The mechanism of age related immunity in cattle

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Abstract

The present study attempted a cellular and transcriptomatic approach to investigate the age-related immune response in cattle during intra-cellular protozoan infection. *Neospora caninum* is an obligate intracellular protozoan parasite that causes abortion and negative economic impacts in cattle worldwide. It is important to understand the protective immune mechanisms in response to *N. caninum* infection and develop an effective and safe vaccine for cattle. There are limited studies available that directly address the age-related immune difference during *N. caninum*. The immune response during *N. caninum* infection is Th1 based and mediated by high expression of pro-inflammatory cytokines; most importantly interleukin 12 (IL-12), interferon gamma (IFN-γ) and nitric oxide (NO) by immune cells predominantly macrophages. Monocytes are pivotal due to the link they form between the innate and adaptive immune response and are one of the first immune cells encountered by intra-cellular parasites during infection.

This study investigating the leukocytes profile of neonates (2 week old) and adult cattle (2-3 year old) by flow cytometry. Data shown suggests that neonates had a significant higher percentage of $CD14^+$ monocytes with higher expression of CD80 cell surface markers. Additionally, these data demonstrated an age-related alteration in granulocytes, T and B cell populations across neonates and adult cattle. The monocyte function was compared between young (6 month old) and adult cattle in response to lipopolysaccharide (LPS), IFN-γ, a combination of both and aluminium hydroxide (Alum). These data confirmed monocytes from young cattle have a higher level of secretion of IL-6, IL-1β and tumor necrosis factor alpha (TNF-α) and caspase 1 activity than adult derived monocytes.

Furthermore, this study attempted to resolve if this age-related difference was maintained in the context of an *in vitro* infection with *N. caninum* infection. The number of parasitized monocytes was determined after infection or co-culture with autologous natural killer (NK) cells. CD80 expression was determined as a marker of cellular activation by flow cytometry.

These results reveal monocytes from adult cattle harbour a higher number of parasites compared to neonates; additionally, a greater reduction of parasitaemia was observed in neonates with a higher level of cytokines IL-1β and IL-6 during *N. caninum* infection. However, NK cells from neonates and adult did not display much difference in cytotoxic activity, measured through perforin and granzyme B production after co-culture with *N. caninum* infected monocytes.

To complement this gene array analysis was also performed which suggests that during infection, neonates have 535 significantly upregulated genes (>2 fold) compared to adults that showed only 23 upregulated genes (>2 fold). Interestingly, only two genes were common in both groups. Further, common biological pathways that involved in the immune response were evaluated and both age groups showed changes in the upregulation of tyrosine phosphorylation of STAT protein and JAK-STAT cascade pathways. These results also indicate a greater magnitude of immune response and a more complex network of upregulated genes in neonates. Overall these comparisons show that there is a fundamental difference in the immune response of neonates and adult cattle during steady-state and in the response to intra-cellular parasite infection in neonatal monocyte led inflammatory responses.

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.

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Conferences and Presentations

Oral Presentations

1. Sharma P, S Egan and R Flynn "Magnitude of the inflammatory response to parasite infection differentiates calf and adult bovine monocytes" to British Society for Parasitology (8-11 April 2018 Spring meeting) at University of Aberystwyth, Wales , UK.

2. Sharma P, S Egan and R Flynn "Innate immune response to *Neospora caninum* infection in Cattle" to British Society for Parasitology (2-5 April 2017 Spring meeting) at University of Dundee, Scotland, UK.

3. Sharma P, S Egan and R Flynn "Innate immune response to *Neospora caninum* infection in Cattle" (12-13 September 2017) 70th annual Association of Veterinary Teaching and Research Work (AVTRW) conference at University of Surrey, UK.

4. Sharma P, T Coffey and R Flynn "Role of Bovine monocytes in the age-dependent immune response against *Babesia*" on XII European European Multicolloquium of Parasitology (EMOP) Conference 20-24th July 2016 at University of Turku, Finland.

5. Sharma P, S Egan and R Flynn "Innate immune response to *Neospora caninum* infection in Cattle" Infection and Global Health Day (24th November 2017) University of Liverpool, UK.

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List of Abbreviations

£	British pound
°C	Degree Celsius
ADCC	Antibody-dependent cell-mediated cytotoxicity
Ag	Antigen
APC	Antigen-presenting cells
BAL	Bronchio-alveolar lavage
BCR	B-cell receptor
BSA	Bovine serum albumin
CD	Cluster of differentiation
CFSE	Carboxyfluorescein succinimidyl ester
CLP	Common lymphoid prescursor
CNS	Central nervous system
DAMP	Damage associated molecular pattern
DCs	Dendritic cells
DMEM	Dulbecco's modified eagle medium
D-PBS	Dulbecco's Phosphate buffered saline
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
FACS	Flow cytometry activated cell sorting
FBS	Fetal bovine serum
GM-CSF	Granulocyte macrophage colony-stimulating factor
hrs	Hours
IFN-γ	Interferon gamma
Ig	Immunoglobulin
IL	Interleukin

iNO	Inducible nitric oxide
IRAK	Interleukin-1 receptor-associated kinase
IRF	Interferon regulatory factor
JAK	Janus Kinase
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
MPP	Multipotent progenitor cells
NLR	NOD- like receptor
NF-κB	Nuclear factor kappa-light chain enhancer of activated B cells
NK	Natural killer cells
NO	Nitric oxide
PAMP	Pathogen associated molecular pattern
PBMC	Peripheral blood mononuclear cells
PMA	Phorbol 12-myristate 13-acetate
PMN	Polymorphonuclear leukocytes
PRR	Pattern recognition receptors
RNA	Ribonucleic acid
RT	Room temperature
rpm	Round per minute

sec	Seconds
STAT	Signal transducer and activator of transcription
TCR	T-cell receptor
Th	T helper cells
TLRs	Toll like receptors
TNF-α	Tumour necrosis factor-alpha
T-reg	Regulatory T cells
TRIF	TIR-domain containing adaptor-inducing interferon-β
TRAF	TNF receptor associated factor
US$	United State dollar
WC1	Workshop cluster 1
WBC	White blood cells
μl	Microlitre

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Chapter 1 Introduction and Literature review

1. Introduction

1.1 Immunity and its component

Immunity is defined as the capacity of a host to identify and remove an infectious agent and providing an immediate as well as memory response for potential future encounters. Immunity can be classified as belonging to the innate and the adaptive system (Fig.1.1). Innate immune mechanisms are inborn and considered non-specific to any particular pathogen and consist of physical barriers such as the skin, mucosa, various chemical barriers such as cell secretions and the functions of phagocytic and granulocytic cells.

Adaptive immunity is antigen specific and possesses a memory that is rapid in its secondary responses and classified into natural and artificial immunity. Natural or acquired immunity is developed when a hosts come into contact with a pathogen and develops antigen specific responses against pathogen derived proteins over the course of infection or disease. However, artificial immunity is best explained through the provision of colostrum or vaccination. Passing of maternal antibodies through colostrum from a mother to offspring is passive immunity which is specific but lasts for a short duration helping to protect from disease in early life. Vaccination is a type of active artificial immunity, where antigens or peptides are deliberately introduced into the system with the intention of developing a specific and long-lasting immune response (Keller and Stiehm, 2000).

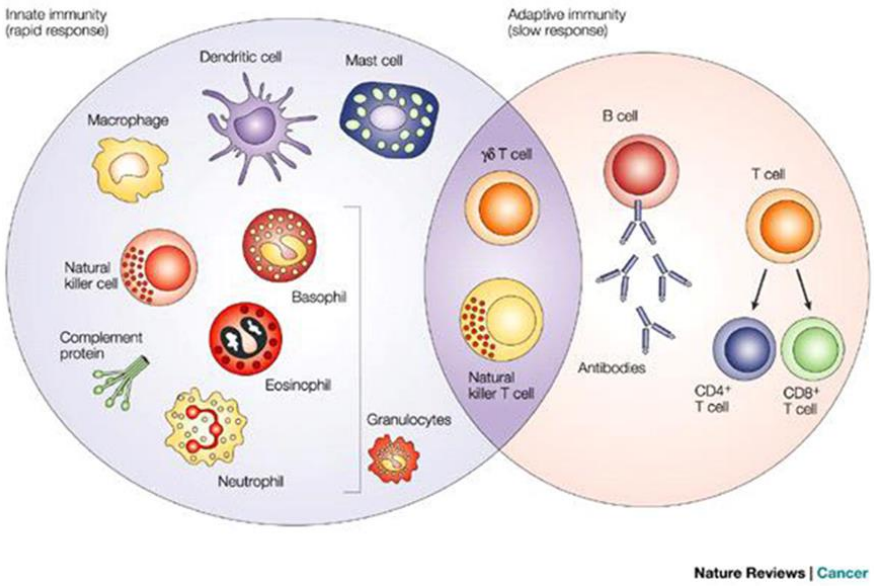


Figure 1.1 Diagram showing the cells of innate and adaptive immunity

The innate immune response is characterised by cells such as macrophages, Natural Killer (NK) cells, dendritic cells (DCs) and neutrophils. The adaptive immune response comprises B cells and T cells where B cells secrete the antibodies and T cells differentiate into $CD4^+$ and $CD8^+$ T cells. Gamma delta T cells and natural killer T cells are prototype of unconventional T cells and consist a small subset of T cells (Adapted from Dranoff, 2004).

1.2 Innate immunity

The innate immune response primarily consists of physical barriers, innate leukocytes and plasma proteins (Rainard & Riollet, 2006) and it is crucial against most diseases such as *Mycobacterium bovis* infection where alveolar macrophages act as the first line of defence via killing of bacteria by phagocytosis. Innate responses actively participates in immunity from the earliest stages of life and includes macrophages, monocytes, neutrophils and NK cells (Oviedo-Boyso et al., 2007). However, reduced innate immune capacity can be related to increased susceptibility to respiratory diseases in cattle (Ackermann et al., 2010). The innate immune system is non-specific but effective against most pathogens in spite of their antigenic variance (Grasso et al., 2002) for example, anatomical barriers such as the skin remove the harmful bacteria by desquamation of skin. Likewise, intestinal peristaltic movements and respiratory tract cilia help in the elimination of pathogens and their toxins through their continuous movement.

1.2.1 PRRs

PRRs often known as pathogen recognition receptors are germline-encoded host sensors that detect conserved molecular patterns of pathogens (Kumar et al., 2011). PRRs evolved before the adaptive immune system and are conserved primitive recognition receptors (Doyle and O'Neill, 2006). They are mainly expressed in innate immune cells such as DCs, macrophages, monocytes, neutrophils and epithelial cells (Alberts et al., 2002; Schroder & Tschopp, 2010). Pathogen specific molecules known as pathogen associated molecular patterns (PAMPs) are identify by certain PRRs and able to distinguishes pathogenic molecules from non-pathogenic (Bannerman et al., 2004). They include lipopolysaccharide (LPS), bacterial or viral DNA and RNA, bacterial peptides, fungal glucans and chitin. While damage associated molecular pattern's (DAMPs) are endogenous stress signals including uric acid and extracellular ATP (Kumar et al., 2011). PRRs are further sub classified on the basis of ligand specificity, function and evolutionary relationship into membrane bound PRRs such as Toll-Like receptors (TLRs) and C-type lectin receptors (CLRs) or Cytoplasmic PRRs including NOD-like receptors (NLRs) and RIG-I- like receptors (RLRs). Ligation of PRRs by their specific PAMPS results in secretion of inflammatory effector cytokines and cytokine which instruct the differentiation of the cellular components of the adaptive system (Doyle and O'Neill, 2006).

1.2.2 TLRs

PAMPs are recognised by TLRs which are situated on the cell surface or in endosomal compartments of host cells (Underhill & Ozinsky, 2002; Werling & Jungi et al., 2003). TLRs are expressed by various immune cells population including circulating monocytes, tissue macrophages and many epithelial cells. In addition to TLR2 expressed in cattle myeloid cell population (Werling et al., 2006) and secrete various cytokines and mediators in response to ligation (Lembo et al., 2003) and acting to increase the protective activity of macrophages and neutrophils toward infectious agents. Moreover, activation or priming of the acquired immune response is another feature of innate immune response (Alluwaimi and Cullor, 2002). In cattle a total 10 TLR are known (Fisher et al., 2011) where TLR1, 2, 6 and 10 are associated with recognition of Gram positive and negative bacteria (Roach et al., 2005).

Some of the common and conserved ligands which bind to TLR2 include herpes simplex virus and *M. bovis* (Zarember and Godowski, 2002; Wang et al., 2011) however, LPS binds to TLR4 facilitated by indirect binding through LPS binding protein (LBP) and CD14. TLRs generate a signal via a specific pathway and adaptor molecule complex such as Myeloid differentiation primary response 88 (MyD88) that is used by all of TLR except TLR3, linked to Interleukin-1 (IL-1) receptor kinase (IRAK) that leads to stimulation of tumour necrosis factor (TNF) receptor associated factor (TRAF) and nuclear factor-κB (NF-κB) (O'Neill, 2004). NF-κB is a key transcription factor that is involved in the activation of various pro-inflammatory cytokines such as TNF-α, IL-1, IL-6 and IL-8. Other cytoplasmic proteins known as NLRs are key elements in the regulation of the inflammatory response via identification of bacterial peptidoglycan and subsequent enhancement of the pro-inflammatory and antimicrobial immune response (Caruso et al., 2014).

1.2.3 Antimicrobial peptides

Antimicrobial peptides are important components of the innate immune response and a variety of antimicrobial peptides are present in cattle where they act on broad range of Gram positive and negative bacteria (Brogden et al., 2003). Antimicrobial peptides such as defensins and cathelicidins have multiple functions in host defence. They are not only secreted by phagocytic cells and lymphocytes but also by a range of gastrointestinal epithelial cells over the course of the innate response and further enhance the adaptive response (Zanetti, 2005; Oppenheim et al., 2003). Studies suggests that defensins and cathelicidins can enhance the production of Th1 and Th2 cytokines from murine splenic cells (Lillard et al., 1999; Tani et al., 2000; Niyonsaba et al., 2001). The first bovine cathelicidins were purified from the neutrophils and named bactenecins 5 and 7 (Gennaro et al., 1989; Skerlavaj et al., 1990; Frank et al., 1990). Furthermore, some anionic peptides are also identified in bronchoalveolar lavage of cattle where neonates have shown 3 times greater concentarion of anionic peptides compared to adult cattle (Caverly et al., 2001). In cattle, 13 β defensins have been identified and are often known as bovine neutrophil beta defensins (BNBD) additionally, BNBD4, 5 and tracheal antimicrobial

peptide (TAP) are highly expressed in bovine alveolar macrophages (Selsted et al., 1993; Ryan et al., 1998).

1.2.4 Chemokines and cytokines

Chemokines and cytokines are primarily chemical mediators that play an important role in innate immune response. They not only control the recruitment of immune cells toward the site of infection but control the release of immune cells from bone marrow (Sokol & Luster, 2015). Chemokine ligands can be classified on the basis of position of their initial cysteine residues such as XC, CC, CXC, and CX3C. In addition, CC chemokine ligands (CCLs) have two adjoining amino-terminal cysteine residues however CX3CL1 has three amino acids separating the two initial cysteine residues. Currently bovine chemokine receptor sequences are available for CCR1, CCR4, CCR5, CCR7, CCR9, CXCR1, CXCR2, CXCR3, CXCR4, CXCR5 and CXCR6 (Widdison et al., 2010). The role of CCR5 has been reported in *T. gondii* and *N. caninum* infection via recruitment of leukocyte in bovine and mice (Kameyama et al., 2012). Furthermore, during protozoan infection such as *Plasmodium, Leishmania, Trypanosoma cruzi* and *T. gondii* high levels of CXCR3 was noticed and trafficking of $CD4^+$ Th1 T cells is CXCL9 (MIG) and CXCL10 (IP-10) ligands dependent (Bonecchi et al., 1998; Sallusto et al., 2004).

Cytokines are small secreted proteins by a broad range of immune cells. Pro-inflmmatory cytokines are mainly secreted by activated macrophages and involved in upregulation of inflammatory reaction. IL-1β, IL-6 and TNF-α are considered as early cytokines and involved during cell injury, infection, invasion, and inflammation. However, IL-4, IL-10, IL-11, and IL-13 are considered anti-inflammatory cytokines and important to control the pro-inflammatory cytokine response. IL-10 is one of the most potent anti-inflammatory cytokine that inhibits the overexpression of TNF-α, IL-6 and IL-1 secreted by activated macrophages (Zhang & An, 2007). Transforming growth factor beta (TGF-β) is the second suppressor cytokine that inhibits the cytokines produced by activated macrophages and Th1 immune cells and act against IL-1, IL-2, IL-6, and TNF (Roberts & Sporn, 1993).

1.2.5 Cells of innate immune response

1.2.5.1 Monocyte

Monocytes are physically the largest leukocyte and under normal conditions they comprise between 3-8 % of the circulating cell population in bovine (Weiss & Wardrop, 2010). Their numbers increase during infection however, their life span is relatively short and after 24 hrs they undergo apoptosis (Monie, 2017). Monocytes, macrophages and DCs are derived from common precursor cells from the bone marrow. Additionally, some monocytes migrate towards sites of infection or injury and differentiate into macrophages and dendritic cells in tissues. Additionally, the spleen serves as a reservoir for monocytes to be deployed during inflammatory conditions (Swirski et al., 2009).

Monocytes express primary phagocytic properties including movement, adherence, endocytosis and anti-microbial capacity. During acute infection an immune response cascade starts that involves monocyte adherence to endothelium via interaction between the integrins (CD11a, CD11b, CD11c and CD18) expressed on monocyte cell membranes to ICAM1 and ICAM2 receptors present on the endothelium resulting in cell activation, secretion of various inflammatory cytokines, superoxide radicals and Nitric Oxide (NO) (Shereck et al., 2012). In addition to phagocytic activity, monocytes are noted for the presence of internal and external PRRs which make them reactive to pathogens, PAMPs and other DAMPs. CD14 is a monocyte surface marker and in combination with TLR4, recongnize the bacterial LPS (Monie, 2017). Bone marrow derived monocytes themselves play a critical role in protective host responses during *in vitro Listeria monocytogenes*, *Leishmania spp*. or fungal infection provoked inflammation (Goncalves et al., 2011; Wuthrich et al., 2012).

Cytokines and chemokines are essential part of the monocyte/macrophage effector response and they produce a variety of inflammatory cytokines such as TNF, IL-1, IL-6, IL-8, and IL-12 (Auffray et al., 2009). They are also responsible for evoking adaptive immune response via antigen presentation to major histocompatibility complex II (MHC-II). Monocytes through their secretion of IL-12 promote the

production of IFN-γ from NK cells and T cells (Goff et al., 2010). IFN-γ is key in priming macrophages and ultimately helping in the removal of intra-cellular pathogens by T cell activation (Brown et al., 2006).

1.2.5.2 Macrophages

Macrophages originate from blood monocytes where they leave the circulation and enters in different tissue such as spleen, liver, lymph node, lungs, peritoneal cavity and subcutaneous tissue (Van Furth & Cohn, 1968; Randolph et al., 2008). Their naming is dependent on varied locations such as histiocytes (Tissue), Kupffer cells (Liver), alveolar macrophages (Lungs) and microglia (Brain) (Ovchinnikov, 2008). Recruited macrophages can be polarised either classically activated (M1) or alternatively activated (M2a, M2b and M2c) macrophages depends on polarisation signal. Th1 cytokine IFN-γ, TNF, or LPS induce M1 macrophages development where they secrete proinflammatory cytokines TNF-α, IL-1β, IL-6, IL-12, IL-23 as well as a higher amount of reactive oxygen species (ROS) and reactive nitrogen species (RNS), iNOS and promote the metabolism of arginine into NO and citrulline. Thus they are highly reactive for distruction and removal of intra-cellular pathogen (Vanden Eijnden et al., 2005).

However, in response to stimulus IL-4 and IL-13 macrophages are polarised toward a M2a phenotype that promote an accumulation of Th2 cells (Gundra et al., 2010) whereas M2b phagocyte are activated by LPS, immune complexes, apoptotic cells and IL-1Ra signal and produce a high level of IL-10 and other pro-inflammatory cytokines TNF and IL-6 (Biswas & Mantovani, 2010). Furthermore, in response to IL-10 and TGF-β, macrophages are converted into M2c phenotype that secrete immunosuppressive cytokine IL-10, TGF-β and induce development of Th2 cells and regulatory T cells (Zhang et al., 2010).

In bovine alveolar macrophages are unique and most abundant innate immune cell which is crucial for clearance of pathogen from lungs via phagocytosis, production of lysozymes and production of IFN (Green & Kass, 1964; Charley, 1982). Macrophages not only important for immunity but also play a major role in tissue homeostasis (Sieweke & Allen, 2013).

The activity of macrophage can be enhance by cytokines secreted by T cells where IFN-γ act as a most potent activator of macrophage. Upon activation macrophage secrete various pro-inflammatory cytokine including TNF, IL-1, IL-6, IL-8, and IL-12. Bovine macrophages are highly phagocytic and first cells that encounter during *Mycobacteria* infection (Bielefeldt ohmann & Babiuk, 1986). They also shows phagocytic activity aganist *Candida parapsilosis* (Forman & Babiuk, 1982), IgG coated sheep erythrocytes (Forman & Babiuk, 1982) and different bacterial species such as *Staphylococcus aureus, Listeria monocytogenes*, *Serratia marcescens, Pasteurella multocida* and killed *Pasteurella haemolytica* (Benson et al, 1978). Alveolar macrophages express low level of F4/80 and CD11b and higher level of CD206. They also participate in antigen cell dependent cytotoxicity and secrete higher amount of IFN titre during viral infection (Forman & Babiuk, 1982).

1.2.5.3 DCs

DCs are an integral part of the mammalian immune system and are derived from hematopoietic bone marrow progenitor cells. It has been assumed that DCs act as an interface between the innate and adaptive immune response (Banchereau & Steinman, 1998). They are heterogeneous for example myeloid and plasmacytoid DCs (found in peripheral lymphoid organs) are responsible for antigen processing as well presentation to the surface of the naïve T cells and often known as professional antigen processing cells (APCs) (Banchereau & Steinman, 1998; Gluckman et al., 2002; Della et al., 2005).

In humans and mice myeloid DCs are mainly located in the skin, where they named Langerhans cells (Larregina et al., 2001), the inner lining of the nose, lungs, stomach and intestines. They are also present in bone marrow (Egner and Hart, 1995), blood (Gluckman et al., 2002), lymph nodes and spleen. Immature DCs are constantly sensing for pathogens via PRRs such as TLRs which capture antigens and leave the tissue. They cross the endothelium of lymphatic vessels and under the influence of the chemokines CCL21 and CCL19 migrate to the lymphatic nodes (Marsland et al., 2005). They also degrade pathogen proteins via phagocytosis and present them at their cell surface using MHC molecules. Additionally, they upregulate the cell surface co-stimulatory molecules such as CD80, CD86 and CD40 during T-cell activation and secrete pro-inflammatory cytokines such as TNF-α and IL-12. DCs are able to prime naïve lymphocytes and can produce polarised immune responses due to their cytokine secretion pattern (Banchereau et al., 2000; Henri et al., 2001).

The role of bovine DCs has been examined through surgical cannulation of peripheral lymphatic vessels (McKeever et al., 1991). Bovine peripheral blood DCs are classified into different subset on the basis of phenotype, morphology and function (Stephens et al., 2003). The plasmacytoid DCs defined as $CD4^+$ and produce higher amount of type 1 IFN in response to TLR7 and TLR9 stimulation (Gibson et al., 2012). However, conventional DCs subset is characterised by expression of MHC-II and produce a number of pro-inflammatory cytokines (Banchereau & Steinman, 1998).

CD172a is an important molecule that expressed on myeloid DCs however, study suggest bovine plasmacytoid DCs do not expressed CD172a that suggest they do not belong to myeloid lineage cells (Sei et al., 2014). Furthermore, DEC205 is a C type lectin that found in DCs and involved in cell apoptosis and necrosis (Shrimpton et al., 2009; Tel et al., 2011) and expressed on bovine plasmacytoid DCs (Sei et al., 2014). In bovine, conventional DCs are defined by expression of $CD11c^{+}$ and DEC205 and upon TLR stimulation they upregulate the CD80 expression as well as secerete large amount of TNF-α. Morover plasmacytoid and conventional DCs are efficient for internalization and degradation of exogenous antigen resulting production of type I IFN (Sei et al 2014).

1.2.5.4 Neutrophils

Neutrophils are the most abundant cell type and contribute to between 40-75 % of the total circulating leukocyte population in both humans and mice. However, in cattle they make up only 18-33 % of the circulating leukocyte population (Weiss and Wardrop, 2012). They are characterised by a multi-lobed nucleus and often known as polymorphonuclear cells (PMNs) with short life span and undergo spontenous apoptosis. Neutrophils provide the first line of defence and recruited at the site of inflammation in response to CXCL8 secreted by infected cells or tissue resident macrophages (Cohen and Burns, 2002). They have a critical role in protective immune responses along with basophils and eosinophils (Nathan, 2006). Neutrophils act as type of phagocytic cell which is primarily found in the blood stream and shows immediate responses in bacterial invasion (Jacobs et al., 2010) and characterised by expression of Intergrin alpha M also known as CD11b which is involved in neutrophil adhesion and migration (Solovjov et al., 2005). Studies suggest that bovine neutrophils have different properties compared to other species such as they stay for longer in the bone marrow at the time of development stage so initial neutropenia is very common during initial stage of inflammation (Valli et al., 1971; Weiss et al., 2010) also reduced proliferative activity shown by low myeloid to erythroid ratio (0.5-1) in bone marrow.

1.2.5.5 Basophils and eosinophils

Basophils and eosinophils are effector cells during allergic inflammation. They express common receptors and cytokines but are functionally different. In bovine basophils make up only a small portion of granulocytes and contributing only 0.0-2.0% in a differential blood counts. (Weiss and Wardrop, 2012). During parasitic infections and allergic conditions basophils numbers increase (Voehringer, 2009) and they contain large cytoplasmic granules and a two lobed nucleus. Basophils secrete histamine and heparin during allergic reactions and possess a surface receptor that binds immunoglobulin E (IgE) that is involved in host defence against helminth infection (Rossi et al., 1993; Estambale et al., 1995; Turner et al., 1999). Studies in mice have demonstrated that basophils contribute to secondary immune responses as well as regulate the behaviour of T cells and importantly they are necessary for conversion of naïve $CD4^+$ T cells into Th2 cells (Nakanishi, 2010). Basophils can express MHC class-II and co-stimulatory molecules such as CD40, CD80/86 and act as a potent APCs under allergic settings (Sokol et al., 2009; Perrigoue et al., 2009; Yoshimoto et al., 2009). Evidence indicates that basophils secrete large amounts of IL-4 that is critical for production of IgE antibody (Janeway, 2001).

Additionally eosinophils comprise 0-20 % of the leukocyte population in cattle (Weiss and Wardrop, 2012). Helminth infection induces Th2 immune responses that is characterised by cytokines such as IL-4, IL-5 and IL-13 (Huang and Appleton, 2016) also infection triggers the bone marrow to release massive numbers of eosinophils into the blood and antibody class-switching to produce IgE. Thus eosinophilia is a central feature of host response to helminth infection (Allen and Maizels, 2011). Eosinophils mature in the bone marrow and IL-5 controls their development from myeloid precursor cells (Lambrecht and Hammad, 2015; Yamaguchi et al., 1988). Some data indicates that eosinophils are capable of acting as APCs due to upregulation of MHC-II during helminth infection (Mawhorter et al., 1993). They migrate towards site of helminth infection in response to certain chemokines such as CCL11, CCL24, CCL5 and leukotrienes B4 and secrete a wide range of cytokines IL-1, IL-2, IL-4, IL-5, IL-6, IL-8, IL-13 and TNF alpha (Hogan et al., 2008; Rothenberg and Hogan, 2006).

1.2.5.6 NK cells

NK cells are a significant part of the innate immune response and classified as a group I innate lymphoid cell descended from common progenitor as T and B cells. The bone marrow, thymus, lymph nodes and spleen are major sites for the differentiation and maturation of NK cells (Iannello et al., 2008) and thereafter they enter to circulation. NK cells make up between 2-10 % of the total lymphocytes population in cattle (Kulberg et al., 2004; Kamden et al., 2006). They are type of cytotoxic lymphocyte which primarily responds to damaged and compromised host cells and are best known for the killing of virus and tumour infected cells via secretion of key cytokines IFN-γ and TNF-α. In addition to they participate to enhance immune responses via polarization of T cells toward Th1 type immune response and maturation of DCs (Arina et al., 2007).

While NK cells originate in the bone marrow, they do not need any prior antigen stimulation and neither are they MHC restricted for cytolytic killing (Hirose et al., 1999). While patrolling, NK cells come into contact with other cells and identify those cells that are missing self MHC-I expression such as cancerous or infected cells are subsequently killing via release of cytotoxic granules such as perforin and the protease granzyme B where perforin creates pores in the cell membrane of target cells helping granzyme B to enter infected cells leading to apoptosis or cell lysis (Lodoen & Lanier, 2005).

1.3 Adaptive immunity

The adaptive immune response also known as the acquired immune response requires a complicated mechanism of activation compared to the innate system. Specific features of the acquired immune system are immunological memory which helps in further encounters of disease causing pathogens. This form of immunity comprises two components such as antibody and cell mediated immune responses. It is long lasting and specific for pathogens but takes time to develop. Antibody and cell mediated immune responses are induced by T and B cells respectively. The adaptive immune system functions via recognition of foreign antigen through the process of antigen presentation and formation of memory B cells and T cells (Janeway

et al., 2001). An adaptive immune response is triggered when pathogens escape the innate immune response and the threshold level of antigen is formed which transmit danger signal via DCs (Janeway et al., 2001).

1.3.1 B cells

B cells are derived from pluripotent haemopoietic stem cells which are mainly found in haemopoietic tissues such as liver in foetus and the bone marrow of adults (Alberts et al., 2002). The process of B cell development begins with migration of multipotent progenitor cells (MPPs) into the liver and then bone marrow. MPPs are subsequently differentiated into common lymphoid precursor (CLP) and then common lymphoid 2 progenitors (CL2P) that leads to the B-cell lineage (Tobon et al., 2013).

1.3.1.1 B cell development and activation

The development of B cells proceeds in different stages with a characteristic gene expression pattern of the immunoglobulin H and L chain genes (Pelanda and Torres, 2012). B cells undergo positive and negative selection where positive signals are antigen independent signalling via a pre B cell receptor and B cell receptor (BCR) while negative selection involves binding of self-antigen to the BCR (Martensson et al., 2010). If binding of BCR to self antigen is too strong then cells undergo clonal deletion, receptor editing and anergy (Tucker & Tedder, 2008). During development, immature B cells migrate from the bone marrow to the spleen and become modified into two transitional stages T1B during migration towards the spleen and T2B within spleen (Chung et al., 2003).

B cells activation takes place in spleen and liver and it can be either T cell dependent or independent. CD21 that is a surface receptor and enhance the activation of B cells via making a B cell co-receptor complex with other surface protein such as CD19 and CD81 (Rengasamy et al., 2013; Zabel and Weis, 2001).

B cells transform to plasma cells upon encountering pathogen antigens and secrete antibodies. Afterwards some B cells act as memory B cells and during second exposure will immediately display a more effective, rapid response (Hesslein et al., 2011). The mechanisms by which T cells and B cells recognize their cognate foreign

antigen is different. For example, T cells identify their target only in association of MHC molecules on APCs unlike B cells which retain the capacity to recognize their target independent of MHC molecule via T cell effector molecules such as CD40. This is an important membrane bound protein belonging to the TNF family and binding of CD40 to CD40L leads to conversion of resting B-cells into active cell cycle and a thymus dependant B cell response (Janeway et al., 2001).

The principal function of B cells is secretion of antibodies that provide long term immunity. Antibodies also known as Ig are glycosylated protein molecules present on the surface of B cells and serving as BCR. They are secreted into extracellular space and capable of binding and neutralization of infectious agents (Janeway et al., 2001). A single antibody molecule made of four protein chains, two heavy and two light that linked together by disulfide bonds. There are five subclasses of antibody produced in mammals such as IgA, IgD, IgE, IgG, and IgM each with unique functions and biological properties. Additionally, four subclasses or isotypes of IgG antibodies is also found, IgG1, IgG2, IgG3 and IgG4. These antibodies exert their effector functions via 3 mechanism, either they neutralize their targets (they bind to a virus and prevent it from entering a cell) or via activation of macrophages and other immune cells by binding to Fc receptors (FcRs) that recognize the constant regions of specific antibody classes. Sometime they are able to activate the classic pathway of the complement system by binding to C1q and destroy the pathogen. Plasma cells are short lived (2-3 days) cells, among them 10 % of plasma cells survive as long lived which become memory B cells (Janeway et al., 2001).

1.3.2 T cells

T cells originate within the bone marrow but mature in the thymus, playing key roles in adaptive immune responses and are characterised by the presence of a T cell receptor (TCR) on their surface. T cell development takes place in the thymus which involves differentiation as well positive and negative selection of T cells. T cell precursors enter the cortex of the thymus and after differentiation move toward the medulla. Approximately, 98 % of precursor T cells die due to apoptosis during the development process in the thymus by failing either positive selection or negative selection.

T cells education takes place during foetal stages of life and afterward birth the thymus can shrink 3 % in a year and results in a reduction of thymic naïve T cells produced in adults (Haynes et al., 2000). Double positive T cells ($CD4^+/CD8^+$) successfully pass positive and negative selection through binding with MHC I or II peptides appropriately (Starr et al., 2003). The outcomes of positive selection determine the fate of a T cell and they eventually become either $CD4^+$ or $CD8^+$ through their interaction with MHC-II and MHC-I respectively (Zerrahn et al., 1997).

Negative selection eliminates T cells by apoptosis which have very high binding affinities with MHC peptides; however some of these are selected to become Treg and some depart from thymus in the form of immature naïve T cells (Pekalski et al., 2017).

1.3.2.1 T cell activation

T cell activation leads to intra-cellular signalling pathways that involves proliferation, effector function or death depending on the intensity of the TCR signal and associated signals. T cells originate in the thymus, leave and then circulate throughout the body until they identify their cognate antigen on the surface of APCs. T cell activation is a multi-step process and full activation requires signals at every step. TCRs present on CD4 and CD8 are key pathways for the initiation of T cell activation via binding to antigenic peptide complexed with MHC. The next signal is generated by either cytokines or involvement of co-stimulatory molecules such as B7.1 (CD80) and B7.2 (CD86) on the APC (Budd and Fortner, 2017) which induces proliferation of T cells. However, in order to prevent over expression of T cell activation, CTLA-4 (CD152) competes with CD28 for B7 binding and decreases activation signals to the T-cell. The induction of the third signal starts when a T-cell receives an antigen and co-stimulatory signal and this determines the fate of T-helper cells and they become Th1 either (through the presence of cytokine IL-12), Th2 (through the presence of IL-4) or Th17 (through the presence of IL-6, IL-23) cells and subsequently migrate to the site of infection or inflammation.

Moreover, some other molecules of the CD45 family are also responsible for activation of T cells (Alexender et al., 1992; Fraser et al 1993). CD45 is known as leukocytes common antigen and in humans it is coded by the protein tyrosine

phosphate receptor type C. There are various isoforms of CD45 present in all blood cells except erythrocytes. Previous studies suggested that in naïve T cells ($CD4^+$ and $CD8^+$) express CD45RA which is higher in molecular weight; however memory T cells expresses the CD45RO isoform which has a lower molecular (Altin and Sloan, 1997).

1.3.2.2 CD4 and CD8 T cells

There are two major forms of T cell comprising cytotoxic T cells (CD8) and helper T cells (CD4). T cells express multiple copies of their specific antigen receptor called the TCR on their cell surface. The basic function of cytotoxic T cells is to destroy the infectious cells and helper T cells function to assists macrophages (amongst other cells) in their activation. Additionally, T cells also stimulate B cells and the cytotoxic processes of T cells (Alberts et al., 2002). The CD8 and CD4 molecule is associated with MHC-class I and II molecule, respectively and important in the recognition of foreign antigen and their processing. Helper T cells have a specific role in the secretion of cytokines, in addition they display co-stimulatory molecules on their surface which affect the activation of different cell types (Gutcher et al., 2007).

1.3.2.3 Th17 cells

It is well known that CD4 T helper cells are able to differentiate into T helper 1 (Th1) or T helper 2 (Th2) where Th1 cells are characterised by expression of IFN-γ that is cell mediated immune response and able to control intracellular pathogen infection. However, Th2 cells are characterised by production of IL-4 and humoral mediated immune response that protect against helminth infection (Harrington et al., 2005; Park et al., 2005). Additionally, Th17 cells are subset of T helper cells and characterised by expression of IL-17 and protect the host when Th1 and Th2 immune response are unable to defend such as extracelluar bacteria and fungi. They also play crucial role in pathogenesis of various autoimmune and inflammatory disorder. IL-17 also produce by other immune cells such as activated $CD4^{+}\alpha\beta$ T cells, $CD8^{+}\alpha\beta$ T cells, NKT cells, γδ T cells, macrophages and neutrophils and play an important role in inflammation by recruiting neutrophils and chemokines production (Jin & Dong, 2013).

Th17 cells differentiation takes place under the influence of TGF-β, IL-6, IL-21 and IL-23. In bovine two distinct cell populations CD4 Th17 and γδ Th17 cells have shown to produce IL-17 that is important in protective immunity against various protozoan infection such as *Toxoplasma gondii*, *Neospora caninum* and *Plasmodium berghei*. (Flynn & Marshall, 2011; Peckham et al., 2014 Ribot et al., 2010).

Furthermore, CD4 Th17 express CCR6 and IL-23 in contrast to γδ Th17 that do not show these molecules.

1.3.2.4 Regulatory T (T_{regs}) cells

Tregs are a specific T cell subpopulation often known as suppressor T cells which act to regulate the immune response through maintenance of homeostasis and self-tolerance. Tregss are associated with the prevention of T cell proliferation and cytokine secretion. They are linked to control of these responses thus preventing auto-immune disease and limiting chronic inflammatory conditions (Kondelkova et al., 2010). Tregs which are produced by a normal thymus are known as natural Tregs and those produced outside of the thymus known as adaptive Treg cells. Natural Treg cells express CD4 and CD25 and are labelled as $CD4^+CD25^+$. Forkhead box protein, Foxp3, is a member of the family of transcriptional factors (Fontenot et al., 2003; Fontenot et al., 2005) that regulate the development and function of natural Treg cells.

Treg cells inhibit the activation, proliferation and cytokine secretion from $CD4^+$, $CD8^+$ T cells, B cells and DCs via cytokines such as TGF-β and IL-10. Another important suppression mechanism of Treg cells includes prevention of co-stimulation via CD28 on effector T cells by binding of CTLA4. Alternatively, they can also supress sterilizing and anti-tumour immunity (Vignali et al., 2008). In recent studies, it was documented that normal adaptive immune response produced Treg cells which were responsible for the suppression of dysfunctional immune responses that could lead to autoimmune and chronic inflammatory disease in animals as well as humans. Moreover, the adaptive immune response not only recruited effector T cells and B cells population but Tregs cells for maintaining homeostasis and optimising the magnitude of the response (Sakaguchi et al., 2008).

1.3.2.5 Gamma delta (γδ) T cells in bovine immune response

γδ T cells comprise a small subset of T cells which have a distinct TCR composed of γ and δ chains. Usually they are present in lower numbers compared to alpha beta (αβ) T cells. γδ T cells most commonly found in the gut mucosa (Holtmeier, 2005). They can act as a bridge between the innate and adaptive immune response

because of ability to rearrange their TCR gene and make a memory phenotype that enable them to act as a PRR, (Born et al., 2006; Morita et al., 2000). The unique feature of γδ T cells is they do not require any antigen processing or MHC presentation for their immune properties and able to identify soluble protein, non-protein antigens and have many cytotoxic function (Carding and Egan, 2002). Evidence suggests that γδT cells activation may be reliant on production of exogenous IL-2 with involvement of other cytokines including IL-12, IL-15, IL-18 and TNF-α (Davis et al., 1996; Skeen et al., 1995).

These cells form a small proportion of between 2-7 % of the total PBMC population in humans and mice compared to other ruminants where they have been shown to contribute to between 25–75 % of the total mononuclear cell population (Clevers et al., 1990 and Hein and Mackay, 1991; Itohara et al., 1989; Bucy et al., 1989). Their number is also found to be greater in neonates and *in vitro* studies indicate development of thymic γδ T cells may be regulated by autocrine or paracrine stimulatory signals provided by IL-4 (Barcena et al., 1991). Moreover, high numbers of γδ T cells may provide an early mechanism of producing Th1 type cytokines in calves (Brown et al., 1994; Baldwin et al., 2000).

The major population of these γδ T cells show expression of Workshop cluster 1 molecule ($WC^{+}1$) (Mackey et al., 1986). $WC1^{+}$ γδ T cells are present in thymus, lymph node, gut and spleen (Clevers et al., 1990 and Hein and Mackay, 1991). The $WC1^{+}$ molecule is involved in proliferation of γδ T cells and production of IFN-γ in response to certain cytokines such as IL-12 and IL- 18 (Brown et al., 1996; Price et al., 2006). The WC1 family is characterised by different isoforms that shown different functions. WC1.1 expression is linked to the ability to proliferate and produce IFN-γ in response to antigen stimulation as well as IFN-γ secretion without proliferation of Th1 cells or IL-12 stimulation. WC1.1 and WC1.2 expression are mutually exclusive and number of WC1.1 expressing cells decreases with age however, the proportion of WC1.2 is persistent with age which suggests that both populations have a different roles in immune responses (Rogers et al., 2005; Price et al., 2007). Furthermore, $WC1^{+}$ cells are involved in secretion of TNF-α and IL-12 which can be involved in early induction of Th1 responses and help to eliminate intracellular infection and act as

link between the innate and adaptive immune response (Collins et al., 1996; Brown et al., 1994).

1.4 *Neospora caninum*

Neospora caninum is an apicomplexan intra-cellular protozoan parasite which causes abortion, reproductive failure and neonatal mortality in the cattle (Dubey, 2003) and neuromuscular disease in dogs. It was mistakenly identified as *Toxoplasma gondii* when it was first observed in dogs at Norway in 1984 (Bjerkas et al., 1984). No evidence of human infection exists to date (McCann et al., 2008) however, some experimental studies have reported establishment of *N. caninum* infection in Rhesus monkeys (*Macaca mulatta*) (Barr et al., 1994).

1.4.1 General life cycle of *N. caninum* and epidemiology

The *N. caninum* life cycle moves between herbivorous and canid hosts as depicted in Fig. 1.3. Herbivorous animals act as an intermediate host while dogs act as either the definitive or intermediate hosts (McAllister et al., 1998; Lindsay et al., 1999a, 1999b, 2001; Basso et al., 2001a; Dubey et al., 2002). It can affect a wide variety of intermediate hosts such as cattle, sheep and goat while a different species of *Neospora hughesi* has been previously isolated from a horse (Marsh et al., 1998). Many aspects of the *N. caninum* sexual life cycle (enteroepithelial) are still not explored (Almeria, 2013) and this includes elucidating the interaction between the dog and parasite during natural infection. The life cycle of *N. caninum* is characterised by three infectious stages commonly known as tachyzoites, bradyzoites (tissue cysts) and oocysts.

Canids become infected by ingestion of infected meat in the form of tissue cysts (McAllister et al., 1998; Lindsay et al., 1999a, 1999b; Dijkstra et al. 2001; Schares et al., 2001; Gondim et al., 2002) and bradyzoites are release into the lumen of duodenum. In the intestinal epithelium, bradyzoites are converted into tachyzoites and form merozoites and eventually become macrogametes and microgametes which undergo a merogony (sexual stage) that form a zygote. The oocyst containing unsporulated sporozyoites are release into the intestinal lumen and shed in the environment with faeces (Sykes, 2014; Greene, 2012) thus farm dogs are a potential source of infection in cattle.

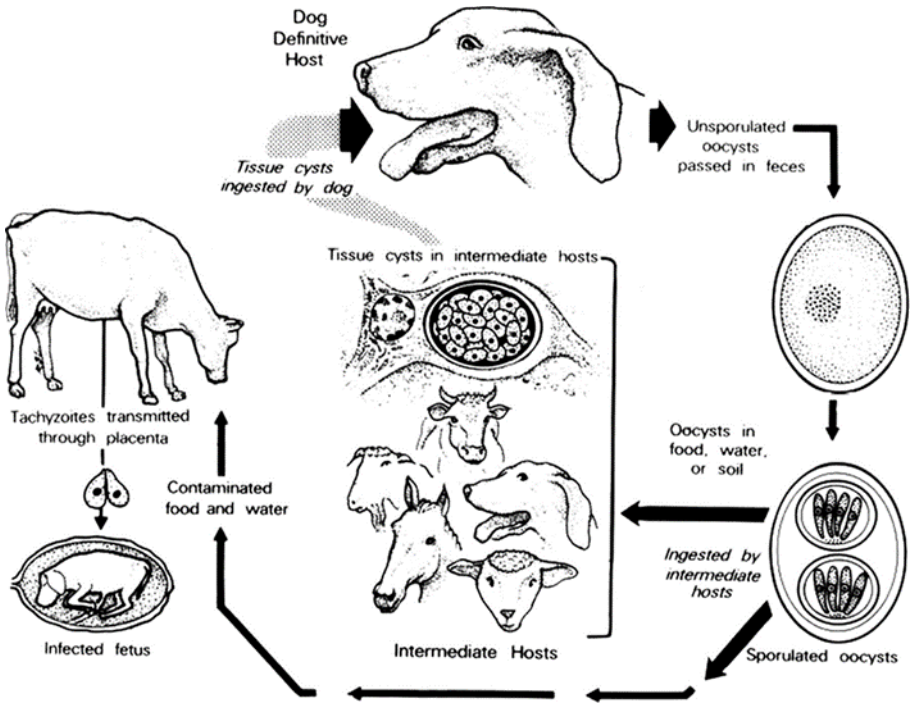


Figure 1.2 Description of the general life cycle of *N. caninum*

The dog acts as the definitive host and herbivorous livestock particularly cattle, are the intermediate host. Unsporulated oocysts are passed in faeces and contaminate the feed and water sources and when ingested by intermediate host sporulation begins and form tachyzoites (fast growing stage) or bradyzoites or tissue cyst stage (slow growing stage). During pregnancy tachyzoites are transmitted via the placenta and the foetus is infected. The life cycle is completed when infected meat (tissue cyst stage) is ingested by dogs (Figure adapted from Dubey J.P 1999).

Sporulation occurs outside the host body and cattle become infected after ingestion of sporulated oocyst via contaminated feed and water. In cattle, sporozoites are release into the duodenum and within the intestinal lumen they transform into tachyzoites that is rapidly multiplying stage and disseminating to different parts of the body including brain, heart, liver, lungs and gravid placenta (Dubey et al., 2006; Dubey et al., 2002; Hemphill et al., 1996). Once the host generates a protective immune response tachyzoites differentiate into bradyzoites that preferentially persist within nervous tissue or skeletal muscle and can remains for long periods within the host and serve as a continual source of infection. The recrudescence of parasites occur when host immune response is suppressed, immunomodulated or animal is pregnant that cause conversion of bradyzoites into tachyzoites stage that cause transplacental transmission. In cattle the major form of transmission is vertical

through transferal of parasites *in utereo* (Dubey et al., 2007). Many studies have been conducted to identify the sero-prevalence of *N. caninum* antibodies in dairy and beef cattle. Globally, Dubey et al (2007) estimated a sero-prevalence ranging from 3-60 % in dairy cattle and 2-30% in beef cattle. An additional longitudinal European study suggested sero-prevalence of *N. caninum* in dairy cattle and beef cattle was around 16-76% and 41-61% respectively (Bartels et al. 2006). In UK dairy cattle, sero-prevalence has been shown to range between 6.6-37% however, economic loss associated with UK beef cattle have not estimated (Trees et al., 1999; Davison et al., 1999a, Williams et al., 1999; Crawshaw and Brocklehurst, 2003; Woodbine et al., 2008; Brickell et al., 2010; Reichel et al., 2013), depending on the studies performed. It has also been reported that sero-positive cows have a greater chance (percentage) of abortion compared to sero-negative animals (Dubey, 1999; Anderson et al., 2000; Buxton et al., 2002). Furthermore, some research groups also suggest cattle between 13-24 months of age had a significantly lower seroprevalence of *N. caninum* compared with 7-12 months old calves or heifers of 24 months old or above (Davison et al., 1999b). Studies have also reported a higher prevalence of neosporosis in farm dogs compared with urban dwelling dogs (Sawada et al., 1998; Wouda et al., 1999; Basso et al., 2001b).

N. caninum associated abortion can either be epidemic where an abortion outbreak is temporary and up to 15% of cows are at risk. Alternatively, endemic abortion can be persistent and sporadic lasting from months to many years. Epidemic abortion occurs mainly due to primary infection of naïve dams through consumption of oocysts via contaminated food and water and abortion of the foetus occurs within a short time frame due to multiple exposures of dam through horizontal route (McAllister et al., 2000; McAllister et al., 2005). Recrudescence of latent infection due to immunosuppression can causes abortion in endemic settings (Guy et al., 2001; Weston et al., 2005). In this context there are a number of risk factors including age of the animal where older cattle have been shown to be at greater risk of becoming seropositive and thus aborting (Rinaldi et al., 2005; Dyer et al., 2000).

1.4.2 Global economic impact of *N. caninum* in the cattle industry

The economic burden of *N. caninum* has been calculated in terms of maternal infertility, abortion, increased inter-calving periods and reduced milk production. However, secondary losses are also incurred through the expense of diagnosis and replacement costs for culled animals (Dubey and Schares, 2006). It has been estimated that *N. caninum* infection can cause a 3-4% reduction in milk yield in seropositive dairy cattle compared to sero-negative cattle (Hernandez et al., 2001; Thurmond and Hietala, 1997a; Hernandez et al., 2001; Romero et al., 2005). In contrast, some Canadian studies have suggested that there was no correlation between the seropositive or seronegative status of cows and milk production (VanLeewan et al., 2002; Hobson et al., 2002). The previous studies estimated that UK dairy industry suffers annual losses of approximately £19M due to abortion related losses alone (Tress et al., 1999; Davison et al., 1999a; Williams et al., 1999; Crawshaw and Brocklehurst, 2003; Woodbine et al., 2008; Brickell et al., 2010; Reichel et al., 2013).

In beef cattle the seropositive status of animal has been associated with early culling, weaning weight, average daily weight and reproductive performance (Kasari et al., 1999; Larson et al., 2004; Barling et al., 2000; Waldner et al., 1998). Unfortunately, there is no appropriate data available that tracks the impact and rate of *N. caninum* induced abortion in beef cattle (Dubey et al., 2007).

1.4.3 Clinical signs of *N. caninum* in cattle and dogs

The clinical signs associated with bovine *N. caninum* include a mummified foetus, stillborn calf or the birth of a congenitally infected that may show neurological symptoms, be underweight or born without any clinical signs of disease. Additionally, sometimes the foetus may be reabsorbed in utero and therefore the infection may go unnoticed (Dubey and Lindsay, 1996). In dogs, more severe forms of the disease has been reported particularly in young dogs of 6 months of age with clinical signs characterised by neuromuscular symptoms including paralysis of hind limb, ataxia and loss of conscious proprioception and in some cases hydrocephalus and narrowing of the spinal cord have been recorded (Dubey, 2003).

Arthrogryposis often occurs, showing scar formation in affected muscles as results of injury to lower motor neuron and myositis (Knowler and Wheeler, 1995; Silva and Machado, 2016). Adult dogs may display encephalomyelitis, focal cutaneous nodules or ulcers, pneumonia, peritonitis, hepatitis, or myocarditis particularly when combined with immunosuppressive drugs (McAllister et al., 1998). Cutaneous forms of neosporosis are only seen during severe cases of disease, characterised by dermatitis due to large numbers of parasites infiltrating the skin. There is an age-associated risk as dogs aged between 8-15 years have been found to be more prone for cutaneous form of neosporosis (Dubey, 2003). In dogs congenital infection is the main route of transmission from infected mothers to pups. However, Barber and Trees (1998) reported that the parasite is not able to sustain vertical transmission for long periods of time in dogs.

1.4.4 Diagnosis of *N. caninum*

N. caninum diagnosis is important and various methods including serological, molecular and histological based examination are exist for diagnosis. The brain, heart, liver, placenta, blood and serum of infected animals can be examined for diagnostic purpose and accuracy can be increase if multiple tissue samples are examined (Dubey, 2003). Histological examination of aborted foetus and placenta material are important where foetal brain lesions are characterised by focal encephalitis, necrosis and non-supportive inflammation (Barr et al., 1991). Serological based tests including Enzyme Linked Immunosorbent Assay (ELISA), Immunofluorescence Antibody Test (IFAT), *Neospora* agglutination test (NAT) and immunoblot assay have been used for detection of *N. caninum* antibodies in serum, blood or milk samples. Additionally, the avidity ELISA test is able to differentiate between current and chronic infection which is a useful aid in epidemic and endemic abortion (Dubey, 2003). Some molecular techniques like polymerase chain reaction (PCR) are also useful to diagnose *N. caninum* infection (Baszler et al., 1999). Due to close similarity with *T. gondii* and *Sarcocyst cruzi* differential diagnosis utilising PCR may be needed (Canada et al., 2002).

1.4.5 Treatment and preventive measures for *N. caninum*

To date there is no approved or efficient treatment available for bovine neosporosis and chemotherapy is likely to be uneconomical due to the prolonged treatment periods required which may render milk unfit for consumption due to the presence of drug residues (Reichel and Ellis, 2002). Some *in vitro* and *in vivo* studies reported the effectiveness of toltrazuril and its derivative ponazuril on *N. caninum* tachyzoites and found treated calves harboured no detectable parasites in the brain or other organs. Mice treated with toltrazuril have been shown to be refractory to trans-placental transmission of *N. caninum* (Gottstein et al., 2005; Haerdi et al., 2006; Kritzner et al., 2002; Darius et al., 2004).

Canine neosporosis can be treated with prolonged administration of clindamycin which is a primary drug of choice and effective against tachyzoites stages, if treatment begins in the early stages of disease (Sykes, 2014). A combination of sulphonamide and clindamycin is very effective for neosporosis, additionally pyrimethamine have shown a synergestic effect on canine neosporosis (Lindsay et al., 1994; Lindsay et al., 1996). However, prognosis is poor in young puppies if treatment is delayed or in those with severe nervous or muscular signs. Furthermore it has noticed that clindamycin is less effective on bradyzoites stage so treatment of chronic neosporosis can be considered for further research where bradyzoites remains for longer time (Lidsay et al., 1994).

Currently no effective vaccination is available for *N. caninum* despite several research groups attempting to develop an attenuated or live vaccine (Innes et al., 2002; Williams and Trees, 2006). A POLYGEN-adjuvanted, killed *N. caninum* tachyzoite preparation was produced and tested in one study but was unable to induce a protective immune response (Andrianarivo et al., 2000). Later a HAVLOGEN-adjuvanted killed vaccine NeoGuard, was developed and widely used in different parts of world however its efficacy was inconsistent (46%) and in some cases increased the abortion risk and so it has been withdrawn from market (Guy et al., 2005; Williams et al., 2007; Heuer et al., 2004; Romero et al., 2004). A recombinant canine herpesvirus expressing the *N. caninum* surface protein (NcSRS2) vaccine was developed and tested in both dogs and cattle where immunised dogs were able to

produce *N. caninum* IgG antibody with no clinical signs of canine herpes virus. Immunised cattle showed parasite specific $CD4^+$ T cells and an IFN-γ response but no further investigation were carried out to assess its protective status (Nishikawa et al., 2000; Staska et al., 2005). Additionally, research suggests that the use of attenuated γ-irradiated tachyzoites in C57BL6 mice was able to induce IFN-γ, IL-10, IgG1, IgG2a in immunised mice during acute challenge with *N. caninum* but a lack of persistence of irradiated tachyzoites within the host body for long periods required further booster doses and this could limit this approach for commercialization (Ramamoorthy et al., 2006; Lindsay et al., 1999b).

1.4.6 Control measures

Control measures are critical with emphasis on preventing vertical and horizontal transmission, environmental contamination and transmission from definitive hosts to intermediate hosts and vice versa. Several preventive measures and control programmes are being conducted in different parts of the world to combat neosporosis (Hall et al., 2005; Reichel & Ellis., 2005). However, there is no standard procedure for prevention of neosporosis because epidemiology of neosporosis is variable according to geographical locations (Dubey et al., 2007). Generally, farm biosecurity is very basic and effective control measures include purchase replacement stock from disease free herd or with records of excellent reproductive performance before using them for production (Dubey et al., 2007). Additionally, control of dogs within the farm environment is the most important control strategy as this can act as a source of oocysts and contaminate the environment. A study also suggested that young dogs pose a greater risk due to greater oocysts shedding rates of compared to older animals (Gondim et al., 2005).

Studies suggest that reproduction management could be another effective way to control *N. caninum* infection in dairy farm and can be achieved by use of embryo transfer, transferring the embryo from an infected cow to healthy recipient cow to reduce the transplacental transmission (Baillargeon et al., 2001; Landmann et al., 2002). Some experimental studies reveals that veneral transmission could be possible by intrauterine inoculation of tachyzoites infected semen (Serrano et al., 2006). Also presence of *N. caninum* DNA has noticed in naturally infected bull semen

that suggest low number of viable organism which is not able to establish disease condition (Caetano-da-Silva et al., 2004; Ferre et al., 2005; Ortega-Mora et al., 2003; Canada et al., 2006). Furthermore, artificial insemination can be a good alternative and it was noticed that use of beef bull semen over dairy bull could decrease the risk of abortion within dairy herd (López-Gatius et al., 2005). *N. caninum* infected animals will always act a reservoir and spread the further infection within herd via endogenous transplacental route so testing and culling of infected animals is the most effective way to control the infection, however it is not always cost effective (Hall et al., 2005).

1.5 Immune response against *N. caninum*

Various *in vitro* and *in vivo* immunological studies on cattle and mice have established that the host immune response against *N. caninum* is primarily a Th1 based response and mediated by some key pro-inflammatory cytokines (Almeria et al., 2017; Baszler et al., 2008; Khan et al., 1997; Innes et al., 1995; Yamane et al., 2000). Studies suggest that during infection IFN-γ production from NK cells and IL-12 play an important role in controlling parasitaemia within infected hosts. Moreover, in vitro studies suggest protective role of $CD4^+$ lymphocytes to lyse *N. caninum* infected cells in cattle (Innes et al., 2002; Innes et al., 2005; Hemphill et al., 2006; Klevar et al., 2007; Monney and Hemphill, 2014). Generally adult cattle do not show any clinical signs of neosporosis except abortion. During pregnancy the host immune response is modulated to support the pregnancy (Raghupathy, 1997) and foetal induced cytokines such as IL-10 and IL-4 favour the recrudescence of the tissue cyst stage (Williams et al., 2006). The switching of bradyzoites stage to tachyzoites stage is a Th2 dependent phenomenon which is characterised by lack of IL-12, IFN-γ, TNF-α and NO as depicted in Fig. 1.3.

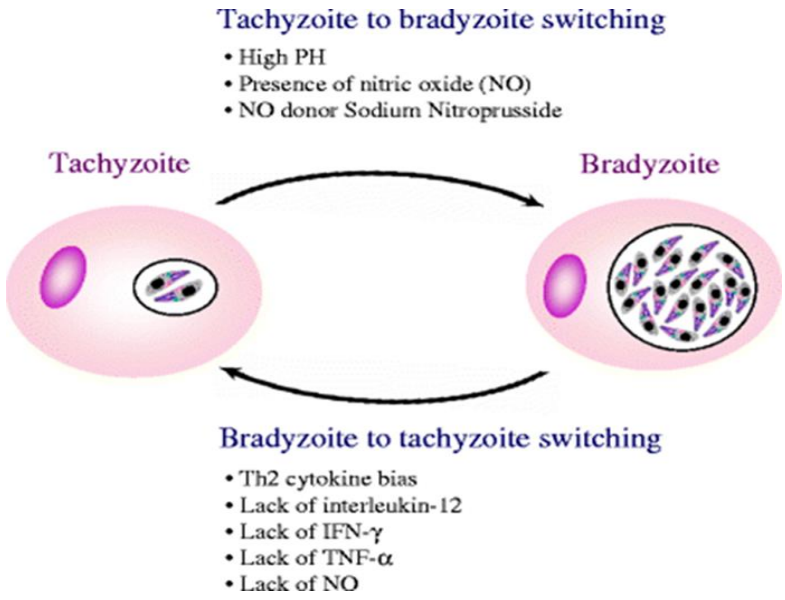


Figure 1.3 Factors mediated the interconversion of tachyzoites stage to bradyzoites

Recrudescence of *N. caninum* is Th2 dependent phenomenon where switching of intracellular bradyzoites (tissue cyst stage) to tachyzoites occur and mediated by IL-10 and IL-4 cytokines. The intercoversion of bradyzoites to tachyzoites is characterised by lack of pro-inflammatory cytokines such as IL-12, IFN- IFN-γ, TNF-α and NO (Adapted from Hemphill et al., 2006).

The reactivated tachyzoites disseminate throughout the body and can cross the placenta and infect the foetus. The resulting infection can cause abortion or birth of a congenital infected calves depending on the stage of pregnancy when animal get the infection (Williams et al., 2000).

It has been established that IL-12 and IFN-γ have an important role in host protection against intracellular parasites *N. caninum* and *T. gondii* (Baszler et al., 1999; Gazzinelli et al. 1993; Khan et al. 1994; Khan et al., 1997; Hunter et al. 1995). Previous data suggests that IFN-γ is an important Th1 mediated cytokine in protective immune response against *N. caninum* in the bovine foetus (Lunden et al., 1998; Tanaka et al., 2000; Yamane et al., 2000). Moreover, upregulation of IFN-γ was noted in naturally or experimentally challenged dams, indicating the induction of an important component of the cell mediated immune response at the early stage of the gestation period (Andrianarivo et al., 2001; Williams et al., 2000). Data indicates that IFN-γ also stimulates the endogenous production of TNF-α which activates IFN-γ primed macrophages to prevent intracellular multiplication of parasite. Furthermore, TNF-α and IFN-γ have a synergistic effect that can induce cell apoptosis and inhibit intracellular parasite replication (Hunter et al., 1994; Nishikawa et al., 2001; Chao et al., 1994).

Upregulation of IL-12 also induces the production of IFN-γ by parasite antigen stimulated T cells in cattle and mice during early stage of *N. caninum* infection (Almeria et al., 2003; Brown et al., 1996; Khan et al., 1997). These data also indicate that Th1 immune response is partially protective in dams during the early stages of gestation but intense production of Th1 cytokines also give rises to excessive inflammation and this can have a detrimental effect on foetal growth and promote abortion. To neutralise the Th1 effects, the foetus, either alone or in combination with the dam, must be able to induce other cytokines such as IL-10 and IL-4 and it appears to be able to control the inflammatory responses by down-regulating IFN-γ production (Liew et al., 1991; Thouvenin et al., 1997). An increase in IL-10 activity was reported during a study of *T. gondii* infection in mice (Gazzinelli et al., 1996)

however due to high secretion of IL-10, hosts may be unable to eliminate *N. caninum* infection (Krishnan et al., 1996).

1.5.1 Maternal immune response

It has been observed that cattle can develop natural immunity after initial infection with *N. caninum* and these animals have a lower risk of abortion compared to those who obtain their first infection just prior to or during pregnancy (McAllister et al., 2000). The risk of abortion due to *N. caninum* is greater during the first pregnancy and it reduce with subsequent pregnancies (Thurmond and Hietala, 1997b) which indicates that cattle can acquire a certain level of protective immune response. However, this protective immune response is not sufficient to prevent vertical transmission. Experimental data reported that a naturally infected pregnant cow could prevent abortion and foetopathy during *N. caninum* challenge infection at mid gestation but not vertical transmission in subsequent further pregnancies (Williams et al., 2003).

Pregnancy is an important event where the dam immune response is either modulated or supressed and this can provide the opportunity for recrudescence of parasites during chronic or latent infection. The host immune response is influenced by pregnancy hormones where progesterone and prostaglandin E2 are considered to bias T-cell immune responses towards a Th2 response, which in turn will allow reactivation of bradyzoites into tachyzoites (Innes et al., 2001; Piccinni et al., 1995; Kalinski et al., 1997; Rosbottom et al., 2011). Th1 immune responses are responsible for the restriction of parasites but at the same time overexpression of these responses can be fatal for the developing foetus during the early stages of gestation (Raghupathy, 1997, Innes et al., 2002). One study suggested that the foetal trophoblast produces IL-10 cytokines that polarise the immune response towards a Th2 cytokine environment at maternal foetal interface (Wegmann et al., 1993).

IL-10 also downregulates the expression of IFN-γ and this can alter the host parasite relationship in favour of the parasites and facilitate invasion of the placenta and foetus. The severity of infection and outcomes of pregnancy depends on the number of parasites and gestational age of the foetus (Innes et al., 2002). Experimental

infection studies indicate that infection at 210 days gestation in cattle leads to less inflammation and characterised by infiltration of T-cell subset ($CD4^+$, $CD3^+$,γδT cells) and lower numbers of $CD8^+$ and NK cells from the placenta (Canton et al., 2013). Moreover, the role of B-cells in *N. caninum* immunity is still not clear and few numbers of B cells expressing surface marker CD79αcy (CD79 form a B-cell antigen receptor complex with IgG and play key role in activation and maturation of B-cells) have been recorded in placental infiltrate in late gestation (Canton et al., 2013) however a murine study reported B-cell deficient mice were more prone to a cerebral form of *N. caninum* infection (Eperon et al., 1999).

1.5.2 Foetal immune response

The outcome of *N. caninum* infection during pregnancy is further depend on immune status of foetus. The foetal cell-mediated immune response to *N. caninum* infection can be detected at early stages of gestation, however it is not considered to be mature enough to prevent abortion and mild placental pathology can be detected (Bartley et al., 2013). The foetus is unable to mount cell mediated immune responses at a70-day gestation period, however a significant amount of IL-10, IFN-γ, IL-4, IL-12 and lymphocyte proliferation activity have been noted (Bartley et al., 2012). Studies have shown that the foetus can mount a *N. caninum* specific cellular and humoral response in mid to late gestation and characterised by lymphocyte proliferation, production of IL-10, IFN-γ, IL-4 and the detection of anti-Neospora IgG antibodies (Osburn et al., 1982; Almeria et al., 2003; Bartley et al., 2004; Bartley et al., 2013).

Studies have also reported the upregulation of the innate immune response through expression of TLR2 and TLR9 in *N. caninum*- infected foetal tissues, as well as in spleen and lymph nodes taken from the dam. Ligation of TLR2 is linked with monocyte and APC activation, while TLR9 ligation is linked with bovine DC and B-cell activation (Werling et al., 2006; Bartley et al., 2013). The foetal immune response is also a determining factor of the fate of ongoing pregnancies, where studies indicate the rapid foetal death during early pregnancy with *N. caninum* associated lesions in the placenta and foetus (Macaldowie et al., 2004) compared to less severe pathology during mid or late gestation (Collantes-Fernández et al., 2006).

Initially some data established that parasites and parasite-associated necrosis of the foetus was widely disseminated at an early stage of gestation when the foetal immune response is not able to limit the parasite burden or pathology (Gibney et al., 2008; Collantes- Fernades et al., 2006). During early pregnancy a large number of parasites circulate into the placenta which leads to induction of Th1 immune responses and abortion occuring at 13 weeks. Additionally, challenge infection during mid gestation did not result in high mortality and this was attributed to the mounting of parasite-specific responses by the foetus (Bartley et al., 2004). However, some *N. caninum* parasites were still detected in the foetal central nervous system (CNS) and placental tissue (Maley et al., 2003).

1.6 Age related immune responses do they exist?

Innate immune mechanisms are key to fight against haemoparasitic diseases such as Malaria and Babesiosis and the spleen is a pivotal in this process. The presence or absence of a spleen also influences the severity and outcome of disease. During an early phase of infection, an antibody-dependent parasite destruction through the phagocytosis of infected erythrocytes and free parasites noticed in the spleen (Goff et al., 2006; Roberts et al., 1972; Hildebrandt, 1981). Some studies have been shown that mononuclear phagocytic cells first encounter *Babesia* merozoites within the spleen and trigger the innate response (Varma and Shartry, 1980).

The most striking feature of Babesiosis is, an inverse age-related immunity has been reported where young animals with an intact immune system suffer fewer severe symptoms which include a febrile response, lethargy, anaemia and display a faster recovery after disease. In contrast, adult cattle will display severe signs of anaemia, vascular congestion, cerebral dysfunction, renal and pulmonary dysfunction and ultimately death (Bock et al., 2004; Wright and Goodger, 1988; Goff et al., 2001). Young calves display inborn immunity against Babesiosis infection which lasts until approximately 6 months of age (Goff et al., 2002).

Initially, innate immunity against Babesiosis was correlated with the passive transfer of maternal antibodies through colostrum however offspring from non-immune dams were also found to exhibit the same level of immunity, suggesting a

greater importance for the cellular components of the immune system providing protective immunity (Hall, 1960; Riek, 1963).

Previous studies reported that resistance in the context of *B. bovis* infection depends upon early expression of several cytokines IL-12, IFN-γ and TNF-α in calves compared to adults (Goff et al., 2010). Bovine macrophages and monocytes secrete NO and IFN-γ with TNF-α acting as a co-stimulus for release of NO from other PMNs (Jungi et al., 1996; Goff et al., 2006). However, in the presence of some PAMPs, NO production is enhanced, and this process may be independent of the co-stimulatory effects of IFN-γ and TNF-α (Goff et al., 2010). Increased populations of NK cells in the spleen of young calves have also been reported during the initial phase of *B. bovis* infection (Goff et al., 2003). Previous studies indicate that induction of NO due to stimulation with *Babesia* merozoites or membrane components of merozoites was an IFN-γ dependent event (Stich et al., 1998). IL-10 acts as an antagonist in the case of abesiosis infection and supresses the production of NO which inhibits the microbicidal activity of macrophages and with higher production of IL-10 observed in adult cattle, this is considered a factor in age-related immunity against the disease (Oswald et al., 1992; Gazzinelli et al., 1992).

1.7 Objectives of current study

The present study sought to address mechanisms of age related immunity during intra-cellular parasitic infection and how this mechanism affects the adaptive response. This study attempted to describe the age related alteration in the number of circulating leukocytes including monocyte, T and B cells and granulocytes among neonates (2 week old) and adult cattle (2-3 year old). Monocyte functional ability was investigated in young (6 month old) and adult (2-3 year old) cattle in terms of cytokine production and their potential role in bovine innate and adaptive immunity. This study also hypothesized that age dependent effects in monocytes could alter their subsequent interaction with NK cells during intra-cellular protozoan infection. It would be a possible explanation of the inverse age related immune responses in cattle. In the present study *N. caninum* was used as a model parasite to determine the role of monocytes during intra-cellular protozoan infection as well as influence of age on autologus monocyte NK cell co-culture system.

The present study had the following specific aims:

- To characterise the cell surface markers of the circulating leukocyte population among neonates and adult cattle by multi-colour flow cytometry analysis.
- To characterise monocyte function response between young and adult cattle in response to LPS, IFN-γ, a combination of both and alum in terms of cytokine secretion.
- To evaluate the neonatal alveolar macrophages in terms of cytokines production and CD80 expression.
- To identify the role of bovine monocyte during *N. caninum* infection and effect of autologous monocyte NK cell co-culture on parasitaemia. The NK cells cytotoxic activity compared between neonates and adult to determine age related immune mechanism.
- To determine if there are global age related changes that may alter neonatal vaccination efficacy by analysis of monocyte gene expression using bovine microarray based analysis.

Chapter 2 General materials and methods

2. General materials and methods

2.1 Collection of whole blood samples from cattle

Whole blood samples from Holstein Friesian cattle of different age groups and sex were collected for separation of peripheral blood mononuclear cells (PBMCs). Approximately, 500 ml of whole blood samples in lithium heparin anticoagulant from young cattle of 6 month old female was purchased from Seralab International Ltd. UK and use to evaluate the monocyte functional response. However, approximately 1 L heparinised (Lithium heparin salt 35 mg/l, H0878, Sigma Aldrich, Dorset, UK) whole blood from adult male beef of cattle were collected from a nearby abattoir (Redferns SK17 9HT, Buxton, Derbyshire) and use to identify the monocyte function response, leukocytes population and challenge infection study. In addition to this, blood was collected from male neonates of 2 weeks age from the dairy animal unit at the School of Veterinary Medicine and Science (SVMS), University of Nottingham and used to identify leukocytes population and challenge infection study. All of the samples were collected and purchased between fall and winter of 2014-2015, 2015-2016 and 2016-2017.

2.2 Isolation of PBMCs from whole blood

Buffy coat was prepared from the whole blood of both adult, young and neonates animals by decanting blood into 50 ml tubes and centrifuged at 400 X *g* for 30 mins. The buffy coat layer between the plasma and erythrocytes was collected with a Pasteur pipette and transferred into 50 ml tubes. 15 ml buffy coats were diluted with an equal amount of sterile Dulbecco's phosphate buffer saline (D-PBS, D8537 Sigma Aldrich, Dorset, UK) and density gradient centrifugation was performed to obtain PBMCs as follows. Approximately, 30 ml of diluted buffy coat was overlaid onto 15 ml of Histopaque 1077 (1.077g/l, 10771, Sigma Aldrich, Dorset, UK) and centrifuged at 400 X *g* for 30 mins with the brake off. The layer between the histopaque and the erythrocytes was collected into another centrifuge tube and erythrocytes were lysed by the addition of 5 ml of erythrocyte lysis buffer (Appendix 1) and mixed cells by pipetting for 10 mins. Thereafter samples were centrifuged at 220 X *g* for 10 mins and the supernatant of lysed RBC was discarded.

To obtain leukocytes from whole blood of neonates and adult cattle for flow cytometry analysis, 5 ml of buffy coat layer was washed twice with 10 ml of cold D-PBS and lysed with erythrocyte lysis buffer as mentioned above. The above procedure was repeated 2-3 times for complete lysis of erythrocytes. The final washing was performed with D-PBS and RPMI-1640 complete media (R8758 Sigma Aldrich, Dorset, UK) supplemented with 10 % fetal bovine serum (FBS, F4135 Sigma Aldrich, Dorset, UK) and 1 % penicillin-streptomycin (10,000 U penicillin & 100 mg/ml streptomycin (P4333 Sigma Aldrich, Dorset, UK).

2.2.1 Storage of PBMC

The total number of PBMCs obtained from adult, young and neonates were kept overnight at -80^{0}C before transferring into liquid nitrogen for long term storage. The number of PBMC were assessed by haemocytometer, using the trypan blue exclusion method and freeze 1x10^{7}cells/ml in a freeze mix that contains RPMI-1640 complete media with 10 % DMSO (Sigma Aldrich, UK) 40 % FBS and transferred into Nunc cryovials (V7884, Sigma Aldrich, Dorset, UK) and stored in liquid nitrogen.

2.3 Harvesting of alveolar macrophages from neonatal lungs

Four Holstein Friesian neonates were obtained at 2 weeks of age from the dairy animal unit at SVMS, University of Nottingham. These neonates were euthanized by captive bolt and jugular exsanguination under schedule 1 procedure dictated by Home Office. All work was reviewed and approved by the SVMS ethical review committee and by the University AWERB committee.

From these neonates, lungs were removed and lung lavage was performed to recover alveolar macrophages. Lungs were cleaned, the oesophagus cut and any extra fat and muscle was carefully removed. 1 L of sterile PBS was poured into lungs and lungs were gently massaged. Lavage fluid was collected by inverting the lungs into sterile glass beakers. Additional washing of lungs was repeated to obtain a final alveolar lavage fluid volume of a liter. Lavage fluid was strained through sterile muslin gauze and placed in 1 L sterile glass bottles and proceed immediately for harvesting of alveolar macrophages.

The harvested liquid was transferred into 50 ml tubes and centrifuged at 112 X *g* for 15 mins. The supernatants were discarded and the cells washed twice with fresh D-PBS at 112 X *g* for 15 mins.

The cell pellets were pooled into two 50 ml tubes/animal, resuspended in D-PBS and centrifuged at 252 X *g* for 15 mins. To harvest macrophages, the cell pellet was resuspended into 30 ml of RPMI-1640 harvesting medium supplemented with, 10 % FBS, 1% penicillin-streptomycin, 0.05 mg/L fungizone (Sigma Aldrich, UK), and 1 µl/ml 2-Mercaptoethanol (2ME, Sigma Aldrich, UK). The numbers of cells were counted and adjusted to 3×10^6 cells per 3 ml in RPMI-1640 complete media supplemented with 10 % FBS and 1 % penicillin streptomycin. A total of 3×10^6 cells were seeded/well into 6 well cell culture plates (83.3920.005, Sarstedt, Germany) and incubated for 24 hrs at 37^0C with 5 % CO_2. Thereafter, culture media and non-adherent cells were removed and replaced with 3 ml RPMI-1640 growth media supplemented with 1 µl/ml 2ME, 3 % FBS but without fungizone and penicillin-streptomycin. Macrophages were cultured for a further 24 hrs at 37^0C with 5 % CO_2.

2.3.1 Culture stimulation of alveolar macrophages

After 24 hrs culture of neonatal alveolar macrophages as described above evaluation of IL-6, IL-1β, TNF-α, IL-12 and IL-10 was perfomed. The cell culture supernatants were discarded after 24 hrs incubation and replaced with fresh RPMI complete media which contains LPS (1 µg/ml), IFN-γ (20 ng/ml) and a combination of LPS/IFN-γ and incubated at 37^0C for 24, 48 and 72 hrs. Aluminium hydroxide (500 µg/ml) stimulated alveolar macrophages were incubated at 37^0C for 8, 18 and 24 hrs. The supernatants were collected at the end of each respective incubation period and transferred into another 6 well plate then stored at -20^0C.

2.4 Magnetic separation of $CD14^+$ monocytes population from PBMC

Stored PBMCs were used for the separation of $CD14^+$ monocyte populations. Cryopreserved PBMCs were removed from liquid nitrogen and thawed at room temperature (RT). The cells were washed in 10 ml of warm RPMI-1640 complete media and centrifuged at 220 X *g* for 10 mins at 22^0C. The supernatant was discarded and cell pellets were resuspended in 1 ml of fresh RPMI-1640 complete media and a

final number of cells were adjusted to 10^7cell/ml. Cells were washed by centrifugation at 300 X *g* for 10 mins and supernatant was completely aspirated. The magnetic labelling of cells was performed by resuspending 10^7cells in 80 µl of MACS running buffer (Appendix 1) and 20 µl of human CD14 microbeads (130-050-201, Miltenyi Biotec, Bergisch-Gladbach, Germany) were added and mixed by pipetting followed by incubation on ice for 15 mins. After incubation cells were washed with 2 ml of MACS running buffer and centrifuged at 300 X *g* for 10 mins. The supernatant was discarded and cells were finally resuspended in 500 µl of fresh MACS running buffer.

The magnetic separation of $CD14^+$ monocytes was performed on a MACS LS column (130-042-401, Miltenyi Biotec, Bergisch-Gladbach, Germany) and magnetic separator. The column was placed in the MACS magnetic separator and rinsed with 3 ml of MACS rinsing buffer (Appendix 1). Thereafter, 500 µl of cell suspension in running buffer was added onto the column and washed three times with 3 ml of MACS rinsing buffer and the unlabelled cell (CD14 negative) fraction was collected in a 15 ml tube. The column was removed from the magnetic separator and placed into a new 15 ml tube followed by rinising of column with fresh 5 ml rinsing buffer. The CD14 labelled cells were eluated by firmly pushing a plunger into the column and collecting the eluate in a fresh 15 ml tube containing 2 ml of RPMI-1640 complete media. The eluated cell fraction was centrifuged at 220 X *g* for 8 mins and the total number of $CD14^+$ monocytes were counted in a haemocytometer using trypan blue exclusion method and the number of cells were adjusted to 2×10^6 cells/ml.

2.5 Isolation of NK cells from PBMC

In a monocytes NK cells co-culture experiments, NK cells were isolated from unlabelled cell fraction obtained during $CD14^+$ monocytes isolation. The numbers of unlabelled cells were counted and number of cells were adjusted to 10^7 cells/100 µl in MACS running buffer. Primary labelling of cells was performed with mouse anti-bovine CD335 antibody (MCA2365GA, AbD serotec) with a 1:50 dilution and incubated on ice for 15 mins. The primary labelled cells were washed with 1-2 ml of running buffer and centrifuged at 300 X *g* for 10 mins.

Supernatant was aspirated and 10^7 cells were resuspended into 80 µl of running buffer and 20 µl rat anti-mouse IgG1 microbeads (130-047-102, Miltenyi Biotec, Bergisch Gladbach, Germany) followed by incubation on ice for 15 mins. Cells were washed with 1-2 ml of running buffer and centrifuged at 300 X *g* for 10 mins and resuspended into fresh 500 µl of running buffer. Magnetic separation of NK cells was performed by rinsing a MACS LS column with 3 ml of rinsing buffer. 500 µl of cell suspension was added into the column and washed 3 times with 3 ml of rinsing buffer. NK cells elution was performed by firmly pushing a plunger into the column and collecting the eluate in a fresh 15 ml tube containing 2 ml of RPMI-1640 complete media.The eluated cell fraction was centrifuged at 220 X g for 8 mins and the total number of NK cells were counted in a haemocytometer using trypan blue exclusion method. NK cells were stimulated with recombinant bovine IL-15 (RP0009B-025, Kingfisher Biotech Inc.) with a concentration 10 ng/ml. The cells were seeded into 24 well plate (CLS3527 Sigma Aldrich, Dorset, UK) ($1x10^4$ cells/well) and incubated at 37^0C overnight.

2.6 Culture stimulation of CD14^{+}monocytes

The CD14^{+} monocyte were stimulated with bacterial lipopolysaccharide (LPS) 1µg/ml (L3012, Sigma Aldrich, Dorset, UK), Interferon gamma (IFN-γ) 20 ng/ml (PHP050A, BioRad, UK) alone and a combination of LPS and IFN-γ to induce the expression of IL-6, IL-1β, TNF-α and IL-12. Aluminium hydroxide (Alum) 500 µg/ml (239186, Sigma Aldrich, Dorset, UK) was used to stimulate the induction of IL-1β from CD14^{+} monocyte. LPS, IFN-γ and a combination of both stimulated CD14^{+} monocytes were seeded with 2×10^{5} cells/well in triplicate in a 48 well plates (150687, Nunc, Thermofisher Scientific, UK) and incubated at 37^{0}C for 24, 48 and 72 hrs. Alum stimulated CD14^{+} monocytes were incubated for 8, 18 and 24 hrs. The supernatants were collected at the end of each respective incubation period and transferred into another 48 well plate, then stored at -20^{0}C. For caspase 1 analysis alum stimulated monocytes were incubated for 4, 6 and 8 hrs. The cell culture lysates were generated after centrifugation of plates at 300 X *g* for 10 mins at 4^{0}C followed by removoal of the cell culture supernatants. 100 µl of chilled cell lysis buffer (ALX-850-212-KI01, Enzo life science, UK) was added to the cells and incubated for 10 mins on ice. The resulting lysate was stored at -20^{0}C for further use.

2.7 Infection of CD14^{+}monocytes with *N. caninum* tachyzoites and autologus co-culture with NK cells

NK cells were isolated from unlabelled fraction of PBMC and cultured in 24 well plates with IL-15 followed by incubation at 37^{0}C for 24 hrs (See section 2.5). The culture media was discarded and cells were rinsed with with 200 µl ice cold D-PBS per well. The cells were collected in fresh RPMI complete media (200 µl/well) by gentle pippetting and transferred into 1.5 ml microcentrifuge tubes and kept at 4^{0}C until use. Purified *N. caninum* tachyzoites were counted and 10^{7} tachyzoites/ml were resuspended in D-PBS (See section 2.11 for *N. caninum* culture information).

The fluorescent dye carboxyfluorescein succinimidyl ester (CFSE, C34570, Invitrogen, Life technologies) was added at a concentration of 5 µm/10^7 parasites and incubated at RT for 10 mins. At the end of the incubation period, 400 µl of RPMI complete media was added and kept parasites on ice for 5 mins then centrifuged at 448 X *g* for 5 mins. The supernatant was aspirated and pellet was resuspended in 1 ml of freshD-PBS. Purified stained *N. caninum* tachyzoites were used to infect $CD14^+$ monocytes at MOI 1:4 in 24 well cell culture plates and plates were incubated at 37^0C for 3 hrs. Afterward the supernatant was collected and replenished with fresh RPMI complete media and $1x10^4$ washed NK cells were added into *N. caninum* infected and uninfected wells, giving a target : effector ratio of 10:1. In control wells only $CD14^+$ monocytes and $CD14^+$ monocytes/tachyzoites were cultured without the addition of NK cells.

2.8 Cytokines ELISA

To measure the cytokine levels in cell culture supernatants, ELISAs were performed. Bovine IL-6, IL-1β (ESS0029 and ESS0027, Thermofisher Scientific, UK) TNF-α (VS0285B-002, KingFisher Biotech), IL-10 (CSB-E12917B, Cusabio, Wuhan, Hubei China), IL-17A (VS0284B-002, KingFisher Biotech) and IFN-γ (3119-1H-6, Mabtech kits were used according to manufacturer's instructions. IL-12 cytokine was evaluated by using paired capture and detection antibody (DS-MB-02653 and DS-MB-02652, RayBiotech) with optimised protocol. All reagent reagents were allowed to thaw at RT before use.

2.8.1 Bovine IL-6 ELISA

The IL-6 coating antibody was diluted 1:100 in carbonate-bicarbonate buffer with pH 9.4 (Appendix 2) and 100 µl added into each well of a 96 well plate (44-2404-21, Nunc MaxiSorp™ flat-bottom Thermofisher Scientific, UK). The plate was covered with plate sealer and incubated overnight at RT. Coating antibody was aspirated and 300 µl of blocking buffer (Appendix 2) was pipetted into each well and incubated at RT for 1 hr. The blocking buffer was aspirated and plate was dried before addition of cell culture supernatants and IL-6 standard. Lyophilized recombinant bovine IL-6 standard was reconstituted according to the manufacture's recommendation and serially diluted from 5000 pg/ml to 39.06 pg/ml. 100 µl of each standard was added into wells in duplicate, and samples were tested in duplicate. The plate was covered with plate sealer and incubated for 1hr at RT with moderate shaking (25/mins).

The plate was emptied and each well washed 3 times using 300 µl ELISA washing buffer and dried by tapping against tissue paper. The anti-bovine IL-6 detection antibody was diluted to 1:100 in reagent diluent and 100 µl was added to each well. The plate was covered with plate sealer and incubated at RT for 1 hr with moderate shaking (25/mins). The washing steps were repeated as above and streptavidin-HRP was diluted to 1:400 in reagent diluent and 100 µl was added in each well and incubated at RT for 30 mins with moderate shaking (25/mins). Each well washed 3 times with 300 µl of washing buffer with subsequent addition of 100 µl TMB substrate buffer to each well of plate and placed in the dark for 20 mins at RT to develop a colour. The reaction was stopped by addition of 100 µl of stop solution containing 0.16 M sulphuric acid and absorbance was recorded immediately on an ELISA plate reader at 450 nm and with a 540 nm reference filters.

2.8.2 Bovine IL-1β ELISA

The IL-1β coating antibody was diluted 1:100 in carbonate-bicarbonate buffer with pH 9.4 and 100 µl added into each well of a 96 well plate. The plate was covered with plate sealer and incubated overnight at RT. Coating antibody was aspirated and 300 µl of blocking buffer was pipetted into each well and incubated at RT for 1 hr The blocking buffer was aspirated and the plate was dried before addition of cell culture supernatants and IL-1β standard. Lyophilized recombinant bovine IL-1β standard was reconstituted according to the manufacturer's recommendation and serially diluted from 2000 pg/ml to 15.625 pg/ml in reagent diluent. 100 µl of each standard was added into wells in duplicate, and samples were tested in duplicate, and incubated at RT for 1hr.

The plate was emptied and each well washed 3 times using 300 µl ELISA washing buffer and dried by tapping against tissue paper. The anti-bovine IL-1β detection antibody was diluted to 1:100 in reagent diluent and 100 µl was added to each well. The plate was covered with plate sealer and incubated at RT for 1 hr. The washing steps were repeated as above and streptavidin-HRP was diluted to 1:400 in reagent diluent and 100 µl was added in each well and incubated at RT for 30 mins. Each well washed with 300 µl of washing buffer 3 times with subsequent addition of 100 µl TMB substrate buffer to each well of plate and placed in the dark for 20 mins at RT to develop a colour.The reaction was stopped by addition of 100 µl of stop solution containing 0.16 M sulphuric acid and absorbance was recorded immediately on an ELISA plate reader at 450 nm and with a 540 nm reference filters.

2.8.3 Bovine TNF-α ELISA

The plates are pre-coated with bovine TNF-α capture antibody. The bovine TNFα standard was prepared with 1:1 serial dilution with RPMI complete media and the lowest standard concentration was set to zero. 100 µl prepared standard of each concentration was added in duplicate and cell culture supernatants were tested in duplicate according to the manufacturer's instructions. The plate was emptied and each well washed 4 times using 300 µl ELISA washing buffer and dried by tapping against tissue paper. The anti-bovine TNF-α detection antibody was diluted to 1:100 in reagent diluent and 100 µl was added to each well. The plate was covered with plate sealer and incubated at RT for 1 hr. The washing steps were repeated as above and streptavidin-HRP was diluted to 1:400 in reagent diluent and 100 µl was added in each well and incubated at RT for 30 mins. Each well washed 3 times with 300 µl of washing buffer with subsequent addition of 100 µl TMB substrate buffer to each well of plate and placed in the dark for 30-40 mins at RT to develop a colour without plate sealer. The reaction was stopped by using 100 µl of stop solution to each well which contain 0.18 M sulphuric acid and absorbance was recorded immediately on an ELISA plate reader at 450 nm.

2.8.4 Bovine IL-10 ELISA

This assay is a competitive inhibition assay which determine the IL-10 secretion in neonatal alveolar macrophages. The plates are pre-coated with polyclonal goat anti-rabbit IL-10 antibody. The standards were ready to use with highest concentration 1000 pg/ml to lowest concentration 0 pg/ml and used according to the manufacturer's instructions. 50 µl of ready to use standard of each concentration as well as cell culture supernatants were added in duplicate into each wells. 50 µl of HRP-conjugate were added to standard and samples well followed by addition of 50 µl of detection antibody and mixed. The plates were incubated at 37^0C for 1 hr. The plate was emptied and each well washed 3 times with 200 µl 1X washing buffer. Thereafter, 50 µl of substrate A and 50 µl of substrate B were added into each well of the plate and mixed by gentle pipetting. The plate was incubated at 37^0C for 15 mins in the dark. 50 µl of stop solution was added to each well with gentle mixing to stop reaction and absorbance was recorded immediately on an ELISA plate reader at 450 nm.

2.8.5 Bovine IL-12 ELISA

In this assay paired mouse anti-bovine IL-12 antibodies were optimised for working concentraion. Purified IgG mouse anti-bovine IL-12 (DS-MB-02653, RayBiotech, UK) was used as a capture antibody and purified IgG biotin conjugated IL-12 antibody (DS-MB-02652, RayBiotech,UK) was used as a detection antibody. The mouse anti-bovine IL-12 coating antibody was diluted 1:100 in carbonate-bicarbonate buffer with pH 9.4 and 100 µl was added into each well of a 96 well plate and incubated overnight at RT. Coating antibody was aspirated and 300 µl of blocking buffer was pipetted into each well and incubated at RT for 1 hr. The blocking buffer was aspirated and plate was dried before addition of cell culture supernatants. 100 µl of cell culture supernatants were added in duplicate and incubated at RT for 1 hr. The plate was emptied and each well washed 3 times with ELISA washing buffer and dried by tapping against tissue paper. The anti-bovine biotin conjugated IL-12 detection antibody was diluted to 1:100 in reagent diluent and 100 µl was added to each well. The plate was covered with plate sealer and incubated at RT for 1 hr.

The washing steps were repeated as above and streptavidin-HRP was diluted to 1:400 in reagent diluent and 100 µl was added in each well and incubated at RT for 30mins. Each well washed with 300 µl of washing buffer with subsequent addition of 100 µl TMB substrate buffer to each well of plate and placed at RT in the dark for 20 mins to develop a colour. The reaction was stopped by addition of 100 µl of stop solution containing 0.16 M sulphuric acid and absorbance was recorded immediately on an ELISA plate reader at 450 nm and with a 540 nm reference filters.

2.8.6 Bovine IL-17A ELISA

ELISA plates are pre-coated with bovine IL-17A capture antibody. The bovine IL-17A standard was prepared with 1:1 serial dilution with complete media and the lowest standard concentration was set to zero according to manufacturer's instruction. 100 µl prepared standard of each concentration as well as cell culture supernatants were added in duplicate and incubated at RT for 1 hr. The plate was emptied and each well washed 4 times with ELISA washing buffer and dried by tapping against tissue paper. The IL-17A detection antibody was diluted to 1:100 in reagent diluent and 100 µl was added to each well. The plate was covered with plate sealer and incubated at RT for 1 hr. The washing steps were repeated as above and streptavidin-HRP was diluted to 1:400 in reagent diluent and 100 µl was added in each well and incubated at RT for 30 mins. Each well washed again with 300 µl of washing buffer with subsequent addition of 100 µl TMB substrate buffer to each well of plate and placed at RT in the dark for 30-40 mins without plate sealer to develop colour. The reaction was stopped by addition of 100 µl of stop solution to each well which contain 0.18 M sulphuric and absorbance was recorded immediately on an ELISA plate reader at 450 nm.

2.8.7 Bovine IFN-γ ELISA

96 well ELISA plate was coated with 100 µl of MT17.1 monoclonal antibody (2 µg/ml in D-PBS) and incubated overnight at 4^0C. Each well washed twice with 200 µl of D-PBS and dried by flicking of plate on tissue towel. The plate was blocked with 200 µl of incubation buffer (Appendix 3) into each well and incubated at RT for 1 hr. The plate was emptied and each well washed 5 times with ELISA washing buffer and dried by tapping against tissue paper. Lyophilized recombinant bovine IFN-γ standard was reconstituted according to manufacturer's recommendation and serially diluted from 50 ng/ml to 15.625 pg/ml in incubation buffer. 100 µl of prepared standard of each concentration as well as cell culture supernatants were added in duplicate incubated at RT for 2 hrs.

The plate was emptied and washed 5 times with ELISA washing buffer and dried by tapping against tissue paper. 100 µl MT-307 biotin labelled monoclonal detection antibody (0.25 µg/ml in incubation buffer) was added to each well. The plate was covered with plate sealer and incubated at RT for 1 hr. The washing steps were repeated as above and streptavidin-HRP was diluted to 1:1000 in incubation buffer and 100 µl was added in each well and incubated at RT for 1 hr. Each well washed with 300 µl of washing buffer 5 times with subsequent addition of 100 µl TMB substrate buffer to each well of plate and placed at RT in the dark for 30 mins to develop colour. The reaction was stopped by addition of 100 µl of stop solution containing 0.16 M sulphuric acid and absorbance was recorded immediately on an ELISA plate reader at 450 nm.

2.9 Enzyme assays

2.9.1 Caspase-1 analysis

Caspase-1 analysis was performed by using a commercial kit (ALX-850-212, Enzo Life Sciences, UK) according to the manufacturer instructions in cell culture lysates which is based on detection of the cleavage of VYAD-AFC (AFC: 7-aminso-4-trifluoromethyl coumarin). Cell culture lysates were generated after centrifugation of plates at 300 X *g* for 10 mins at 4^0C and the cell supernatants were removed

followed by addition of 100 µl chilled cell lysis buffer and incubated on ice for 10 mins. The resulting lysate was stored at -20^{0}C for further use.

For caspas 1 analysis, 50 µl of cell lysate was added to each well of 96 well black fluorimeter plate (M5061-40EA, Sigma Aldrich, Dorset, UK) in duplicate in the dark followed by addition of 50 µl 2X reaction buffer and 5 µl 1 mM YFAD-AFC substrate buffer to achieve a final concentration 50 µM of YFAD-AFC. The standard curve was generated by addition of 1 µM of AFC in 2X reaction buffer to achieve a final concentration of 100 nM and serially diluted 1:2 over a range of 100 nM to 1.5 nM. The plate was incubated at 37^{0}C for 2 hrs in the dark and absorbance was recorded immediately by a fluorimeter with 400 nm excitation and 505 nm emission filters.

2.9.2 Assessment of Lytic activity of NK cells

The specific lytic activity of NK cells was determine using granzyme B and perforin assay from *N. caninum* infected and uninfected CD14$^+$ monocytes NK cell co-culture of neonates and adult cattle (section in 2.7). NK cells upon contact with target cells secrete granzyme B that enters into target cell with the assistance of perforin which is a pore forming protein.

2.9.2.1 Granzyme B assay

Granzyme B was measured from monocytes NK cells co-culture supernatants using a commercial fluorometric kit (Ab157403, Abcam, UK) according to the manufacturer instruction. Cell culture supernatants were collected from infected and uninfected neonates and adult cattle 24 hrs post infection. 50 µl of cell culture supernatants were added in duplicate from each individual samples in a 96 well black fluorimeter plate (M5061-40EA, Sigma Aldrich, Dorset, UK) followed by addition of 50 µl reaction mix (AFC substrate: assay buffer, 1:10). 2 µl reconstituted positive control (1:10) in assay buffer was added per well in duplicate followed by addition of 50 µl reaction mix and final volume was adjusted upto 100 µl with granzyme assay buffer. The standard curve was generated by dilution of 10 µl of 1 mM AFC standard with 990 µl of granzyme assay buffer to achieve a final concentration 10 µM and serially diluted from 250 pmol/well to 0 pmol/well in assay buffer according to

manufacturer protocol. 100 µl of each concentration of standard was added in duplicate into each well with subsequent incubation of plate at 37^0C in the dark. The sample absorbance was recorded at 0 min and 40 mins by a fluorimeter with 380 nm excitation and 500 nm emission filters.

2.9.2.2 Perforin assay

Cytolytic activity was determined by haemoglobin released from red blood cells (RBC). The RBC pellet was prepared by centrifugation of 15 ml of sheep whole blood in a 50 ml tube at 400 X g for 15 mins with brake off. The upper plasma and buffy coat layer was carefully removed and remaining pellet was centrifuged at 400 X g for 10 mins and supernatant was discarded. The pellet was washed 3 times with cold PBS containing 15 mM EDTA and bottom 2/3 layer of RBC was collected in a fresh tube and kept at 4^0C for further use. The numbers of RBC were counted in a haemocytomter and final number were adjusted $1.5X10^8$/ml in perforin buffer (Appendix 3).

50 µl of RBC ($7.5X10^6$) in 100 µl of perforin buffer was added into a flat bottom 96 well plate in duplicate followed by addition of 50 µl of monocytes NK-cell co-culture supernatants of *N. caninum* infected as well uninfected neonates and adult cattle. For positive control 50 µl of sheep RBCs were lysed with RBC lysis buffer at the ratio of 1:1, 1:2, 1:3 and 1:4 and final volume was adjusted 300 µl by addition of D-PBS. Negative control sheep RBCs were incubated with perforin buffer. The samples were mixed by pipetting and incubated at 37^0C for 15 mins followed by centrifugation at 400 X g for 5 mins. The supernatants were collected and 100 µl tranferrred into a new 96 well flat bottom plate with a further 15 mins incubation at 37^0C. The sample OD was measured at 405 nm absorbance filter in an ELISA plate reader.

2.10 Flow cytometry analysis of leukocytes and *N.caninum* infected CD14^{+}monocytes

2.10.1 Flow cytometry analysis of leukocytes with different antibody panels

Flow cytometric analysis was performed to determine the expression of cell surface markers on neonates and adult bovine leukocytes and neonatal alveolar macrophages. Analysis of different antibody panels targeting the monocyte, lymphocyte and granulocyte populations within circulating leukocytes as well as alveolar macrophages was performed on a BD FACSCanto II flow cytometer and flow cytometric results were analysed using Weasel 3.1.2 software.

2.10.2 Preparation of sample for flow cytometry analysis

Cell samples were prepared by washing of 1x10^{7} leukocyte in 10 ml of RPMI-complete media and centrifuged at 252 X *g* for 10 mins. The number of cells was adjusted to 2×10^{6} cells/ml in FACS buffer (Appendix 2). 100 µl of cell suspension was placed in 2ml tubes and to avoid the binding of non-specific antibodies to the Fc receptor, blocking was performed by addition of 100 µl FACS blocking buffer (Appendix 2) to cell samples. The samples were incubated on ice for 20 mins.

2.10.3 Direct labelling of cells

The samples were removed from the ice and washed with 200-300 µl FACS buffer by centrifugation at 252 X *g* for 5 mins. The supernatants were discarded and 0.1-10 µg/ml primary antibodies were added to each tubes depending on optimal antibody concentration (Table 2.1) followed by incubation on ice for 45 mins in the dark. Thereafter, cells were washed 3 times with 200-300µl FACS buffer by centrifugation at 220 X *g* for 5 mins. The cells were resuspended in a final volume of 400-600 µl FACS buffer and kept on ice in the dark until analysis.

2.10.4 Indirect (secondary) labelling of cells

Indirect/secondary labelling of leukocyte was performed for granulocyte (CD11c), γδT-cell (WC1.1) and memory B-cell (CD45RO). Cell preparation was performed as described in section 2.10.2.

The diluted unlabelled antibodies (refer to Table 2.1 for antibody concentrations) were added to each cell sample in 2 ml tube and incubated on ice for 45 mins in the dark. The cells were washed with addition of 200-300 µl FACS buffer by centrifugation at 220 X g for 5 mins followed by labelling with diluted secondary antibodies (refer to Table 2.1 for antibody concentrations) and incubated on ice for 45 mins in the dark. Cells samples were washed three times with 200-300µl with FACS buffer by centrifugation at 220 X *g*. The samples were resuspended in a final volume of 400-600 µl FACS buffer in 2 ml tube and kept on ice in the dark until analysis.

2.10.5 Permeabilization and fixation of cells

To determine FoxP3 expression, cell membranes were permeabilized and subsequently fixed using the BD Cytofix/Cytoperm kit (554714, BD Bioscience, UK) according to manufacturer's instruction. Leukocytes were prepared and stained with primary antibody as described above (2.10.2 & 2.10.3). The cells were washed with addition of 200-300 µl of FACS buffer by centrifugation at 220 X g for 5 mins and fixed with 250 µl of perm/fix buffer for 20 mins on ice. Cells were washed twice with 1X BD wash buffer containing saponin at 220 X *g* for 5 mins. Supernatants were discarded and anti Foxp3 antibody was added and incubated on ice for 45 mins in the dark. Cells were washed three times with 400-600µl of BD wash buffer by centrifugation at 220 X g for 5 mins and resuspended in a final volume of 300-600 µl BD wash buffer and kept on ice until analysis.

2.10.6 Staining of *N. caninum* infected / uninfected monocyte and monocyte NK cell co-culture for flow cytometry

Flow cytometry was performed on *N. caninum* infected and uninfected $CD14^+$ monocytes as well as $CD14^+$ monocytes NK cells co-cultured. The supernatants from cultures were removed and kept at -20^0C for cytokine analysis. The monolayer of cells were rinsed with cold D-PBS to remove growth media. The cells monolayer was treated with 200 µl (0.25%) trypsin-EDTA per well and incubated at 37^0C for 5 mins for cell detachment. The trypsin activity was neutralized by adding 500 µl of D-PBS and mixed genetly by pipetting. The cells were collected in 1.5 ml tubes and centrifuged at 220 X *g* for 5 mins. The supernatant was discarded and cells were resuspended into 100 µl of FACS buffer. The infected and uninfected monocytes as

well as monocytes NK cells were fixed with 200 µl of 4% paraformaldehyde at 4^0C for 10 mins and washed with 400 µl of cold D-PBS and centrifuged at 220 X g for 5 mins. A sample of unlabelled (CFSE free) *N. caninum* tachyzoites were also fixed with 4% PFA, washed with D-PBS and centrifuged at 220 X g for 5 mins. The cell samples were resuspended in a final volume of 200 µl in FACS buffer.

To prevent non-specific binding, 100 µl FACS blocking buffer was added into each samples and kept on ice for 20 mins. At the end of the incubation period cells were washed with 200 µl of FACS buffer and centrifuged at 220 X *g* for 5 mins. R-phycoerythrin (RPE) labelled mouse anti bovine CD80 antibody was added into cell samples according to optimized concentration (Table 2.1) and incubated on ice for 45 mins in the dark followed by washing with 200 µl of FACS buffer and centrifuged at 220 X g for 5 mins. The cells were resuspended in a final volume of 300 µl of FACS buffer and kept at 4^0C in the dark until analysis.

Table 2. 1 List of monoclonal antibodies and their respective isotype control used for monocytes, lymphocytes and granulocytes markers

Antibody	Conjugate	Dilution	Isotype	Clone	Isotype Control	Manufacture
CD14	FITC	1/50	IgG1	CC-G33	IgG1	BIORAD
CD80	RPE	1/50	IgG1	IL-A159	IgG1	BIORAD
CD86	RPE	1/50	IgG1	IL-A190	IgG1	BIORAD
CD11b	FITC	1/10	IgG2b	CC-126	IgG2b	BIORAD
MHC-II	RPE	1/10	IgG2a	IL-A21	IgG2a	BIORAD
CD11c	Unlabelled**	1/10	IgM	BAQ153A	IgM	Kingfisher,Biotech
CD4	RPE	1/10	IgG2a	CC8	IgG2a	BIORAD
CD25	FITC	1/10	IgG1	IL-A111	IgG1	BIORAD
Foxp3	Alexa fluor 647	1/10	HuCAL fab	7627	HuCAL Fab	BIORAD
WC1	FITC	1/100	IgG2a	CC-101	IgG2a	BIORAD
WC1.1	Unlabelled*	1/100	IgG1	BAQ159A	IgG1	Monoclonal antibody center
CD45RO	Unlabelled*	1/50	IgG1	CC1	IgG1	BIORAD
CD21	FITC	1/50	IgG1	CC51	IgG2b	BIORAD

**IgM conjugated with APC was used for secondary labelling of CD11c.

* IgG1 Alexa fluor 647 conjugated was used for secondary labelling of WC1.1 and CD45RO

2.11 *Neospora caninum* culture

N. caninum (NcLiv1) isolate (P47) provided by Dr. Diana Williams (University of Liverpool) was maintained in Vero cell lines (P37) by serial passage. NcLiv1 is most common European isolate obtained from cerebrum of a dog and extensively use for *in vivo* and *in vitro* studies (Barber et al., 1995).

2.11.1 Vero cells propagation from frozen stock

Vero cells (kidney of African green monkeys) were used for the maintenance of *N. caninum*. Frozen Vero cells were removed from liquid nitrogen and thawed at RT. The contents of the cryovial was transferred into a 15 ml tube containing DMEM media (D5671, Sigma Aldrich, Dorset, UK) supplemented with 10% FBS and 1% 10,000 U/ml penicillin 100 mg/ml streptomycin. The content was centrifuged at 448 X *g* for 10 mins then supernatant was aspirated and the cell pellet was resuspended in fresh 5 ml of DMEM complete media. The Vero cell suspension was transferred into 25 cm^2 tissue culture flask with vented cap (CLS430639, Sigma Aldrich, Dorset, UK) and incubated at 37^0C with 5% CO_2.

2.11.2 Maintenance of Vero cells

Confluent monolayer growth of Vero cells was achieved after 2-3 passages before they were suitable for infection. Growth media was removed from confluent layers of Vero cells grown in 25cm^2 flask and rinsed with 5 ml of warm D-PBS to remove remaining growth media. 1 ml of warm 0.25% trypsin EDTA (25200-072, gibco life technologies) was added into tissue culture flask followed by incubation at 37^0C for 5 mins and checked the cells for detachment from the flask surface. The cells were collected in fresh 10 ml of DMEM complete media to inactivate the effects of trypsin. The cell suspension was transferred into a 15 ml tube and centrifuged at 448 X *g* for 10 mins. The supernatant was discarded and the pellet was resuspended in 10 ml of fresh DMEM media. The number of cells were counted by haemocytometer using the trypan blue exclusion method. A total $3x10^5$ cells per 25 cm^2 tissue culture flask were seeded in 5 ml DMEM complete media.

2.11.3 *In vitro* culture of frozen *N. caninum*

In vitro culture of *N. caninum* tachyzoites was performed in Vero cells line (P37). The frozen parasite was thawed in a water bath at 37^0C and centrifuged at 448 X *g* for 10 mins with 10 ml of DMEM complete media. The supernatant was discarded and parasites were resuspended in 2 ml of fresh DMEM complete media for counting of total number of tachyzoites by haemocytometer using trypan blue exclusion method. *In vitro* culture of *N. caninum* was performed on a confluent monolayer of Vero cells. The media from 25 cm^2 tissue culture flask was removed and replaced with fresh 5ml DMEM complete media a total $9x10^5$ parasites per 25 cm^2 flask was used.

2.11.4 Maintenance and purification of *in vitro* culture of *N. caninum*

Cultures of *N. caninum* were regularly monitored and media was replaced on day 2 with DMEM media supplemented with 2% FBS and 1% penicillin (10,000 U/ml)/ streptomycin (100 mg/ml). The growth of tachyzoites was examined microscopically and on day 7 post culture, growth was determined to be optimum with free tachyzoites visible. For purification of *N. caninum* and passage new cultures, the parasites cell monolayer was harvested using cell scraper (CLS3010, Corning® Sigma Aldrich, UK) and content was transferred into a 15 ml of tube. The cells were centrifuged at 448 X *g* for 10 mins and supernatant was removed. The cells pellet was resuspended in 10 ml of warm D-PBS and mixed by gentle pipetting followed by centrifugation at 448 X g for 10 mins, supernatant discarded and cell pellet was resuspended in 5 ml of warm D-PBS and disintegrate using a blunt 25 G needle. PD10 desalting columns (GE17-0851-01, GE Healthcare Bio-Science AB, Sweden) were used for the purification of *N. caninum* tachyzoites. The column was rinsed with 10 ml of D-PBS to remove any preservative left in the PD10 column. Afterwards, 5 ml of parasites and cell suspension was added and rinsed twice with addition of 5 ml D-PBS and elution was collected in a fresh 15 ml tube. The Vero cells were retained on the column due to big size whereas free tachyzoites were washed through the column. The eluted parasites were centrifuged at 448 X g for 10 mins and supernatant was removed. The tachyzoites were resuspended in 5 ml of fresh D-PBS and total numbers of tachyzoites were counted by haemocytometer using trypan blue

exclusion method. A total $3x10^5$ tachyzoites were used for further infection of new Vero cell cultures in $25cm^2$ flask and $5X10^4$ tachyzoites were used for monocyte and monocyte NK cell co-culture infection study.

2.12 Preparation of RNA samples for bovine microarray

2.12.1 RNA isolation

RNA isolation was performed from *N. caninum* infected and uninfected $CD14^+$ monocytes of neonates (n=3) and adult cattle (n=3) grown in a monolayer in duplicate. Isolation of RNA was performed using a commercial RNeasy mini kit (74104, QIAGEN) according to the manufacturer instruction with some modification. The monolayer of cells were washed with fresh cold D-PBS after aspiration of growth media to remove residual media. The cells were directly lysed by adding of 350 µl of lysis buffer (RLT) in a 24 well plates. The cell lysates were collected into RNAse free microcentrifuge tube and vortex for 30-60 sec. The complete homogenization was performed using QIAshredder homogenizers (79654, QIAGEN) by loading of 350 µl cell lysates into QIAshredder column and placed into a 2 ml collection tube followed by centrifugation at 17000 X g for 2 mins. An equal volume of 70% molecular grade ethanol (51976, Sigma-Aldrich) was added in homogenized lysate and mixed by pipetting followed by transferred into an RNeasy spin column.

700 µl homogenized sample was loaded into RNeasy spin column placed in a 2 ml collection tube and centrifuged at 8,000 X g for 30 sec. The column was washed with 700 µl wash buffer (RW1) by centrifugation at 8,000 X g for 30 sec and this step was repeated as above. The flow was discarded along with collection tube and carefully placed the spin column in a fresh collection tube to reduce solvent carry over. 500 µl of wash buffer (RPE) was placed into column and centrifuged at 8,000 X g for 30 sec and washing step was repeated at 8,000 X g for 2 mins.

The spin column was placed into new a collection tube and centrifuged at 17,000 X g for 1 min to remove any residual ethanol from the column membrane. The RNA spin column was placed into a new fresh RNAse free 1.5 ml microcentrifuge tube and 20 µl RNAse free water was added followed by brief incubation at RT by centrifugation at 8,000 X g for 1 min. The centrifugation process was repeated as above with 20 µl RNAse free water.

2.12.2 DNase treatment

Eluted total RNA was treated with Ambion DNA free DNase kit (AM1906, Ambion, UK) to eliminate genomic DNA traces from samples according to the manufacturer's instruction. The total volume of eluted RNA (40 µl) was adjusted to 46 µl using 5 µl of 10X buffer and 1 µl of rDNase was added and incubated at 37^0C for 30 mins. DNase activity was inactivated by adding 6 µl of DNase inactivation reagent at the end of the incubation period and mixed thoroughly by pipetting followed by brief incubation at RT for 2 mins. DNase treated samples were centrifuged at 10,000 X g for 2 mins and approximately 40 µl supernatants RNA was carefully removed and transferred into new RNase free microcentrifuge tubes. 1 µl of RNA was used for quality analysis while remaining was stored at -80^0C.

2.12.3 Quality analysis

The quality and quantity of RNA samples was evaluated by using NanoDrop 2000/2000 c Spectrophotometer. 1 µl DNA free eluted RNA sample was loaded in a NanoDrop well and read the ratio of absorbance at 260/280 nm and 260/230 nm. Purity of RNA was determine at absorbance 260 /280 nm and a ratio near 1.8- 2.0 was accepted as a pure RNA. If the ratio is below to this range it was considered as contaminated with protein and phenol that absorb 280 nm. The 260/230 nm ratio was used as a secondary measure of RNA purity and a range of 2.0-2.2 was accepted as a good quality RNA. If the ratio is below this range it was considered as contaminated with EDTA, carbohydrate and phenol which absorb 230 nm. Before processing the sample for microarray the quality assurance of RNA was performed (Centre Genomic Research, University of Liverpool). An agilent bioanalyser was used to check the RNA integrity number (RIN) and value near to 7 were considered as good quality RNA.

Chapter 3 Characterization of monocyte, granulocyte and lymphocyte surface markers and their association with age dependent immunity

3.1 Introduction

The circulating leukocyte population is heterogeneous in composition and can change depends on infection or inflammatory stimuli. In a naïve animal the different proportions of leukocytes are not fixed from birth but dynamic until an animal reaches maturity.

3.1.1 Monocytes changes with age

Monocytes represent between 0-8% of the total white blood cell (WBC) population in cattle (Weiss & Wardrop, 2010). Bovine monocytes consists of distinct subsets of $CD14^{+}CD16^{-}$ (classical monocytes) and $CD14^{-}CD16^{++}$ (non classical monocytes) (Corripio-Miyar et al., 2015). Previous studies on Japanese black cattle reported an alternation in the monocyte population with age and found an elevated number of cells at 2 months of age and thereafter gradually reduces from 6 month to adult levels (Tanaka et al., 2008). Monocytes constantly under take migration into tissues and convert into dendritc cells depends on pro-inflammatory stimulation. In neonates, 1 week old, the dendritic cell population appears to possess lower antigen presentation capacity (Morein et al., 2002), suggestive of the idea that cell number and function are sometimes independent.

3.1.2 Granulocyte changes with age

In adult cattle, neutrophils comprise 15-33% of the total WBC population (Weiss & Wardrop, 2010) where they act as a primary defence against bacterial and fungal infection via phagocytosis and generation of respiratory burst (Nauseef & Clark, 2000; Paape et al., 2003). Neutrophils become functionally active at 1 week of age in neonates and cell numbers can reach adult levels by 5 weeks of age (Kampen et al., 2006; Hauser et al., 1986). Previous research comparing adult and neonatal cattle neutrophils have shown a differential age-related response to zymosan compared with IgG or phorbol myristate acetate (PMA) and neonatal neutrophils having a significantly greater response to zymosan which is related to increase protein kinase C (PKC) signalling levels (Higuchi and Nagahata, 1998). It has been reported that the capcity to generate respiratory burst activity and phagocytic capacity against *Staphylococcus aureus* is higher in young calves compared to older

cattle (Menge et al., 1998; Hauser et al., 1986). Despite this, little data exists about changes in absolute or proportional numbers of cells with age. In terms of cattle granulocyte proportions, data suggests that granulocyte proportions may be more dynamic than other cell types with a reduction recorded from birth until 2 months of age and then a significant increase in granulocyte proportions in the animal up to 18 months of age (Tanaka et al., 2008).

3.1.3 T cell changes with age

T cells are defined by the composition of the TCR present on the surface and involved in recognition of antigen in MHC complexes. Cells with an alpha (α) and beta (β) chain comprise the major proportion (95%) of T cells (αβ T cells) being further divided into $CD4^+$ and $CD8^+$ cells, while gamma delta chains identify γδ T cells making up the final 5% of the cell population. The proportion of CD4 and CD8 becomes established during the first 10-12 weeks of life and there is evidence which show changes in the total T cells proportions of cattle with age. Ultimately, there is a consistently larger $CD4^+$ population in adult cattle with no alteration in $CD8^+$ population between young and adult subjects (Wilson et al., 1996, Wyatt et al., 1994).

γδ T cells are important mediators of immunity during early life and their contribution is evident in the major portion of the total circulating lymphocytes population (35-75%) in young cattle compared to adults where they make up only 17% of the population (Wilson et al., 1996; Davis et al., 1996). The proportions of γδ T cells follow the same pattern and their counts declined in older sheep compared to lambs (Nussey et al. 2012). The high proportion of γδ T cells at early life is a likely indicator of their importance in early immune events due to their capacity to produce large quantities of Th1 like cytokines (Brown et al., 1994; Baldwin et al., 2000).

3.1.4 B cell changes with age

B cells are central to the adaptive immune response, producing protective antibodies, presenting antigen and also having regulatory functions (Gruver et al., 2007). The number of circulating B cells represent only 4% of total lymphocytes in neonatal calves of 1 week age, compared to adult cattle where they represent approximately 20-30 % of total lymphocytes (Chase et al., 2008). Past studies on

cattle B cell populations have documented a correlation between age and B cell counts and notice a gradual increase with age from 7.4 % to 28.2 % (Tanaka et al., 2008). In support of this lower expression of CD21, a marker of B cell was reported in neonates compared with adults (Kamden et al., 2006). The levels of CD21 increase steadily in young calves and the absolute number of B cells also increases between birth and 11 weeks of age (Kamden et al., 2006). Evidence found that young cattle have lower proportions of mature B cells which increase with age suggesting that B cells undergo maturation slowly via antigen engagement but make up a relatively small population of circulating lymphocytes in young (Wyatt et al., 1994). A number of studies have shown differences in the B cell composition of bone marrow where adult bone marrow has fewer CD79α$^+$ B-cells (4.7 %) compared to fetal bone marrow (26.0%) suggesting that B cell lymphopoiesis declines in adult cattle (Ekman et al., 2012).

3.1.5 Aim of current study

There is a lack of a comprehensive comparative dataset for many leukocyte populations in cattle where age is a factor. To address this, present study has attempted to characterize age related changes in the proportion of circulating monocytes, granulocytes, B cells, CD4$^+$ and WC1$^+$ γδ T cells by flow cytometry in neonates and adult cattle.

3.2 Materials and Methods

3.2.1 Leukocytes expression

Flow cytometry was performed to analyse the cell surface expression of leukocyte obtained from Holstein Friesian male neonates of age 2 week old while adult animal were 2-3 year old male cattle.

3.2.2 Flow cytometry and data analysis of leukocytes

Fluorophore labelled leukocytes were kept at 4^0C and flow cytometry was performed on a BD FACS Canto-II. The cells were gated on the basis of forward scatter (FSC) and side scatter (SSC) parameter to exclude dead cells or debris. Unstained cells and appropriate isotype controls were used to set a threshold and identify the stained cells population from unstained one. For multiple stained i,e FITC, RPE, APC and Alexa Fluor 647 appropriate compensation setting was applied. A minimum of 10,000-30,000 events were recorded per samples.

Acquired flow cytometry data were analysed in weasel software 3.0.2. Results were expressed as median with 95 % confidence interval and mean ± SEM where appropriate. The statistical analysis was performed by Mann Whitney's and 2 way ANOVA using Graphpad prism 7.2. Results were considered statistically significant with $P\ values < 0.05$.

3.3 Results

3.3.1 Characterization of monocyte surface markers

In order to characterise the monocyte pool, flow cytometry analysis of neonates and adult cattle leukocytes was performed. Initial gating of leukocyte was performed on the basis of a dot plot of FSC and SSC. Monocytes were identified from the FSC/SSC dot plot within an unstained leukocytes population. Expression of $CD14^+$ monocytes was determined in the pre-gated monocyte population. Thereafter dual stained $CD14^+CD86^+$, $CD14^+CD80^+$ and $CD14^+$MHC-II^+ cell populations were compared between neonates (n=3) and adults (n=4) gated monocyte population (Fig. 3.1 & 3.2).

The representative flow cytometry data (Fig 3.1 b & 3.2 b) revealed that within the monocyte gate of neonates there was a higher percentage of $CD14^+$ cells compared to adults (30.7% vs 6.71%). Results from dual staining indicated that neonatal $CD14^+$ monocytes had greater expression of CD80 compared to the same population in adults (Fig. 3.1 c & 3.2 c). Interestingly neither neonates nor adult $CD14^+$ monocytes showed detectable expression of $CD86^+$ population (Fig 3.1 d & 3.2 d). From an examination of the $CD14^+$ monocyte population for MHC class II it was found that adult animals had a higher percentage of dual positive CD14 and MHC-II compared to their neonatal counterparts (Fig 3.1 e & 3.2 e). Together, these results indicate that neonatal monocytes displayed higher expression of CD14 and co-expression of CD14 and CD80. However, these data found an individual to individual variation among neonates and no statistical significant difference was observed in terms of expression of $CD14^+$, $CD14^+CD80^+$ and $CD14^+$MHC-II^+ (*P value= 0.0571,* Fig. 3.3).

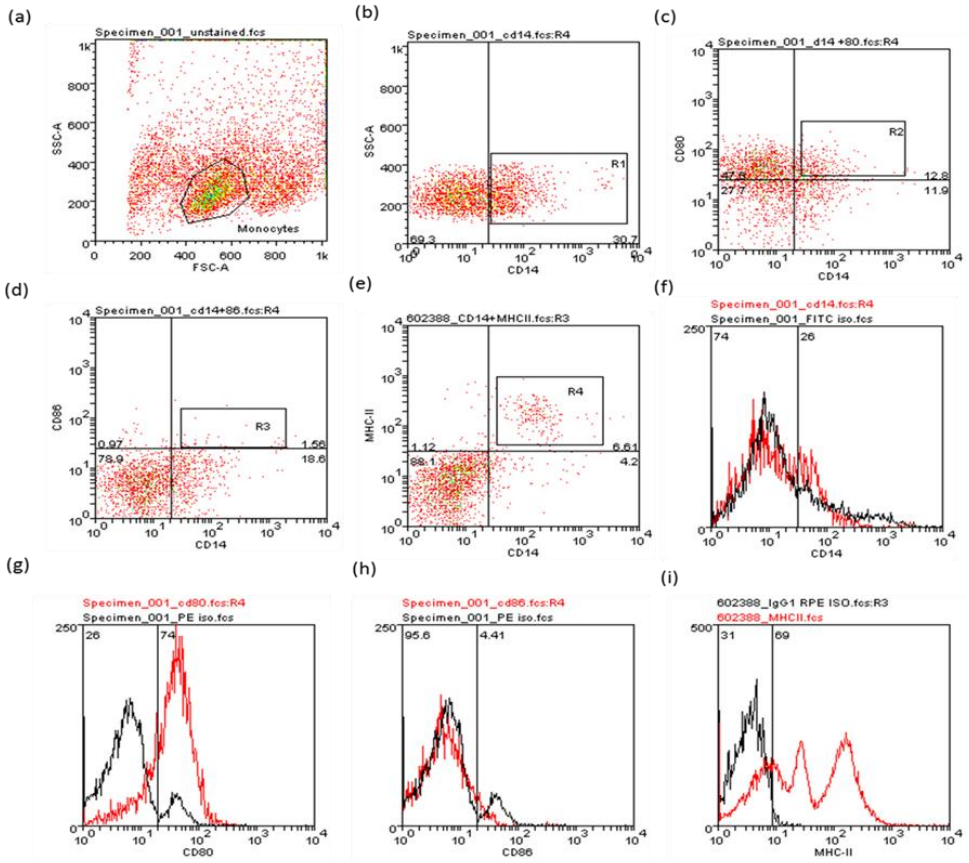


Figure 3.1 Gating strategy to identify the expression of monocyte surface markers in neonates

The dot plots and histogram are representative data from one neonate among three. **(a)** Unstained leukocytes of neonates (n=3) were gated based on forward and side scatter to identify a monocyte population by flow cytometry. **(b)** FITC labelled $CD14^+$ monocytes were identified in a monocyte gated population. **(c, d & e)** Two colour flow cytometry was performed with mouse anti-bovine FITC labelled CD14 and RPE labelled CD80, CD86 and MHC-II antibody to identify the expression of dual stained $CD14^+CD80^+$, $CD14^+CD86^+$ and $CD14^+$MHC-II^+ as a subpopulations of monocytes (right upper corner R2, R3 and R4 respectively). **(f, g, h & i)** CD14, CD80, CD86 and MHC-II positive control were overlaid with their respective negative isotype control to check the background.

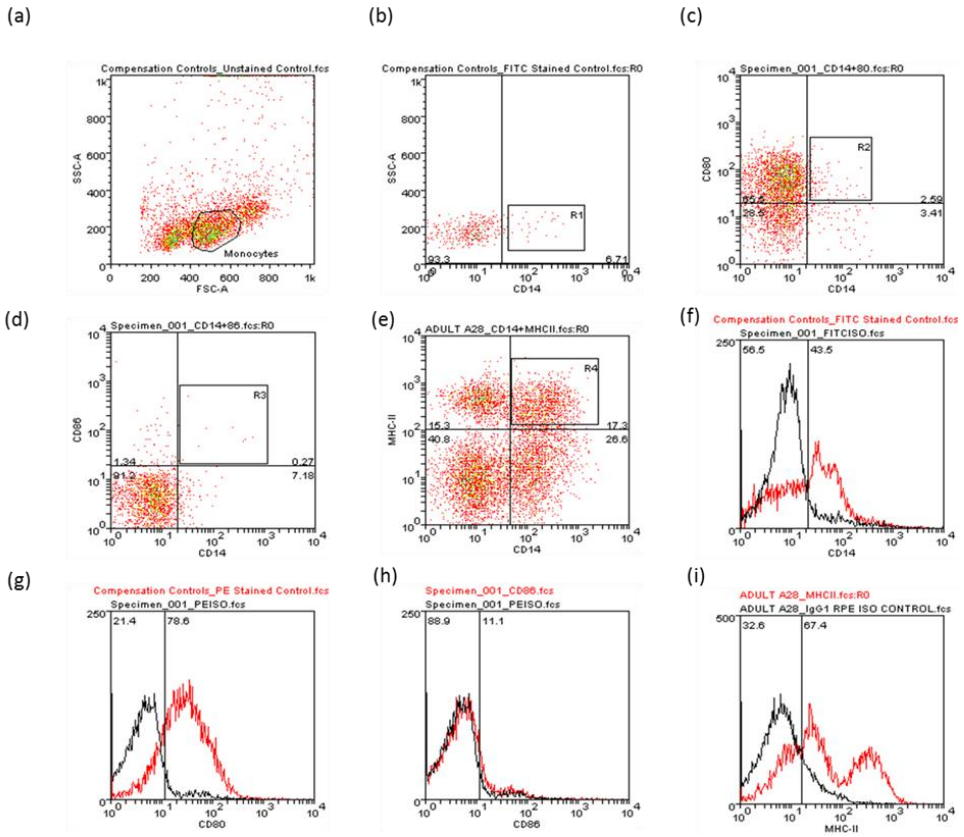


Figure 3.2 Gating strategy to identify the expression of monocyte surface markers in adults

The dot plots and histogram are representative data from one adult cattle among four. **(a)** Unstained leukocytes of adult cattle (n=4) were gated based on forward and side scatter to identify a monocyte population by flow cytometry. **(b)** FITC labelled $CD14^{+}$ monocytes were identified in the monocyte gated population. **(c, d & e)** Two colour flow cytometry was performed with mouse anti-bovine FITC labelled CD14 and RPE labelled CD80, CD86 and MHC-II antibody to identify the expression of dual stained $CD14^{+}CD80^{+}$, $CD14^{+}CD86^{+}$ and $CD14^{+}MHC\text{-}II^{+}$ as a subpopulations of monocytes (right upper corner R2, R3 and R4 respectively). **(f, g, h & i)** CD14, CD80, CD86 and MHC-II positive control were overlaid with their respective negative isotype control to check the background.

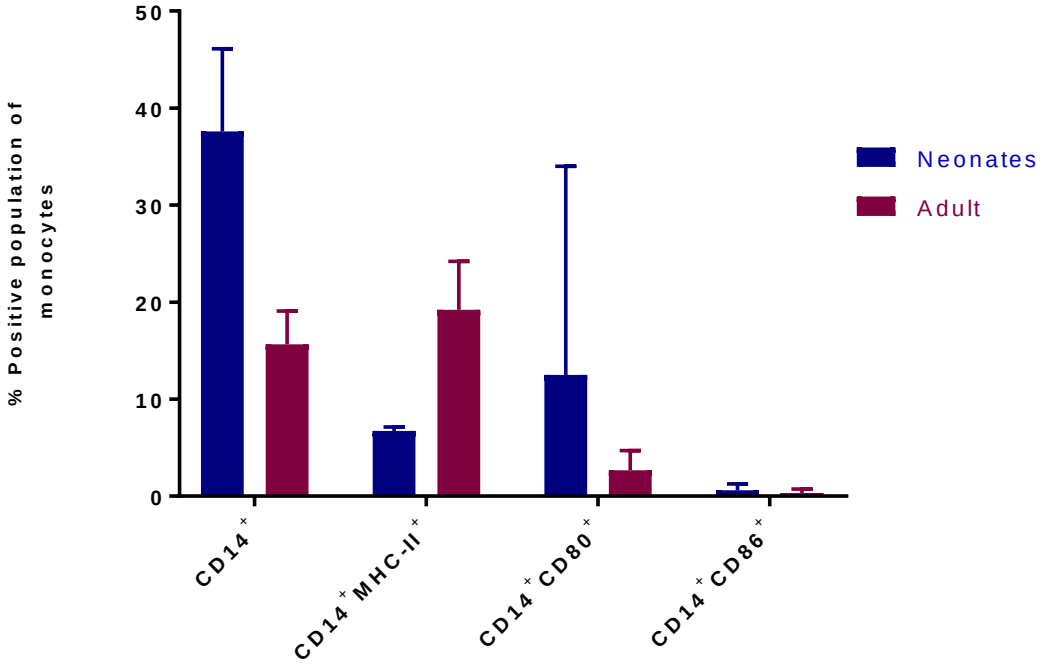


Figure 3.3 Expression of monocyte surface markers of neonates and adults by flow cytometry

Neonatal and adult leukocytes were stained with FITC labelled mouse anti-bovine CD14 and RPE labelled mouse anti-bovine CD80, CD86 and MHC-II. Leukocytes were gated for the $CD14^+$ monocyte population with co-expression of cell surface markers of CD80, CD86 and MHC-II was determined using flow cytometry. The bars show median with 95% confidence interval of neonates (n=3) and adult (n=4). Results were analysed using GraphPad Prism 7.02 and statistical analysis was performed using a Mann-Whitney test with *P value=0.0571* for $CD14^+$, $CD14^+MHC\text{-}II^+$ and $CD14^+CD80^+$.

3.3.1.1 Characterization of neonatal alveolar macrophages surface markers

In order to examine the expression of surface markers on tissue macrophages, alveolar macrophages were also examined for expression of CD80 and CD86 that were stimulated with LPS/IFN-γ or unstimulated as controls. The baseline CD80 expression was higher when compared to CD86 at 48 hrs post stimulation. These data suggest that a combination of LPS/IFN-γ were not able to induce the expression of CD80 or CD86 at 24 hrs post stimulation. The level of CD80/86 was similar at 24 hrs but there is a continued expression of CD80 at 48 hrs (Fig. 3.4).

Expression of CD80 in the alveolar macrophage population was increased in the presence of LPS/IFN-γ (Figure 3.4, *P value=0.0024*) 48 hrs post stimulation indicating monocyte activation. However, expression of CD86 was not affected by stimulation and unstimulated controls showed the same degree of CD86 expression. These results revealed that during the transition from blood monocytes to tissue macrophages there was continuous expression of CD80 in the cell population and this could be significantly upregulated in the presence of LPS/IFN-γ.

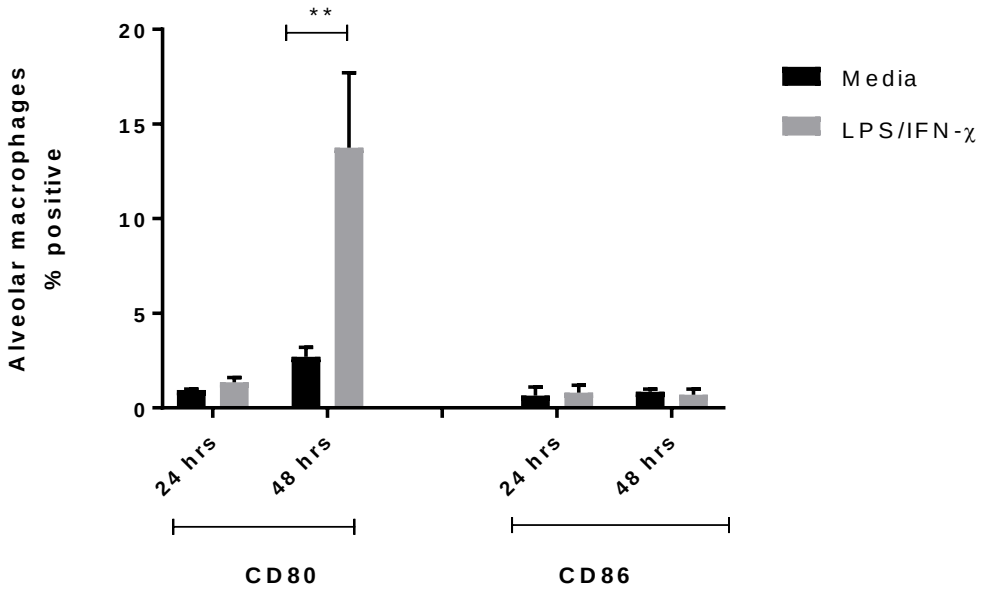


Figure 3.4 Expression of CD80 and CD86 cell surface markers on alveolar macrophages from neonates by flow cytometry

Neonatal alveolar macrophages were stimulated with LPS/IFN-γ and then stained with RPE labelled mouse anti-bovine CD80 and CD86 before analysis. Percent of alveolar macrophage population positive for surface CD80 and CD86 were identified at 24 and 48 hrs post incubation using flow cytometry. The bars show mean ± SEM of neonatal alveolar macrophages (n=3). Results were analysed using GraphPad Prism 7.02 and statistical analysis was performed out by 2 way ANOVA with Sidak's multiple comparisons test to determine the significance difference between stimulation time and stimulants. The results revealed significant difference in terms of expression of CD80 surface markers at 48 hrs incubation time with ***P value=0.0024*.

3.3.2. Characterization of granulocyte surface markers

In order to characterise the granulocyte cell surface markers flow cytometry analysis of cattle leukocytes was performed. FSC and SSC dot plot from unstained leukocyte was used to gate a granulocyte cell population. The granulocyte population was initially gated on MHC-II and representative flow cytometry data show that 94.6 % of granulocytes of neonates were positive for MHC-II surface markers and only 5.24 % were MHC-II negative (Fig. 3.5 b). Whereas in adult cattle the percentage of MHC-II negative subset was higher on average 31.6 % (Fig. 3.6 b).

In order to further characterise these populations, expression of $CD11b^{+}CD11c^{+}$ was analysed across adults and neonates in the context of MHC-II positive or negative cells. MHC-II positive cells were identified among the gated granulocyte population to define a putative DC population. CD11bCD11c positive subsets were determined in the potential DC pool and then within the non-DC MHC-II negative pool. The data indicates that amongst putative DCs ($MHC\text{-}II^{+}$) both neonates and adult cattle had an equal proportions of single positive $CD11b^{+}$ cells (Fig. 3.7). When $CD11c^{+}$ DCs were examined it was found that adults had a greater proportion of these cells compared with neonates (22% vs 5%); while in the dual positive DC population the reverse trend was seen with more dual positive DCs in neonates compared with adults (19.03% vs 7.33%). When the MHC-II negative population was examined it was seen that in all combinations of CD11b or CD11c neonates expressed lower levels compared with adults. While in adults the most defined sub-population was the $CD11b^{+}$ $MHC\text{-}II^{-}$ pool comprising ~20 % of the $MHC\text{-}II^{-}$ cells. With equal proportions of adults being either $CD11c^{+}$ single positive or dual positive.

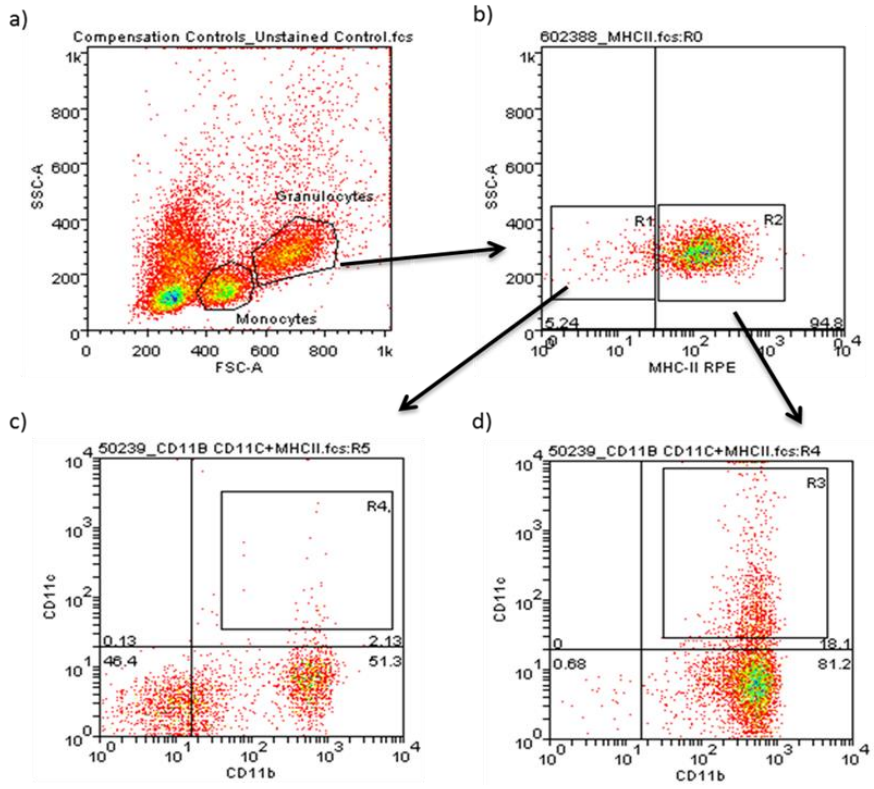


Figure 3.5 Granulocytes gating strategy to identify CD11b^{+}CD11c^{+} population within MHC-II subset in neonates

The dot plots are representative data from one neonate among three. **(a)** Unstained leukocytes population of neonates (n=3) were gated based on forward and side scatter to identify a granulocyte population by flow cytometry. **(b)** Granulocyte population were selected from leukocytes population to identify the RPE labelled mouse anti-bovine MHC-II positive (R1) and negative subset (R2). **(c)** Three colour flow cytometry was performed with FITC labelled mouse anti-bovine CD11b and APC labelled mouse anti-goat CD11c antibody to identify the expression of dual stained CD11b^{+}CD11c^{+} subset (R3) was determine within MHC-II positive subset. **(d)** CD11b^{+}CD11c^{+} subset (R4) was determine in MHC-II negative subpopulation of granulocytes.

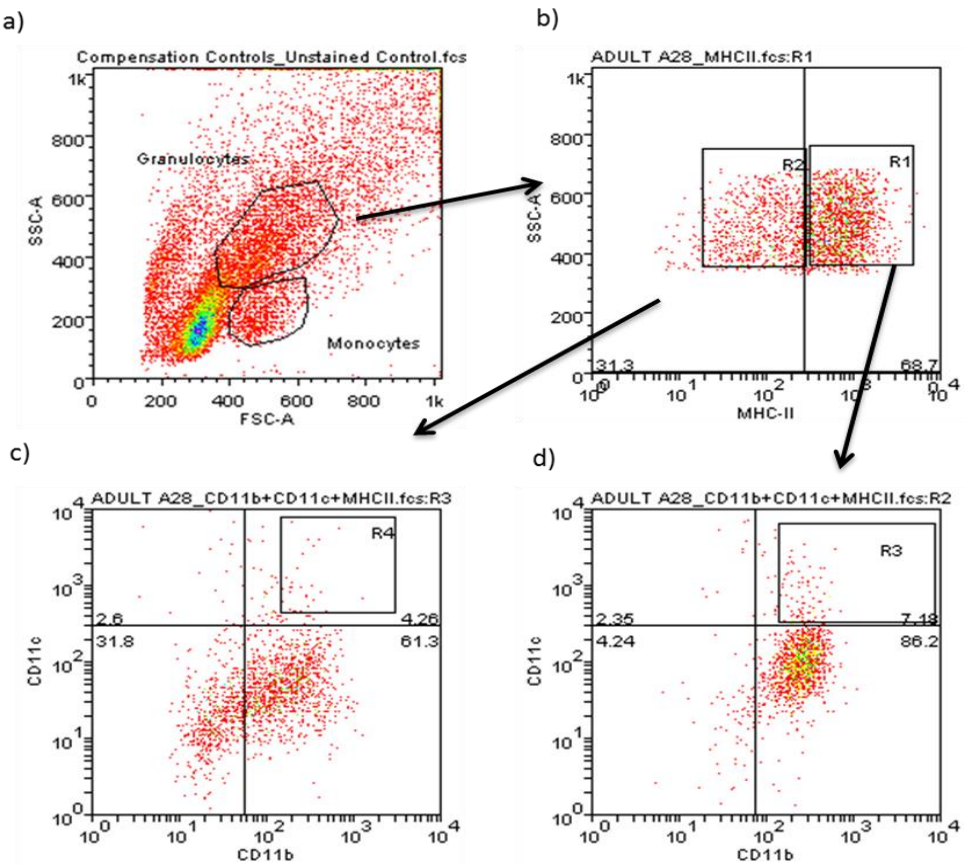


Figure 3.6 Granulocytes gating strategy to identify CD11b$^+$CD11c$^+$ population within MHC-II subset in adults

The dot plots are representative data from one adult cattle among two. **(a)** Unstained leukocytes population of adult cattle (n=2) were gated on the basis of forward and side scatter to identify a granulocyte population by flow cytometry. **(b)** Granulocytes population were selected from leukocytes population to identify the MHC-II positive (R1) and negative subset (R2). **(C)** Three colour flow cytometry was performed with FITC labelled mouse anti-bovine CD11b and APC labelled mouse anti-goat CD11c antibody to identify the expression of dual stained CD11b$^+$CD11c$^+$ subset (R3) was determine within MHC-II positive subset. **(d)** CD11b$^+$CD11c$^+$ subset (R4) was determine in MHC-II negative subpopulation of granulocytes.

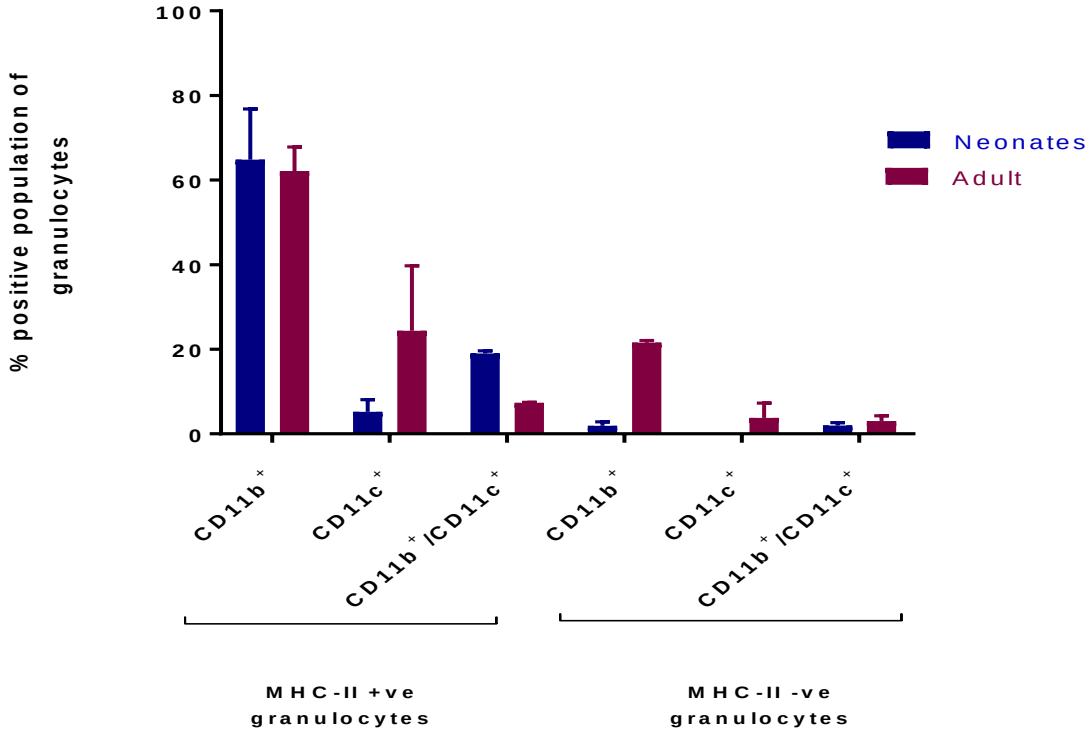


Figure 3.7 Expression of granulocytes cell surface marker on leukocytes population from neonates and adults by flow cytometry

Neonatal and adult leukocytes were stained with FITC labelled mosue anti-bovine CD11b and APC labelled mouse anti-goat CD11c. Leukocytes were gated for the granulocytes population and MHC-II positive and negative subsets were identified. Expression of $CD11b^{+}$, $CD11c^{+}$ and dual positive $CD11b^{+}CD11c^{+}$ subset were determined within MHC-II positive and negative subset using flow cytometry. The bars show median with 95% confidence interval of neonates (n=3) and adult (n=2) animals. Results were analysed using Graphpad prism 7.02 and statistical analysis was performed using a Mann-Whitney's test.

3.3.3 Characterization of γδ T cell surface markers

Leukocytes obtained from neonates and adult cattle were analysed on the basis of FSC and SSC dot plot to identify the lymphocyte population. $WC1^+$ γδ T cells expression was determined on gated lymphocyte population of neonates and adult (Fig. 3.8 b & 3.9 b), furthermore a dual stained $WC1^+WC1.1^+$ subset was identified among $WC1^+$ γδ T cell population (Fig. 3.8 c & 3.9 c).

The representative flow cytometry data indicate a greater proportion of $WC1^+$ γδ T cell within the lymphocyte gate of neonates compared to adults and 42.7 % of lymphocyte were positive for $WC1^+$ in the neonates whereas in adults only 17.3 % of lymphocytes were positive for $WC1^+$ γδ T cells. The result suggests a statistical significant difference in terms of proportions of $WC1^+$ γδ T cells (Fig 3.10, *P value = 0.0286).* The major subpopulation of $WC1^+$ γδ T cells was previously shown to be the $WC1.1^+$subset and data confirms this to be the case in both neonates and adults. However, this proportion was relatively higher in the $WC1^+$ γδ T cell pool of neonates when compared with adults (Fig. 3.10). The proportion of $WC1^+WC1.1^-$ γδ T cells was relatively lower in adult cattle and only neonates demonstrated a detectable pool of these cells.

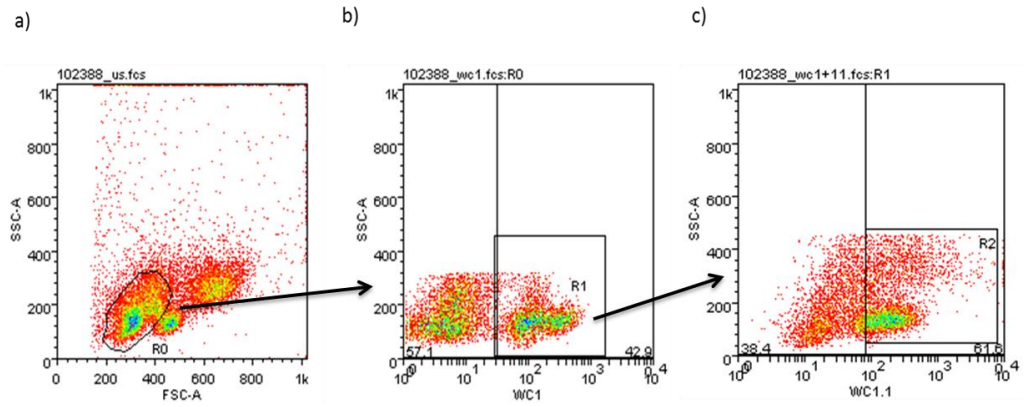


Figure 3.8 Lymphocyte gating strategy to identify WC1$^+$ and WC1.1$^+$ subset in neonates

The dot plots are the representative data of one neonate among four. **(a)** Unstained leukocytes population of neonates (n=4) were gated based on forward and side scatter to identify the lymphocyte population by flow cytometry. **(b)** Gated lymphocyte population were examined for the expression of WC1$^+$ T-cell subset (R1) labelled with mouse anti-bovine FITC antibody. **(c)** Two colour flow cytometry was performed with FITC labelled mouse anti-bovine WC1 and Alexa fluor 647 mouse anti-goat WC1.1 antibody to identify the expression of WC1$^+$WC1.1$^+$ subset. The upper right corner (R2) show WC1.1$^+$ subset within WC1$^+$ positive T-cell.

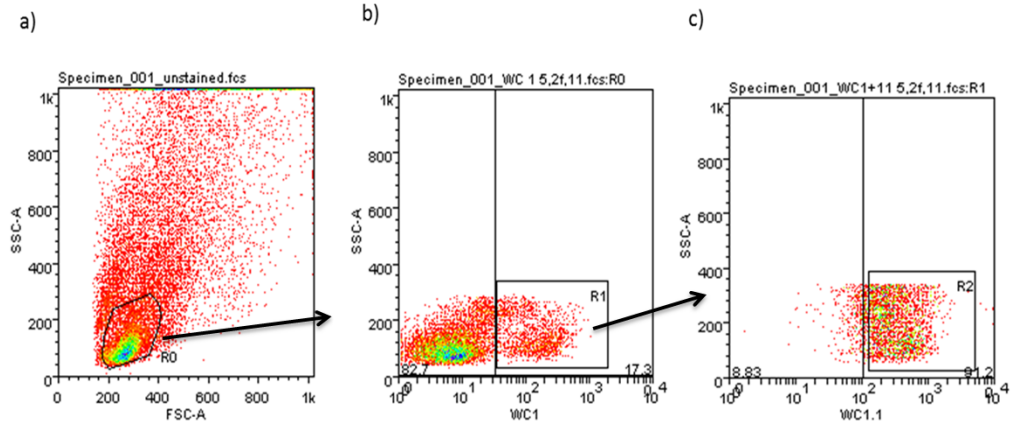


Figure 3.9 Lymphocyte gating strategy to identify WC1$^+$ and WC1.1$^+$ subset in adults

The dot plots are the representative data of one adult cattle among four. **(a)** Unstained leukocytes population of adult cattle (n=4) were gated based on forward and side scatter to identify the lymphocyte population by flow cytometry. **(b)** Gated lymphocyte population were examined for the expression of WC1$^+$ T cell subset (R1) labelled with mouse anti-bovine FITC antibody. **(c)** Two colour flow cytometry was performed with FITC labelled mouse anti-bovine WC1 and Alexa fluor 647 mouse anti-goat WC1.1 antibody to identify the expression of WC1$^+$WC1.1$^+$ subset. The upper right corner (R2) show WC1.1$^+$ subset within WC1$^+$ positive T cell.

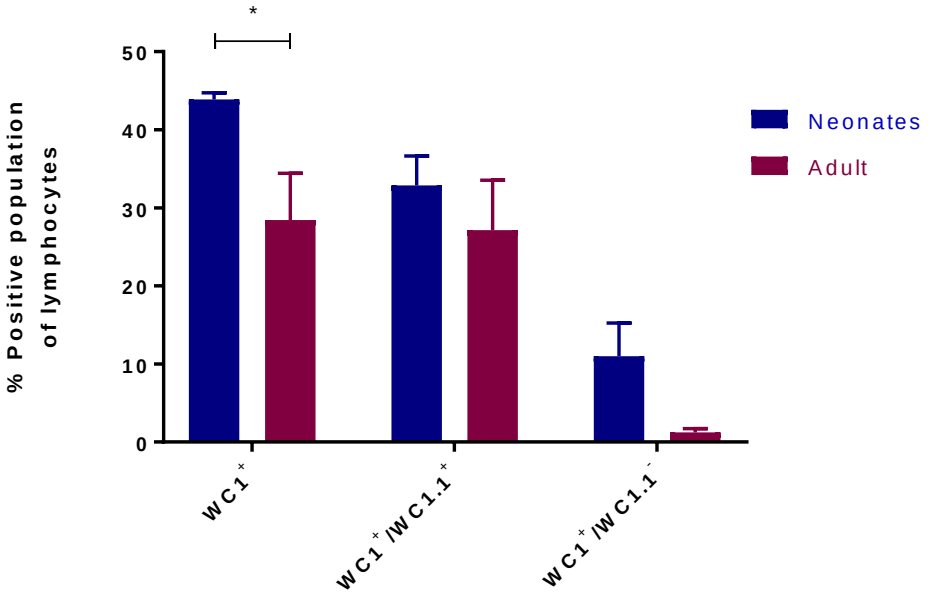


Figure 3.10 Expression of WC1$^+$ and WC1.1$^+$ γδT cell surface markers on lymphocyte from neonates and adults by flow cytometry

Neonatal and adult leukocytes were stained with FITC labelled mosue anti-bovine WC1 and Alexa fluor 647 labelled mouse anti-goat WC1.1 antibody. Leukocytes were gated for the lymphocytes population and expression of WC1$^+$ T cells were determined. Expression of WC1.1$^+$ and WC1$^-$subset were determined in gated WC1$^+$ T cells population using flow cytometry. The bars show median with 95% confidence interval of neonates (n=3) and adult (n=4) cattle. Results were analysed using GraphPad Prism 7.02 and statistical analysis was performed using a Mann Whitney's test. The results revealed a significant difference in terms of expression of WC1$^+$ T cell surface marker among neonates and adult with * *P value= 0.0286*.

3.3.4 Characterization of CD4$^+$ T cells population in cattle

In order to characterise the CD4$^+$ T cell markers, flow cytometry analysis of cattle leukocytes was performed. Leukocytes were gated on the basis of FSC and SSC to identify a lymphocyte population. The proportion of CD4$^+$ T cell was determined in neonates and adult lymphocyte gate (Fig. 3.11 b & 3.12 b). The representative flow cytometry data revealed that adult cattle have fractionally more CD4$^+$ T cells than neonates (16.5 % 13.5 %).

The dual positive CD4$^+$CD25$^+$ population was also higher in adult cattle compared to neonates (Fig. 3.12 c). The proportions of Foxp3$^+$ Tregs was determine

within $CD4^+CD25^+$ T cells subset and observed a low level of circulating Tregs in both neonates and adults (Fig. 3.13, 0.22% vs 0.12%). $Foxp3^+$ Treg was examined in $CD4^+$ T cell subset and found approximately 2.57 % of neonates CD4 T cells were $Foxp3^+$ compared with adults where this proportion was only 0.61 % (Fig. 3.13). However, these data were not showing any statistical significant difference in terms of expression in neonates and adult.

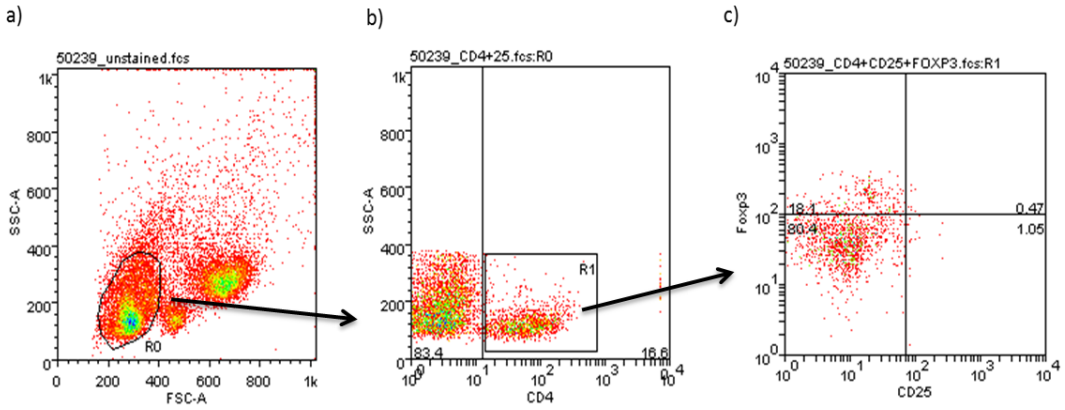


Figure 3.11 Lymphocyte gating strategy to identify $Foxp3^+$ Treg population in neonates

The dot plots are representative data of one neonate among four. **(a)** Unstained leukocytes of neonates (n=4) were gated based on forward and side scatter to identify the lymphocyte population by flow cytometry. **(b)** Gated lymphocyte population were examined for the expression of RPE labelled mouse anti-bovine CD4 positive T-cell subset (R1). **(c)** Three colour flow cytometry was performed with RPE and FITC labelled mouse anti-bovine CD4 and CD25 respectively as well as human anti-bovine Alexa fluor 647 Foxp3 antibody to identify the expression of Foxp3 Treg cell population within $CD4^+$ and $CD4^+CD25^+$ T cells subset.

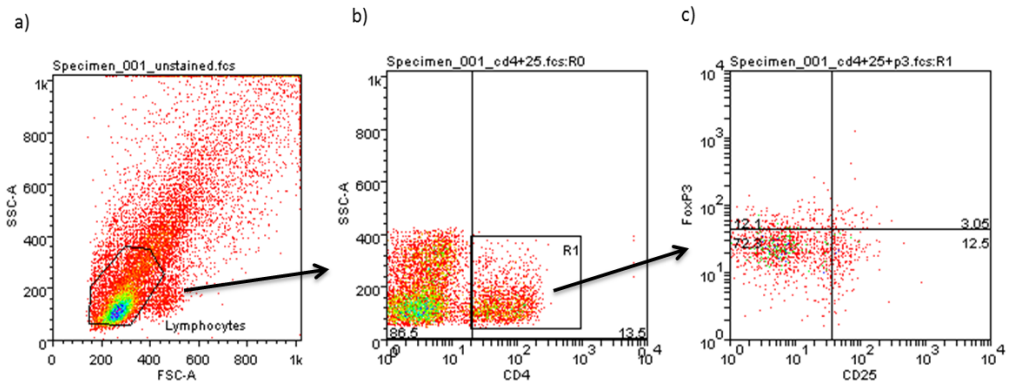


Figure 3.12 Lymphocyte gating strategy to identify Foxp3$^+$ Treg population in adults

The dot plots are representative data of one adult among three. **(a)** Unstained leukocytes of adult cattle (n=3) were gated based on forward and side scatter to identify the lymphocyte population by flow cytometry. **(b)** Gated lymphocyte population were examined for the expression of RPE labelled mouse anti-bovine CD4 positive T cell subset (R1). **(c)** Three colour flow cytometry was performed with RPE and FITC labelled mouse anti-bovine CD4 and CD25 respectively as well as human anti-bovine Alexa fluor 647 Foxp3 antibody to identify the expression of Foxp3 Treg cell population within CD4$^+$ and CD4$^+$CD25$^+$ T cells subset.

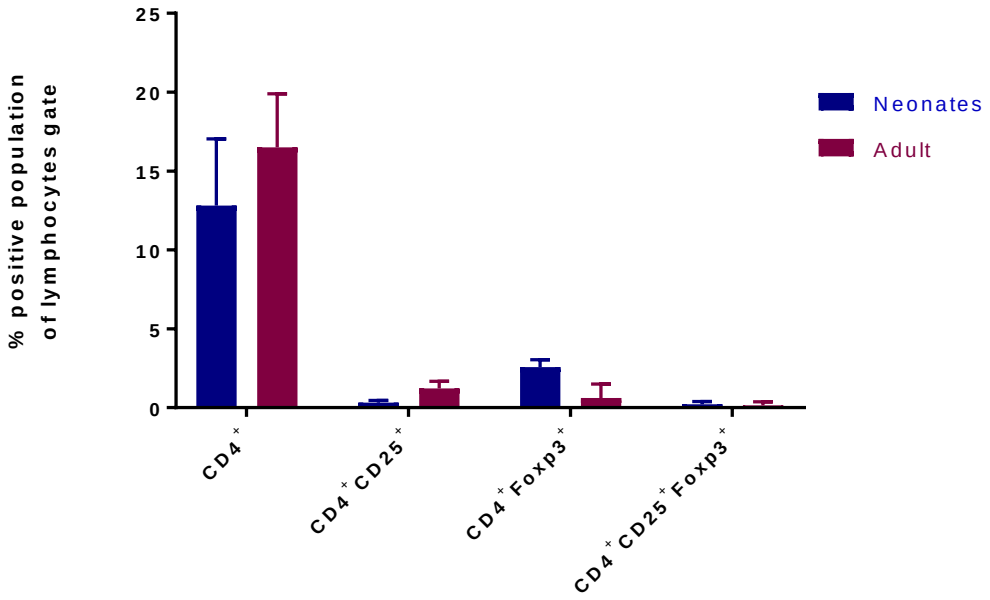


Figure 3.13 Expression of Foxp3⁺ Treg surface markers on lymphocyte from neonatal and adults by flow cytometry

Neonatal and adult leukocytes were stained with RPE labelled mouse anti-bovine CD4, FITC labelled mouse anti-bovine CD25 and Alexa fluor 647 labelled Foxp3 antibodies. Leukocytes were gated for the lymphocytes population. $CD4^+$ T cells subset were identified from lymphocytes population and $Foxp3^+$ Treg cell proportions were identified within $CD4^+$ and $CD4^+CD25^+$ cell subset using flow cytometry. The bars show median with 95% confidence interval of neonates (n=3) and adult (n=3) cattle. Results were analysed using Graphpad Prism 7.02 and statistical analysis was performed using a Mann-Whitney's test.

3.3.5 Characterization of $WC1^+Foxp3^+$ population within non-CD4 cells

Evaluation of γδ T cell population was performed for evidence of Foxp3 expression. Dual positive WC1Foxp3 cells were identified within the CD4 negative lymphocyte gate (Fig. 3.14 c & 3.15 c). The data indicates neonates and adult cattle have a very low proportions of WC^+Foxp3^+ subset within CD4 negative T cells. However, it was found that neonates had comparatively higher levels of $WC1^+Foxp3^+$ T cells than adults (Fig 3.16, 1.79% vs 0.34%).

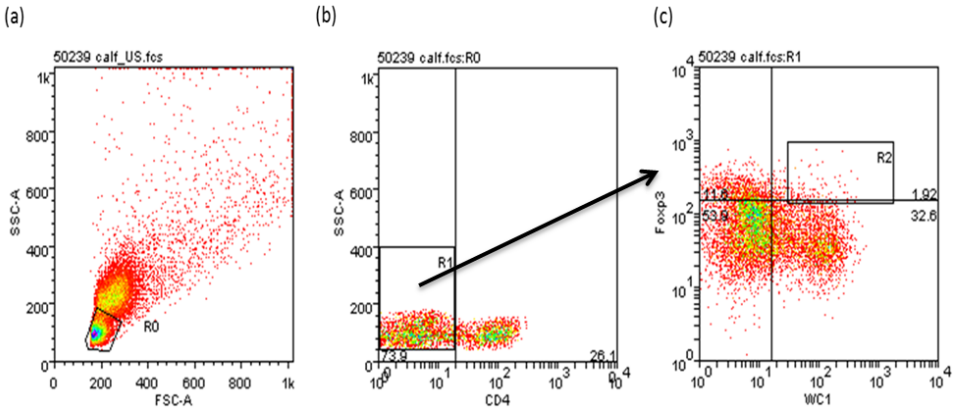


Figure 3.14 Lymphocyte gating strategy to identify WC1^{+}Foxp3^{+} Treg population in neonates

The dot plots are representative result of one neonate among two. **(a)** Unstained leukocyte population of neonates (n=2) were gated based on forward and side scatter to identify a lymphocyte subpopulation by flow cytometry. **(b)** Gated lymphocyte population were examined for the expression of RPE labelled mouse anti-bovine CD4 negative T cell subset (R1). **(c)** Three colour flow cytometry was performed with RPE and FITC labelled mouse anti-bovine CD4 and WC1 respectively as well as human anti-bovine Alexa fluor 647 labelled Foxp3 antibody to identify the expression of WC1^{+}Foxp3^{+} stained subset within CD negative lymphocyte (R2).

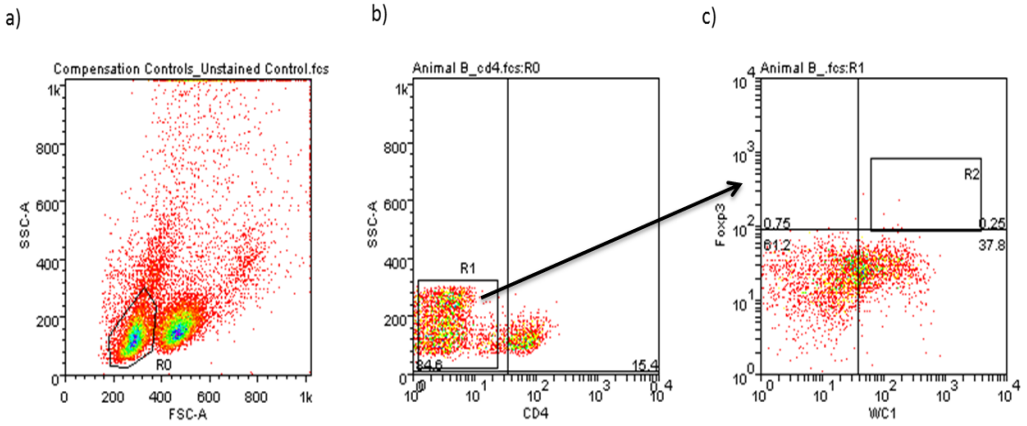


Figure 3.15 Lymphocyte gating strategy to identify WC1^{+}Foxp3^{+} Treg population in adults

The dot plots are representative result of one adult among two. **(a)** Unstained leukocyte population of adult cattle (n=2) were gated based on forward and side scatter to identify a lymphocyte subpopulation by flow cytometry. **(b)** Gated lymphocyte population were examined for the expression of RPE labelled mouse anti-bovine CD4 negative T-cell subset (R1). **(c)** Three colour flow cytometry was performed with RPE and FITC labelled mouse anti-bovine CD4 and WC1 respectively as well as human anti-bovine Alexa fluor 647 labelled Foxp3 antibody to identify the expression of WC1^{+}Foxp3^{+} stained subset within CD negative lymphocyte (R2).

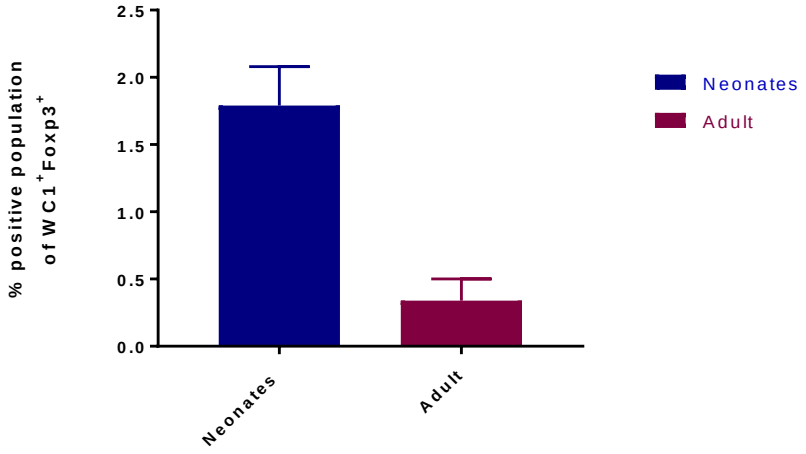


Figure 3.16 Expression of WC1$^+$Foxp3$^+$ Treg surface marker on CD4 negative T cells from neonates and adults by flow cytometry

Neonatal and adult leukocytes were stained with RPE labelled mosue anti-bovine CD4 and Alexa fluor 647 labelled human anti-bovine Foxp3 antibody. Leukocytes were gated for the lymphocytes population. CD4 negative cell proportions were gated within lymphocyte population and expression of dual stained WC1$^+$Foxp3$^+$ subset was identified within CD4 negative lymphocyte population using flow cytometry. The bars show median with 95% confidence interval of neonates (n=2) and adult (n=2) cattle. Results were analysed using GraphPad Prism 7.02.

3.3.6 Characterization of B cell surface markers

In order to characterise the B cells population flow cytometry analysis of cattle leukocytes was performed. B cells were identified within the lymphocyte gate as CD21$^+$ cells and representative flow cytometry results revealed a difference in CD21$^+$ cell proportions between neonates and adult leukocytes (25.4 % vs 6.06 %, Fig. 3.17 b & 3.18 b). Adults had a higher proportion of CD21$^+$ B cells within the lymphocyte gate compared to neonates as indicated in Fig. 3.19. However, no statistically significant difference was detected in terms of CD21 proportions between age groups (*P value= 0.0571*). Memory B cells, expressing CD45RO were identified within the CD21$^+$ population and observed, CD45RO positive memory B cells made up a greater proportion of the CD21$^+$ population in adult cattle than neonates (18.3 % vs 4.32 %) but no statistical significant difference was noticed between both age group (Fig. 3.19, *P value=0.0571*).

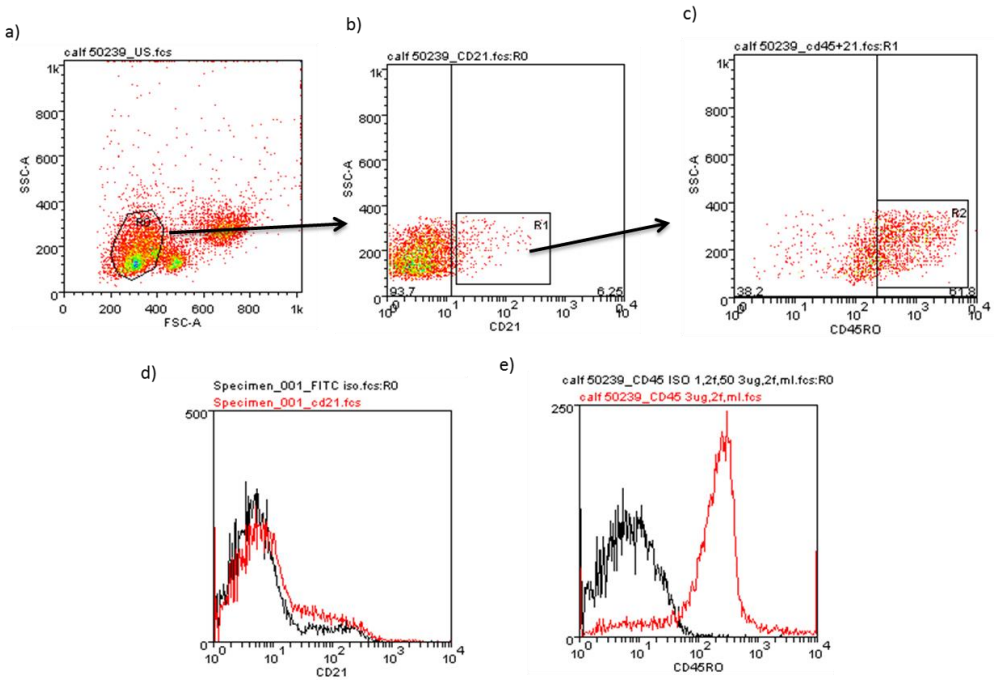


Figure 3.17 Lymphocyte gating strategy identify B cell population in neonates

The dot plots and histograms are representative data from one neonate among three. **(a)** Unstained leukocytes of neonates (n=3) were gated based on forward and side scatter to identify a lymphocyte population by flow cytometry. **(b)** Expression of FITC labelled $CD21^+$ cell subset within gated lymphocyte population was identified (R1 region). **(c)** Two colour flow cytometry was performed with FITC labelled mouse anti-bovine CD21 and secondary conjugated Alexa fluor 647 mouse anti-goat CD45RO antibody to identify the expression of $CD45RO^+CD21^+$ subset within $CD21^+$ gated population showing in the R2 region. **(e & f)** Histogram showing expression of $CD21^+$ and $CD45RO^+$ cell population within lymphocyte gating and overlaid with CD21 and CD45RO isotype control to check the background.

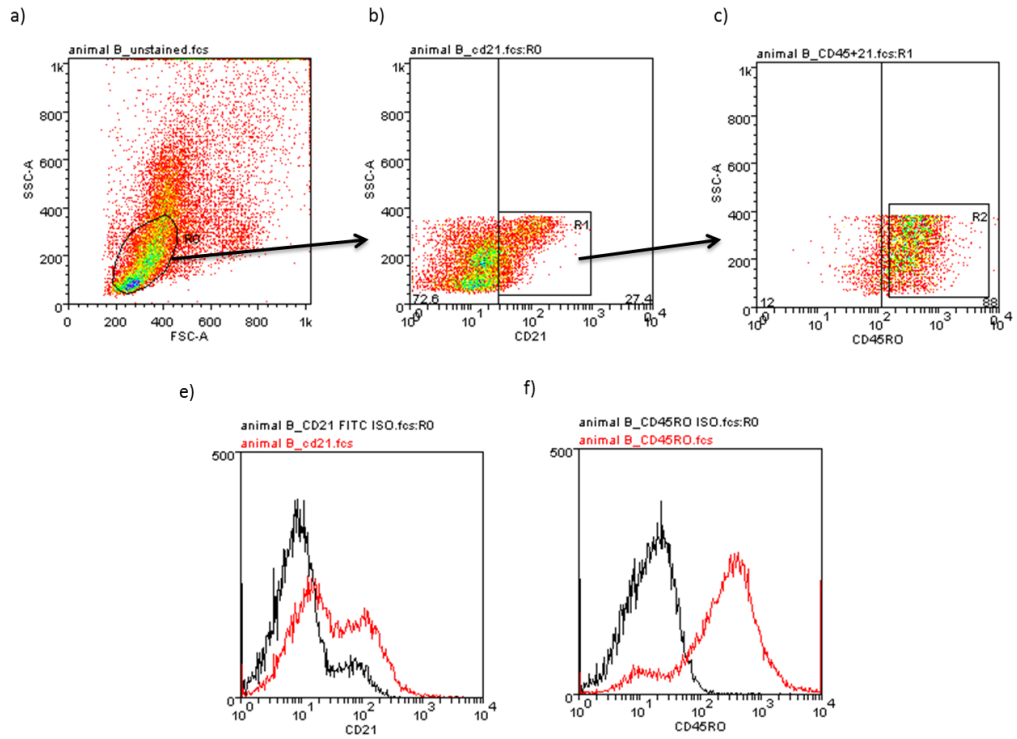


Figure 3.18 Lymphocyte gating strategy to identify B cell populations in adults

The dot plots and histograms are representative result of one adult cattle among three. **(a)** Unstained leukocytes of adult cattle (n=3) were gated to identify the lymphocyte population by flow cytometry. **(b)** Expression of FITC labelled $CD21^+$ cell subset within gated lymphocyte population was identified (R1 region). **(c)** Two colour flow cytometry was performed with FITC labelled mouse anti-bovine CD21 and secondary conjugated Alexa flour 647 mouse anti-goat CD45RO antibody to identify the expression of $CD45RO^+CD21^+$ subset within $CD21^+$ gated population showing in the R2 region. **(e & f)** Histogram showing expression of $CD21^+$ and $CD45RO^+$ cell population within lymphocyte gating and overlaid with CD21 and CD45RO isotype control to check the background.

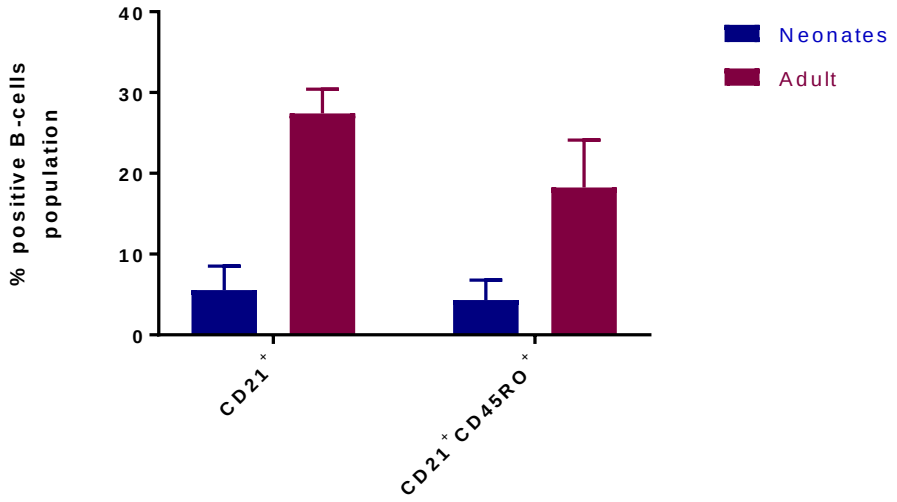


Figure 3.19 Expression of $CD21^+$ and $CD21^+CD45RO^+$ cell surface marker on lymphocyte from neonatal and adult cattle by flow cytometry

Neonatal and adult leukocytes were stained with FITC labelled mouse anti-bovine CD21 and Alexa fluor 647 labelled mouse anti-goat CD45RO antibody. Leukocytes were gated for the lymphocyte population. Expression of CD21 positive subset was identified from lymphocyte population using flow cytometry. Dual stained $CD21^+CD45RO^+$ cells subset was determined within gated $CD21^+$ B cells. The bars show median with 95% confidence interval of neonates (n=4) and adult (n=3) cattle. Results were analysed using Graphpad Prism 7.02 and statistical analysis was performed using a Mann-Whitney's test.

3.4 Discussion

3.4.1 Monocyte changes with age

This study aimed to provide an overview of the age-related difference in cattle leukocyte subsets. A major finding reported here is that there is higher proportion of $CD14^+$ monocytes in neonates compared to adults with greater co-expression of CD80 (Fig. 3.3). In neonates, blood monocytes were also compared with alveolar macrophages and results revealed that neonatal alveolar macrophages as well as blood monocytes showed high expression of CD80 but very little expression of CD86 in either cell populatons. CD80 and CD86 are co-stimulatory molecules for T cell activation (Romo-Tena et al., 2013). Previous findings indicate that CD80 engagement skews T cell cytokine production towards IFN-γ thereby negatively regulating CD86/IL-10 in an autocrine fashion (Balkhi et al., 2004). The monocyte functional analysis (chapter 4) supports these findings where IL-10 secretion were not detectable. It is also hypothesised that high levels of CD80 expression on $CD14^+$ monocyte subsets may leads to increased activation of T cells or NK cells (Tatari-Calderone et al., 2002), again supported by the findings in Chapter 5.

To date no comprehensive studies comparing of monocyte subsets in cattle with respect to age have been performed. However, some studies reported a higher proportion of monocytes in calves 2 months of age compared with adult cattle (Tanaka et al., 2008) that supports the findings presented here. Some evidence suggests that there are different phenotypes of circulating blood monocytes where a $CD14^+/CD16^-$ (classical) subset contributes more than 80% of total bovine blood monocytes and the remaining cells can be catagorised into $CD14^-/CD16^-$ (non-classical) (Hussen et al., 2013). CD16 is involved in mediating antibody dependent cellular cytotoxicity (ADCC) by human monocytes (Mandelboim et al., 1999; Yeap et al., 2016) and shifting of the monocyte pool from classical to non-classical with age may suggest an increase in adaptive immune functions with age, an idea supported by an existing study of cattle monocytes (Seidler et al., 2010).

This study also investigated MHC class-II expression on $CD14^{+}$ monocytes subset and interestingly, proportions of $CD14^{+}$MHC-II^{+} cells were higher in adult cattle compared to neonates; indicating increased antigen presentation capacity to T cells with age. Previous studies on human and cattle monocytes subsets, has documented an intermediate monocytes subset ($CD14^{++}/CD16^{+}$) showing high levels of MHC-II (Wong et al., 2011; Hussen et al., 2013). However, other studies suggest that $CD14^{+}CD16^{-}$, $CD14^{+}CD16^{+}$ and $CD14^{-}CD16^{++}$populations displays similar levels of MHC-II expression in cattle (Corripio-Miyar et al 2015) making this an unresolved issue at present. Though, this study did not compare different monocyte subsets with respect to age in cattle, this comparison and the resolution of MHC-II expression should be an issue for cattle research.

3.4.2 Granulocyte changes with age

This study utilised MHC-II expression in the granulocyte gate to distinguish neutrophils (negative) from dendritic cells (positive). It was found that proportions of MHC-II bearing cells change with age and thus assumed that adult cattle had a greater proportion of dendritic cells compared to neonates. In both age groups, CD11b expression was conserved in dendritic cells. These data also revealed that adult cattle contain greater proportions of $CD11c^{+}$ cells within neutrophils and dendritic cells population in comparison to neonates (24.3% & 3.7%, Fig. 3.7). However, neonates displayed high levels of co-expression of $CD11b^{+}CD11c^{+}$ within dendritic cells indicating a high potential for neonates to undertake chemotaxis, adhesion and transmigration across endothelium; leading to clearance of pathogens through phagocytosis and cell-mediated killing (Dana et al., 1991). The neutrophils proportion which was dual positive for CD11b/CD11c subset was consistently lower in both age groups of cattle.

Previous studies of bovine granulocytes also indicates a reduction in the pool at the very early stages of life immediately after birth and that it gradually increased thereafter (Tanaka et al., 2008; Hicks et al., 1983). Furthermore, previous studies of the neutrophil pool shows similar proportions in both young and adult cattle (Kamden et al., 2006). However, some conflicting data indicates a functional difference in bovine neutrophils with respect to age. Reduced phagocytic activity has

been noted against *Escherichia coli* in neonatal neutrophils. Conversely, increased respiratory burst and the capacity to ingest *Staphylococcus aureus* was recorded in young calves (Menge et al., 1998; Hauser et al., 1986). This work and prior studies would suggest that a functional examination of granulocytes, in particular neutrophils is warranted as the age-related difference in this pool is unclear due to multiple conflicting studies.

3.4.3 γδ T cells change with age

This present study addressed the relative abundance of γδ $WC1^+$ T cells between neonates and adults and we found a significant difference, where approximately 45 % of neonatal lymphocytes being γδ $WC1^+$ T cells compared to adults. However, expression of dual positive $WC1^+WC1.1^+$ γδ T cells did not alter in neonates and same pattern was seen in adults (Fig. 3.10). This supports many previous findings where γδ T cells can compose up to 70 % of the circulating T cells pool in young ruminants unlike mice and human where γδ T cells comprises only 1-10 % of total T cells and are further reduced with age (Clevers et al., 1990; Hein et al., 1990; Hein and Mackay, 1991). The higher percentage of γδ T cells in neonates indicates an important role in early protective immunity that gradually decrease as the adaptive immune response becomes increasingly active (Brown et al., 1996; Price et al., 2007).

A further of the $CD4^-$ T cells subpopulation was evaluated to see whether γδ $WC1^+$ T cells express $Foxp3^+$. Less than 1 % percent of γδ $WC1^+$ cells were positive for Foxp3 expression in adults, also neonates displayed a small proportion (1.9%) of γδ $WC1^+$ T cells positive for Foxp3. Recent work has strongly implicated a γδ T cells as a major regulatory and suppressive T cell populations in ruminants instead of the classic $CD4^+CD25^+Foxp3^+$ (Hoek et al., 2009; Guzman et al., 2014). The presence of γδ T cells in large abundance in young ruminants makes this finding interesting in terms of resistance and immunity for certain infectious disease.

3.4.4 T cells change with age

Examination of the lymphocyte pool revealed that adult cattle have higher proportions of $CD4^+$ T cells compared to neonates; suggesting subpopulations of lymphocytes alter with age this being in agreement with earlier findings where higher CD4 proportions was seen in adult cattle (Ayoub and Yang, 1996; Wilson et al., 1996; Wyatt et al., 1993).

The percentage of Tregs, characterised by expression of CD4, CD25 and Foxp3, was examined. A small proportion of $CD4^+CD25^+$ was detected in adult and neonatal cattle but expression of $Foxp3^+$ cell within this population was quite low in both age groups. These findings suggest there is a small pool of natural Treg proportion in healthy animals. Interestingly, the pool of Treg expressing $CD4^+Foxp3^+$, but not CD25, was relatively higher in neonates. It could therefore be assumed that healthy animals do not possess high levels of $Foxp3^+$ Treg cells. Previous study suggests *Psoroptes ovis* infected sheep did not shown much increase (12.5 %) in proportions of T cells expressing Foxp3 during course of infection (McNeilly et al., 2010). Some human study also noted smaller proportions of the natural Treg ($CD4^+CD25^+FOXP3^+$) population in young individual (Hou et al., 2017).

3.4.5 B cells change with age

An evaluation of B cells dynamics with age was perfomed. CD21, complement receptor 2, was used as a marker of mature B cells. B cell populations were seen to change with age and adult cattle lymphocytes express higher proportions of $CD21^+$ cells compared to neonate. All leukocytes express the common leukocyte antigen CD45 but specific isoforms can denote different stages of maturity or activation with CD45RO distinguishing memory cells. On this basis we found that mature B cells expressing CD45RO was also higher in adults than neonates, indicating a memory B cells population increasing along a neonatal to adult gradient. This is in agreement with previous cattle studies suggested that neonates have low numbers of $CD21^+$ B cells (Van Kampen & Mallard, 1997; Mengen et al., 1999) also the number of dual positive CD21 and CD32 (a second B-cell marker) increased from birth to 90 days.

Moreover the absolute number of $CD21^+$ cells was also higher in mature cattle (Chattha et al., 2009; Kamden et al., 2006).

Taken together these data suggest that monocyte, lymphocyte and granulocyte populations expressed a dynamic shift with age and neonates have a distinct subset of $CD14^+$ monocytes and γδ T cells that may indicate neonates mainly reliant on innate immune component however, an increase B cells subset with age suggest a well develop adaptive immune response in adult.

Chapter 4 Comparative evaluation of monocyte cytokines response in young and adult cattle and estimation of alveolar macrophages cytokines response in neonates

4. Introduction

4.1 Monocyte and its subset

Monocytes are an essential part of the innate immune response and also serve to replenish the pool of tissue macrophages and DCs. They activate or regulate the adaptive immune response via initiation and resolution of inflammatory process. Monocytes and subsequently macrophages are derived from the common myeloid precursor in the bone marrow and differentiate into macrophages in response to differentiation factors such as granulocyte macrophage colony stimulating factor (GM-CSF) and macrophages colony stimulating factor (M-CSF) (Wiktor-JedrzejczaK and Gordon, 1996).

Previous bovine studies have identified that the circulating monocytes pool is heterogeneous subsets and can be classified based on expression of CD14 and CD16 into classical (cM, $CD14^{+}CD16^{-}$), intermediate (intM, $CD14^{+}CD16^{+}$) and non-classical monocytes (ncM, $CD14^{-}CD16^{+}$) (Hussen et al., 2013). It is estimated that 89 % of circulating monocytes belong to the classical monocyte subset and display prominent phagocytic activity compared with the non-classical and intermediate subsets (Hussen et al., 2013; Ziegler-Heitbrock, 2007; Ziegler-Heitbrock et al., 2010). Monocytes are able to differentiate into M1 inflammatory and M2 anti-inflammatory macrophage subsets during infection and injury (Auffray et al., 2007). M1 macrophages are responsible for secretion of pro-inflammatory cytokines that are are pivotal for the regulation and activation of the immune response (Kurihara et al., 1997; Yasaka et al., 1981). However, M2 macrophages are responsible for anti-helmitnh immunity and tissue repair (Passlick et al., 1989; Ziegler-Heitbrock et al., 2010).

Classical activation of M1 macrophages is mediated by IFN-γ and these cells are characterised by their secretion of pro-inflammatory cytokines, whereas M2 (alternatively activated) exhibit anti-inflammatory functions activated by IL-4 (Flynn and Mulcahy, 2008). This indicates that the M1 macrophages phenotype profile could be more important during infection with intracellular pathogens such as

Mycobacterium tuberculosis or *Listeria monocytogenes* for effective clearance of pathogens (Werling et al., 2004).

It has been noted that inflammatory monocytes have specific receptors like TLRs and scavengers receptors which can able to identify PAMPs and perform phagocytosis of pathogen, dead cells and debris (Yang et al., 2014). Data from human and mice indicates that monocyte subset proportions alter with age and their functional and chemotaxis activity also change with age (Tacke & Randolph, 2006; Seidler et al., 2010). Studies suggest that expression and functional capability of TLR1 receptors on monocytes and TLR3 on macrophages declines in older human individuals and also that there is less production of TLR1/2 associated cytokines in aged individuals (Kong et al., 2008; Van Duin et al., 2007). Several murine studies also described the functional impairment of TLRs with lower production of some cytokines IL-6, IL-1β and TNF-α in older C57BL/6 and BALB/c mice macrophages when stimulated with known TLR ligands such as LPS (Renshaw et al., 2002; Boehmer et al., 2004; Chelvarajan et al., 2005). This would suggest that TLR ligand responsiveness alters with age and may contribute to lower responsiveness to infection.

This evidence supports the broad reports of vaccination strategies are more successful in younger animals, in part because older animals have depleted immune resources leading to lower priming capacity of the adaptive immune response and several vaccination studies have shown diminished protective immune response in older animals including horses and monkeys in comparison to young counterparts (Goronzy and Weyand, 2013, Coe et al., 2012; Muirhead et al., 2009).

4.2 Cytokine associated with monocyte

A number of bovine studies address the monocytes response where LPS stimulated inflammatory monocytes produce elevated levels of IL-1β, TNF-α, and IL-12 compared to unstimulated cells; however anti-inflammatory monocytes, as expected, show greater expression of IL-10 (Corripio-Miyar et al., 2015). Whilst there is little fundamental evidence of monocyte differences in healthy cattle across an age spectrum. However, inverse age-related immunity is an exception that is only documented in *Babesia* spp. infection of cattle, where young calves have been shown

to exhibit strong pro-inflammatory responses with the early induction of IL-12 by calf macrophages thought to contribute to the early elimination of parasites (Goff et al., 2002; Goff et al., 2001; Goff et al., 2010; Brown et al., 2006). Protective immunity was also observed in subsequent challenge infections, with an inability to detect parasites in circulation (Homer et al., 2000). The differences in the immune responses in terms of cytokine production, activation of immune cells and clearance of pathogen are a distinguishing feature of aging.

4.3 Aim of study

This chapter describes the variation in the responsiveness of bovine monocytes, isolated from young and adult cattle to stimulation with well characterised ligands and the implications for age-related immune development to infection as a result of this. The present study also aimed to evaluate the cytokines response from tissue (alveolar) derived macrophages of neonatal calves that could have important implications for the elimination of pathogens.

4.2 Materials and Methods

4.2.1 Monocyte cytokines response

Monocyte cytokines response was evaluated from young and adult cattle where young cattle were 6 month old female Holstein Friesian cattle however, adult cattle were 2-3 year old male cattle.

4.2.2 Alveolar macrophages cytokines response

Alveolar macrophage cytokines response was evaluated from 2 week old neonates Holstein Friesian male cattle.

4.2.3 Statistical analysis of ELISA results

Cytokine expression result were analysed using Microsoft Excel. Linear graph was plotted from serially diluted standards and their respective optical density (OD) to obtain the equation of the line and an R^2 value. The cytokine protein concentration was calculated from the line equation and subsequent graphs generated using GraphPad Prism version 7.02 software. The results represent the median with 95% confidence interval and mean ± SEM where appropriate. The statistical analysis was performed using Mann Whitney's test for non parametric data distribution. In addition to this, 2 way ANOVA with multiple comparison using Tukey's (within stimulant at different post incubation period) and Sidak's test (between age group at different post incubation and stimulants) where $P\ value < 0.05$ was considered as statistically significant.

4.2.4 Caspase-1 analysis

The standard curve was generated as described in section 4.2.2 and caspase- 1 activity was calculated in alum stimulated cell lysates as pmol / min / 10^5 cells used per well.

4.3 Results

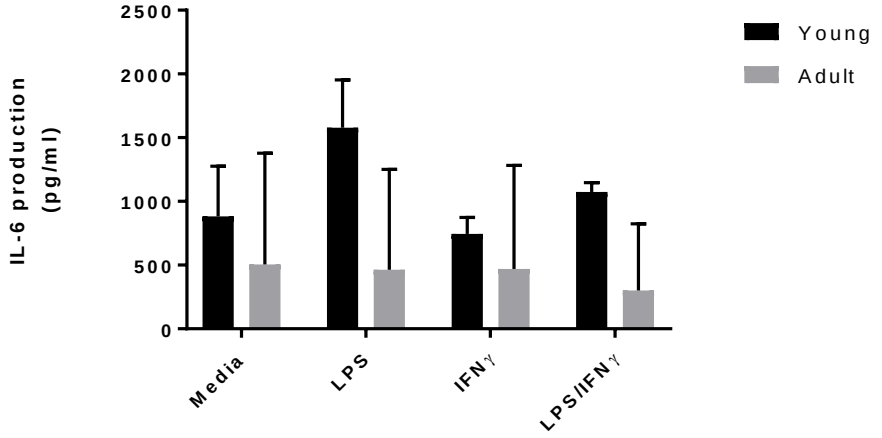
4.3.1. Cytokine production in blood monocytes

4.3.1.1 Secretion of IL-6 in blood monocytes

IL-6 secretion was investigated in $CD14^+$ monocytes from young and adult cattle. Comparing IL-6 induction between young and adult cattle (Fig. 4.1 a & b), the results show that there was a comparatively high production of IL-6 in monocytes from young calves at 24 hrs post incubation. In adult cattle levels of IL-6 did not change much in respect to different stimulus and a range between 300-469 pg/ml was observed. However, young cattle were displayed a higher level of IL-6 and LPS was found to be the most potent stimulant with production of 1578 pg/ml (Fig 4.1 a). These data also suggested that unstimulated monocytes shown a high degree of IL-6 induction among young and adult cattle.

Moreover, young cattle expressed a great animal to animal variation in response to LPS and IFN-γ. Data recorded at 48 hrs (Fig 4.1 b) showed a reduction in IL-6 within monocytes taken from young cattle in response to LPS and IFN-γ but an increase (1700 pg/ml) in response to stimulation with a combination of both. These data indicate that adult cattle sustain IL-6 production at 24 and 48 hrs post incubation. However, a higher baseline was also observed in unstimulated monocytes from young and adult cattle. These results are not statistically significantly different between young and adult cattle for either different stimulants or incubation times.

(a)



(b)

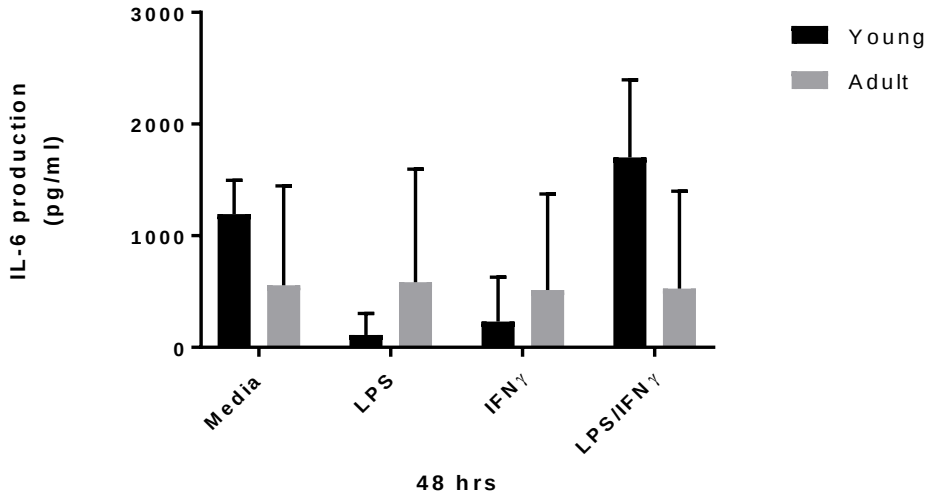


Figure 4.1 IL-6 production in CD14$^+$ monocytes from young and adult cattle in response to LPS, IFN-γ and LPS/IFN-γ

Expression of IL-6 in blood monocytes was examined in young and adult bovine in response of LPS (1 µg/ml), IFN-γ (20 ng/ml) and LPS/IFN-γ together at **(a)** 24 and **(b)** 48 hrs post incubation. The supernatants were collected at end of incubation time and analysed in duplicate by ELISA for IL-6 secretion. These data showing mean ± SEM of young (n=3) and adult cattle (n=3). The statistical analysis was conducted by 2 way ANOVA using GraphPad Prism 7.02 and multiple comparison was performed using Sidak's multiple test.

4.3.1.2. Inflammasome activation in blood monocytes

To investigate activation of the inflammasome, alum stimulated $CD14^+$ monocytes of young and adult cattle were examined at 4 hrs, 6 hrs and 8 hrs post incubation. These results (Fig 4.2), revealed that caspase-1 activity was relatively higher in alum treated young bovine $CD14^+$ monocytes compared to adult however, no difference was observed between the different time points and caspase-1 activity for adult and young cattle. The unstimulated monocytes showed same level of caspase-1 activity in terms of incubation time within young and adult cattle. This result suggests that there was no correlation between caspase-1 activity and incubation time; however these data are not statistically significant due to variation among individual animals.

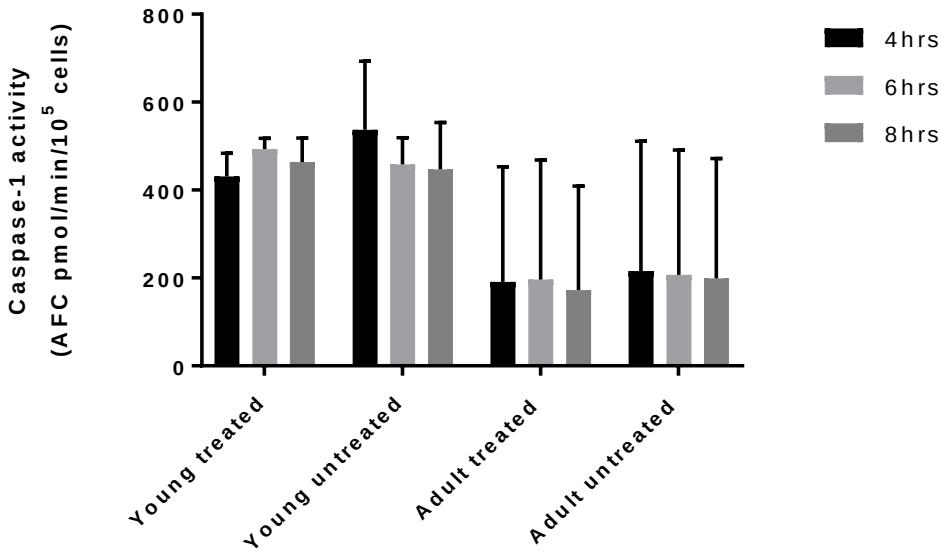


Figure 4.2 Caspase-1 activity in $CD14^+$ monocytes from young and adult cattle in response to alum

Caspase-1 activity was examined in young and adult $CD14^+$ monocytes stimulated with alum (500 µg/ml) at different time intervals as indicated above. At the end of each incubation cells were lysed and cell lysates were collected. Caspase-1 activity was measured in cell lysates of young and adult cattle in duplicate after 120 mins incubation period in the dark at 37^0C. These results showing mean ± SEM of young (n=3) and adult (n=5) bovine and statistical analysis was conducted, by 2 way ANOVA using GraphPad Prism 7.02 and multiple comparison was perfomed using Sidak's multiple test.

The levels of IL-1β were measured on a delayed time frame to allow caspase-1 to cleave pro-IL-1β. The monocyte response to alum induced production of IL-1β was relatively at higher concentrations in young cattle monocytes compared to adult monocytes. However no difference was detected in alum treated and untreated monocytes in terms of IL-1β production among young and adult cattle monocytes (Fig. 4.3). The optimum induction of IL-1β was observed 18 hrs post incubation in young animals and afterward IL-1β secretion was decreased. In adult cattle production of IL-1β was not time dependent event and same level was detected at 8 18 and 24 hrs post incubation.

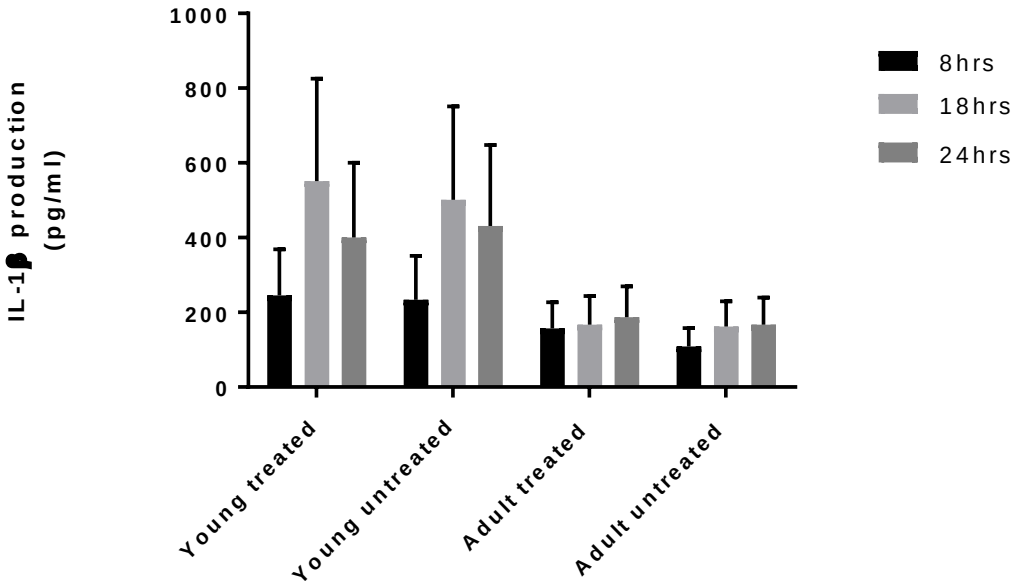


Figure 4.3 IL-1β production in CD14^{+} monocytes from young and adult cattle in response to alum

Expression of IL-1β production was examined in aluminium hydroxide (alum with 500 µg/ml) stimulated CD14^{+} monocytes of young and adult bovine at different interval of time as indicated above. The supernatants were collected at end of incubation time and analysed in duplicate by ELISA for IL-1β secretion. These result showing mean ± SEM of young (n=3) and adult (n=9) and statistical analysis was conducted by 2 way ANOVA using GraphPad Prism 7.02 and multiple comparison was performed with Sidak's multiple test.

4.3.1.3. TNF-α production in blood monocytes

TNF-α production was measured in $CD14^+$ monocytes from young and adult cattle. The results of this study revealed a differences between TNF-α levels in monocytes from adult and young at 48 hrs (Fig. 4.4). Young cattle showed comparatively higher levels of TNF-α in respect to IFN-γ. These data also suggest that LPS alone was not able to elicit significant amounts of TNF-α in young or adult animals. The combination of LPS and IFN-γ were able to induce TNF-α production from monocytes in both groups. Adult cattle demonstrated lower amounts of TNF-α with a greater variation between individual animals. In young cattle TNF-α production was more consistent, especially with respect to IFN-γ stimulation. These results indicate that, unstimulated monocytes of adult cattle did produce baseline level of TNF-α whereas young cattle monocytes did not express detectable level of TNF-α (minimum detection range 78 pg/ml). However, these results were not statistically significant either in terms of age (*P value= 0.8613)* or in terms of various stimulants *(P value = 0.1540)* due to large variation among animals.

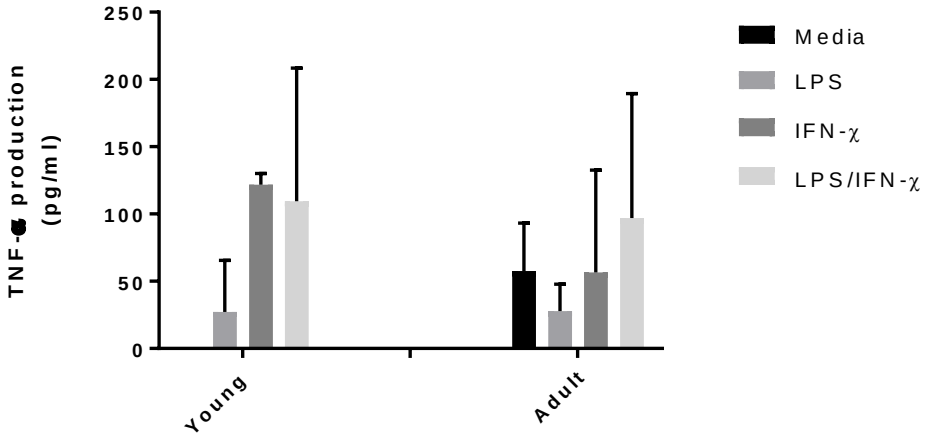


Figure 4. 4 TNF-α production in $CD14^+$ monocytes from young and adult cattle in response to LPS, IFN-γ and LPS/IFN-γ

Expression of TNF-α in blood monocytes was examined in young and adult cattle in response of LPS (1 μg/ml), IFN-γ (20 ng/ml) and LPS/IFN-γ together at 48 hrs post incubation. The supernatants were collected at end of incubation time and analysed in duplicate by ELISA for TNF-α production. These data showing mean ± SEM of adult (n=2) and young bovine (n=2) and statistical analysis was performed by 2 way ANOVA using GraphPad Prism 7.02 and multiple comparison was performed with Sidak's multiple test.

4.3.2 Cytokine expression of neonatal alveolar macrophages

Using alveolar macrophages harvested from lungs of neonates, cytokine expression profile of macrophages were evaluated in responses to stimulation with LPS, IFN-γ, LPS/IFN-γ and alum.

4.3.2.1 Expression of IL- 6, IL-1β, and TNF-α

The induction of IL-6 was evaluated from alveolar macrophages in response to LPS, IFN-γ and a combination of both at 24 hrs, 48 hrs and 72 hrs (Fig. 4.5). There was a delayed induction of IL-6, however alveolar macrophages were not able to produce a greater amount of IL-6 and <100 pg/ml was observed as maximum at 72 hrs post stimulation in response to LPS/IFN-γ. Expression of IL-6 was dependent on the stimulus type as LPS and a combination of LPS/IFN-γ were able to stimulate IL-6 production from alveolar macrophages but IFN-γ alone was unable to induce IL-6. These results are statistically not significant.

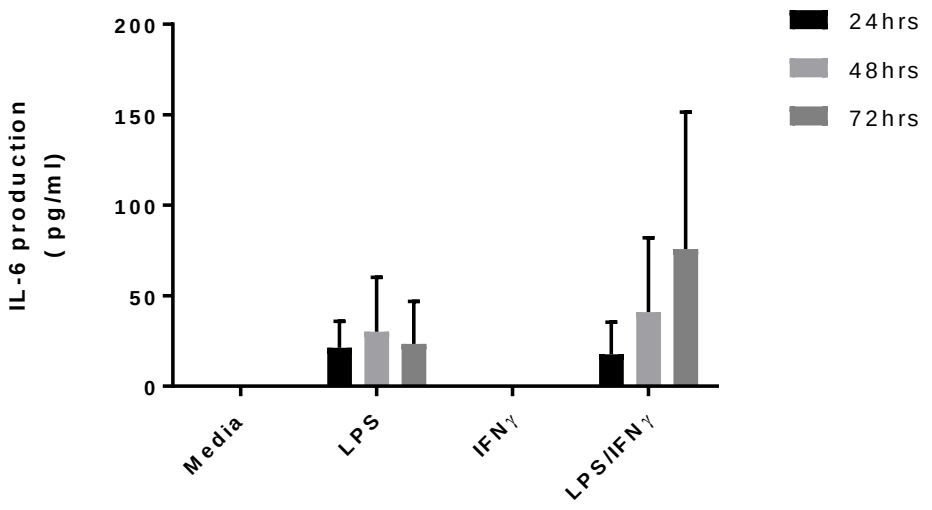


Figure 4.5 IL-6 production in alveolar macrophages of neonates in response to LPS, IFN-γ and LPS/IFN-γ

Expression of IL-6 was examined in neonatal alveolar macrophages stimulated with LPS (1 µg/ml), IFN-γ (20 ng/ml) and LPS/IFN-γ together at different interval of time as indicated above. The supernatants were collected at end of incubation time and analysed in duplicate by ELISA for IL-6 secretion. These data showing mean ± SEM of neonates (n=4) and statistical analysis was conducted by 2 way ANOVA using GraphPad Prism 7.02 and multiple comparison was performed using Tukey's multiple test.

The expression of IL-1β was evaluated from alveolar macrophage of neonates (Fig 4.6). It was observed that given LPS/IFN-γ was most consistent for IL-6 expression; these stimulants were preferentially assessed for ability to induce IL-1β cytokine expression. These data showed increase IL-1β production 24 hrs and 48 hrs post incubation in response to LPS/IFN-γ; suggesting that LPS/IFN-γ in combination was able to induce cytokine production at each of the time points tested. Whilst 24 and 48 hrs post stimulation appeared to be the optimum time points for induction, but at 72 hrs post incubation it gradually declined. Significant differences were determined between media control and stimulated macrophages at 24, 48 and 72 hrs with respective *P values* of *0.0042, 0.0027 and 0.0294*.

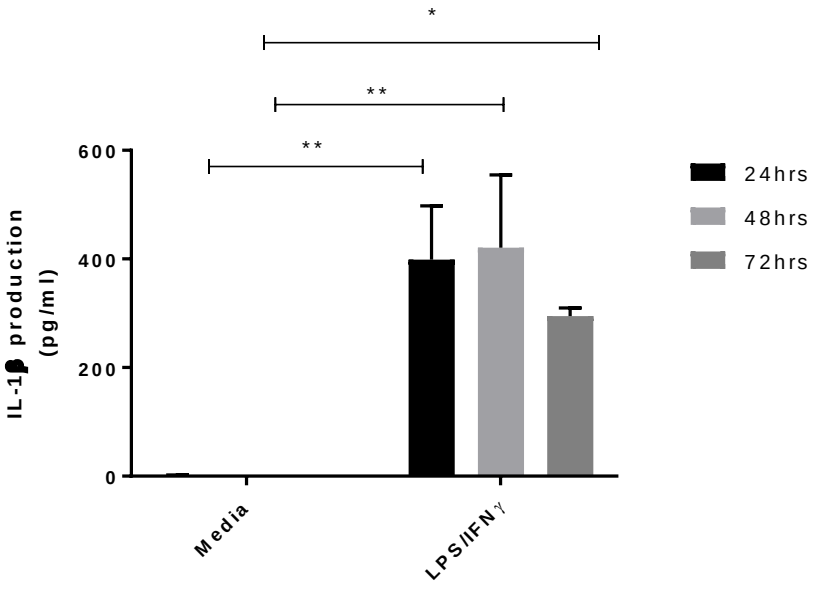


Figure 4.6 IL-1β production in neonatal alveolar macrophages in response to LPS/IFN-γ

IL-1β production was examined in neonatal alveolar macrophages stimulated with LPS/ IFN-γ together at different interval of time as indicated above. The supernatants were collected at end of incubation time and analysed by ELISA in duplicate for IL-1β production. These data showing mean ± SEM of neonates (n=4) and statistical analysis was conducted by 2 way ANOVA using GraphPad Prism 7.02 and multiple comparison was performed by Sidak's multiple test. The statistical significant differences were found between the media control vs LPS/IFN-γ stimulation at 24, 48 and 72 hrs post stimulation with *P values* of *** 0.0042, **0.0027* and **0.0294* respectively.

In terms of alum, used as an alternative stimulant that activate caspase-1 enzyme to cleave immature form of IL-1β into mature form. These data revealed that alum can act as a more potent stimulant and does not require TLR signal for activation of immune response and triggers early induction of IL-1β at 18 hrs and thereafter it reduced with time (Fig. 4.7). However, this data did not show any statistically significant difference between stimulation times (*P value= 0.4539)* due to variation among animals

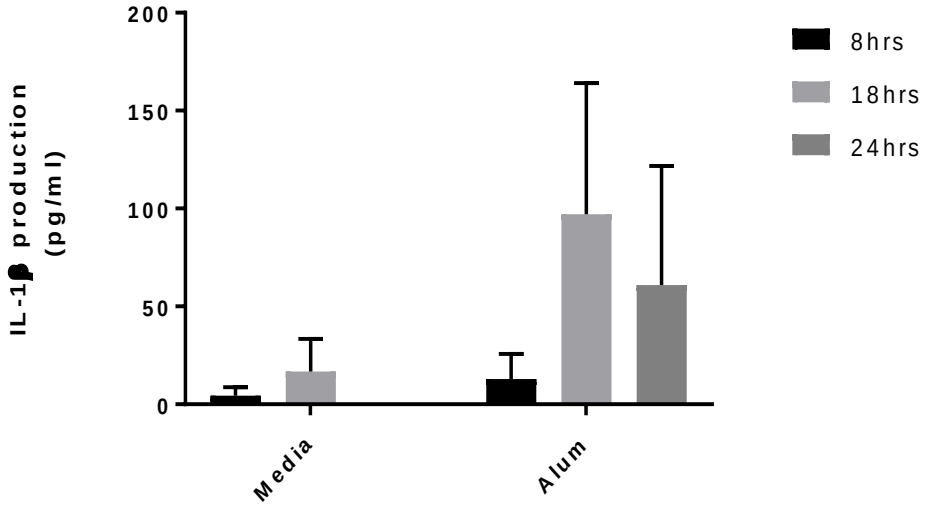


Figure 4.7 IL-1β production in neonatal alveolar macrophages in response to alum

IL-1β production was examined in neonatal alveolar macrophages stimulated with alum (500 µg/ml) at different interval of time as indicated above. The supernatants were collected at end of incubation time and analysed by ELISA in duplicate for IL-1β secretion. These data showing the mean ± SEM of neonates (n=4) and statistical analysis was conducted by 2 way ANOVA using GraphPad Prism 7.02 and multiple comparison was performed by Sidak's multiple test.

The production of the inflammatory cytokine TNF-α was measured in alveolar macrophages of neonates over time in response to stimulus LPS, IFN-γ and LPS/IFN-γ. The optimum stimulation time was found to be 24 hrs and after this, production of TNF-α began to decrease (Fig. 4.8). These data also suggests that LPS and a combination of LPS/IFN-γ were able to induce maximum level of TNF-α.

Additionally, IFN-γ alone was not able to induce any detectable TNF-α at different time periods; however together LPS/IFN-γ produced elevated amounts of TNF-α compared to LPS and followed the same pattern of secretion as LPS with peak production at 24 hrs and gradually decrease at 72 hrs. These data revealed a statstically significant differences among LPS stimulated alveolar macrophages at 24 and 72 hrs *(P value=0.0019*) and LPS/IFN-γ stimulated alveolar macrophage shows a significant difference between 24 and 72 hrs (*P value=0.002*) and 48 and 72 hrs (*P value=0.0035*).

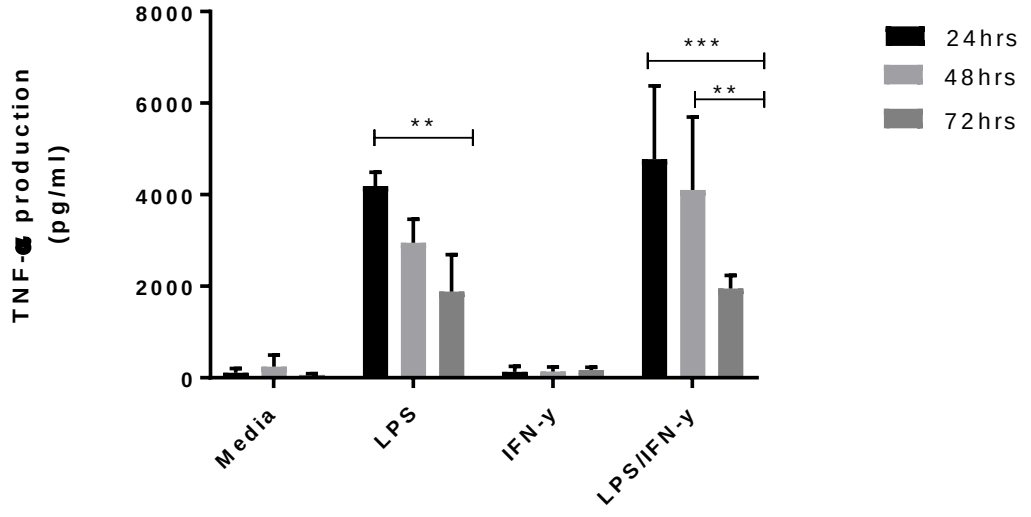


Figure 4.8 TNF-α production in alveolar macrophages from neonates in response to LPS, IFN-γ and LPS/IFN-γ

TNF-α production was examined in neonatal alveolar macrophages stimulated with LPS (1 µg/ml) and IFN-γ (20 ng/ml) and a combination of both at different time interval as indicated. The supernatants were collected at end of incubation time and analysed by ELISA in duplicate for TNF-α secretion. These data showing mean ± SEM of neonates (n=4) and statistical analysis was conducted by 2 way ANOVA using GraphPad Prism 7.02 with multiple comparisons using Tukey's multiple test. The significant difference was noticed at 24 and 72 hrs post incubation in terms LPS *(P value**0.0019*) and in terms of LPS/IFN-γ at 24 and 72 hrs *(P value ***0.0002)* and 48 and 72 hrs post incubation *(P value **0.0035).*

4.3.2.2 IL-12 and IL-10 expression in alveolar macrophages

IL-12 production in neonatal alveolar macrophages was evaluated by paired mouse anti-bovine IL-12 antibodies. In neonates IL-12 induction was compared by fold induction over media alone stimulation due to a lack of availability of an IL-12 recombinant standard. IL-12 production showed a similar pattern to those of TNF-α and IL-1β with the maximum amount of IL-12 detected at 24 hrs post stimulation with LPS/IFN-γ (Fig. 4.9) and decreased thereafter. These data suggest that production of IL-12 was greater in response to LPS and LPS/IFN-γ however, IFN-γ alone was unable to produce significant amounts of IL-12. The statistical significant difference was noticed between stimulus LPS and IFN-γ (*P value = 0.0012*) and LPS/IFN-γ versus IFN-γ *(P value = 0.0039)* at 24 hrs. In terms of incubation time LPS stimulated macrophages showed the significant difference between 24 and 48 hrs (*P value = 0.019*).

IL-12 production was also evaluated in young and adult bovine monocytes which was comparatively less (Appendix 4, 1.5 fold over media) than alveolar macrophages (Fig. 4.9, 4-2.5 fold over media). However, the level of IL-12 within blood monocytes was same in young and adult cattle.

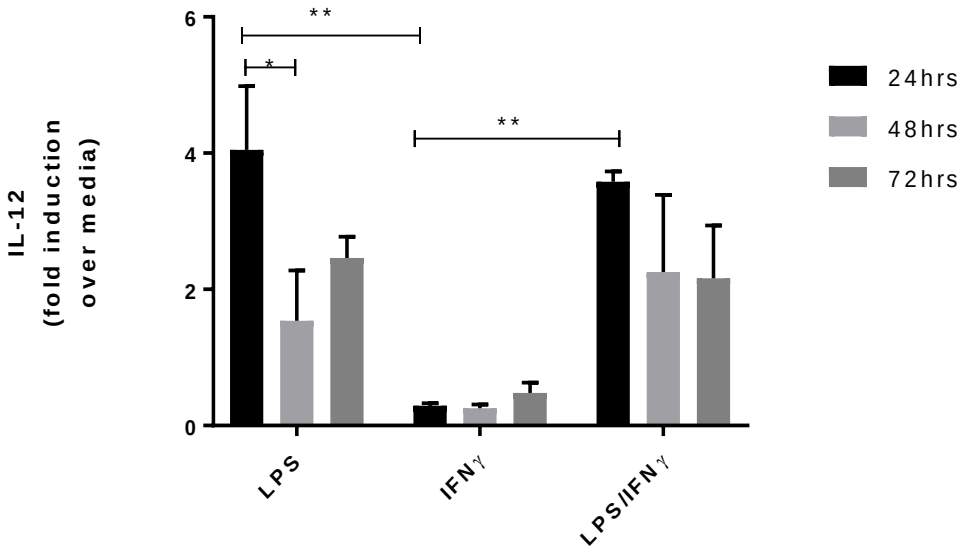


Figure 4. 9 IL-12 production in alveolar macrophages from neonates in response to LPS, IFN-γ and LPS/IFN-γ

IL-12 production (fold induction over media) was examined in neonatal alveolar macrophages stimulated with LPS (1 µg/ml), IFN-γ (20 ng/ml) and a combination of both at different interval of time as indicated above. The supernatants were collected at end of incubation time and analysed by ELISA in duplicate for IL-12 secretion. These data showing mean ± SEM of neonates (n=3) and statistical analysis was conducted by 2 way ANOVA using GraphPad Prism 7.02 with multiple comparison by Tukey's test. The significant difference noticed with ***P value 0.0012 and 0.0039* between LPS versus IFN-γ and LPS/IFN-γ versus IFN-γ respectively and ***P< 0.0197* between LPS stimulated alveolar macrophages at 24 hrs and 48 hrs incubation time.

The production of anti-inflammatory IL-10 from stimulated neonatal alveolar macrophages was also determined. Measurements of IL-10 cytokine were below the detectable limits of the kit (detection range 5 pg/ml -1000 pg/ml) after stimulation with LPS, IFN-γ or LPS/ IFN-γ at different time points (data not shown). These results indicate that alveolar macrophages did not express any measurable IL-10 in response to these stimuli over the 24 to 72 hrs post incubation. Overall these data demonstrated LPS and IFN-γ together elicit maximum induction of pro-inflammatory cytokines. An increase amount of pro-inflammatory cytokines such as IL-12 and TNF-α from alveolar macrophage was noticed compared to blood monocytes.

4.4 Discussion

The present study focused on an evaluation of blood monocyte responses to stimulation in young and adult cattle in terms of pro-inflammatory cytokine secretion. This study also examined the cytokine expression from neonatal alveolar macrophages.

4.4.1 Inflammasome activation in blood monocyte of young and adult cattle

These data show that young cattle expressed higher levels of caspase-1 activity compared with adult in response to alum that leads to the conversion of immature pro-IL-1β into the mature form. The induction of the inflammasome linked caspase-1 activity was seen over a short time frame (4-8 hrs) which may offer an explanation for early induction of IL-1β in alum stimulated $CD14^{+}$ monocytes, where an optimum stimulation time of 18 hrs was recorded with a gradual reduction at 24 hrs. Additionally, alum can achieve inflammatory effect through secretion of IL-1β in a brief time period compared to LPS/IFN-γ in young cattle.

These findings are also supported by previous *in vitro* studies that indicate early expression (24 hrs) of IL-1β from alum stimulated bovine PBMCs and a caspase-1 dependent IL-1β secretion. Moreover a combination of alum and LPS elict optimum IL-1β secretion compared to LPS alone (Harte et al., 2017). Alum stimulated bone marrow derived macrophages of murine also showed a peak production of IL-1β between 8-10 hrs (Eisenbarth et al., 2008).

It is known that alum adjuvant activates the NALP3 inflammasome does not require MyD88 or TRIF signalling pathways that act on TLRs to stimulate innate immunity (Gavin et al., 2006; Hanfen et al., 2008; Kool et al., 2007). NALP3 is an important regulator of age-related inflammation and it can become activated in older human patients in response to DAMPs such as extracellular ATP, excess glucose and urate crystal that leads to chronic inflammation (Eisenbarth and Flavell., 2009; Goldberg and Dixit, 2015).

4.4.2 Pro-inflammatory cytokines secretion from blood monocytes of young and adult cattle

The present study reveals that blood derived monocytes of young cattle are more responsive to a variety of immune stimulants and an upward trend was noted for IL-6, IL-1β and TNF-α when compared to adults that indicate a higher inflammatory activity in monocytes obtained from young cattle. As previous study confirmed that monocyte is a hetrogenous population and consist of classical and non classical subset based on the expression of $CD14^{+}$ and $CD16^{+}$ surface markers in human monocyte (Ziegler-Heitbrock et al., 2010) and identical in bovine (Huessen et al., 2013). It is further explain by age dependent shift from classical to non-classical monocytes indicating a decline in circulating monocytes population with age in human (Seidler et al., 2010) that cause reduce production of pro-inflammatory cytokines. Given the strong inflammatory response identified in young cattle in this study, it would be reasonable to hypothesis that the majority of young bovine blood monocytes may also correspond to the classical blood monocytes profile.

These data in agreement with previous findings where a functional alteration was reported in monocyte subset that suggests classical subset of bovine monocyte have high phagocytic, ROS and more expression of inflammatory cytokines such as TNF-α and IL-1β (Hussen et al., 2013). Previous murine studies suggest that the classical monocytes ($Ly6C^{high}$/ $CCR2^{high}$) subset is able to produce high levels of TNF-α, IL-6 and IL-1β in response to LPS. Moreover, the same study showed a high F4/80 expression on $Ly6C^{high}$ monocytes of young mice that belongs to macrophage maturity marker (Puchta et al., 2016).

The functional alteration in monocyte could be due to lower expression of TLR receptors in older animals as suggested in previous mouse studies that demonstrates, macrophages obtained from older mice have lower expression of TLR4 receptor and responses to LPS stimulation gave reduced amounts of IL-6, IL-1β, TNF-α, IL-6, IL-12 and elevated levels of IL-10 compared to young mice (Chelvarajan et al., 2005; Gomez et al., 2010). Suggesting age-related dysregulation of bone marrow derived macrophages and supporting the finding of relatively higher IL-10

production in older mice (Kim et al., 2017) which inhibits the synthesis of other pro-inflammatory cytokine (Moore et al., 2001).

4.4.3 Cytokines secretion from neonatal alveolar macrophage

Tissue derived macrophages from neonates were also examined for cytokines expression and a continuous trend towards greater inflammatory responses in alveolar macrophages of neonates was noted. The alveolar macrophages taken from neonates indicate that during the transition from blood monocytes to tissue macrophages, the capacity to mount a strong inflammatory response is retained in neonates. Alveolar macrophages elicited a greater amounts of TNF-α, however level of IL-1β was same and a reduction in IL-6 induction was noticed compared to blood derived monocytes in response to LPS, IFN-γ and alum. Additionally, IFN-γ alone was not able to induce production of these cytokines and required the presence of additional stimulatory molecules, in this case LPS to enhance cytokine secretion.

It is known that during infection monocytes rapidly migrate from blood to the site of infection where they elicit a prompt immune response and mature into macrophages (Auffray et al., 2007). In the transition from monocytes to macrophages, a M1/M2 phenotype may also change cells functional properties. *In vitro* bovine studies have shown that LPS stimulated macrophages differentiate into the M1 phenotype and make them efficient producers of ROS, NO and inflammatory cytokines TNF-α, IL-1β, and IL-6 (Werling et al., 2004). In the present study, induction of pro-inflammatory cytokines from blood monocytes and alveolar macrophages illustrates tendency towards transition to M1 macrophages. Some bovine studies are in agreement with our findings where alveolar macrophages elicited a response characterised by rapid and greater TNF-α production compared to blood monocytes during *M. bovis* infection (Piercy et al., 2007). To further confirm this, evaluation of arginase-1 or NO production by these cell types could provide more evidence to help to specify the M1/M2 phenotypes of cattle macrophages.

The study presented here indicates early induction of IL-12 (24 hrs) in neonatal macrophages in response to LPS and LPS/IFN-γ while production of IL-10 was not detectable at 24-72 hrs post stimulation. This suggests delayed expression of IL-10 could allow neonates to exert a prolonged inflammatory response leading to an optimal Th1 response allowing them to lower their parasitaemia. Goff et al (2002) demonstrates that IL-10 act as an inhibitory factor for induction of *iNOS* from *B. bovis* merozoites infected cattle monocyte. It is possible that the timeframe used to measure IL-10 induction was too short in this study. Another explanation could be that alevolar macrophages tend towards M1 conversion rather than M2 and more likely expressed higher levels of pro-inflammatory cytokines when stimulated with LPS/IFN-γ.

These findings are in agreement with previous bovine studies where the kinetics of IL-12 mRNA expression was evaluated in *B. bovis* infected adult and young spleen mononuclear cells (SMC) and observed an early (24-96 hrs) and strong expression of IL-12 in young SMC compared with adult (168 hrs) (Goff et al., 2002; Goff et al 2010; Schneider et al., 2010). An earlier study demonstrated IL-12 production in bovine macrophages cultured with *B. bovis* regardless of the presence or absence of IFN-γ (Shoda et al., 2000) suggesting that PPRs are effective, in isolation, in young macrophage in line with above mentioned findings using LPS and alum. Moreover, during *M. avium* infection, neutralization of IL-10 has positive impact on production of IL-12 from macrophages demonstrated that a relation between IL-10 and IL-12 exists in cattle (Weiss et al., 2005). The mechanism of interaction between IL-10 and IL-12 during protozoan infection could be explain as upregulation of intracellular pathogen killing; however production of IL-10 would be potential benefit to both the host (limiting pathology) and the pathogen.

While exploring potential differences in reactivity of alveolar macrophage in neonates; a comparative study was not performed using adult alveolar macrophages. The difficult nature of obtaining alveolar macrophages from live adult animals which requires sedating animals with recoverable anaesthesia and performing bronchoalveolar lavage (BAL), a procedure which requires both a UK Home Office licence and high degree of skill and competence to be carried conducted.

Alternatively, acquiring BAL samples at post mortem is complicated by the high degree of colonisation of the respiratory tract by pathogens such as *Pasturella* confounding the measurement of any subsequent immune response (RJ Flynn Pers. Commun.).

From this study, it can be concluded that there is evidence of an association between age and the innate immune response to non-infectious stimuli. The response of blood monocytes during the induction of inflammatory cytokines indicated that the innate immune response is more pronounced in young cattle and this may have implications for immunity to infections during early life however, source of samples and sampling time should be considered as it can affect the immune response by interfering on health status of animals. Neonatal alveolar macrophage have significant role in the defense against respiratory tract infections by activation and release of pro-inflammatory cytokines however, excessive production of inflammatory cytokines is likely to be deleterious and cause damage to the lung tissues. Thus whether this pattern of responses is beneficial remains to be examined.

This study indicates that there is likely to be an association between monocyte activity and the innate immune response during intra-cellular parasitic infection that is often seen in *Babesia, Toxoplasma*, *Neospora* infection.

Table 4.1 A summary describing the leukocyte population alteration with age in cattle

Immune cells	Neonates (2 week old)	Adult (2-3 yrs old)	Functions
$CD14^+$ Monocytes	37.6 % ↑	15.65 % ↓	Producing inflammatory cytokines and clearing pathogen.
Monocyte CD80 expression	12.5 % ↑	2.6 % ↓	Provide co-stimulatory signal for T-cell activation
ΓδT- cells expression	43.8 % ↑	28.4 % ↓	Act as a regulatory T-cell in cattle and secrete cytokine IL-17.
Treg cells expression ($CD4^+CD25^+Foxp3^+$)	0.22 % ↑	0.14 % ↓	Modulate the immune response, maintain tolerance to self antigen and prevent autoimmune disease
$CD4^+$ T cells	12.8 % ↓	16.5 % ↑	Help other immune cells by release T-cell cytokines
MHC-II expression	6.75 % ↓	19.2 % ↑	Antigen presentation and interaction with $CD4^+$ T cells and initiate immune response
B-cells CD21 CD45RO	 5.5 % ↓ 4.3 % ↓	 27.4 % ↑ 18.3 % ↑	CD21 also known as Complement receptor 2 markers present in mature B-cells population and CD45 is common leukocyte receptor and associated with T- cell activation

Table 4.2 Monocytes cytokine expression in response to LPS, IFN-γ, a combination of both and alum in cattle

Cytokine expression	Young (6 month) Optimum induction and potent stimulant	Adult (2-3 year) Optimum induction and potent stimulant	Functions
IL-6	1578 pg/ml LPS at 24 hrs post stimulation	469 pg/ml LPS at 24 hrs post stimulation	Important role in acquired immunity via stimulation of antibody production and effector T cell development
Caspase-1 activity	496 pmol/min 4, 6 and 8 hrs post stimulation	196 pmol/min 4, 6 and 8hrs post stimulation	It is essential for conversion of immature form of IL-1β into mature form
IL-1β	550 pg/ml Alum at 18 hrs post stimulation	187 pg/ml Alum at 24 hrs post stimulation	It is a potent pro-inflammatory cytokine that is crucial for host defense during intra-cellular infection
TNF-α	120 pg/ml LPS at 48 hrs post stimulation	97 pg/ml LPS/IFN-γ at 48 hrs post stimulation	An inflammatory cytokine that induce cellular immune response cause parasite destruction and reduction of parasitaemia

Chapter 5 Innate immune response to *N. caninum* infection in cattle

5.1 Introduction

N. caninum is an obligate intra-cellular protozoan parasite responsible for abortion and infertility in cattle, as well as neuromuscular diseases in dogs (Dubey and Lindsay, 1996). Unlike *T. gondii*, which is a noted zoonotic disease, there is no evidence of human infection reported to date (McCann et al., 2008).

5.1.1 Cell mediated immune response during *N. caninum* infection

Cell mediated immune responses associated with bovine *N. caninum* infection involve antigen specific cell proliferation and secretion of IFN-γ, TNF-α and IL-12, have been shown to be essential for defence against *N. caninum* infection (Williams et al., 2000; Marks et al., 1998; Staska et al., 2003; Innes et al., 2002). It is believed that *N. caninum* infection in mice leads toTLR2 dependent activation of DCs resulting in enhanced secretion of pro-inflammatory cytokine IL-12 and providing essential signals for initiation of effective Th1 immune response against infection (Kopp and Medzhitov, 2003; Mineo et al., 2010). Detailed studies of the innate immune response in *N. caninum* infection in cattle has been somewhat limited to date but previous murine studies have shown increased parasite killing activity by peritoneal macrophages in the presence of IFN-γ (Nishikawa et al., 2001; Mineo et al., 2010a; Adams et al., 1990; Dion et al., 2011) and also IFN-γ stimulated antigen-presentation on splenic DCs of infected BALB/c mice is correlated with increased production of NO (Veeraseatakul and Chutipongvivate, 2005).

The mechanism underlying protective immunity to *N. caninum* functions via induction of IL-12 which leads to expansion and differentiation of parasite specific $CD4^+$ and $CD8^+$ T cells a defining feature of Th1 immune responses (Staska et al., 2003). The protective role of $CD8^+$ T cell themselves has been more readily shown in intra-cellular protozoan infection such as those with *T. gondii* (Denkers & Gazzinelli, 1998; Jordan et al., 2010). Past studies using $CD8^+$ deficient mice suggest a role for $CD8^+$ T cells in providing protective immunity with CD8 deficient mice showing impaired IFN-γ production and being more susceptible to *N. caninum* infection with aggravated pathology after infection in comparison with immune competent mice (Correia et al., 2015).

More conclusively, the role of IFN-γ in host defence during *N. caninum* infection has been illustrated in both *in vivo* murine models and *in vitro* bovine tissue culture models (Khan et al., 1997; Nishikawa *et al.,* 2001; Yamane *et al*., 2000; Innes et al., 1995) where IFN-γ producing $CD4^+$ and $CD8^+$ T cells have been shown to provide protection specifically, through IFN-γ priming of mononuclear cells to limit multiplication of *N. caninum* infected fibroblasts in a iNOS and L-tryptophan partially-dependent fashion (Baszler et al., 1999; Tuo et al., 2005). Alternatively, IFN-γ produced by NK cells elicited CD4 Th1 cells, these can lead to control of *N. caninum* tachyzoite multiplication through the production of NO from infected macrophages (Nishikawa et al., 2001).

5.1.2 Role of NK cells in *N. caninum* infection

NK cells contribute to between 2-10% of the total lymphocyte population in cattle with a greater number of cells present in neonates aged between 8-120 days (Kulberg et al., 2004; Kamden et al., 2006). The role of NK cells as effector cells in achieving protective immunity has been studied in a bovine model where NK cells were shown to produce IFN-γ during the early stages of infection (Boyson et al., 2006). Studies suggests that, during *N. caninum* infection, the initial peripheral pool of NK cells was reduced at 4-6 days post infection in calves and rose thereafter (Klevar et al., 2007) that could be explained by the recruitment through the bone marrow and proliferation in the spleen during parasitic infection (Goff et al., 2003; Antunez and Cardoni, 2004).

The importance of NK cells have been established in *N. caninum* infection models and studies have indicated that NK cells are amongst the first cells recruited to the site of infection, where they have two major functions; production of cytokines and lysis of infected cells before activation of T and B cells medicated immunity (Klevar et al., 2007; Korbel et al., 2004). Bovine *in vitro* studies showed IFN-γ production from *N. caninum* infected calf NK cells (Korbel et al., 2004).

How crucial the role of NK cells is during *N. caninum* infection still requires confirmation, however the importance of NK cells in bovine neosporosis as a potent source of IFN-γ even without IL-12 priming is known and indicating that *N. caninum*

tachyzoites are capable of triggering IFN-γ alone (Boyson et al., 2006). Moreover, the role of NK cells as an early producer of IFN-γ has been confirmed in other protozoan infections such as Leishmaniasis and Malaria (Scharton-Kersten and Sher, 1997; Artavanis Tsakonas and Riley, 2002).

5.1.3 Study Aim

In previous chapters an age-dependent alteration was observed in terms of monocyte number, function and expression of the co-stimulation ligand CD80; all of which were higher in neonates. These age-dependent effects in monocytes may alter their subsequent response to intracellular parasitic infection and interaction with NK cells. This chapter attempts to explore the interaction of monocytes and NK cells during *N. caninum* infection in the context of neonates and adult cattle through analysis of the percentage of infected monocytes NK cells in a co-culture system, markers of cell activation and cytokine secretion during infection. To further explore these findings a bovine microarray was performed to compare the gene expression profile across neonates and adult cattle in response to *N. caninum* infection and identified pathways and genes that may be involved in the age-related immune response in the context of *N. caninum* infection.

5.2 Materials and methods

5.2.1 *N. caninum in vitro* infection study

N. caninum in vitro study was perfomed from $CD14^+$ monocytes obtained from Holstein Friesian male neonates of age 2 week old and 2-3 year old male adult cattle.

5.2.2 Flow cytometry, sample gating and data analysis

Fluorophore labelled monocytes and CFSE labelled tachyzoites were analysed by flow cytometry on a MACSQuant Analyzer 10. The monocytes were gated on the basis of forward and side scatter parameters to exclude dead cells or debris. Unstained monocytes, *N. caninum* tachyzoites and appropriate isotype controls were used to set a threshold and to differentiate the stained cell population from the unstained population. For dual staining i.e. CFSE and RPE, appropriate compensation setting was applied and a minimum of 20000-30000 events were recorded per sample. Acquired flow cytometry data were analysed using WEASEL software 3.0.2. Results were expressed as median with 95% confidence interval and mean ± SEM where appropriate. The statistical analysis were performed using Graphpad prism 7.2 with multiple comparisons were performed using either by Mann Whitney's test (Comparison among neonates and adult) and 2 way ANOVA with Sidak's multiple comparison test (Comparison among neonatal and adult monocytes with and without NK cell co-culture). Results were considered statistically significant *with P values < 0.05*.

5.2.3 Enzyme assays

The standard curve was generated as described in section 4.2.2 and granzyme b activity was calculated monocyte NK cell co-culture supernatants in pmol/min/ml. Perforin activity was assessed as capacity to release haemoglobin from RBCs and OD was recorded as respective monocyte NK cell co-culture supernatants.

5.2.4 Micrarray analysis

Microaray analysis was perfomed from *N. caninum* infected and uninfected monocytes obtained from Holstein Friesian male neonates of age 2 week old and 2-3 year old male adult cattle. Prepared RNA samples were submitted to Centre Genomics Research (CGR, University of Liverpool) for microarray processing.

5.2.4.1 Microarray preparation, hybridisation and scanning

Duplicate total RNA samples from neonates (n=3) and adult monocytes (n=2) in duplicate were used for Agilent One-Color Microarray-based Gene Expression Analysis using cyanine 3-labeled targets to measure gene expression in *N. caninum* infected and control samples. The Bovine (V2) Gene Expression Microarray, 4×44K chip (G2519F-023647) was used in this experiment. cDNA samples were prepared from total RNA samples of infected and control cattle monocytes using Affinity Script RT oligo dT promoter primers by reverse transcription and then RNA was generated from cDNA via transcription using T7 RNA polymerase and cyanine 3 CTP (Fig. 2.1). Cyanine 3 labelled amplified RNA was purified and quantified using NanoDrop. The Cyanine 3-labeled, linear amplified cRNA was hybridised onto microarray slides and washed to remove unbound RNA from the slides. The slides were scanned using an Agilent C Scanner for feature extraction.

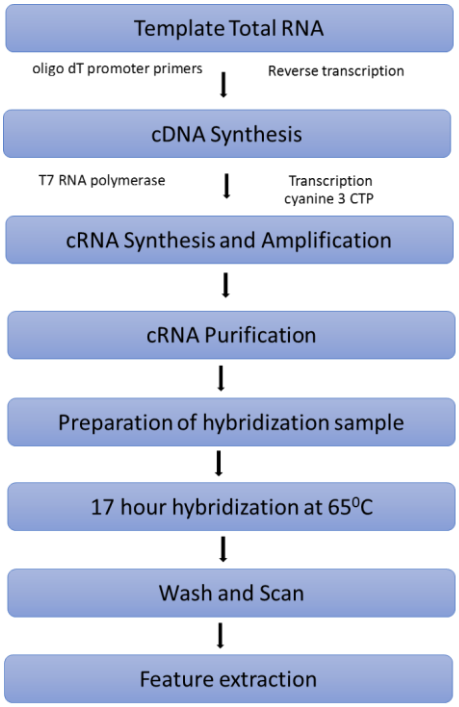


Figure 5.1 Work flow for Agilent one colour microarray sample preparation and analysis

Total RNA samples from *N. caninum* infected and uninfected monocytes of neonates and adult cattle were processed for microarray analysis. cDNA was prepared from template RNA and transcripted for cRNA synthesis and purified. Purified cRNA was hybridized with cDNA probes into array slides. These array slides containing hybridized samples were washed and scanned.

5.2.4.2 Microarray data analysis

Scanned microarray images were extracted in GeneSpring GX 9.0. Data text files were converted into Excel files and background mean signals were subtracted from mean signals. The raw data were analysed by uploading the mean files into network analyst a web based tool for gene expression profiling (Xia et al., 2015). Data were normalised by a log2 transformation and low variance genes were filtered on base of interquartile range (IQR) for equal difference among upper and lower quartile. The differential gene expression was identify by limma method by using adjusted *P* value <0.05 and fold change >2.0 (Mariani et al., 2003). Specific comparisons were performed between infected and control group of young and adult cattle to determine the set of differentially expressed genes. The interaction across young and adult cattle was examined via nested comparison in infected and control group.

5.2.4.3 Evaluation of gene of interest

An evaluation of genes of interest was performed among neonates (n=3) and adult (n=2) infected versus uninfected monocytes in duplicate. The obtained mean signal after subtracting background signal from each replicate with corresponding gene were evaluated after uploading files in a web based R programme (Xia et al., 2015) from neonates and adult cattle to evaluate the gene expression profile through one class analyses.

Initially, 44,469 genes were analysed to determine comparison among neonates infected versus uninfected and adult infected versus uninfected. Out of these, 25,642 annotated genes identity were matched and used to determine any upregulated or downregulated differentially expressed genes in each age group after removal of duplicate genes IDs. To see the specific interaction between adult infected versus neonates infected and adult uninfected versus neonates uninfected, nested comparison was performed with same annotated genes profiles. Identification of differentially expressed genes was performed to check the involvement of gene in different biological process. A total 44,449 gene were uploaded and among them 25,642 annotated gene identity were matched and used to find out the differentially

expressed gene after removal of duplicate gene IDs. Annotated gene data base and visual exploration was performed to determine the protein-protein interactions for identification of different pathways that were associated in biological process. Additionally, a full list of differentially expressed gene was reviewed for genes known to be involved in age related immune response and functions of various immune pathways related genes across young and adult cattle.

5.3 Results

5.3.1 CFSE labelling of parasite population

N. caninum tachyzoites were examined for CFSE labelling by flow cytometry. A parasite population was identified and gated on the basis of forward and side scatter profile of parasites. Figure 5.2 shows the gating analysis of CFSE labelled and unlabelled parasites; these data reveal a high percent (69.1%) of *N. caninum* tachyzoites were CFSE positive (Fig. 5.2d) and there was little to no autofluoresence (0.02%) from unstained parasites (Fig. 5.2b).

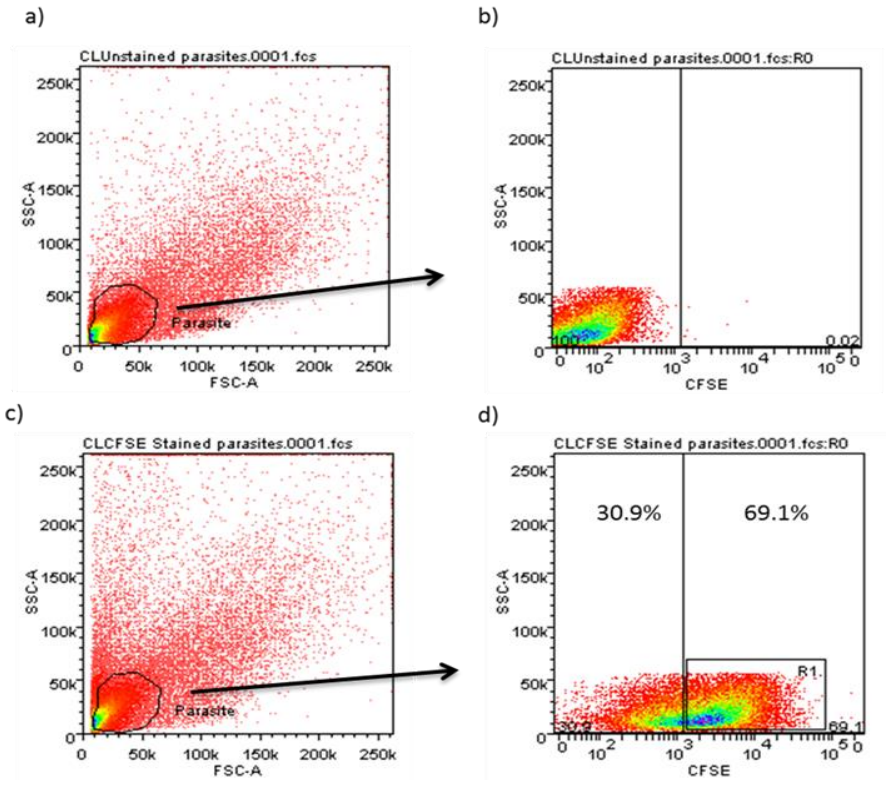


Figure 5.2 Gating strategy to determine the percent of CFSE labelled parasites

(a) A parasite population was gated on the basis of forward and side scatter dot plot of unstained parasites. **(b)** Gated unstained parasite population showing no CFSE staining. **(c)** CFSE labelled parasite population were gated based on forward and side scatter dot plot. **(d)** Gated stained parasite population showing CFSE labelled parasites (R1) population (69.1%).

5.3.2 *In vitro* infection of cattle CD14^{+}monocyte

CD14^{+} monocytes obtained from neonates and adults were infected using CFSE labelled *N. caninum* tachyzoites for 24 hrs and percentage of infected CD14^{+} monocytes were identified by flow cytometry analysis. Forward and side scatter dot plots of monocytes were used to identify the percentage of infected monocytes by identification of CFSE positive monocytes. In terms of monocyte infection, the results demonstrated that adult cattle monocytes harboured more number of *N. caninum* tachyzoites with a range between 11.2-45.3% (Fig. 5.3b) compared with neonates where a range of 4.92-10.2 % infected monocytes was noticed (Fig. 5.3d).

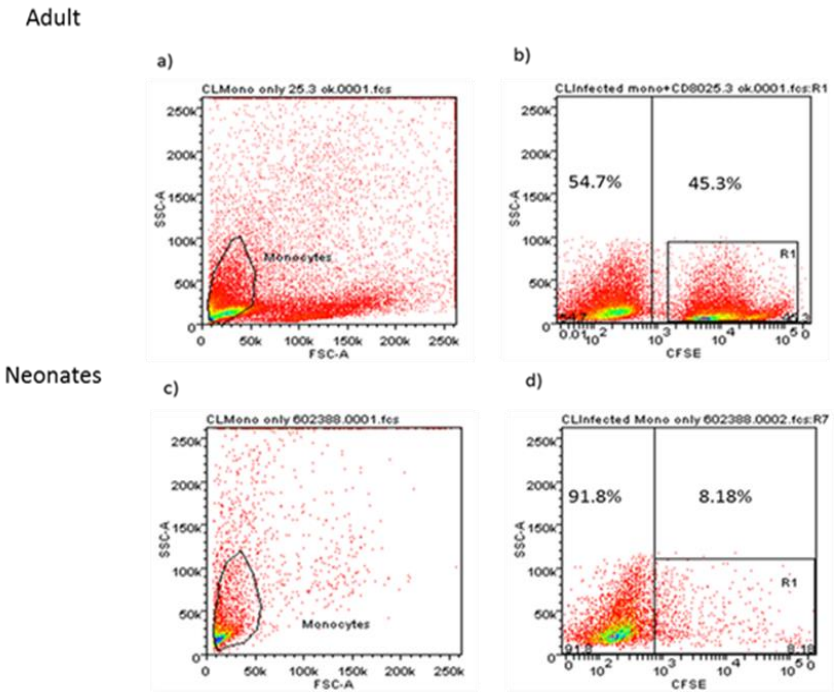


Figure 5.3 Gating strategy to identify the infected CD14^{+} monocytes population of adult and neonatal cattle

These dot plots are representative data from one adult and neonate cattle **(a & c)** Unstained CD14^{+} monocytes from (adult n=6) and (neonates n=4) were gated on the basis of forward and side scatter dot plot respectively. (**b & d)** CFSE positive infected CD14^{+}monocytes were identified within gated population of monocytes of adult and neonates.

The monocytes from adult cattle harbouring more number of *N. caninum* tachyzoites with a mean ± SD; 31.1 ± 5.08 whereas in neonates the mean ± SD was 9.02 ± 1.37 within infected monocytes. Additionally, this difference was found to be statistically significant (Fig. 5.4, *P value=0.0095*). This data was collected from a total of four neonates and six adults cattle where all neonatal monocytes displaying similar levels of parasitaemia (parasitized monocytes) whereas the percentage of infected $CD14^{+}$ monocytes was subject to larger animal-to-animal variation for infected monocytes from adult animals (Fig. 5.4).

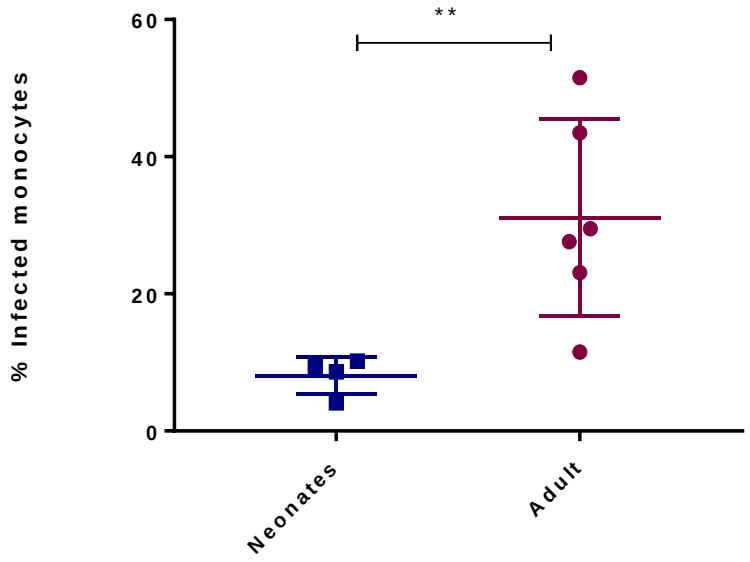


Figure 5.4 Percent of *N. caninum* infected $CD14^{+}$ monocyte from neonates and adult cattle 24 hrs post infection

Cattle monocytes were infected with CFSE labelled *N. caninum* tachyzoites and parasites uptake was determined by flow cytometry 24 hrs post infection. These results shows median with 95% confidence interval of neonates (n=4) and adult (n=6) cattle. Data were analysed using GraphPad Prism 7.02 and statistical analysis was performed using a Mann Whitney test. These result shows significant difference within percentage of infected $CD14^{+}$ monocytes across neonates and adult with *P value=0.0095.*

5.3.3 Expression of CD80 after *N. caninum* infection

In order to determine if activation of the global monocyte population occurred after infection, CD80 expression was evaluated before and after *N. caninum* infection to determine the fold change difference in expression. These results showed an increase of CD80 expression such that activation of $CD14^+$ monocytes was greater in adults leading to a greater fold increase of CD80 expression (mean ± SD; 73.54 ± 11.23) compared to neonates (mean ± SD; 40.5 ± 33.28). However, these results were not significantly different and there was a great deal of individual variation within the neonates was examined (Fig. 5.5).

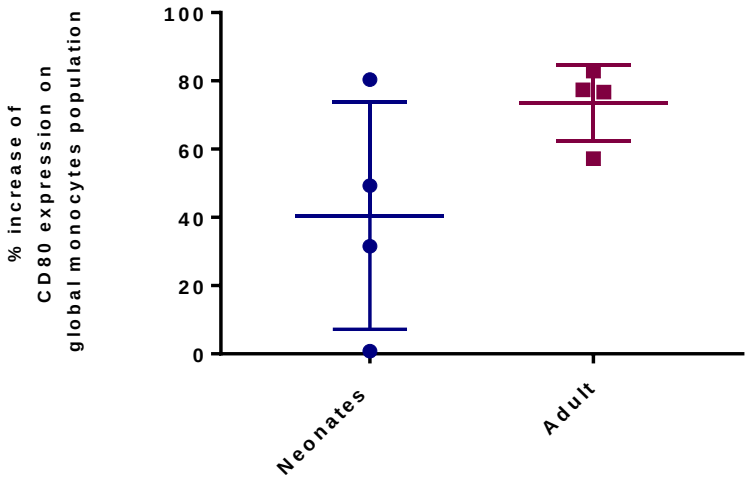


Figure 5.5 Percent increase of CD80 expression before and after *N. caninum* infection within global $CD14^+$ monocyte population

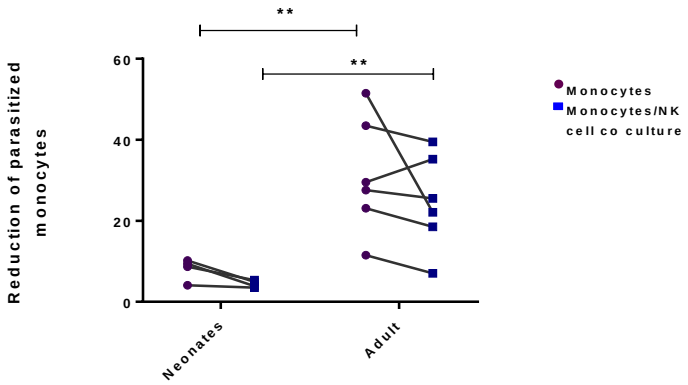
The percentage increase of CD80 expression was determined within the global $CD14^+$monocyte population of neonates (n=4) and adult (n=4) cattle before and after *N. caninum* infection. These result shows median with 95% confidence interval of neonates and adult and analysed using GraphPad Prism 7.02. The statistical analysis was performed using a Mann-Whitney test to determine the significant difference between neonates and adult.

5.3.4 Effects of NK cells co-culture on monocyte parasitaemia

The effects on parasitaemia (parasitized monocytes) were evaluated in the context of co-culture with autologous NK cells. Using the same flow cytometry strategy as above the percentage of infected monocytes with CFSE labelled parasites was determined after co-culture. These results demonstrate that adult cattle have more parasitized monocytes compared to neonates which were also found to be statistically significant (Fig 5.6a, *P value=0.0095*). After NK cell co-culture reduction of parasitized monocytes was observed in both age group; 4 adult among 5 have shown reduction of parasitaemia whereas all neonates shown reduction; additionally, in neonates the reduction was constant than adults (Fig. 5.6a).

To normalise the effect of different baseline of parasitaemia, the fold reduction after NK cell co-culture was also evaluated where infected neonatal monocytes showed a greater fold reduction (40 fold) when compared to adult infected monocytes (20 fold) however, due to the variation seen in responses from adult monocytes this was not statistically significant (Fig. 5.6b).

(a)



(b)

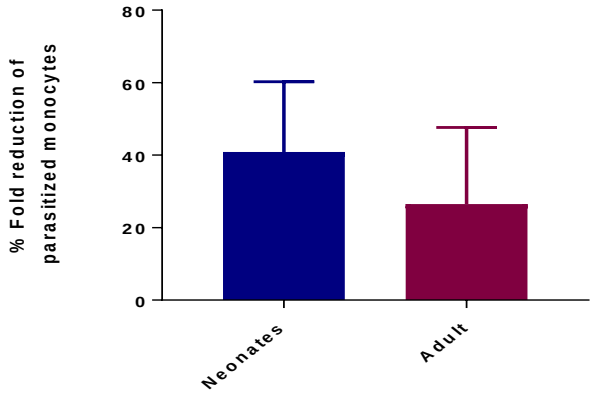


Figure 5.6 Parasitized monocyte before and after NK cell culture from neonates and adult cattle. Percent fold reduction of parasitized monocyte with subsequent NK cell co culture of neonates and adult cattle

(a) $CD14^{+}$ monocytes of neonates (n=4) and adult (n=6) were infected with CFSE labelled *N. caninum* parasites at a ratio of 4:1 and cultured alone or with NK cells at a ratio of 10:1 for 24 hrs at 37^{0}C. Cells were collected and flow cytometry was performed to identify the percent of infected monocytes before and after NK cell co- culture. This graph represents the percentage reduction of parasitized monocyte after NK cell co-culture. Results were analysed using GraphPad Prism 7.02 and statistical analysis was performed using a Mann-Whitney test. A significant difference was noted between neonates and adult monocyte before and after NK cell co-culture with *P value =0.0095* and *0.0095* respectively.

(b) The fold reduction of CFSE labelled *N. caninum* positive monocytes after NK cell co-culture was determined in neonates and adult cattle. These result shows median with 95% confidence interval of neonates (n=4) and adult (n=6). Results were analysed using GraphPad Prism 7.02 and statistical analysis was performed with a Mann-Whitney test to examine the significant difference among neonates and adult.

5.3.5 Effect of NK cell co-culture on CD80 expression

To evaluate whether NK cells upregulate markers of monocyte activation after co-culture, CD80 expression was examined. These data revealed increased CD80 expression post infection in both the adult and neonates global monocyte population and neonates were more responsive to infection in terms of CD80 expression compared to adult. However, monocyte NK cell co-culture did not significantly appear to affect CD80 expression in neonates and adult cattle. Additionally, a very little upregulated CD80 expression was observed in infected monocytes of neonates with NK cell co-culture. However, adult cattle displayed a reduction of CD80 expression with NK cell co-culture in infected and uninfected monocyte (Fig. 5.7). On the basis of CFSE positive labelling, monocytes were categorized into parasitized and non-parasitized monocytes; assigning CFSE positive cells as parasitized cells. An analysis of these cells indicated that only parasitized (CFSE positive) monocytes expressed CD80 and none of the CFSE negative, non-parasitized, monocytes displayed any CD80 expression either in adults or in calves.

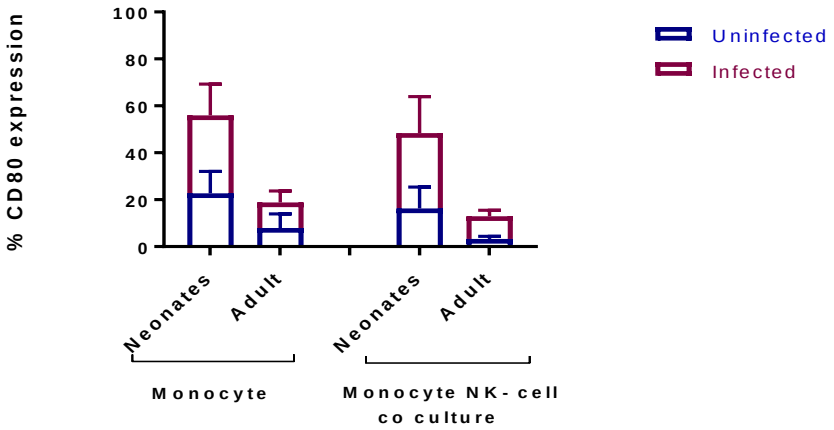


Figure 5.7 Percent CD80 expression on global monocytes population before and after *N. caninum* infection in neonates and adult cattle

CD80 expression was identified using flow cytometry analysis of monocyte and monocyte-NK cell co-cultured cells from *N. caninum* infected and uninfected control samples 24 hrs post infection. Data shows the mean ± SE of neonates (n=4) and adult (n=5) cattle CD80 expression where high CD80 expression was recorded within infected monocytes. Result were analysed using GraphPad Prism 7.02 and statistical analysis was performed by 2 way ANOVA with Sidak's multiple comparison to determine the significance difference between neonates and adult.

The results indicate that adults have shown a fold reduction in expression of CD80 following NK cell culture within parasitized monocytes and most of the cattle did not show any noticeable CD80 expression (Fig. 5.8). Moreover neonates sustained CD80 expression after NK cell co-culture and a low degree of increase CD80 expression was noticed after NK cell culture which may indicate neonatal monocytes have a greater responsiveness to NK cell co-culture.

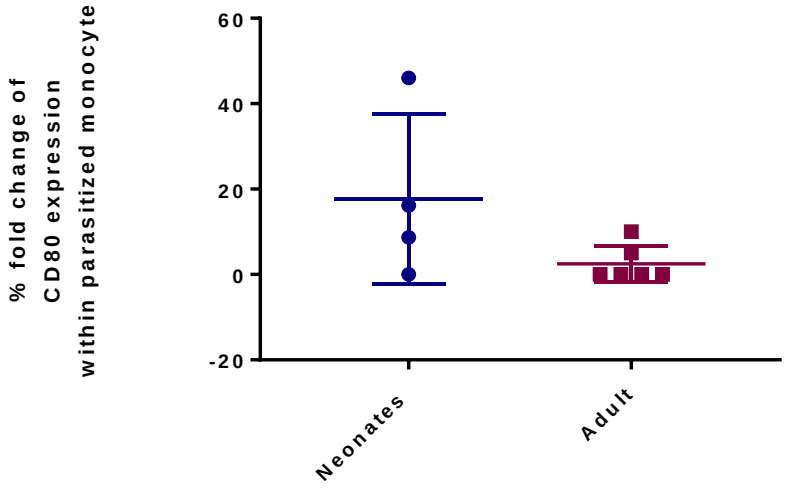


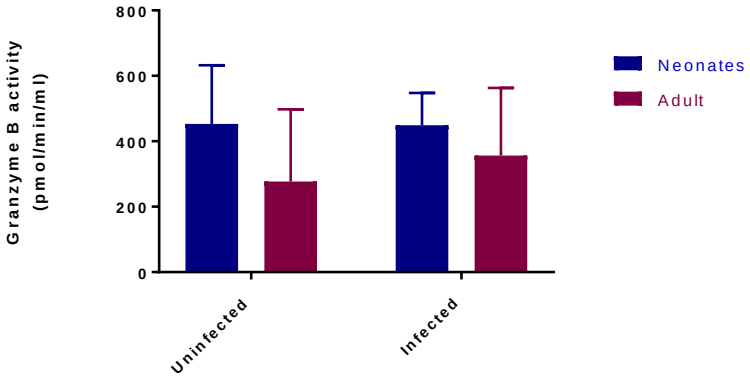
Figure 5.8 Percent fold change of CD80 expression on parasitized monocyte population after NK cell co-culture

CD80 expression was determined within parasitized monocyte population of neonates and adult before and after NK cell co-culture. The Percent fold change of CD80 expression was evaluated using flow cytometry of *N. caninum* infected monocyte after NK cell co-culture of neonates and adult cattle 24 hrs post infection. These data shows median with 95% confidence interval of neonates (n=4) adult (n=6). Results were analysed using Prism Graphpad 7.02 and statistical analysis was performed using a Mann-Whitney test.

5.3.6 Lytic activity of NK cells

Lytic activity of NK cells was evaluated by granzyme B and perforin measurement in co-culture supernatants at 24 hrs post infection. The data demonstrated a relatively higher level of granzyme B enzyme activity in infected NK cells from neonates compared to adults (Fig 5.9 a). However, due to a large variation among neonates no statistical significant difference was observed. Perforin activity was also measured by the ability of collected supernatants to lyse sheep RBCs (as a traditional method). These data suggests that neonates and adult had a similar level of perforin activity at 24 hrs post infection (Fig 5.9 b). Moreover, lytic ability of NK cells were same in both age group with no statistical significant difference among infected *(P value= 0.20)* and uninfected neonates versus adult (*P value = 0.99)*.

(a)



(b)

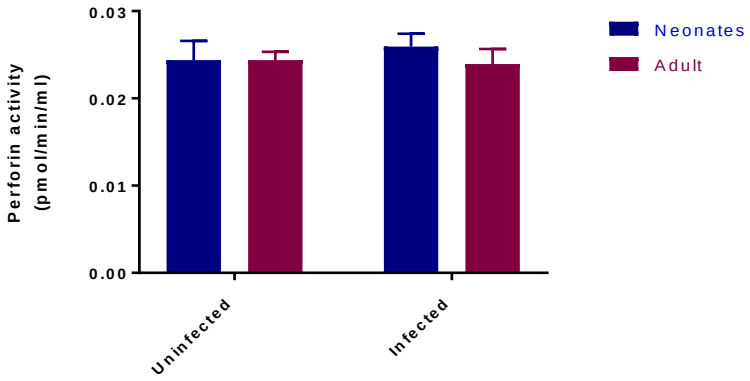


Figure 5.9 Measurements of granzyme B and perforin activity from neonates and adult cattle NK cells

Cytotoxic activity of NK cells was measured by granzyme B activity and perforin activity.

(a) Granzyme B was measured following monocyte NK cell co-culture and *N. caninum* infection. Cell supernatants were collected 24 hrs post infection from infected and uninfected samples and incubated with the AFC substrate in the presence of Granzyme B assay buffer for 30 mins in the dark at 37^0C. The optical density was recorded at 550 nm emission and 365nm excitation filter. These result displayed median with 95% confidence interval of neonates (n=4) and adult (n=5) cattle.

(b) Perforin activity was measured by incubating cell supernatants with 50 µl of diluted sheep RBC in perforin buffer ($1.5x10^8$/ml) for 20 mins at 37^0C. The optical density, as a proxy of released haemoglobin, was recorded at 405 nm absorbance. These data showing median with 95% confidence interval from neonates (n=4) and adult cattle (n=4). Result were analysed using Prism Graphpad 7.02 and statistical analysis was performed by Mann Whitney's test to determine the significance difference between neonates and adult cattle.

5.3.7 Monocyte specific cytokine production- IL-1β and IL-6

To further assess the responses within the infected culture system, the cytokine response of monocytes was determined (Fig. 5.10). Following infection with *N. caninum* tachyzoites, level of pro-inflammatory cytokines increase. These data revealed that neonates were more responsive to *N. caninum* infection and produced greater amounts of IL-1β during infection when compared to adults, which was found to be statistically significant (, *P value= 0.0028*). These data indicates that adult cattle displayed a low level of IL-1β from monocytes during infection as well as control.

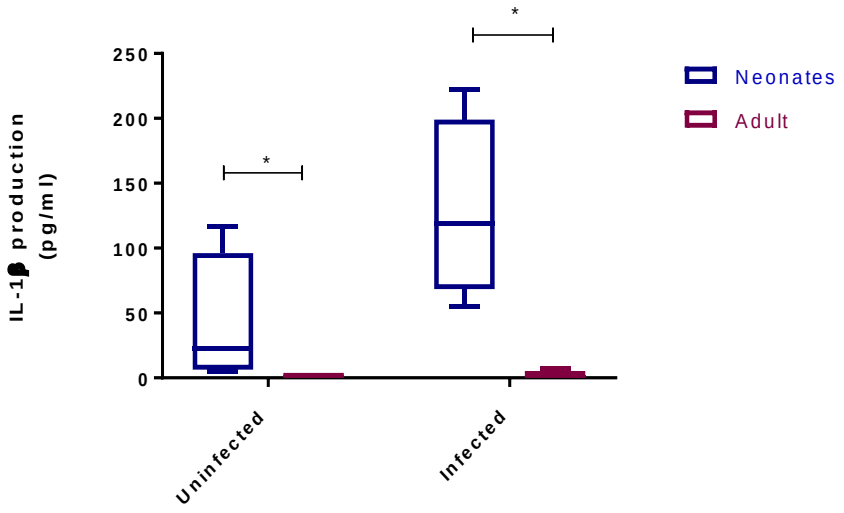


Figure 5.10 IL-1β production from *N. caninum* infected and uninfected monocyte of neonates and adult cattle

IL-1β production was determined from *N. caninum* infected and uninfected $CD14^{+}$ monocytes of neonates and adult cattle at 24 hrs post infection, supernatants were collected and analysed by ELISA in duplicate. These data showing median with 95% confidence interval of neonates (n=4) and adult cattle (n=4). Result were analysed using Prism Graphpad 7.02 and statistical analysis was performed by using Mann-Whitney test. The significance difference was noticed between infected and uninfected monocytes of neonates and adult with *P value=0.0286*.

IL-6 was also evaluated from infected and uninfected monocytes of neonates and adult cattle. These data indicated that adult cattle were not very responsive for IL-6 production also and during an *in vitro N. caninum* infection only one animal among 4 showed IL-6 production from infected and uninfected monocytes (Fig. 5.11). However, neonatal monocytes secreted marginally higher IL-6 when compared with adults; however this was not found to be statistically significant.

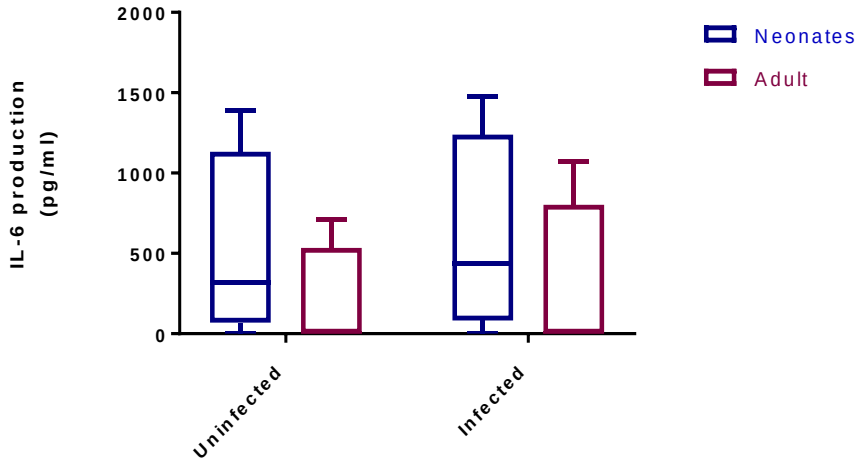


Figure 5.11. IL-6 production from *N. caninum* infected and uninfected CD14^{+} monocyte of neonates and adult cattle

IL-6 production was determined from *N. caninum* infected and uninfected CD14^{+} monocytes of neonates and adult cattle at 24 hrs post infection, supernatants were collected and analysed by ELISA in duplicate. These data showing median with 95% confidence interval of neonates (n=4) and adult cattle (n=4). Result were analysed using GraphPad Prism 7.02 and statistical analysis was performed by using a Mann Whitney test.

The presence of NK cell cytokines such as IL-17A and IFN-γ were also examined from culture supernatants, however results revealed that both the neonates and adult cattle did not produce detectable amounts of either cytokine which may due the relatively brief incubation time period (data not shown).

5.3.8 Microarray analyses

5.3.8.1 Differentially expressed genes in neonates and adult cattle in response to *N. caninum* infection

One-class analysis identified a total of 535 upregulated differentially expressed genes in the infected neonatal monocytes compared to uninfected. While adult cattle showed only 23 upregulated differentially expressed genes using an infected versus non-infected monocyte control comparison (fold change >2). In common to both groups is that all differentially genes were upregulated. The data presented here also compared the baseline set of genes that were differentially expressed between uninfected neonates and adult cattle monocytes where 3764 differentially expressed genes were identified (fold change >2).

The differentially expressed genes were ordered in terms of fold change expression and the top 10 differentially expressed genes are presented in Table 5.1 a & b (see appendix 7 and 8 for full list of differentially expressed genes in neonates and adult respectively). These data demonstrate that the *ICAM3* and *RPL10* genes were the most highly expressed in neonates and adult cattle respectively in response to *N. caninum* infection with a 7.1 fold expression increase over non-infected controls. Among these *ICAM3, CCL2* and *SOCS2* were highly expressed genes in neonates and directly associated with initiation of the immune response; however, potential roles for *TMED4*, a trafficking protein is postulated in the early protein secretory pathway. Furthermore, the *CCDC50* and *CTTN* genes upregulated in neonates are negative regulators of *NF-kB* as well as effectors of epidermal growth factor (EGF) induced-cell migration, respectively. Additionally, *CCDC69* and *SMAP2* are known to be highly expressed within tissues such as fat and lymph nodes respectively (Table 5.1 a).

The annotated data also indicate that only *RPL10* and *TOMM7* genes were common in neonates and adult cattle. This result reveals that the top 10 highly expressed genes in adult cattle fall into multiple biological pathways where immunity is not the primary function. In adult, these genes had functions associated with the cell cycle checkpoint (*SFN*), regulation of cell proliferation (*PHB*), transcriptional

activation (*S1PR2*), cellular adhesion as well as migration (*FN1*) and regulation of actin polymerisation (*PFN2*). *FGR1* is found to be associated with downstream signalling functions via the *PI3K* and *MAPK* pathways that are involved in cell proliferation and cell cycle regulation. While *TOMM7* is associated with mitochondrial protein import towards cells (Table 5.1 b).

Table 5.1 a. Summary of highly expressed differentially genes in neonatal monocytes involved in immune response pathways during *N. caninum* infection

Gene name and symbol (Neonates)	Fold change expression	*P value*	Functions
Intercellular adhesion molecule 3 (ICAM3)	7.1	0.0026784	It is an intercellular adhesion molecule that present in all leukocytes and ligand for lymphocyte function associated antigen -1 in the initiation of the immune response
C-C motif chemokine ligand 2 (CCL2)	5.0	0.0026784	Chemotactic factor that attracts and involved in recruitment of monocytes.
Transmembrane p24 trafficking protein 3 (TMED3)	4.7	0.0026784	It is related to transport to the Golgi and potential role in vesicular protein trafficking, mainly in the early secretory pathway.
Coiled-coil domain containing 69 (CCDC69)	4.7	0.0026784	Act as a scaffold to regulate the recruitment and assembly of spindle midzone components in chromosomal segregation during cell cycle.
Suppressor of cytokine signaling 2 (SOCS2)	4.7	0.0026784	Negative regulators of cytokine receptor signaling via the Janus kinase/signal transducer and activation of transcription pathway (the JAK/STAT pathway)
Coiled-coil domain containing 50 (CCDC50)	4.1	0.0028224	Involved in epidermal growth factor (EGF) signalling
Cortactin (CTTN)	4.1	0.0074129	Contributes to the organization of the actin cytoskeleton and cell shape
Ribosomal protein L10 like (RPL10L)	4.1	0.0074129	Plays a role in the formation of actively translating ribosomes
Cytochrome P450 family 1 subfamily B member 1 (CYP1B1)	4.0	0.0084992	Plays an important role in the regulation of perivascular cell proliferation, migration, and survival through modulation of the intracellular oxidative state and NF-kappa-B expression and/or activity, during angiogenesis
Small ArfGAP2 (SMAP2)	3.9	0.0084992	Important role in endocytosis and activates ADP-ribosylation factor 6.

This table showing the top 10 most highly expressed gene by microarray analysis in neonatal monocytes in response to *N. caninum* infection. This list showing the upregulated genes in *N. caninum* infected monocytes versus uninfected control of neonates with their fold expression (>2) and *P value =0.05*.

Table 5.1 b Summary of highly expressed differential expressed genes in adult monocytes involved in immune response pathways during *N.caninum*

Gene name and symbol (Neonates)	Fold change expression	*P value*	Functions
Ribosomal protein L10 like (RPL10L)	7.1	0.0001338	Plays a role in the formation of actively translating ribosomes
Profilin 2 (PFN2)	5.6	0.024019	Regulates actin polymerization in response to extracellular signals
Translocase of outer mitochondrial membrane 7 (TOMM7)	5.3	0.024019	Regulates the assembly and stability of the translocase complex during metabolism of protein
Stratifin (SFN)	5.3	0.024019	Cell cycle checkpoint protein and plays a role in preventing DNA errors during mitosis
Peptidyl-prolyl cis-trans isomerase A Peptidyl-prolyl cis-trans isomerase A, N-terminally processed PPIA)	5.3	0.024019	Accelerates the folding of proteins and acts as a cyclosporin binding-protein may play a role in cyclosporin A-mediated immunosuppression
Fibronectin 1 (FN1)	5.3	0.024019	Involved in cell adhesion and motility, opsonization, wound healing and maintenance of cell shape
Ubiquitin C-terminal hydrolase L1 (UCHL1)	5.3	0.024019	Involved both in the processing of ubiquitin precursors and of ubiquitinated proteins
Sphingosine-1-phosphate receptor 2 (S1PR2)	5.3	0.024019	Participates in cell proliferation, survival, and transcriptional activation
Prohibitin (PHB)	5.2	0.024019	It has a role in regulating proliferation and inhibits DNA synthesis
Fibroblast growth factor receptor 1 (FGFR1)	5.2	0.024019	Mitogenic signaling molecules that have roles in angiogenesis, wound healing, cell migration, neural outgrowth and embryonic development

This table showing a summary of highly expressed top 10 genes in adult monocytes in response to *N. caninum* infection by microarray analysis. This list showing the upregulated genes in *N. caninum* infected monocytes versus uninfected control of adult with their fold expression (>2) and *P value =0.05*.

5.3.8.2 Biological process related with immune response in neonates and adult cattle

The biological processes associated with the immune response were evaluated those identified in the array. The visual exploration was performed from the list of upregulated differentially expressed genes of neonates and adult cattle to see the protein-protein interaction in various biological pathways by network analyst programme (Xia et al., 2015). These microarray data identified a total of 120 altered biological pathways in neonatal monocytes and 53 pathways in adult cattle monocytes. Of these pathways, 25 pathways in neonates and 10 pathways in adult monocytes were directly related to the immune response (see Appendix 9 and 10 for complete list).

A side-by-side comparison of the immune related biological processes indicated that in both neonates and adult monocytes, the JAK-STAT cascade, tyrosine phosphorylation of STAT and regulation of NF-kB transcription factor activity were key pathways altered during infection with *N. caninum* tachyzoites. These pathways were related to production of pro-inflammatory cytokines and reduction of parasitaemia; however magnitude of expression is more in neonates compared to adult cattle (Fig. 5.12). Additionally, regulation of cyclin-dependent protein kinase activity and Rho protein signal transduction pathways demonstrate that neonates and adult cattle have a regulated cell cycle and cell proliferation activity. However, a highly transcribed lysosomal transport and apoptosis signalling pathway in neonates indicate a greater cytotoxic killing during *N. caninum* infection compared to adult.

These common biological pathways also demonstrate that neonates have more number of upregulated genes associated with each pathways (Table 5.2). These data indicate that all biological pathways were upregulated to a greater degree in neonates compared to adults cattle and those have the potential to make a greater contribution to the immune response.

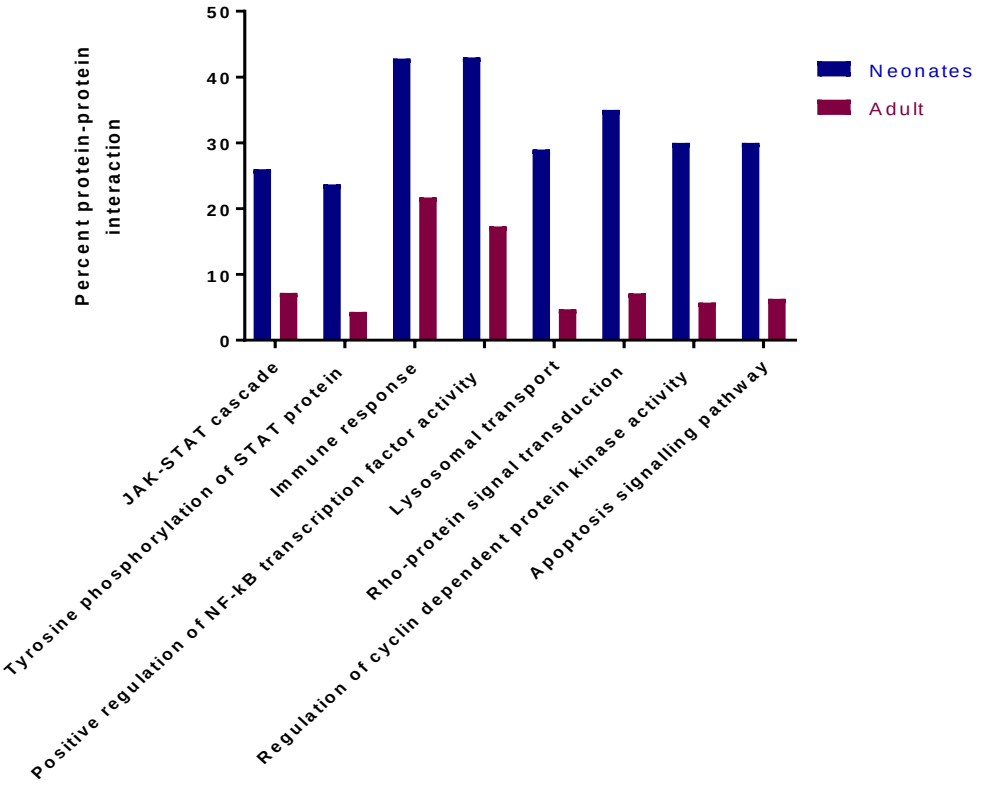


Figure 5.12 Schematic representation of different biological pathway in neonates and adult cattle by microarray analysis

Common immune related KEGG pathways which were upregulated in neonatal and adult cattle monocytes infected with *N. caninum* versus uninfected monocytes. These bars represent the percentage of estimated protein-protein interactions in common biological immune responses pathway of neonates (n=3) and adult (n=2) cattle.

(% change in protein-protein interaction is estimated in microarray as follows the number of upregulated genes in the pathways/Total number of genes in the pathway X100).

Table 5.2 Description of common biological pathway which were upregulated in neonates and adult cattle monocytes in response to *N. caninum* infection (24 hrs post infection)

Biological pathway associated with immune response	Age group	Total no. of gene in pathway	No. of upregulated gene	% of microarray estimated protein-protein interaction
JAK-STAT cascade	Adult	291	21	7.2%
	Neonates	291	77	26%
Tyrosine phosphorylation of STAT protein	Adult	232	10	4.3%
	Neonates	232	55	23.7%
Immune response	Adult	28	5	21.7%
	Neonates	28	12	42.8%
Positive regulation of NF-kB transcription factor activity	Adult	23	4	17.3%
	Neonates	23	10	43%
Lysosomal transport	Adult	62	3	4.7%
	Neonates	62	18	29%
Rho-protein signal transduction	Adult	28	2	7.14%
	Neonates	28	10	35%
Regulation of cyclin dependent protein kinase activity	Adult	52	3	5.7%
	Neonates	52	16	30%
Apoptosis signalling pathway	Adult	63	4	6.3%
	Neonates	63	19	30%

Common biological pathways associated with primarily immune response during *N. caninum* infection in adult and neonates monocytes. This table shows no. of gene present in each pathways and no. of upregulated genes in corresponding pathways in adult and neonates. Percent of microarray estimated protein-protein interaction was determined by no. of total genes present in pathway/ no. of upregulated genes in pathwayX100.

5.3.8.3 Important protein-protein interaction pathway in neonates and adult cattle

5.3.8.3.1 JAK-STAT Cascade pathway

To evaluate the impact of upregulated genes in the major biological pathway identified above, protein-protein interaction analysis was performed by using network analyst programme. These interaction data suggest that neonates have larger and more complicated networks involving larger numbers of upregulated genes in comparison to adult cattle where the same networks display simpler and fewer interaction (Fig. 5.13). JAK-STAT cascade network in neonates was characterised by upregulation of 77 gene including *IL-6ST, ILK, MYC, BCL2* and *RPL11*. In adult cattle 3 upregulated genes *CCND1 RPS9* and *FGFR1* found that were upregulated in neonates also. In adult cattle the JAK-STAT cascade and positive regulation of cytokines pathway were associated with the cell cycle and growth related gene *CCND1*.

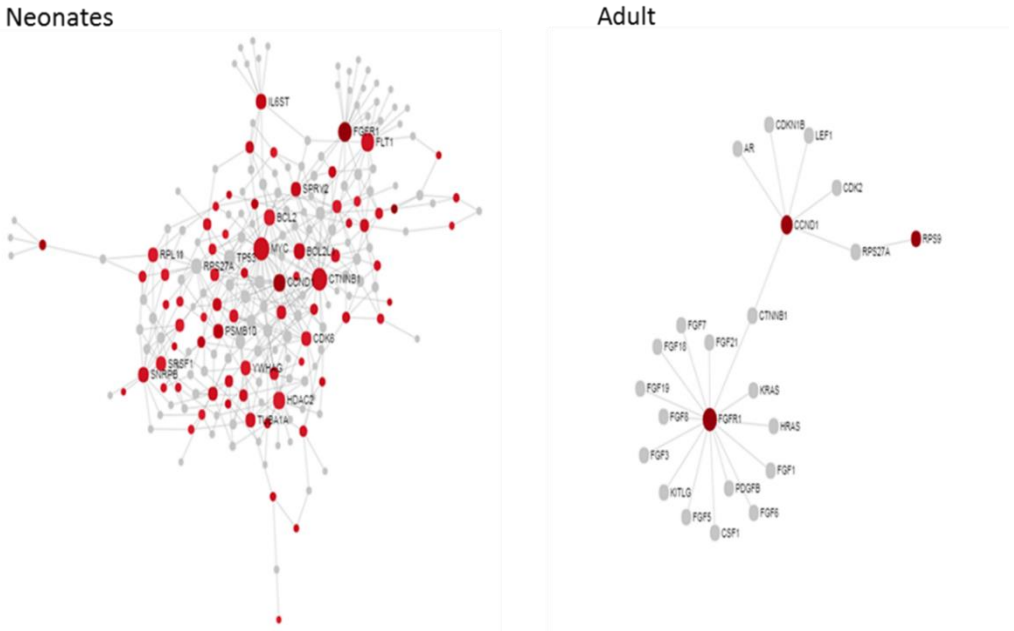


Figure 5.13 Upregulated genes network of JAK -STAT cascade pathway in neonates and adult cattle

Changes in the JAK-STAT gene network as analysed by microarray to estimate and visualise the upregulation in protein-protein interactions. The network diagrams show changes in members of the JAK-STAT cascade and their interactions in response to *N. caninum* infected versus uninfected control neonatal and adult cattle monocytes 24 hrs post infection. These red nodes represent the upregulation of various genes during protein-protein interaction while grey nodes represent no change of genes expression in corresponding pathway. These data show a high number of genes upregulated in neonates, with subsequent increases in network interactions, while adult cattle harboured fewer upregulated genes and fewer interactions in the pathway**.**

5.3.8.3.2 Tyrosine phosphorylation of STAT protein

Analysis of the tyrosine phosphorylation of STAT proteins pathway indicated involvement of *CCND1* and *FGFR1* genes in the adult, these also being important in the cell cycle and cell proliferation. However, neonates have shown a wider range of upregulated gene including *IL-6ST*, *MAP3K1, FGFR1, MYC, CCND1, RPL11, SPRY2, HDAC2, FLT1, CTNNB1* and *BCL2* (Fig. 5.14). Among them *HDAC2* was associated with transcription regulation and cell cycle however, *RPL11* enhances the *TP53* activation that is also known as tumor protein P53 gene and act as a tumor suppresser via controlling over proliferation and regulation of cell division and aids in synthesis of protein in cells

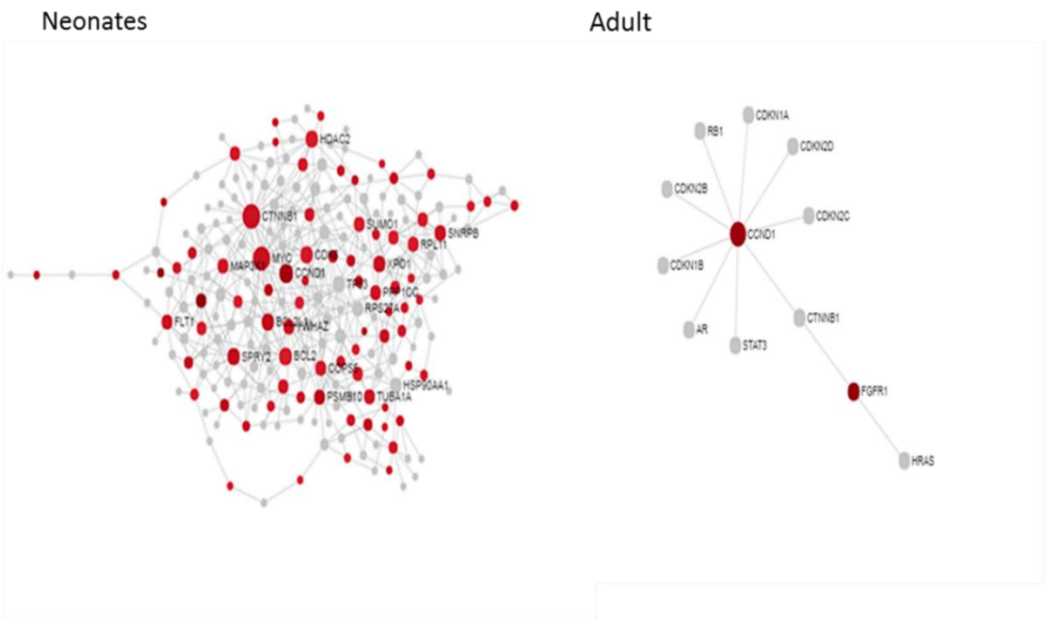


Figure 5.14 Upregulated genes network of tyrosine phosphorylation of STAT protein in neonates and adult cattle

Changes in the tyrosine phosphorylation of STAT protein network as analysed by microarray to estimate and visualise the upregulation in protein-protein interactions. The network diagrams show changes in members of the tyrosine phosphorylation of STAT protein and their interactions in response to *N. caninum* infected versus uninfected control neonatal and adult cattle monocytes 24 hrs post infection. These red nodes represent the upregulation of various genes during protein-protein interaction while grey nodes represent no change of genes expression in corresponding pathway. These data show a high number of genes upregulated in neonates, with subsequent increases in network interactions, while adult cattle harboured fewer upregulated genes and fewer interactions in the pathway**.**

5.3.8.3.3 Positive regulation of cytokine secretion

Positive regulation of cytokines production represents the largest and most altered pathway during *N. caninum* infection in neonatal monocytes and it was characterised by upregulation of *BCL2* and *BCL2L1* that play important role in protection against apoptosis, auto-immune disease and cancers (Fig. 5.15). c-*MYC* is an important transcription factor and regulates expression of 15% of genes and play important roles in proliferation of B-cells. While in adult cattle positive regulation of cytokines secretion pathway is only characterised by upregulation of *CCND1* gene.

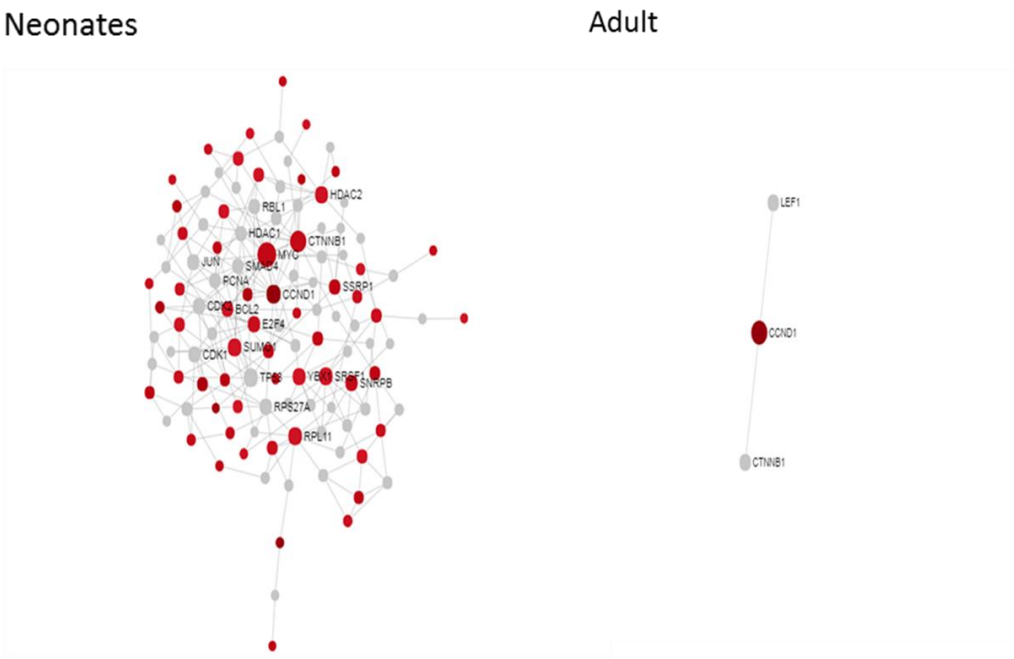


Figure 5.15 Upregulated genes network of positive regulation of cytokines secretion in neonates and adult cattle

Changes in the positive regulation of cytokines secretion network as analysed by microarray to estimate and visualise the upregulation in protein-protein interactions. The network diagrams show changes in members of the positive regulation of cytokines secretion and their interactions in response to *N. caninum* infected versus uninfected control neonatal and adult cattle monocytes 24 hrs post infection. These red nodes represent the upregulation of various genes during protein-protein interaction while grey nodes represent no change of genes expression in corresponding pathway. These data show a high number of genes upregulated in neonates, with subsequent increases in network interactions, while adult cattle harboured fewer upregulated genes and fewer interactions in the pathway**.**

5.3.8.3.4 Immune response pathway

Immune response pathway related genes were also compared in neonates and adult and found *CCND1, FGFR1, MYC, IL6ST*, *SPRY2, STRAP* and *FLT1* genes were upregulated in neonates however, only *CCND1* and *FGFR1* upregulated in adult cattle. These genes were associated with cell cycle, cell division and regulation of cell growth (Fig. 5.16). *SPRY2* was seen as a negative regulator of fibroblast growth factor (*FGR*) and *STRAP* was associated with negative regulation of TGF-β signalling pathway and positive regulation of *PDPK1* kinase activity.

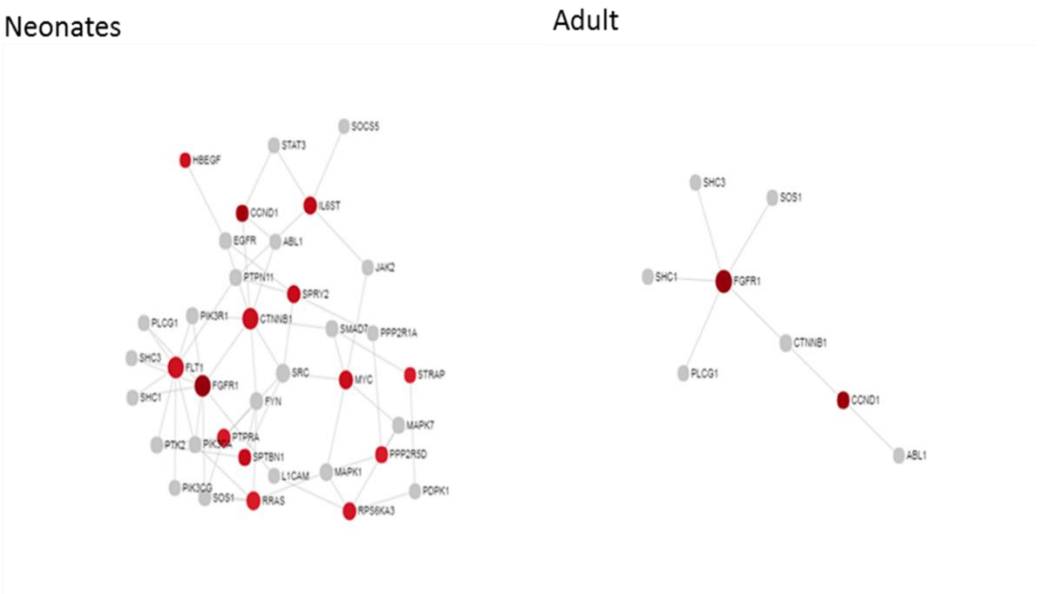


Figure 5.16 Upregulated genes network of immune response in neonates and adult cattle

Changes in immune response network as analysed by microarray to estimate and visualise the upregulation in protein-protein interactions. The network diagrams show changes in members of the immune response and their interactions in response to *N. caninum* infected versus uninfected control neonatal and adult cattle monocytes 24 hrs post infection. These red nodes represent the upregulation of various genes during protein-protein interaction while grey nodes represent no change of genes expression in corresponding pathway. These data show a high number of genes upregulated in neonates, with subsequent increases in network interactions, while adult cattle harboured fewer upregulated genes and fewer interactions in the pathway**.**

5.3.8.4 Interaction between neonates and adult cattle control group to identify the baseline difference in monocyte response

5.3.8.4.1 IL-1 secretion pathway

In order to investigate the baseline differences across neonates and adult cattle, control group were compared. The data presented here shows a greater degree of interaction in uninfected neonates compared to adult cattle and a total 3764 differentially expressed upregulated genes (Fc>2) identified when neonatal monocytes were compared to adult cattle monocytes. However, none of genes were differentially expressed in infected neonates versus infected adult.

These analysed data displays a wider variety of biological pathways were upregulated in uninfected control neonatal monocytes compared to adult cattle; notable amongst this the IL-1 secretion pathway (Fig. 5.17). IL-1 secretion pathway was analysed across control group of neonates and adult cattle and 19 genes were significantly expressed in neonates cattle, compared to adults including *CARD9, BCL10, NKKB1, NKFB2* and *TRAF6* that are important genes related to innate immune response and cytokine secretion.

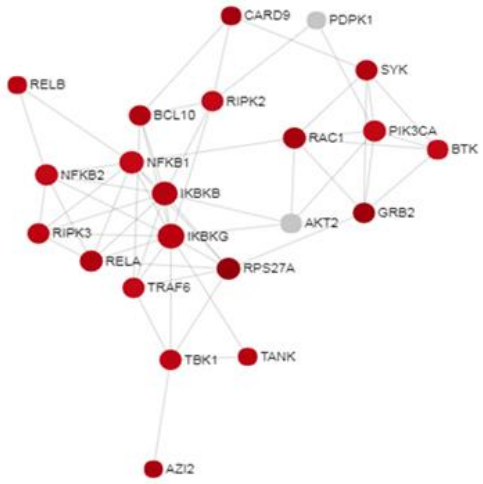


Figure 5.17 Upregulated genes network of IL-1 secretion in uninfected neonates versus adult cattle

Changes in IL-1 secretion network as analysed by microarray to estimate and visualise the upregulation in protein-protein interactions. The network diagrams show changes in members of the IL-1 secretion and their interactions in response to *N. caninum* uninfected neonates versus uninfected control adult cattle monocyte after 24hrs post infection. These red nodes represent the upregulation of various genes during protein-protein interaction while grey nodes represent no change of genes expression in corresponding pathway. These data showing a high baseline expression in neonates compared to adult cattle in corresponding pathway**.**

5.3.8.4.2 Positive regulation of NF-kappa B transcription activity

Regulation of NF-kappa B transcription activity was also analysed and a variety of genes were found to be highly expressed in neonates compared to adults including *STAT3, NFKB1, RAC1, MAPK3, RAP1A, IRAK1, CDK2*, *MED14, MED28, CCNC, RPS8, RPS3, CCNT2, CDK9* and *SFN* genes (Fig. 5.18). Furthermore, SFN gene expression was also seen during NF-kappa B transcription pathway of infected neonates and adult cattle.

These analysed data indicate a much more complex pathways and transcription of new range of genes in uninfected control group of neonates. Additionally, these results indicate transcription of more number of genes includes *CD40, CARD9, TLR4, MYD88, IRF3, TLR4, TRAF6, TRAF3 and CASP1*. Among them

TLR4, CARD9, CD40, CASP1 and *MyD88* play important role in activation of innate immune response via pathogen recognition and antigen presentation and secretion of pro-inflammatory cytokine IL-1β. Additionally, *TRAF3* and *TRAF6* are *TNF* family associated receptor factors and involved in activation of innate immune response via *CD40* and *TLR* and *IL-1*.

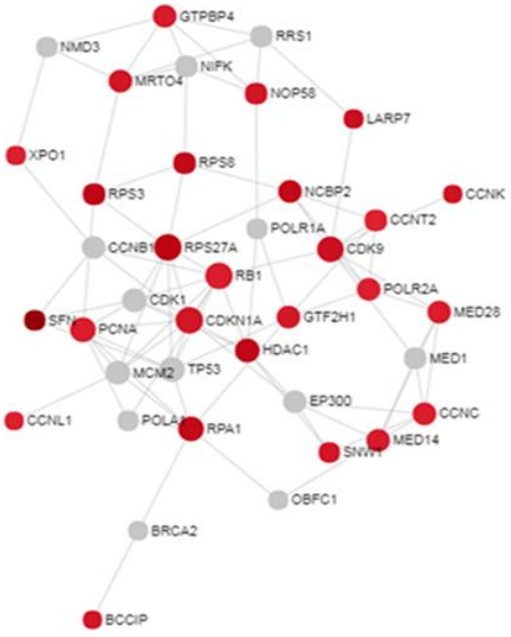


Figure 5.18 Upregulated genes network of positive regulation of NF-kappa B transcription activity in uninfected neonates versus adult cattle

Changes in the positive regulation of NF-kappa B transcription activity network as analysed by microarray to estimate and visualise the upregulation in protein-protein interactions. The network diagrams show changes in NF-kappa B transcription members and their interactions in response to *N. caninum* uninfected neonates versus uninfected control adult cattle monocyte after 24 hrs post infection. These red nodes represent the upregulation of various genes during protein-protein interaction while grey nodes represent no change of genes expression in corresponding pathway. These data showing a high baseline expression in neonates compared to adult cattle in corresponding pathway**.**

5.4 Discussion

5.4.1 Degree of monocyte invasion

Neosporosis is a primary cause of abortion and reproductive failure in cattle. In the absence of effective treatment, better understanding of the immune response will facilitate future success in vaccination. To further this understanding the age-dependent differences in monocyte and monocyte NK cells interaction was evaluated in the context of infection. The most striking finding was that $CD14^{+}$monocyte from adult bovine were more susceptible to invasion by *N. caninum* tachyzoites and the total percentage of parasitized monocytes was higher when compared to neonates indicating adult monocytes sustained higher levels of parasite growth.

Prior studies also support this primary finding, rodent studies of parasite infection using oral inoculation of a virulent strain of *T. gondii* in mice showed that 1-5 day old mice had a parasitaemia confined to the brain, liver, lungs and spleen however 30 day old mice presented with disseminated *T. gondii* tachyzoites in all organs suggesting an inability to contain parasite replication (Guerrero et al., 1995). Similar findings was also reported in 11 month old mice which harbour higher numbers of *T. gondii* tissue cysts compared to 4 month old mice (Gardner and Remington, 1978) thus suggesting that host age is an important factor in control of parasite replication and number of latent cysts may also increase with age.

Experimental infection suggests timing of pregnancy and the age of foetus are two major influences in the different outcomes in *N. caninum* infection (Williams et al., 2000) thus the precedent for age-related responses in *N. caninum* infection has been established. It is assumed that the foetus becomes immunocompetence by 100 days of gestation period and immune response is fully developed at 120 days where it can exhibit cellular immune responses (Tierney and Simpson-Morgan, 1997; Hein et al., 1988; Almeria et al., 2003; Almeria et al., 2010). Foetal immunocompetence could explain the restriction of *N. caninum* to the foetal central nervous system, skeletal muscles and heart at stages of 20-32 weeks of pregnancy (Rosbottom et al., 2011).

Moreover, a direct comparative study allowed researchers to conclude that cattle infected in early gestation (day 70) showed extensive disseminated necrosis including brain, spinal cord as well as liver that cause tissue destruction and abortion of foetus; while later in gestation (day 210) the foetus appears to become immunologically mature and thus able to limit infection within brain and spinal cord thereby preventing abortion (Williams et al., 2003; Innes et al., 2001; Gibney et al., 2008). These studies support the concept that foetal immune competence could inhibit multiplication of parasites and as suggested by data presented here, this heightened competence continues after birth. Thus, it could be assumed that increasing age of the foetus and immediately in the neonatal period there is a reduced risk of abortion and infection respectively with the parasite load being significantly lower during third trimester of pregnancy compared to first and second trimester (Collantes-Fernández et al., 2006; Williams et al., 2000; Gibney et al., 2008) or later in life.

5.4.2 Monocyte response in term of CD80 expression and cytokine secretion

The study presented here also evaluated the role of CD80 which is a co-stimulatory molecule and necessary for activation of T cells. CD80 expression was determined on *N. caninum* infected and control monocytes with and without presence of NK cells. These data reveal a high increase in CD80 expression after *N. caninum* infection where neonates displayed a high level of expression compared to adult cattle (Fig. 5.7 a). Previous studies correlate with these results, where *N. caninum* infected murine macrophages and splenic DCs showed increased expression of CD80 compared to uninfected controls (Veeraseatakul & Chutipongvivate, 2005; Abe et al., 2014; Abe et al., 2015). However infected monocyte NK cell co-culture was not able to maintain the expression of CD80 and it was continuously decreased in both neonates and adults (Fig. 5.8). This could be evidence of parasites modulation of signalling, diminishing the expression of CD80 in bovine monocyte. This hypothesis could be supported by evidence that parasite secreted/excreted antigen down regulates the PI3K/AKT and p38 MAPK signalling pathways downstream of GPCR signalling thereby increasing parasites replication (Mota et al., 2016).

Monocyte specific function was also assessed through detection of the cytokine response to *N. caninum* infection in adult and neonatal monocytes. The results obtained have shown induction of IL-1β was significantly higher in *N. caninum* infected monocytes of neonates compared to adult. This result is supported by microarray findings where the IL-1 production pathway was upregulated in uninfected neonates groups with increased expression of *CARD9, BCL10, NFKB1, NFKB2, TRAF6, IKBKB* and *IKBKG* genes. It is a key pathway during acute inflammatory response and upregulation of this pathway would underlie the enhanced secretion of IL-1 β from *N. caninum* infected monocytes. IL-1β has important role in innate immune response and impairment of *NALP3* inflammasome leads to decreased production of IL-1β during *N. caninum* infected macrophage (Wang et al., 2017). Furthermore, previous work suggests increased IL-1β production from LPS primed and *N. caninum* infected bovine macrophages (Flynn and Marshall, 2011).

Additionally, a high level of IL-6 was found in neonatal monocyte, indicating a trend towards higher pro-inflammatory processes (Fig. 5.11) that is supported by the microarray findings related to the upregulated JAK-STAT pathway in neonates and characterised by high levels of expression of genes such as *IL6-ST* that is an important ligand to activate tyrosine phosphorylation of stat protein while other genes *such as ILK, FGFR1, MYC, BCL2 and CCND1* were associated with integrin mediated signal transduction and cell cycle progression, apoptosis and cellular transformation. Interestingly overexpression of these genes leads to cancer and other tumors. These data indicate that IL-1β and IL-6 are important pro-inflammatory cytokines of monocytes/macrophages that helps to achieve protective immune response during intra-cellular parasitic infection. Previous bovine studies noted elevated production of IL-6 and IL-1β from *N. caninum* infected adult fibroblast at 72 hrs post infection (Peckham et al., 2014) but did not make a direct comparison with neonatal fibroblasts, the divergence in this work might stem from the different cells types used. From murine studies, it is well established that macrophages are the main responder cell controlling intra-cellular protozoan infection such as *N. caninum and T. gondii* multiplication via secretion of IL-1β (Wang et al., 2017; Gorfu et al., 2014).

Furthermore, macrophage deficient mice are more sensitive to infection and show severe signs of *N. caninum* pathology (Abe et al*.,* 2014).

5.4.3 Effect of monocyte NK cell co-culture on parasites

In this study, monocytes and NK cells were co-cultured with *N. caninum* tachyzoites and a reduction in parasitaemia was apparent. This reduction was more pronounced in neonates compared to adults (Fig. 5.4). Previous work demonstrated that during intra-cellular protozoan infection, NK cells carry out a key roles in lysing infected fibroblast cells (Korbel et al., 2004) and an age impairment of NK cells function has been reported in terms of their cytotoxic activity (Solana et al., 1999; Tarazona et al., 2012). To determine if cytotoxic granules of NK cells were released in the co-culture system granzyme B and perforin activity was measured. Results indicated a relatively higher level of NK cells cytotoxicity in neonates which could be an additional mechanism underlying the age related difference in immune responses to *N. caninum*. Previous studies showed that neonates NK cells have greater proliferation activity compared to adult cattle (Elhmouzi-Younes et al., 2009). A peak NK cells cytotoxic activity can be detected after few hours to days (11) post *N. caninum* infection which correlated with an increased number of NK cells in peripheral blood during infection (Biron et al., 1999; Klevar et al., 2007). The higher granzyme activity could be linked to neonatal NK cells expressing greater levels of NKp46; as this ligand is directly associated with cytotoxic activity of NK cells (Elhmouzi-Younes et al., 2009).

There is some evidence that NK cells are among the first cells which produce IFN-γ that prime resting macrophages into activated, lysed infected cells and are responsible for control multiplication of *N. caninum* in calves (Klevar et al., 2007). Previous murine studies reported a substantial age related decline in the competence of NK cells to effect lysis of YAC-1 target cells in response to *Trypanosome musculi* infection (Albright and Albright, 1983). Moreover, studies demonstrated that depletion of NK cells prior to infection, rendered mice highly susceptible to *T. gondii* (Goldszmid et al., 2007). Hence, it can be concluded that aging not only affects the number of NK cells but also their functional abilities where NK cells provide a key to anti-protozoal immunity.

Expression of IL-17A and IFN-γ was analysed as specific cytokines produced by NK cells from monocytes NK cells co-culture supernatants (where NK cells were pre-treated with IL-15) but cytokine levels were below the limit of detection, which may be due to the short incubation period (24 hrs) of culture. Supporting this finding that the JAK-STAT pathway did not show upregulation of IFN-γ related gene (*IRF* or *STAT*) during *N. caninum* infection of monocytes in adult or neonates that could be possible reason to show low level of IFN-γ secretion during monocytes NK cell co-culture. Prior work has shown that *IRF* gene expression is essential in macrophages for downstream IL-17 or IFN-γ induction (Krausgruber et al., 2011). Even non-detectable levels of IFN-γ and IL-17A level cannot rule out the importance of NK cells during *N. caninum* infection. The lytic activity of NK cells via granzyme B able to support the important role of NK cells in controlling parasites. Other *in vitro* bovine study elucidated a difference in the responses of NK cells in neonates (6-7 days) and calves (6 month) where lower production of IFN-γ was reported by neonatal bovine NK cells which were stimulated with IL-15, compared to IL-2, for 7-10 days and further incubated with IL-12 for 24hrs (Elhmouzi-Younes et al., 2009).

5.4.4 Monocytes gene expression

In order to further explore our results, gene expression was performed using microarray from *N. caninum* infected $CD14^{+}$ monocytes of neonates and adult cattle. It was observed that neonates were more responsive to infection, judged by high number of differentially expressed genes. Additionally, most of the genes in neonates were related to the immune response however, adult cattle have shown lower levels of differentially expressed genes with fewer immune-related targets. Some important biological pathways were also identified in neonates and adult cattle but it was noticed a greater range of pathways were affected in neonates compared to adult cells. Immune response related pathways were compared in neonates infected/control and adult infected/control and it was found that a high magnitude of networks in neonates were related to activation and regulation of the immune response.

The JAK-STAT cascade pathway was upregulated in neonates as well as adult cattle and this is an important signalling pathway in immune regulation and cell proliferation. The data showed that JAK-STAT signalling pathway was much more functional in neonates with upregulation of *IL6ST, CDK6, FGFR1, MYC, BCL2, BCL2L1* and *CCND1* genes. The function of *IL-6ST* gene was associated with IL-6 secretion, whereas *BCL2* was involved in prevention of apoptosis of lymphocytes. Furthermore, regulation of cell cycle and proliferation to supress oncogenesis through expression of *CDK6, FGFR1, MYC, SPRAY2, HDAC2, RPL11, PBMB10, CTNND1* was observed. However, adult cattle showed upregulation of *FGFR1, RPS9* and *CCND1* genes that are associated with cell cycle and cell growth related events.

The array data did not show direct upregulation of *STAT3* in neonates or adult in response to *N. caninum* infection. This may be explained by the finding that *SOCS2* was upregulated and noted as top 10 highly expressed gene in neonates. *SOCS2* is also a well established negative regulator of JAK-STAT signalling (Yoshimura et al., 2007; Johnston and O'Shea, 2003) that may explain the greater induction of IL-6 from *N. caninum* infected monocyte of neonates. However, prolonged activation of *STAT3* leads to induction of anti-inflammatory cytokine IL-10 secretion that can cause apoptosis of host cell. Previous data indicate that *STAT3,* induced by IL-10 has negative impact on macrophages anti-inflammatory functions (Takeda et al., 1999).

The study presented here found upregulated *STAT3* in the positive regulation of T cells proliferation pathway of uninfected neonates group; that is well known for the regulation of IL-12, IL-10, cellular respiration and regulation of *CCND1* and *BCL* that control cell cycle (Ellis et al., 2010; Wegrzyn et al., 2009 ; Kortylewski et al., 2009; Acosta-Iborra et al., 2009). Further studies confirm that continuous phosphorylation of *STAT3* can cause NcROP16 induced host cell apoptosis *during N. caninum* infection (Ma et al., 2017).

The tyrosine phosphorylation of STAT Protein and immune response pathways were also upregulated in neonates and displayed higher expression of various genes including *HDAC2, CCND1, TP53, CDK1, CDK2, RPL11, E2F4, SUMO1, MAPK3, MYC, RPS27, XPO1* and *SNRPB*. These genes were associated with regulation of cell cycle and suppression of tumor or cancer cells.

However, adult cattle have shown upregulation of *FGFR1* and *CCND1*. Cell proliferation, migration, differentiation and apoptosis were the key functions affected by these genes in the pathway. These process are crucial for haematopoiesis and formation of immune cells however overexpression of these pathways can leads to neoplastic growth and auto-immune diseases (Calò et al., 2003; O'Shea and Plenge, 2012).

Another major finding in this study was the upregulation of large numbers of genes involved in the positive regulation of cytokine production in neonates compared to adult in response to *N. caninum* infection. In the support of these data, previous studies reported upregulation of well known pathways in immune response against *N. caninum* such as JAK-STAT cascade, *MyD88* and regulation of *NF-kB* transcription in the mouse (Mineo et al., 2009; Ellis et al., 2010). Additionally, mice infected with *T. gondii* only mount a sufficient inflammatory response through the presence of intact *TLR, MyD88, NF-kB*, and *MAPK* signalling (Denkers, 2010; Kim et al., 2006). A high baseline of differentially expressed genes (3764 genes) within the uninfected group was noticed across neonates and adult cattle. Interestingly, a wide range of biological pathways were upregulated in neonates that indicate a fundamental difference in immune capacity.

NF-kB transcription factor activity that plays a central role in immune and inflammatory processes showed an upregulation of *RPS3, MED14, MED28, LARP7, CCNC, HDAC1,SFN, RB1, CDK9* and *CCND1* in neonates. However, only *SFN* gene was upregulated in adult cattle which may explain why neonates more effectively reduce parasitaemia. NF-kB transcription factor often associates with the stress response, regulation of cell proliferation and apoptosis and dysregulation of NF-κB pathways leads to immunodeficiency, autoimmunity and cancer (Courtois and Gilmore 2006). The important role of NF-kB during *N. caninum* infection was described in previous work where NF-kB inhibition before priming of macrophages leads to reduce protection and higher parasitaemia (Flynn and Marshall, 2011), furthermore, an increase activity of NF-kB was seen during *T. cruzi* infection that directly associated with clearance of parasites from host body (Hall et al., 2000).

The positive immune response pathway also showed that there was greater expression of *TRAF6* and *CD40* both associated with *TNF* receptor family signalling; *CD40* activates *TRAF1, TRAF2* and *TRAF6*. They are associated with wide range of immune and inflammatory response such as switching of T cell dependent immunoglobulin and memory B cell development (Grewal and Flavell, 1998). Finally, the positive immune response pathway showed upregulation of *TLR4, MyD88* and *TRAF3* that play a basic role in pathogen recognition and innate immunity. The upregulated expression of *MyD88* in uninfected monocytes from neonates could suggest a higher pro-inflammatory immune response in neonates upon infection. Moreover, they also support previous work that demonstrated a role for *MyD88* in murine *N. caninum* infection. Mice deficient in *MyD88* produced only basal levels of IL-12 and IFN-γ but higher levels of IL-10 from 3-7 day post infection (Mineo et al., 2009).

From these data it can be concluded that the neonatal immune response is significantly different to that of the adult upon *N. caninum* infection. Differential gene expression and functional analysis shows a more wide ranging robust response in neonates leading to increased monocyte cytokine production, reduced parasitemia and stronger monocyte NK cell interactions. There is a fundamental difference in how neonates and adults respond and this is evident from the baseline network analysis. The data here presents an opportunity to tailor vaccines such that they use pre-primed immune responses.

Chapter 6 General discussion and conclusions

6. General Discussion

The present work focused on age-related differences in the immune responses of cattle during *N. caninum* infection.

6.1 Monocyte functional study

The immune response upon stimulation was assessed to determine if monocyte function across young and adult cattle was altered in terms of pro-inflammatory cytokine secretion. These results confirmed that in young cattle blood-derived monocytes were more responsive to stimulants such as alum and LPS/IFN-γ. Additionally, neonatal alveolar macrophages continued to express significant levels of IL-1β, TNF-α and IL-12 secretion with low levels of IL-10 production. These data suggest a high pro-inflammatory cytokine environment in monocyte from young cattle and neonatal alveolar macrophage populations which may explain the effective elimination of parasitaemia in young cattle in comparison with responses seen in adult cattle. The inverse age-related immune response is an exception phenomenon and well established in *B. bovis* infection where calves show a strong protective response with early induction of Th1 cytokines with later stages associated with production of IL-4 and IL-10 (Goff et al., 2001; Goff et al., 2002; Chen et al., 2000). The M1 inflammatory monocyte pool is involved in the rapid clearance of intra-cellular parasites (Goncalves et al., 2011; Bosschaerts et al., 2010). Murine studies indicated that during *B. microti* and *T. gondii* parasitic infection, there is a need for early elevation of Th1 cytokines and monocytes are able to produce the early signals such as IL-12 and IFN-γ required for the initial control multiplication of intra-cellular parasites (Aguilar-Delfin et al., 2003; Chen et al., 2000).

This study suggests that monocytes undergo a functional change with age and the presence of a higher degree of pro-inflammatory cytokines indicate a predominant classical monocyte phenotype in young cattle compared to adults. Some bovine studies indicate that Bacillus Calmette-Guérin (BCG) vaccinated neonatal calves protect themselves much efficiently during challenge infection of *M. bovis* compared to adult cattle (Hope et al., 2005; Hope et al., 2011).

The increased susceptibility to infection and decreased response to vaccination in older individuals is associated with immunosenescence, where both the altered proportions and activities of innate immune cells have been reported (Panda et al., 2009; Loubet et al., 2016). The aging of immune cells also reduced the IFN-γ : IL-10 ratio via secretion of more IL-10 within influenza affected peripheral blood mononuclear cells and associated reduction in cytolytic capacity of $CD8^+$ T cells responsible for clearing of influenza virus (Mcelhaney et al., 2012).

Moreover, some human studies demonstrate an alteration in phenotype and functionality of monocytes and a link with decreased levels of the activation molecule (human leukocyte antigen-antigen D related) HLA-DR and chemokine receptor CX3CR1 (Seidler et al., 2010). A number of murine based studies support finding as well, this indicating a low expression of CX3CR1 on non-classical monocytes affects the life span of tissue derived macrophages, as well as reducing recruitment of monocytes to the site of an infection (Tacke et al., 2007; Auffray et al., 2009 b; Gautier et al., 2009). This study compared the cytokine environment during activation of blood monocytes and tissue macrophages in young and neonates respectively but it did not address same profile for alveolar macrophages from adult cattle due to difficulty in obtaining these cells from adult animals.

Alum stimulated young monocytes showed marginally higher caspase-1 activity compared to adult cattle. It has been documented that the alum adjuvant uses a NALP3 mediated inflammasome based activation for caspase-1 activity where caspase-1 cleaves pro-IL-1β into mature IL-1β (Li et al., 2008). However, persistence of low grade of inflammation can lead to age related metabolic disorders which are characterised by elevated amounts of macrophages producing TNF in fatty tissues of both mice and humans (Weisberg et al., 1993; Hotamisligil et al., 1993; Stienstra et al., 2011; McGillicuddy et al., 2011). Additionally, evidence suggests that prolonged production of TNF-α also contributes to the priming of NALP3 inflammasome for subsequent activation in older individuals and mice (Baylis et al., 2013; Bauernfeind et al., 2016).

Furthermore, there are studies which demonstrate that caspase-1 and its induction of IL-1β is essential for the control of *in vitro* replication of *T. gondii* (Marshall et al., 2011). Zheng et al (1995) have shown *in vivo* that, mice lacking IL-1β are incapable of protecting themselves against protozoan infection. Therefore it would be interesting to investigate this for bovine parasites such as *N. caninum*. Evidence suggests that the NALP3 inflammasome derived pathway induces a protective immune response during *N. caninum* infection in mouse macrophages and has been linked to the control of parasite multiplication (Wang et al., 2017). In the present study adult cattle blood samples were obtained from local abattoir and assuming that animal were apparently healthy. However, the exact health status of animals is unknown that should be consider because it could affect the immune response in terms of inflammatory activities well as variation among animals. Additionally, blood samples were collected during fall and winter terms, where immunity could be supressed due to seasonal change.

6.2 Changes of leukocyte population with age

Circulating blood leukocytes of neonates and adult cattle were examined to build a comparative profile of monocytes, granulocytes, T and B cells. These data confirmed that neonatal $CD14^+$ monocytes had greater CD80 expression. However, CD86 expression was not detectable on $CD14^+$ monocytes of either neonates or adult cattle. In terms of CD80/86 expression on alveolar macrophages, this study established that continuous CD80 expression could be maintained in neonates in response to a stimulus (LPS/IFN-γ). It also indicated that during the transition from blood monocytes to tissue macrophages these cells became more responsive to inflammatory signals. This is also supported by higher levels of pro-inflammatory cytokine secretion from neonatal alveolar macrophages (as described chapter 4) that suggest neonatal immune response is become activated. CD80 and 86 are co-stimulatory molecules and during microbial sepsis; their expression on monocytes is differentially regulated where expression of CD80 increases compared to CD86 (Nolan et al., 2008). Several other studies also documented the role of CD80 and CD86 in the activation of T cells and it has been observed that *T. gondii* infected

human monocytes express elevated levels of CD80 as well as upregulating CD86 (Subauste et al., 1998).

The circulating blood $CD14^+$ monocytes from adult cattle expressed a significantly greater level of MHC-II that indicates adult animals have a greater capacity for antigen presentation to $CD4^+$ T cells. Several other studies also support these findings where the classical monocyte subset ($CD14^+/CD16^-$) showed greater MHC-II expression; however the study here only addressed MHC-II expression on the global $CD14^+$ monocytes population instead of any specific monocyte subset.

Results of this study can confirm that proportions of γδ T cells change with age and neonates compromise a higher percentage of γδ T cells at about 45% compared to adult cattle were it comprises 29% of the total lymphocyte pool. Additionally, 80-90 % of the $WC1^+$ γδ T cells population was positive for WC1.1 subset in both age groups. These findings support previous studies where young ruminants harboured larger populations of γδ T cells and their function is determined by expression of the scavenger receptors WC1 and WC1.1 (Rogers et al., 2005). The $WC1^+$ subpopulation play an important role in modulating the immune response towards Th1 phenotype via production of IFN-γ that would able to control intra-cellular pathogen infection (Pollock and Welsh, 2002).

In this study, evaluation of T cells also indicates an alteration of the $CD4^+$ subset with age and a relatively higher proportion of these cells was found in adult cattle. However, the natural Treg population that is derived from thymus was very low in both age groups whilst the $CD4^+Foxp3^+$ pool was relatively high (2.57%) in neonates compared to adult (0.61%). These results agree with previous studies indicating low expression of $CD4^+Foxp3^+$ during a number of parasite infections such as *Psoroptes ovis* in sheep and *Fasciola hepatica* in cattle (McNeilly et al., 2009; Sachdev et al., 2017). Interestingly, bovine studies would suggest that $WC1^+$γδ T cells subset may serve as a primary Treg cells (Hoek et al., 2009; Guzman et al., 2014). Therefore, it could be assumed that healthy animals do not show high levels of Treg expression regardless of their age. The number of dual positive $CD4^+CD25^+$ also alters with age and a relatively higher percentage was noticed in adult (1.22% vs 0.31%) compared to neonates that is in agreement of human studies where natural

$CD4^+CD25^+$T reg cells pool increase in peripheral circulation with aging (Gregg et al., 2005).

Characterisation of the granulocyte cellular profile indicated that most of the granulocytes were DCs as characterised by the presence of a MHC II positive population in both neonates and adult cattle however, a small pool of neutrophils was also detected as an MHC II negative population. The low proportion of neutrophils may expected because cells being recruited to the tissues during bacterial, viral, rickettsial (*Anaplasma phagocytophilum, Ehrlichia ruminantium*) and protozoan (*Theileria sp.*) infection and resulting neutropenia have been noted in early inflammatory conditions (Stuen, 2007; Tornquist and Rigas, 2010; Omer et al., 2002). The present study, attempted a phenotypic comparison of leukocyte population among neonates and adult but due to small size of samples it may be not a representative study and it can be improve by adding more number of samples. However, obtaining blood samples from live animals raise certain ethical issues that limit the use of more animals.

6.3 *In vitro N. caninum* challenge study

In the *N. caninum* challenge infection study, CFSE labelled purified *N. caninum* tachyzoites were used to infect $CD14^+$ monocytes of neonates and adult cattle. This study demonstrates a greater uptake of parasites by adult monocytes compared to neonates that suggest increase recruitment of monocytes in adult cattle during infection however they were not able to produce sufficient level of pro-inflammatory cytokine IL-1β and IL-6 responsible for elimination for parasitaemia. The naïve $CD14^+$ monocytes were co cultured with autologous NK cell co-culture post infection that indicate reduction of parasites was relatively higher in neonates. It accompanying with higher activity of cytotoxic granule granzyme B and perforin secreted by NK cells and expected to eliminate infected cells. However, these data did not shows an age related significant difference in granzyme or perforin activity. Age associated decline in NK cell phenotype and functions through secretion of less perforin and granzyme in human studies has reported; moreover due to change in phenotype they are less likely to produce IFN-γ and affect pathogen killing abilities (Hazeldine et al., 2013;

Lopez-verges et al., 2010). In humans granulysin, perforin and granzyme are all responsible for rapid killing of intra-cellular bacteria (Walch et al., 2014) and it has been reported that granulysin can kill protozoa such as *T. cruzi, T. gondii* and *L. major* through the production of superoxide and inhibition of oxidative defense enzymes (Dotiwala et al., 2016). However these data addressed an *in vitro N. caninum* challenge infection that could be validate by *in vivo* clinical infection study.

6.4 Bovine gene expression data

The *N. caninum in vitro* challenge infection data is complemented by microarray gene expression profiles of neonates and adult cattle where neonates showed extensive upregulated gene networks for multiple immune response pathways. The most salient upregulated pathways were the JAK-STAT cascade and tyrosine phosphorylation of STAT protein pathway in infected versus uninfected neonates and adult. The upregulation of IL-6 was confirmed by ELISA in neonatal monocytes and was further confirmed by high levels of expression of the *IL-6ST* gene in the JAK-STAT pathway of neonatal monocytes. Interestingly, adult cattle did not show any expression of IL-6 neither directly through the proteinfrom stimulated monocytes nor gene array data. Furthermore, secretion of IL-1β protein was significantly higher in neonates during monocytes specific response in infected as well uninfected monocytes; which was further validated by gene array profile with upregulation of IL-1 secretion pathway in neonates. The presence of higher levels of IL-6 and IL-1β at the protein level as well as the gene array data of neonates may demonstrate a strong trend towards a greater in magnitude inflammatory response compared to adults.

However, neonates and adult cattle only showed upregulated differential gene expression without any downregulation and this may be due to the short time frame (24 hrs) of incubation which was used in the challenge study. This is also in agreement with monocytes/macrophage functional assays where the highest levels of IL-1β, IL-6 and TNF-α was detected at 24 hrs post infection and thereafter their secretion was reduced by 72 hrs. The gene array profile of uninfected neonates versus adult monocytes highlighted the activation of *STAT3* in neonates and is a

major signal transducer downstream of the *gp130* (IL-6) receptor (Fig. 6.1a). The major concern is how a single transcription factor can be involved in different contradicted outcomes. From this study it may concluded, a short term burst of *STAT3* protein in neonates that would participate in the secretion of IL-1β and IL-6 and due to high level of these cytokines there was a greater reduction of parasitaemia in neonatal infected monocytes.

However, *SOCS2* expression was detected in neonates that could potentially inhibits *STAT3* activation thereby avoiding continuous phosphorylation that may lead to the secretion of IL-10. Conversely, adult cattle did not activate *STAT3*; neither did they express *SOCS2* that would make them likely to secrete IL-10 and increase parasitaemia (Fig. 6.1b). From these data it can be assumed that there is a fundamental role for *STAT3* in mediating cytokine induction during an acute response. Previous work has shown the *STAT3* signalling pathway plays a major role in *T. gondii* infection, a close relative to *N. caninum,* and studies investigating the expression of *STAT3* in murine bone marrow derived macrophages found rapid and continuous expression of *STAT3* leading to anti-inflammatory cytokine environment. Additionally, *STAT3* knockout macrophages have shown enhanced TLR mediated clearance of parasites (Butcher et al., 2005; Kim et al., 2006; Hitziger et al., 2005). However, the activity of *STAT3* is not linear it has a clear bi-phasic response with multiple regulatory elements (Wang et al., 2013). There is also evidence that *STAT3* signalling can give rise to inflammatory events in macrophages (Samavati et al., 2009). In the future determining the specifics of *STAT3* expression and kinetics by qPCR and western blotting would be required. Within the context of infection however, the role of *STAT3* could be determined by use of chemical inhibitors or RNAi. Specifically if *STAT3* is central to the age-related mechanism present here it would be postulated that reversal of *STAT3* in adult monocytes restores parasite clearance or over expression in neonates would give rise to greater parasite loads.

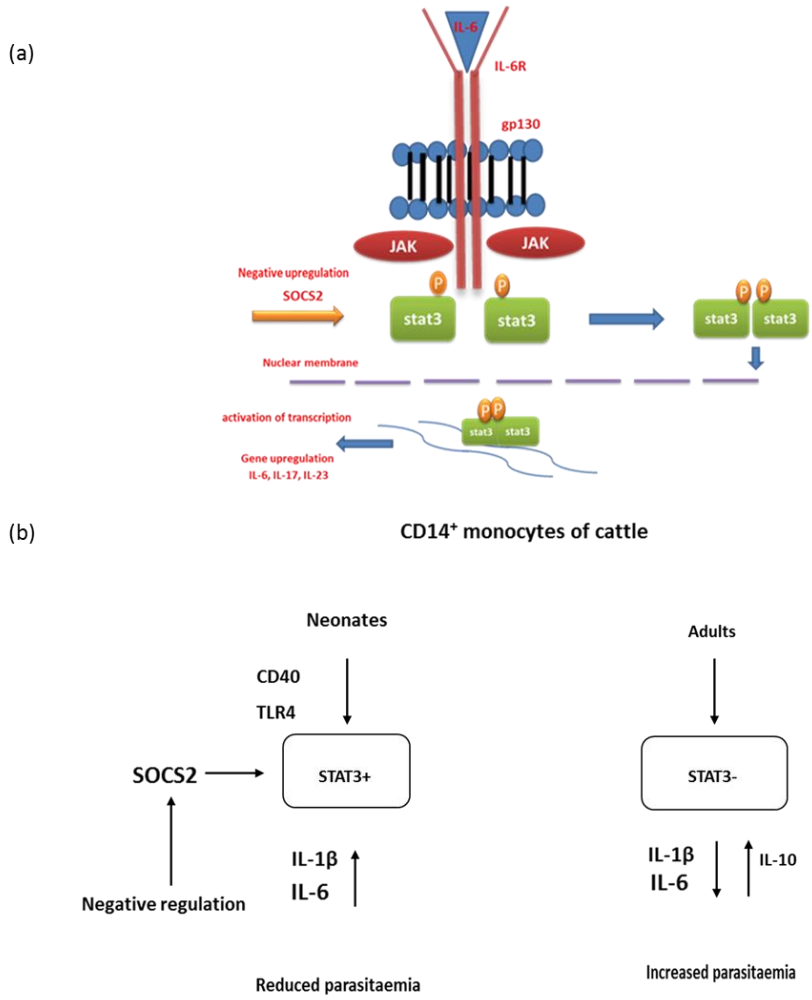


Figure 6.1 Mechanism of *STAT3* signalling pathway in neonates and adult monocytes

a) JAK-STAT signalling pathway where ligand IL-6 bind to receptor that activates the associated JAK. Activation of *STAT* dependent upon tyrosine phosphorylation that start dimerization of *STAT* protein that translocate into the nucleus. It binds to consensus promoter sequences and causes the transcriptional induction of target genes IL-6, IL-17 and IL-23.

b) The *STAT3* signalling pathway is transient and upregulated in neonatal monocytes that leads to expression of IL-6 and IL-1β and leads to the elimination of intracellular parasites. Additionally, *SOCS2* act as negative regulator of *STAT3* that inhibits continuous phosphorylation of *STAT* protein and supresses the secretion of the anti-inflammatory cytokine IL-10. This response was absent in adult monocytes, resulting expression of IL-10 and increased parasitaemia.

6.5 Conclusions

All together these data demonstrate an age related immune mechanism across neonates and adult cattle. It can be also concluded that number of circulating leukocytes population changes with age and it can also affect functional capacity of immune cells to response against pathogen. Neonates have shown a higher percent of $CD14^{+}$ monocytes cell populations. Γδ T cells are important regulatory components of circulating lymphocytes and due to presence of high numbers in neonates are likely to make them more resistant to intra-cellular parasitic infection. Additionally, thymus derived Treg pool were not so obvious in either neonates or adult cattle where it makes only a small proportion of the total T cells population. The memory B-cells population was also found to differ with age and adult cattle were found to have more CD45RO positive lymphocytes compared to neonates. Neonatal monocytes have shown a greater pro-inflammatory cytokine environment that contributed to elimination of more parasites. In contrast, adult cattle were more prone to infection that was evident by greater numbers of parasitized monocytes during *N. caninum* infection. These data also indicate that monocytes becomes more activated once they reached into tissue and produce high levels of IL-1β, TNF-β, IL-6 and IL-12 and it may elucidate the role of monocytes during infection conditions.

Additionally, the cytotoxic killing activity of NK cells also play a major role in clearance of parasitaemia. However there is not much difference was noticed in granzyme B and perforin activity of neonates and adult. A higher degree of cellular activation of neonatal monocytes in an autologous co-culture of NK cells was determined during *N. caninum* infection via CD80 expression. Finally, these findings are supported by microarray gene expression data where neonatal monocytes displayed a robust network for preliminary immune response pathways. An upregulated biological pathway such as tyrosine phosphorylation and JAK-STAT cascade shows the role of cytokines IL-1β and IL-6 during *N. caninum* infection.

In summary, the mechanism of age-related immunity can be compared as a fundamental difference in the steady-state and in the response to intracellular parasite infection in neonatal monocyte led inflammatory responses.

7 Appendices

Appendix 1

MACS running buffer (pH = 7.2)

Phosphate buffer saline	1 Litre
Bovine serum albumin	5 g
EDTA	2 mM

Adjusted pH to 7.2, Filter sterilize through 0.2µm filter

MACS rinsing buffer (pH= 7.2)

Phosphate buffer saline	1 Litre
EDTA	2 mM

Adjusted pH to 7.2, Filter sterilize through 0.2 µm filter

RPMI complete media

RPMI-1640	89%
Fetal bovine serum	10%
Penicillin streptomycin	1 %

Freeze mix

Fetal bovine serum	80%
DMSO	20%

Erythrocyte lysis buffer

KHCO3	1.0 g
NH4Cl	8.26 g
EDTA (pH 8.0)	37 mg

Dissolved the following in 800 ml distilled H_2O and adjusted pH to 7.4. Qs to 1000 ml with distilled H_2O. Filter sterilize through 0.2 µm filter.

Appendix 2

ELISA Blocking buffer

Bovine serum albumin	4%
Sucrose	5%

Dissolved in D-PBS and filter sterilize through 0.2 µm filter.

Reagent diluent

Bovine serum albumin	4%

Dissolved in D-PBS and filter sterilize through 0.2 µm filter.

Incubation Buffer

Bovine serum albumin	0.5%
Tween 20	0.05%

Dissolved in D-PBS and filter sterilize through 0.2 µm filter

ELISA wash buffer

Tween 20	0.05%

Dissolved in 1X phosphate buffer saline

FACS buffer

Fetal bovine serum	5-10%

Dissolved in 1X D-PBS

FACS blocking buffer

Bovine serum albumin	0.5%

Dissolved in 1X D-PBS

Appendix 3

Perforin Buffer

HEPES	10 mM
NaCl	0.15 M
Bovine serum albumin	10 μg/ml
$CaCl_2$	4 mM

Adjusted pH to 7.4, Filter sterilize through 0.2 μm filter

Appendix 4

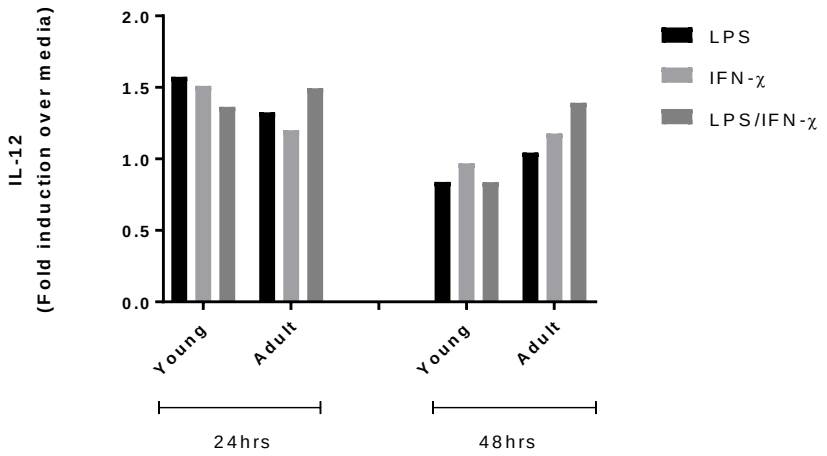


Figure 1. IL-12 production in CD14^{+} monocytes of cattle

IL-12 production was examined in LPS (1μg/ml) and IFN-γ (20ng/ml) stimulated CD14^{+} monocytes of young (n=1) and adult (n=1). The supernatants were collected at end of incubation time (24hrs and 48hrs) and analysed by ELISA.

Appendix 5 Complete list of significantly upregulated differential genes in young *N. caninum* infected and uninfected cattle

EntrezID	logFC	adj.P.Val	Symbols	Name
538748	7.1952	0.002678	ICAM3	intercellular adhesion molecule 3 [Source:HGNC Symbol;Acc:HGNC:5346]
539034	5.0739	0.002678	CCL2	C-C motif chemokine ligand 2 [Source:HGNC Symbol;Acc:HGNC:10618]
614074	4.7438	0.002678	TMED3	transmembrane p24 trafficking protein 3 [Source:HGNC Symbol;Acc:HGNC:28889]
280794	4.7298	0.002678	CCDC69	coiled-coil domain containing 69 [Source:HGNC Symbol;Acc:HGNC:24487]
281768	4.702	0.002678	SOCS2	suppressor of cytokine signaling 2 [Source:HGNC Symbol;Acc:HGNC:19382]
537907	4.1423	0.002822	CCDC50	coiled-coil domain containing 50 [Source:HGNC Symbol;Acc:HGNC:18111]
539299	4.1343	0.007413	CTTN	cortactin [Source:HGNC Symbol;Acc:HGNC:3338]
524530	4.1145	0.007413	RPL10L	ribosomal protein L10 like [Source:HGNC Symbol;Acc:HGNC:17976]
616227	4.057	0.008499	CYP1B1	cytochrome P450 family 1 subfamily B member 1 [Source:HGNC Symbol;Acc:HGNC:2597]
613524	3.9767	0.008499	SMAP2	small ArfGAP2 [Source:HGNC Symbol;Acc:HGNC:25082]
513445	3.9663	0.008499	PGLS	6-phosphogluconolactonase [Source:HGNC Symbol;Acc:HGNC:8903]
280881	3.9318	0.008499	SDF2L1	stromal cell derived factor 2 like 1 [Source:HGNC Symbol;Acc:HGNC:10676]
614606	3.9172	0.008499	VMA21	VMA21 vacuolar H+-ATPase homolog (S. cerevisiae) [Source:HGNC Symbol;Acc:HGNC:22082]

493717	3.9154	0.008499	ASS1	argininosuccinate synthase 1 [Source:HGNC Symbol;Acc:HGNC:758]
508490	3.8547	0.008499	TMEM158	transmembrane protein 158 (gene/pseudogene) [Source:HGNC Symbol;Acc:HGNC:30293]
529759	3.837	0.008499	ARHGDIB	Rho GDP dissociation inhibitor beta [Source:HGNC Symbol;Acc:HGNC:679]
617129	3.7414	0.008499	BAK1	BCL2 antagonist/killer 1 [Source:HGNC Symbol;Acc:HGNC:949]
541028	3.6327	0.008499	AP2S1	adaptor related protein complex 2 sigma 1 subunit [Source:HGNC Symbol;Acc:HGNC:565]
281592	3.568	0.008499	NXPH3	neurexophilin 3 [Source:HGNC Symbol;Acc:HGNC:8077]
617707	3.5661	0.008499	COX7A2	cytochrome c oxidase subunit 7A2 [Source:HGNC Symbol;Acc:HGNC:2288]
530409	3.5293	0.008499	CRELD2	cysteine rich with EGF like domains 2 [Source:HGNC Symbol;Acc:HGNC:28150]
508941	3.5136	0.008499	PSD3	pleckstrin and Sec7 domain containing 3 [Source:HGNC Symbol;Acc:HGNC:19093]
516908	3.4973	0.008499	STK25	serine/threonine kinase 25 [Source:HGNC Symbol;Acc:HGNC:11404]
505680	3.4655	0.008499	RGS14	regulator of G-protein signaling 14 [Source:HGNC Symbol;Acc:HGNC:9996]
280858	3.4611	0.008499	IL1RN	interleukin 1 receptor antagonist [Source:HGNC Symbol;Acc:HGNC:6000]
1E+08	3.4394	0.008499	NDUFA12	NADH:ubiquinone oxidoreductase subunit A12 [Source:HGNC Symbol;Acc:HGNC:23987]
507536	3.4232	0.008499	CHCHD10	coiled-coil-helix-coiled-coil-helix domain containing 10 [Source:HGNC Symbol;Acc:HGNC:15559]
521073	3.4133	0.008499	FLT1	fms related tyrosine kinase 1 [Source:HGNC Symbol;Acc:HGNC:3763]

510898	3.3929	0.008499	BCL2	B-cell CLL/lymphoma 2 [Source:HGNC Symbol;Acc:HGNC:990]
532605	3.3913	0.008499	SUCLG1	succinate-CoA ligase alpha subunit [Source:HGNC Symbol;Acc:HGNC:11449]
281043	3.3731	0.008499	TPST1	tyrosylprotein sulfotransferase 1 [Source:HGNC Symbol;Acc:HGNC:12020]
507342	3.3515	0.008499	ACOT7	acyl-CoA thioesterase 7 [Source:HGNC Symbol;Acc:HGNC:24157]
539060	3.2995	0.008499	IDH3A	isocitrate dehydrogenase 3 (NAD(+)) alpha [Source:HGNC Symbol;Acc:HGNC:5384]
508412	3.2974	0.008499	SNRPA1	small nuclear ribonucleoprotein polypeptide A' [Source:HGNC Symbol;Acc:HGNC:11152]
514788	3.2878	0.008499	SRPK2	SRSF protein kinase 2 [Source:HGNC Symbol;Acc:HGNC:11306]
286857	3.2753	0.008499	C3	complement component 3 [Source:HGNC Symbol;Acc:HGNC:1318]
280765	3.2705	0.008499	EPRS	glutamyl-prolyl-tRNA synthetase [Source:HGNC Symbol;Acc:HGNC:3418]
1.05E+08	3.2637	0.008499	XPO1	exportin 1 [Source:HGNC Symbol;Acc:HGNC:12825]
507792	3.2596	0.008499	SCAMP3	secretory carrier membrane protein 3 [Source:HGNC Symbol;Acc:HGNC:10565]
614921	3.255	0.008499	RABAC1	Rab acceptor 1 [Source:HGNC Symbol;Acc:HGNC:9794]
515652	3.2508	0.008499	CKAP5	cytoskeleton associated protein 5 [Source:HGNC Symbol;Acc:HGNC:28959]
787125	3.2351	0.008499	DPY30	dpy-30, histone methyltransferase complex regulatory subunit [Source:HGNC Symbol;Acc:HGNC:24590]
504371	3.2307	0.008499	IMPA2	inositol monophosphatase 2 [Source:RefSeq peptide;Acc:NP_001179211]

520327	3.223	0.008499	THBD	thrombomodulin [Source:HGNC Symbol;Acc:HGNC:11784]
508132	3.2203	0.008499	E2F4	E2F transcription factor 4 [Source:HGNC Symbol;Acc:HGNC:3118]
535439	3.2198	0.008499	MAFF	MAF bZIP transcription factor F [Source:HGNC Symbol;Acc:HGNC:6780]
535222	3.2154	0.008499	FIS1	fission, mitochondrial 1 [Source:HGNC Symbol;Acc:HGNC:21689]
282328	3.2139	0.008499	SIL1	SIL1 nucleotide exchange factor [Source:HGNC Symbol;Acc:HGNC:24624]
407125	3.1975	0.008499	CFL1	Cofilin-1 [Source:UniProtKB/Swiss-Prot;Acc:Q5E9F7]
514357	3.1963	0.008499	RPN2	ribophorin II [Source:HGNC Symbol;Acc:HGNC:10382]
615846	3.1945	0.008499	LRRC59	leucine rich repeat containing 59 [Source:HGNC Symbol;Acc:HGNC:28817]
614497	3.1769	0.008499	SND1	staphylococcal nuclease and tudor domain containing 1 [Source:HGNC Symbol;Acc:HGNC:30646]
506480	3.1695	0.008499	GALNT1	polypeptide N-acetylgalactosaminyltransferase 1 [Source:HGNC Symbol;Acc:HGNC:4123]
767985	3.1644	0.008499	CS	citrate synthase [Source:HGNC Symbol;Acc:HGNC:2422]
540176	3.1594	0.008499	BACE1	Beta-secretase 1 [Source:UniProtKB/Swiss-Prot;Acc:Q2HJ40]
540179	3.1525	0.008499	FUS	FUS RNA binding protein [Source:HGNC Symbol;Acc:HGNC:4010]
514739	3.1404	0.008499	LMAN1	protein ERGIC-53 precursor [Source:RefSeq peptide;Acc:NP_001092413]
780878	3.1346	0.008499	MCUR1	mitochondrial calcium uniporter regulator 1 [Source:HGNC Symbol;Acc:HGNC:21097]

522269	3.1331	0.008499	HMGN2	Non-histone chromosomal protein HMG-17 [Source:UniProtKB/TrEMBL;Acc:F2Z4H2]
529878	3.1245	0.008499	PDIA6	protein disulfide-isomerase A6 precursor [Source:RefSeq peptide;Acc:NP_001193274]
511041	3.1122	0.008499	ILK	integrin linked kinase [Source:HGNC Symbol;Acc:HGNC:6040]
515309	3.1015	0.008499	SYNCRIP	synaptotagmin binding cytoplasmic RNA interacting protein [Source:HGNC Symbol;Acc:HGNC:16918]
618849	3.098	0.008499	NDUFS4	NADH:ubiquinone oxidoreductase subunit S4 [Source:HGNC Symbol;Acc:HGNC:7711]
510004	3.061	0.008499	RPRD1A	regulation of nuclear pre-mRNA domain containing 1A [Source:HGNC Symbol;Acc:HGNC:25560]
514997	3.025	0.008499	KIF5B	kinesin family member 5B [Source:HGNC Symbol;Acc:HGNC:6324]
538649	3.0174	0.008499	ANKRD28	ankyrin repeat domain 28 [Source:HGNC Symbol;Acc:HGNC:29024]
508319	3.0071	0.008499	GSTP1	glutathione S-transferase pi 1 [Source:HGNC Symbol;Acc:HGNC:4638]
506043	3.003	0.008499	CNN3	Calponin-3 [Source:UniProtKB/Swiss-Prot;Acc:Q32L92]
509889	2.9864	0.008499	TSPAN4	tetraspanin 4 [Source:HGNC Symbol;Acc:HGNC:11859]
539449	2.97	0.008499	DUS1L	tRNA-dihydrouridine synthase 1-like [Source:RefSeq peptide;Acc:NP_001071552]
535182	2.9432	0.008499	ACSL4	acyl-CoA synthetase long-chain family member 4 [Source:HGNC Symbol;Acc:HGNC:3571]
789464	2.9329	0.008499	AMPD2	adenosine monophosphate deaminase 2 [Source:HGNC Symbol;Acc:HGNC:469]

512401	2.9299	0.008499	GRB10	growth factor receptor bound protein 10 [Source:HGNC Symbol;Acc:HGNC:4564]
540138	2.9254	0.008499	FUBP1	far upstream element-binding protein 1 [Source:RefSeq peptide;Acc:NP_001070314]
512666	2.9173	0.008499	ARF3	ADP-ribosylation factor 1 [Source:UniProtKB/Swiss-Prot;Acc:P84080]
513061	2.9117	0.008499	CAPZB	capping actin protein of muscle Z-line beta subunit [Source:HGNC Symbol;Acc:HGNC:1491]
511918	2.9063	0.008499	PRDX1	peroxiredoxin 1 [Source:HGNC Symbol;Acc:HGNC:9352]
540040	2.9045	0.008499	MID1IP1	MID1 interacting protein 1 [Source:HGNC Symbol;Acc:HGNC:20715]
539633	2.9024	0.008499	HM13	histocompatibility (minor) 13 [Source:HGNC Symbol;Acc:HGNC:16435]
511245	2.8998	0.008499	NUTF2	nuclear transport factor 2 [Source:HGNC Symbol;Acc:HGNC:13722]
522155	2.8963	0.008499	STUB1	STIP1 homology and U-box containing protein 1 [Source:HGNC Symbol;Acc:HGNC:11427]
522473	2.8915	0.008499	EGR1	early growth response 1 [Source:HGNC Symbol;Acc:HGNC:3238]
1E+08	2.8884	0.008499	CKB	Creatine kinase B-type [Source:UniProtKB/Swiss-Prot;Acc:Q5EA61]
528603	2.8831	0.008499	CTPS1	CTP synthase 1 [Source:HGNC Symbol;Acc:HGNC:2519]
539591	2.8784	0.008499	DNAJC15	DnaJ heat shock protein family (Hsp40) member C15 [Source:HGNC Symbol;Acc:HGNC:20325]
1.02E+08	2.8775	0.008499	ATP5G3	ATP synthase, H+ transporting, mitochondrial Fo complex subunit C3 (subunit 9) [Source:HGNC Symbol;Acc:HGNC:843]
504867	2.869	0.008499	NAXE	NAD(P)HX epimerase [Source:HGNC Symbol;Acc:HGNC:18453]

511087	2.8672	0.008499	TOMM7	translocase of outer mitochondrial membrane 7 [Source:HGNC Symbol;Acc:HGNC:21648]
507804	2.8607	0.008499	ZEB1	zinc finger E-box-binding homeobox 1 [Source:RefSeq peptide;Acc:NP_001193519]
514394	2.8454	0.008499	HSP90B1	heat shock protein 90kDa beta family member 1 [Source:HGNC Symbol;Acc:HGNC:12028]
281418	2.8452	0.008499	AVEN	apoptosis and caspase activation inhibitor [Source:HGNC Symbol;Acc:HGNC:13509]
513918	2.844	0.008499	OSGIN2	oxidative stress induced growth inhibitor family member 2 [Source:HGNC Symbol;Acc:HGNC:1355]
613575	2.8408	0.008499	PURA	purine rich element binding protein A [Source:HGNC Symbol;Acc:HGNC:9701]
528033	2.8349	0.008499	ILF3	interleukin enhancer binding factor 3 [Source:HGNC Symbol;Acc:HGNC:6038]
514271	2.8344	0.008499	CDIPT	CDP-diacylglycerol--inositol 3-phosphatidyltransferase [Source:HGNC Symbol;Acc:HGNC:1769]
509111	2.8321	0.008499	HIKESHI	Hikeshi, heat shock protein nuclear import factor [Source:HGNC Symbol;Acc:HGNC:26938]
538668	2.8267	0.008528	AK2	adenylate kinase 2 [Source:HGNC Symbol;Acc:HGNC:362]
282119	2.8147	0.008571	LIMS1	LIM zinc finger domain containing 1 [Source:HGNC Symbol;Acc:HGNC:6616]
526819	2.7987	0.008592	MPHOSPH 6	M-phase phosphoprotein 6 [Source:HGNC Symbol;Acc:HGNC:7214]
513830	2.7924	0.008592	DYNC1LI2	cytoplasmic dynein 1 light intermediate chain 2 [Source:RefSeq peptide;Acc:NP_001193081]
282479	2.7878	0.008592	SLC44A3	solute carrier family 44 member 3 [Source:HGNC Symbol;Acc:HGNC:28689]

783763	2.7778	0.008592	ARPC5L	actin related protein 2/3 complex subunit 5 like [Source:HGNC Symbol;Acc:HGNC:23366]
513004	2.7766	0.008592	YBX1	nuclease-sensitive element-binding protein 1 [Source:RefSeq peptide;Acc:NP_777240]
538903	2.772	0.008592	PREP	prolyl endopeptidase [Source:HGNC Symbol;Acc:HGNC:9358]
281867	2.7642	0.008592	CCDC150	coiled-coil domain containing 150 [Source:HGNC Symbol;Acc:HGNC:26834]
531770	2.764	0.008592	CBX1	chromobox 1 [Source:HGNC Symbol;Acc:HGNC:1551]
280715	2.757	0.008592	MRPL22	mitochondrial ribosomal protein L22 [Source:HGNC Symbol;Acc:HGNC:14480]
524779	2.7562	0.008592	VPS54	VPS54, GARP complex subunit [Source:HGNC Symbol;Acc:HGNC:18652]
532422	2.7543	0.008592	JOSD1	Josephin domain containing 1 [Source:HGNC Symbol;Acc:HGNC:28953]
327669	2.7538	0.008592	RILPL1	Rab interacting lysosomal protein-like 1 [Source:HGNC Symbol;Acc:HGNC:26814]
281719	2.7452	0.008592	AIFM1	apoptosis inducing factor, mitochondria associated 1 [Source:HGNC Symbol;Acc:HGNC:8768]
506970	2.7386	0.008592	REXO2	Oligoribonuclease, mitochondrial [Source:UniProtKB/Swiss-Prot;Acc:A2VE52]
534935	2.7372	0.008592	NT5DC1	5'-nucleotidase domain containing 1 [Source:HGNC Symbol;Acc:HGNC:21556]
281529	2.7317	0.008592	RBMS1	RNA binding motif single stranded interacting protein 1 [Source:HGNC Symbol;Acc:HGNC:9907]
404186	2.7272	0.008592	ACAA2	acetyl-CoA acyltransferase 2 [Source:HGNC Symbol;Acc:HGNC:83]
509802	2.7243	0.008592	SRI	sorcin [Source:HGNC Symbol;Acc:HGNC:11292]

509966	2.7114	0.008592	ZNRF1	zinc and ring finger 1, E3 ubiquitin protein ligase [Source:HGNC Symbol;Acc:HGNC:18452]
618226	2.7112	0.008592	IDH2	isocitrate dehydrogenase (NADP(+)) 2, mitochondrial [Source:HGNC Symbol;Acc:HGNC:5383]
526027	2.7061	0.008592	MLF2	myeloid leukemia factor 2 [Source:HGNC Symbol;Acc:HGNC:7126]
533297	2.7034	0.008592	KLF3	Kruppel like factor 3 [Source:HGNC Symbol;Acc:HGNC:16516]
522406	2.6939	0.008592	ERGIC1	endoplasmic reticulum-golgi intermediate compartment 1 [Source:HGNC Symbol;Acc:HGNC:29205]
786041	2.6873	0.008592	DNAJC6	DnaJ heat shock protein family (Hsp40) member C6 [Source:HGNC Symbol;Acc:HGNC:15469]
535183	2.686	0.008592	MDH1	malate dehydrogenase 1 [Source:HGNC Symbol;Acc:HGNC:6970]
520639	2.6799	0.008592	NDUFA8	NADH:ubiquinone oxidoreductase subunit A8 [Source:HGNC Symbol;Acc:HGNC:7692]
282152	2.6713	0.008592	POLR3B	polymerase (RNA) III subunit B [Source:HGNC Symbol;Acc:HGNC:30348]
539090	2.6678	0.008592	DHX15	DEAH-box helicase 15 [Source:HGNC Symbol;Acc:HGNC:2738]
509253	2.6675	0.008592	VDAC1	voltage dependent anion channel 1 [Source:HGNC Symbol;Acc:HGNC:12669]
513699	2.6585	0.008592	ELAVL1	ELAV like RNA binding protein 1 [Source:HGNC Symbol;Acc:HGNC:3312]
534217	2.6582	0.008592	LDHB	lactate dehydrogenase B [Source:HGNC Symbol;Acc:HGNC:6541]
540026	2.657	0.008592	HAX1	HCLS1 associated protein X-1 [Source:HGNC Symbol;Acc:HGNC:16915]

524770	2.6503	0.008592	PMVK	phosphomevalonate kinase [Source:HGNC Symbol;Acc:HGNC:9141]
338437	2.6422	0.008592	MTFR1L	mitochondrial fission regulator 1 like [Source:HGNC Symbol;Acc:HGNC:28836]
511470	2.642	0.008714	SERPINB10	serpin family B member 10 [Source:HGNC Symbol;Acc:HGNC:8942]
505916	2.6413	0.008714	ARHGDIA	Rho GDP dissociation inhibitor alpha [Source:HGNC Symbol;Acc:HGNC:678]
512578	2.6367	0.008714	RPL28	ribosomal protein L28 [Source:HGNC Symbol;Acc:HGNC:10330]
444872	2.636	0.008735	MCM10	minichromosome maintenance 10 replication initiation factor [Source:HGNC Symbol;Acc:HGNC:18043]
506605	2.635	0.0088	ORMDL3	ORMDL sphingolipid biosynthesis regulator 3 [Source:HGNC Symbol;Acc:HGNC:16038]
514355	2.6331	0.008805	SPATS2L	spermatogenesis associated serine rich 2 like [Source:HGNC Symbol;Acc:HGNC:24574]
539132	2.6312	0.008822	UBE2N	ubiquitin conjugating enzyme E2 N [Source:HGNC Symbol;Acc:HGNC:12492]
539524	2.6305	0.008857	HDAC2	histone deacetylase 2 [Source:RefSeq peptide;Acc:NP_001068614]
326598	2.6288	0.008893	HSPD1	heat shock protein family D (Hsp60) member 1 [Source:HGNC Symbol;Acc:HGNC:5261]
534576	2.6284	0.008893	HLTF	helicase like transcription factor [Source:HGNC Symbol;Acc:HGNC:11099]
281833	2.6271	0.008893	MCM5	minichromosome maintenance complex component 5 [Source:HGNC Symbol;Acc:HGNC:6948]
540191	2.6264	0.008893	DDX39B	DEAD-box helicase 39B [Source:HGNC Symbol;Acc:HGNC:13917]

529670	2.6239	0.008893	MYO1C	myosin IC [Source:HGNC Symbol;Acc:HGNC:7597]
539229	2.6236	0.008893	PHB	prohibitin [Source:HGNC Symbol;Acc:HGNC:8912]
615869	2.6223	0.008893	PPIA	Peptidyl-prolyl cis-trans isomerase A Peptidyl-prolyl cis-trans isomerase A, N-terminally processed [Source:UniProtKB/Swiss-Prot;Acc:P62935]
540139	2.6154	0.008983	MAD2L2	MAD2 mitotic arrest deficient-like 2 (yeast) [Source:HGNC Symbol;Acc:HGNC:6764]
540247	2.5988	0.009018	POLA2	polymerase (DNA) alpha 2, accessory subunit [Source:HGNC Symbol;Acc:HGNC:30073]
617316	2.5891	0.009018	HBEGF	heparin binding EGF like growth factor [Source:HGNC Symbol;Acc:HGNC:3059]
538485	2.589	0.009018	FADS3	fatty acid desaturase 3 [Source:HGNC Symbol;Acc:HGNC:3576]
539547	2.5879	0.009018	MRPL51	mitochondrial ribosomal protein L51 [Source:HGNC Symbol;Acc:HGNC:14044]
520167	2.5876	0.009018	YWHAZ	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta [Source:HGNC Symbol;Acc:HGNC:12855]
505828	2.5851	0.009018	MYEF2	myelin expression factor 2 [Source:HGNC Symbol;Acc:HGNC:17940]
512508	2.5813	0.009082	ASNA1	arsA arsenite transporter, ATP-binding, homolog 1 (bacterial) [Source:HGNC Symbol;Acc:HGNC:752]
282082	2.5794	0.009082	ZFHX3	zinc finger homeobox 3 [Source:HGNC Symbol;Acc:HGNC:777]
614579	2.5785	0.009082	PSMA7	proteasome subunit alpha 7 [Source:HGNC Symbol;Acc:HGNC:9536]

618429	2.5784	0.009082	TCEAL8	transcription elongation factor A like 8 [Source:HGNC Symbol;Acc:HGNC:28683]
510245	2.5767	0.009082	KTN1	kinectin 1 [Source:HGNC Symbol;Acc:HGNC:6467]
505005	2.5757	0.009082	CORO1C	coronin 1C [Source:HGNC Symbol;Acc:HGNC:2254]
326581	2.5736	0.009082	DYNLT1	Bos taurus dynein, light chain, Tctex-type 1 (DYNLT1), mRNA. [Source:RefSeq mRNA;Acc:NM_174620]
534231	2.5673	0.009082	HNRNPAB	heterogeneous nuclear ribonucleoprotein A/B [Source:HGNC Symbol;Acc:HGNC:5034]
539670	2.5672	0.009082	CSNK2A1	casein kinase II subunit alpha [Source:RefSeq peptide;Acc:NP_777060]
506551	2.5649	0.009082	MED29	mediator complex subunit 29 [Source:HGNC Symbol;Acc:HGNC:23074]
507012	2.5634	0.009082	PARP1	poly(ADP-ribose) polymerase 1 [Source:HGNC Symbol;Acc:HGNC:270]
281840	2.5596	0.009082	DES	desmin [Source:HGNC Symbol;Acc:HGNC:2770]
540272	2.5581	0.009178	HNRNPA3	heterogeneous nuclear ribonucleoprotein A3 [Source:HGNC Symbol;Acc:HGNC:24941]
286764	2.5566	0.009178	GNPNAT1	glucosamine-phosphate N-acetyltransferase 1 [Source:HGNC Symbol;Acc:HGNC:19980]
616293	2.5562	0.009189	TWF1	twinfilin actin binding protein 1 [Source:HGNC Symbol;Acc:HGNC:9620]
505585	2.5506	0.009191	FKBP11	FK506 binding protein 11 [Source:HGNC Symbol;Acc:HGNC:18624]
504411	2.549	0.009191	NEDD8	NEDD8 precursor [Source:RefSeq peptide;Acc:NP_777189]
618204	2.5471	0.009191	ATP23	ATP23 metallopeptidase and ATP synthase assembly factor homolog (S. cerevisiae) [Source:HGNC Symbol;Acc:HGNC:29452]

508124	2.5451	0.009191	SFPQ	splicing factor proline and glutamine rich [Source:HGNC Symbol;Acc:HGNC:10774]
511077	2.541	0.009191	HNRNPD	heterogeneous nuclear ribonucleoprotein D [Source:HGNC Symbol;Acc:HGNC:5036]
539073	2.5358	0.009191	UBAP2L	ubiquitin associated protein 2 like [Source:HGNC Symbol;Acc:HGNC:29877]
404132	2.5312	0.009191	BHLHE40	basic helix-loop-helix family member e40 [Source:HGNC Symbol;Acc:HGNC:1046]
513843	2.5305	0.009191	LASP1	LIM and SH3 protein 1 [Source:HGNC Symbol;Acc:HGNC:6513]
511763	2.5273	0.009276	AKAP2	A-kinase anchoring protein 2 [Source:HGNC Symbol;Acc:HGNC:372]
517539	2.5262	0.009341	MLEC	malectin [Source:HGNC Symbol;Acc:HGNC:28973]
540564	2.5253	0.009343	FAM60A	family with sequence similarity 60 member A [Source:HGNC Symbol;Acc:HGNC:30702]
510568	2.5215	0.009343	PPP2R5D	serine/threonine-protein phosphatase 2A 56 kDa regulatory subunit delta isoform [Source:RefSeq peptide;Acc:NP_001193287]
536628	2.5183	0.009398	ANXA2	annexin A2 [Source:HGNC Symbol;Acc:HGNC:537]
535844	2.5138	0.009404	DPCD	deleted in primary ciliary dyskinesia homolog (mouse) [Source:HGNC Symbol;Acc:HGNC:24542]
505011	2.5054	0.00947	USP14	ubiquitin specific peptidase 14 [Source:HGNC Symbol;Acc:HGNC:12612]
613674	2.5032	0.00947	PSMB10	proteasome subunit beta 10 [Source:HGNC Symbol;Acc:HGNC:9538]
282419	2.5016	0.009475	RUFY2	RUN and FYVE domain-containing protein 2 [Source:RefSeq peptide;Acc:NP_001193636]

280726	2.4966	0.009484	SUZ12	SUZ12 polycomb repressive complex 2 subunit [Source:HGNC Symbol;Acc:HGNC:17101]
505945	2.4953	0.009484	FKBP4	FK506 binding protein 4 [Source:HGNC Symbol;Acc:HGNC:3720]
614936	2.4899	0.009484	HSBP1	heat shock factor binding protein 1 [Source:HGNC Symbol;Acc:HGNC:5203]
617624	2.4896	0.009484	NUDC	nudC nuclear distribution protein [Source:HGNC Symbol;Acc:HGNC:8045]
404150	2.487	0.009484	HMGA1	high mobility group AT-hook 1 [Source:HGNC Symbol;Acc:HGNC:5010]
281351	2.4858	0.00949	GMPPB	GDP-mannose pyrophosphorylase B [Source:HGNC Symbol;Acc:HGNC:22932]
614777	2.4855	0.00949	SLC25A5	Bos taurus solute carrier family 25 (mitochondrial carrier; adenine nucleotide translocator), member 5 (SLC25A5), mRNA. [Source:RefSeq mRNA;Acc:NM_174659]
616981	2.4831	0.00949	TECR	trans-2,3-enoyl-CoA reductase [Source:HGNC Symbol;Acc:HGNC:4551]
616239	2.4771	0.00949	PPP1CC	protein phosphatase 1 catalytic subunit gamma [Source:HGNC Symbol;Acc:HGNC:9283]
280955	2.4722	0.00949	LZTS2	leucine zipper, putative tumor suppressor 2 [Source:HGNC Symbol;Acc:HGNC:29381]
1.02E+08	2.4684	0.00949	HSPE1	heat shock protein family E (Hsp10) member 1 [Source:HGNC Symbol;Acc:HGNC:5269]
533980	2.4666	0.00949	ATP2A2	ATPase sarcoplasmic/endoplasmic reticulum Ca2+ transporting 2 [Source:HGNC Symbol;Acc:HGNC:812]
539003	2.4661	0.00949	DCUN1D5	defective in cullin neddylation 1 domain containing 5 [Source:HGNC Symbol;Acc:HGNC:28409]

338052	2.4565	0.00949	CRLF3	cytokine receptor like factor 3 [Source:HGNC Symbol;Acc:HGNC:17177]
511200	2.4556	0.00949	CHD7	chromodomain helicase DNA binding protein 7 [Source:HGNC Symbol;Acc:HGNC:20626]
538915	2.4553	0.00949	EIF4H	eukaryotic translation initiation factor 4H [Source:RefSeq peptide;Acc:NP_001069220]
510230	2.4546	0.009504	ZMYND11	zinc finger MYND-type containing 11 [Source:HGNC Symbol;Acc:HGNC:16966]
518136	2.4545	0.009509	ADAM33	ADAM metallopeptidase domain 33 [Source:HGNC Symbol;Acc:HGNC:15478]
513410	2.4524	0.009629	METAP2	methionyl aminopeptidase 2 [Source:HGNC Symbol;Acc:HGNC:16672]
280677	2.4503	0.009694	HSPA4	heat shock protein family A (Hsp70) member 4 [Source:HGNC Symbol;Acc:HGNC:5237]
507455	2.4503	0.009694	KATNB1	katanin regulatory subunit B1 [Source:HGNC Symbol;Acc:HGNC:6217]
519573	2.4485	0.009694	TSEN54	tRNA splicing endonuclease subunit 54 [Source:HGNC Symbol;Acc:HGNC:27561]
1E+08	2.445	0.009694	STRAP	serine/threonine kinase receptor associated protein [Source:HGNC Symbol;Acc:HGNC:30796]
786013	2.4412	0.009694	PA2G4	proliferation-associated 2G4 [Source:HGNC Symbol;Acc:HGNC:8550]
538975	2.441	0.009694	VIM	vimentin [Source:HGNC Symbol;Acc:HGNC:12692]
1E+08	2.4396	0.009694	CHTF8	chromosome transmission fidelity factor 8 [Source:HGNC Symbol;Acc:HGNC:24353]
359720	2.4337	0.009694	KDELR1	KDEL endoplasmic reticulum protein retention receptor 1 [Source:HGNC Symbol;Acc:HGNC:6304]
512161	2.433	0.009694	SNRPB	small nuclear ribonucleoprotein polypeptides B and B1 [Source:HGNC Symbol;Acc:HGNC:11153]

281937	2.4308	0.009694	NUP133	nucleoporin 133 [Source:HGNC Symbol;Acc:HGNC:18016]
511649	2.4288	0.009694	NMT1	N-myristoyltransferase 1 [Source:HGNC Symbol;Acc:HGNC:7857]
503620	2.4281	0.009694	CNIH1	cornichon family AMPA receptor auxiliary protein 1 [Source:HGNC Symbol;Acc:HGNC:19431]
281103	2.4262	0.009694	UCKL1	uridine-cytidine kinase 1 like 1 [Source:HGNC Symbol;Acc:HGNC:15938]
539498	2.4217	0.009694	PPP4R1	protein phosphatase 4 regulatory subunit 1 [Source:HGNC Symbol;Acc:HGNC:9320]
282641	2.4196	0.009694	MSN	moesin [Source:HGNC Symbol;Acc:HGNC:7373]
527471	2.4187	0.009694	PLPP3	phospholipid phosphatase 3 [Source:HGNC Symbol;Acc:HGNC:9229]
539313	2.4177	0.009694	TIMM9	translocase of inner mitochondrial membrane 9 [Source:HGNC Symbol;Acc:HGNC:11819]
508912	2.4152	0.009694	YBX3	Y-box binding protein 3 [Source:HGNC Symbol;Acc:HGNC:2428]
505840	2.4136	0.009694	HINT1	histidine triad nucleotide binding protein 1 [Source:HGNC Symbol;Acc:HGNC:4912]
532990	2.4107	0.009694	SUMO1	small ubiquitin-like modifier 1 [Source:HGNC Symbol;Acc:HGNC:12502]
337887	2.4083	0.009694	NLRP5	NLR family pyrin domain containing 5 [Source:HGNC Symbol;Acc:HGNC:21269]
281499	2.4065	0.009694	CHST14	carbohydrate sulfotransferase 14 [Source:HGNC Symbol;Acc:HGNC:24464]
286818	2.4052	0.009694	MRPL14	mitochondrial ribosomal protein L14 [Source:HGNC Symbol;Acc:HGNC:14279]
768311	2.4042	0.009694	POMGNT1	protein O-linked mannose N-acetylglucosaminyltransferase 1 (beta 1,2-) [Source:HGNC Symbol;Acc:HGNC:19139]

281945	2.401	0.009747	TCP1	t-complex 1 [Source:HGNC Symbol;Acc:HGNC:11655]
614279	2.3983	0.009758	TADA2A	transcriptional adaptor 2A [Source:HGNC Symbol;Acc:HGNC:11531]
508312	2.3941	0.009758	VEZT	vezatin, adherens junctions transmembrane protein [Source:HGNC Symbol;Acc:HGNC:18258]
615873	2.3926	0.009863	TP53BP1	tumor protein p53 binding protein 1 [Source:HGNC Symbol;Acc:HGNC:11999]
613304	2.3907	0.00987	HSD17B8	estradiol 17-beta-dehydrogenase 8 [Source:RefSeq peptide;Acc:NP_001039789]
532851	2.3892	0.00987	AAR2	AAR2 splicing factor homolog [Source:HGNC Symbol;Acc:HGNC:15886]
511844	2.3845	0.00987	ST7	Suppressor of tumorigenicity 7 protein [Source:UniProtKB/Swiss-Prot;Acc:A4D7R9]
540281	2.3819	0.00987	HOMER3	homer scaffolding protein 3 [Source:HGNC Symbol;Acc:HGNC:17514]
521650	2.3772	0.009876	ACTN1	actinin alpha 1 [Source:HGNC Symbol;Acc:HGNC:163]
513592	2.3742	0.009876	FAF1	Fas associated factor 1 [Source:HGNC Symbol;Acc:HGNC:3578]
768015	2.3736	0.009881	BCL2L1	BCL2 like 1 [Source:HGNC Symbol;Acc:HGNC:992]
1E+08	2.3708	0.009881	EDC4	enhancer of mRNA decapping 4 [Source:HGNC Symbol;Acc:HGNC:17157]
522921	2.3701	0.009973	MRPL24	mitochondrial ribosomal protein L24 [Source:HGNC Symbol;Acc:HGNC:14037]
514793	2.3688	0.010036	SLC25A39	solute carrier family 25 member 39 [Source:HGNC Symbol;Acc:HGNC:24279]
788085	2.3679	0.010081	SLC17A9	solute carrier family 17 member 9 [Source:HGNC Symbol;Acc:HGNC:16192]

510781	2.3671	0.010083	CHMP4B	charged multivesicular body protein 4B [Source:HGNC Symbol;Acc:HGNC:16171]
338054	2.3643	0.010085	PFN2	profilin 2 [Source:HGNC Symbol;Acc:HGNC:8882]
1E+08	2.3633	0.010085	DYNC1I2	cytoplasmic dynein 1 intermediate chain 2 [Source:RefSeq peptide;Acc:NP_001069351]
509583	2.3625	0.010085	CCT7	T-complex protein 1 subunit eta [Source:RefSeq peptide;Acc:NP_001039636]
1E+08	2.3568	0.010089	EIF4E2	eukaryotic translation initiation factor 4E family member 2 [Source:HGNC Symbol;Acc:HGNC:3293]
616055	2.3519	0.010089	MAGED1	MAGE family member D1 [Source:HGNC Symbol;Acc:HGNC:6813]
287022	2.3518	0.010094	EIF4G1	eukaryotic translation initiation factor 4 gamma 1 [Source:HGNC Symbol;Acc:HGNC:3296]
518062	2.3513	0.010114	TSPAN5	tetraspanin 5 [Source:HGNC Symbol;Acc:HGNC:17753]
507270	2.3454	0.010131	NUP93	nucleoporin 93 [Source:HGNC Symbol;Acc:HGNC:28958]
327710	2.3435	0.010131	ABCF2	ATP binding cassette subfamily F member 2 [Source:HGNC Symbol;Acc:HGNC:71]
281997	2.3394	0.010131	TCN1	transcobalamin 1 [Source:HGNC Symbol;Acc:HGNC:11652]
529404	2.3372	0.010131	ETFA	electron transfer flavoprotein alpha subunit [Source:HGNC Symbol;Acc:HGNC:3481]
282646	2.3353	0.010131	OLA1	Obg-like ATPase 1 [Source:HGNC Symbol;Acc:HGNC:28833]
514185	2.334	0.010131	MAGED2	MAGE family member D2 [Source:HGNC Symbol;Acc:HGNC:16353]
618415	2.3288	0.010131	TSPAN3	tetraspanin-3 [Source:RefSeq peptide;Acc:NP_001030564]

517496	2.325	0.010131	RSAD1	radical S-adenosyl methionine domain containing 1 [Source:HGNC Symbol;Acc:HGNC:25634]
511349	2.3227	0.010156	RPL29	ribosomal protein L29 [Source:HGNC Symbol;Acc:HGNC:10331]
539332	2.3194	0.010164	DNAJC19	mitochondrial import inner membrane translocase subunit TIM14 [Source:RefSeq peptide;Acc:NP_001029630]
404098	2.3182	0.010183	CACYBP	calcyclin binding protein [Source:HGNC Symbol;Acc:HGNC:30423]
509857	2.3158	0.010194	CFDP1	craniofacial development protein 1 [Source:HGNC Symbol;Acc:HGNC:1873]
614333	2.3147	0.010233	KPNA2	karyopherin subunit alpha 2 [Source:HGNC Symbol;Acc:HGNC:6395]
527211	2.3143	0.010235	RANGAP1	Ran GTPase activating protein 1 [Source:HGNC Symbol;Acc:HGNC:9854]
286880	2.3137	0.010247	PAM16	presequence translocase-associated motor 16 homolog (S. cerevisiae) [Source:HGNC Symbol;Acc:HGNC:29679]
282271	2.3131	0.010307	ACTA1	actin, alpha 1, skeletal muscle [Source:HGNC Symbol;Acc:HGNC:129]
536676	2.3122	0.010314	SRSF2	serine and arginine rich splicing factor 2 [Source:HGNC Symbol;Acc:HGNC:10783]
527885	2.3118	0.010463	ARF5	ADP ribosylation factor 5 [Source:HGNC Symbol;Acc:HGNC:658]
404053	2.3089	0.010463	BOP1	block of proliferation 1 [Source:HGNC Symbol;Acc:HGNC:15519]
509591	2.3045	0.010463	BCKDK	branched chain ketoacid dehydrogenase kinase [Source:HGNC Symbol;Acc:HGNC:16902]
505524	2.3001	0.010463	HNRNPDL	heterogeneous nuclear ribonucleoprotein D like [Source:HGNC Symbol;Acc:HGNC:5037]

523962	2.2979	0.010463	POU2F1	POU class 2 homeobox 1 [Source:HGNC Symbol;Acc:HGNC:9212]
783497	2.2944	0.010463	RPS6KA3	ribosomal protein S6 kinase A3 [Source:HGNC Symbol;Acc:HGNC:10432]
618047	2.2941	0.010463	RAB4A	RAB4A, member RAS oncogene family [Source:HGNC Symbol;Acc:HGNC:9781]
526135	2.2922	0.010463	MYC	v-myc avian myelocytomatosis viral oncogene homolog [Source:HGNC Symbol;Acc:HGNC:7553]
616365	2.2915	0.010485	ADPRHL2	ADP-ribosylhydrolase like 2 [Source:HGNC Symbol;Acc:HGNC:21304]
1.01E+08	2.2914	0.010499	NAGK	N-acetylglucosamine kinase [Source:HGNC Symbol;Acc:HGNC:17174]
614343	2.2914	0.010512	ILF2	interleukin enhancer binding factor 2 [Source:HGNC Symbol;Acc:HGNC:6037]
281419	2.2912	0.010696	BICD2	BICD cargo adaptor 2 [Source:HGNC Symbol;Acc:HGNC:17208]
521004	2.2906	0.010696	SCRIB	scribbled planar cell polarity protein [Source:HGNC Symbol;Acc:HGNC:30377]
519920	2.2891	0.010696	MED25	mediator of RNA polymerase II transcription subunit 25 [Source:RefSeq peptide;Acc:NP_001075914]
534315	2.289	0.010696	ARL3	ADP ribosylation factor like GTPase 3 [Source:HGNC Symbol;Acc:HGNC:694]
540369	2.2873	0.010711	PLA2G15	phospholipase A2 group XV [Source:HGNC Symbol;Acc:HGNC:17163]
533011	2.2855	0.010715	PABPC4	poly(A) binding protein cytoplasmic 4 [Source:HGNC Symbol;Acc:HGNC:8557]
541130	2.2843	0.010772	PLK2	polo like kinase 2 [Source:HGNC Symbol;Acc:HGNC:19699]
530070	2.2832	0.010805	SRSF1	serine and arginine rich splicing factor 1 [Source:HGNC Symbol;Acc:HGNC:10780]

514372	2.2801	0.010805	TUBA1A	tubulin alpha 1a [Source:HGNC Symbol;Acc:HGNC:20766]
514465	2.2784	0.010805	NUP37	nucleoporin 37 [Source:HGNC Symbol;Acc:HGNC:29929]
522006	2.2749	0.010805	GMPS	guanine monophosphate synthase [Source:HGNC Symbol;Acc:HGNC:4378]
614626	2.2747	0.010856	PPIB	Peptidyl-prolyl cis-trans isomerase B [Source:UniProtKB/Swiss-Prot;Acc:P80311]
512534	2.2725	0.010858	GSS	glutathione synthetase [Source:HGNC Symbol;Acc:HGNC:4624]
444880	2.2709	0.010951	DHX36	DEAH-box helicase 36 [Source:HGNC Symbol;Acc:HGNC:14410]
282318	2.2704	0.010985	RFX7	regulatory factor X7 [Source:HGNC Symbol;Acc:HGNC:25777]
534011	2.268	0.011037	MRPL48	mitochondrial ribosomal protein L48 [Source:HGNC Symbol;Acc:HGNC:16653]
540839	2.2667	0.011168	FOSL1	FOS like 1, AP-1 transcription factor subunit [Source:HGNC Symbol;Acc:HGNC:13718]
617293	2.2658	0.011168	FARS2	phenylalanyl-tRNA synthetase 2, mitochondrial [Source:HGNC Symbol;Acc:HGNC:21062]
785113	2.2648	0.011168	CTNNB1	catenin beta 1 [Source:HGNC Symbol;Acc:HGNC:2514]
517962	2.2632	0.011225	DYNLL2	dynein light chain LC8-type 2 [Source:HGNC Symbol;Acc:HGNC:24596]
281806	2.2623	0.011272	KIF2A	kinesin heavy chain member 2A [Source:HGNC Symbol;Acc:HGNC:6318]
524920	2.2621	0.011278	TMEM223	transmembrane protein 223 [Source:HGNC Symbol;Acc:HGNC:28464]
281860	2.2611	0.011313	RAN	GTP-binding nuclear protein Ran [Source:RefSeq peptide;Acc:NP_001029877]

508759	2.2592	0.011313	NAA30	N(alpha)-acetyltransferase 30, NatC catalytic subunit [Source:HGNC Symbol;Acc:HGNC:19844]
782669	2.2586	0.011313	ZNF664	zinc finger protein 664 [Source:HGNC Symbol;Acc:HGNC:25406]
507997	2.2561	0.011313	PPM1G	protein phosphatase, Mg2+/Mn2+ dependent 1G [Source:HGNC Symbol;Acc:HGNC:9278]
1E+08	2.2519	0.011313	NAA15	N(alpha)-acetyltransferase 15, NatA auxiliary subunit [Source:HGNC Symbol;Acc:HGNC:30782]
615842	2.2508	0.01138	SERBP1	SERPINE1 mRNA binding protein 1 [Source:HGNC Symbol;Acc:HGNC:17860]
615368	2.2497	0.01138	SNX17	sorting nexin 17 [Source:HGNC Symbol;Acc:HGNC:14979]
513777	2.2471	0.01138	MALSU1	mitochondrial assembly of ribosomal large subunit 1 [Source:HGNC Symbol;Acc:HGNC:21721]
506895	2.2462	0.011381	FAM120A	family with sequence similarity 120A [Source:HGNC Symbol;Acc:HGNC:13247]
614347	2.2432	0.011447	ZMAT2	zinc finger matrin-type 2 [Source:HGNC Symbol;Acc:HGNC:26433]
515056	2.2418	0.011465	KLC2	kinesin light chain 2 [Source:HGNC Symbol;Acc:HGNC:20716]
504589	2.2402	0.011587	FAM98A	protein FAM98A [Source:RefSeq peptide;Acc:NP_001076924]
507436	2.2392	0.011587	ELP2	elongator acetyltransferase complex subunit 2 [Source:HGNC Symbol;Acc:HGNC:18248]
508852	2.2391	0.0116	SRSF9	serine and arginine rich splicing factor 9 [Source:HGNC Symbol;Acc:HGNC:10791]
782743	2.2388	0.01177	ZNF706	zinc finger protein 706 [Source:HGNC Symbol;Acc:HGNC:24992]
511190	2.2372	0.011938	LSM14A	LSM14A mRNA processing body assembly factor [Source:HGNC Symbol;Acc:HGNC:24489]

529972	2.236	0.011938	ACTN4	actinin alpha 4 [Source:HGNC Symbol;Acc:HGNC:166]
616120	2.2321	0.011938	ARMC8	armadillo repeat containing 8 [Source:HGNC Symbol;Acc:HGNC:24999]
536553	2.2318	0.011938	YWHAQ	14-3-3 protein theta [Source:UniProtKB/Swiss-Prot;Acc:Q3SZI4]
286796	2.2308	0.012019	SLC25A22	solute carrier family 25 member 22 [Source:HGNC Symbol;Acc:HGNC:19954]
618702	2.2285	0.012086	TPM1	Tropomyosin alpha-3 chain [Source:UniProtKB/Swiss-Prot;Acc:Q5KR47]
504586	2.2282	0.012099	FBL	fibrillarin [Source:HGNC Symbol;Acc:HGNC:3599]
327688	2.2278	0.012164	G0S2	G0/G1 switch 2 [Source:HGNC Symbol;Acc:HGNC:30229]
527595	2.2277	0.012169	FSCN1	fascin actin-bundling protein 1 [Source:HGNC Symbol;Acc:HGNC:11148]
525428	2.2264	0.012169	CAMK2N2	calcium/calmodulin dependent protein kinase II inhibitor 2 [Source:HGNC Symbol;Acc:HGNC:24197]
537619	2.2249	0.012169	RBMX	RNA binding motif protein, X-linked [Source:HGNC Symbol;Acc:HGNC:9910]
513649	2.2248	0.012185	PTPRA	protein tyrosine phosphatase, receptor type A [Source:HGNC Symbol;Acc:HGNC:9664]
531389	2.2247	0.012185	RPS29	40S ribosomal protein S29 [Source:RefSeq peptide;Acc:NP_777229]
525680	2.2224	0.012206	SSRP1	structure specific recognition protein 1 [Source:HGNC Symbol;Acc:HGNC:11327]
617998	2.2216	0.01224	ATP5G2	ATP synthase, H+ transporting, mitochondrial Fo complex subunit C2 (subunit 9) [Source:HGNC Symbol;Acc:HGNC:842]

1.01E+08	2.2198	0.01224	PDCD10	programmed cell death 10 [Source:HGNC Symbol;Acc:HGNC:8761]
327676	2.219	0.01224	TNPO2	transportin 2 [Source:HGNC Symbol;Acc:HGNC:19998]
538357	2.2187	0.01224	TACC1	transforming acidic coiled-coil containing protein 1 [Source:HGNC Symbol;Acc:HGNC:11522]
511124	2.2187	0.012297	COPS5	COP9 signalosome subunit 5 [Source:HGNC Symbol;Acc:HGNC:2240]
535351	2.2179	0.012298	EXOSC5	exosome component 5 [Source:HGNC Symbol;Acc:HGNC:24662]
541058	2.2137	0.01238	PCBP4	poly(rC) binding protein 4 [Source:HGNC Symbol;Acc:HGNC:8652]
541093	2.2098	0.01238	TSPAN6	tetraspanin 6 [Source:HGNC Symbol;Acc:HGNC:11858]
512327	2.2073	0.012439	HNRNPLL	heterogeneous nuclear ribonucleoprotein L like [Source:HGNC Symbol;Acc:HGNC:25127]
529062	2.2047	0.012439	FN1	fibronectin 1 [Source:HGNC Symbol;Acc:HGNC:3778]
540207	2.2044	0.012444	TYW5	tRNA-yW synthesizing protein 5 [Source:HGNC Symbol;Acc:HGNC:26754]
517409	2.2017	0.012482	ZDHHC9	zinc finger DHHC-type containing 9 [Source:HGNC Symbol;Acc:HGNC:18475]
533351	2.2015	0.01265	PDZD8	PDZ domain containing 8 [Source:HGNC Symbol;Acc:HGNC:26974]
528204	2.1997	0.01265	H2AFY	H2A histone family member Y [Source:HGNC Symbol;Acc:HGNC:4740]
768240	2.1994	0.012693	PARP2	poly(ADP-ribose) polymerase 2 [Source:HGNC Symbol;Acc:HGNC:272]
613421	2.1978	0.012701	RHEB	Ras homolog enriched in brain [Source:HGNC Symbol;Acc:HGNC:10011]

614105	2.1971	0.01282	SIGMAR1	sigma non-opioid intracellular receptor 1 [Source:HGNC Symbol;Acc:HGNC:8157]
507196	2.197	0.012857	SLC39A10	solute carrier family 39 member 10 [Source:HGNC Symbol;Acc:HGNC:20861]
515222	2.1948	0.012883	GPS2	G protein pathway suppressor 2 [Source:HGNC Symbol;Acc:HGNC:4550]
513562	2.1946	0.012904	UBE2H	ubiquitin conjugating enzyme E2 H [Source:HGNC Symbol;Acc:HGNC:12484]
509437	2.193	0.012909	TMEM109	transmembrane protein 109 [Source:HGNC Symbol;Acc:HGNC:28771]
540417	2.1929	0.013193	RAD23A	RAD23 homolog A, nucleotide excision repair protein [Source:HGNC Symbol;Acc:HGNC:9812]
506018	2.1894	0.013282	RPL11	ribosomal protein L11 [Source:HGNC Symbol;Acc:HGNC:10301]
512584	2.1873	0.013341	FOSL2	FOS like 2, AP-1 transcription factor subunit [Source:HGNC Symbol;Acc:HGNC:3798]
444858	2.1863	0.013341	SLC35A1	solute carrier family 35 member A1 [Source:HGNC Symbol;Acc:HGNC:11021]
511913	2.184	0.013341	MIF	Macrophage migration inhibitory factor [Source:UniProtKB/Swiss-Prot;Acc:P80177]
532659	2.1832	0.013347	HNRNPR	heterogeneous nuclear ribonucleoprotein R [Source:HGNC Symbol;Acc:HGNC:5047]
510050	2.1829	0.013419	ELOVL5	ELOVL fatty acid elongase 5 [Source:HGNC Symbol;Acc:HGNC:21308]
536558	2.1827	0.013521	CAMK2N1	calcium/calmodulin dependent protein kinase II inhibitor 1 [Source:HGNC Symbol;Acc:HGNC:24190]
780877	2.1825	0.013543	MAP3K1	mitogen-activated protein kinase kinase kinase 1 [Source:HGNC Symbol;Acc:HGNC:6848]
532203	2.1793	0.013623	UBE2R2	ubiquitin-conjugating enzyme E2 R2 [Source:RefSeq peptide;Acc:NP_001193039]

514014	2.177	0.013623	RBBP7	RB binding protein 7, chromatin remodeling factor [Source:HGNC Symbol;Acc:HGNC:9890]
513162	2.1769	0.013671	ZNHIT1	zinc finger HIT-type containing 1 [Source:HGNC Symbol;Acc:HGNC:21688]
280796	2.1766	0.013814	SH3BP4	SH3 domain binding protein 4 [Source:HGNC Symbol;Acc:HGNC:10826]
506066	2.1751	0.013822	SNRPA	small nuclear ribonucleoprotein polypeptide A [Source:HGNC Symbol;Acc:HGNC:11151]
353301	2.1728	0.013827	CCDC43	coiled-coil domain containing 43 [Source:HGNC Symbol;Acc:HGNC:26472]
506889	2.1725	0.013827	CCND1	cyclin D1 [Source:HGNC Symbol;Acc:HGNC:1582]
512043	2.1682	0.013844	EIF2D	eukaryotic translation initiation factor 2D [Source:RefSeq peptide;Acc:NP_001030276]
509158	2.1612	0.013906	PPP1R14C	Bos taurus protein phosphatase 1, regulatory (inhibitor) subunit 14C (PPP1R14C), mRNA. [Source:RefSeq mRNA;Acc:NM_001078068]
787995	2.1599	0.013907	MRPS25	mitochondrial ribosomal protein S25 [Source:HGNC Symbol;Acc:HGNC:14511]
531459	2.1579	0.013949	PDAP1	PDGFA associated protein 1 [Source:HGNC Symbol;Acc:HGNC:14634]
525059	2.1578	0.013949	DNAJC9	dnaJ homolog subfamily C member 9 [Source:RefSeq peptide;Acc:NP_001192615]
520462	2.1574	0.014011	KPNB1	karyopherin subunit beta 1 [Source:HGNC Symbol;Acc:HGNC:6400]
280716	2.154	0.014151	AMMECR1	Alport syndrome, mental retardation, midface hypoplasia and elliptocytosis chromosomal region gene 1 [Source:HGNC Symbol;Acc:HGNC:467]
528839	2.1536	0.014151	CLPB	ClpB homolog, mitochondrial AAA ATPase chaperonin [Source:HGNC Symbol;Acc:HGNC:30664]

513533	2.1511	0.014279	TMEM106C	transmembrane protein 106C [Source:HGNC Symbol;Acc:HGNC:28775]
782132	2.1495	0.014315	CNIH4	cornichon family AMPA receptor auxiliary protein 4 [Source:HGNC Symbol;Acc:HGNC:25013]
1E+08	2.1493	0.014642	PNPT1	polyribonucleotide nucleotidyltransferase 1 [Source:HGNC Symbol;Acc:HGNC:23166]
509422	2.1492	0.014664	EEF1G	eukaryotic translation elongation factor 1 gamma [Source:HGNC Symbol;Acc:HGNC:3213]
510315	2.1474	0.014764	ZNF410	zinc finger protein 410 [Source:HGNC Symbol;Acc:HGNC:20144]
534553	2.1471	0.014947	SMARCAL1	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a like 1 [Source:HGNC Symbol;Acc:HGNC:11102]
286884	2.1469	0.014949	UCK2	uridine-cytidine kinase 2 [Source:HGNC Symbol;Acc:HGNC:12562]
618184	2.1465	0.015044	DTX3	deltex 3, E3 ubiquitin ligase [Source:HGNC Symbol;Acc:HGNC:24457]
519789	2.1435	0.015096	SLC44A2	solute carrier family 44 member 2 [Source:HGNC Symbol;Acc:HGNC:17292]
534046	2.1418	0.015187	PIGF	phosphatidylinositol glycan anchor biosynthesis class F [Source:HGNC Symbol;Acc:HGNC:8962]
373543	2.1391	0.015419	NUCKS1	nuclear casein kinase and cyclin dependent kinase substrate 1 [Source:HGNC Symbol;Acc:HGNC:29923]
282092	2.1388	0.01548	DNTTIP1	deoxynucleotidyltransferase, terminal, interacting protein 1 [Source:HGNC Symbol;Acc:HGNC:16160]
510994	2.1372	0.01548	UGCG	UDP-glucose ceramide glucosyltransferase [Source:HGNC Symbol;Acc:HGNC:12524]

513277	2.1372	0.015562	ACTL6A	actin like 6A [Source:HGNC Symbol;Acc:HGNC:24124]
514541	2.1361	0.015629	PSME2	proteasome activator subunit 2 [Source:HGNC Symbol;Acc:HGNC:9569]
507924	2.1348	0.015686	SPP1	secreted phosphoprotein 1 [Source:HGNC Symbol;Acc:HGNC:11255]
327693	2.1344	0.015741	UXT	ubiquitously expressed prefoldin like chaperone [Source:HGNC Symbol;Acc:HGNC:12641]
537885	2.1321	0.015809	PPIF	peptidylprolyl isomerase F [Source:HGNC Symbol;Acc:HGNC:9259]
282384	2.1282	0.015868	RPS25	40S ribosomal protein S25 [Source:UniProtKB/Swiss-Prot;Acc:Q56JX5]
515766	2.1268	0.015898	WDR43	WD repeat domain 43 [Source:HGNC Symbol;Acc:HGNC:28945]
338072	2.1256	0.015898	TNPO1	transportin-1 [Source:RefSeq peptide;Acc:NP_001070008]
509684	2.125	0.015898	TGIF1	TGFB induced factor homeobox 1 [Source:HGNC Symbol;Acc:HGNC:11776]
281661	2.1244	0.015956	HMX1	H6 family homeobox 1 [Source:HGNC Symbol;Acc:HGNC:5017]
788039	2.1235	0.016052	METTL12	methyltransferase like 12 [Source:HGNC Symbol;Acc:HGNC:33113]
508251	2.1235	0.016099	ARL2	ADP ribosylation factor like GTPase 2 [Source:HGNC Symbol;Acc:HGNC:693]
509076	2.1231	0.016153	MRPS21	mitochondrial ribosomal protein S21 [Source:HGNC Symbol;Acc:HGNC:14046]
1E+08	2.1228	0.016193	INHBA	inhibin beta A subunit [Source:HGNC Symbol;Acc:HGNC:6066]
327680	2.1219	0.01621	PPP5C	protein phosphatase 5 catalytic subunit [Source:HGNC Symbol;Acc:HGNC:9322]

281275	2.1214	0.01621	UTP4	UTP4, small subunit processome component [Source:HGNC Symbol;Acc:HGNC:1983]
534850	2.1207	0.016213	OBSL1	obscurin-like protein 1 [Source:RefSeq peptide;Acc:NP_001068959]
507179	2.1199	0.016213	NTS	neurotensin [Source:HGNC Symbol;Acc:HGNC:8038]
514090	2.1197	0.016264	VASN	vasorin precursor [Source:RefSeq peptide;Acc:NP_001077265]
537402	2.1187	0.016265	IL6ST	interleukin 6 signal transducer [Source:HGNC Symbol;Acc:HGNC:6021]
512321	2.1184	0.016265	FAM212A	family with sequence similarity 212 member A [Source:HGNC Symbol;Acc:HGNC:32480]
617914	2.1147	0.016281	MCTS1	malignant T-cell amplified sequence 1 [Source:HGNC Symbol;Acc:HGNC:23357]
521892	2.1147	0.016429	ANAPC13	anaphase promoting complex subunit 13 [Source:HGNC Symbol;Acc:HGNC:24540]
533922	2.1116	0.016617	CBX5	chromobox 5 [Source:HGNC Symbol;Acc:HGNC:1555]
317659	2.1111	0.01669	AATF	apoptosis antagonizing transcription factor [Source:HGNC Symbol;Acc:HGNC:19235]
508149	2.1108	0.016695	GRAMD1A	GRAM domain containing 1A [Source:HGNC Symbol;Acc:HGNC:29305]
282446	2.1074	0.016708	CCDC58	coiled-coil domain containing 58 [Source:HGNC Symbol;Acc:HGNC:31136]
538838	2.1068	0.016708	MTHFD1	methylenetetrahydrofolate dehydrogenase, cyclohydrolase and formyltetrahydrofolate synthetase 1 [Source:HGNC Symbol;Acc:HGNC:7432]
540426	2.1053	0.016708	OCRL	OCRL, inositol polyphosphate-5-phosphatase [Source:HGNC Symbol;Acc:HGNC:8108]

540406	2.1049	0.016766	LGALS1	galectin 1 [Source:HGNC Symbol;Acc:HGNC:6561]
1E+08	2.1041	0.016931	UFSP1	UFM1-specific peptidase 1 (inactive) [Source:HGNC Symbol;Acc:HGNC:33821]
616881	2.1037	0.016936	PPCDC	phosphopantothenoylcysteine decarboxylase [Source:HGNC Symbol;Acc:HGNC:28107]
507027	2.1033	0.016953	RPS9	ribosomal protein S9 [Source:HGNC Symbol;Acc:HGNC:10442]
505390	2.1022	0.017017	NGLY1	N-glycanase 1 [Source:HGNC Symbol;Acc:HGNC:17646]
1.01E+08	2.0982	0.017017	SH2B1	SH2B adaptor protein 1 [Source:HGNC Symbol;Acc:HGNC:30417]
528860	2.0972	0.01714	GJB2	gap junction protein beta 2 [Source:HGNC Symbol;Acc:HGNC:4284]
506325	2.097	0.017234	CRYAB	Alpha-crystallin B chain [Source:UniProtKB/Swiss-Prot;Acc:P02510]
505134	2.0942	0.017326	FGFR1	fibroblast growth factor receptor 1 [Source:HGNC Symbol;Acc:HGNC:3688]
514161	2.0938	0.017423	HMGN3	high mobility group nucleosomal binding domain 3 [Source:HGNC Symbol;Acc:HGNC:12312]
523627	2.0916	0.017702	TACC2	transforming acidic coiled-coil containing protein 2 [Source:HGNC Symbol;Acc:HGNC:11523]
618428	2.0898	0.017757	ZNF618	zinc finger protein 618 [Source:HGNC Symbol;Acc:HGNC:29416]
534770	2.0892	0.017967	FGGY	FGGY carbohydrate kinase domain containing [Source:HGNC Symbol;Acc:HGNC:25610]
506185	2.0882	0.018101	CHST11	carbohydrate sulfotransferase 11 [Source:HGNC Symbol;Acc:HGNC:17422]
282380	2.0864	0.018102	ADAR	adenosine deaminase, RNA specific [Source:HGNC Symbol;Acc:HGNC:225]

508535	2.0852	0.018132	FKBP7	FK506 binding protein 7 [Source:HGNC Symbol;Acc:HGNC:3723]
512653	2.0851	0.018952	ATP1B1	ATPase Na+/K+ transporting subunit beta 1 [Source:HGNC Symbol;Acc:HGNC:804]
508877	2.0842	0.019347	MECR	trans-2-enoyl-CoA reductase, mitochondrial precursor [Source:RefSeq peptide;Acc:NP_858055]
282447	2.083	0.019467	CAPN1	calpain 1 [Source:HGNC Symbol;Acc:HGNC:1476]
511754	2.0817	0.019534	RTN2	reticulon 2 [Source:HGNC Symbol;Acc:HGNC:10468]
506683	2.0812	0.019645	CFAP36	cilia and flagella associated protein 36 [Source:HGNC Symbol;Acc:HGNC:30540]
540568	2.0806	0.019645	NASP	nuclear autoantigenic sperm protein [Source:HGNC Symbol;Acc:HGNC:7644]
539760	2.0796	0.019662	CKS2	CDC28 protein kinase regulatory subunit 2 [Source:HGNC Symbol;Acc:HGNC:2000]
515798	2.0788	0.019753	LAS1L	LAS1-like, ribosome biogenesis factor [Source:HGNC Symbol;Acc:HGNC:25726]
767620	2.077	0.019913	RRAS	related RAS viral (r-ras) oncogene homolog [Source:HGNC Symbol;Acc:HGNC:10447]
414346	2.0768	0.020008	XPO7	exportin 7 [Source:HGNC Symbol;Acc:HGNC:14108]
533768	2.0759	0.020008	GTF2IRD1	GTF2I repeat domain containing 1 [Source:HGNC Symbol;Acc:HGNC:4661]
520650	2.071	0.020102	ST5	suppression of tumorigenicity 5 [Source:HGNC Symbol;Acc:HGNC:11350]
513622	2.0707	0.020312	GTF3C3	general transcription factor IIIC subunit 3 [Source:HGNC Symbol;Acc:HGNC:4666]
281682	2.0702	0.020424	SHC1	SHC adaptor protein 1 [Source:HGNC Symbol;Acc:HGNC:10840]

517756	2.0693	0.020473	NUSAP1	nucleolar and spindle associated protein 1 [Source:HGNC Symbol;Acc:HGNC:18538]
407223	2.0686	0.020631	F3	coagulation factor III, tissue factor [Source:HGNC Symbol;Acc:HGNC:3541]
534382	2.0679	0.020905	ATP2B1	ATPase plasma membrane Ca2+ transporting 1 [Source:HGNC Symbol;Acc:HGNC:814]
505106	2.0674	0.020905	EXOSC1	exosome component 1 [Source:HGNC Symbol;Acc:HGNC:17286]
533736	2.0673	0.020967	MTMR4	myotubularin related protein 4 [Source:HGNC Symbol;Acc:HGNC:7452]
533175	2.0665	0.020971	LRP3	LDL receptor related protein 3 [Source:HGNC Symbol;Acc:HGNC:6695]
504355	2.0662	0.021147	MIOS	meiosis regulator for oocyte development [Source:HGNC Symbol;Acc:HGNC:21905]
538885	2.066	0.021198	OGFOD3	2-oxoglutarate and iron dependent oxygenase domain containing 3 [Source:HGNC Symbol;Acc:HGNC:26174]
407210	2.0651	0.021596	PKIA	protein kinase (cAMP-dependent, catalytic) inhibitor alpha [Source:HGNC Symbol;Acc:HGNC:9017]
512299	2.065	0.022	MTHFD2	methylenetetrahydrofolate dehydrogenase (NADP+ dependent) 2, methenyltetrahydrofolate cyclohydrolase [Source:HGNC Symbol;Acc:HGNC:7434]
506945	2.0642	0.022	SYNGR3	Synaptogyrin-3 [Source:UniProtKB/Swiss-Prot;Acc:A2VE58]
505288	2.0637	0.022374	VAMP5	vesicle associated membrane protein 5 [Source:HGNC Symbol;Acc:HGNC:12646]
510205	2.0623	0.022528	ZNF777	zinc finger protein 777 [Source:HGNC Symbol;Acc:HGNC:22213]

616164	2.0623	0.02376	ARL4C	ADP ribosylation factor like GTPase 4C [Source:HGNC Symbol;Acc:HGNC:698]
524528	2.0621	0.024203	SMOX	spermine oxidase [Source:HGNC Symbol;Acc:HGNC:15862]
534894	2.0621	0.02531	XRCC5	X-ray repair cross complementing 5 [Source:HGNC Symbol;Acc:HGNC:12833]
1E+08	2.062	0.025374	TBL3	transducin beta-like protein 3 [Source:RefSeq peptide;Acc:NP_001040084]
280933	2.0609	0.025572	UCHL1	ubiquitin C-terminal hydrolase L1 [Source:HGNC Symbol;Acc:HGNC:12513]
782746	2.0607	0.025714	YWHAG	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein gamma [Source:HGNC Symbol;Acc:HGNC:12852]
538774	2.0597	0.025722	B3GALT6	beta-1,3-galactosyltransferase 6 [Source:HGNC Symbol;Acc:HGNC:17978]
1E+08	2.0592	0.02578	FERMT2	fermitin family member 2 [Source:HGNC Symbol;Acc:HGNC:15767]
282052	2.0586	0.02586	HOXB4	homeobox protein Hox-B4 [Source:RefSeq peptide;Acc:NP_001071582]
508864	2.0577	0.02626	TMEM98	transmembrane protein 98 [Source:HGNC Symbol;Acc:HGNC:24529]
540318	2.0576	0.026621	KRT18	keratin 18 [Source:HGNC Symbol;Acc:HGNC:6430]
508813	2.0567	0.026775	L3HYPDH	trans-L-3-hydroxyproline dehydratase [Source:HGNC Symbol;Acc:HGNC:20488]
616028	2.0558	0.026923	MAPRE3	microtubule associated protein RP/EB family member 3 [Source:HGNC Symbol;Acc:HGNC:6892]
614957	2.0557	0.026985	LARP4B	La ribonucleoprotein domain family member 4B [Source:HGNC Symbol;Acc:HGNC:28987]

509983	2.0552	0.027028	HSPG2	heparan sulfate proteoglycan 2 [Source:HGNC Symbol;Acc:HGNC:5273]
514741	2.0552	0.027128	TACO1	translational activator of cytochrome c oxidase I [Source:HGNC Symbol;Acc:HGNC:24316]
280682	2.0498	0.02763	ITGA3	integrin subunit alpha 3 [Source:HGNC Symbol;Acc:HGNC:6139]
281020	2.0491	0.027817	LAMB2	laminin subunit beta 2 [Source:HGNC Symbol;Acc:HGNC:6487]
512840	2.0489	0.027931	CDK6	cyclin dependent kinase 6 [Source:HGNC Symbol;Acc:HGNC:1777]
505723	2.0477	0.028069	MAP1B	microtubule associated protein 1B [Source:HGNC Symbol;Acc:HGNC:6836]
615565	2.0473	0.028115	PTRH1	peptidyl-tRNA hydrolase 1 homolog [Source:HGNC Symbol;Acc:HGNC:27039]
515135	2.0463	0.028208	SPC25	SPC25, NDC80 kinetochore complex component [Source:HGNC Symbol;Acc:HGNC:24031]
618186	2.0458	0.028589	USP13	ubiquitin specific peptidase 13 (isopeptidase T-3) [Source:HGNC Symbol;Acc:HGNC:12611]
504950	2.0457	0.028685	IGFBP6	insulin like growth factor binding protein 6 [Source:HGNC Symbol;Acc:HGNC:5475]
518494	2.0456	0.028792	CYR61	cysteine rich angiogenic inducer 61 [Source:HGNC Symbol;Acc:HGNC:2654]
768014	2.0439	0.028821	AIFM2	apoptosis inducing factor, mitochondria associated 2 [Source:HGNC Symbol;Acc:HGNC:21411]
287023	2.0435	0.029886	ELAC2	elaC ribonuclease Z 2 [Source:HGNC Symbol;Acc:HGNC:14198]
407154	2.0435	0.030462	CDC14A	cell division cycle 14A [Source:HGNC Symbol;Acc:HGNC:1718]

515925	2.0421	0.030462	MAP2K5	dual specificity mitogen-activated protein kinase kinase 5 [Source:RefSeq peptide;Acc:NP_001193699]
282689	2.0419	0.030832	SNRPN	small nuclear ribonucleoprotein polypeptide N [Source:HGNC Symbol;Acc:HGNC:11164]
783762	2.0409	0.031192	SPTBN1	spectrin beta, non-erythrocytic 1 [Source:HGNC Symbol;Acc:HGNC:11275]
539779	2.0402	0.031288	CFL2	cofilin 2 [Source:HGNC Symbol;Acc:HGNC:1875]
510844	2.0385	0.031347	NPM3	nucleophosmin/nucleoplasmin 3 [Source:HGNC Symbol;Acc:HGNC:7931]
616503	2.0371	0.031401	ZBTB20	zinc finger and BTB domain containing 20 [Source:HGNC Symbol;Acc:HGNC:13503]
1.05E+08	2.0361	0.032095	EFEMP2	EGF containing fibulin like extracellular matrix protein 2 [Source:HGNC Symbol;Acc:HGNC:3219]
510201	2.0358	0.03308	SRM	spermidine synthase [Source:HGNC Symbol;Acc:HGNC:11296]
504565	2.0344	0.033162	TMEM205	transmembrane protein 205 [Source:HGNC Symbol;Acc:HGNC:29631]
514261	2.0335	0.033616	FAT1	FAT atypical cadherin 1 [Source:HGNC Symbol;Acc:HGNC:3595]
530358	2.0301	0.034317	CMTM4	CKLF like MARVEL transmembrane domain containing 4 [Source:HGNC Symbol;Acc:HGNC:19175]
533892	2.0301	0.034366	TCF4	transcription factor 4 [Source:HGNC Symbol;Acc:HGNC:11634]
513171	2.0291	0.03459	FBXL16	F-box and leucine rich repeat protein 16 [Source:HGNC Symbol;Acc:HGNC:14150]
526329	2.028	0.034696	CXXC5	CXXC finger protein 5 [Source:HGNC Symbol;Acc:HGNC:26943]

527939	2.0279	0.034717	PAX6	paired box 6 [Source:HGNC Symbol;Acc:HGNC:8620]
613927	2.0277	0.035805	SMARCA1	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 1 [Source:HGNC Symbol;Acc:HGNC:11097]
615796	2.027	0.035905	AK1	adenylate kinase 1 [Source:HGNC Symbol;Acc:HGNC:361]
534385	2.027	0.037637	LAMA4	Bos taurus laminin, alpha 4 (LAMA4), mRNA. [Source:RefSeq mRNA;Acc:NM_001205965]
281066	2.0264	0.037811	CENPW	Bos taurus centromere protein W (CENPW), mRNA. [Source:RefSeq mRNA;Acc:NM_001111261]
1.02E+08	2.0258	0.038051	HES1	hes family bHLH transcription factor 1 [Source:HGNC Symbol;Acc:HGNC:5192]
767979	2.0254	0.038164	HSPB8	heat shock protein family B (small) member 8 [Source:HGNC Symbol;Acc:HGNC:30171]
615755	2.0253	0.038277	ARHGEF17	Rho guanine nucleotide exchange factor (GEF) 17 [Source:RefSeq peptide;Acc:NP_001269515]
506939	2.0235	0.038652	RAB13	RAB13, member RAS oncogene family [Source:HGNC Symbol;Acc:HGNC:9762]
540457	2.0221	0.038662	AP1M2	adaptor related protein complex 1 mu 2 subunit [Source:HGNC Symbol;Acc:HGNC:558]
512479	2.0216	0.038788	SPRY2	sprouty RTK signaling antagonist 2 [Source:HGNC Symbol;Acc:HGNC:11270]
506411	2.0207	0.038967	PHYHIPL	phytanoyl-CoA 2-hydroxylase interacting protein like [Source:HGNC Symbol;Acc:HGNC:29378]
531945	2.0183	0.039298	FAM171A1	protein FAM171A1 precursor [Source:RefSeq peptide;Acc:NP_001095650]
1E+08	2.0171	0.039621	TRIP6	thyroid hormone receptor interactor 6 [Source:HGNC Symbol;Acc:HGNC:12311]

510525	2.0168	0.040039	CDK5	cyclin dependent kinase 5 [Source:HGNC Symbol;Acc:HGNC:1774]
512427	2.0165	0.04049	RSRC1	arginine and serine rich coiled-coil 1 [Source:HGNC Symbol;Acc:HGNC:24152]
506131	2.015	0.041859	SDC1	syndecan 1 [Source:HGNC Symbol;Acc:HGNC:10658]
509256	2.0141	0.042002	NR2F2	nuclear receptor subfamily 2 group F member 2 [Source:HGNC Symbol;Acc:HGNC:7976]
616632	2.0137	0.042654	LHX1	LIM homeobox 1 [Source:HGNC Symbol;Acc:HGNC:6593]
535714	2.0128	0.042687	CTGF	connective tissue growth factor [Source:HGNC Symbol;Acc:HGNC:2500]
614967	2.0107	0.04301	KYNU	kynureninase [Source:HGNC Symbol;Acc:HGNC:6469]
519758	2.0093	0.04408	GPC4	glypican 4 [Source:HGNC Symbol;Acc:HGNC:4452]
286862	2.0093	0.044623	PQBP1	polyglutamine binding protein 1 [Source:HGNC Symbol;Acc:HGNC:9330]
533865	2.0091	0.044623	IRX3	iroquois homeobox 3 [Source:HGNC Symbol;Acc:HGNC:14360]
511091	2.0082	0.045199	FAM69B	family with sequence similarity 69 member B [Source:HGNC Symbol;Acc:HGNC:28290]
512562	2.0071	0.045393	KLHDC9	kelch domain containing 9 [Source:HGNC Symbol;Acc:HGNC:28489]
505050	2.0062	0.045652	TIMP1	TIMP metallopeptidase inhibitor 1 [Source:HGNC Symbol;Acc:HGNC:11820]
536838	2.0047	0.046137	TM7SF2	transmembrane 7 superfamily member 2 [Source:HGNC Symbol;Acc:HGNC:11863]
505401	2.004	0.046983	LHX2	LIM homeobox 2 [Source:HGNC Symbol;Acc:HGNC:6594]

615849	2.0037	0.047263	CBX6	chromobox 6 [Source:HGNC Symbol;Acc:HGNC:1556]
615572	2.0007	0.049024	SCN1B	sodium voltage-gated channel beta subunit 1 [Source:HGNC Symbol;Acc:HGNC:10586]
538548	2.0004	0.049227	KCTD15	potassium channel tetramerization domain containing 15 [Source:HGNC Symbol;Acc:HGNC:23297]

Appendix 6 Complete list of significantly upregulated differential genes in adult *N. caninum* infected and uninfected cattle

EntrezID	logFC	adj.P.Val	Symbols	Name
538748	7.1	0.000134	RPL10L	ribosomal protein L10 like [Source:HGNC Symbol;Acc:HGNC:17976]
539034	5.6	0.024019	PFN2	profilin 2 [Source:HGNC Symbol;Acc:HGNC:8882]
614074	5.3	0.024019	TOMM7	translocase of outer mitochondrial membrane 7 [Source:HGNC Symbol;Acc:HGNC:21648]
528453	5.3	0.024019	SFN	stratifin [Source:HGNC Symbol;Acc:HGNC:10773]
281418	5.3	0.024019	PPIA	Peptidyl-prolyl cis-trans isomerase A Peptidyl-prolyl cis-trans isomerase A, N-terminally processed [Source:UniProtKB/Swiss-Prot;Acc:P62935]
280794	5.3	0.024019	FN1	fibronectin 1 [Source:HGNC Symbol;Acc:HGNC:3778]
514394	5.3	0.024019	UCHL1	ubiquitin C-terminal hydrolase L1 [Source:HGNC Symbol;Acc:HGNC:12513]
540305	5.3	0.024019	S1PR2	sphingosine-1-phosphate receptor 2 [Source:HGNC Symbol;Acc:HGNC:3169]
530409	5.2	0.024019	PHB	prohibitin [Source:HGNC Symbol;Acc:HGNC:8912]
281768	5.2	0.024019	FGFR1	fibroblast growth factor receptor 1 [Source:HGNC Symbol;Acc:HGNC:3688]
537907	5.2	0.024019	ST5	suppression of tumorigenicity 5 [Source:HGNC Symbol;Acc:HGNC:11350]
540907	5.2	0.024019	EPHB3	ephrin type-B receptor 3 precursor [Source:RefSeq peptide;Acc:NP_001179725]
540176	5.2	0.024019	ATP5G3	ATP synthase, H+ transporting, mitochondrial Fo complex subunit C3 (subunit 9) [Source:HGNC Symbol;Acc:HGNC:843]

529759	5.2	0.024019	SDC1	syndecan 1 [Source:HGNC Symbol;Acc:HGNC:10658]
519758	5.2	0.024019	ATP1B1	ATPase Na+/K+ transporting subunit beta 1 [Source:HGNC Symbol;Acc:HGNC:804]
533892	5.1	0.025002	RPS9	ribosomal protein S9 [Source:HGNC Symbol;Acc:HGNC:10442]
282262	5.0	0.038547	IGFBP4	insulin like growth factor binding protein 4 [Source:HGNC Symbol;Acc:HGNC:5473]
508490	4.9	0.044061	ITGA3	integrin subunit alpha 3 [Source:HGNC Symbol;Acc:HGNC:6139]
326581	4.9	0.044061	EEF1G	eukaryotic translation elongation factor 1 gamma [Source:HGNC Symbol;Acc:HGNC:3213]
618849	4.9	0.044061	HMGA1	high mobility group AT-hook 1 [Source:HGNC Symbol;Acc:HGNC:5010]
281997	4.9	0.046164	PRDX1	peroxiredoxin 1 [Source:HGNC Symbol;Acc:HGNC:9352]
510325	4.8	0.046699	FAM214B	family with sequence similarity 214 member B [Source:HGNC Symbol;Acc:HGNC:25666]
524530	4.8	0.046699	CCND1	cyclin D1 [Source:HGNC Symbol;Acc:HGNC:1582]

Appendix 7 Significantly upregulated biological pathways in calf infected versus uninfected

Pathway	Total no. of genes	No. of Upregulated genes	*P Value*
nucleosome assembly	233	97	4.16E-28
hemopoiesis	53	34	5.39E-18
base-excision repair	68	36	3.34E-15
regulation of secretion	137	54	6.04E-15
positive regulation of transferase activity	166	59	7.76E-14
Rho protein signal transduction	94	41	2.07E-13
DNA-dependent transcription, elongation	106	44	2.31E-13
DNA catabolic process	16	15	6.91E-13
DNA damage checkpoint	487	117	9.42E-12
nitrogen compound metabolic process	227	68	1.06E-11
alcohol metabolic process	87	36	3.91E-11
regulation of transcription from RNA polymerase II promoter	498	117	4.37E-11
energy reserve metabolic process	206	62	7.03E-11
hormone secretion	81	34	8.53E-11
DNA damage response, signal transduction by p53 class mediator	317	83	1.30E-10
positive regulation of cell adhesion	120	43	1.40E-10
DNA replication initiation	604	133	2.22E-10
organic acid metabolic process	13	12	2.61E-10
JAK-STAT cascade	291	77	3.62E-10
steroid metabolic process	349	87	6.96E-10
intrinsic apoptotic signaling pathway	36	20	1.58E-09
tRNA metabolic process	27	17	1.89E-09
lipid biosynthetic process	374	90	2.40E-09
phagocytosis	103	37	2.48E-09
regulation of cytokine biosynthetic process	708	146	3.48E-09
epithelial cell differentiation	34	19	3.57E-09
regulation of protein metabolic process	147	44	4.90E-08
viral reproduction	17	12	7.48E-08
actin filament-based process	161	45	3.17E-07
protein polymerization	54	22	3.44E-07
cell maturation	426	92	3.90E-07
aerobic respiration	72	26	5.15E-07
response to hypoxia	105	33	6.69E-07
actin filament organization	30	15	1.11E-06
regulation of MAPK cascade	93	30	1.15E-06
homeostasis of number of cells	406	86	2.18E-06
DNA-dependent DNA replication	68	24	2.25E-06

post-Golgi vesicle-mediated transport	18	11	2.32E-06
tyrosine phosphorylation of STAT protein	232	55	5.15E-06
cell-cell adhesion	49	19	5.20E-06
apoptotic DNA fragmentation	62	22	5.35E-06
transcription, DNA-dependent	45	18	5.47E-06
pyrimidine nucleotide metabolic process	58	21	6.05E-06
DNA repair	338	73	6.25E-06
positive regulation of cellular component organization	11	8	9.28E-06
double-strand break repair	27	13	9.96E-06
receptor-mediated endocytosis	51	19	1.03E-05
carbohydrate transport	146	38	1.60E-05
positive regulation of transcription, DNA-dependent	28	13	1.64E-05
stress-activated protein kinase signaling cascade	66	22	1.69E-05
mitotic sister chromatid segregation	25	12	2.29E-05
neutral amino acid transport	12	8	2.47E-05
mRNA processing	19	10	4.10E-05
nucleotide-excision repair	89	26	4.20E-05
positive regulation of T cell proliferation	126	33	4.95E-05
humoral immune response	48	17	6.37E-05
phosphatidylinositol biosynthetic process	270	58	6.47E-05
multicellular organismal development	44	16	7.23E-05
apoptotic nuclear changes	178	42	7.32E-05
chromatin remodeling	49	17	8.59E-05
embryo implantation	36	14	8.73E-05
immune response	28	12	9.20E-05
cation transport	17	9	9.53E-05
cellular aromatic compound metabolic process	8	6	0.000107
response to drug	8	6	0.000107
tissue remodeling	41	15	0.000112
RNA catabolic process	18	9	0.000169
cytoskeleton organization	214	47	0.00018
fatty acid oxidation	292	60	0.00018
DNA-dependent transcription, initiation	22	10	0.000198
one-carbon metabolic process	22	10	0.000198
steroid biosynthetic process	215	47	0.000202
M phase of mitotic cell cycle	57	18	0.000212
negative regulation of transcription from RNA polymerase II promoter	15	8	0.000223
muscle organ development	15	8	0.000223
regulation of endocytosis	15	8	0.000223
positive regulation of cell differentiation	35	13	0.000267
regulation of pH	12	7	0.000272
apoptotic signaling pathway	63	19	0.00028
chromosome condensation	9	6	0.000285

regulation of small GTPase mediated signal transduction	4	4	0.000286
positive regulation of NF-kappaB transcription factor activity	23	10	0.00031
small GTPase mediated signal transduction	36	13	0.000369
reciprocal meiotic recombination	145	34	0.000389
rhythmic process	28	11	0.000451
regulation of body fluid levels	24	10	0.000469
regulation of DNA binding	47	15	0.000619
regulation of cyclin-dependent protein kinase activity	52	16	0.00065
lysosomal transport	62	18	0.000668
regulation of cell growth	43	14	0.000746
androgen receptor signaling pathway	54	16	0.00103
MAPK cascade	22	9	0.00106
meiotic cell cycle	65	18	0.00123
cell migration	5	4	0.00128
positive regulation of DNA binding	5	4	0.00128
regulation of cell differentiation	5	4	0.00128
macromolecule biosynthetic process	252	50	0.00136
inorganic anion transport	115	27	0.00145
vesicle-mediated transport	8	5	0.00148
cellular homeostasis	23	9	0.00154
protein processing	46	14	0.00157
RNA 3'-end processing	46	14	0.00157
regulation of Rho protein signal transduction	28	10	0.00192
positive regulation of cysteine-type endopeptidase activity involved in apoptotic process	42	13	0.00192
cell cycle checkpoint	3	3	0.0022
response to toxin	3	3	0.0022
anion transport	53	15	0.00242
anatomical structure morphogenesis	91	22	0.00261
positive regulation of binding	169	35	0.00327
isoprenoid metabolic process	60	16	0.00342
protein modification process	21	8	0.00342
DNA metabolic process	6	4	0.00345
lipoprotein metabolic process	13	6	0.00365
G2/M transition of mitotic cell cycle	77	19	0.00389
positive regulation of cytokine secretion	1160	181	0.00397
establishment of organelle localization	31	10	0.00451
ribosome biogenesis	22	8	0.00477
positive regulation of protein metabolic process	10	5	0.00531
protein localization	32	10	0.0058
RNA splicing, via transesterification reactions	121	26	0.00631

Appendix 8 Significantly upregulated biological pathways in adult infected versus uninfected

Pathway	Total no. of genes	No. of Upregulated genes	*P value*
nucleosome assembly	233	68	1.29E-80
JAK-STAT cascade	291	21	4.08E-11
intrinsic apoptotic signaling pathway	36	8	8.75E-09
regulation of endocytosis	15	6	1.32E-08
hormone secretion	81	10	4.51E-08
positive regulation of hydrolase activity	59	8	5.07E-07
actin filament organization	30	6	1.34E-06
response to drug	8	4	1.4E-06
base-excision repair	68	8	1.54E-06
epithelial cell differentiation	34	6	2.92E-06
gene silencing	13	4	1.37E-05
immune response	28	5	1.93E-05
Hemopoiesis	53	6	4.13E-05
positive regulation of NF-kappaB transcription factor activity	23	4	0.000154
response to hypoxia	105	7	0.000283
inorganic anion transport	115	7	0.000492
tyrosine phosphorylation of STAT protein	232	10	0.000522
positive regulation of transferase activity	166	8	0.00093
stress-activated protein kinase signaling cascade	66	5	0.00121
apoptotic process	20	3	0.00172
cell projection assembly	6	2	0.00213
DNA-dependent transcription, initiation	22	3	0.00228
chromatin remodeling	49	4	0.00289
actin filament-based process	161	7	0.00346
cell-matrix adhesion	8	2	0.00391
chromosome condensation	9	2	0.00499
interleukin-2 production	9	2	0.00499
cell maturation	426	12	0.00565
apoptotic signaling pathway	63	4	0.00714
small GTPase mediated signal transduction	36	3	0.00935
negative regulation of apoptotic process	37	3	0.0101
proteoglycan biosynthetic process	70	4	0.0103
negative regulation of transcription, DNA-dependent	1	1	0.0122
DNA damage response, signal transduction by p53 class mediator	317	9	0.0152
regulation of cytokine production	16	2	0.0157
RNA splicing, via transesterification reactions	121	5	0.0159
lipid catabolic process	17	2	0.0177

DNA repair	338	9	0.022
sexual reproduction	2	1	0.0242
regulation of cyclin-dependent protein kinase activity	52	3	0.0252
protein modification process	21	2	0.0265
actin polymerization or depolymerization	140	5	0.0279
embryonic morphogenesis	22	2	0.0289
cellular homeostasis	23	2	0.0314
cytoskeleton-dependent intracellular transport	23	2	0.0314
regulation of body fluid levels	24	2	0.034
synaptic transmission	59	3	0.0348
DNA damage checkpoint	487	11	0.0348
mitotic sister chromatid segregation	25	2	0.0366
lysosomal transport	62	3	0.0395
meiotic cell cycle	65	3	0.0444
regulation of Rho protein signal transduction	28	2	0.0451
interleukin-8 biosynthetic process	29	2	0.0481

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