Shifting ruminant monocyte subsets in the neonate to adult transition

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by

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Abbreviations

AAM – alternatively activated macrophage

Ag - antigen

APCs – antigen presenting cells

BMDM – bone marrow derived macrophages

BSA – bovine serum albumin

bTB – Bovine tuberculosis

CAM – classically activated macrophage

CCR2 – chemokine receptor type 2

CD – cluster of differentiation

COX2 – cyclo-oxygenase 2

DAMPs – damage associated molecular patterns

DCs – dendritic cells

DNA – deoxyribonucleic acid

dsRNA - double stranded RNA

FACS – fluorescence activated cell sorting

FBS – foetal bovine serum

IFN-y – Interferon gamma

IL – Interleukin

iNOS – inducible nitic oxide synthase

KO – knock out

LPS - lipopolysaccharide

MCSF – macrophage colony stimulating factor

MHCII – major histocompatibility complex II

MPS – mononuclear phagocyte system

Mtb – *Mycobacterium tuberculosis*

NK cells – natural killer cells

NLRs – nod like receptors

PAMPS – pathogen associated molecular patterns

PBLs – peripheral blood leukocytes

PBMCs – peripheral blood mononuclear cells

PBS – phosphate buffered saline

PHA - phytohaemagglutinin

PMA - Phorbol 12-myristate 13-acetate

PRR – pattern recognition receptor

RIG receptor - retinoic acid-inducible gene receptor

RNA – ribonucleic acid

SCID – severe combined immunodeficiency

ssRNA - single stranded RNA

Th – T helper

TLR – toll like receptor

TNFα – tumour necrosis factor α

µl – microlitre

X g – times gravity

Abstract

Monocytes are a critical part of the innate immune system, responsible for immune activation and therefore disease control. These cells exist in multiple subsets but the proportions and functions of these are poorly defined within cattle. To date there has been little research as to the proportions of the different monocyte subsets that exist within the bovine neonates compared to the adult population, especially within the $CD16^{+}$ subsets. Previous studies have demonstrated that adults and neonates differ in their response to infection challenge. The predominance of classical or non-classical monocyte subsets can have significant effects on the outcome of disease and has potential implications for disease transmission, both from a herd and zoonotic perspective. In addition, this may have an effect on the impact of coinfection and the ability of helminth parasites to dysregulate the immune response and upregulate arginase expression. This current study aimed to identify monocyte subsets within both adult and neonate cattle and establish any differences that exist. Peripheral blood leucocytes (PBLs) were first isolated from adult and neonatal blood samples before fluorescent activated cell sorting (FACS) was used to identify CD14, CD16 and MHCII surface markers present on the monocyte population. Neonate cattle were found to have significantly higher proportions of $CD14^{+}$, $CD14^{+}CD16^{-}MHCII^{-}$ and a higher proportion of the triple negative subset, alongside greater overall quantities of monocytes. Functionality assays demonstrated that neonates have a significantly higher expression of both CCR2 and arginase which both play a role in the response to infection. Cluster analysis showed that the adult and neonates form two separate clusters, with neonates showing a higher degree of homogeneity amongst the subsets, indicating that neonatal development follows a predictable pattern. Overall, this indicates that neonates are likely to have greater plasticity within their monocyte population and are therefore able to adapt to different infection challenges within their cellular repertoire better than the adult populations.

Chapter 1. Introduction

The innate immune system exists as a rapid non-specific first response to infection, with the adaptive immune system becoming activated subsequent to these initial innate encounters. The adaptive immune system is characterised by the possession of antigen specific properties and the ability to develop memory responses. This memory response enables a more rapid and specific response to repeat pathogen challenge and is the basis for successful immunisation.

The innate immune system provides the first crucial barrier against pathogens, initiates tissue repair processes and serves to prime and shape the adaptive immune response via antigen presentation and localised cytokine production. It consists of a variety of cellular and biochemical defence mechanisms that recognise common structures on pathogens and requires no prior exposure to provide effective protection. It has a broad spectrum to ensure that the greatest number of pathogens are detected as possible. The importance of the innate immune system is demonstrated repeatedly by the use of SCID mice, or similar, who show resistance to multiple infectious disease challenges despite an inability to mount an adaptive immune response. This is demonstrated by infection with *Listeria monocytogenes*; early production of cytokines IFN-γ and TNF by innate immune cells (for example Natural Killer (NK) cells and macrophages) is essential and without these cytokines SCID mice rapidly die[1].

1.1 Components of Innate immunity

The innate immune system can be divided into three main components: 1) physical and chemical barriers such as epithelia and their locally secreted products, antimicrobial peptides such as defensins and cathelicidins at the barrier surface; 2) phagocytic or granulocytic cells such as macrophages and neutrophils, dendritic cells (DCs), natural killer (NK) cells and innate lymphoid cells and 3) blood protein components such as complement[2].

1.2 Pattern Recognition Receptors

Cells of the innate immune system possess Pattern Recognition Receptors (PRRs) which detect Pathogen Associated Molecular Patterns (PAMPs) and Damage Associated Molecular Pathogens (DAMPs). PRRs are ubiquitously expressed across cell types throughout lymphoid and non-lymphoid tissues. They are situated in various locations throughout the cell: on the

cell membrane, cytosol, and within phagocytic vesicles. PAMPs are highly conserved patterns found in microorganisms that are absent from host cells such as bacterial and fungal cell wall components e.g. lipopolysaccharide (LPS), fungal glucans, and viral nucleic acids. DAMPs are products released by damaged or dead cells, such as alarmins released by stressed or dying cells. Binding to a PRR activates signal transduction pathways that upregulates cellular functions, such as the secretion of inflammatory cytokines, chemokines and type 1 interferons[3].

Different pathogens are recognised by different receptors depending on where they first enter the host. Intracellular bacteria, for example, *Mycobacterium tuberculosis* (Mtb), are recognised through CD14, TLRs, NLRs, mannose receptors and DC SIGN, mainly through the mycobacterial cell wall components. For example, mycobacteria are rich in lipoarabinomannan (Lam), which, alongside lipopeptides, is detected by TLR2. This is demonstrated by blockade of MyD88 and TLR2 leading to a lack of TNFα production[4]. Recognition of the helminth liver fluke, *Fasciola hepatica*, for example, is largely through recognition of glycosylated proteins by c-type lectin receptors such as the mannose and dectin receptors on DCs and macrophages. Blockade of the mannose receptor leads to partial inhibition of the immunomodulatory effects of *F. hepatica*, and together with the dectin 1 receptor, may be crucial for its survival within the host[5]. DAMPs also play a role in enabling recognition of parasites via damage of host tissue; newly encysted juvenile fluke (NEJs) enter the peritoneal cavity through the gut endothelium which triggers release of DAMPs from the epithelial lining[6,7].

The mechanisms of viral and bacterial recognition are better defined than for parasites. Virus infected cells are recognised by the immune system due to the production of double stranded RNA (dsRNA) and other viral nucleic acids[8]. TLR 3, 7, 8 and 9 are expressed within cells associated with the endoplasmic reticulum and endosomal membranes and detect nucleic acid ligands typical of viruses. For example, TLR 7 and 8 recognise single stranded RNA (ssRNA) and TLR 9 dsDNA, which are produced by host cells but not present in the endosome under normal conditions. Dual TLR 2 and 9 expression are needed for effective resistance against herpes virus infection; dual ablation of both led to high mortality, and ablation of one or other leads to an increased viral cell load. High mortality was associated with a reduction in early monocyte and NK cell infiltration[9]. RIG receptors are cytoplasmic RNA helices which detect viral dsRNA and RNA-DNA duplexes within the cytosol. RIG-I is essential for the induction of type 1 interferon in various cells such as fibroblasts and DCs, whereas plasmacytoid DCs use TLRs for recognition and do not require RIG-I[10]. MDA5 is a

cytosolic helicase that detects dsRNA ligands, and, in contrast to RIG-I, binds dsRNA internally. RIG-I is essential for the recognition of ssRNA viruses such as influenza and paramyxoviruses, and the MDA5 critical for picornavirus detection as demonstrated by the respective KO mice being more susceptible to infection[11].

1.3 Mononuclear phagocyte system

The mononuclear phagocyte system (MPS), comprises a network of cells derived from progenitor cells in the bone marrow. Originally these cells were thought to be derived from a common lineage, however they are now also known, within the mouse, to develop in multiple waves; phagocytes arising from erythro-myeloid progenitors (EMPs) in the yolk sac, monocytes produced in the foetal liver and finally, monocytes derived from haemopoietic stem cells[12]. These cells share common functional features, such as the ability to phagocytose cells, release of cytotoxic granules, migration in response to chemokines and secretion of cytokines. The system is made up of monocytes, macrophages and dendritic cells[13]. Monocytes are the main mononuclear phagocytic cell predominately found circulating within the blood, with macrophages and dendritic cells found within tissues. This system is critical for innate immunity, controlling inflammation and maintaining tissue tolerance. IL-4 receptor KO mice show an absence of IL-4 and IL-13 signalling and an increase in Th1 cytokine responses when infected with the helminth *Schistosoma mansoni*. Macrophages make up a large component of the *S. mansoni* egg granuloma; with IL-13 being responsible for inducing arginase activity within macrophages and so critical for regulation of granuloma formation and maintenance. Absence of both these responses leads to 100% mortality, despite the presence of regulatory IL-10, due to the absence of alternatively activated macrophages (AAMs, see Section 1.5) to control disease associated pathology. Mortality was independent of neutrophils, eosinophils and antigen specific Th2 responses; highlighting the crucial role of the MPS in controlling inflammation in acute helminth infection[14]. In their role as antigen presenting cells both macrophages and DCs are critical in allowing the development of self-tolerance. For example, DCs within the gut become conditioned with gut flora and develop into $CD103^+$ DCs that induce regulatory T cells via a process of antigenic tolerance[15] .

1.4 Monocytes

Monocytes have bean shaped nuclei and a finely granular cytoplasm possessing lysozymes and phagocytic vacuoles. In adult cattle, monocytes comprise between 0-8% of the

circulating white blood cell compartment[16]. Monocyte Colony Stimulating Factor (M-CSF) stimulates monocyte precursors in the bone marrow to mature (Figure 1). These cells are then released into the bloodstream where they circulate until they are recruited to site of injury or inflammation by a combination of chemokines and selectin/integrin interactions.

It was previously thought that monocytes leave the bloodstream and act as a reservoir for tissue macrophages and DCs within the tissues, differentiating on extravasation. During stress or exercise the number of monocytes present within the bloodstream can increase in minutes and return to baseline rapidly. This is due to the existence of a marginal pool, cells in close contact with the venuole endothelium from which cells are mobilised in response to catecholamines. This is facilitated by differential expression of adhesion molecules compared to those found on circulating monocytes, with cells in the marginal pool possessing high levels of PECAM1[17].

Table 1. Monocyte receptor expression between subsets in different species. Common CD markers used to phenotype monocytes are listed. +/- denotes expression level.

	Classical Monocytes			Intermediate Monocytes			Non-classical Monocytes		
	Human	Mouse	Bovine	Human	Mouse	Bovine	Human	Mouse	Bovine
CD14	+		+	+		+	+	+	-
CD16	-		-	+		+	+	+	+
MHCII	+		+	+		+	+		+
CD172a			+			+			+
CD163	+		+	+		+			+/-
Ly6C (Gr1)		+			+			+	
CD43	+	+		+	+		+	+	
CCR2	+	+	+	+	+	+	-	-	-
CX3CR1	-	-	-				+	+	+
CD62L	+	+	+	+	+	+	-	-	-

18,19,20,21,22,23,24

Monocyte subsets were first identified in the 1980s when $CD16^+$ and $CD14^+$monocytes were characterised. This led to the identification of three subsets in humans; $CD14^+CD16^-$ classical monocytes, $CD14^+CD16^+$ intermediate monocytes and $CD14^{low}CD16^+$ non classical monocytes[25]. Bovine monocyte subsets were identified more recently using the same CD14 CD16 classification, and, as with the human subsets, identified classical monocytes as being the largest subset (89%)[19](Table 1).

In recent years monocytes have been characterised as being a heterogeneous population of cells distinguished by their expression of cell surface markers, functions, and cell size. Monocytes can be identified by their expression of CD11b and CD11c in humans, and CD11b, CD115 and F4/80 in mice[26]. Bovine monocytes can be identified based on CD172a expression[19,24], all express CD115 and have similar levels of CD40 and 80 expression[24]. Both subsets express CXCR4. They lack B, T, NK and DC markers, and are morphologically and phenotypically heterogeneous[22].

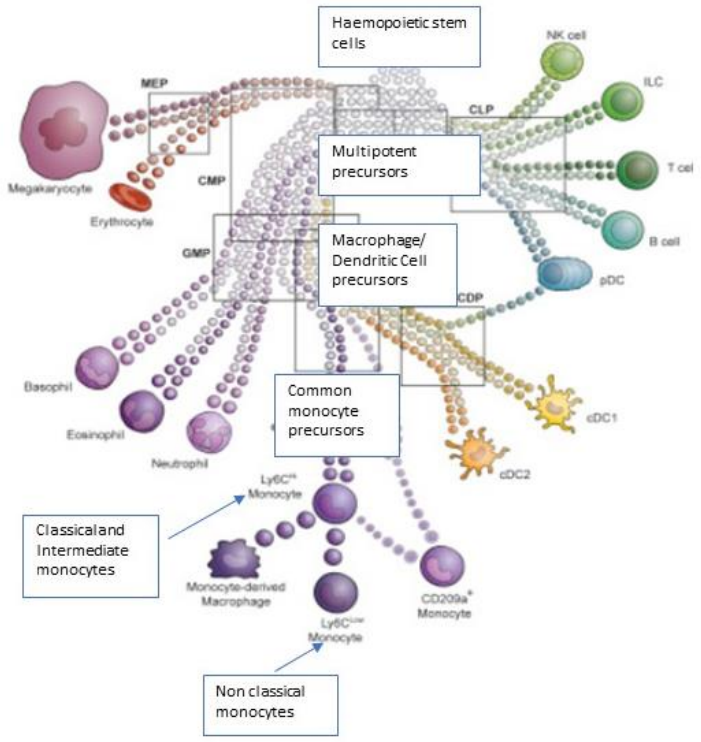


Figure 1. Monocyte development. Adapted from Guilliams et al, 2018[27]This image shows the development of monocyte subsets from their origin as stem cells through the different stages of myeloid cell differentiation.

1.4.1 Classical monocytes

Classical monocytes are the most numerous monocyte subset, and in mice and humans, produce inflammatory mediators and are rapidly recruited to tissue following injury (or

insult). They possess low levels of the CX3CR1 receptor but express high quantities of the CCR2 receptor. In humans these cells express high surface levels of CD14 and lack CD16 and in addition are $CD62L^{+}$, $CD64^{+}$ and express the inflammatory chemokine receptors CCR1, CXCR1 and CXCR2[20,28]. In mice these cells express CD62L, Ly6C/G (Gr1), VLA2 and 4 and LFA1[22]. In cattle a majority of classical monocytes express low levels of CD16 and have high expression of TLR2[24].

Physically these cells are usually larger than the non-classical monocytes, having a diameter of 10-14μm compared to 8-12μm for the non-classical subtype. These cells have been demonstrated as being present in the spleen under steady state, but upon inflammation in the absence of infection invade the peritoneum, which corresponds with their expression of CD62L and CCR2[22]. Classical monocytes express high levels of CCR2 which can bind to several chemokines, most importantly CCL2 (MCP-1). Murine studies demonstrate that recruitment occurs when tissues express CCL2 in response to infection which then mediates monocyte mobilisation from the bone marrow and migration into both the peritoneal cavity and lymph nodes via the high endothelial venuoles (HEVs)[29]. However the role of CCR2 in recruitment to tissues is not absolute as studies using CCR2 KO mice showed that monocytes were still able to travel to sites of infection in the absence of CCR2[30]. Likewise, CD62L expression has been demonstrated to play a key role in directing monocytes to sites of inflammation and lymph nodes by inducing rolling along the endothelium, initiating adhesion. In non-inflammatory states CD62L is responsible for directing monocytes towards non lymphoid tissue such as the bone marrow[31]. In addition, human studies demonstrate that classical monocytes will also migrate in response to MIP-1α[23]. It has also been demonstrated in mice that CCR6/CCL20 are required to recruit $Gr1^{hi}$ monocytes into epithelial tissue after skin immunisation, and that this is essential for CD8 T cell priming by monocyte derived DCs[32]. In mice, they show a greater tendency to migrate to sites of inflammation compared with the non-classical subset[22]. In humans and mice, they have been demonstrated to give rise to macrophages and DCs in inflammatory states[33], and replenish resident macrophage populations as demonstrated by the ability to differentiate into Langerhans cells *in vitro* in response to CXCL14 and epidermal stem cells[34].

Classical monocytes are capable of extravasating from the blood into non inflamed tissue without differentiation, retaining their monocyte characteristics, and becoming tissue monocytes. These monocytes upregulate MHCII and COX2, induced by interactions with the

endothelium, and enter tissues in a CD62L dependant manner to survey and transport molecules from tissues to draining lymph nodes during steady state conditions[35]. Their expression of CD62L means they can enter HEVs in both steady state and inflammatory conditions[36], though most of the recruitment is thought to occur directly to the tissue and subsequent entry to the lymph node is through the afferent lymphatics[37]. In response to IL-4 stimulation and GM-CSF they have been shown to acquire both DC surface marker expression and morphology; and stimulate T cell proliferation, in contrast to the other subsets, which failed to undergo similar changes[38].

In humans, classical monocytes have been demonstrated to have a higher phagocytic capacity than other subsets[19], generate high levels of ROS[21] and secrete the highest levels of inflammatory cytokines including TNFα, IL-6 and IL-1β which are important in immunity to microbes[38]. In cattle they secrete higher levels of IL-10 and the lowest amounts of IL-12 compared to the other subsets[24], alongside higher levels of arginase[19]. They also possess the highest levels of IL-1β, IL-8 (a neutrophil chemoattractant) and DEFB1 (an antimicrobial peptide)[17]. In mice, classical monocytes can be further subdivided into $CD43^{++}$ and $CD43^{+}$. The $CD43^{++}$ subset displayed 5 fold higher TNF production in response to Salmonella LPS stimulation compared to the $CD43^{+}$ subset[39]. TNF functions to regulate the immune response by causing cell proliferation, activating cells, receptor expression and migration, as well as regulating production of other cytokines. During infection with *Listeria monocytogenes*, recruitment of granulocytes and monocytes has been demonstrated to be essential in containing early infection and for bacterial killing through TNF, IFN-γ, IL-12 and IL-18 production[40].

1.4.2 Non-classical monocytes

Physically non-classical monocytes are smaller and less granular than the classical monocytes. In humans they are defined as $CX3CR1^{++}CD14^{+}CD16^{++}$. They also express CD206, but no CD62L, CCR1, CCR2, CXCR1, CXCR2, and low levels of CCR5[23,22]. These cells have higher levels of MHCII and CD32[33]. They also possess TLR 7 and 8 receptors, which allow the detection of viruses and nucleic acids[41]. In mice, non-classical monocytes are $CX3CR1^{++}CCR2^{-}$ $CD62L^{-}$ $CD43^{++}$ and express low levels of Gr1 and Ly6C and high levels of LFA[2,22,42]. In cattle, they demonstrate a lower expression of CXCL1 with higher CD1b and little CD163 expression[24]. $CD16^{+}$ monocytes express higher CD1b levels[24], which are involved in presentation of lipid antigens. These double positive cells have been shown to be important

in immunity against mycobacterial infections in cattle during natural infection[43]. Human studies demonstrate non-classical monocytes have the capacity to induce more effective T cell responses than other subsets[44] as shown by higher levels of T-cell derived IL-4 being detectable after stimulation with non-classical monocytes[45].

Non-classical monocytes are present in a wide variety of organs throughout the body in a steady state, a distribution that is maintained during inflammation. They possess a longer half-life, persisting longer in tissues, and thus are frequently more involved in tissue homeostasis. They serve as a precursor for resident myeloid cells such as $CD11c^{+}1\text{-}A^{+}$ DCs in non-inflamed tissues including liver, lung, brain and spleen, replenishing the tissue resident pool of macrophages and DCs[22].

Non-classical monocytes express higher levels of CX3CR1 and CXCR4, whose ligand is CX3CL1 (fractalkine). In a resting state they patrol along the endothelial lining in an LFA-1 and CX3CR1 dependant manner[46]. CX3CL1 acts to mediate cell adhesion and trigger chemotaxis in response to soluble fractalkine released by enzymatic cleavage and is expressed on endothelial cells, epithelium and neurons following inflammation. CX3CR1 is expressed on blood monocytes, NK cells and a subset of T lymphocytes. Non-classical monocytes are preferentially recruited to sites expressing CX3CL1, and hence during inflammation they may contribute to vascular and tissue injury due to population expansion. In addition to CX3CL1, non-classical monocytes will also migrate through the endothelium in response to CXCL12, but not CCL2[23]. Once in the tissues however, their retention is poor and mostly transient[44]. Binding of CX3CL1 to its receptor may also deliver a survival signal to non-classical monocytes, as demonstrated by the absence of CX3CR1 leading to failure to persist in the blood[22].

Non-classical monocytes are responsible for tissue repair following injury and inflammation, as demonstrated by high gene transcription of MER Proto-Oncogene, Tyrosine Kinase (MerTK). MerTK is responsible for uptake of dead cells and acts as a negative regulator of T cell activation[17], contributing to the role of non-classical monocytes in tolerance. In mice, tissue remodelling genes such as arginase1, Fizz1 and mannose receptor are upregulated following infection, which demonstrates the role of non-classical monocytes in tissue remodelling and repair[46], functions which are also linked to alternatively activated macrophages. Human studies have also shown high levels of SIRPα expression on non-

classical monocytes, a receptor which supresses phagocytosis, DC differentiation and cytokine production, which demonstrates the regulatory, or anti-inflammatory, function of the non-classical subset[38].

Strikingly, in humans, these cells produce large amounts of pro-inflammatory cytokines, including IL-1β in response to infection[47,48], and have been shown to produce equal amounts of ROS compared to the other subtypes[49]. They demonstrate a higher endocytic capacity compared to $CD14^+$ cells[47] and secrete little IL-10[50] and high amounts of IFN-α in response to TLR3 stimulation[38]. Thus, human and murine non-classical monocytes would appear to have opposing characteristics and functions. In contrast, the classical and intermediate monocytes in cattle appear to be responsible for inflammatory responses[19], whereasnon-classical monocytes secrete negligible levels of IL-1β[24]. Bovine non-classical monocytes are more endocytic and induce higher T cell responses compared to the other subtypes[24] and have relatively poor inflammatory responses with lower expression of IL-8 and arginase-1 compared to classical and intermediate monocytes[19]. They have lower phagocytic activity, but higher antigen presenting capacity than classical monocytes[28]; this is reflected in the lack of ability to generate significant levels of ROS in cattle[19]. There were low levels of IL-12 secretion in cattle monocytes, however the non-classical monocytes showed a greater increase after stimulation compared to the other subsets[24].

TNFα is mainly produced by the $CD16^+$ monocytes[47], however, within these two subsets there is conflicting evidence as to the cytokine production. In humans, one study demonstrated that non-classical monocytes were poor producers of a wide variety of cytokines, but the intermediate subset produced most TNFα, IL-1β and IL-6[41]. However, Wong *et al* demonstrated that non-classical monocytes produced the highest levels of TNFα and IL-1β, with equal amounts of IL-6 and IL-8 produced by all three subsets[48]. Non classical monocytes in mice have also been demonstrated to be responsible for high TNFα production following infection[46].

1.4.3 Intermediate monocytes

More recently a third subset has been identified which represents a transitional population of monocytes moving from the classical to non-classical subtypes. These are a population of $CD64^+CD16^+CD14^{++}$ cells that have intermediate expression of other cell surface receptors present on both classical and non-classical monocytes. They tend to express a range of CCRs

that are present in both monocyte subsets, in addition to other CCRs; hence the term intermediate.

Intermediate monocytes represent a population of cells that are transitioning from classical to non-classical monocytes[31]; during the course of infection or following M-CSF stimulation, there is an increase in intermediate monocytes followed by non- classical monocytes[51,19]. A study in mice looked at the relationships between the three subsets of monocytes by eliminating blood monocytes from the circulation. The order in which the subsets reappeared from the bone marrow was then examined. Phagocytic cells were depleted using clodronate loaded liposomes and re-emerging cells were labelled using fluorochrome loaded liposomes. This demonstrated that the first subset to reappear were the Ly6C^{hi} population (equivalent to the classical monocyte subset). Ly6C^{lo} monocytes were present in significant numbers from 7 days after monocyte elimination and, via fluorescent labelling, were shown to have developed from Lyc6^{hi} monocytes[31]. Blocking the M-CSF pathway leads to depletion of the non-classical subtype, implying that CD115 (MCSF-R) signalling is needed for the differentiation of classical to non-classical monocytes[52].

In mice intermediate monocytes have been demonstrated to express CCR7 and CCR8 in the blood, which are the same chemokine pathways used by monocyte derived DCs during movement from skin to lymph nodes (LN), and as such it has been hypothesised that these intermediate monocytes are more likely to give rise to LN DCs[53]. In cattle they have a high expression of MHCII and CD86, low expression of CD11b and produce high levels of IL-12 and can upregulate expression of CXCL8, CXCL1, IL1β and TNF[19]. With this, in humans, they have shown the capacity of intermediate monocytes to induce a Th1 response to viral and bacterial antigens alongside antigen mediated T cell proliferation, potentially due to expression of high levels of expression of molecules such as CD40 involved in antigen presentation[54].

These cells have been demonstrated to have the highest inflammatory potential of the subsets, producing the highest basal production of ROS within both human and cattle monocytes[49,19]. Human studies have also linked intermediate monocytes to roles in angiogenesis through the increase in TIE2 protein expression and VEGF receptor 2 and CD105[49].

Human intermediate monocytes produce the highest levels of IL-10 in response to LPS[55,54], however other studies have stated that IL-10 production is mainly found in classical monocytes[48]. Cattle non-classical monocytes have shown lower constitutive levels of IL-10 compared to the other subsets with high inter-animal variation[24].

1.5 Macrophages

Historically it was thought that macrophages were derived from monocytes, and the sole function of monocytes was to replenish tissue resident macrophages. It is now understood that a large proportion of tissue resident macrophages, such as Kupffer cells in the liver, are derived from yolk sac precursors prior to birth. These are self-maintained (i.e., capable of proliferating and sustaining the population without the need for other cells) throughout life and proliferate in response to inflammation[56,57,27]. The exception to this are populations such as the intestinal macrophages, which are solely replenished from classical monocytes, likely due to the bacterial stimulation from the gut microflora leading to constant low level inflammation[58]. Many tissues contain both self-renewing and monocyte derived populations, and it is currently unclear as to whether the monocyte derived macrophages perform identical functions to the tissue derived macrophages[59].

Macrophages function in the primary response to pathogens, tissue homeostasis, iron processing, resolution and repair, including clearance of dead cells. They also act to mediate local and systemic inflammation as well as the innate and adaptive immune responses. Macrophages are also capable of fusing to create multinucleated giant cells in response to IL-4 and IL-13, a process which is seen with infection by Mtb. It has been hypothesised that the formation of these giant cells may lead to increased killing ability and the ability to destroy larger parasites[60].

Macrophages express receptors such as C-type lectins and scavenger receptors which recognise bacteria and trigger the active process of phagocytosis. They also express high levels of an Fc receptor which mediates antibody dependant phagocytosis. This leads to transcription of several genes coding for enzymes such as phagocyte oxidase, reactive oxygen species, iNOS (which creates NO through L-arginine conversion) and proteolytic enzymes such as cathepsins which act to destroy the pathogen within the phagolysosome[61].

Macrophages can be split into two distinct subsets: classically activated macrophages and alternatively activated macrophages. There are also other minor subsets such as regulatory macrophages which secrete high levels of IL-10, and tumour associated macrophages, although due to their immunosuppressive activity they are often grouped under the alternatively activated macrophage subset[62].

Classically activated macrophages (CAMs) develop in response to dual stimulation with IFN-γ and microbial products such as LPS, or in the adaptive immunity context through IFN-γ and CD40-CD40L interaction. In addition, some TLR ligands can also activate pathways signalling through IRF3, which results in IFNβ production, which together with TNF, can activate macrophages in an endogenous fashion[63]. CAMs upregulate their production of cytokines such as TNF, IL-1, IL-23, and IL-12 alongside chemokines, iNOS, ROS, prostaglandins, leukotrienes, and platelet activating factor. TNF and IL-1 act to increase leucocyte recruitment. IL-12 promotes Th1 differentiation and increases IFN-γ production from NK and T cells. Expression of co-stimulation molecules on the surface is upregulated, and they also become more efficient at antigen presentation due to upregulation of MHCII. Together with the production of cytokines such as IL-12, this leads to amplification of cell mediated immune responses such as T cell differentiation. The result of this is inflammation and fever, as IL-6, TNFα and IL-1 act as pyrogens. IL-1 and IL-6 also induce hepatocytes to produce acute phase proteins and fibrinogen, which are released into the circulation. Alongside this is concurrent tissue damage due to the secretion of acid hydrolases and TNFα. Combined, large quantities of cytokines, in particular TNF, can lead to the development of septic shock[64].

In contrast, alternatively activated macrophages (AAM) are primarily involved in tissue reorganisation and repair, alongside some roles in parasite killing. IL-4 and IL-13, primarily Th2 associated cytokines, act to supress CAM activation and lead to AAM formation. Suppression of CAM occurs partially by IL-4 stimulation of IL-10 and TGFβ production by AAMs; which inhibits Th1 development and function, thus decreasing CAM populations. In the course of wound healing AAMs also induce fibrosis and scarring, due to their secretion of platelet derived growth factor that stimulates fibroblast proliferation and collagen synthesis through IL-13 and TGFβ production. IL-4 induces MHCII expression and upregulates mannose receptor expression[60]. IL-10 acts to decrease antigen presentation ability through the decrease in expression of costimulatory molecules on macrophages and inhibiting production of TNFα, IL-6 and IL-1β and downregulating Th1 responses through the induction

of Tregs. High levels of arginase-1 are also produced by AAMs which alongside IL-10 and RELMα acts to promote wound healing and restore homeostasis[62]. Mice deficient in RELMα expression and infected with *Schistosoma mansoni* eggs showed exacerbated lung inflammation, increased granuloma formation and fibrosis due to an increase in Th2 cytokine responses. *In vitro*, RELMα was demonstrated to act selectively on Th2 cells to inhibit the production of Th2 cytokines with no effect on IFN-γ production by Th1 cells[65]. AAMs have been demonstrated to have a vital role in parasite killing in mice infected with *H. polygyrus* showing that arginase-1 is involved in protective responses, and AAMs are needed for parasite clearance; depletion of AAMs led to a lack of effective parasite explusion[66].

Macrophages also express genes involved in angiogenesis, such as VEGF and FGF2, though there is conflicting evidence as to whether these are primarily produced by the CAM or AAMs or a potential hybrid phenotype involved in wound healing[60,67], or whether CAM and AAMs are involved at different stages of tissue repair. It has also been suggested that these are a transitional macrophage subgroup with a unique gene expression profile responsible for fibrotic response to injury, both pathological and healing[68]. VEGF secretion by monocyte derived macrophages is essential for the induction of angiogenesis in mice, and is dependent on CCR2 signalling to recruit circulating monocytes and macrophages[69].

1.6 Arginase

As mentioned above, macrophages possess 2 inducible enzymes, iNOS and arginase, that both use L-arginine as a substrate and are induced by Th1 or Th2 cytokines respectively (Figure 2). Arginase hydrolyses L-arginine to urea and L-ornithine. L-ornithine is a metabolite for the production of polyamines and prolines, needed for cell proliferation and collagen production, the excess deposition of which is key in causing fibrosis. This contributes to the function of AAMs in repair and fibrosis[70].

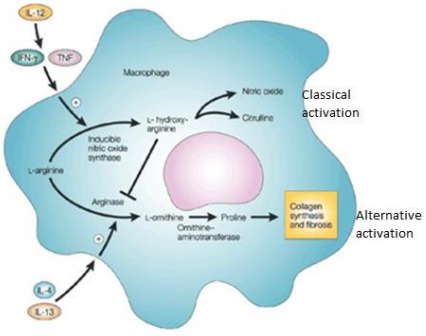


Figure 2. Macrophage classical and alternative activation pathways. Adapted from Pearce et al, 2002 [71]**.** This shows the pathways of iNOS (classical activation) and arginase (alternative activation) which both compete for the same substrate (l-arginine)

The induction of arginase activity by macrophages is dependent on secretion ofTh2 cytokines IL-4 and IL-13 and also arginase reciprocally enhances Th2 cytokine production. The induction of arginase is a common method for parasites to evade the immune response and engineer their survival within the host[72,73].

High arginase activity has been demonstrated to have a detrimental effect in NOS deficient mice infected with *Schistosoma mansoni*, who fail to control the response. Schistosome eggs, in contrast to the adult worm, induce arginase production through a Th2 type response. Liver pathology caused by granulomatous reaction and periportal fibrosis has been demonstrated to be reduced in mice that have a Th1 polarised response, with type 1 cytokines and NOS2 being important to controlling the response[74]. However, mice genetically deficient in AAMs die in the acute stages of infection due to increased histopathology and endotoxaemia from a high Th1 and NOS2 response. This is suggestive that the Th2 response is protective due to its ability to cause differentiation to AAMs, and their role in reducing inflammation[14]. During chronic *Schistosoma mansoni* infection, arginase deficient mice display significant increase in fibrosis compared to control mice, alongside evidence of portal hypertension. Survival in the chronic stage of infection was markedly reduced and blocking the action of iNOS had no effect on the survival; suggestive that arginase protects from the effects of Th2 pathology through regulation of granuloma size and pathology. This was consistent with the finding of larger quantities of T cells present, and an increase in Th2 cells, IFN-γ and IL-5 independent of IL-10 or TGFβ. Data suggest that the AAMs deplete the stores of arginine, which is required for T cell proliferation and CD4 T cell responses, hence the increased pathology[75].

Studies have also demonstrated the impact of arginase in macrophages limiting allergic responses and resolving inflammation in allergic skin disease. An upregulation of iNOS and IL-6 occurred if arginase deficient macrophages were present, leading to an exaggerated inflammatory response[76].

Arginase has been demonstrated to have a detrimental effect on any intracellular infection that relies on TLR signalling. As discussed above, TLR signalling is required for recognition of pathogens and activation of protective immune responses. *T. gondii* has been shown to use multiple mechanisms to induce arginase; more rapid expression was found in STAT6 deficient mice, in contrast to the usual production of arginase by AAMs, which requires STAT6

signalling. C/EBPβ and MyD88 were also required for arginase production by mycobacteria, however these are not required for production by AAMs. Arginase-1 was induced in macrophages by mycobacterial infection within CAMs, resulting in the suppression of NO production, due to the competition for arginine substrate, which is needed to combat intracellular infection. This suggests that persistence of chronic intracellular infection is associated with arginase expression; in the absence of arginase increased NO production occurred and mycobacteria and *T. gondii* infections were controlled[73]. Studies in mice infected with *Mycobacterium avium* demonstrate this concept; a stereotypical Th1 immune environment was present during infection, with increased levels of IFN-γ and little IL-4, correlating with reduced arginase activity. Tuberculosis granulomas have been demonstrated to have a distinct organisation, designed to both contain the infection but prevent pathology within the lung. Macrophages with higher iNOS expression locate towards the core of the granuloma, in order to contain the spread of bacteria, and AAMs towards the outer regions to limit pathology and enhance fibrosis[77].

Within the field, coinfection with multiple pathogens is common, and, within cattle, infections have been shown to have impacts on other disease processes due to modulating effects on the host immune system. Through experimental infection, animals coinfected with *Fasciola hepatica* and bovine tuberculosis (bTB) have shown reduced sensitivity to both diagnostic assays; the single intradermal comparative tuberculin test (SICTT) and whole-blood IFN-γ assay[78]. Moreover, this finding has been replicated in the field at a national level[79]. Distribution of *Fasciola hepatica* infection within the UK overlaps significantly with bovine tuberculosis, and thus this has the potential to have significant implications for disease control and spread. In livestock the interaction of helminths with zoonotic bacteria, e.g. bTB, offers the potential to amplify the risk of spillover zoonotic infection into human populations. An effective Th1 type response is required to eradicate TB infection. Fasciola acts to counteract the Th1 response and instead induces a Th2 type response, and hence AAMs, to facilitate survival. Significantly, it is known that alternatively activated macrophages (AAM) induced by *F. hepatica* are a major feature of these dysregulated responses[80,81,82], leading to an upregulation of arginase-1[66].

1.7 Aging and monocyte kinetics

Bovine neonates are born with a naïve adaptive compartment, with little to no memory T- or B-cells. They have yet to clonally expand and thus lack the basis of protective secondary,

adaptive immunity which is thought to make the neonate more susceptible to infection. Maternal transfer of immunity acts to bridge the gap to an extent, with antibody transfer occurring via colostrum post parturition. Maternal leucocytes have been demonstrated to be present in the bovine neonatal immune system, though this has little effect on the continuing immune response as these are absent from the circulation after 36 hours. Additionally, the percentage of these present in the peripheral blood of the neonate was around 1% of the circulating cells and the function of these cells is questionable[83].

Within the neonatal immune system, γδ T cells form a predominant portion of the T cells within the blood. This population expands and accounts for 60% of the T cell pool in the first 1-2 weeks after birth[84]. In humans neutrophil numbers have been shown to rise after birth within the first 24 hours and then gradually decline to adult levels within 72hours of birth[85]. These cells show decreased functionality compared to adults, such as a delayed phagocytic capacity and decreased myeloperoxidase levels. Neonates also possess increased numbers of immature granulocytes; this may lead to increased risk of infection as the functional capacity may not be maximal in cattle until four months of age, although effective responses are seen within a week after birth[86]. A majority of a calves immune system is mature by 5 to 8 months of age, with $CD4^+$, $CD8^+$ and γδ T cell percentages stabilising within 10-12 weeks and $CD21^+$ lymphocytes increasing in 6-8 weeks with a consequent increase in gamma globulin at 10-12 weeks[87,86].

Development of the neonatal immune system is partially dependant on exposure to microbes, with dysbiosis negatively affecting immune system development[88]. Human neonates have been shown to differ in their immune make-up at birth and then converge to a shared path of immune development at around three months, potentially through multiple immunological inputs decreasing the potential for variation. With the exception of T cell populations, immune cell populations were found to reach adult phenotypes within the first three-month period. Divergent environmental influences and various microbial stimuli are likely to be the reasoning behind immunological variation in both neonates and adults[88]. The overall dampened immune response within the neonate is also required to develop immune tolerance and toward a healthy microbiome[89,85]. DCs have been shown to be responsible for promoting this regulatory T cell induction through inhibition of TNFα production via arginase-2 activity[90]. In addition, decreased levels of caspase activity and impaired IL-1β secretion are seen in human and animal preterm neonates and foetus' that are consequently restored to

adult levels shortly after birth[91]. As IL-1β has a proinflammatory effect, the lack of IL-1β production in gestation provides a protective effect on the developing foetus, preventing the onset of inflammatory disease, organ injury, and allowing the development of self-tolerance[92].

In humans, monocytes represent a greater percentage of blood mononuclear cells in infants compared to adult populations[91]. Proportions of classical macrophages have been shown to increase in adulthood compared to paediatric populations, and then decrease with older age (>50 years of age); demonstrating that the monocyte populations fluctuate with age. The same study demonstrated that airway macrophages are predominately derived from peripheral cells following lung transplant, indicative of the ability of peripheral monocytes to replace resident monocyte populations during adulthood[93].

It has been demonstrated that human neonates and adults display different immune responses to the same pathogens, which can result in both protective immune responses and detrimental effects, depending on the pathogen. Human monocytes have demonstrated age related differences in responses to *Listeria monocytogenes* infection *in vitro*, with neonates demonstrating a reduction in interferon response factor 3 (IRF3), and adults demonstrating an increase in expression of inflammatory genes at baseline[94]. Given that IRF3 is required for induction of interferon (IFN) this would lead to a reduction in IFN production within neonates and so an increased susceptibility to infection with intracellular organisms. Differences in responses to TLR ligation in neonates have been established, with TLR1 and 4 within the monocyte population being expressed at lower levels in older populations, and TLR5 expression increasing with age[95]. Newborns produce large quantities of IL-6 and IL-23, supporting Th17 differentiation alongside reduced levels of Type 1 IFN and costimulatory molecules from pDCs[96] and high IL-10 production up to one month of age[97]. Decreased levels of the IL-12 and IL-23 p40 subunit are also observed, which has been correlated with an increased sepsis risk in preterm neonates due to the consequent lack of Th1 and Th17 differentiation and so lack of response to microbial infections[98]. Some of the observed differences in response to receptor binding are thought to result from reduced MyD88, IRF5 [99], and decreased levels of CD14 expression.[91] In addition to the above neonatal immune cells are less capable of producing multiple cytokines in response to TLR binding[100].

A majority, ~90%, of the peripheral blood monocytes in both humans and cattle are the more uniform $CD14^+$ monocytes, and about 10% are $CD16^+$[19,28], although these subset ratios have been shown to vary in cases of disease. Patients with tuberculosis have demonstrated an increase in the $CD16^+$ monocyte population with the $CD16^+$ population showing fewer maturation features and producing lower levels of IL-10 and higher TNFα[101]. A similar pattern has also been observed in human sepsis[102] and also within HIV infection with up to 40% of the monocyte population being $CD16^+$[103]. This may be indicative of the function of the $CD16^+$ monocytes in inflammation, infection, and repair, especially during the innate immune response. It may also be a direct effect of bacterial infection inducing $CD16^+$ monocyte production via cytokine induction[104].

In contrast to the picture seen with the human monocyte population during infection, bovine neonatal monocytes have been shown to have an increased $CD14^+$ population with higher CD80 expression and higher levels of IL-1β and IL-6 production in response to *Neospora caninum* infection both at baseline and during challenge. This resulted in a decreased parasite burden due to the upregulation of the JAK-STAT pathway which could be coupled with enhanced NK cell activation[105]. In vivo calves have been demonstrated to have an increase in proinflammatory cytokines such as IFN-γ, lower parasitaemia and higher survival when infected with *Babesia bovis*, due to increased IL-12 and NO production by NK cells[106]. In adults, IL-12 and IFN-γ production occurs later, with higher and longer lasting IL-10 production and greater TGFβ production earlier on in infection[107]. Together, this implies that bovine neonates are more resistant to protozoal invasion, though the upregulation of the immune response may lead to detrimental host inflammation.

The differing immune responses seen to infection between adults and neonates have the potential to impact on the causes of neonatal mortality and the ability to better understand the innate response to pathogen interactions and the immunological development. The variability in monocyte subsets may help explain the differences in responses seen to pathogens between adult and neonates.

1.8 Research Rationale

To date there has been little research as to the proportions of the different monocyte subsets that exist within the bovine neonates compared to the adult population. As discussed above, the predominance of classical or non-classical subsets can have significant effects on the

outcomes of disease and has potential implications for disease transmission, both from a zoonotic and herd perspective. In addition, this may have an effect on the impact of coinfection and the ability of helminth parasites to dysregulate the immune response and upregulate arginase expression. Knowledge of the changing monocyte populations with age and the effects of this also provides the potential for the development of therapeutics to exploit this innate immune response.

Therefore, I propose to undertake a comparison of bovine neonate and adult monocyte subsets in blood and quantify the proportions of the subsets present and the impact this has on the potential to respond to infection.

1.9 Aims

The aims of this project are to:

- Investigate the changes in the proportion of monocyte subsets between neonate and adult cattle
- Investigate proxies of immunity which may reflect monocyte subsets or their function and how these change from neonates to adults and the impact this has on the potential to respond to infection via measurement of monocyte activity

Based on the current evidence available in human studies and the knowledge of bovine monocytes, we hypothesise that neonatal monocytes have a larger proportion of classical monocytes, due to their ability to initiate a greater inflammatory response, alongside a greater diversity of subset proportions within the population. Given the responses within humans, we would anticipate that the neonates have less of a polyfunctional response than the adult monocytes.

Chapter 2. Materials and Methods

Ethical approval was provided by the University of Liverpool AWERB, approval #AWC0174, for collection of blood samples post-mortem.

Whole blood samples were collected from adult cows of varying ages and sexes from a nearby abattoir (CH62 1AB) in 9ml lithium heparin blood tubes.

Neonate blood from 4 Holstein-Friesian male calves at 14 days of age was collected into a 1 litre heparinised container via jugular exsanguination after slaughter under a schedule 1 approved method.

2.1 Isolation of Peripheral Blood Leukocytes (PBLs) from whole blood

Whole blood collected in the lithium heparin tubes was decanted into 15ml centrifuge tubes before being centrifuged at 350 *x g* for 25 minutes with the deceleration set at 1. The buffy coat between the plasma and erythrocyte layers was then collected using a sterile Pasteur pipette. This was placed in a 15ml tube before further centrifugation at 250 *x g* for 10 minutes. The supernatant was then discarded leaving the pellet of remaining erythrocytes and buffy coat. Next 1ml of ACK Lysis buffer (Appendix 1) was added to the pellet and the cells mixed before leaving for 5 minutes to lyse the remaining erythrocytes. 10mls of RPMI complete media (Appendix 1) was then added and samples centrifuged at 250 *x g* for 10 minutes. If required, the process of erythrocyte lysis was then repeated to ensure removal of all remaining erythrocytes leaving a white pellet. The remaining cells were then washed twice further using RPMI complete media to ensure full removal of the ACK lysis buffer from the cells. The pellet was then resuspended in 5mls of RPMI complete media and cells were counted using a haemocytometer and trypan blue exclusion method. The cells were then spun down before addition of freeze mix (Appendix 1). 1ml of freeze mix per 1mL of cells (2 x 10^6 cells/mL) was added. Cells were kept at -80°C overnight before transfer to liquid nitrogen.

2.2 Preparation of cells for flow cytometry

On removal from liquid nitrogen cells were first thawed in a water bath at 37°C before addition of an equal amount of RPMI complete media to dilute the DMSO. The sample was then centrifuged at 250 *x g* for 5 minutes and the supernatant discarded. The pellet was then

resuspended in FACS buffer (Appendix 1) to adjust the cell count to a concentration of $2x10^6$ cells/ml. Due to the high background obtained with the CD16 isotype three different blocking methods were trialled; (i) FACS blocking buffer (Appendix 1) (ii) Bovine Gamma Globulin at 1: 10000 dilution (iii) 50% FACS blocking buffer: 50% Bovine Gamma Globulin (at 1:10000 dilution). 200µl of the buffer was then placed in an Eppendorf tube with 100µl of the cell suspension, and this was then further centrifuged at 250 *x g* for 5 minutes. The supernatant was discarded and a further 150µl of FACS blocking buffer (or equivalent) was added to reduce the non-specific binding of antibodies to the Fc receptor. The samples were then placed on ice for 20 minutes.

After removal from the ice, the cells were further washed in the FACS blocking buffer (or equivalent) by centrifugation at 250 *x g* for 5 minutes. After removal of the supernatant a further 100µl of FACS blocking buffer (or equivalent) was added alongside optimal concentrations of the primary antibody or isotype (Table 2). 5µl of CD14 and 10µl of MHCII primary antibody was used. 5µl of CD16 primary antibody or Fab Fragment Anti-Human IgG (H+L) at 1:1000 dilution was used. The cells were then incubated on ice for 30 minutes in the dark. The cells were then washed twice in 100µl of FACS blocking buffer (or equivalent) by centrifugation before being resuspended in 250µl of FACS buffer for analysis. Flow cytometry was performed using a MacsQuant Analyser (Miltenyi Biotech). Compensation was applied when using dual staining and a minimum of 10,000-30,000 events was recorded. Cells were gated on the basis of FCS and SSC and monocyte populations identified, after which single cells were then identified on an FSC-A vs FSC-H plot. Unstained and isotype controls were used to set positive staining thresholds and FACS blocking buffer was used for all future experiments.

Table 2. Antibody isotypes and controls

Antibody/ Marker	Target Species	Conjugate	Clone	Source	Isotype	Working Dilution
CD14	Bovine	FITC	CC-G33	BioRad	IgG1	1:20
MHC II DR	Bovine	RPE	CC-108	BioRad	IgG1	1:10
CD16	Human	Alexa-Fluor 647	KD1	BioRad	IgG2a	1:20
	Bovine	Unconjugated		Jackson ImmunoResearch	Bovine Gamma Globulin	1:10000
	Human	Unconjugated	Polyclonal	Jackson ImmunoResearch	AffiniPure Fab Fragment Goat Anti-Human IgG (H+L)	1:10000
Arginase-1	Human	Alexa-Fluor 405	658922	R&D Systems	IgG2b	1:200
CCR2 C-terminal region (ARP58410_P050)	Human	Unlabelled		Aviva Systems Biology		1:1000
IgG	Rabbit	HRP		Aviva Systems Biology		1:1000

2.3 Stimulation of PBLs

Phorbol 12-myristate 13-acetate (PMA, Sigma Aldrich) was added at a concentration of 10ng/ml with Ionomycin (EMC Millipore Corp) at 500ng/ml to one sample and phytohaemagglutinin (PHA, Sigma Aldrich) at a concentration of 2.5µg/ml with Ionomycin at 500ng/ml to another sample from the same animal. An unstimulated sample was also run alongside the stimulated samples for comparison. The cells were then incubated at 4°C for 4 hours prior to preparation for intracellular arginase staining as detailed below.

2.4 Intracellular Arginase Staining

To stain for intracellular arginase, the cells were fixed and permeabilised using the BD Cytofix kit according to the manufacturer's instructions. PBLs were thawed as per the flow cytometry protocol. Cells were prepared for flow cytometry as detailed above before the addition of 250µl of Fixation/Permeabilization solution for 20 minutes at 4°C. The cells were then washed twice in 250µl of BD Perm/Wash buffer at 220 *x g* for 5 minutes. The supernatant was discarded and then the pellet resuspended in 50µl of Perm/Wash buffer alongside the arginase antibody or isotype (Table 2) at concentrations of 1:100, 1:150 and 1:200. The cells were then incubated at 4°C in the dark for 30 minutes. Following this, cells were then washed

twice using 250µl of Perm/Wash buffer before being resuspended in 200µl of FACS buffer for analysis.

2.5 Magnetic separation of $CD14^+$ monocytes from PBLs

PBLs previously prepared using the methods described in Section 2.1 were thawed prior to the addition of an equal amount of RPMI complete media to dilute the DMSO. The cells were then centrifuged at 220 *x g* for 10 minutes and then the supernatant was discarded. The remaining cells were resuspended in 1ml of RPMI complete media and centrifuged for a further 10 minutes at 300 *x g*.

The remaining pellet was resuspended in 80µl of MACS running buffer (Appendix 1) and 20µl of anti-CD14 human microbeads (130-050-201, MACS Miltenyi Biotec) per 10^7 of cells was added. This was then mixed and left on ice for 15 minutes prior to washing in 2ml of MACS running buffer. The cells were then centrifuged for 10 minutes at 300 *x g*.

A MACS LS column and magnetic separator (Miltenyi Biotec) was used to separate the $CD14^+$ monocytes. The column was first rinsed with 3ml of MACS rinsing buffer (Appendix 1). The cells were then added onto the column and rinsed three times with 3ml of MACS rinsing buffer. After the unlabelled cell suspension was discarded the column was then removed from the magnetic separator and placed over a 15ml centrifuge tube. 5ml of rinsing buffer was added to the column and the $CD14^+$ cells collected by pushing the plunger into the column to elute the cells. The cells were then counted using a haemocytometer.

2.6 Arginase Assay

An arginase assay was performed using the method established by Modolell *et al*[108]. $CD14^+$ cells collected using magnetic separation described above were added to 1ml of 1% Triton (Sigma Aldrich) and centrifuged at max speed for 2 minutes. 50µl of lysate was then added to 50µl of 10mM $MnCl_2$/50mM Tris-HCl buffer (pH 7.5) (Sigma Aldrich) in an Eppendorf and mixed. Beef liver homogenate was used as a control in a 1:50 dilution. The cells were then incubated at 55°C in a heat block for 10 minutes for enzyme activation.

50µl of 0.5M L-arginine substrate (pH 9.7) (Sigma Aldrich) was added to each Eppendorf and then incubated at 37°C for 1 hour. The reaction was then stopped by addition of acid stop solution (H_2SO_4 (96%), H_3PO_4 (85%) and H_20 in a 1:3:7 ratio) (Sigma Aldrich). 400µl was added to each sample and control and 600µl to each urea standard. 9% α- isonitrosopriopherone

(diluted in ethanol) was added to develop the colour, with 25µl added to each sample and 37.5µl to each standard. These were then heated to 103°C for 45 minutes before being left to cool in the dark for 10 minutes afterwards.

200µl aliquots were then transferred to a 96 well plate (triplicates for the standards and duplicates for samples) and ODs measured at 540nm using a plate reader.

2.7 Western blot

An equal amount of Laemmli sample buffer (Sigma Aldrich) was added to the cell lysate formed from the $CD14^+$ cells (as described in Section 2.6) along with 1:10 volume of β-mercaptoethanol (Sigma Aldrich). The sample was then heated at 95°C for 5 minutes before being placed on ice.

The resolving gel mix (Appendix 1) was poured, overlain with isopropanol and left to set at room temperature for thirty minutes after which the isopropanol was poured off and stacking gel mix (Appendix 1) was then poured onto the resolving gel and combs added before being left to set for a further thirty minutes at room temperature. The gels were placed in the tank and running buffer (Appendix 1) was then added. Combs were then removed and 20µl of each sample and 2µl of protein ladder were loaded. The gel was then run at 100V for 1hr initially, or until the dye front had run to the bottom of the gel. The gel was then removed from the tank and transferred to the PVDF membrane using the semi-dry method. A Trans-Blot SD Semi-Dry Cell (Bio-Rad) was used to perform the transfer of the protein from the gel to the membrane. The PVDF membrane was activated in methanol for 2 minutes then rinsed in transfer buffer (Appendix 1). 2 pieces of blotting paper were soaked in transfer buffer (Appendix 1) and the gel placed in-between the membrane and blotting paper. Further transfer buffer was added on top and a pipette tip used to smooth the surface and ensure no air bubbles were present. The voltage was then set to 25V for 30 minutes. The membrane was then removed and blocked in 5% milk with TBST (Appendix 1) for 1 hr on the plate shaker before being washed twice with TBST for 5 minutes. 1:1000 dilution of the CCR2 primary antibody (Table 2) in 5% milk solution was then added and left overnight on the plate shaker in the cold room.

The membranes were then washed 3 x with TBST for 5 minutes before secondary antibody (Table 2; Rabbit IgG HRP conjugate at 1:1000 dilution) in 5% milk solution was then added and left on the plate shaker for 1 hour. Following this the membrane was washed 3 x with

TBST and then imaged using ECL solution (ThermoFisher) on a ChemiDoc Imaging System (Bio-Rad).

2.8 Dot Blot

Nitrocellulose membrane was cut into 0.5cm wide strips, placed in a tray and 10µl of test lysate or control was added to the membrane. Lysed bone marrow derived macrophages (BMDM) were used as a positive control. The membrane was left to dry completely before 1ml of blocking buffer (5% milk in PBS) was added to each strip for 90 minutes at room temperature on a plate shaker. The membrane was then washed 3 times with PBST (0.05% Tween) for 3 minutes. 1ml of CCR2 primary antibody solution (Table 2; 1:1000 dilution in blocking buffer) was then added to the strip and incubated for 90 minutes at room temperature on the plate shaker before being washed 3 x as previously described. 1ml of secondary antibody solution was then added (Table 2; 1:1000 dilution in blocking buffer) and incubated for 60 minutes at room temperature. The membrane was then washed 3x as previously before imaging using ECL solution as above.

2.9 Statistical Analysis

Data Analysis was conducted using Prism 8 (GraphPad). FACS data was analysed using FlowJo v10.6.1. A two-way ANOVA with Sidaks multiple comparison test performed in Prism 8 was used to compare the monocyte subset data obtained from FlowJo analysis. ImageJ was used to quantify pixel density following dot blot analysis. The images obtained from the dot blot were inverted prior to measuring the mean density for all the samples and background, the central area of each dot blot was selected, and the resulting pixel intensity recorded.. The background mean density was subtracted from each of the samples. Using the cell counts obtained prior to conducting the assay, the values were then calculated to give a mean density value per 10^4 cells. Arginase and CCR2 data were analysed using a Mann Whitney U test to compare the means between groups. In all cases p values <0.05 were considered significant. Using R Studio version 3.3.1, hierarchical clustering was performed on the full data set, incorporating subset data alongside CCR2 and arginase activity for each individual animal. Clustering was performed using calculation of the Euclidean distance and Wards criterion for formation of linkages. Code used was modified from workshop notes from the Computational Biology Facility at the University of Liverpool alongside code in Data Science for Immunologists[109] (Appendix 2).

Chapter 3. Results

3.1 Optimisation of antibody staining

Differing concentrations of primary antibodies were used to determine the optimal concentration of each to use for FACS identification of monocyte subsets. Dilutions of 1:10, 1:20, and 1:50 were used for CD14, CD16 and MHCII alongside their respective isotypes. Initial gating was performed on an unstained FSC vs SSC plot to identify the leucocyte population and exclude doublets. Within the gated population each antibody was then compared to the respective isotype at different concentrations and the optimal identified. Final concentrations of 1:10 for MHCII (Figure 3) and 1:20 for CD14 (Figure 4) and CD16 (Figure 5) were used for all future experiments.

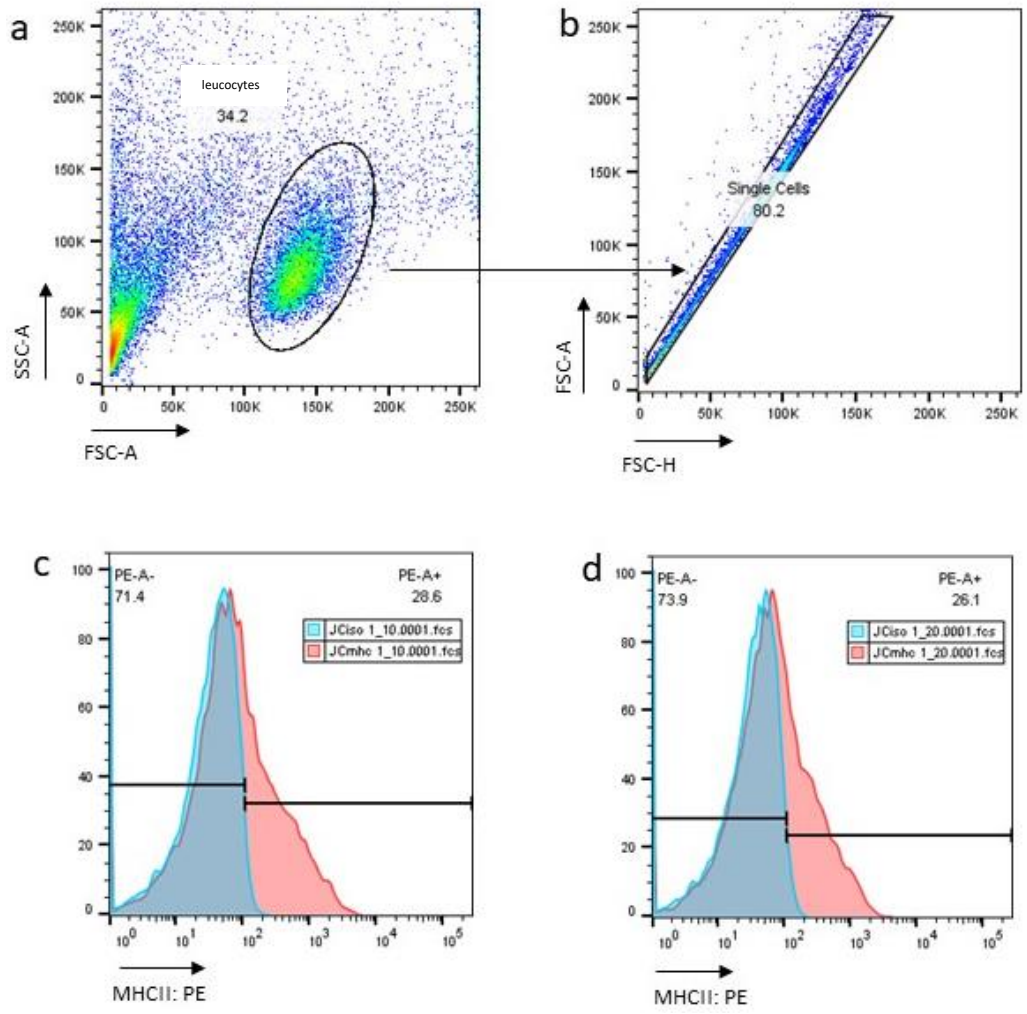


Figure 3: Example of optimisation performed for MHCII staining. Flow cytometry was performed on isolated PBLs from fresh blood samples. Cells were gated on the lymphocyte population based on the FSC and SSC in the unstained sample (a), and then further gated to eliminate doublets (b). Dilutions of 1: 10 (c) and 1:20 (d) were performed, alongside isotype controls, to establish the optimal concentration of antibody to use.

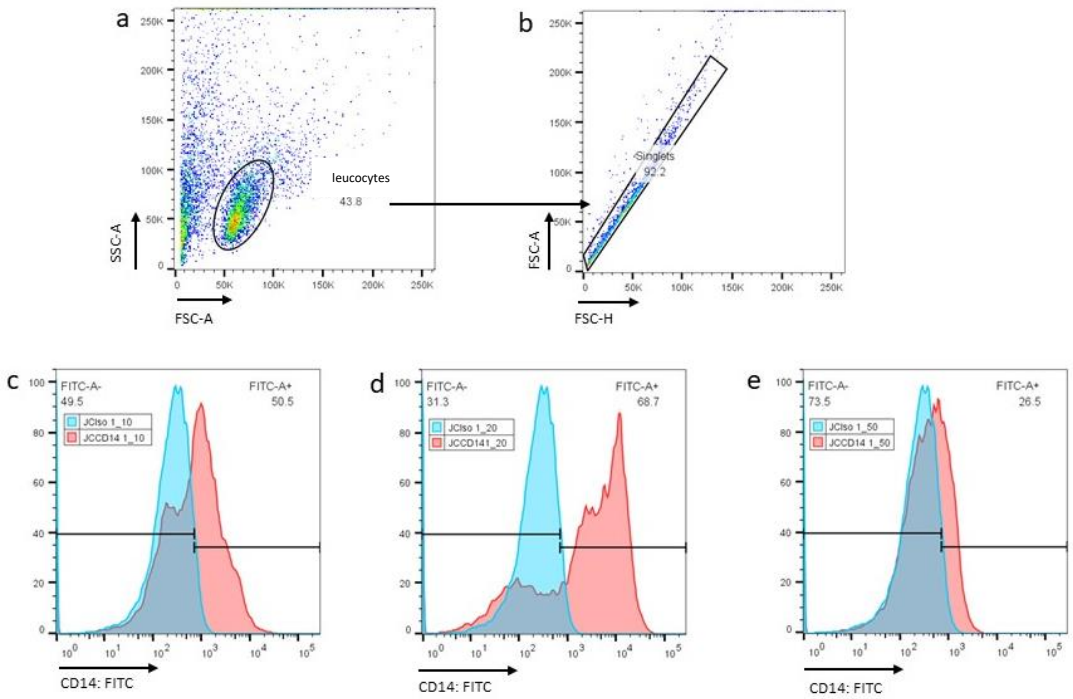


Figure 4: Example of optimisation performed for CD14 staining. Flow cytometry was performed on isolated PBLs from fresh blood samples. Cells were gated on the lymphocyte population based on the FSC and SSC in the unstained sample (a), and then further gated to eliminate doublets (b). Dilutions of 1: 10 (c), 1:20 (d) and 1:50 (e) were performed, alongside isotype controls, to establish the optimal concentration of antibody to use.

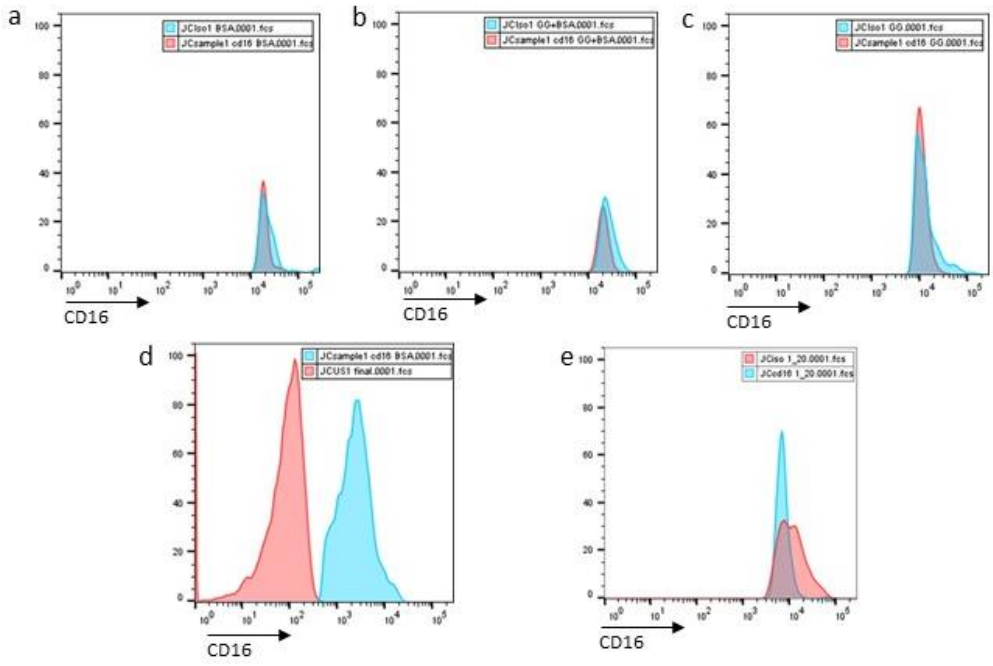


Figure 5: CD16 optimisation. Cells were gated on the lymphocyte population on the basis of an FSC vs SSC plot and doublets removed on an FSC-A vs FSC-H plot. Samples were then incubated using different blocking agents to try and reduce the background and stained using the CD16 isotype and antibody; using BSA alone (a), using gamma globulin and BSA (b), using gamma globulin alone (c) and using no isotype and comparing the BSA blocking to the unstained sample (d). A Fab fragment IgG was also trialled as an alternative isotype (e).

The isotype used for CD16 demonstrated high background staining and thus different blocking buffers were used to attempt to reduce this i) FACS blocking buffer (Appendix 1) (ii) Bovine Gamma Globulin at 1: 10000 dilution (iii) 50% FACS blocking buffer: 50% Bovine Gamma Globulin (at 1:10000 dilution) (Section 2.2). None of the buffers trialled significantly reduced the background (Figure 5) and using a Fab fragment as an alternative isotype failed to reduce the background staining of the isotype. During all future experiments CD16 antibody staining was compared to the unstained population to identify a positive population.

3.2 Monocyte subsets in neonatal and adult cattle

To investigate the changes between neonate and adult monocyte populations triple stained FACS analysis was performed and the results compared between adult (n=24) and neonates (n=4). As above, the monocyte population was identified on an FSC vs SSC plot and doublets excluded on an FSC-H vs FSC-A plot, from here a $CD14^{+}$ and $CD14^{-}$ population was identified (Fig 6 & 7 f-h). From the $CD14^{+}$ population cell subsets were gated for CD16 and MHCII staining, and the same gating applied to the $CD14^{-}$ population. Dual staining populations of $MHCII^{+}$ and $CD16^{+}$ were identified within the $CD14^{+}$ population alongside the triple positive population. Neonates overall displayed a higher proportion of monocytes, based upon FSC/SSC, compared to the adult samples (Fig 6a & 7a) along with a larger percentage of $CD14^{+}$ monocytes (Fig 6c & 7c). Within the $CD14^{-}$ gate $CD16^{+}MHCII^{+}$ and $CD16^{+}MHCII^{-}$ populations were identified, alongside a triple negative subset. Less individual variation was apparent between the neonate samples, with the exception of neonate 2, adult samples displayed more individual variation in the monocyte subsets as was apparent from the heatmaps constructed from the data (Appendix 3).

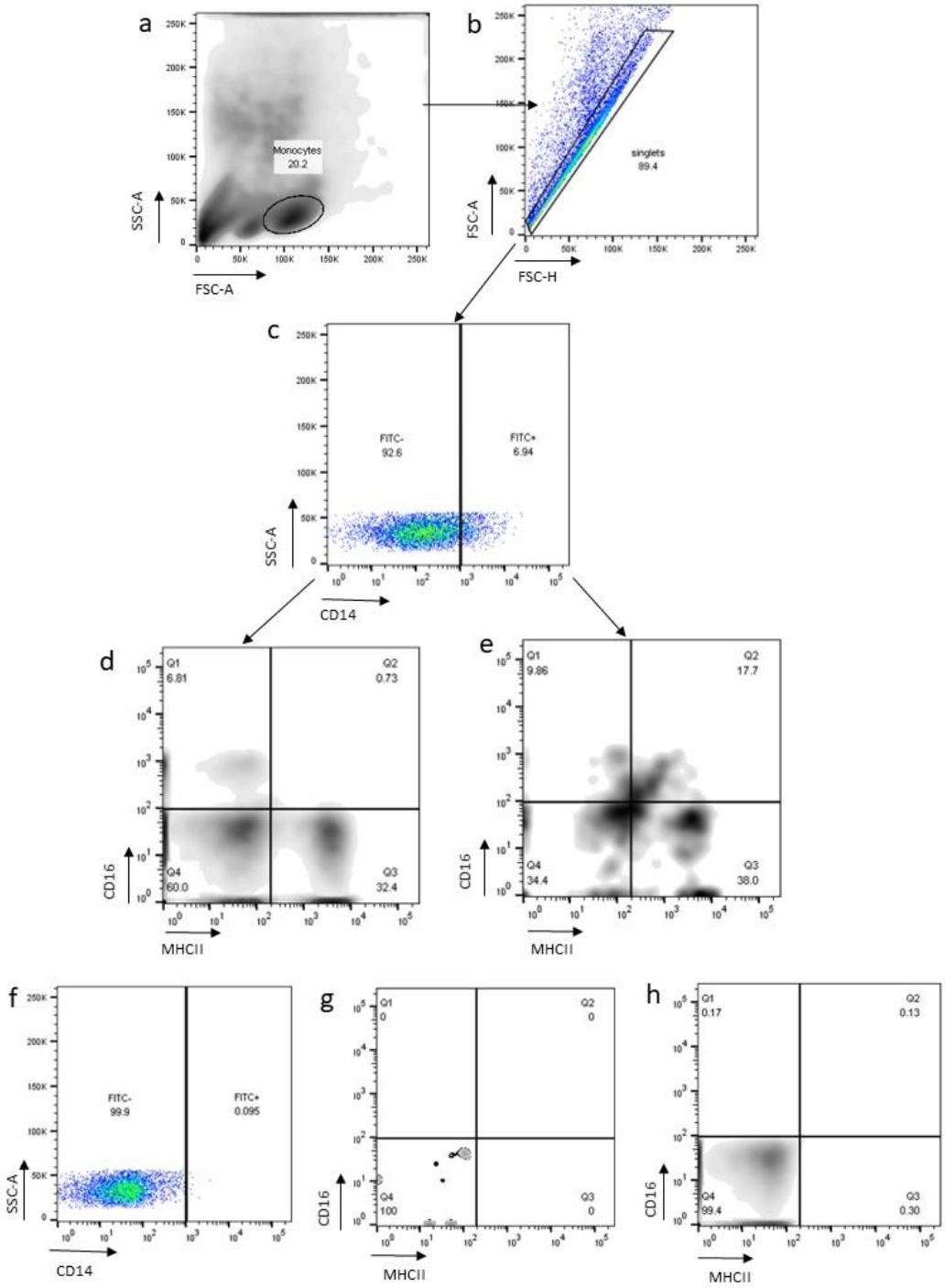


Figure 6. Representative example of a FACS plot for adult blood sample. The monocyte population was identified on an FSC vs SSC plot (a). Doublets were then eliminated on an FSC-H vs FSC-A plot (b). The sample was then gated on $CD14^{+}$ and $CD14^{-}$ cells (c). The MHCII and CD16 populations were then identified within the CD14 negative (d) and positive populations (e). Isotype controls were used to set the threshold for $CD14^{+}$ staining (f) and then gated for MHCII and CD16 within the $CD14^{+}$ (g) and $CD14^{-}$ (h) populations.

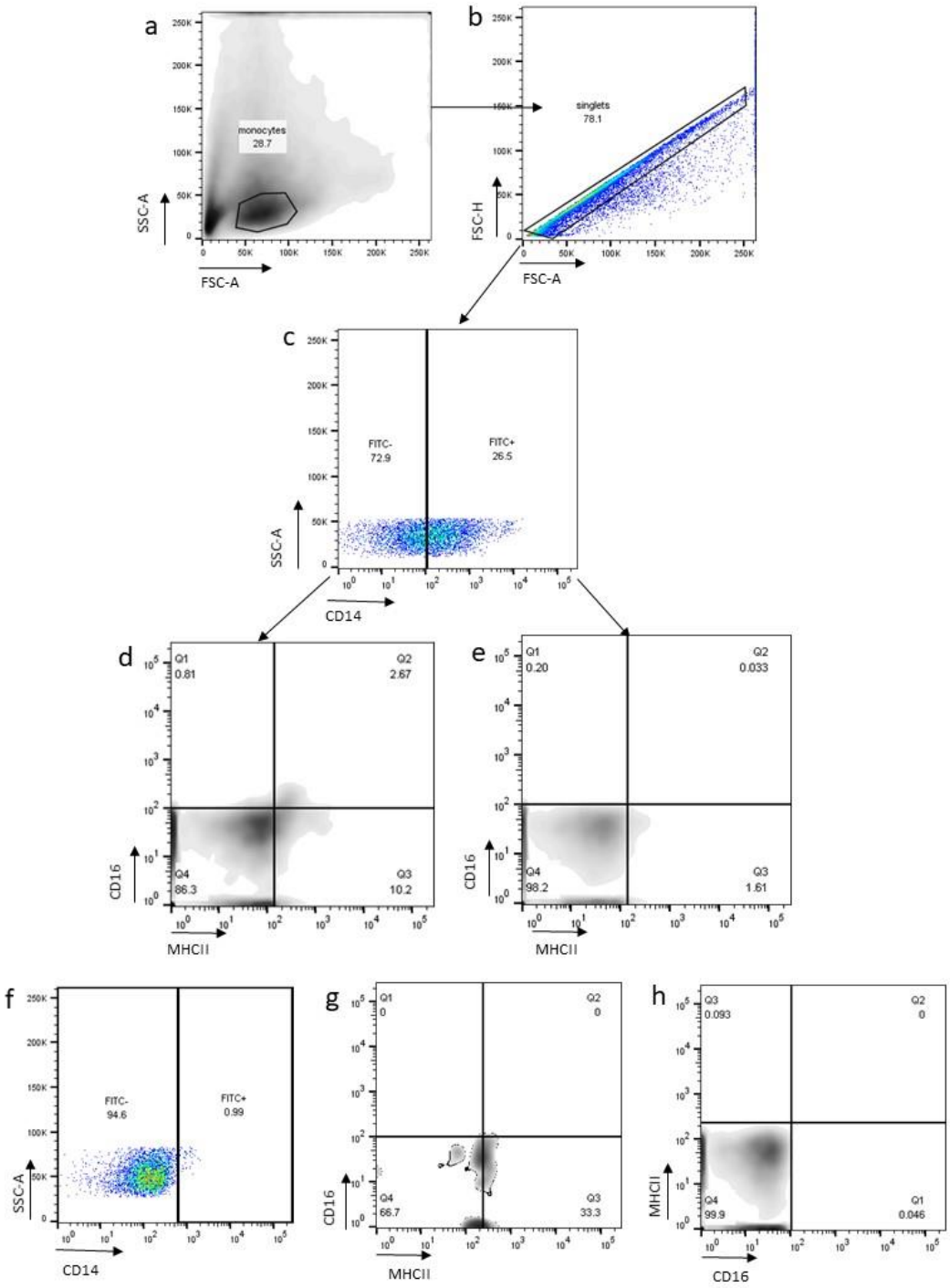


Figure 7. Representative example of a FACS plot for neonate blood sample. The monocyte population was identified on an FSC vs SSC plot (a). Doublets were then eliminated on an FSC-H vs FSC-A plot (b). The sample was then gated on $CD14^{+}$ and $CD14^{-}$ cells (c). The MHCII and CD16 populations were then identified within the CD14 negative (d) and positive populations (e). Isotype controls were used to set the threshold for $CD14^{+}$ staining (f) and then gated for MHCII and CD16 within the $CD14^{+}$ (g) and $CD14^{-}$ (h) populations.

The data from individual adults and neonates was compared using a two-way ANOVA. The monocyte subpopulations identified based on their cell surface staining were grouped into classical, non-classical and intermediate monocyte populations based on previous literature and conventions used in human phenotyping[21]. This demonstrated significant differences in the $CD14^{+}16^{-}MHCII^{-}$ population between the adults and neonates, with neonates having a higher percentage ($p<0.0001$; Fig 8a). Neonates demonstrated a larger proportion of the triple negative subset in comparison to the adult population ($p<0.05$; Fig 9). Adults demonstrate a tendency towards higher percentages of CD16+ populations, though this was non-significant on statistical analysis. Looking at the overall CD14+ population, a significant difference was observed between the adults and neonates with the neonates displaying a higher percentage ($p<0.05$; Fig 10). No significant difference was observed between the other subsets.

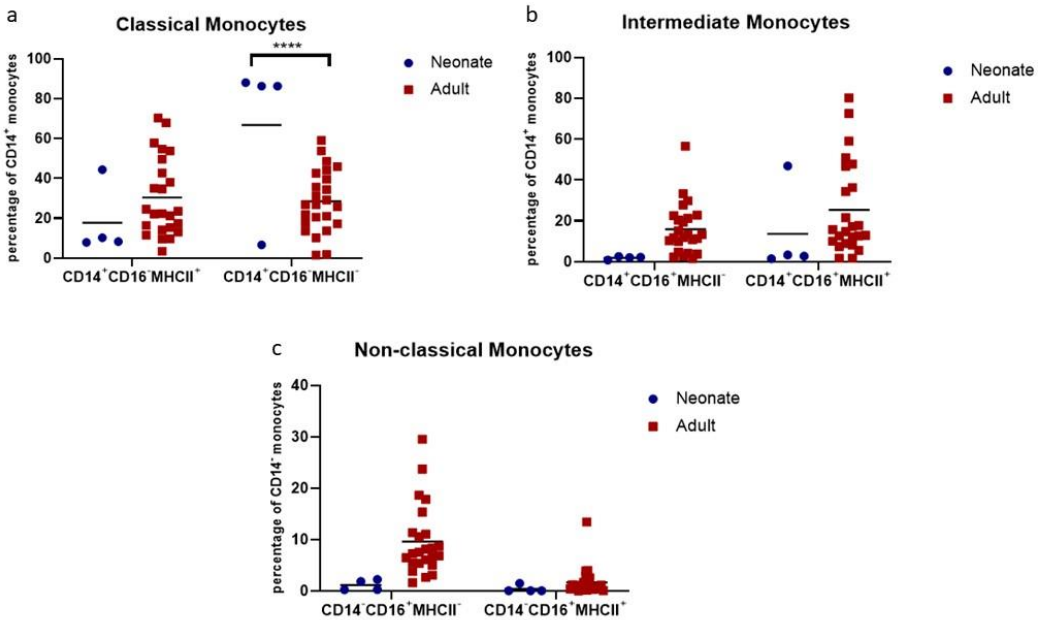


Figure 8. Distribution of monocytes within neonate and adult populations. PBLs were triple stained with FITC labelled CD14, PE labelled MHC-II and APC labelled CD16. Monocytes were divided into subpopulations a) Classical Monocytes; b) Intermediate Monocytes; c) Non-classical Monocytes following FACS gating. Results were analysed using a two-way ANOVA (****, $P<0.0001$)

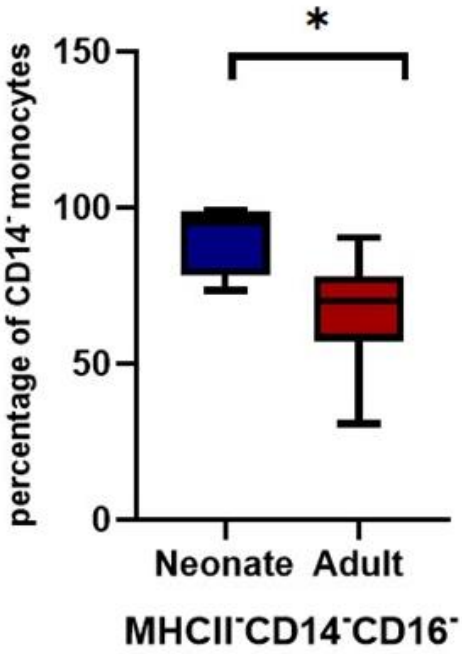


Figure 9. Expression of triple negative subset in neonate and adult PBLs by flow cytometry. PBLs were triple stained with FITC labelled CD14, PE labelled MHC-II and APC labelled CD16. Monocytes were divided into subpopulations following FACS gating, including a triple negative subset. Results were analysed using a two-way ANOVA. A significant difference was observed between adult and neonate triple negative subsets (*, P<0.05).

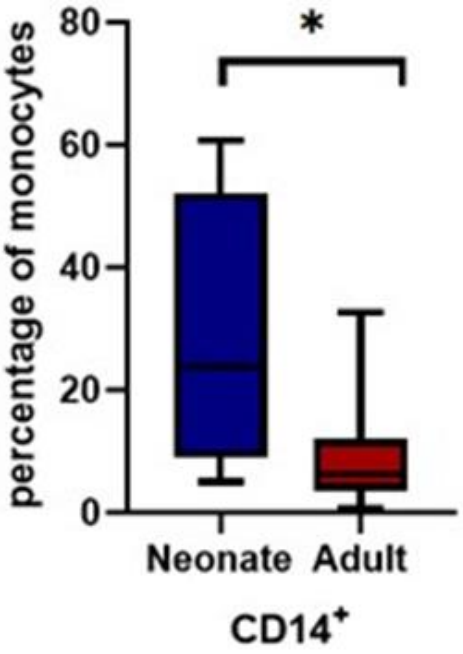


Figure 10. Expression of $CD14^+$ in neonate and adult PBLs by flow cytometry. PBLs were stained using FITC labelled mouse anti-bovine CD14. Cells were gated on the monocyte population and $CD14^+$ population identified. Results were analysed using a Mann Whitney test. A significant difference was observed between adult and neonate $CD14^+$ expression (*, P<0.05)

3.3 Arginase expression in monocytes

Expression of arginase has been associated with and used as a marker of the non-classical monocyte subset. Measurement of intracellular arginase was attempted (Section 2.4), with and without in vitro stimulation with PMA or PHA and culture with Ionomycin (Section 2.3). Differing concentrations of anti-arginase antibody were used; however, the isotype control showed greater staining than the primary anti-arginase antibody throughout all concentrations, and little difference was apparent in either the isotype or arginase antibody staining compared to the unstained (Fig 11 a-c). Using a 1:100 concentration of antibody the isotype gave 72.5% positive arginase staining and the anti-arginase antibody 30.6% positive in comparison to the unstained sample. The 1:150 gave 50.6% positive staining for the isotype and 12.0% positive for the anti-arginase antibody and 1:200 42.9% and 9.35% respectively. To overcome low, and non-specific, levels of staining, stimulation of cells prior to intracellular staining with PMA/PHA and Ionomycin was attempted. Stimulation of cells showed no increase in the arginase staining (Fig 11d). Importantly, when trialled alongside surface staining for CD14, both PHA/PMA and Ionomycin stimulation demonstrated alterations in the CD14 expression compared to the unstimulated control (Fig 11e).

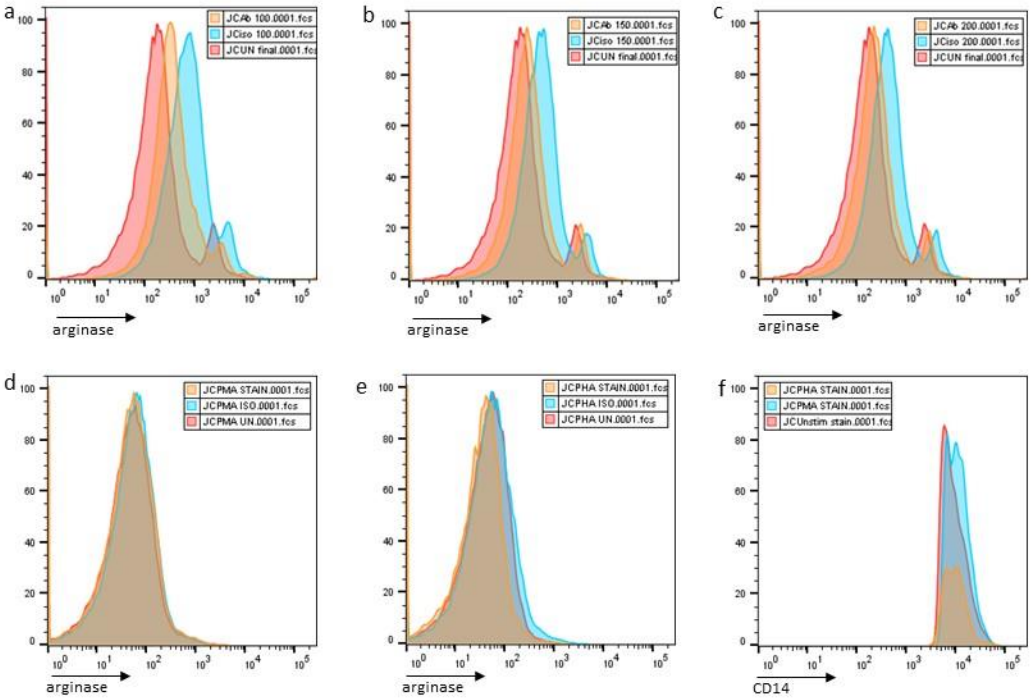


Figure 11: Intracellular arginase staining. Following fixing and permeabilization, PBLs were stained using human arginase 1 Alexa Fluor 405 conjugated antibody or isotype (mouse IgG2B Alexa Fluor 405 conjugated antibody). Different concentrations of arginase antibody and isotype were used to identify the optimal antibody concentration to use; 1:100 (a), 1:150 (b), 1:200 (c). Samples were stimulated for four hours prior to intracellular arginase staining with either PMA and Ionomycin (d) or PHA and Ionomycin (d) which had no effect on the arginase expression. PBLs were stained with FITC conjugated CD14 prior to stimulation and intracellular arginase staining, which demonstrated alteration in the CD14 expression following stimulation (f). Figures a-c; orange denotes arginase antibody, blue isotype and red unstained sample. Figure d; orange is PMA staining, blue isotype and red unstained. Figure d; orange is PHA staining, blue isotype and red unstained. Figure d; orange is PHA staining, blue PMA staining and red unstained.

An enzymatic assay was then used to determine arginase activity as described in Section 2.6 using $CD14^+$ monocyte cell lysate, using urea production as a proxy for arginase activity. The urea production was calculated per 10^4 cells for each sample to allow comparisons to be made between animals. Results demonstrated significant differences between adults and neonates with neonates displaying higher urea production per minute ($p<0.01$) (Fig 12), corresponding to higher arginase activity.

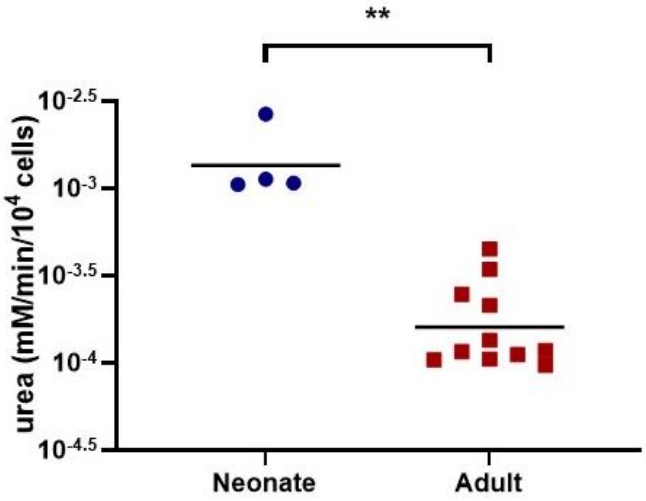
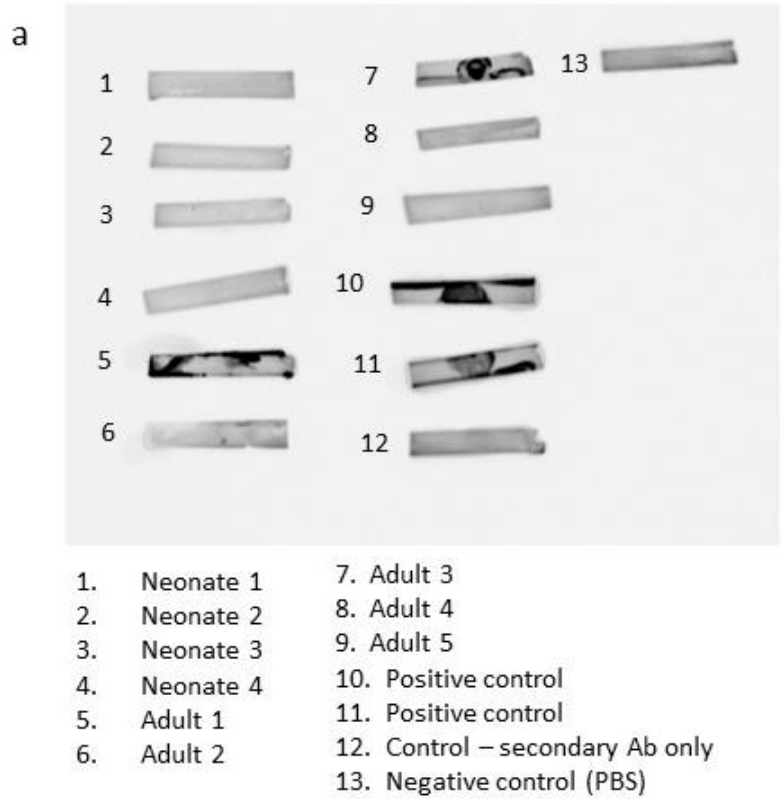


Figure 12. Expression of arginase activity in neonate and adult CD14$^+$ monocytes via arginase assay. Magnetic separation was used to separate CD14$^+$ monocytes from PBLs. Cell counts were performed and an arginase assay performed on the cell lysate to measure urea production. Data was analysed using a Mann Whitney test. Significant differences in arginase activity are observed between neonate and adult CD14$^+$ monocytes (**, P< 0.01**)**

3.4 CCR2 expression

Previous studies have shown CCR2 to be a marker of classical monocyte subsets, and so quantities of CCR2 were measured within the adult and neonate monocytes. Measurement of CCR2 was initially trialled using Western blotting (Appendix 4) of the cell lysate as described above. Separating the CD14$^+$ cells from the PBLs (Section 2.4) required using BSA within the MACS running buffer (Appendix 1); this prevented the cell lysates from being separated within the blot. Following this, dot blots were then used to measure CCR2 expression within the lysate (Fig 13a). These blots were then inverted (reversing the colours) and analysed using Image J. The resulting values were then adjusted to give a mean density value per 10^4 of cells. Once the values were adjusted for the cell counts, this showed that neonates have a significantly higher expression of CCR2 compared to the adults as measured by pixel density ($p<0.01$; Fig 13b).



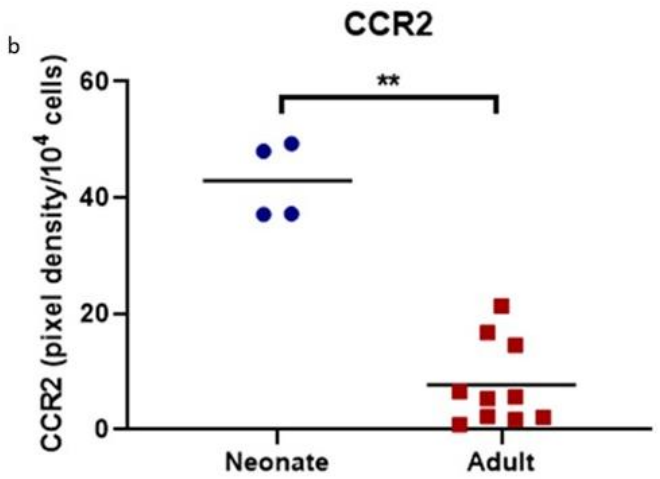


Figure 13. Expression of CCR2 in neonate and adult CD14$^+$ monocytes via dot blot. Magnetic separation was used to separate CD14$^+$ monocytes from PBLs. Cell counts were performed and dot blots performed on the cell lysate. Data was analysed using a Mann Whitney test. a) Representative example of dot blot performed on cell lysate. b) Significant differences in CCR2 pixel density are observed between neonate and adult CD14$^+$ monocytes (**, P< 0.01**)**

3.5 CCR2 and Arginase activity correlation

In order to determine if a relationship between arginase activity and CCR2 expression in monocytes existed linear regression analysis was performed on the data. This gave an R^2 value of 0.7486 and a p value <0.01, indicating a significant correlation (Fig 14). However, when plotted it is clear from the data that the neonates and adults form two separate clusters indicating that linear regression is likely not appropriate to analyse the data sets.

Hierarchical clustering was performed on the full data set, incorporating subset data alongside CCR2 and arginase activity for each individual animal (Fig 15). Clustering was performed using calculation of the Euclidean distance and Wards criterion for formation of linkages. The output demonstrates that the adult and neonate samples form two separate clusters, with the exception of Neonate 2 which clusters within the adult grouping. The height for each linkage corresponds to the value for the Euclidean distance between clusters. The starting nodes for the major clusters with the greatest distance between them contain Neonates 1,3 and 4 in cluster one and Neonate 2 and all adults in the second cluster. This indicates that monocyte profiling can predict the immunological age of animals.

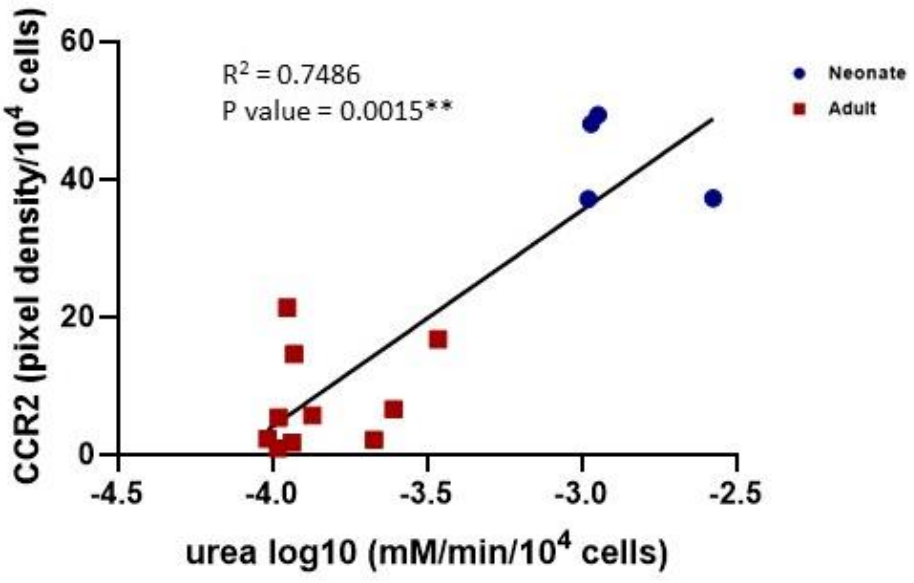


Figure 14. CCR2 and arginase activity correlation in adult and neonate monocytes. Linear regression analysis was performed on the CCR2 and urea values for both adult and neonates. This demonstrates that two separate clusters are formed.

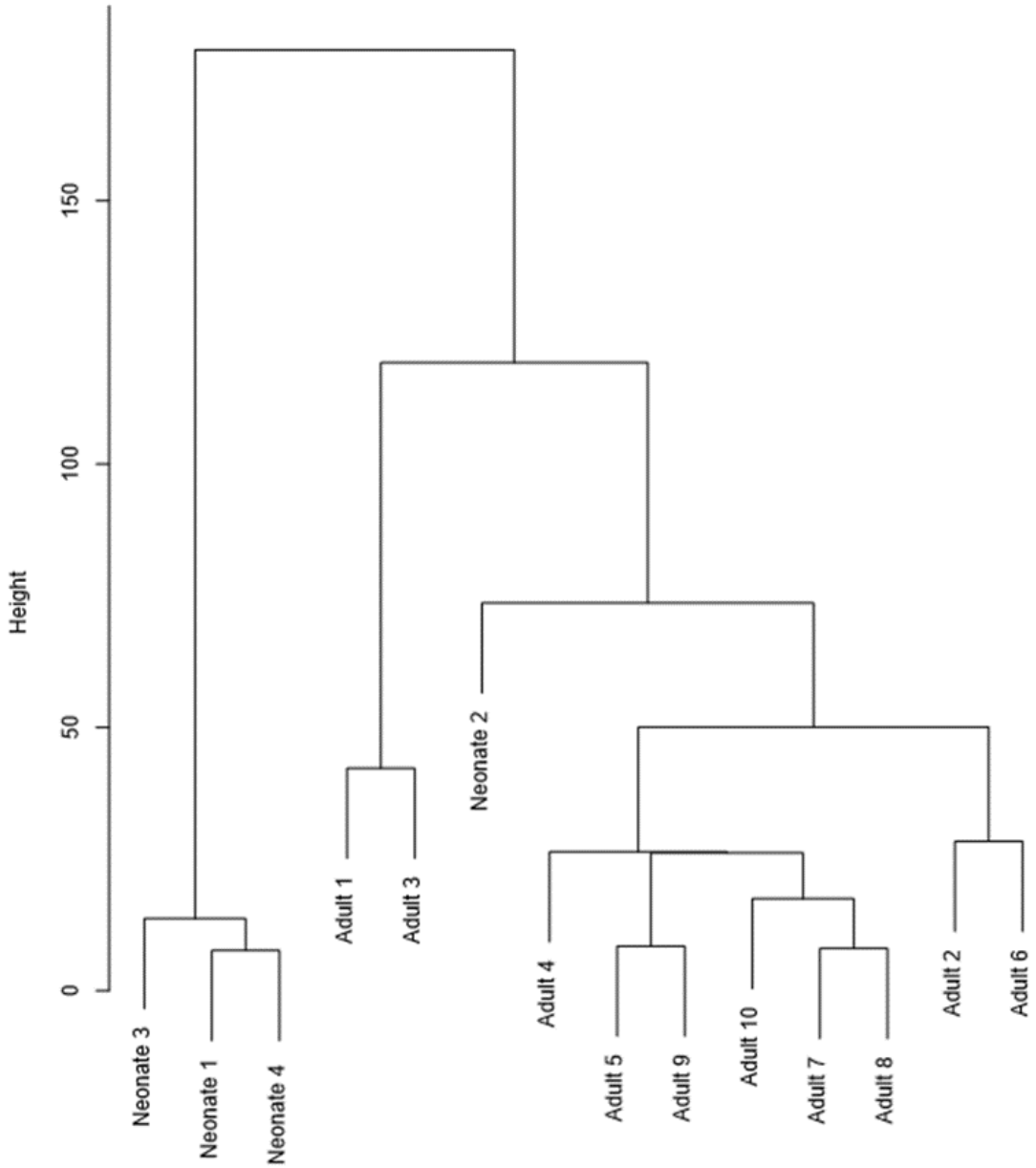


Figure 15. Cluster dendrogram of adult and neonate data. A cluster map was created using R Studio version 3.3.1 using the hierarchical method, Euclidean distance, and Wards criterion for linkages. The height for each linkage corresponds to the value of the Euclidean distance between clusters. With the exception of Neonate 2, adults and neonates form two separate clusters.

Discussion

Currently little is known about the development of the monocyte subset populations within the context of the development of the innate immune system in cattle. Differing proportions or absolute numbers of monocyte subsets may give rise to differing disease outcomes and disease transmission potential within herds. To address this, we set out to describe the changes in monocytes populations between adult and neonate cattle. We found that neonates, compared to adults, displayed a significantly higher proportion of $CD14^{+}$, $CD14^{+}CD16^{-}MHCII^{-}$ and triple negative monocyte subsets. Functional assays demonstrated that neonates have significantly higher expression of both CCR2 and arginase. We confirmed that age is a distinguishing factor between high versus low expression of these functional markers; monocyte subset presence does not correlate with either CCR2 or arginase expression. Given these findings, we can confirm the hypothesis that neonatal monocytes have higher classical monocyte populations, however they displayed a more uniform distribution of monocyte subsets and greater polyfunctionality, and therefore the second part of the hypothesis can be rejected.

Previous studies from our group have shown that neonate cattle possess a higher proportion of monocytes compared to adult cattle[105], a finding which has been repeated within this study. Human neonate cord blood has lower levels of non-classical (patrolling) monocytes ($CD14^{dim}CD16^{+}$) and no significant differences in the other subsets[110], though other studies have found classical (inflammatory) monocytes ($CD14^{+}CD16^{+}$) to be lower in cord blood[111]. Lower quantities of inflammatory monocytes seen in human neonates may reflect the lack of exposure to infection or other inflammatory stimuli whilst *in utero*. As placental transfer of immunity occurs in humans, there may also be a lack of reliance on the neonatal innate immune system, and therefore less need for a heightened response. Newborn dromedary calves have significantly reduced numbers of $CD14^{+}CD163^{+}MHCII^{+}$ subset; a subset identified as having the highest phagocytic and ROS capacity, and overall less CD14 and MHCII expression on all monocytes[112]; contrasting with our findings here.

Development of the immune system has been well defined in humans. Studies suggest that neonates are immunologically diverse at birth and then later converge onto a similar pathway by three months of age, whereon the environmental influence impacts over the life course[88], a pattern which is also observed within the neonate cattle samples. Preterm infants show an exaggerated response to oxygen radicals despite reduced production, countered by

increased IL-10 production and a reduced TLR response[113], especially TLR4 and co-receptor CD14. Newborns, especially preterm infants, show a shift towards Th2 type responses and heightened overall reaction characterised by CXCL8 production with consequential neutrophil and γδ T cell activation[114]. A lack of IL-12 and IL-23 has been associated with increased risk of developing neonatal sepsis[98]. Dysregulated responses by the premature neonatal immune system to bacterial stimuli such as pathogenic *E. coli* has the potential to cause an infection driven inflammatory response and subsequent tissue damage[115] such as necrotising enterocolitis (NEC). Monocytes in infants with NEC have higher TLR4 expression and higher TNFα and IL-6 expression and reduced TGFβ. These eventually promote the differentiation of Th17 cells and so further enhancing the inflammation[116].

In contrast, infants born at term show increased IL-6 and IL-23 compared to preterm neonates, causing a resulting Th17 dominance, and an overall reduction in polyfunctionality compared to adults. A gradual postnatal shift then occurs with a decrease in IL-10 and an increase in pro-inflammatory cytokines such as TNFα and IL-12[95]. Age related changes in adaptive immunity, and the development of memory responses are thought to influence TLR mediated innate immune responses, and thus have an indirect effect on innate immune development[95]. Human monocytes cultured from neonates and the elderly have been shown to have reduced TNFα, IL-6 and IL-1β concentrations in response to dengue viral infection when compared to the response from young adults, with neonates showing the least increase in cytokine concentrations[117]. Likewise a similar response has been observed in neonatal monocytes after LPS stimulation, with lower quantities of TNF and IL-6[118] and a reduced response to *Staphylococcus epidermidis* and group B *Streptococcus*[115]. This contrasts with our observations of neonate cattle showing greater polyfunctionality, and potentially greater context specific immunity, than the adult subsets. As discussed earlier, this may be due to *in utero* infection pressures seen in cattle. The lack of placental transfer in cattle compared to humans and subsequent reliance on colostrum for passive transfer of immunity may result in the need for heightened innate immune responses in neonatal cattle. Postnatally, bovine neonates are subject to greater infection pressures than that seen within human neonates; the increased infection pressures seen over time may have resulted in differences in neonatal immune responses.

Exposure to pathogens is known to influence the response of the innate immune system, with antigenic exposure driving the development of the adaptive compartment early in life. It has been hypothesised that neonates exposure to microbes is large, diverse and occurs

simultaneously and is therefore predictable[88]. With the exposure to microbes being predictable, this equates to immunological development also following a predictable course. This pattern is observed within the cluster analysis, with the exception of Neonate 2, and may explain the homologous results seen within the neonates. In contrast, a large degree of diversity is seen within the adults in our study; this may reflect parasite exposure, diet or differing exposure to pasture/housing and therefore being subject to differing infection pressures. Environmental influences are known to exert cumulative effects over the lifecourse in humans, from vaccination to infection and nutrition, and have been shown to dominate over heritable factors with this variation in the immune system increasing with age[119]. This pattern of neonatal homology and increasing diversity with age is also detectable within our cattle samples.

Genetic defects are also known to have an impact on innate immune development, with diseases such as atopy having a familial history and TLR polymorphisms demonstrating altered BCG responses[120]. Previous studies have suggested that neonatal epigenetics are primarily determined in utero, and, with increasing age, genetic and environmental factors play more of a role, with differing sensitivity between individuals[121]. However, within cattle, miRNA expression profiles have been shown to alter in response to diets of differing fat content[122], showing that it is possible to alter the genomic profiles of cattle postnatally through husbandry changes, which may account for some of the diversity seen within our adult population. Epigenetic changes are also largely responsible for the changes seen during trained immunity, the concept that the innate immune system has a limited capacity for memory alongside the adaptive immune system.

The concept of trained immunity is a recent one, previously it was thought that only the adaptive response had memory capacity. Innate immune cells have now been shown to have enhanced resistance on reinfection with the same pathogen, a process mainly driven by epigenetic modifications, potentially through cytokine or PRR signalling. The increased responsiveness is non-specific, mediated through transcription factors and epigenetic signalling and lasts for weeks/months[27123]. Within a mouse model, infection with BCG within the bone marrow has been demonstrated to cause local cell expansion and myelopoiesis. Macrophages that are generated are epigenetically modified to give better protection against Mtb than naive macrophages to provide long term innate protection. This requires IFN-γ signalling to generate trained monocyte populations[124]. Likewise, exposure of monocytes to *C. albicans* enhanced the subsequent response to stimulation with unrelated pathogens via reprogramming of chromatin marks which led to increased cytokine

production[125]. Enhanced monocyte responses have also been observed in parasite infection via neutrophil priming a Th2 type response, giving accelerated parasite clearance up to 45 days post infection[126].

Early gut dysbiosis has been shown to effect innate immune system development alongside overall deviation from the stereotypical development at three months of age[88]. Germ-free mice have been shown to have lower levels of monocyte recruitment to the intestine at 3 weeks of age with adults having fewer of all monocyte subsets. Administration of broad-spectrum antibiotics also resulted in a deficit of immature macrophages within the intestine[127]. This demonstrates the importance of the presence of a diverse microbiome on the healthy development of the immune system. Within our sample population, Neonate 2 showed deviation from the other neonate samples; on post mortem evidence of petechial haemorrhage and sloughing of the intestinal lining was apparent; this may indicate potential gut and microbial abnormalities that, comparing with human populations, may have influenced the make-up of the immune system. Further study using 16S sequencing to analyse intestinal contents in addition to analysis of monocyte subsets in neonates would enable a direct within animal comparison of the effects of gut flora on the innate immune system development.

Plasticity of the innate immune system is a concept that has been recently discussed, with classical monocytes developing to non-classical monocytes, or as previously mentioned, into tissue resident macrophages under certain conditions. Within our study, neonates displayed a significantly higher proportion of the triple negative subset in comparison to adult monocytes. These are likely to represent immature monocytes that are yet to develop expression of cell surface markers and may indicate a greater potential for the neonatal immune system to adapt to differing challenges or infections. Under inflammatory conditions human monocytes are able to display different functional phenotypes in response to different infection challenge; classical and intermediate monocytes responding to bacterial infection and non-classical monocytes responding to viral infection[38].

Classical monocytes are recruited to sites of inflammation, alongside their transition into the non-classical monocyte subset. They are thought to represent a more plastic subset than their non-classical counterpart, being able to transition between subsets, into tissues and replenish macrophage populations. Neonates were found to have a significantly higher proportion of $CD14^+$ monocytes and, within this, $CD14^+CD16^-MHCII^-$ monocytes. Neutrophil degranulation products have been shown to induce calcium influx in classical and

intermediate monocytes via cross linking of CD18 in bovine monocytes, alongside inducing migration of intermediate monocytes. Macrophages formed from classical or intermediate monocytes during the presence of neutrophil degranulation products show reduced MHCII expression, more IL-10 and IL-12 production and enhanced ROS and phagocytosis[128].

$CD14^+$ classical monocytes are associated with a higher expression of CCR2, which is important for allowing extravasation and migration during inflammatory processes. During this study neonates were found to have significantly higher expression of CCR2, potentially a reflection of the higher proportion of $CD14^+$ monocytes that neonates possessed. Patterns of increased CCR2 are also seen in human neonates, thought to be influenced by the placental environment[129]. Expression of CCL2 and CCL5 by stellate cells is responsible for the engraftment of monocytes following infection[130]. Likewise, in the lung, during infection the alveolar macrophage pool is replenished by monocytes recruited by CCL2 interaction[131]. Replacement of embryonic monocytes by circulating $Ly6C^{hi}$ monocytes within the murine intestine at weaning is also dependant on CCR2, driven largely by intestinal microbes, demonstrated by CCR2 KO mice being deficient in colonic macrophages[127]. The increased CCR2 seen within neonate cattle may be driving increased intestinal monocyte turnover, further investigation would be needed to see the effects of weaning on the expression of CCR2. The measurement of CCR2 undertaken during this study using dot blots and Image J necessitated the adjustment of the resultant cell density measurements for cell counts present within the lysate; ideally further investigation to confirm this finding, using samples with identical counts would be undertaken, alongside a greater number of neonate samples.

Previously, adult bovine classical monocytes have been shown to have the highest quantities of arginase-1 expression[19], which correlates with the data obtained here, with arginase being measured in $CD14^+$, and therefore classical, monocytes. Neonates show both higher quantities of classical monocytes and higher arginase expression. The higher quantities of both CCR2 and arginase expression within the neonate population may also be a result of the larger quantities of triple negative monocytes, and the ability of these cells to be plastic and polyfunctional. The significant positive correlation that is apparent between CCR2 and arginase activity is indicative of this. However, the adult population also shows a positive correlation between arginase and CCR2. The R^2 value for the neonates alone shows a higher degree of correlation than the adult subset alone; potentially indicating that with increasing age there is a divergence in the expression of CCR2 and arginase activity within a single cell.

CCR2 KO mice have reduced numbers of classical monocytes emigrating from the bone marrow with and without infection with *L. monocytogenes* but this also has the potential to lead to reduced host tissue damage[30]. In contrast, a lack of arginase-1 induction (using KO mice) during *T gondii* infection led to increased host survival[73]. Our study demonstrated that neonates show an increased arginase activity; this may therefore mean that neonates are more at risk of infection by intracellular pathogens, however Sharma *et al* demonstrated that neonate monocytes harbour fewer parasites during infection[105]. As discussed above, this may be a reflection of the large proportion of triple negative subset present, harbouring the ability to switch and upregulate arginase.

The potential ability of the neonate immune system to switch and upregulate production of arginase/CCR2 dependant on infection may also have impacts on the response to coinfection with different pathogens. It has previously been established that cattle coinfected with *F. hepatica* and *M. bovis* have a reduction in IFN-γ responsiveness[78] and a negative association between exposure to *F. hepatica* and TB diagnosis[79]. The role of AAMs induced by *F. hepatica* is a major feature of these dysregulated responses,[80,81,82] leading to an upregulation of arginase 1[66]. With neonates possessing larger quantities of both triple negative and $CD14^+$ classical monocytes, future work to look at the difference in responsiveness to coinfection between adults and neonates would be interesting.

In addition, further analysis measuring kinase activity would enable measurement of phosphorylation and therefore changes resulting to alterations in signalling in response to incoming stimuli that occur between adult and neonates and whether the CCR2 and arginase measured is active. As these adult samples were collected from the abattoir, the exact age, health and parasite status of the cattle were unknown, and so it may be that different ages were represented within the results, which may also account for some of the variation seen.

Overall, this study has shown that significant differences exist between bovine neonate and adult monocyte populations, both in terms of subset quantities and functions. This work builds on previous studies demonstrating neonates have greater quantities of $CD14^+$ monocytes. We have also demonstrated that neonates have an increased quantity of triple negative subsets, and increased arginase and CCR2 activity compared to the adult populations. This provides the potential for therapeutic interventions to be targeted depending on the age, and therefore immune status of the animal, to exploit the potential of the innate immune system to be heightened earlier in life. Likewise, targeted pathogen exposure, for example ensuring that calves are exposed early on in life during a period of

heightened immune responsiveness, may mean that a better and more effective immune response is achieved. It also offers the potential for immune modulation via trained immunity, neonates exposed earlier in life to pathogens, who have a larger classical and triple negative monocyte pool, have the potential to mount a better and longer lasting innate immune response.

Appendices

Appendix 1. Reagents

Ack lysis buffer

Makes 500ml of ACK lysis buffer:

- NH_4CL 4gms=0.15M
- $KHCO_3$ 0.5gms =10mM
- EDTA 0.0186gms=0.1mM
- DH_2O-500ml

pH between 7-8

RPMI complete media:

90mls RPMI (R8758 Sigma Aldrich)

10% FCS (F4135 Sigma Aldrich)

1% penicillin- streptomycin (10,000 U penicillin & 100 mg/ml streptomycin) (P4333 Sigma Aldrich)

Freeze mix

10% DMSO,

45% RPMI,

45% FCS (Sigma Aldrich)

FACS buffer

10% FCS (Sigma Aldrich)

80% PBS (Sigma Aldrich)

FACS blocking buffer

1% BSA dissolved in PBS

MACS Running Buffer:

0.5% BSA (Sigma Aldrich)

1 litre PBS

2mM EDTA

MACS Rinsing Buffer:

1 litre PBS

2mM EDTA

Resolving Gel

Acrylamide/Bisacrylamide -4ml

1.5M Tris pH8.8 – 2.5ml

10% SDS - 50µl

dD H_20 – 3.4ml

10% ammonium persulphate (100mg to 1ml) - 75µl

TEMED – 75µl

Stacking Gel

Acrylamide/Bisacrylamide – 0.65ml

0.5M Tris pH6.8 – 1.25ml

10% SDS - 50µl

dD H_20 – 3.025ml

10% Ammonium persulphate - 25µl

TEMED – 2.5µl

Running buffer

Tris -HCl 25mM

Glycine 200mM

SDS 0.1%

Transfer Buffer (10x)

30.3g L Tris

144g L Glycin

TBST

20mM Tris

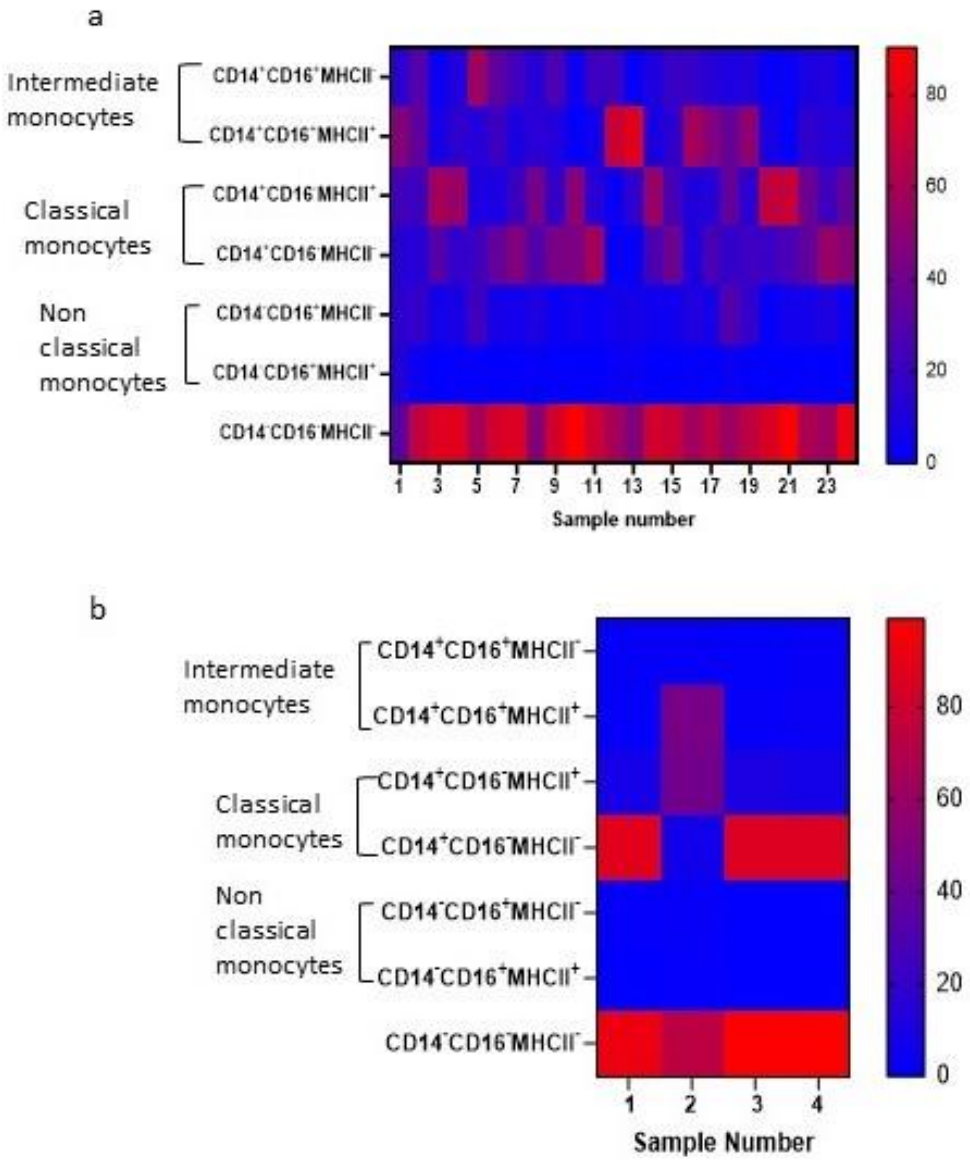
150mM NaCl

0.1% Tween

Appendix 2. R Studio code

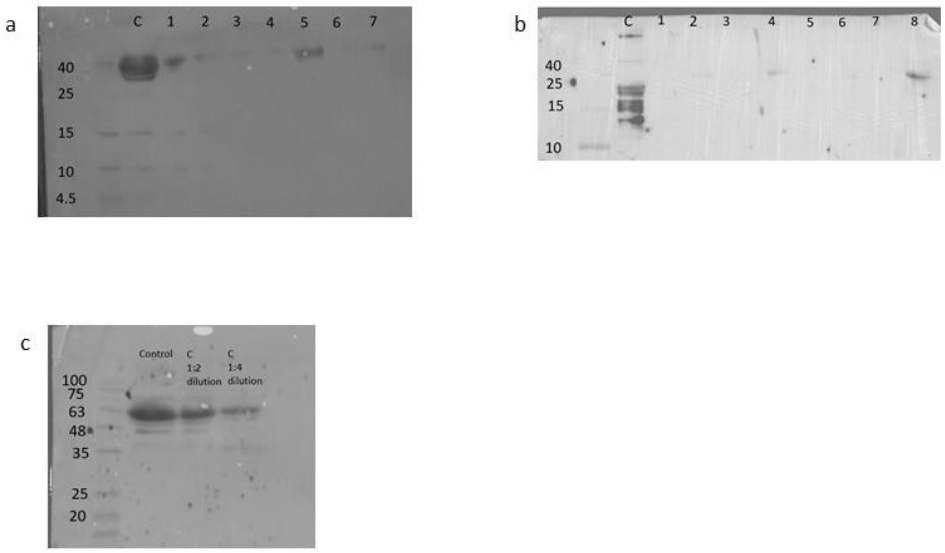
```
> dot_touse<- dot[, 2:ncol(dot)]
> rownames(dot_touse)<- paste(dot[, 2], dot[, 1], sep = "_")
> dot_dist_samples<- dist(x = dot_touse, methods = "euclidean")
> dot_hc_samples <- hclust(d = dot_dist_samples, method = "ward.D2"
                           + )
   > plot(dot_hc_samples)
```

Appendix 3. Heatmaps of adult and neonate monocyte subset distribution



Heat maps of data from adult (a) and neonate (b) cattle. This demonstrates variation in monocyte subsets with neonate samples possessing higher quantities of triple negative subsets and a greater homology within subset distribution.

Appendix 4. Western blots



CCR2 expression via Western blot. $CD14^{+}$ lysate from adult samples (labelled 1-8) and positive control (BMDM lysate, labelled C) were run on a Western blot and stained using CCR2 primary antibody followed by secondary antibody (rabbit HRP conjugated antibody) (a) or secondary antibody alone (b). Control samples were run alone at differing concentrations (c).

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