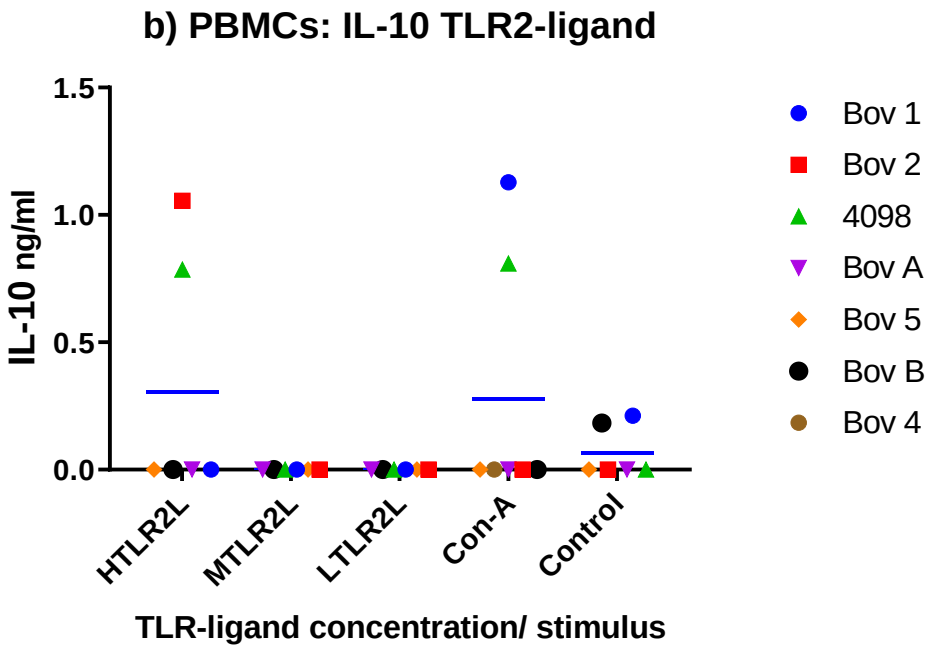
Figure 6.479: IL-4 concentrations measured in the supernatant of γδ T cells cultured in response to TLR3, TLR8, and TLR9 agonists in 5 animals (Figures 6.69 a, b and c respectively.

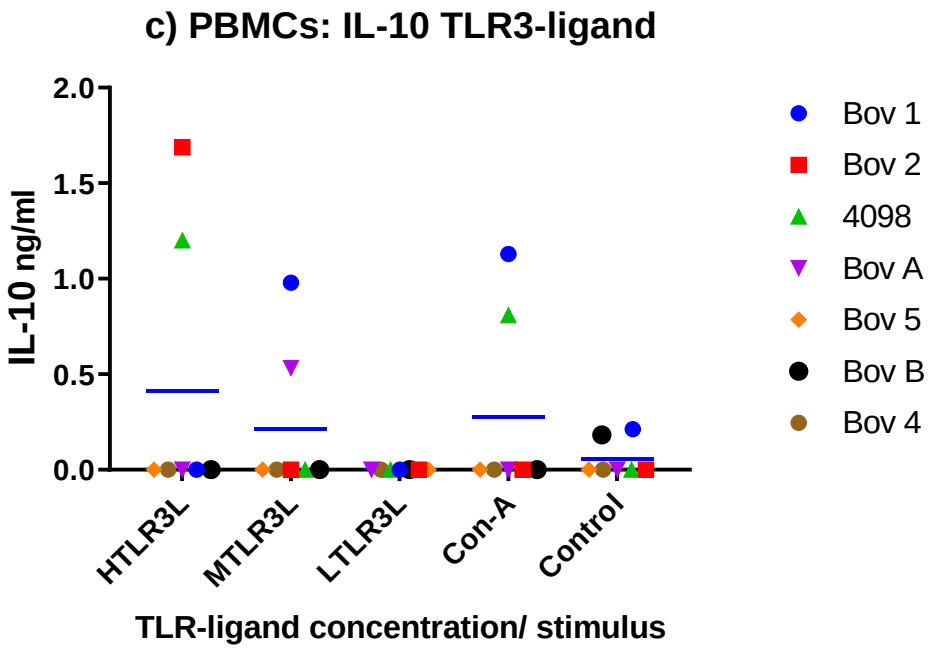
6.7 IL-10 results of PBMCs and T cell subsets stimulated with TLR ligands

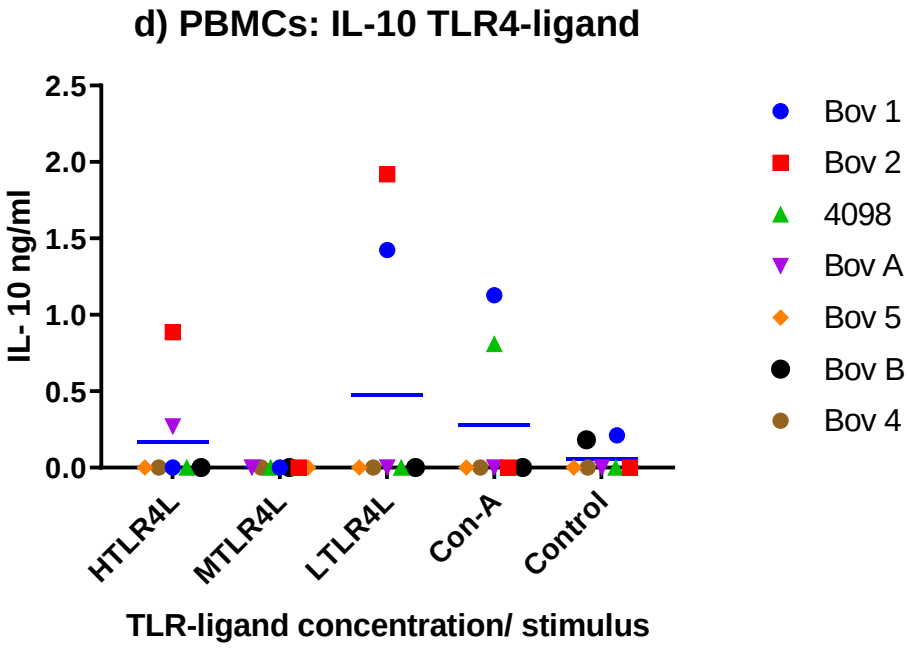
The comparison between PBMC TLR ligand-stimulated groups and unstimulated controls (Mean ± Standard error) showed no significant differences at the group level, but there were individual animal samples that exhibited a significant response and these are highlighted below. Figure 6.70a) shows a significant increase in IL-10 in animals stimulated with the high concentration of TLR1 ligands (Bov 2 and Bov 4098 P=0.0478 and P=0.0482 compared to unstimulated control) and significant increase in IL-10 in those stimulated with medium concentrations of TLR 1 in 2 animals (Bov1 and Bov2) over controls (P=0.0461 and P=0.0453). Figures 6.70b and c) show significant increases of IL-10 in those cells stimulated with the high concentration of TLR2 and TLR3 ligands in 2 animals (Bov2 and Bov 4098) over controls (P=0.0452 and P=0.0466). Figures 6.70d and e show significant increase of IL-10 in those stimulated with low concentrations of TLR4 ligand in 2 animals (Bov1 and Bov 2) over controls (P=0.0447 and P=0.0419). Figure 6.70e shows secretion of IL-10 from cells stimulated with low concentrations of TLR5 ligands (P=0.0422 and P=0.0438) vs unstimulated control in 2 animals (Bov1 and Bov2). Figure 6.70f shows secretion of IL-10 from cells stimulated with medium concentrations of TLR6 agonist in 2 animals (Bov1 and Bov 2) over controls (P=0.0456 and P=0.0436). In figure 6.70g, cells from 3 animals (Bov 1, Bov2, Bov A) gave significant increases in IL-10 stimulated with the high concentration of TLR7 ligand compared to unstimulated controls (P=0.0438, P=0.0377 and P=0.0426). The low concentration of TLR7

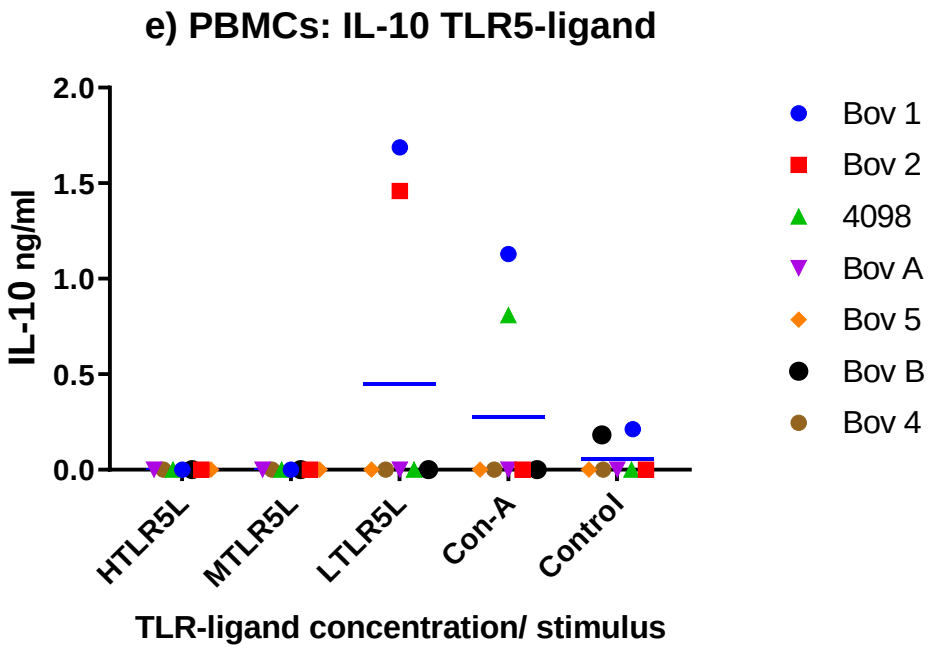
ligand caused a significant increase in IL-10 over controls in 2 animals (Bov1 and Bov2, P=0.0468 and P=0.0479). In figure 6.70h, only one sample from Bov 1 gave a significant IL-10 response to TLR8 ligand at medium concentration over controls (P=0.0475). Finally, figure 6.70i shows only one sample (Bov 2) responded significantly to stimulation with high concentration of TLR9 over controls (P=0.0443).

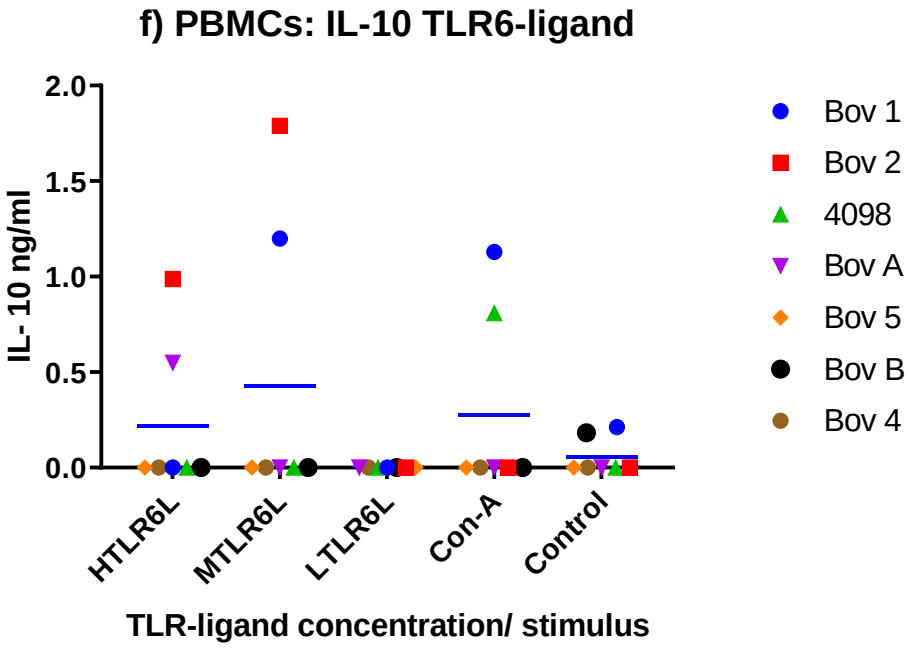

a) PBMCs: IL-10 TLR1-ligand
IL- 10 ng/ml
2.5
2.0
1.5
1.0
0.5
0.0
HTLR1L
MTLR1L
LTLR1L
Con-A
Control
TLR-ligand concentration/ stimulus
Bov 1
Bov 2
4098
Bov A
Bov 5
Bov B
Bov 4

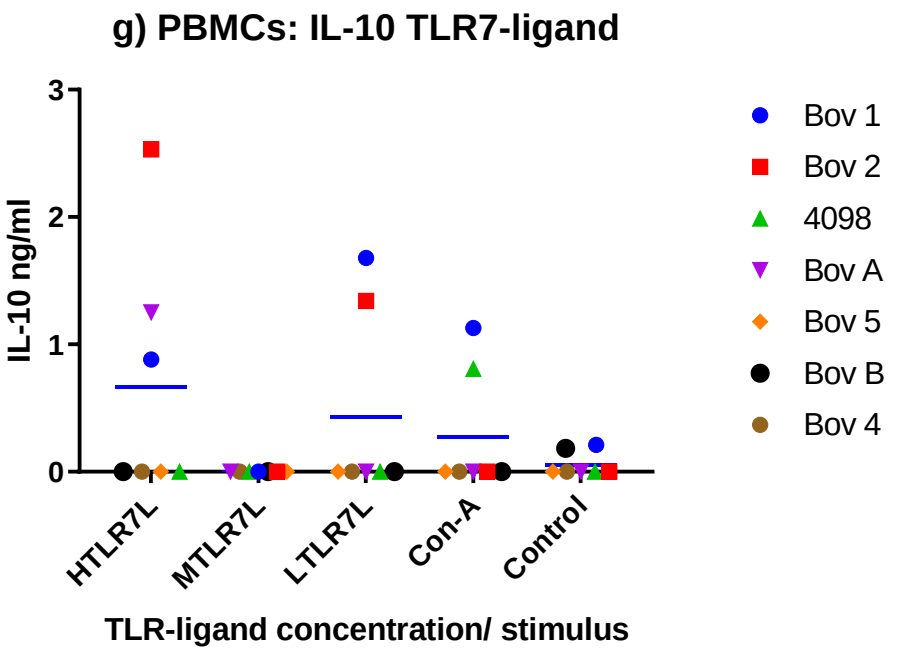

b) PBMCs: IL-10 TLR2-ligand
IL-10 ng/ml
1.5
1.0
0.5
0.0
HTLR2L
MTLR2L
LTLR2L
Con-A
Control
TLR-ligand concentration/ stimulus
Bov 1
Bov 2
4098
Bov A
Bov 5
Bov B
Bov 4

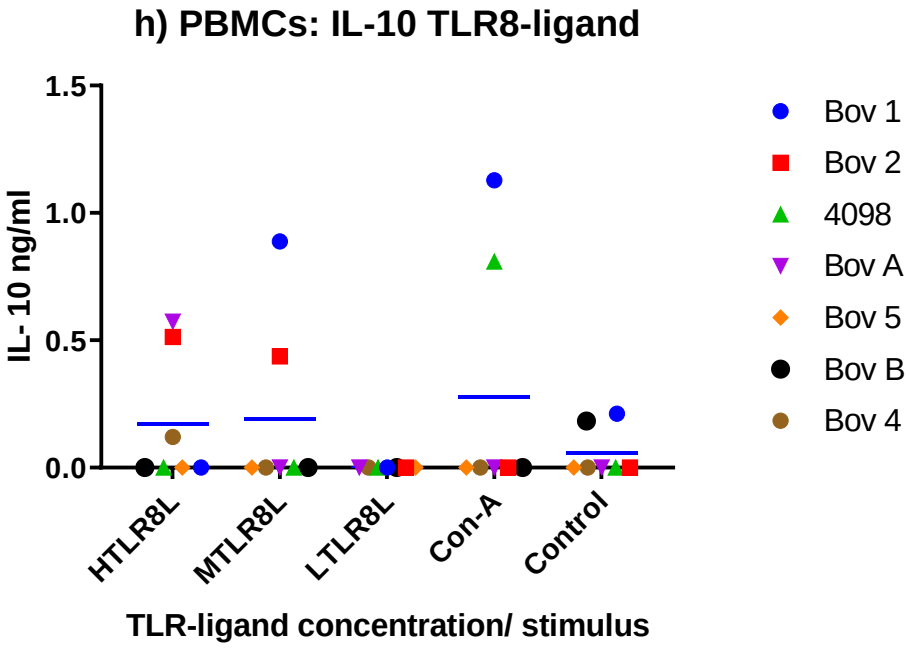

c) PBMCs: IL-10 TLR3-ligand
IL-10 ng/ml
2.0
1.5
1.0
0.5
0.0
HTLR3L
MTLR3L
LTLR3L
Con-A
Control
TLR-ligand concentration/ stimulus
Bov 1
Bov 2
4098
Bov A
Bov 5
Bov B
Bov 4


d) PBMCs: IL-10 TLR4-ligand
IL- 10 ng/ml
2.5
2.0
1.5
1.0
0.5
0.0
HTLR4L
MTLR4L
LTLR4L
Con-A
Control
TLR-ligand concentration/ stimulus
Bov 1
Bov 2
4098
Bov A
Bov 5
Bov B
Bov 4


e) PBMCs: IL-10 TLR5-ligand
IL-10 ng/ml
2.0
1.5
1.0
0.5
0.0
HTLR5L
MTLR5L
LTLR5L
Con-A
Control
TLR-ligand concentration/ stimulus
Bov 1
Bov 2
4098
Bov A
Bov 5
Bov B
Bov 4


f) PBMCs: IL-10 TLR6-ligand
IL- 10 ng/ml
2.0
1.5
1.0
0.5
0.0
HTLR6L
MTLR6L
LTLR6L
Con-A
Control
TLR-ligand concentration/ stimulus
Bov 1
Bov 2
4098
Bov A
Bov 5
Bov B
Bov 4


g) PBMCs: IL-10 TLR7-ligand
IL-10 ng/ml
3
2
1
0
HTLR7L
MTLR7L
LTLR7L
Con-A
Control
TLR-ligand concentration/ stimulus
Bov 1
Bov 2
4098
Bov A
Bov 5
Bov B
Bov 4


h) PBMCs: IL-10 TLR8-ligand
IL- 10 ng/ml
1.5
1.0
0.5
0.0
HTLR8L
MTLR8L
LTLR8L
Con-A
Control
TLR-ligand concentration/ stimulus
Bov 1
Bov 2
4098
Bov A
Bov 5
Bov B
Bov 4

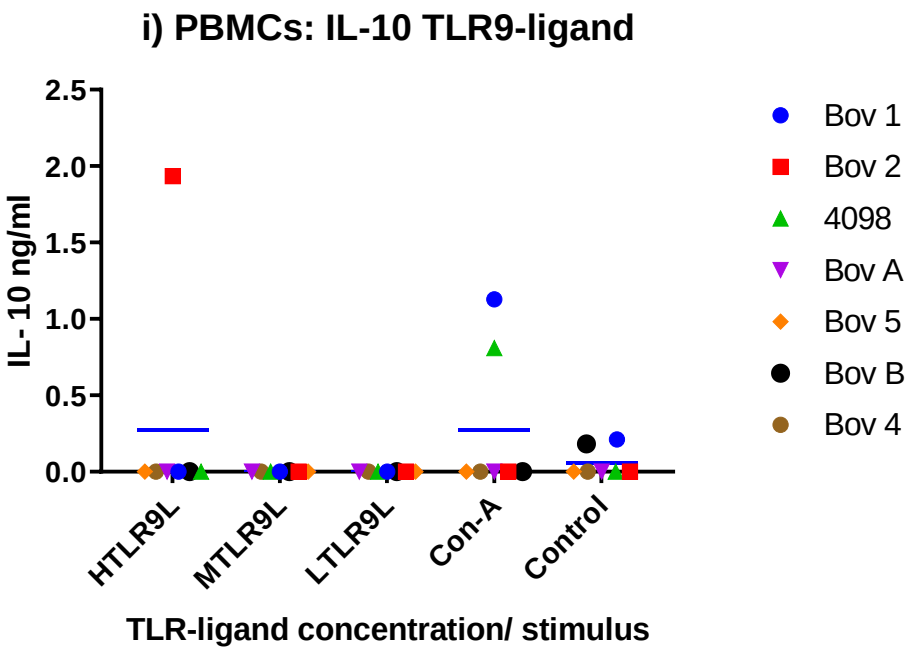


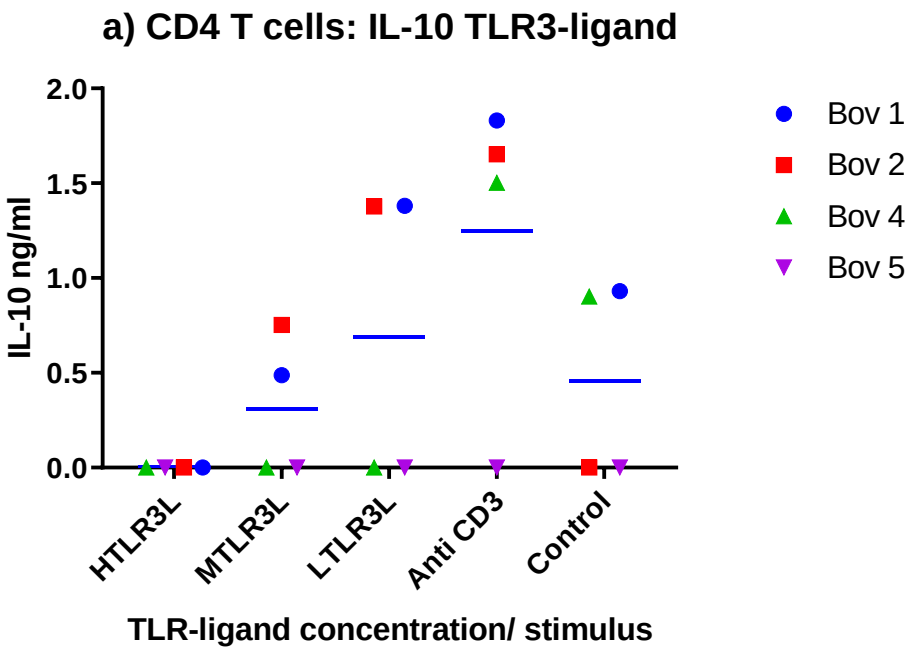
Figure 6.70: IL-10 concentrations measured in the supernatant of PBMCs cultured in response to TLR1-9 agonists in 7 animals. (a) LR1 ligand. (b) TLR2 ligand. (c) TLR3 ligand. (d) TLR4 ligand. (e) TLR5 ligand. (f) TLR6 ligand. (g) TLR7 ligand. (h) TLR8 ligand. (i) TLR9 ligand.

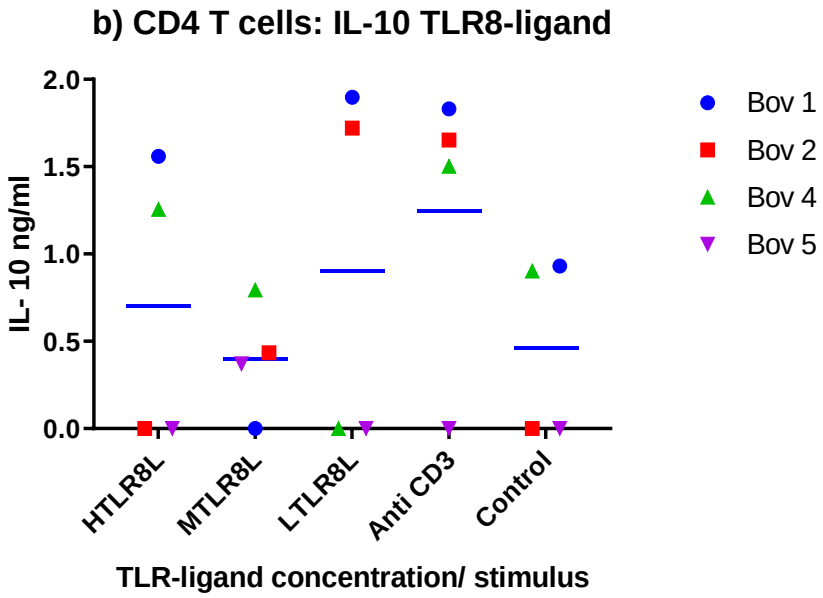
Figure 6.71 (a) shows (that there was no significant production of IL-10 secretion by the CD4 T cells to any concentration of TLR3 agonist by comparison with the control negative group. The low concentration of TLR8 ligand significantly induced IL-10 in 1 animal (Bov1) over controls (P=0.0452). In figure 6.71c, CD4 T cells stimulated with high concentrations of TLR9 ligand showed a significant increase of IL-10 in 1 animal (Bov4) over controls (P=0.0476).

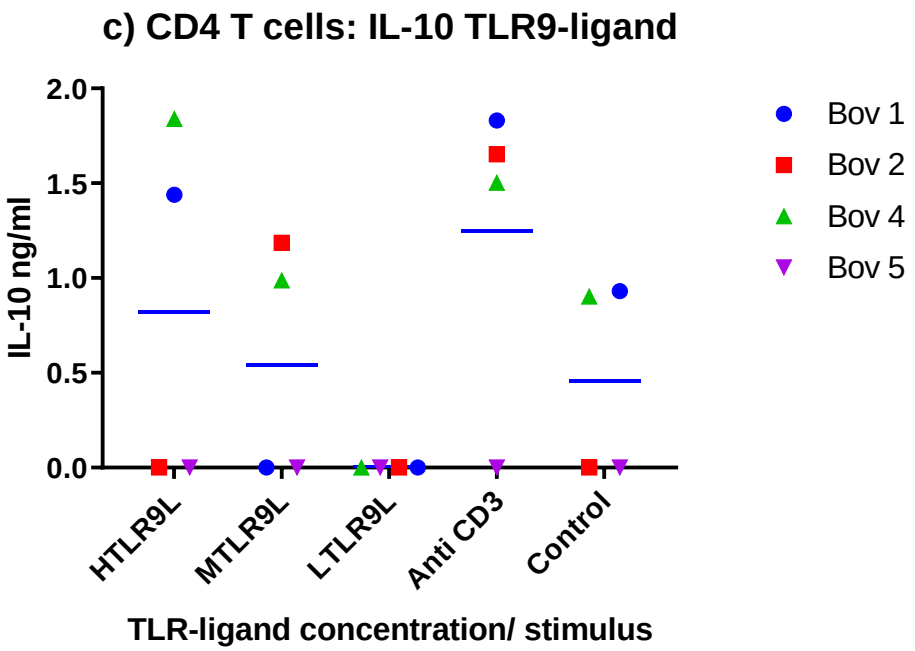
Figure 6.71d reveals that CD8 T cells stimulated with medium concentration of TLR3 ligand induced IL-10 in Bov 1 when stimulated with medium concentrations of TLR3 ligand over controls (P=0.041 compared to control). Figure 6.71e, shows no significant increase of IL-10 from CD8 T cells stimulated with TLR8 ligand at 3 concentrations.

Figure 6.71f shows the response in CD8 T cells of 2 animals (Bov1 and Bov 5) stimulated with high and medium concentrations of TLR9 ligand respectively by comparison with the control group (P=0.0477 and P=0.0433).

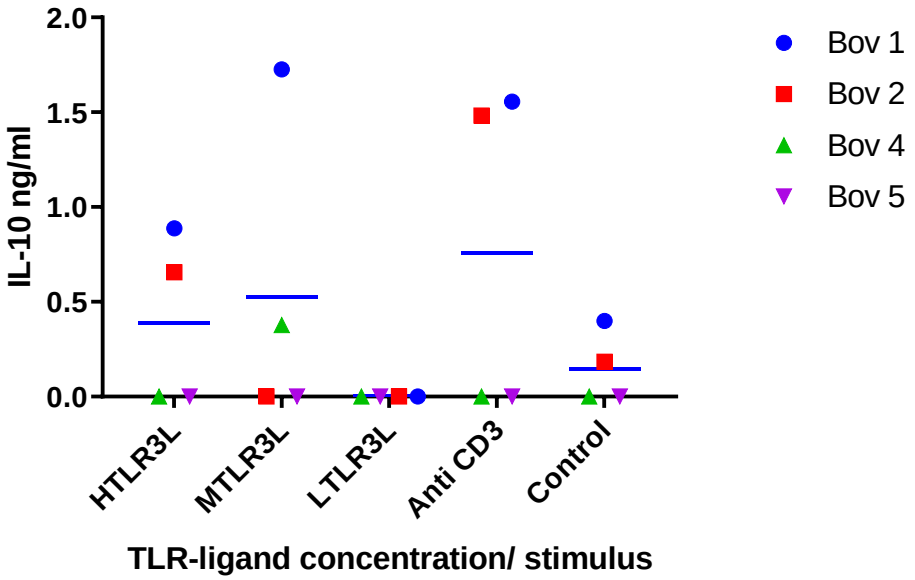
Figure 6.71g, shows gamma delta T cells stimulated with the high concentration of TLR3 ligand which significantly induced IL-10 in 2 animals (Bov1 and Bov2) over controls (P=0.0448 and P=0.0461). Figure 6.71h, shows a significant increase of IL-10 secreted from gamma delta T cells stimulated with medium concentrations of TLR8 ligand in 2 animals (Bov1 and Bov4) over controls (P=0.0482 and P=0.0466). Finally, figure 6.71i, shows no significant production of IL-10 from gamma delta T cells stimulated with TLR9 ligand at 3 concentrations by comparison with controls.


a) CD4 T cells: IL-10 TLR3-ligand
IL-10 ng/ml
2.0
1.5
1.0
0.5
0.0
HTLR3L
MTLR3L
LTLR3L
Anti CD3
Control
TLR-ligand concentration/ stimulus
Bov 1
Bov 2
Bov 4
Bov 5

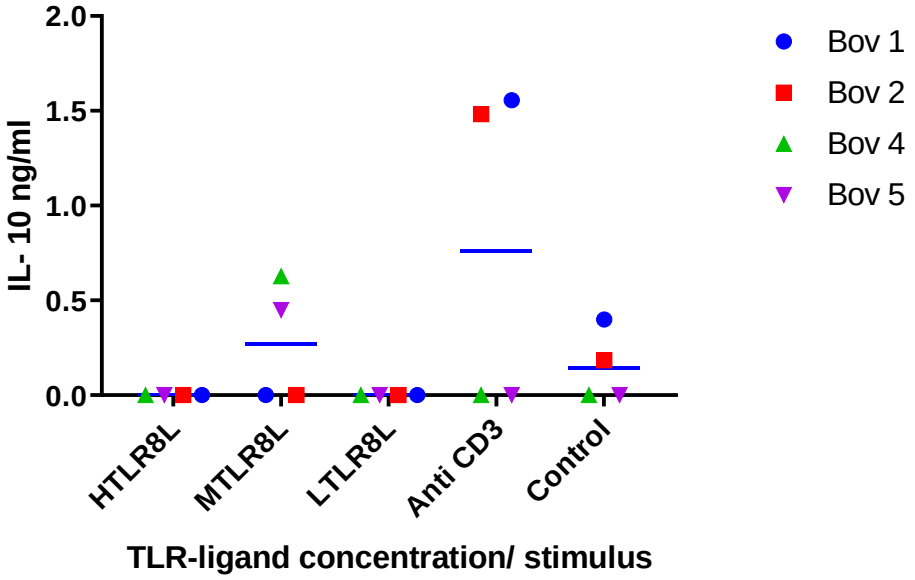

b) CD4 T cells: IL-10 TLR8-ligand
IL- 10 ng/ml
2.0
1.5
1.0
0.5
0.0
HTLR8L
MTLR8L
LTLR8L
Anti CD3
Control
TLR-ligand concentration/ stimulus
Bov 1
Bov 2
Bov 4
Bov 5


c) CD4 T cells: IL-10 TLR9-ligand
IL-10 ng/ml
2.0
1.5
1.0
0.5
0.0
HTLR9L
MTLR9L
LTLR9L
Anti CD3
Control
TLR-ligand concentration/ stimulus
Bov 1
Bov 2
Bov 4
Bov 5

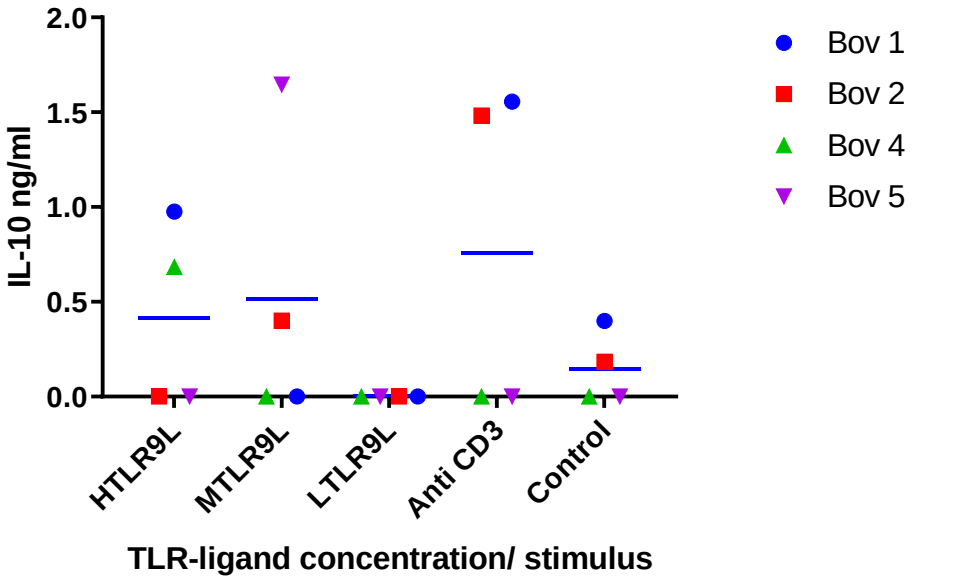
d) CD8 T cells: IL-10 TLR3-ligand



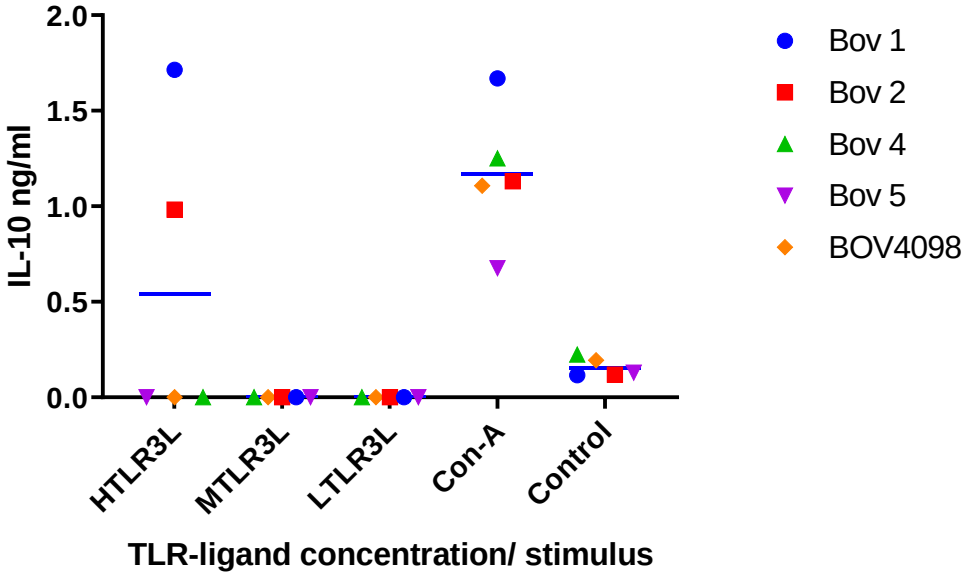
e) CD8 T cells: IL-10 TLR8-ligand

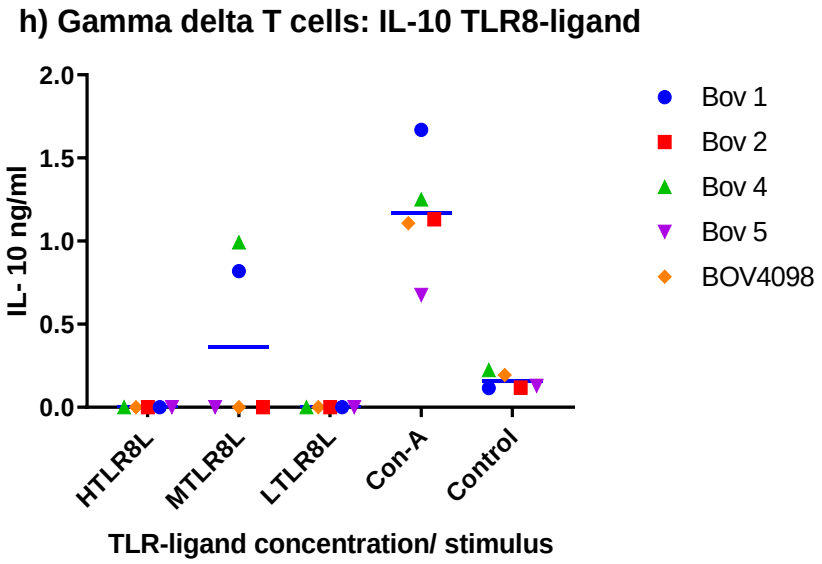


f) CD8 T cells: IL-10 TLR9-ligand



g) Gamma delta T cells: IL-10 TLR3-ligand





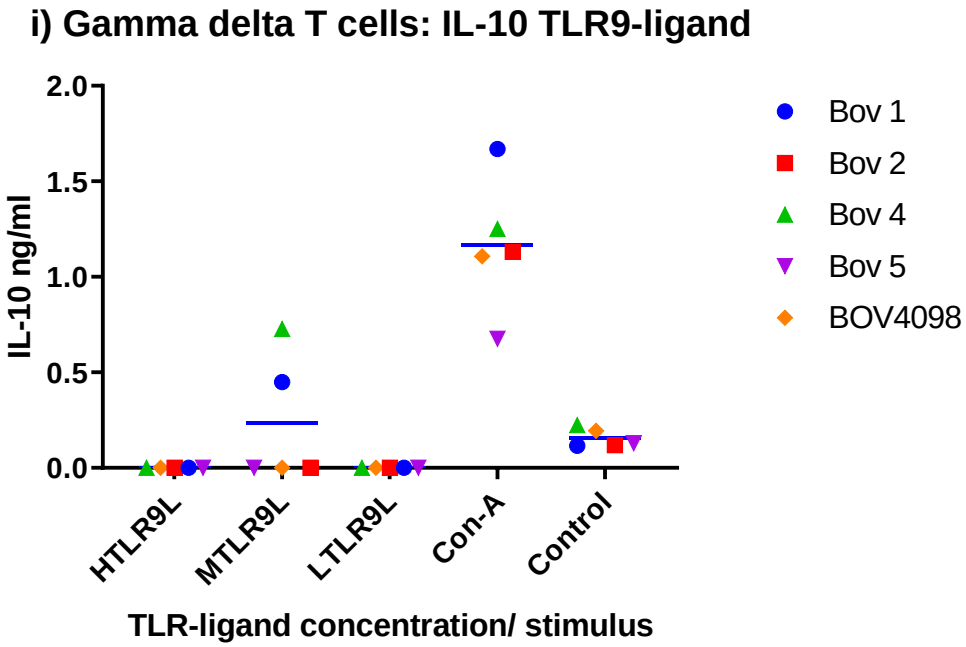


Figure 6.71: IL-10 concentrations measured in the supernatant of T cell subsets cultured in response to TLR3, TLR8, and TLR9 agonists in 4 animals (for CD4 , figures a-c, and CD8 T cell samples – figures d-f) and 5 animals (for γδ T cell samples – figures g-i).

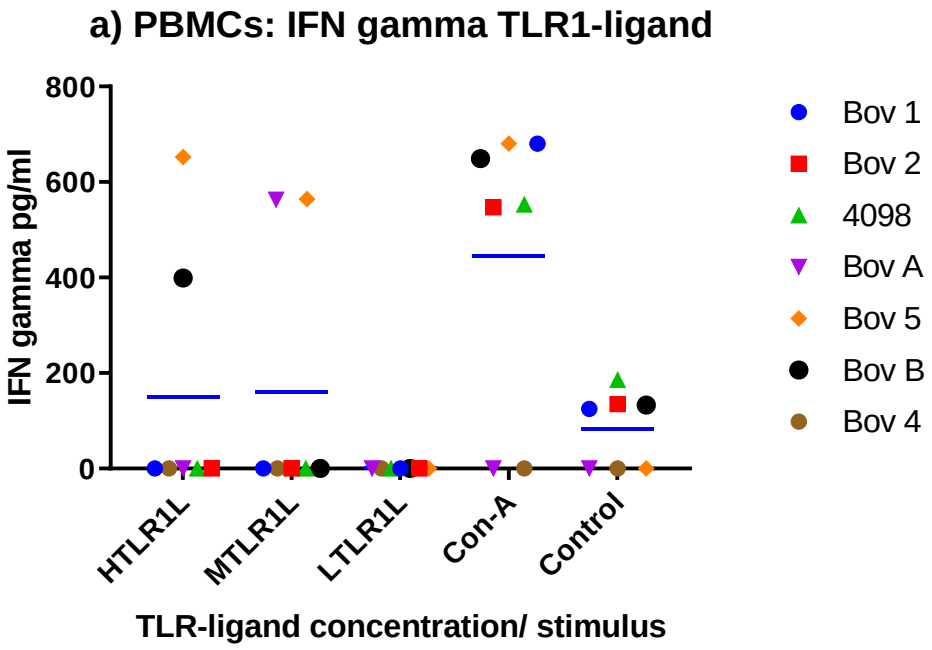
6.8 IFNγ results of PBMCs and T cell subsets stimulated with TLR ligands

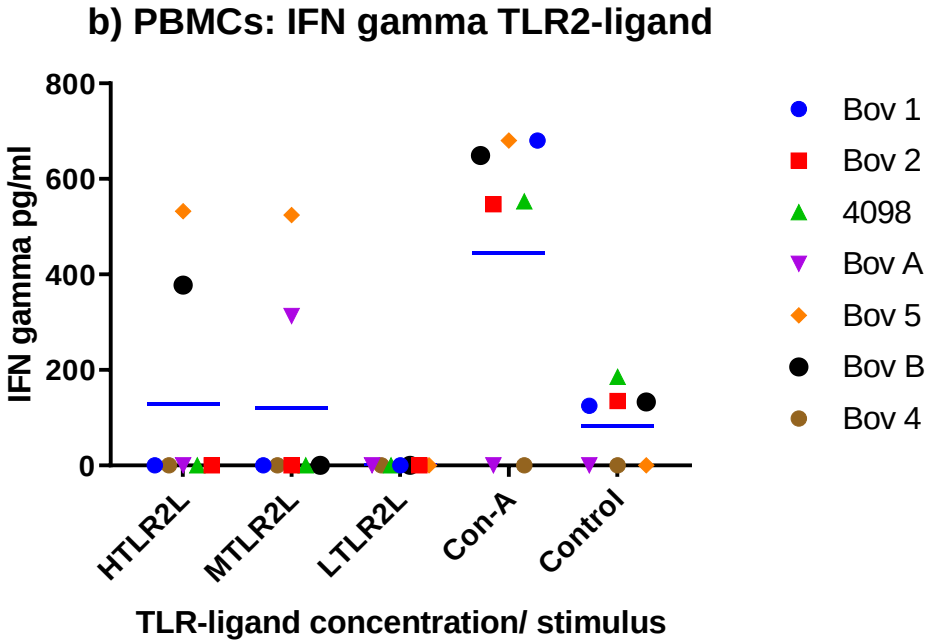
6.8.1 IFNγ results of PBMCs stimulated with TLR ligands

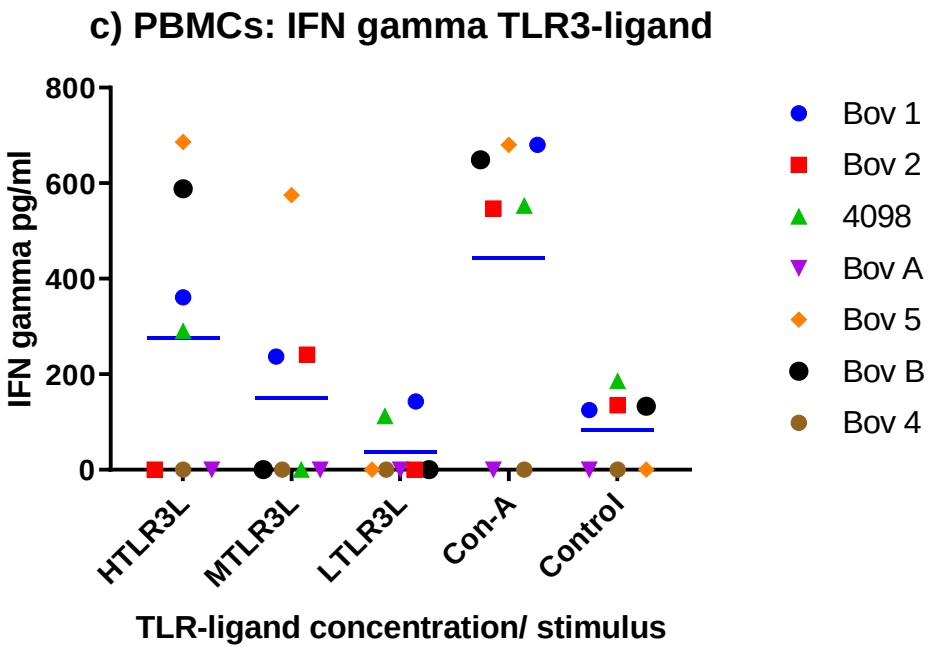
The comparison between PBMC TLR ligand-stimulated groups and unstimulated controls (Mean ± sem) showed no significant differences at the group level, but there were individual animal samples that exhibited a significant response and these are highlighted below. Figure 6.72a shows that a significant IFNγ response was detected in the PBMCs of only 1 animal (Bov 5) ($P=0.0339$ compared to controls) stimulated with high concentrations of TLR1 ligands and in bov A and Bov5 for medium TLR1 stimulation ($P<0.04$). Figure 6.72b shows a significant IFNγ response in Bov 5 stimulated with either high or medium concentrations of TLR2 agonists over controls ($P<0.316$). In figure 6.72c and d, 2 animals yielded significant amounts of IFNγ in response to simulation with high and medium concentrations of TLR3 and TLR4 agonists (Bov B and Bov 5), ($P=0.0349$ and $P=0.0356$ compared with controls). In figure 6.72e, f and g, PBMCs yielded significant amounts of IFNγ in response to stimulation with high and medium concentrations of TLR5, TLR6 and TLR7 agonists in 3 animals (Bov 5, Bov A and Bov B) respectively by comparison with control negative groups ($P=0.0361$, $P=0.0377$ and $P=0.0395$ respectively).

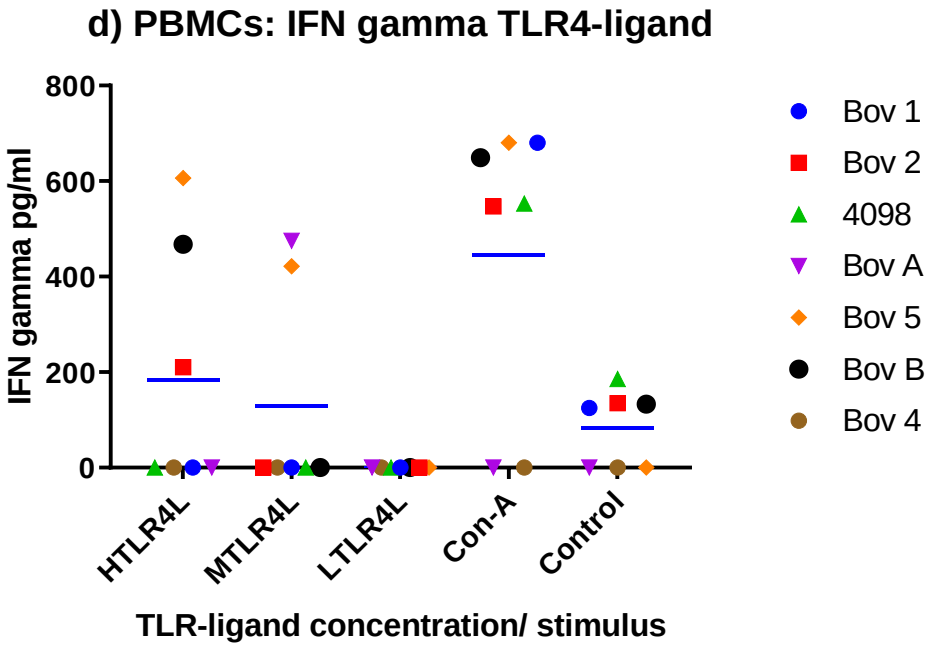
Figure 6.72h, shows significant production of IFNγ in 3 animals (Bov5, Bov A and Bov B) stimulated with high and medium concentrations of TLR8 ligands by comparison with the unstimulated control groups ($P=0.0348$, $P=0.0366$ and $P=0.0371$). Finally, figure 6.72i reveals a significant production of IFNγ in the supernatant of PBMCs stimulated with high and medium concentrations of TLR9 ligands in 3 animals (Bov 5, Bov A and Bov

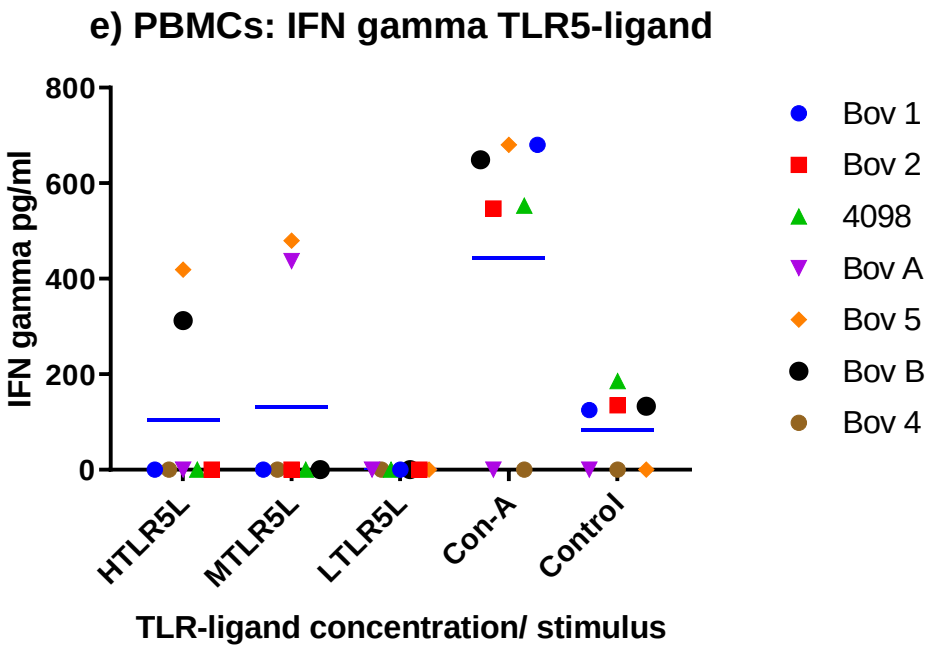
B). by comparison with control negative group (P=0.0327, P=0.0355 and P=0.0387 respectively).

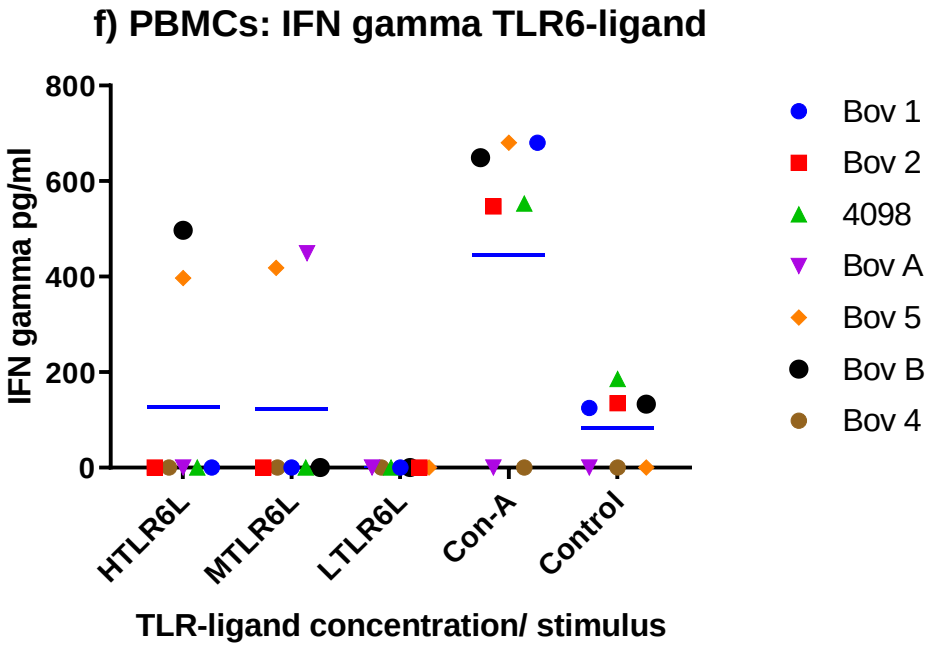


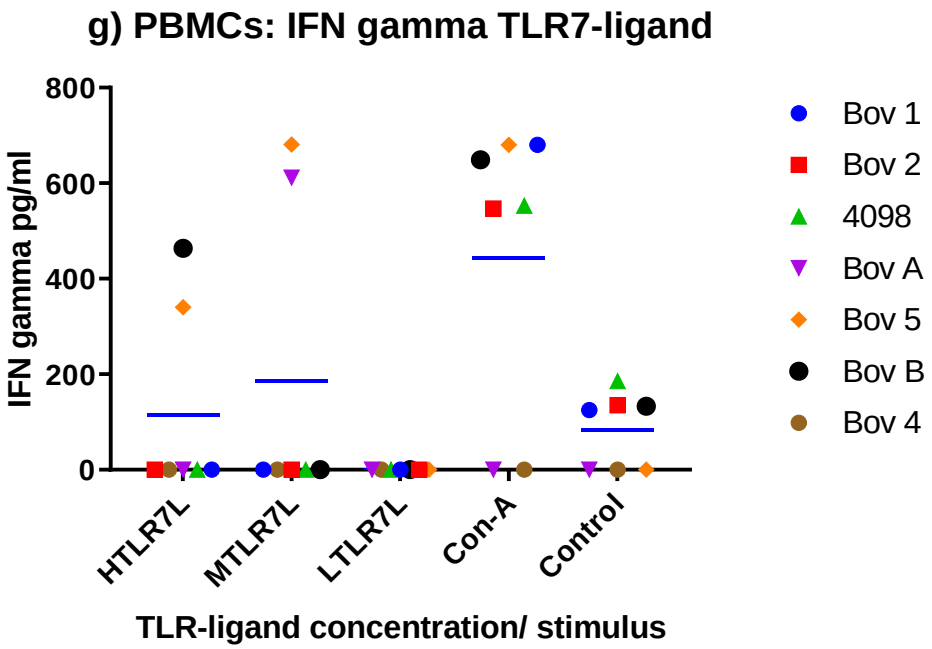


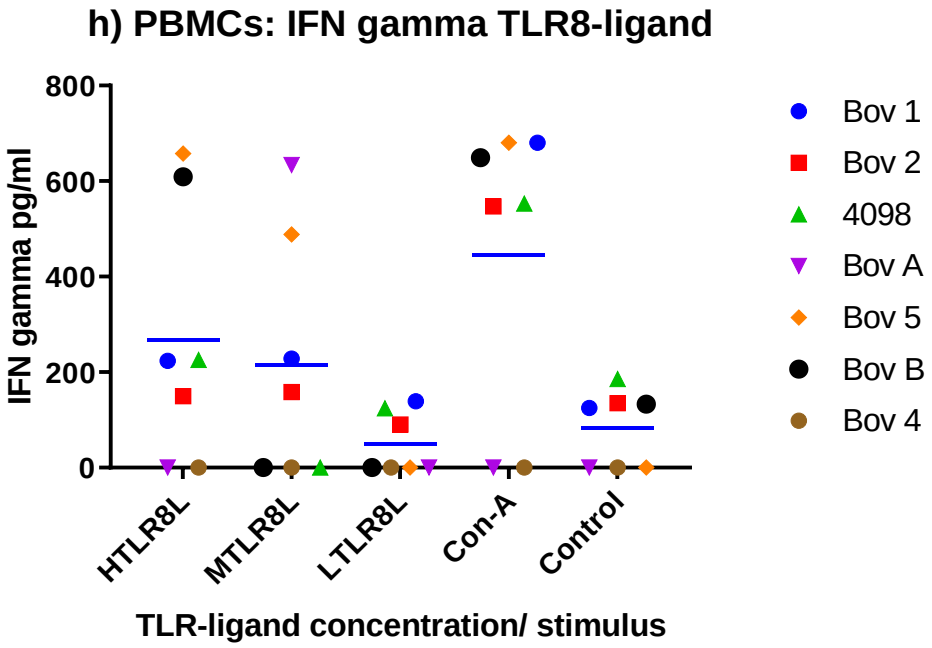

c) PBMCs: IFN gamma TLR3-ligand
IFN gamma pg/ml
800
600
400
200
0
HTLR3L
MTLR3L
LTLR3L
Con-A
Control
TLR-ligand concentration/ stimulus
Bov 1
Bov 2
4098
Bov A
Bov 5
Bov B
Bov 4


d) PBMCs: IFN gamma TLR4-ligand
IFN gamma pg/ml
800
600
400
200
0
HTLR4L
MTLR4L
LTLR4L
Con-A
Control
TLR-ligand concentration/ stimulus
Bov 1
Bov 2
4098
Bov A
Bov 5
Bov B
Bov 4


e) PBMCs: IFN gamma TLR5-ligand
IFN gamma pg/ml
800
600
400
200
0
HTLR5L
MTLR5L
LTLR5L
Con-A
Control
TLR-ligand concentration/ stimulus
Bov 1
Bov 2
4098
Bov A
Bov 5
Bov B
Bov 4


f) PBMCs: IFN gamma TLR6-ligand
IFN gamma pg/ml
800
600
400
200
0
HTLR6L
MTLR6L
LTLR6L
Con-A
Control
TLR-ligand concentration/ stimulus
Bov 1
Bov 2
4098
Bov A
Bov 5
Bov B
Bov 4


g) PBMCs: IFN gamma TLR7-ligand
IFN gamma pg/ml
800
600
400
200
0
HTLR7L
MTLR7L
LTLR7L
Con-A
Control
TLR-ligand concentration/ stimulus
Bov 1
Bov 2
4098
Bov A
Bov 5
Bov B
Bov 4


h) PBMCs: IFN gamma TLR8-ligand
IFN gamma pg/ml
800
600
400
200
0
HTLR8L
MTLR8L
LTLR8L
Con-A
Control
TLR-ligand concentration/ stimulus
Bov 1
Bov 2
4098
Bov A
Bov 5
Bov B
Bov 4

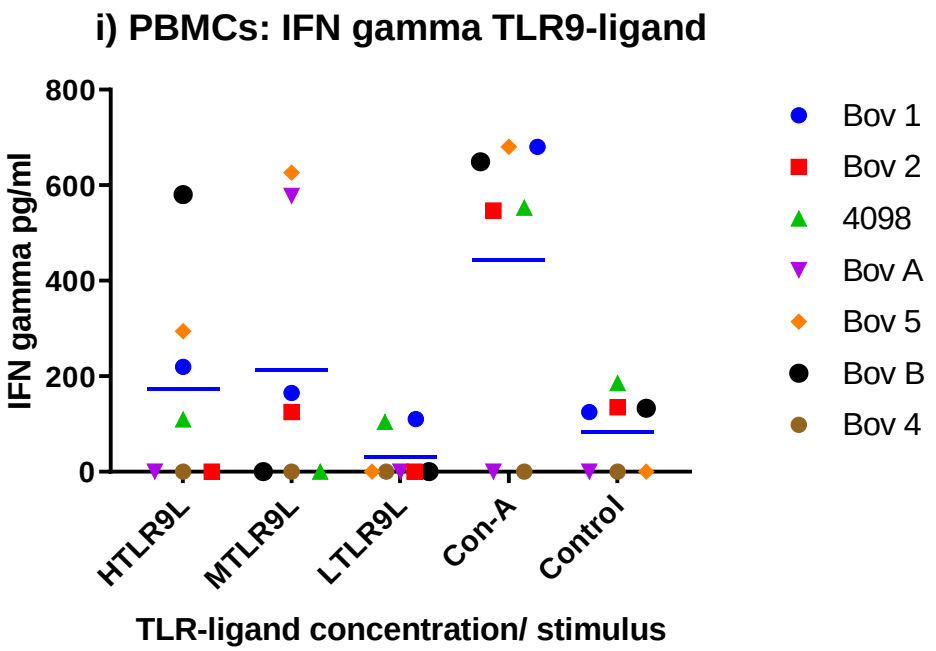


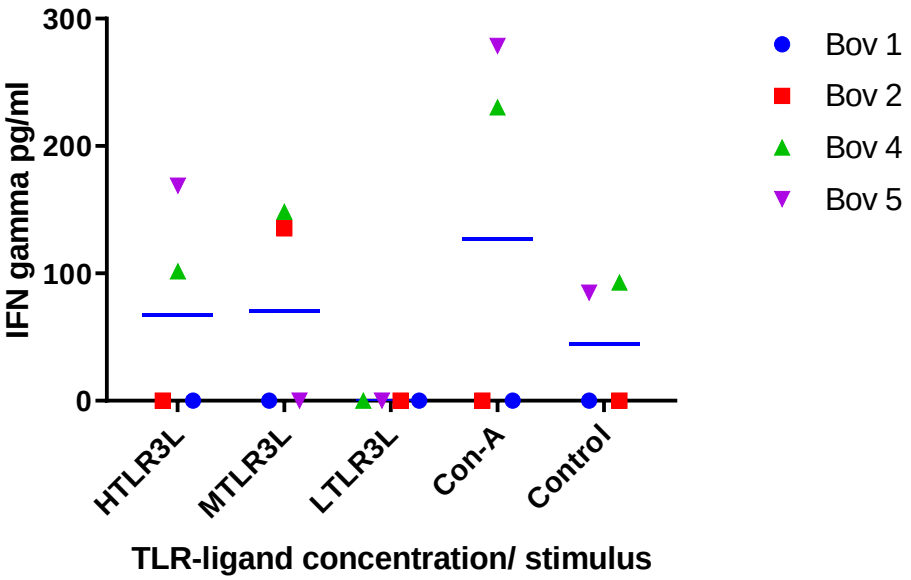
Figure 6.48 (a-i, TLR ligands 1-9 respectively): IFNγ concentrations measured in the supernatant of PBMCs cultured in response to TLR1-9 agonists in 7 animals.

6.8.2 IFNγ results of T cell subsets stimulated with TLR ligands

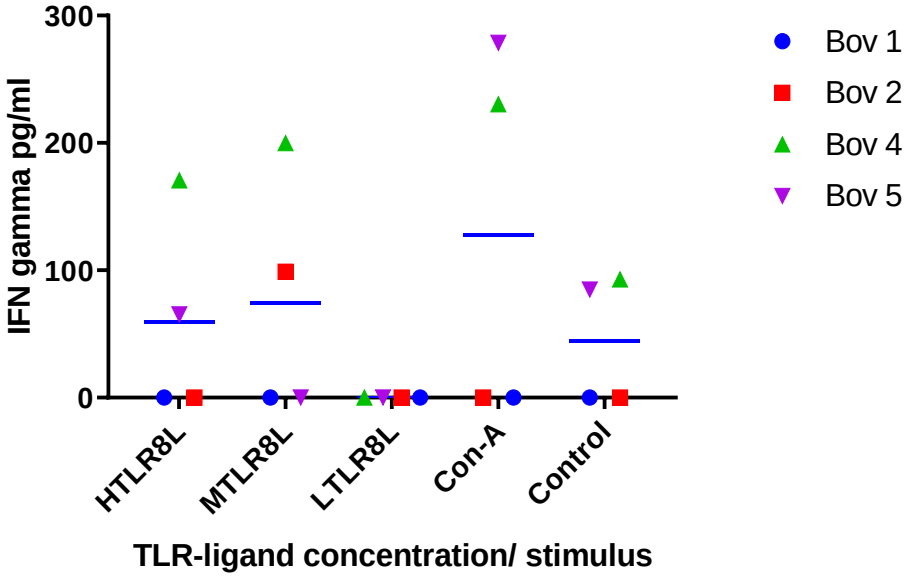
IFNγ was not secreted by neither CD4 T cells, nor CD8 T cells stimulated with TLR3, TLR8, and TLR9 agonists, while gamma delta T cells produced some IFNγ (figure 6.73).

The comparison between γδ T cell TLR ligand-stimulated groups and unstimulated controls (Mean ± Standard error) showed no significant differences at the group level, but there were individual animal samples that exhibited a significant response and these are highlighted below.

a) Gamma delta T cells: IFN gamma TLR3-ligand



b) Gamma delta T cells: IFN gamma TLR8-ligand



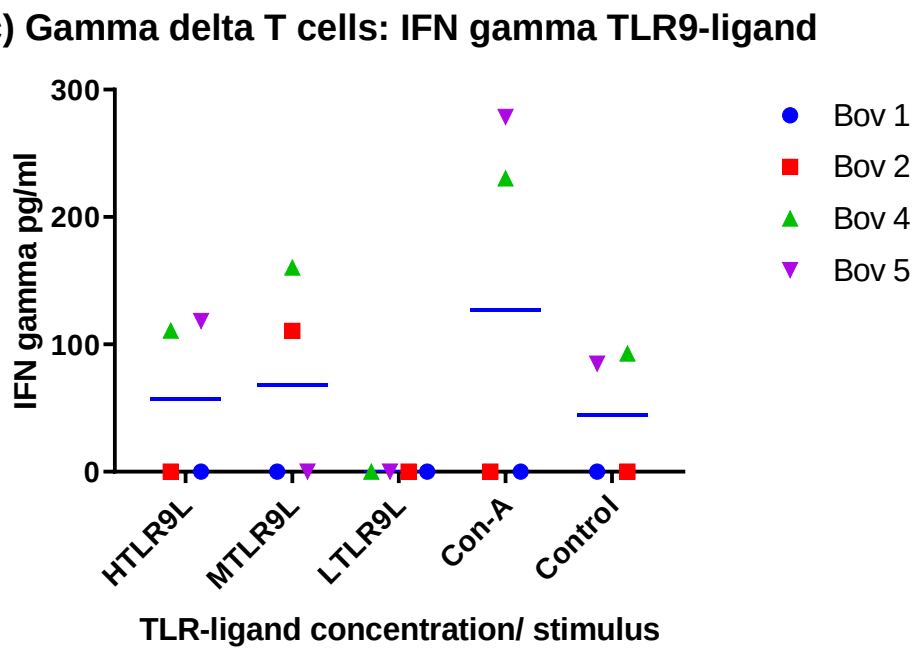


Figure 6.73. Response of γδ T cells to TLR-agonists (a-c, TLRs 3, 8 and 9 agonists respectively.

6.9 Discussion

In this chapter, TLR activation by TLR-ligand stimulation was assessed in PBMC and the T cell subsets (CD4, CD8, γδ T cells) by the production of a range of cytokines. Some analyses are incomplete owing to time constraints, but there are interesting results from what has been done. CXCL8 has been used most often as a read out for TLR activation in a variety of cell types (Allavena *et al*., 2008, Fonseca *et al*., 2011, Mills, 2011, Russell *et al*., 2012, Lee *et al*., 2015, Karthikeyan *et al*., 2016, Salvi *et al*., 2016, Tombácz *et al*., 2017).

Secretion of CXCL-8 from bovine APCs such as monocytes, macrophages, dendritic cells and B cells upon stimulation with different microbes has previously been recorded (Widdison *et al*., 2008, Taubert *et al*., 2009, Metcalfe *et al*., 2010, Düvel *et al*., 2014).

What was immediately apparent was that only a subset of cell samples from the bovine animals gave a response in the assays. This meant that there was no significant cytokine response at the entire group level for any cytokine, but that individual samples did give a significant response for some analyses (expanded below). In particular cell samples from bovs 1,2, 4, 5 and 4098 were generally more responsive to a range of TLRLs for cytokine production that the other samples. The reasons for this are not known. However it could be that the immune status of the animal may have been responsible. All animals were checked for clinical disease and overall good health prior to entering to the abattoir, but the immune system of outbred animals in the environment are constantly being challenged and this is normal. We were unable to perform a thourogh analysis of this.

In this study CXCL8 was the cytokine amongst those tested that gave the most comprehensive results. Both PBMC and T cell subsets of a proportion of the animal samples responded to the TLR-Ls (PBMC) or a subset of them (see below) respectively to give a CXCL8 response. In this study ELISAs specific for the bovine animal were used whereas in the above references, assays cross –reactive with bovine molecules but using antibody raised against murine or human cytokines were used. PBMC contain cells other than T cells that can produce TLR-activated cytokines. These include monocytes and B cells and probably NK cells. The PBMCs as in the work of previous chapters was a positive control for TLR-ligand activity and TLR engagement (although this latter was not specifically molecularly measured). Using PBMCs as target cells for TLR-agonist stimulation measured by CXCL8 production confirmed that the TLR agonists were active on bovine cells, although whether the optimum concentration range was used or not was not further explored. Within the PBMC, monocytes, B cells, possibly NK cells as well as T cells amongst other cell types are all capable of responding to the TLR agonists.

Using the purified T cell subsets, only TLR3L, TLR8L and TLR9L were used to stimulate the cells for cytokine analyses to accommodate the capacity of the ELISA kits and also due to the fact that these analyses were performed at the end of the study period, where there was not time to do a comprehensive analysis. They were prioritised as they are involved in anti-viral immunity, an interest of our research group. CD4+ T cells, CD8+ T cells and γδ T cells from bov 1, bov 4 responded to TLR3L, TLR8L and TLR9L in all the T cell subsets to produce CXCL8, and bov 2 and bov 5 to specific TLR agonists to produce CXCL8. Why these animals were

responsive in these assays and not (for the most part) other animals is not known. This is discussed further in the general discussion.

Amongst the other cytokine responses studied, both PBMC and T cell subsets of a proportion of animal samples responded to TLRLs to produce IL-4, and IL-10, and TNF-alpha and IFN-alpha to a much lesser extent. TGF-beta was not detected in a preliminary analysis (although the number of samples analysed was too low to draw any conclusions).

Within the T cell subsets, the production of IL4 and IL10 by a proportion of the samples analysed is interesting given that T cell subset (CD4+ T cells, CD8+ T cells, γδ T cells) production of IFN-gamma was not detected. Interferons are a family of low molecular weight proteins secreted by many host cells in response to intracellular bacteria, viruses, or against tumours. We noticed an absence or low levels of type I interferons (IFNα) in the supernatants of PBMC or CD4+ or CD8+ T cell subset samples stimulated with TLR ligands. It had been recorded by Charleston *et al*. (2001) who found confusing results, with IFNα produced from cells infected with cytopathic BVD virus but not produced when non-cytopathic BVD virus was used. Herath *et al*. (2009) studied the expression of TLRs1-10 in the uteri of fertile and non-fertile cattle and discovered that IFNα was at similar levels in both, unlike IL1α, IL1β, or IL10 which were elevated in the infertile cattle compared to fertile cattle.

Interestingly PBMCs and T cell subsets of some animals produced IL-4 to TLR-ligand stimulation. IL-4 is difficult to detect in bovine samples (except in some specific disease states). This is also of interest as IFNγ responses were not prevalent. The reason for this is not clear, but could reflect a Th2 dominance of immune response (IL-4 mediated) over a Th1 response (IFN-

γ mediated) in the T cells from these animals. However, this is highly speculative with no supporting evidence.

IFNγ is a Th1 cytokine which is derived from CD4+ T cells amongst other cells (Ike *et al*., 2005) and was secreted in PBMCs, but not CD4+, CD8+ or γδ T cells stimulated with TLR agonists. IFNγ is known to be produced by activated NK cells, CD4 T cells (particularly T helper 1 cells), and CD8 T cells predominantly (Harty *et al*., 1992, Ike *et al*., 2005). γδ T cells also produce this cytokine (Skeen and Ziegler, 1995) as do monocytes/ macrophages under some circumstances (Munder *et al*., 1998). All of above mentioned potentially IFNγ secreting cells are part of the PBMC pool. The lack of IFNγ by TLR-ligand stimulated T cells remains to be explained. One of the major cytokines released by cytotoxic T cells is IFNγ (Kennedy *et al*., 2002, Wherry and Ahmed, 2004, Shin *et al*., 2005, Stabel *et al*., 2007). Baldwin *et al*. (2000) detected IFNγ secreting γδ T cells (phenotype WC1+, which is the same as the cells we use, fractionated using the WC1-specific CC15 mAb) by two methods; flow cytometry and ELISA. Vesosky *et al*. (2004) expanded purified γδ T cells co-cultured with cell wall particles of *Mycobacterial mycolylarabino-galactan* (a peptidoglycan molecule similar to TLR2 agonist we used). These γδ T cells produced significant levels of IFNγ. This was also seen by Price and Hope, (2009) but using another species of *Mycobacterium* in an APC (*M. bovis*-infected dendritic cells) instead. Later studies continued to support the concept of secreting IFNγ by γδ T cells upon stimulation (Toka *et al*., 2011, Plattner *et al*., 2013).

T cells, mainly Th2 cells, are a source of IL-4 (they also respond to IL-4 stimulation), but this is not a generic rule (Nelms *et al*., 1999, Chatila,

2004). We stimulated PBMCs (that includes T cells) with TLR1-9 agonists but recorded IL-4 secretion in the supernatant only in a proportion of the bovine samples. IL-4 secretion was detected from purified T cell subsets stimulate with TLR-agonists (novel and original observation). In this regard, Waldvogel *et al*., (2000) measured very low but inconsistent levels of IL-4 and IFNγ by ELISA in PBMCs stimulated with non-cytopathic BVD virus in pregnant and non-pregnant cattle although the mRNA of both cytokines was positively detected by RT-qPCR method.

Sopp and Howard (2001) detected IL-4 secretion (by flow cytometry) from lymphocytes (collected from bovine lymph nodes and peripheral blood) *in vitro* activated by PMA (a T cell mitogen). They found that only CD4+ T cells produced IL-4 but not CD8+ nor γδ T cells. In this study IL-4 was secreted by γδ T cells from a couple of animal cells in response to TLRs 3 and 8 but not at all by CD8+ T cells.

IL-10 was detected in some PBMC samples and in some TLR3L, TLR8L and TLR9L stimulated CD4 T cells, CD8 T cells and γδ T cells (bov 1 bov 2 , bov 4 and bov 5 in particular). This cytokine is known to possess immunoinhibitory activity, notably in autoimmune disease or cancer. This raises the possibility that Under some circumstances (not defined) TLR-agonist engagent could lead to T cell expression of IL-10 and a suppressive local environment in vivo for ongoing immune responses.

In conclusion, it is difficult to draw robust conclusions from this analysis, but there are indications of interesting results that should be followed up with a more incisive study.

Chapter Seven

General discussion,

conclusions and recommendations

7.1 General overview

In this study, the expression and function of TLRs on bovine T cells has been researched. The T cell subsets studied included CD4+ T cells, CD8+ T cells and γδ T cells from peripheral blood samples of cattle. We originally wished to include Tregs as well, but the work on rabbit T cells (to inform cattle Treg development in culture) indicated that this would be time consuming and the cells did not in any case show suppressive activity, so this was dropped.

TLRs1-10 were analysed on the T cell subsets by RT-qPCR. Some did not express TLR5 and TLR6, but the other samples did express all of the TLRs. This is the first description of this that we are aware of in cattle. Cell purities were adequate to allow allocation of TLRs to the T cell subsets but with the caveat that the few non T cells in the preparations could have contributed to the analysis. There is a lack of bovine-specific TLR blocking antibodies to confirm TLR-ligand specificity for the TLR, but there are conclusions that can drawn, nevertheless. Differences between whole PBMCs and the T cell subsets with respect to TLR expression are likely attributable in the main to the presence in the PBMCs of monocytes, NK cells and B cells, all of which are known to express TLRs in cattle (and other animals) (Werling *et al*., 2006, Werling *et al*., 2017).

An important observation is that differences in TLR expression and more obviously function (see below) were seen in the different animals of PBMC and their derived T cell subsets. The reasons for this are not known but may indicate previous immune activation status of the donor animals or involvement of other co-factors or molecules for TLR expression and

function not present in PBMC or the T cell subsets *in vitro*. This is discussed further below.

Functionally, the CFSE assay revealed proliferation in response to TLR agonists in T cell subsets in a non-consistent way but not in PBMCs. TLR3-agonist, TLR8-agonist, and TLR9-agonist stimulation of CD4+, CD8+ and γδ T cells in 3-4 animals induced measurable cell division /proliferation by the CFSE assay. However, this aspect of the study requires further validation of techniques and time constraints did not allow this, focussing instead on cytokine production as a read out for TLR-agonist activated cells. The cytokine assays revealed some interesting results, were a better read out of TLR-ligand activation of the cells and indicated differential expression of the cytokines at the T cell level and the TLR-agonist used. This is discussed below. Time constraints meant that some of the work is incomplete and in particular for T cell subset activation studies the number of TLR-ligands used was reduced from all those stimulating all 9 TLRs to those targeting TLRs 3 (dsRNA as ligand), 8 (single stranded viral RNA ligand) and 9 (unmethylated CpG oligodeoxynucleotide DNA) that are important in anti-virus responses, an interest of our research group.

7.2 Putative Rabbit Tregs

Phenotypic properties of regulatory T cells in animals and humans have been mostly identified as (CD4+CD25hiFOXP3+). They have been investigated in laboratory animals such as mice (Fontenot *et al*., 2005b, Ziegler, 2006, Murai *et al*., 2009, Waight *et al*., 2015), guinea pigs (Shang *et al*., 2011, Kato-Maeda *et al*., 2012, Clark *et al*., 2015), rats (Donia *et al*., 2009, Fujiki *et al*., 2010, Jin *et al*., 2013) but not in rabbits. We

attempted to generate putative Tregs in rabbit MLN cells by adding titrated levels of ConA, IL-2, and TGFβ to cells in IMDM medium and we successfully obtained up to 25% of putative rabbit nTreg cells in the total cell population after 6 days of culture (detected by flow cytometry and confirmed by RT-qPCR as FOXP3+). Therefore, we documented the phenotype for the first time in rabbits.

Our results show that rabbit MLN cells responded well to IL-2 which is essential for development of CD25+ external marker (Zheng *et al*., 2007), and we can attribute FOXP3+ (a key feature and intracellular marker of nTregs) in the generated cells to TGFβ, agreeing with (Chai *et al*., 2005), while ConA provided a non specific T cell proliferation (Wang *et al*., 2012, Ando *et al*., 2014).

With regard to the functional role of the putative rabbit Tregs, there was no suppression of the proliferation of autologous MLN cells. This is at variance with the generally accepted view that Tregs function as immunosuppressive cells through secretion of immune-inhibitory cytokines (mostly IL-10) (Anderson *et al*., 2007, Murai *et al*., 2009, Nylén *et al*., 2007, Sakaguchi *et al*., 2009). We did not measure this cytokine in the supernatant.

There has been a description of human FOXP3+ gene detected in some T effector cells and also in CD4+CD25+ Tregs that did not show any suppressive action or inhibitory cytokine production (Allan *et al*., 2007). Liu *et al*. (2006) found that CD127+ was essential for CD4+FOXP3+ human Tregs function. We did not look for this. Tran *et al*. (2007) tested the effect of TGFβ on FOXP3 expression which was enhanced (FOXP3+) in Tregs but

these cells were not inhibitory in spite of the secretion of immuno-suppressive cytokines.

Finally, it was found that induced human Tregs (FOXP3+) in the intestine play different role represented by anti-inflammatory role rather than immune-suppression (Round and Mazmanian, 2010). In summary, we discontinued the Treg culture approach for bovine Tregs.

7.3 Bovine T cell TLR gene expression

We reported a higher frequency of γδ T cells compared to other T cell subsets in the bovine blood samples from seven animals. This along with the lower frequencies of B cells, CD8+ T cells and CD4+ T cells is consistent with the results of others, who also point out that young ruminants have the highest frequency of γδ T cells that decline in number with age (Hayday and Tigelaar, 2003, Telfer and Baldwin, 2015, Gillespie *et al*., 2017).

The expression of TLR1-10 genes in bovine PBMCs was demonstrated, as well as in CD4+, CD8+, and γδ T cells, which was demonstrated in this study for the first time. However, TLR5 and TLR6 were not expressed in 2 animals. These results are roughly in line with studies on T cell TLR expression in other species (mostly humans and mice). In these studies TLR2, TLR3, TLR4, TLR7, and TLR8 expression was generally most prevalent on T cells amongst the TLRs analysed (Peng *et al*., 2005, Kabelitz, 2007, González-Navajas *et al*., 2010, Reynolds *et al*., 2010, Flaherty and Reynolds, 2016, Pacheco *et al*., 2016).

In this study, bovine CD8+ T cells expressed TLR1-10 genes. Observations on cytotoxic T cells (in humans and mice) have revealed intracellular TLR3, and TLR7-9 genes (Kabelitz, 2007, Vanders *et al*., 2015, Wu *et al*., 2015b)

while recently, purified CD8+ T cells were found to express some extracellular TLRs (TLR1, TLR2, and TLR4-6 genes) in certain disease conditions (McCarron and Reen, 2009, Hammond *et al*., 2010, Freeman *et al*., 2013, Portou *et al*., 2015).

TLR1-10 genes were detected in γδ T cells. Research on human γδ T cells has indicated the presence of TLR1-9 genes (Pietschmann *et al*., 2009, Bonneville *et al*., 2010, Hannani *et al*., 2012, Zheng *et al*., 2013, Dar *et al*., 2014). In conclusion, this study demonstrates that in common with other studied mammalian species, bovine T cells express a range or TLRs, conferring on them the potential to respond directly to TLR-agonists.

7.4 The activation status of bovine TLRs assessed by CFSE and cytokine assays and functional aspects of γδ T cells

The non responsiveness of PBMCs in the CFSE assay could be due to preferential uptake of the TLR agonists by monocytes, B cells and other non T cells, that would not be stimulated to proliferate, or in the case of monocytes an activation involving immunosuppression (e.g by IL-10 production). Activated monocytes can cause death of T cells (apoptosis) (Beck *et al*., 2011, Sepulcre *et al*., 2011). This is highly speculative though. In contrast, CD4+, CD8+, and γδ T cells showed propagation in response to TLR3, TLR8, and TLR9 agonists in certain animals which indicates that these TLR are functional on these cells. TLR3, TLR8, and TLR9 agonists consist of dsRNA, ssRNA, and DNA nucleic acids (Bhardwaj *et al*., 2010).

Recently, based on the literature (Hoek *et al*., 2009, Guzman *et al*., 2014), it is clear that γδ T cells may function as regulatory T cells which could work alongside or instead of Tregs CD4+CD25hi FOXP3+ T cells in cattle. However, in this study, the γδ T cells were stimulatory for autologous T cells (both unstimulated and ConA activated. It is possible that this is due to residual Con-A in the assay although steps were taken to wash this away. Alternatively, the γδ T cells may need to be stimulated in a different way, are the wrong subset or the target cells are inappropriate. There was not time to explore these various alternatives.

7.5 Cytokine responses

Proliferation requires particular signalling pathways and is not the only measure of cell activation. We could have looked at signalling pathways, but the easiest method available to us was to look for cytokine production from the T cell subsets as a measure of TLR activation by agonists (CXCL-8, IFNα, IFNγ, TNFα, TGFβ, IL-4, and IL-10). This also allows us to speculate on differential function (mediated by different cytokines).

Results of CFSE assays (proliferation assay) were incomplete and although some information was obtained from a few animal T cell samples (bov 1 bov 4 and bov 5 for CD4 T cell and CD8 T cell responses to TLR3 ligand and TLR8 ligand, and bov A and bov B and bov 1 for γδ T cells stimulated with TLR8 ligand and TLR3 ligand) time constraints meant that refining this assay was abandoned in favour of developing the cytone assays as a read out for T cell subset responses to TLR-ligands.

The pro-inflammatory CXCL-8 is used as a major readout of TLR signalling processes (Fonseca *et al*., 2011, Mills, 2011, Russell, 2012, Russell *et al*.,

2012, Smith, 2012). CXCL8 production was observed in TLRs 1-9 ligand-stimulated PBMCs from 3 to 5 of seven cattle. Bov 1, 2, 4 and 5 T cell subsets responded to TLR 3, 8 and 9 ligands to produce CXCL8. In these animals this is good evidence of TLR-ligand activation of the cells.

Secretion of the other (above-mentioned) cytokines might indicate some further specific functional role. Of interest was the production of IL-4 by CD4+ T cells and γδ T cells (but not CD8+ T cells) from bov 1 and bov 2 animals, to TLR3, 8 and 9 ligands (CD4 T cells) and to TLR 3 and 8 ligands (CD8 T cells). This could indicate a Th2 bias in the responder animals by T cells, and this result should be validated and followed up. However the production of IFN-γ by PBMCS in 3 animals (bov 5 , bov B and bovine A) needs to be explained.

IL-10 production was produced by T cell subsets to TLR3 Ligand and differentially to TLR8 and TLR9 ligand in bovs 1, 2 and 4 and 5 (to a lesser extent). This suggests that, in these animals T cells could have a local suppressive function. Once again this is highly speculative. TNF-α and IFN-α responses to TLR-ligands in PBMC and T cells was a very limited study and the results are equivocal. TGF-β analyses were pilot only and limited to two animals. Time constraints limited this aspect of the study.

7.6 Conclusions and recommendations

Putative rabbit nTregs phenotypically identified as CD4+CD25hiFOXP3+ cells were generated in culture. The putative rabbit Tregs lacked immunosuppressive activity and equivalent cattle Tregs were not developed for inclusion in the main objectives of the study. Importantly, this study has identified for the first time the expression of TLRs on

fractionated CD4+ T cells, CD8+ T cells and γδ+ T cells. Functional studies (cytokine production by the T cell subsets to TLR agonist stimulation) indicated that this approach has value and should be followed up.

The most important outcome was that only a proportion of animal T cells responded to TLR-ligands. It was interesting to note that cells from same animals consistently gave a result in the various functional assays. These were: bov 1, bov 2, bovine 4098, and to a lesser extent bovine 4, bovine 5 then bovine A and bovine B. The other animals were poor or non responders. The two main reasons for this are likely to be (A) that the TLR-ligands are not active on bovine cells and (B) that the immune status of the animals is vaiable within the cohort. We can prett well rule out (A) because the CXCL8 responses of responder animal PBMCs to all 9 TLR-ligands were positive. It is still possible that the concentration range used may not be optimal. More likely is that the animal cells were differentially responsive to the TLR-ligands as a result of ongoing inflammatory or immune responses that may have inhibited responses to the TLR-ligands. The animals listed above as good all round responders could not be differentiated from any of the others at the clinical level as all were passed as clinically disease-free and healthy. To get round this, in future in may be prudent to use pathogen-free animals of a given breed, gender and age.

Future work would be to refine the techniques and study the consequences of TLR agonist stimulation of T cells in the context of immune responses to antigen *in vitro* and possibly later *in vivo*.

Appendices

8.1 Preparations of generic reagents, media, and compounds

8.1.1 RPMI complete medium

Material	Catalogue number/supplier
500 ml RPMI 1640 medium	11544526 – Fisher Scientific, Loughborough, UK
55 ml heat inactivated foetal calf serum (FCS)	10073772 – Fisher Scientific, Loughborough, UK
5.5 ml of 10,000 (U/ml) Penicillin/ 10 (mg/mL) Streptomycin	15140122 – Fisher Scientific, Loughborough, UK
1 ml Gibco Amphotericin B (Fungizone®) 0.25 µg/ml	15290-018 – Fisher Scientific, Loughborough, UK
5M of L-glutamine	G7513 - SIGMA-ALDRICH, Dorset, UK

8.1.2 Freeze-mix medium

Material	Catalogue number/supplier
45 ml heat inactivated foetal calf serum (FCS)	10073772 – Fisher Scientific, Loughborough, UK
5 ml DMSO	276855 - SIGMA-ALDRICH, Dorset, UK

8.1.3 MACS running buffer

This was made up by mixing 4 ml ethylenediaminetetra-acetic acid (EDTA 500mM) + 5 gm bovine serum albumin (BSA) dissolved in 1 L PBS (commenced with 800 ml then finalised to 1 L). pH adjusted to 7.2 then sterile filtered and finally stored at 4℃ in the fridge.

8.1.4 MACS rinsing buffer

This was made up by following the same steps in above section (8.1.3) to prepare MACS running buffer, but without adding BSA.

8.1.5 Tris Acetate EDTA buffer (50X TAE)

It was made up by mixing 242g of Tris base plus 57.1 ml of glacial acetic acid plus 0.5M EDTA in 800 ml of distilled water (DW) then topped up to form a total volume of 1L and stored at room temperature.

8.1.6 Tris Acetate EDTA buffer (1X TAE)

It was made up by diluting 50X TAE (described in above section 8.1.5) as follows: to prepare 1L of 1X TAE, 20 ml of 50X TAE dissolved in 980 ml DW.

8.1.7 Erythrocyte lysis buffer (ELB)

To make up 1L of ELB, a mixture of 8.3 g ammonium chloride (NH_4Cl at 0.15M working conc) plus 1 g potassium carbonate (KHCO3 at 10mM working conc) was dissolved in 200 ul of 0.5M EDTA at 0.1mM working conc, all dissolved in 800 ml double distilled water (ddH2O), and pH adjusted to 7.2 - 7.4, then volume adjusted to 1 Litre with ddH2O and sterile filtered then stored in the fridge.

8.2 Antibodies used in putative rabbit Treg project

Antibody	**Catalogue number/supplier**
Mouse IgG2a isotype control:FITC	MCA929F- BIO-RAD, Watford, UK
Mouse anti rabbit CD4:FITC	MCA799F - BIO-RAD, Watford, UK
Mouse IgG2b purified CD25 isotype control	MG2b00 - Thermofisher Scientific, Rugby, UK
Mouse anti rabbit CD25 (unconjugated)	MCA1119GA - BIO-RAD, Watford, UK
Goat anti−Mouse IgG2b Human Adsorbed R−PE	M32404 – Life technologies, Carlsbad, USA
Anti-Mouse/Rat FOXP3 APC	17-5773-82 - Thermofisher Scientific, Rugby, UK
Rat IgG2a K Isotype Control APC	17-4321-41 - Thermofisher Scientific, Rugby, UK

8.3 Antibodies used in bovine TLR project

Antibody	**Catalogue number/supplier**
Mouse anti-bovine CD4 T cells	MCA834GA - BIO-RAD, Watford, UK
Mouse anti-bovine CD8 T cells	MCA837GA - BIO-RAD, Watford, UK
Mouse anti-bovine γδ T cells	MCA838G - BIO-RAD, Watford, UK
Goat anti-mouse IgG microbeads	130-048-401 - Miltenyi Biotec, Woking, UK
Mouse anti-bovine CD11b FITC	MCA1425F - BIO-RAD, Watford, UK
Mouse anti-bovine CD21 FITC	MCA1424F - BIO-RAD, Watford, UK
Mouse anti-bovine CD4 FITC	MCA1653F - BIO-RAD, Watford, UK
Mouse anti-bovine CD8 FITC	MCA2216F - BIO-RAD, Watford, UK
Mouse anti-bovine WC1 (CC15 clone) FITC	MCA838F - BIO-RAD, Watford, UK
Mouse IgG2a Negative Control	MCA929F - BIO-RAD, Watford, UK
Mouse IgG2b Negative Control	MCA691F - BIO-RAD, Watford, UK
MOUSE ANTI BOVINE CD4:RPE	MCA1653PE - BIO-RAD, Watford, UK
MOUSE IgG2a NEGATIVE CONTROL:RPE	MCA929PE - BIO-RAD, Watford, UK
MOUSE ANTI BOVINE CD8:Alexa Fluor® 647	MCA837A647 - BIO-RAD, Watford, UK
MOUSE IgG2a NEGATIVE CONTROL: Alexa Fluor® 647	MCA929A647 - BIO-RAD, Watford, UK

8.4 ELISA kits used for cytokines' measurements

Kit name	**Catalogue number/supplier**
Bovine Interleukin 8 ELISA kit	DIY1028B-003, Kingfisher Biotech Inc, St. Paul, USA
Bovine IFN-αA ELISA kit	DIY0663B-003, Kingfisher Biotech Inc, St. Paul, USA
Bovine TNF-α ELISA kit	DIY0675B-003, Kingfisher Biotech Inc, St. Paul, USA
Bovine IFN-γ ELISA development kit	3119-1H-6 / Mabtech, Cincinnati, USA
TGFβ ELISA kit	ABIN996501, Antibodies-online, Aachen, Germany
"Matched Antibody Pairs" used for detection of IL-4 and IL-10 plus their standards (recombinant proteins)	
Mouse anti bovine interleukin-4	MCA2371, BIO-RAD, Watford, UK
Mouse anti bovine interleukin-4: Biotin	MCA2372B, BIO-RAD, Watford, UK
Recombinant bovine interleukin-4	PBP010, BIO-RAD, Watford, UK
Mouse anti bovine interleukin-10	MCA2110, BIO-RAD, Watford, UK
Mouse anti bovine interleukin-10: Biotin	MCA2111B, BIO-RAD, Watford, UK
Recombinant bovine interleukin-10	PBP016A, BIO-RAD, Watford, UK

8.5 Kits used for gene expression and molecular work

Kit name	**Catalogue number/supplier**
RNeasy Plus Mini Kit	74136 - Qiagen, Manchester, UK
RNase-Free DNase Set	79254 - Qiagen, Manchester, UK
Transcriptor First Strand cDNA Synthesis Kit	04897030001 – Roche, Burgess Hill, UK
Taq PCR Kit	E5000S - New England Biolabs, Hitchin, UK
QIAquick® Gel Extraction Kit	28704 - Qiagen, Manchester, UK
Probes Master Kit (for qPCR)	04707494001 – Roche, Burgess Hill, UK
Low molecular weight DNA ladder	N3233S - New England Biolabs, Hitchin, UK
Gel loading dye	N3233S - New England Biolabs, Hitchin, UK

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