

**Immunoregulation in bovine parasitic infection,
mechanisms and implications**

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Abstract

One of the characteristic features of parasitic infections is the chronicity and persistence of the infection for long time. Their success is due to its ability to deviate the immune response of their host toward the immunoregulatory or immunosuppressive state. IL-10 is a multifunctional cytokine with both immunosuppressive and immunostimulatory effects during parasite infection, playing a crucial role in the regulation of the immunity by ameliorating the destructive immunopathology associated with excessive inflammation but often suppressing effective immune responses against pathogens and impairing parasite clearance. Several transcription factors have been characterised that act on the promoter region of human and mouse IL-10 and their role in the transcription of this cytokine has been experimentally verified. In our study, a 570 bp fragment of the 5' UTR region flanking the bovine *il-10* gene was cloned and characterised by sequencing. Several SNPs relative to the reference sequence are described, the putative polymorphisms lie within the transcription factor binding sites Sp1, Cap, HSF, ADR1, MZF1, GATA1 and Hb. We have investigated the role of GATA1 in regulation of IL-10 expression from fluke naïve PBMCs in response to LFH and FhTLM and we demonstrate that both LFH and FhTLM treatment induced GATA1 expression from PBMCs. These molecules showed distinct effects on PBMC functions i.e. stimulation of IL-10 in response to FhTLM and inhibition when LFH is used. However, both factors inhibit PBMCs proliferation and IFN- γ expression. We have also found that FhTLM binds to bovine TGF β -RIED and TGF β -RIED fusion proteins but with a higher avidity to TGF β -RIED. These results highlight the role of FhTLM as a

potent immunomodulatory of the bovine immune system by binding to bovine cytokine receptors.

Neospora caninum infection is the major cause of abortion in dairy cattle. Infection with this parasite leads to a polarized Th1 response with high IFN- γ and IL-12 which provide protection against *N. caninum* infection. We have determined the effects of IL-10 in the regulation of IFN- γ and its association with production parameters (milk yield, lactation numbers) by measuring the levels of these cytokines in serum of infected animals and relating these to the key production parameters. Our results have shown that 84 % of high seropositive dams gave birth to seropositive calves and IL-10 concentration was higher in seropositive animals compared to seronegative. However, no correlation was found between the IL-10 concentration of seropositive dams and their calves. Together these indicated that IL-10 is an important cytokine during neosporosis, which modulate the immune response and allow the transmission of the infection to the offspring. Finally, our results for association of the IL-10 with lifetime daily yield demonstrated that IL-10 has a negative association with milk production during infection with *N. caninum*.

Results of our study demonstrate a key role for bovine IL-10 in the regulation of multiple facets of parasite infection and immunity.

Declaration

I confirm that the work in this dissertation is my own work and contain no material that has been submitted for any other degree at the University of Nottingham or other institution.

All of the procedures in this thesis have been done under the regulation of the University of Nottingham.

Azad Sulaiman

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Conferences, Posters and Oral Presentations

1. Sulaiman AA., Japa OH., Emes RD., Flynn RJ. (2015) The role of a growth factor like protein from *Fasciola hepatica* in host immunomodulation (Poster Presentation). Immunity to Veterinary Pathogen: Informing Vaccine Development. Kystone Symposia. Kystone Resort, Colorado, USA. 20-25 January 2015.
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List of Abbreviation

£	British pound
°C	Degree Celsius
ADCC	Antibody-dependent cell-mediated cytotoxicity
Ag	Antigen
APC	Antigen-presenting cells
CD	Cluster of differentiation
DC	Dendritic cells
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
ERK	Extracellular signal-regulated protein kinase
GM-CSF	Granulocyte macrophage colony-stimulating factor
hrs	Hours
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
iNO	Inducible nitric oxide
IRAK	Interleukin-1 receptor-associated kinase
Kb	Kilobase
KC	Keratinocyte chemoattractant
Kg	Kilogram
LPS	Lipopolysaccharide

MAPK	Mitogen-activated protein kinase
MCP	Monocyte chemoattractant proteins
mg	Milligram
MHC	Major histocompatibility complex
mins	Minutes
MIP	Macrophage inflammatory proteins
ml	Millilitre
MΦ	Macrophages
NF-κB	Nuclear factor kappa-light chain enhancer of activated B cells
NK	Natural killer cells
NK	Natural killer cells
NO	Nitric oxide
OVA	Ovalbumin
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PHA	Phytohaemagglutinin
PMA	Phorbol 12-myristate 13-acetate
PMN	Polymorphonuclear leukocytes
RNA	Ribonucleic acid
s	Seconds
STAT	Signal transducer and activator of transcription
TCBZ	Triclabendazole
Th	T helper cells
TNF-α	Tumour necrosis factor-alpha

T-reg	Regulatory T-cells
TRIF	TIR-domain containing adaptor-inducing interferon- β
US\$	United State dollar
μ l	Microlitre

1 Introduction and Literature Review

1.1 Parasite infection in veterinary medicine

The term ‘parasite’ is used to describe a wide range of organisms including viruses, bacteria, protozoa, fungi, helminths and arthropods that depend on other organisms to survive and develop (Perry and Randolph, 1999). Parasites are considered a major cause of animal mortality and loss of production worldwide. For example, fascioliasis caused by liver fluke *Fasciola hepatica* (*F. hepatica*), is directly associated with mortality, weight loss and liver condemnation (Ngategize et al., 1993). Furthermore, fluke infection compromises the immune response and increases the susceptibility of their host to infection with a number of bacterial pathogens such as *Salmonella dublin* (Aitken et al., 1979). Abortion and stillbirth are the major consequence of neosporosis in dairy cattle that leads to devastating economic losses in dairy herds (Maley et al., 2003). In addition, the expensive methods used for diagnosis of *Neospora. caninum* (*N. caninum*) and premature culling of infected animals are another form of economic losses (Graham et al., 1996, Thurmond and Hietala, 1996, Barling et al., 2000, Reichel and Ellis, 2006). Another impact of parasitic infection is the high cost of treatment and the emergence of some strains of parasite with resistance to anthelmintic drugs, this has added significantly to productivity losses associated with parasite infection (Brennan et al., 2007).

1.2 Immune response to protozoa infection

The interaction of the host immune system and the parasite leads to the evolution of distinct immune response to particular parasites. These immune responses have developed to allow

mammals to actively identify and eliminate the invading parasites using the most appropriate effector mechanisms to minimise pathology. However, at multiple junctures parasites have developed their own mechanisms to overcome the mammalian innate and adaptive immune responses. This varies from antigenic variation to targeted secreted modulators that actively deactivate or alter the phenotype of leukocytes.

1.2.1 Innate immunity

The innate immune system is the second line of host defence against infection after the anatomic and physiologic barriers and consists of distinct populations of cells such as macrophages, dendritic cells, neutrophils, eosinophils, mast cells, and natural killer cells, which are differentially activated at the site of infection according to the type of invading microorganisms (Janeway and Medzhitov, 2002). Innate immune recognition depends on a number of germline-encoded receptors, termed pattern recognition receptors (PRRs). These receptors have evolved to recognise conserved products of microbial pathogens, referred to as pathogen-associated molecular patterns (PAMPs) (Kumar et al., 2011). Several families of PRRs have been demonstrated to play critical role in immunity including Toll-like receptors (TLRs), NOD-like receptors (NLRs), RIG-like receptors (RLRs) and DNA receptors (Takeuchi and Akira, 2010). To date 10 TLR members (TLR1-10) have been identified in human and 13 in mice (Kawai and Akira, 2010). These TLRs are either expressed on the cell surface (TLR-1, 2, 4, 5, and 6), or in the intracellular compartment (endosomal) (TLR-3, 7, 8 and 9) (Kang and Lee, 2011, Singh et al., 2012).

Signalling via all types of TLRs except TLR-3 and TLR-4 results in recruitment of the adaptor protein myeloid differentiation factor 88 (MyD88) (Ghosh and Stumhofer, 2013).

In the case of TLR-3 and the TLR-4 alternative pathway, MyD88 is replaced by the Toll/IL-1 receptor domain containing-adaptor inducing IFN- β (TRIF) signalling pathway (Ghosh and Stumhofer, 2013). These two adaptors are responsible for the activation of distinct signalling pathways, MyD88 recruits IRAK4 which is necessary for the activation of NF- κ B and MAPK and initiation of the signalling cascade results in production of pro-inflammatory cytokines (Kawagoe et al., 2008). While, TRIF interact with receptor interacting serine/threonine protein kinase 1 (RIP1) and this in turn activate NF κ -B and induces the production of type I interferons (IFNI) (Meylan et al., 2004, Perry et al., 2004). TLR and the MyD88 dependent pathways are important in mediating immune responses against different parasitic infection (Krishnegowda et al., 2005), and often contributes to the outcome of pathology associated with infections. Thus, a good understanding of these pathways is important for designing strategies to control these infections.

Studies of mice deficient in TLRs have shown that each TLR recognises distinct PAMPs and initiate specific immune responses against parasitic infection. For example TLR-2 recognises *Plasmodium falciparum* glycosyl-phosphatidylinositol (GPI) and triggers the production of pro-inflammatory cytokines (TNF- α and IL-1) via the MyD88-dependent pathway (Krishnegowda et al., 2005). In addition, TLR-9 binds to hemozoin (haemoglobin digested by the parasite) and induces the production of TNF- α and IL-12 and cellular co-stimulatory factors (CD40 and CD86) from murine dendritic cells (DCs) (Coban et al., 2005). The production of pro-inflammatory cytokines is associated with the development of the symptoms of malaria; fever, rigor and severe anaemia (Miller et al., 2002). While, protection against the acute stage of *plasmodium. chabaudi* infection requires the production of IFN- γ (Su and Stevenson, 2000). Furthermore, IL-12 is crucial for antibody-mediated immunity against blood-stage *P. chabaudi* (Su and Stevenson, 2002).

Several TLRs have been implicated in mediating the immune response against *Toxoplasma gondii* infection in mice; TLR-11 recognises the parasite derived profilin-like protein, which is crucial in induction of IL-12 (Yarovinsky et al., 2005). In addition, a TLR-11/TLR-12 heterodimer is also able to bind to profilin and induce the production of IL-12 and IFN- α . Moreover, DC derived IL-12 is important for triggering IFN- γ expression by NK cells, which is necessary for mediating protection (Koblansky et al., 2013). Neutralization of IFN- γ in mice infected with *T. gondii* is associated with parasitemia and death (Suzuki et al., 1988). While in humans, due to the lack of TLR-11 and TLR-12, other endosomal receptors such as TLR-7 and TLR-9 have been implicated in sensing parasite RNA and DNA and subsequent activation of the effective immune response against *T. gondii* (Andrade et al., 2013).

In *Leishmania. major* infection TLR-2 has been shown to bind to lipophosphoglycan (LPG) of the parasite and promote the production of IFN- γ and TNF- α by M Φ s and NK cells (Becker et al., 2003). It has been shown that NK derived IFN- γ is an important mediator of innate resistance by increasing cytotoxic activity against the parasite (Akuffo et al., 1993, Barral-Netto et al., 1995).

The roles of TLR-3, TLR-7 and TLR-9 in the recognition of nucleic acids of *L. major* has been well documented. Mice deficient in TLR-9 or TLR-7 experienced increased susceptibility to Leishmania infection, while TLR-3 deficient mice showed resistance to parasite infection. Surprisingly, triple knockout mice showed more severe lesions than MyD88 deficient mice, indicating the role of TRIF signalling pathway in mediating resistance to Leishmania infection in the absence of MyD88 (Schamber-Reis et al., 2013). Furthermore, in human patients TLR-2, TLR-4 and TLR-9 have important roles in

determining the outcome of infection with several *Leishmania* spp. (Tuon et al., 2010, Tuon et al., 2012, Rasouli et al., 2012).

During *Trypanosoma. cruzi* infection, TLR-2 has been shown to bind to glycosylphosphatidylinositol (tGPI)-mucin and induce the expression of NO and pro-inflammatory cytokines (IL-12 and TNF- α) (Camargo et al., 1997, Campos et al., 2001). The production of nitrogen intermediates is important for clearance of the parasite (Holscher et al., 1998). TLR-9 recognises parasite DNA and triggers the production of TNF- α , IL-12 and NO by M Φ s (Shoda et al., 2001, Bafica et al., 2006). Other endosomal TLRs including TLR-3 and TLR-7 are also important in response to *T. cruzi*; TLR-7^{-/-} mice produce a low level of IL-12 and IFN- γ and are susceptible to *T. cruzi* infection and TLR-3^{-/-} mice produce normal amounts of these cytokines and cured from the infection, indicating that differential recognition of the parasite can influence disease outcome (Caetano et al., 2011).

Taken together, these studies indicate that engagement of the parasite PAMPs with TLRs of DC results in the activation of the adaptor MyD88, leading to presentation of MHC class II-peptide complexes to T-cells (signal 1) and the induction of expression of CD40, CD80 and CD86 costimulatory molecules (signal 2) and production of pro-inflammatory cytokines IL-12 (signal 3) (Kapsenberg, 2003). Several leukocytes have been shown to produce IL-12 during intracellular protozoan infection including macrophages, neutrophils and DCs (Locksley, 1993, Romani et al., 1997, Reis e Sousa et al., 1997). DCs are the most important cell subset in initiating the Th1 immune response to intracellular parasites, due to their ability to produce IL-12 and to present antigen to naïve T-cells (Banchereau et al., 2000, Ng et al., 2008).

1.2.2 Adaptive immunity

Despite the important role of innate immunity in providing resistance to certain acute infections, the adaptive immune response that is characterised by selective expansion of T-cells and B-cells has a major role in providing long-term immune response against chronic protozoal infection. IL-12 is a key cytokines for development of Th1 cells; it induces the production of IFN- γ and TNF- α from NK in response to *T. gondii* and *L. major* infection (Trinchieri, 1995, Gazzinelli et al., 1993, Scharton-Kersten et al., 1995). Recently, it has been demonstrated that IL-12 is also critical for production of IFN- γ from T-cells during *L. donovani* infection (Robert-Gangneux et al., 2012). IFN- γ has been shown to induce the activation of classically activated macrophages (CAM Φ) that produce NO, important for controlling the intracellular parasite (Aliberti, 2005). Other studies indicated that IFN- γ has another role in protection against intracellular parasites by stimulating antigen presentation via the MHC complex (Boehm et al., 1997). These studies indicated that IFN- γ is important for mediating the protective Th1 immune response to protozoan. Importantly, continued production of IL-12 is required for the maintenance of effective Th1 response (Stobie et al., 2000).

Despite the protective role of NK cells during intracellular parasitic infection that is linked to the production of IFN- γ , other studies have shown that these cells can also produce regulatory cytokines such as IL-10 and TGF- β (Hunter et al., 1994, Grant et al., 2008). IL-10 functions to inhibit the accessory cells required for the activation of NK and T cells, and TGF- β inhibits IFN- γ production by NK cells and thus modulates the resistance to *T. gondii* (Hunter et al., 1995). Furthermore, IL-10 and TGF- β indirectly inhibit macrophage effector functions toward the ingested organisms and enhance the survival of the parasite (Roilides

et al., 1997). It is unclear whether signalling via the TLRs by the parasite is a mechanism used to escape the protective host immune response or a balanced response to minimise immunopathology. In summary intracellular parasites induce a Th1 immune response characterised by production of IL-12, IFN- γ , IgG2 and nitric oxide and classical activation of macrophages.

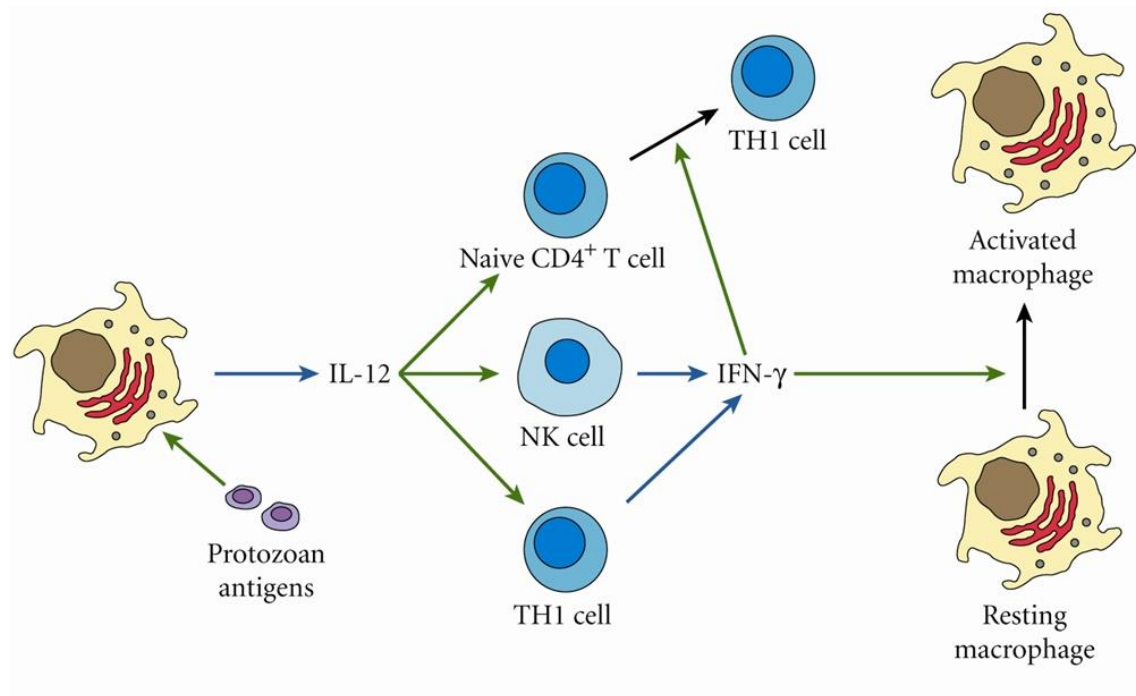


Figure 1.1: IL-12 during protozoan infection.

Infection with intracellular parasites triggers the production of IL-12 from phagocytic cells and induces the expression of IFN- γ by NK cells and T-cells. The production of IFN- γ induces activation of resting macrophages and promotes the differentiation of parasite specific Th1 cells which are crucial for controlling the infection. Figure adapted from www.ppdictionary.com (Hamza et al. 2010) [accessed online 03/03/2015].

1.3 Immune responses to helminth infection

It is well documented that helminth parasites are long-lived organisms, surviving for several years within their animal or human host. Their success is associated with their ability to modulate and/or suppress the host immune response to ensure their own survival (Allen and Maizels, 2011, McSorley et al., 2013). The immune response to helminth infections is also very complex and occurs as a result of the interaction between the host immune cells and the variety of molecules that are released from live or dead parasite. The influence of these helminth molecules on host immune responses have been extensively examined (Berasain et al., 2000).

1.3.1 Innate immunity

As stated above, the interaction of microbial PAMPs with TLRs on DCs are mainly associated with signals that drive the development of type-1 DCs, characterised by production of IL-12 and induction of Th1 cell differentiation (Medzhitov and Janeway, 2000, Schulz et al., 2000). Conversely, the interaction of helminth products with TLR-2, TLR-3 and TLR-4 are mainly suppressive to the TLR signalling cascades or may initiate an immunosuppressive environment during helminth infection (Semnani et al., 2008). For example, the soluble products of *Trichuris suis*, *Trichinella spiralis* and *Schistosoma mansoni* differentially suppressed the production of IL-12 and TNF- α from TLR stimulated DCs (Kuijk et al., 2012). Moreover, *Fasciola hepatica* tegument antigen inhibited the production of pro-inflammatory cytokines such as IL-6, IL-12 and TNF- α and inhibits IL-10 and costimulatory molecules CD40, CD80 and CD86 by targeting a range of TLR on

murine DCs (Hamilton et al., 2009). The excretory-secretory products of *F. hepatica* have been shown to interact with macrophage TLR-2 and TLR-4 and suppresses the TLR-2-induced IFN- γ and TLR-4-induced NO *in vitro* (Flynn and Mulcahy, 2008a). Moreover, *F. hepatica* cathepsin L1 (CL1) and glutathione transferase (GST) partially activated DCs through TLR-4 to produce IL-6, IL-12 and macrophage inflammatory protein 2 (MIP-2), and DCs exposed to these products suppressed the development of Th17 and Th2 cells (Dowling et al., 2010). Recently, *F. hepatica* fatty acid binding protein has been shown to induce the development of alternatively activated macrophage (AAM Φ) that produce high levels of IL-10 (Figueroa-Santiago and Espino, 2014). Subsequent studies demonstrated that this molecule suppresses LPS-induced production of IL-6, IL-12, and TNF- α and the phagocytic capacity of M Φ through inhibiting TLR-4 activation and the downstream signalling events in mice bone marrow derived M Φ (Martin et al., 2015).

Furthermore, Th2 polarization can also occur following interaction of parasite antigen and TLR that normally promotes Th1 response. For instance, the carbohydrate LNFPIII of *Schistosoma* promotes a Th2 response through TLR-4 and activation of the extracellular signal-regulated kinase (ERK), which induces type 2 DCs (Thomas et al., 2003). Moreover, the *Schistosoma* lysophosphatidylserine (lyso-PS) binds to TLR-2 on DCs and promotes the development of IL-10 producing T-reg cells (van der Kleij et al., 2002), suggesting that helminths can exploit multiple TLR pathways to induce immune suppression.

Another explanation for Th2 polarisation by helminth is that some helminth products such as *Schistosoma* egg antigen (SEA) and excretory/secretory products (ESP) of *Nippostrongylus brasiliensis* induces partial activation of DCs termed muted DCs characterised by expression of low levels of pro-inflammatory cytokines and costimulatory molecules (MacDonald and Maizels, 2008). However, these muted DCs are still potent Th2

inducer both *in vitro* and *in vivo* (Whelan et al., 2000, Jankovic et al., 2004). In addition, the signalling events activated downstream of TLRs in DCs in response to helminth products are different from those stimulated by protozoan products. For example, ERK, JNK and p38 are activated down stream of TLR in DCs stimulated with LPS, but in DCs activated with SEA no JNK phosphorylation occurs (Kane et al., 2004).

The glycoprotein omega-1 that is found in Schistosome eggs has been reported to promote Th2 responses. It functions by inhibiting DC activation and decreasing the strength of the interaction between DCs and naïve CD4⁺ T cells receptors leading to induction of GATA3 expression and Th2-cell differentiation (Steinfelder et al., 2009). In addition, omega-1 inhibited IL-12 production by DCs and in turn, suppressed Th1 cells induction *in vivo* (Everts et al., 2009).

It has been demonstrated that epithelial cells are the main initiators of the Th2 response, damage to the intestinal epithelium induces the production of several innate Th2 cytokine or alarmins such as IL-25, IL-33, and thymic stromal lymphopoietin (TSLP) (Smithgall et al., 2008, Ramalingam et al., 2009, Saenz et al., 2010). These are important in shaping the subsequent adaptive immune response. TSLP activates DCs and induce the expression of Th2-attracting chemokines thymus and activation-regulated chemokine (TARC) and macrophage-derived chemokine (MDC) and the TSLP-activated DC induce the production of IL-4, IL-5, IL-13 and TNF- α (Soumelis et al., 2002). Furthermore, TSLP promotes the development of IL-4-producing basophils (Siracusa et al., 2011). IL-33 induces the expression of MHC class II, CD86 and IL-6 from DC which in turn trigger the Th2 response (Rank et al., 2009). In addition, IL-33 promotes the differentiation of alternatively activated macrophages (Kurowska-Stolarska et al., 2009). The important roles of IL-33 and TSLP in expulsion of *T. muris* has been demonstrated (Humphreys et al., 2008, Taylor et

al., 2009). Several studies indicated the role of IL-25 in mediating helminth induced Th2 response. Mice that expressed IL-25 within their gut successfully expelled *T. muris* while IL-25^{-/-} mice failed to expel the parasite and developed a chronic infection due to suppression of protective Th2 response (Owyang et al., 2006). Finally, IL-25 and IL-33 are necessary for the recruitment of group 2 innate lymphoid cells (ILC2s) termed nuocytes which produce IL-13 (Moro et al., 2010, Price et al., 2010) and the absence of these cytokines and subsequent deficit in ILC2s results in severe defects in expulsion of *N. brasiliensis* (Neill et al., 2010). The suppression of Th2 response by *F. hepatica* CL1 and the stimulation of this response by SEA, indicate that the immune response vary with particular parasite species, and the responses to gastrointestinal helminths are different from those to tissue helminths.

1.3.2 Adaptive immunity

The data above indicate that the damage associated with helminth infection is one of the important mechanisms by which the host recognises the invading helminth and responds with an appropriate Th2 immune response. In addition, helminth products modulate dendritic cells to suppress the TLR-mediated Th1 response, further enhancing the anti-helminth response (Carvalho et al., 2009, Everts et al., 2012). The central player in the Th2 response is the CD4⁺ Th2 cells which produce IL-4, IL-5, IL-9 and IL-13 (Paul and Zhu, 2010). It has been shown in multiple systems, that *F. hepatica* induce a Th2 response associated with production of IL-4, IL-5, IL-10 and IgG1 and promote the activation of alternative type of macrophages which lead to fibrosis.

These cytokines further induce the recruitment of eosinophils, basophils and mast cells to the site of infection that are the main source of IL-4, IL-5 and IL-13, which are important in promoting the Th2 response. Among these cytokines, IL-4 remains the main cytokine produced by Th2-cells that promotes B-cells class switching to IgE (Zhu et al., 2010, Paul and Zhu, 2010). IgE in turn cross links the high affinity Fc receptor (FcεRI) on the surface of basophils and mast cells leading to mast cell degranulation and secretion of various inflammatory mediators including heparin, histamine and serotonin (Zhu et al., 2010, Paul and Zhu, 2010). These factors induce a range of functions such as smooth muscle contraction, increased vascular permeability, mucus hypersecretion and recruitment of AAMΦ (Zhu et al., 2010, Paul and Zhu, 2010). Another characteristic feature of helminth infection is eosinophilia and mast cells hyperplasia induced by the Th2- cell cytokines IL-5 and IL-9, respectively (Paul and Zhu, 2010). It has been shown that cercaria of *S. mansoni* induced the release of histamine from mast cells *in vitro* (Catto et al., 1980) which is necessary for recruitment of inflammatory cells to the path of helminth migration. Infection with *T. spiralis* is associated with mucosal mastocytosis that leads to increased permeability of the intestinal epithelium and thereby mediates parasite expulsion from the intestine (McDermott et al., 2003). Eosinophilia induced by IL-5 has also been associated with protection against helminths infection such as *Heligmosomoides polygurus* and *Toxocara canis* (Svetic et al., 1993, Takamoto et al., 1997).

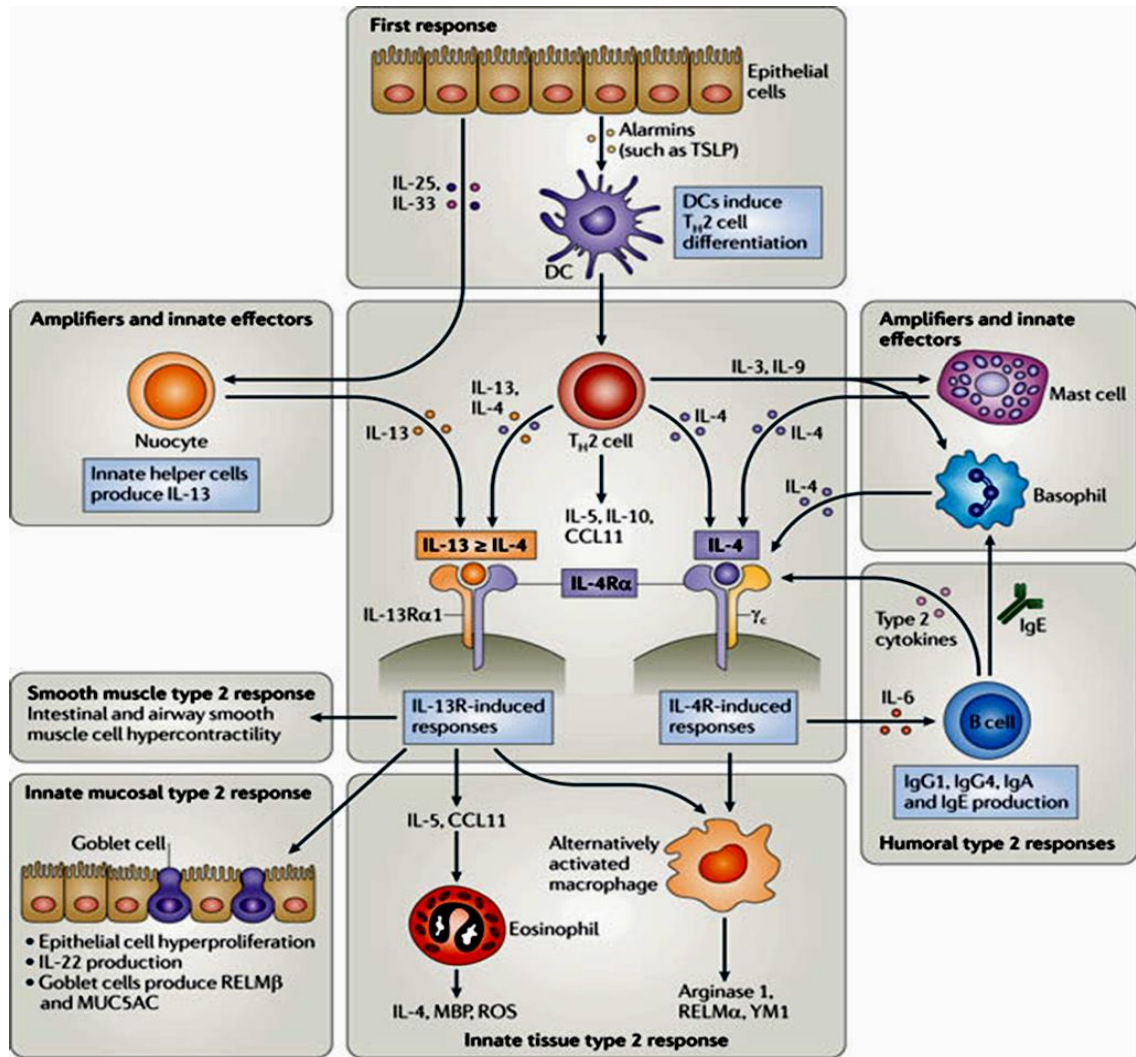


Figure 1.2: The central role of IL-4 in promoting the Th2 immune response during helminth infection.

Following penetration of helminth to the epithelium cells, alarmins (IL-25, IL-33 and TSLP) promote the development of a Th2 response by DCs. IL-4 and IL-13 are the main cytokines that direct the Th2 immune response by inducing the AAMΦ and promoting IgE expressing B-cells. This figure adapted from Nature Review (Allen and Maizels, 2011) [accessed online 01/04/2015].

1.4 *Fasciola hepatica*

The liver fluke *F. hepatica* is a digenetic trematode responsible for the disease fascioliasis that causes significant economic losses in important farmed ruminants such as cattle and sheep. Fascioliasis is commonly caused by two species; *F. hepatica*, which is dominant in temperate areas and *F. gigantica*, which is commonly found in tropical regions (Andrews, 1999). The major losses are caused by direct production losses (milk and meat), anthelmintic treatment and condemnation of infected livers. This loss is estimated at over US\$ 2-3 billion worldwide per year to agri-business sectors (Spithill et al., 1999) and in the UK it costs the livestock industry around £300 million annually (<http://www.eblex.org.uk>). Additionally, the disease is also present in goats, buffalo, swine, camels, equids and wild mammals meaning the parasite can circulate through sylvatic cycle (Haroun and Hillyer, 1986).

Recently the disease has been classed as an emerging human pathogen with an estimated 2.4 million people infected worldwide and over 180 million are at risk of infection (Mas-Coma et al., 2009). Human fascioliasis is endemic in Peruvian Altiplano, the highlands of Lima, the Man taro and Cajamarca valley, parts of Western Europe, South East Asia, the Caspian sea and parts of Africa (Marcos et al., 2006).

1.4.1 Life cycle

F. hepatica has a complex life cycle which requires an intermediate host (Andrews, 1999). The fresh water snail *Galba truncatula* acts as a natural intermediate host (Mas-Coma et al., 2001) in which parasite asexual multiplication and development occurs. Whereas,

vertebrate hosts serve as the definitive host in which the parasite reproduces sexually. The life cycle of *F. hepatica* is initiated when the infective stage (metacercaria) are ingested by the final host (Andrews, 1999). In the small intestine a newly excysted juvenile (NEJ) emerges and penetrates the intestinal wall, migrating through the peritoneal cavity moving towards the liver (Figure 1.3). Within the liver parenchyma, the fluke begin to tunnel and feed causing extensive tissue damage. Subsequent bleeding can lead to death in heavily infected susceptible animals such as young lambs. It can take approximately 6-8 weeks from the infection before the fluke are present in the bile ducts (Kaplan, 2001). The adult parasites are hermaphrodite and self-fertilization may occur (Andrews, 1999), after 8 weeks of infection mature worms begin to release un-embryonated eggs within the bile which pass out in the faeces of the final host (Kaplan, 2001). Outside the vertebrate host, the eggs undergo embryonation and hatch into miracidia which infect the snail host (Andrews, 1999). Within the snail three developmental stages take place; transformation from miracidia to sporocyst, to redia and then finally to cercaria taking approximately five to seven weeks (4a, 4b and 4c in Figure 1.3). In each of these developmental stages, there is a multiplication of the number of parasites leading to large number of cercaria being released compared to the number of infecting miracidium; each miracidium producing more than 600 cercaria. The free-swimming cercaria emerge from the snail body and become encysted on vegetation as the infective metacercaria (Andrews, 1999).

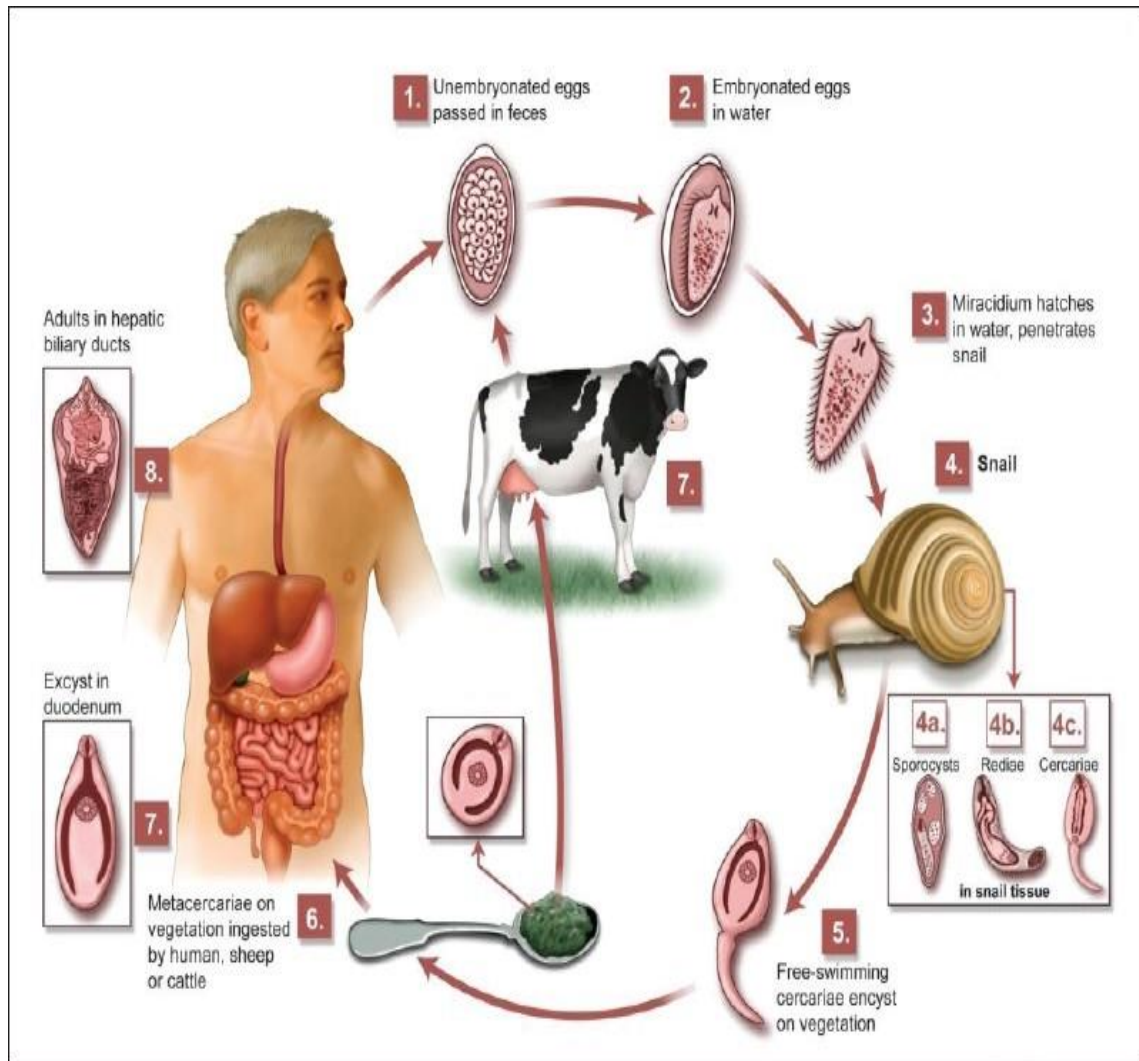


Figure 1.3: life cycle of *F. hepatica*.

Figure adapted from www.clinicalimagingscience.com (Dusak et al., 2012) [accessed online 20/04/2015].

1.4.2 Epidemiology

F. hepatica epidemiology depends on factors that affect the parasite larvae and intermediate host such as temperature and moisture. In addition, the availability of large numbers of intermediate hosts increases the likelihood of infection (Torgerson and Claxton, 1999). In the United Kingdom the prevalence of fascioliasis is increasing amongst livestock (Daniel and Mitchell, 2002), and it is estimated to have a 76 % prevalence in dairy herds (McCann et al., 2010). This is mainly due to increased movement of livestock around the country and changes in weather patterns which favour the development of the parasite (Poulin, 2006). The presence of adequate moisture and temperatures above 10°C are necessary for development of the free-living life stages of the parasite and for the snail population. In temperate climates such as in the UK, temperature is often the only limiting factor for the parasite life cycle, while in warmer climates both moisture and temperature are the limiting factors (McCann et al., 2010). In the UK there are two traditional periods for fascioliasis outbreaks, summer and winter (Fox et al., 2011). The incidence of liver fluke infection increases in summer from April to October due to the temperature required for the development of the parasite and summer rainfall that helps snail development (Mitchell, 2002).

1.4.3 Control and Treatment

One aspect of fascioliasis control is to reduce the snail population and pasture contamination with metacercaria. This can be achieved by improving drainage systems, or to close off the wet areas of pasture to decrease the number of eggs hatching and the

development of free-living stages of parasite (miracidium) and the snail population. Pasture management to prevent contact between the infective stage and definitive host is ideal but often impractical (Sargison, 2012). Moreover, whilst using molluscicides such as copper sulphate is effective, it can lead to accumulation of toxic residues in the water and soil which adversely affects the environment and has thus limits their use. In addition, resistant strains of snails may appear, so the frequent application of molluscicides will not eradicate all snails (Haroun and Hillyer, 1986).

Anthelmintic drugs are the only specific treatment for clinical fascioliasis. To date several fasciolicides have been used for treatment of ruminant fascioliasis and they are classified into four main groups. These vary in safety, efficacy and the stage of parasite they target (Table 1.1).

Table 1.1: Efficacy of different group of drugs used for treatment of *F. hepatica*.

(Fairweather and Boray, 1999)

Drug groups	Drug names	Age of fluke targeted (weeks)	Drug Efficacy (%)
Halogenated phenols	nitroxynil	7, 8 and 9	50-90
		10, 11, 12, 13 and 14	91-99
Salicylanilides	closantel	7,8 and 9	50-90
		10, 11, 12, 13 and 14	91-99
	rafoxanide	4, 5, and 6	50-90
		7 - 14	91-199
	oxyclozanide	10 and 11 12, 13 and 14	50-70 80-99
Benzimidazoles	triclabendazole	2 and 3	90-99
		4 - 14	99-100
	albendazole	10 and 11 12, 13 and 14	50-70 80-99
Sulphonamides	clorsulon	8 - 14	90-99

Among all of these agents triclabendazole (TCBZ) is the most effective and widely used drug for the control of fascioliasis worldwide (Gaasenbeek et al., 2001) and it is the only drug that effectively kills the early immature fluke (Boray et al., 1983). Therefore, it is considered as the drug of choice for treatment of the acute fascioliasis, which is more commonly seen in sheep than in cattle. Other drugs such as nitroxylnil and closantel kill the late immature and adult fluke 4 week post infection (Fairweather and Boray, 1999). Recently, Shrifi et al.,(2014) demonstrated that TCBZ reduced the protease enzyme activity of the excretory-secretory products (ESP) of *F. hepatica* in vitro, indicating the role of this drug in compromising the migration and feeding ability of the parasite and, in turn, survival. TCBZ-resistant strains of liver fluke have been reported in Australia (Overend and Bowen, 1995) in Scotland (Mitchell et al., 1998) in Ireland (Lane, 1998) in western areas of the UK (Mitchell et al., 1998, Thomas et al., 2000) and in the Netherlands (Moll et al., 2000). To date, attempts to develop new alternative drugs for the treatment of immature flukes that could replace TCBZ have been unsuccessful (Fairweather, 2011). Others showed that the combination of two drugs may increase efficacy against the immature flukes: combination of clorsulon (2.0 mg/kg) and nitroxylnil (10.2 mg/kg) produced an efficacy of 95 % to 2 week old fluke and 99 % against 4 week old fluke (Hutchinson et al., 2009). However, the cost and remaining subclinical infections caused by immature flukes when undertreated with TCBZ present an ongoing problem with its use (Haroun and Hillyer, 1986, Overend and Bowen, 1995).

In recent years, the use of vaccines has become an alternative, cost effective and desirable method for the control of liver fluke (Almeida et al., 2003). A number of experimental vaccine candidates have been used to induce immunity in multiple species, but these show varied efficacy in different species. For example, an irradiated attenuated vaccine is

effective in cattle (Acosta et al., 1998) but not in sheep (Creaney et al., 1995) and mice (Spithill et al., 1999). The protease CL1, which is a virulence protein found in ESP, is a first generation recombinant protein vaccine. A second member of this protease family, CL2, has been studied as a potential vaccine candidate due to its functions during the lifecycle: it facilitates the migration of the fluke within the host tissues, contributes to feeding and immune evasion (Dalton et al., 2003). Moreover, Dalton et al. (1996) found that the use of CL1 and CL2 induced high levels of protection through reducing the fluke burden and egg viability. Others reported that CL2 in cattle is responsible for a high IgG2 antibody response leading to a reduction of fluke numbers (Mulcahy et al., 1998).

Regardless of the time needed for the development of an effective candidate, much work is still to be done on the most appropriate and effective adjuvant (Hacariz et al., 2009b). The recent year experienced the use of CL, GST and fatty acid binding protein as vaccine candidates against *F. hepatica* in cattle and sheep. Majority of these vaccine reduced liver pathology without affecting the fluke burden (Toet et al., 2014). To date, no vaccine candidate has been commercialised to provide protection for humans or animals against *F. hepatica* infection.

1.5 *Neospora caninum*

Neospora caninum is an apicomplexan protozoan parasite closely related to *Toxoplasma gondii*, it was first identified as a parasite responsible for a neuromuscular disorder in dogs that had no antibodies to *T. gondii* (Bjerkas et al., 1984). In 1987 it was described as a *T.*

gondii-like parasite causing encephalomyelitis and nervous signs in calves, the animals testing negative for toxoplasmosis and sarcocystis in an immunoperoxidase test (O'Toole and Jeffrey, 1987).

N. caninum was first recognised as a new protozoan species belonging to the genus *Neospora* in 1988 and attributed as causing fatal disease in dogs (Dubey et al., 1988). The first case of abortion outbreak in dairy cattle due to *N. caninum* was reported in 1989 in New Mexico (Thilsted and Dubey, 1989). Recently this parasite has been regarded as one of the most important causes of abortion in cows worldwide.

1.5.1 The importance of *N. caninum*

N. caninum is reported as a major cause of abortion in both dairy and beef cattle worldwide (Dubey and Lindsay, 1996, Hoar et al., 1996, Dubey and Schares, 2011). It is estimated that 12.5 % of bovine abortions in UK dairy cattle in 1997 (Davison et al., 1999b) and 20 % of abortions in the Netherlands (Wouda et al., 1997) were related to *N. caninum* infection. In California 42.5 % of aborted foetuses tested positive for neosporosis (Anderson et al., 1995), this costs the cattle industry around 1 billion USD per year (Reichel et al., 2013). Abortion as an outcome is influenced by several factors such as the time of primary infection, maternal immunity during pregnancy, host susceptibility, parasite strain and stage of pregnancy (Williams et al., 2000, Innes et al., 2005). Abortion may occur in any stage of gestation but the majority of abortions usually occur during early (<3 months) gestation (Williams et al., 2000, Regidor-Cerrillo et al., 2014). Repeated abortions can occur in herds (Obendorf et al., 1995, Dannatt et al., 1995), but the rate of repeated abortion in individual animals cattle is very low (estimated to be around 5 %) thus

indicating that infected animals may develop protective immunity against *N. caninum* infection (Anderson et al., 1995). However, the exact frequency of repeat abortion due to *N. caninum* is unknown, as aborting cows are often culled (Obendorf et al., 1995), and one study indicates that the risk of subsequent abortion increased by 5.6 fold following the first abortion (Thurmond and Hietala, 1997b). However, abortion outbreaks in infected cattle are one of the major and most obvious causes of economic losses, reduction in milk yield is another impact of neospora infection; this may be due to abortion that increases the calving interval or prolongs the lactation (Hobson et al., 2002). An example of the conflicting results in the few studies examining the effects of infection on the milk production. A study in California and Florida from dairy herds found a reduction in milk yield among seropositive cows by 1.3 Kg/cow daily which is accounted for a loss of \$128 per cow per lactation (Thurmond and Hietala, 1997a). Conversely, in New Zealand a study of dairy herd revealed an increase in milk yield by 0.4/cow daily among seropositive cows. All these impacts may be an important reason for increased culling rate of animals in many countries (Thurmond and Hietala, 1996, Esslemont and Kossaibati, 1997). The rate of culling was increased in herd with a history of *N. caninum*-associated abortion (Tiwari et al., 2005). The losses are not only restricted to dairy cattle; some data in beef cattle indicated that *N. caninum* can also result in significant abortion losses in these herds (Barr et al., 1994, Boulton et al., 1995). In addition, another study demonstrated that infection was associated with significant reduction in the weight gain and final carcass weight of beef calves (Barling et al., 2000). However the reported prevalence of the disease among dairy cattle was around (36 %) and in beef cattle it was (18 %) (Quintanilla-Gozalo et al., 1999, Bartels et al., 2006) and so the reported abortion rate in beef cattle is lower compared to dairy cows (De Meerscham et al., 2000).

In addition to these direct economic costs, there are also several indirect costs such as the costs of diagnosis, and rebreeding of the infected animals (Thurmond and Hietala, 1996, Dubey and Schares, 2006).

1.5.2 Life cycle and transmission

Dogs were first identified as a natural definitive host for *N. caninum* by McAllister et al. (1998). However, other canids such as coyotes, grey wolves and Australian dingos were then considered as a potential definitive host for this parasite (Gondim et al., 2004b, King et al., 2010, Dubey et al., 2011). Dogs usually acquire infection by ingestion of tissue cysts, within the small intestine bradyzoites are released and invade the intestinal cells. These bradyzoites developed into the sexual stage (gametocytes) in the duodenum and unsporulated oocysts produced 8 to 20 days post infection for 5 to 17 days (Figure 1.4). Oocysts sporulate outside the host, this can occur within 24hrs but may take up to 3 days post shedding to form 2 sporocysts, each having four sporozoites (McAllister et al., 1998, Lindsay et al., 1999). Intermediate hosts ingest oocyst-contaminated food, water or soil and sporozoites develop in the intestine then penetrate the epithelial cells and develop into motile rapidly dividing tachyzoites (asexual stage). These tachyzoites can spread to different cells and tissues of the host e.g. neural cells, fibroblasts, macrophages, hepatocytes, (Bartels et al., 2005) muscle cells, myocardium and the placenta of pregnant cows (Shivaprasad et al., 1989, Dubey et al., 1996). During unfavourable conditions, e.g. strong host immune responses, tachyzoites transform to a slowly dividing cyst stage termed bradyzoites. These are surrounded by a cyst wall for protection and remain inactive until the host immune response is suppressed. These cysts have been identified in the brain,

spinal cord and retina of the infected host (McAllister et al., 1998). The life cycle is completed if these cysts or the infected aborted foetus are ingested by the definitive host (Figure 1.4).

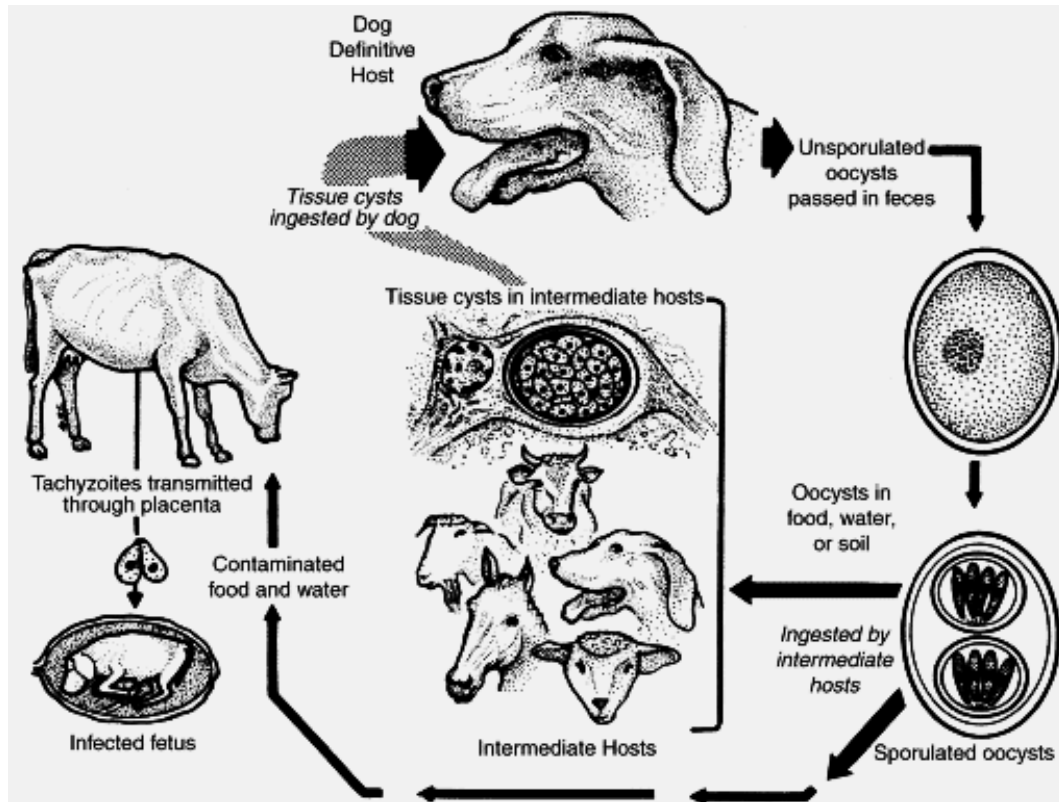


Figure 1.4: Life cycle of *N. caninum*.

Figure adapted from afsdairy.ca.uky.edu (Dubey, 2005) [accessed online 02/05/2015].

Several studies indicate there is an association between the presence of the dogs in the herd and the prevalence of the disease, suggesting the role of the dogs in transmission of neosporosis (Pare et al., 1998, Bartels et al., 1999). Transmission of *Neospora* may be horizontal or vertical. Horizontal transmission occurs by ingestion of contaminated food or

water with oocysts (Dubey et al., 2007). However, vertical transmission is the most important method by which the infection is transmitted through generation (from dam to the foetus) and maintained within the herd (Pare et al., 1994, Lopez-Gatius et al., 2004, Dubey et al., 2007). Much data has been generated that shows that calves born from seropositive cows were also seropositive; more than 95 % of the calves born from infected mothers have antibodies against neospora and remain infected for life (Piergili Fioretti et al., 2003, Dubey et al., 2007). However, transmission from cow to cow has not been yet demonstrated; calves may be infected by ingestion of milk contaminated with neospora tachyzoites (Davison et al., 2001).

1.5.3 Diagnosis

Several serological assays have been used to measure antibodies to *N. caninum* in serum or milk samples, such as indirect fluorescent antibody test (IFAT) and ELISA (Bartels et al., 2005, Bjorkman et al., 2006). Another useful tool for diagnosis of Neosporosis is through the detection of immunoreactive parasite antigen in the host sera (Atkinson et al., 2000). In addition, western blotting has been used to confirm the IFAT results (Alvarez-Garcia et al., 2002). Recently, avidity tests were developed to differentiate between acute and chronic infections in cattle, which induce low and high avidity antibody, respectively. These methods are important in controlling neosporosis and preventing the introduction of the disease to naïve herds or to areas free from the disease (Dubey et al., 2007). Using an agglutination test, it has been found that the level of specific IgM peaks 2 week post infection and declines below the detection limits at 4 week post infection (De Marez et al., 1999). After experimental infection, the level of IgG increases during the first week and

remains high until 6 months, this is characterised by an initial rise in specific IgG1, followed by IgG2 (Andrianarivo et al., 2001, De Marez et al., 1999). A whole tachyzoites mastazyme ELISA test has been developed by Williams et al., (1997) to detect *N. caninum* antibodies in serum with high sensitivity and specificity compared to IFAT.

1.5.4 Immune response to *N. caninum* infection

It is well known that intracellular protozoan parasites elicit a polarized Th1 immune response dominated by production of IL-12, IFN- γ and TNF- α which in turn activates free oxygen radicals and the nitric oxide pathway that are effective killers of many protozoans parasites (Hunter and Reiner, 2000). It has been demonstrated multiple times that IFN- γ is crucial in mediating protection against the closely related intracellular parasite, *T. gondii* (Suzuki et al., 1988, Suzuki and Remington, 1990). The majority of data on the immune response to *N. caninum* comes from murine studies, less being known about bovine Neospora immunity. Splenocytes from mice experimentally infected with *N. caninum* proliferate in response to parasite soluble antigen *in vitro*, and these cells produce high levels of IL-12 and IFN- γ mRNA within a few hrs of infection. Neutralization of these two cytokines in infected mice inhibited the natural resistance of these mice to infection, indicating the important role of these cytokines in protective immunity to *N. caninum* (Khan et al., 1997). Other studies have proved the importance of IFN- γ in resistance to *N. caninum* by comparing the cytokine profile in three strains of mice. They found that the resistance of B10.D2 mice was related to high IFN- γ :IL-4 ratios, while susceptible BALB/c and C57BL/6 mice induce a low IFN- γ :IL-4 ratio (Long et al., 1998). Tanaka et al., (2000) found that the depletion of CD4⁺, CD8⁺ and IFN- γ from *N. caninum* infected mice, using

monoclonal antibodies, led to a high mortality rate in these mice. Infection of rat astrocytes with *N. caninum* produces high levels of IL-10 in culture within 24 hrs of infection (Pinheiro et al., 2006). Other studies on rat indicated that treatment of *N. caninum* infected neuron/glia co-culture with IFN- γ reduced the tachyzoites proliferation and increased NO production in the culture (De Jesus et al., 2014). However, there is only limited data on the bovine immune response to *N. caninum* infection. These studies demonstrate that *N. caninum* antigens induce proliferation of PBMC and CD4⁺ T cells and IFN- γ production in both naturally and experimentally infected cattle (Lunden et al., 1998, Marks et al., 1998, De Marez et al., 1999, Williams et al., 2000, Andrianarivo et al., 2001). Recombinant IFN- γ suppressed the multiplication of *N. caninum* within ovine fibroblast cells *in vitro* (Innes et al., 1995). A similar observation was found in primary bovine brain cells; IFN- γ inhibited the proliferation of tachyzoites in cultures (Yamane et al., 2000). During pregnancy, the dynamic of the host immune response alters towards a regulatory Th2 response with high level of IL-10, IL-4 and TGF- β that abolish the inflammatory response induced by Th1 cytokines such as IFN- γ which is detrimental to pregnancy (Tangri and Raghupathy, 1993, Entrican, 2002). Regidor-Cerrillo et al.,(2014), observed that stimulation of PBMCs from pregnant cows with parasite soluble antigen stimulated high levels of IFN- γ and IL-4 and this occur rapidly following infection. Also the expression of IFN- γ , IL-12, TNF, IL-4 and IL-10 mRNA from placenta of infected heifers were significantly increased at 70 days of gestation. This indicates a mixed Th1/Th2 response during early gestation, which is usually associated with abortion. In contrast, the ability of the infected cow to mount a parasite-specific cell proliferation and IFN- γ responses was reduced significantly during the mid-gestation; this is beneficial in maintaining the pregnancy (Innes et al., 2001). Almeria *et al.*, (2014) investigated the foetal and maternal immune responses following experimental

infection of cows at 110 days of gestation and demonstrated that the cytokine expression in the spleens of dams and foetus was varied, the infection stimulated the expression of IFN- γ , IL-12, TNF- α , IL-6 and IL-10 mRNA and inhibited the IL-4 expression in spleen and placenta of dams. The highest IFN- γ expression was observed in the spleen and caruncle of dams carrying the dead foetus indicating that excessive placental response are deleterious. While, the competent fetal immune response associated with increased survival.

In summary, it is clear that the generation of a Th1 immune response and accompanying pro-inflammatory cytokines play a critical role in controlling *N. caninum* infection. As stated above abortion is the main consequence of *N. caninum* infection in early gestation, which occurs because of placental damage mediated by the immune response of the dam and the foetus (Maley et al., 2003). Placental damage is mediated by different immune cells including CD3, CD4, CD8, $\gamma\delta$ T-cells and NK cells and the production of IFN- γ (Maley et al., 2006).

1.6 Parasite effectors/immune-modulator

A wide range of immunomodulation strategies have been demonstrated during parasitic infection (Playfair, 1982). Successful immunomodulation to limit excessive immunopathology and inflammation is advantageous for the long term existence of both the host and the parasite. As a host, it is critical to produce an effective immune response to control the parasite but also to limit damage to self. For the parasite, it is important to complete its life cycle by either damping down the immune response or converting it to an ineffective response (Pearce and MacDonald, 2002, Gause et al., 2003). An important

characteristic feature of the parasite is the biological diversity in their *in vivo* niches; this underlines the different immune responses, mediated against an array of species of parasites ranging from intracellular protozoa e.g. *T. gondii* which induce a Th1 responses (Gazzinelli et al., 1991) to extra cellular helminths e.g. *F. hepatica* that trigger Th2 responses (Finkelman et al., 1991). The second distinguishing feature is the chronicity and persistence of the antigenic stimulation within the host which leads to direction of the immune responses toward an immunoregulatory or immunosuppressive state promoting parasite survival (Mitchell, 1991).

With respect to *F. hepatica*, various processes of immunomodulation have been reported. For example, in cattle incubation of the juvenile fluke with immune serum and eosinophils or neutrophils allowed the attachment of granulocytes to the surface of the parasite, but the rapid turnover and shedding of the outer glycocalyx prevented cell mediated damage to the parasite (Duffus and Franks, 1980).

F. hepatica produces a variety of cysteine proteinase termed CLs within hours of infection and the most important enzymes in this group are CL1 and cathepsin L2 (Smith et al., 1993). These enzymes are expressed by the gut epithelial cells of the newly excysted juvenile (NEJ) and adult parasite and they are involved in a wide range of biological activities. For example, cathepsin Ls are important in the digestion of the ingested blood and host tissue (Tort et al., 1999). Also, the regurgitated protease has the ability to degrade host collagen, fibronectin and laminin which in turn facilitate the burrowing of the parasite through the tissues (Berasain et al., 1997). In addition, these CLs are also able to cleave the host immunoglobulin in the hinge region and inhibit the antibody-mediated attachment of eosinophils to the parasite surface (Carmona et al., 1993, Berasain et al., 1997). Moreover, CLs play an important immunosuppressive role by inhibiting the protective Th1 responses

and induction of Th2 responses in the host (O'Neill et al., 2001). Recently, it has been shown that CL1 inhibits TRIF dependent activation of macrophages and production of pro-inflammatory cytokines IL-6, IL-12 and TNF- α via degradation of TLR-3 in macrophage endosomes (Donnelly et al., 2010).

The antioxidant thioredoxin peroxidase (TPx/Prx) secreted from adult fluke induces the development of alternatively activated macrophages (AAM Φ) both *in vitro* and *in vivo*. AAM Φ being characterised by the production of prostaglandin and IL-10 (Donnelly et al., 2005).

One studies highlighted the role of protein derived from *F. hepatica* tegument in mediating degranulation and histamine release from rodent peritoneal mast cells (Trudgett et al., 2003). It has been shown that human mast cells produce IL-4 and IL-5 (Bradding et al., 1993) which are important in the differentiation of the Th2 immune response (Mosmann et al., 1986). The tegumental antigens of the parasite inhibit the protective effects of mast cells against parasite infection by polarizing the immune response toward the antibody-dependent Th2 response, which is un-effective.

It has been found that *F. hepatica* larva is also able to produce antioxidant proteins such as superoxide dismutase (SOD) which induce the dismutation/partitioning of super oxide anion (O_2^-) into hydrogen peroxide (H_2O_2) and oxygen (Fridovich, 1983). Therefore, these are regarded as important defence mechanisms that protect the larvae from the lethal effects of reactive oxygen species (ROS) of the host (Hahn et al., 2001).

The various life cycle stages of *Schistosoma. mansoni* different life stages i.e. larvae, adults and eggs are all implicated in diverse mechanisms of modulation of the immune system. It has been found that the ES products of the larval stage induced the partial maturation of DCs and polarisation of the immune responses toward Th2 response *in vivo* (Jenkins and

Mountford, 2005). IgE is important in mediating protection against helminths parasites. However, the cercaria and larval ESP of *Schistosoma* contain an elastase-like protease that cleaves IgE in human, mouse and rats and inhibits its protective functions and also facilitates skin penetration (Pleass et al., 2000, McKerrow et al., 2006). During skin penetration *Schistosoma* cercaria secrete a protein Sm16 which inhibits TLR signalling in monocytes by inhibiting the degradation of the IRAK1 signalling protein (Brannstrom et al., 2009).

Proteomic analysis of *S. mansoni* eggs revealed the presence of a cationic fraction (CEF6) which contains 2 glycoproteins (alpha 1 and omega1) (Dunne et al., 1991). It has been proved that *S. mansoni* egg antigen (SEA) alpha 1 is a potent trigger for IL-4 production from naïve human basophils, and it induces the degranulation of basophils and release of histamine and leukotrienes (Falcone et al., 1996, Haisch et al., 2001, Schramm et al., 2003), indicating that alpha-1 is an important inducer of Th2 responses. Omega-1 has been shown to bind to certain human chemokines, neutralising their biological functions e.g. inhibition of chemokine-mediated neutrophils recruitment. Neutralisation of this protein in experimentally infected mice enhanced egg-induced inflammation, indicating the role of this factor in the modulation of egg granulomatous response (Smith et al., 2005). Moreover, it is suggested that omega-1 is responsible for liver damage in T-cell deficient mice, as the passive transfer of anti-omega-1 serum to immunodeficient *S. mansoni*-infected mice prevented hepatocyte damage (Dunne et al., 1991).

Heligmosomoides polygyrus is another gastrointestinal nematode of mice that has evolved several mechanism to escape the protective Th2 immune response (Urban et al., 1992). Several proteins with immunosuppressive activity have been identified in the ES products of this parasite (Pritchard et al., 1994). Telford et al.,(1998) demonstrated that the ES

products of *H. polygyrus* contain immunomodulatory factors that inhibited the *in vitro* production of IgE, IgG1, and IgG4 Ab from murine and human PBMCs. This effect may be mediated by T-cells as the depletion of T-cells from the culture failed to replicate the effect. However, the ES also induced the expansion of the CD8⁺ T-cells in the culture. Others investigated the functions of adult worm extracts fractionated by gel chromatography and proposed that one of these fractions inhibited antigen-specific T-cell proliferation within murine mesenteric lymph node and downregulated iNOS production from IFN- γ stimulated macrophages (Rzepecka et al., 2006). Calreticulin (CRT) is a protein secreted normally from the endoplasmic reticulum and has a variety of important roles in the regulation of cellular functions (Michalak et al., 1999). *H. polygyrus* expresses this protein at all parasite stages, the highest concentration being observed in tissue larvae (L4). Adult worm CRT induced high levels of IL-4 and IL-10 in CD4⁺ T-cells isolated from *H. polygyrus* infected mice, which suggests the Th2-polarizing property of parasite CRT (Rzepecka et al., 2009). The role of *H. polygyrus* derived products in modulation of DC functions has been investigated; treatment of DC with ES products inhibited the TLR9-induced cytokines (IL-12p40, IL-10, IL-6) and co-stimulatory molecules (CD40, CD86 and MHC II) on bone marrow derived-dendritic cells (BMDC). In addition, the adoptive transfer of ES pre-treated BMDC pulsed with ovalbumin suppressed the specific antibody response in recipient mice. Also, in this study it was found that ES-treated DCs co-cultured with CD4⁺ T-cells stimulated the expansion of IL-10-producing CD4⁺ T regulatory cells, which inhibited T-cell proliferation and IFN- γ release in malaria co-infection (Segura et al., 2007).

1.7 Interleukin 10 (IL-10)

Fiorentino et al. (1989) first described IL-10 as a cytokine synthesised by Th2 cells that inhibited the production of Th1 cytokines including IFN- γ , IL-2, IL-3 and GM-CSF, therefore it was termed cytokine synthesis inhibitory factor at that time. IL-10 had several other alternative names such as B cell derived T-cell growth factor (B-TCGF) or T-cell growth inhibitory factor (TGIF).

1.7.1 Sources of IL-10

It is well known that IL-10 production is not restricted to a certain type of immune cell but is a product of almost all immune cells (Wolk et al., 2002). In mice, however, the main source of IL-10 is the CD4⁺ Th2 cell (Fiorentino et al., 1989). Other significant producers include macrophages and B-cells (O'Garra et al., 1990, Fiorentino et al., 1991b, Seki et al., 1998). Furthermore, some non-hematopoietic cells are considered a source of IL-10 in mice such as thymocytes and keratinocytes (MacNeil et al., 1990, Enk and Katz, 1992). Several studies demonstrated the role of both CD4⁺ CD25⁺ and CD4⁺ CD25⁻ T-reg cells in the production of IL-10 (Belkaid et al., 2002, Sundstedt et al., 2003, O'Garra and Vieira, 2004). Additionally, Th1 cells can also produce this cytokine (Anderson et al., 2007).

In humans the main sources of this cytokine are activated monocyte (de Waal Malefyt et al., 1991b). However, it is also expressed by different CD4⁺ T-cells including Th0, Th1 and Th2 cells and by CD8⁺ T-cells (Yssel et al., 1992). Subsequent studies showed that this cytokine is also produced by natural killer cells, activated B-cells, dendritic cells,

eosinophils and neutrophils (Mehrotra et al., 1998, Blanco et al., 2008, Grant et al., 2008, Zhang et al., 2009).

In recent years there has been the description of the IL-10-producing T-r1 cell in humans and mice, these are differentiated in the presence of IL-10 and characterised by production of large amounts of IL-10 (Battaglia et al., 2006, Awasthi et al., 2007).

During active infection several populations of IL-10 producing cells have been identified (Couper et al., 2008). A new subset of regulatory DCs have been identified which are different from normal DCs that produce pro-inflammatory cytokines in response to bacterial LPS. These regulatory DCs produce IL-10 in response to LPS treatment *in vitro* (Fujita et al., 2006). Furthermore, macrophages are also considered the main source of IL-10 during infection with *Leishmania major*.

Naturally occurring T-regs have the ability to produce copious amount of IL-10 during multiple infections with pathogens such as *L. major* and *S. mansoni* (Belkaid et al., 2002, Hesse et al., 2004).

In cattle IL-10 is expressed by the majority of Th-cells including Th0, Th1 and Th2 cells clone in response to *Babesia bovis* or *F. hepatica* antigen (Brown et al., 1994b).

1.7.2 IL-10 functions

IL-10 has varying effects on the function of a number of different immune cells including T-cells, B-cells, monocytes, DCs and NK cells.

1.7.2.1 Effects on monocytes/macrophages

IL-10 has important effects on the modulation of cytokine production and the function of monocytes/macrophages. It inhibits the production of pro-inflammatory mediators such as IL-1 α , IL-1 β , IL-6, IL-8, IL-12, TNF- α , G-CSF, GM-CSF and M-CSF from monocyte/macrophages (de Waal Malefyt et al., 1991a, Fiorentino et al., 1991a, D'Andrea et al., 1993, Gruber et al., 1994, Moore et al., 2001). Moreover, IL-10 upregulates the expression of anti-inflammatory mediators including IL-1 receptor antagonist (IL-1Ra) and soluble p55 and p75 TNF receptors in monocytes (Jenkins et al., 1994, Linderholm et al., 1996, Hart et al., 1996). The anti-inflammatory effects of IL-10 is mainly due to its inhibitory effects on IL-1 and TNF- α which are both responsible for the stimulation of secondary mediators like platelet-activating factor (PAF) and prostaglandins, while the IL-10- mediated inhibition of IFN- γ is related to its ability to inhibit IL-12 production which in turn suppresses CD4⁺ Th1 development (D'Andrea et al., 1993). Interestingly, IL-10 is able to suppress its own production from LPS-activated monocytes (de Waal Malefyt et al., 1991a). IL-10 can also downregulate the production of IL-23 from macrophages, necessary for development of Th17 immune responses (Schuetze et al., 2005). Recently, it has been found that IL-10 reduced the LPS- induced production of IL-1 β , IL-8 from carp monocyte/macrophages via the inhibition of NF- κ B activation (Wei et al., 2015). Demonstrating a species conserved role and mechanism for IL-10 in immunoregulation.

The production of pro-inflammatory cytokines from activated monocytes/macrophages are crucial in clearing the invading microorganisms and recruiting and activating other immune cells (Fujiwara and Kobayashi, 2005). For example, TNF- α inhibits bacterial growth and macrophage death in early infection, IL-1 β induces lymphocyte activation which enhances

thymocyte proliferation and IL-8 is essential in recruitment of neutrophils during acute inflammation (Lonnemann et al., 1989, Akahoshi et al., 1994, Clay et al., 2008). Conversely, overproduction of these cytokines may lead to inflammatory diseases with detrimental effects. Therefore, IL-10 play important role in controlling the inflammatory process by limiting the production of these pro-inflammatory cytokines (Fiorentino et al., 1991a, Mendez-Samperio et al., 2002).

IL-10 also inhibits the production of members of the CC chemokine family; MIP-1 α , MIP-3 α , MIP-3 β , Rantes, MCP-1, and MCP-5 and the CXC chemokine family; IP-10, MIP-2 and KC from LPS-stimulated monocyte (Berkman et al., 1995, Marfaing-Koka et al., 1996, Rossi et al., 1997, Kopydlowski et al., 1999). The main functions of these chemokines is the recruitment of leukocytes to the site of inflammation (Baggiolini et al., 1994). CC chemokines attract monocytes, basophils, eosinophils and T-cells (Taub et al., 1993, Hedrick and Zlotnik, 1996, Kitauro et al., 1996). Whilst, CXC chemokines mainly induce the recruitment of neutrophils (Baggiolini et al., 1994). Thus, the anti-inflammatory function of IL-10 is also mediated through the inhibition of chemokines. However, IL-10 can also increase the expression of some chemokine receptors e.g. CCR1, 2 and 5 on monocytes leading to an increased susceptibility of these monocyte to infection with HIV (Sozzani et al., 1998).

IL-10 induces the expression of IgG-Fc receptors, Fc γ RIII (CD16) and Fc γ RI (CD64), on monocytes/ macrophages which are essential for the uptake and opsonisation of antibody-coated pathogens. This in turn enhances the antibody-dependent cellular cytotoxicity (ADCC) response and the phagocytic activity of these cells for bacteria and fungi (te Velde et al., 1992, Capsoni et al., 1995, Calzada-Wack et al., 1996) demonstrating that not all of the effects of IL-10s are anti-inflammatory.

In contrast, IL-10 indirectly inhibits the superoxide anion and nitric oxide production from macrophages and subsequently reduces the killing ability of the cells toward the ingested organisms (Oswald et al., 1992, Cunha et al., 1992, Roilides et al., 1997). IL-10 also inhibits the production of O_2^- from PMA or FMLP primed monocytes and also reduced the ADCC effect of neutrophils (Capsoni et al., 1997). In addition, IL-10 downregulates the expression of MHC class II antigen, and costimulatory molecules including CD80 (B7), CD54 (ICAM-1), and CD86 (B7.2) on monocytes (de Waal Malefyt et al., 1991b, Ding et al., 1993, Willems et al., 1994, Creery et al., 1996). The suppression of the antigen presenting activity of monocyte/macrophages mediated by IL-10 mainly occurs via inhibition of the vesicular transport of the MHC II complex to the plasma membrane and is not due to downregulation of transcription of MHC II (Koppelman et al., 1997). These effects indirectly inhibit the capacity of T-cells and B-cells to respond to infection. Together, these data indicated that IL-10 mediates multiple direct and indirect actions of the monocyte/macrophage lineage.

1.7.2.2 Effects on Dendritic cells

Several studies indicate that IL-10 plays an important role in modulating the ability of DCs to activate and prime naive T-cells and the initiation of the immune response. IL-10 inhibits IL-12 production and the expression of costimulatory molecules by DCs (Ludewig et al., 1995, Buelens et al., 1995, Chang et al., 2004). Similarly, IL-10 inhibited the primary alloantigen-specific T-cell proliferation response (Bejarano et al., 1992, Caux et al., 1994). The addition of IL-10 to the monocyte culture with the presence of GM-CSF and IL-4 promoted the differentiation of monocyte derived macrophages and inhibited the

differentiation of monocyte derived myeloid DC. In addition, IL-10 suppressed the IL-13 dependent proliferation and survival of the lymphoid DCs (Rissoan et al., 1999). On the other hand, IL-10 has no detectable effects on mature DCs due to the lack of IL-10 receptor on these cells (Allavena et al., 1998, Fortsch et al., 2000). Interestingly, distinct populations of IL-10 producing DCs have been identified in lung, intestine and monocyte-derived DCs (Iwasaki and Kelsall, 1999, Bouloc et al., 2000, Akbari et al., 2001). Others demonstrated that IL-10 treatment of immature DCs was associated with inhibition of the expression of MHC class II, CD58, CD86 costimulatory molecules and CD83. The reduced capacity of these cells resulted in an inability to induce T-cell proliferation and induced a state of anergy in CD4 T-cells (Steinbrink et al., 1997). Collectively these data indicates that IL-10 inhibits the Th-1 mediated inflammatory response. In recent years there has been much interest in the use of tolerogenic DCs in the treatment of autoimmune diseases; one mechanism utilised to generate these cells is by the use of IL-10 (Rutella et al., 2006, Torres-Aguilar et al., 2010, Van Brussel et al., 2014).

1.7.2.3 Effects on Neutrophils

It has been demonstrated that IL-10 is a potent inhibitor of several pro-inflammatory cytokines including IL-1, IL-8 and TNF- α secretion by LPS-stimulated PMN (Cassatella et al., 1993, Wang et al., 1994). The inhibition of IL-1 is mainly due to the IL-10-mediated upregulation of IL-1Ra production by PMN (Cassatella et al., 1994, Marie et al., 1996). Other studies indicated that IL-10 inhibits the production and expression of MIP-1 α , MIP-1 β and IL-8 from LPS-treated PMN (Kasama et al., 1994). IL-10 also inhibited the extracellular production of IP-10 from neutrophils stimulated with IFN- γ and LPS or TNF-

α (Cassatella et al., 1997, Gasperini et al., 1999). Furthermore, IL-10 reduced the phagocytic and bactericidal capacity of PMN effects which were associated with inhibition of CD11-b expression and reduced production of O_2^- (Laichalk et al., 1996). IL-10 also inhibits the production of cyclo-oxygenase2 (COX)-2 and prostaglandin E2 from LPS-activated neutrophils (Niirio et al., 1997). Recently, it has been shown that IL-10 inhibits the recruitment of monocytes to the lung during infection with *Streptococcus pneumonia* (Penaloza et al., 2015).

1.7.2.4 Effects on T cells

Initially, IL-10 was first described by Fiorentino *et al.*, (1989) as a cytokine produced from Th2 cells which inhibited the proliferation of Th1 cells by suppressing the production of IL-2, IFN- γ , IL-3, lymphotoxin, TNF and GM-CSF production from Th1 cells. Subsequent studies indicated that human IL-10 was produced by, and has inhibitory effects upon both Th1 and Th2 cells. For example, IL-10 inhibited the proliferation of Th1 and Th2 subtypes in response to specific antigen or to phytohaemagglutinin (PHA) mitogen stimulation but did not inhibit IL-2-induced proliferation of Th cells. In addition, IL-10 inhibited the production of IFN- γ from Th1 cells and IL-4 and IL-5 from Th2 cells in response to specific antigen (Del Prete et al., 1993). IL-10 also inhibits the production and expression of IFN- γ by memory T cells in the presence or absence of APCs (Naundorf et al., 2009). In addition, the ability of IL-10 to inhibit IL-1, IL-6, IL-12, IL-8 and TNF- α by different immune cells has been shown to impair the activation and differentiation of Th1 cells subsets (de Waal Malefyt et al., 1991a, Fiorentino et al., 1991a, D'Andrea et al., 1993). In some situation, IL-10 enhance Th2 and reduces Th1 response e.g. IL-10 inhibit IL-12

expression on the surface of APCs and this was associated with the suppression of IFN- γ production and development of Th2 immune responses (Steinbrink et al., 2002). Also, IL-10 inhibited the ability of Langerhans cells to induce the Th1 cells proliferation in response to peptide Ag while enhancing the proliferation of Th2 cells (Enk et al., 1993). Moreover, it has been shown that IL-10 inhibited human antigen specific T cell proliferation, either indirectly via downregulating the MHC class II on the surface of monocytes, dendritic cells and Langerhans cells (de Waal Malefyt et al., 1991b, Caux et al., 1994, Peguet-Navarro et al., 1994), or directly by suppression of endogenous IL-2 production from T cells (Taga and Tosato, 1992, de Waal Malefyt et al., 1993). It has been shown that IL-10 inhibits apoptosis of T-cells deprived of IL-2 *in vitro* (Taga et al., 1993). IL-10 inhibits the expression of costimulatory factors CD54, CD80 and CD86 by monocytes and macrophages, which are important for T cells activation and differentiation (Ding et al., 1993, Willems et al., 1994). Others have shown that IL-10 induces a state of anergy or non-responsiveness in CD4⁺ T cells (Groux et al., 1996). In contrast to the inhibitory effects of IL-10 on CD4⁺ T cells as mentioned above, IL-10 has stimulatory effects towards the CD8⁺ T cells. IL-10 induced IL-2 and IL-4 dependent proliferation of CD8⁺ T cells in mice (Chen and Zlotnik, 1991). However, Groux et al., (1998) demonstrated that IL-10 has different effects on human CD8⁺ T cells depending on their activation state; IL-10 can inhibit alloantigen-specific CD8⁺ T cells proliferation in the presence of APCs via inhibition of the costimulatory functions of APCs. In contrast in the absence of APCs, IL-10 induces the proliferation of these cells. Furthermore, IL-10 enhances the cytotoxic activity of CD8⁺ T cells towards tumour cells (Berman et al., 1996, Fujii et al., 2001).

1.7.2.5 Effects on B cells

IL-10 has variable effects on proliferation and antibody production from B-lymphocytes. IL-10 upregulated the expression of MHC class II and enhanced viability of splenic B-cells in mice (Go et al., 1990). Several workers demonstrated that IL-10 is a potent inducer of survival, proliferation, differentiation and isotype switching of human B-cells activated by *Staphylococcus aureus* Cowan (SAC) or CD40 (Levy and Brouet, 1994, Rousset et al., 1995). It has been shown that production of IgM, IgG1 and IgA were enhanced by the addition of IL-10 to cultures while the presence of both IL-10 and IL-4 favour the production of IgG4 and IgE from these system (Armitage et al., 1993, Uejima et al., 1996). In others studies it was found that IL-10 inhibited the IL-4-induced IgE production from CD40L-stimulated B-cells (Akdis et al., 1998). Furthermore, IL-2 synergised with IL-10 to induce the proliferation and differentiation of Ig-producing B-cells mainly through upregulation of the IL-2 receptor in B-cells (Fluckiger et al., 1993). Subsequent studies indicated that IL-10 induces apoptosis of *Staphylococcus aureus*-activated B-cells when added in the initial stage of activation, whereas IL-10 promotes the survival of mature SAC-activated B-cells and the differentiation of such cells into Ig producing cells (Itoh and Hirohata, 1995). Recently, distinct populations of regulatory B-cells that produce IL-10 and IgG4 have been demonstrated and the role of these cells in suppression of Ag specific CD4⁺ T cell proliferation was mainly related to the overexpression of IL-10 (van de Veen et al., 2013).

The stimulatory effects of IL-10 on B-cell proliferation and Ig production may indicate a use for this cytokine in the treatment of various immunodeficiency syndromes e.g. IgA deficiency (Briere et al., 1994). Conversely, the use of IL-10 antagonists are potentially

important treatments for some antibody-mediated autoimmune diseases such as systemic lupus erythematosus (Liorente et al., 2000).

1.7.3 IL-10 in parasitic infection

During infection, IL-10 mainly acts as an immunoregulatory cytokine, ameliorating the excessive immunopathology associated with bacteria, viruses, fungi, protozoa and helminth infection but it may impair pathogen control. For example, during protozoan infection, IL-10 regulates resistance to infection mainly through deactivating macrophages and inhibiting NO expression (Gazzinelli et al., 1992b, Vieth et al., 1994). The ablation of IL-10 during *T. gondii* and *Trypanosoma cruzi* infections was associated with excessive Th1 responses resulting in lower parasite burdens, but severe fatal immunopathology (Gazzinelli et al., 1996, Hunter et al., 1997, Suzuki et al., 2000). Immunopathology observed in the absence of IL-10 during parasitic infection mainly results from the overexpression of IL-12 which lead to increase production of IFN- γ and TNF- α (Gazzinelli et al., 1996, Holscher et al., 2000, Murphy et al., 2001). Furthermore, neutralisation of IL-10 during *L. major* infection was associated with complete clearance of the parasite from murine skin lesions (Belkaid et al., 2001). However, during *Plasmodium chabaudi* infection, IL-10 is crucial for preventing pathology by inhibiting IL-12 secretion (Perona-Wright et al., 2009) without compromising the IFN- γ mediated parasite killing (Jankovic et al., 2007). This Indicates that there is a balance between the production of IL-10 and IFN- γ during parasitic infection. Thus, a high parasite burden is associated with production of high level of IFN- γ , which requires increased production of IL-10 (Karp et al., 1993).

Several studies indicate that helminth infection is associated with strong Th2 responses with the production of IL-4, IL-5, IL-9, IL-10 and IL-13 together with eosinophilia and high IgE production (Finkelman et al., 1991, Van Milligen et al., 1998, Allen and Maizels, 2011). In addition, allergic diseases demonstrate a similar immunological pattern; allergen induces the secretion of IL-4, IL-5 and IL-13 from primed T-cells and these in turn induce the recruitment of eosinophils and mast cells. However, an inverse correlation has been found between helminth infections and the incidence of several allergic conditions such as airway hypersensitivity and asthma (van den Biggelaar et al., 2000, Nyan et al., 2001). In addition, the burden and the chronicity of helminth infection may also determine the outcome of allergic conditions; it has been shown that the proportion of positive skin test reactions to atopic allergens in non-infected individuals is higher than in individuals infected with *S. mansoni* (Araujo et al., 2000). One of the mechanisms for this negative association is the stimulation of IL-10 production by the parasite. For example, parasite-induced IL-10 inhibited allergen-mediated airway eosinophilia and reduced the OVA-specific IgG1 and IgE during infection with *Neospora brasiliensis* (Wohlleben et al., 2004). IL-10 reduces skin reactivity to mites during *S. mansoni* infection (Nyan et al., 2001). And, it has been demonstrated that *S. mansoni* protects mice from IgE-induced fatal anaphylaxis through the induction of IL-10 producing B-cells (Fallon et al., 2001, Mangan et al., 2004).

It has been shown that infection with *S. mansoni* protects non-obese diabetic (NOD) mice from spontaneous autoimmune diabetes (Cooke et al., 1999) with SEA-induced IL-10 having an important role (Zaccone et al., 2003).

One of the characteristic features of *S. mansoni* infection is the induction of strong Th2 immune responses to eggs laid in the portal veins and subsequently trapped in the host liver

and intestine (Grzych et al., 1991). These eggs induce Th2 responses associated with production of IL-4, IL-5, IL-10 and IL-13 (Grzych et al., 1991, Pearce et al., 1991, Wynn et al., 1993), and downregulation of Th1 cytokines including IFN- γ and IL-2 (Pearce et al., 1991). The various experimental studies that examined the role of Th2 in mediating host survival during *S. mansoni* infection, have reported conflicting results. IL-4 protects mice from TNF- α -mediated cachexia (Brunet et al., 1997). Subsequent studies indicate that IL-4-dependent Th2 response is important for survival of the mice during *Schistosoma* infection (Patton et al., 2001). However, this response may lead to hepatic fibrosis during the chronic stages of disease, mainly due to IL-13, which acts as a pro-fibrotic factor and the IL-13-deficient mice showed enhanced survival indicating that Th2 response does not increase survival during *Schistosoma* infection (Chiaramonte et al., 1999, Fallon et al., 2000). In addition, this polarized immune response is associated with the formation of granulomatous liver disease leading to hepatic fibrosis, portal hypertension, intestinal haemorrhage and death (Cheever, 1993, Pearce and MacDonald, 2002). On the other hand, resistance to *S. mansoni* infection is correlated to Th1 response (Vella and Pearce, 1992). It has been shown that IL-10 is critical for maintaining the balanced Th1 and Th2 response during schistosomiasis, and ablation of this cytokine from mice infected with *S. mansoni* promotes a mixed Th1/Th2 immune response (Wynn et al., 1997). Hoffmann et al. (2000) showed that the excessive Th1 response induced in mice deficient in IL-10/IL-4 associated with high mortality rate that linked to overexpression of IFN- γ , TNF- α and NO and the formation of non-fibrotic granuloma. While mice deficient of IL-10/IL-12 generated a highly polarized Th2 response and associated with excessive hepatic fibrosis and formation of large granuloma, indicating that IL-10 regulate the pathogenesis of schistosomiasis by inhibiting the excessive Th1 and Th2 response. Initial investigators demonstrated IL-10 is

the main cytokine responsible for polarization of egg-induced Th2 responses by inhibiting the Th1 cytokines IFN- γ (Sher et al., 1991). Furthermore, IL-10 suppressed the antigen presenting capacity of macrophages and this in turn leads to inhibition of SEA-specific Th1 proliferation (Flores Villanueva et al., 1993). Subsequent studies have demonstrated that IL-10 inhibits macrophages and dendritic cells activation and control the excessive Th1 and Th2 responses that lead to inflammation and tissue damage (Flores Villanueva et al., 1994). Systemic administration of recombinant monoclonal IL-10 was found to down regulate delayed type hypersensitivity reaction to SEA and granuloma formation in lung and liver by suppressing the IL-2 and IFN- γ (Flores-Villanueva et al., 1996). In humans, IL-10 has been shown to modulate granuloma formation and control the severe morbidity to *Schistosoma* infection (Falcao et al., 1998). These data indicate that *Schistosoma*-induced IL-10 impairs the parasite antigen-specific lymphocyte proliferation and IFN- γ production which in turn both enhance parasite persistence and minimises the disease pathology and morbidity. Fairfax et al. (2012) showed that IL-10 is important for development and recruitment of IgG1-secreting B-cells in the liver of chronically infected mice, and neutralization of IL-10 using anti-IL-10R antibody leads to decrease the hepatic IgG1 secreting cells and the development of portal hypertension and dissemination of parasite eggs to the lung and heart. A more recent study demonstrated that the repeated penetration of skin by the larvae of *S. mansoni* leads to accumulation of IL-10 producing CD4⁺T-cells, and IL-10 has important role in ameliorating the inflammation and limiting tissue damage associated with repeated Schistosome infection (Sanin et al., 2015).

Infection with *F. hepatica* is associated with early production of IFN- γ and followed by high level of IL-4 and IL-10 as the disease progress to the chronic stage suggesting a biased Th2 response during chronic fascioliasis (Moreau et al., 1998, Tliba et al., 2002, Ingale et al.,

2008, Mendes et al., 2013). IL-10 produced by hepatic lymph node mononuclear cells in response to *F. hepatica* ESP suppressed the recombinant bovine IFN- γ induced NO production from monocytes (Moreau et al., 1998). In addition, *F. hepatica* was capable of inducing the development of AAM Φ both *in vitro* and *in vivo*. (Donnelly et al., 2005, Flynn et al., 2007a). These cells were characterised by expression of YM1, FIZZ and acid mammalian chitinase (AMCase) and had a high arginase activity. Moreover, AAM Φ serve to prime the Th2 response by suppressing IFN- γ and inducing production of IL-10 in response to *F. hepatica* ES (Flynn and Mulcahy, 2008a). These macrophages had reduced innate immune responses to bacterial infection leading to increase susceptibility to secondary bacterial infection (Gordon, 2003). It has been shown that the skin test used for diagnosis of bovine tuberculosis was compromised in animals co-infected with *F. hepatica* and this mainly correlated to the presence of AAM Φ , indicating the role of IL-10 in modulating the immune response during helminth co-infection with secondary pathogen. (Flynn et al., 2007b). Hacariz et al, (2009a) demonstrated the role of IL-10 in compromising the resistance of sheep to infection with *F. hepatica* by examining the fluke burden; there was found to be a negative correlation between the IL-10 level and fluke number.

Several studies in mice and cattle have demonstrated that infection with *N. caninum* is associated with Th1 response with production of high level of IFN- γ and IL-12 (Khan et al., 1997, Lunden et al., 1998, Tanaka et al., 2000, Williams et al., 2000). However, during pregnancy the immune response is biased toward the Th2 response, which is important for maintaining the pregnancy (Wegmann et al., 1993, Marzi et al., 1996). The most important Th2 cytokines that dominate during pregnancy and are associated with its maintenance are IL-4 and IL-10. IL-4 and IL-10 are important for suppressing Th1 responses and maintain

the pregnancy (Quinn et al., 2004). However, in *N. caninum* infected animals this biased Th2 immune response is responsible for transmission of the infection to the foetus (Long and Baszler, 2000). These data together indicate that IL-10 induced during *N. caninum* infection modulate the immune response to provide an environment which maintain the pregnancy and allow transmission of the pathogen to the foetus.

1.7.4 Regulation of IL-10 expression

Given the role of IL-10 as a crucial immune-regulatory cytokine, an understanding of how IL-10 expression is regulated is important for designing immune intervention strategies. Regulation of IL-10 expression occurs at several stages including remodelling of the chromatin structure of the IL-10 locus, transcriptional regulation and post-transcriptional regulation (reviewed in Gabryšová et al. 2014). Several studies indicate that the changes in the chromatin structure of IL-10 locus play an important role in controlling the expression of the IL-10 gene in cells producing this cytokine (Im et al., 2004, Kang and Im, 2005).

It is well known that IL-10 production is not restricted to the adaptive immune system; it is also produced by the innate immune system (Moore et al., 2001, O'Garra and Vieira, 2004, Saraiva and O'Garra, 2010). Cells of the innate immune systems have been shown to produce IL-10 following stimulation of PRRs by the pathogen derived products (Saraiva and O'Garra, 2010). TLR are the most important type of PRRs that induce IL-10 production, and several TLRs including 2, 4, 5, 7 and 9 have been identified as producers of IL-10 in response to stimulation (Agrawal et al., 2003, Boonstra et al., 2006, Hacker et al., 2006, Chi et al., 2006, Kaiser et al., 2009). The downstream signalling of the TLR adaptor proteins MyD88 and TRIF are both essential for signal transduction and production of IL-

10 (Boonstra et al., 2006). Signalling of TLRs through these adaptor proteins induces the activation of several signalling pathways such as phosphatidylinositol 3 kinase (PI3K)-AKT pathway, the mitogen activated protein kinase (MAP Kinase) pathway, the nuclear factor kappa B (NF- κ B) pathway and the activation of interferon regulatory factor (IRF). All of these downstream pathways regulate TLR-induced IL-10 production (Kawai and Akira, 2010).

It has been shown that activation of the MAP kinase proteins including ERK1 and ERK2 are essential for IL-10 production from TLR stimulated macrophages and DCs (Dillon et al., 2004, Kaiser et al., 2009). In addition, ERK activation can stimulate the production of IL-10 from non-TLR stimulated DCs (Slack et al., 2007). The transcription factor activator protein 1 (AP-1) that is activated downstream of the MAP kinase signalling pathway positively regulates IL-10 production from TLR stimulated macrophages and DCs (Agrawal et al., 2003, Dillon et al., 2004, Hu et al., 2006). Moreover, other transcription factors have been shown to positively regulate IL-10 production. For example, Sp1 binds to the proximal part of the IL-10 promoter and this site is critical for transactivation of the *il-10* gene (Brightbill et al., 2000). Sp3 has also been shown to bind to the IL-10 promoter and trans activate the *il-10* gene in the *Drosophila* SL2 cell line (Tone et al., 2000). CCAAT/enhancer binding protein (CEBP) β and IRF-1 are also found to bind and trans-activate the *il-10* gene locus (Liu et al., 2003, Ziegler-Heitbrock et al., 2003). The MAP kinase p38 is also a positive regulator for IL-10 production from TLR-stimulated macrophages and DCs (Yi et al., 2002, Chi et al., 2006). It has been shown that p38 is essential in downstream activation and binding of Sp1 to the IL-10 promoter, which in turn transactivates *il-10* in lipopolysaccharide stimulated human macrophages (Ma et al., 2001). Other studies showed that both ERK and p38 activate the MSK1 and MSK2, which both

contribute to the downstream activation of CREB and ATF1, which subsequently bind, and transactivate the IL-10 promoter (Ananieva et al., 2008).

The PI3K-AKT pathway also has an important role in regulation of the IL-10 production from monocytes, macrophages and DCs (Martin et al., 2003). It has been shown that PI3K-AKT pathway activates ERK and mammalian target of rapamycin (mTOR) (Ohtani et al., 2008). mTOR regulates IL-10 production through the activation of STAT3, which in turn trans activates *il-10* (Benkhart et al., 2000, Weichhart et al., 2008). STAT3 is also important in induction of IL-10 production from human monocyte-derived macrophages in response to IL-10 itself (Staples et al., 2007).

The third signalling pathway, NF- κ B, activated downstream of the TLR system has also been implicated in the regulation of IL-10 production (Kanters et al., 2003). The binding of the NF- κ B p65 to the conserved DNase I hypersensitive site (HSS-4.5) upstream of the IL-10 transcription site in TLR-4 stimulated macrophages is critical in regulation of IL-10 production in this system (Saraiva et al., 2005). The binding of this NF- κ B subunit to the IL-10 promoter upregulated the production of IL-10 in dsRNA-activated protein kinase R stimulated macrophages (Chakrabarti et al., 2008). Furthermore, NF- κ B p105 regulates IL-10 production through direct NF- κ B and indirect TPL-2/ERK- dependent pathways (Banerjee et al., 2006). Several other transcription factors have been associated with regulation of *il-10* expression in TLR stimulated macrophages and/or DCs. For example, c-MAF can induce IL-10 production in LPS-activated macrophages, this regulation is IL-4 dependent and induces c-MAF production in macrophages (Cao et al., 2005). Moreover, the role of the C/EBP transcription factor in mediating *il-10* expression in response to cAMP has been well-documented (Brenner et al., 2003).

In contrast to the innate immune cells, which express IL-10 directly after stimulation with pathogen associated molecules, the expression of the IL-10 from the adaptive immune cells including T-cells requires the activation and differentiation of naïve T-cells into effector Th-cells subsets such as Th1, Th2, and Th17 which produce IL-10 (Zhu et al., 2010). The differentiation of the CD4⁺ T cells phenotypes primarily depends on the cytokines present during activation, the downstream STAT family protein activated and the lineage specific master regulators. Differentiation of the Th1 phenotype requires IL-12 signalling through STAT4 and the expression of T-bet transcription factor that in turn induces the production of IFN- γ (Murphy et al., 2000, Szabo et al., 2000). Th2 development is governed by IL-4 signalling through STAT6 and the expression of the GATA 3 that induces the production of IL-4, IL-5 and IL-13 (Zheng and Flavell, 1997, Mowen and Glimcher, 2004). Differentiation of Th17 cells depends on TGF- β and IL-6 signalling through STAT3 and the expression of ROR- γ that induces the production of IL-17 (Veldhoen et al., 2006, Ivanov et al., 2006). Despite the differences in the development of Th subsets, IL-10 is produced by all of these Th cells phenotypes (Saraiva and O'Garra, 2010). The production of IL-10 from these cells is highly associated with their development programmes. For example, IL-12 and STAT4 are both important for production of IL-10 from Th1 cells, but only in the presence of high concentration of antigen (Saraiva et al., 2009). Similarly, IL-4 signalling via STAT 6 and GATA 3 are required for IL-10 expression from Th2 cells (Shoemaker et al., 2006, Chang et al., 2007), while TGF- β , IL-6 and STAT 3 are essential in driving the production of IL-10 from Th-17 cells (Stumhofer et al., 2007). Following activation of the T cell receptors, several signalling pathways are initiated which are linked to the regulation of IL-10 production from Th cells. Among these pathways, ERK is an important positive regulator for IL-10 in different Th cells subsets (Saraiva et al., 2009).

The most important transcription factors activated downstream of the TCR is AP-1 (Wang et al., 2005), this factor consists of JunB or c-Jun which bind and trans-activate the *il-10* gene locus in Th2-cells (Jones and Flavell, 2005). Furthermore, another member of the AP-1 family, BATF has a significant role in regulation of IL-10 and IL-4 expression in Th2 cells (Tussiwand et al., 2012, Murphy et al., 2013). Similarly, IRF4 factor has the ability to bind and trans-activate the *il-10* and *il-4* promoter leading to upregulation the expression of these cytokines from Th2-cells (Ahyi et al., 2009). The transcription factor NFAT1 positively regulates IL-10 expression from Th1 and Th2 as measured in a luciferase reporter system (Im et al., 2004, Lee et al., 2009). It has been demonstrated that GATA3 induces upregulation of IL-10 production from CD4⁺ T cells and enhance the development of IL-10 producing T cells from naïve T cells culture (Nawijn et al., 2001, Shoemaker et al., 2006).

Interestingly, IL-10 induces its own expression from T cells via activation of STAT3 (Staples et al., 2007). It has been shown that binding of STAT4 to the *il-10* locus in Th1 cells and STAT6 in Th2 cells turned the *il-10* gene locus into a transcriptionally active site (Wei et al., 2010). Moreover, the SMAD proteins activated downstream of the TGF- β signalling pathways have a role in the regulation of IL-10 production from Th cells. Among these proteins SMAD4 induces the expression of IL-10 from Th1 cells (Kitani et al., 2003) and SMAD3 upregulates IL-10 production from Th2 cells (Blokzijl et al., 2002). Several other transcription factors have been implicated in the regulation of IL-10 expression from Th cells, including c-MAF factors which are essential for production of the IL-10 in T reg cells (Apetoh et al., 2010) and Th17 cells (Xu et al., 2009). It has been shown that IL-12 induces the production of IL-10 from Th1 through inhibition of GSK3- β and the subsequent activation of c-jun, which trans activates the *il-10* gene (Garcia et al., 2009). IL-27 induces

the development of IL-10 producing Tr1 from naïve T cells via induction of the AHR receptor that binds to c-maf and activates the IL-10 promoter (Apetoh et al., 2010).

1.8 Project aims

The overall aims of this project were:

1. To characterise the bovine IL-10 promotor for the presence of transcription factors binding site and evaluate the role of these factors in regulation of IL-10 expression.
2. To examine the *in vitro* effects of liver fluke homogenates on the functions of naïve bovine PBMCs in terms of proliferation, cytokines secretion and transcription factors expression.
3. To determine the ability of the FhTLM to bind to bovine receptors and induce IL-10 production via TGF- β pathway.
4. To measure the effects of serum IL-10 on the dairy production parameter (milk yield and lactation numbers) during *N. caninum* infection.

2 General Material and methods

2.1 Isolation of bovine genomic DNA from whole blood

Bovine blood from healthy female adult Holstein-Frisian cows was purchased from Sera Labs, UK. Genomic DNA was extracted from whole blood using a Wizard® Genomic DNA Purification kit (Promega, Madison, USA) following the manufacturer's instructions as per below:

30 ml of cell lysis solution was added to 10 ml of blood in a sterile 50 ml tube and mixed by inverting the tube 5-6 times. The mixture was incubated at room temperature for 10 min, the tube being inverted 2-3 times during the incubation period to aid lysis of the red blood cells. The suspension was centrifuged for 10 mins at 2000 rpm. The supernatant was discarded without disturbing the visible white pellet leaving around 1 ml of liquid. The cells were completely re-suspended by vigorous vortexing for 10-15 s, 10 ml of nuclei lysis solution was added to the cells, then mixed by pipetting 5-6 times to lyse the white blood cells. An additional 3ml of nuclei lysis solution was added and incubated at 37 °C, as recommended by the protocol, to lyse any clumps of cells remaining after adding the nuclei lysis solution. RNase was added at a final concentration of 20 µg/ml and the mixture incubated at 37 °C for 15 mins. 4 ml of protein precipitation solution was added and vortexed vigorously for 20 s and then centrifuged at 2000 rpm for 10 mins, a brown protein pellet became apparent. The supernatant was transferred to a new tube containing 10 ml isopropanol (100 %). The solution was mixed by inverting the tube until white thread-like strands of DNA became visible. The solution was then centrifuged at 2000 rpm for 1 min and the supernatant was removed. 10 ml of ethanol (70 %) was added to the DNA and mixed by inverting the tube several times to wash the DNA and the side of the tube. Then

ethanol was removed carefully and the tube was left opened for 15 mins to allow air drying of the DNA pellet. Finally, 800 µl of DNA rehydration solution was added to the DNA then incubated at 65 °C for 1 hr and stored at 4°C.

2.2 Isolation of Bovine peripheral blood mononuclear cells (PBMCs)

Heparinized bovine blood (500 ml) was purchased as above. PBMCs were isolated from blood by density gradient centrifugation over Histopaque® with a specific density of 1077g/ml (Sigma-Aldrich, Dorset, UK). An equal volume of sterile D-PBS (Sigma Aldrich, Dorset, UK) was added to whole blood and mixed. 15 ml of Histopaque 1077 was pipetted into 50 ml conical tubes. Then 30 ml of diluted blood was gently layered onto the histopaque and centrifuged at 400 g for 30 mins at room temperature (20 °C) with the brake off (deceleration zero). The white buffy layer of PBMCs which lay on the top of red blood cells were aspirated and washed with warm complete medium, then centrifuged at 400 g for 10 mins at room temperature. Then 10 ml of sterile ACK RBC lysis buffer was added to the PBMCs and incubated for 10 mins at room temperature. Afterward PBMCs were washed three times with complete medium. The viable number of PBMCs was assessed by counting the cells using the trypan blue exclusion method and a Neubauer counting chamber. Finally the cells were re-suspended at a concentration of 1×10^6 cells/ml in a 1:1 mix of complete medium and freeze mix medium and transferred to cryovial tubes which were stored at -80 °C. The vials then transferred to a liquid nitrogen tank for long-term storage.

2.3 Isolation of Monocytes (CD14⁺)

Following isolation of PBMCs by density gradient over Histopaque® (described above). Cells were suspended at a concentration of 10^7 /ml in complete media and centrifuged at 300 g for 10 mins. The supernatants were discarded and cells were re-suspended in 80 µl of MACS running buffer and 20 µl of paramagnetic microbeads conjugated with human CD14 (Catalog no. 130-050-201; Miltenyi Biotec, Bergisch-Gladbach, Germany) and incubated for 15 mins in darkness at 4 °C. Cells were then washed by centrifugation with 2 ml of MACS running buffer and re-suspended in 500 µl of MACS running buffer. Cells bound to Ab conjugated beads were isolated by positive selection on a MACS column (Miltenyi Biotec, Germany) placed in a magnetic field. Briefly, the columns were applied to a suitable magnetic field Macs separator and the column was rinsed with 3 ml of the MACS rinsing buffer. The cell suspension was loaded onto the column and the column was washed 3 times with MACS rinsing buffer and unlabelled cells were collected from the flow through. The column was removed from the magnet and placed in a new 15 ml tube and monocytes were collected by loading 5 ml of MACS running buffer onto the column and firmly pushing the plunger into the column.

To determine the purity of monocyte cell, flow cytometric technique with forward and side scatter was employed. 10 µl of FITC conjugated mouse anti bovine CD14 (CC-G33) or mouse IgG1 antibody (AbD Serotec, Oxfordshire, UK) were added to 10^6 monocyte in PBS and incubated for 30 mins on the orbital shaker at 4 °C in darkness. The cell suspension was then analysed using FACSCanto II analyser (BD Bioscience, California, USA).

2.4 Isolation of genomic DNA from bovine monocytes

The Wizard® Genomic DNA Purification kit (Promega, USA) protocol was used for isolation of monocyte genomic DNA in this study. Monocytes were isolated by positive selection (described in section 2.3) and washed with sterile PBS. 600 µl of nuclei lysis solution was then added to the cells and mixed by pipetting up and down to lyse the cells. 17.5 µl of 20 mg/ml proteinase K was added to the mixture and incubated at 55 °C for 3 hrs with vortexing every 1 hr. Following this, 3 µl of RNase solution was added to the nuclear lysate and mixed by inverting the tube several times and the mixture was incubated at 37 °C for 30 mins. The sample was then cooled to room temperature and 200 µl of protein precipitation solution was added and vortexed at high speed for 20 s. The sample was incubated on ice for 5 mins then centrifuged at 16000 g for 5 mins to precipitate the protein. Afterward the supernatant was transferred to a new tube and 600 µl of isopropanol was added and mixed until a white thread of DNA formed. The mixture was then centrifuged at 16000 g for 1 min and the supernatant removed. The DNA pellet was washed by adding 600 µl of ethanol (70 %) and centrifugation at 16000 g for 1 min. The supernatant was carefully removed and the DNA pellet exposed to air to dry. Finally, 100 µl of DNA rehydration solution was added to the pellet, incubated overnight at room temperature and the DNA stored at -20 °C for long term storage.

2.5 Gel electrophoresis analysis

agarose gel (1.5 %) was prepared by mixing 1.5 gram of agarose (Thermo Fisher Scientific, Leicestershire, UK) with 100 ml TAE buffer. Heating in microwave to dissolve, then allowing to cool for 5 mins, 6 µl of Nancy-520 (Sigma-Aldrich, UK) were added to the mixture which was poured into a casting tray and allowed to solidify for 50 mins. The tray was placed into the gel tank (Thermo Fisher Scientific, UK) and TAE buffer was added until the top of the gel was submerged. 24 µl of DNA sample was mixed with 4 µl of 6X loading dye and the mixture was loaded into one well of the gel adjacent to the 1 Kb plus DNA ladder (Thermo Fisher Scientific, UK). The gel was run at 110 volt for 60 mins. The gel was visualised under a UV illuminator and ImageQuant 300 (GE Healthcare, UK) was used for image capture.

2.6 Purification of DNA from agarose gel

DNA fragments were recovered from agarose gel by using Genelute™ Gel extraction kit (Sigma-Aldrich, Saint Louis, USA) as follows: DNA bands, visible under UV trans-illuminator, were excised using a sterile scalpel blade. The gel portions were weighed and 3 gel volumes of the gel solubilisation solution added to each tube. The mixture was incubated at 60 °C for 10 mins with vortexing throughout to completely dissolve the gel. A binding column for each reaction was prepared by placing the binding column G into a 2 ml collection tube. 500 µl of column preparation solution was added to each binding column and centrifuged at 15000 g for 1 min. The flow through was discarded and the binding

columns returned to the collecting tubes. After the gel slice was completely dissolved, 1 gel volume of isopropanol was added (100 %) to the tubes and mixed thoroughly. The dissolved gel mixture was then added to the binding columns and centrifuged for 1 min. The flow through was discarded and the binding columns returned to the collecting tubes. 700 µl of wash solution was added to the binding columns and centrifuged as above. The flow through was discarded and the binding columns returned to the collection tubes and centrifuged as above without wash solution. The binding tubes were then transferred to new collection tubes and 50 µl of elution solution added to the centre of the membrane of each tube and incubated for 1 min at RT. The tubes were centrifuged for 1 min and the through flow was collected and stored at -20 °C.

2.7 Preparation of competent DH5α *E. coli*

A stock of DH5α *E. coli* was a kind gift from (Dr. Allegrucci Cinzia; University of Nottingham). The chemical competent *E. coli* cells were prepared by growing a colony of plated cells in 10 ml LB broth (Invitrogen, UK) supplemented with Ampicillin (50 µg/ml) overnight at 37 °C with shaking. 5 ml of this overnight culture was inoculated into 200 ml LB broth with ampicillin and grown until an OD of 600 was obtained. After this, the cells were pelleted by centrifuging at 3500 rpm for 15mins at 4 °C. The supernatant was discarded and the cells re-suspended in 50ml of cold CaCl₂ (75 mM), incubated on ice for 35 mins and centrifuged as above. Afterward 3 ml of cold CaCl₂ was added to the cells and transferred to a flask containing 0.488 ml of glycerol (100 %) and swirled to mix. Cells

were left on ice for 1hr. Finally, 50 μ l of these cells were transferred to sterile 1.5 ml Eppendorf tubes and stored at -80 °C for future use.

2.8 Transformation of plasmid DNA to DH5 α competent cells

E. coli were removed from -80 °C and thawed on ice. Each ligation reaction was added to a separate tube containing 50 μ l of competent cells and mixed gently by tapping. The tubes were incubated on ice for 30 mins. The reactions were incubated for 30 s on a heat block adjusted to 42 °C prior to returning to ice. 250 μ l of warm super optimal broth with catabolite repression (SOC) medium (Sigma-Aldrich, USA) was added to each vial under aseptic conditions. The vial was transferred to a shaking incubator and incubated at 37 °C and 225 rpm for 1 hr. Subsequently 200 μ l of each mixture was plated onto an LB agar plate containing Ampicillin (50 μ g/ml), 40 μ l Xgal (20mg/ml) and 4 μ l of IPTG (200 mg/ml). Plates were incubated at 37 °C overnight.

2.9 Peripheral blood mononuclear cells culture and stimulation

For cytokine measurement and lymphocyte proliferation, PBMCs were suspended in complete medium and the cell number adjusted to 1×10^6 cells/ml. 200 μ l of PBMCs were cultured in triplicate in 96 well cell culture plates (Thermo Fisher Scientific, UK). Cells were incubated at 37 °C for 72 h at CO₂ (5 %) under the following conditions:

- A. without addition of ConA (Alpha Diagnostic International, Texas, USA) and LFH (negative control)
- B. stimulation with ConA (5 µg/ml)
- C. stimulation with LFH (20 µg/ml)
- D. stimulation with recombinant FhTLM (200 ng/ml)
- E. stimulation with ConA + LFH
- F. stimulation with ConA + FhTLM

Following incubation the supernatants were removed following centrifugation of the plates at 300 g for 10 mins to remove cell debris and stored at -20 °C until cytokine measurement.

2.10 Cytokine measurement

2.8.1 IL-10

The concentration of IL-10 in cell culture supernatants was measured using commercially available bovine IL-10 ELISA kit (Cusabio, Wuhan, Hubei Province, China) as per manufacturer's instruction. Briefly, all reagents and samples were brought to room temperature prior to use. The plate provided in the kit was pre-coated with a polyclonal goat –anti-rabbit IL-10 antibody. 50 µl of samples and standard were added in triplicate to the plate wells. The plate was washed 3 times with wash buffer, 20X stock supplied, and dried by tapping against tissue paper. 50 µl of horseradish peroxidase enzyme HRP-conjugate were then added to each well, and then 50 µl of detection antibody and the plate sealed and incubated at 37 °C for 1 hr. The plate was washed 3 times and dried by tapping against tissue paper. 50 µl of substrate solution A and 50 µl of substrate solution B were

added to each well and the plate was incubated for 15 mins at 37 °C in the dark. Finally, 50 µl of stop solution was added to all well and the optical density (OD) was measured at 450 nm using a micro-plate reader (LT-4000, Labtech, UK). The OD of the all samples was blank corrected: (average OD samples-average OD negative control). A regression line was calculated using bovine IL-10 standards ranging from zero to 1000 pg/ml (Figure 2.1).

The concentration of IL-10 from each test samples was determined from the standard curve using the following equation: $Y=281.65x^2-57.95x+0.2393$ ($R^2=0.9986$).

The sensitivity of the assay was 2.5 pg/ml and results were expressed as pg/ml.

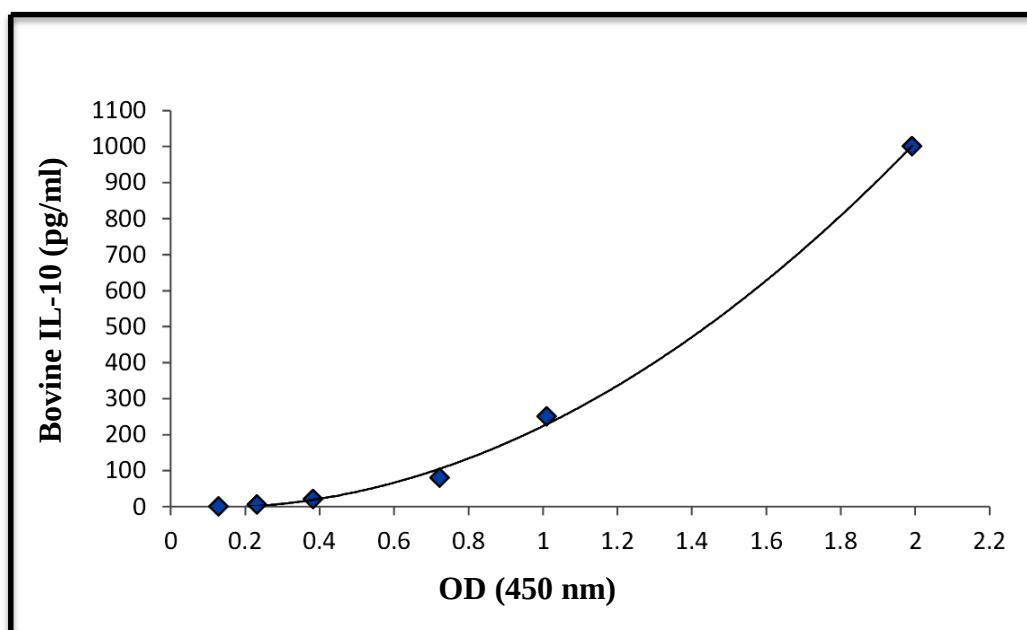


Figure 2.1: Standard curve for Bovine IL-10 ELISA.

Mean OD values determined at various concentrations of IL-10 (0, 5, 20, 80, 250, and 1000 pg/ml).

2.8.2 IFN- γ

IFN- γ from cell culture supernatant was measured using the Bovine IFN- γ ELISA kit (Thermo Fisher scientific, UK) as per manufacturer's instructions. 100 μ l of anti-bovine IFN- γ coating antibody diluted 1:100 in carbonate-bicarbonate buffer were added to each well of 96 well plates, which were sealed and incubated overnight at room temperature. The plates were washed three times after each step using TweenTM-20 (0.05 %) in D-PBS (pH 7.4). Then, the plate was blocked with 300 μ l of BSA (4 %) in D-PBS (pH 7.4), sealed and incubated for 1 hr at room temperature. After washing, the supplied standard was reconstituted with reagent diluent to prepare a top standard of 2000pg/ml. This was serially diluted 1:2 to prepare a range of standards from 2000 to 15.6 pg/ml. 100 μ l of standards and samples were added in duplicate to the plate and incubated for 1 hr at room temperature. Following this, 100 μ l of 1:100 diluted detection antibodies were added to each well and incubated for 1 hr at room temperature. Then, 100 μ l of 1:400 diluted streptavidin-HRP were added to each well and incubated for 30 mins at room temperature. After washing, 100 μ l of TMB substrate were added to each well and the plate incubated in darkness for 20 mins at room temperature. The reaction was stopped by adding 100 μ l of stop solution to each well. Absorbance was measured at 450nm with reference wavelength of 550nm using micro-plate reader (LT-4000, Labtech, UK). The OD of the all samples was blank corrected: (average OD samples –average OD negative control). A regression line was calculated using bovine IFN- γ standards ranging from 15.6 to 2000 pg/ml (Figure 2.2). The concentration of IFN- γ from each test samples was determined from the standard curve using the following equation: $Y=233.31x^2+319.35x+35.536$ ($R^2=0.9974$). The results were expressed as pg/ml.

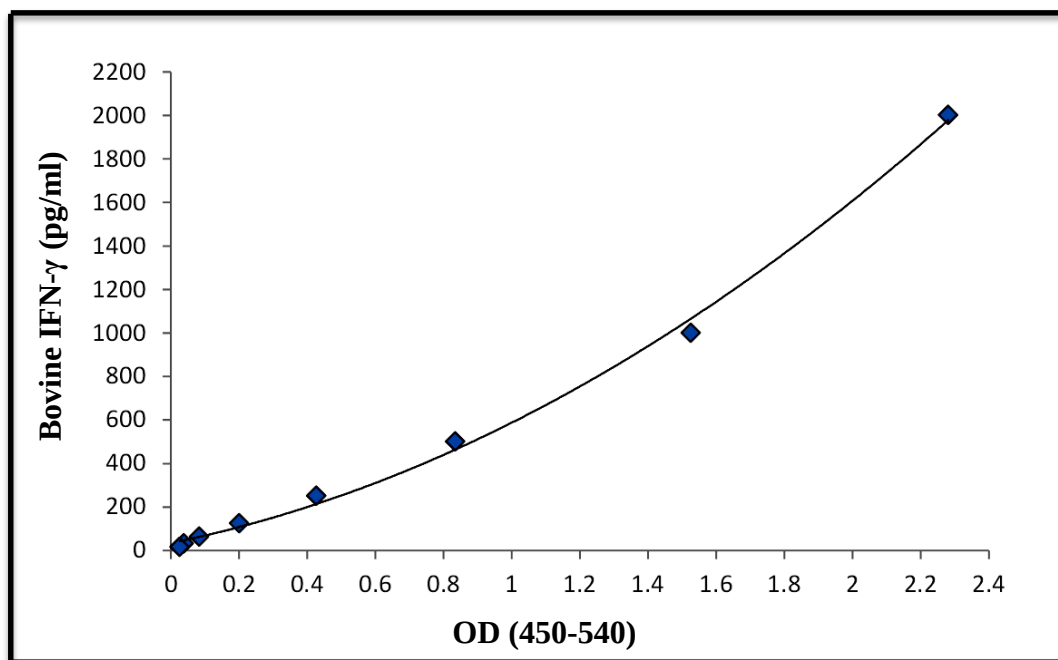


Figure 2.2: Standard curve for Bovine IFN- γ ELISA.

Mean OD values determined at various concentrations of IFN- γ (15.6, 31.25, 62.5, 125, 250, 500, 1000 and 2000 pg/ml).

2.11 Restriction enzyme digestion

For all endonuclease digests, Promega enzymes and a generic protocol were used as per below: In a sterile PCR tube the following were mixed

Table 2.1: Restriction digestion reaction

Component	Volume
Sterile deionised water	16.3 μ l
Restriction enzyme buffer 10X	2 μ l
Acetylated BSA (10 μ g/ μ l)	0.2 μ l
DNA (1 μ g/ μ l)	1.0 μ l
Restriction enzyme (10u/ μ l)	0.5 μ l
Final volume	20 μ l

The reaction mixture was mixed and centrifuged at 4000 g for 30 s. Afterward, this was incubated at enzyme optimum temperature for 1.5 hrs.

3 Regulation of bovine IL-10 and its role in helminth immune responses

3.1 Introduction

Cattle infected with *F. hepatica* show a polarized Th2 immune response dominated by eosinophilia, IL-4 and IgG1 production (Brown et al., 1994a, Bossaert et al., 2000). Others have found the presence of AAM Φ during *F. hepatica* infection (Donnelly et al., 2005, Flynn et al., 2007a). This type of macrophage is associated with high levels of IL-10 and expression of the mannose receptor (Gordon, 2003). These findings indicate that liver fluke infection modulates the host immune response toward a Th2 response with production of high levels of IL-10. This would appear to be a critical strategy that allows the survival of the parasite while maintaining the health of infected host for long periods. This is evidenced in vaccine studies that show that susceptibility of cattle to *F. hepatica* infection is correlated with a Th2-dominated immune response and the protection is associated with Th1 response (Mulcahy et al., 1999).

Several studies have demonstrated that IL-10 was upregulated during *F. hepatica* infection (Moreau et al., 1998, Cervi et al., 2001, Flynn and Mulcahy, 2008b, Hacariz et al., 2009a, Mendes et al., 2013). IL-10 is a pleiotropic cytokine produced by a variety of cells such as T-cells, B-cells, monocytes, macrophages, dendritic cells, epithelial cells and keratinocytes (Fiorentino et al., 1989, Moore et al., 1993). It has a dichotomous function by acting as an immunosuppressive and immune-stimulatory cytokine during infections. Primarily IL-10 functions to inhibit the proliferation of antigen-induced Th1 cells in the presence of macrophages, and suppress the production of IFN- γ by Th1 clones stimulated by dendritic cells (Macatonia et al., 1993). The suppression of Th1 is mediated by the inhibition of IL-12 and IFN- γ from macrophages and natural killer cells, respectively (Mosmann and

Moore, 1991, Moore et al., 1993). Also IL-10 has been shown to reduce the expression of MHC class I on the surface of cytotoxic T cells (Matsuda et al., 1994) and MHC class II (de Waal Malefyt et al., 1991b) and the co-stimulatory molecules CD80 and CD86 on monocytes and macrophages (Fiorentino et al., 1991b). Thus, IL-10 regulates immune effector cells and modulates inflammation to limit host tissue damage by multiple pathways during the immune response. During infection IL-10 is known to ameliorate the excessive Th1 response and the subsequent immunopathology related to infection with *Trypanosoma cruzi* (Hunter et al., 1997), *Toxoplasma gondii* (Gazzinelli et al., 1996) and *Mycobacterium tuberculosis* (Bekker et al., 1998). It can also regulate the Th2 response and prevent pathology associated with *S. mansoni* induced fibrosis (Wynn, 2004).

In helminth infection, the role of IL-10 in controlling the host-parasite immune response has been examined by several workers. For example, the *in vitro* role of IL-10 in controlling the *F. hepatica* specific IFN- γ production from peripheral blood mononuclear cells (PBMCs) of infected cattle has been demonstrated (Flynn and Mulcahy, 2008b). IL-10 is also known to regulate the allergic response accompanying helminth infection (Wohlleben et al., 2004). Moreover, over production of IL-10 may mediate susceptibility to some protozoan parasite such as *Leishmania donovani* and *T. cruzi* (Reed et al., 1994, Murphy et al., 2001, Roque et al., 2007). Therefore, understanding the mechanisms that regulate IL-10 secretion is important to clarify its function during parasitic infection. The regulation of IL-10 expression varies among different species and within individuals in the same species, these differences are crucial in determining the outcome of any inflammatory response (Marincola, 2006). The majority of studies on the molecular regulation of IL-10 expression have been carried out using monocyte/macrophages lineage cells as a

predominant cellular source for IL-10 secretion following LPS stimulation *in vivo* and *in vitro* (de Waal Malefyt et al., 1991a).

In humans, it has been shown that polymorphisms in the IL-10 promoter region have an influence on the transcription rate, and thus production, of this cytokine (Eskdale et al., 1997, Turner et al., 1997, Kube et al., 2001). Confirmation of this is seen when the expression level of IL-10 has been correlated to the severity of rheumatoid arthritis (Hajeer et al., 1998), cancer (Kim et al., 1995) and schistosomiasis (Carneiro-Santos et al., 2002).

Regulation of IL-10 expression occurs at both transcriptional and post-transcriptional levels. Post-transcriptional mechanisms include the 3' untranslated region (UTR) providing stability and delaying the decay rate. The 5' UTR allows for binding to the 40s ribosomal subunit and both the length and structure of the UTR will influence the expression of IL-10 (Forte et al., 2009).

At the transcriptional level, the role of Sp1 in activation of the IL-10 promoter in macrophages stimulated by LPS has been examined (Brightbill et al., 2000). The requirement of Sp3 for IL-10 transcription was confirmed by luciferase reporter assay using *Drosophila* SL2 cells (Tone et al., 2000). By using gel shift assay, C/EBP was found to be responsible for LPS-induced IL-10 expression in murine macrophages (Liu et al., 2003). knockdown of c-Maf significantly inhibits IL-10 protein synthesis and mRNA expression in macrophages stimulated with LPS (Cao et al., 2005). In humans IFN- α induces IL-10 expression via IFN regulatory factor (IRF) 1 and signal transducer and activator (STAT) 3 (Ziegler-Heitbrock et al., 2003, Benkhart et al., 2000). In human schistosomiasis differences in the levels of IL-10 produced by cells during the intestinal and hepato-splenic form of disease is related to the levels of p38 MAP kinase and the activation of

transcription factor 2 (ATF2) (Carneiro-Santos et al., 2002). GATA3 plays a major role in the regulation of IL-10 expression in murine Th2 cells (Nawijn et al., 2001). The presence of GATA3 within the IL-10 locus switches the locus into a transcriptionally active state and directly upregulates the transcription of the *il-10* gene in mice (Shoemaker et al., 2006).

Parasitic infection is associated with the release of immunomodulatory molecules which differ among parasite species, this include hypodermin A of *Hypoderma bovis* (Nicolas-Gaulard et al., 1995), onchocystatin of *Onchocerca volvulus* (Schonemeyer et al., 2001), ESP of *F. hepatica*, including CL1 (Carmona et al., 1992), GST and peroxiredoxin (Prx) (Goose, 1978).

During fascioliasis several mechanisms of host immunomodulation have been investigated, such as modulation of circulating lymphocyte and splenocyte proliferation (Poitou et al., 1993). Suppression of the proliferative response of PBMCs to mitogen post infection has been reported in lambs infected with *F. hepatica* (Zimmerman et al., 1983). In cattle experimentally infected with *F. hepatica*, fluke antigen was able to increase PBMC proliferation and increase IL-2 levels during the first 4 weeks post-infection (Oldham and Williams, 1985). The proliferative response of rat splenocytes and thymocytes to Concanavalin A (ConA) or Pokeweed mitogen (PWM) or different fluke antigens such as metacercariae antigen or ES from adult fluke increased during the second week of infection (Poitou et al., 1992). As infection progresses, the bovine host response changes from an IFN- γ led response with lymphocyte proliferation during the first 4 weeks of infection, to one where IL-4 dominates before parasite-specific lymphocyte proliferation declines and the regulatory cytokines IL-10 and TGF- β dominate at 12 week post infection (Flynn & Mulcahy 2008b). In contrast, the proliferation of ovine PBMCs stimulated with the mitogen

ConA was inhibited in cultures treated with ES in a dose-dependent manner (Moreau et al., 2002). In sheep infected with *F. hepatica*, IFN- γ production in the culture supernatant of PBMCs stimulated with *F. hepatica* ESP increased during the first two week of infection while IL-10 secretion from monocytes remained high across the first six week of infection. Circulating monocyte numbers were reduced after 35 day post infection (Moreau et al., 1998). Following experimental *F. hepatica* infection of cattle, the highest proliferation index and IFN- γ levels were observed in PBMCs stimulated with ConA during 2 to 5 week post infection (Clery and Mulcahy, 1998).

A greater understanding of the regulatory mechanisms of bovine IL-10 and its function in response to *F. hepatica* antigen in naïve animals are required if effective vaccines capable of overcoming immunomodulation are to be designed. In particular, an understanding of which transcription factors drive IL-10 expression in naïve lymphocytes to *F. hepatica* antigens is essential.

3.2 Material and Methods

3.2.1 Isolation of Bovine IL-10 promotor fragment

The fragment flanking the bovine *il-10* gene 5' sequence from whole blood and monocytes was obtained and cloned using GenomeWalkerTM Universal kits (Clontech, USA) as per below.

3.2.1.1 Isolation of genomic DNA

Bovine gDNA was isolated from blood and monocytes as described in section 2.1 and 2.4 respectively.

3.2.1.2 Quality of genomic DNA

To check the size of genomic DNA, 20 µl of purified DNA was analysed by gel electrophoresis on a agarose gel (1.5 %) using Nancy-520 dye (Sigma -Aldrich, UK) at 100 V. 1kb plus DNA ladder (Thermo Fisher Scientific, UK) was used to approximate the band size, and images were captured using an ImageQuant 300 (GE Healthcare, UK). The concentration of DNA was measured using Thermo scientific Nanodrop 8000 and OD ratios at 260 nm and 280 nm were recorded.

3.2.1.3 Digestion of DNA

For construction of a DNA library, five digestion reactions were set up for each sample of gDNA. Four blunt end digestions using Dra I, EcoRV, Pvu II and Stu I restriction enzymes were used to digest gDNA, and one positive control digestion using Pvu II and human gDNA.

For each reaction tube the following reagents were mixed:

25 µl of gDNA (0.1 µg/µl), 8 µl of restriction enzyme (10 units/µl), 10 µl of restriction enzyme buffer (10X) and 57 µl of deionized water.

These mixtures were incubated at 37 °C for 2 hrs and vortexed at slow speed for 10 s then returned to 37 °C overnight.

3.2.1.4 DNA purification

100 µl of phenol (Sigma Aldrich, USA) was added to each digestion reaction, prior to centrifugation at 12000 g for 1 min to separate the aqueous and organic layers. Then the upper aqueous layer was transferred to a new tube and the lower organic layer was discarded. 100 µl of chloroform (Sigma Aldrich, USA) was added to each tube which was centrifuged as before, then the upper layer was transferred to a new tube the lower layer discarded. 200 µl of cold ethanol (95 %), 10 µl sodium acetate (3M), and 20 µg of glycogen (Invitrogen, USA) was added to each reaction tube which was then centrifuged for 15 mins at 14000 g. The supernatants were removed and the pellets washed by addition of 100 µl of cold ethanol (80 %) and centrifugation at 14000 g for 10 mins. The supernatants were

discarded and the tubes left opened to dry. Finally, 20 µl of TE buffer was added to each tube and mixed to re suspend the purified DNA.

3.2.1.5 Ligation of DNA to genome walker adaptors

4 µl of purified DNA was transferred to a new tube. Then, to each tube the following was added: 1.9 µl genome walker adaptor (25 µM), 1.6µl ligation buffer (10X), and 0.5 µl T4 DNA ligase. This was incubated at 16 °C overnight. The reaction was stopped by incubation at 70 °C for 5 mins. Finally 72 µl of TE added to each tube and mixed for 15 s.

3.2.1.6 Primer design

A pair of reverse nested PCR primers, GSP1 and GSP2 (Table 3.1) was designed based on the DNA sequence upstream to the translation initiation site (ATG) of the bovine IL-10 gene region located on the chromosome 16 (AC_000173.1) and amplifies in a 5' direction. Primers were synthesised by Sigma-Aldrich. The GenomeWalker™ Universal kit includes two adaptor primers, AP1 and AP2 (Table 3.1) which amplify the adaptors ligated to the both ends of genomic DNA fragments to create a GenomeWalker library. Also, two positive control primers, PC1 and PC2 (Table 2) included in the kit to amplify exon 1 of the tissue-type plasminogen activator (tPA) from the positive control human gDNA (Table 3.1).

Table 3.1: Primers used for the amplification of bovine IL-10 promoter

Primer name	Sequence (5'-3')
GSP1(Gene specific primer 1)	CCCCTCCCTTCCCTAGACCTCTCCAGCTTT
GSP2(Gene specific primer 2)	AACTCTTCAGGCCTCAGTTTCCCCAGAA
AP1(Adaptor primer 1)	GTAATACGACTCACTATAGGGC
AP2(Adaptor primer 2)	ACTATAGGGCACGCGTGGT
PCP1(positive control primer 1)	AGAAACCCGACCTACCACGGCTTGCTCCTT
PCP2(positive control primer 2)	CCCTTTCCTCGCAGAGGTTTTCTCTCCAGC

3.2.1.7 PCR amplification

The protocol for gene walking amplification includes constructing eight primary and eight secondary PCR reactions: four experimental templates, two positive controls and two negative controls.

For the first round PCR amplification a primary PCR master mix for all eight reactions was prepared as follows:

Table 3.2: PCR master mix for the genome walking amplification

Component	Per reaction	Per 8 reaction
PCR grade water	40 μ l	320 μ l
10X PCR Buffer (15Mm Mgcl ₂)	5 μ l	40 μ l
dNTP (2 mM each)	1 μ l	8 μ l
AP1 (10 μ M)	1 μ l	8 μ l
Taq DNA polymerase (Qiagen GmbH)	1 μ l	8 μ l
Total volume	48 μ l	384 μ l

To each of the eight tubes 48 μ l of primary PCR master mix was added. Then 1 μ l of GSP1 (Table 3.1) was added to each tube from 1 to 5, and 1 μ l of PCP1 (Table 3.1) to tubes from 6 to 8. To each tube from 1 to 4, 1 μ l of each gDNA library was added. To the positive control number 1, 1 μ l of control human genomic DNA (0.1 μ g/ μ l) added, and 1 μ l of pre-constructed control library added to the positive control number 2. 1 μ l of H₂O was added to the both negative control tubes.

The reaction was mixed briefly and a typical PCR cycle was followed; this consisted of a two-step cycle as follows:

- A. 12 cycles of 25 s at 94 °C and 3 mins of 72 °C
- B. 37 cycles of 25 s at 94 °C and 3 mins at primer annealing temperature 65 °C
- C. 1 cycle of 10 mins final extension at 72 °C.

For the second PCR reaction the same protocol as above was used for preparation of the master mix, using the AP2 instead of AP1 primer. 1 μ l of 1:50 diluted primary PCR product

was used as a nested PCR template. GSP2 and PCP2 (Table 3.1) were used in place of GSP 1 and PCP1. The PCR reaction was performed using the following parameters:

- A. 10 cycles of 25 s at 94 °C and 3 mins of 72 °C
- B. 25 cycles of 25 s at 94 °C and 3 mins at primer annealing temperature 65 °C
- C. 1 cycle of 10 mins final extension at 72 °C.

After each PCR round 20 µl of the PCR product was analysed by electrophoresis on a agarose gel (1.5 %) (as above).

3.2.1.8 Gel purification

Upon completion of the 2nd stage PCR the amplified IL-10 gene fragments were purified from the agarose gel using GeneluteTM Gel extraction kit (Sigma-Aldrich, USA) as in 2.6

3.2.2 Blunt end cloning

The IL-10 promotor sequence from nucleotides (nt) -550 to -1120 relative to the translation initiation site (+1) was PCR amplified (3.2.1.7) and inserted into a pGEM-T vector (Promega) between the SacII and SpeI site as per bellow:

3.2.2.1 Ligation of the DNA with the pGEM-T vector

A ligation reaction for all purified DNA samples and one for the positive control was set up as follows:

Table 3.3: Ligation mixture for pGEM-T vector

Components	Standard reaction	Positive control
2X rapid ligation buffer	5 µl	5 µl
pGEM-T vector	1 µl	1 µl
PCR products	X µl	
Control DNA		2 µl
T4 DNA ligase	1 µl	1 µl
Nuclease free water to final volume	10 µl	10 µl

A 3:1 ratio of PCR insert to vector was used and the volume of the inserts was calculated by using the following equation:

$$\frac{ng\ vector \times Kb\ insert}{3.0\ Kb\ vector} \times \frac{3}{1} = ng\ insert$$

The final volume was adjusted to 10 µl with dH₂O and the reaction was mixed by pipetting and incubated at 4 °C overnight.

3.2.2.2 Transformation of plasmid DNA to DH5α competent cells

Each ligation reaction were transformed to the competent E. coli as described in (2.8) section.

3.2.2.3 Examination of plate for identification of positive insertion

The positive recombinants (yellow colonies) were identified through an anti-Ampicillin selection (blue/white screening). Single positive colonies were harvested by using sterile tips and grown in 10 ml of LB broth containing ampicillin (50 µg/ml), in a shaker incubator at 37°C and 225 g overnight.

3.2.2.4 Purification of plasmid DNA from recombinant *E. coli* culture

Purification was done using GenElute™ Five-Min Plasmid Miniprep Kit (Sigma-Aldrich, USA) instructions. Briefly, 1 ml of overnight culture was centrifuged for 5 mins at 400 g , 600 µl of supernatant were removed and 40 µl of cooled lysis reagent was added to 400 µl of the cell suspensions and mixed thoroughly, this was then incubated at room temperature for 2 mins. The binding column was prepared by placing into the collection tubes and adding 500 µl of column preparation solution to each tube. The tubes were centrifuged for 10 s at 12000 g and the flow through was discarded. Afterward, 400 µl of binding solution was added to each lysate reaction and mixed briefly. This mixture was transferred to the binding column, centrifuged at 12000 g for 20 s and the flow through discarded. After this, 700 µl of wash solution was added to each column and centrifuged as above. The flow through was discarded and the washing repeated by adding 200 µl of wash solution and re-centrifuged for 30 s for effective washing and drying of the column. Finally, the binding column transferred to a new collecting tube and 400 µl of elution solution was added to the surface of the filter. The column was centrifuged for 30 s at 12000 g and eluted plasmid DNA was collected and stored at -20°C.

3.2.2.5 Validating insertion of plasmid DNA

BstZI restriction digest (Promega, UK) were used to cleave the plasmid fragment from the pGEM-T vector. In a sterile tube (one for each sample) the following were mixed:

Table 3.4: BstZI digestion reaction mixture

Components	Volume
RE 10X Buffer	2 μ l
Acetylated BSA (10 μ g/ μ l)	0.2 μ l
DNA (1 μ g/ μ l)	1 μ l
BstZI Enzyme (10 u/ μ l)	0.5 μ l
Sterile deionized water	16.3 μ l
Final volume	20 μ l

The reactions were then mixed well by pipetting and incubated at 50 °C for 1h. Finally, 20 μ l from each sample were analysed on agarose/Nancy gel (1.5 %) to visualize the DNA bands.

3.2.3 DNA sequencing and sequence analysis

The entire sequence of the positive recombinant plasmids were determined using Sanger sequencing (Source Bioscience Lifescience, Nottingham, UK). T7F and SP6 specific primers were used for amplification and sequencing of plasmids.

After receiving the sequence results, it was checked for vector contamination by blasting against the pGEM-T vector sequence (<https://www.promega.com/~media/files/vector%20sequences/t%20vectors/pgemt.txt?la=en>). Then recombinant bovine IL-10 sequences were blasted against the DNA sequence upstream to the ATG of bovine IL-10 gene region located on the chromosome 16 (AC_000173.1) using (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Sequences with a high similarity to the bovine IL-10 promoter were then used as a reference for alignment against IL-10 clone from monocyte sequences using the same software (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). To detect nucleotide polymorphisms between the whole blood and monocyte, Geneious 7.0.5 software was used. Putative transcription factor binding site (TFs) were assigned by using TFSEARCH ver.1.3 software from Yutaka Akiyama 1995 (Kyoto Univ.). The sites of the polymorphisms were detected and checked for the presence of transcription factor binding sites and whether it makes differences in the scoring scheme.

3.2.4 Lymphocyte proliferation assay

For measuring percentage PBMCs proliferation in response to stimulation with LFH, alamarBlue (Thermo Fisher Scientific, UK) was used as per below:

PBMCs were suspended in complete medium and the cell number adjusted to 1×10^6 cells/ml. 200 μ l of PBMCs suspension was added to wells of 96 well cell culture plates (Thermo Fisher Scientific, UK) in triplicate. Cells were incubated for 72 h at 37 °C and CO₂ (5 %) in air under the following condition:

- A. without addition of ConA and LFH (negative control)
- B. stimulation with ConA (5 μ g/ml)

C. stimulation with LFH (20 µg/ml)

D. stimulation with ConA+ LFH

20 µl of alamarBlue reagent was added to each well of the 96 well plate and incubated for 4 h at 37 °C in dark after the experimental period. Thereafter the absorbance was measured at 540 nm minus 630 nm using an LT-4000 plate reader.

The percentage reduction in alamar blue represented the percentage proliferation of PBMCs and calculated using this equation:

$$\% \text{ Proliferation} = \frac{(\text{sample OD} - \text{control PBMCs OD})}{\text{control PBMCs OD}} \times 100$$

3.2.5 Detection of cytokines production in PBMCs by ELISA

The level of IFN-γ and IL-10 in culture supernatant of PBMCs stimulated as described in 2.7 were measured using commercially available ELISA kit (2.8).

3.2.6 Immunofluorescence assay

PBMCs were suspended in complete media at 1×10^6 /ml and 200 µl aliquots of cells were grown in 24 well cell culture plate (Thermo Fisher Scientific., UK) containing glass cover slips. Cells were stimulated with ConA (5 µg/ml), LFH (20 µg/ml) and the combination of ConA and LFH or without stimulation. The plate was incubated for 72 hrs at 37 °C and CO₂ (5 %) and centrifuged at 300 g for 10 mins following incubation to allow collection of cells on the cover slips. The medium was removed and cells were washed three times with PBS. PBMCs were then fixed in paraformaldehyde (4 %) for 15 mins at room temperature and

washed with PBS. Afterward, cells were permeabilized for 10 mins at room temperature with Triton X-100 (0.5 %) in PBS and washed with PBS. Blocking was with BSA (3 %) in PBS for 45 mins at room temperature. Cells were incubated for 1hr at room temperature with a 1:100 dilution of polyclonal rabbit anti-human GATA1 H-200 (Santa Cruz Biotechnology, INC). After extensive washing with PBS, the cells were incubated for 1 hr at room temperature in the dark with 1:1000 dilution of donkey anti-rabbit IgG-FITC (Alpha Diagnostic International). Slides were then washed with PBS, mounted with Vectashield (Vector Labs Ltd., UK) containing 4',6-diamidino-2-phenylindole (DAPI) staining reagent. Images were captured using Leica DM500B microscope and DFC 350FX camera (Leica Microsystem Ltd., UK) using X40 and X63 magnification.

3.2.7 Statistical analysis

PBMCs proliferation results were expressed as percentage of proliferation increase in response to each treatment. Results were graphed and data analysed using GraphPad prism version 6.0 software. One-way ANOVA was used for analysis and the significant differences between control and each treatment were determined using Dunnett's multiple comparison test. All bars represent mean values $\pm$ SDM of two independent experiments.

3.3 Results

3.3.1 Construction of Genome walker library

Four restriction enzymes (Dra I, EcoR V, Pvu II, Stu I) were used for construction of genome walker libraries and for the positive control library Pvu II used to digest human genomic DNA supplemented in the kit. This step was repeated for all gDNA obtained from whole blood and monocytes. Following visualization on agarose gel (1 %), smears were found in all five lanes which indicate complete digestion of the DNA in each library (Figure 3.1).

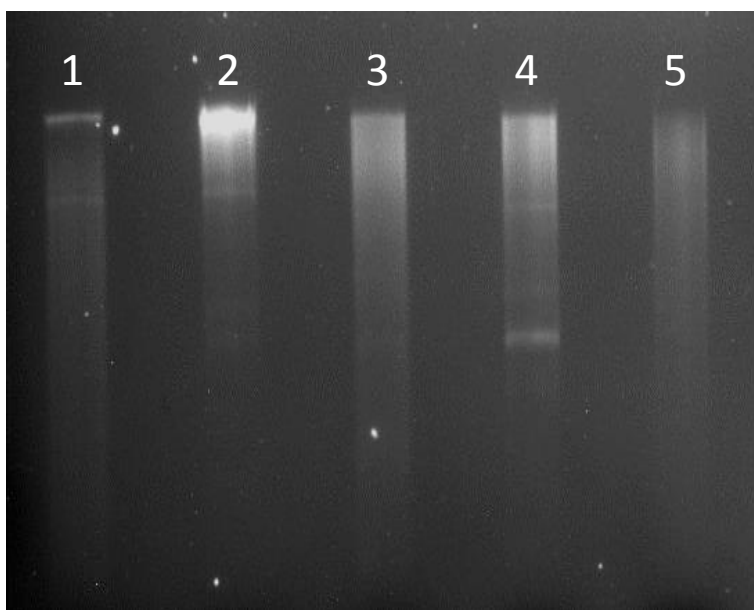


Figure 3.1: Agarose gel (1 %) of Bovine gDNA digestion. Lane 1 shows gDNA digested with Dra I. Lane 2 shows gDNA digested with EcoR V. Lane 3 shows gDNA digested with Pvu II. Lane 4 shows gDNA digested with Stu I. Lane 5 shows positive control human gDNA digested with Pvu II.

3.3.2 Genome walking PCR amplification of bovine IL-10 promoter using gDNA from whole blood as template

The results of the first and second gene walking PCR amplification obtained are shown below in figure 3.2. One distinct band was visible among the four digested gDNA libraries.

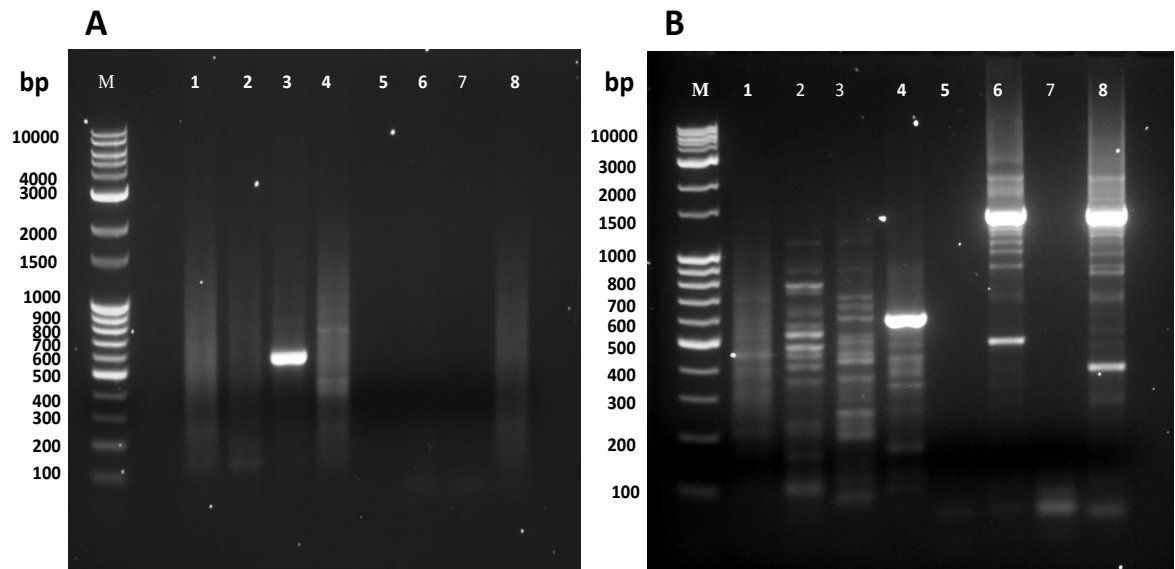


Figure 3.2: Agarose gel (1.5 %) demonstrating the first (A) and second (B) round genome walking PCR results of bovine IL-10 from whole blood. Lane M: 1Kb plus DNA ladder. The first round PCR shows 600 bp PCR products in lane 3. The second round PCR shows multiple PCR products with different sizes and a visible 650 bp product in lane 4. Lane 5: negative control (1); Lane 6: positive control (1); Lane 7: negative control (2); Lane 8: positive control (2).

3.3.3 BstZI restriction digestion for validating the insertion of IL-10

As shown above in figure 3.2, the distinct visible band (650 bp) from the *Stu* I library (lane 4) was gel purified and subsequently cloned into the Promega pGEM-T vector using blunt end cloning and ligation. Ligated vector/PCR product was used to transform DH5 α *E. coli* cells over night before positive colonies were picked, sub cultured in LB broth and screened for insertion by digestion with BstZI (Promega). Our digestion indicated that ligation and transformation had occurred (Figure 3.3).

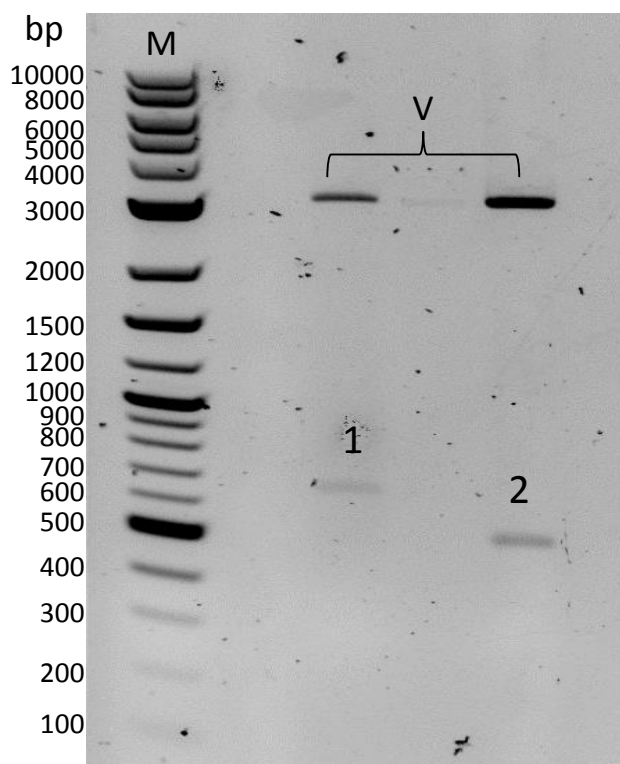


Figure 3.3: Agarose gel (1.5 %) of the BstZI digestion of the bovine IL-10 from whole blood. Lane M: 1Kb plus ladder. V: pGEM-T vector. 1: 650 bp gDNA insert. 2: 500 bp positive control insert.

The purified plasmid DNA was sent for sequencing at (Source Bioscience, Lifescience), when results were received, sequences were checked for vector contamination prior to blasting against the NCBI database of IL-10 promoter and only 570 bp of the cloned IL-10 plasmid showed 100 % similarity.

3.3.4 Monocyte purity

Following isolation of monocytes by MACS, the purity of these cell populations was analysed by measuring the percentage of CD14 positive cells via flow cytometry using FL-1 (FITC) channel (Figure 3.4).

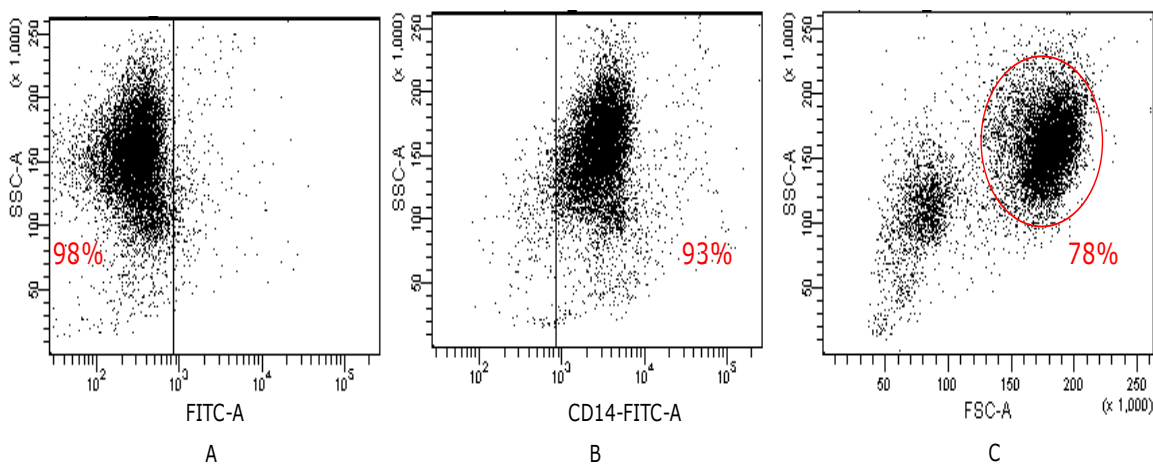


Figure 3.4: Percentage of bovine monocyte cells using flow cytometry analysis.

(A): Isotype control with no CD14 expression. (B): Shifting the dot plot to the right side showing 93 % CD14 positive cells incubated with FITC conjugated anti-CD14 Ab. (C): Analysis of monocyte using the forward FSC and side SSC scatter shows that the isolated cells population contained > 78 % monocytes.

3.3.5 Gene walker PCR amplification of bovine IL-10 promoter using gDNA from monocytes as template

The results of the first and second genome walking PCR amplification of monocyte DNA, are shown below in figure 3.4. Several distinct band (150, 200, 1000 and 1200 bp) were visible in lane 2.

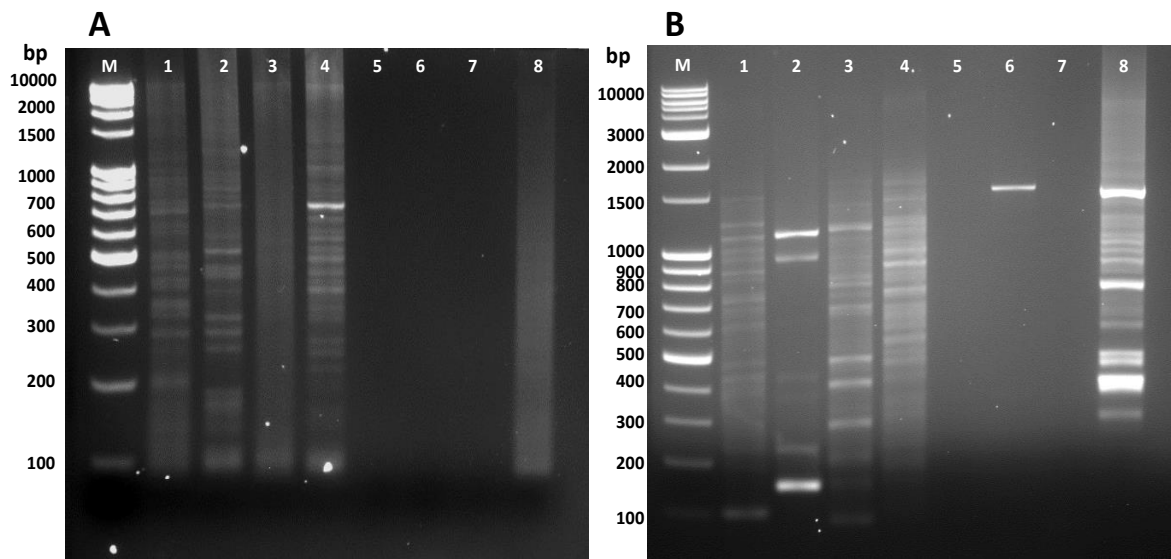


Figure 3.5: Agarose gel (1.5 %) demonstrating the first (A) and second (B) round genome walking PCR of bovine IL-10 from monocyte. Lane M: 1Kb plus DNA ladder; Lane 1; 2; 3 and 4 with different size bovine IL-10 amplicons. Lane 5: negative control (1); Lane 6: positive control (1); Lane 7: negative control (2); Lane 8: positive control (2).

3.3.6 BstZI restriction digestion for validating the insertion of IL-10

As shown above in figure 3.5, the distinct visible band from the second PCR: 100 bp from the Dra I library (lane 1); 150, 200, 1000 and 1200 bp from the EcoRV library (lane 2) were gel purified and subsequently cloned into the pGEM-T vector using blunt end cloning and ligation. Ligated vector/PCR products was used to transform to DH5 α *E. coli* cells over night before positive colonies were picked, sub cultured and screened for insertion by digestion with BstZ I enzyme. Our digestion indicated that ligation and transformation had occurred in three samples (150, 200 and 1200 bp) from EcoRV library (Figure 3.5).

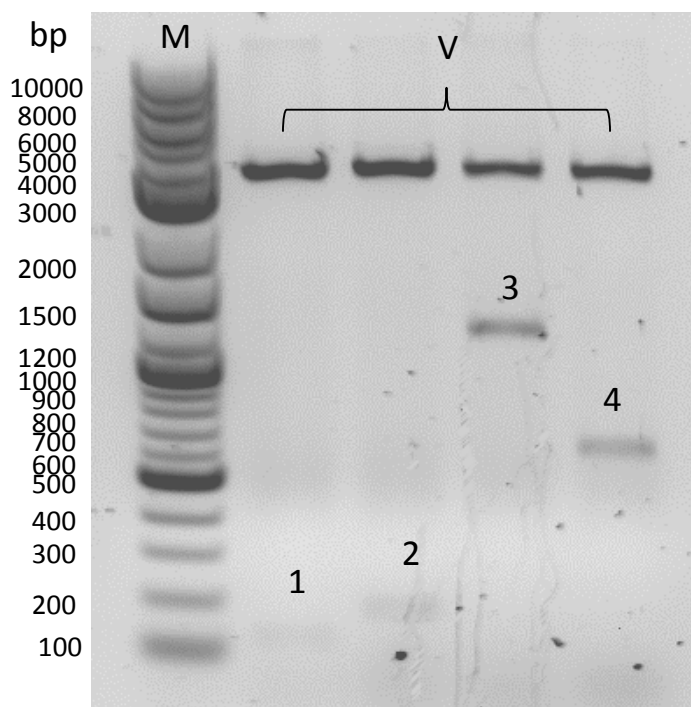
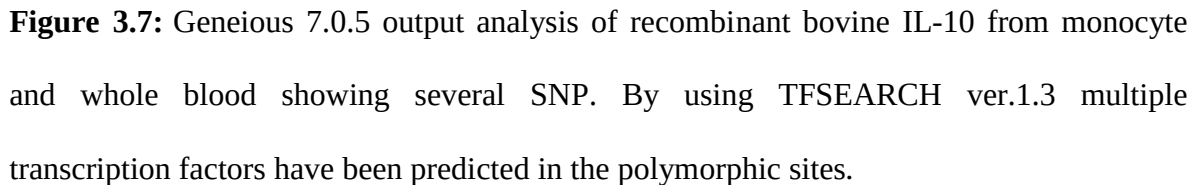


Figure 3.6: Agarose gel (1.5 %) of the BstZI digestion of bovine IL-10 from monocyte.

Lane M: 1Kb ladder. V: pGEM-T vector. 1, 2 and 3: 150, 200 and 1200 bp IL-10 insert. 4: 500 bp positive control insert

3.3.7 Detection of single nucleotide polymorphisms (SNPs) and TFs in cloned 5' UTRs of whole blood and monocyte gDNA

We successfully isolated and cloned several DNA fragment of IL-10 promotor from whole blood and monocytes genome walking method. The IL-10 fragment from whole blood (570 bp) showed 100 % similarity to NCBI sequence of bovine IL-10 promotor from nucleotide -550 to -1120 relative to the translation initiation site (ATG) of bovine IL-10 gene. Alignment of IL-10 from whole blood and monocyte sequence for SNP detection using Geneious 7.0.5 scan, allowed identification of several SNPs between the cloned IL-10 from whole blood and monocyte. Three T to C, five G to A, two C to T and one T to G substitution have been found within the different putative transcription binding site. The putative transcription factors that lie within the polymorphic region were predicted by TFSEARCH. The Cap binding site located at position -932 was only found in monocytes while at position -1106 Cap found on both whole blood and monocyte. At position -1106 the Sp1 binding site is only found on blood. The HSF binding site was found at position -1065 on monocyte, and at position -701 on whole blood, ADR1/MZF1, NkX-2 AND Lyf-1 reserved the same position -1054 on blood and monocyte, CdxA binding site which found on blood changed to GATA 1 binding site on monocyte at position-921 (Figure 3.7).



In the second amplified fragment of monocyte and by using TFSEARCH ver.1.3 the score of Hb varied from 89.3 for gDNA and 93.8 for monocyte. While, cap only has been found in monocyte with a score of 86.5 (Figure 3.8).

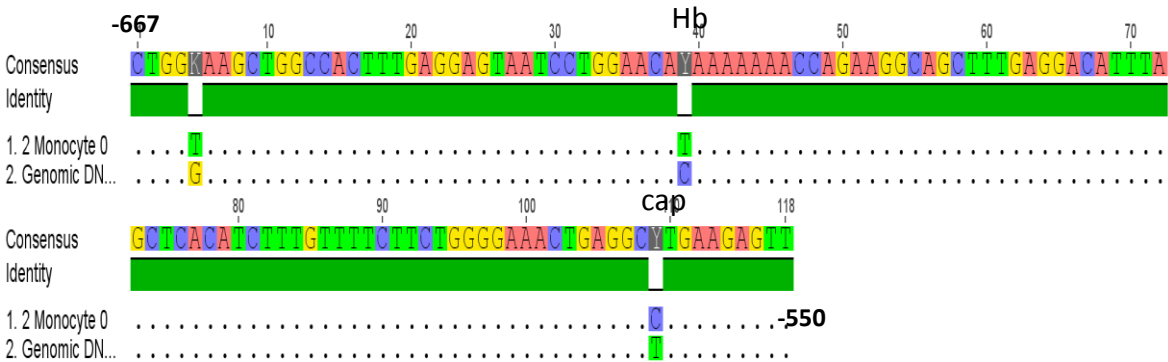


Figure 3.8: Geneious 7.0.5 output analysis of recombinant bovine IL-10 from monocyte and whole blood showing 3 SNP area at nucleotide -559, -629 and -663. By using TFSEARCH ver.1.3. two transcription factors Hb and cap have been identified with in the polymorphic sites -559 and -629 respectively.

3.3.8 *In vitro* Effect of LFH on bovine PBMCs proliferation

PBMC proliferation following treatment with LFH and ConA was measured using alamarBlue dye. After 4 h of incubation of the PBMCs culture with alamarBlue, the proliferation of the cells was measured and compared in each treatment (Figure 3.9).

As shown the greatest PBMCs proliferation was seen in cultures stimulated with ConA (5 µg/ml). In cells stimulated with LFH (20 µg/ml) a significant reduction (~4-fold) in the percentage of PBMCs proliferation was found ($P<0.001$). In addition, LFH significantly reduced the proliferative ability of ConA on PBMCs in culture treated with ConA and LFH ($P<0.05$).

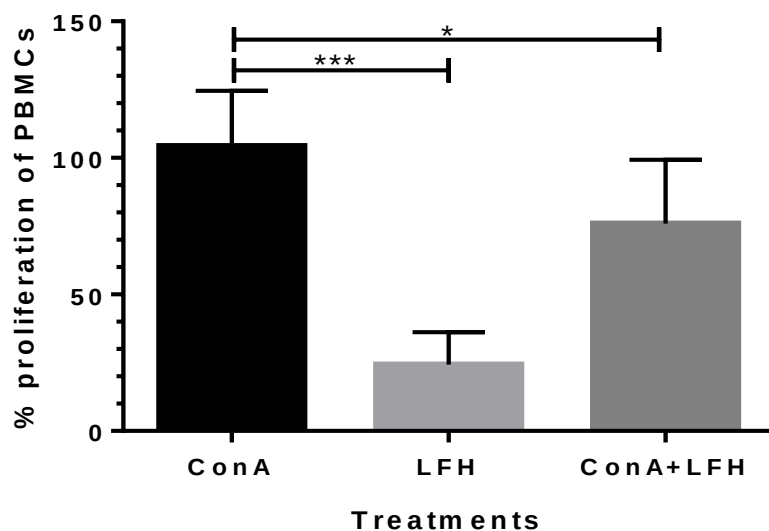


Figure 3.9: Percentage of PBMCs proliferation in response to ConA (5 µg/ml), LFH (20 µg/ml) and combined ConA and LFH using alamarBlue assay. Results were analysed using one-way ANOVA. * indicates $P<0.05$, and *** indicates $P<0.001$. Data represent the mean PBMCs percentage $\pm$ SDM of two independent experiments performed in triplicate. Results present data normalised to the proliferation of unstimulated PBMCs.

3.3.9 *In vitro* effect of LFH on IFN- γ production from bovine PBMCs

IFN- γ concentration in PBMCs culture supernatant was measured by ELISA and compared between treatments (Figure 3.23). It is clear that the highest level of IFN- γ was observed in PBMCs supernatant in response to ConA (5 μ g/ml) as compared to non-stimulated PBMCs (control). Stimulation with LFH significantly reduced the IFN- γ production in the culture supernatant ($P<0.001$) when compared to ConA stimulated culture. Furthermore, LFH treatment showed the ability to suppress the stimulatory effect of ConA on IFN- γ secretion in culture stimulated with ConA and LFH ($P<0.01$).

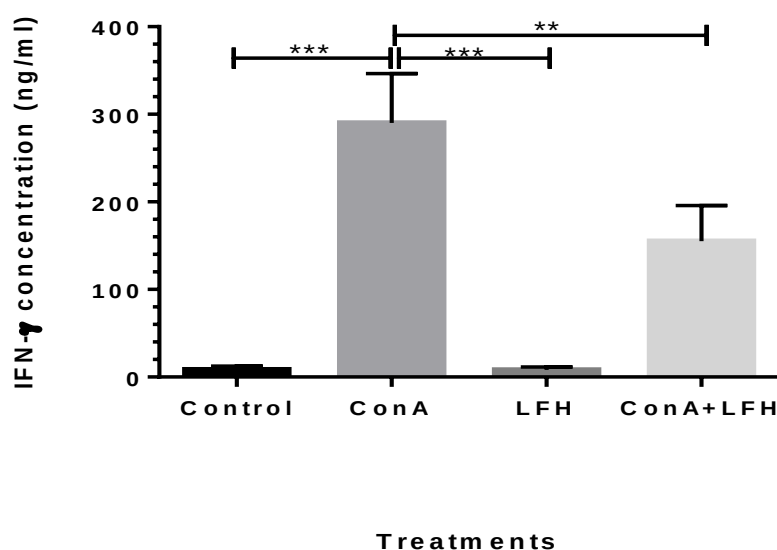


Figure 3.10: IFN- γ concentration in PBMCs treated with ConA (5 μ g/ml), LFH (20 μ g/ml) and combined ConA and LFH using ELISA. Results were analysed using one-way ANOVA. **indicates $P<0.01$ and *** indicates $P<0.001$. Data represent the mean of IFN- γ concentration $\pm$ SDM of two independent experiments performed in triplicate.

3.3.10 *In vitro* effect of LFH on IL-10 production from bovine PBMCs

PBMCs were stimulated as per proliferation experiments and the IL-10 concentration in culture supernatants was measured by ELISA and compared between different treatments (Figure 3.21).

The results show that ConA stimulated cultures produced the greatest IL-10 levels. Stimulation with LFH significantly reduced the IL-10 level in culture supernatant ($P < 0.05$), with the high reduction of IL-10 release was seen in the cultures stimulated with ConA and LFH ($P < 0.01$).

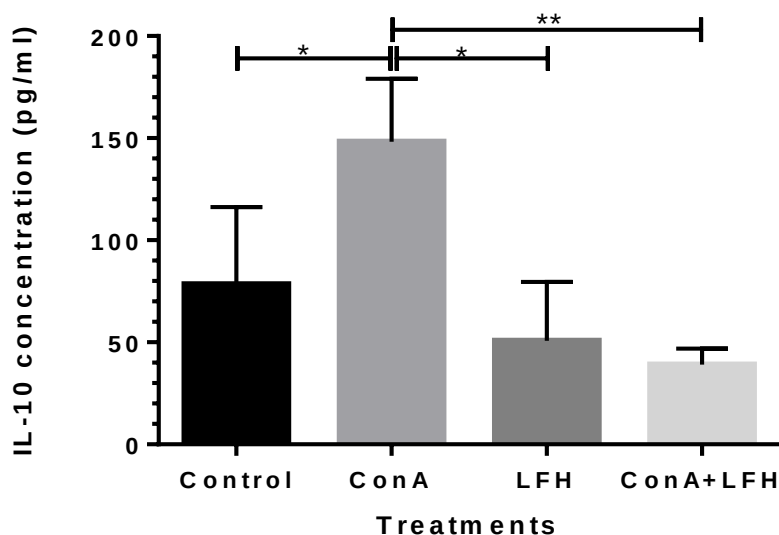


Figure 3.11: IL-10 level in PBMCs treated with ConA (5 $\mu\text{g/ml}$), LFH (20 $\mu\text{g/ml}$) and combined ConA and LFH using ELISA. Results were analysed using one-way ANOVA. * indicates $P < 0.05$, ** indicates $P < 0.01$. Data represent the mean of IL-10 concentration $\pm$ SDM of two independent experiments performed in triplicate.

3.3.11 *In vitro* effect of LFH on GATA1 expression from bovine PBMCs

Expression of GATA 1 from bovine PBMCs stimulated with ConA, LFH and ConA and LFH was examined by immunofluorescence. The percentage of GATA1 expression was determined by examination of ≥ 500 cells from each 5 field of the slide using Leica DM500B microscope at X400 and X630 objective (Figure 3.13).

PBMCs nuclei were stained by DAPI (blue) and GATA1 was indirectly labelled with FITC (green fluorescence) (Figure 3.12).

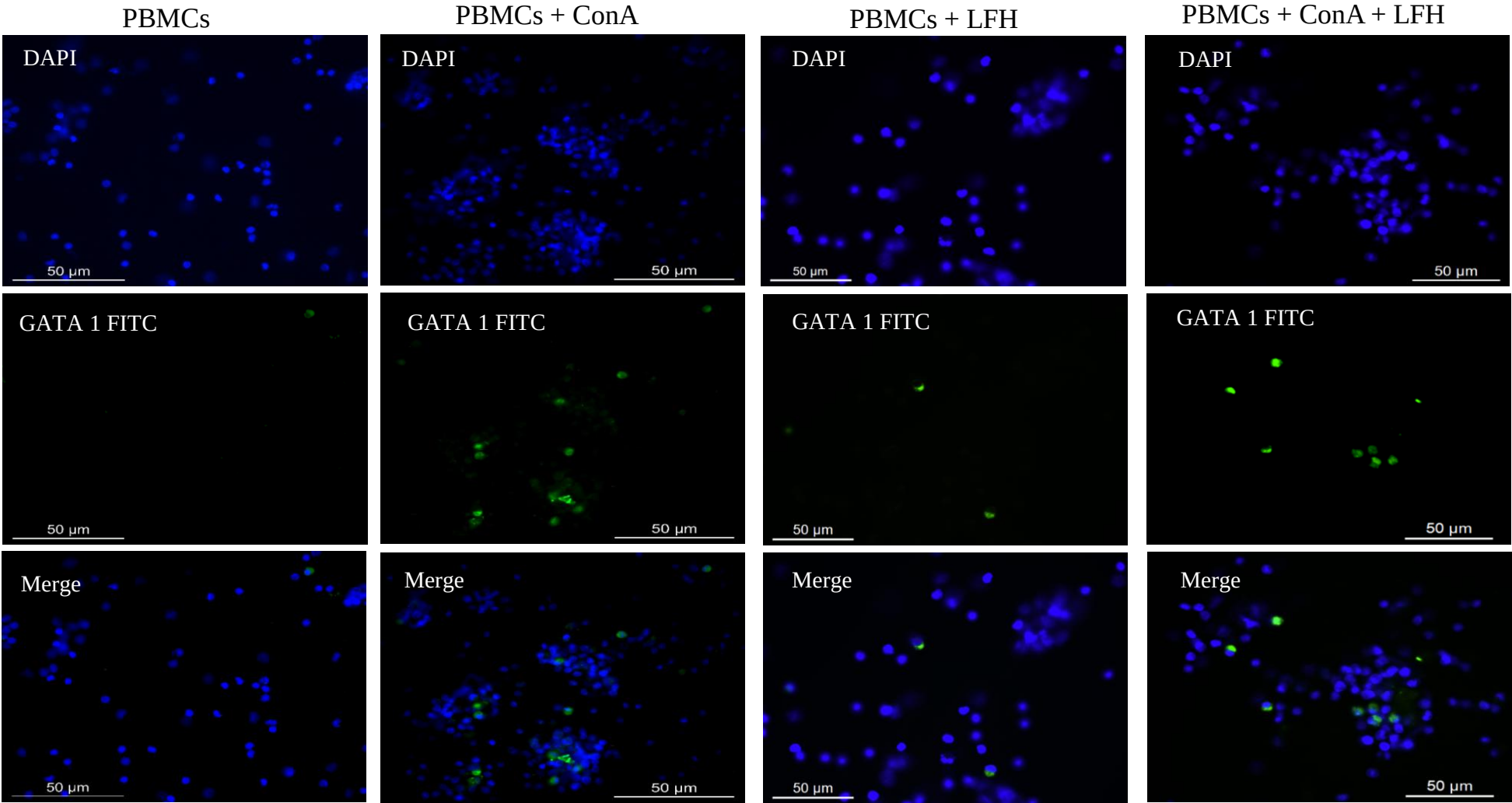


Figure 3.12: Immunofluorescence of PBMCs stimulated with ConA, LFH and combined ConA and LFH.

Nuclei stained with DAPI and GATA1 protein with FITC. Cells were visualised under a Leica DM 500B microscope at X 400 and X 630 objective.

The percentage of GATA1 expressing cells was significantly greater ($P<0.01$) in cells treated with ConA when compared to unstimulated controls (Figure 3.13). Treatment with LFH significantly increased ($P<0.05$) GATA1 expression to levels above unstimulated controls but not to the same degree as seen with ConA. Combination of the two stimulants, while having lower levels compared to ConA alone, still had significantly ($P<0.05$) higher levels of GATA1 when compared to unstimulated controls.

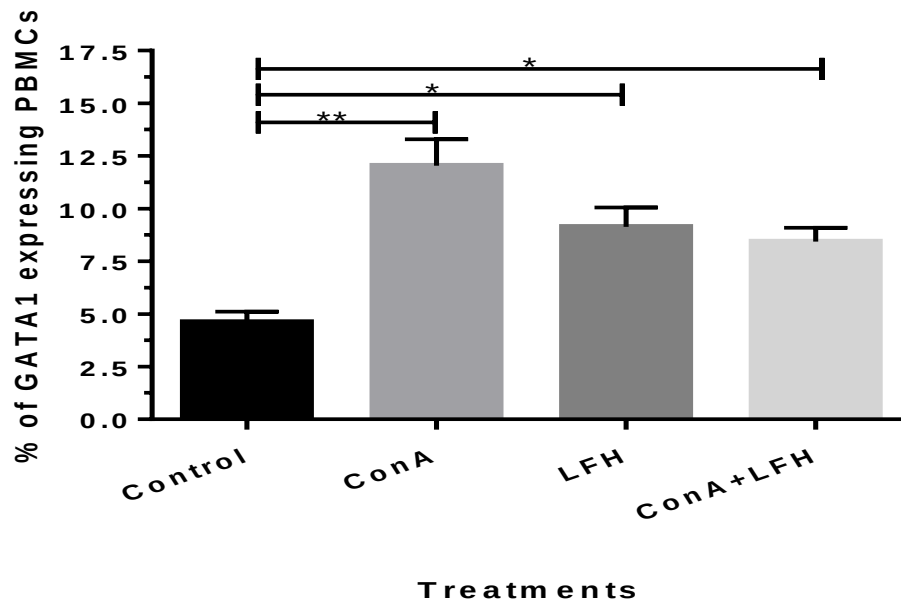


Figure 3.13: Percentage of GATA1 expressing PBMCs in response to ConA, LFH, and combined ConA and LFH as determined by immunofluorescence and inspection of ≥ 500 cells under microscope. Results were analysed using one-way ANOVA. ** indicates $P<0.01$, * indicates $P<0.05$. Data represent the mean percentage $\pm$ SDM of ≥ 500 GATA1 expressing PBMCs from two independent experiments.

3.4 Discussion

IL-10 gene expression can be controlled at both transcriptional and post transcriptional level (Brightbill *et. al.*, 2000; Powell *et. al.*, 2000). Interestingly, the changes in the effects of IL-10 on inflammation are mainly dependent on the transcriptional level of the gene (reviewed in Sciesielski *et. al.* 2010). This makes the genetic regulation of IL-10 extremely important in a disease such as *F. hepatica* where it already has a proven effect. In this study for the first time, we isolated and characterised the bovine IL-10 promoter from gDNA and compared this with the UTR isolated from the monocyte subset. A genome walking method with a 2 round PCR approach was utilised followed by blunt end cloning into the pGEM-T vector system to characterise the 5' promoter region sequence of the bovine *il-10* gene. To date several studies on the characterisation of the IL-10 promoter of human, mice, pig, chicken and guinea pig have been published (Eskdale and Gallagher, 1995, Eskdale *et al.*, 1997, Brightbill *et al.*, 2000, Rothwell *et al.*, 2004, Quan *et al.*, 2012). In our study the bovine IL-10 promoter fragment consisting of 570 bp from nucleotide -550 to -1120 relative to the translation initiation site (ATG) was amplified and cloned by this method. By using Geneious 7.0.5 scan, several single nucleotide polymorphisms (SNPs) were identified between the cloned IL-10 from whole blood and monocyte. Several mapping and characterization studies of the human IL-10 5' UTR have been undertaken, and analysis of the promoter region revealed that there are several polymorphic areas which influence IL-10 protein production *in vitro* (Turner *et al.*, 1997). Transcriptional turnover depends on the binding of regulatory elements to specific sequences within the promoter region. Studies of the promoter region of IL-10 have identified several positive and negative regulatory

sequences upstream of the transcription initiation site, and more than 74 transcription factor binding sites have been found within this region (Kube et al., 1995, Eskdale et al., 1997). In mice, the transcription of the IL-10 gene is positively regulated by the transcription factors Sp1 and Sp3 (Tone et al., 2000). In our study, we predicted putative transcription factors binding site using TFSEARCH to find transcription factor binding site that lie within the polymorphic region. Several transcription factor site, each with different validity scores, were identified within the IL-10 promotor of whole blood and monocyte data sets including Cap, Sp1, HSF, ADR1/MZF1, NkX-2, Lyf-1, CdxA, GATA 1 and Hb. Other studies also found Sp1 in mice (Brightbill et al., 2000) and pig (Quan et al., 2012). Interestingly, we found that the polymorphic allele change G to A at nucleotide -917 changed GATA 1 that binds to monocytes IL-10 promotor into CdxA factor in whole blood IL-10 promotor. We speculate that the differences in transcription factors within the IL-10 promoter between monocyte and whole blood may explain the differences in IL-10 gene expression among the different cell subsets. Our results demonstrated an *in vitro* role for LFH in modulating PBMC proliferation responses and IL-10 expression. Treatment of PBMCs with LFH significantly reduced the proliferative response of lymphocytes to the mitogen ConA; the highest percentage proliferating cells was observed in culture treated with ConA and the lowest in the LFH treated group. Others have shown that ConA induces the production of IL-2 and the expression of IL-2 receptor on the CD4 and CD8 T-cells in the presence and absence of accessory cells (Hunig et al., 1983). These signals, along with the presence of accessory cells are required for T-cells activation and proliferation (Mueller et al., 1989). Similarly, Flynn and Mulcahy, (2008b) found that ConA treatment of the bovine PBMCs induced the proliferation of PBMCs in non-infected animals. In contrast, the proliferation of PBMCs was reduced at 4 and 12 weeks post *F. hepatica* infection in response to ConA

stimulation. The suppression of PBMC proliferation mediated by LFH treatment that we found in our study agreed with those observations in lambs by Zimmerman et al., (1983) and in sheep (Chauvin et al., 1995, Moreau et al., 2002). In contrast, Oldham and Williams (1985) observed that stimulation of bovine PBMCs with fluke antigen increased cell proliferation during the first 4 week post infection. In infected rats, proliferation of splenocytes and thymocytes was increased in response to ConA, PWM or fluke antigen (Poitou et al., 1992). It has been shown that the excretory secretory protein from *F. hepatica* suppressed the proliferative response of normal rat spleen cells to ConA *in vitro*, which may explain the suppression that we have seen in response to LFH. (Cervi et al., 1998). Similarly, in cultures treated with LFH and ConA, the proliferation of PBMCs was reduced compared to ConA stimulation alone. Several studies have indicated the role of IL-10 in suppression of cell proliferation. In mice it has been found that IL-10 inhibited ConA-induced T-cells proliferation only when macrophages were used as antigen presenting cells, this was mainly due to the inhibition of costimulatory molecules expression on macrophages (Ding and Shevach, 1992). In addition, in cattle IL-10 inhibited the antigen-induced proliferation of *Babesia bovis* and *F. hepatica* specific Th-cells (Brown et al., 1994b). Therefore, we examined IL-10 production from PBMCs in response to parasite specific (LFH) and nonspecific (ConA) stimulation. Our ELISA results demonstrated an unexpected high IL-10 concentration in the culture of unstimulated PBMC (control), this may indicate that these cows may have been naïve with respect to liver fluke but not with respect to other infections. We demonstrated a significant reduction in IL-10 production from PBMCs in response to LFH treatment. This was in contrast to ConA stimulated cells that secreted a higher amount of IL-10. These results agreed with those observed by (Flynn and Mulcahy, 2008b), who, using infected animals, found that the IL-10 levels in PBMCs

stimulated with ConA was greater in comparison to LFH treated culture at 4 weeks post infection. In this investigation, in cultures treated with ConA plus LFH a further reduction of IL-10 level was seen. Therefore we could not correlate the suppressive effects of LFH on PBMCs proliferation with parasite antigen induced IL-10. Our finding is in contrast with the recently reported roles of IL-10 in mediating the suppressive effects of parasite antigen, as is the case in basophils stimulated with antigen of *L. sigmodontis* in infected mice (Larson et al., 2012). We must assume that there are two mechanisms mediating the immunosuppressive effects of LFH in fluke naïve and primed PBMCs during *F. hepatica* infection. For example, the tegument antigen of the parasite suppress the production of IL-10 from murine DC by targeting TLR (Hamilton et al., 2009). Moreover, IL-10 has the ability to down-regulate its own synthesis from monocytes via a negative feedback mechanism (de Waal Malefyt et al., 1991a). This finding lead us to suggest that the low IL-10 levels found in cultures stimulated with LFH may be due to the induction of a negative feedback loop induced by parasite-specific IL-10.

We also examined the *in vitro* role of LFH on IFN- γ production from fluke naïve PBMCs and, as before, LFH treatment reduced the IFN- γ level in the culture supernatant as compared to ConA treated groups which displayed the highest IFN- γ concentration. Our results agreed with those using lymphocytes from chronically infected cattle which failed to show any increased production of IFN- γ in response to fluke antigen and produced a minimally detectable level of this cytokine in response to ConA stimulation (Clery et al., 1996).

GATA 1 is a protein encoded by *gata 1* gene and is mainly involved in cell growth and has been implicated in cancer progression. This transcription factor is expressed by erythroid cells, mast cells, eosinophil and megakaryocyte lineages (Martin et al., 1990, Romeo et al.,

1990) and has a major role in development and differentiation of these cells (Pevny et al., 1991, Shivdasani et al., 1997, Yu et al., 2002). It has been shown that GATA 1 is one of the three primary transcription factors that regulates eosinophil lineage specification (McNagny and Graf, 2002). In support of this, GATA1 has been shown to be important in the development of mature eosinophils from eosinophil progenitors during *Nippostrongylus brasiliensis* infection (Milanovic et al., 2008). However, little data surrounding the role of GATA1 during helminth infection is available. Recently a role for GATA1 in the generation and function of basophils has been demonstrated, the absence of GATA1 in basophils significantly reduced IL-4 expression from these cells following stimulation with IgE and OVA Ag, and this in turn impaired the protective immune function of basophils during infection with *Nippostrongylus brasiliensis* due to the essential role of this cytokine in differentiation of monocytes or macrophages into M2-type macrophages that destroy skin larvae (Nei, 2013). The *st2* gene encoding for the IL-33 receptor is preferentially expressed by murine Th2 cells which produces high levels of IL-10, IL-5 and IL-4 (Lohning et al., 1998), has been shown to be positively regulated by GATA1 in murine mast cells. Indicating the role of GATA1 in regulation of IL-10 expression (Gachter et al., 1998).

Our transcription factor search demonstrated that GATA1 may play an important role in mediating IL-10 production from fluke naïve PBMCs. In our study we measured the expression of GATA1 in fluke naïve PBMCs using IF assays. We found that treatment with LFH induced GATA1 expression in PBMCs but not to the same level as ConA treatment which induced the highest level of GATA1 protein.

Collectively we can conclude that IL-10 is not responsible for the immunosuppressive effects of LFH on fluke naïve PBMCs in terms of proliferation or IFN- γ secretion, and LFH

induced GATA 1 may have a role in regulation of IL-10 expression from fluke naïve PBMCs. However, this needs to be confirmed by using GATA1 mutant IL-10 promotor sequences in a luciferase reporter plasmid system.

4 Role of a parasite effector, FhTLM on IL-10 and immunoregulation

4.1 Introduction

The transforming growth factor beta (TGF- β) superfamily includes a large number of multifunctional proteins; TGF- β isoforms (1-5), activins, inhibins, bone morphogenic proteins (BMPs), growth and differentiation factor (GDF) and mullerian inhibitory factor (MIF). These proteins are secreted from a variety of cell types in a latent inactive form (Lawrence et al., 1984). Activation of these forms is mediated by cleaving the pro-peptide (latency associated peptide-LAP) from the mature domain (Kingsley, 1994) and this final product is responsible for the varied biological functions in target cells. In mammals the TGF- β family consists of three protein members; TGF- β 1, TGF- β 2 and TGF- β 3 (Massague, 1990). In avian species (e.g. chickens) four isoform have been identified (Jakowlew et al., 1988) and five in amphibians (e.g. frog) (Kondaiah et al., 1990). TGF- β was first described as a homo-dimer of a 25 kDa protein that induced the anchorage-independent growth of normal rat fibroblasts in soft agar culture (Assoian et al., 1983). Later it was shown that TGF- β is a potent growth inhibition factor of lymphoid, epithelial, endothelial, fibroblast, neuronal and hematopoietic cells (Tucker et al., 1984, Moses et al., 1985, Roberts et al., 1985). However this factor also plays a crucial role in the regulation of differentiation and function of cells of the immune system (Letterio and Roberts, 1998). Within the TGF- β superfamily, TGF- β 1 has notable inhibitory effects on cells of the immune system. It has been shown that TGF- β released from human T-cells was able to down-regulate IL-2 dependent T-cell proliferation (Kehrl et al., 1986b). Also TGF- β 1 suppresses NK cell activity *in vitro* (Rook et al., 1986), and inhibits *in vitro* activation of murine peritoneal macrophages (Tsunawaki et al., 1988). In contrast, other literature has

proved the stimulatory capacity of this cytokine on T-cell proliferation (Cerwenka et al., 1994). Moreover, TGF- β induces the development of CD8⁺ T-cells from thymic precursors and from IL-2 dependent cytotoxic T-cell lines *in vitro* (Suda and Zlotnik, 1992, Inge et al., 1992). TGF- β 1, with GM-CSF, induces the development of Langerhans dendritic cells (Geissmann et al., 1998). Various studies have provided contrasting data with regard to the effects of TGF- β on CD4⁺ T-helper (Th) cell differentiation; initial studies indicated that TGF- β favours the differentiation of Th1 from naïve CD4 T cells (Espevik et al., 1990b). Conversely, administration of TGF- β to mice experimentally infected with *L. amazonensis* or *L. braziliensis* resulted in development of several non-healing lesion and overproduction of IL-4 (Barral-Netto et al., 1992). More recently, studies have identified a new role for TGF- β ; in combination with IL-6 and IL-23, induces the development of the Th17 cell subset (Veldhoen et al., 2006).

In parasitic infection, TGF- β , together with IL-4 and IL-10, acts as an important immunoregulator that down-regulates the cell mediated immune response to a variety of intracellular infections such as *Leishmania* spp. (Barral et al., 1993, Letterio and Roberts, 1998), *T. gondii* (Hunter et al., 1995) 1995) and *T. cruzi* (Gazzinelli et al., 1992a). In extracellular helminth infections, TGF- β exerts the same inhibitory effects in *Schistosoma mansoni* infection (Oswald et al., 1992). Furthermore, the role of TGF- β and IL-10 in ameliorating the pathology and promoting tissue repairs during helminth infection has been demonstrated (Belkaid et al., 2006). It has been shown that TGF- β is important in mediating the production of collagen and fibrosis (Cutroneo, 2007). Fibrosis is an important mechanism that limits the invasion of *F. hepatica* within the liver parenchyma and increases resistance to infection (Behm and Sangster, 1999). In cattle experimentally infected with *F. hepatica*, it has been found that TGF- β and IL-10 are produced at high

levels and are involved in parasite survival (Flynn and Mulcahy, 2008b). In sheep experimentally infected with *F. hepatica* the level of TGF- β 1 gene expression from hepatic lymph nodes was negatively correlated to the fluke burden at 23 weeks post infection indicating that TGF- β 1 may increase resistance to *F. hepatica* infection (Hacariz et al., 2009a). In mice, *F. hepatica* induced-TGF- β inhibited autoimmune encephalomyelitis mediated by autoantigen-specific Th1 and Th17 responses (Walsh et al., 2009). Together, these studies suggest that TGF- β inhibits a partial protective Th1-like response in *F. hepatica* infection.

Interestingly, the TGF- β family cytokines initiate their biological effects through binding to shared/related cell surface receptors. Using radio-labelled TGF- β 1 and an affinity-binding assay, three cell surface glycoprotein receptors were identified and termed TGF- β RI, TGF- β RII and TGF- β RIII (Massague, 1992, Lin and Lodish, 1993). The structures of these receptors have been examined by recombinant cloning and this has revealed that type I and type II receptors have an intracellular serine/threonine kinase domain which is regarded as the most important in mediating signal transduction by TGF- β (Boyd and Massague, 1989). The type III receptor is a beta-glycan receptor consisting of a short cytoplasmic domain (Wang et al., 1991) and has a distinct biological function from type I and type II (Massague, 1990). Several studies have examined the mechanism by which these receptors mediate TGF- β activity, by generating a negative dominant mutant line, it has been found that the deletion of TGF- β RII from murine CD4⁺ T cells induced uncontrolled T-cell activation and multi-organ inflammation in transgenic mice, which also indicates an anti-proliferative effects of TGF- β RII (Gorelik and Flavell, 2000). Conditional deletion of the TGF- β RI in murine T-cells showed the requirement for TGF- β RI during the development of thymic Foxp3⁺ cell one week after birth (Liu et al., 2008). Collectively these studies

demonstrated that intact TGF- β signalling controls T-cell autoimmune pathologies via CD4⁺ Foxp3⁺ T-reg cells. Moreover, using mutant cells chemically resistant to the growth inhibitory effects of TGF- β indicated that the absence of both TGF- β RI and TGF- β RII are responsible for mediating this resistance. Transfection of mutant TGF- β resistant cells with TGF- β RII restored the responsiveness of these cells to TGF- β and this response is linked to the presence of TGF- β RI which forms a complex with TGF- β RII (Boyd and Massague, 1989, Laiho et al., 1990).

The signal transduction pathway is conserved within the TGF- β family. Activation of this pathway starts by binding of the specific ligand to a type II receptor at the cell surface, this complex then recruits and phosphorylates a type I receptor. The activated receptors then phosphorylate the cytoplasmic receptor regulated Smads (R-Smads) protein which then binds to cooperative Smad (Co-Smad) to form a complex which migrate to the nucleus and initiates transcription of target genes (Wrana et al., 1992, Wrana et al., 1994, Massague et al., 2000).

Several TGF- β homologues have been identified in different species. In the nematode *Caenorhabditis elegans* three homologues have been characterised and these mainly influence the development of the nematode; DAF7 controls dauer larva development (Ren et al., 1996), UNC-129 is important for axon guidance during growth (Colavita et al., 1998) and CET-1 regulates the body length and tail formation in males (Morita et al., 1999). In the parasitic helminth *Brugia malayi*, TGH-1 and TGH-2 have been identified and the latter plays a role in modulation of the host immune response and share similarity to *Caenorhabditis elegans* DAF-7 and human TGF- β (Gomez-Escobar et al., 2000). Furthermore, *Caenorhabditis elegans* DAF-7 homologues have been identified in *Strongyloides stercoralis*, *Strongyloides ratti* and *Parastrongyloides trichosuri* (Crook et

al., 2005, Massey et al., 2005). In humans the glycoprotein endoglin which is expressed on vascular endothelial cells, shares a sequence similarity with TGF- β and binds TGF- β 1 receptors with a high affinity (Cheifetz et al., 1992). In the mollusc *Crassostrea gigas*, a homolog to vertebrate TGF- β and activin has been found to be involved in the immune response in the mollusc (Lelong et al., 2007).

In parasites, the majority of TGF- β homologue show similarity to mammalian activin/inhibin proteins. The first TGF- β homologue found in *S. mansoni* was an inhibin/activin (SmInAct)-like ligand which is associated with the embryogenesis of this parasite (Freitas et al., 2007). Also *S. mansoni* bone morphogenic protein (BMP) has been characterised to have a high similarity to the mammalian BMP subfamily (Freitas et al., 2009). The cross interaction of the ligand and receptors between host and parasite is considered as an important survival strategy for the parasite by mediating its development, or manipulating the host immune response and physiology in their favour (Brehm and Spiliotis, 2008). Recently, a novel TGF- β homologue has been characterised from *F. hepatica* in our laboratory termed transforming growth factor like molecule (FhTLM), and shows 32% similarity to bovine and human TGF- β 1 (Japa et al., 2015). This study demonstrated that FhTLM shows restricted expression across life stages and has developmental effects whilst also binding to a mammalian luciferase reporter cell line.

In order to understand the exact mechanism by which bovine TGF β -R1 and TGF β -R2 is activated by FhTLM we have cloned the common extracellular domain of bTGF β -R1 and TGF β -R2 and expressed it in HEK cells to compare the binding affinity of these receptors to human TGF- β 1 and to FhTLM. We have demonstrated that FhTLM is able to bind to mammalian receptors and that it also induces signal transduction, as demonstrated by changes in cytokine secretion and lymphocyte proliferation.

4.2 Material and methods

4.2.1 Bioinformatics

Bioinformatics analysis was carried out using NCBI databases and NCBI's conserved domain finder tool. Sequences of bovine TGF β -RI and TGF β -RII were retrieved from NCBI database and the extracellular domains (ED) were identified by conserved domain. The corresponding protein sequences were then obtained using BLASTX in ensemble genome browser and compared with the human protein sequence using NCBI's blastn algorithm.

4.2.2 Isolation of bovine TGF β -RIED and TGF β -RIED

The extracellular domains of both bTGF β -RI and TGF β -RII were cloned from bovine PBMCs by the following method.

4.2.2.1 RNA extraction

Extraction of bovine total RNA from PBMCs was carried out using Nucleospin[®] RNA (MACHEREY-NAGEL, Germany) as per manufacturer's protocol. Bovine PBMCs were removed from storage (-80 °C), thawed and washed twice with sterile PBS. Then the cells were suspended in complete media and the number adjusted to 5×10^6 /ml. 350 μ l of buffer RA1 and 3.5 μ l of β -mercaptoethanol were added to the cell pellet and vortexed vigorously. The lysate was added to a violet ring nucleospin filter placed in a 2 ml collection tube and

centrifuged for 1 min at 11000 g. The filter was removed and 350 μ l of ethanol (70 %) were added to the lysate and mixed by pipetting up and down. The mixture was then loaded to the light blue ring column, placed in a collection tube and centrifuged for 30 second at 11000 g. Afterward 350 μ l of membrane desalting buffer was added and centrifuged as above for 1 min. The DNA was removed by adding 95 μ l of DNase mixture (90 μ l reaction buffer and 10 μ l reconstituted rDNase) directly to the surface of the silica membrane and incubating for 15 mins at RT. The membrane was washed with 200 μ l of buffer RAW2 and 600 μ l of RAW3 and the flow through was discarded after each wash. Finally, the RNA was eluted by addition of 40 μ l of RNase free water and spinning for 1 min at 11000 g. The eluted RNA was stored at -20 °C for further use.

4.2.2.2 cDNA synthesis

The first strand cDNA was synthesised using Transcriptor first strand cDNA synthesis kit (Roche Applied Science, Germany) following manufacturer's instruction. In a sterile nuclease free PCR tube the following were mixed for each sample:

Table 4.1: cDNA master mix

Component	Volume
Random hexamer primer (600 pmol/ μ l)	2 μ l
Reverse transcriptase reaction buffer	4 μ l
Protector RNase inhibitor (40 U/ μ l)	0.5 μ l
Deoxynucleotide mix (10 mM)	2 μ l
Reverse transcriptase (20 U/ μ l)	0.5 μ l

11 μ l of RNA was added to this mix and centrifuged to collect the sample at the bottom of the tube. The reaction mixture was incubated for 10 mins at 25 °C and then at 55 °C for 30 min. The reverse transcriptase was inactivated by heating at 85 °C for 5 mins and the reaction was stored at -20 °C.

4.2.2.3 Amplification of bovine TGF β -RIED and RIIED

The b TGF β -RIED sequence from nucleotide (nt) +88 to +330 (relative to the translation initiation site +1), which gives rise to a 243 bp fragment, and the TGF β -R2ED sequence from nt +139 to +453 that gives rise to a 315 bp fragment, were PCR amplified using specific primers. The forward primer had a *NCOI* restriction site incorporated and the reverse primer had a *BglII* restriction site incorporated for the generation of bTGF β -RIED. The forward primer had an *EcoRI* restriction site incorporated and the reverse primer had a *BglII* restriction site incorporated for the generation of bTGF β -RIIED.

Table 4.2: Primer sequence used for amplification of bTGF β -RIED and TGF β -RIED.

Restriction sites shown in red.

Primer name	Sequence 5'-3'
Forward TGFβ-R1 <i>NCOI</i>	TGGC CATGG CAGTGTTTTTGCCACCTTG
Reverse TGFβ -R1 <i>BglII</i>	GCC GATCT TGATGGCTTTCCAACAGTTG
Forward TGFβ-R2 <i>EcoR1</i>	ATTG AATTC GCTGTCGCAGCTGTGTAAGT
Reverse TGFβ-R2 <i>BglII</i>	ACCAG GATCT TTACTGCTGGTGTACTCTTC

Amplification was performed as follows: 1 μ l of bovine cDNA was used as a template in 50 μ l PCR reaction mixtures containing Tris-HCl (10 mM), MgCl₂ (1.5 mM), dNTP (200 μ M), primers (0.1 μ M) and *Taq* DNA polymerase (2.5 units) (Qiagen). Amplification of each domain was carried out separately in a Techne TC-512 thermocycler with an initial denaturation of 3 mins at 94 °C followed by 35 cycles of 94 °C for 30 seconds, 68 °C for 1 min and 72 °C for 1 min. The reaction was completed by 1 cycle of 72 °C for 10 min. The amplified product was loaded into 1.5 % agarose gel as described in 2.5 and visualised on a UV trans illuminator. The amplicons were gel purified using GenElute™ Gel Extraction Kit (Sigma-Aldrich, UK) as described in 2.6, the concentration was checked by Nanodrop 8000 (Thermo Scientific, UK) and stored at -20 °C for subsequent use.

4.2.3 Production of recombinant TGF β -RIED-Fc and TGF β -RIED-Fc

The amplified extracellular domain of bTGF β -RIED was sub-cloned into the *NCOI* and *BglIII* cut vector and TGF β -RIED into *EcoRI* and *BglIII* cut vector, pFUSE-hIgG1-Fc2 (InvivoGen, UK), so that the Fc portion of human IgG1 was fused to the ED of bTGF β -RI and bTGF β -RII respectively. The double digested purified products were used to ligate to the linearized vector using 1 μ l T4 DNA ligase (Promega, UK), 5 μ l rapid ligation buffer, 1 μ l pFUSE-hIgG1-Fc2 vector (50 ng/ μ l) and 3 μ l of the digested product. The reactions were incubated at 4 °C overnight. 5 μ l of the ligation reaction were used to transform 50 μ l *E. coli* DH5 α chemical competent as described in section 3.2.2.3. The transformed cells were grown on LB agar plates supplemented with 50 μ g/ml zeocinTM (InvivoGen, UK) at 37 °C overnight.

4.2.4 Verification of recombinant pFUSE-TGF β -RIED-Fc and pFUSE-TGF β -RIED-Fc colonies

The positive white recombinant bacteria colonies were inoculated into 10 ml LB broth supplemented with 50 μ g/ml zeocinTM in a shaker incubator at 37 °C overnight. Recombinant plasmid DNA was purified from cultures using the GenEluteTM Five-Minute Plasmid MiniprepKit (Sigma-Aldrich, USA) as described in (3.2.2.5). The purified DNA plasmids were subsequently checked for insertion using *NCOI*, *BglIII* enzymes to release the bTGF β -RIED insert and *EcoRI*, *BglIII* enzymes to release the bTGF β -RIED insert from the pFUSE vector. The digested reactions were separated on 1.5 % agarose gel alongside with 1Kb plus DNA ladder for 60 mins and visualised using a UV trans-

illuminator. The concentration of the positive inserts were checked by Nanodrop 8000 (Thermo Scientific, UK) and stored at -20 °C.

To confirm correct insertion and orientation of the both receptors, plasmids with the correct insert, by digest analysis, were sent for sequencing using Sanger sequencing (Source Bioscience Life science, Nottingham, UK). The plasmids were sequenced bi-directionally using specific forward and reverse primers (Table 4.2) for each domain. Sequence results were trimmed for vector contamination and compared to held NCBI reference sequences of bovine TGF β -RIED and TGF β -RIED to confirm correct sequence and orientation.

4.2.5 Expression of recombinant pFUSE-TGF β -RIED-Fc and pFUSE-TGF β -RIED-Fc protein

Human embryonic kidney cells (HEK) 293 cells were cultured in Dulbecco's Modified Eagle's Medium (Sigma Aldrich, USA) supplemented with FCS (10%) (Sigma-Aldrich, USA), penicillin (100 μ g/ml), streptomycin (100 μ g/ml) and allowed to reach 80% confluency at 37 °C and CO₂ (5 %). Transfection of HEK cells with recombinant plasmids was done using jetPRIME[®] DNA and siRNA transfection reagents (Polyplus-transfection, USA) as bellow;

The day before transfection, HEK 293 cells were seeded into a 6 well culture plates (Thermo Fisher Scientific, UK) at 2×10^5 cells/well. DMEM medium was added to give a final volume of 2ml per well and the cells were incubated at 37 °C and CO₂ (5 %) o/n. 4 hrs before transfection the media were replaced with serum free media pre-warmed to 37 °C. In sterile tubes, one for each plasmid, 2 μ g of plasmid with the correct insert for the bTGF β -RIED and TGF β -RIED was diluted into 200 μ l of jetPRIME[™] buffer and mixed by

vortexing. Then 4 μ l of jetPRIME[®] was added to the mixture, vortexed for 10 s and incubated at room temperature for 10 mins. 200 μ l of each transfection mix was added drop by drop onto the cells and the plate was gently shaken and returned to the incubator. The transfection medium was replaced with complete DMEM medium after 4 hrs and the cells incubated at 37 °C for 72 hrs. A positive control GFP reporter plasmid, Pc-gfp-c2 (Clontech, UK), was used in parallel to confirm transformation. After 72 hrs the positive controls were assessed under an inverted microscope (LEICA DMIL) for the presence of green fluorescence inside HEK cells, images were captured using a LEICA DFC 490 camera connected to the microscope.

4.2.6 Limiting dilution of zeocin-selected transfected HEK cells

After confirming transfection under the fluorescent microscope, cells were collected from the 6 well plates and re-suspended at 3×10^5 cells/ml in DMEM media (Sigma-Aldrich, USA) supplemented with FCS (10 %) and zeocin[™] (400 μ g/ml). 400 μ l of these cells were transferred into the top left well of 96 well plate and limiting dilution applied by a 1:2 dilution of this suspension down the first column to a final volume of 100 μ l. Also 1:2 dilution were carried out across the plate to a final volume of 100 μ l, cells were incubated at 37 °C and CO₂ (5 %) for 72 hrs.

4.2.7 Identification of the TGF β expressing HEK clone by ELISA

All 96 well of the plate of zeocinTM selected, transfected HEK were examined using a light microscope (Nikon EFD-3) and 6 wells without viable cells were excluded. Supernatant from wells with viable colonies of cells were used in an ELISA to detect clones secreting the correct ED-Fc fusion protein as below:

Flat bottom 96-well plates (Thermo Fisher Scientific, UK) were coated with 50 μ l of human TGF- β 1 (Catalog Number: 100-21, PeproTech, USA) at a concentration of 500 ng/ml, the plate was sealed and incubated at room temperature overnight. The following day the plate was washed three times with TweenTM-20 (0.05 %) in D-PBS (pH 7.4) this was repeated after each step. 200 μ l of BSA (4 %) in D-PBS (Sigma-Aldrich, USA) was added to block all wells prior to incubation for 1 hr at room temperature. Afterward, 100 μ l supernatant from zeocinTM selected transfected HEK cells were added to the ELISA plate and incubated for 1 hr at RT. Supernatant from non-transfected HEK cells were used as negative controls. Then, 100 μ l of HRP conjugated anti-human IgG1 (HP6070, Thermo Fisher, UK) at a concentration of 5 μ g/ml was added to each well and incubated for 1 hr and the plate washed. The color was developed by adding 100 μ l of TMB substrate to each well and incubating in the dark for 30 min. The reaction was stopped by applying 100 μ l of stop solution and absorbance was measured at 450 nm using a micro-plate reader (LT-4000, Labtech, UK). After blank correction, the clones with a positive OD value were collected from the wells of 96 well plates, transferred to T75 flask and allowed to expand in zeocinTM supplemented media. Supernatant from these clones was collected and stored at -20 °C for further use.

4.2.8 Detection of interactions of rTGF β -RIED-Fc and RIIED-Fc fusion with human TGF- β 1 and FhTLM

The efficiency of binding of the b TGF β -RIED-Fc and TGF β -RIIED-Fc to hTGF- β 1 and FhTLM was quantified by direct ELISA as per bellow:

A flat bottom 96-well plate (Thermo Fisher Scientific, UK) was coated with 50 μ l of hTGF- β 1 (100 μ g/ml), plate was sealed and incubated at room temperature overnight. The plate was washed three times with TweenTM-20 (0.05 %) in D-PBS (pH 7.4) and this was repeated between each step. Additional protein binding site were blocked by adding 200 μ l of BSA (4 %) in D-PBS to all wells and incubating for 1 hr at room temperature. Un-purified bTGF β -RI and RII Fc fusion proteins in the tissue culture supernatant were used as neat or diluted 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128 and supernatant from non-transfected HEK were used as negative control and added to the wells of the plate and incubated for 1 hr at room temperature. Then, 100 μ l of HRP-conjugated Anti-human IgG1 Ab (HP6070, Life Technology) at a concentration of 5 μ g/ml was added to each well and incubated for 1 hr and the plate washed. The color was developed by addition of 100 μ l of TMB substrate to each well and incubating in the dark for 30 min. The reaction was stopped by applying 100 μ l of HCL (1 %) and absorbance was measured at 450 nm using micro-plate reader (LT-4000, Labtech, UK).

The same steps as above were used to detect the binding of the FhTLM protein to the bTGF β -RI and RII by using 600, 500, 400, 300, 200, 100 and 0 ng/ml of hTGF- β 1 or FhTLM for coating and purified bTGF β -RIED-Fc and RIIED-Fc fusion protein at concentration (1.25 μ g/ml).

4.2.9 Avidity of the FhTLM to bTGF β -RIED-Fc and TGF β -RIED-Fc

To estimate the avidity of the FhTLM interaction with Fc fusion proteins of the bTGF β -RIED and RIIED, potassium thiocyanate (KSCN) was introduced into the ELISA to disrupt the binding between the bovine receptors and the recombinant FhTLM protein. ELISA were performed as stated above (section 4.2.8) using FhTLM as coating antigen at concentration of (500 ng/ml). After addition and incubation with (1.25 μ g/ml) of Fc fusion proteins, different concentrations (1, 2, 3, 4, 5 and 0 M) of KSCN were added to each well and incubated for 1 hr at RT. Thereafter the binding and optical densities were measured as stated above (4.2.8).

4.2.10 Lymphocyte proliferation assay

The percentage of PBMCs proliferation in response to ConA (5 μ g/ml), FhTLM (200ng/ml) and a combination of ConA and FhTLM was measured using alamarBlue (Thermo Fisher Scientific, UK) as previously described (3.2.4).

4.2.11 Detection of cytokines production in PBMCs by ELISA

The levels of IFN- γ and IL-10 in culture supernatant of PBMCs stimulated as previously described (2.9) were measured using commercially available ELISA as previously described (Section 2.8).

4.2.12 Immunofluorescence assay

To determine the levels of GATA1 expression in FhTLM-stimulated PBMCs, we performed immunostaining as previously described in section (3.2.6). After staining, slides were mounted with Vectashield (Vector Labs Ltd., UK) containing DAPI staining reagent. Images were captured using a Leica DM 500B microscope and DFC 350 FX camera (Leica Microsystem Ltd., UK) using X 400 and X 630 magnification.

4.2.13 Statistical analysis

PBMC proliferation was expressed as percentage of proliferation increase in response to each treatment. Results were graphed and data analysed using GraphPad prism version 6.0 software. A one-way ANOVA was used for analysis and the significant differences between control and each treatments were determined using Dunnett's multiple comparison test. All bars represent mean values $\pm$ SDM of two independent experiments.

4.3 Results

4.3.1 Identification of the putative extracellular domain sequence of bovine TGF β -RI

The nucleotide sequence of bovine TGF β -RI was retrieved from the NCBI (NM_174621.2). The sequence of TGF β -RIED consists of 243 nt (from 88 bp to 330 bp) encoding a protein region of 81 AA (30-110 AA) (Figure 4.1).



Figure 4.1: Nucleotide and deduced amino acid sequences of bovine TGF β -RIED.

The putative ED nucleotide sequence is shown in blue and the corresponding protein sequence in red.

An alignment of the amino acid sequence of bovine and human TGF β -RIED shows that the cysteine residues are highly conserved between the two species. Globally there is 98 % homology between the amino acid sequences of bovine and human TGF β -RIED (Figure 4.2).

B

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Bovine  LQCFCHLCTKDNFTCVTDGLCFVSVTETTDKVIHNSMCIAEIDLIPRDRPFVCAPSSKTGS
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Human   LQCFCHLCTKDNFTCVTDGLCFVSVTETTDKVIHNSMCIAEIDLIPRDRPFVCAPSSKTGS

Bovine  ITTTYCCNQDHCNKIELPTV
          ||||||||||||||||
Human   VTTYCCNQDHCNKIELPTT
  
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Figure 4.2: Comparison between the protein sequence of bovine and human TGF β -RIED.

The different in amino acid sequence between the two receptors is shown in red.

4.3.2 Identification of the putative extracellular domain sequence of Bovine TGF β -RII

The nucleotide sequence of bovine TGF β -RII was retrieved from the NCBI (NM_001159566.1). The sequence of TGF β -RIIED consists of 315 nt (from 139 to 453) encoding a protein of 105 AA (47-151 AA) (Figure 4.3).

A

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1  ATGGGCCGGGGGCTGCTCGGGGGCCTGTGGCCGCTGCACGTCGTCTCTGTGGACGCGCATC
   -M--G--R--G--L--L--G--G--L--W--P--L--H--V--V--L--W--T--R--I--
61  GCCAGCACGATCCCGCCGCACGTTCCCAAGTCGGTTAACAGCGATATGATGGTCACTGAC
   -A--S--T--I--P--P--H--V--P--K--S--V--N--S--D--M--M--V--T--D--
121  CACAACGGCGCCATCAAGCTGTGCGCAGCTGTGTAAGTTCTGCGACGTGCGGTCTGCCACC
   -H--N--G--A--I--K--L--S--Q--L--C--K--F--C--D--V--R--S--S--T--
181  TGTGACAACCAGAAGTCCTGCTGGAGCAACTGCAGCATCACGGCCATCTGCGAGAAGCCG
   -C--D--N--Q--K--S--C--W--S--N--C--S--I--T--A--I--C--E--K--P--
241  GAGGAGGTCTGCGTGGCTGTCTGGAGGAAGAATGATGAGAACATCACGCTGGAGACAGTC
   -E--E--V--C--V--A--V--W--R--K--N--D--E--N--I--T--L--E--T--V--
301  TGCCATGACCCCAAGATTGCCTACCATGGATTTGTCTCTGGACGATGCTGCTTCTTCAAAG
   -C--H--D--P--K--I--A--Y--H--G--F--V--L--D--D--A--A--S--S--K--
361  TGTATCATGAAGGAAAGAAAGGGGTCCGGAGAGACTTTCTTCATGTGCTCCTGCAGCTCC
   -C--I--M--K--E--R--K--G--S--G--E--T--F--F--M--C--S--C--S--S--
421  GAAGAATGCAATGACCACATTATCTTCTCCGAA
   -E--E--C--N--D--H--I--I--F--S--E--

```

Figure 4.3: Nucleotide and deduced amino acid sequences of bovine TGF β -RIIED.

The putative ED nucleotide sequence is shown in blue and the corresponding protein sequence in red.

B

Figure 4.4: Comparison between the protein sequence of bovine and human TGF β -RII. The different in amino acid sequence between the two receptors is shown in red.

4.3.3 PCR amplification of bTGF β -RIED and TGF β -RIED

The 243 bp bTGF β -RIED and 315 bp bTGF β -RIED fragments were obtained by PCR amplification using specific forward and reverse primer (Table 4.2). These fragments were visualised by agarose gel electrophoresis (Figure 4.5) prior to purification for subsequent cloning.

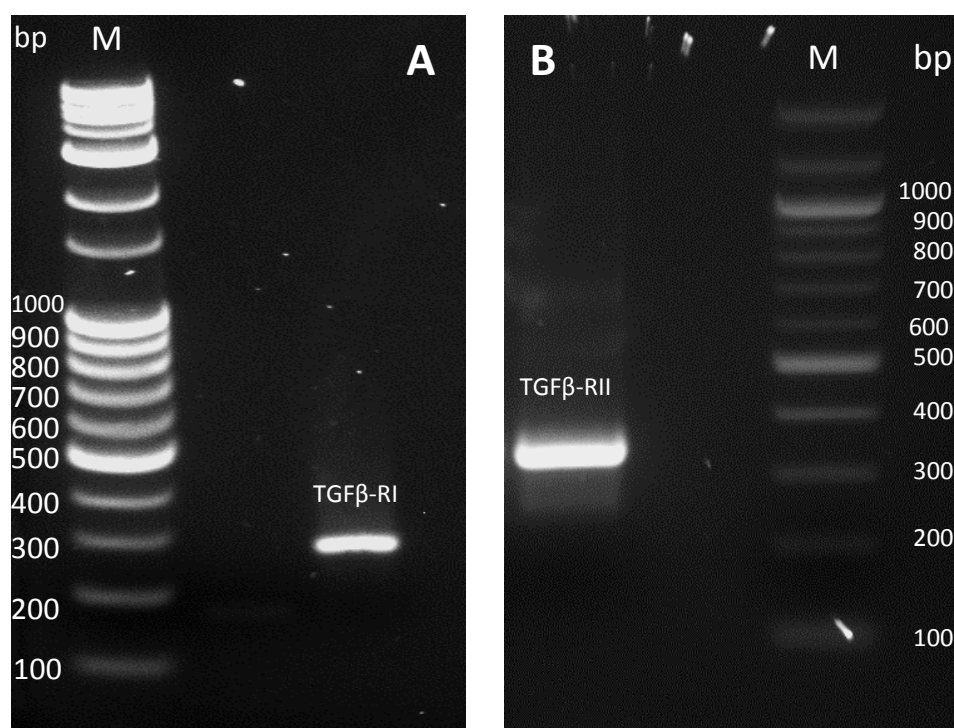


Figure 4.5: Agarose gel electrophoresis (1.5 %) showing the PCR products of bTGF β -RIED (A) and bTGF β -RIED (B). Lane M, 1Kb plus DNA ladder. A, 243 bp TGF β -RIED with *NCOI* and *BglII* overhangs. B, 315 bp TGF β -RIED with *ECORI* and *BglII* overhang.

4.3.4 Construction of recombinant pFUSE-TGF β -RI-hIgG1-Fc and pFUSE-TGF β -RII-hIgG1-Fc

The gel purified bTGF β -RIED PCR product was ligated into a *NCOI*/*Bgl**III* double digested pFUSE-hIgG1-Fc vector. Endonuclease digestion of the pFUSE-hIgG1-Fc2 and bTGF β -RIED PCR products produced *NCOI* and *Bgl**III* 5' overhangs and allowed our product to be inserted at the multiple cloning site between 640 nt which encodes the second nt of *NCOI* site and 648 nt encoding the second nt of *Bgl**III* (Figure 4.6).

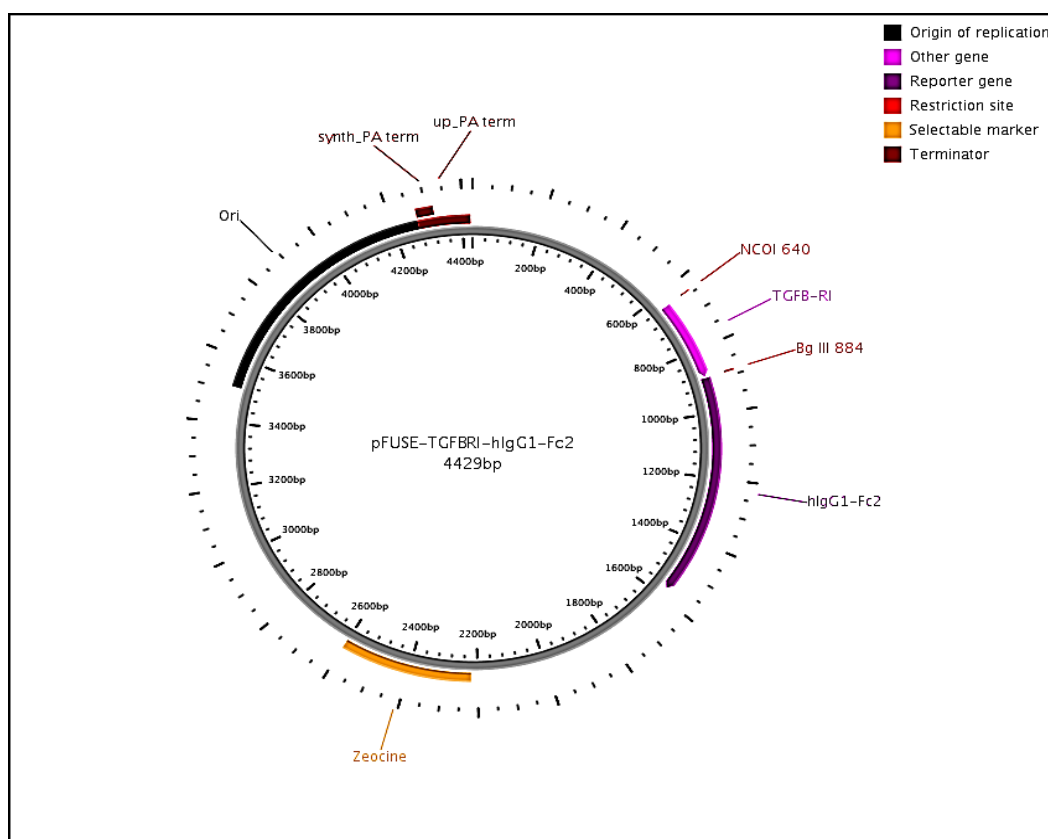


Figure 4.6: Map of pFUSE-TGF β -RIED-hIgG1-Fc2. Showing the human IgG1 Fc2 (889-1569), *NCOI* (640) and *Bgl**III* (884-888) restriction sites, bTGF β -RIED inserts (641-883), origin of replication (3512-4272) and zeocin resistance selection gene (2220-2601).

The gel purified bTGF β -RIIED PCR product was ligated into a *ECORI/BglIII* double digested pFUSE-hIgG1-Fc vector. The digested bTGF β -RIIED PCR fragment was inserted between the *ECORI* overhang at 626 nt and the second nt of *BglIII* at nt 648 (Figure 4.7).

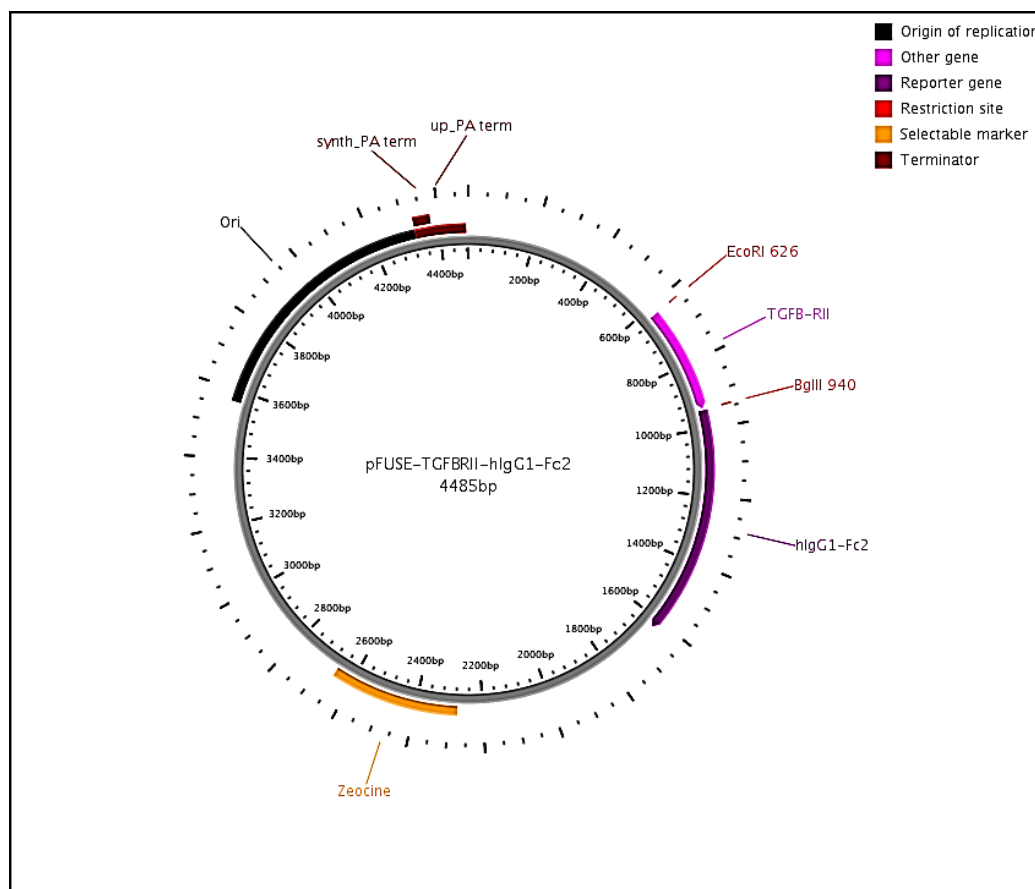


Figure 4.7: Map of pFUSE-TGF β -RII-hIgG1-Fc2. Showing the human IgG1 Fc2 (945-1625), EcoRI (626) and BglIII (940-944) restriction sites, bTGF β -RIIED insert (627-939), origin of replication (3568-4328) and zeocin resistant gene (2276-2657).

4.3.5 Conformation of the rTGF β -RIED-Fc and rTGF β -RIED-Fc plasmid

Ligated plasmids were transformed into competent DH5 α E. coli cells. Multiple white colonies (positive-insertions) from both plasmids were examined for the presence of the correct insert by endonuclease digestion. The digests indicated that ligation and transformation had occurred for both constructs (Figure 4.8).

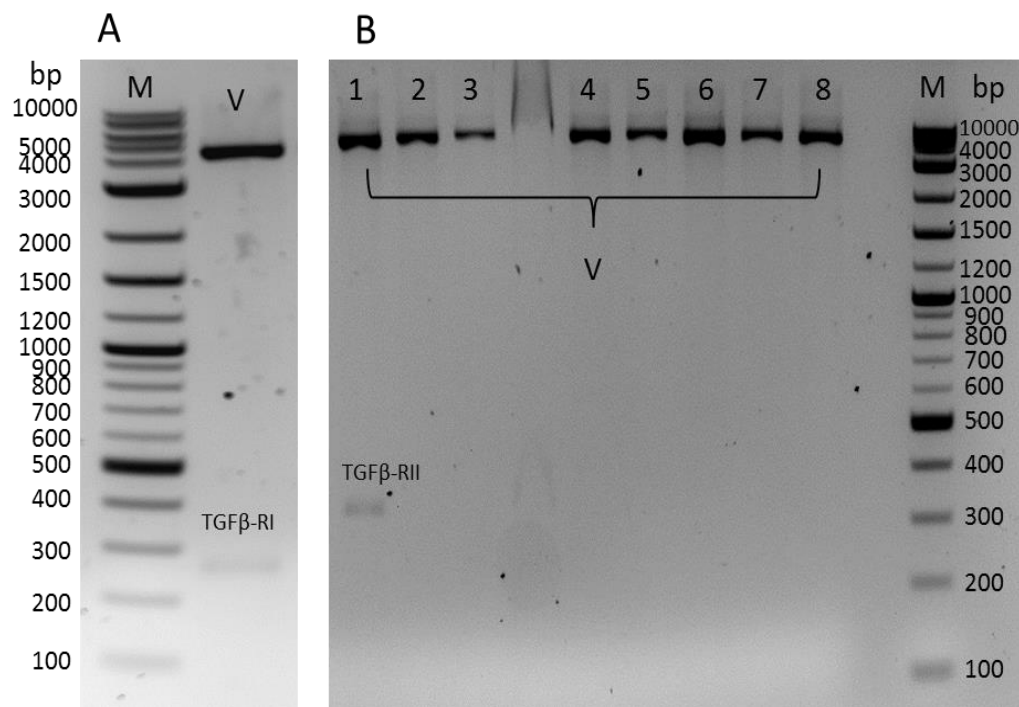


Figure 4.8: Agarose gel electrophoresis (1.5 %) of digested bTGF β -RIED-Fc (A) and bTGF β -RIED-Fc (B). Lane M: 1Kb plus DNA ladder. 1, 2, 3, 4, 5, 6, 7, 8 and V: pFUSE-hIgG1-Fc vector. A: 243 bp TGF β -RIED-Fc. B: 315 bp TGF β -RIED-Fc.

4.3.6 Transfection of HEK 293 cells and conformation of transfection using a pEGFP-C2 reporter plasmid

A reporter plasmid, pEGFP-C2 (Clontech, USA), was used in a co-transformation to confirm transfection by the presence of green fluorescence when examined by inverted fluorescence microscope 72 hrs after transfection (Figure 4.9).

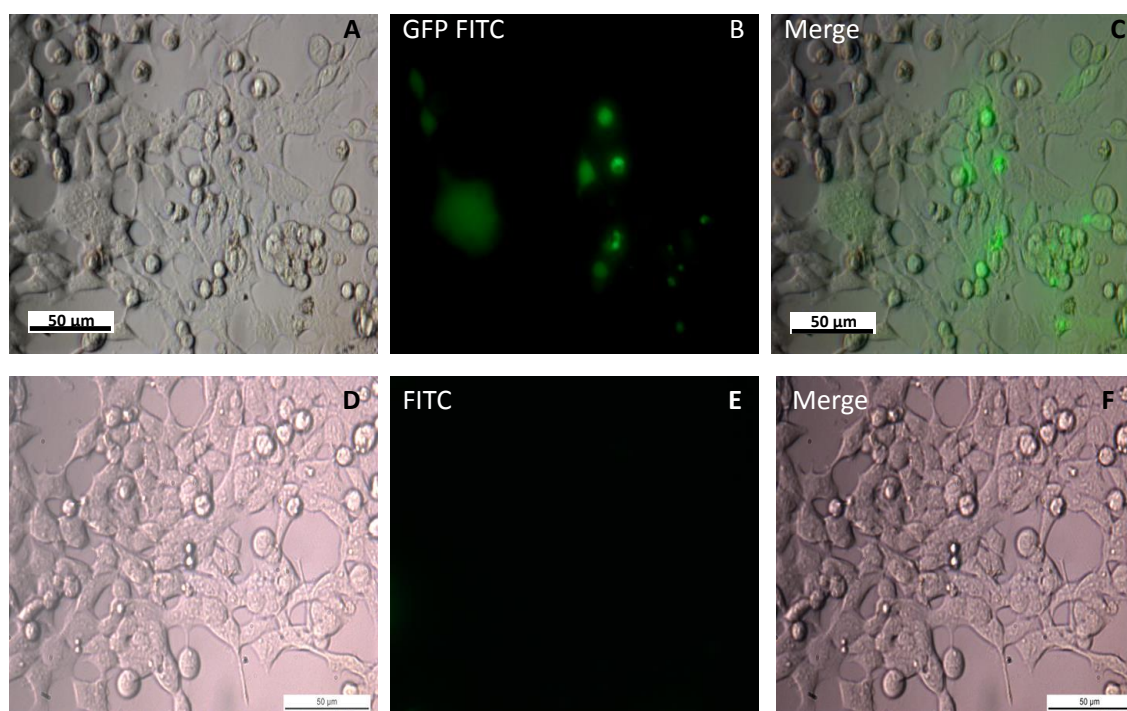


Figure 4.9: Expression of pEGFP-C2 in HEK-293 cells. Transfected HEK cells visualised using contrast phase (A) or FITC fluorescence (B, C) microscope showing the green fluorescent pEGFP-C2 protein. Non-transfected HEK cells visualised using contrast phase (D), or fluorescence (E, F) microscopy.

4.3.7 Clonal selection of bTGF β -RIED-Fc and TGF β -RIED-Fc fusion protein secreting HEK 293 cells

After confirming the transfection of HEK 293 cells using the control pEGFP-C2 plasmid, a limiting dilution protocol was applied to the transfected cells and supernatants from these cells were screened with ELISA to identify the clones secreting fusion proteins as below:

To screen supernatants from selected transfected HEK cells, human TGF- β 1 was used in a capture ELISA format. Anti-human Fc:HRP was used to detect bound fusion proteins. Supernatants from 96 well limiting dilution plates were screened and seven clones from TGF β -RIED-Fc and one TGF β -RIED-Fc transfected clone gave a positive OD reading above the back ground level of the non-transfected HEK cells. These were then transferred into T75 flasks and allowed to expand for further use.

4.3.8 Bovine TGF β -RIED-Fc bind human TGF- β 1 with high affinity

In order to determine and compare the binding capacity of the bTGF β -RIED-Fc and bTGF β -RIED-Fc to human TGF- β 1, a binding assay with a fixed concentration of hTGF- β 1 (100 ug/mL) was used in an ELISA. Analysis of the binding data revealed that the soluble bTGF β -RIED-Fc and bTGF β -RIED-Fc bound to hTGF- β 1 coated to the 96-well plate in a dose dependent manner. However, the binding affinity of bTGF β -RIED-Fc is ~2 fold higher than the binding of the bTGF β -RIED-Fc to hTGF- β 1. Moreover, the binding of both receptors were abolished at 1 in 64 dilutions (Figure 4.10).

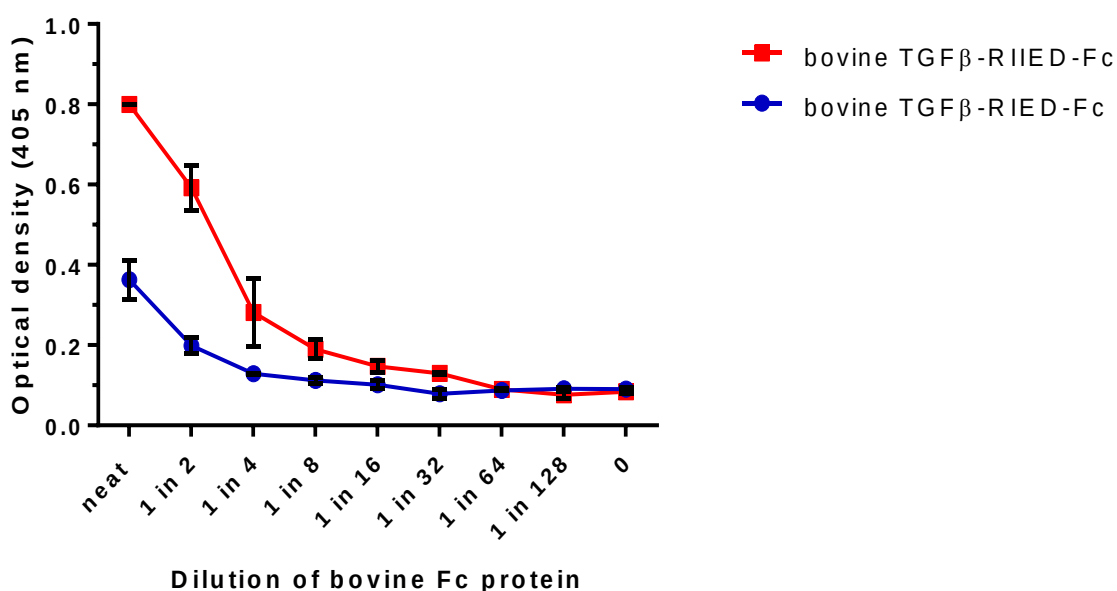


Figure 4.10: Binding assay of the hTGF- β 1 to different dilution of unpurified bTGF β -RIED-Fc and bTGF β -RIED-Fc fusion proteins. Data represent the mean OD $\pm$ SDM of two independent experiments.

4.3.9 Bovine TGF β -RIED-Fc bind FhTLM with high affinity

The binding of bTGF β -RIED-Fc and bTGF β -RIED-Fc to FhTLM protein was determined by ELISA, using decreasing concentrations of FhTLM and hTGF- β 1. As previously shown, bTGF β -RIED-Fc at concentration of (1.25 μ g/ml) binds to hTGF- β 1 with high affinity when compared with bTGF β -RIED-Fc. Interestingly, the binding of the bTGF β -RIED-Fc to FhTLM at a high coating concentration of FhTLM (600 and 500 ng/ml) was $\sim$ 2 fold higher than that of the bTGF β -RIED-Fc at the same concentration. The binding of bTGF β -RIED-Fc with FhTLM remained high for all concentrations and declined only in the absence of FhTLM. While, bTGF β -RIED-Fc only showed binding to 600 and 500 ng/ml of FhTLM and declined thereafter. However, the binding of bTGF β -RIED-Fc to hTGF- β 1 was $\sim$ 4 fold higher than the binding to FhTLM (Figure 4.11).

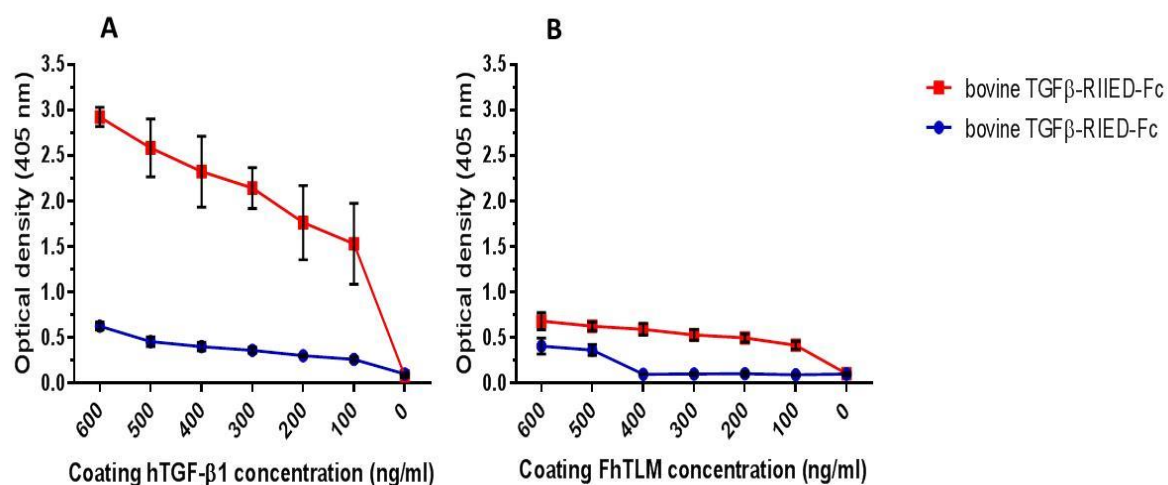


Figure 4.11: Binding assay of different concentration of hTGF- β 1 (A) and FhTLM (B) to (1.25 μ g/ml) bTGF β -RIED-Fc and bTGF β -RIED-Fc. Data represent the mean OD $\pm$ SEM of two independent experiments.

4.3.10 Bovine TGF β -RIIED-Fc binds FhTLM with high avidity

To test the avidity of FhTLM (500 ng/ml) with our fusion proteins, FhTLM was used as a capture antigen, in combination with bTGF β -RIED-Fc and TGF β -RIIED-Fc fusion proteins at concentration of (1.25 μ g/ml). To this, decreasing concentrations of KSCN were introduced into the ELISA after addition of Fc fusion proteins (Figure 4.12).

It is clear that the avidity of the bTGF β -RIED-Fc to FhTLM was significantly stronger than the bTGF β -RIED-Fc and the avidity reduced as the concentration of the KSCN increased. At 2 M KSCN, the binding of bTGF β -RIED-Fc was completely abolished. While the dissociation of bTGF β -RIED-Fc was abolished by 3M of KSCN.

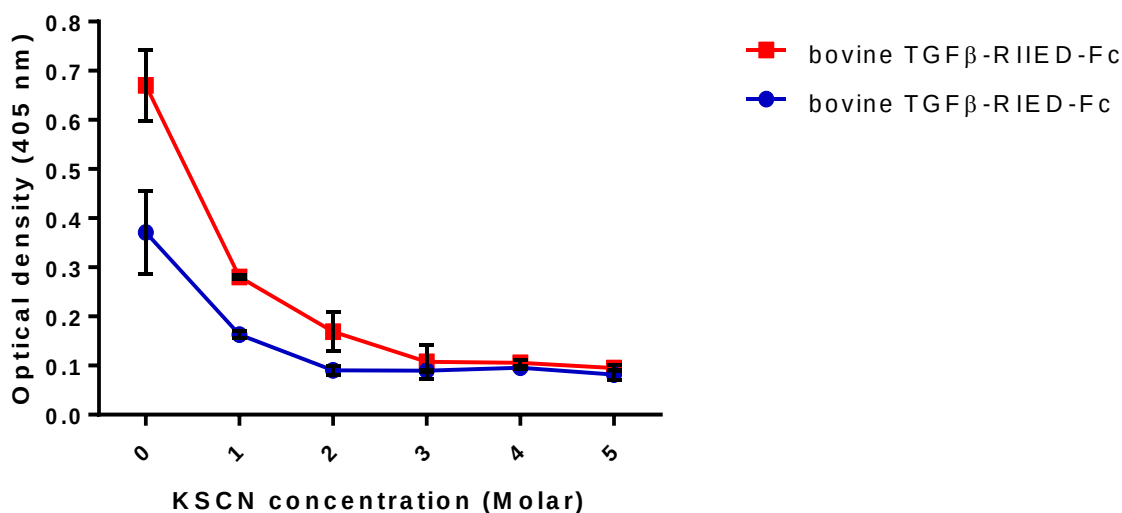


Figure 4.12: Avidity of the FhTLM to bTGF β -RIED-Fc and bTGF β -RIIED-Fc using different concentration of KSCN. Data represent the mean OD $\pm$ SDM of two independent experiments.

4.3.11 *In vitro* effect of FhTLM on bovine PBMCs proliferation

Proliferation of PBMCs in response to FhTLM was determined using alamarBlue, with ConA used as a positive control. Following 4 hrs incubation with alamarBlue, the proliferation of the PBMCs was measured and compared in each treatment (Figure 4.13). The highest percentage of PBMC proliferation was seen in cultures stimulated with ConA. Treatment with FhTLM significantly reduced the percentage proliferation of PBMCs ($P < 0.05$). However, FhTLM reduced the proliferative capacity of ConA in culture treated with ConA and FhTLM, this effect was not statistically significant. Dose response to FhTLM was previously determined (Japa 2014, Sulaiman et al., under review) and the optimal dose was determined from this work and used here in.

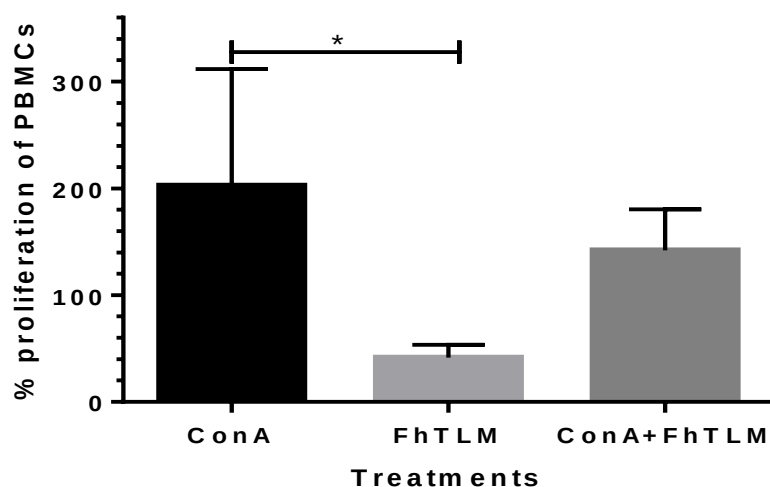


Figure 4.13: Percentage of PBMCs proliferation in response to ConA (5 $\mu\text{g/ml}$), FhTLM (300 ng/ml) and combination of ConA and FhTLM. Results were analysed using one-way ANOVA. * indicates $P < 0.05$. Data represent the mean PBMCs percentage $\pm$ SDM of two independent experiments performed in triplicate. Results present data normalised to the proliferation of unstimulated PBMCs.

4.3.12 *In vitro* effect of FhTLM on IFN- γ production from bovine PBMCs

IFN- γ concentration in PBMC cultures treated as detailed in (4.2.10) was measured by ELISA and compared between treatments (Figure 4.14). A mean increase of 76.68 ng/ml in the concentration of IFN- γ was seen in ConA treated cultures. IFN- γ production was significantly inhibited ($P<0.001$) in the culture treated with FhTLM when compared to ConA treated culture. However, it was slightly increased (13.65 ng/ml) when compared to unstimulated PBMCs (11.39 ng/ml). In addition, FhTLM suppressed the stimulatory effect of ConA on IFN- γ production by significantly ($P<0.01$) reducing the IFN- γ levels in culture treated with ConA and FhTLM.

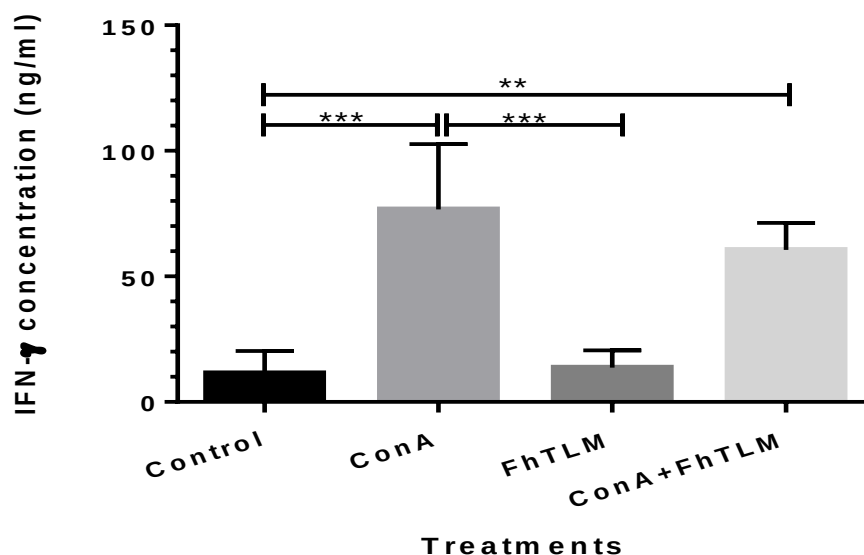


Figure 4.14: IFN- γ concentration in PBMCs treated with ConA (5 μ g/ml), FhTLM (300 ng/ml) and combined ConA and FhTLM was measured by ELISA. Results were analysed using one-way ANOVA. ** indicates $P<0.01$, and *** indicates $P<0.001$. Data represent the mean of IFN- γ concentration $\pm$ SDM of two independent experiments performed in triplicate.

4.3.13 *In vitro* effect of FhTLM on IL-10 production from bovine PBMCs

The concentration of IL-10 in culture supernatants of PBMC treated as detailed in (4.2.10) was measured by ELISA and compared between different treatments (Figure 4.15).

Results shows that IL-10 concentration in unstimulated cultures produced the lowest IL-10 concentration means (100.46 pg/ml) and ConA stimulated PBMCs culture produced a mean of 9120.72 pg/ml). In cultures treated with FhTLM, a significant increase ($P<0.05$) in the IL-10 concentration was demonstrated. However, in cultures treated with a combination of ConA and FhTLM, a non-significant increase was found in the IL-10 concentration when compared to non-treated culture.

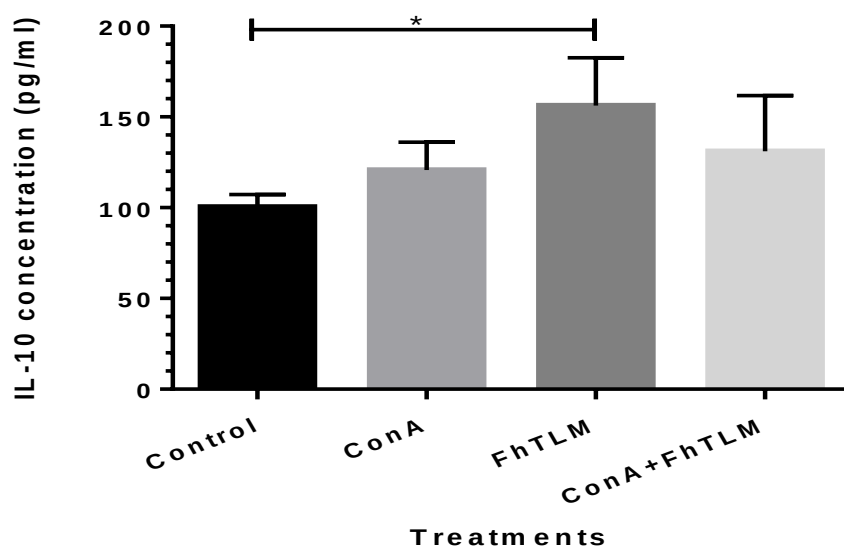


Figure 4.15: IL-10 concentration in PBMC treated with ConA (5 μ g/ml), FhTLM (300 ng/ml) and ConA and FhTLM was measured by ELISA. Results were analysed using one-way ANOVA. * indicates $P<0.05$. Data represent the mean of IL-10 concentration $\pm$ SDM of two independent experiments performed in triplicate.

4.3.14 *In vitro* effect of FhTLM on GATA1 expression from bovine PBMCs

As we had previously identified GATA1 as a potential regulatory transcription factor for IL-10 (chapter 3), we sought to determine if FhTLM could modulate GATA1 expression. Bovine PBMCs stimulated with ConA, FhTLM and a combination of both treatments were tested for changes in GATA1 expression by immunofluorescence. The percentage of GATA1 expression was determined by examination of ≥ 500 cells from each 5 field of the slide using Leica DM500B microscope at X400 objective (Figure 4.17). PBMC nuclei were stained by DAPI (blue) and GATA1 was labelled with FITC (green fluorescence) (Figure 4.16).

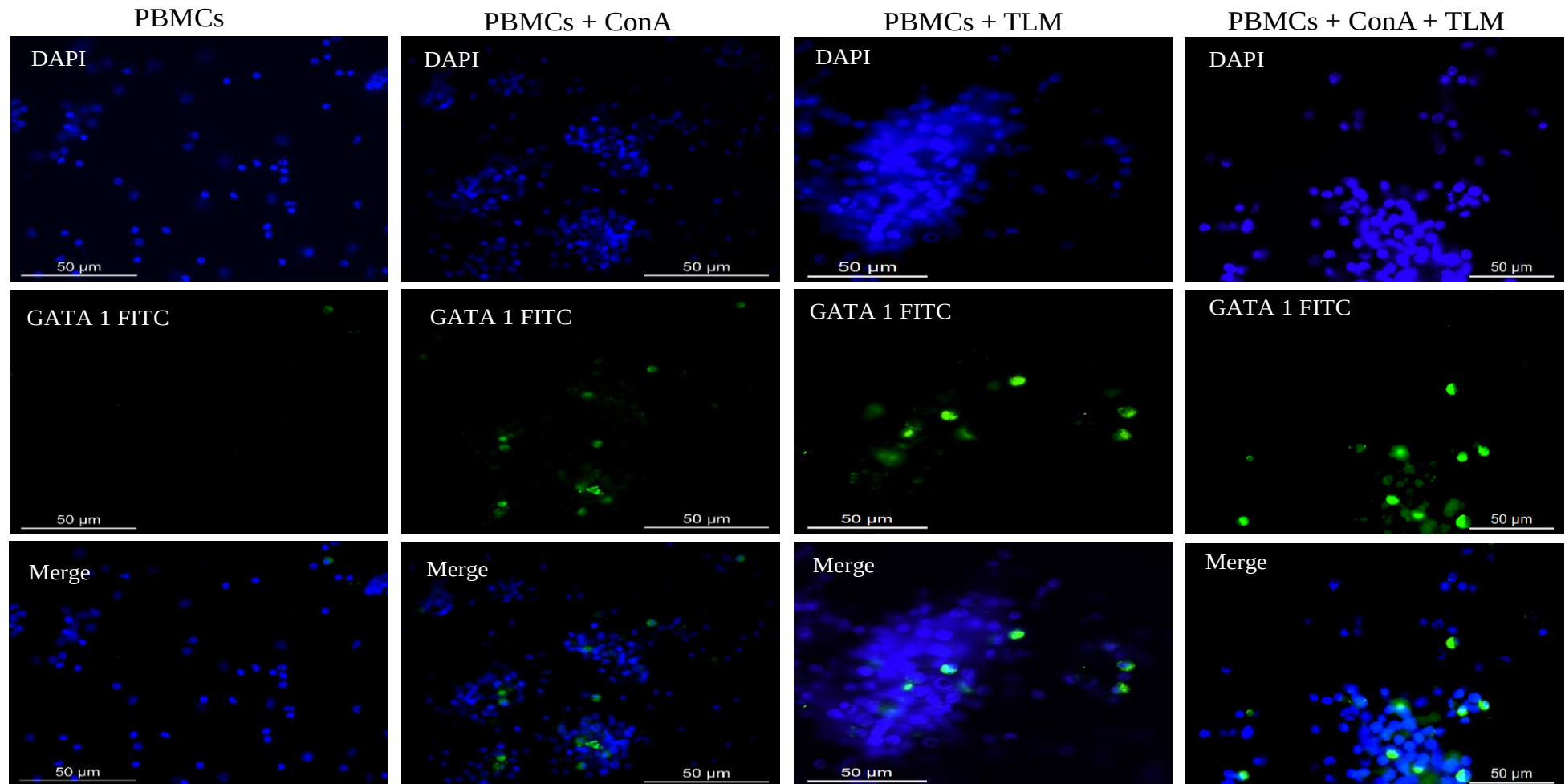


Figure 4.16: Immunofluorescence of PBMC stimulated with ConA, Fh TLM and a combination of ConA and FhTLM.

Nuclei stained with DAPI and GATA1 protein with FITC. Cells were visualised under a Leica DM 500B microscope at X 400 objective.

The percentage of GATA1 positive cells was significantly ($P < 0.01$) higher in cells treated with ConA when compared to the unstimulated and FhTLM stimulated PBMCs. GATA 1 expression in response to FhTLM was significantly ($P < 0.05$) increased in FhTLM-stimulated culture when compared to unstimulated PBMCs. There was no significant difference between the FhTLM and combination FhTLM and ConA treatments (Figure 4.15).

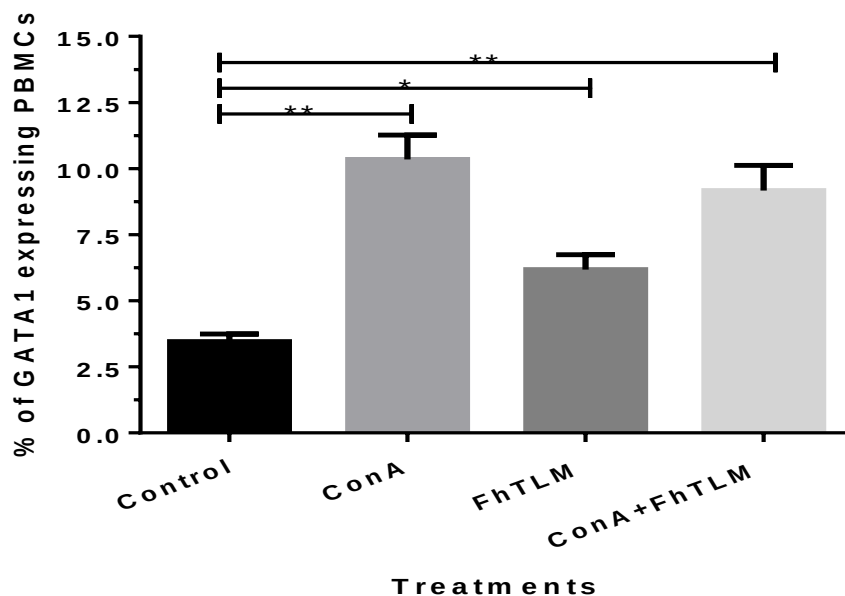


Figure 4.17: Percentage of GATA1 expressing PBMCs in response to ConA, FhTLM, and combined ConA and FhTLM as determined by immunofluorescence and inspection of ≥ 500 cells under microscope. Results were analysed using one-way ANOVA. * indicates $P < 0.05$, and ** indicates $P < 0.01$. Data represent the mean percentage $\pm$ SDM of ≥ 500 GATA1 expressing PBMCs from two independent experiments.

4.4 Discussion

The pleotropic functions of TGF- β are initiated following binding to the type I and type II cell surface receptors. One of the main functions of TGF- β in the immune system is the regulation of cellular proliferation and differentiation (Massague, 1990). To understand the molecular signalling and the structure of TGF- β receptors, expression cloning studies and cross binding of iodinated TGF- β to cell surface receptors have been widely used. In these studies the classification of the receptors depended on the size of the cross linked complex formed between iodinated TGF- β and different receptors (Franzen et al., 1993, Ebner et al., 1993). Radio-iodinated TGF- β studies indicated that iodinated human TGF- β bind with high affinity to rat fibroblasts and BALB/c 3T3 cells *in vitro* and scatchard analysis of the binding of these two cells indicated that there is only a single site for high affinity binding in these cells (Massague and Like, 1985).

To examine the role of the FhTLM in signalling via the bovine TGF- β system, we cloned bovine TGF β -RIED and TGF β -RIIED as -hIgG2-Fc fusion proteins. HEK 293 cells were transfected with the recombinant plasmids for the bTGF β -RIED-Fc and bTGF β -RIIED-Fc fusion proteins. Following visualization of the positive HEK 293 cells using the pEGFP-C2 reporter gene and ELISA we successfully distinguished positive clones from the negative clones. Supernatant from the positive HEK clones was used to determine the binding affinity of the recombinant bTGF β -RIED-Fc and TGF β -RIIED-Fc fusion proteins to human TGF- β 1. Our ELISA results indicated that bTGF β -RIIED-Fc bind with high affinity to hTGF- β 1. In contrast, the affinity of interaction of bTGF β -RIED-Fc to hTGF- β 1 was lower when using the same approach. As FhTLM shares sequence similarity with mammalian

TGF- β we sought to determine whether this protein will bind to the ED of bovine TGF β -RI/II with the same affinity as hTGF- β 1. We found that FhTLM binds bTGF β -RII-ED-Fc with a higher affinity than to bTGF β -RI-ED-Fc. This in agreement with that observation for the binding measurement recorded for TGF- β 1 and TGF- β 3 to TGF β -RII-ED using a surface biosensor approach (De Crescenzo et al., 2003). Previously, it has been identified that both TGF β -RI and RII bind TGF- β I and TGF- β II with higher affinity than TGF- β III (Cheifetz et al., 1987). We also tested the avidity (strength of interaction) of the bTGF β -RII-ED-Fc and bTGF β -RII-ED-Fc to FhTLM by introduction of KSCN into our ELISA. We found that 2M of KSCN was able to dissociate the binding of the bTGF β -RII-ED-Fc to FhTLM and 3M induced dissociation of the bTGF β -RII-ED-Fc. Altogether, these data indicate that FhTLM is able to bind to the bovine TGF- β receptors with greater avidity to bTGF β -RII.

A growing body of literature has identified a role for TGF- β in immune regulation, mainly through the inhibition of function of lymphoid, epithelial, endothelial, fibroblast, neuronal and hematopoietic cells (Tucker et al., 1984, Moses et al., 1985, Roberts et al., 1985). Several workers have demonstrated the crucial role of the type I and type II receptors in mediating TGF- β functions. For example TGF- β mediated inhibition of mink lung epithelial cell proliferation is primarily associated with the presence of TGF β -R1 on the surface of these cells (Boyd and Massague, 1989).

Several TGF- β homologues have been identified in different parasite species implying an important role for these molecules in modulation of the host immune response (Hewitson et al., 2009). In our study we examined the *in vitro* effects of FhTLM on bovine PBMC proliferation and propose that FhTLM significantly impaired PBMC proliferation. As FhTLM is homologous to TGF- β this in agreement with several studies including a recent

study that demonstrated that TGF- β produced by regulatory B-cells is responsible for the inhibition of T-cell proliferation in a co-culture model (Nouel et al., 2015). In addition, we have confirmed that FhTLM binds TGF β -RII with high affinity. Moreover, the stimulation of Smad 2/3 seen in the previous studies (Sulaiman et al., under review). We therefore hypothesize that TGF β -RII may be indirectly responsible for mediating the suppressive effect of FhTLM on PBMCs. This is supported by a study demonstrating that *H. polygyrus* excretory secretory antigens (HES) induced Foxp3 T-reg cells via TGF- β signalling pathways, and inhibition of T-reg cells was seen in the absence of TGF- β RII (Grainger et al., 2010). Other studies have indicated that the inhibitory effects of TGF- β on cell proliferation may be linked to its ability to block cytokine production such as inhibition of IL-2 mediated T-cell proliferation, IL-1 induced thymocyte proliferation and growth factor induced B-cell proliferation (Espevik et al., 1990a, Fargeas et al., 1992, Espevik et al., 1987, Kehrl et al., 1986b, Ristow, 1986, Kehrl et al., 1986a). We have also sought to test these mechanisms by measuring cytokine production from PBMCs in response to FhTLM. Our IFN- γ ELISA results demonstrate that FhTLM is a potent inhibitor of IFN- γ production from PBMCs. This data is supported by the results of Espevik et al., (1987) who demonstrated that TGF- β treatment inhibited IFN- γ production by PBMCs. The inhibitory effects of FhTLM on IFN- γ production from PBMCs may suggest a role for this protein in suppression of the host immune response. Moreover, it is well known that IFN- γ , a Th1 cytokine, is responsible for partial protection against liver flukes (Pleasant et al., 2011, Mulcahy et al., 1999, Flynn and Mulcahy, 2008b). Our results indicate that suppression of IFN- γ production by FhTLM protein may be crucial in maintaining parasite survival in the face of ongoing immune responses. We also measured the *in vitro* level of IL-10 production from PBMCs in response to FhTLM and we demonstrated an increase in the IL-10 release

from cells treated with FhTLM. This is in agreement with previous results that reported that TGF- β induces IL-10 production from macrophages in normal and tumour bearing mice (Maeda et al., 1995). The SMAD protein activated downstream of the TGF- β pathway are necessary for production of IL-10 from Th cells (Blokzijl et al., 2002, Kitani et al., 2003).

It has been demonstrated that IL-10 inhibited *F. hepatica* specific IFN- γ production from PBMCs during the chronic stage of infection (Flynn and Mulcahy, 2008b). Recently it has been shown that regulatory B-cell produced IL-10 which inhibited IFN- γ production from T-cells *in vitro* (Blair et al., 2010). Our observation indicates that FhTLM alone induces IL-10 production from PBMCs but not when FhTLM is in combination with ConA, suggesting that competition for cytokine production and turnover between FhTLM and ConA responses utilises IL-10 quicker than in FhTLM stimulated cultures. Thus IL-10 level appear lower than expected in comparison to FhTLM treated cultures. The inhibition of IFN- γ and the stimulation of IL-10 seen in response to FhTLM alone indicated that FhTLM may inhibit IFN- γ through the production of IL-10 from bovine PBMCs.

Our transcription factor search identified the presence of a GATA1 binding site in the promotor region of the bovine *il-10* gene (chapter 3). Therefore, we sought to determine if the expression of this factor is altered in PBMCs in response to FhTLM. It has been shown that GATA1 induces regulation of the *st2* gene in murine mast cells, the *st2* gene being responsible for induction of high levels of IL-10, IL-5 and IL-4 (Lohning et al., 1998, Gachter et al., 1998). A more recent study indicated that TGF- β signalling is important in the regulation of GATA1, using SB431542, a known inhibitor of TGF β -RI, for generation of mice displaying reduced levels of GATA1 *in vivo* (Martelli et al., 2005). The high GATA 1 expression seen in PBMCs culture treated with FhTLM led us to suggest that

FhTLM may induce TGF- β signalling pathways in a way that leads to stimulation of GATA1 expression and indirectly induces IL-10 expression.

In conclusion, our findings indicated that the FhTLM protein has the ability to bind to bovine receptors and induce TGF- β signalling. Several factors have been identified in parasites that show a similarity to mammalian TGF- β and have the same Smad pathways (Massague and Chen, 2000). These proteins have important roles in regulation of the immune system and might be associated with *F. hepatica* survival by suppressing PBMCs proliferation, IFN- γ release and enhancing IL-10 production. Finally, we suggest that the signalling of the TGF- β pathway by FhTLM may be a factor in mediating IL-10 production via GATA1 activation in the PBMC culture. These observations on FhTLM may be important in the development of an innovative vaccine candidate for treatment of the liver fluke.

**5 Regulation of IL-10 and IFN- γ during *Neospora caninum* infection,
and its effects on dairy production parameters**

5.1 Introduction

Neospora caninum is an intracellular protozoan parasite primarily associated with neurological disorders in dogs and abortion in cattle (Dubey, 2005). *N. caninum* has a complex life cycle, where dogs, coyotes and other canids act as the definitive host and cattle, buffalo, goats, sheep, horses and deer are all recognised as intermediate hosts (Gondim et al., 2004a, Dubey and Lindsay, 1996, Moore, 2005, Gondim, 2006). To date there is no evidence of human infection by this parasite (McCann et al., 2008) and until 1988 *N. caninum* was misidentified as the closely related *Toxoplasma gondii* due to extensive morphological similarity. Analysis of the ribosomal DNA sequence revealed that *N. caninum* and *T. gondii* are closely related but distinct species (Ellis et al., 1994, Marsh et al., 1995). Neosporosis is common among several ungulate species, but in pregnant cattle abortion is the main consequence of infection. Abortion which occurs as a result of the placental damage caused by the replicating parasite and/or due to an immune mediated inflammatory response of the dam and the foetus (Maley et al., 2003). Moreover, along with abortion, *N. caninum* is also responsible for major economic losses represented by stillbirths, low milk yield, premature culling and loss of condition (Graham et al., 1996, Thurmond and Hietala, 1997a, Thurmond and Hietala, 1996, Barling et al., 2000). Surprisingly, few studies have examined the effects of *N. caninum* infection on milk production, and where these studies have been published, they have reported conflicting results. Results from three studies in dairy herds in California, Florida (USA) and Galicia indicated a reduction in milk yield among seropositive cows (Thurmond and Hietala, 1997b, Hernandez et al., 2001, Gonzalez-Warleta et al., 2011). Conversely, in New Zealand

a study of dairy herds revealed an increase in milk yield by 0.4 litre /cow per day among seropositive cows (Pfeiffer et al., 2000), while, in Canada a study of 90 randomly selected dairy herds failed to show any differences in the milk yield between seropositive and seronegative animals (Keefe and VanLeeuwen, 2000). The differences in the observed correlation between *N. caninum* infection and milk production in these studies may be influenced by the differing management systems between countries. For example, in New Zealand pasture-based herd management systems are more common which differ greatly from the dry-lot system used in United States (Hobson et al., 2002). Furthermore, herd-specific routes and timing of infections and consequent immune responses may play an important role in these effects.

Cattle can acquire *N. caninum* infection by two main routes: ingestion of food or water contaminated with oocysts (Dubey et al., 2007), or by congenital infection from the dam to the foetus through the placenta during pregnancy. This latter route appears to be the most efficient way of infection and more than 95 % of the calves born from infected mothers have antibodies against *N. caninum* and remain seropositive for life (Dubey et al., 2007, Piergili Fioretti et al., 2003). However, another route of transmission has been demonstrated; calves may be infected by ingestion of milk contaminated with *N. caninum* tachyzoites (Davison et al., 2001). Several workers have indicated a vital role for the immune system in trans-placental transmission and abortion associated with *N. caninum* infection. Immunization of pregnant mice with a single dose of crude *N. caninum* tachyzoite lysate prior to infection reduced the trans-placental transmission of the infection to offspring (Liddell et al., 1999). Experimental studies have demonstrated the importance of cell mediated immunity and IFN- γ in mediating protection against intracellular parasite such as *T. gondii* (Suzuki et al., 1988, Suzuki and Remington, 1990). The classic Th1

immune response is dominated by production of IL-12, IFN- γ and TNF- α , activated free oxygen and nitric oxide radical production pathways that are fatal for many protozoans (Hunter and Reiner, 2000). Several workers have demonstrated that parasite-specific cell mediated immunity represented by cellular proliferation and IFN- γ can be induced in both naturally and experimentally infected cattle (Lunden et al., 1998, Marks et al., 1998, De Marez et al., 1999, Williams et al., 2000, Andrianarivo et al., 2001, Khan et al., 1997). The pro-inflammatory cytokines including IFN- γ and IL-12 have been shown to have an important protective role against *N. caninum* (Baszler et al., 1999). Initial evidence for the role of cell mediated immunity in protection against *N. caninum* came from *in vitro* studies showing that IFN- γ suppressed the intracellular multiplication of *N. caninum* within ovine fibroblast cells, bovine brain cells and recently in rat neuron/glial co-culture (Innes et al., 1995, Yamane et al., 2000, De Jesus et al., 2014). Several *in vivo studies* have shown that the absence of IFN- γ and IL-12 in mice inhibited the natural resistance of animals to *N. caninum* infection (Khan et al., 1997). Tanaka et al., (2000) highlighted the role for IFN- γ and the cellular component of the immune response in the immunopathology of the infection. Several studies demonstrated a mixed Th1/Th2 response during early gestation (Entrican, 2002) which was associated with abortion (Tangri and Raghupathy, 1993). Whereas, the ability of the infected cow to induce parasite specific cell proliferation and IFN- γ production was reduced significantly during the mid-gestation period which is beneficial in maintaining the pregnancy (Innes et al., 2001).

In summary, it is clear that the generation of a Th1 immune response and the associated pro-inflammatory cytokines play a critical role not only in controlling *N. caninum* infection but also in abortion.

The aims of the current study were to determine the effects of the regulatory cytokine IL-10 on IFN- γ and production parameters (milk yield, lactation numbers) following infection. We also examined the congenital transmission of the *N. caninum* infection from dams to calves to determine if IL-10 was a factor in this. Our data adds further insight into the effect of immunoregulation upon key production parameters and overall animal performance.

5.2 Material and methods

5.2.1 Source of samples and serology

Serum samples were collected from non-pregnant cows belonged to a closed dairy herd on the Shropshire Cheshire border. The herd consisted of 105 dairy cows; all animals were serum sampled, of these 82 were used due to a lack of production data for the remaining animals. The serological status of animals had already been established using a commercial ELISA kit (IDEXX). Levels of antibodies to *N. caninum* were expressed as a sample to positive (SP) ratio, calculated by using the following equation:

$$SP = \frac{OD \text{ Sample} - OD \text{ Negative Control}}{OD \text{ Positive Control} - OD \text{ Negative Control}}$$

Samples with SP ratio ≤ 1.2 were considered as negative and samples with SP ratio >1.2 were classified as positive according to the manufacturer's recommendation. Production data including the lactation number (Lac No), lifetime daily yield (LDY), and calf-dam relationships was obtained from the national milk records, Herd Companion database. No data recorded for the age of the calves and the length of lactation from the cows. Animals with no milk yield were classified as 'no yield', those with yield from 0.59-11 litre were considered as 'low yield', from 11-15 litre were classified as 'medium yield' and from 15-20 were 'high yield' cows.

5.2.2 Detection of IL-10 and IFN- γ from serum samples by ELISA

The level of IL-10 in the serum samples of all 82 animals was measured using the commercially available bovine interleukin 10 ELISA kit (Cusabio Biotech Co. UK) and IFN- γ concentration was measured using bovine IFN- γ ELISA reagent kit (Thermo scientific, USA) as described in (2.8).

5.2.3 Statistical analysis

Results were graphed and data analysed using GraphPad prism version 6.0 software. The Unpaired t test was used for analysis of the differences between the SP ratio of seropositive and seronegative groups. Significant differences were accepted at (95 % confidence limit). The Chi-square test was used to analyse the differences in the percentage of calves born to seropositive and seronegative dams. A Pearson r test was used to test the relationship of the (Lac No), and (LDY) to cytokine levels and SP ratios in both seropositive and seronegative animals. All bars represent the means $\pm$ SDM of seropositive and seronegative animals.

5.3 Results

5.3.1 Seropositivity to *N. caninum*

Initially all animals were grouped as seropositive and seronegative according to their SP ratio in accordance with manufacturer's instructions. Screening of the herd of 82 animals revealed that 41 animals (50 %) were seropositive and 41 seronegative to *N. caninum* infection. The mean SP ratio of seropositive animals was (2.455) and the mean SP ratio of seronegative cows was (0.2577)

5.3.2 Family analysis

Of the 82 cows enrolled in the study we found 29 dams with calves within the herd and of these 12 (41 %) were seropositive and 17 (59 %) were seronegative. To analyse the impact of dam-calf relationship on congenital transmission and SP ratio we used a Chi-square test. Of 12 seropositive dams, 7 (58 %) gave birth to seropositive calves which is significantly ($P < 0.001$) higher than the number of dams that gave seronegative calves 5 (42 %). Surprisingly, of 17 seronegative dams, 5 (29 %) gave birth to seropositive calves (Table 5.2).

Table 5.1: Serostatus of calves born to seropositive and seronegative dams

Dams	Calves		Significant
	Seropositive	Seronegative	
Seropositive (No/%)	7 (58 %)	5 (42 %)	Yes
Seronegative (No/%)	5 (29 %)	12 (71 %)	Yes

The seropositive cows were arranged into ‘high seropositive’ (SP ratio higher than the median SP ratio 2.6273 of the all 12 seropositive cows), and ‘low seropositive’ (SP ratio is <2.6273) as previously used to find the association of anti-*N. caninum* antibodies in milk and abortion in dairy cattle (Gonzalez-Warleta et al., 2011). The number of high seropositive cows was equal to the low seropositive cows number (6). (84 %) of the high seropositive cows give birth to seropositive calves which is significantly ($P<0.001$) higher than the number of dams that gave seronegative calves (16 %). In contrast, only (33 %) of the low seropositive cows gave birth to seropositive calves which is significantly ($P<0.001$) lower than dams with seronegative calves (67 %) (Table 5.3).

Table 5.2: Calves percentage differences born to high and low seropositive dams

Dams serostatus (No)	Seropositive calves (No/%)	Seronegative calves (No/%)	Significant
High seropositive (6)	5 (84 %)	1 (16 %)	Yes
Low seropositive (6)	2 (33 %)	4 (67 %)	Yes

Using Pearson r test, no significant correlation ($R^2 = 0.225$, $P = 0.119$) was found between the dams and calves SP ratio, indicating that high dam SP ratio, and hence antibody levels, had no association with the transmission of infection to the offspring. The average IL-10 level in high seropositive animals (291.719 pg/ml) was slightly higher than the average of the low seropositive dams (256.371 pg /ml).

The seropositive calves had a mean SP ratio of 2.384 and their corresponding dams had a mean SP of 2.667. Five seronegative calves with a mean SP ratio of 0.516 were born to seropositive dams with a mean SP ratio of 2.465. No significant difference was seen between the mean SP ratio of seropositive dams that give birth to seropositive and seronegative calves using unpaired t test with Welch's correction. However, a significant ($P < 0.001$) difference was observed between the mean SP ratio of seronegative dams (0.0) that gave birth to seropositive calves compared to mean SP ratio of cows (0.2655) that gave birth to seronegative calves (Table 5.4).

Table 5.3: Mean SP ratio of the 29 dams as divided according to the serostatus of their calves.

Dams SP ratio (Mean ±SD)	Calves		Significant
	Seropositive	Seronegative	
Seropositive	2.667218±0.122	2.465±0.239	N. S.
Seronegative	0.0±0.0	0.2655±0.145692	YES

5.3.3 IL-10 and IFN- γ level in sera of seropositive and seronegative calves born from infected and non-infected dams

Animals were stratified according to the serological results of the dams and the calves and IL-10 and IFN- γ concentration from calf serum born to infected and non-infected mothers were analysed (Figure 5.1). No significant differences ($P > 0.05$) were identified in the concentration of IL-10 from seropositive calves from either class of dam, Whereas, seronegative calves born to seropositive dams produced significantly higher concentration ($P > 0.05$) of IL-10 than those born from negative dams. IFN- γ concentration was significantly higher ($P > 0.01$) in seropositive calves than seronegative born from seropositive dams. However, no significant differences ($P > 0.05$) were found between seropositive and seronegative calves born from seronegative mothers. Using the Pearson r test, no significant correlation ($R^2=0.512$, $P= 0.109$) was found between the IL-10 level of positive dams and positive calves, and no correlation ($R^2=0.817$, $P=0.280$) was found between the IL-10 level of seropositive dams and their negative calves. No correlation ($R^2=0.006$, $P=0.875$) was found between the IFN- γ level of seropositive dams and corresponding positive calves.

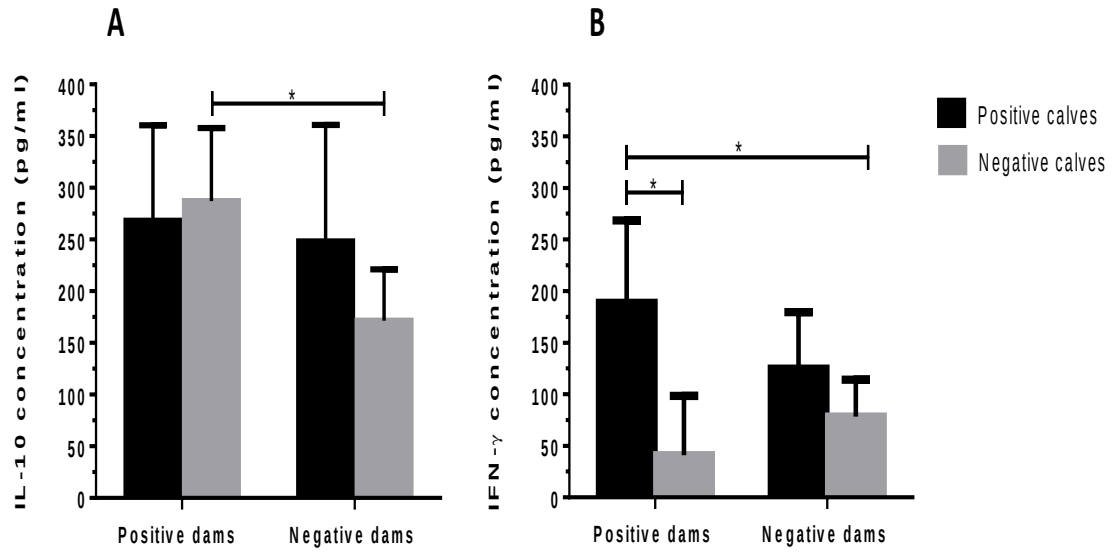


Figure 5.1: IL-10 and IFN- γ concentration in sera of calves born to dams of different serological status. Results were analysed using 2-way ANOVA. * indicates $P < 0.05$, and ** indicates $P < 0.01$. Data represents the mean $\pm$ SDM of IL-10 and IFN- γ concentration in sera of groups of animals.

5.3.4 IL-10 and IFN- γ levels in seropositive and seronegative cows (82) used in the study

Serum IL-10 and IFN- γ concentration of all positive and negative animals were measured and compared between the two major groupings (Figure 5.2). High concentration of IL-10 (255.70 pg/mL) were observed in seropositive cows, whereas, IL-10 concentration in seronegative animals were significantly ($P < 0.05$) lower. Seropositive animals produced a significantly higher ($P < 0.05$) concentration of IFN- γ when compared to seronegative animals (Figure 5.2).

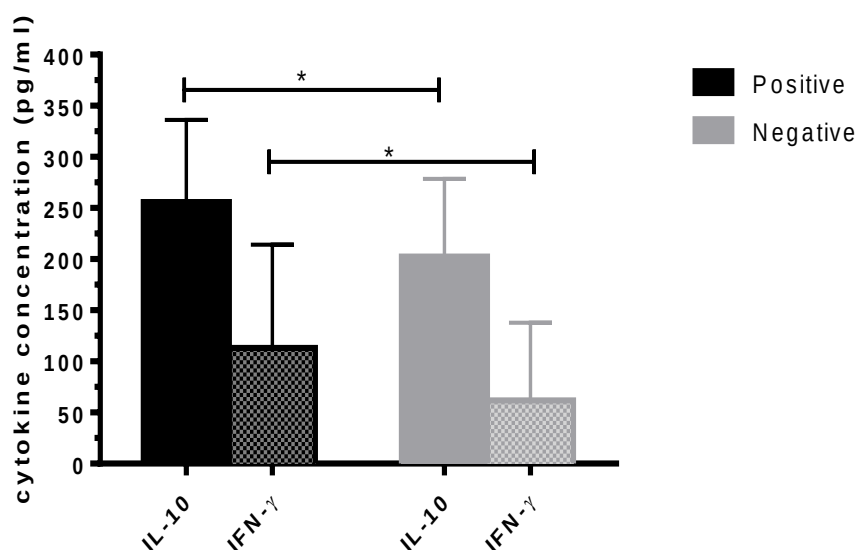


Figure 5.2: IL-10 and IFN- γ concentration in sera of cows with different serological status.

Results were analysed using 2-way ANOVA. * indicates $P < 0.05$. Data represent the mean $\pm$ SDM of serum IL-10 and IFN- γ concentration from 82 cows.

5.3.5 Association of the lactation number to the SP ratio of seropositive and seronegative animals

Animals were grouped according to lactation number (Lac No) data obtained from the NMR interherd database and serology status (Figure 5.3). Analysis of the Lac No with regard to corresponding SP ratio was conducted using Pearson r test and indicated that there was no correlation between the Lac No and SP ratio in either seropositive ($R^2 = 0.03009$; $P = 0.78$) or seronegative animals ($R^2 = 0.000563$; $P = 0.99$).

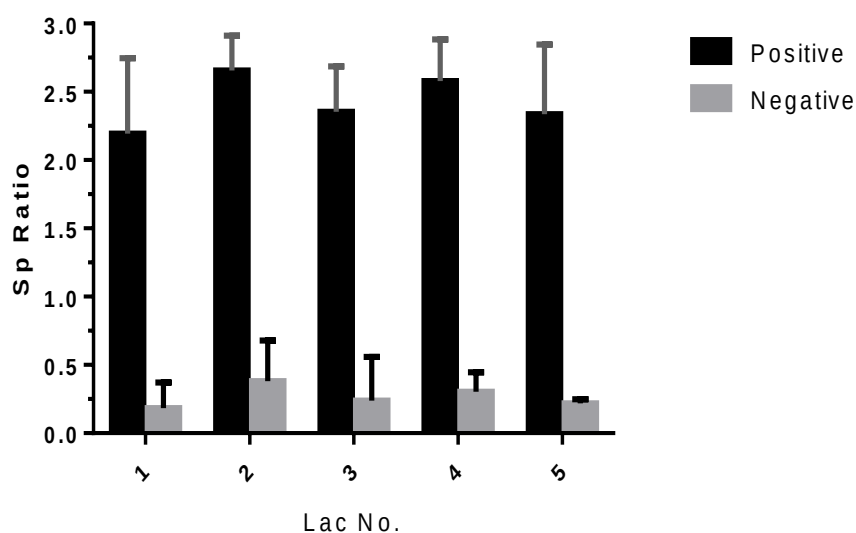


Figure 5.3: Association of the Lac No to SP ratios of seropositive and seronegative cows.

Results were analysed using Pearson r test. Data represent the mean $\pm$ SDM of SP ratio of 82 cows.

5.3.6 Association of Lac. No to IL-10 and IFN- γ in seropositive and seronegative animals

IL-10 and IFN- γ concentration in serum from all 82 animals were compared between the different Lac No (Figure 5.4). High concentration of IL-10 were consistently detected in the serum of positive cows among the different lactation groups. The IFN- γ concentration fluctuated amongst the different lactation groups with the highest mean concentration observed in seropositive animals and the lowest in seronegative animals in third lactation group. No significant differences ($P > 0.05$) in IFN- γ and IL-10 concentration were found among the groups using 2-way ANOVA. No correlations was seen between Lac No and IL-10 concentration in seropositive ($R^2 = 0.169$; $P = 0.49$) nor in seronegative ($R^2 = 0.512$; $P = 0.173$) animals. Also, no correlation was identified between the Lac No and IFN- γ concentration in seropositive ($R^2 = 0.344$; $P = 0.298$) and seronegative animals ($R^2 = 0.356$; $P = 0.287$) using Pearson r test.

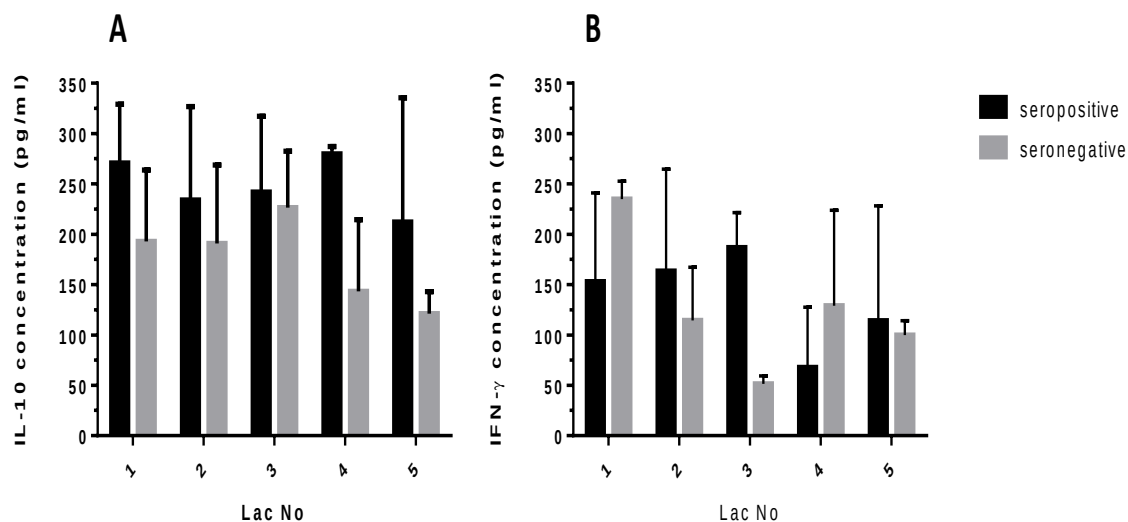


Figure 5.4: IL-10 (A) and IFN- γ (B) concentration of groups of animals grouped by Lac No and seropositive and seronegative status. Results were analysed using 2 way ANOVA and Pearson r test. Data represent the mean $\pm$ SDM of serum IL-10 and IFN- γ concentration of 82 cows.

5.3.7 Lifetime daily milk yield in seropositive and seronegative animals

The mean lifetime daily milk yield of the 37 seropositive cows (11.073 ± 5.263 litre) was slightly lower than that of 31 seronegative cows (12.144 ± 4.622 litre). Analysis of the milk production results using an Unpaired t test showed no significant differences between the seropositive and seronegative cows (Table 5.5).

Table 5.4: Mean lifetime daily milk yield (litre/cow per day) in seropositive and seronegative cows

	Seropositive	Seronegative	Significant
Animal No	37	31	
LDY Mean $\pm$ SD	11.073 ± 5.263 (litre)	12.144 ± 4.622 (litre)	N.S

5.3.8 Association of the Lac No to LDY of seropositive and seronegative animals

Both Lac No and LDY were obtained from NMR database and animals stratified according to serology status (Figure 5.4). As shown in the figure the LDY of cows increased as the Lac No increased in both seropositive and seronegative animals. Using Pearson r test a significant correlations was found between the Lac No and LDY in seropositive animals ($R^2 = 0.944$; $P = 0.005$) and in seronegative animals ($R^2 = 0.968$; $P = 0.002$). In both Lac No 1 and 2 the milk yield of seropositive cows was higher than that of seronegative animals. Conversely, in Lac No 3, 4 and 5 the seronegative cows produce more milk than seropositive animals. However these differences were not significant ($P > 0.05$).

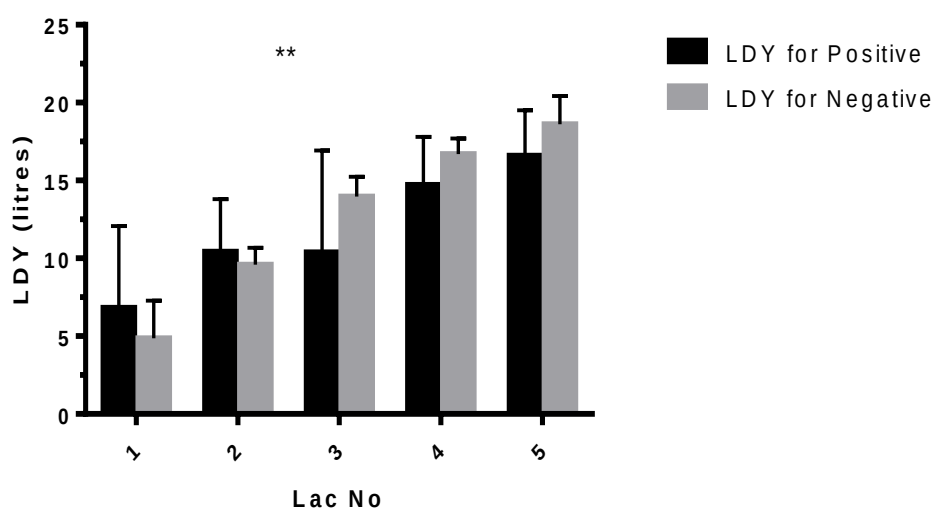


Figure 5.5: Lifetime daily milk yield of groups of cows grouped by Lac No and serology status. Results were analysed using Pearson r test; ** $p = 0.005$ indicate significant correlation between Lac No and LDY for positive samples and ** $p = 0.002$ indicate significant correlation between Lac No and LDY for negative samples. Data represent the mean $\pm$ SDM of LDY of 82 cows.

5.3.9 Association of the LDY.to the IL-10 and IFN- γ in seropositive and seronegative animals

Animals were grouped according to the LDY and IL-10 and IFN- γ concentration corresponding to these groups were matched and analysed (Figure 5.5). Analysis of the individual IL-10 concentrations against LDY groups using Pearson r test revealed that there was a significant negative correlation between the LDY and IL-10 level in seropositive animals ($R^2 = 0.9791$; $P = 0.01$), but not in seronegative animals ($R^2 = 0.4$; $P = 0.367$). Analysis of the correlation between IFN- γ and milk yield in different groups did not show any significant correlations in seropositive ($R^2 = 0.643$; $P = 0.197$) nor in seronegative groups ($R^2 = 0.269$; $P = 0.48$).

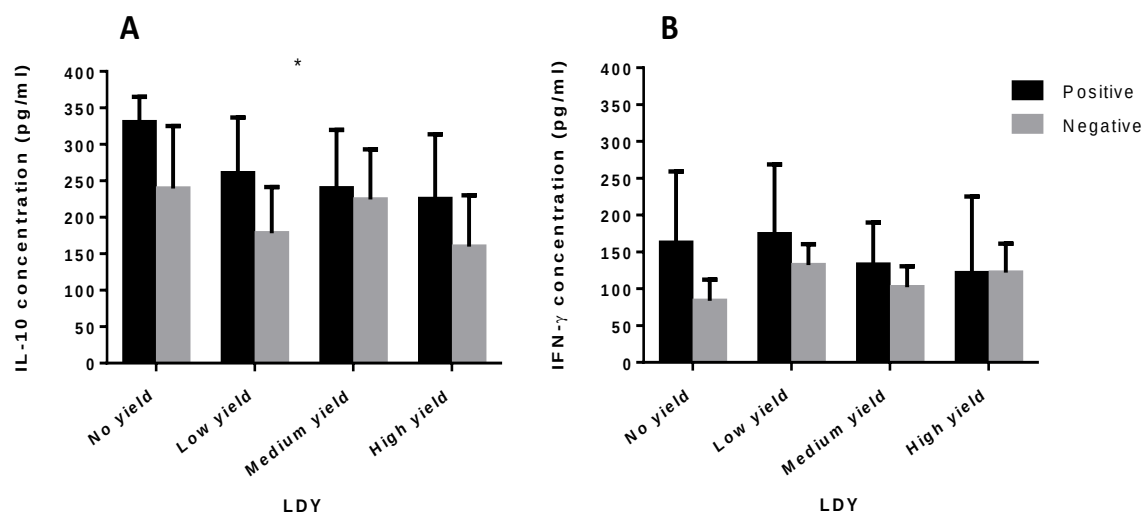


Figure 5.6: Concentration of IL-10 (A) and IFN- γ (B) in the serum of cows grouped by LDY and serology status. Results were analysed using Pearson r test; * $p = 0.01$ indicate significant correlation between LDY and IL-10 of positive cows. Data represent the mean $\pm$ SDM of cytokine concentration of 82 cows.

5.3.10 Correlation of LDY to the SP ratio in seropositive and seronegative animals

Analysis of the correlation of lifetime daily milk yield against the SP ratio of seropositive and seronegative groups was conducted (Figure 5.6). The SP ratio of the seropositive and seronegative cows remained relatively consistent among the different groups. No correlation was found between the daily milk yield and SP ratio in seropositive ($R^2 = 0.21$; $P = 0.533$) nor in seronegative cows ($R^2 = 0.46$; $P = 0.319$).

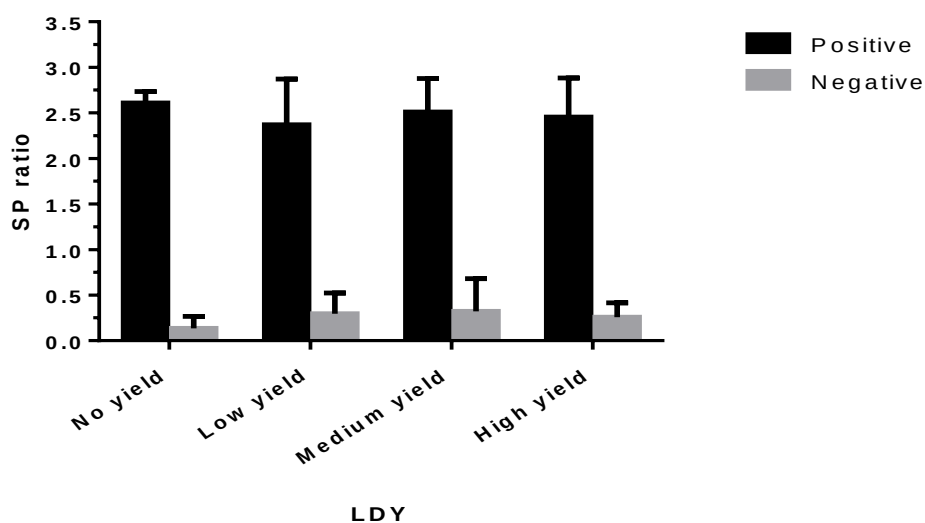


Figure 5.7: SP ratios of groups of animals grouped by LDY and seropositive and seronegative status. Results were analysed using Pearson r test. Data represent the mean $\pm$ SDM of SP ratio of 82 cows.

5.4 Discussion

Several studies have indicated that *N. caninum* infection induced a Th1 immune response with high levels of IFN- γ in cattle (Lunden et al., 1998, De Marez et al., 1999, Williams et al., 2000, Andrianarivo et al., 2001). In addition, the importance of a strong T-cell response and, in particular, IFN- γ in immunity to intracellular parasite has been well characterised (Suzuki et al., 1988, Suzuki and Remington, 1990). The protective role of IFN- γ during *N. caninum* infection has been demonstrated through its suppression of the intracellular multiplication of tachyzoites in different animal species (Innes et al., 1995, De Jesus et al., 2014). Moreover, neutralization of this cytokine in mice reversed the natural resistance of mice to *N. caninum* infection (Khan et al., 1997).

We sought to examine the levels of IL-10, and its known regulatory effects on IFN- γ , in the sera of animals used in this study. To the best of our knowledge, this is the first study that has measured the levels of IL-10 and IFN- γ in non-pregnant cows naturally infected with *N. caninum*. Several earlier studies have examined cytokine levels in *N. caninum*-infected animals during the course of pregnancy (Almeria et al., 2014). Our results indicated that the IFN- γ level in seropositive calves were significantly higher than in seronegative calves irrespective of the serological status of the dams. The lowest IFN- γ levels that we have seen were in seronegative calves born to seropositive dams. With the lack of correlation between IFN- γ levels of the seropositive dams and their corresponding calves, it is suggestive that IFN- γ may not be the only factor in trans placental transmission. In addition, the results of the whole herd analysis showed that infection with *N. caninum* induced high serum concentration of IFN- γ compared to non-infected animals. We also

demonstrated a high level of IL-10 in the sera of *N. caninum* infected cows as compared to non-infected animals. Our findings suggest a mixed Th1/Th2 response is induced during *N. caninum*, this is a common feature of the immune response against different intracellular parasites (Silva et al., 1992, Beresford et al., 1991, Denis and Ghadirian, 1993). The precise role of the IL-10 during infection with intracellular pathogens has not been well defined. The most plausible hypothesis is that the induction of IL-10 may be used by the parasite to escape the destructive immune response mediated by IFN- γ (Khan et al., 1995, Sher et al., 1991). It has been demonstrated that increased production of IL-10 down regulates the toxic IFN- γ -mediated inflammatory response during acute intracellular infection; in mice infected with *T. gondii*, IL-10 deficient mice died from the severe pathology associated with overproduction of IFN- γ , IL-12 and TNF- α (Gazzinelli et al., 1996). On the other hand, this may prevent the complete removal of the parasite and facilitate the development of chronic infection and transmission of the parasite to the foetus during pregnancy (Quinn et al., 2002). Therefore, a balanced IFN- γ /IL-10 response is beneficial for both the long persistence of the parasite and survival of the host.

Analysis of the dam-calf relationship results demonstrated that the percentage of positive calves born to seropositive dams was significantly higher than the percentage of negative calves and supported the hypothesis of congenital transmission of *N. caninum* as a main mode of transmission and persistence of *N. caninum* within the herds (Dubey et al., 1992). This in agreement with the results observed in both the USA and UK showing that up to 81 % and 95 % respectively of calves born from infected mothers are infected (Pare et al., 1996, Davison et al., 1999a). Furthermore, when the SP ratio of the seropositive cows was classified into 'high' and 'low' seropositive, the percentage of animals with high SP ratio that give birth to seropositive calves was high (84 %). However, no significant association

was identified between the SP ratio of the dams and the probability of calves' serological status indicating that the parasite antibody level had no association with the transmission of infection to the calves. This classification was important as a high ELISA serological status indicates an active infection (Dubey et al., 2006). In addition, the levels of IL-10 in seropositive animals were significantly higher than seronegative animals. However, no significant correlation was identified between the IL-10 of seropositive dams and corresponding positive calves. Together this data may suggest that IL-10 is an important factor during *Neospora* infection; promoting the survival of the calves by minimising the immune pathology associated with *N. caninum* infection whilst maintaining infection within the herd. It has been shown that IL-4 and IL-10 are dominant cytokines during pregnancy and these cytokines being responsible for maintaining the pregnancy while transmitting the infection to the foetus (Long and Baszler, 2000, Quinn et al., 2004). Moreover, the observation that 30 % of seronegative cows gave birth to seropositive calves indicated that these seronegative cows were infected but have low antibody concentration. Also, the possibility of postnatal infection in these animals by horizontal route is possible, since the experimental infection of cattle with *N. caninum* oocysts by oral route is possible (De Marez et al., 1999).

Furthermore, we determined the effects of IL-10 and IFN- γ on key production parameters of cows infected with *N. caninum*. Our results showed no correlation between the different lactation numbers and the concentration of serum IL-10 and IFN- γ . Whilst we only found 5 distinct lactation groups in the seronegative cows, there were 8 similar groups of seropositive cows. The seropositive cows in the Lac No 8 group produced the highest level of IFN- γ (data not shown) and this may suggest an increased frequency of abortion is induced in seropositive animals with high level of IFN- γ resulting in an increased

frequency of calving and lactation compared to non-infected animals with only 5 lactation during the same period. Thus in part, our finding support the suggestion that IFN- γ has detrimental effects on the pregnancy leading to abortion (Innes et al., 2005, Almeria et al., 2010).

Our analysis of daily milk production level with the IFN- γ level demonstrated no correlation between these variables. However, it was clear that the level of IL-10 was negatively associated with the LDY in seropositive animals. Our findings are similar to those in healthy dairy cows, where animals with high IL-10 plasma concentration have lower milk yields than those with low plasma IL-10 (Caroprese et al., 2012).

However, the seropositive animals in lac no 1 and 2 make more milk, and no correlation were seen between IL-10 level and different lac no , this indicates that there is no impact of IL-10 on the milk production among different age groups.

The lack of association of the SP with the Lac No indicated no association between infection and the age of the animals. This supported the suggestion that *N. caninum* infection in cattle is chronic with little chance of host developing sterile immunity (Anderson et al., 1995, Thurmond and Hietala, 1997b). Our results are similar to those studies conducted on dairy cattle that demonstrated no association of the age of the animals to the seroprevalence of *N. caninum* infection (Schaes et al., 1998, Kamga-Waladjo et al., 2010).

The few studies that have analysed the effects of the *N. caninum* infection on milk production have provided conflicting results. The results of the current study indicated that there were no significant differences in milk production between seropositive and seronegative cows. Indicating, that infection has no effect on milk production. This in agreement with the study of large numbers of herds conducted in Canada which showed no

significant differences in the milk production between seropositive and seronegative cows (Keefe and VanLeeuwen, 2000). On the other hand, analysis of the results of milk production after adjusting for the effects of lactation number revealed that the milk yield in seropositive cows during the first and second lactation was higher than seronegative animals, but this was not significant. This results supports the finding from a crossbred dairy herd in New Zealand which demonstrated an increase in the milk production of 0.4 litre in seropositive cows during two successive lactation years (Pfeiffer et al., 2000). Moreover, our data contrasted with the results of single herd studies in California and Florida which demonstrated a significant reduction in milk production among *N. caninum* infected cows during first lactation (Thurmond and Hietala, 1997c, Hernandez et al., 2001). We found that of cows in lactation number three or subsequent lactations, seronegative cows produce more milk than seropositive, these differences were not significant. Our findings in general are similar to a Canadian study in which no significant differences in milk production were found between first lactation seropositive and seronegative cows in non-aborting cows (Hobson et al., 2002)

It has been demonstrated that cell mediated immunity is important in controlling systemic *N. caninum* (Innes et al., 2000) and during mid-gestation this form of immunity is reduced in infected animals (Innes et al., 2001). This immunosuppression may induce the recrudescence of the tachyzoites thus enhancing tissue damage and impairing milk production in infected animals. The absence of a significant association between *N. caninum* serological status and milk yield in our study may be in part due to the immunocompetent status of the animals. In the present study, by considering each lactation group as representing a different age group, we did find a significant increase in the milk production as the lactation number increases or as the age of animals increases regardless to

the serological status of the animals. This supports the hypothesis that the profitability and production of a milking cow increases with age (Esslemont and Kossaibati, 1997, Romero et al., 2005), but this was not influenced by *N. caninum* infection in our study.

In conclusion, a mixed IFN- γ /IL-10 response dominated in *N. caninum* infected cattle in this study. IL-10 concentration in sera of infected cowss had a negative association with milk production. Milk production of the current dairy herd depends on the immune status and age of the animals, regardless of the infection status of the animals. Furthermore, we demonstrated the possibility of postnatal/horizontal infection in 30 % of the calves born to seronegative dams. However further evidence is required to confirm this mode of transmission of *N. caninum* in these calves.

6 General Discussion and conclusion

6.1 General discussion

In the work detailed here we have cloned and characterised a 570 bp fragment of the bovine IL-10 promoter lying upstream of the translation initiation site +1 (ATG). For the first time, we have identified multiple transcription factor binding sites within the cloned IL-10 promoter including: Cap, Sp1, HSF, ADR1/MZF1, NkX-2, Lyf-1, CdxA, GATA 1 and Hb. Our results was similar to previous studies in mice, transcriptional regulation of the IL-10 promotor has been shown to rely on several transcription factors; SP1, SP1/SP3, C/EBP, c-Maf and GATA3 (Brightbill et al., 2000, Tone et al., 2000, Liu et al., 2003, Cao et al., 2005). Analysis of the human IL-10 promoter region revealed several polymorphic alleles that influence IL-10 gene expression (Turner et al., 1997). SNPs have been shown to occur within the transcription factor region affecting the expression of this cytokine (Higuchi et al., 1998). This is a regulatory element yet to be explored in cattle.

No previous data linking GATA1 to IL-10 production has been published. However, several workers demonstrated the important role of this transcription factor in the development and differentiation of erythroid cells, mast cells, and eosinophils (Pevny et al., 1991, Yu et al., 2002, Milanovic et al., 2008). It has been found that the absence of GATA 1 from basophils reduced the protective effects of basophils during infection with *N. brasiliensis* by impairing IL-4 production essential to the differentiation of protective M2-type macrophages (Nei et al., 2013). Moreover, in murine mast cells, GATA1 positively regulates the expression of the *st2* gene (Gachter et al., 1998), this gene has being shown to be indirectly responsible for subsequent production of IL-10, IL-5 and IL-4 (Lohning et al., 1998).

In the present study, we measured the expression of GATA1 in bovine PBMCs in response to ConA, LFH and FhTLM using an IF approach. We demonstrated that stimulation of fluke naïve PBMCs with LFH or FhTLM significantly increased the GATA1 expression compared to non-stimulated fluke naïve PBMCs. However, the highest percentage of GATA1 expression was seen in PBMCs treated with ConA. Several studies have demonstrated that IL-10 is up regulated during *F. hepatica* infection (Moreau et al., 1998, Cervi et al., 2001, Flynn and Mulcahy, 2008b, Hacariz et al., 2009a, Mendes et al., 2013). Our results indicated that LFH treatment suppressed IL-10 production from PBMC culture, while ConA induced high levels of this cytokines. This is in keeping with the previous findings observed by Flynn and Mulcahy, (2008b) who, using infected animals, found that the IL-10 levels in PBMC stimulated with ConA was greater in comparison to LFH treated cultures at 4 weeks post infection. Strikingly, our data presented in chapter 4 demonstrated the production of high IL-10 *in vitro* in response to FhTLM leading us to believe that LFH and FhTLM may have divergent mechanisms of action.

The differences in the regulation of IL-10 expression in PBMCs stimulated by LFH and FhTLM raises an important question about the different methods of interaction and signalling that these molecules induce. Our results indicate that FhTLM binds TGF- β receptors, and FhTLM is a product of NEJ flukes, while LFH is an adult fluke extract and data to date would suggest that protease immunomodulators dominate this antigenic extract (Smith et al., 1993), which may lead to degradation of IL-10 and IFN- γ . Moreover, the induction of IL-10 production seen in response to FhTLM, but not in the presence of ConA, may indicate that the rate at which IL-10 is utilised might increase in co-stimulation.

The enhanced GATA1 expression found following stimulation of PBMCs with FhTLM indicates the important role of this factor in the induction of IL-10. It has been shown that

FhTLM stimulate the smad 2/3 expression from fluke naïve PBMCs *in vitro* (Sulaiman et al., under review). The response to FhTLM, a novel TGF- β homologue isolated from *F. hepatica*, indicates the important role of TGF- β signalling in regulation of GATA1 expression. This concurs with findings in mice where inhibiting TGF β -RI resulted in low levels of GATA1 (Martelli et al., 2005). Our results indicate that FhTLM induces the TGF- β signalling pathway leading to stimulation of GATA1 expression from PBMCs.

We demonstrated the *in vitro* role of LFH and FhTLM in modulating fluke naïve PBMCs proliferation response. Treatment of fluke naïve PBMCs with LFH or FhTLM significantly reduced PBMC proliferation and reduced the proliferative response of lymphocytes to the mitogen ConA. Suppression of PBMC proliferation by LFH treatment concurs with similar observations in lambs by (Zimmerman et al., 1983) and in sheep (1995, Chauvin et al., 1995, Moreau et al., 2002). Conversely, our findings contrasted with the results of Oldham and Williams (1985) who observed that stimulation of bovine PBMCs with fluke antigen increased cells proliferation during the first 4 week post infection. In infected rats, proliferation of splenocytes and thymocytes was increased in response to ConA, PWM or fluke antigen (Poitou et al., 1992). However, it must be noted that in contrast to all previous findings we used fluke naïve PBMCs in our study. One of the main composition of LFH is CL and this proteinase has been shown to inhibits the Th1 response and activation of macrophages (Donnelly et al., 2010, O'Neill et al., 2001) explaining the suppression that we have seen *in vitro* in response to LFH. Furthermore, the suppressive effects of the FhTLM on the proliferation of PBMCs in our study was similar to the finding in a recent study showing that TGF- β produced by regulatory B-cells is responsible for the inhibition of T-cell proliferation in a co-culture model (Nouel et al., 2015). Other studies have shown that TGF- β has an inhibitory effect on the proliferation of a variety of immune cells such as T-

cells, B-cells and thymocytes (Espevik et al., 1990a, Kehrl et al., 1986a, Ristow, 1986). Thus, we can conclude that FhTLM, and to a lesser extent, LFH negatively regulates PBMC proliferation in fluke naïve animals.

Our ELISA binding assay results provided evidence that FhTLM bind TGF β -RII with high affinity, leading us to conclude that the suppressive effect of FhTLM on PBMCs proliferation was most likely mediated through the TGF β -RII, Indicating an important role for this parasite protein in the helminth-host interaction.

We have also examined the *in vitro* role of LFH and FhTLM on IFN- γ production from fluke naïve PBMCs and we found that both LFH and FhTLM treatment reduced the IFN- γ level in the culture supernatant as compared to ConA treated cells which displayed the highest IFN- γ concentrations. The inhibition of IFN- γ in response to LFH seen in our study was similar to the finding of Clery et al.(1996), who observed that lymphocytes from cattle chronically infected with *F. hepatica* failed to show any production level of IFN- γ in response to fluke antigen but produced detectable levels in response to ConA stimulation. The inhibition of IFN- γ that we have seen implies that FhTLM may inhibit IFN- γ through the production of IL-10 from bovine PBMCs. Blair et al., (2010) found that regulatory B-cells-induced IL-10, thereby inhibiting IFN- γ production from T-cells *in vitro*. IL-10 suppresses proliferation of different cell types by impairing antigen presenting capacity and inhibition of costimulatory molecules in monocytes and macrophages (Mosmann and Moore, 1991, de Waal Malefyt et al., 1991a, Moore et al., 1993).

Despite the well-documented role of IL-10 as a potent immunosuppressive cytokine inhibiting the proliferation of variety of immune cells (de Waal Malefyt et al., 1991a, Fiorentino et al., 1991b), our results indicated that PBMC suppression in response to LFH was not correlated to IL-10 expression as stimulation with LFH was associated with

inhibition of IL-10. This finding is interesting since the previous studies have shown that IL-10 mediates suppression of lymphocyte proliferation in many infections including *Leishmania major* (Bacellar et al., 2000) and *L. sigmodontis* (Larson et al., 2012). Moreover, IL-10 also has the ability to down regulate its own synthesis from monocytes via a negative feedback mechanism (de Waal Malefyt et al., 1991a), therefore we must also consider this as an explanation. Moreover, PBMC suppression in response to FhTLM was accompanied by high level of IL-10, suggesting that FhTLM leads to IL-10 expression and in turn mediated suppression of PBMCs.

Further studies are required to confirm that GATA1 is necessary for the induction of the IL-10 promoter. This could be achieved by using a mutant IL-10 promoter containing wild type or mutant GATA 1 binding sequences cloned into luciferase reporter plasmids and subsequently transfected into macrophages. Detection of luciferase activity following stimulation with ConA and/or LFH would then confirm if GATA1 leads to IL-10 transcription directly.

During *F. hepatica* infection, the presence of IL-10 impairs the immune response against the pathogen allowing the persistence of infection (Cervi et al., 2001). IL-10 suppress both IFN- γ and NO production during infection with liver fluke (Moreau et al., 1998, Mendes et al., 2013). However, high IL-10 and IL-4 levels induced during *F. hepatica* infection were associated with immunopathology such as liver fibrosis which is important for the survival of the host by minimising the damage of hepatic parenchyma associated with migration of fluke (Mendes et al., 2013). The induction of IL-10 from PBMCs in response to FhTLM (chapter 4) suggests a role for this parasite protein in mediating suppression of lymphocyte proliferation and IFN- γ production from PBMCs. All of these mechanisms favour the

survival of the parasite and the host by limiting the damage associated with the potent Th1 responses (Wynn et al., 1998, Hoffmann et al., 2000, Schopf et al., 2002).

We further studied the role of regulatory IL-10 on IFN- γ and production parameters (milk yield, lactation number) of dairy cows naturally infected with *N. caninum*. The results of the whole herd analysis revealed that infection with *N. caninum* induced a high concentration of IFN- γ and IL-10 compared to non-infected animals indicating a mixed Th1/Th2 response. This is a common feature of the immune response against intracellular parasites (Silva et al., 1992, Beresford et al., 1991, Denis and Ghadirian, 1993). When the seropositive dams were grouped into high seropositive and low seropositive, 84 % of high seropositive cows transmitted the infection to their calves and the concentration of IL-10 in these dams was higher than low seropositive cows. However, no significant correlation existed between the IL-10 concentration of seropositive dams and their positive calves which may suggest that IL-10 is an important factor in *N. caninum* infection suppressing the immune response and allowing the infection to be transmitted to the foetus. The role of the immune system in mediating abortion during *N. caninum* infection has been demonstrated in a number of experimental infection of pregnant mice and it has been shown that abortion is not associated with the presence of parasite in the placenta or foetal tissue (Long and Baszler, 1996). Despite the protective role of IFN- γ during *N. caninum* infection (Baszler et al., 1999). The precise role of IL-10 during infection with *N. caninum* has not been well defined. However, we suggest that the production of IL-10 is important to counteract the severe pathology associated with over production of IFN- γ as was demonstrated when mice deficient in IL-10 died from pathology associated with overproduction IFN- γ , IL-12 and TNF- α during *T. gondii* infection (Gazzinelli et al., 1996). However, this may prevent the complete removal of the parasite and facilitate the

development of chronic infection and transmission of the parasite to the foetus during pregnancy (Quinn et al., 2002). In addition to the role of IL-10 in trans-placental transmission, these data indicate that IL-10 play a crucial role both in protecting the calves from the destructive immune pathology and in maintaining the infection within the herd.

Our results showed no correlation between the number of lactations and the concentration of serum IL-10 or IFN- γ . Furthermore, a negative correlation was observed between IL-10 and lifetime daily yield of milk in seropositive animals. Our results was similarly to a previous study of healthy dairy cow showed that cows with high IL-10 plasma level have less milk than those with low plasma IL-10 (Caroprese et al., 2012).

6.2 Conclusion and future works

In summary, our data demonstrates that both LFH and FhTLM have distinct mechanisms to direct IL-10 production by fluke naïve PBMCs *in vitro*. Notably, IL-10 expression was stimulated in response to FhTLM and inhibited by LFH. The binding of FhTLM to bTGF- β receptors indicated that this parasite protein is able to drive bovine TGF- β signalling. We have also demonstrated the induction of GATA1 in response to FhTLM. Altogether, these findings indicate that FhTLM stimulates GATA1 expression via the TGF- β pathway which in turn induces IL-10 production. This is distinct from those pathways mediated by LFH. It will be interesting to further confirm if GATA1 regulates IL-10 production in response to LFH or FhTLM by constructing an IL-10 promotor with mutant GATA1 and detecting luciferase activity in the reporter plasmid following stimulation with LFH or FhTLM. IL-10 plays an important role in modulation of the immune response by supressing PBMCs

proliferation and inhibiting IFN- γ *ex vivo*. It is well known that IFN- γ is important in mediating protection against *F. hepatica* and *N. caninum*. While, IL-10 is crucial for regulation of the host immune response by minimising tissue damage but this is at the expense of improved parasite survival (Beiting et al., 2007, Hoffmann et al., 2000). Collectively these data support the role of FhTLM in the generation of a suppressive environment favourable to both parasite persistence and survival of the host, making this an important mechanism to understand for successful vaccination. Finally we have also demonstrated that infection with *N. caninum* is associated with high IL-10 concentration. The high IL-10 concentration measured in high seropositive dams which had 84 % seropositive calves, suggested that IL-10 is an important factor in trans-placental transmission and is a good indicator of active infection. However we also saw an increase in serum IFN- γ in infected animals indicating a mixed IFN- γ /IL-10 response which is protective against immune pathology, but may mediate transmission. Moreover, animals with high IL-10 level produced lower amounts of milk, indicating the negative association of this cytokine on milk production during infection. Due to the crucial role of IL-10 during parasitic infections outlined above, it is necessary to identify the dominant cellular sources of IL-10 in healthy animals.

7 Appendices

Buffer and media formulation

Complete cell culture medium

RPMI 1640 (Sigma Aldrich, Ireland).....	450 ml
Heat inactivated foetal calf serum (Sigma Aldrich, Ireland).....	50 ml
Penicillin-streptomycin (10 mg/ml).....	5 ml
L-glutamine (5 M).....	1 ml

HEK cell culture medium

DMEM with L glutamine (Sigma Aldrich, Ireland).....	450 ml
Heat inactivated foetal calf serum (Sigma Aldrich, Ireland).....	50 ml
Penicillin-streptomycin (10 mg/ml).....	5 ml

Freeze mix medium

Heat inactivated foetal calf serum (Sigma Aldrich, Ireland).....	450 ml
DMSO.....	50 ml

MACS running buffer

Bovine serum albumin	5g
0.5M EDTA (pH 8.0)	4 ml
Bring the volume to 1 litre with phosphate buffer saline and store at room temperature.	

MACS rinsing buffer

0.5 M EDTA (pH 8.0).....4 ml

Bring the volume to 1 litre with phosphate buffer saline and autoclave then store at room temperature

Tris Acetate EDTA buffer (TAE) 50X

Tris base 242 g

Glacial acetic acid 57.1 ml

0.5 M EDTA (pH 8.0) 100 ml

Bring the volume to 1 litre with distilled water and store at room temperature.

Tris EDTA buffer (TE) 10 X

1 M Tris-Cl (pH 7.5).....100 ml

0.5 M EDTA (pH 8.0).....20 ml

Bring the volume to 1 litre with distilled water and store at room temperature

ACK RBC lysis buffer

Ammonium chloride (NH₄Cl).....8.3 g

Potassium bicarbonate (KHCO₃).....1.0 g

0.5M EDTA..... 200 µl

Bring the volume to 1litre with distilled water and adjust the PH to 7.2 - 7.4.

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