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Transcriptional control of macrophage function in the pig and its relationship to infectious disease susceptibility

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Declaration

The thesis presented is the work of the author except where stated otherwise by reference and/or acknowledgement. Any work presented, which has been conducted by (or in collaboration with) others is explicitly acknowledged. No part of this work has been submitted in candidature for any other degree or qualification.

Name: …….. Date: ……..

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Abstract

The biology of cells of the mononuclear phagocyte system has been studied extensively in the mouse. Studies of the pig as an experimental model have commonly been consigned to specialist animal science journals. This thesis considered some of the many ways that pigs may address the shortcomings of mice as models for the study of macrophage differentiation and activation *in vitro*, and the biology of sepsis and other pathologies in the living animal.

Flow cytometry was used initially to phenotype cells from the porcine lung, peritoneal cavity, blood and bone marrow using the LPS receptor CD14 and the FC receptor CD16, markers frequently employed to differentiate human monocytes into subsets. The expression of SIRP-alpha (SWC3a, CD172a), which is present on all cells of myeloid origin, and the haemoglobin scavenger receptor, CD163 which has previously been used to study monocyte differentiation in the pig was also studied. The findings validated previous work where blood monocytes were divided into subsets on the expression of CD14 and CD163. Furthermore, like human and mouse, pig monocytes also exhibited variation in CD16 expression, having a subset which was $CD14^{hi}CD16^{lo}$ and another which was $CD14^{lo}CD16^{hi}$. A whole genome approach was then used to study the differences between the monocyte subsets in the pig, using monocytes sorted into two populations based on the expression of CD14 and CD163. The gene expression profiles obtained were then compared to publically available data from monocyte subsets in human and mouse.

This thesis also investigated the expression of genes that are known to be differentially expressed between human and mouse. To do this gene expression in porcine bone marrow derived macrophages was analyzed across an LPS time course. Like human macrophages, pig macrophages did not induce nitric oxide nor any arginine metabolizing genes in response to LPS. Instead they responded with robust induction of indoleamine 2,3-dioxygenase (IDO) and other enzymes of the tryptophan metabolism pathway such as kynurenine hydroxylase, kynureninase and tryptophan-tRNA synthetase. The tryptophan metabolism pathway has been implicated in sepsis in man and the absence of this pathway in the mouse may be one

of the reasons why an adequate rodent model of sepsis has not been developed. The IDO inhibitor 1-methyl-tryptophan (1-MT) has been used to treat mouse macrophages where it had a protective effect after LPS administration. Similar experiments on pig macrophages did not show the same protective effect and induction of key immune genes was increased after treatment with 1-MT suggesting IDO is involved in feedback control of the immune system.

With the completion of the genome sequence and the characterisation of many key regulators and markers, the pig has emerged as a tractable model of human innate immunity and disease that should address the limited predictive value of rodents in preclinical studies. This project aimed to address the gap in our knowledge of the control of innate immunity in the pig and provided further evidence that the pig can function as an ideal model to study innate immunity.

Abbreviations

ACD	Acid Citrate Dextrose
AIM2	Absent In Melanoma 2
AM	Alveolar Macrophage
AL	Argininosuccinate Lyase
APC	Antigen Presenting Cell
ARG	ARGinase
AS	Argininosuccinate Synthetase
ASF	African Swine Fever
ATP	Adenosine TriPhospahte
BAL	BrochoAlveolar Lavage
BH_4	tetraHydroBiopterin
BMC	Bone Marrow Cell
BMDM(s)	Bone Marrow Derived Macrophage(s)
BSA	Bovine Serum Albumin
bp	base pair
CAGE	Cap Analysis Gene Expression
CAT2	Cationic Amino acid Transporter 2
CCL	C-C motif Ligand
CCR	C-C motif Receptor

cDNA	complementary DNA
CFTR	Cystic Fibrosis Transmembrane conductance Regulator
CORG	COmparative Regulatory Genomics
CpG	C-Phosphate-G
CT	Cycle Threshold
cRNA	copy RNA
(rh)CSF1	(recombinant human) Colony Stimulating Factor 1
CSF1R	Colony Stimulating Factor 1 Receptor (CD115)
CXCL	C-X-C Chemokine Ligand
CXCR	C-X-C Chemokine Receptor
CYP27B1	cytochrome P450, family 27, subfamily B, polypeptide
DAVID	Database for Annotation, Visualisation and Integrated Discovery
DC(s)	Dendritic Cell(s)
DExH	Asp-Glu-X-His
DNA	DeoxyriboNucleic Acid
dNTP	DeoxyriboNucleic triPhosphate
dsDNA	double stranded DNA
DTT	DiThioTreitol
ECR	Evolutionary Conserved Regions
EDTA	EthyleneDiamineTetraAcetic acid

EGFP	Enhanced Green Fluorescent Protein
EMR1	EGF-like module-containing Mucin-like hormone Receptor-like 1
ENCODE	ENCyclopedia Of DNA Elements
eNOS	endothelial NOS
FC	Fragment Crystallizable
FCGR	FC Gamma Receptor
FCS	Foetal Calf Serum
FSC	Forward SCatter
GADD45B	Growth Arrest and DNA-Damage-inducible β
GCH1	Gtp CycloHydrolase1
GCSF	Granulocyte Colony Stimulating Factor (CSF3)
GEO	Gene Expression Omnibus
GMCSF	Granulocyte Macrophage Colony Stimulating Factor (CSF2)
GO	Gene Ontology
GPI	GlycoPhosphatidyl-Inositol
GTP	Guanosine TriPhosphate
HIN200	Hemopoietic IFN-inducible Nuclear protein 200
HMGB1	High-Mobility Group Box protein1
HPRT	Hypoxanthine PhosphoRibosylTransferase
HSP	Heat Shock Protein

IDO	Indoleamine 2,3-DiOxygenase
IFN	InterFeroN
IL	InterLeukin
iNOS	inducible NOS
i.p	intraperitoneal
IRF	Interferon Regulatory Factor
IRG	p47 Immunity-Related Gtpases
IRI	Ischemia Reperfusion-Injury
ISG	Interferon Stimulated genes
i.v	intravenous
IVT	In Vitro Transcription
JAG1	Jagged 1
JNK	Jun N-terminal Kinase
Ka/Ks	non synonymous v synonymous base change
KMO	Kynurenine hydroxylase
KYNU	Kynureninase
LBP	LPS Binding Protein
LFA	Lymphocyte Function-associated Antigen
LPS	LipoPolySaccharide
LRR	Leucine Rich Repeats

mAb	Monoclonal Antibody
MAPK	Mitogen-Activated Protein Kinase
MCL	Markov Cluster Algorithm
MDM	Monocyte Derived Macrophage
MDP	Muramyl DiPeptide
MEK	Mitogen-activated protein Kinase 1
MHC	Major Histocompatibility Complex
MLR	Mixed Lymphocyte Reaction
miRNA	micro RNA
MNDA	Myeloid Nuclear Differentiation Antigen
MPS	Mononuclear Phagocyte System
mRNA	Messenger RNA
1MT	1-Methyl-Tryptophan
Mx	Myxo-virus resistant, IFN-inducible GTPase
NACHT	NAip, Ciita, Het-e and Tp1
NaN_3	Sodium Azide
NFκB	Nuclear Factor κB
NK	Natural Killer
nNOS	Neuronal NOS
NO	Nitric Oxide

NOD2	Nucleotide-binding Oligomerization Domain containing 2
NOS	Nitric Oxide Synthase
OAT	Ornithine AminoTransferase
PAMP	Pathogen Associated Molecular Pattern
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PiGMaP	European Pig Gene Mapping Project
PKR	Protein Kinase R
PM	Peritoneal Macrophage
PRR	Pattern Recognition Receptor
PRRS(V)	Porcine Respiratory and Reproductive Syndrome (Virus)
RANKL	Receptor Activator of NFκB Ligand
RBC	Red Blood Cell
RNA	RiboNucleic Acid
RNAseq	RNA Sequencing
RPMI	Rose-Parkes Memorial Institute media
RT qPCR	Real Time quantitative PCR
SCF	Stem Cell Factor
SD	Standard Deviation

SIRP	Signal Regulatory Protein
SIRS	Systemic Inflammatory Response Syndrome
SLA	Swine Leukocyte Antigen
SNP	Single Nucleotide Polymorphism
SSC	Side SCatter
STAT	Signal Transducer and Activators of Transcription
STO	SIM mouse embryo fibroblasts resistant to Thioguanine and Oubain
SWC	Swine Workshop Cluster
TEPM	Thioglycolate Elicited Peritoneal Macrophage
TGF	Transforming Growth Factor
TIR	Toll/IL1 Receptor
TLR	Toll Like Receptor
TNF	Tumour Necrosis Factor
TREM	Triggering Receptor Expressed on Myeloid cells
TRIF	TiR-domain-containing adapter-Inducing interFeron-β
tRNA	transfer RNA
UTR	UnTranslated Region
WARS	tryptophan-tRNA synthetase

Chapter 1: Introduction

1.1 Macrophage biology and transcription control

In vertebrates, the first line of defence against pathogens is the resident tissue macrophage. Macrophages represent the tissue compartment of the mononuclear phagocyte system (MPS), which also includes blood monocytes and their bone marrow progenitors, as well as the closely-related myeloid dendritic cells. They comprise 10-15% of the total cells in most organs of the body, and are especially concentrated at locations of exposure to pathogen infiltration, lining all epithelia and the microvasculature (Hume, 2006; Hume, 2008a; Hume et al., 2002).

Macrophage activation in response to microbial products has been studied extensively in mouse and human systems, most recently using genome-scale approaches generating complex interaction networks (Ghisletti et al., 2010; Nilsson et al., 2006; Ramsey et al., 2010). In each species, the activation process involves induction and repression of thousands of genes across an extended time course. Amongst the most inducible genes in macrophages responding to a microbial stimulus are feedback control genes that act at every conceivable level of the activation cascade. These inflammation suppressor genes are highly polymorphic within species and inactivating mutations in many of them are known to generate a hyper-inflammatory phenotype (Gilchrist et al., 2008; Gilchrist et al., 2006; Nathan, 2002; Wells et al., 2005). Although this complex effector network has many levels of post-transcriptional regulation, including inducible microRNAs (miRNAs) (El Gazzar and McCall, 2010; Li et al., 2010; Fontana et al., 2010; Fontana et al., 2007), the major control point occurs at the level of transcription initiation; the point at which the production of individual mRNAs is switched on and off. Accordingly, a detailed knowledge of transcription control in macrophages is central to understanding all major human and animal infectious diseases and the molecular basis of genetic variations in disease resistance amongst species and individuals. A

number of recent studies have approached the issues of macrophage differentiation and activation through genome-scale network analyses (Aderem, 2005; Ramsey et al., 2008; Ravasi et al., 2007). This thesis investigates the transcriptional control of macrophage function in the pig and its relationship to infectious disease susceptibility.

An effective response to pathogen challenge requires the host to distinguish self from non-self. This involves the recognition of pathogen-associated molecular patterns (PAMPS). PAMPS are classes of molecules that are generally intrinsic and essential to pathogen biology (and therefore not readily modified or eliminated in response to selection by the host) and are not found in the host. There are many different pattern recognition receptor families, including toll-like receptors (TLRs), C-type lectins, DExH box family members and the NACHT-domain family (Takeuchi and Akira, 2010). The pattern recognition receptors, as well as many of the innate immune effector families (e.g. chemokines, defensins, proteases) produced by activated macrophages, comprise large multigene families that vary greatly even between mammals in terms of the numbers of members, sequences of functional orthologs, and pattern of expression.

Comparative analysis of RNA extracted from mouse and human macrophages cultured under the most similar conditions possible has revealed extensive differences. The array data confirmed a known difference: human macrophages do not induce the effector enzyme, inducible nitric oxide synthase (iNOS, NOS2), which generates the toxic radical nitric oxide (NO), instead inducing indoleamine dioxygenase (IDO) in response to LPS (Roshick et al., 2006; Thoma-Uszynski et al., 2001). In a direct comparison of mouse and human macrophages responding to lipopolysaccharide, only around 30% of definitive orthologous genes that were induced in one species were also induced in the other (Schroder et al., 2012). The differences in gene expression between species arise in part from functional promoter

evolution at some level, either in the promoters of the transcription factors themselves, or in the promoters of their target genes, or both. Indeed, TLRs 2, 3 and 4 each have operative promoter differences between mouse and human (Heinz et al., 2003). Human macrophages express the TLR9 receptor for bacterial DNA at lower levels than mouse and further induction of message is not observed in response to interferon-gamma priming because the human promoter lacks a key binding site for the macrophage transcription factor, IRF8 (Schroder et al., 2007). The hypothesis of this thesis was that pig macrophages are phenotypically and genotypically closer to human macrophages than the more commonly used mouse model systems.

Phylogenetic footprinting has been used in many genome-wide comparative studies of mammalian promoters to highlight conserved elements (Birney et al., 2007). In genes where the regulation and function is shared amongst the species and there are clear orthology relationships, this approach is powerful. Resources such as the ECR browser and CORG (Dieterich et al., 2007; Loots and Ovcharenko, 2007) provide important tools in promoter annotation, in identifying candidate enhancers in the vicinity of genes of interest, and in producing transcriptional networks. A significant part of the pilot stage of the ENCODE project (Birney et al., 2007) which aims to understand the control of transcription at the molecular level, focused on comparative sequence analysis of functional elements across multiple mammalian species. This thesis further aims to highlight the value of the pig as a model for human macrophage biology, and also as a third species to enable an understanding of evolution of innate immune responses.

1.2 Macrophage biology in the pig

Pigs are the most important meat-producing livestock species world-wide. Because of the intensive mode of breeding and rearing, they are highly susceptible to pathogen epidemics, and consequently selection of animals for increased disease resistance is of major economic importance. Amongst the major pathogens that cause economic losses in pigs, many viral (e.g. vesicular stomatitis virus, porcine

circovirus 2, swine pox virus, African swine fever (ASF), classical swine fever, porcine adenovirus, swine vesicular disease virus, porcine respiratory and reproductive syndrome (PRRS), parainfluenza) and bacterial (e.g. *Salmonella spp*, *Yersinia enterocolitica*, *Mycoplasma hypopneumoniae*, *Actinobacillus pleuropneumoniae*) pathogens replicate within macrophages and/or profoundly alter macrophage gene expression. In addition to the economic and welfare issues these viral and bacterial species impart on the pig, many of these pathogens are candidate zoonoses.

Like human and mouse macrophages, pig monocytes and monocyte-derived macrophages detect pathogens through receptors that recognise a range of pathogen-associated molecular patterns, including cell wall components (lipopolysaccharide, peptidoglycan) and nucleic acids (Raymond and Wilkie, 2005). They respond with rapid changes in expression of proinflammatory cytokines and other effectors. Macrophage gene products are clearly required for effective innate immunity, but they also initiate the symptoms of infection (sickness, behaviour, weight loss etc.) that cause morbidity or mortality. Studies of viral (PRRS) and bacterial (actinobacillus) pathogens of pigs (Ait-ali et al., 2007; Moser et al., 2008) suggest that variation in disease susceptibility or pathology between breeds, or between individuals within a breed, is correlated with differences in macrophage activation.

Despite the economic importance of the pig, and the clear relevance as a model for human biomedicine (Schook et al., 2005) there has not previously been an attempt to overview the similarities and differences between the pig, more commonly studied rodent models, and humans. The publication of the pig genome and the advent of microarray technologies for this species mean that it is timely to examine this question. This thesis examines in detail the knowledge of macrophage biology and the molecular basis of innate immunity and disease susceptibility in the pig, a species

that, unlike mice and rats, is of economic and welfare importance to humans, and shares many infectious challenges with us.

1.3 Markers for the mononuclear phagocyte system

The visualisation of mononuclear phagocytes in the circulation, and in tissues, depends upon the identification of cell surface proteins, many of them endocytic receptors, which are present only on these cells. The advent of monoclonal antibody (mAb) technologies made it possible to identify cells bearing these proteins on their cell surface by immunohistochemical approaches. Monoclonal-defined markers are now commonly used in rodent and human systems to identify mononuclear phagocytes and subsets of these cells with specialised functions or locations. Only recently have such monoclonal antibodies been made explicitly for the study of pigs, in many cases advantage has been taken of the cross reaction of mouse monoclonal antibodies against human macrophage-expressed gene products (Ezquerra et al., 2009). Owing to the extensive functional differences between different macrophage populations, there are few gene products that are common to all members of the MPS of mouse or human. In fact, many surface markers, such as the integrins CD11b and CD11c, lectin-like molecules such as sialoadhesin and macrosialin, EMR1 (F4/80), and certain chemokine receptors e.g. CCR1, CCR2 and CX3CR1 are used rather arbitrarily to divide the MPS into putative functional subsets (Gordon and Taylor, 2005). However, many of the gene products that have been used to define macrophage identity in one species are not definitive markers of this cell type in other species. For example, the F4/80 antigen, the product of the *Emr1* gene which is a member of the EGF-TM7 family of G-protein coupled receptors, has been used extensively as a marker of macrophages in the mouse (Austyn and Gordon, 1981). There are only two members of the EMR family in mice, EMR1 and EMR4. In humans four family members, EMR1-4, have been identified, although surprisingly EMR4 has acquired a truncation when compared to other primates (Kwakkenbos et al., 2004) and is now considered a pseudogene. EMR1, 2 and 3 are all expressed in human monocytes (biogps.gnf.org), but have acquired specific functions in

granulocytes (Matmati et al., 2007). This situation is not only true for markers; but for gene products that execute macrophage cell functions. Although humans, mice and pigs diverged at approximately the same point in evolution (Springer et al., 2003; Jorgensen et al., 2005), the short generation time in rodents may have accelerated evolution in this species meaning that pigs and humans are closer in sequence than the mouse. Overall, compared to the mouse, the pig immune system has been found to more closely resemble human for more than 80% of parameters examined while mice were more similar to human than pigs for less than 10% (Schook et al., 2005). The extent of functional orthology between pigs, mice and humans in macrophage biology will be considered further throughout this chapter.

One subset of mononuclear phagocytes that has been studied in detail is the so-called dendritic cell (DC), a cell population originally defined by its ability to present antigen to naive T lymphocytes. It is not clear that this function is linked to any particular marker, and a growing body of opinion believes DCs are simply another cell of the MPS. They can derive from the same precursor as macrophages, share many functions (including antigen presentation), respond to the same growth factors and share the same markers (Geissmann et al., 2010b; Geissmann et al., 2010a; Hume, 2008a; Hume, 2008b). One factor that appears to be important for the production of the subset of DC found within the T cell areas of lymphoid organs is Flt3 ligand (Flt3L) (Li et al., 2010). Guzylack-Piriou et al. (Guzylack-Piriou et al., 2010) recently characterised the pig Flt3L, and showed that, as in mice (Merad and Manz, 2009) expression of the receptor for this factor, Flt3, in lymphoid organs was restricted to interdigitating cells of T cell areas; the classical DC. Amongst other candidate markers of DC from mouse or human, the C type lectin CD205 retains high level expression in epidermal DC (Langerhans cells) and lymphoid tissues of the pig (Flores-Mendoza et al., 2010). Conversely, the integrin CD11c, which is often used as a marker for DC in mice despite its extensive expression in tissue and inflammatory macrophages (Hume, 2008a; Hume, 2008b), is expressed differently in pigs from humans or mice; being restricted to monocytes and absent from granulocytes (Ezquerra et al., 2009; Bailey, 2009).

1.4 Growth factors and myelopoiesis in the pig.

Macrophages share committed progenitors with the other major mammalian phagocyte population, neutrophilic granulocytes (Metcalf, 2008). Indeed, mature inflammatory neutrophilic granulocytes in mice and humans may retain the ability to convert into macrophages (Sasmono et al., 2007). The differentiation of both cell types in mice is controlled by the haemopoietic growth factors, stem cell factor (SCF, Kit-ligand), interleukin 3 (IL3), macrophage colony-stimulating factor (CSF1), granulocyte macrophage colony-stimulating factor (GMCSF, CSF2) and granulocyte colony-stimulating factor (GCSF, CSF3) (Hume, 2006; Hume et al., 2002; Metcalf, 2008; Sasmono et al., 2007). Three of these factors in the pig, SCF, IL3 and GMCSF have been cloned and expressed, and like their human counterparts, were able to mobilise haemopoietic stem cells when injected into pigs and promote engraftment in bone marrow transplantation (Kozlowski et al., 1999). One molecule that is expressed on the vast majority of mouse and human cells designated as mononuclear phagocytes is the receptor responsible for responding to CSF1. The CSF1 receptor (CSF1R, CD115) is a type III integral member protein tyrosine kinase encoded by the c-fms protooncogene and displays restricted expression in mononuclear phagocytes and their precursors (Sasmono et al., 2003). In the mouse, CSF1 alone is not sufficient for differentiation of early myeloid progenitor cells; however, it can function in synergy with the other haematopoietic stem cell factors to generate mononuclear phagocyte progenitor cells from multipotent progenitor cells. CSF1 synergises with GMCSF, GCSF and IL3 along with other growth factors such as SCF to regulate the maturation of hematopoietic progenitor cells into mature immune effector cells (Barton and Mayer, 1989). CSF1 also acts with the receptor activator of NFκB ligand (RANKL) to regulate the generation of osteoclasts from mononuclear progenitors (Yao et al., 2002)

CSF1 and GMCSF are both 4 helix bundle growth factors, but they have different tissue distribution and act through different receptor classes. In the mouse, a *Gmcsf*

null mutant is not globally macrophage deficient (Hibbs et al., 2007), whereas both *Csf1* and *Csf1r* mutants in mice or rats have severe macrophage deficiencies and multiple pleiotropic defects in growth and development (Gow et al., 2010; Dai et al., 2002; Cecchini et al., 1994; Ryan et al., 2001) The major non-redundant function of GMCSF appears to be in the production or maintenance of macrophages and their function in the lung, and deficiency leads to pulmonary alveolar proteinosis (Carey and Trapnell, 2010; Hibbs et al., 2007). Additionally, there are substantial deficiencies in resistance to a wide range of pathogens, suggesting that GMCSF is primarily involved in emergency production of macrophages and activation of several effector functions (Carey and Trapnell, 2010; Hibbs et al., 2007). Cells grown in GMCSF, either from bone marrow or peripheral blood in mice or humans, have been widely studied as putative "immature DC" (Hume, 2008a; Hume, 2008b). However, the gene expression profiles of these cells does not distinguish them significantly from other macrophage populations (Mabbott et al., 2010). Others have suggested that macrophages differentiated in the presence of GMCSF display a "classically activated" phenotype while those differentiated in the presence of CSF1 have a phenotype closer to "alternatively activated" macrophages (Fleetwood et al., 2009). GMCSF has been used to generate monocyte derived DCs in the pig (Carrasco et al., 2001; Summerfield et al., 2003); the cells elicit T cell activation *in vitro,* although unlike human monocyte-derived DC, the pig "DC" retained the macrophage markers CD14 and CD16. Pig GMCSF encoded within plasmid DNA has been used to boost antigen presenting cell activity in vaccines (Melkebeek et al., 2008). In mouse and human, some protocols for the generation of culture-derived DC also employ IL4 as an additional stimulus. IL4 is not a prominent cytokine in the blood in pigs; the function apparently being replaced by the related IL13. GMCSF in combination with IL13 was found to generate active antigen-presenting cells from pig peripheral blood monocytes (Bautista et al., 2007).

CSF1, and its receptor, have evolved rather rapidly across vertebrates suggesting that it is under immune selection (Garceau et al., 2010). CSF1 is commonly used to generate bone marrow-derived macrophages (BMDMs) from the mouse, and to

mature peripheral blood monocytes from humans to give rise to monocyte derived macrophages (MDM). Interestingly, a recently-discovered second ligand for the CSF1R, named Interleukin-34 (IL34) was identified in a high through-put functional ligand-receptor expression screen (Lin et al., 2008). IL34 is much more conserved across species than CSF1. It probably binds a different part of the CSF1 receptor (Garceau et al., 2010) and has quite distinct expression pattern in the mouse (Wei et al., 2010).

The major non-redundant function of CSF1 in mice is to promote the maturation of peripheral blood monocytes to become the immediate precursors of resident tissue macrophages (Macdonald et al., 2010). Surprisingly, there has been relatively little study of actions of CSF1 in the pig and until recently, CSF1 had not been widely-studied in other species. Human CSF1 can act on all mammals tested, but human macrophages do not respond to the mouse ligand (Lin et al., 2008; Wei et al., 2010). Pig peripheral blood or bone marrow cells have been differentiated into macrophages *in vitro* using L929 conditioned media, a source of CSF1, or CSF1 purified from L929 conditioned media (Denham et al., 1996; Genovesi et al., 1990; Mayer, 1983). SIM mouse embryo fibroblasts resistant to thioguanine and oubain (STO cells), which express CSF1, GMCSF, IL3, SCF and leukaemia inhibitory factor, have also been used as a feeder layer upon which to grow porcine macrophages (Talbot et al., 1998). It would appear that unlike human macrophages, porcine macrophages respond to murine CSF1. Our laboratory has isolated and characterised pig CSF1 and IL34(D Gow, unpublished), and developed techniques to generate macrophages from bone marrow material and peripheral blood monocytes. Factor-dependent cell lines expressing the pig CSF1R have also been produced, which respond to recombinant pig and human CSF1 and are currently producing monoclonal antibodies against the pig CSF1R, which is commonly used as a marker in studies of human monocytes (Ingersoll et al., 2010). The receptor diverges quite rapidly across species, and anti-human CD115 antibodies tested thus far have not cross-reacted with the pig. These reagents will allow well-defined comparative studies of macrophage transcriptional responses and function in the pig, mouse and human

under comparable conditions. Such comparisons are of particular interest because it is known that mice and human monocyte-macrophages respond in different ways to human CSF1 (Irvine et al., 2009). The use of a third species such as the pig will help to identify whether such differences are due to changes in the rodent and primate lineages since their divergence or intrinsic differences between the rodent and primate immune system.

1.5 Cell surface markers on porcine monocytes and macrophages

The swine leukocyte consortium has tested a wide range of anti-human monoclonal antibodies, and antibodies generated against pig macrophages, for reactivity with pig macrophages. Many human antibodies cross-react and a number of antibodies are commercially available (Ezquerra et al., 2009). Of these, a useful pan-monocyte marker for the pig (in the absence of anti-CD115) is SWC3, which recognises the regulatory molecule SIRP alpha, also known as SHPS-1, the macrophage fusion receptor or CD172a (Alvarez et al., 2007). Antibodies against the endosomal protein, CD68, have also been useful for visualising pig macrophages in tissue sections (Frich et al., 2006; Ackermann et al., 1994).

In both humans and mice, it has become clear that peripheral blood monocytes can be divided into at least two functionally distinct classes (Ingersoll et al., 2010; Cros et al., 2010; Geissmann et al., 2010a; Geissmann et al., 2010b) and the most recent classifications have introduced a third, intermediate subset of monocytes (Wong et al., 2011; Ziegler-Heitbrock et al., 2010). From mouse studies, it has been inferred that one population, defined by expression of a surface marker shared with granulocytes (Ly6C) is the immediate precursor of inflammatory macrophages, where the other population, defined by a lack of Ly6C and expression of the chemokine receptor, CCR2, and high expression of another chemokine receptor, CX3CR1, is the precursor of most resident tissue macrophages (Geissmann et al.,

2003). In mice, each population makes up around 50% of the total. Several lines of evidence indicate that the "resident" monocytes mature from the "inflammatory" Ly6C-positive monocytes (Sunderkotter et al., 2004) and, as noted above, this process is CSF1 dependent in vivo (Macdonald et al., 2010). In both mouse and human the cDNA microarray profiles of the "resident" monocytes support the view that monocyte heterogeneity reflects a maturation profile (Ancuta et al., 2009; Ingersoll et al., 2010; Wong et al., 2011).

In humans, the key markers of monocyte maturation are the lipopolysaccharide receptor CD14 and the low affinity immunoglobulin Fc receptor, *FCGR3*, detected with antibodies against CD16. The functional equivalent of mouse "resident" monocytes in humans appears to be those that express high CD16 and low CD14. By contrast to the mouse, these cells make up only around 10% of monocytic cells in humans (Ancuta et al., 2009; Ingersoll et al., 2010). In mice, CD16 is present on all peripheral blood monocytes, albeit at different levels in the two subpopulations (Ingersoll et al., 2010). The data on humans cannot be translated to other species, because humans have at least a duplication of the *FCGR33* gene (some individuals have further copies), and the second gene (*FCGR3B*) encodes a GPI-anchored isoform. In common with the mouse, most monocytes in the pig are $CD16^{+}$ (Chamorro et al., 2005; Sanchez et al., 1999) although the levels vary significantly (Chapter 3).

Similar to mouse and human, pig monocytes have been divided into two major subpopulations on the basis of marker expression, in this case CD163 (also known as haptoglobin scavenger receptor and p155) which is a transmembrane glycoprotein and member of the scavenger receptor cysteine-rich family of proteins (Sanchez et al., 1999). As in mice the numbers of each monocyte subset are close to 50% of the total therefore it is unclear at present whether monocyte subsets in the pig can be directly compared to similar populations of cells in other species. This thesis aimed

to address this concern by examining surface marker expression on porcine monocytes and differential gene expression between previously identified sub populations. Previous findings on monocyte subsets in the pig are covered in more detail in Chapters 3 and 4.

1.6 The mononuclear phagocyte system in host defence, the pattern recognition receptors

Analysis of the transcriptomic response of macrophages to PAMPS in mouse and human has been greatly expedited in the post-genomic era (Aderem, 2005; Ramsey et al., 2008; Ravasi et al., 2007). The completion of genome sequences in the pig has recently enabled similar approaches (Uthe et al., 2007; Wang et al., 2007) such as described in this thesis (Chapter 4, 5 and (Kapetanovic et al., 2012)).

The best-characterised PRRs are the Toll-like receptors (TLRs), of which the archetype is TLR4 that functions as a receptor for LPS (**Figure 1**). TLRs are type I trans-membrane proteins with a large extra-cellular domain containing multiple leucine rich repeats (LRRs), a trans-membrane domain and a cytoplasmic Toll/IL1R (TIR) domain. Generally TLR1, 2, 4, 5, 6 and 10 are expressed on the cell surface and recognise microbial compounds although TLR4 can also be found intracellularly (Guillot et al., 2004; Ueta et al., 2004). TLR3, 7, 8 and 9 are found on the membranes of intracellular organelles e.g. endosomes. These patterns are conserved in pigs as in other mammals (Chaung et al., 2008). TLR4, previously named hToll, was the first mammalian TLR discovered and implicated in pro-inflammatory signalling in macrophages (Medzhitov et al., 1997). Its role in LPS detection was demonstrated when C3H/HeJ mice, which are resistant to LPS, were found to have naturally occurring mutations in their copy of TLR4 (Poltorak et al., 1998; Qureshi et al., 1999). LPS binding protein (LBP), synthesised in the liver, binds LPS then the LPS/LBP complex binds to CD14 which forms a complex with TLR4 (Jiang et al., 2000). MD2, a 160 amino acid (aa) protein, associates with the extracellular portion

of TLR4 and is required for LPS responsiveness (Dziarski and Gupta, 2000). Ligand binding to TLRs promotes the recruitment of adaptor protein(s), MyD88 and/or TRIF to the highly conserved intracellular TIR domain which leads to a well-studied signalling cascade (Doyle and O'Neill, 2006) (Akira, 2006; Kawai and Akira, 2007; Takeuchi and Akira, 2010) that ultimately leads to generation of numerous proinflammatory and anti-pathogen effector molecules. Different TLRs differ in the ability to recruit the adaptors. TLR signalling is MyD88 dependant with the exception of TLR3 which signals independently of MyD88 through TRIF, whilst TLR4 can signal through MyD88 or TRIF. Most of the components of TLR signalling have been studied in the pig. For example, Porcine MyD88 has a high degree of identity with the human protein, and its involvement in the TLR2-dependent and TLR4/MD2 signalling pathway has been demonstrated (Tohno et al., 2007).

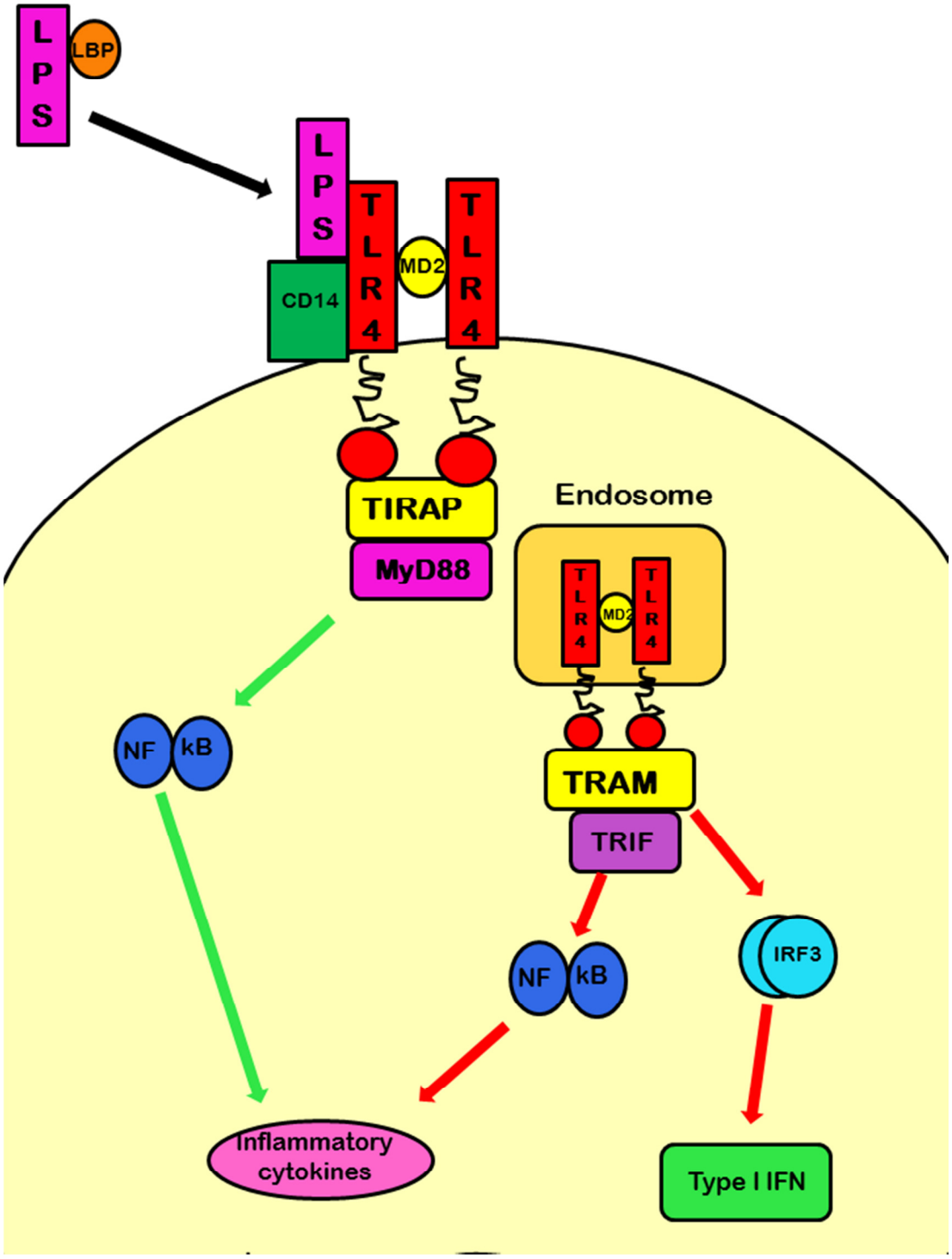


Figure 3-1 LPS signals through TLR4.

Adapted from Kawai *et al.* (2010)

TLRs form part of the pathogen recognition system in organisms as simple as drosophila, and they have expanded and diversified in different vertebrate species (Jann et al., 2009). In humans, 10 functional TLRs have been identified, as well as RP105, a protein similar to TLR4 but which is preferentially expressed on mature B-cells and does not have a TIR domain (Yazawa et al., 2003). The orthologs of the human genes have been cloned and characterised in the pig and shown to be more closely related to human than the orthologous murine genes (Morozumi and Uenishi, 2009; Roach et al., 2005; Tohno et al., 2007). The similarity may be especially important for studies of TLR8, which was initially thought to be non-functional in mice, but was recently found to contribute to viral DNA recognition (Martinez et al., 2010). Another gene with quite distinct functions in mice and humans is the receptor for CpG DNA, TLR9 (Bauer et al., 2001). The TLR9 gene is discordantly-regulated between mice and humans (Schroder et al., 2007) and there is distinct DNA sequence specificity in binding specific CpG-containing oligonucleotides (Roberts et al., 2005). Pigs are mainly human-like in both the cellular expression and DNA sequence specificity of responses to CpG containing oligonucleotides (Dar et al., 2010). Nevertheless, pig-selective CpG oligonucleotides have been selected and tested for optimal ability to stimulate cytokine production by peripheral blood cells (Kamstrup et al., 2001). As in humans, these oligonucleotides varied in their activity across individuals, suggesting that there might be functional polymorphism in TLR9 sequence specificity. Studies examining LPS regulated orthologous genes in mice and humans have shown that divergently regulated orthologues were enriched for genes encoding “cellular inputs" such as cell surface receptors (eg. TLR6, IL7R) and their “functional outputs" such as inflammatory cytokines/chemokines (eg. CCL20, CXCL13). Conversely, the intracellular signaling components activated by the “cellular inputs” were mainly convergently regulated (Schroder et al., 2012). Several of these “cellular inputs” have been shown to be similarly LPS regulated in the pig and humans (Schroder et al., 2012; Kapetanovic et al., 2012). Such divergence in cell surface receptors between species suggests that LPS may have differing effects on sensitivity to further immune challenge in mice versus humans and pigs. TLR6 was upregulated in mice macrophages but not human after LPS stimulation. LPS priming boosted IL6 production in response to a TLR6 agonist in mouse but not

human macrophages (Schroder et al., 2012). Such species differences are likely to play a part in infections or inflammation where multiple immune receptors are required.

Structural alterations to TLRs affect their ability to recognise PAMPs and single nucleotide polymorphisms (SNPs) in human TLR genes can increase or decrease the risk of disease e.g. tuberculosis (TLR1) or legionnaires disease (TLR5) (Misch and Hawn, 2008). Similar polymorphisms in TLR genes in pigs may explain the differences in susceptibility to disease observed in some animals (Jann et al., 2009; Uenishi and Shinkai, 2009). Distribution of SNPs in TLR1, TLR2, TLR4, TLR5, and TLR6 across 11 different breeds of pig were found to be mainly in the LRRs which are important in detection of PAMPs. This maintenance of diversity in TLRs must have some advantage for the immune system that ensures its preservation across breeds despite the pressures of intensive breeding. Studies looking at SNPs in TLR3, TLR7 and TLR8 revealed they have fewer polymorphisms leading to amino acid substitutions than TLRs found on the cell surface (Morozumi and Uenishi, 2009; Shinkai et al., 2006). The authors suggest these could have been eliminated by intensive breeding although the extent of heterogeneity found in LRRs of cell surface TLRs would suggest otherwise.

Functional polymorphisms in TLR4, the receptor for lipopolysaccharide, have been associated with increased susceptibility to a wide range of human diseases (Ferwerda et al., 2008a). Porcine and human TLR4 proteins share around 52% identity in the ligand-binding sites which rises to over 90% in the TIR domain, reflecting possible diversity in the ligands but a very highly conserved signalling pathway. The gene structure, 3 exons and 2 introns alternatively, is highly conserved in human, mouse and pig. The porcine and human promoters are more related to each other than to the mouse, but all three species contain TATA-less, purine-rich promoter typical of myeloid-expressed genes (Thomas et al., 2006). Consistent with this view, TLR4 in

the pig can be detected in the liver, spleen, bone marrow and lymph nodes, as well as on monocytes and macrophages (Thomas et al., 2006). Diverse function of TLR4 amongst pig breeds could provide models for humans, as well as identifying possible disease susceptibility alleles in the pig. Palermo *et al. (Palermo et al., 2009)* identified 34 SNPs including 17 in the non-coding sequence and 17 in the coding sequence, in particular 5 non-synonymous substitutions within exon 3, which codes for the LRR ligand-binding domain. Shinkai *et al.* investigated TLR SNPs in 96 individual pigs and found 7 non-synonymous SNPs within TLR4, also predominantly in the LRR region (Shinkai et al., 2006). There were actually fewer SNPs within TLR4 than within other TLRs sequences; the presence of ligand-binding and important co-receptor-binding domains (CD14, MD2) imposes constraints on divergence. However, the ligand-binding domain of TLR4 diverged most rapidly between all species. Such variation can generate species-specific responses; for example forms of lipopolysaccharide that are antagonists of TLR4 in humans, are agonists in horse (Bryant et al., 2007). Hence, it will be of interest to explore the structural constraints on LPS recognition in the pig model to determine how accurately it can model human biology. Recently, a cyanobacterial LPS-like TLR4 antagonist that blocks LPS-induced shock in mice was found also to block actions of pure *E.coli* LPS in pig blood (Thorgersen et al., 2009).

1.7 Other pattern recognition receptors

Aside from the TLRs, there are many other pattern recognition receptors on the cell surface and within innate immune cells. There has recently been considerable interest in C type lectin receptors, such as Dectin and Mincle (Kerrigan and Brown, 2010; Kerrigan and Brown, 2009; Wells et al., 2008; Yamasaki et al., 2008) that recognise complex polysaccharides and beta-glucans found in cell walls of bacterial and fungal pathogens. Sonck *et al.* (Sonck et al., 2010) have reported that a range of β-glucans can activate cytokine secretion and reactive oxygen production by pig monocytes and neutrophils. The spectrum of intracellular pattern recognition receptors that bind to microbial products (nucleic acids, microbial peptidoglyans)

(RIG-I, MDA5, the NOD/Nacht family) has also been delineated in the pig and to an even greater extent than the TLRs, these gene appear to contain many non-synonymous amino acid substitutions amongst pig breeds (Kojima-Shibata et al., 2009). One of these genes, NOD2, has been implicated in human Crohn's disease (Hugot et al., 2001). Genetic manipulation of NOD2 in the mouse has failed to provide either a useful model of small intestinal pathology in the mouse (Maeda et al., 2005) nor resolution of whether NOD2 mutations associated with Crohn's disease result in gain of function or loss of function (Eckmann and Karin, 2005). Tohno *et al.* have cloned and characterised pig NOD2 (Tohno et al., 2008) which is more closely related to human (81.6%) than to mouse. Furthermore, they confirmed that pig NOD2 was, like human NOD2, localized intracellularly and induced NF-κB activation after stimulation with the ligand muramyl dipeptide (MDP). NOD2 detects peptidoglycan motifs (Girardin et al., 2003) and in both mouse and human recognizes multiple pathogens like *Mycobacterium tuberculosis* or *Staphylococcus aureus* (Deshmukh et al., 2009; Divangahi et al., 2008; Kapetanovic et al., 2010). Recently, using a porcine model of *Salmonella typhimurium* infection, Meurens et al. (Meurens et al., 2009) demonstrated induction of NOD2 in the Peyer's patch and the gut wall. Studies on the distribution of SNPs have revealed that NOD2 has seven nonsynonymous SNPs in the LRR domain (Kojima-Shibata et al., 2009). Indeed, a substitution in NOD2 appears to alter sensitivity to MDP (Jozaki et al., 2009). There is clearly potential within the pig model to understand the biology of NOD2-related disease susceptibility in humans, and potentially vice versa.

Another class of proteins involved in the pattern recognition process includes members of the HIN200 family of interferon-inducible genes, which form a genomic cluster that is clearly syntenic between mouse and humans. Of these HIN200 domain-containing genes present in the mouse, absent in melanoma 2 (AIM2) acts as a detector of cytoplasmic dsDNA (Burckstummer et al., 2009; Hornung et al., 2009; Roberts et al., 2009) linking recognition to activation of the inflammasome with IL1b processing and macrophage cell death, whilst a second family member, p202, acts as a repressor of activation (Roberts et al., 2009). The number of members of this

family varies substantially amongst species and even within species. All of the mammals appear to have a copy of the myeloid nuclear differentiation antigen (MNDA), a gene implicated in cell cycle control in myeloid cells. Some mouse strains have an additional copy, called MNDAL (Zhang et al., 2009). Variation in the number of, sequence and gene complement have been linked to autoimmunity in mice and humans (Choubey and Panchanathan, 2008). In the pig, no evidence can be found for the existence of clear functional orthologs of either AIM2, or the putative repressors, despite conservation of the syntenic genomic region where these genes reside in mice and humans (www.ensembl.org). However, pig macrophages do undergo cell death in response to transfected dsDNA (D Sester, unpublished).

1.8 Macrophage activation in the pig

Mononuclear phagocytes recruited into tissues are plastic in their function. Macrophages are the major infiltrating population during development, in sterile wounds, tissue injury and foreign body reactions. Their activities are biased towards phagocytosis, extracellular proteolysis and production of growth factors that promote growth and repair. Infectious agents and/or immunological stimuli attract macrophages that differ greatly depending on the precise nature of the challenge. These phenotypes have come to be broadly classified into two classes of "activation"; Classical activation, which is often dependent on a priming signal, is associated with production of pro-inflammatory cytokines, antimicrobial and tumoricidal effectors along with a polarization of the T-cell compartment to that of a Th1 phenotype (Gordon and Martinez, 2010; Gordon and Taylor, 2005; Martinez et al., 2008; Taylor and Gordon, 2003; Taylor et al., 2005). The archetypal macrophage-activating cytokine in both mouse and human is interferon-gamma, which acts in part to prime responses through the various TLRs (Schroder et al., 2004; Schroder et al., 2006). There is only a limited literature on classical and alternative activation in pig systems, although there is no reason to suspect any distinct pattern of responsiveness to cytokines. In fact, most published work on the pig focuses on antiviral functions of interferon-gamma. Interferon-gamma

production is controlled by a number of quantitative trait loci in the pig (Lu et al., 2010) and correlated with effective resistance to the macrophage-trophic viral pathogen PRRSV (Lunney et al., 2010). Early studies of interferon gamma in pigs focussed on its ability to induce an antiviral state in macrophages (Esparza et al., 1988) and subsequently the classical priming activity was demonstrated, wherein interferon-gamma pretreatment of monocytes enhanced subsequent activation of interleukin 1 production (Charley et al., 1990). More recently, interferon gamma has been shown to protect against foot and mouth disease virus in pigs (Diaz-San Segundo et al., 2010).

Alternative activation of macrophages leads to the induction of sets of genes that promote direct toxicity against macropathogens, antibody-mediated host defence, suppression of classical T cell response, resolution of inflammation and promotion of wound repair (Martinez et al., 2008; Sica et al., 2008). It is associated with production of cytokines such as IL10 and TGFβ, and in pathological situations, with fibrosis. In general, alternative activation is associated with increased secretion of IL4 by Th2 cells and mast cells, and the induction of IL4 target genes in macrophages. As noted above, one confusing feature of the literature is that IL4 is commonly used in culture with GM-CSF to increase the production of monocyte-derived dendritic cells. Bautista *et al.* (Bautista et al., 2007) have suggested that in pigs, the IL4-related cytokine IL13 is more abundant and more physiologically relevant than IL4.

1.9 Inflammatory cytokine production in pigs

Macrophages respond to the ligation of pattern recognition receptors with induction of numerous inflammatory mediators, including chemokines and cytokines that recruit and activate other immune cells. The cytokines produced will differ depending on the pathogen present and the manner in which it activates the macrophage. There have been numerous studies of pig macrophage responses to

activation that largely recapitulate findings from mouse or human systems. Porcine macrophages from various locations treated with LPS increase production of the major pro-inflammatory cytokine, TNFα (Izeboud et al., 2000; Taylor et al., 1993; Vezina et al., 1995) and expression of IL1β (Charley et al., 1990; Foss et al., 1999; Sacco et al., 1996) IL6 (Choi et al., 2002; Lee et al., 2004), IL8 (Cagiola et al., 2006; Lin et al., 1994a) and IL12 (Foss et al., 1999; Pappaterra Mendoza et al., 2000). Useful microarray platforms are in their comparative infancy in pigs (see below), but have already been used in a study of transcriptional responses within the lymph nodes of pigs infected with salmonella (Wang et al., 2007) and with monocytes infected with African swine fever virus (Afonso et al., 2004). A recent microarray study of the activation of pig peripheral blood mononuclear cells by LPS identified the inflammatory chemokines, CCL2, CCL8, CXCL2, CXCL3, CXCL5 and IL8 (for which there is not a clear mouse ortholog) as the most inducible genes (Gao et al., 2010). Interestingly, LPS also induced the calgranulins, S100A8, S100A9 and S100A12. There has been considerable interest in the role of these proteins in inflammation (Goyette and Geczy, 2010; Perera et al., 2009) and S100A12 does not have a clear ortholog in mice (Ravasi et al., 2004).

1.10 Of mice and men... and pigs.

Mice are commonly used as models to study fundamental features of innate and acquired immunity. But the innate and acquired immune systems of mouse and human have clearly diverged substantially (Mestas and Hughes, 2004). An obvious example that highlights the difference between the two animals is the cellular constituents of the circulation. The neutrophil population represents 50 to 70% of peripheral blood cells in humans but only 10 to 25% in the mouse. In contrast to rodents, human neutrophils produce defensins (Eisenhauer and Lehrer, 1992) and no direct homologue of IL8, a neutrophil chemoattractant, has been identified in the mouse (KC, MIP-2 or LIX can be considered functionally similar although they are still quite different in their sequences) (Lehrer et al., 1993). As noted above, there is a clear difference in relative abundance of the two populations of monocytes

(Ingersoll et al., 2010) less than half of the genes that distinguish subsets are conserved between mouse and human, and some markers (CD36, CD9, TREM-1) are completely divergent. This thesis is the first description of differential gene expression between monocyte subsets in the pig, such studies provide useful evidence on whether subpopulations of monocytes in different species may be comparable in their functions.

One striking and well characterised example of an innate immune effector pathway, downstream of TLR activity that is differentially regulated in mouse and human is the nitric oxide (NO) pathway. In the mouse NO is an important antimicrobial molecule produced via the action of the calcium-independent/inducible NO synthase (NOS2, iNOS) following activation of macrophages by pathogens. A protective role for this effector has been described in numerous infection models in mouse. Optimal induction of NOS2 in mice requires the combined actions of interferon-gamma and TLR signalling a reflection of a more general intersection between the two signalling pathways in which interferon sensitises to TLR signals (Schroder et al., 2006; Schroder et al., 2004). Mouse macrophages activated with LPS, with or without interferon, also induce an arginine transporter to bring the substrate into the cells, and arginase, which converts L-arginine, a by-product of NO synthesis, into L-ornithine. They also induce enzymes such as GTP cyclohydrolase1 (GCH1) required to produce the cofactor for NOS, tetrahydrobiopterin (BH_4). None of these pathways is active in human macrophages activated *in vitro* (Liu et al., 2006). There has been a considerable inconsistency in the literature as to whether human macrophages actually express NOS2, and produce nitric oxide via a NOS2-catalysed mechanism, under any circumstances (Fang and Nathan, 2007; Liu et al., 2006; Schneemann and Schoeden, 2007). What is clear is that the control of NOS2 gene expression is different between the species. Like most groups, our laboratory has failed to find NOS2 induction by human macrophages in response to LPS, and like previous authors (Jungi et al., 1996; Zelnickova et al., 2008), this thesis reports similar results with pig macrophages from multiple tissue sources. So in this respect, the pig resembles a human.

Whether or not NO is a functional antimicrobial effector in some contexts, NOS2 is not a pseudogene in humans or pigs. But there are substantial insertions rearrangements in the genomic interval comparing the mouse and human syntenic regions surrounding NOS2 and the promoter is poorly conserved (www.ensembl.org). A change in the regulation of the NO pathway in humans could be compensated by alternative antimicrobial pathways. One example is the TLR-mediated up-regulation of the vitamin D receptor which facilitates induction of the antimicrobial peptide cathelicidin (LL37) in humans but not mice (Liu et al., 2006). As vitamin D_3 is produced after exposure to ultraviolet B light, this pathway is presumed to have either evolved in diurnal humans who are active during the day, or was not maintained in nocturnal mice since splitting from our common ancestor. This thesis provided some evidence that this pathway may also be active in pigs (**Chapter 5**) .

Another alternatively regulated immune pathway of mouse and human macrophages is the regulation and production of indoleamine 2,3-dioxygenase (IDO). IDO is produced after stimulation with different pathogens in IFN-γ-treated human macrophages but not mouse macrophages, supposedly due to the inhibitory effects of NO (Murray et al., 1989; Thomas et al., 1994). As with iNOS and arginine metabolism, induction of IDO is part of the induction of an entire pathway of tryptophan metabolism, including a tryptophan transporter, and the enzymes kynureninase and kynurenine hydroxylase, which leads to production of potentially toxic metabolite, quinolinic acid. IDO and tryptophan metabolism have been implicated in numerous aspects of immune regulation, and indeed the serum tryptophan/kynurenine ratio is an index of immune activation (Mellor and Munn, 2004; Schrocksnadel et al., 2006). In this respect, pig macrophages again resemble human, in that IDO is one of the most LPS-inducible genes in multiple different macrophage populations ((Gao et al., 2010; Kapetanovic et al., 2012), our unpublished observations).

One final striking example of divergent immune regulation includes the use of family members of the p47 immunity-related GTPases (IRG) family; there are 23 members in mice and they are immune-regulated and clearly required in host defence (Bekpen et al., 2005). Surprisingly, only a full length gene for one family member is present in humans, IRG1, with an additional truncated pseudogene being present. IRG1 is profoundly inducible in human macrophages after IFN-g and LPS treatment (Martinez et al., 2006). The human and pig IRG1 loci are very similar, flanked in close proximity by ceroid-lipofuscinosis neuronal protein 5 (CLN5) and potassium channel tetramerisation domain containing 12 (KCTD12) (www.ensembl.org).

Type I IFNs are the primary immune defense against viruses (Stark et al., 1998) and have additional roles in modulating the adaptive immune system and controlling cellular proliferation and death among other functions (Goodbourn et al., 2000). Perhaps due to their many roles in the immune system, Type I IFNs have diverged into at least eight distinct subfamilies: IFNκ (*IFNK*), IFNβ (*IFNB*), IFNε (*IFNE*), IFNδ (*IFND*), IFNζ (*IFNZ*), IFNα (*IFNA*), IFNω (*IFNW*), and IFNτ (*IFNT*) (Krause and Pestka, 2005). All Type I IFN genes in human and mouse, except *IFNK* are clustered in an approximately 400 kb length of DNA between *IFNB* and *IFNE*. There is some evidence that species-specific evolution of IFN families has occurred, *IFND* has only been identified in the pig and is absent in the mouse and human (Lefevre et al., 1998), while *IFNZ* is represented in the mouse, but is completely absent in humans (Oritani et al., 2001; Takahashi et al., 2001) and *IFNW*, which is present as a single functional gene and at least two pseudogenes in humans, has only been described as a single pseudogene in mice (Hardy et al., 2004). *IFND* and *IFNT* appear to have evolved different functions in swine and ruminants. They are not induced by viruses but instead are instead involved in pregnancy (Roberts et al., 2008; Lefevre et al., 1998). Differences between type I IFN responses have been described between man and mice (Rogge et al., 1998). Furthermore the type I IFN, IFNα which is secreted by several cell types including macrophages, provide an important link between the innate and adaptive immune system by inducing Th1 differentiation. This process requires STAT4 activation through recruitment to the

IFNα receptor by STAT2. In mice, however, IFNα does not activate STAT4 or cause Th1 differentiation due to an insertion in the STAT2 gene (Farrar et al., 2000) suggesting the control of the adaptive immune system may differ between mice and humans.

A recent study carried out systematic comparisons of transcriptional regulation in mouse versus human macrophages responding to LPS. Broadly-speaking more than 10% of genes (including the examples above) showed absolutely divergent regulation, and around 25% were quantitatively very divergent (Schroder et al., 2012). Similar analysis in pigs using arrays and RNAseq is underway in our laboratory, but it is already clear that for a set of genes that show the greatest divergence (iNOS, CCL20, STAT4, IDO), pigs resemble humans (D Beraldi, unpublished).

Aside from the differences in gene regulation, mice and human are also divergent at the protein sequence level, especially amongst genes expressed by innate immune cells. Murphy et al (Murphy, 1993) assembled a large database of human and rodent orthologous proteins. The majority of proteins (79%) varied little between the species, this included structural and cytoskeletal proteins, cell cycle regulators, neurotransmitters, growth factors and associated receptors. In contrast proteins involved in host defence were approximately 3-fold more divergent than the average. The highly divergent proteins were mainly the extracellular or transmembrane components of the intracellular signalling pathways which mediate the host response to infection, such as the interleukins, interferons, colony-stimulating factors, chemokines, chemoattractants and Fc receptors, and plasma or exocrine proteins such as opsonins, thrombogens and proteins which bind to hormones, lipids, metabolites, drugs and heme. Such evolutionary divergence in cell surface receptors and secreted effectors activated via the pathogen recognition systems has been reported by others (Schroder et al., 2012). The divergence among these immune

mediators demonstrates the strong evolutionary selection that genes involved in innate immunity are under. Generally the average non synonymous v synonymous (Ka/Ks) base change was 3-4 fold higher in immune related genes in primates versus rodents demonstrating enhanced fixing of mutations due to the advantage provided by altered protein function (Ellegren, 2008). A comparison of such genes across species is provided at ImmTree (http://bioinf.uta.fi/ImmTree)(Ortutay et al., 2007).

Systematic large scale genomic analysis of the pig was initiated in the 1990s with the formation of the European Pig Gene Mapping Project (PiGMaP) (Archibald et al., 1995) and generation of a large databank that continues to be maintained at the Roslin Institute (http://www.thearkdb.org). Even the earliest comparisons indicated that the pig genome is more closely related to the human genome than the mouse (Federico et al., 2004). A more detailed comparison of shotgun reads established that the pig bisects the evolutionary branch between mouse and human, with the mouse at a three-fold greater evolutionary distance, regardless of whether one considers coding, UTR or intergenic regions (Wernersson et al., 2005). This analysis identified a set of 50 conserved miRNAs, of which 23 were more closely-related to human than mouse, and 25 equidistant. The small effective population size of humans and pigs is associated with accumulation of slightly deleterious mutations which have no adaptive benefit but are fixed essentially by chance. Accordingly, humans have an overall greater functional genetic diversity than mice, manifested by a greater Ka/Ks ratio within the species. Similarly, the Ka/Ks ratio is higher for comparisons between primate species than between rodent species. In simple terms, rodents are more highly evolved than humans or pigs, perhaps due to their shorter generation time, and pigs are more like humans than mice.

1.11 The pig as an inflammatory model

The use of pigs to model the human immune system is becoming increasingly common. Pigs are already widely used to study gastroenterology, cardiology,

xenotransplantation and nutrition (Lunney, 2007; Spurlock and Gabler, 2008; Truty and Smoot, 2008) and there is some evidence that the porcine response to infectious challenge is more similar to mans than the more commonly used mouse (Kapetanovic et al., 2012). There are many valid reasons for using pigs; they are economically important in their own right, are physiologically similar to humans and share many infectious challenges with us. *Streptococcus suis*, a common porcine pathogen, can cause meningitis or sepsis in humans (Wertheim et al., 2009) and the 2009 H1N1 influenza pandemic demonstrated the thin barrier between the species (Mehle and Doudna, 2009). The larger size of pigs compared to rodents provides an additional benefit for drug trials that require invasive monitoring, as the animals can be treated in a similar manner to patients in an intensive care unit, healthy pigs subjected to prolonged mechanical ventilation have developed ventilator-acquired pneumonia (Marquette et al., 1999).

Although the limitations of the commonly-used rodent models for understanding the nuances of innate and acquired immunity are becoming widely appreciated, mice and rats are still the animal models of choice for the vast majority of biomedical research. Rodents have many advantages, they are relatively inexpensive to buy and maintain, they do not take up a large amount of space and so can be easily housed. The vast array of transgenic animals and bio-molecular tools available for rodents ensures that they remain a useful tool but this thesis aims to show they may not always be the best choice for studying the human immune system. Like humans, pigs are largely out-bred, but domestication and selection has generated major breeds in which individuals are genetically similar to each other. In the process, different breeds have acquired breed-specific phenotypic traits, including specific disease resistance traits, which may offer insights into similar genetic variation in humans (Hoeltig et al., 2009; Doeschl-Wilson et al., 2009; Vincent et al., 2005).

One area in which rodents have severe limitations as an experimental model is sepsis. Sepsis is defined as a systemic inflammatory response syndrome (SIRS) with either suspected or proven (e.g. positive blood culture) infection (Annane et al., 2005). A diagnosis of SIRS is given when there is a combination of two or more of the following symptoms: fever or hypothermia, tachycardia, tachypnea and leukocytosis or neutropenia. Sepsis is one of the principal causes of mortality in U.S, where it is responsible for 9.3% of all deaths, similar to the mortality rate from myocardial infarction (Angus et al., 2001). In terms of morbidity, sepsis is responsible for 50-95 cases per 100,000 patients and its incidence has increased by 9% each year (Martin et al., 2003).

The pathology of sepsis is attributable to sustained activation of macrophages and the production of numerous secreted products that cause disseminated intravascular coagulation and multiple organ failure. Despite numerous trials, there are still no effective therapies (Stearns-Kurosawa et al., 2011) which can at least partly be attributed to the lack of predictive animal models. Mice have been used extensively to simulate sepsis and endotoxin shock however they have many faults as a model for these human conditions (Dyson and Singer, 2009). The murine response to endotoxin challenge generally results in hypothermia; a consequence of peripheral vasodilatation, while fever is the more common outcome in humans (Habicht, 1981). Mice are also approximately 10^5 more resistant than humans to endotoxin shock (Munford, 2010) and the lethal dose (LD) for mice is 5-12 times more (mg/kg) than for men, or pigs (Berczi et al., 1966). The underlying cause of this resistance is currently unknown; however the involvement of circulating protein(s) present in sera has been proposed (Warren et al., 2010). The inherent resistance of mice can be overcome with the administration of priming agents such as D-galactosamine but the resulting pathology in this model is more representative of acute hepatotoxicity than a true model of sepsis (Mignon et al., 1999). It is hoped that comparative genetic studies in a third species such as the pig, as outlined in this thesis, may provide some clues towards why humans are so much more sensitive to bacterial products than mice and hence provide targets for the therapeutic use.

The failure of the mouse to adequately mimic the pathologies observed in sepsis in man has led researchers to turn to the pig as a possible model. Multiple different experimental approaches have been used to mimic the human pathophysiology of sepsis: intravenous (iv) or intraperitoneal (ip) injection of LPS or bacteria or ip injection or "release" of faeces are the most commonly used (Rittirsch et al., 2007). Perhaps the simplest sepsis models results from the iv infusion of LPS (Carlsson et al., 2009). In this model, reducing the endotoxin concentration had little effect on TNF production but an improvement in respiratory function was noted. Lipcsey *et al.* (Lipcsey et al., 2008) used the same model to demonstrate the correlation between the speed of LPS infusion and the physiological responses. The infusion model in pigs has been used in several studies to investigate the effect of different drugs on long-term, hyperdynamic porcine endotoxemia (Hauser et al., 2006; Konrad et al., 2004; Murphey and Traber, 2000). Various studies have validated bacterial injection as a sepsis model using a variety of bacteria. Injection of *Pseudomonas aeruginosa* (Rimmele et al., 2006) produced pigs with inflammatory cytokine profiles and a cardiovascular profile similar to human pathology while continuous infusion of serogroup A streptococci led to acute septic shock with pulmonary hypertension, artery hypotension and reduced cardiac output (Saetre et al., 2000). More recently, Nielsen *et al* (Nielsen et al., 2009) described a model for human meningococcal sepsis by injection of *Neisseria meningitidis* in pigs. Similar to human sepsis, this porcine model resulted in hemodynamic and cardiovascular output failures as well as increased inflammatory cytokine production (TNF, IL1b and IL6).

Blood poisoning or sepsis is perhaps the most serious complication arising from an inflammation of the peritoneum (peritonitis) in human patients. A surgical model of caecal ligation and perforation of the bowel, or inoculation of faecal material or bacteria into the peritoneal cavity can be used to simulate peritonitis. Several laboratories have validated a porcine model by injecting either *E.coli* or autologous faecal content into the peritoneal cavity (de Azevedo et al., 2007; Goldfarb et al.,

1996). Although the resulting peritonitis appeared to have no impact on circulating creatinine levels, it induced coagulation disturbance, leukopenia, decrease in arterial pressure, acute lung injury and increased levels of IL6 resembling many of the physiological outcomes observed in human patients. Kazarian *et al*. (Kazarian et al., 1994) compared ip inoculation with *E. coli* or autologous faecal material. There were distinct responses, with the latter proving a better model for human peritonitis. Injection of faecal material resulted in formation of abdominal abscesses and pyogenic granuloma and the slow onset of sepsis.

1.12 Inflammatory models

The close physiological and immunological resemblance of pigs to humans has allowed the modelling of many inflammatory lung disorders. Cystic Fibrosis (CF) is a genetic disease caused by mutations in the gene encoding a chloride channel; the protein product of this gene is called the cystic fibrosis transmembrane conductance regulator (CFTR) protein (Lyczak et al., 2002). Mutations in CFTR result in chronic pulmonary inflammation. Models using the CFTR-deficient mouse have proven useful in providing a basic understanding of CF however mice do not develop the same pancreatic, liver, intestinal or lung abnormalities as human CF patients. Rogers *et al.* developed CFTR-/- pigs which have defective chloride transport and develop intestinal obstruction (meconium ileus), exocrine pancreatic destruction and focal biliary cirrhosis. This model more closely mimics the pathology commonly shown by CF patients. The development of a genetically modified pig model for CF will aid understanding of the pathology associated with this condition (Rogers et al., 2008).

Pigs can be useful for modelling many human inflammatory disorders. Infection with *Erysipelothrix rhusiopathiae* has been used as to study human chronic polyarthritis. The resulting pathology (IL1 beta production, infiltration of cells and increased expression of ICAM1 and MHC II) was found to be similar to the human

disease and the use of larger animals allowed a clearer view of the inflamed joint (Davies et al., 1994; Davies et al., 1992). Pigs infected with *Mycobacterium bovis* have proven useful to study tuberculosis (Bolin et al., 1997) and a model using *Bordetella pertussis* has been established to study whooping cough in newborn infants (Elahi et al., 2005). *B. pertussis* infected pigs displayed similar symptoms to infected humans such as nasal discharge, nonparoxysmal cough, breathing difficulties, and growth retardation. A pig model for the gram-negative bacterium *Helicobacter pylori* has also been developed. *H. pylori* colonises the human stomach and can be detected in more than 50% of people. Infection leads to gastric or duodenal ulcers and atrophic gastritis in a small percentage of patients. Poutahidis *et al.* (Poutahidis et al., 2001) developed a promising model of crossbred (Large White and Pietrain) pigs which were infected with *H. Pylori* leading to stomach lesions and adhesion to the gastric epithelium resembling the pathology in human patients.

1.13 Conclusions

We now know so much about mouse biology that there are few mouse diseases that could not be cured. But in terms of translation into the clinic, mice lie! Perhaps it is time to utilise an animal that may be more beneficial to us. This thesis aims to determine whether transcriptional control of the mononuclear phagocyte system in the pig is closer to humans than the more commonly used mouse. Macrophage biology underlies most of the pathology of the disease that afflict us. **Table 1** summarises some of the advantages of the pig as a model system for the study of human disease. The study of pig macrophages is practical (one can obtain very large numbers of cells from multiple sites of the same animal, and they can be stored frozen), and with completed genomes, genetics of breeds, transcriptomic resources, increasing availability of cytokines and antibodies, and even transgenics, the technical advantages of the mouse as a model are evaporating. As in the words of the popular French addage, “tout est bon dans le cochon” (everything is good in the pig). Most importantly, studies in the pig are actually much more likely to be

predictive of therapeutic efficacy in humans, and there are economic reasons to study pigs in their own right. The market for mouse therapies is rather more limited.

General Comments	Pig Model		Mouse Model		References
Biological	+	large litter	+	small facility, cheaper	(Zelnickova et al., 2008; Pampusch et al., 1998; Rothkotter, 2009)
	+	physiology closer to human (blood volume, blood pressure, etc.)	+	large literature	
	+	bigger organs, lymphoid organs similar to human	+	large litter size	
	+	repeat blood sample and invasive observation possible	+	easy to handle	
	+	pig macrophages do not produce NO	+	large knowledge of physiology	
	+	many cells and large volume of tissue per animal, many experiments can be performed per animal	+	large number of immunological tools	
	-	large animal, difficult to handle	-	Mouse macrophages produce NO	
	-	expensive	-	low no. of cells and small volume of tissue per animal, many animals needed for one experiment	
	-	large facility required (especially for pathogen-free studies)	-	metabolism can be very different to human	
	-	limited availability of immunological tools			
Genetics	+	outbred, closer to the genetic variation seen in humans	+	inbred, good to understand basic molecular biology	(Archibald et al., 1995; Federico et al., 2004)
	+	genetically closer to human	+	large number of transgenic or knock out animals available	
	+	genome sequencing almost finished	+	genome sequenced	
	-	outbred can be problematic for some experiments	-	evolving at a higher rate than human	
			-	mostly inbred, no variation in the genome	

Human Inflammatory Pathology					
Sepsis	+	Strong reaction to endotoxin	-	endotoxin resistant	(Habicht, 1981; Rittirsch et al., 2007; Rimmele et al., 2006; de Azevedo et al., 2007; Jiang et al., 2000)
	+	haemodynamic and cardiovascular values close to human	-	no fever, mostly hypothermia	
			-	Young mice mainly used, sepsis patient are often elderly	
Lungs inflammation	+	lung size and functional capacity are similar to man	+	molecular and immunological tools available	(Kirschvink and Reinhold, 2008; Reinhold et al., 2008; Salez et al., 2000)
	+	similar respiratory mechanics, gas exchange, glycoprotein composition of submucosal gland	-	4 right lobes, 1 left lobe	
	+	can use similar diagnostic and experimental techniques as used in humans (endoscopies, alveolar lavages)	-	macrophages do not respond to TLR9 stimuli nor produce IL-10	
	-	4 right lobes, 2 left lobes (humans have 3 right lobes and 2 left lobes)	-	breathing patterns different, no respiratory bronchioles (Dixon al, 1999)	
	-	have interstitial macrophages which are not present in human lungs			
Cystic Fibrosis (CFTR -/- animals)	+	develop human-like symptoms (lung or pancreatic disease)	+	small, good for studying molecular proprieties	(Rogers et al., 2008)
			-	no human-like symptoms	
Rheumatoid arthritis	+	easy to view the cartilage	-	small joints are hard to study	(Davies et al., 1992; Davies et al., 1994)
	+	human-like symptoms			
	+	natural host for Ery. Rhusiopathice			
	+	Presence of rheumatoid factor in the serum			
Infection models (*Mycobacterium bovis, Salmonella enterica*,	+	good model for tuberculosis (similar resistance, lesions, histology, natural host)	+	helpful to understand mechanisms	(Bolin et al., 1997; Elahi et al., 2005)
	+	major histocompatibility complex quite similar to human	-	mice are often not natural host and are more resistant	

Influenza, Hepatitis E)	+	similar clotting mechanism to humans	-	inbreed (no genetic variation)	
			-	different physiology	
Other Human Pathology					
Nutrition Studies	+	omnivorous	-	different food intake, coprophagy	(Spurlock and Gabler, 2008; Patterson et al., 2008; Gabler and Spurlock, 2008)
	+	physiology and metabolic processes similar to human	-	different lifespan and body proportion	
	-	require more space and more food	-	different intestinal microbiota and intestinal morphology	
	-	pig intestines differ in length - body fat depending on age			
	-	coprophagy rare but possible			
Neurodegenerative Diseases	+	relatively large brain, human-like blood supply and immunologic response characteristics	-	small brain	(Hu et al., 1996; Imai et al., 2006; Bjarkam et al.,
	+	good model for stroke and MPTP model of Parkinsons disease	-	complexity and the development of cognitive circuitry are different	

					2008)
Atherosclerosis	+	growth and aging of aorta similar	+	apolipoprotein E (apoE) and LDL receptor knockout mice	(Ratcliffe and Luginbuhl, 1971; Cullen et al., 2003)
	+	coronary artery distribution is similar to humans	-	mice do not develop atherosclerosis without genetic manipulation	
	+	morphology and biochemistry of plaque is similar to humans	-	lipid physiology that is radically different	
			-	lesions in the mouse coronary artery often extend beyond the elastic lamina	
			-	do not exhibit plaque rupture leading to vessel occlusion	
Wound healing	+	same epidermal and dermal thickness ratios and turnover as human	+	accelerated modes of healing (experiment are quicker)	(Sullivan et al., 2001)
	+	porcine dermal collagen similar to human	-	bandages or treatments may be problematic due to dense fur	
	+	similar healing processes, similar physiology	-	very thin epidermis and dermis	
	+	porcine model generally in agrement with human data 78% (53% for small mammals)	-	rodents mainly heal through wound contraction rather than migration of epidermal cells as in human	

	-	pig dermis relatively elastic but still less than human			
	-	no eccrine glands on pigs			
Ophtalmology	+	similar time course for retinal degeneration	-	small size of the eye	(Chandler et al., 1999; Ruiz-Ederra et al., 2005; Lei and Yao, 2006)
	+	size well suited to sub-retinal injections and somatic gene therapy	-	differences in dimensions of the the lenses (thickness and diameter)	
	+	good model for glaucoma	-	transmission of the mouse lens different	
	+	similar size/thickness of the eye/retina and distribution of the cells			
	-	pigs do not possess a cone-exclusive true macula or fovea as in human			
Bone metabolism, skeleton	+	anatomy, morphology, composition close to human	+	investigation of molecular processes simple	(Cardis et al., 2007; Pearce et al., 2007)
	+	reliable and duplicable model for scoliosis	-	small bone size	
	-	large animal when older	-	bone density measurement more complicated	
	-	denser trabecular network			
Xenotransplantation	+	easy supply		Not Possible in mice	(Ekser and Cooper, 2010; Truty and Smoot, 2008)
	+	genetic manipulation possible			
	-	viral recombination not ruled out			
	-	transmission of swine-pathogens to human host need to be evaluated			

Table 6.1 Description of Animal Models: Advantages and Disadvantages of the Mouse and Pig Models in General, Inflammatory Pathologies, and Other Human Pathologies

Chapter 2: Materials and Methods

2.1 Materials

2.1.1 General reagents

Lipopolysaccharide (LPS) (from *Salmonella minnesota* rough strain RE595) was used at a final concentration of 100ng/mL. Recombinant human colony stimulating factor-1 (rhCSF1) (a gift from Chiron, Emeryville, CA, USA) was used at a final concentration of 1x104 U/mL (100ng/mL). 1-methyl-DL-trptophan (Sigma) was dissolved in DMSO (Sigma) and used at a final concentration of 1:1000 (100μM). L-tryptophan (Sigma) was diluted in RPMI 1640 (Invitrogen) and used at a final concentration of 250μM.

2.1.2 Antibodies

All antibodies and working antibody concentrations used in these studies are described in Appendix 1.

2.2 Mammalian cell culture

2.2.1 Primary macrophages

Porcine monocyte derived macrophages (MDM) and bone marrow derived macrophages (BMDM) were obtained by ex vivo differentiation from peripheral blood mononuclear cells (PBMCs) and bone marrow cells (BMC) in the presence of rhCSF1 as described previously (Kapetanovic). Animals used are detailed in Appendix 1. The pigs were sedated with ketamine and euthanized using a captive bolt. Two different freezing Media were assessed, FM1 (10% DMSO, 90% FCs) and FM2 (20%DMSO, 80% FCS). FM1 was used for all experiments.

2.2.1.1 Monocyte derived macrophages

Blood was collected by intra-cardiac puncture into a vacuum sealed glass bottle containing 100mLs Acid Citrate Dextrose (ACD) buffer. Whole blood was separated into 10, 50mL falcon tubes (Greiner Bio one) which were centrifuged at 1200g for 15 minutes with no brake. The buffy coat was removed and 25mLs mixed with 25mLs RPMI (Sigma). 30 mLs of the buffy coat/RPMI mix was layered on top of 15mLs of Lymphoprep (Axis-Shield, Norway) and centrifuged at 1200g for 25 minutes with no brake. PBMCs separate out into a distinct layer which can be easily removed. This was washed twice with RPMI 1640 and centrifuged at 600g for 10 minutes then 400g for 10 minutes. Red cells were lysed using 5mL of erythrocyte lysis buffer (10mM KHCO3, 155mM NH4Cl, 0.1M EDTA, sterile 0.2uM filtered) for 5 minutes then the cells washed and centrifuged at 400g for 10 minutes. The pellet was collected, re-suspended in PBS (Mg2+ Ca2+ free) and counted before being slow frozen at -80°C in an isopropanol bath then moved to -155°C for long term storage.

2.2.1.2 Bone marrow derived macrophages

Five posterior ribs were removed from each side of the rib cage The outer surface of the bone was cleaned with alcohol, both extremities were cut and, using a 20ml syringe with an 18g needle, bone was flushed in both sides with RPMI-1640 (Sigma, UK) containing 5 mM EDTA to prevent clotting. Cells were spun, suspended in red cell lysis buffer (10mM $KHCO_3$, 150mM NH_4Cl, 0.1mM EDTA pH 8.0) for 2min, spun again and washed with phosphate buffered saline followed by RPMI-1640. Bone-Marrow cell (BMC) were finally suspended in a freezing medium (90% heat-inactivated Fetal Calf Serum (hiFCS) – 10% DMSO) and frozen as per MDMs.

2.2.1.3 Peritoneal macrophages

A small incision was made in the peritoneal cavity and a catheter with a 30cc concentric balloon that allows internal sealing of the entry site by inflation with a 50mL syringe was inserted. A sterile i.v. bag containing 1.5L PBS was attached and

held above the pig to allow PBS (Mg2+ Ca2+ free) to drain into the peritoneal cavity. The pig's peritoneal cavity region was massaged then the i.v bag lowered to below the pig and the PBS drains out into the sterile i.v. bag. The peritoneal lavage was poured into 500mL centrifuge tubes (Corning Incorporated, USA) and centrifuged at 400G for 10 minutes. Cells were counted and frozen as MDMs

2.2.1.4 Alveolar macrophages

Once the peritoneal lavage has been collected the pig was cut down the middle and the lungs removed. The trachea was clamped with locking forceps to stop blood entering the lungs. The lungs were filled with 750mL PBS, massaged for 20 seconds and emptied and this process repeated. The lavage was collected in 500mL centrifuge tubes and centrifuged at 400xg for 10 minutes. The pellet was collected, re-suspended in PBS and the cells processed for freezing as MDMs, if necessary red blood cells are lysed as MDMs

2.2.1.5 Macrophages from septic pigs

Four Goettingen minipigs (Ellegaard, Denmark) were anaesthetised, intubated and 10^5 cfu Actinobacillus pleuropneumoniae (provided by Moredun Scientific) delivered by direct bronchoscopic inoculation into the right lower lobe of the lung. Following extubation and recovery pigs were returned to their enclosure and temperature and heart rate monitored remotely by radiotelemetry (Data Sciences International). On advice of the veterinarian the animals were euthanased four to six hours after initial infection due to the severity of clinical symptoms and the death of one animal. Blood was collected and PBMCs isolated as described above

2.2.2 Culture conditions

Cells were retrieved from cryopreservation and seeded at $5x10^6$ cells/$10cm^2$ in a complete media consisting of RPMI 1640 (Invitrogen) supplemented with 10% heat

inactivated FCS (Sigma), penicillin/streptomycin (25U/25µg/mL, Gibco), 2mM Glutamax (Invitrogen).

Primary macrophages were seeded on bacteriological plates (Sterilin, UK). AMs and PMs were left at 37˚C for 2 hours in complete media, non-adherent cells removed by washing then cells used within 24 hours. MDM and BMDMs were incubated for in complete media supplemented with rhCSF1, Cells were maintained for a maximum of 10 days in culture.

Prior to experiments, cells were split, using an 18-gauge hypodermic needle and syringe, and seeded onto tissue culture plastic (Nunc) at 1million cells/mL and allowed to adhere for 24 hours. Cells were grown in 37˚C humidified air venting 5% CO2.

2.3 Flow cytometric analysis

Cells for FACS analysis were prepared using pre-chilled solutions and all centrifugation steps were carried out at 4˚C. Cells were recovered from cryopreservation as previously described or harvested from culture using an 18G needle, washed, pelleted and incubated 5M cells/mL in hi block (1x PBS, 0.1% NaN_3, 2% heat inactivated FCS, 0.1% BSA) with 2% heat inactivated normal mouse serum on ice for 15 minutes. Cells are pelleted, the hi block removed then resuspended in 100µL lo block (1x PBS, 0.1% NaN_3, 0.2% heat inactivated FCS, 0.1% BSA) containing the appropriate antibody or isotype control (appendix 1). Samples were incubated at 4˚C in the dark for 30 minutes before washing 3 times with lo block. Cells were resuspended in 200ul lo block with 0.1% Sytox Blue (Invitrogen) for FACS analysis using the CyAn (Dako). Data collection and analysis was performed using Summit 4.1 software (Dako).

2.4 RNA analysis

2.4.1 RNA extraction

RNA extraction was carried out using the Qiagen RNeasy mini kit using on column DNase treatment (Qiagen, UK) and RNA quantified using a Nanodrop (Nanodrop, USA). RNA quality was assessed using Agilent nanochip (Agilent)

2.4.2 RT qPCR

2.4.2.1 cDNA synthesis

cDNA was synthesized from 1µg RNA using Superscript III (Invitrogen). 11µL reactions containing 500ng random primers (Invitrogen) and RNA were prepared and heated to 70˚C for 10 minutes then chilled on ice. Reverse transcription occurred in a reaction containing 1x first strand buffer, 2µL 1M DTT, 1µL 10mM dNTP and 1µL Superscript III (Invitrogen). This was incubated at 50˚C for 50 minutes then 70˚C for 10 minutes. Negative controls were performed with the omission of Superscript III to check for genomic contamination. The cDNA was diluted 1:20 for use in RT qPCR reactions.

2.4.2.2 Primer design and optimisation

Primer pairs used for RT qPCR are detailed in appendix 2. All oligonucleotides were designed using Primer3 (Steve Rozen, Helen J. Skaletsky (1998) Primer3. Code available at http://www-genome.wi.mit.edu/genome_software/other/primer3.html.) and synthesised by Invitrogen (Paisley, UK). Primers were designed with an optimal amplicon size between 80-150 bp. Primer pairs were designed so that one of the primers overlapped an exon junction to prevent possible amplification of any remaining genomic DNA. Primers are designed with an annealing temperature of 60˚C and optimised using a pool of cDNA consisting of the samples to be tested. The

Dissociation curve analysis for each primer pair ensured that only a single amplification product was produced and efficiency was between 95-105%. Primers were used at 500nM

2.4.2.3 Quantification of mRNA expression for specific genes

mRNA expression was quantitated using the SybrGreen quantitative PCR system (Invitrogen, Carlsbad, CA, USA) in 25 µL reactions of a 96 well plate. Each reaction contained 9.5 µL diluted cDNA and 0.5µM forward and reverse primers in 1 x SyberGreen reagent. Samples were measured by real-time quantitative PCR using a Stratagene MX300P real-time PCR system (LaJolla, CA) at 1 cycle of 50 °C for 2 minutes then 95 °C for 2 minutes, followed by 40 cycles of 15 seconds at 95 °C then 30 seconds at 60 °C. One cycle of 95°C for 1 minute, 60°C for 30 seconds, 95°C for 15 seconds then 25°C for 30 seconds was used for melting curve analysis. Transcript abundance relative to HPRT and standard deviation (SD) was determined using the following equations (as recommended by Applied Biosystems). Standard error of the mean was used where appropriate

gene/HPRT = $2^{-\Delta Ct}$; where ΔCt = geneCt – HPRTCt (the difference in cycle threshold)

SD = $2^{-\Delta Ct} - 2^{-x}$; where $x = \Delta Ct + SQRT (SDgene2 + SDHPRT2)$

2.4.3 BMDM microarray

Gene expression levels in BMDM were anlaysed by 3' Affymetrix array performed by ARK genomics (Roslin, Edinburgh). RNA was first reverse transcribed using an Oligo-dT Promoter Primer in a first strand cDNA reaction. Following RNaseH mediated second strand cDNA synthesis the double stranded cDNA was purified and served as a template in the subsequent in vitro transcription (IVT) reaction. The IVT reaction was carried out in the presence of T7 RNA Polymerase and a biotinylated

nucleotide analog/ribonucleotide mix for cRNA amplification and biotin labelling. The biotinylated cRNA targets were then cleaned up, fragmented and hybridised to the Affymetrix GeneChips as described in user manual of Ambion® WT expression Kit (Affymetrix). Probe set expressions were normalized using the robust multi-array average (RMA) algorithm as implemented in the Bioconductor package (Affymetrix). The annotation of the genes was obtained thanks to Prof. Chris K. Tuggle, using the Affymetrix porcine annotation in addition to the Affymetrix human annotation. Differentially expressed probes were detected by comparing un-stimulated macrophages with each of the other time points then fitting a linear model through each probe using the Bioconductor package LIMMA. The design matrix for the linear models included a coefficient for each of the three pigs and a coefficient for the comparisons of interest: 0 vs 2 h, 0 vs 7 h and 0 vs 24 h (statistical analysis by Dario Beraldi). Normalized array data were uploaded to the software Biolayout Express(3D) (http://www.biolayout.org/) and a graph was created using parameters of *R* mean 0.95, Markov clustering algorithm of 2.2, and a minimum number of 6 nodes per cluster. The 500 most inducible probesets were identified using fold change ($p<0.01$) then compared to available array data from mouse and human macrophages treated in exactly the same manner (Schroder et al., 2012)

2.4.4 Monocyte microarray

Monocytes gene expression data levels were analysed by use of a whole genome array performed by ARK Genomics (Roslin, Edinburgh). The array was commissioned by the Roslin Institute (Freeman *et al.* Manuscript in preparation) and prepared by Fios Genomics and Affymetrix. Data was sorted to extract all genes differentially expressed between subsets (> 1.5 fold change or <0,67 fold change) Normalized, sorted array data was then uploaded to the software Biolayout Express(3D) (http://www.biolayout.org/) and a graph was created using parameters of *R* mean 0.95, Markov clustering algorithm of 2.2, and a minimum number of 6 nodes per cluster. Genes which were differentially expressed in mouse and human monocyte subsets was identified from the literature (Ingersoll et al., 2010) and

expression levels of these genes analysed in pig monocyte subsets. Probes were selected as detailed below.

2.4.4.1 Design of the 'Snowball' array and annotation of the probesets

Porcine expressed sequences (cDNA) were collated from public data repositories (ENSEMBL, RefSeq, Unigene and the University of Iowa ANEXdb database) to create a non-overlapping set of reference sequences. A series of sequential BLASTN analyses, using the NCBI blastall executable, were performed with the -m8 option. The initial subject database comprised 2,012 sequences manually annotated *S. scrofa* gene models from Havana provided by Jane Loveland on 29 July 2010, plus 21,021 sequences acquired using Ensembl BioMart *Sscrofa* (build 9, version 59 on 22 July 2010). For each iteration query sequences that did not have an alignment with a bitscore in excess of 50 were added to the subject database prior to the next iteration.

The iterations involved the following query datasets:

1. 35,171 mRNA sequences from NCBI, downloaded on 15 July 2010; 6,286 added to subject database
2. 7,882 RefSeq sequences from NCBI, downloaded on 15 July 2010: 0 added to subject database
3. 43,179 Unigene sequences from NCBI, downloaded on 15 July 2010 (filtered to include only those longer than 500 bases): 10,125 added to subject database
4. 121,991 contig sequences, downloaded from Iowa Porcine Assembly v1 (annexdb.org) on 30 July 2010 (filtered to include only those longer than 500 bases): 10,536 added to subject database
5. 2,370 miRNA sequences (pig, cow, human, mouse), downloaded from miRbase, 30 July 2010 (Release 15, April 2010, 14197 entries): all added without blastn analysis.

The final subject database comprised 52,355 expressed sequences.

To facilitate the design of array probes that were uniformly distributed along the entire length of transcripts, each transcript was split into several probe selection regions (PSRs), each of which was then the target for probe selection. The size of each PSR, typically ~150 nucleotides, was determined by the length of the input sequence, with the ultimate aim being to obtain 20-25 probes per transcript. Oligo design against the ~343,000 PSRs was performed by Affymetrix. In addition, standard Affymetrix controls for hybridisation, labelling efficiency and non-specific binding were included on the array (a total of 123 probesets), together with complete tiling probesets for 35 porcine-related virus genome sequences (both strands, centre-to-centre gap of 17 nucleotides) for possible future infection-based studies. The final array is comprised of 47,845 probesets, with a mean probe coverage of 22.

Initial annotation of the gene models was obtained from the sequence sources and converted into an annotation set using the AnnotateDbi Bioconductor package. However, following this exercise many probesets were without useful annotation. Therefore the original sequences from which the probes had been designed were blasted against NCBI Refseq in order to try and impute the most likely orthologous gene of the 'unannotated' pig transcripts. In order to have one gene per query sequence the following annotation pipeline was followed:

1. For each query the hit with lowest e-value within each species was chosen
2. Genes with e-value hits <1e-9 against *H. sapiens* were annotated with HGNC names/descriptions, however genes with matches starting with 'LOC' were not used.

3. Step 2 was repeated using in order: *Sus scrofa, Bos taurus, Pan troglodytes, Mus musculus, Canis lupus familiaris, Pongo abelii, Equus caballus, Rattus norvegicus, Macaca mulatta*.
4. Step 3 was repeated using any other species (in no particular order) to which a hit could be obtained.
5. For the remaining probes use LOC genes from (in order of priority): Homo sapiens, Sus scrofa, Bos taurus, Pan troglodytes, Mus musculus
6. Use everything else, in no particular order.

Out of 47,845 sequences represented on the array, 27,322 probesets now have annotations that correspond to a current (15th Dec. 2011) HGNC symbol for human protein coding gene, 14,426 of which are unique (out of a total 19,219 listed by HGNC). The remaining probesets were annotated with the information available for those sequences.

2.5 Investigation of the nitric oxide pathway

2.5.1 Nitrite assay

Activity of the inducible nitric oxide synthetase (iNOS/NOS2) enzyme generates the unstable effector molecule, nitric oxide. iNOS activity was determined by the detection of nitrite, the stable metabolite of nitric oxide using the Greiss reagent (0.1% α-napthyl-amine, 1% sulfanilimide, 2.1% phosphoric acid). Differentiated macrophages were seeded at 1M/mL in a 6 well plate and allowed to adhere for 24 hours before treatment with 100ng/ml LPS. Supernatants were removed and stored at -20˚C. Nitrite concentration was assayed against standards by incubating 50µL of supernatant/standard with 50µL Greiss reagent for 10 minutes. Absorbance was measured at 540nm.

2.6 Cloning and sequencing of RT qPCR gene products

cDNA was synthesised as previously using primers for the IDO, STAT4, CCL20 and HPRT. The pGEM-T easy kit (Promega) was used as per instructions to clone the pcr products in XL-1 blue competent cells. Plasmid DNA was purified using the Miniprep kit (Quiagen). DNA sequencing was performed by DNA Sequencing & Services (MRCPPU, College of Life Sciences, University of Dundee, Scotland, www.dnaseq.co.uk) using Applied Biosystems Big-Dye Ver 3.1 chemistry on an Applied Biosystems model 3730 automated capillary DNA sequencer. Sequencing results were blasted against the pig genome to ensure the correct gene product was amplified by the prime

Chapter 3: CSF1 and Maturation of Porcine Macrophages

3.1 Introduction

As discussed previously the LPS receptor CD14 and Fc gamma receptor CD16 are commonly used as markers to distinguish human monocytes subsets (Ziegler-Heitbrock, 2007) while the glycophosphatidyl-inositol (GPI)-anchored glycoprotein Ly6C and the chemokine receptors CCR2 and CXCL3 have been used for the same purpose in mice (Auffray et al., 2009). Monocyte heterogeneity has also been shown in rats where CD43 is used to distinguish monocytes (Ahuja et al., 1995). In both mouse and human, "intermediate" monocytes populations between the two extremes have also been distinguished (Sunderkotter et al., 2004; Cros et al., 2010; Grage-Griebenow et al., 2001a; Grage-Griebenow et al., 1993; Grage-Griebenow et al., 2000; Wong et al., 2011). A consortium of investigators has proposed that the CD14 high population, which is more numerous in humans, be referred to as "classical" monocytes; the minor CD16 positive expression being "non-classical" and monocytes which express both CD14 and CD16 be termed intermediate (Ziegler-Heitbrock et al., 2010; Wong et al., 2011). This nomenclature has not yet been widely accepted and it is not clear how well it translates across species other than humans, where the monocyte populations are more similar in relative abundance. The presumption in all such efforts is that the marker-defined subpopulations also have distinct functions, differing in inflammatory and differential potential, cytokine production and recognition of pathogens. This chapter will review the markers used to distinguish monocyte populations in other species, discuss the functions of monocyte subsets and examine markers in the pig and their regulation during monocyte differentiation.

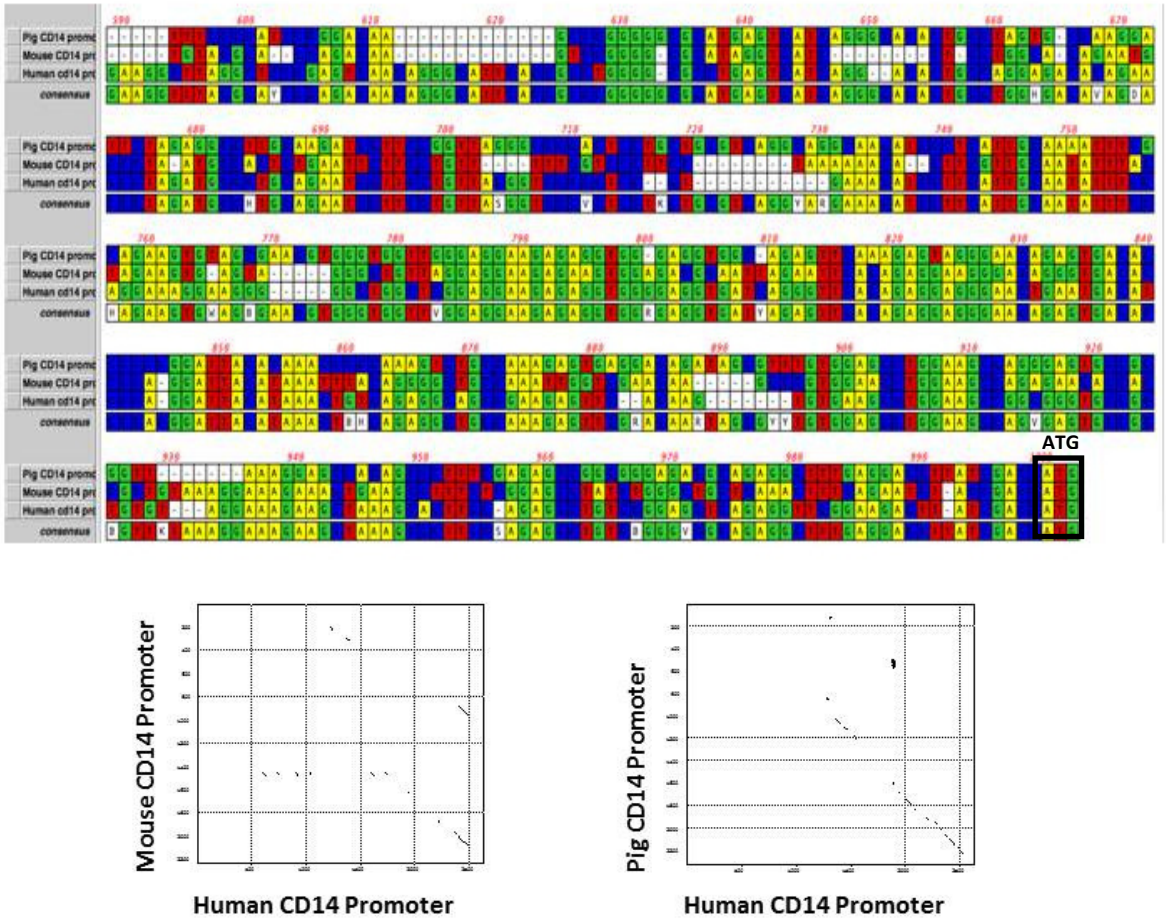


Figure 3-1 Comparison of CD14 promoters in human, mice and pigs.

Promoters for the *CD14* gene in human, mice and pigs were extracted and analysed by Pustell and ClustalW Alignments. The human and pig *CD14* promoters were more conserved than the pig and mouse.

3.1.1 Monocyte markers

3.1.1.1 CD14

CD14 is a GPI-linked protein and acts as a receptor for LPS and LPS/LPS binding protein (LBP) complexes. It is required to detect low concentration of endotoxin and for signalling through TLR4 and MD2 (Pugin et al., 1993). Like humans, pigs require CD14 for detection of LPS (Thorgersen et al., 2009). However, as in mice and men, higher concentrations of LPS (100µg/mL) can facilitate binding in a CD14 independent manner (Kielian et al., 1995). Porcine *CD14* cDNA has been cloned and the sequence encodes a 373 aa peptide, which shows 70% homology with the human protein. Porcine CD14 was first identified using human anti-CD14 monoclonal antibodies which show cross-reactivity with primates and pig but not mouse. Different anti-CD14 antibodies display different staining patterns, although it is not known whether this is due to differences in the affinities of the antibodies or because different antibodies recognise different forms of CD14 (Ziegler-Heitbrock et al., 1994).

CD14 is commonly used in humans as a marker for monocytes/macrophages but is also detected on granulocytes, albeit at much lower levels (Antal-Szalmas et al., 1997). Similarly porcine CD14 is highly expressed on monocytes, tissue macrophages and at lower levels on granulocytes (Sanz et al., 2007; Qiu et al., 2007; Petersen et al., 2007b). As in human and mice, porcine CD14 expression is induced relatively late during macrophage differentiation, it is not present on early myeloid progenitors but is expressed after cells have differentiated into blood monocytes (Summerfield and McCullough, 1997; Chamorro et al., 2004). CD14 is highly expressed in human liver, by hepatocytes as well as Kupffer cells, but no mRNA is produced in murine liver (Hetherington et al., 1999; Pan et al., 2000) (see also cDNA array data on biogps.gnf.org). CD14 also has tissue specific expression in the pig, most notably it is expressed at high levels in the liver as it is in man (Sanz et al., 2007; Qiu et al., 2007; Petersen et al., 2007b). The lack of *Cd14* expression in mouse liver is a fundamental difference between mice and humans (and pigs) which may have some importance to understanding the difference in LPS sensitivity between the species. Production of NO varies greatly between man and mice and there is some evidence this may be due to fundamental differences in the promoters (Taylor and Geller, 2000; Zhang et al., 1996; Yu et al., 2005).

Figure 3.1 shows Pustell and ClustalW alignments of mouse, human and pig CD14 promoters. As has been noted previously when comparing genes which have differential expression between species (Perez et al., 2006; Yu et al., 2005), the mouse and human promoters for *CD14* show little conservation. By contrast the pig and human promoters are much more closely conserved. This difference in promoter architecture could be responsible for the different expression patterns of CD14 between men and pigs, and mice which may be a contributing factor in the vast differences in LPS sensitivity between the species.

3.1.1.2 CD16

In man, CD16 (Fc gamma receptor type III, encoded by the *FCGR3* gene) is found in two forms, a transmembrane form expressed on monocytes and NK cells and a phosphatidylinositol linked form expressed on neutrophils (Ravetch and Perussia, 1989). Copy number variation in *FCGR3* has been linked to susceptibility to disease in humans and rats (Aitman et al., 2006; Willcocks et al., 2008) although humans appear to be the only animal to have two isoforms of the gene. Previous studies in the pig found CD16 expression predominantly on NK cells, monocytes and macrophages and at lower levels on neutrophils and immature granulocytes (Chamorro et al., 2004). CD16 was found at slightly higher levels on $CD163^{+}$ cells (Sanchez et al., 1999). CD16 is perhaps most commonly used as a marker of monocyte differentiation in man. $CD14^{hi}CD16^{-}$ monocytes can be matured into $CD14^{lo}CD16^{hi}$ monocytes and MDMs generated *in vitro* increased expression of CD16 as they matured into macrophages (Ziegler-Heitbrock et al., 1993). Mouse monocyte subsets, previously categorised by the presence of Ly6C, can also be split on the basis of CD16 with the more mature $Ly6C^{lo}$ subset expressing higher levels of CD16 (Ingersoll et al., 2010), suggesting CD16 is also a marker of monocyte maturation in the mouse.

3.1.1.3 CD163

In man, CD163 has been identified as a receptor involved in clearance of hemoglobin-haptoglobin-haptoglobin complexes by resident macrophages (Ritter et al., 1999). More recently identified functions include as a receptor for TNF-like weak inducer of apoptosis (TWEAK) (Bover et al., 2007), as an erythroblast adhesion receptor (Fabriek et al., 2007) and

as a bacterial innate immune sensor (Fabriek et al., 2009). CD163 expressing human macrophages have also been shown to secrete anti-inflammatory cytokines (Philippidis et al., 2004). Like CD14, CD163 exists in a membrane bound and soluble form and pro-inflammatory stimulation leads to shedding from the cell surface (Weaver et al., 2007; Sulahian et al., 2004). It is highly expressed on tissue macrophages and is considered a candidate innate immune sensor for bacteria, generating an anti-inflammatory signal (Fabriek et al., 2009; Weaver et al., 2007). CD163 expression is restricted to myeloid cells in man and is more strongly expressed by $CD14^{hi}$ $CD16^{lo}$ monocytes than by the $CD14^{lo}CD16^{hi}$subset. However treatment of monocytes with CSF1 increased expression on all human subsets (Tippett et al., 2011). Mouse Ly6C ^{hi} and Ly6C ^{lo} subsets do not appear to have differential expression of CD163 (Ingersoll et al., 2010). In rats, CD163 was originally detected with the ED2 monoclonal antibody, which recognises most tissue macrophages. ED2 antigen has not been detected on rat monocytes (Polfliet et al., 2006; Dijkstra et al., 1985). As in man CD163 expression in the pig is restricted to myeloid cells and is higher on tissue macrophages and a subset of blood monocytes which were considered more mature. CD163 ^{hi} monocytes were claimed to express lower levels of CD16, converse to what was observed in man, (Sanchez et al., 1999; Ingersoll et al., 2010). Like CD14 and CD16, CD163 is an import immune receptor. CD163 is a receptor for Porcine Reproductive and Respiratory Virus Syndrome (PRRVS) (Duan et al., 1997) and African Swine Fever Virus (ASFV) (Sanchez-Torres et al., 2003) and higher expression is associated with increased susceptibility to these viral pathogens (Calvert et al., 2007; Sanchez-Torres et al., 2003; Patton et al., 2009). In man HIV infected macrophages and microglia have been shown to up-regulate CD163 expression (Kim et al., 2006) and expression on blood monocytes was also increased in HIV infected patients (Tippett et al., 2011)

3.1.2 Monocyte subsets

The markers described above are commonly used to distinguish monocytes in humans, rodents and pigs and the current view of the most commonly used subsets are summarized in **Figure 3.2**. More recently an intermediate subset of human monocytes has been identified which expressed intermediary levels of markers previously used to distinguish classical and non-classical monocytes. Only a few markers have proven to be specific for intermediate

monocytes (e.g. GFRα2, CLEC10A) and these were expressed at low levels on these cells making isolation difficult (Wong et al., 2011). A second Fc receptor, CD64, has also been used to further sub-divide human $CD16^+$ monocytes (Grage-Griebenow et al., 2001b; Grage-Griebenow et al., 1993; Grage-Griebenow et al., 2000) while a Ly6C intermediate population of mouse monocytes has been reported (Sunderkotter et al., 2004). Human $CD16^+$ monocytes produced higher levels of TNFα and IL1β and low levels of IL10 while $CD16^-$ monocytes produced low levels of TNFα and appear to be the main producers of IL10 (Belge et al., 2002; Ziegler-Heitbrock et al., 1992; Ziegler-Heitbrock, 2007). Mouse $Ly6C^{lo}$ monocytes resemble Human $CD16^+$ monocytes based on surface marker expression and gene array studies (Ingersoll et al., 2010; Geissmann et al., 2003). Distinct cytokine expression profiles give clues to the function of these subsets *in vivo* and will be discussed in more detail in **Chapter 4**, however the specific function of each monocyte subset has not yet been fully elucidated. Murine $Ly6C^{lo}$ monocytes, which are defined by high expression of the fractalkine receptor CX3CR1 and absence of another chemokine receptor, CCR2, have been shown to have an LFA1 (CD11a/CD18) dependent patrolling function. In response to tissue injury $CX3CR1^+$ cells left the circulation and travel to the site of injury where they were responsible for the early inflammatory response. CX3CR1 was itself required for tissue invasion (Auffray et al., 2007). $CD14^{dim}CD16^{hi}$ monocytes which also lack CCR2 and have high expression of CX3CR1, appeared to fulfill this function in man (Cros et al., 2010). Human $CD16^+$ monocytes also expressed CX3CR1 and migrate in response to CX3CL1 expressed by inflamed endothelial cells. $CD16^-$ monocytes expressed CCR2 and CD62L and responded to CCL2 which mediated migration from the bone marrow and to sites of inflammation, suggesting the different subsets are recruited into different sites during the inflammatory response (Ancuta et al., 2003). Relative numbers of the $CD14^{lo}CD16^{hi}$ subset varied in some forms of infection or inflammation. Excessive exercise led to an increase in the $CD14^{lo}CD16^{hi}$ population, the infiltrating cells coming from the marginal pool rather than peripheral blood. $CD14^{lo}CD16^{hi}$ monocytes preferentially homed to the marginal pool due to high expression of adhesion molecules which were then down regulated by the exercise induced release of catecholamines which resulted in them moving back into the circulation (Steppich et al., 2000). The $CD16^+$ monocyte subset also increased in inflammatory disease such as rheumatoid arthritis (Baeten et al., 2000) and atherosclerosis (Wildgruber et al., 2009) and in bacterial and viral infections such as tuberculosis (Vanham et al., 1996) and HIV

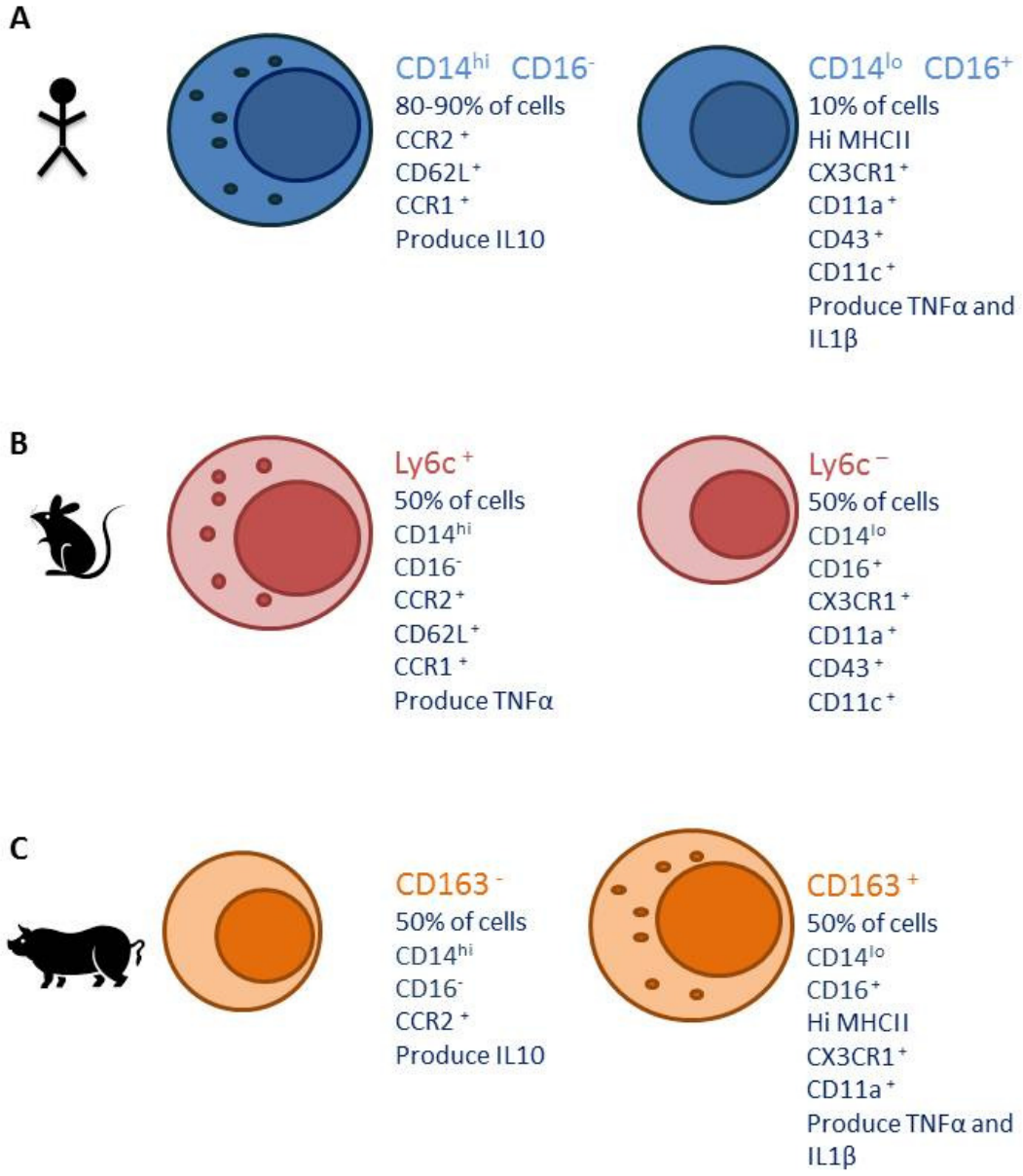


Figure 3-2 Monocyte subsets in man, mouse and pig

CD14 and CD16 are commonly used to define monocyte subsets in man (**A**) while Ly6C and CD14 and CD163 fulfill the same function in mice (**B**) and pigs (**C**) respectively.

(Allen et al., 1991). In sepsis as many as 50% of circulating blood monocytes were $CD16^+$ (Blumenstein et al., 1997; Fingerle et al., 1993). The $CD16^+$ monocyte subset also increased with age while total leukocyte and monocyte numbers remained static (Seidler et al., 2010). Different functions have not yet been ascribed to monocyte subsets in the pig although previous authors have described higher expression of CD16 (Sanchez et al., 1999) as well as high CX3CR1 and low levels of CCR2 (Moreno et al., 2010) on $CD163^{hi}$ pig monocytes which suggest they are therefore approximately equivalent to the human $CD14^{lo}CD16^{hi}$ monocyte subset (Ziegler-Heitbrock, 2007).

It is not entirely clear that "subset" is an appropriate term, since the subdivisions of monocytes depend somewhat on exactly where one places gates on a flow cytometer. The prevailing view is that the different subsets are maturation stages of the same cells and this has been backed-up by recent genetic studies (Wong et al., 2011). *In vitro* generated human MDM expressed lower levels of CD14 and high levels of CD16. They also displayed increased MHC class II expression and decreased expression of CD11b and CD33, similar to the $CD14^{lo}CD16^{hi}$ subset. Removal of all $CD16^+$ cells, including the $CD14^{lo}CD16^{hi}$ subset, from PBMCs prior to cell culture did not affect the generation of $CD16^+$ cells indicating that the $CD14^{lo}CD16^{hi}$ cells were derived from $CD14^+CD16^-$ monocytes (Ziegler-Heitbrock et al., 1993). In the mouse, down-regulation of Ly6C on $Ly6C^{hi}$ circulating monocytes has been described, suggesting that as in man, the murine monocyte subsets represent a continuum of different stages of maturation (Sunderkotter et al., 2004).

3.1.3 Expression of monocyte markers in the pig

The proposed functional equivalence of the human $CD16^+$ and mouse $Ly6C^-$ cells are mainly supported by the differential expression of a few key markers which are summarised in **Appendix 3**. More recently, some groups have used microarray technology to acquire a global view of differences between monocyte subsets across different species (Ingersoll et al., 2010; Cros et al., 2010) and this will be discussed with relevance to the pig in **Chapter 4**. Of the markers that are differentially expressed between monocyte subsets in man and mouse, only a few have been studied in the pig. CD14 and CD16 were differentially expressed although the expression of CD16 has not been fully studied (Sanchez et al., 1999), most groups instead focused on differential expression of CD163 (Chamorro et al., 2004;

Chamorro et al., 2000; Chamorro et al., 2005). CD11a had a similar expression pattern in the pig to that found in humans. Human anti CD11b and CD11c antibodies recognise the porcine molecules wCD11R1 and wCD11R2 respectively although both had different expression patterns to that observed in humans. wCD11R1 was expressed by 50% of granulocytes and was found on some blood monocytes, alveolar macrophages, NK cells and DCs. wCD11R2, the human counterpart of which is often used as a marker of DCs, was expressed on monocytes, DCs and AM but not granulocytes and was up-regulated following differentiation of PBMCs into tissue macrophages (Chamorro et al., 2000; Ezquerra et al., 2009). As in humans and mice, expression of CX3CR1 was higher on $CD163^+$ monocytes while CCR2 expression was low and the converse was true for $CD163^-$ monocytes (Moreno et al., 2010). Unfortunately the lack of commercially available anti-porcine antibodies means that expression of CCR2 and CX3CR1 have only been studied at the mRNA level and data on their expression at the protein level is not available. Similarly expression of CD43, CD62L and CCR1 have not been studied in the pig, probably due to the lack of porcine specific antibodies. Studies looking at cytokine expression of pig monocyte subsets found all porcine monocytes produced TNF and IL1α after LPS stimulation, however $CD163^+$ monocytes produce higher levels of these pro-inflammatory cytokines and only $CD163^-$ monocytes produced IL10, suggesting that, like human $CD16^-$ monocytes, $CD163^-$ monocytes have an anti-inflammatory role while $CD163^+$ monocytes were more pro-inflammatory(Chamorro et al., 2005).

3.1.4 Expression of porcine specific markers

A number of pig specific myeloid cell markers have been studied by previous groups (Chamorro et al., 2005; Chamorro et al., 2004; Chamorro et al., 2000; Ezquerra et al., 2009). The most commonly used are swine workshop cluster (SWC) 1, 3, 8 and 9. SWC1 was expressed on resting lymphocytes and myelomonocytic cells and was down-regulated as monocytes differentiate (Basta et al., 1999; Chamorro et al., 2004; Chamorro et al., 2000; Ezquerra et al., 2009). SWC3 is the porcine homologue of the receptor-type transmembrane glycoprotein signal-regulatory protein alpha (SIRPα ,CD172a) and has a high degree of homology with the human protein (75% to 80%). CD172a was expressed on every cell of myeloid origin from the earliest bone marrow pre-cursors (Summerfield and McCullough,

1997). SWC8 is commonly used as a granulocyte marker. Myeloid cells from the jejunal lamina propria, blood and bone marrow granulocytes were $SWC8^+$ while freshly isolated monocytes were negative (Thacker et al., 2001). SWC9 has 89% homology with human phosphodiesterase I / ecto-nucleotide pyrophosphatase 1 (NPP1/CD203a). The transcript was highly expressed in the thymus, pulmonary alveolar macrophages and lung tissues. Expression was up-regulated during monocyte differentiation into macrophages and its expression was restricted to mature macrophages (Petersen et al., 2007a). CD14, CD163, SWC9, SWC3 and SLA DR were used to define multiple myeloid cell subsets and as in man, different subsets have been proposed to have different immune-regulatory roles (Chamorro et al., 2004; Chamorro et al., 2000; Chamorro et al., 2005). An excellent review of porcine monocyte markers is given by Ezquerra et al. (Ezquerra et al., 2009).

3.1.5 CSF1

Colony stimulating factor 1 (CSF1, MCSF) was the first haemopoietic growth factor to be isolated and promotes the growth of mature macrophages from immature progenitors (Stanley et al., 1976). The non-redundant functions of CSF1 have been studied in the osteopetrotic/osteopetrotic (*op/op)* mouse, which has a mutation in the *Csf1* gene or an introduced mutation in the CSF1 receptor (CSF1R) gene (*Csf1r*). *Op/op* mice have skeletal abnormalities, a shorter life span, a number of neurological and reproductive defects, and lower resistance to infection underlining the importance of macrophages in regulating development and homeostasis in a number of physiological systems. They were not completely devoid of all macrophages but their numbers were substantially reduced (Ryan et al., 2001; Dai et al., 2002; Cecchini et al., 1994). CSF1 has the ability to differentiate mouse BMC into mature macrophages in culture and this has been widely utilised in studies of myeloid cell development (Hume et al., 1987; Warren and Vogel, 1985; Hume and Gordon, 1983). The CSF1 gene has evolved rather rapidly across species (Garceau et al., 2010). Human CSF1 is active in mice, and indeed recombinant human CSF1 was used in the first *in vivo* studies (Hume et al., 1988). Conversely, murine CSF1 does not act on the human CSF1R (Stanley, 2001). The pig CSF1R binds both mouse and human CSF1 and both factors are able to stimulate pig macrophage differentiation and proliferation (Gow *et al.* manuscript in preparation). The colony forming effects of CSF1 are increased when cells are cultured in

a combination of growth factors including GMCSF, IL3 and IFNγ (Breen et al., 1991; Breen et al., 1990; Miyauchi et al., 1988; Caracciolo et al., 1987). CSF1 has a number of immunological effects; it enhanced cytotoxicity, superoxide production, phagocytosis, chemotaxis and cytokine production in monocytes and macrophages (Chitu and Stanley, 2006). Moreover the innate immune response can be affected by CSF1-dependent modulation of TLRs and CSF1 enhanced the cytokine response to LPS in the mouse (Sweet et al., 2002) although not in man (Irvine et al., 2009) or the pig (Kapetanovic *et al.* manuscript under review). There are three isoforms of CSF1, the secreted proteoglycan, a secreted glycoprotein and a cell-surface membrane anchored form, all of which have different effects in immunity and inflammation (Chitu and Stanley, 2006). In cancer the different isoforms of CSF1 have been shown to have both pro (Aharinejad et al., 2002; Lin et al., 2001; Pyonteck et al., 2011) and anti-tumour effects (Lu et al., 1991; Graf et al., 1999; Jadus et al., 0 AD; Hoa et al., 2007) although generally CSF1 is seen to have a cancer promoting role (Pollard, 2005). CSF1 up-regulated expression of CD14 and CD16 on human monocytes (Ji et al., 2004; Li et al., 2004; Kawanaka et al., 2002) and elevated levels of this growth factor may lead to increased numbers of $CD16^+$ monocytes which can worsen the pathology of rheumatoid arthritis among other diseases (Kawanaka et al., 2002). Elevated numbers of $CD16^+$ monocytes are also observed in cancer where treatment with recombinant human CSF1 (rhCSF1) further increased their numbers (Saleh et al., 1995). Treatment with an anti-CSF1 antibody caused a decrease in $CD16^+$ monocytes in non-human primates (Radi et al., 2011) suggesting blockade of CSF1 could improve the pathology of disease where an abnormally high number of $CD16^+$ monocytes contribute to the pathology (Kawanaka et al., 2002; Baeten et al., 2000; Wildgruber et al., 2009; Vanham et al., 1996; Allen et al., 1991; Blumenstein et al., 1997; Fingerle et al., 1993).

It was originally hoped that CSF1 administration would improve the outcome of many disease such as cancer (Munn et al., 1990). However much of the pathology of infectious or inflammatory diseases are macrophage mediated and increased levels of CSF1 led to increased numbers of macrophages (Weiner et al., 1994). Interest has therefore turned to antagonists of CSF1. There is some indication that blocking CSF1 may have a beneficial effect on some human pathologies (Macdonald et al., 2010; Hashimoto et al., 2011; Lenzo et

al., 2011; Hume and MacDonald, 2011). The actions of CSF1 can be blocked by inhibitors against the protein tyrosine kinase activity of the receptor or by anti-CSF1R antibodies which block binding of the ligand(s) to the receptor. In normal physiological conditions CSF1 is cleared from the circulation by receptor mediated endocytosis or by binding to CSF1R. This ensures that monocytopoesis is linked to the immune state of the body (Shaposhnik et al., 2010; Bartocci et al., 1987). Blocking CSF1R therefore causes a huge increase in circulating CSF1; as the antibody dissipates and CSF1 begins to be cleared from the circulation a correspondingly large increase in monocyte numbers occurs. This does not happen when CSF1R kinase inhibitors are used as they do not inhibit receptor-mediated endocytosis so some CSF1 will still be cleared from the circulation. They do however block autocrine CSF1 which anti-CSF1R antibodies cannot do (Irvine et al., 2006; Hume and MacDonald, 2011).

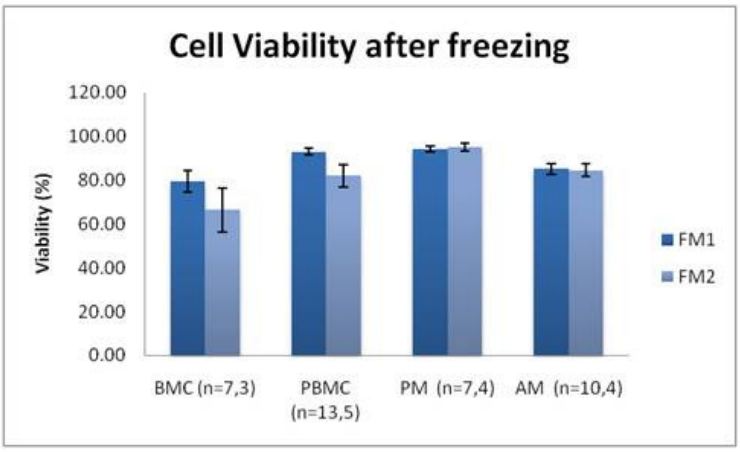
A recently developed anti-CSF1R antibody (M279) specifically depleted Ly6C^{lo} monocytes in a mouse model. A corresponding increase in Ly6C^{hi} monocytes was observed although total monocyte numbers were not affected (Macdonald et al., 2010). M279 selectively depleted several tissue macrophage populations including macrophages from established tumours but had no effect on macrophage numbers in inflammatory models of disease in the mouse. These results have been partly replicated using a different anti-CSF1R antibody (AFS98) (Lenzo et al., 2011; Hashimoto et al., 2011). These studies confirmed the CSF1-dependant decrease in Ly6C^{lo} cells which Macdonald *et al*. (2010) observed. They also report a decrease in total monocyte numbers. Lenzo *et al* (2011) used AFS98 at lower doses than Hashimoto *et al* (2011) and correspondingly observed a more selective reduction in the mature monocyte subset. The difference in total monocyte numbers when treated with M279 versus AFS98 is probably due to intrinsic differences in the antibodies, since M279 has a much higher affinity for CSF1R than AFS98(Hume and MacDonald, 2011). Additionally AFS98 is immunoglobulin IgG2a type and may bind to the Fc receptor CD64 which is constitutively expressed by macrophages. This could lead to the death or removal of AFS98 labelled monocytes by other CD64 expressing macrophage (Hume and MacDonald, 2011). All three studies demonstrated a loss of peritoneal macrophages after anti-CSF1R antibody administration, probably due to the reduced numbers of Ly6C^{lo} circulating monocytes which are the precursors of mature tissue macrophages. The differentiation of Ly6C^{hi} cells into the

more mature Ly6C^{lo} subset and subsequent loss of selected tissue macrophage populations was the only non-redundant function of CSF1. This suggests that, in the mouse at least, the two monocyte subsets are different maturation stages of the same cell and that CSF1 is essential for maturation of resident Ly6C^{hi} cells into the more mature Ly6C^{lo} subset and thence to tissue macrophages but not for generation of monocytes (Macdonald et al., 2010).

There is a large body of work studying the heterogeneous expression of surface markers on human and mouse monocytes (Gordon and Taylor, 2005; Geissmann et al., 2003; Ziegler-Heitbrock, 1996). The expression of similarly heterogeneously expressed markers has been previously studied in the pig, mainly on blood monocytes (Chamorro et al., 2004; Chamorro et al., 2000; Chamorro et al., 2005; Gordon and Taylor, 2005; Sanchez et al., 1999). One of the major advantages of the pig as a model for human immunity is the ability to compare MDM and BMDM with mature tissue macrophages from the same animal. Tissue macrophages are generally agreed to derive from circulating monocytes and are important for conservation of tissue homeostasis through the repair or remodelling of tissue after inflammation or injury (Gordon and Taylor, 2005). They show a high degree of heterogeneity which is related to the specialised functions they fulfil in their particular anatomical niche. AMs are a mature tissue macrophage population, known to be dependent upon continuous CSF1 signaling, in both the steady state (Macdonald et al., 2010) and in an LPS-induced model of lung inflammation (Lenzo et al., 2011), in the mouse at least. They are distinct from macrophages in other sites and play a central role in the innate immune system. AMs are the first line of defense against inhaled pathogens and are involved in the pathology or control of diseases such as Pneumonia (Broug-Holub et al., 1997), Chronic Obstructive Pulmonary Disease (Hodge et al., 2003) and Legionnaires Disease (Shin and Roy, 2008). AMs also function as an important link with the adaptive immune system as they function as antigen presenting cells (APC) (Vecchiarelli et al., 1994). The numbers of alveolar macrophages isolated from a mouse are small and limit the scope of *in vitro* experiments. Human AMs can only be isolated by bronchoalveolar lavage (BAL), an unpleasant procedure patients may be unwilling to undergo and are therefore also in short supply. The large size of the pig will provide a unique resource permitting the isolation and analysis of a large number of alveolar macrophages from a single animal, enabling models of

human lung pathology to be developed to a far greater degree than is possible with the mouse. Many of the problems working with AMs described above also apply to the study of PMs. Thioglycolate medium can be used to induce sterile peritoneal inflammation (Gallily et al., 1964) and thioglycolate elicited mouse peritoneal macrophages (TEPMs) are a commonly used model of inflammatory macrophages (Sester et al., 2005; Irvine et al., 2006; Halili et al., 2010). The numbers of peritoneal macrophages isolated from a mouse without the use of thioglycolate is however very small, making the study of non-inflammatory PMs difficult. Additionally the use of thioglycolate does not allow these elicited cells to be seen in any other role than as their classic function as APCs, murine PMs collected by peritoneal lavage have been shown to suppress T cell activation through IFNγ mediated catabolism of arginine (by iNOS) and tryptophan (by IDO) (Matlack et al., 2006). Human PMs represent one of the few human resident macrophage populations that can be

A



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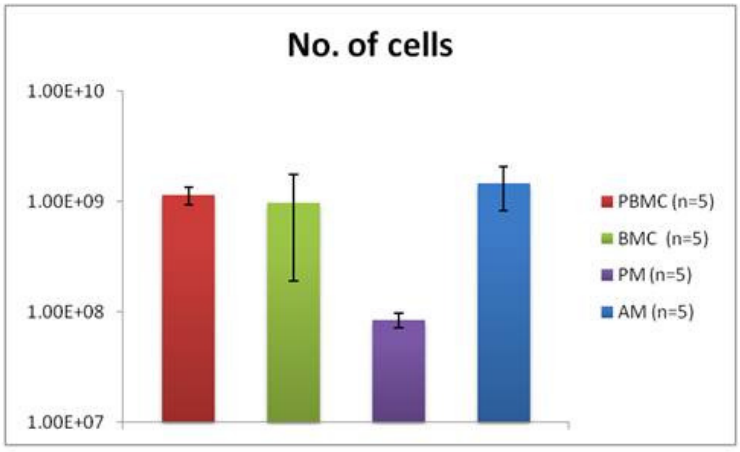

Figure 3-3 Cells were harvested from 4 different compartments in the pig.

BMCs (**A-C**) and PBMCs (**D-F**) treated with rhCSF1 for up to 7 days. **A** and **D** freshly isolated cells, **B** and **E** grown in CSF1 for 3 days, **C** and **F** grown in CSF1 for 7 days. **G** PBMCs grown in the absence of rhCSF1 for 3 days.

easily isolated and studied (Xu et al., 2007; MacGowan et al., 1983) and there is a literature on their study (Jha et al., 1996; Chang et al., 2010; Peterson et al., 1983). Unfortunately human AMs and BMC are not readily available limiting comparisons between macrophages from different anatomical niches in man. The porcine peritoneal cavity is a sterile environment from which large numbers of macrophages can be easily isolated and will enable the comparison of macrophages from different anatomical locations (e.g. AMs versus PMs) and in different states of maturation (e.g. MDMs or BMDMs versus AMs or PMs)

3.2 Results

3.2.1 Establishing a model of macrophage activation in large animals

3.2.1.1 Isolation and cryopreservation of porcine macrophages from blood, bone marrow, lungs and peritoneal cavity

As stated above, the ability to compare macrophages from different locations is one of the advantages of using the pig as a model. A large number of cells were successfully isolated from the lungs, peritoneal cavity, bone marrow and blood of a pig (**Figure 3.3B**). The number of cells collected from one animal was very large, on average 1E+09 PBMCs, 9E+08 BMCs, 8E+07 PMs and 1E+09 AM (n=5) (**Figure 3.3B**). Thus cells from one animal could be stored and used in many experiments without pooling samples from different animals as is common in the mouse. The large number of cells obtained from one animal also allows repetition of experiments using the same cells.

In order to best utilise the large number of cells isolated from the pig a cryopreservation protocol was optimised. Two different freezing media were assessed, FM1 and FM2 (**Chapter 2**). Viability after cryopreservation was above 80% for all cells using FM1 and higher in BMCs and PBMCs using FM1 (**Figure 3.3A**) therefore FM1 was used for all future work.

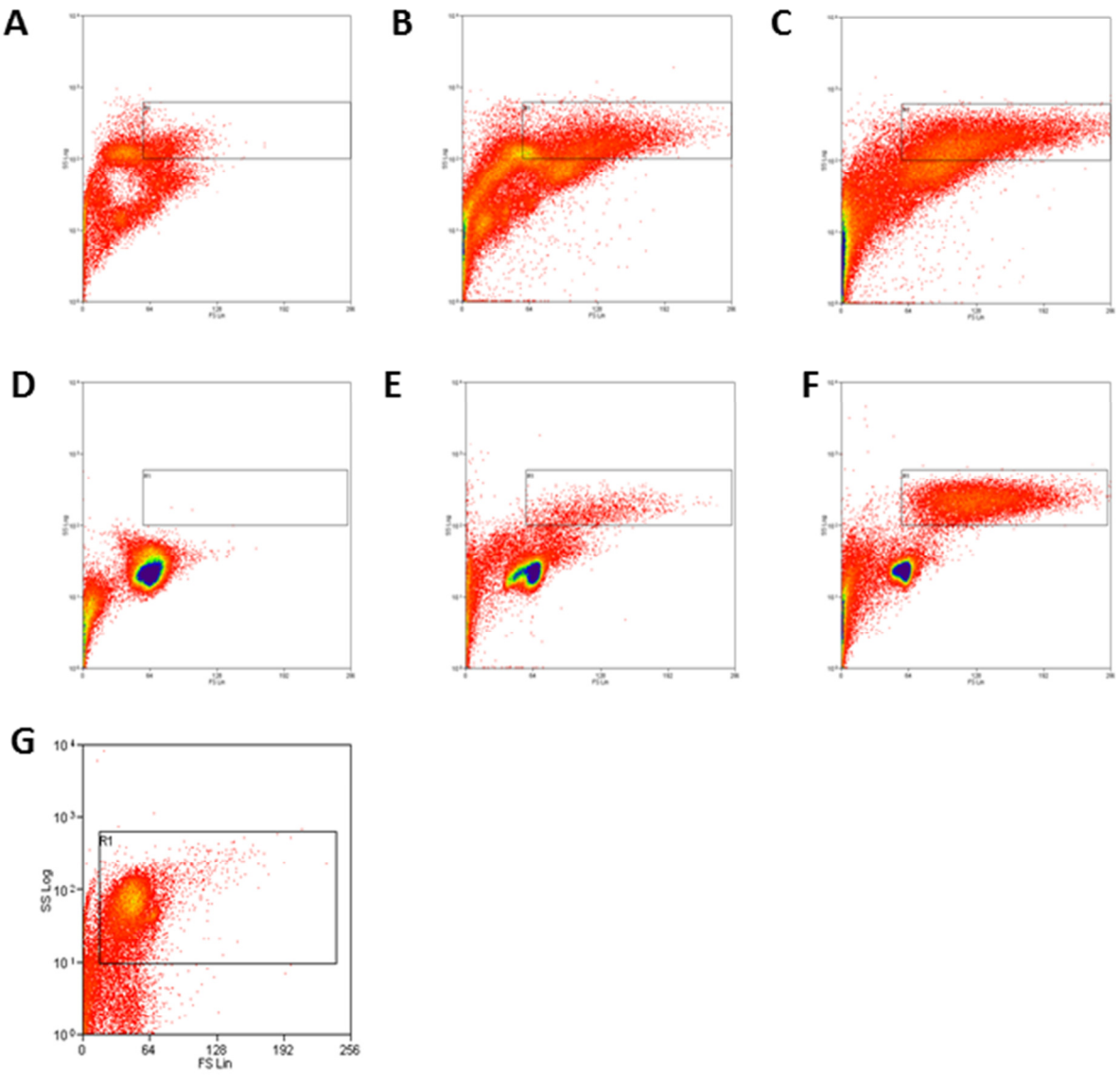

Figure 3-4 rhCSF1 caused BMCs and PBMCs to increase in granularity.

BMCs (**A-C**) and PBMCs (**D-F**) treated with rhCSF1 for up to 7 days. **A** and **D** freshly isolated cells, **B** and **E** grown in CSF1 for 3 days, **C** and **F** grown in CSF1 for 7 days. **G** PBMCs grown in the absence of rhCSF1 for 3 days.

3.2.1.2 Recombinant human CSF1 induces differentiation

Previous authors have used mouse L929 conditioned medium as a source of mouse CSF1 to grow pig macrophages. Recombinant human CSF1 potentially provides a more potent and reproducible system, as previously employed in studies of the mouse. Accordingly, pig bone marrow cells and PBMCs were cultured in rhCSF1 as described previously (Stacey et al., 1993). Cells from both populations grown in

rhCSF1 for 3 days at concentrations previously found to be maximally active for mouse, increased in size and granularity (**Figure 3.4B, E**) compared to freshly isolated cells (**Figure 3.4A, D**). After 7 days growth in rhCSF1 this population of larger, more granular cells increased further. Expression of the maturation associated marker CD16 also increased on rhCSF1 cultured PBMCs. Cells grown in normal media without the addition of rhCSF1 for 3 days did not show this change in morphology or marker expression (**Figure 3.4G**).

3.2.2 Characterisation of porcine BMCs

3.2.2.1 Expression of CD14, CD16, CD163 and CD172a on BMC

Murine BMDM are one of the most commonly used cells when modeling the human immune system. They are often compared to human MDM as similarly immature macrophage/monocyte populations. Blood and bone marrow monocytes are readily available in large quantities from the pig. Examining the expression levels of key immune markers on porcine BMDM and MDM will allow a fuller characterisation of these immature cells from the same animal. Previous studies have validated the use of Flow assisted cytometry (FACS) to study cell populations. Forward scatter (FSC) vs. side scatter (SSC) plots have been shown to compare favourably to manual bone marrow differential counts in the dog, and distinct populations of erythroid and myeloid cells could be distinguished as well as megakaryocytes. Cells of the same lineage but at different points of maturation were also detectable (Weiss et al., 2000). Our freshly isolated porcine BMC showed heterogeneous cell populations as has been described for other species (Weiss et al., 2000; Lee, 1991; KOLLER

et al., 1996) when studied by FSC vs SSC FACS plots (**Figure 3.5A**) showing several populations of varying size and granularity.

The expression of CD14 and CD16 has been studied extensively on immature monocytes (Ziegler-Heitbrock, 2007; Ziegler-Heitbrock, 1996; Ziegler-Heitbrock et al., 1993; Ziegler-Heitbrock and Ulevitch, 1993). Likewise CD163 has been used as a monocyte marker in pig blood derived monocytes (Chamorro et al., 2005; Chamorro et al., 2004; Chamorro et al., 2000). The expression of these surface markers was therefore studied on BMCs to enable comparisons of these two undifferentiated monocyte populations in the same animal. Approximately half of the BMCs were $CD14^+$ $CD16^+$ and this large double positive population was composed of two distinct smaller populations (**Figure 3.4C, D**). One population expressed higher levels of both CD14 and CD16 while the second population had lower levels of both molecules and some $CD14^-$ $CD16^{lo}$ cells. Just over half of $CD14^+$ BMCs expressed CD163 (**Figure 3.4E, F**) and only 6% of $CD163^+$cells were $CD14^-$, showing CD163 to be a good marker of a subset of monocytes from the bone marrow as well as its previous use as a marker of blood monocyte subsets. By contrast CD172a was expressed by all $CD14^+$ cells at high levels (56% of total gated cells). Gating on the larger, more granular cells of this population showed they had a higher expression of all the markers studied suggesting expression increased as the cells matured

3.2.2.2 Effects of CSF1 on expression of CD14, CD16, CD163 and CD172a

As discussed previously (**Figure 3.4**), addition of rhCSF1 caused an increase in the average size and granularity of bone marrow cells with time (**Figure 3.4A-C**). It also caused an increase in auto-fluorescence which is typical of differentiated macrophages and can be seen in the isotype control for BMCs cultured for 3 (**Figure 3.6G-J**) and 7 days (**Figure 3.6L-O**). Increased auto-fluorescence is probably due to increased numbers of lipid droplets in the mature macrophages and/or uptake of phenol red from culture medium. The increased number of mature, differentiated cells was reflected in the FSC vs. SSC plots (**Figure 3.6A, F, K**) where a new population of cells could be seen which was larger, more granular and more auto-fluorescent than the original population of BMCs.

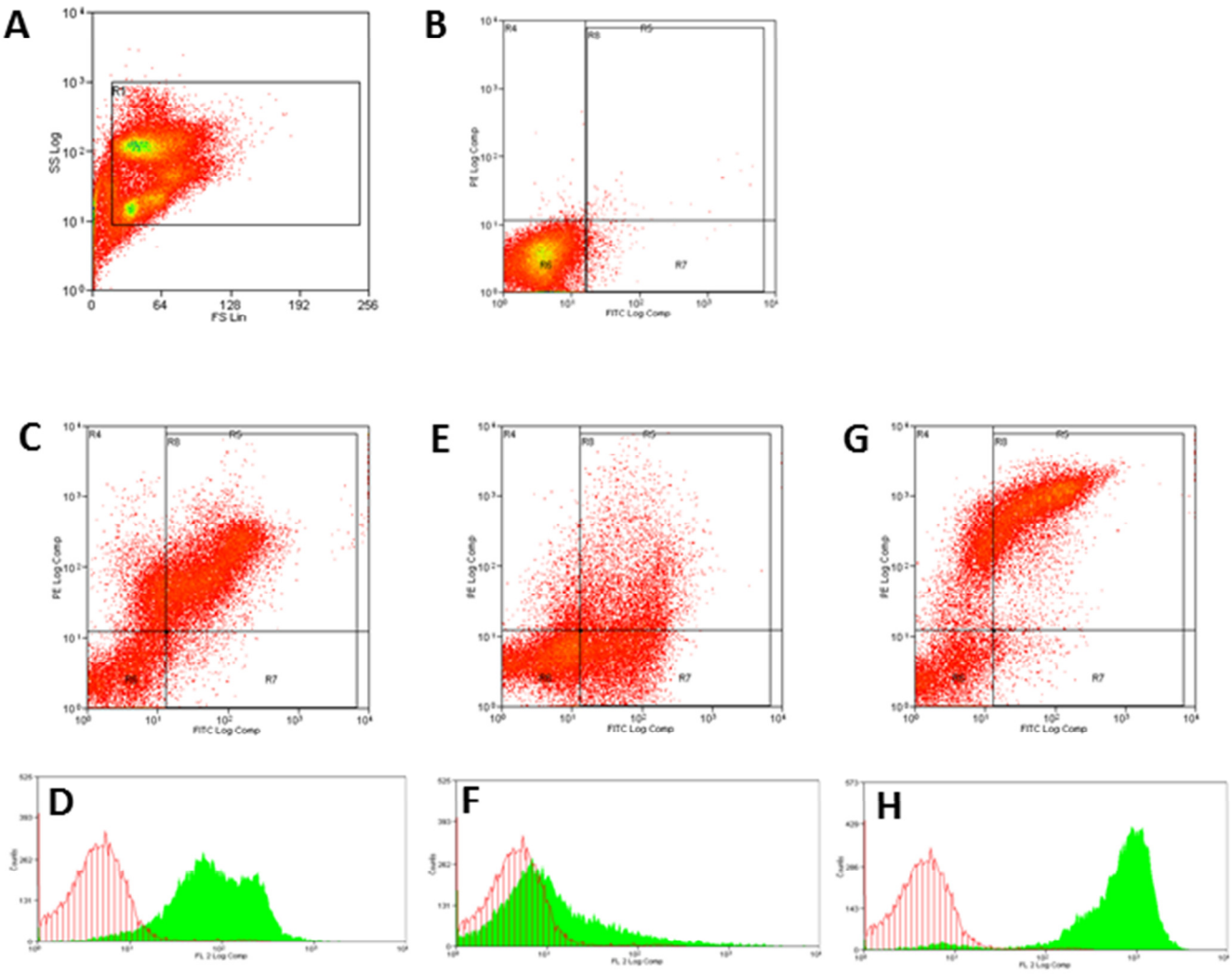

Figure 3-5 Cytometry characterization of freshly harvested BMCs.

Freshly harvested BMCs from young male Large-White x Landrace pigs were analysed by forward-scatter (FS Lin) and side-scatter (SS Log; **A**), stained with an isotype control (**B**) or antibodies CD14-FITC and CD16-PE/CD163-PE/CD172a-PE (**C**, **E**, and **G**, respectively). Graphs show CD16, CD163, or CD172a cells gated on the CD14 population isolated (**D**, **F**, and **H**, respectively).

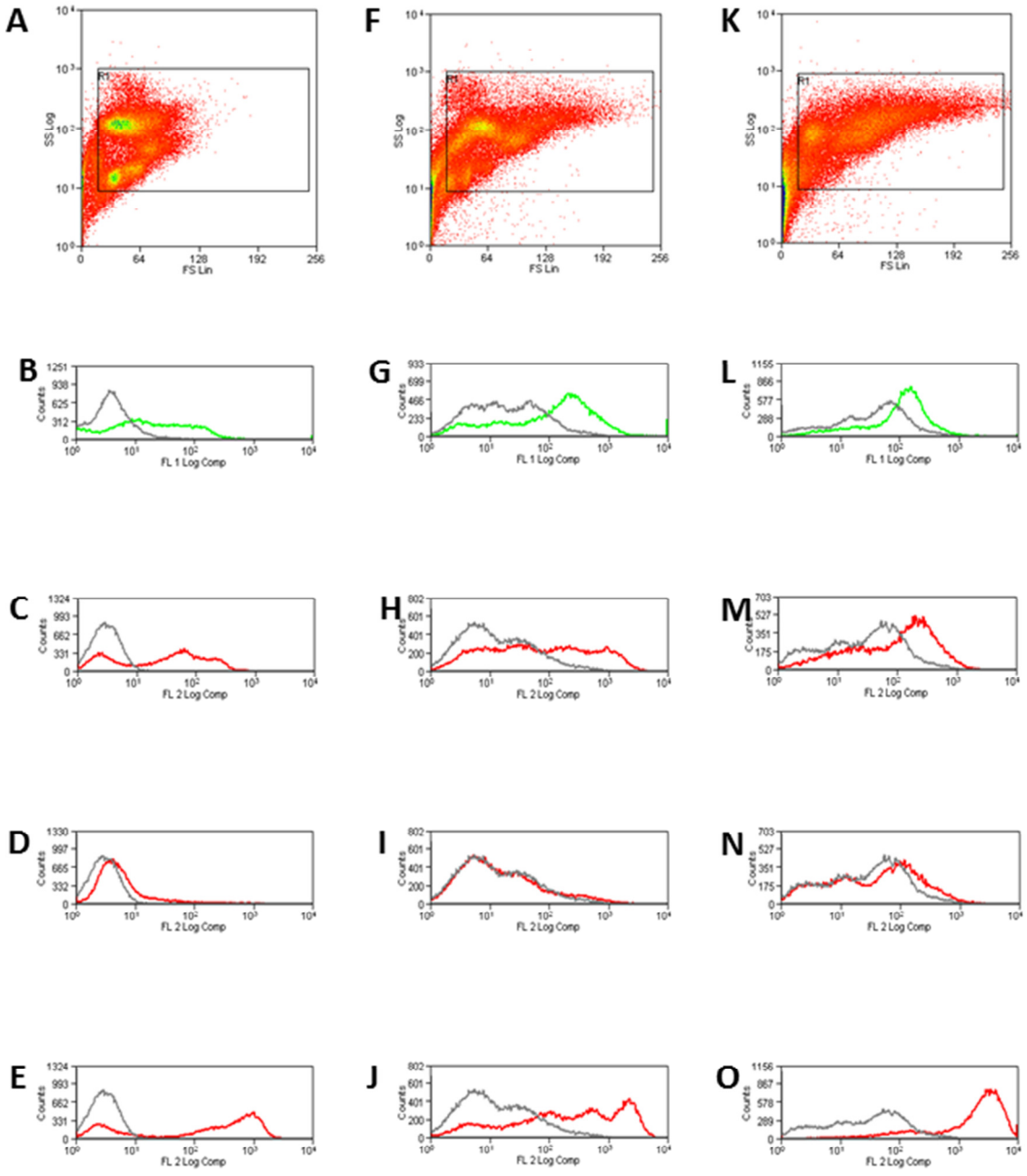

Figure 3-6 Cytometry characterization of BMCs grown in rhCSF1.

Freshly harvested BMCs (**A-E**), 3 day old (**F-J**) and 7 day old (**K-O**) MDM from young male Large-White x Landrace pigs were analysed by forward-scatter (FS Lin) and side-scatter (SS Log), and stained with an isotype control or antibodies CD14-FITC (**B, G, L**), CD16-PE (**C, H, M**), CD163-PE (**D, I, N**) or CD172a-PE (**E, J, O**) .

The increases in apparent cell size and granularity in response to rhCSF1 were associated with altered expression of cell surface markers detected by FACS. The classical monocyte marker CD14 increased after 3 days culture in rhCSF1 with the $CD14^{hi}$ population in particular increasing. This increase was associated with the appearance of larger cells after culture in rhCSF1 (**Figure 3.6G**). Furthermore additional culture in rhCSF1 increased the number of larger more granular cells (**Figure 3.6** k) which corresponded to a further increase in $CD14^{hi}$ cells (**Figure 3.6L**). Expression of the Fc receptor CD16, which is increased on mature human monocytes, was also increased by culture in rhCSF1. Freshly isolated bone marrow contained two populations of $CD16^{+}$ cells and a distinct population of $CD16^{-}$ cells (**Figure 3.6C**). As with CD14 culture in rhCSF1 increased the number of $CD16^{hi}$ cells (**Figure 3.6I, M**). As described above, CD163 is used as a marker of monocyte maturation, it is also expressed by some mature macrophages. Nevertheless culturing BMCs in rhCSF1 for up to 7 days did not greatly alter levels of expression of this marker. The proportion of $CD163^{+}$ cells at day 7 was very similar to what was observed in freshly isolated BMC (**Figure 3.6D, I, N**). As described above, CD172a is expressed by all myeloid cells. Some freshly isolated BMC were $CD172a^{-}$ although the majority of cells expressed high levels (**Figure 3.6E**). Culture with rhCSF1 increased levels of this marker (**Figure 3.6**) until after 7 days culture all cells were $CD172a^{+}$ (**Figure 3.6O**). Culturing pig BMC in rhCSF increased the expression of CD14, CD16 and CD172a and perhaps surprisingly did not increase levels of CD163 which has been noted by others to increase on blood derived monocytes as they mature (Chamorro et al., 2005; Sanchez et al., 1999). As discussed above, CD16 is seen as a marker of macrophage differentiation in humans and mice, it would appear that it can also be used as a marker of macrophage maturation in the pig.

3.2.3 Characterisation of porcine AMs

3.2.3.1 Expression of CD14, CD16, CD163, CD172a on AMs

Analysis of AMs isolated from rodents and humans has shown that lung macrophages are not a homogeneous cell population (Nicod et al., 1987; Shellito and Kaltreider, 1984). Subpopulations have been characterized by the use of density separation techniques in combination with mAb staining with different immunological functions assigned to these subpopulations (Spiteri and Poulter, 1991). Previous experiments have divided pig AMs by density (Zeidler et al., 1987). Expression of the scavenger receptor CD163 has been shown

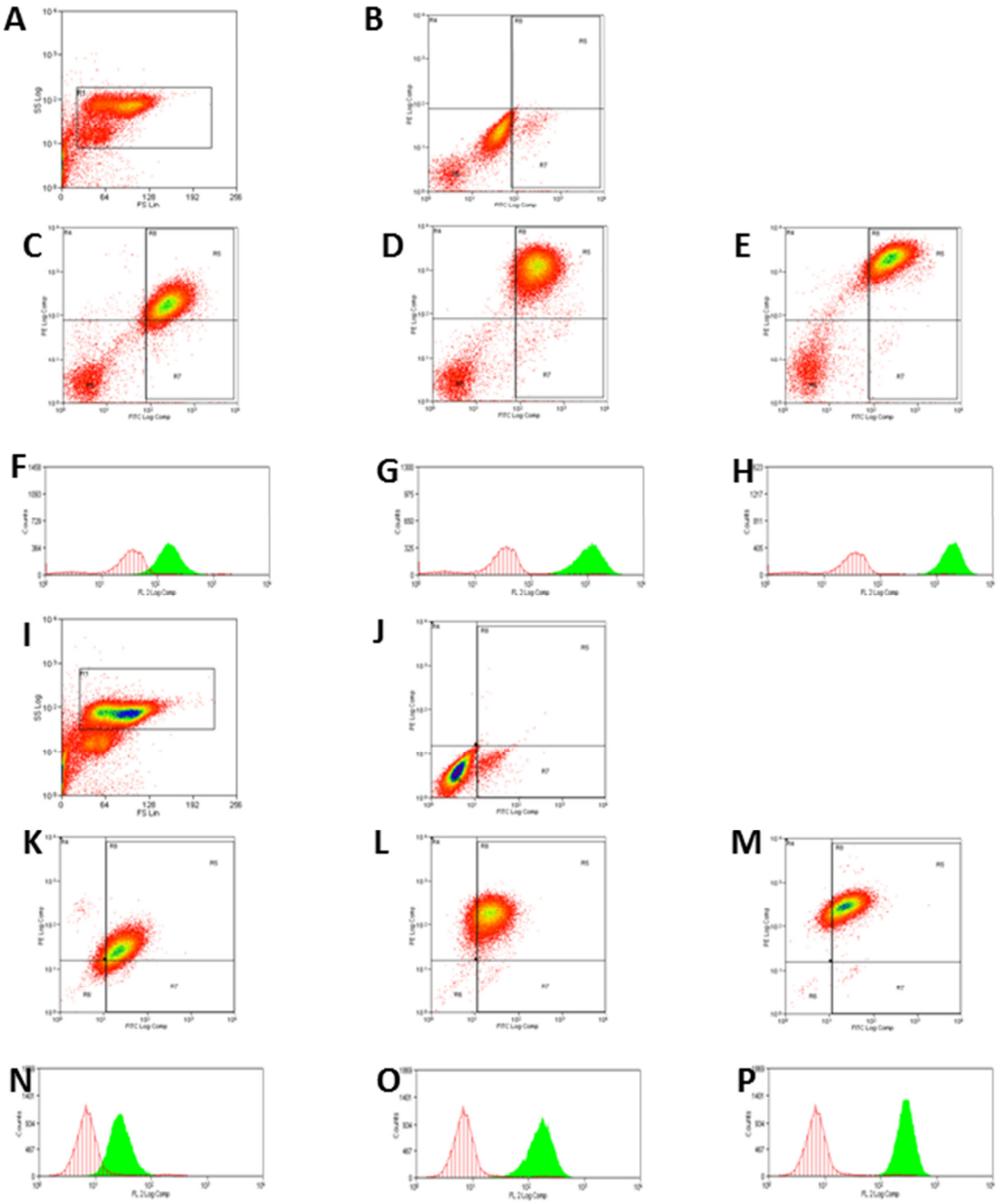

Figure 3-7 Cytometry characterization of freshly harvested AMs.

AMs, from young male Large-White x Landrace pigs were analysed by forward-scatter (FS Lin) and side-scatter (SS Log; **A, I**), stained with an isotype control (**B, J**) or antibodies CD14-FITC and CD16-PE/CD163-PE/CD172a-PE (**C&K**, **D&L**, and **E&M**, respectively). Graphs show CD16, CD163, or CD172a cells gated on the CD14 population isolated (**F&N**, **G&O**, and **H&P**, respectively).

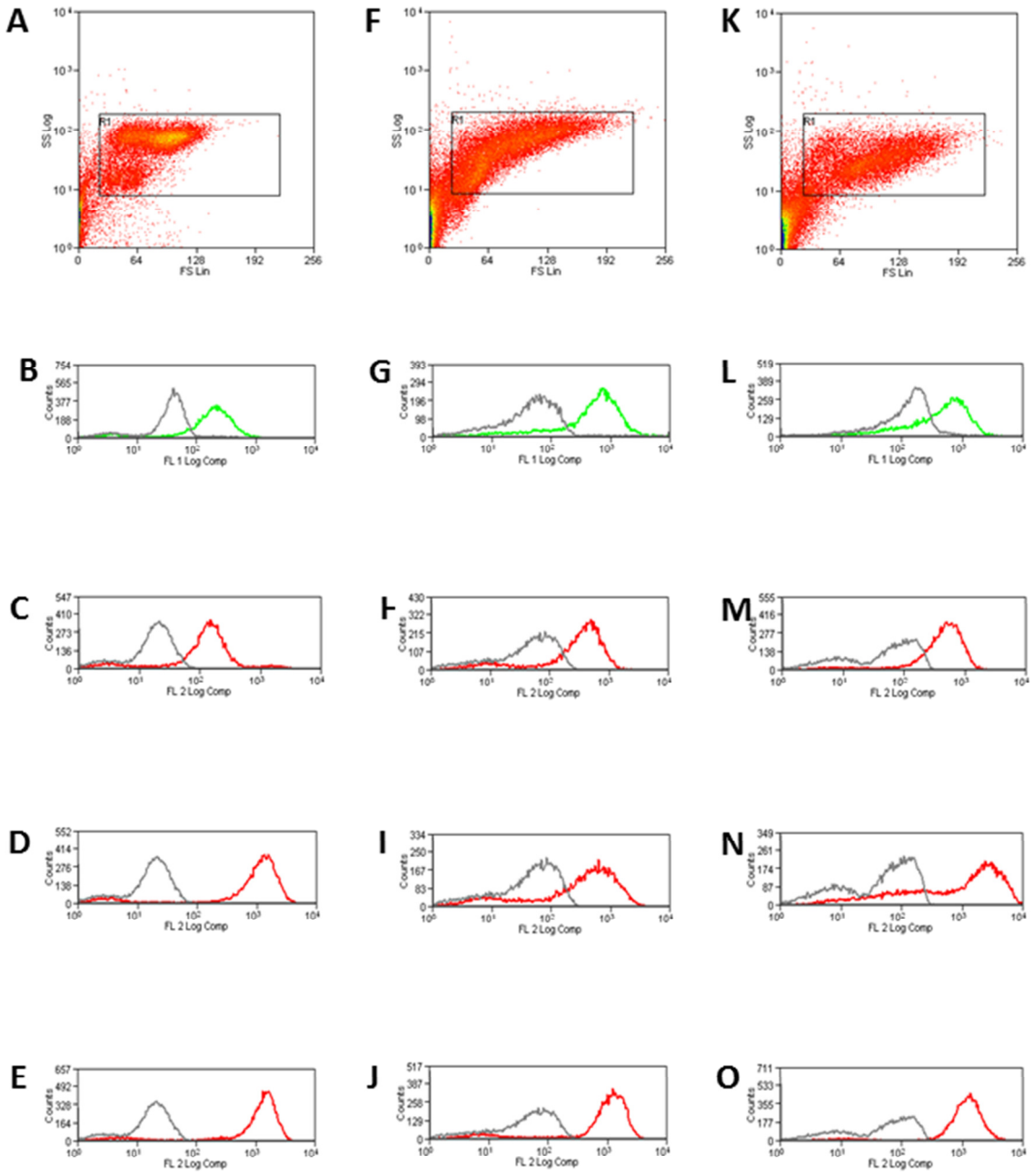

Figure 3-8 Effects of CSF1 on pig AMs.

Freshly harvested AMs (**A-E**), 3 day old (**F-J**) and 7 day old (**K-O**) AMs grown with rhCSF1 from young male Large-White x Landrace pigs were analysed by forward-scatter (FS Lin) and side-scatter (SS Log), and stained with an isotype control or antibodies CD14-FITC (**B, G, L**), CD16-PE (**C, H, M**), CD163-PE (**D, I, N**) or CD172a-PE (**E, J, O**) .

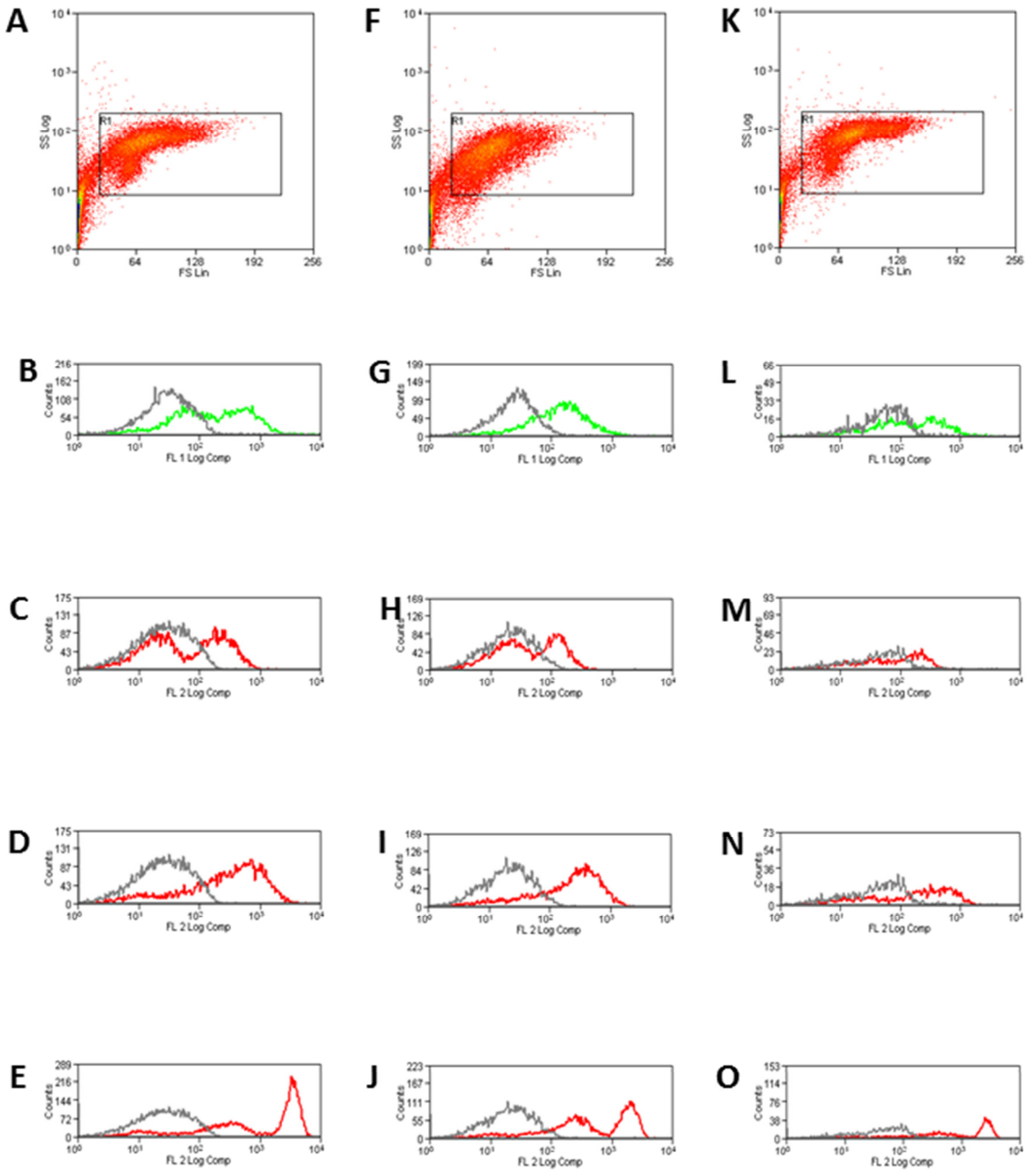

Figure 3-9 Effects of CSF1 inhibitor on pig AMs – day 3.

3 day old AMs grown in the absence of CSF1 (**A-E**), with CSF1 and inhibitor (**F-J**) without CSF1 and with inhibitor (**K-O**) from young male Large-White x Landrace pigs were analysed by forward-scatter (FS Lin) and side-scatter (SS Log), and stained with an isotype control or antibodies CD14-FITC (**B, G, L**), CD16-PE (**C, H, M**), CD163-PE (**D, I, N**) or CD172a-PE (**E, J, O**) .

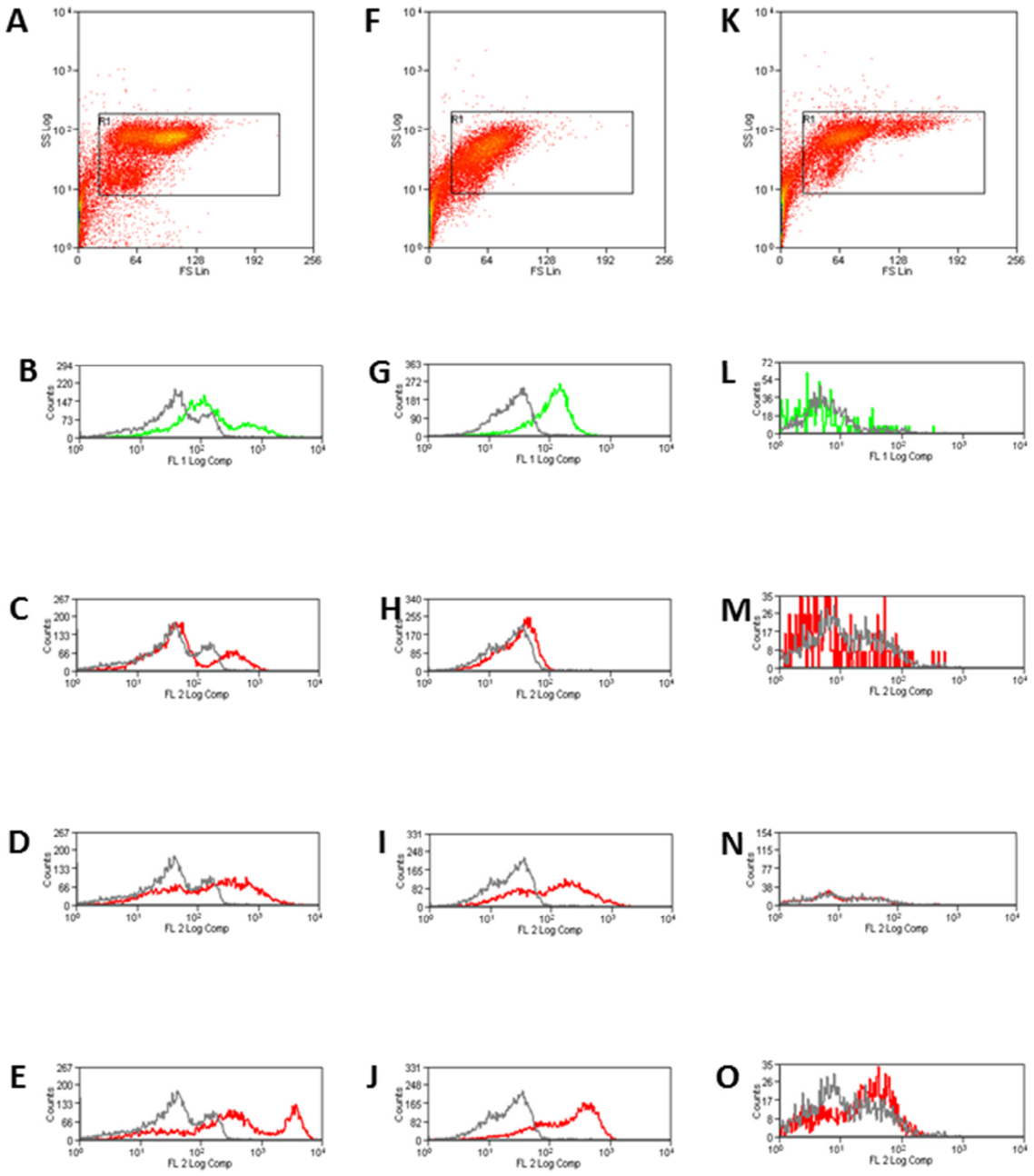

Figure 3-10 Effects of CSF1 inhibitor on pig AMs - day 7.

7 day old AMs grown in the absence of CSF1 (**A-E**), with CSF1 and inhibitor(**F-J**) without CSF1 and with inhibitor (**K-O**) from young male Large-White x Landrace pigs were analysed by forward-scatter (FS Lin) and side-scatter (SS Log), and stained with an isotype control or antibodies CD14-FITC (**B, G, L**), CD16-PE (**C, H, M**), CD163-PE (**D, I, N**) or CD172a-PE (**E, J, O**) .

to correlate with permissiveness to infection as discussed above (Sanchez-Torres et al., 2003; Calvert et al., 2007). To determine whether pig AMs showed similar heterogeneity to that observed in human and mice the expression of the immune markers CD14, CD16, CD163 and CD172a was analysed by FACS.

Freshly isolated AMs were composed of at least 2 populations when looking at FSC versus SSC plots. When looking at the porcine lung macrophages as a whole (**Figure 3.7A**) the majority of cells were $CD14^+$ $CD16^+$ $CD163^+$ $CD172a^+$ (**Figure 3.7C-E**). As with the differentiated BMCs, many of the cells were highly auto-fluorescent, as these cells were freshly isolated and had not been cultured it must be assumed this was due to increased lipid droplets within the cytoplasm of the macrophages. All of the $CD14^+$ AMs were also positive for CD16, CD163 and CD172a (**Figure 3.7F-H**). Like the BMC, it was the larger, more granular cells (**Figure 3.7I**) which were $CD14^+$ $CD16^+$ $CD163^+$ $CD172^+$ (**Figure 3.7K-P**). These are the mature tissue macrophages, the smaller $CD14^-CD16^-CD163^-CD172a^-$ were likely to be B-cells due to the absence of either CD14 or CD172a. This confirmed that porcine AMs are composed of two populations of cells although it seems unlikely the smaller population belongs to the macrophage lineage. The CD14+ cells were relatively homogeneous for marker expression suggesting that if there is two populations of AMs in the porcine lung then the expression of the immune markers studied is not sufficient to differentiate them

3.2.3.2 Effects of CSF1 on expression of CD14, CD16, CD163 and CD172a

As described in Chapter 1, GMCSF is necessary for the production or maintenance of alveolar macrophages. Although superficially healthy, the lungs of *Gmcsf* deficient mice do not develop normally (Stanley et al., 1994) ensuring that the mice suffer from a pathology very similar to that displayed by human patients suffering from alveolar proteinosis, a disease of the lungs caused by accumulation of surfactant proteins in the lungs, (Carey and Trapnell, 2010; Hibbs et al., 2007). Mice deficient in both *Gmcsf* and *Csf1* have a more severe phenotype than *Gmcsf* deficient mice alone suggesting an important relationship between these two growth factors in lung development (Lieschke et al., 1994). The CSF1 deficient

op/op mouse has a severe reduction in the numbers of macrophages in the lung although they are not completely absent (5-15% of normal lung macrophage numbers) (Wiktor-Jedrzejczak et al., 1992) Therefore to determine whether alveolar macrophage function in the pig was regulated by CSF1, expression of surface markers was examined on cells grown in the presence or absence of CSF1 and the selective CSF1R kinase inhibitor GW2580.

Culture of porcine AMs in rhCSF1 for up to 7 days increased their size and granularity but did not greatly alter the expression of CD14 (**Figure 3.8B, G, L**) or CD16 (**Figure 3.8C, H, M**). Expression of CD163 (**Figure 3.8D, I, N**) and CD172 (**Figure 3.8E, J, O**) decreased slightly. In particular the number of $CD163^{lo}$ cells increased. Suggesting CSF1 did not further differentiate porcine AMs

Mouse macrophages require CSF1 for survival. To assess the effects of rhCSF1 on survival of porcine AMs cells were grown in the presence of the selective CSF1R kinase inhibitor GW2580. After 3 days in rhCSF1 52% of cells analysed were alive while only 39% of cells grown with rhCSF1 plus GW2580 and 29% of cells grown without rhCSF1 and with GW2580 were alive showing rhCSF1 promoted survival of porcine AM. AMs would be expected to continue replicating in culture so these numbers refelect not just decreased survival but presumably decreased replication in the absence of CSF1. The inhibitor did not kill all the cells, suggesting either CSF1 is not the only signal that keeping porcine AMs alive or that the inhibitor did not completely inhibit the receptor. Cells grown without rhCSF1 (**Figure 3.9A**) or in the presence of the inhibitor GW2580 (**Figure 3.9F, K**) were smaller and less granular than cells grown with CSF1 (**Figure 3.8F**) after 3 days (**Figure 3.9A, F, K**). The effect was more marked after 7 days deprivation (**Figure 3.10A, F, K**) versus 7 days culture with rhCSF1 (**Figure 3.8K**). The expression of CD14 was lower in rhCSF1 deprived cells after 3 days (**Figure 3.9B, G, L**) as was CD16 (**Figure 3.9C, H, M**) and CD163 (**Figure 3.9D, I, N**) suggesting CSF1 promoted their expression. The effects on CD172a expression were not as marked (**Figure 3.9E, J, O**). Staining patterns for AMs grown in rhCSF1 for 7 days (**Figure 3.8K-O**) were very similar to those seen after 3 days growth indicating that additional culture did not lead to a further differentiated phenotype. Cells grown without

rhCSF1 expressed CD14, CD16, CD163 and CD172a although the population of $CD14^{hi}$ (**Figure 3.10B**), $CD16^{hi}$ (**Figure 3.10C**), $CD163^{hi}$ (**Figure 3.10D**) and $CD172a^{hi}$ (**Figure 3.10E**) cells was less than those grown with rhCSF1 (**Figure 3.8L-O**). AMs grown with the inhibitor for 7 days expressed CD14 (**Figure 3.10G**), CD163 (**Figure 3.10 I**) and CD172a (**Figure 3.10J**), albeit at lower levels, but CD16 expression had almost completely disappeared suggesting CD16 expression is CSF1 dependent (**Figure 3.10H**). CD16 is commonly used as a marker of mature macrophages and its loss may suggest a loss of the mature macrophage phenotype. The most marked phenotypic change was seen with cells grown in the absence of rhCSF1 and with the inhibitor for 7 days (**Figure 3.10L-O**). The majority of cells died and the ones that remained were $CD14^{-}$ $CD16^{-}$ $CD163^{-}$ and largely $CD172a^{-}$ .These cells were unlikely to be macrophages, since CD172a is expressed on all cells of myeloid origin. This also suggested that the inhibitor did not completely block all CSF1R activity as the presence of exogenous rhCSF1 led to a less severe phenotype. These results suggest that although porcine AMs are a fully differentiated cell type, mature AMs may require continuous CSF1 signaling. The inhibitor used in these experiments may also have off target effects therefore the reduced cell viability seen in AMs grown in the presence of GW2580 may have been due to blockade of growth factors other than CSF1. The use of further controls such as AMs grown in the presence of a range of inhibitor which target alternative receptors would have provided a clearer answer to which growth factors are necessary for survival of AMs. The presence of CSF1 did not alter the expression of the surface markers studied, nevertheless cells gown without an exogenous source of CSF1 or in the presence of a CSF1 inhibitor showed lower expression of all marker studied. The expression of these markers is likely to have functional significance therefore loss of markers can most likely be related to loss of function suggesting CSF1 would be required to maintain the *in vivo* functions of mature AMs in the pig.

3.2.4 Characterisation of porcine PM

3.2.4.1 Expression of CD14, CD16, CD163, CD172a on PMs

In this study, large numbers of PMs were isolated from the pig without the use of an eliciting stimulus (**Figure 3.11B**) enabling a model of non-inflammatory differentiated macrophages to be studied.

Freshly isolated PM expressed uniformly high levels of all markers examined. 90% of PMs were positive for both CD14 and CD16 (**Figure 3.11C**), 80% were $CD14^+$ $CD163^+$ (**Figure 3.11E**) and 91% were $CD14^+$ $CD172a^+$ (**Figure 3.11G**). This confirmed the presence of CD14, CD16, CD163 and CD172a on mature porcine PMs in addition to the AMs examined suggesting these markers may be present on many mature porcine macrophage populations. As with the AMs examined, this population was relatively homogeneous and phenotypically similar to AMs. Microscopic examination of porcine AMs and PMs showed visible differences in cell morphology however examination of surface marker expression showed striking similarity between these two tissue macrophage populations and further surface markers will be needed to differentiate the two populations by FACS.

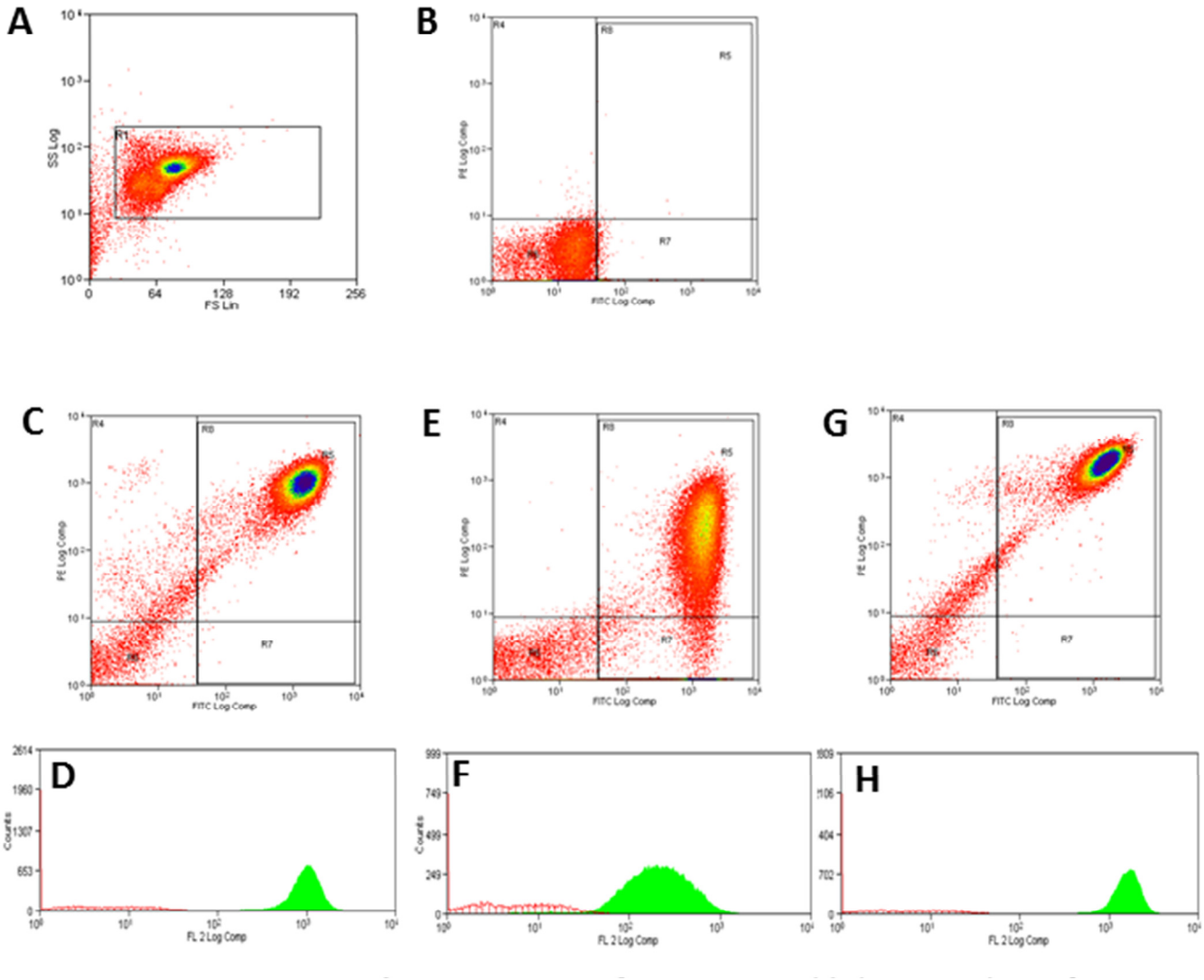

Figure 3-11 Cytometry characterization of pig PMs.

Freshly harvested PMs from young male Large-White x Landrace pigs were analysed by forward-scatter (FS Lin) and side-scatter (SS Log; **A**), stained with an isotype control (**B**) or antibodies CD14-FITC and CD16-PE/CD163-PE/CD172a-PE (**C**, **E**, and **G**, respectively). Graphs show CD16, CD163, or CD172a cells gated on the CD14 population isolated (**D**, **F**, and **H**, respectively).

3.2.5 Characterisation of porcine MDMs

3.2.5.1 Expression of CD14, CD16, CD163 and CD172a on pPBMCs from six different breeds

The level of $CD16^+$ monocytes varies considerably in man, whether due to ill health or natural variation between subjects. There is also considerable interest in humans in the association between genetic variation at the CD14 (Yu et al., 2011; Mason et al., 2010) and CD16 loci (Molokhia et al., 2011; Aitman et al., 2006; Fanciulli et al., 2007) and susceptibility to a wide range of inflammatory and infectious diseases.

Particular pig breeds are more sensitive to some viral and bacterial pathogens (Wang et al., 2011b; Reiner et al., 2010; Ait-ali et al., 2007), for instance pigs from the Landrace line are less susceptible to PRRVS and ASF. AMs are the primary cell target of PRRVS *in vivo* and expression of CD163 has been shown to confer susceptibility to PRRVS (Calvert et al., 2007) and to ASFV (Sanchez-Torres et al., 2003). AMs from the more resistant Landrace pigs showed no difference in the expression of the PRRVS receptor CD163 (Ait-ali et al., 2007)

To survey for possible genetic variation in these key receptors in pigs, PBMCs were isolated from at least two individual animals from 6 different breeds (animals detailed in appendix 3). This small number of animals precludes any conclusions being drawn from this experiment as true species variation could not be assessed. It was hoped however that any large differences between breeds would be picked up by this small sample All of the breeds studied had populations which were $CD14^-$ $CD16^+$ (presumed to be NK cells or immature monocytes), $CD14^+CD16^+$, $CD14^+$ $CD163^+$ and $CD14^+$ $CD172a^+$ although there was subtle variation between breeds. The percentage of cells in each population is summarized in **Appendix 3**. All animals showed differential expression of CD16 versus CD14 (**Figure 3.12C, 3.13C, 3.14C, 3.15C, 3.16C, 3.17C**), having a population which expressed high levels of CD14 and lower levels of CD16, hereafter called $CD14^{hi}$ $CD16^{lo}$, and another population

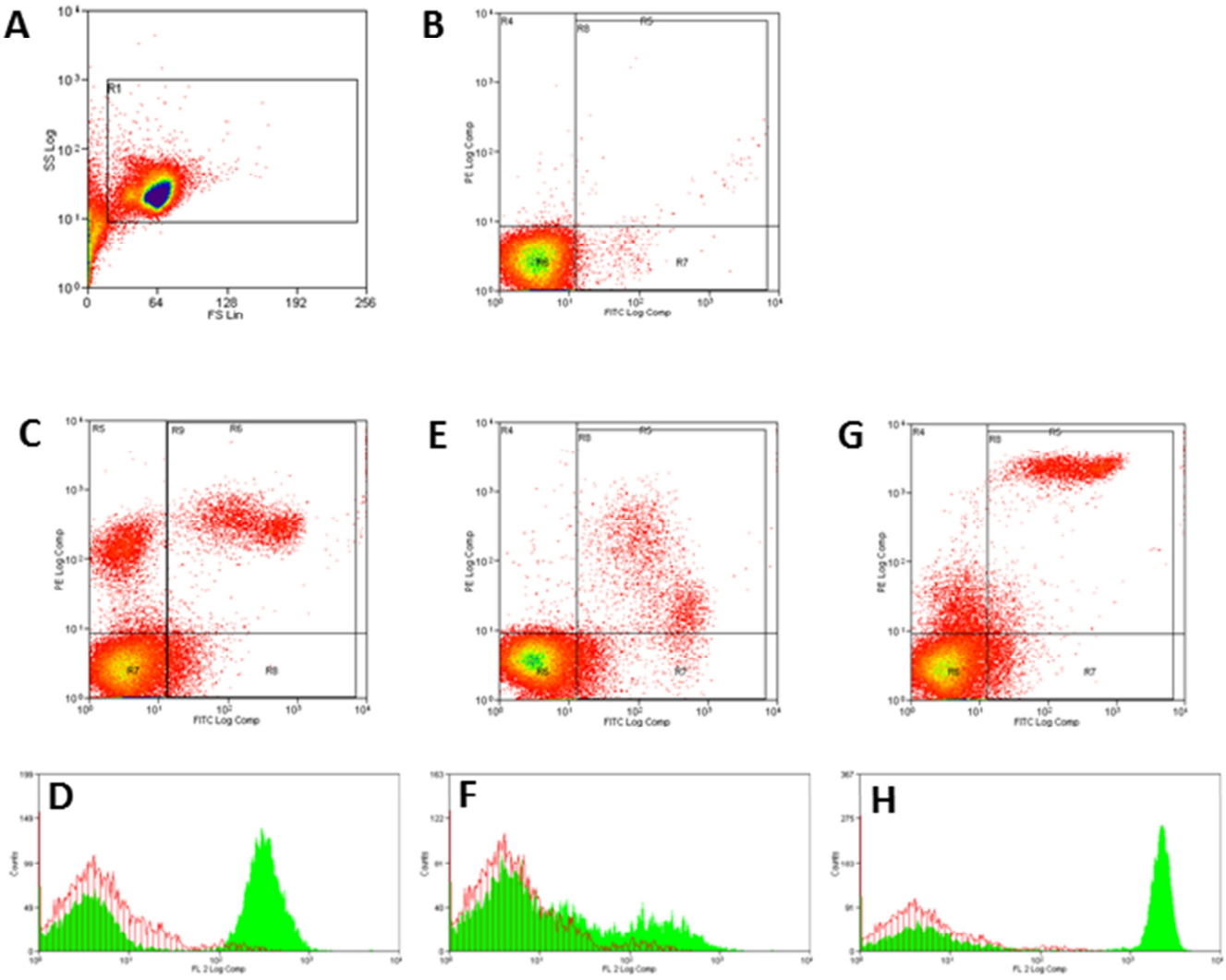

Figure 33-12 Cytometry characterization of freshly harvested PBMCs - Large-White x Landrace.

Freshly harvested PBMCs from young male Large-White x Landrace pigs were analyzed by forward-scatter (FS Lin) and side-scatter (SS Log; **A**), stained with an isotype control (**B**) or antibodies CD14-FITC and CD16-PE/CD163-PE/CD172a-PE (**C**, **E**, and **G**, respectively). Graphs show CD16, CD163, or CD172a cells gated on the CD14 population isolated (**D**, **F**, and **H**, respectively).

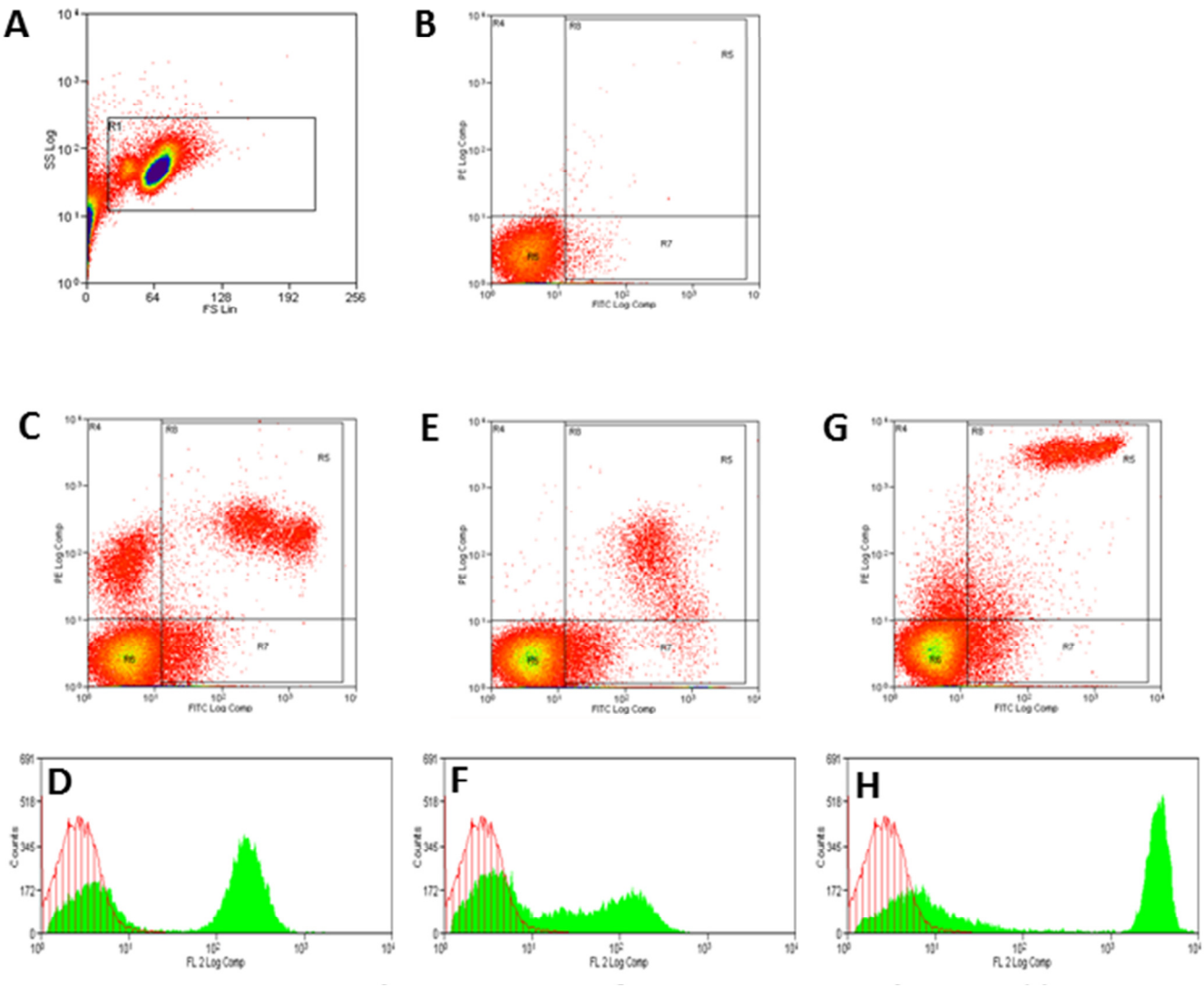

Figure 33-13 Cytometry characterization of pig PBMCs - Hamshire.

Freshly harvested PBMCs from young male Hampshire pigs were analyzed by forward-scatter (FS Lin) and side-scatter (SS Log; **A**), stained with an isotype control (**B**) or antibodies CD14-FITC and CD16-PE/CD163-PE/CD172a-PE (**C**, **E**, and **G**, respectively). Graphs show CD16, CD163, or CD172a cells gated on the CD14 population isolated (**D**, **F**, and **H**, respectively).

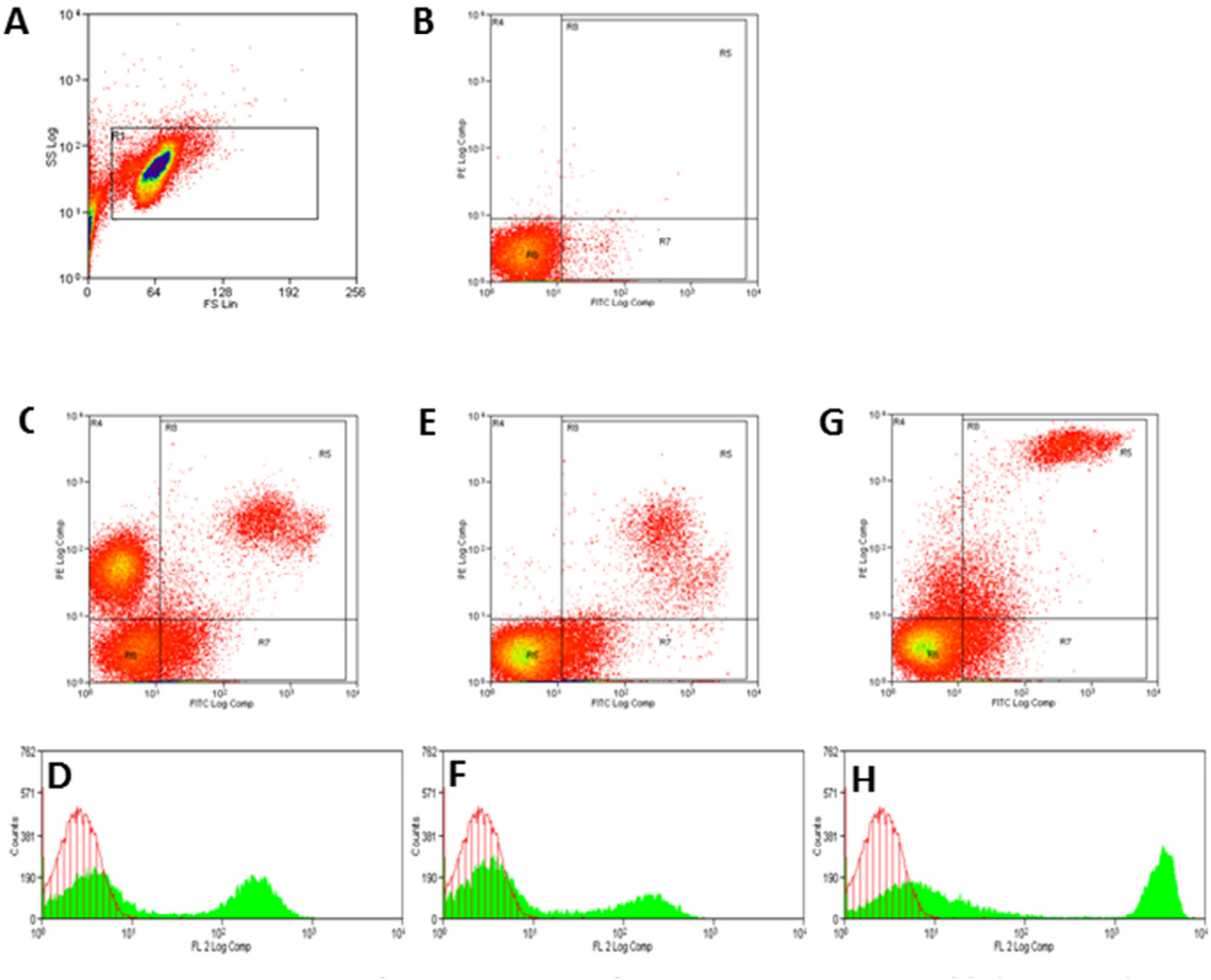

Figure 3-14 Cytometry characterization of pig PBMCs - Duroc.

Freshly harvested PBMCs from young male Duroc pigs were analyzed by forward-scatter (FS Lin) and side-scatter (SS Log; **A**), stained with an isotype control (**B**) or antibodies CD14-FITC and CD16-PE/CD163-PE/CD172a-PE (**C**, **E**, and **G**, respectively). Graphs show CD16, CD163, or CD172a cells gated on the CD14 population isolated (**D**, **F**, and **H**, respectively).

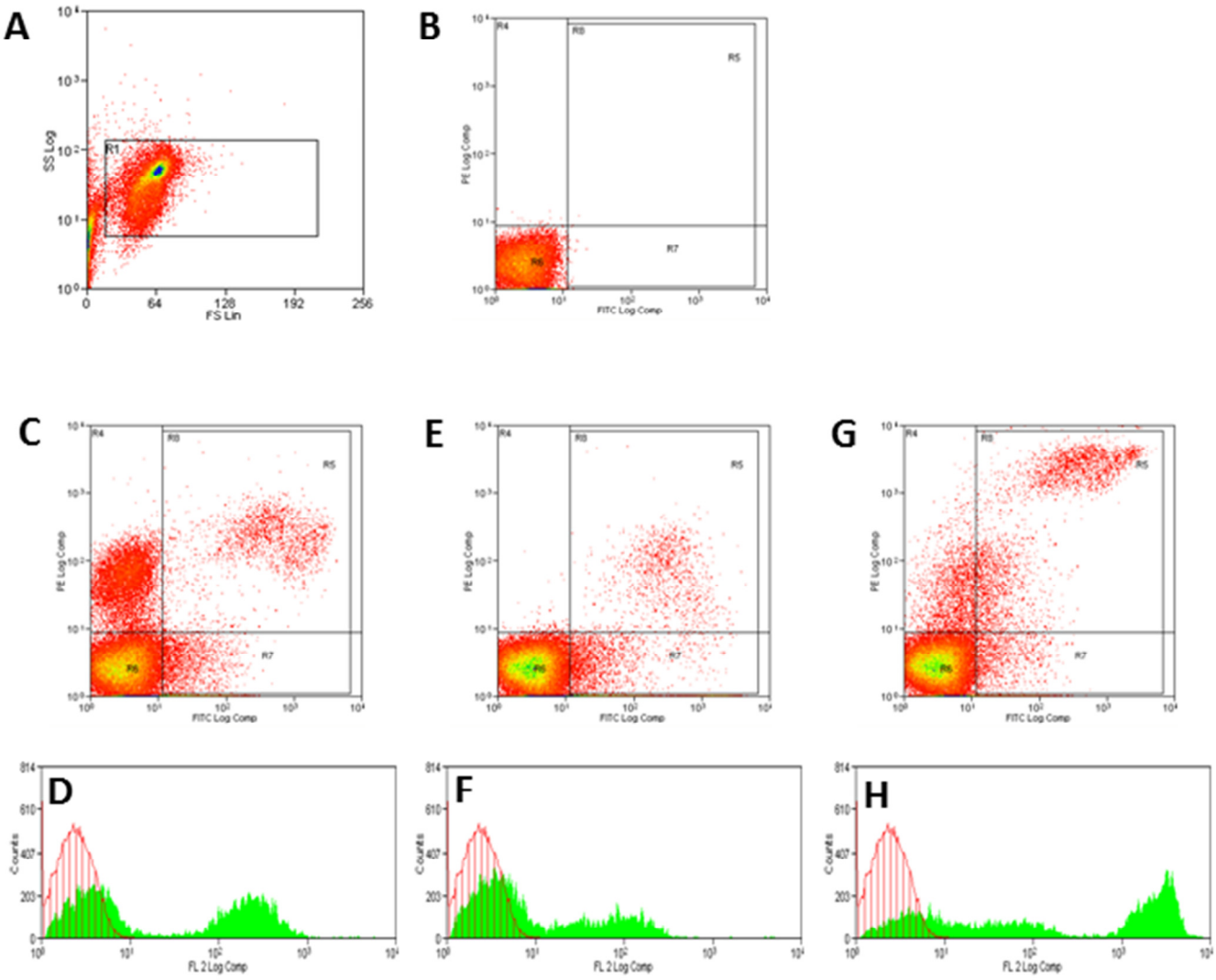

Figure 3-15 Cytometry characterization of pig PBMCs - Large White

Freshly harvested PBMCs from young male Large-White pigs were analyzed by forward-scatter (FS Lin) and side-scatter (SS Log; **A**), stained with an isotype control (**B**) or antibodies CD14-FITC and CD16-PE/CD163-PE/CD172a-PE (**C**, **E**, and **G**, respectively). Graphs show CD16, CD163, or CD172a cells gated on the CD14 population isolated (**D**, **F**, and **H**, respectively).

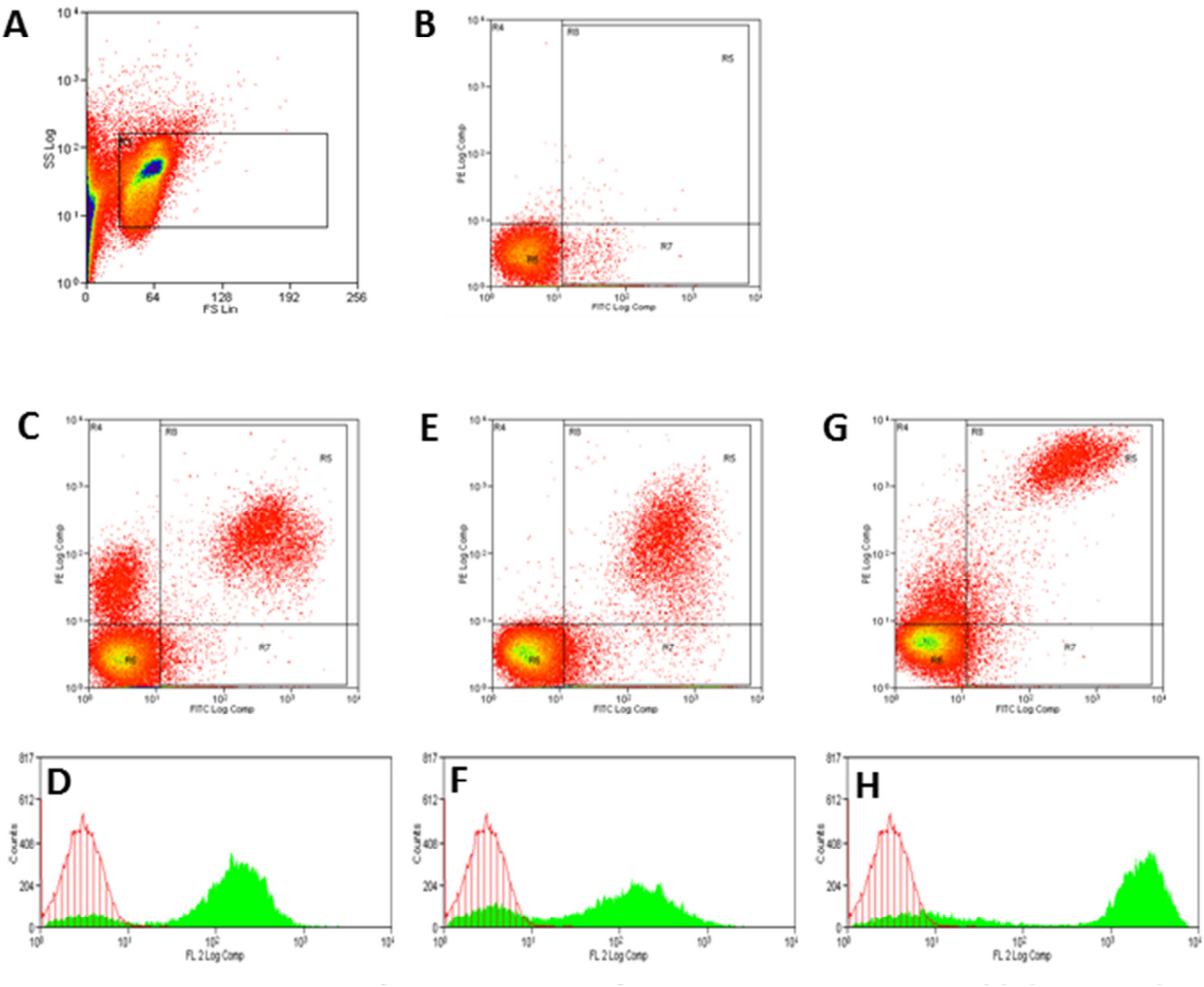

Figure 3-16 Cytometry characterization of pig PBMCs – Pietrain

Freshly harvested PBMCs from young male Pietrain pigs were analyzed by forward-scatter (FS Lin) and side-scatter (SS Log; **A**), stained with an isotype control (**B**) or antibodies CD14-FITC and CD16-PE/CD163-PE/CD172a-PE (**C**, **E**, and **G**, respectively). Graphs show CD16, CD163, or CD172a cells gated on the CD14 population isolated (**D**, **F**, and **H**, respectively).

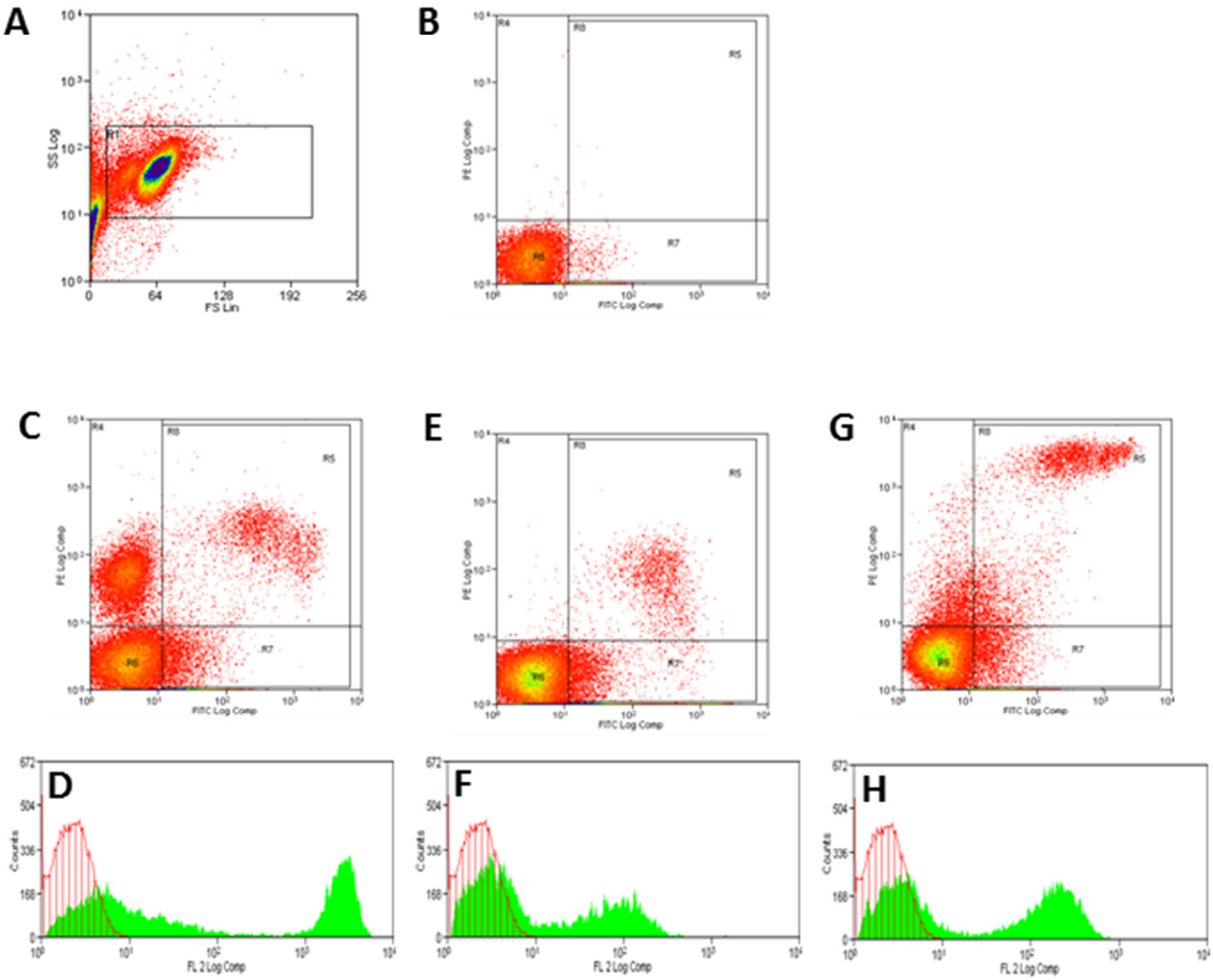

Figure 3-17 Cytometry characterization of pig PBMCs – Landrace

Freshly harvested PBMCs from young male Landrace pigs were analyzed by forward-scatter (FS Lin) and side-scatter (SS Log; **A**), stained with an isotype control (**B**) or antibodies CD14-FITC and CD16-PE/CD163-PE/CD172a-PE (**C**, **E**, and **G**, respectively). Graphs show CD16, CD163, or CD172a cells gated on the CD14 population isolated (**D**, **F**, and **H**, respectively).

which expressed lower levels of CD14 and higher levels of CD16, $CD14^{lo}$ $CD16^{hi}$. With some breeds e.g. Hampshire, there appeared to be more distinct populations (**Figure 3.13C**) while other breeds e.g. Pietrain (**Figure 3.16C**), had more of a continuum of cells with the extremes of the population expressing different levels of CD14 and CD16. Unlike humans, the $CD16^{+}$ population represented around half of the $CD14^{+}$ monocytes (**Appendix 3**). Similar variation was seen in CD163 staining as previously reported (Chamorro et al., 2000; Chamorro et al., 2004; Chamorro et al., 2005) (**Figure 3.12E, 3.13E, 3.14E, 3.15E, 3.16C, 3.17C**). CD172a expression showed little variation, all $CD14^{+}$ monocytes also expressed high levels of CD172a (**Figure 3.12G, 3.13G, 3.14G, 3.15G, 3.16C, 3.17C**). The variation in expression of CD163 on $CD14^{+}$ monocytes confirmed previous work in this field (Chamorro et al., 2004; Chamorro et al., 2000; Chamorro et al., 2005). Furthermore, in common with human and mice, this study showed pig monocytes can be divided into populations based on differential expression of CD16 by $CD14^{+}$ monocytes although how these populations relate to each other is unknown.

3.2.5.2 Effects of CSF1 on expression of CD14, CD16, CD163, CD172a

Like BMCs, PBMCs are a heterogeneous population of immature cells. Culture with growth factors such as CSF1 can cause the cells to differentiate into a mature macrophage population. As with BMCs, rhCSF1 caused PBMCs to increase in size and granularity (**Figure 3.18A, F, K**) and as previously an increase in auto-fluorescence was also observed (**Figure 3.18L-O)** compared to freshly isolated PBMCs (**Figure 3.18B-E)**. The increase in apparent cell size and granularity in response to rhCSF-1 was also associated with altered expression of cell surface markers. The new population of larger cells was predominantly $CD14^{+}$. (**Figure 3.18 A, B, G, L**). These cells also increased expression of CD16 (**Figure 3.18 C, H, M).** CD172a was expressed at high levels by a small proportion of freshly isolated

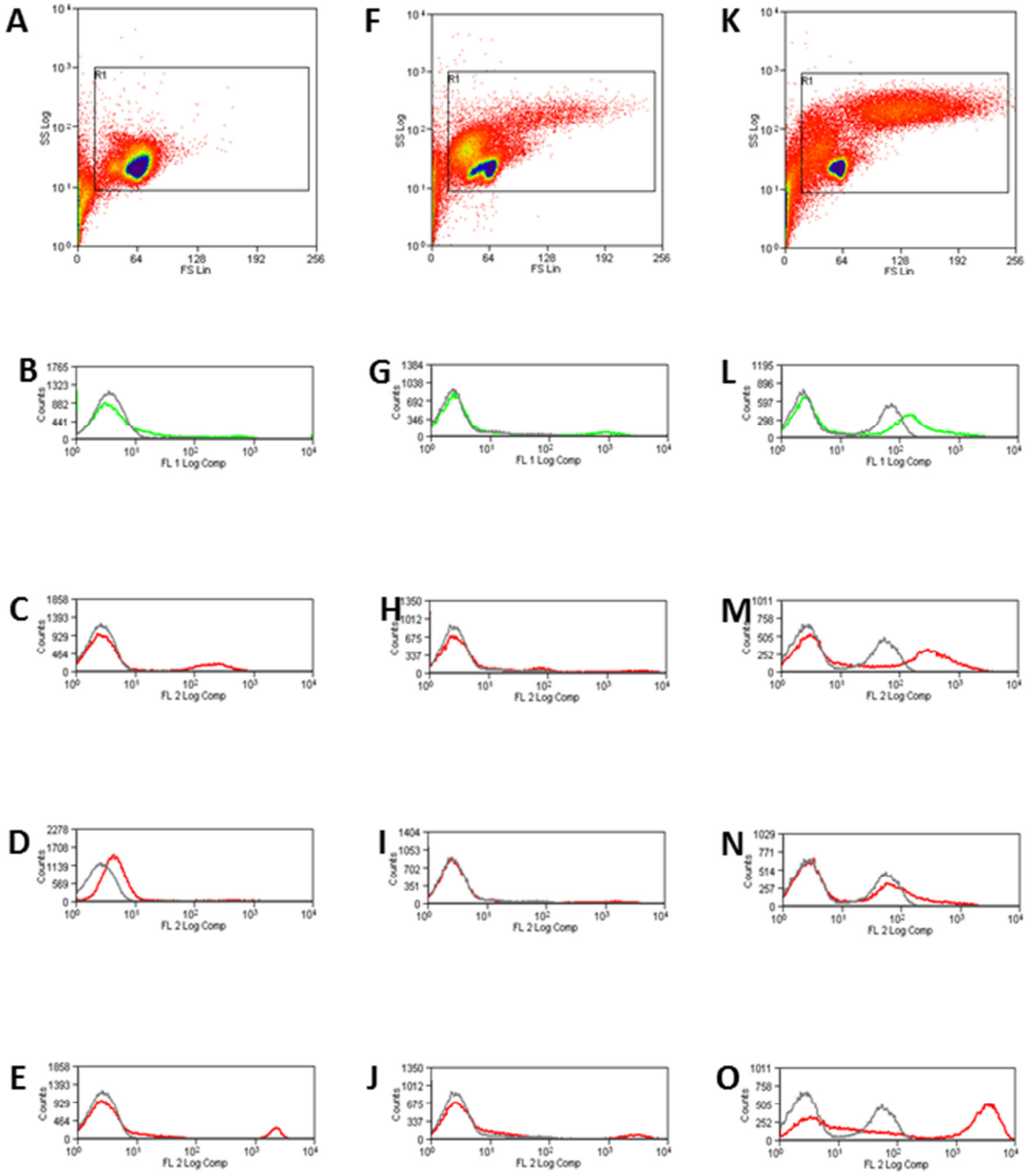

Figure 3-18 Effects of CSF1 on PBMCs.

Freshly harvested PBMCs (**A-E**), 3 day old (**F-J**) and 7 day old (**K-O**) MDM from young male Large-White x Landrace pigs were analysed by forward-scatter (FS Lin) and side-scatter (SS Log), and stained with an isotype control or antibodies CD14-FITC (**B, G, L**), CD16-PE (**C, H, M**), CD163-PE (**D, I, N**) or CD172a-PE (**E, J, O**) .

PBMCs (**Figure 3.18 E**), culture with CSF1 did not increase the intensity of expression but did however increase the proportion of the cells expressing CD172a (**Figure 3.18 O**). Perhaps most surprisingly given its use as a marker of maturation previously, CD163 expression decreased with culture in the growth factor CSF1 (**Figure 3.18 D, I, N)**. CSF1 marginally increased CD14 expression in human monocytes (Asakura et al., 1996) but it appears to have had a more marked effect on CD14 expression on pig monocytes and also increased expression of the maturation marker CD16.

3.2.6 Expression of surface markers on PBMCs from pigs with severe sepsis

PBMCs from pigs infected with *Actinobacillus pleuropneumoniae* that later developed severe sepsis were anlaysed for the expression of CD14, CD16, CD163 and CD172a to see whether, as in humans, there was a major change in the monocyte subpopulations in response to infectious challenge. This was an opportunistic experiment due to a mistake by another laboratory. A sample was obtained before the pigs developed sepsis but after surgical intervention (to fit monitoring devices). Ideally a sample would have been obtained when the pigs were healthy before any surgical intervention. Further control animals would have been desirable to assess the inflammatory effects of surgical intervention and the infection process, for example animals fitted with telemetry devices and not infected, animals undergoing mock infection with for instance heat killed bacteria and animals infected with gram negative bacteria whch are commonly found as commensal bacteria in the gut. Unfortunately the unplanned nature of this experiment meant these controls were not available. The number of $CD14^+$ monocytes which were negative for CD16, CD163 or CD172a was increased in freshly harvested PBMCs from minipigs compared to other breeds studied. The pigs had already undergone surgical intervention which may have changed the cellular composition of the blood, for instance by recruiting immature $CD14^+$ monocytes. Nevertheless, as previously (**Figures 3.12, 3.13, 3.14, 3.15, 3.16, 3.17**), a $CD14^-CD16^+$ NK or immature monocyte population was present and $CD14^+$ cells exhibited heterogeneous expression of CD16 and CD163. A more homogenous population of $CD14^{hi}$ or $CD14^{lo}$ and $CD172a^+$ cells (**Figure 3.19C-H**) was also present as observed in the healthy pigs from the 6 other breeds

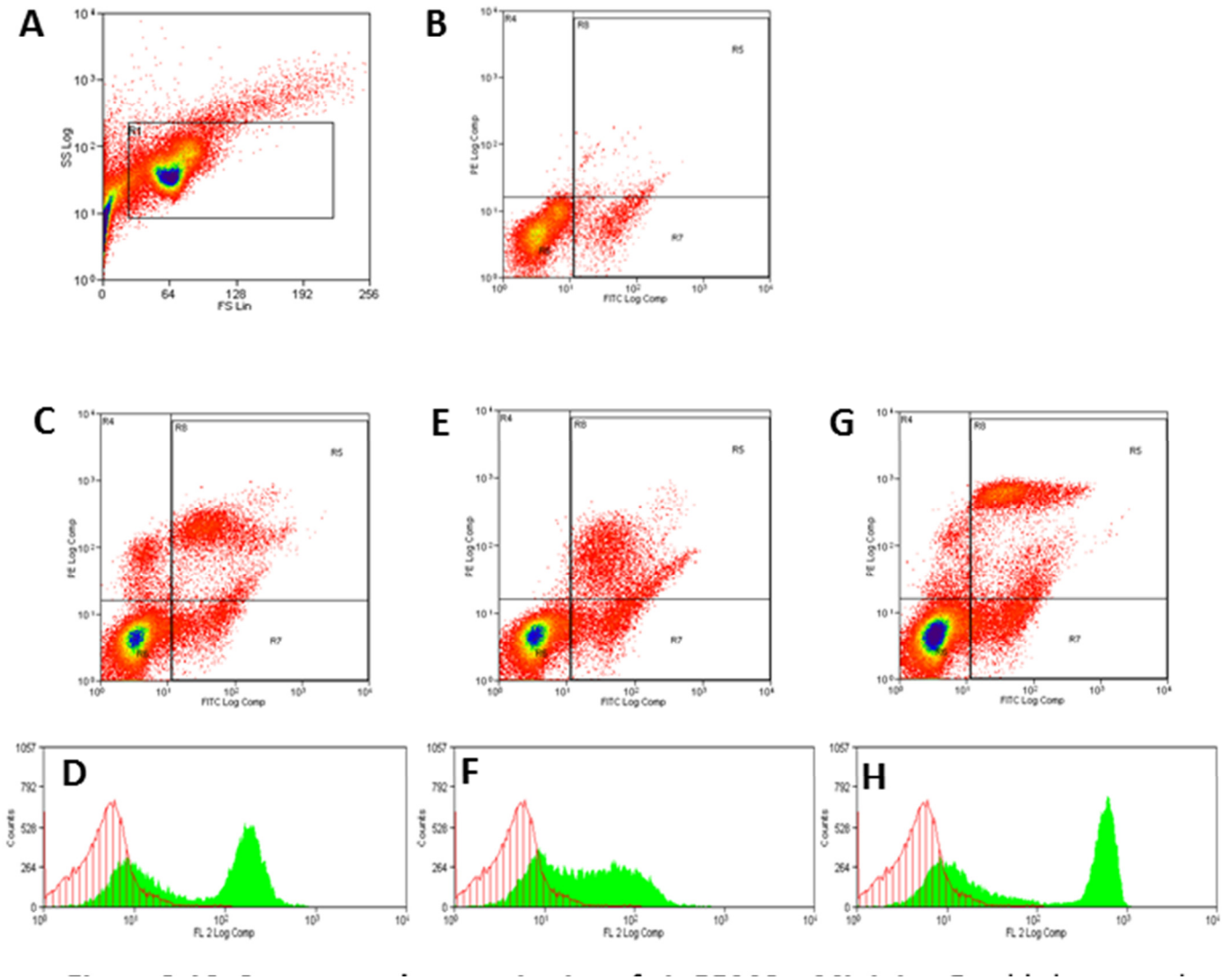

Figure 3-19 Cytometry characterization of pig PBMCs – minipigs

Freshly harvested PBMCs from young male minipigs were analysed by forward-scatter (FS Lin) and side-scatter (SS Log; **A**), stained with an isotype control (**B**) or antibodies CD14-FITC and CD16-PE/CD163-PE/CD172a-PE (**C**, **E**, and **G**, respectively). Graphs show CD16, CD163, or CD172a cells gated on the CD14 population isolated (**D**, **F**, and **H**, respectively).

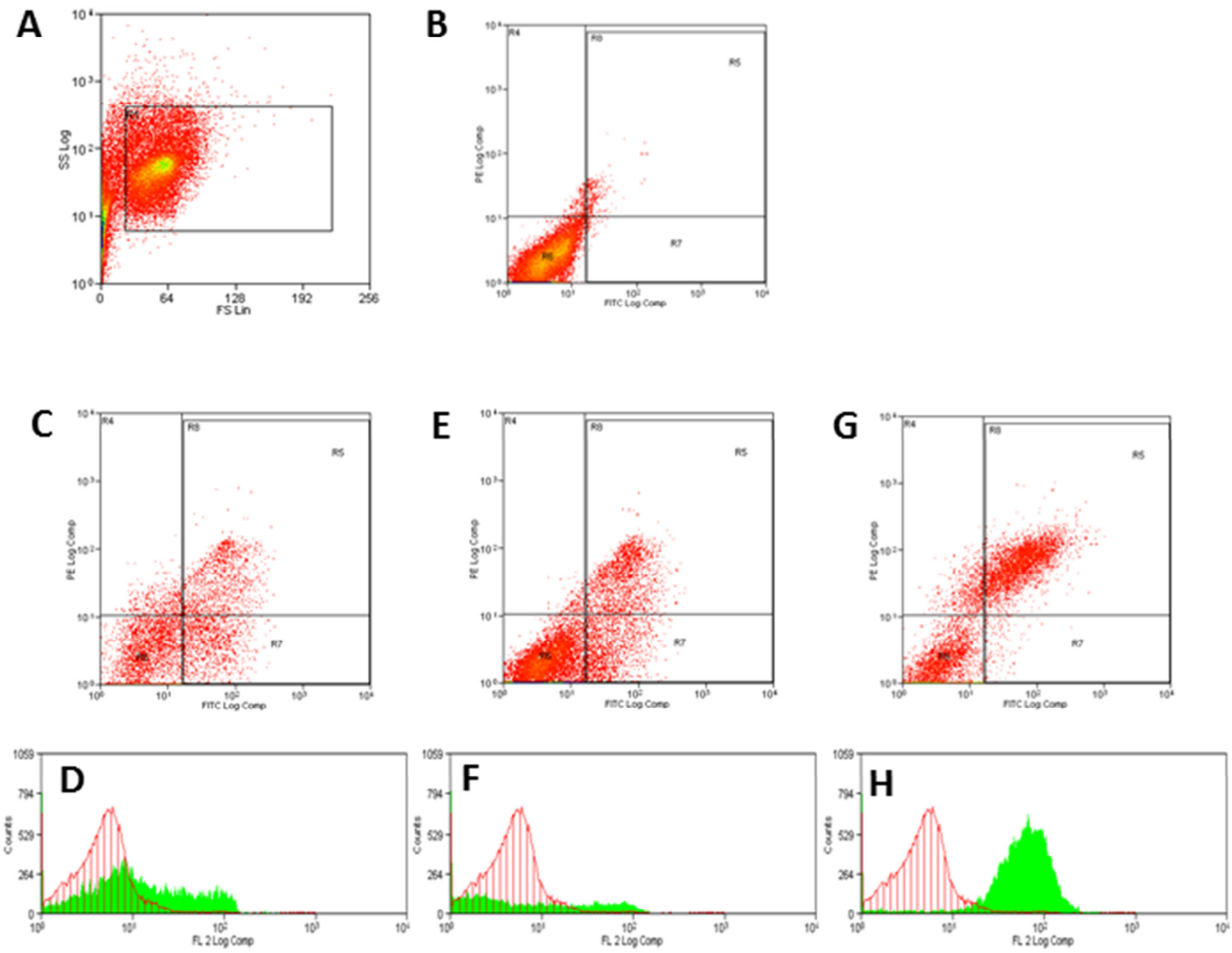

Figure 3-20 Cytometry characterization of PBMCs from minipigs suffering from Septic Shock.

Freshly harvested PBMCs from young male minipigs, infected with AP for 12 hours then euthanised, were analysed by forward-scatter (FS Lin) and side-scatter (SS Log; **A**), stained with an isotype control (**B**) or antibodies CD14-FITC and CD16-PE/CD163-PE/CD172a-PE (**C**, **E**, and **G**, respectively). Graphs show CD16, CD163, or CD172a cells gated on the CD14 population isolated (**D**, **F**, and **H**, respectively).

examined (**Section 3.2.5.1**). As expected the pigs were severely leukopenic after septic shock (**Figure 3.20C-H**). This experiment was only carried out once, on two animals without adequate controls meaning conclusions cannot be drawn, nevertheless the drop in circulating leukocytes serves as a good indication of the severity of septic shock against which to measure changes in gene expression (**Chapter 5**).

3.3 Discussion

The pig is an ideal animal for modelling human innate immunity. There are many benefits to using the pig as a model over the more commonly used rodent models, not least the large numbers of cells available from one animal (**Figure 3.3**). Mouse cells have been frozen previously to more easily enable the transportation of knock-out animals, derived from the transported cells, between research groups (Lattin et al., 2009). This study utilised a similar protocol to store porcine cells from blood, bone marrow, lungs and peritoneal cavity. Cells were frozen and stored at -80˚C enabling multiple experiments from the same animal to be carried out over a long period of time. This will enable inter-animal variability to be studied, something more difficult in the mouse due to the small number of cells harvested from each animal. Genes that vary between animals of the same species often rapidly evolve between species and may be important immune effectors (Murphy, 1993). Analysing gene expression from a single animal rather than pooled samples, as may occur with murine samples, will more readily allow the discovery of such inter-animal variability. It is important to note that generally speaking, pooling murine samples is not desirable as this will mask any inter-animal variation and make it impossible to spot any outliers which may skew results for the whole population. Research on primary human cells has often utilised blood derived monocytes or MDMs (Irvine et al., 2009). Research on these cells in the mouse is hampered by technical difficulties and the extremely small amount of blood available from a single animal. Mouse BMDMs are therefore frequently used (Sester et al., 2005) and although a useful tool they introduce another variable when trying to apply knowledge from animal studies to humans; comparing pig and human MDM removes any differences which may be

due to cell type rather than species. This study showed that cells can be harvested from the lung, peritoneal cavity, blood and bone marrow in the pig. Like for like comparisons can be made between porcine and human monocytes and monocyte derived macrophages in order to develop a better model for studying the human innate immune system. Thus the pig provides an ideal model for studying innate immunity in man. Immature monocytes and mature macrophages are readily harvested and available in large numbers facilitating the study of multiple human pathologies and will allow differences between monocytes/macrophages from different anatomical niches and in different states of activation to be studied in more detail.

3.3.1 Pig monocytes can be divided into subsets

As discussed previously, porcine blood monocytes can be split into subsets, previous groups have used differential expression of CD14 and CD163 to do so (Chamorro et al., 2000). Heterogeneous expression of CD16 has been noted but not studied in detail (Sanchez et al., 1999). This study showed that the differential expression of CD16 observed in both man and mice (Ingersoll et al., 2010) was conserved in pigs (**Figure 3.12-17**) although the proportion of $CD16^{+}$ monocytes was far greater in pig (**Appendix 3**) than in man where only around 10% of $CD14^{+}$ monocytes express CD16 (Passlick et al., 1989). In this aspect pigs appear more like mice having a near even number of $CD14^{hi}$ and $CD14^{lo}$ monocytes. There was also a population of monocytes which expressed very low levels of CD14 and did not express CD163 or CD16 (this can be seen most clearly in the Hampshire and Duroc, **Figures 313E & 3.14E**). It is not entirely clear how these monocyte populations relate to similar populations in man or mice, if at all. PBMCs from 6 different breeds of pig were examined for expression of CD14, CD16, CD163 and CD172a to detect possible genetic variation in these key immune receptors. As discussed above, variation in humans in *CD14* (Mason et al., 2010; Yu et al., 2011) and *CD16* (Fanciulli et al., 2007; Molokhia et al., 2011; Aitman et al., 2006) has been linked to susceptibility to disease. All breeds examined had variable expression of CD16 and CD163 and high

expression of CD172a on CD14 positive cells. As with $CD14^+CD16^+$ monocytes, the number of $CD14^+CD163^+$ cells was approximately half the total number of $CD14^+$ monocytes. The similar expression of these key immune markers between different pig breeds confirmed similar studies looking at monocyte markers in Meishan and Large White pigs (Clapperton et al., 2005). Moreover the slight heterogeneity between individual pigs was similar to the variation observed between humans (Passlick et al., 1989). There were no major differences in expression of any of the molecules examined although there was some variation, for example Hampshires and Durocs had a larger population of monocytes expressing very low levels of CD14. The small number of animals in this study limits any conclusions which may be drawn about breed specific genetic variation at any loci. A larger study may pick up individual variation at one or more of these loci which would almost certainly affect susceptibility to disease, as has been discovered in man (Aitman et al., 2006; Fanciulli et al., 2007; Molokhia et al., 2011; Yu et al., 2011). The number of $CD16^+CD14^-$ cells, which were presumed to be NK cells or possibly immature monocytes, also differed between animals. Like the expression of CD14 and CD16, this varied by animal rather than breed. NK cell phenotype in humans has previously been correlated to age, older subjects had lower levels of the more mature $CD3^-$ $CD56^{dim}$ NK cells which expressed only low levels of CD16 (Hayhoe et al., 2010). Expression of Killer cell Ig-like receptors (KIR), which inhibit and activate NK cell functions, has also been shown to be controlled at the genome level and varies between individuals (Shilling et al., 2002; Guinan et al., 2009). This ensures that the repertoire of NK cells is slightly different for each individual which will likely affect NK-cell mediated immunity. It appears there is similar variation in the NK cell population among pigs. In humans the $CD16^+$ population of monocytes can vary with health (Baeten et al., 2000; Blumenstein et al., 1997; Fingerle et al., 1993; Steppich et al., 2000; Ziegler-Heitbrock, 2007; Zimmermann et al., 2010; Allen et al., 1991). Unfortunately the number of pigs examined was too low to assess if the relative levels of each population, whether studied with CD16 or CD163, changed in the minipigs with the onset of sepsis. Severe leucopenia was present in the post-septic samples suggesting the samples were collected too close to natural death to assess relative levels of monocytes. Samples taken at an earlier time

point may have shown some differences in relative numbers of monocytes. The $CD14^{lo}CD16^{hi}$ population (**Figure 3.19C**) appeared larger in the minipigs prior to the development of sepsis. This initial sample was obtained after these animals had already undergone surgery to insert telemetry devices which itself would have caused an inflammatory response. This may suggest that the $CD14^{lo}CD16^{hi}$ population increases in an inflammatory response in the pig. The relative numbers of CD14 and CD163 positive cells appeared closer to what was observed in the additional breeds studied. Of the other pigs studied (**Figure 3.12-17**), all appeared healthy although post-mortem examination showed several had lesions on the lungs. Rather than showing an increase or decrease in one particular subset these pigs had less $CD14^{+}CD16^{+}$, $CD14^{+}$ $CD163^{+}$ and $CD14^{+}$ $CD172a^{+}$ cells overall. Unfortunately again the number of animals was too small to determine if the relative numbers of the monocyte subsets vary with disease in the pig and data on the health of the animals was not collected as they all appeared healthy pre-mortem. Monocyte subsets have also been shown to vary with age (Tacke and Randolph, 2006) and health in mice (Tacke et al., 2007) therefore it seems likely that a more detailed study may show variation in relative monocyte numbers in pigs.

3.3.2 Markers of monocyte maturation are common to humans, mice and pigs

CD163 was expressed selectively on $CD14^{lo}$ monocytes. These cells also expressed marginally higher levels of CD16. As discussed above, others have reported that $CD163^{+}$ pig monocytes were heterogeneous, with mainly low CD14 expression and high MHC II expression, similar to human $CD14^{lo}CD16^{+}$ human monocytes. $CD163^{+}$ cells expressed adhesion molecules, CD11a, wCD11c, wCD29 and CD49d, and co-stimulatory molecules, such as B7, at higher levels than $CD163^{-}$ cells (Chamorro et al., 2005; Chamorro et al., 2004; Ezquerra et al., 2009). This may have accounted for their strong antigen presenting ability and higher allostimulatory capacity, and could lead to differences in the ability of the cells to migrate to tissues. By contrast to humans, these "resident" type monocytes in the pig were approximately 50% of total monocytes (**Appendix 3**). Taken together, the data

suggest that by contrast to human and in common with mice, pig monocytes mature towards a "resident" phenotype to a greater extent in the circulation. Previously surface CD163 has been reported to be more highly-expressed in the $CD14^{lo}CD16^{hi}$ "resident" monocyte subset in human blood, and inducible in the $CD14^{hi}$ monocytes during CSF1-induced differentiation *in vitro* (Buechler et al., 2000). However recent studies have shown CD163 to be more highly expressed in the $CD14^{hi}CD16^{-}$ populations (Ingersoll et al., 2010; Cros et al., 2010) and expression can be induced in human $CD16^{+}$ monocytes by CSF1 (Tippett et al., 2011). As in humans, expression of CD163 in pigs is restricted to monocytes and macrophages and clearly defines a monocyte subset.

3.3.3 rhCSF1 induced differentiation of pig monocytes

Like mouse (Hume et al., 1987; Warren and Vogel, 1985; Hume and Gordon, 1983), pig monocytes could be differentiated into macrophages using rhCSF1 (**Figure 3.4**). As described above, CSF1 is present in the circulation and is important for macrophage differentiation, growth and survival. It is also induced in inflammatory states and has a key role in regulating macrophage activation (Sweet and Hume, 2003) therefore CSF1 differentiated macrophages are a good model for studying *in vivo* process *in vitro* (Pollard, 2009). Exogenous CSF1 was required for survival of mouse macrophages in culture although TEPMs, an inflammatory macrophage type, expressed *Csf1* mRNA and survived without a source of exogenous CSF1 (Irvine et al., 2006). *In vitro* human macrophages did not require CSF1 for survival due to autocrine production of CSF1 (Becker et al., 1987) although freshly-isolated monocytes did not express CSF1, (Biogps.org.info; (Hume and MacDonald, 2011)) suggesting expression was induced by culture conditions. rhCSF1 induced differentiation and promoted proliferation in pig monocytes but was not essential for survival once the cells had differentiated, suggestive of autocrine production by mature porcine macrophages (Kapetanovic et al, 2011, in press). In both mouse and human, culture in CSF1 alone promoted an immunosuppressive role for macrophages. However continuous exposure in humans drove macrophages towards a pro-atherogenic phenotype which was not replicated in mouse macrophages (Irvine

et al., 2009). CSF1 primed mouse macrophages to respond to LPS stimulation with a pro-inflammatory response but had no effect on the production of TNFα and IL6 by human macrophages. Instead it induced a set of pro-atherogenic genes including CXCL10, CCL2 and CCL7 and repressed the anti-atherogenic chemokine CXCR4 (Irvine et al., 2009). Similarly rhCSF1 did not increase LPS-induced TNFα production by pig (Kapetanovic *et al*. manuscript under review) suggesting like humans, CSF1 did not prime for a pro-inflammatory response in the pig. It should also be remembered that CSF1 is not present in the circulation in isolation so *in vivo* other factors may modify the extremes of phenotypes seen *in vitro.*

Culture with rhCSF1 caused porcine BMCs and PBMCs to increase in size and granularity which had a corresponding increase in expression of surface markers. Imature bone marrow progenitors expressed only low levels of CD14 and CD16 (**Figure 3.6B, C**) which confirmed findings in human (Passlick et al., 1989; Lee, 1991) and bovine bone marrow (Adler et al., 1994). Studies on human monocytes likewise reported an increase in CD14 expression as cells matured (Rigby et al., 1984), consistent with the findings of this study in the pig (**Figure 3.6B, G, L**). Furthermore CD14 was absent from monocyte precursor cell lines (Rigby et al., 1984) but found on more mature monocytic cell lines such as monomac 6 (Ziegler-Heitbrock et al., 1988) in agreement with its role as a marker of macrophage maturation. Macrophages derived *in vitro* from blood or bone marrow progenitors are frequently used to simulate the responses of tissue macrophages *in vivo*. The increase in expression of CD14, CD16 and CD172a suggests that rhCSF1 matured t pig monocytes into a more differentiated cell type, similar to AMs or PMs. One of the major benefits of pig over the more commonly used rodent models is the ability to compare macrophages/monocytes from different locations from the same animal. Pigs will allow us to more easily determine differences between immature monocytes, *in vitro* generated macrophages and mature tissue macrophages. Freshly isolated BMC did not express high levels of CD163 (**Figure 3.6D**) in common with previous findings (Sanchez et al., 1999). Furthermore rhCSF1-matured bone marrow or blood derived macrophages did not express high levels of CD163 (**Figure 3.6D, I,**

N and 3.18D, I, N) when compared to AMs (**Figure 3.8B**) or PMs (**Figure 3.11F**). CD163 has been used as a marker of monocyte maturation in the pig (Chamorro et al., 2005) is expressed on mature human macrophages (Fabriek et al., 2005) and was highly expressed by mature macrophages in this study and previous studies (Sanchez et al., 1999). The lack of CD163 on CSF1-derived macrophages is therefore surprising and highlights an important difference between *in vitro* generated pig BMDMs or MDMs and the naturally occurring tissue macrophages in this study. CD163 is expressed on a large number of macrophages, although not all tissue macrophages express CD163, for example macrophages from the marginal zone and white pulp of the spleen and those found in lymph node follicles are CD163 negative (Ezquerra et al., 2009)

There was an obvious difference in the size of immature and mature monocytes in men and pigs. Contrary to our study, where $CD16^{+}$ monocytes were larger and more granular than $CD16^{-}$ monocytes, human $CD16^{+}$ monocytes were smaller than their $CD16^{-}$ counterparts (Passlick et al., 1989) though like our cells they were more granular. This is not altogether surprising in man as promonocytes are larger than $CD14^{++}$ circulating blood monocytes which in turn are larger than the more differentiated $CD14^{+}$ $CD16^{+}$ blood monocytes (van Furth et al., 1979). It is uncertain why human monocytes should decrease in size as they differentiate while pig monocytes increase although it is clear that monocytes from both species undergo CSF1-driven differentiation which results in increased expression of the maturation markers CD14 and CD16.

3.3.4 Porcine AMs and PMs expressed high levels of CD14, CD16, CD163 and CD172a

The expression of CD14, CD16, CD163 and CD172a was universally high among porcine PMs and AMs. Previous investigations detected only low levels of CD16 in human PMs, differing from the high CD16 expression previously reported on human AMs (Passlick et al., 1989). Human peritoneal macrophages have been compared to CSF1-derived anti-inflammatory type II macrophages. Among other characteristics

they both expressed CD163 and lacked CD16, and after stimulation with LPS both macrophage types produced IL10 and down-regulated the co-stimulatory molecule CD86 (Xu et al., 2007). Human AMs also expressed CD163 as do most other differentiated macrophage populations (ZENG et al., 1996; Van den Heuvel et al., 1999; Fabriek et al., 2005) and high expression has previously been reported on AMs in the pig (Sanchez et al., 1999). Human PMs have been shown to phagocytose opsonized cells (MacGowan et al., 1983) although they are also able to phagocytose non-opsonized bacteria (Boner et al., 1989). Resident mouse PMs expressed low levels of the β-glucan receptor dectin1 which is important for the non-opsonic recognition of fungal derived β-glucans. In contrast mouse AMs expressed high levels of dectin1 (Taylor et al., 2002). The expression of CD16 by porcine PMs suggests that unlike human PMs they may phagocytose opsonized cells. Alternatively opsonized cells in the human peritoneal cavity may be cleared by other cells or the equivalent cells may not be opsonized at all and dealt with in a non Fc receptor-mediated manner. Pig PMs and AMs appeared relatively homogeneous, at least when comparing expression of surface markers. Nevertheless previous investigations have identified different pathways of immune activation. Porcine AMs stimulated with double stranded RNA (dsRNA) expressed high levels of type I IFNs, TNFα and RANTES. By contrast PMs stimulated with dsRNA expressed lower levels of type I IFNs but higher levels of the antiviral mediators protein kinase receptor (PKR) and myxovirus resistant, IFN-inducible GTPase (Mx) mRNA. The authors suggested AMs respond to dsRNA through a TLR3 dependent pathway while PMs respond through a PKR mediated pathway (Loving et al., 2006). Further studies using a broader array of surface markers may detect molecules specific to one type of macrophage and analysis at the genome level would almost certainly increase knowledge of the differences between these two mature macrophage populations in the pig.

3.3.5 Porcine AMs produce CSF1

Porcine AMs, a mature macrophage cell type, were largely positive for CD14, CD16, CD163 and CD172a upon isolation (**Figure 3.7)** and retained these markers up to 7 days in culture with rhCSF (**Figure 3.8**). Human AMs were reported to have much lower levels of CD14 but similar levels of CD16 (Passlick et al., 1989). As described above, mice deficient in *Gmcsf* did not show normal lung development (Stanley et al., 1994) while *Csf1* deficient mice have reduced numbers of lung macrophages (Wiktor-Jedrzejczak et al., 1992). Mice deficient in both *Gmcsf* and *Csf1* show a more severe phenotype than knockouts of either growth factor alone (Lieschke et al., 1994) demonstrating that both GMCSF and CSF1 have non redundant functions, in mice at least. AMs are a differentiated cell type and culturing them in the presence of rhCSF1 did not affect expression of surface markers to the extent that was seen with undifferentiated monocytes from bone marrow and blood suggesting that CSF1 did not regulate the function of already differentiated pig AMs. The loss of CD14 and CD16, and to a lesser extent CD163 and CD172a on AMs grown without CSF1 (**Figure 3.9A-E, Figure 3.10A-E**) or with a CSF1 inhibitor (**Figure 3.9F-J, Figure 3.10F-J**) suggested that although CSF1 did not change surface marker expression on already differentiated AMs it was necessary for maintenance of the differentiated phenotype. The expression of the surface markers was most reduced in AMs grown without rhCSF1 and with the inhibitor suggesting autocrine production of CSF1 was able to partially save the differentiated phenotype. This was validated by the increased rate of death among cells grown in the presence of the selective CSF1R kinase inhibitor GW2580 which blocked autocrine and exogenous CSF1(Irvine et al., 2006) although the survival of some AMs after 7 days culture in the presence of the inhibitor suggests it did not fully block CSF1R. As described above AMs would have been expected to continue replicating in culture so the increased cell numbers in the presence of CSF1 could also indicate replication that did not occur in the absence of CSF1. RNASeq data (unpublished, Dario Beraldi) showed constitutive expression of CSF1 at very low levels by AMs. The absence of an external source of CSF1 may have triggered a mechanism for increased expression in the AMs. The CSF1 inhibitor used in this study also inhibited FLT3. However it was likely that the effects reported here were

due to inhibition of CSF1, as FLT3L was expressed only at very low levels by AMs although it is possible that cells grown in the absence of CSF1 could have increased expression of FLT3L and quantification of FLT3L expression in the presence and absence of CSF1 would have answered this question. The AMs also failed to produce the alternative ligand for the CSF1R, IL34 (unpublished, Dario Beraldi). These findings suggest that porcine AMs can produce autocrine CSF1 and it may be required for maintenance of a mature phenotype. As stated above further experiments using additional inhibitors may have given a clearer picture of the requirements for AMs, for instance the *Gmcsf* knockout mouse has shown the importance of GMCSF in the developing lung so it would have been interesting to block the GMCSF receptor. It would also be interesting to determine if cells grown in the presence of an inhibitor could be rescued (ie. display the same phenotype as cells in normal culture conditions) by the addition of growth factors after a period of starvation or whether the addition of a GMCSF could rescue CSF1 starved cells and vice versa. The mechanism of renewal of AMs is not fully understood (Gordon and Taylor, 2005). AMs have been shown to be both self-renewing locally (Tarling et al., 1987) and to be replenished from the bone marrow (Thomas et al., 1976). It may be that the two populations of macrophages found in the lung fulfill different functions with one population proliferating to provide new cells while the others "mature" into the resident macrophages. Pulmonary macrophages obtained by enzymatic digestion of murine lungs could be divided into loosely adherent (LAMS) and firmly adherent mononuclear cells (FAMS). LAMs were more effective at stimulating allogeneic mixed lymphocyte reactions (MLR) than FAMs, AMs obtained by BAL or blood monocytes. LAMS were Fc receptor negative and poorly phagocytic (Nicod et al., 1987). These experiments showed that adherence to plastic can be a useful method of differentiation macrophage populations from the lung. More recent studies have assigned different immunological functions to different subpopulations separated by density, staining and adherence. Non-adherent cells had APC capacity while adherent cells expressed C3b and Fc receptors and were strongly phagocytic (Spiteri and Poulter, 1991). The AMs isolated from this study had remarkably similar expression of surface markers and FACS analysis did not show two clear populations of macrophages. The population of cells which were $CD14^{+}$

was remarkably homogeneous with regards to expression of CD16, CD163 and CD172a. Additional experiments in this laboratory utilizing differential adherence to plastic as previously described (Nicod et al., 1987) identified loosely adherent and firmly adherent macrophages and confirmed that expression of the markers studied are similar for both populations (unpublished, Ronan Kapetanovic). These differences in adherence are likely to relate to functional differences not reflected in the surface markers examined suggesting that as described in mice and humans, pig AMs are composed of two populations of cells.

Expression was examined for a few key molecules commonly used to study human monocytes which may also be used to differentiate mouse monocytes into subsets (Ingersoll et al., 2010). The work presented in this chapter shows that pig monocytes can be classified on the basis of CD14 and CD163 or CD16 expression. Work on phenotyping pig monocytes and macrophages is still in its infancy compared to what is known about human and mouse cells. Further studies are needed to increase our knowledge of monocyte subsets in the pig. The phagocytic ability of the different subsets is as yet unknown, as is the expression of other molecules found to have heterogeneous expression in human and mouse monocyte subsets, the lack of suitable antibodies makes this difficult for now. For instance previous work on CCR2 did not use specific anti-CCR2 antibodies but chemokine binding assay instead and RT qPCR was used to show differential expression of CCR2 and CX3CR1 rather than examination of expression at the protein level (Moreno et al., 2010). Previous work has shown variation between mRNA levels and protein expression (Ingersoll et al., 2010; Zhao et al., 2009) specific antibodies would enable differences identified at the mRNA level to be confirmed at the protein level. Genetic studies may identify new markers which could be useful for further phenotyping macrophage populations from different site within the pig. AMs, PBMCs and BMCs were differentiated with CSF1 for 7 days. However only AMS were cultured without rhCSF1 and in the presence of a CSF1 inhibitor. It would be interesting to study the effects of a lack of CSF1 on the other cell types. Production of a pig specific CSF1 antibody is also required before the importance of CSF1 in

monocyte maturation can be assessed in the pig. The rest of this thesis will focus on MDM and BMDM as these are the commonly used cells from man and mouse respectively.

Chapter 4: Analysis of porcine monocyte subsets

4.1 Introduction

Until recently the proposed homology between human $CD16^{hi}$ and mouse $Ly6C^{lo}$ monocytes was based mainly on the surface expression of a few molecules as described in **Chapter 3**. More recent research using a microarray approach to fully investigate the extent of any similarities has shown that a core set of genes is expressed selectively in the same pattern in the homologous subsets in both species (Ingersoll et al., 2010; Cros et al., 2010). The discovery of differential gene expression between the subsets lends credence to the hypothesis that distinct monocyte subsets may have conserved functions as has already been established with lymphocytes (e.g. Th_1, Th_2). Many human diseases involve alterations in either total or relative monocyte numbers (Baeten et al., 2000; Blumenstein et al., 1997; Ellery et al., 2007; Fingerle et al., 1993; Moniuszko et al., 2009). Discoveries of differences between monocyte subsets at the gene level provide possible targets for manipulating disease outcome or alternatively identify genes which may function as markers of disease progression. Studies on mice or other laboratory animals are therefore necessary to determine if the expression of genes which are selectively expressed in human monocytes are conserved in other species. Examination of monocyte gene expression in other species will therefore provide useful information which will enable better modelling of human inflammatory diseases.

4.1.1 Differentially expressed genes

The first efforts to dissect the functions of monocyte subsets looked for genes which were differentially expressed. Two studies from 2009 (Zhao et al., 2009; Ancuta et al., 2009) found clear differences in the gene expression profiles of human monocyte subsets and reported a whole host of enriched functions or pathways in each subset. Functions consistently assigned to human monocyte subsets are summarised in

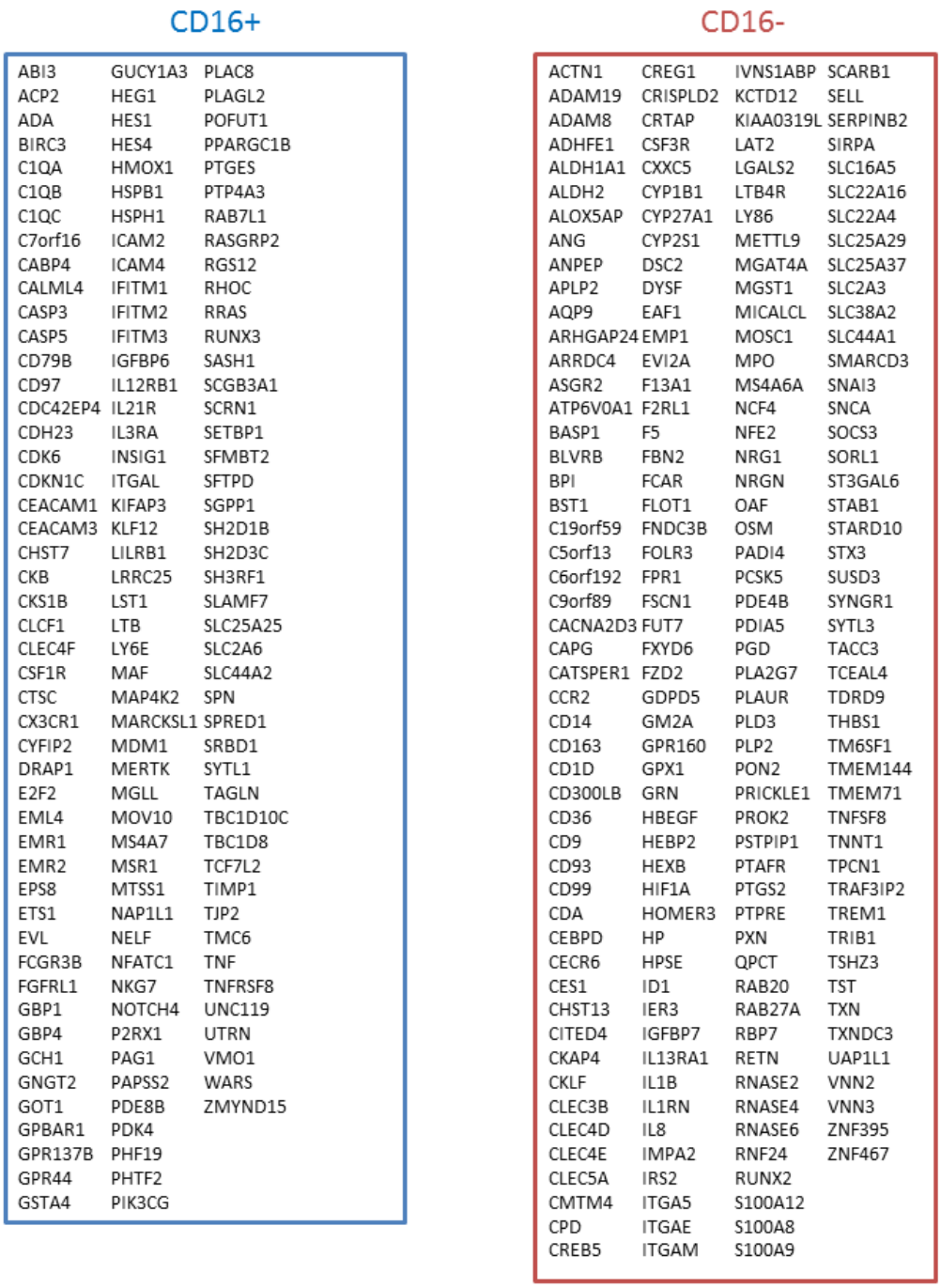

Figure 4-1 A core set of genes were consistently expressed preferentially by one monocyte subset

Data from Ancuta *et al.* (2009), Ingersoll *et al.* (2010) and Zhao *et al*. (2009) was re-analysed to find genes which were preferentially expressed in the same pattern in all studies.

Table 4.1. Among other functions Zhao *et al.* suggested that $CD16^+$ monocytes were enriched for genes involved in FcγR-mediated phagocytosis, B cell receptor signalling and apoptosis signalling while $CD16^-$ monocytes expressed genes associated with antimicrobial function and IL8 signalling. Ancuta *et al.* found similar differential expression including the granulocyte colony-stimulating factor receptor (*CSF3R, GCSFR, CD114*) which was expressed more highly by $CD16^-$ monocytes, and *CSF1R* which was more highly expressed by $CD16^+$ monocytes. They suggested that $CD16^+$ monocytes expressed higher levels of genes related to NK cell mediated toxicity, actin binding and oxidative stress. $CD16^-$ monocytes were enriched in genes relating to hematopoietic cell lineage, receptor mediated endocytosis and lipid binding molecules. Re-analysis of data from Ancuta *et al.,* Zhao *et al.,* and Ingersoll *et al.* identified 140 genes which were preferentially expressed by $CD14^{lo}CD16^+$ monocytes and 196 which were more highly expressed by $CD14^{hi}CD16^-$ monocytes in all three studies (**Figure 4.1**). Use of the online bioinformatics resource DAVID (the Database for Annotation, Visualization and Integrated Discovery) (Huang et al., 2008; Andersson et al., 2000; Huang et al., 2009) showed $CD14^{lo}CD16^+$ were enriched for genes involved in negative regulation of immune functions, regulation of apoptosis, the inflammatory response, regulation of cytokine production and the complement pathway. $CD14^{hi}CD16^-$ monocytes were enriched in genes involved in signalling, wound healing and chemotaxis (**Table 4.1**). The full gene lists can be found in **Appendix 5.** Interestingly Ancuta *et al.* also identified a third, intermediate phenotype of $CD14^+CD16^+$ monocytes which expressed both CD14 and CD16 and intermediate levels of many other surface markers such as *CSF1R* and *CSF3R*. These "intermediate" monocytes have become part of the accepted nomenclature of human monocyte subsets (Ziegler-Heitbrock et al., 2010) and corresponding subsets have been identified in macaques (Kim et al., 2010). The most recent studies have therefore examined gene expression between all three subsets of human monocytes; classical $CD14^{hi}CD16^-$, non-classical $CD14^{lo}CD16^{hi}$ and intermediate $CD14^+CD16^+$ monocytes (Wong et al., 2011; Cros et al., 2010). Like the gene expression data examined above, Wong *et al.* found classical monocytes to be enriched for genes associated with wound healing and coagulation, response to stimuli and

Monocyte subset	Functions	Reference
Classical $CD14^{hi}CD16^{-}$	Wound healing, coagulation, response to infectious stimuli, angiogenesis	(Wong et al., 2011; Ingersoll et al., 2010; Ancuta et al., 2009; Zhao et al., 2009)
Intermediate $CD14^{+}CD16^{+}$	MHC Class II processing and presentation	(Wong et al., 2011)
Non-Classical $CD14^{lo}CD16^{hi}$	Cell movement, Fc receptor mediated phagocytosis, complement components	(Wong et al., 2011; Ingersoll et al., 2010; Ancuta et al., 2009; Zhao et al., 2009)

Table 4.1 Suggested functions of human monocyte subsets
Adapted from (Wong et al., 2011)

angiogenesis; intermediate monocytes were enriched for genes associated with MHC class II processing and presentation and non-classical monocytes expressed genes associated with cell movement, Fc receptor mediated phagocytosis and complement components (**Table 4.1**). All four microarray approaches (Wong et al., 2011; Ingersoll et al., 2010; Ancuta et al., 2009; Zhao et al., 2009) found broad similarities between genes preferentially expressed by classical monocytes. Wong *et al.* suggested the broad range of genes associated with classical monocytes showed they were highly versatile cells capable of responding to a variety of external cues while non-classical monocytes expressed many genes involved in cytoskeletal rearrangement which may explain their patrolling function described previously (Cros et al., 2010). There was however more disagreement in genes preferentially expressed by $CD16^{+}$ monocytes, Wong *et al.* (2011) subdivided this group into non classical $CD14^{lo}$ and intermediate $CD14^{+}$ monocytes making comparisons between previous studies difficult; it is not clear with previous reports whether intermediate monocytes would have fallen into the classical or non-classical subsets. Wong *et al.* also found evidence for the maturation of monocytes as they moved from expressing high levels of CD14 and low levels of CD16 through intermediate expression and thence to high CD16 and low CD14 expression they also modulated expression of genes. Classical monocytes expressed anti-apoptotic genes and genes associated

with proliferation while non-classical monocytes displayed a pro-apoptotic and anti-proliferative expression profile.

4.1.2 Conservation of gene expression differences between monocyte subsets in men and mice

More recent studies have compared gene expression between human and mouse monocyte subsets to determine if the phenotypic similarities observed in man were conserved at a genomic level in the mouse. Ingersoll *et al.* discovered 269 genes in humans and 561 in mice which were differentially expressed between monocytes subsets. Of these 132 were differentially expressed in the same pattern between the species; 69 were more highly-expressed in $Ly6c^+$ and $CD16^-$ monocytes and 63 in $CD16^+$ and $Ly6C^{lo}$ monocytes. The array data confirmed functional differences which had been observed at the protein level such as comparatively restricted expression of *CCR2, CD62L* and *CD64* by $CD16^-$ and $Ly6C^+$ monocytes and of *CD43, CD11a* and *CD11c* by $CD16^+$ and $Ly6C^{lo}$ monocytes. Genes where differential expression in the subsets was reversed between the species were also discovered. *CXCR4, TREM1* and *CD36* were more highly expressed by $CD16^-$ human monocytes and $Ly6C^{lo}$ mouse monocytes while *CD9* was more highly expressed by $CD16^+$ and $Ly6C^+$ monocytes. Finally, some differentially-expressed genes were unique to one species or the other. Mouse $Ly6C^{lo}$ monocytes were identifiable by higher expression of genes related to the transcription factor peroxisome proliferator-activated receptor γ (*Pparg*) such as fatty acid binding protein 4 (*Fabp4*) which is regulated at the transcriptional level by *Pparg* along with other associated genes including *Cd36, Hsd11b1* and *G0s2*. This signature was not conserved in human $CD16^+$ monocytes. $Ly6C^{lo}$ mouse monocytes were also enriched for genes involved in recognition and engulfment of apoptotic cells including *Cd36, Tgm2, Treml4* and *Itgav*. The authors concluded that the major difference between monocyte subsets in man and mouse was a higher expression of classical scavenger receptors and apoptotic cell recognition molecules on $Ly6C^{lo}$ monocytes which was not found on human $CD16^+$ monocytes and the strong *Pparg* signature found in murine $Ly6C^{lo}$ monocytes alone. Both human and mouse

monocytes shared differential expression of the macrophage-specific transcription factor EC (Tcfec/TFEC) (Rehli et al., 1999) which was more highly expressed by human $CD16^-$ and murine $Ly6C^+$ monocytes and *TCF7l2* (TCF4) and Pou2f2/Oct2 which were both elevated in $CD16^+$ and $Ly6C^{lo}$ monocytes.

Similarly Cros *et al*. (2010) compared gene expression in human and mouse subsets and divided human monocytes into three populations which were broadly similar to those proposed by Ancuta *et al.* (2009) and Wong *et al*. (2011). Hierarchical clustering split the samples into two main groups both of which included human and mouse samples. The $CD14^{dim}$ subset, called non-classical subset by Wong and Ancuta, was proposed to be the closest homologue to mouse $Ly6C^-$ cells. This contrasted with previous studies which suggested the expression or absence of CD16 should be used to differentiate human monocytes into subsets and that mouse $Ly6C^-$ monocytes were the closest homologs (Ingersoll et al., 2010; Ziegler-Heitbrock, 2007; Geissmann et al., 2003). Array data since has not agreed with the findings of Cros *et al.* and has concluded that human $CD16^{hi}$ and mouse $Ly6C^{lo}$ monocytes are most probably the nearest homologous cell types (Wong et al., 2011). The data presented by Cros *et al*. does however raise the question of whether mouse monocytes might also be subdivided further, and whether there is any rational basis for the setting of gates that distinguish the different subsets.

4.1.3 Functional differences

In addition to groups of functionally-related differentially-expressed genes, *in vivo* or *in vitro* functional differences between monocyte subsets have been described. Expression of specific surface molecules suggests that particular subsets will be trafficked to distinct sites of inflammation or injury in response to their specific ligands. Human $CD16^-$ and mouse $Ly6C^+$ monocytes express CCR2 and so respond to secretion of the CCR2 chemokine ligand, CCL2, at sites of infection. Similarly, the expression of CX3CR1 by $CD16^+$ or $Ly6C^-$ monocytes results in trafficking in

response to CX3CL1 (Serbina et al., 2008). Ly6C^{+} monocytes were recruited to peripheral tissue in response to inflammation and can migrate to draining lymph nodes where they have been shown to promote T cell proliferation (Geissmann et al., 2003). In the absence of inflammation they were not found in peripheral tissues and homed to the bone marrow when injected into the circulation (Varol et al., 2007). Conversely Ly6C^{-} cells were recruited to peripheral tissue under non-inflammatory conditions where they may renew local tissue macrophage populations (Geissmann et al., 2003). Cros *et al.* (2010) proposed that human CD14^{dim} and mouse Ly6C^{-} monocytes were most closely related due to their similar response to bacterial and viral stimuli. Studies carried out in different laboratories have provided differing results on cytokine response of various subsets, summarised in **Table 4.2**. Cros *et al.* provided some functional evidence of the similarity of human CD14^{dim} and mouse Ly6C^{-} monocytes; fluorescently labelled CD14^{dim} monocytes exhibited LFA1-dependent patrolling behaviour in a transgenic mouse model similar to what had previously been ascribed to mouse Ly6C^{-} monocytes (Cros et al., 2010; Auffray et al., 2007).

Cros *et al.* (2010) described how in their hands CD14^{dim} monocytes responded primarily to whole virus or TLR7 or TLR8 agonists with production of the pro-inflammatory cytokines TNFα and IL1β in a MyD88 dependent pathway which led to phosphorylation of p42 mitogen-activated protein kinase 1 (MEK1) and Jun N-terminal kinases (JNK). Production of the "lymphocyte helper" cytokines and chemokines IL6 and IL8 by CD14^{+}CD16^{-} monocytes was also MyD88 dependent but led to phosphorylation of p38 mitogen-activated protein kinase (MAPK) suggesting different signalling pathways were active in the different subsets. Similarly mouse Ly6C^{-} monocytes responded strongly to TLR7 agonists but not LPS suggesting they were like human CD14^{dim} monocytes in their strong response to virus and weak response to bacterial products. More recent analysis of the cytokine profiles of human monocyte subsets tended to support the traditional view of the non-classical

Subset	Function	References
Human $CD14^{hi}CD16^{-}$	Inhibit fungal germination	(Serbina et al., 2009)
	Produced high levels of CCL2, CCL3, Il6,IL10 and IL8 after LPS stimulation	(Cros et al., 2010)
	High TNFα production after LPS, zymosan and *S.aureus* stimulation	(Skrzeczynska-Moncznik et al., 2008)
Human $CD14^{lo}CD16^{+}$	Respond to viruses with production of proinflammatory cytokines, poor phagocytes, did not produce ROS, MPO or lysozyme. Produced little cytokines in response to LPS, exhibited patrolling behaviour	(Cros et al., 2010)
	High TNFα production after LPS, zymosan and *S.aureus* stimulation	(Skrzeczynska-Moncznik et al., 2008)
Human $CD14^{+}CD16^{+}$	Produced high levels of TNFα and IL1β after LPS stimulation	(Cros et al., 2010)
	Produced high levels of IL10 after LPS stimulation	(Skrzeczynska-Moncznik et al., 2008)
Mouse $Ly6C^{hi}$	Control fungal infections	(Traynor et al., 2000; Traynor et al., 2002)
	Respond to viral ligands with production of type I IFNs through TLR2	(Barbalat et al., 2009)
Mouse $Ly6C^{lo}$	Patrolling behaviour responded strongly to TLR7 agonists but not LPS	(Auffray et al., 2007) (Cros et al., 2010)

Table 4.2 Proposed roles in infection of human and mouse monocyte subsets

$CD16^{hi}$ monocytes as being the main producers of pro-inflammatory cytokines in response to LPS (Wong et al., 2011). The studies summarised in **Table 4.2** demonstrate the variable results achieved in studies of monocyte subsets from different laboratories. This could be due to several reasons; different isolation methods are often employed meaning $CD14^{hi}CD16^{-}$ monocytes could be composed of slightly different cells depending on the laboratory the experiment is conducted in. Furthermore some of the anti-CD14 antibodies used to isolate the subsets have been

described to have a blocking action (Power et al., 2004) which would affect subsequent studies on response to TLR4 ligands.

The differential expression of CD14 and CD163 on pig monocyte subsets has been demonstrated previously by others (Chamorro et al., 2004; Chamorro et al., 2000; Chamorro et al., 2005) and this thesis also clearly showed differential expression of CD16 in the pig, as in man and mouse (**Chapter 3**). Unfortunately there are many problems associated with cross-species comparisons, for instance the same markers often cannot be used across species. In the pig CD163 is differentially expressed by CD14 positive monocytes with those cells that express highest levels of CD14 expressing lower levels of CD163. CD163 is also differentially expressed by human CD14 positive monocytes but in the converse pattern to in pig and it is not differentially expressed in the mouse at all (Ingersoll et al., 2010). Furthermore in humans CD16 is a duplicated gene which shows copy number variation amongst individuals (Zhou et al., 2010), and although it is also differentially expressed in both pig and mouse monocytes, it is not useful as a marker. CD14 also differs amongst species, in that it is an acute phase protein expressed by the liver in humans but not in mice (Hetherington et al., 1999; Pan et al., 2000) and finally, Ly6C is a marker only in mice. Despite these problems there are likely to be homologous cell populations across species, particularly in those as closely related as humans and pigs. There has been no previous analysis of the gene expression profiles of pig monocyte subsets therefore the aim of this chapter was to determine if the recently reported differences between monocyte subsets in mouse and human were conserved in the pig. The pig may function as good model for many inflammatory human conditions. The identification of shared differential gene expression between human and pig monocyte subsets may assist in finding targets for disease or markers of disease progression

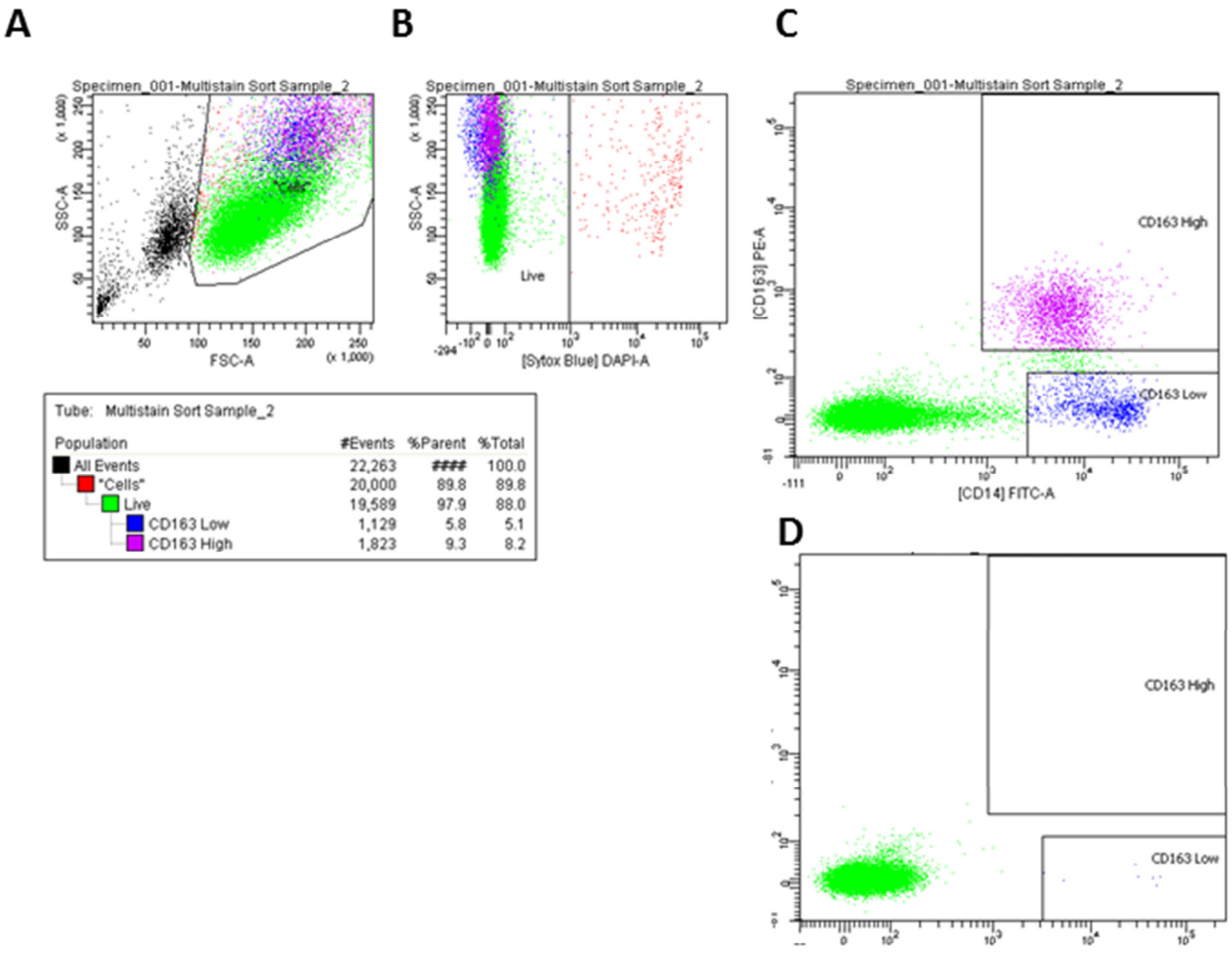

Figure 4-2 Monocytes were FACS sorted on CD163.

Monocytes were gated (**A**), dead cells excluded (**B**) and cells sorted on expression of CD14 and CD163 (**C**). Isotype control is also shown (**D**)

4.2 Results

4.2.1 Microarray analysis of porcine monocyte subsets

Pig monocytes were stained with CD14 and CD163 and FACS sorted (FACS Aria) into two populations of $CD14^{hi}CD163^{lo}$ and $CD14^{lo}CD163^{hi}$ monocytes (**Figure 4.2**). RNA was extracted and analysed by microarray and normalization and statistical analysis was performed by Fios Genomics. **Figure 4.3** shows a workflow diagram for the analysis of the microarray data. Three animals were examined separately. Statistical analysis of the microarray showed many probes were differentially expressed between the $CD14^{hi}CD163^{lo}$ and $CD14^{lo}CD163^{hi}$ populations. Probes were sorted and only those which were differentially expressed by ≥ 1.5 or ≤ 0.67 fold change between the monocyte subsets were included. This normalised and filtered dataset was then analysed using Biolayout *Express*^{3D} (Freeman et al., 2007) with an MCL of 1.7, R=0.95, smallest cluster = 3. This generated 69 clusters which were grouped into two distinct shapes, one of which was composed of clusters of probes which were expressed more highly in $CD14^{hi}CD163^{lo}$ monocytes and one composed of clusters which were more highly expressed in $CD14^{lo}CD163^{hi}$ monocytes (**Figure 4.4**). The expression profiles of *CD163, CD16* and *CD14* shown in **Figure 4.4** confirmed the successful separation based upon the markers and the differential expression of CD16 seen previously (**Chapter 3**). CCR2 and CX3CR1 are differentially expressed on mouse and human monocytes and so far only limited studies have investigated their expression in the pig (Moreno et al., 2010). Probes for CX3CR1 were not included in this array but CCR2 was expressed more highly by $CD14^{lo}CD163^{hi}$ monocytes which is the converse to what is seen in human and mice and what has been reported previously in the pig (Moreno et al., 2010; Ingersoll et al., 2010). Data from Biolayout can more easily be represented in a 2D format using the graph editor yEd (**Figure 4.5**). In addition to the variation between the monocyte subsets that was reproducible amongst each of the animals, **Figure 4.5** also shows that there was variation between the individual animals. For example genes in cluster 6 were most highly segregated between monocyte subsets in pig 3, similar distinct expression can be seen for pig 2 in cluster 3. The three animals used for this

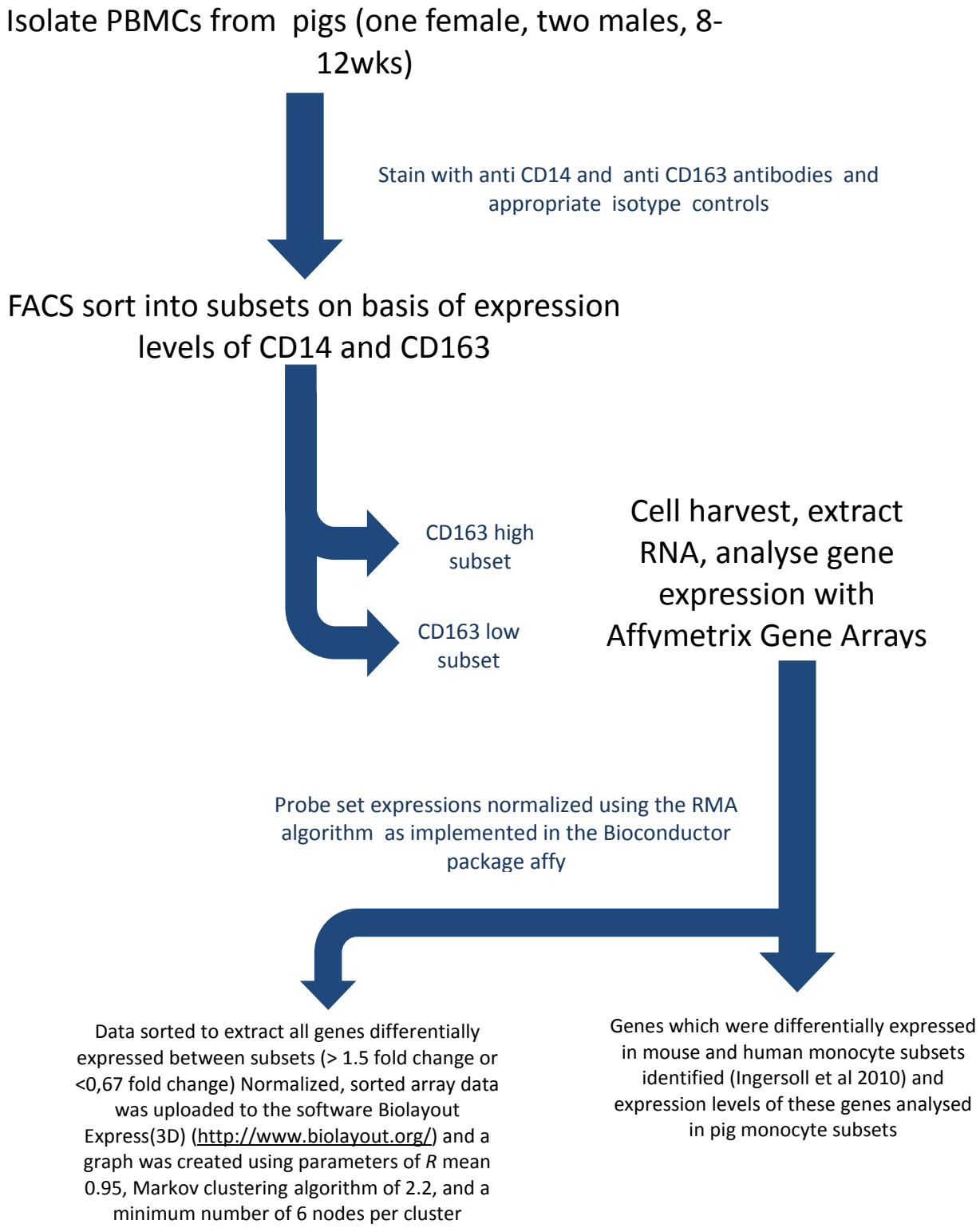

Figure 4-3 Work flow for studying genes which were differentially expressed in porcine monocte subsets. PBMCs isolated from blood of pigs 8-12 week old LW x Landrace F1 cross (2 males 1 female), stained with anti-CD14 and anti-CD163 antibodies and FACS sorted into subsets to obtain RNA for analysis by Affymetrix array.

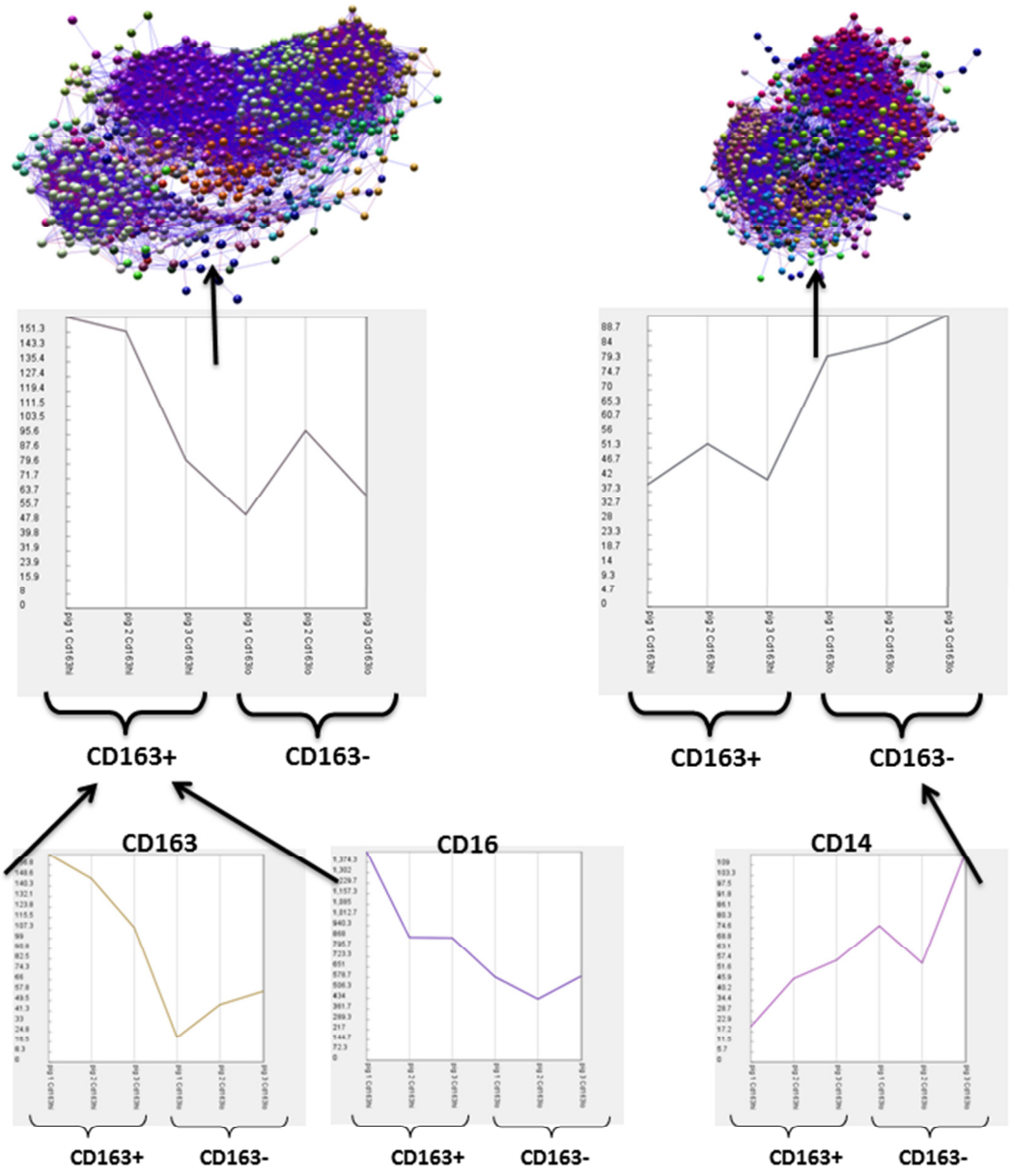

Figure 4-4 Micro-array data clustered into two distinct groups containing genes which were more highly expressed by CD163hi or CD163lo monocytes.

Expression of the surface markers CD163, CD14 and CD16 are shown to verify successful sorting of monocytes.

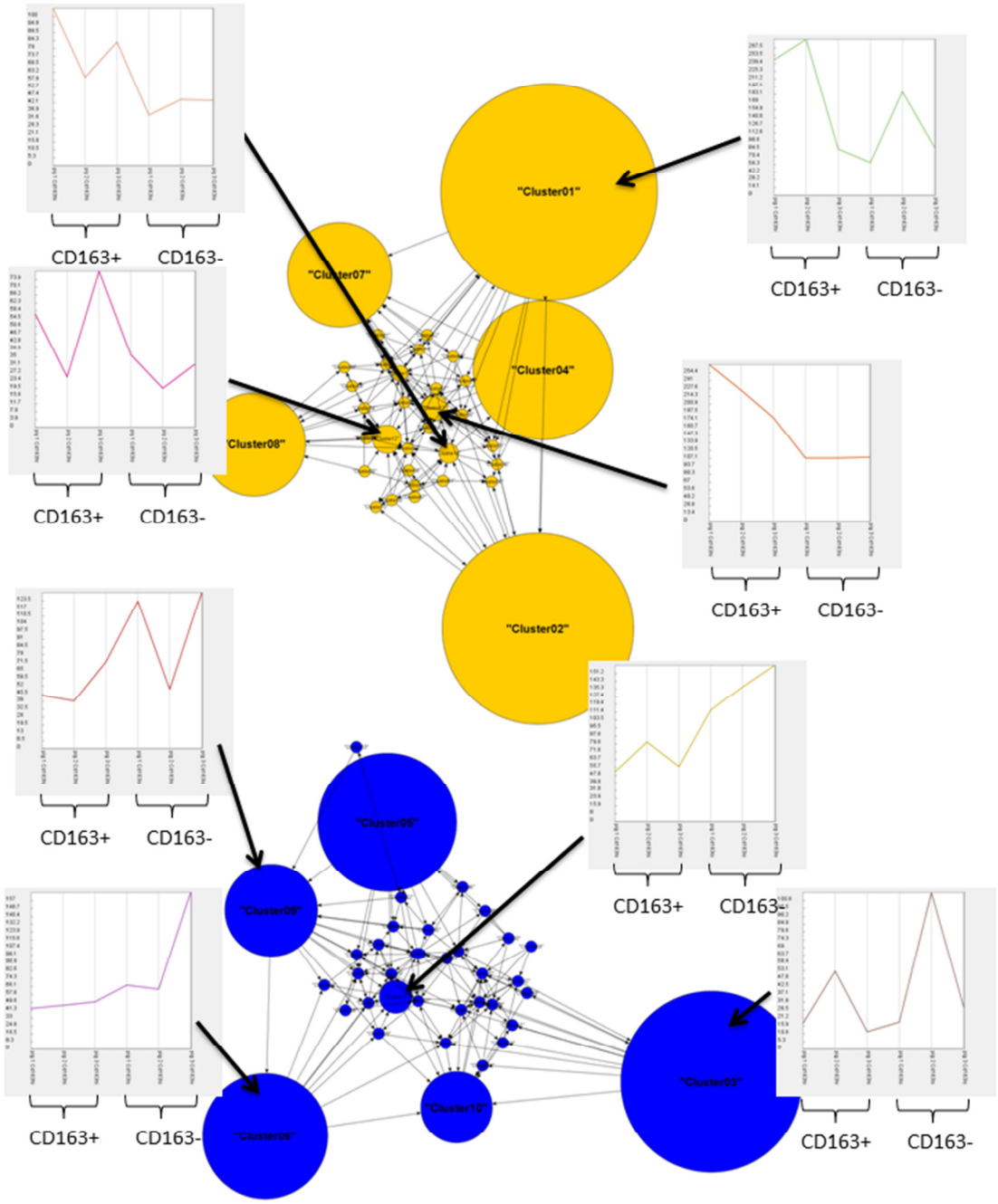

Figure 4-5 Clusters of co-expressed genes were more highly expressed by CD163hi or CD163lo monocytes

Data also showed some variation between individual animals.

study were from different genetic backgrounds and clusters such as these may highlight variation between breeds which may results in differential response to infection. Alternatively variation between samples may be due technical or biological variation and has been reported to be common in clinical data (Theocharidis et al., 2009).

To highlight the most robust differences between CD163^{hi} and CD163^{lo} pig monocytes, the top 50 most differentially expressed genes were identified (**Figure 4.6)**. CD163^{lo} monocytes expressed higher levels of the neutrophil chemotactant chemokine *IL8* and its receptors IL8 receptor 2 fragment and IL8 receptor β (*IL8RB, CXCR2*) as well as the tetraspanin *CD82* which is involved in T cell activation. The IFN inducing cytokine *IL18* was also more highly expressed in CD163^{lo} monocytes as were the pro-inflammatory genes *S100A8* and prostaglandin-endoperoxide synthase 2 (*PTGS2*, COX2) . CD163^{hi} monocytes expressed high levels of legumain (*LGMN*) which is essential for antigen presentation. The class II genes of the swine leukocyte antigen (SLA) genes, like human MHC class II genes, are involved in control of immune response to foreign antigen. *SLA-DRB1* was one of the most highly expressed genes on CD163^{hi} pig monocytes suggesting they may have a higher antigen presenting role than CD163^{lo} monocytes. Other immune genes preferentially expressed by CD163^{hi} monocytes included the C-type lectin *CLEC7A* (dectin1) which recognises a variety of beta glucans from fungi and plants and kynurenine 3-monooxygenase (*KMO*) which the catalyses the metabolism of kynurenine to 3-hydroxykynurenine in the *IDO* mediated metabolism of tryptophan to kynurenine which is discussed in more length in **Chapter 5**. Dectin1 has also been described to function as a co-receptor for recognition of various microbial products (Yadav and Schorey, 2006; Dillon et al., 2006; Ferwerda et al., 2008b). The miRNA mir-29c (ssc-mir-29c) was also up-regulated in CD163^{hi} monocytes as was the receptor for angiopoeitin-1 (*TEK*). The differential expression of key immune response genes suggests specialised functions for monocyte subsets in the pig as has been reported previously in other species (Zhao et al., 2009; Wong et al., 2011; Ingersoll et al., 2010; Ancuta et al., 2009). For instance CD163^{lo} monocytes

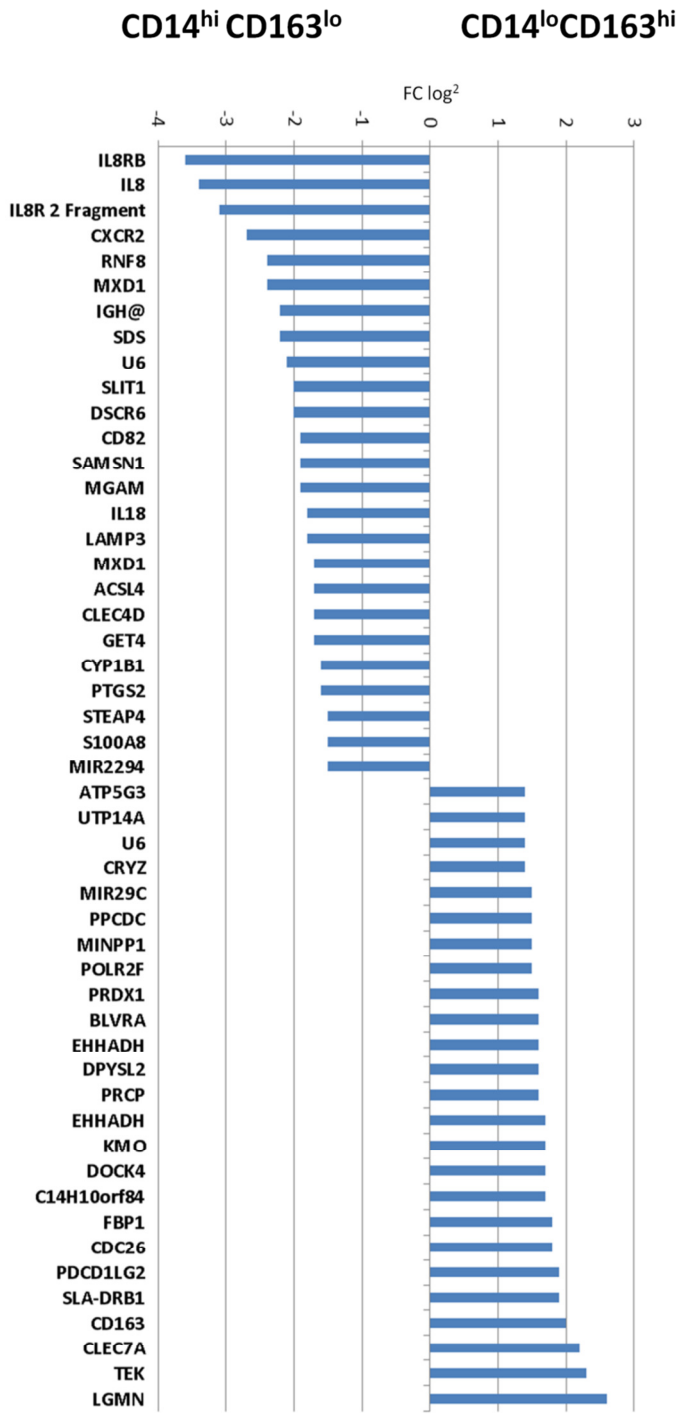

Figure 4-6 The top 50 most differentially expressed genes between pig monocyte subsets.

Genes were sorted by fold change and the 50 most differentially expressed genes (by fold change, average across all three samples, ($p>0.01$)) are shown.

expressed many genes involved with recruitment of other immune cells while CD163^{hi} monocytes appeared specialised for antigen presentation. To enable a better understanding of any functional differences between the subsets all probes which were more highly expressed in CD163^{hi} monocytes by at least 1.5 fold change were identified (2082 individual genes). Analysis of these genes using DAVID (Huang et al., 2008; Huang et al., 2009) showed enrichment for mitochondrial components, translation, cellular metabolic processes, intracellular protein transport, localization and binding, nuclear parts, cytoplasmic membrane bound vesicles and RNA processing. This suggests CD163^{hi} monocytes are more metabolically active so are actively growing unlike CD163^{lo} monocytes. They are responding to growth factors by increasing protein synthesis and RNA synthesis and were enriched for genes involved in anabolic metabolism, the TCA cycle and respiration. The top ten clusters of GO terms which were enriched in CD163^{hi} monocytes can be found in **Appendix 6**. Similar analysis of probes which were expressed more highly in CD163^{lo} monocytes provided 1485 unique genes. Analysis with DAVID showed enrichment for genes involved in leukocyte activation and differentiation such as *CSF2, CSF3R, CXCR2, IL8, TGFB1, TLR2, TNF* (**Figure 4.7A-G**) and the inflammatory response and response to wounding including *CCL5, CCR1, CD14, CXCR2, IL1B, TGFB1, THBS1, TLR2, TLR9,TNF, IL8, CSF3R* (**Figure 4.7H-L**). This suggests that CD163^{lo} monocytes are the immune effector cells and that this function is switched off as the cells mature to CD163^{hi} monocytes. The full list of genes found in the top ten clusters can be found in **Appendix 6**.

As with previous studies in human and mouse (Ingersoll et al., 2010; Cros et al., 2010) there did appear to be some lymphocyte contamination, *KLRG1* which is mainly expressed on T cells was in cluster 6 and *BANK1* and *BCL7A* which are expressed by B cells were in cluster 1 and 9 respectively.

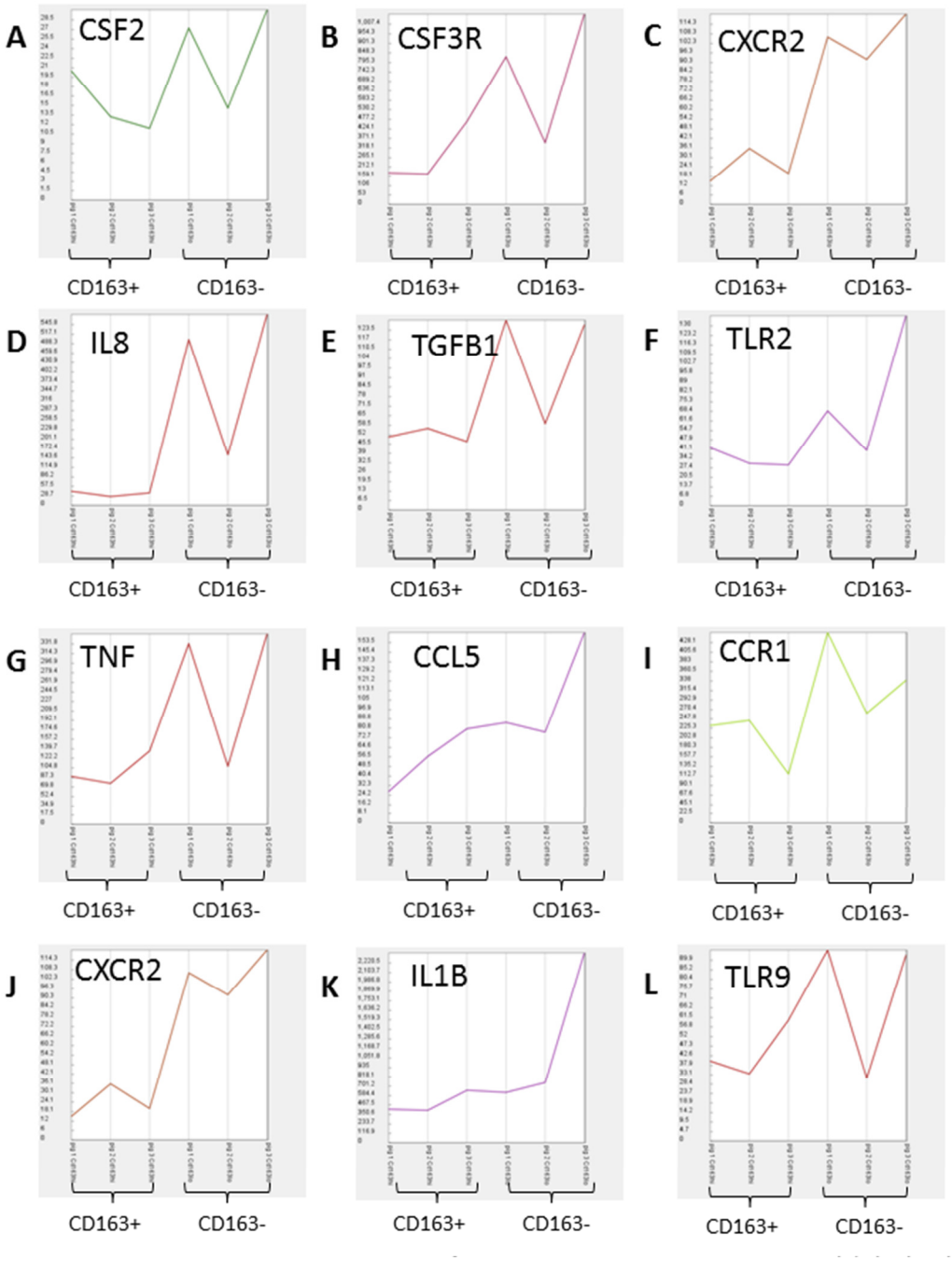

Figure 4-7 Expression profiles of immune associated genes

4.2.2 Analysis of human and mouse data sets

To compare gene expression of pig monocytes to the better characterised human and mouse monocyte subsets, microarray data comparing human and mouse monocytes was downloaded from the Gene Expression Omnibus (GEO) database (ncbi.nlm.nih.gov/gds) (GSE16836, E-MEXP-2544, E-MEXP-2545, GSE18565, GSE17256, GSE17256). Probes were sorted and only those which were differentially expressed by ≥ 1.5 or ≤ 0.67 fold change between the monocyte subsets were included. This data was analysed using Biolayout *Express*^{3D} (Freeman et al., 2007) with an MCL of 1.7, R=0.95, smallest cluster = 3

4.2.2.1 Human monocyte subsets

There have been several studies comparing gene expression of the traditional monocyte subsets in man (Zhao et al., 2009) and more recent studies have examined differences between three human monocyte subsets (Wong et al., 2011). Cros *et al.* (2010) examined how differences between these three monocyte subsets could relate to differentially expressed genes in the mouse. In other studies, Mabbott et al. (Mabbott et al., 2010) have taken microarray datasets from multiple laboratories and analysed them using Biolayout to identify clusters of genes that track together consistently. Analysis of this human monocyte microarray data highlights many sample specific rather than cell type specific clusters, suggesting that the different datasets from different labs are not strictly comparable. For example the largest cluster of co-expressed probes, cluster 1 was composed of genes which were expressed more highly in one sample (CD14_s32) (**Figure 4.8**). This is common when there is technical or biological variation between samples and as discussed above may be observed with clinical data (Theocharidis et al., 2009). Two samples appeared to have been switched at some stage as sample CD1416_s38 was labelled as $CD14^{hi}CD16^{hi}$ although the gene expression pattern more closely resembled the other 4 $CD14^{+}CD16^{-}$ samples. Correspondingly the expression pattern of sample CD14_s37 more closely fit the other $CD14^{hi}CD16^{hi}$ samples (**Figure 4.8, cluster 4, 9 and 17**). Cluster 1 contained some B-cell associated genes (*BANK1, BCL7A, CD22, CD72*) which as above may suggest contamination. The interferon regulated

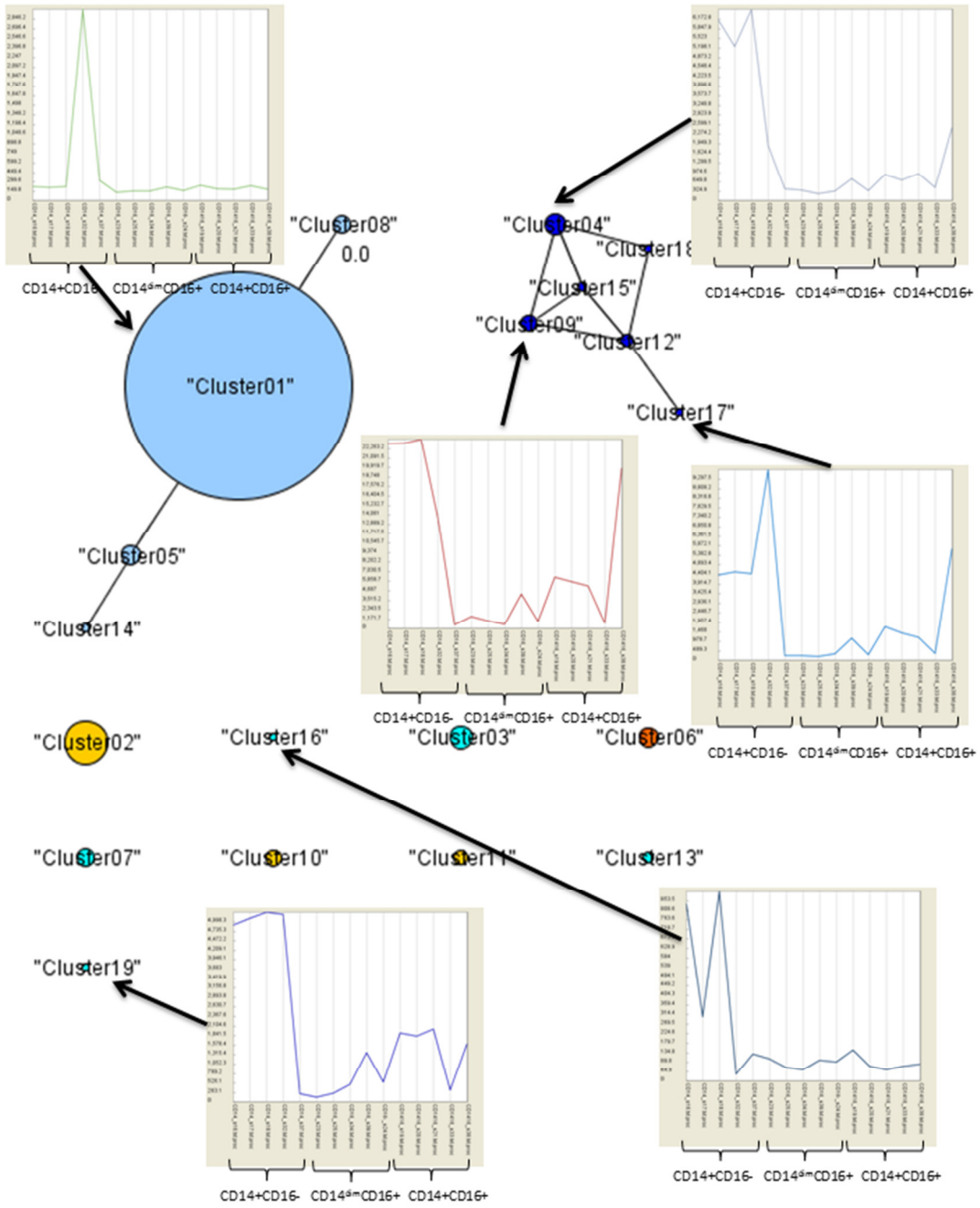

Figure 4-8 Human monocyte subset array data from Cros *et al.* was re-analysed using Biolayout Express.

gene *SIGLEC1* was highly expressed in this cluster as was *CD22* which belongs to the siglec family of lectins. *SIGLEC1* was highly expressed on $CD14^{hi}$ monocytes from patients with systemic sclerosis (York et al., 2007). Cluster 4, 9, 12, 15, 16, 17 and 19 (**Figure 4.8**) contained genes which were highly expressed in the $CD14^{+}CD16^{-}$ samples and which were only expressed at low levels by the $CD14^{dim}$ monocytes, these include *CCR2, IL1RN, CD163*, *TREM1, FOS, S100A8* and *S100A12*. Many of the clusters containing genes which were highly expressed by $CD14^{+}CD16^{-}$ monocytes (Clusters 4, 9, 12, 15, 17 and 19) contained genes which were expressed at intermediate levels by $CD14^{+}CD16^{+}$ monocytes (**Figure 4.8**). Genes in these clusters included *CCR2, IL1RN, CD163, S100A8* and *S100A12*. Unlike more recent analysis of the proposed three human monocyte subsets (Wong et al., 2011) there were no clusters in this dataset which were specific for genes which were expressed more highly by $CD14^{dim}CD16^{hi}$ or $CD14^{hi}$ $CD16^{hi}$ monocytes.

Similar studies comparing differences between the two traditional monocyte subsets have also been carried out (Ingersoll et al., 2010; Ancuta et al., 2009). As these may be more comparable to the two monocyte subsets in the pig, genes which were differentially expressed in these studies were identified. Comparison of data from Ingersoll *et al.* (2010) did not show the sample specific variation seen in the Cros *et al.* data, instead it clustered into genes which were more highly expressed in specific subsets (**Figure 4.9**). The majority of genes clustered into two main clusters. The largest cluster, cluster 1, contained 1819 probes for genes which were expressed at higher levels in $CD16^{+}$ monocytes (including *CD16*) such as, *CX3CR1, CD11a, CD11c*, *CD43* and *CSF1R*. Similarly Cluster 2 contained 1447 probes for genes which were expressed more highly in $CD16^{-}$ monocytes (including *CD14*) such as *CCR2, CD163, CCR1, CD62L, CSF3R, CSF2RA, FCGR1B, CD64, CD36.* Analysis of gene expression data from Ancuta *et al.* was examined in a similar manner and clustered into two main groups which were differentially expressed between the monocyte subsets (**Figure 4.10**) again as reported with other data above there were some sample specific clusters (**Figure 4.10, cluster 1, cluster 5**). The largest cluster, cluster 1 contained 3211 probes which were more highly expressed in one sample,

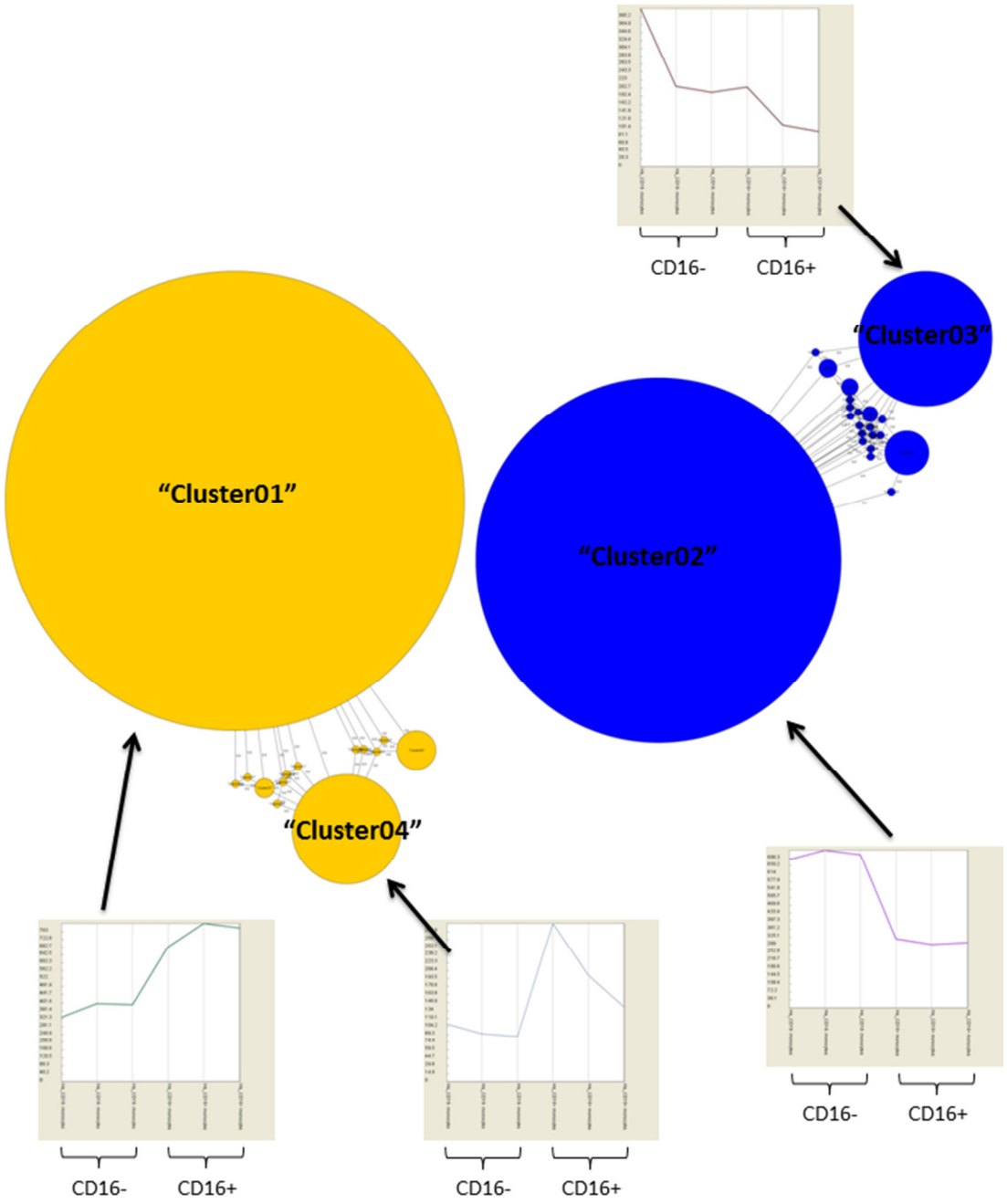

Figure 4-9 Human monocyte subset array data from Ingersoll *et al*. was re-analysed using Biolayout Express.

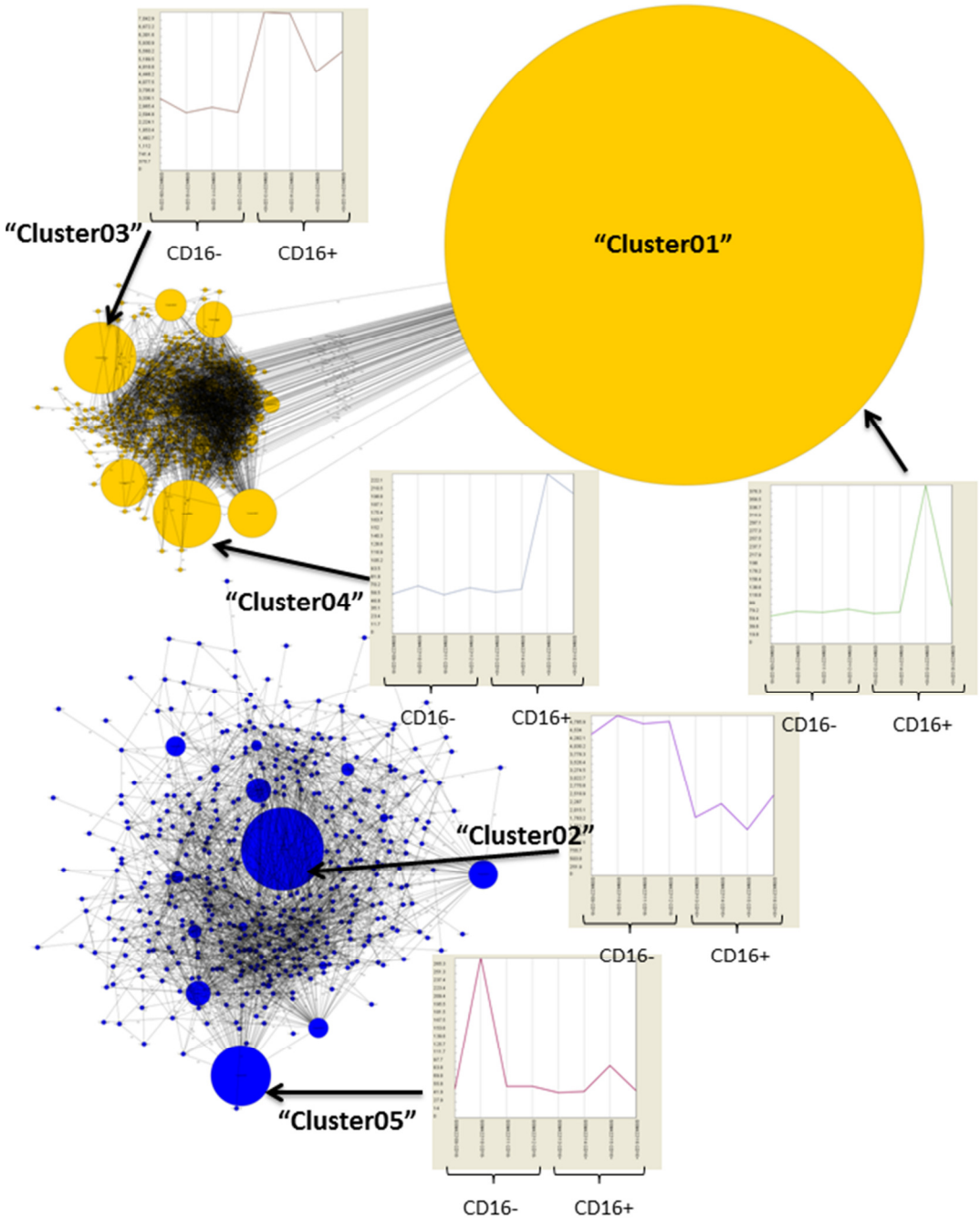

Figure 4-10 Human monocyte subset array data from Ancuta *et al.* was re-analysed using Biolayout Express.

CD43 was included in this cluster. Genes which were more highly expressed by $CD16^+$ monocytes, in all data examined (Ingersoll et al., 2010; Zhao et al., 2009; Ancuta et al., 2009) included *CD62L*, *CX3CR1, CSF1R, TNF, FCGR3B* (CD16) and *ITGAL* (CD11a) while probes for genes such as *CCR1*, *CD9, CD36, IL8, CD163, CSF3R, S100A8, S100A9* and *CCR2* were expressed more highly by $CD16^-$monocytes (**Figure 4.1**). Data from Cros *et al.* (2010) was not included in this comparison due to the high sample specific clusters (**Figure 4.8**)

4.2.2.2 Mouse

Until recently the proposed homology between human $CD16^+$ and mouse $Ly6c^{lo}$ monocytes mainly rested on the expression of a few key surface markers (detailed in **Appendix 3**). Recently several groups have compared gene expression data from human and mouse monocyte subsets to determine if the similarities extend beyond expression of a few key molecules. The array data from these studies was downloaded from GEO and analysed as above. Gene expression levels for mouse monocytes from Cros *et al.* (Cros et al., 2010) and Ingersoll *et al.* (Ingersoll et al., 2010) clearly clustered into cell type specific clusters (**Figure 4.11** and **Figure 4.12**). Genes which were more highly expressed in $Ly6C^-$ monocytes in both studies included *Ccl5*, *Pparg*, *Tlr8*, *Il1rn*, *Il18bp*, *Il9r*, *Irf6*, *Sirpα*, *Csf1r, Csf2rb1, Clec4e, Serpinb2, Smurf2, Pde4b, Rtn1, Jag1, Ear3, Cd36* and *Trem1*. Similarly both studies discovered genes which were more highly expressed in $Ly6C^+$ cells such as *Ifngr2*, *Il11ra1*, *Tgfbi*, *Tgfb1*, *S100a13*, *Traf5*, *Ccl9*, *Cd92*, *Ccr2*, *Icam1*, *Casp2, Usp18* and *Oas2*. There were some genes which were shown to be differentially expressed between the subsets in a converse manner between the two studies. For instance the transcription factor *Tcfec* was only highly expressed by one $Ly6C^+$ sample in the study by Cros *et al.* while Ingersoll *et al.* found it increased in all samples. Cros *et al.* found high expression of the transcription factor *Tcf4* and *Cd9* in $Ly6C^+$ samples while Ingersoll *et al.* found it to be more highly expressed by $Ly6C^-$ cells. Similarly Cros found higher expression of *Ifit2, C3* and *Msr* in $Ly6C^-$ monocytes while Ingersoll *et al.* found higher expression in $Ly6C^+$ monocytes. Differences in gene expression levels between the studies could be down to the different isolation

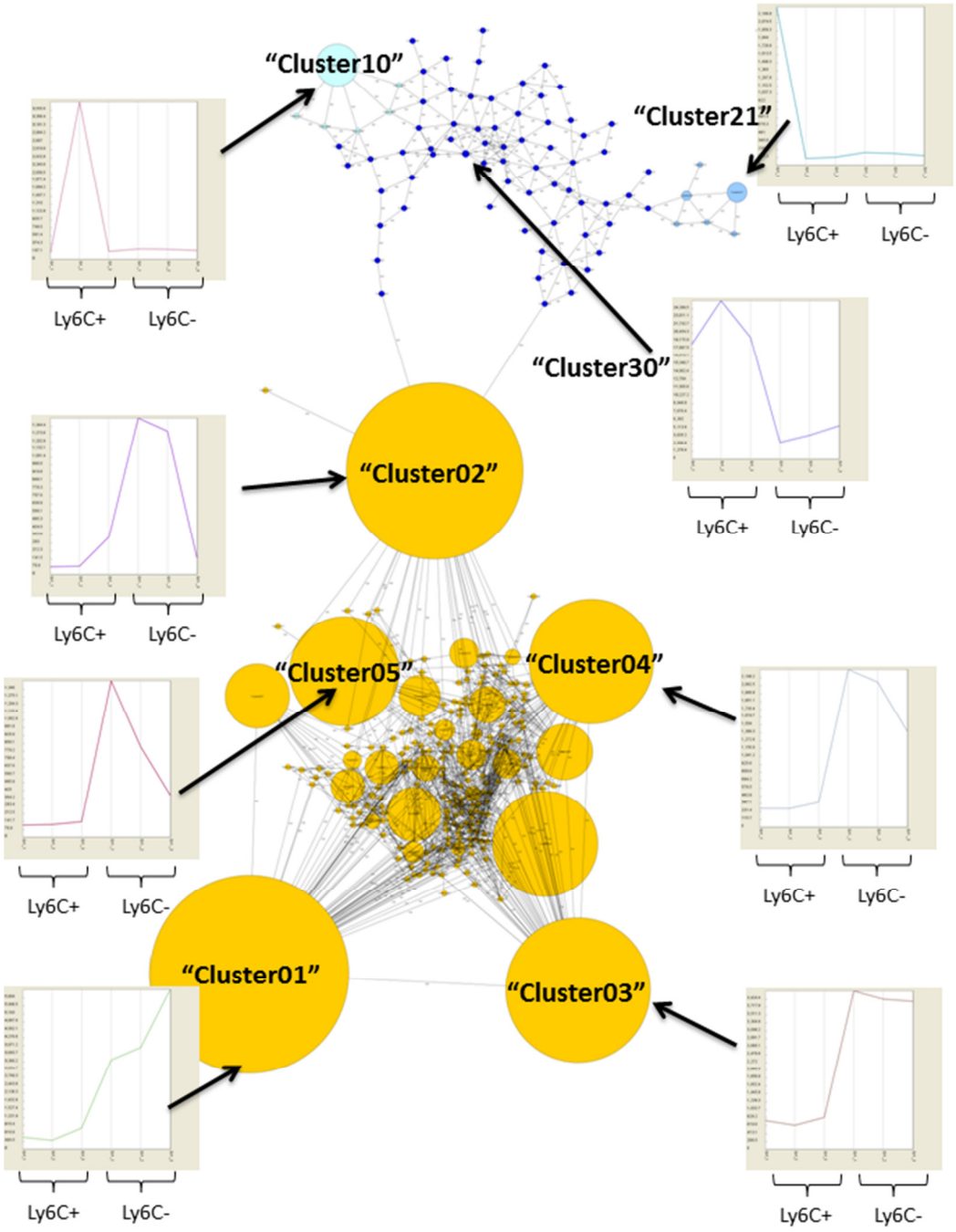

Figure 4-11 Murine monocyte subset array data from Cros *et al.* was re-analysed using Biolayout Express.

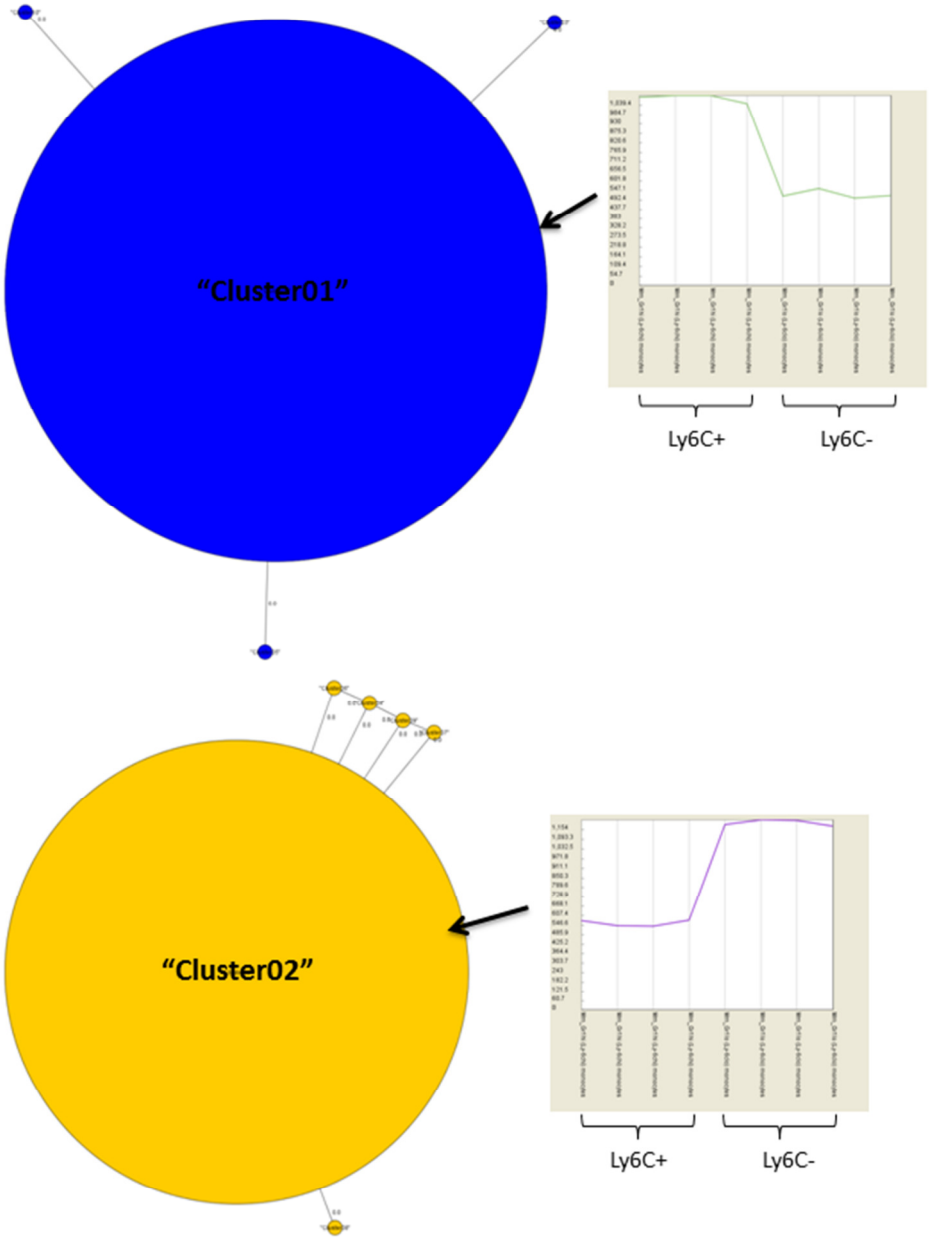

Figure 4-12 Murine monocyte subset array data from Ingersoll *et al*. was re-analysed using Biolayout Express.

methods used or the different platforms used to analyse gene expression however this data provides a list of genes which are consistently expressed by one specific monocyte population in the mouse and which will prove useful for future mouse models of human disease.

4.2.3 Comparison of human, mouse and pig monocyte subsets.

Ingersoll *et al.* (2010) previously identified 132 genes which were differentially expressed in the same subsets in both human and mice monocytes and 33 genes which were differentially expressed but in a converse pattern between the two species (Ingersoll et al., 2010). To determine if the differential expression of these genes was also conserved in pig monocyte subsets expression was examined in monocytes FACS sorted on the basis of CD14 and CD163 expression. **Figure 4.13** shows genes where the differential expression was conserved between human and pig (80 genes) and **Figure 4.14** shows genes which were differentially expressed in pig and human but in a converse manner to each other (44 genes), 43 genes which were identified by Ingersoll *et al.* were not in the pig array. Among those genes more highly expressed on $CD14^{hi}$ human ($CD14^{hi}CD16^{-}$), pig ($CD14^{hi}CD163^{lo}$) and mouse ($Ly6C^{hi}$), monocyte subsets was *CD14, S100A8, CCR1, IL18, LBR* and *CSF3R.* The expression of *JAG1, TREM1 SGK1, PDE4B, SERBINB2, CLEC4E* and *CD36* was conserved in human and pig but not mouse, these genes were more highly expressed by human and pig $CD14^{hi}$ monocytes and $CD14^{lo}$ mouse monocytes. $CD14^{lo}$ human ($CD14^{lo}CD16^{+}$), pig ($CD14^{lo}CD163^{hi}$) and mouse ($Ly6C^{lo}$) monocyte subsets expressed higher levels of *CD16, ICAM2, TGFBR3, ITGAL, LTB.* Human and pig $CD14^{lo}$ monocytes shared high expression of interferon-induced proteins with tetratricopeptide repeats 2 and 3 (*IFIT2, IFIT3*) and the scavenger receptor *MSR1* which were all more highly expressed on mouse $CD14^{hi}$ monocytes. Although expression was conserved between the three species the degrees of differential expression was often different between the species, for instance CD14 was more highly expressed by Human $CD16^{-}$ monocytes compared to $CD16^{+}$ monocytes than when comparing the two subsets in pig or mouse and although CD16 was differentially expressed by CD14 positive pig monocytes, both populations expressed it at relatively high levels compared to human $CD16^{lo}$ monocytes. Differential gene expression can help identify functions which are specific to one monocyte subset. Mouse $Ly6C^{lo}$ and human $CD16^{+}$

monocytes have been described as better stimulators of mixed lymphocyte reactions. The finding that genes encoding MHC class II molecules were enriched in mouse Ly6C^{lo} and human CD16^{+} monocytes

validated this (Ingersoll et al., 2010). Similarly others have reported pig CD163^{hi} monocytes as better accessory cells (Sanchez et al., 1999) and this study showed that *SLA-DQ* and *DR* were expressed more highly on CD163^{hi} monocytes (*SLA-DQA, SLA-DQB1, SLA-DRA, SLA-DRB1, SLA-DRB2*).

Ingersoll *et al.* (2010) found expression of phagocytic and scavenger receptors to differ between human and mouse monocyte subsets. Generally these were more highly expressed on mouse Ly6C^{lo} and human CD16^{-} monocytes suggesting different roles for the monocyte subsets in human and mice. The expression of these receptors was therefore examined in the pig monocyte subsets to determine if a specific subset appeared to be better adapted for recognition and engulfment of apoptotic cells. The expression of the adhesion molecule thrombospondin 1 (*THBS1*) and the thrombospondin receptor *CD36* was higher on CD163^{lo} monocytes as was expression of triggering receptor expressed on myeloid cells 1 (*TREM1)* (**Figure 4.13**). Expression of similar adhesion molecules or scavenger receptors such as the α_v integrin (*ITGAV*, CD51) did not differ between the subsets and mRNA levels of the apoptotic cell recognition molecule *SIRPA* were varied, expression was high on CD163^{hi} monocytes from pig 2, slightly high on CD163^{hi} monocytes from pig 1 but there was no difference in expression between monocyte subsets in pig three. Furthermore expression of SIRPA at the protein level (**Chapter 3**) showed homogeneous expression on all CD14^{+} monocytes. Expression of some scavenger receptors and receptors for apoptotic cells does seem to be differentially expressed as in humans although analysis with DAVID (Huang et al., 2008) did not find enrichment of either cluster for genes relating to phagocytosis suggesting that the

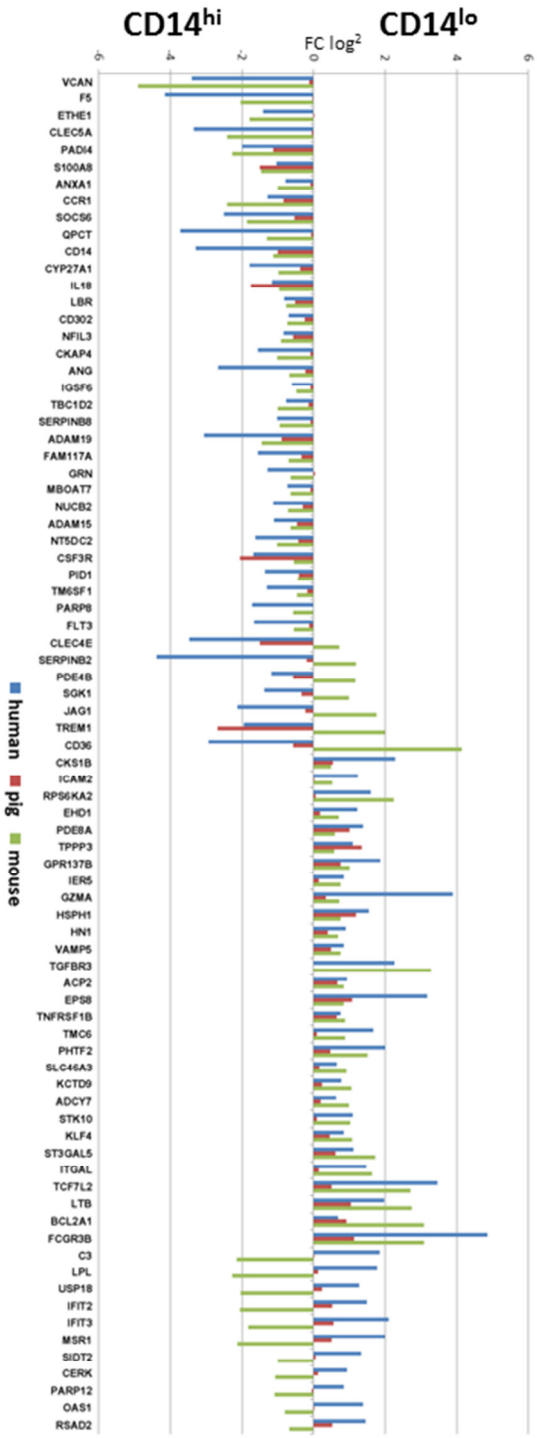

Figure 4-13 Genes which were differentially expressed in the same pattern between human and pig monocyte subsets.

Mouse and human data from Ingersoll *et al.* (2010).

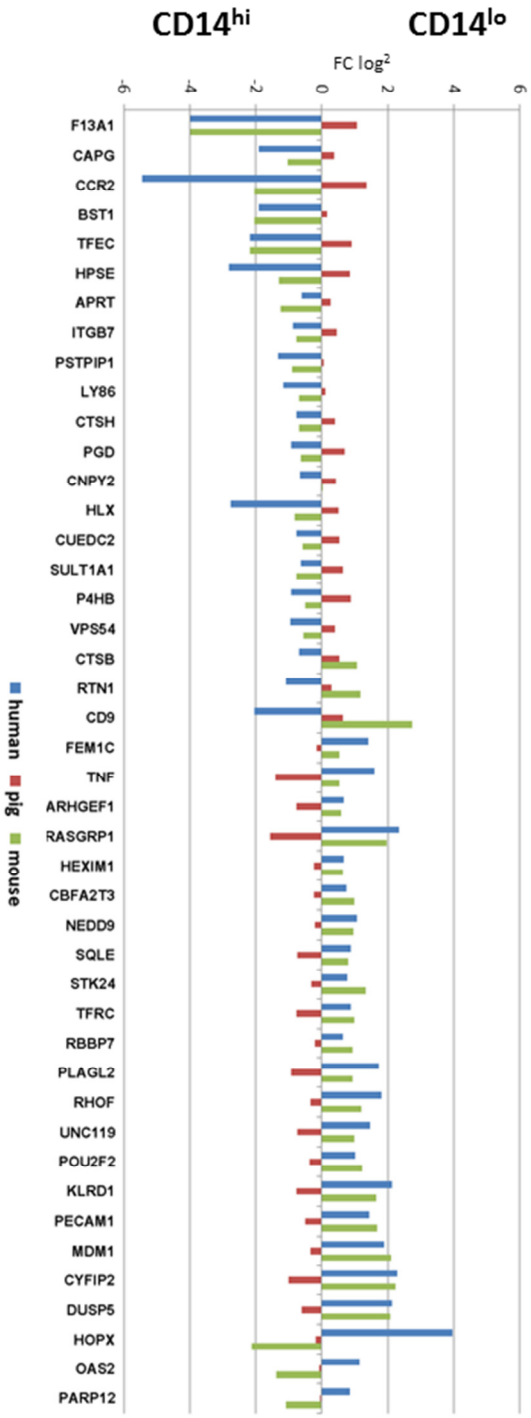

Figure 4-14 Genes which were differentially expressed in the converse pattern between human and pig monocyte subsets.

Mouse and human data from Ingersoll *et al.* (2010).

specialisation of one subset that was observed in man and mouse does not happen to such an extent in the pig.

Surprisingly *CCR2* was shown to be on average more highly expressed by $CD14^{lo}$ pig monocytes in contrast to the high expression on $CD14^{hi}$ human and mouse monocytes (**Figure 4.14**) and to previous reports (Moreno et al., 2010). Analysis of the array data show there were two probes for *CCR2* (SNOWBALL_014576.1_st, SNOWBALL_014576.2_st) one of which was more highly expressed by $CD163^{hi}$ monocytes and one by $CD163^{lo}$ monocytes. Again contrary to previous reports (Chamorro et al., 2005) *TNF* was more highly expressed by pig $CD14^{hi}$ monocytes converse to the high expression by human and mouse $CD14^{lo}$ monocytes. The transcription factor *TFEC* was also more highly expressed on $CD14^{lo}$ pig monocytes and $CD14^{hi}$ human and mouse monocytes. Ingersoll *et al.* have reported differential expression of *CD9* between human and mouse monocyte subsets and this array showed that like mice, it was pig $CD14^{lo}$ monocytes which expressed higher levels of this adhesion molecule. The transcription factor *POU2F2* was also differentially expressed between pig versus human and mouse. CD64 has been used to divide human monocytes into subsets (Grage-Griebenow et al., 1993; Grage-Griebenow et al., 2000; Grage-Griebenow et al., 2001b) and differential expression has been shown between murine monocytes however differential expression was not seen in the pig. Finally Ingersoll *et al.* also noted a PPARγ signature in mouse $Ly6C^{lo}$ monocytes, *PPARG* was expressed only at very low levels suggesting that pig monocytes were like human monocytes in this aspect too.

This data suggests $CD14^{hi}$ monocytes are perhaps the most closely conserved between different species. The porcine $CD163^{hi}CD14^{lo}$ monocyte may not be the functional equivalent of human $CD14^{lo}$ population. Examination of porcine monocyte FACs plots show that the expression of CD14 on $CD163^{hi}$ monocytes is still relatively high compared to that seen on $CD16^{hi}$ human monocytes, these cells may be better termed as intermediate expressers of CD14. Examination of FACS plots from **Chapter 3** and **Figure 4.2** show there is a true $CD14^{lo}$ population which is also negative for CD163. CD163 may be better utilised to simply subdivide the $CD14^{hi}$ population in the pig. One of the biggest differences

between monocyte subsets in humans and other species examined is the relative numbers of each subset; in man around 90% of monocytes are classical $CD14^{hi}$ and only 10% express low levels of CD14. The $CD14^{lo}CD163^{-}$ porcine monocyte population identified in **Chapter 3** may be more similar to human $CD14^{lo}$ monocytes and the numbers of porcine $CD14^{lo}$ monocytes may be closer to the percentages in humans. Previous investigations have so far focussed on subdivisions of $CD14^{lo}$ populations in man, subdivision of the $CD14^{hi}$ population has not been examined in humans and the use of further antibodies may show a sub-populations of $CD14^{hi}$ monocytes. $CD14^{hi}$ monocytes they make up 90% of circulating monocytes and heterogeneity in this population does not seem unlikely.

4.3 Discussion

Previous groups have used the LPS receptor CD14 and the scavenger receptor CD163 to divide pig monocytes into subsets. As with similar work in humans and rodents the assumption is that differing surface expression of such key immune molecules results in functional differences such as inflammatory role, cytokine production and pathogen recognition. Work on monocyte subsets in the pig has so far been limited to analyses of surface markers (Chamorro et al., 2004; Chamorro et al., 2000; Chamorro et al., 2005) or smaller studies looking at expression of specific genes (Moreno et al., 2010). This chapter aimed to identify differences at the genomic level between monocyte subsets in the pig and hypothesise how they may relate to functional differences between the cells *in vivo*.

4.3.1 Gene expression differences between pig monocyte subsets

In order to determine possible functions of pig monocytes, the 50 most differentially expressed genes were identified (**Figure 4.5**). $CD163^{lo}$ monocytes expressed between 6-12 fold higher levels of the chemokine IL8 and its receptors (*IL8RB, IL8R2, CXCR2*) than $CD163^{hi}$ monocytes. IL8 was first identified as a powerful neutrophil chemoattractant (Yoshimura et al., 1987) and has been demonstrated to have similar effects in several species (Beaubien et al., 1990; Hassfurther et al., 1994; Lindley et al., 1988; Caswell et al., 1999; Seow et al., 1994). IL8 has been shown to have a wide variety of effects on neutrophils (Mukaida et al., 1998) including increasing expression of the adhesion molecules

CD11b/CD18, CD11c/CD18 and CR1 (Detmers et al., 1990), and induction of superoxide and hydrogen peroxide and release of granule enzymes (Walz et al., 1987). Neutrophil infiltration is one of the hallmarks of acute inflammation and injection of IL8 resulted in rapid neutrophilia (Larsen et al., 1989; Laterveer et al., 1996). The relatively high expression of *IL8* by $CD163^{lo}$ monocytes suggests they may have an important role in recruiting neutrophils to sites of inflammation or infection. Moreover increased expression of IL8 receptors suggests IL8 may have an auto-stimulatory or regulatory role in $CD163^{lo}$ monocytes as has previously been described in T cells (Gesser et al., 1996). The recruitment of neutrophils to sites of inflammation is also of therapeutic interest as it is common in ischemia reperfusion injury (IRI) which can lead to complications in organ transplantation, cerebral stroke, myocardial infarction and bowel surgery (Litt et al., 1989). Depletion of neutrophils resulted in attenuation of IRI in an animal model (Korthuis et al., 1988). Identification of the $CD163^{lo}$ subset as important mediators of neutrophil influx may provide new therapeutic targets. IL8 also induced angiogenesis (Koch et al., 1992) although it has been suggested this was through the recruitment of leukocytes rather than direct actions of IL8 (Petzelbauer et al., 1995). Nevertheless IL8 production at sites of inflammation may lead to increased wound healing whether through direct or indirect formation of new blood vessels. In addition to its many effects on neutrophils, IL8 has also been shown to inhibit IgE production by B cells (Kimata et al., 1992), stimulate release of histamine from basophils (White et al., 1989) and to be chemotactic for T cells (Larsen et al., 1989). IL8 has been particularly implicated in Th_1 responses; Th_1 cytokines up-regulated IL8R expression on T cells *in vitro* while Th_2 cytokines inhibited T cell chemotaxis to IL8 (Jinquan et al., 1995). Moreover IL8 has been shown to be involved in regulation of T helper cell activities, suppressing IL4 and up-regulating its own production by CD4 T cells (Gesser et al., 1996). Expression of the IL8 receptor CCR1 has also been shown to define a subset of highly cytotoxic CD8 T cells with high levels of perforin, granzyme B and IFNγ which were primed to hone for early recruitment into sites of infection or inflammation (Hess et al., 2004). Expression of IL8 by $CD163^{lo}$ monocytes could provide an important link between the innate and acquired immune system through regulation of T cell function in addition to its neutrophil attracting properties. At present no direct murine homolog of IL8 has been identified and mice appear to lack the IL8 receptor CXCR1. Nevertheless the murine chemokines CXCL1 (KC), CXCL2 (MIP2) and CXCL5 (LIX) signal through a receptor

homologous to human CXCR2 and mediate neutrophil chemotaxis (Bozic et al., 1994; Lee et al., 1995) suggesting a similar mechanism for neutrophil recruitment also exists in the mouse despite the lack of a direct homolog of IL8. In man $CD16^-$ monocytes have previously been reported to be enriched for genes involved in production of IL8 (Ancuta et al., 2009; Zhao et al., 2009; Ingersoll et al., 2010) and **Figure 4.1**) and to respond to LPS with production of IL8 (Cros et al., 2010). A recent study examining gene expression differences between three monocyte subsets (classical $CD14^{hi}CD16^-$, non-classical $CD14^{lo}CD16^{hi}$ and intermediate $CD14^+CD16^+$) found IL8 to among one of the most differentially expressed genes, being strongly expressed by the classical human subsets (Wong et al., 2011) suggesting that in men and pigs at least $CD14^{hi}$ monocytes are important mediators of the immune response by influencing neutrophil and T cell response to infection.

$CD163^{lo}$ monocytes also expressed higher levels of the cytokine IL18. IL18 is a member of the IL1 family, is structurally related to IL1β and was originally described as an inducer of IFNγ (Okamura et al., 1995). Like IL8, IL18 can regulate the adaptive immune response functioning as a regulator of both Th_1 and in the absence of IL12, Th_2 responses (Nakanishi et al., 2001). Autocrine IL18 has been shown to have pro-inflammatory functions by enhancing production of IL1β and TNFα and up-regulating expression of the adhesion molecules VCAM1 and ICAM1 on monocytes (Dai et al., 2004). IL18 has also been shown to have a role in resolution of the inflammatory immune response by preventing monocyte apoptosis and promoting differentiation into macrophages. IL18 increased production of the angiogenesis-regulating and immune cell chemotactic chemokines CXCL8, CXCL9 and CXCL10 (Coma et al., 2006) again suggesting $CD163^{lo}$ monocytes may serve as a link between the innate and adaptive immune system. Similarly the co-stimulatory molecule CD82 was more highly expressed by $CD163^{lo}$ monocytes. CD82 has been shown to function as an accessory molecule in T cell activation by associating with CD4 or CD8 to deliver co-stimulatory signals for the T cell receptor (TCR)/ CD3 pathway (Imai and Yoshie, 1993; Nojima et al., 1993). Signalling through CD82 links to the actin cytoskeleton, so may have a particular role in T cell co-stimulation through induction of morphological changes (Lagaudriere-Gesbert et al., 1998; Delaguillaumie et al., 2004). $CD163^{lo}$ monocytes expressed a large number of genes associated with control of the adaptive immune response

suggesting they may serve as an important bridge between the innate and acquired immune system.

A pro-inflammatory role was also suggested for CD163^{lo} monocytes due to high expression of the pro-inflammatory genes *PTGS2* and *S100A8*. *PTGS1* and *PTGS2* catalyze the first step in the synthesis of the fatty acid derivatives prostaglandins, thromboxanes and prostacyclins (KOSAKA et al., 1994). Prostaglandins play key roles in regulation of immune function, angiogenesis and kidney development (Williams et al., 1999) and contribute to T cell development (Rocca et al., 1999). *PTGS2* is generally seen as pro-inflammatory and is a major target for the treatment of inflammatory disease (Willoughby et al., 2000). S100A8 has also been implicated in inflammation (Odink et al., 1987; Roth et al., 2003; Goyette and Geczy, 2010; Perera et al., 2009; Ravasi et al., 2004) and excessively high levels have been associated with recurrent infections and inflammation (Sampson et al., 2002). S100A8 has also been shown to modulate cystoskeletal-membrane interactions during macrophage activation and to be involved in phagocyte migration (Roth et al., 1993). Similar high expression of genes of the S100 family has been described in human CD14^{hi}CD16^{-} monocytes (Wong et al., 2011) suggesting the proposed pig and human "classical" monocytes share a similar pro-inflammatory role.

The lysosomal asparaginyl endopeptidase legumain (*LGMN*) was the most highly expressed gene in CD163^{hi} pig monocytes. *LGMN* can be found in lysosomes and degrades antigenic proteins for presentation to CD4^{+} cells so is responsible for a key step in antigen presentation (Manoury et al., 1998; Antoniou et al., 2000). CD163^{hi} monocytes also expressed the highest levels of the SLA class II gene *SLA-DRB1*, levels of other SLA genes were also higher in CD163^{hi} monocytes (*SLA-DQA, SLA-DQB1, SLA-DRA, SLA-DRB1, SLA-DRB2*) suggesting a role for porcine CD163^{hi} monocytes in class II processing and presentation. A similar role in MHC processing has been described for human "intermediate" CD14^{+} CD16^{+} monocytes (Wong et al., 2011).

Research in the last 10 years has revealed that Micro RNAs (miRNAs) control gene expression at several different levels including mRNA stability and translation. miRNAs are a class of non-coding, single stranded RNA molecules of around 19-25 nucleotides; each targets a spectrum of target genes at a post-transcriptional level by imperfect base-pairing with the 3'-UTR (David, 2004). Distinct miRNAs control several steps within the acquired and innate immune responses, development of disease and immune cell differentiation (Baltimore et al., 2008; O'Connell et al., 2010). The miRNA miR-29c was highly expressed in pig CD163^{hi} monocytes. miR-29c has been shown to suppress responses to intra-cellular pathogens by targeting expression of the archetypal Th_1 cytokine, IFNγ. Mice infected with intracellular bacteria down-regulated miR-29 expression in NK cells, and $CD4^+$ and $CD8^+$ T cells which enabled these cells to produce higher levels of IFNγ. miR-29 also directly targeted IFNγ mRNA which suppressed production of IFNγ (Ma et al., 2011). The miR-29 family have also been shown to target the zinc finger protein TNFα-induced protein 3 (*TNFAIP3*) (Wang et al., 2011a). *TNFAIP3* inhibits NFκB activation and TNF-mediated apoptosis (Vereecke et al., 2009). Such negative regulation of the immune response is an import method of limiting inflammation. Expression of miR-29c has been shown to inversely correlate with *TNFAIP3* in hepatocellular carcinoma cell lines and clinical samples and over-expression of miR-29c was shown to suppress *TNFAIP3*, inhibit cell proliferation and induce apoptosis in a hepatitis B virus-related hepatocellular carcinoma cell line. miR-29 family members may also target genes involved in fibrosis in organs such as the lungs (Cushing et al., 2011). CD163^{hi} monocytes are thought to mature from CD163^{lo} monocytes therefore high expression of miR-29c by CD163^{hi} monocytes may inhibit monocyte proliferation as the cells mature to eventually become tissue macrophages.

CD163^{hi} monocytes also expressed higher levels of kynurenine 3-monooxygenase (*KMO*) which is involved in the *IDO* mediated metabolism of kynurenine to 3-hydroxykynurenine (**Figure 5.1**). *IDO1* was also expressed at higher levels in CD163^{hi} monocytes. *IDO1* is highly LPS inducible in mature macrophage populations and is discussed in more detail in **Chapter 5**.

4.3.2 Differences between human and mice monocyte subsets

Many diseases are associated with monocytosis or alterations in the proportions of the monocyte subsets, increases in the $CD16^+$ subset is particularly associated with inflammatory disease (Baeten et al., 2000; Blumenstein et al., 1997; Ellery et al., 2007; Fingerle et al., 1993; Kawanaka et al., 2002; Mosig et al., 2009). Increases in the intermediate subset have also been reported in asthmatic patients (Moniuszko et al., 2009) and $CD14^+CD16^+$ "intermediate" monocytes have been shown to be most permissive to infection with HIV (Kim et al., 2010; Ellery et al., 2007). Mice are often used to model human inflammatory disorders therefore there is great interest in determining any similarities or differences between monocyte subsets in the two species in order to better model human conditions which affect relative monocyte numbers or which may result from alternations in a particular subset of monocytes.

Human $CD14^+CD16^-$ monocytes produced a broad range of cytokines including IL10 and IL6 but little TNFα after stimulation with LPS while $CD14^+CD16^+$ monocytes were the main producers of the inflammatory cytokines TNFα and IL1β (Belge et al., 2002; Wong et al., 2011). TNFα production has also been shown to be higher in a $CD43^{hi}$ subset of $Ly6C^{lo}$ mouse monocytes (Burke et al., 2008) although recruited $Ly6C^{hi}$ monocytes have also been shown to produce TNFα (O'Dea et al., 2011; Serbina et al., 2008). While the role of $Ly6C^-$ monocytes in infection is unclear they have been ascribed a patrolling behaviour (Auffray et al., 2007) which has been mirrored by human $CD16^+$ monocytes (Cros et al., 2010) giving important clues about their functions *in vivo*. As described previously new classification divides the continuum of CD14 and CD16 expressing monocytes seen in a FACS plot into three monocyte subsets with cells at either end of the spectrum being the most diverse in terms of expression of surface markers, gene expression, cytokine profiles and maturation state (Wong et al., 2011). It is also not yet clear how this new classification will transfer to other species where the number of "non-classical" monocytes are far higher than in humans. The high TNFα production by murine $CD43^{hi}$ monocytes does however raise the possibility that further subdivisions of the established monocyte subsets may be possible in species other than humans. Cros *et al.* (2010) analysed mouse and human monocyte subsets using microarray technology and segregated the data into three clusters corresponding to the

subsets they identified. They proposed that human $CD14^{hi}CD16^{-}$ and $CD14^{+}CD16^{+}$ monocytes were most closely related to each other and mouse $Ly6C^{lo}$ monocytes in contrast to previous reports (Ingersoll et al., 2010). Reanalysis of the data of Cros *et al.* using Biolayout *Express*^{3D} (Freeman et al., 2007) showed many genes that were expressed at high levels in $CD14^{hi}CD16^{-}$ monocytes were expressed at intermediary levels in $CD14^{+}CD16^{+}$ and expressed only at low levels or not at all in $CD14^{lo}CD16^{hi}$ monocytes suggesting that this really is a continuum. This included genes such as *CCR2, IL1RN, CD163, TREM1, S100A8* and *S100A12*. These genes have consistently been shown to be selectively expressed by $CD14^{hi}$ monocytes (**Figure 4.1**). There were no clusters which contained genes which were most highly expressed by $CD14^{+}CD16^{+}$ or $CD14^{lo}CD16^{hi}$ monocytes. By contrast Ingersoll *et al.* (2010) determined human $CD14^{hi}CD16^{-}$ monocytes to be most closely related to mouse $Ly6C^{hi}$ monocytes. In agreement with Ingersoll *et al*. Wong *et al.* (2011) determined the closest relationship to be between classical $CD14^{lo}CD16^{hi}$ and intermediate $CD14^{+}CD16^{+}$ monocytes. Additionally, unlike the gene expression studies by Cros *et al.*, array data from Wong *et al.* showed genes which were specifically expressed by both $CD16^{+}$ subsets. They also found $CD14^{lo}CD16^{hi}$ monocytes to respond to LPS with induction of pro-inflammatory cytokines, in contrast to Cros *et al.* and in agreement with many previous studies (Skrzeczynska-Moncznik et al., 2008; Belge et al., 2002). Skrzeczynska-Moncznik *et al.* (2008) and Cros *et al.* both claim differences in the expression of TLRs between $CD14^{hi}CD16^{+}$ and $CD14^{dim}CD16^{+}$ subsets but it is unclear from the data presented by Cros *et al.* whether this was significant for all differences claimed, Skrzeczynska-Moncznik *et al.* did however show significance for TLR2 and TLR4 expression suggesting different monocyte subsets would respond to different infectious stimuli.

Cros *et al.* (2010) and Ingersoll *et al.* (2010) compared gene expression between human and mouse monocyte subsets but came to different conclusions about the relationship between them. Some of the differences between the data from both studies could lie in the isolation methods used. Cros *et al.* isolated monocytes based on expression of CSF1R (CD115) positive cells while Ingersoll *et al*. purified on size/density and then removed NK cells using anti-CD56, they then positively enriched using magnetic beads. This may have resulted in Ingersoll *et al.* failing to purify the $CD14^{dim}CD16^{+}$ cells that Cros *et al.* emphasised.

Additionally the anti-CD14 antibody used by Cros *et al,* M5E2, has been show to block LPS activity (Power et al., 2004) explaining the differences in cytokine profiles reported by Cros *et al.* compared to previous studies (Belge et al., 2002; Wong et al., 2011; Skrzeczynska-Moncznik et al., 2008). This suggests that contrary to the findings by Cros *et al.* human $CD14^{hi}CD16^{lo}$ and mouse $Ly6C^{hi}$ monocytes are most probably the closest comparable cell types between the two species. Furthermore the differences between these two papers show that experimental data from different laboratories is not strictly comparable, differences in isolation methods, antibodies used and the rather arbitrarily defined gates used to separate populations can all lead to different results.

4.3.3 A core set of genes is conserved in human, mouse and pig monocyte subsets

Monocytosis or alterations in relative numbers of monocytes are features of a many inflammatory conditions (Ancuta et al., 2009; Blumenstein et al., 1997; Ellery et al., 2007; Fingerle et al., 1993; Kawanaka et al., 2002; Moniuszko et al., 2009; Steppich et al., 2000). As discussed at length in the introduction to this thesis, pigs may function as a better model for many inflammatory human conditions than many of the more commonly used rodent models. The comparison of pig and human monocytes on a genome wide scale may therefore provide targets to manipulate or markers to affect disease outcome. Differential expression of CD14 and CD16, which have traditionally been used to divide human monocytes into subsets (Ziegler-Heitbrock et al., 1993; Ziegler-Heitbrock, 1996), was somewhat conserved between human, mouse and pigs (**Figure 4.13**). However, CD16 has not been used as a subset marker in other species, so the subpopulations defined by this marker may not be strictly comparable. It is worth recalling that whereas $CD16^{+}$ monocytes account for only 10% of human monocytes, CD14 low populations (with differential expression of Ly6C or CD163) are more like 50% of monocytes in mice and pigs. There is however another population of $CD14^{lo}$ monocytes in the pig which did not express CD163 or CD16. If this population was viewed as the "true" $CD14^{lo}$ population and CD163 seen merely as a further marker to differentiate $CD14^{hi}$ monocytes then the numbers of $CD14^{hi}$ and $CD14^{lo}$ monocytes between pigs and humans may be more comparable.

As discussed above, expression of many genes which control interactions with the adaptive immune system (*IL8, IL18*) were conserved between human and pig CD14^{hi} monocytes. Expression of granulocyte-colony stimulating factor (GCSF, CSF3) receptor was also higher on human, pig and mouse CD14^{hi} monocytes (**Figures 4.1, 4.13**). CSF3 controls the production, differentiation and function of granulocytes (Avalos, 1996) and has been described as a maturation factor for monocytes as well (Jiang and Schwarz, 2010; Rossetti et al., 2010). Higher expression of *CSF3R* on CD14^{hi}CD16^{-} monocytes has been previously reported (**Figure 4.1**) and may suggest that production of this monocyte subset in the bone marrow is co-ordinated with granulocytes (Ingersoll et al., 2010; Mobley et al., 2007). Moreover IL8 is a powerful neutrophil chemotactant produced by CD14^{hi} monocytes in humans and pigs suggesting an important relationship between CD14^{hi} monocytes and neutrophils. Mobely *et al.* suggested numbers of CD14^{hi}CD16^{-} monocytes may be correlated to numbers of granulocytes in order to ensure sufficient numbers were available to clear apoptotic neutrophils. Tying the production of these immature monocytes with the production of neutrophils by co-expression of CSF3R would appear to be a mechanism to do so. There appears to be a clear immune function for CD14^{hi} monocytes in humans and pigs and this subset perhaps appears to be the most consistent across the species. This suggests immature monocytes can be compared between species but markers in use at present do not allow strict comparisons between the more mature CD14^{lo} subsets. CD163 may function better as a differentiator of CD14^{hi} monocytes in the pig, this would be more comparable with its role in man where it has been described to define a subset of CD14^{hi} monocytes (reports suggest between 7-20% of monocytes expressed CD163) (Asleh et al., 2003; Zwadlo et al., 1987)

One of the archetypal features of blood monocytes is their phagocytic ability. Studies have suggested mouse Ly6C^{lo} monocytes were more efficient phagocytes (Tacke et al., 2006; Randolph et al., 2008) and gene expression differences suggested a similar role for human CD16^{+} monocytes as discussed in the introduction (Zhao et al., 2009). Functional studies have provided conflicting results, these are largely summarised in **Table 4.1**. Experiments using latex beads suggested human CD16^{+} monocytes possessed greater phagocytic ability (Mosig et al., 2009) while comparing the ability to phagocytose *Aspergillus fumigate* found

both $CD16^+$ and $CD16^-$ monocytes had similar phagocytic ability although only $CD16^-$ monocytes inhibited germination (Serbina et al., 2009). Similarly earlier reports ascribed higher phagocytic capacity to human $CD64^+$ monocytes which were either $CD16^+$or $CD16^-$ (Grage-Griebenow et al., 2000; Grage-Griebenow et al., 1993). More recent studies using latex beads showed both subsets were able to phagocytose the beads but $CD16^-$ monocytes had higher uptake of the beads (Wildgruber et al., 2009). These sometimes conflicting reports suggest that both subsets have some phagocytic ability depending on the stimuli. One of the main differences noted by others between human and mouse monocyte subsets was relative expression of phagocytic and scavenger receptors. In mice these were found on $Ly6C^{lo}$ monocytes (*SIRPA, MSR1, CD36, THBS1*) while in humans they were expressed more highly on $CD16^-$ monocytes, or were not differentially expressed at all, again suggesting that in man neither subset preferentially functions as phagocytes (Ingersoll et al., 2010). The scavenger receptors *MSR1* and *CD36* are involved in clearance of apoptotic cells (Erwig and Henson, 2007) and expression was conserved in human and pig but not mouse. CD36 has a role as scavenger receptor for oxidised low-density lipoproteins and CD36 deficiency is frequently associated with atherosclerotic cardiovascular disease (Hirano et al., 2003). Although expression of these molecules was conserved between humans and pigs *CD36* was more highly expressed by $CD14^{hi}$ monocytes while *MSR1* was expressed by $CD14^{lo}$ monocytes in both species, again suggesting that one subset is not specialized for phagocytic ability in humans or pigs as appeared to be the case in the mouse (Ingersoll et al., 2010). Similarly *TREM1* was more highly expressed by human and pig $CD14^{hi}$ monocytes and by mouse $CD14^{lo}$ monocytes. TREM1 activates cells for pro-inflammatory cytokine release and is a crucial mediator of septic shock (Bouchon et al., 2001). Expression of the notch 2 ligand jagged 1 (*JAG1)* was also conserved in human and pig $CD14^{hi}$ monocytes but expressed more highly by mouse $CD14^{lo}$ monocytes. *JAG1* has been shown to promote cell survival (Choi et al., 2009) and has been described as a potential Th_2-promoting factor (Goh et al., 2009). Expression of JAG1 on APC promoted Th_2 development through a STAT6-independent pathway (Amsen et al., 2004). The conservation of gene expression between human and pig $CD14^{hi}$ monocytes noted above may suggest these subsets are specialized for specific functions in both human and pig that is not conserved to the same degree in the mouse.

Conservation of gene expression in $CD14^{lo}$ human ($CD14^{lo}CD16^{+}$), pig ($CD14^{lo}CD163^{hi}$) and mouse ($Ly6C^{lo}$) monocyte subsets was seen for *CD16, ICAM2, TGFBR3, ITGAL* and *LTB*. Human and pig $CD14^{lo}$ monocytes alone shared high expression of the complement component 3 (*C3*). The C3 receptor 1 (*C3AR1*) has previously been reported to be more highly expressed by $CD14^{lo}$ monocytes (Mobley et al., 2007; Ancuta et al., 2009). C3 is an ancient molecule being found in sea urchins and may have a role outside of the immune system (Al-Sharif et al., 1998). In the immune system, C3 contributes to innate immunity by promoting phagocytosis and supporting local inflammatory responses. It also provides an important link to the acquired immune system by modulating complement-dependent leukocyte functions and enhancing the humoral response to antigen (Sahu and Lambris, 2001). Naturally occurring C3 deficiency leads to increased susceptibility to bacterial infections (Lachmann, 1975) and certain immune disorders such as systemic erythematosus (SLE) (Matsuyama et al., 2001).

Previous reports have described mouse $Ly6C^{lo}$ and human $CD16^{+}$ monocytes as better stimulators of mixed lymphocyte reactions moreover both mouse $Ly6C^{lo}$ and human $CD16^{+}$ monocytes expressed higher levels of genes encoding MHC class II molecules than $Ly6C^{lo}$ and $CD16^{-}$ monocytes respectively (Ingersoll et al., 2010; Wong et al., 2011). Similar reports have been published describing better T cell stimulating ability of pig $CD163^{hi}$ monocytes (Sanchez et al., 1999) and the increased class II gene expression (SLA-DQ and DB) on $CD163^{hi}$ monocytes is consistent with this view.

Gene expression data also identified some transcription factors which were differentially expressed between monocyte subsets in the pig. The macrophage specific transcription factor *TFEC* (Rehli et al., 1999) was more highly expressed on $CD14^{lo}CD163^{hi}$ pig monocytes, expression of *TFEC* was converse to what was observed in human and mouse monocytes where it was more highly expressed by $CD14^{hi}$ monocytes in both species. *TFEC* has been shown to be induced upon stimulation of macrophages with LPS or Th2 cytokines in a STAT6 dependent manner. Interestingly *Tfec* mutant mice had much lower induction of *Csf3r* after stimulation with IL4 (Rehli et al., 2005). Additionally *ETS2* was more highly

expressed on $CD14^{lo}CD163^{hi}$ pig monocytes. *ETS2* has been shown to be involved in expression of the macrophage scavenger receptor gene (Wu et al., 1994). $CD14^{hi}$ $CD163^{lo}$ monocytes expressed higher levels of the transcription factors *IRF5* and *POU2F2*, *POU2F2* expression was converse to what was reported in human and mice monocytes (Ingersoll et al., 2010). *IRF5* has been shown to promote polarization of macrophages towards an inflammatory phenotype with high expression of IL12 genes and repression of IL10 (Krausgruber et al., 2011). *POU2F2* has been described to act as a cell survival factor by directly activating BCL2 (Heckman et al., 2005). Differential expression of transcription factors has not been described in porcine monocyte subsets before.

Perhaps the most unexpected finding of this study was the higher mRNA levels of *CCR2* on $CD14^{lo}CD163^{hi}$ pig monocytes. This contradicts previous reports (Moreno et al., 2010) and is in contrast to the low expression of CCR2 on human and mouse $CD14^{lo}$ monocytes and high expression on $CD14^{hi}$ monocytes from both species (**Figure 4.14**). High expression of CCR2 is one of the defining features of $CD14^{hi}$ monocytes in both mice and man and *CCR2* was one of the most differentially expressed genes between human monocyte subsets (Ingersoll et al., 2010). Porcine expression of CCR2 converse to what has been described in human and mice would have important consequences for homing in response to CCL2 expression at site of infection or inflammation. For instance $CCR2^{+}$ monocytes are preferentially recruited in atherosclerosis (Veillard et al., 2005). However the array probes for *CCR2* did not show consistent expression, one was more highly expressed by $CD163^{hi}$ monocytes and one by $CD163^{lo}$ monocytes. *CCR2* is not annotated on the pig genome and porcine specific anti-CCR2 antibodies are not available, previous studies have relied on indirect methods to assess its expression (Moreno). Correct identification of the porcine *CCR2* gene or development of porcine specific anti-CCR2 antibodies are essential. Similarly previous reports (Chamorro et al., 2005) identified porcine $CD14^{lo}CD163^{hi}$ monocytes as being the main producers of TNFα (Chamorro et al., 2005). This study found higher levels of *TNF* mRNA produced by the $CD14^{hi}$ $CD163^{lo}$ subset. Previous reports have described increased TNFα production from a subset of mouse monocytes expressing high levels of CD43 and low levels of Ly6C (Burke et al., 2008) suggesting TNFα production may be limited to a smaller subset of monocytes. Furthermore Cros *et al.* found intermediate

$CD14^{+}CD16^{+}$ monocytes to be the main producers of TNFα in response to LPS while non-classical $CD16^{hi}$ monocytes produced TNFα only in response to virus (Cros et al., 2010). This study suggests that monocytes previously referred to as $CD14^{lo}CD163^{hi}$ may in fact be more similar to human intermediate monocytes and the higher levels of TNFα produced may support this view. Finally it is unclear whether these differences at the mRNA level are reflected at the protein level. Previous studies have shown discrepancies between mRNA and protein levels (Zhao et al., 2009). Moreover this study analysed monocytes in their resting state, stimulation with LPS may results in higher induction of TNFα by a different population of monocytes altogether.

Chapter 5: Response of pig macrophages to LPS

5.1 Introduction

Mice and humans diverged approximately 70 million years ago (Waterston et al., 2002) therefore as discussed at length in **Chapter 1**, it is unsurprising that their response to infectious stimuli often differs dramatically (Fairbairn et al., 2011; Mestas and Hughes, 2004). Nevertheless rodents are widely used to model the human immune system. Many important immune effectors are differentially expressed between human and mouse e.g. Indoleamine 2, 3-dioxygenase (*IDO*), inducible nitric oxide synthase (iNOS*, NOS2*), this means that despite their widespread use, mice may not always function as the best animal to model the human immune response. Indeed the literature is full of animal trials which have appeared promising in rodents only to fail to translate to meaningful improvements for human patients; Cancer and Alzheimer's are just two of the diseases which have been cured in mice (Harris, 1999) and multiple murine trials for sepsis spectacularly failed to reduce the death rate among human patients (Poli-de-Figueiredo et al., 2008; Lopez et al., 2004). Mice challenged with either live bacteria or a non-infectious molecule, such as LPS, react in a very different way to the heterogeneous group of patients classified as having sepsis: they are relatively resistant to LPS mediated toxicity (Copeland et al., 2005). Mice treated with LPS fail to develop the organ failure commonly seen in humans and die quite suddenly. Indeed mice are much more resistant to LPS than humans, commonly used doses (1-25mg/kg) are 1000 to 10,000 times what is needed to induce severe septic shock in humans (Warren, 2009; Munford, 2010). It has been suggested that the differences in sensitivity to LPS between man and mouse is due to proteins found in serum rather than differing responses of immune effector cells (Warren et al., 2010). Some researchers have produced "humanised" mice which may function as a better model than many commonly used non transgenic mice (Shultz et al., 2007). This provides a

solution to some of the issues associated with working on rodents although there may be many as yet unknown differences between man and mouse.

5.1.1 Murine production of nitric oxide

Mice respond to microbial challenge with robust production of nitric oxide (NO). Macrophages are the principal effector cell for the production of NO in the mouse (Stuehr and Marletta, 1985) and the cytotoxic effects of NO have been well documented in rodents (Bogdan, 2001; MacMicking et al., 1997). As discussed in Chapter 1, NOS mediates the production of NO from arginine. Alternatively arginase-1 (*ARG1*) metabolises arginine generating ornithine. This is a classic pathway of M2 or alternatively activated macrophages as opposed to the NOS2 mediated metabolism of arginine to NO that predominates in M1 or classically activated macrophages (Hassanzadeh Ghassabeh et al., 2006; Munder et al., 1998; Martinez et al., 2006). In addition to the extensively studied anti-microbial effects of NO (Nathan and Hibbs Jr, 1991), anti-tumour (Xie et al., 1996; Brantley et al., 2010) and immunosuppressive (DAI and GOTTSTEIN, 1999) effects have been described. *Nos2* $^{-/-}$ mice failed to clear *Listeria monocytogenes* infection illustrating the importance of NO in clearance of intracellular pathogens. Furthermore macrophages from these animals failed to suppress lymphoma replication *in vitro* showing the important anti-tumour effects of NO (MacMicking et al., 1995). NOS inhibitors have had adverse effects on disease outcome in the majority of mouse models tested. In tuberculosis (Chan et al., 1995), malaria (Nussler et al., 1993), herpes simplex (Croen, 1993), and Cryptococcus neoformans infections (Lovchik et al., 1995) NOS inhibitors worsened disease outcome demonstrating the importance of NO in the immune response of the mouse to a wide range of pathogens. The effects of NO in the mouse are however double edged. NO defends the host against bacterial infection at the risk of damage to tissues or induction of shock. It is therefore not entirely surprising that NO inhibitors have also had protective effects in some mouse models for instance *Nos2* $^{-/-}$ mice did not experience hypotension in a model of endotoxemia (MacMicking et al., 1995). Similarly protective effects have

been observed with NO inhibitors in murine models of septic shock (Bahar Tunctan et al., 1998). Unfortunately these promising results in the mouse have not translated into benefits for man. Hypotension is one of the most serious clinical symptoms of septic shock. NO has vasodilating effects, researchers have therefore tried to block NO production by inhibiting NOS2 as a possible treatment for sepsis in humans, unfortunately inhibiting NOS2 did not improve patient survival in clinical trials and in some cases even worsened the outcome (Bakker et al., 2004; Lopez et al., 2004; Poli-de-Figueiredo et al., 2008). It is likely NO inhibitors in humans inhibited eNOS instead of iNOS which could interfere with vasodilation leading to an increase in hypotension instead of the desired decrease.

There is a large amount of evidence for the importance of NO as an immune effector in the mouse but the evidence for its importance in man is less compelling. Experiments detecting NO from human macrophages were often unable to be replicated in other laboratories (MacMicking et al., 1997) although some laboratories have reported *NOS2* expression in directly isolated macrophages from patients suffering from inflammatory disorders (Nicholson et al., 1996; Hunt and Goldin, 1992; Anstey et al., 1996; St Clair et al., 1996). It appears that some of the divergence between production of NO in man and mice may be due to differential control of transcription (Taylor and Geller, 2000). Transcription of *Nos2* in mouse macrophages is controlled by a basal promoter and an enhancer element. Both regions are LPS responsive and the enhancer region is also responsive to IFNγ. A series of inactivating nucleotide changes in the enhancer element in human macrophages resulted in failure to initiate transcription. Furthermore κB binding sites in both regions of the mouse *Nos2* gene have previously been shown to be important in regulation of expression. The comparative κB binding site in the basal promoter region of the human *NOS2* gene did not bind to NFκB/Rel complexes with the same specificity as mouse macrophages while the κB binding site in the enhancer region failed completely to bind NFκB/Rel complexes (Zhang et al., 1996). Additional experiments in transgenic mice carrying an insertional human *NOS2* promoter-reporter gene construct showed differing endogenous and LPS induced

expression of the transgene when compared to murine *Nos2*. This suggests many of the differences between *NOS2* expression in man and mouse may be due to differential regulation of the promoters in the different species (Yu et al., 2005). What is clear is there is an intrinsic difference in mouse and human macrophages. In culture mouse macrophages produce *Nos2* mediated NO which human macrophages fail to do. It appears that whatever is "different" about human macrophages is also "different" in pig macrophages; they too failed to produce NO in culture (**Figure 5.15**).

5.1.2 Human production of Indoleamine 2, 3-dioxygenase

As discussed above the classic murine response to infectious stimuli involves metabolism of arginine to produce NO. By contrast to murine macrophages, human macrophages preferentially metabolize tryptophan. Indoleamine 2, 3-dioxygenase-1 (*IDO1*) is a heme-containing enzyme which catalyses the degradation of tryptophan through the kynurenine pathway (**Figure 5.1**) (Taylor and Feng, 1991). Activated human macrophages internalise tryptophan and metabolise it via IDO to kynurenine and thence to several metabolites that have been implicated in regulation of immune cell function and antimicrobial actions. Interestingly tryptophan is the only amino acid which is specifically regulated in response to signals from the immune system. For tryptophan to be metabolized in macrophages by IDO it must first enter the cell by means of a solute carrier although the specific transporter for tryptophan has not yet been identified. System L is a widely expressed transport system which accepts tryptophan, among other amino acids, however system L is not specific enough to explain the level of tryptophan depletion by MDM in culture. It appears likely there may be two different tryptophan transport systems. One low affinity system (System L) and another as yet unidentified system with 100-fold higher affinity which is specific for tryptophan and up-regulated during MDM differentiation (Seymour et al., 2006).

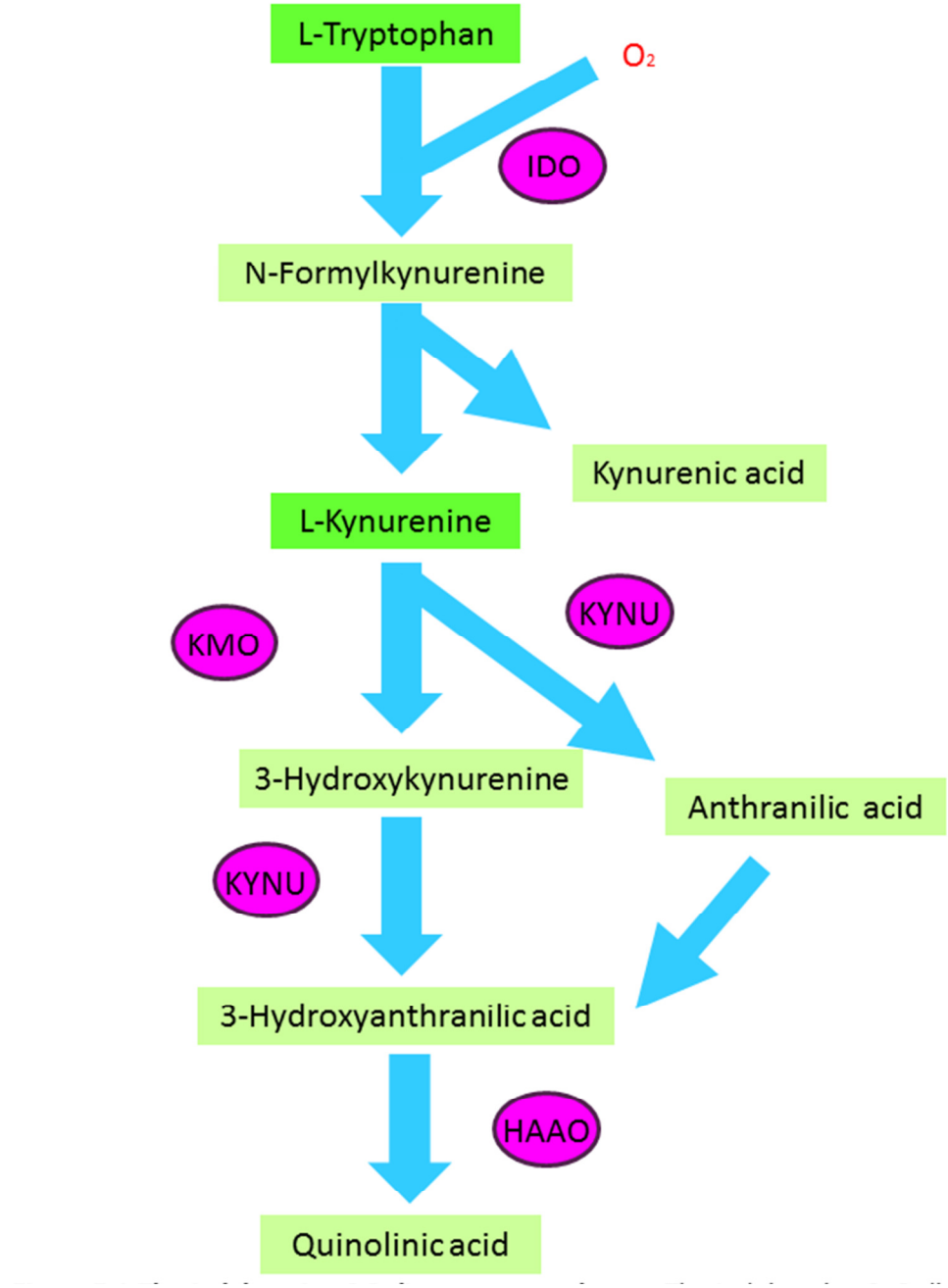

Figure 5-1 The Indoleamine 2,3-dioxygenase pathway.

The Indoleamine 2, 3-dioxygenase mediate catabolism of tryptophan to Kynurenine and its metabolites. **IDO**; indoleamine 2, 3-dioxygenase, **KYNU**; kynureninase, **KMO**; kynurenine 3-monooxygenase, **HAAO**; 3-hydroxyanthranilate 3, 4-dioxygenase.

The enzyme IDO1 and the metabolism of tryptophan can have immune regulatory properties; this pathway is involved in suppression of T-cell responses, activation of regulatory T cells and inhibition of natural killer cells (Mellor and Munn, 2004; Mellor et al., 2002). It is not clear exactly how the IDO mediated metabolism of tryptophan effects the immune response, whether depletion of tryptophan suppresses T cell proliferation (Mellor and Munn, 2004) or the kynurenine metabolites have a suppressive function (Moffett and Namboodiri, 2003) has yet to be proven. Certainly the kynurenine metabolites 3-hydroxyanthranilic acid (3-HAA) and quiniloinic acid (QUIN) have been shown to induce apoptosis specifically in Th1 cells (Fallarino et al., 2002), effects have also been shown on NK cells (Frumento et al., 2002; Song et al., 2011) and possibly B cells (Terness et al., 2002). The IDO1 pathway has been implicated in many diseased states. IDO1 is expressed by most human tumours and has tumour promoting properties in mice (Uyttenhove et al., 2003). Lower levels of tryptophan in the serum of cancer patients suggests IDO may be chronically active in some cancers. It was also suggested that constitutive expression of IDO by these patients may contribute to depression through disruption of serotonin metabolism (Schroecksnadel et al., 2007). The IDO1 pathway has also been implicated in sepsis, increased kynurenine levels in plasma after trauma were indicative for the later development of sepsis (Logters et al., 2009) and the ratio of quinolinic acid to tryptophan (Zeden et al., 2010) or kynurenine to tryptophan (Logters et al., 2009) have been used to discriminate between non septic and pre septic patients. Patients already suffering from septic shock have also shown good correlation between plasma kynurenine to tryptophan ratios and plasma IFNγ, IL10 and lymphopenia (Darcy et al., 2011). IDO1 activity can lead to increased vasodilation which may cause the drop in blood pressure which often leads to organ dysfunction and death for many septic patients. Additionally the tryptophan metabolite kynurenic acid had an inhibitory effect on TNFα and the DNA-binding protein high-mobility group box protein1 (HMGB1) production by monocytes *in vitro* (Wang et al., 2006; Tiszlavicz et al., 2011). HMGB1 can function as a positive control of pro-inflammatory cytokine production (Andersson et al., 2000) and high levels have also been found in septic patients suggesting the kynurenine produced by

the IDO pathway is not being further metabolised to kynurenic acid (Wang et al., 1999).

Recent studies have focused on the vasodilatory effects of kynurenine. Wang *et al.* showed systemic inflammation induced tryptophan metabolism which led to endothelial expression of *Ido1* in mice after infection or treatment with LPS. *Ido1* expression resulted in kynurenine dependent vasodilation and hypotension and inhibition of *Ido1* led to an increase in blood pressure (Wang et al., 2010). *Ido1* was not routinely expressed by mouse macrophages after stimulation with LPS or IFNγ (biogps.gnf.org) although there is some evidence that it can be induced in murine macrophages when NO synthesis is inhibited using arginine free medium (Thomas et al., 1994; Alberati-Giani et al., 1997). Any vasodilating effects reported by Wang *et al.* must therefore have been due to endothelial *Ido1* expression as they did not inhibit NO production. They also found that *Ido1*$^{-/-}$ mice were less hypotensive than wild type mice but that *Nos2a* $^{-/-}$ mice were even less hypotensive. This illustrates one of the issues of using mice as a model for sepsis, not only are mice far more resistant to endotoxin than man but any response involves the NO pathway, a pathway not active in human macrophages. The same authors showed that tryptophan and kynurenine also had *IDO1* dependent vasodilatory effects on pig arteries and as pigs do not produce NO (**Figure 5.15**) it seems clear that they can provide a better model to study the complicated immune interactions that result in sepsis in man.

A major aim of this thesis was to examine the pig response to LPS under similar conditions to those used in mouse and human studies. Affymetrix array chips were used to analyse the gene expression profile of LPS stimulated pig BMDMs with regard to differentially expressed genes in human and mouse. There are many genes which are known to be differentially regulated but which were not detected by the Affymetrix array, a selection of these were investigated with RT qPCR (*IDO1,*

CCL20). The nitric oxide pathway and IDO mediated catabolism of tryptophan are LPS inducible pathways in mice and men respectively and the activation of these pathways in the pig was examined in more detail and possible implications of blocking IDO as treatment for sepsis in man were investigated.

5.2 Results

Figure 5.2 shows a workflow diagram for the analysis of microarray data presented in this chapter

5.2.1 Microarray analysis of Porcine BMDM

5.2.1.1 Gene expression after LPS stimulation in porcine BMDM – analysis by microarray

Changes in gene expression at the transcript level were examined using RNA from BMDM stimulated with LPS for up to 24 hours (0, 2, 7 and 24h) from 3 Large White x Landrace F1 cross (1 female, 2 males) and Affymetrix porcine microarrays. As previously observed in humans and mice (Wells et al., 2003; Hume et al., 2007; Nilsson et al., 2006) the expression of a large number of genes was affected. Genes that were initially expressed at high levels were mainly repressed by LPS, while transcription of other genes was initiated or increased in response. LPS stimulation induced the expression of a very large number of genes – 3,343 probesets corresponding to 2,159 genes - peaking at 7 hours with 70.3 % of the total up-regulated genes (2,340 probesets corresponding to 1,518 up-regulated genes) including numerous known LPS-responsive cytokines and chemokines. The LPS response was very well regulated; 30% of the probesets were up-regulated at 7 hours alone, 25.7% were up-regulated only at 24 hours and 26.4% remained up-regulated between 7 and 24 hours. The software package BioLayout Express^{TM} (Freeman et al., 2007) was used to analyse genes that were co-expressed across the three individual pigs examined. Data was normalised as described in **Chapter 2** then were clustered using a correlation of R=0.95 and MCL of 1.7, generating 99 distinct

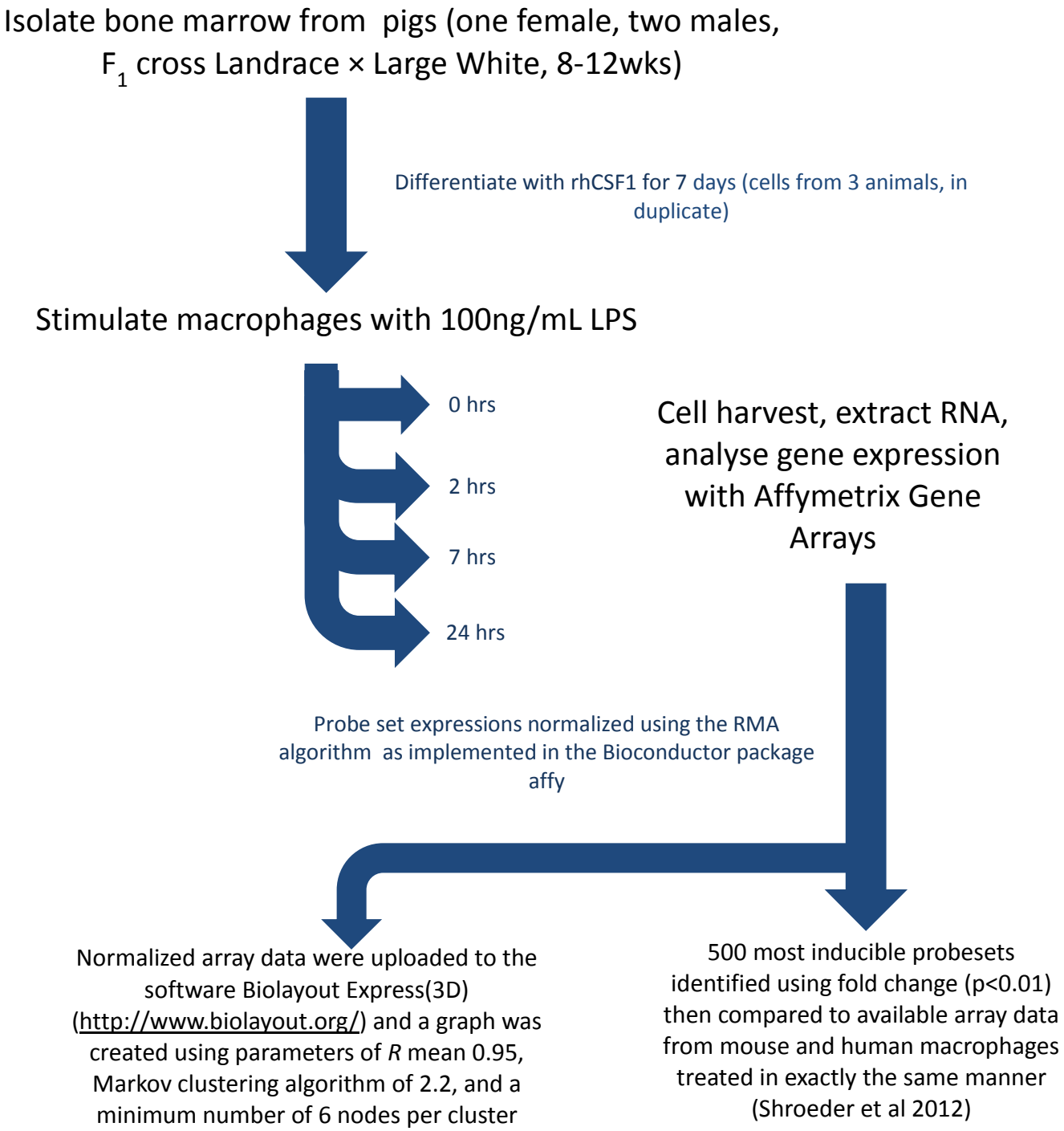

Figure 5-2 Work flow for studying the response of porcine BMDM to LPS.

Bone marrow derived macrophages were cultured by obtaining cells from 8-12 week old LW x Landrace F1 cross (2 males 1 female) and differentiated using rhCSF1. Following 7 days of differentiation cells were stimulated with 100ng/mL LPS then harvested at 0, 2, 7 or 24 hours post treatment to obtain RNA for analysis by Affymetrix array.

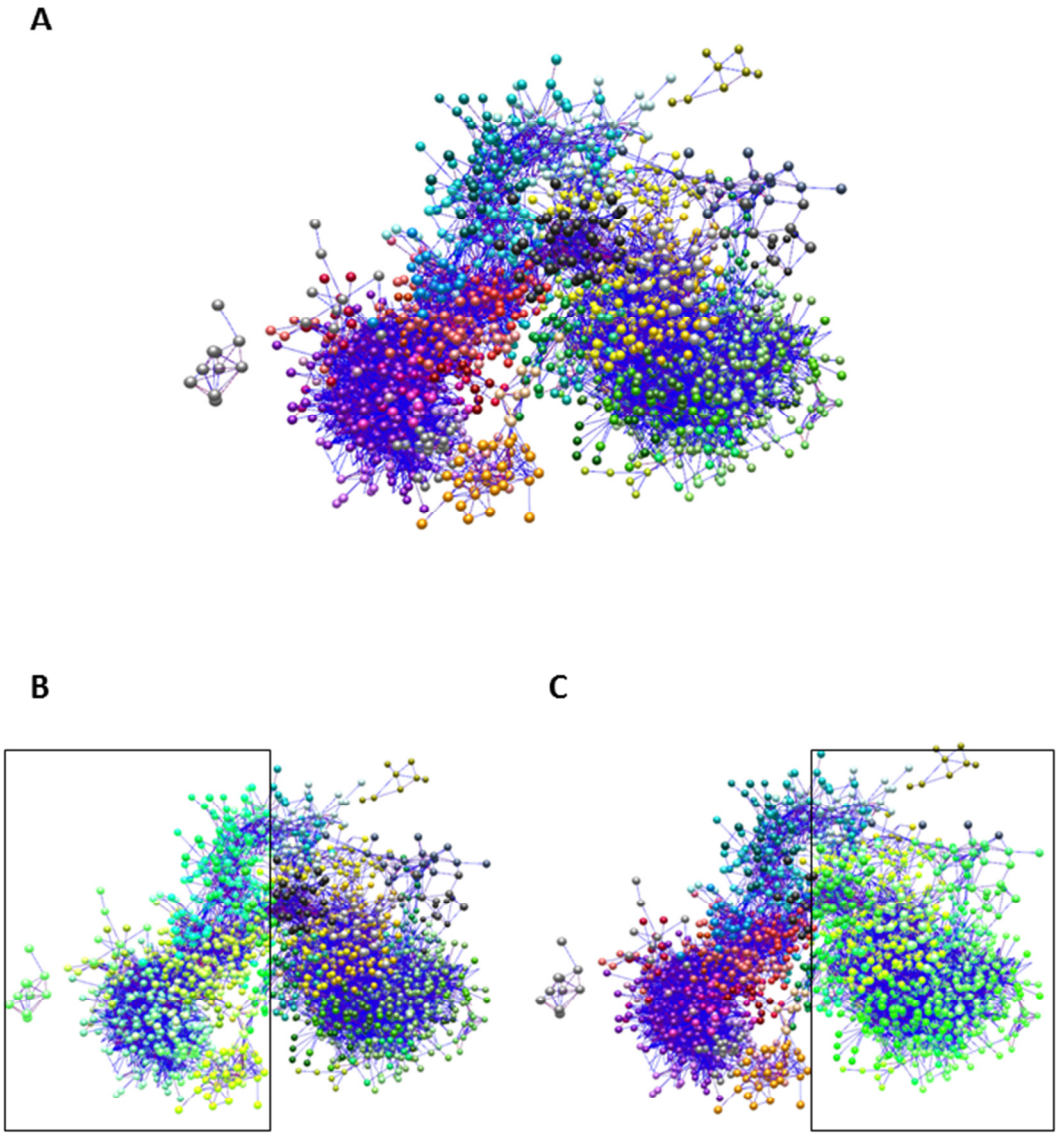

Figure 5-3 LPS regulated genes were clustered using BioLayout Express.

This generated 99 distinct clusters (**A**). Genes which were up-regulated in response to LPS (**B**) clustered together as did genes which were down-regulated (**C**).

clusters (**Figure 5.3A**). In keeping with the view that negative regulation and feedback control is a critical determinant of the duration and magnitude of LPS responses (Coll and O'Neill, 2010), LPS stimulation of pig BMDM triggered even more down-regulation of genes than up-regulation (a total of 5721 down-regulated probesets, corresponding to 4258 genes) (**Figure 5.3B).** The two largest clusters were one group of down-regulated genes (**Figure 5.3B**) and one group of up-regulated genes (**Figure 5.3C**). Genes clustered using Biolayout Express can be represented in a 2D format (**Figure 5.4**) using the graph editor yEd (yWorks.com) which allows the relationship between clusters to be more easily visualised. The largest cluster in this graph, Cluster 1 (**Figure 5.4D**) consisted of 699 probes which were down-regulated after 7 hours LPS stimulation. The third largest cluster, Cluster 3 (**Figure 5.4C**) contained 182 genes which were also down-regulated after 7 hours LPS stimulation but which returned to base levels by 24 hours. At 2 h, 14.3% of the probesets (609 genes) were already down-regulated, including the top down-regulated Hairy/Enhancer of Split 1 (*HES1*) a transcriptional repressor/activator, the Growth-Arrest and DNA damage-inducible gene alpha (*GADD45A*) and the oncogene MYC. Down-regulated genes from the 2 largest clusters included the TGFβ receptor 2 (*TGFBR2*), the angiogenic regulator vascular endothelial growth factor B (*VEGFB*) and the regulator of MAPK1/ERK2 kinase, suppression of tumorigenicity 5 (*ST5*). The multidrug resistance associated protein, *ABCC5* and several heat shock proteins (*HSPD1, HSP90, HSPA4*) were also down-regulated by LPS in the pig. *TLR8* was down-regulated while *TLR2* and *TLR6* were induced by LPS. The modification of TLRs in the porcine macrophages after activation with LPS demonstrates the modification of regulatory networks in response to PAMPS which has previously been reported in the mouse (Nilsson et al., 2006).

Cluster 2 (**Figure 5.4B**) contained 481 probes which were up-regulated by 7 hours LPS stimulation. A large number of these genes are associated with apoptosis and programmed cell death such as the ubiquitin specific peptidase and proteases and several caspases (*CASP3, CASP4, CASP7* and *CASP8)*. There were also a number of

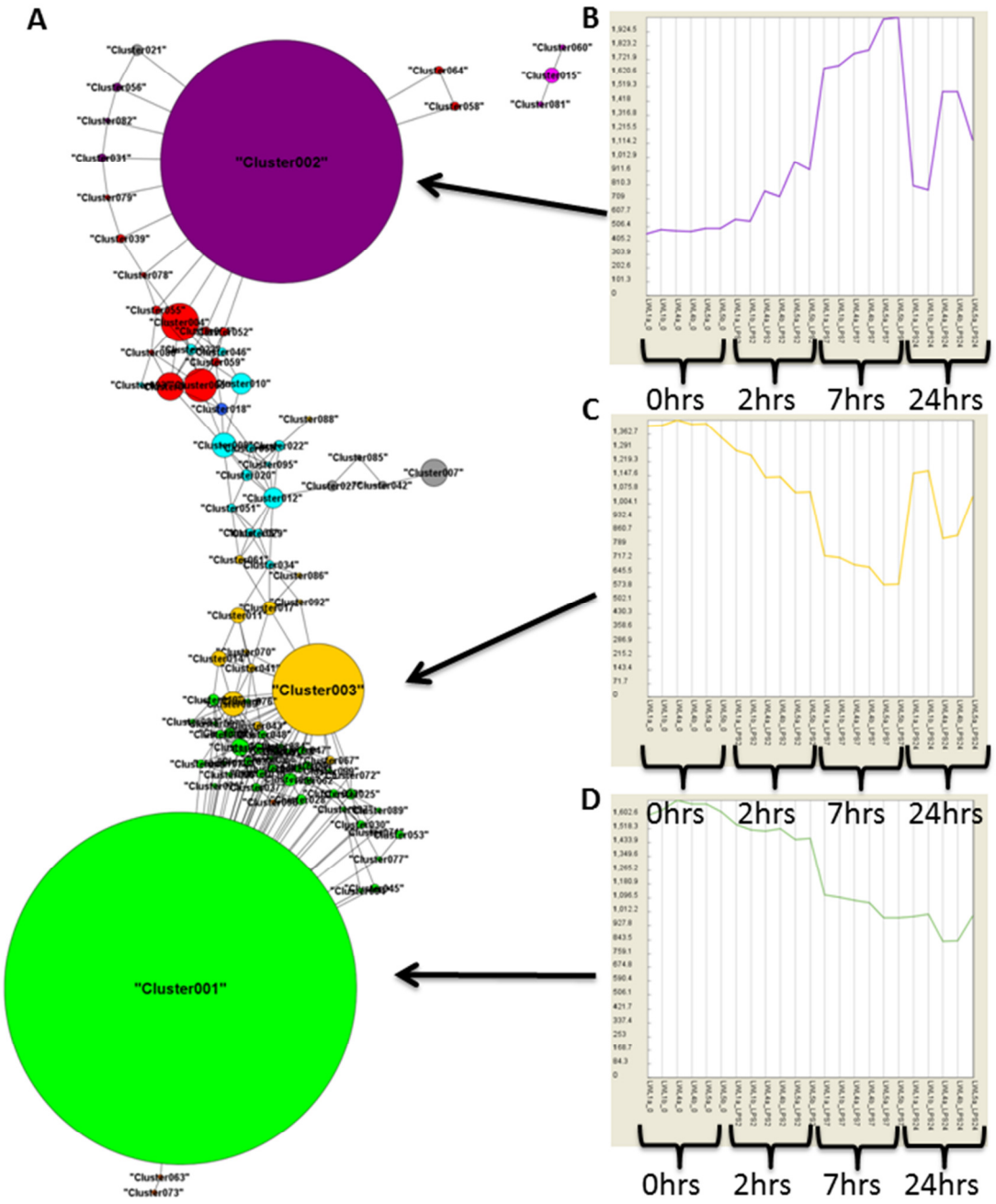

Figure 5-4 The three largest clusters of LPS regulated genes.

Cluster 1 (**A**) consisted of 193 genes (**B**) which were down-regulated by LPS after 7 hrs. 19 un-annotated genes were present.

transcription factors or genes associated with control of transcription such as *STAT1*, *STAT2, ETV6, ETV7, IFI16, IRF2* and *SP100*. Immune response associated genes included the thrombospondin receptor *CD47*. As discussed by Wells *et al.* (Wells et al., 2005), a major component of the inducible genes in mice comprised feedback repressors. In the pig such genes included inhibitory transcription factors (NFkBI) and co-repressors (*NCOR*), members of the SOCS family, dual specificity phosphatase 1 (*DUSP1*), *ATF3*, tristetraprolin (TTP, *ZFP36*) and several inhibitory cytokines (IL1RA (*IL1RN*), *TGFB*, *IL10*). *IL1RN* inhibits the activities of IL1α and IL1β both of which peaked after 2hrs LPS stimulation of the pig BMDM and declined thereafter. There were also a number of genes associated with control of lymphocyte function such as *IL27RA* which regulates Th1 type immune responses through a system involving STAT1; *IL7R* which is involved with lymphocyte development, and the positive regulator of Th1 type cytokine gene expression *PHF11*. *TGFB1* and *TGFB3* which are involved in cell differentiation were also found in cluster 2. Up-regulated genes from other clusters included many components of the TLR and other signalling pathways, including multiple transcription factors as NFkB1, the IRF family (*IRF1, 2, 3, 5, 7, 8* and *9*), CEBPβ (*CEBPB*) and delta (*CEBPD*) and PU.1 (*SPI1*) and co-regulatory factors such as JMJD3 (*KDM6B*), and demethylase 6B (Satoh et al., 2010).

Interestingly, two hours stimulation with LPS produced a relatively small number of probes affected by LPS; however, proportionally more genes had a larger fold change at this time point than at seven or 24 hours. After 2 hours of LPS treatment not many genes were affected, but those that were up-regulated, changed their expression to a large extent, thus suggesting a particularly crucial role for these genes. **Figure 5.5** shows the early response clusters, genes which were induced by 2 hours of LPS stimulation. There were three clusters of early response genes which clustered together (**Figure 5.5A&B**). The expression profile of the largest of these clusters (cluster 15) is shown along with the most related probes which consisted of two additional clusters (cluster 60 & 81) and some genes which did not fall into any

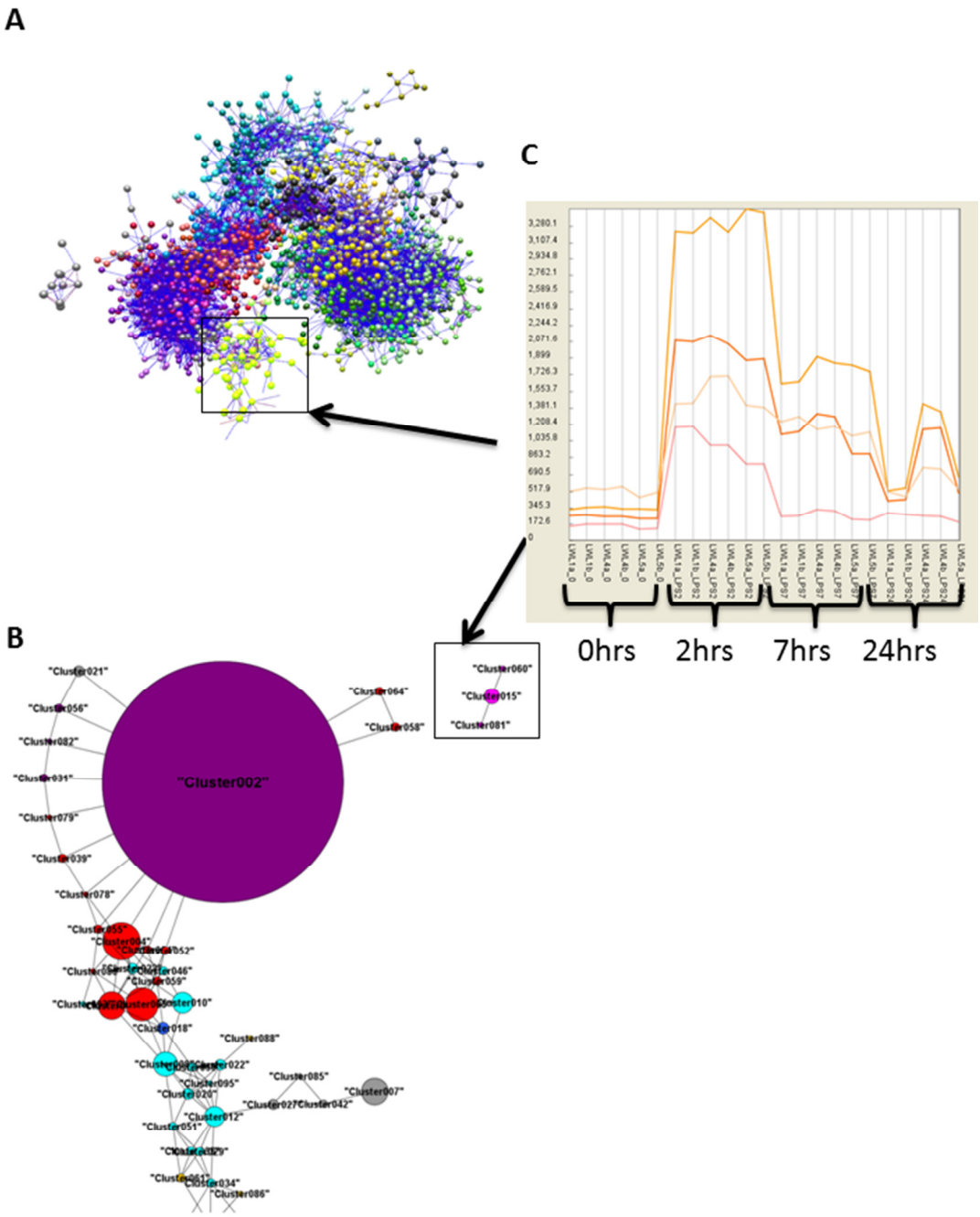

Figure 5-5 LPS early response genes.

Cluster 1 (**A**) consisted of 193 genes (**B**) which were down-regulated by LPS after 7 hrs. 19 un-annotated genes were present.

of the clusters but which were also induced by 2 hours LPS stimulation (**Figure 5.5C**). Many early response genes known to be LPS inducible in the mouse were in this group including the archetypal LPS response gene *TNF*, Interferon β (*IFNBI),* interferon regulatory factor 3 (*IRF3*), the interferon receptor (*IFNAR2*), and Jagged 1 (*JAG1*) – a molecule that is known to be a Notch ligand and a potential Th2-promoting factor. The Growth-Arrest and DNA damage-inducible gene beta (*GADD45B*) was also up-regulated by 2 hours. *GADD45B* was shown to inhibit the apoptosis signal of TNF by interfering with the JNK cascade (De Smaele et al., 2001). Up-regulation of *GADD45B* may allow the macrophages to release TNF without themselves undergoing apoptosis.

5.2.1.2 The pig response to LPS was similar to man

To compare the responses of pig BMDM to mouse BMDM, a list of the 500 most induced probe sets (comprising 174 gene names) in porcine BMDM (at any time point) was compiled and the regulation of these genes was examined in mouse BMDM or TEPM from earlier studies now displayed on biogps.gnf.org. From this list, 200 probes (63 genes) were identified that were not significantly regulated by LPS in mouse BMDM at any time point. The list of discordantly-regulated genes was then compared to publicly available similar human data sets (GSE8608 and GSE5099). Of those 63 genes, 29 were highly induced in activated human monocyte-derived macrophages (**Figure 5.6**). Some of them are related to the IDO and vitamin D3 pathway (*KYNU* and *CYP27B1*), active pathways in human but not in mouse. The heparin-binding EGF-like growth factor (*HBEGF*) was also up-regulated in human and pig but not in mouse. *HBEGF* is also known as the diphtheria toxin receptor (DTR) and interestingly human and pig are sensitive to the toxin whereas mouse are more resistant (Mitamura et al., 1995). **Figure 5.7A & B** show RT qPCR validation of the microarray data for the differentially expressed genes *IL7R* and *CXCL13*. To determine if the expression of these genes was cell type specific rather than species specific, expression was also examined in LPS activated porcine MDM. **Figure 5.7C & D** shows that *IL7R* and *CXCL13* were also LPS inducible in porcine MDM. This strongly suggests that the differing expression

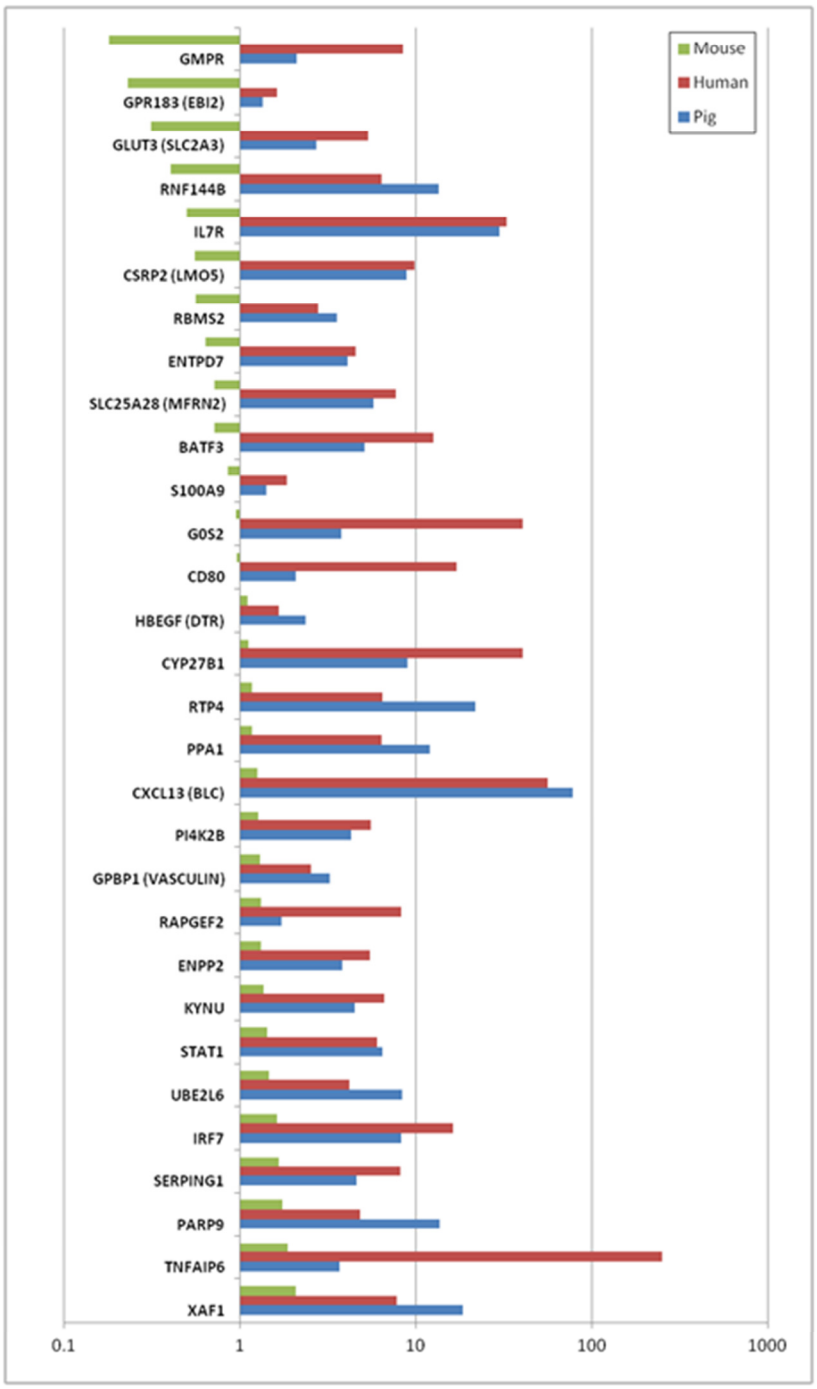

Figure 5-6 The pig response to LPS was similar to human.

Expression of genes was compared between pig, human MDM +LPS (GSE5099 and GSE8608) and mouse BMDM + LPS (unpublished data and biogps.gnf.org). Genes up-regulated in pig and human but not in mouse are shown.

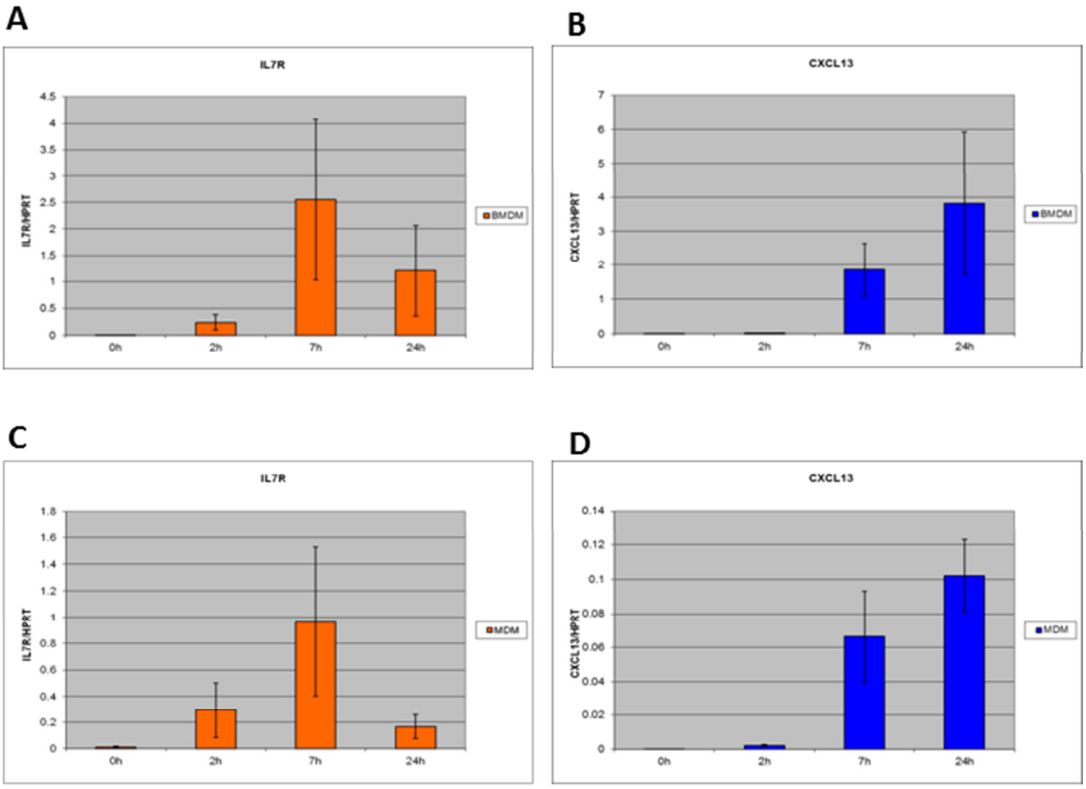

Figure 5-7 IL7R and CXCL13 were LPS inducible in BMDM and MDM

BMCs and PBMCs were treated with rhCSF1 for 7 days to differentiate them into macrophages. BMDM (**A & B**) and MDM (**C & D**) were then activated with LPS (100ng/ml) for 2, 7 or 24 hours. RNA was extracted and reverse transcribed. mRNAs for (**A & C**) IL7R, (**B & D**) CXCL13 and (**C**) CYP27B1 were measured by quantitative PCR. Results are representative of three experiments using RNA from different animals

between human MDM and mouse BMDM is due to species specific rather than cell type specific differences.

5.2.2 Analysis of gene expression profile in pig with regard to differentially expressed genes in human and mouse

5.2.2.1 LPS induced *STAT4*, *IDO1* and *CCL20* in BMDM

The Affymetrix array provided a large scale overview of porcine BMDM response to LPS which can be used to compare gene expression across species. Nevertheless there are some genes which are known to be differentially expressed between man and mouse which are not present in the microarray. Previous work in our laboratory had identified *IDO1*, the chemokine *CCL20* (also known as MIP-3-alpha) and the transcription factor signal transducer and activator of transcription (STAT) 4 as being up-regulated in human monocyte-derived macrophages but not in mouse in response to LPS (Schroder et al., 2012) ;www.biogps.gnf.org;(Martinez et al., 2006; Nilsson et al., 2006)). The lack of induction of these genes in mouse BMDM responding to LPS is also evident from genome-scale 5'RACE (CAGE) expression profile in the FANTOM project (Carninci et al., 2006) and in expression profiling of the RAW264 macrophage cell line (Murray et al., 2005). The mRNA expression of these genes was therefore investigated in porcine BMDM after LPS stimulation using RT qPCR **(Figure 5.8)**. *IDO1* expression was massively up-regulated after 7h and slowly decreased by 24h. The expression of *CCL20* and *STAT4* were also substantially induced after LPS stimulation continuing to increase up to 24 hours. The PCR products for *IDO1*, *STAT4*, *CCL20* and *HPRT* were cloned and sequenced and blasted against the pig genome to ensure the correct gene product was detected by the primers. In addition, the innate immune effector lipocalin-2 (*LCN2*, also known as NGAL) has been shown previously to be highly LPS inducible in mouse but not human macrophages (Schroder et al., 2012). *LCN2* was not induced in porcine BMDM by RT qPCR (data not shown).

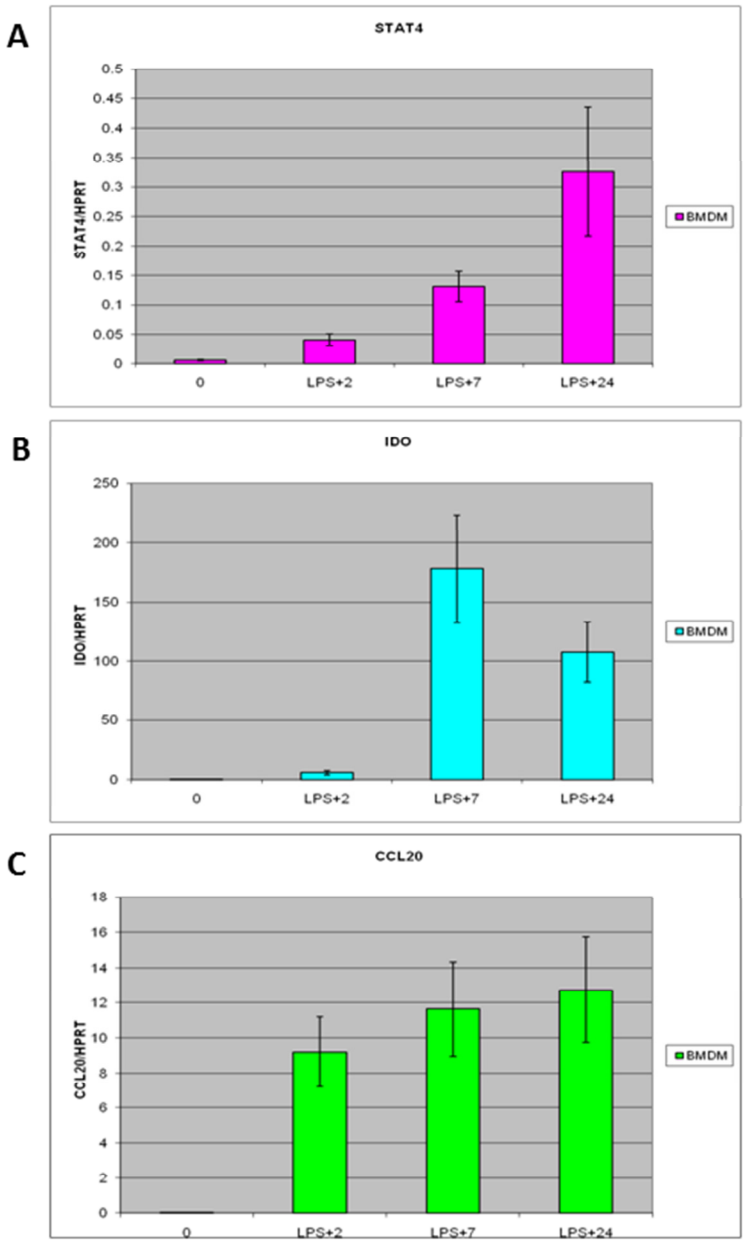

Figure 5-8 LPS induced *STAT4*, *IDO* and *CCL20* in porcine BMDM.

BMCs were treated with rhCSF1 for 7 days then activated with LPS (100ng/ml) for 2, 7 or 24 hours. RNA was extracted and reverse transcribed. mRNAs for (**A**) STAT4, (**B**) IDO and (**C**) CCL20 were measured by quantitative PCR. Results are representative of seven experiments using RNA from different animals

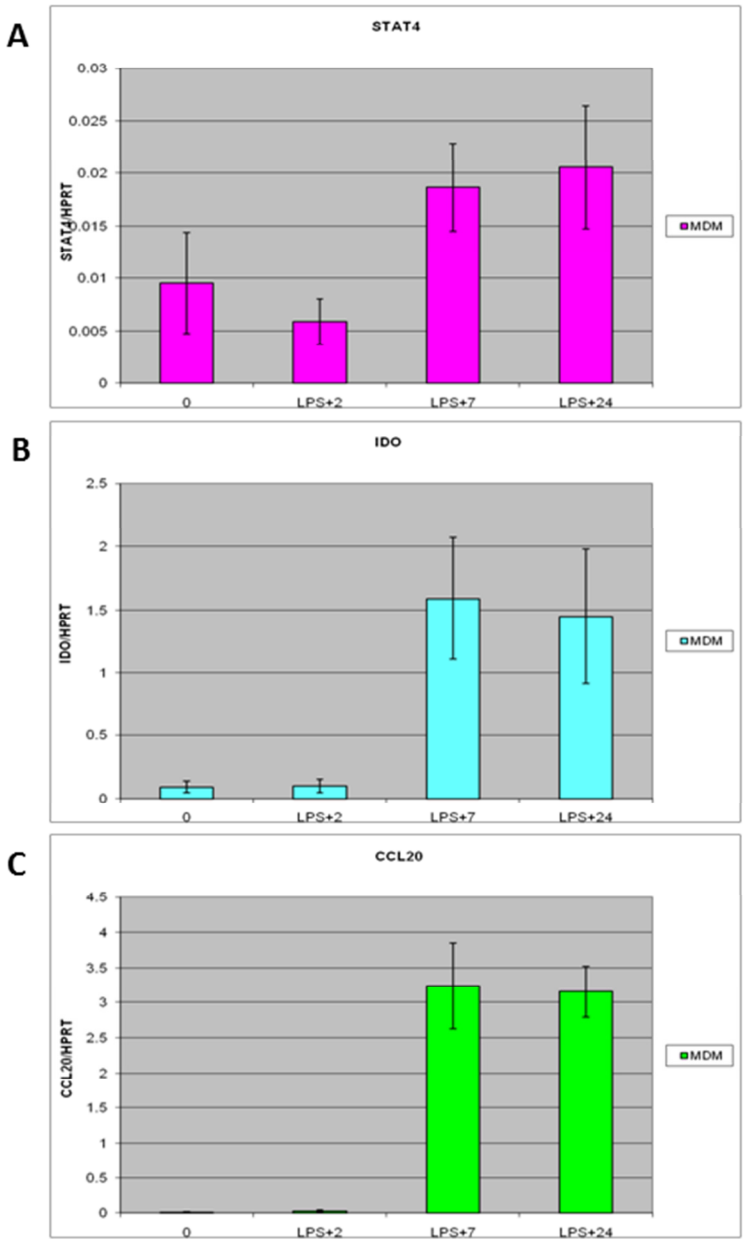

Figure 5-9 LPS induced *STAT4*, IDO and *CCL20* in porcine MDM.

PBMCs were treated with rhCSF1 for 7 days then activated with LPS (100ng/ml) for 2, 7 or 24 hours. RNA was extracted and reverse transcribed. mRNAs for (**A**) STAT4, (**B**) IDO and (**C**) CCL20 were measured by quantitative PCR. Results are representative of four experiments using RNA from different animals

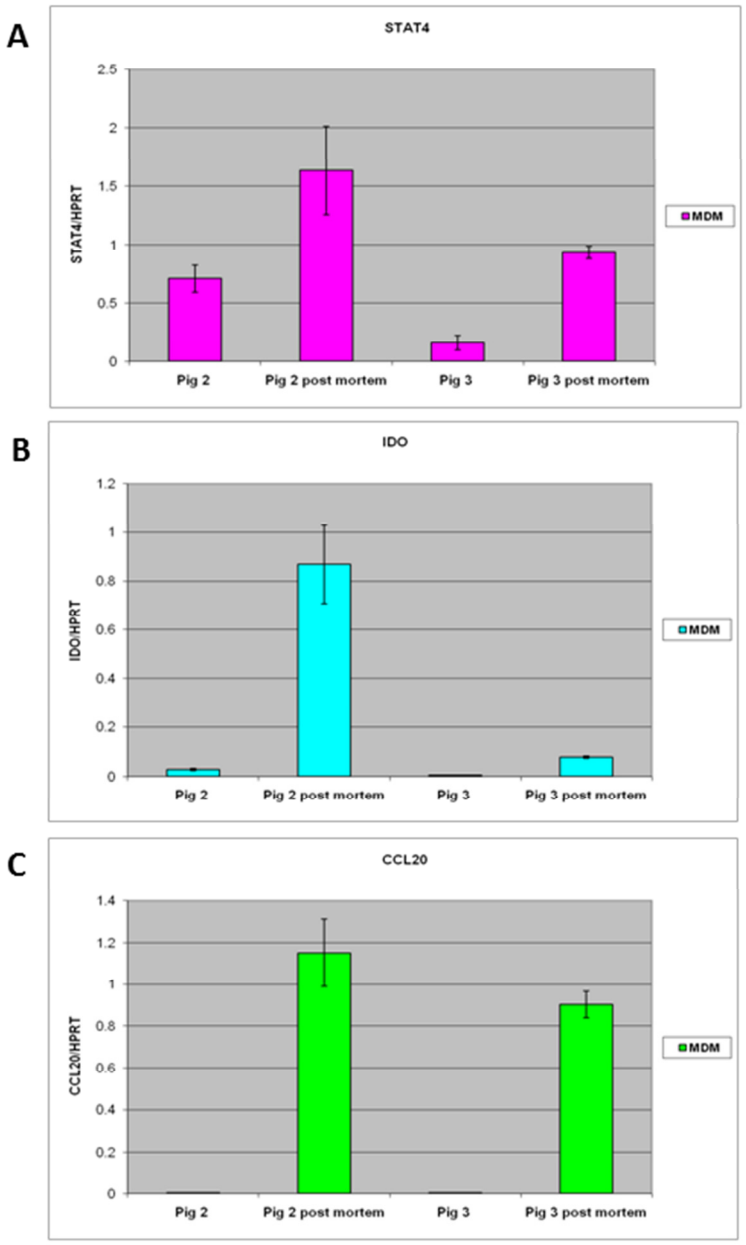

Figure 5-10 LPS induces *STAT4*, IDO and *CCL20* in BMDM from pigs with severe sepsis.

PBMCs were harvested from minipigs with severe sepsis. RNA was extracted and reverse transcribed. mRNAs for (**A**) STAT4, (**B**) IDO and (**C**) CCL20 were measured by quantitative PCR.

5.2.2.2 LPS induced *STAT4*, *IDO1* and *CCL20* in MDM

One difficulty when examining the immune response of mice and men is that comparisons must frequently be made between macrophages from different anatomical locations. The expression of *IDO1*, *STAT4* and *CCL20* in porcine MDM was examined by RT qPCR to investigate if the expression of genes identified as being differentially regulated between human and mouse macrophages could be due to cell specific rather than species specific differences as discussed above. All three genes were up-regulated in MDM although expression was lower than in BMDM (**Figure 5.9**), this may have been due to higher constitutive expression of these genes in MDM (relative to HPRT). This laboratory has also identified *IDO1* as one of the most LPS inducible transcripts in pig AMs using RNA-seq, *CCL20* was also LPS inducible (unpublished, Dario Beraldi). This provides further evidence that the reported differences between LPS inducible genes in human and mice are species specific rather than cell specific.

5.2.2.3 *STAT4*, *IDO1* and *CCL20* were up-regulated in pigs with severe sepsis

In vitro experiments are only ever an approximation of what is occurring *in vivo*. What happens under real inflammatory conditions is often far more complicated than anything that can be simulated in the laboratory environment. Samples from genuine patients or animals are therefore important to validate experimental hypothesis. Pre and post mortem samples from minipigs which were euthanized due to developing severe sepsis were collected and the expression of genes which were highly LPS inducible *in vitro* was examined. Expression of *IDO1*, *STAT4* and *CCL20* was increased in MDM from these pigs by RT qPCR (**Figure 5.10**) suggesting that the *in vitro* model of macrophage inflammation established is a good model for studying what occurs in the whole animal in an inflammatory state.

5.2.2.4 The promoters of LPS regulated genes were more closely conserved in pig and human

As described in the introduction, there is some evidence that the differential expression of immune genes in humans and mice may be controlled at the level of transcription. The promoters of such genes (such as *NOS2*) have therefore been examined for men and mice and differences which could be responsible for the divergent expression of such genes identified (Taylor and Geller, 2000; Zhang et al., 1996; Yu et al., 2005). The microarray data presented in this thesis suggested that many genes which are LPS inducible in man but not mice are also regulated by LPS in the pig, irrespective of cell type studied (**Figures 5.6-10**). The *CYP27B1* promoter, which is LPS inducible in men and pigs but not mice, was therefore analysed in MacVector (MacVector Inc., Cary, NC, USA). The protein product of this gene was conserved 81% in mice and 87% in pigs (www.ensembl.org), but the promoter region was considerably more divergent. All sequences were extracted from the Ensembl database and Pustell DNA matrix alignments were constructed for human v mouse and human v pig, ClustalW alignment of the sequences are also shown (**Figure 5.11**). The Pustell DNA matrix clearly showed that the pig and human promoter for *CYP27B1* was more closely conserved than human and mouse. The software Alibaba2.1 was used to predict the most probable transcription factor binding sites by constructing matrices automatically from TRANSFAC 4.0 sites (http://www.gene-regulation.com). Analysis of the sequences show a gap caused by a repeat insertion in the pig, but otherwise there is very substantial conservation extending more than 1kb upstream. A detailed ClustalW alignment showed subtle variation even in the proximal promoter conserved across all three species. At - 70 bp (relative to the start codon - ATG), the TATA box sequence was identical in pig and human but varied from the consensus in mouse. TATA-boxes are commonly associated with highly-regulated mammalian promoters (The FANTOM Consortium et al., 2005). At -120 bp, a consensus CCAAT-enhancer-binding protein (C/EBP) binding site was found in all 3 species, but NFκB site at -425 in the human was conserved in pig but not in mouse. On the reverse strand at the same position is a GAS (STAT1/3) binding site which was similarly conserved in human and pig but not mouse. Additionally, at -440 bp, a SP1 binding site is identical in human and pig.

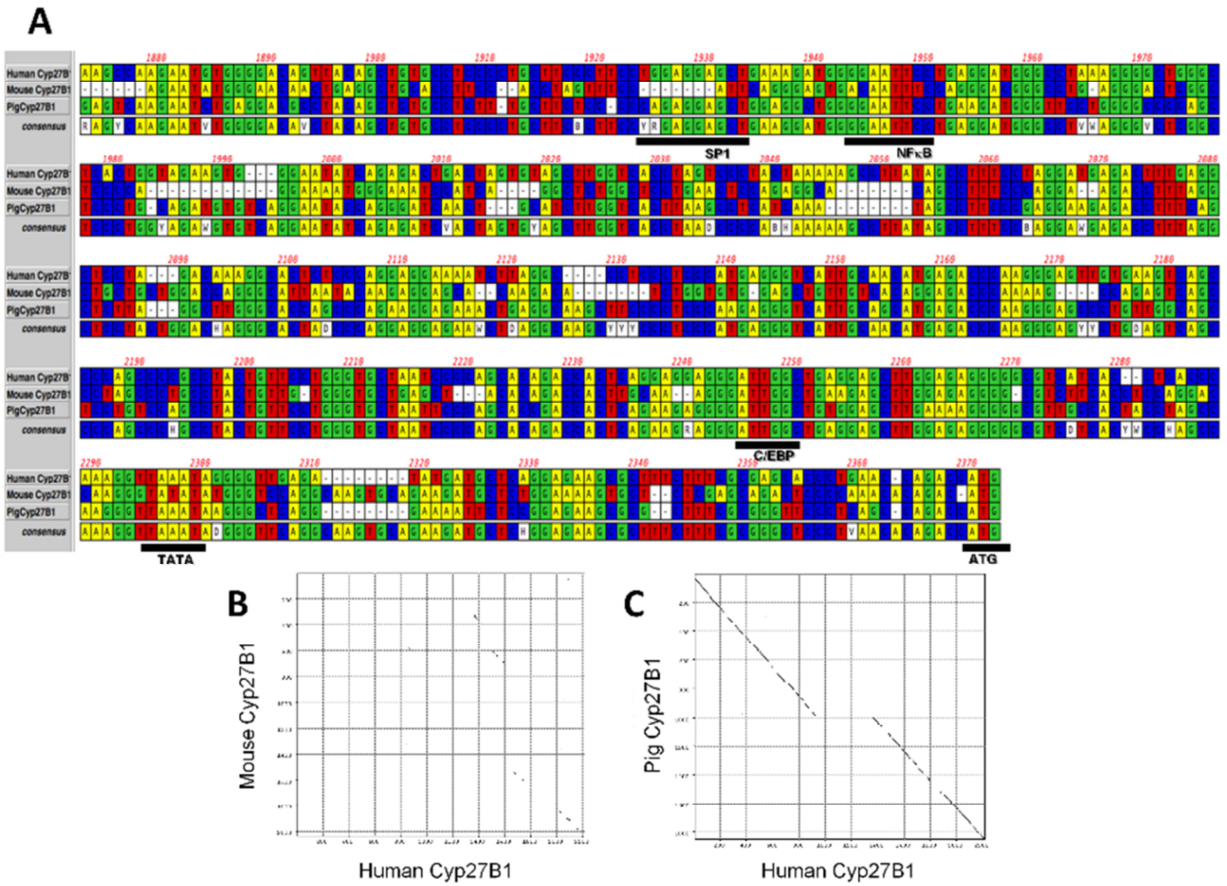

Figure 5-11 The human and pig *CYP27B1* promoter were conserved

To explain the concordance of pig and human inducible gene expression, sequence conservation of promoter regions of divergently-expressed genes was examined across the three species. ClustalW alignments (**A**) for all three species and Pustell aligments of the human and mouse (**B**) and human and pig (**C**) promoters highlight the similarities between the promoters for CYP27B1 in human and pig

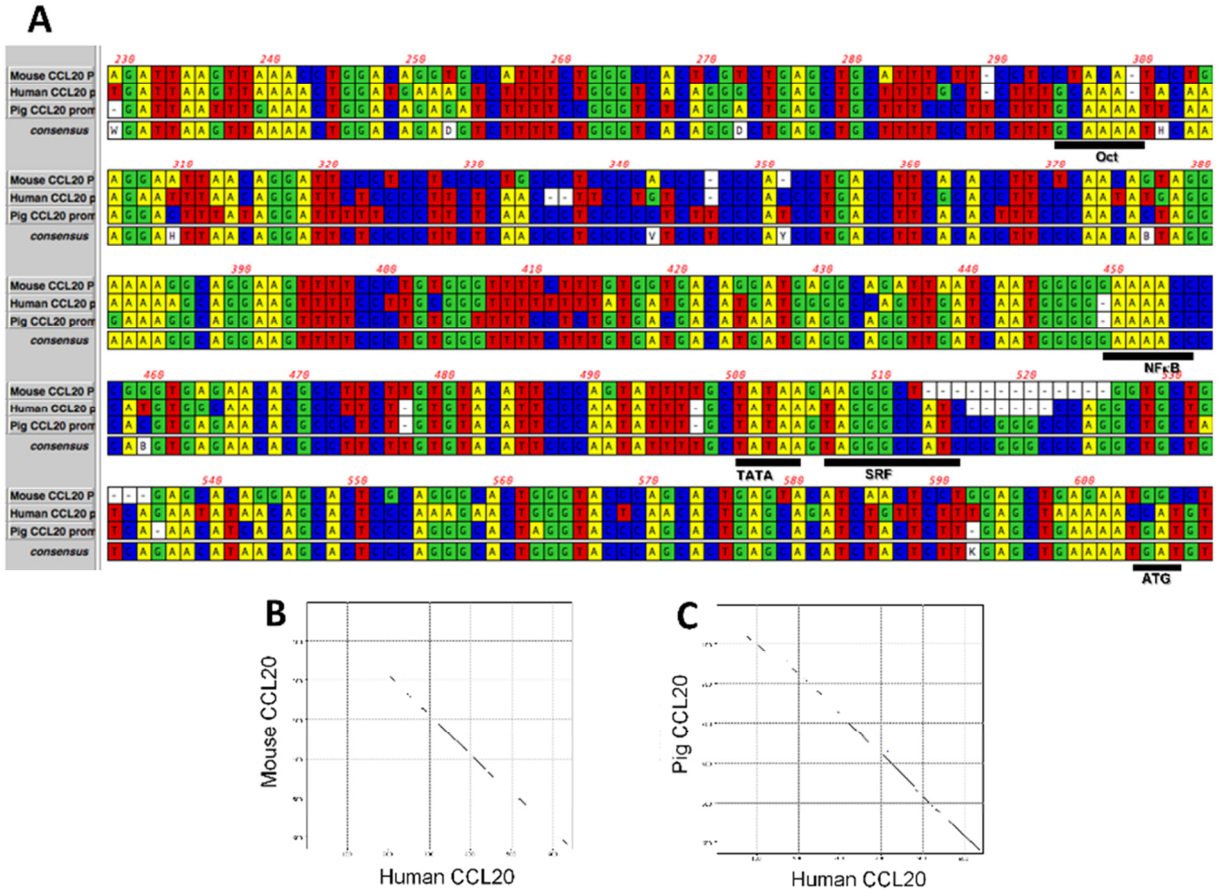

Figure 5-12 The human and pig *CCL20* promoter were conserved

To explain the concordance of pig and human inducible gene expression, sequence conservation of promoter regions of divergently-expressed genes was examined across the three species. ClustalW alignments (**A**) for all three species and Pustell aligments of the human and mouse (**B**) and human and pig (**C**) promoters highlight the similarities between the promoters for CCL20 in human and pig

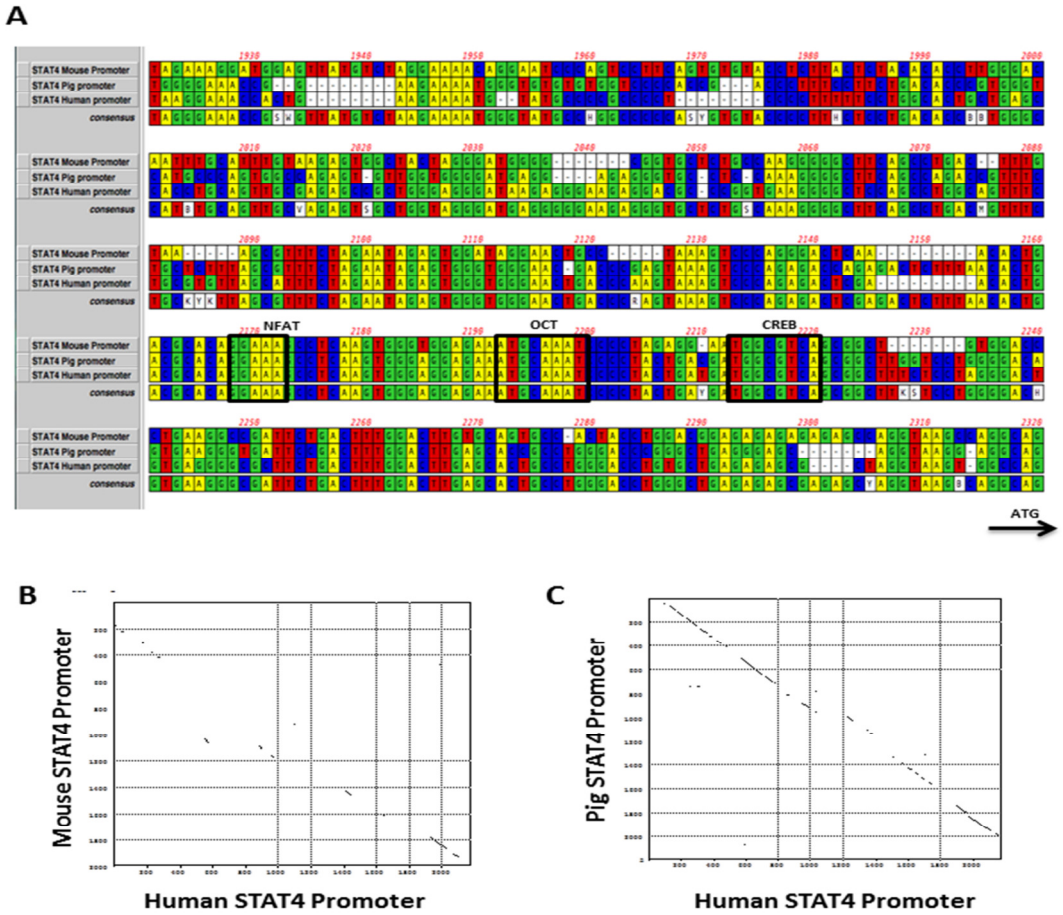

Figure 5-13 The human and pig *IDO1* promoter were conserved

To explain the concordance of pig and human inducible gene expression, sequence conservation of promoter regions of divergently-expressed genes was examined across the three species. ClustalW alignments (**A**) for all three species and Pustell aligments of the human and mouse (**B**) and human and pig (**C**) promoters highlight the similarities between the promoters for STAT4 in human and pig

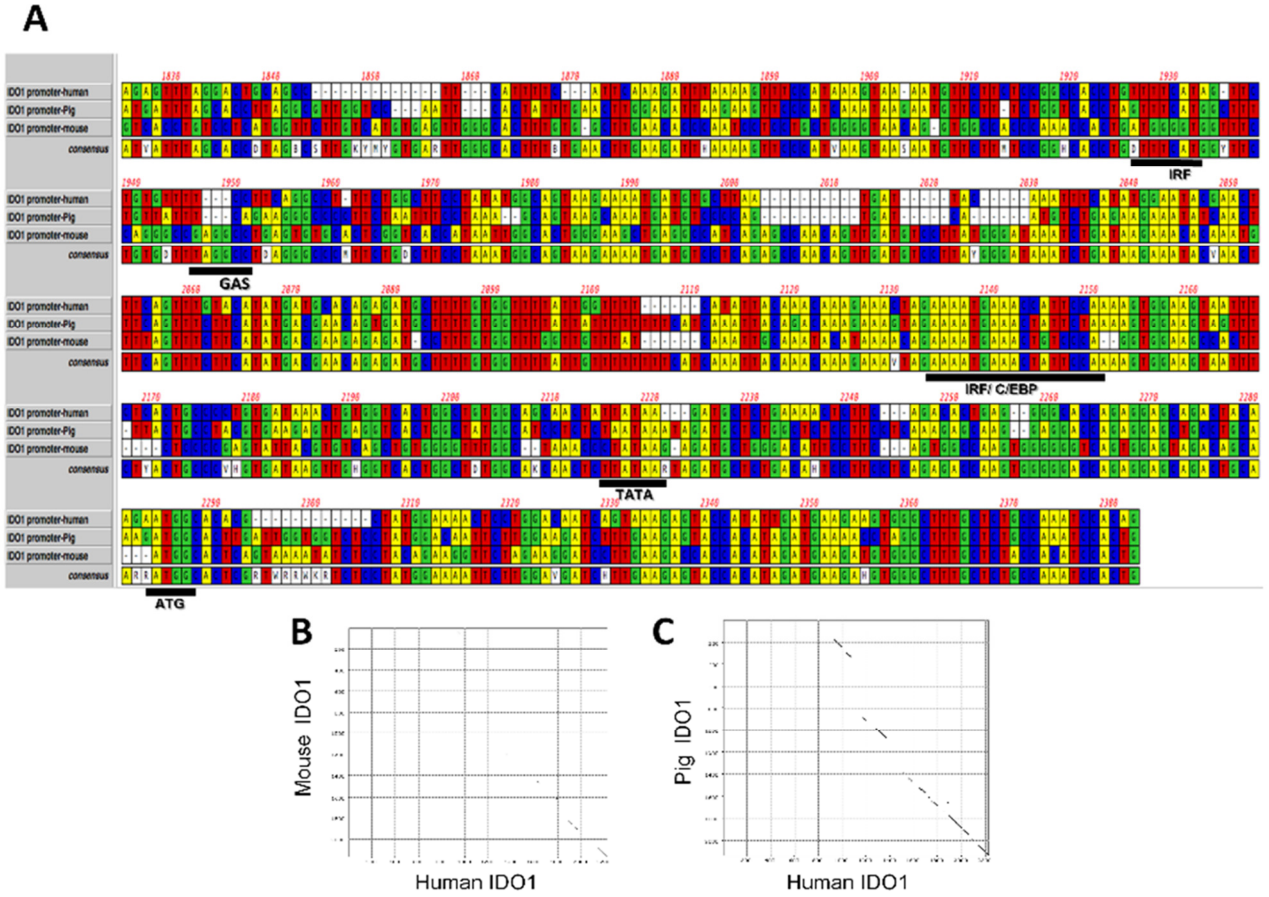

Figure 5-14 The human and pig *STAT4* promoter were conserved

To explain the concordance of pig and human inducible gene expression, sequence conservation of promoter regions of divergently-expressed genes was examined across the three species. ClustalW alignments (**A**) for all three species and Pustell aligments of the human and mouse (**B**) and human and pig (**C**) promoters highlight the similarities between the promoters for IDO in human and pig

In mouse, the part of the sequence was deleted and an IRF1 binding site was identified instead. The promoters of other LPS regulated genes *STAT4, IDO1* and *CCL20* were analysed as above to see if the human and pig promoters for these genes were similarly conserved (**Figure 5.12-14**). There is a TATA box in the *CCL20* promoters from all three species and the immediate proximal promoter is reasonably conserved (**Figure 5.13**). Differences include a loss of sequence immediately downstream of the TATA box in the mouse, and the insertion of a large repeat at around –100 in the mouse that is not in human or pig. Moreover the start site is conserved in pig and human but not mouse. Similarly the proximal promoter for *STAT4* is quite highly conserved in all three species. In this case, there is no TATA box although around 2190, there is an Octamer site in all three species (**Figure 5.13**). Upstream elements such as a NFAT site at 2170 are also conserved in all three species but the Pustell alignment shows far greater sequence conservation between human and pig than human and mouse. The human and mouse *IDO1* promoters have a TATA box around 2220 (**Figure 5.14**) and an IRF binding site is conserved in all three species around 2140. There are two GAS sites at 2148 and 1950 and an IRF binding site in the reverse strand which are not present in the mouse. Overall as can clearly be seen from the Pustell alignments the promoters of these LPS regulated genes are more closely conserved in pig and human than for human and mouse suggesting the differential LPS expression between the species is regulated at the transcription level.

5.2.3 Pig macrophages did not produce NO

5.2.3.1 Analysis of genes involved in NO pathway in the pig

As discussed in the introduction, mouse macrophages respond to LPS with induction of arginine metabolism, inducible nitric oxide synthase, and production of NO where human macrophages do not. **Figure 5.15** compares mouse BMDM and pig BMDM, grown under identical conditions in rhCSF1. As expected, LPS-stimulated mouse macrophages produced large amounts of NO, whereas there was no detectable NO in

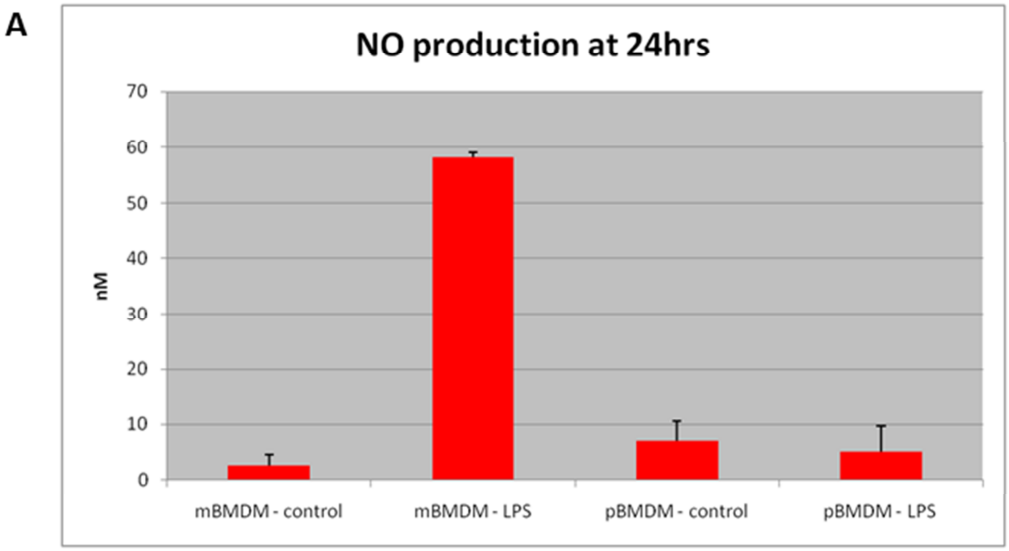

Figure 5-15 NO was induced in mouse but not pig macrophages by LPS stimulation.

Mouse and pig BMDM were grown under identical conditions. LPS stimulated mouse BMDM produced large amounts of NO but pig BMDM did not

Transcript	Gene name	0	2hrs LPS	7hrs LPS	24hrs LPS
Ssc.22478.1.A1_at	CAT2	8.9	9.4	9.7	9.7
Ssc.24908.1.S1_at	CAT2	14.5	15.1	16.0	15.8
Ssc.29566.1.A1_at	CAT2	18.4	16.4	18.7	13.0
Ssc.7852.1.A1_at	CAT2	55.6	53.5	53.2	56.2
Ssc.16117.1.A1_at	NOS2	11.8	13.4	13.2	12.9
Ssc.16117.1.S1_at	NOS2	24.2	27.3	26.8	23.7
Ssc.16188.1.A1_at	NOS2	61.3	60.4	62.7	61.9
Ssc.16188.1.S1_at	NOS2	42.8	43.3	43.9	43.0
Ssc.17717.1.S1_at	ASS1	41.9	49.2	54.6	55.2
Ssc.20648.1.S1_at	ASL	637.4	610.3	409.9	355.7
Ssc.21975.1.S1_at	GCH1	210.6	484.3	206.4	211.0
Ssc.14444.2.S1_at	ARG2	97.6	261.1	148.6	282.3
Ssc.14444.3.A1_a_at	ARG2	176.3	621.8	316.0	732.1
Ssc.29654.1.A1_at	ARG2	26.6	28.0	24.6	24.7
Ssc.25198.1.A1_at	OAT	1045.2	1000.7	864.9	805.3

Table 5.1 – Transcripts involved in NO pathway.

CAT2; cationic amino acid transferase 2, **NOS2**, inducible nitric oxide synthase, **ASL**; argininosuccinate lyase, **ASS1**; argininosuccinate synthetase. **GCH1**; GTP cyclohydrolase, **ARG2**; arginase 2, **OAT**; ornithine aminotransferase

LPS-stimulated pig macrophages. The expression of other key genes of the NO pathway, GTP cyclohydrolase (*GTCH1*), inducible nitric oxide synthase (*NOS2*), argininosuccinate synthetase (*ASS1*), cationic amino acid transporter 2 (*CAT2*), argininosuccinate lyase (*ASL*), arginase 2 (*ARG2*) and ornithine aminotransferase (*OAT*), was obtained from the Affymetrix microarray on LPS stimulated pig BMDM. Expression of these genes was generally at very low levels (<50) or did not change with LPS stimulation (**Table 5.1)**. To validate the microarray, primers were designed against these 7 genes. Again the genes were not detectable by RT qPCR, most of the transcripts were only present at very high CT values (>30, data not shown). The lack of induction of these genes, particularly CAT2 the rate limiting step, confirmed that the NO pathway was not induced by LPS in the pig.

5.2.4 An indoleamine 2, 3-dioxygenease inhibitor was not protective in the pig

As described previously the indoleamine 2, 3-dioxygenease pathway has been implicated in sepsis, kynurenine a by-product of this pathway was increased in patients who later developed septic shock. The IDO inhibitor 1-methyl-tryptophan (1-MT) has been used to treat mouse macrophages where it had a protective effect after LPS administration (Jung et al., 2009). Pig MDMs were treated with LPS or LPS plus either 1-MT or excess tryptophan to determine if inhibition of IDO would have a similarly protective effect as previously described in the mouse.

5.2.4.1 The effects of the IDO inhibitor 1-MT on production of key immune response genes after LPS stimulation

The expression of three key immune response genes was examined by RT qPCR to assess the effects of blocking IDO on LPS activation of pig MDM. Primers were designed for *TNFA*, the archetypal LPS response gene, *IL8*, a neutrophil chemoattractant, and *JAG1*, a notch 1 ligand involved in hematopoeis and Th2 responses. 1-MT massively increased the response of the three genes to LPS in pig 1 and pig 2 versus LPS alone (**Figure 5.16A&B**, **5.17A&B, 5.18A&B**). The effect of

excess tryptophan was however very different between these two animals. In MDM from pig one excess tryptophan pushed the *TNFA* response to LPS forward so it peaked at 2 hours rather than 7 hours (**Figure 5.16A**) while increasing the late response of *IL8* (**Figure 5.17A**) and *JAG1* (**Figure 5.18A**). Excess tryptophan had a much more dramatic effect in MDMs from pig two, here excess tryptophan increased the response of all genes to the same level as the IDO inhibitor (**Figure 5.16B**, **5.17B, 5.18B**). 1-MT initially displayed a protective effect on MDMs from pig three, reducing production of *TNFA* (**Figure 5.16C**), *IL8* (**Figure 5.17C**) and *JAG1* (**Figure 5.18C**) to levels below that of LPS alone. The protective effect disappeared after prolonged exposure to LPS, by 12 hours levels of *TNFA* and *IL8* were the same for cells treated with LPS alone or LPS plus 1-MT and by 24 hours MDMs treated with LPS plus 1-MT produced in excess of five-fold more *TNFA* and *IL8* mRNA than cells treated with LPS alone. 1-MT did reduce the levels of *JAG1* in MDMs from pig three (**Figure 5.18C**). Data from pig four was slightly different. Baseline levels of all three genes studied were higher in pig four than in the other three animals suggesting the macrophages were already activated before the administration of LPS (**Figure 5.16D**, **5.17D, 5.18D**). Initial levels of *TNFA* mRNA were similar in both LPS and LPS plus 1-MT treated MDMs from pig four with excess tryptophan plus LPS having a far greater effect (**Figure 5.16D**). Surprisingly treatment with 1-MT alone causes a constant increase in levels of *TNFA* until 24 hours when it increases massively. Base levels of *IL8* were particularly high and here again excess tryptophan plus LPS increased *IL8* mRNA above treatment with LPS alone or in combination with 1-MT (**Figure 5.17D**). Treatment with excess tryptophan plus LPS also increased levels of *JAG1* although only to similar levels as LPS plus 1-MT, both were higher than LPS alone (**Figure 5.18D**). Treatment with the IDO inhibitor 1-MT did not protect pig macrophages from activation by LPS. The kinetics of response were different in each animal, possibly due to breed specific differences, but overall 1-MT increased production of the immune mediators *TNFA, IL8* and *JAG1* suggesting that treating patients suffering from sepsis with an IDO inhibitor may worsen their condition.

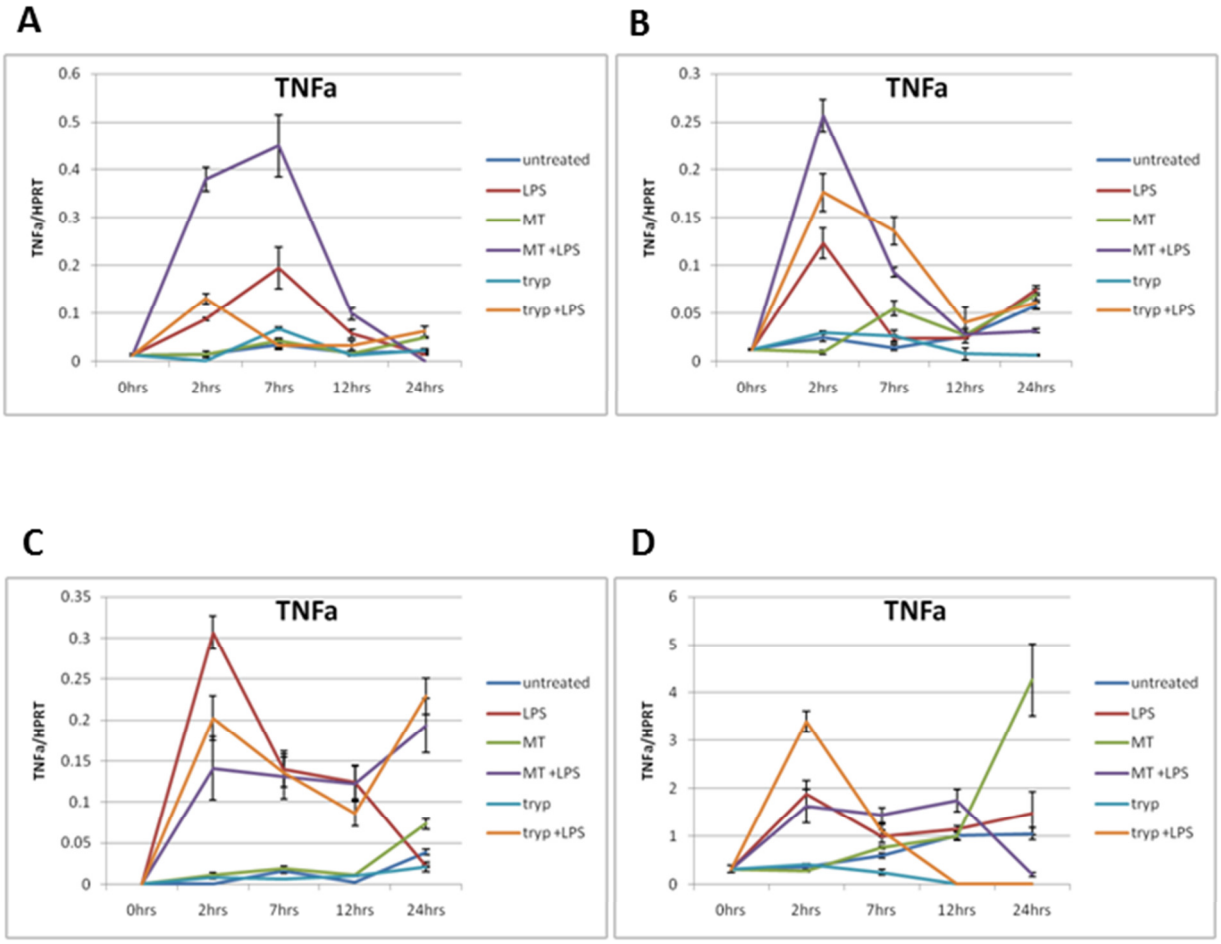

Figure 5-16 1MT increased the TNFA response to LPS.

rhCSF1 derived MDM from 4 animals, (**A**) LWL2 ,(**B**) LW2 ,(**C**) LW3 and (**D**) LAN1 were treated with LPS (100ng/ml), the IDO inhibitor 1-methyltryptophan (100uM) or tryptophan (250uM). TNFA was measured by quantitative PCR.

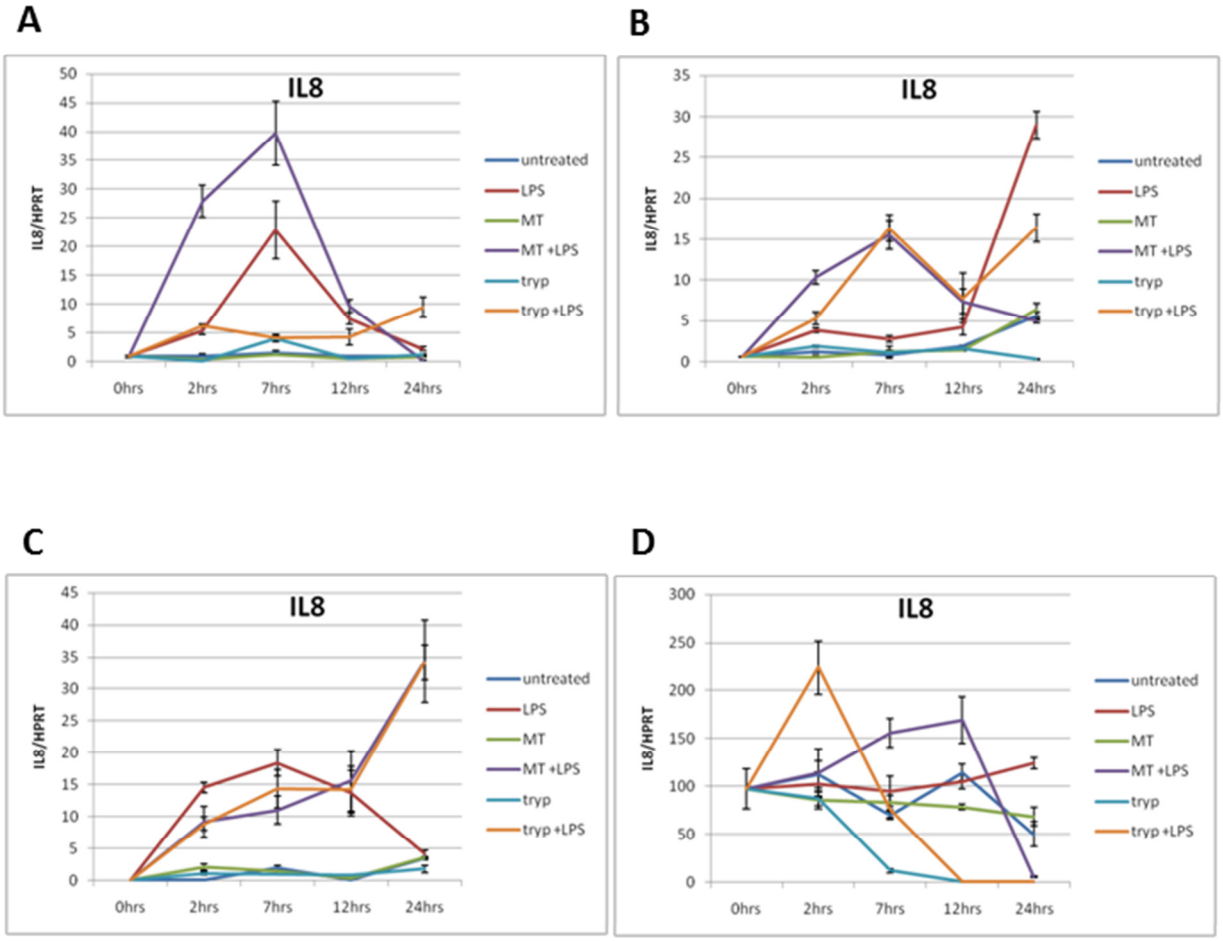

Figure 5-17 1MT increased the IL8 response to LPS.

rhCSF1 derived MDM from 4 animals, (**A**) LWL2 ,(**B**) LW2 ,(**C**) LW3 and (**D**) LAN1 were treated with LPS (100ng/ml), the IDO inhibitor 1-methyltryptophan (100uM) or tryptophan (250uM). IL8 was measured by quantitative PCR.

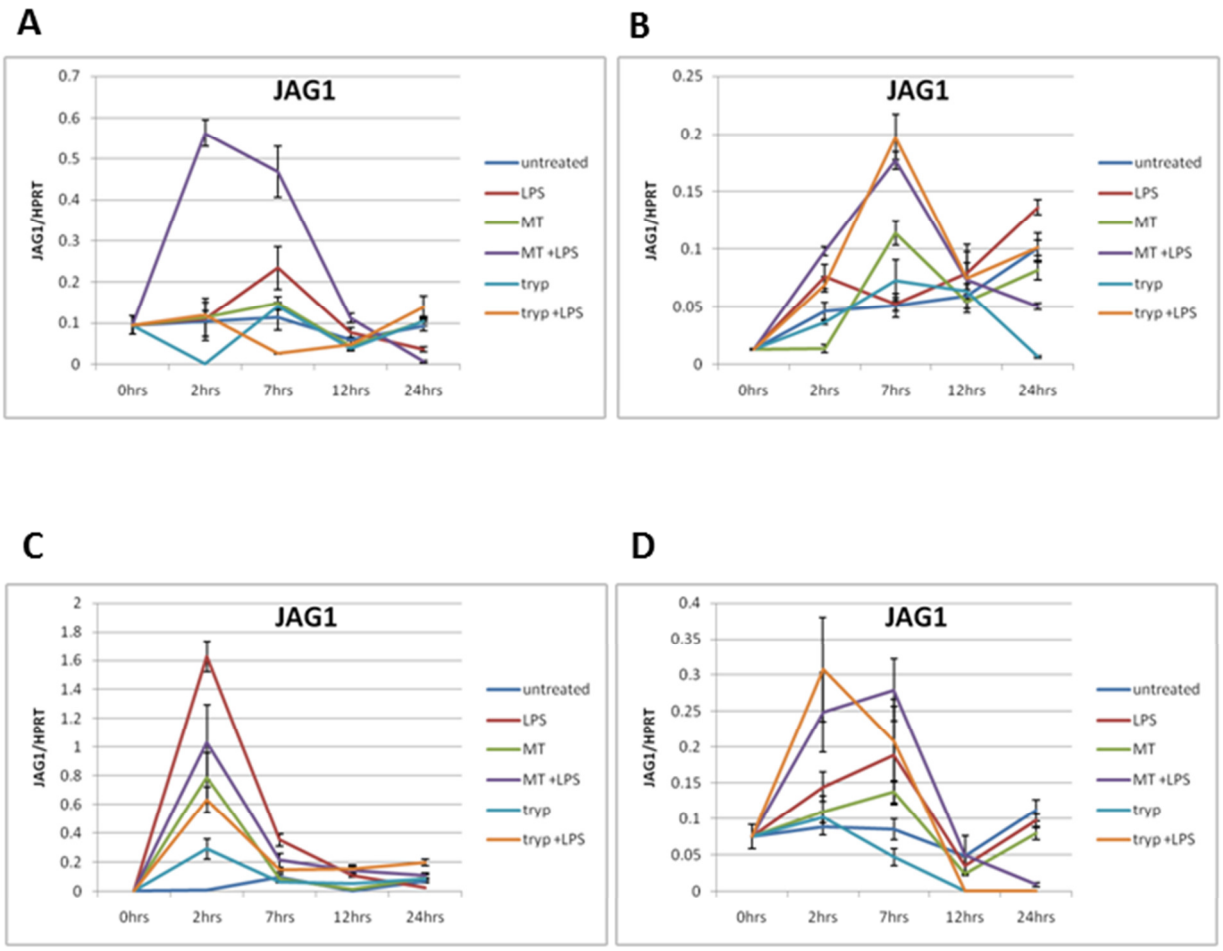

Figure 5-18 1MT increased the JAG1 response to LPS.

rhCSF1 derived MDM from 4 animals, (**A**) LWL2 ,(**B**) LW2 ,(**C**) LW3 and (**D**) LAN1 were treated with LPS (100ng/ml), the IDO inhibitor 1-methyltryptophan (100uM) or tryptophan (250uM). JAG1 was measured by quantitative PCR.

5.3 Discussion

5.3.1 A core set of genes were LPS inducible in the pig

There is a well reported sequential cascade of primary and secondary responses to LPS in mice and men (Nilsson et al., 2006; Gilchrist et al., 2006; Ghisletti et al., 2010). Previous studies have identified a core set of genes that responded in a similar manner regardless of the genetic background of the mouse (Wells et al., 2003). Similarly a core set of genes were induced in human PBMCs in response to PAMPs from different organisms showing that a stereotypical immune response exists regardless of the inflammatory stimulus (Boldrick et al., 2002). The segregation of porcine LPS-regulated genes into clusters using BioLayout Express^{TM} (Freeman et al., 2007) generated 99 distinct clusters. The second largest cluster of co-expressed genes, cluster 2 (**Figure 5.5**) consisted of 481 transcripts which were induced by LPS. Many of these genes relate to control of cell death such as the BCL2 associated athanogene *BAG1* and several caspases (*CASP3, CASP4, CASP7, CASP8*), *CASP7* has previously shown to be LPS inducible in the mouse (Wells et al., 2003). As expected the cellular response to LPS required the switching on of many genes so it was unsurprising to see transcription factors, or genes involved with control of transcription in this cluster such as *STAT1*. *STAT1* is involved in the induction of *IDO*, one of the most LPS inducible genes in both pigs and men, through co-operation with interferon regulatory factor 1 (*IRF1*) (Chon et al., 1996) which was also one of the early response genes in pig BMDM (**Figure 5.5**). Many of the transcription factors seen in the early response clusters may regulate the response of genes induced at a later point. Cluster 2 also contained some genes associated with negative regulation of the immune response such as *IL1RN* which was induced after 7 hours of LPS stimulation and functions as a negative regulator of IL1α (*IL1A*) and IL1β (*IL1B*), both of which were early response genes in this study. Wells *et al.* found that the largest proportion of LPS inducible genes in mouse macrophages were cytoskeletal or phagosomal components, perhaps unsurprising when considering the morphological changes observed in LPS treated BMDM *in vitro*. Genes induced immediately in response to LPS, the "early" cluster, were often involved in

activation of the inflammatory response. Typical early genes in human and mouse endotoxin activated macrophages include known targets of NF-κB, immediate-early gene 3 (*IER3*) and *GADD45B* (Wells et al., 2003) as well as cytokines such as *IL1A* and *IL1B*, the chemokine *IL8* and cell surface receptors such as *CD40* (Boldrick et al., 2002). All of these genes were induced by 2 hours LPS treatment in the pig suggesting many of the "core" LPS inducible genes of mice and men were also induced in the pig. Furthermore *RELA* which binds to NFκB1 or NFκB2 to form the NFκB complex, and *NFKBIA* which inhibits the NFκB complex were both induced immediately in endotoxin activated pig BMDM reflecting the auto-regulation of this signalling pathway which is known to occur in both man and mice (Boldrick et al., 2002). In the mouse many LPS response genes were induced "early" and remained elevated by 21 hours stimulation. The induction of such genes demonstrated that continuous exposure to LPS resulted in continual amplification of the inflammatory response in the mouse (Wells et al., 2003). By contrast in the pig transcription of the early response genes had mostly returned to base levels after 24 hours LPS stimulation (**Figure 5.5**) suggesting that the pig response to LPS is more readily resolved than that of the mouse. This is in contrast to the clinical response of the animals; most strains of mice are resistant to endotoxin while pigs, like man, are exquisitely sensitive (Munford, 2010). This data is the first large scale analysis of LPS response genes in the pig and demonstrates that regulation of many of the "core" LPS response genes in man and mouse were conserved in the pig.

Although LPS activation is often associated with the induction of genes to mount an effective response, typically as many if not more genes were repressed by LPS. The largest cluster of co-expressed genes, Cluster 1 (**Figure 5.4**) contained genes which were initially expressed at high levels and which were down-regulated in response to LPS. Many of these genes are involved in control of protein synthesis and have GO terms relating to control of mitochondrial processes, ATP binding and protein biosynthesis. In the mouse many cell-cycle related genes were down-regulated upon cellular activation confirming the anti-proliferative effect of LPS (Nilsson et al., 2006). Similar down-regulation of cell cycle associated proteins (*CDC34, CDC5L,*

CDC25B, CDCA7, CDC123, CDC42BPA, CDCA7L) cyclin-dependent kinase inhibitors and associated proteins (*CDKN1A, CDK2AP2, CDK2AP1*) and cyclins (*CCNY, CCNF, CCNC*) were observed in the LPS treated pig BMDM. As with the LPS induced genes this set of LPS-repressed genes demonstrates that pig macrophages switch off many of the cellular processes which are down-regulated by LPS in other species.

Large scale genome analyses such as this provide a large amount of data to analyse. It is therefore important to look at all the data, not just that which fits the original hypothesis. Correspondingly the list of genes down-regulated in cluster one disclosed some potential differences between pigs and humans. Expression of the anion transporter *ABCC5* which was down-regulated by LPS in pig BMDM has been shown to increase in HIV infected human macrophages (Jorajuria et al., 2004). Studies of other drug transporters in LPS-activated human macrophages have shown a wide range of effects (Moreau et al., 2010) suggesting expression of some solute transporters may be altered in LPS treated macrophages versus untreated cells. This could have important implications for use of antibiotics and anti-viral drugs in any pig model of human disease. If a solute carrier is repressed by LPS this could result in increased resistance due to unavailability of the treatment within the cell. Indeed LPS has previously been shown to induce resistance to a neomycin-related antibiotic and two chemotherapy drugs in a murine macrophage cell line (Sweet and Hume, 1996). Similarly heat shock proteins are generally up-regulated after cellular stress in humans (Lang et al., 2005; Barreto et al., 2003) but were down-regulated by LPS in pig BMDM in this study. Heat shock protein 1 (*HSPD1*, Hsp60) is often thought of as an endogenous danger signal for the innate immune system. It has been shown to enhance TLR signaling by binding LPS which led to an increase in production of IL12p40 by macrophages and IFNγ by T cells, thereby stimulating both the innate and adaptive immune systems (Osterloh et al., 2007). There were four heat shock proteins in cluster 1 which were all down regulated upon LPS stimulation, an additional 3 HSPs were detectable in the array data *(HSPB11, HSPBP1, HSP90AB1*) of which *HSPB11*, which was in cluster 2, was the only one up-regulated upon

stimulation with LPS. HSPs stabilize proteins and are involved in folding and unfolding, they are generally induced in response to stress. Their repression in pig macrophages suggests that these cells may have another way of dealing with unwanted protein aggregation or perhaps more simply other HSPs which are induced in response to stress and which were not included in this array. Differences between the guanine nucleotide binding (G) protein expression in endotoxin activated pig macrophages and what has been previously reported in human and mouse cells was also observed. *GNAI2* was down-regulated by LPS in pig BMDMs contrary to both mouse macrophages and human macrophages where it was constitutively expressed at high levels and largely unaffected by LPS treatment (Biogps.gnf.org; (Schroder et al., 2012)). Experiments in human monocytes demonstrated an association between CD14 and the α subunits of various G proteins which regulated CD14-dependent endotoxin-activated p38 MAPK activation and cytokine production (Solomon et al., 1998). Furthermore p38 phosphorylation can be induced by LPS interacting with G α agonist while pertussis toxin, which inactivates G proteins, can inhibit this. LPS tolerant cells therefore showed impairment of p38 and endotoxin tolerance has also been associated with decreased G protein function and the subsequent decrease in p38 phosphorylation (Coffee et al., 1992; Ferlito et al., 2001). Down-regulation of *GNAI2* in pig macrophages would suggest decreased p38 phosphorylation and subsequent MAPK activation however LPS induced a strong reaction in pig BMDM which would suggest that *GNAI2* at least does not regulate MAPK activation in pig BMDMs, another G protein may however fulfill this function. It is important when using any animal as a model for human immunity that genetic differences as well as similarities are taken into account. Microarray data such as presented in this chapter allows researchers to integrate the expression patterns of a large number of genes and tease out any important differences between species.

5.3.2 The pig response to LPS was more like the human response

The microarray analysis revealed that around 1/3 of the most regulated genes in the pig BMDM were not induced in the mouse. Of these, approximately 1/2 were

induced in endotoxin stimulated human monocyte-derived macrophages. When comparing LPS inducible genes across species, it was notable that the porcine and human response to LPS seemed to have some involvement with the adaptive immune response, for example the lymphotactic chemokines *CXCL9, CXCL13, CXCL11* and *CCL20* were highly LPS inducible in pigs and humans but not mice (**Figure 5.6-10**). *CCL20* which causes chemotaxis of CCR6 expressing cells to sites of inflammation (Ito et al., 2011), was one of the most LPS inducible genes in human MDM but was not induced by LPS in mouse BMDM or TEPMs ((Martinez et al., 2006; Nilsson et al., 2006)Biogps.gnf.org; (Schroder et al., 2012)). *CCL20* was highly inducible in pig MDM and BMDM as well as being up-regulated in PBMCs from pigs with septic shock (**Figures 5.8-10**). Moreover, the most up-regulated gene at 7h in the pig was CXCL11, an IFN-inducible T cell alpha chemoattractant (Widney et al., 2000). Looking at Cluster 2 from the microarray dataset provided additional evidence for this theory. The lymphocyte associated receptors *IL27RA* and *IL7R* were both switched on by LPS as was *PHF11* which regulated Th1-type cytokine gene expression. *IL7R* was repressed by LPS in the mouse but highly inducible in man (**Figure 5.6 & 5.7**). In addition to *IDO1*, pig BMDM also shared with human macrophages the induction of other tryptophan pathway enzymes, kynurenine hydroxylase (*KMO*), kynureninase (*KYNU*) (**Figure 5.5**) and tryptophan-tRNA synthetase (*WARS*). This pathway is commonly referred to as interferon-gamma inducible, but was clearly responsive to LPS alone in the pig and there was some evidence that it is inducible by LPS alone in man (Taylor and Feng, 1991). Another well-known human-specific inducible gene shared with pigs is *CYP27B1*, the enzyme that regulates the levels of biologically active vitamin D. In humans, vitamin D has been firmly implicated in the control of antibacterial defence (Liu et al., 2006) and the vitamin D receptor was expressed constitutively by human monocytes and macrophages, but not by mouse (biogps.gnf.org). Others have reported that the vitamin D3 receptor was expressed in pig bone marrow (Hittmeier et al., 2006). Although it is not annotated in the microarray, constitutive expression was detected in the pig BMDM by qRT-PCR (data not shown) and *CYP27B1* was highly inducible in pig BMDM (**Figure 5.6**). Analysis of the promoters of genes which were LPS inducible in pigs and humans but not mice (*CYP27B1, IDO, STAT4,*

CCL20) showed striking conservation between the human and pig promoters and a lack of conservation when comparing human and mouse promoters (**Figures 5.11-14**) suggesting the divergent expression of such genes may be due to differences at the transcription level as has been previously reported with *NOS2* (Taylor and Geller, 2000; Zhang et al., 1996; Yu et al., 2005). Additionally promoters were enriched for motifs which bind LPS-regulated transcription factors such as NFκB, C/EBP, IRF and STATS (which bind to GAS sites). Consistent with this *STAT4* and *STAT1* were both LPS inducible in human and pig macrophages but not in mouse macrophages.

This comparison also highlighted several genes which were LPS inducible in pigs but not in mice or humans (data not shown). *IL33*, which is a member of the IL1 family, is one such. IL33 signals through the IL1 receptor-related protein ST2 and can induce Th2 associated cytokines through a MyD88 dependant pathway which results in the phosphorylation of MAPKs (Hazlett et al., 2009). IL33 was traditionally thought to be involved in host defence against nematode infection and allergic diseases such as asthma and dermatitis however more recently it has been implicated in the pathogenesis of some immune diseases and has been shown to drive production of Th2 cytokines through ligation with its receptor ST2 (Schmitz et al., 2005; Dinarello, 2005). IL33 is thought to function as a cytokine and a nuclear factor and can repress transcription (Carriere et al., 2007) but has also been shown to have an enhancing effect on the LPS response in mice by increasing expression of TLR4 and MD2 (Espinassous et al., 2009). By contrast binding of the IL33 receptor, ST2, to murine BMDMs has been shown to lead to the down-regulation of TLR4 preventing over-production of the proinflammatory cytokines IL6, IL12 and TNFα (Sweet et al., 2001). Cyclooxygenase-1 (PTGS1, *COX1*) was also LPS inducible in pig macrophages but not in human or mouse cells. *COX1* and *COX2* convert arachidonic acid to prostaglandin (PG)H2 and regulate angiogenesis in endothelial cells (Tsujii et al., 1998). *COX1* is known to be present in most tissues while *COX2* is found at sites of inflammation and has previously been shown to be LPS inducible in human macrophages (Ariasnegrete et al., 1995). Several of the genes which were LPS inducible in the pig alone (*LAMP2* and *LRM*) were constitutively expressed by

human macrophages suggesting they may still be involved in the human response to LPS.

Fifteen genes were LPS inducible in pigs and mice but not human (data not shown) including *ARG1*. As described in the introduction *ARG1* is the classical gene of alternatively activated macrophages and is normally produced via STAT6 (Pauleau et al., 2004). *ARG1* has also been induced in classically activated mouse macrophages by intracellular pathogens through a MyD88 dependent pathway which was partially dependent on TLR2 (El Kasmi et al., 2008). In mice *Arg1 c*ompetes with *Nos2*; it may be that the absence of active *NOS2* in pig BMDMs allowed the *ARG1 p*athway to become dominant. Again several of the genes found to be LPS inducible in pigs and mice were constitutively expressed by human macrophages (*IFI16, ARHGEF3, LGALS9, STK38L, TOR3A*).

5.3.3 Pig Macrophages did not induce NO in response to LPS

In common with human macrophages LPS did not induce *NO* nor any of the genes involved in the NO pathway (excepting *GCH1* and *ARG2*) in pig macrophages (**Figure 5.15, Table 5.1**). The cationic amino acid transferase (*CAT2*) is the rate limiting step in this reaction so its lack of induction by LPS is clear evidence that this pathway was not active in LPS activated porcine BMDM. *GCH1*, one of the few enzymes involved in the NO pathway which was LPS inducible in the pig, is the rate limiting enzyme in tetrahydrobiopterin (BH_4) biosynthesis. BH_4 is an essential co-factor required for the synthesis of the neurotransmitters dopamine, noradrenaline, adrenaline and serotonin as well as all three forms of NOS. Neopterin, a by-product of BH_4 synthesis, is a common marker of inflammation (Gieseg et al., 2008) and like kynurenine, higher levels of neopterin in patients has been shown to be indicative of developing sepsis or septic shock (Baydar et al., 2009). *GCH1* was also highly expressed in human $CD14^+$ monocytes (Biogps.gnf.org). Human macrophages do not accumulate BH_4, instead *GCH1* metabolises guanosine triphosphate to neopterin

(**Figure 5.19**). It seems probable that the endotoxin-mediated activation of *GCH1* in the pig may lead to the production of neopterin, as in man, rather than NO as in mice. The lack of expression in human monocytes of sepiaterin reductase (*SPR*) and 6-pyruvoyltetrahydropterin synthase (*PTPS*) ((The FANTOM Consortium et al., 2005), FANTOM5 published data (DAH Personal communication)), which are required for the formation of BH_4 from guanosine triphosphate, will lead to the formation of neopterin over biopterins in these cells. In the pig *PTPS* and *SPR* were constitutively expressed at low levels and actually slightly repressed by LPS therefore BH_4 was highly unlikely to be produced by pig macrophages in the normal state or after activation with LPS. Previous studies in the pig have found synthesis of neopterin in response to stress (Breinekova et al., 2007) and LPS (Myers et al., 2003) suggesting that as in humans *GCH1* induction leads to the formation of neopterin rather than BH_4 as in mice. The lack of NO induction in pig macrophages is an important similarity with human macrophages which makes the pig a more suitable model for studying human inflammatory disease than the more commonly used mouse.

ARG2 was one of the few genes of the NO pathway which was LPS inducible in pig BMDMs. *ARG1* is found in the cytosol while *ARG2* is mitochondrial and both catalyse the hydrolysis of L-arginine to L-ornithine and urea (**Figure 5.19**). *ARG1* has been reported to be specifically expressed by M2 macrophage while *ARG2* was expressed by M1 macrophages (Khallou-Laschet et al., 2010). An excess of proline, which is a key component of collagen, can occur in the absence of *NOS2* and arginase activity in the absence of *NOS2,* which has been associated with M2 macrophages, has been proposed to be an important component of wound healing (Hesse et al., 2000; Shearer et al., 1997; Munder et al., 1998). Interestingly, Benga *et al.* (Benga et al., 2009) have claimed that pig macrophages from some breeds can produce NO, but only in response to live microorganisms. As discussed above, others

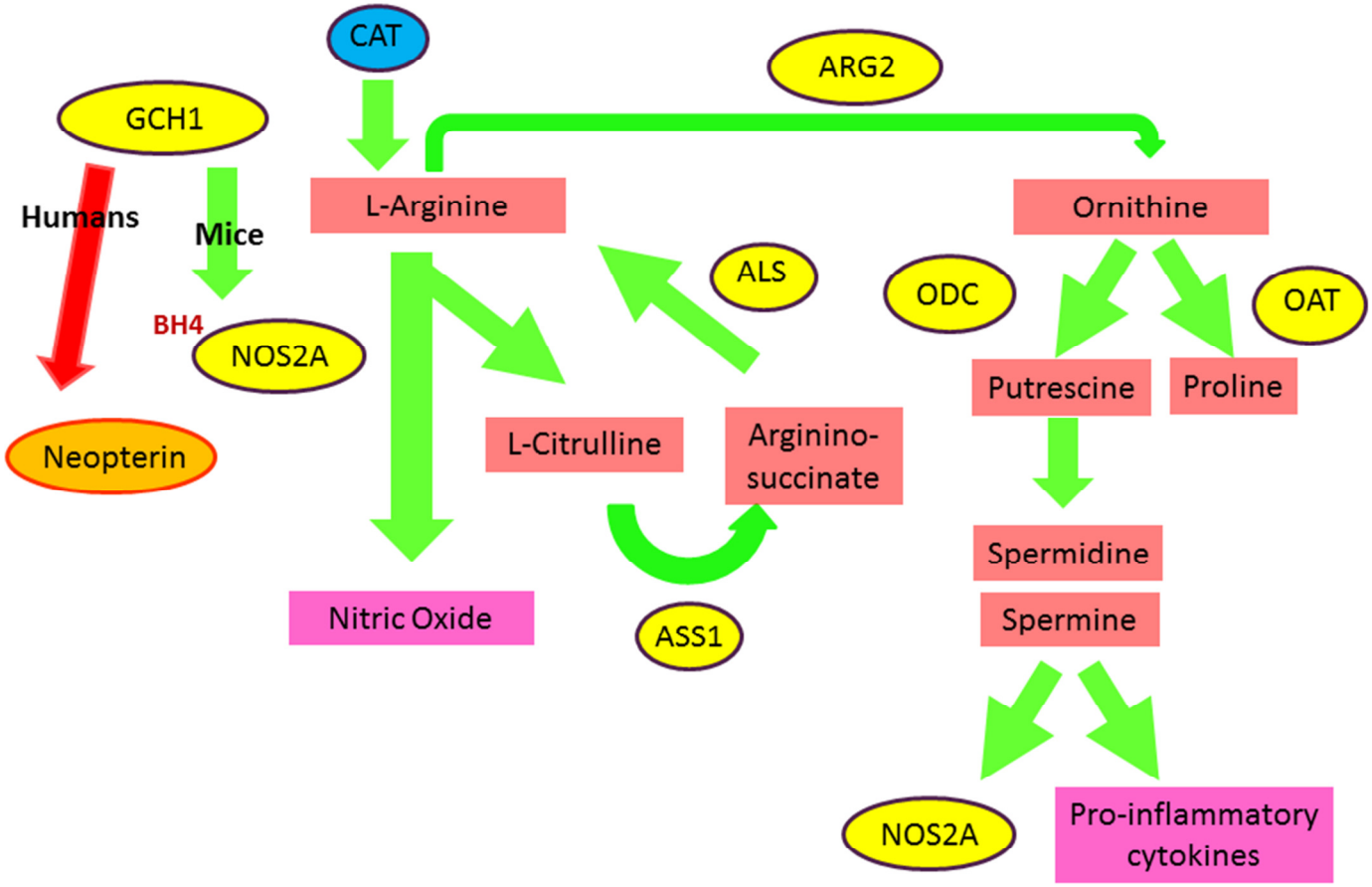

Figure 5-19 The Nitric Oxide pathway

have made similar claims for human macrophages (St Clair et al., 1996; Nicholson et al., 1996; Hunt and Goldin, 1992; Anstey et al., 1996; St Clair et al., 1996).

5.3.4 The IDO pathway was involved in feedback control of the immune response in the pig

Most researchers now agree that human macrophages do not produce NO and this thesis has shown that the NO pathway was not induced in pig macrophages by LPS (**Figure 5.15, Table 5.1**). Instead the IDO mediated metabolism of tryptophan to kynurenine was induced by LPS in both pig and human macrophages. Interestingly IFNγ-induced tryptophan degradation has been shown to occur in parallel to the induction of *GCH1* in human macrophages (Schroecksnadel et al., 2005; Werner et al., 1989) and this thesis has shown that pig macrophages stimulated with LPS induced *GCH1* and *IDO1,* which metabolises tryptophan. Furthermore the degradation of tryptophan and the formation of neopterin, which human macrophages make in preference to NO, are closely correlated (Huber et al., 1984; Murr et al., 2001). Pig MDMs were activated with LPS and the IDO pathway blocked by the use of a specific inhibitor 1-MT. 1-MT exists as two stereoisomers, 1-D-MT and 1-L-MT. There is evidence to show that 1-L-MT preferentially targets IDO1 while 1-D-MT inhibits the novel IDO isoform IDO2 (Metz et al., 2007; Lob et al., 2009). In man *IDO1* and *IDO2* were both highly LPS inducible in macrophages ((The FANTOM Consortium et al., 2005), FANTOM5 published data (DAH Personal communication)). 1-D-MT has been used in mice where, contrary to what was seen in the pig, it led to a decrease in TNFα, IL6 and IL12 and an increase in IL10 after treatment with LPS (Jung et al., 2009). The racemic mix of 1-MT was used in the experiments described in this thesis which Opitz *et al*. found to have a similar, if slightly lesser effect to 1-L-MT alone and should inhibit both isoforms of IDO (Opitz et al., 2011). Inhibiting IDO did not have a protective effect in the pig (**Figure 5.16-18**). This suggests that in the pig induction of *IDO* is a feedback control mechanism of the immune response. In addition kynurenine, the metabolite of this pathway, may have some anti-inflammatory effects. That induction of *IDO* should result in regulation of the immune response isn't entirely surprising; IDO-

mediated effects can only occur after the enzyme has been induced and tryptophan metabolized. Interestingly trials of 1-D-MT in human cancer cells did not show the protective effects previously described in the mouse. Instead *IDO* mRNA and protein expression increased which led to increased kynurenine production by the cancer cells although 1-L-MT did have an inhibitory effect on kynurenine (Opitz et al., 2011). Porcine MDMs were treated with either the IDO inhibitor or an excess of tryptophan in the absence or presence of LPS. Perhaps surprisingly treatment with LPS in the presence of an excess of tryptophan had quite varied effects on the production of the immune mediators measured (**Figures 5.16-18**). The differences in response of LPS stimulated pig MDMs to tryptophan may have been due to the quantity of excess tryptophan initially available. Significant endogenous tryptophan may have caused a large early response to LPS and the addition of further tryptophan to the media would not have affect this however it may have prevented resolution and prolonged the response. If there was not excess tryptophan in the media then the effects of the inhibitor may have taken effect before the LPS response and in this case the addition of further excess tryptophan may have made the initial response worse. Tryptophan concentrations have previously been demonstrated to affect the state of cells *in vitro*. High concentrations of tryptophan (22-400μg/mL) can be toxic to cells and cells grown in tryptophan rich media were more resistant to the anti-proliferative effects of IFNγ (Taylor and Feng, 1991; de la Maza and Peterson, 1988).

This study showed *IDO1* was LPS inducible in pig macrophages and there was some evidence *IDO2* was also LPS inducible (unpublished observations). Neither *Ido1* nor *Ido2* were expressed by mouse macrophages accordingly the inhibitor used on these cells by Jung *et al.* would have affected *Ido* production by non-immune cells e.g. endothelial cells. In murine lungs *Ido* was inhibited by inhibitors of fatty acid cyclo-oxygenase such as indomethacin, phenylbutasol and aspirin, and glucocorticoids such as dexamethasone, betamethasone and cortisone (Sayama et al., 1981). By contrast glucocorticoids have been shown to have a potentiating effect on induction of *IDO1* by human monocytes (Ozaki et al., 1987). *Ido1* was most highly

expressed by white adipose tissue in the mouse (Biogps.org). White fat expresses glucocorticoids receptors which may explain why glucocorticoids had an inhibitory effect in the mouse. *Ido2* was only expressed by mouse liver and at relatively low levels and neither isoform was LPS inducible in the mouse (Biogps.gnf.org). By contrast *IDO1* was one of the most LPS inducible genes in human MDM (Schroder et al., 2012; Hissong et al., 1995). Mouse models seeking to simulate and uncontrolled TLR4 response must therefore always be viewed in light of the lack of induction of this highly inducible human response gene.

In summary, the generation of pig BMDM provides a model system for studying macrophage functional genomics that more closely resembles human biology than traditional mouse models. Like humans, pigs did not induce NO after microbial challenge and instead responded with induction of IDO. Induction was conserved for many other immune-response genes, particularly those associated with the adaptive immune response, between man and mouse. The promoters of such LPS-regulated immune response genes showed striking conservation when comparing the pig and human promoters and a loss of conservation when comparing human and mouse which is most likely responsible for the divergent regulation of these genes between man and pig versus mice. It is likely that much of the difference in immune response of primates and rodents may originate at the transcription level. The domestic pig also offers a potential intersection of genomics and genetics. For example, Benga *et al.* (Benga et al., 2009) have reported significant variation in the response of individuals within and between pig breeds to microbial challenge. The use of BMDM in future studies will permit the assessment of macrophage autonomous variation in populations of animals from diverse sources, separated from the influence of husbandry, nutrition and other variables. This is turn may contribute to understanding genetic control of disease susceptibility in humans.

Chapter 6: Conclusions and future work

The work described in this thesis explores the use of the pig as a model to study human innate immunity. The pig is important in its own right as a production animal, so the comparison also underpins the possible view of humans, which have been much more extensively studied, as models for pig immune responses. There are many advantages to using the pig as a model; a large number of cells can be isolated from one animal and stored for future use, many experiments can be performed from the same animal and macrophages from different anatomical locations and in different states of differentiation from the same animal can be compared. Unlike commonly-used mouse models, the pig is not inbred (although inbred lines do exist), but breed diversity provides genetic resources for the study of within-breed variation in innate immunity and macrophage biology.

Chapter 3 demonstrated some of the major advantages to the pig as a model over the more commonly used rodents and analysed the expression of widely studied immune markers on porcine monocytes and macrophages from different locations and the effects of rhCSF1 on macrophage differentiation in the pig. Expression of the LPS receptor CD14 and Fc receptor CD16 have been extensively studied in man (Ziegler-Heitbrock, 2007). Despite the fact that CD16 exists as multiple copies in the human genome, and there is copy-number variation amongst individuals (Aitman et al., 2006; Mamtani et al., 2010; Molokhia et al., 2011) recent studies have shown that both CD14 and CD16 are also differentially expressed on mouse monocytes (Ingersoll et al., 2010). As well as delineating human monocytes into subsets which are presumed to have specific functions *in vivo,* there is evidence that genetic variation in CD14 and CD16 (including the copy number variation) is associated with increased susceptibility to disease (Mamtani et al., 2010; Molokhia et al., 2011; Zhou et al., 2010; Yu et al., 2011). The scavenger receptor CD163 is expressed by most macrophage populations in man (Fabriek et al., 2005) and also differentially-

expressed amongst human monocyte subsets, being highest on the $CD16^-$ subset (Tippett et al., 2011). CD163 has been used previously to differentiate porcine monocytes into subsets (Chamorro et al., 2000). CD163 functions as a receptor for PRRSV (Calvert et al., 2007) and expression levels have been correlated to permissiveness to ASF (Sanchez-Torres et al., 2003) in pigs. In man HIV infected subjects had higher levels of CD163 on the intermediate $CD14^{hi}CD16^+$ monocyte subset than uninfected individuals and culture with CSF1 increased expression in the same subset (Tippett et al., 2011).

CD172a (SWC3, SIRPα) is expressed on all cells of myeloid origin in the pig from early myeloid progenitors and along with SWC1 and SWC8 (discussed in **Chapter 1**) has been used to separate out the main peripheral blood leukocyte populations (Lunney, 1993; Saalmuller, 1996) and define populations in porcine bone marrow (Summerfield and McCullough, 1997). Knowledge of the different populations present in porcine bone marrow and blood is important to help understand the role these cells may play under normal conditions or during disease. All early porcine myeloid progenitors expressed low levels of CD172a and expression continued on cells which were committed to both the granulocyte or monocyte lineage then increased as cells matured. Low level expression of CD172a was also seen on plasmacytoid DCs (Summerfield and McCullough, 1997; Summerfield et al., 2003). The early expression and retention on all myeloid cells of CD172a suggest it may be important in control of proliferation, differentiation and activation. CD172a is a member of the immunoglobulin superfamily and functions as a receptor for CD47 in humans and rodents (Vernon-Wilson et al., 2000; Ezquerra et al., 2009; Seiffert et al., 1999). The expression of CD47 on murine red blood cells (RBCs) has been reported to act as a "marker of self" by interacting with CD172a on macrophages (Oldenborg et al., 2000) although similar effects have not been reported in humans (Arndt and Garratty, 2004) and the lack of interactions between CD47 expressing human mesenchymal stem cells and CD172a suggest that any such interactions must be at least cell type specific if not species specific (Subramanian et al., 2006). Lack of conservation in the sequence of key areas of CD172a and CD47 between human

and mice also suggests function may not be conserved between the species (Subramanian et al., 2006; Jiang et al., 1999; Subramanian et al., 2007). By contrast human CD172a bound strongly to both human and pig CD47 but not rat or mouse CD47. Similarly porcine specific CD172a antibodies have been shown to react across species (Summerfield and McCullough, 1997) and several human anti-CD47 antibodies bound to pig RBCs but not RBCs from rodents (Subramanian et al., 2006). This thesis found CD172a to be highly expressed by all $CD14^{+}$ monocytes (**Chapter 3**) while others detected higher expression at the mRNA and protein level by human $CD16^{-}$ monocytes and at the protein level alone by mouse $Ly6C^{lo}$ monocytes (Ingersoll et al., 2010).

6.1 Comparison of monocytes/macrophages in the pig

Macrophages originate in the bone marrow from stem cells that differentiate into promonocytes before moving to the blood stream as monocytes, and thence into tissues where they further differentiate into specialised tissue macrophages (Gordon and Taylor, 2005). Distinct tissue macrophage populations are found in specific sites in the body and heterogeneity has been identified between macrophage populations in the lungs (Nicod et al., 1987; Prokhorova et al., 1994) . Previous investigations have compared rodent macrophages from different anatomical niches to determine any differences and possible functional roles *in vivo*. Rat alveolar macrophages displayed higher phagocytic ability and were better able to kill bacteria and fungi than peritoneal macrophages. By contrast rat PMs functioned as better accessory cells and were stronger stimulators of T cell proliferation than AMs suggesting the two mature macrophage populations probably fulfil specific roles *in vivo* (Gjomarkaj et al., 1999).

With the availability of large numbers of macrophages from different anatomical sites in the pig, it will be interesting to determine their antigen-presenting capacities.

This is important for the development of vaccine strategies. There is a limited literature on this topic linking sialoadhesion (CD169, siglec1) and CD163 with antigen presentation to T cells (Poderoso et al., 2011; Revilla et al., 2009). Linking of antigens to antibodies against CD169 or CD163, endocytic receptors expressed by APC, led to increased T cell proliferation (Revilla et al., 2009; Poderoso et al., 2011) The minimal requirements for antigen-presentation are expression of class II MHC and co-stimulator molecules. Others in the laboratory are in the process of array profiling isolated alveolar macrophages which will provide data on the expression levels of Class II genes and co-stimulatory molecules.

It will also be of interest to compare the phagocytic ability, cytokine production and response to challenge of different macrophage populations. This would enable differences to be fully elucidated between macrophages from the lungs and peritoneal cavity or between different macrophage populations from the same location. Peritoneal lavage in the rat resulted in a far greater variety of cell types than alveolar lavage and Gjomarkaj *et al.* (1999) suggested the stronger T cell stimulatory function of rat PMs may result from simply encountering a wider variety of cells than AMs. The composition of non-macrophage cells from either of these locations in the pig was not examined so it would be interesting to determine if the ability to promote lymphocyte proliferation relates to the cell types encountered as it may do in rodents. Rat AMs also expressed higher levels of the antigen ED9 (SIRPα) than PMs (Gjomarkaj et al., 1999). Similarly murine AMs have been shown to produce higher levels of the pro-inflammatory cytokine TNFα after stimulation with LPS than resident or elicited PMs (Tachibana et al., 1992) while PMs produced the anti-inflammatory cytokine IL10 which was not produced by AMs under similar conditions suggesting each population may have a different function in the inflammatory state (Salez et al., 2000). The larger size and relative availability of tissues also presents the possibility of isolating large numbers of tissue macrophages from key organs such as the spleen, liver and gastrointestinal tract. The latter location contains a very abundant macrophage population (Pavli et al., 1990), which is relatively unresponsive to TLR ligands (Bain and Mowat, 2011).

Expression of the immune markers CD14, CD16, CD163 and CD172a was examined on several porcine monocytes/macrophage populations. The mature macrophage populations from the lungs and peritoneal cavity expressed uniformly high levels of the immune markers studied in this thesis. FACS analysis using an extensive array of antibodies will most likely identify markers specific to each population or differences within a cell type. Further unpublished experiments in our laboratory have identified two populations of AMs based on adherence (unpublished, Ronan Kapetanovic) as has been noted previously in man (Nicod et al., 1987). The markers used in this study were unable to differentiate between these populations (unpublished, Ronan Kapetanovic). One might expect that expression of adhesion molecules would differ between the two populations and explain the differential adhesion to plastic; for instance several integrins as well as the CSF1R have been shown to have adhesive properties (Lin et al., 1994b; Rosen and Gordon, 1987; Elsegood et al., 2006). The level of adhesion has also been related to differences in proliferation suggesting the two AM populations may differ in this respect (Xaus et al., 2001). Previous investigations have suggested porcine AMs and PMs respond to viral products through different pathways (Loving et al., 2006) suggesting intrinsic differences between the two cell types. The immune response to viral infection is characterised by induction of type I interferons (IFNα/β) which leads to inhibition of viral replication. Porcine AMs and PMs responded to the synthetic dsRNA analog poly(I:C) with induction of transcription of type I IFNs although the "fold change" increase in transcription was greater in AMs. In contrast induction of transcription of the dsRNA-dependent protein kinase R (PKR) and Myxo-virus resistant, IFN-inducible GTPase (Mx) was higher in PMs. Loving *et al.* refer to "fold change" when comparing expression levels throughout this paper, so it is not clear whether the difference is due to high basal expression or lack of induction. Interestingly the AM response to poly(I:C) was still elevated after 24 hours. Unfortunately, these workers used only a single time point. Similar experiments in our laboratory comparing the TLR4 response in porcine AMs and PMs demonstrated a more sustained response from AMs. Production of TNFα by LPS-stimulated MDMs and BMDMs peaked at between 7-12 hours then declined thereafter. Production of TNFα by PMs stimulated with LPS remained constant for up to 55 hours after

initially increasing sharply while TNFα production by LPS-stimulated AMs was till increasing at the last time point assayed (55 hours) (unpublished, Ronan Kapetanovic). Similarly this thesis showed a clear time-dependent profile in the response of BMDMs to LPS. It is therefore likely that hyper-responsiveness of AM to TLR3 stimulation, which uses the same co-receptors as TLR4 (Takeuchi and Akira, 2010), would be even more obvious in an extended time course.

AMs are critical in the immune response to influenza (Tumpey et al., 2005) and the recent emergence of highly pathogenic strains highlights the need for a good model of pulmonary anti-viral responses. Pig AMs are readily available in large numbers and pigs, unlike mice (where one must use specifically adapted strains) are highly susceptible to influenza. The study described above by Loving *et al*. (2006) investigated the response of AMs to dsRNA. The authors suggested that recognition of and subsequent response to dsRNA was mediated by TLR3 in AMs and PKR in PMs. The IFN response of AMs needs to be tightly regulated in order to avoid uncontrolled inflammation which could lead to pathological effects in the lung. Loving *et al.* therefore suggested that transcription of the interferon stimulated genes (ISG) PKR and Mx could be inhibited by constitutive expression of the suppressors of cytokine signalling (SOCS) proteins or by the transcription factor NFκB which could block binding by other transcription factors to the relevant promoters specifically in AMs. Differences in type I IFN responses have been described between rodents and humans (Rogge et al., 1998) meaning rodent models of viral infections may not always accurately replicate what would occur in a human patient. Further studies on porcine AMs could more fully elucidate the control of type I IFNs in the pig and lead to better models of lung macrophage response to virus.

6.2 Breed diversity

Another advantage of the pig system is the availability of different breeds which differ in their disease susceptibility. This variation is of both intellectual and

practical significance. An ongoing project in our laboratory seeks to identify differentially-expressed genes and macrophage-expressed sequence variants (snps), that distinguish the major breeds. In this thesis, freshly isolated blood monocytes from six different pig breeds were divided into populations based on differential expression of CD14 and CD163 as previously (Chamorro et al., 2000) or CD14 and CD16 as in human and mice (Ingersoll et al., 2010). Expression of the markers examined showed only subtle variation between breeds suggesting no major polymorphisms were present at any of the genetic loci despite previous reports suggesting some pig breeds are more/less susceptible to PRRSV (Ait-ali et al., 2007) and variation in CD14 and CD16 noted among humans (Molokhia et al., 2011; Yu et al., 2011). A minimum of 2 animals from each breed were analysed which would have detected absolute breed specific variation but is clearly too small a sample size to detect animal specific variation. Polymorphisms at CD14 and CD16 are correlated with disease in some human ethnicities and similar genetic diversity may be reflected among breeds of pigs.

6.3 Effects of CSF1 in the pig

In mice and humans, CSF1 has been shown to promote monocyte and macrophage differentiation and proliferation (Hume and MacDonald, 2011). Work in this thesis extended these studies to the pig. Analysis of surface marker expression by AMs grown in the presence or absence of CSF1 suggested an exogenous source of CSF1 was required for the maintenance of the mature AM phenotype. Macrophages grown in the absence of CSF1 or with a CSF1R inhibitor expressed lower levels of CD14, CD163 and CD172a and almost entirely lost expression of CD16. Cells cultured with the inhibitor had further loss of surface marker expression compared to those simply deprived of CSF1 suggesting production of autocrine CSF1 was able to partially rescue the mature macrophage phenotype. Expression of immune markers is likely to relate to differences in immune function *in vivo* and suggests CSF1 is necessary for maintenance of full functionality of lung macrophages.

Mice lacking *Csf1* had reduced numbers of lung macrophages compared to wild type (Wiktor-Jedrzejczak et al., 1992) while *Gmcsf*$^{-/-}$ mice displayed impaired lung development (Stanley et al., 1994). Mice deficient in both growth factors however displayed a more severe phenotype than mice lacking either growth factor alone (Lieschke et al., 1994). This thesis showed that CSF1 was necessary for maintaining the mature AM phenotype. Thus far, there have been relatively few studies of pig GMCSF. One study highlighted the role of GMCSF in improving the immune response to vaccine in pigs and suggested it could a protective role in lung pathology. Adenoviral constructs fusing PRRSV proteins with GMCSF resulted in enhanced immune response in pigs. Moreover pigs pre-treated with the constructs then later infected with PRRSV had less lung damage than pigs pre-treated with constructs expressing only the PRRSV proteins (Wang et al., 2009). GMCSF was not constitutively expressed by the BMDMs examined in this thesis but was induced by LPS. Array data for porcine AMs is currently being analysed in our laboratory and will allow the level of expression of GMCSF in lung macrophages to be determined (Kapetanovic, unpublished).

As in humans and mice, it is possible to grow "dendritic cells" from pig bone marrow in GMCSF (Guzylack-Piriou et al., 2010). In the mouse at least (Mabbott et al., 2010), these kinds of cells are largely indistinguishable from macrophages based upon their gene expression profile, and should probably be called antigen-presenting macrophages. A comparison of pig macrophages, from bone marrow, blood or lung, grown in CSF1 or GMCSF will be an interesting future direction to elucidate the biological importance of the two factors. In addition porcine specific anti-CSF1R monoclonal antibodies are currently being developed in our laboratory (Gow, unpublished) which will allow analysis of expression levels of CSF1R on different macrophage populations.

Previous studies have shown murine AMs to be dependent upon continuous CSF1 signaling, (Macdonald et al., 2010; Lenzo et al., 2011). Similarly murine BMDM required exogenous CSF1 for survival although expression of *Csf1* mRNA has been shown in the inflammatory macrophage population, TEPMs (Irvine et al., 2006). By contrast although freshly-isolated human monocytes did not express CSF1, (Biogps.org.info;(Hume and MacDonald, 2011)) differentiated human macrophages did produce autocrine CSF1 in culture (Becker et al., 1987). As described in **Chapter 3**, inhibitors against the protein tyrosine kinase activity of the CSF1R or anti-CSF1R antibodies have been used to block the actions of CSF1 (Irvine et al., 2006; Hume and MacDonald, 2011; Macdonald et al., 2010; Lenzo et al., 2011; Hashimoto et al., 2011). The anti-CSF1R antibody (M279) selectively depleted several tissue macrophage populations, including macrophages from established tumours but had no effect on macrophage numbers in the lung or in inflammatory models of disease in the mouse (Macdonald et al., 2010). Macdonald *et al.* found the differentiation of $Ly6C^{hi}$ cells into the more mature $Ly6C^{lo}$ subset to be the only non-redundant function of CSF1. Administration of anti-CSF1R antibodies also resulted in loss of peritoneal macrophages (Hashimoto et al., 2011; Lenzo et al., 2011; Macdonald et al., 2010) although this was most probably due to reduced numbers of $Ly6C^{lo}$ circulating monocytes, which are the precursors of mature tissue macrophages, rather than direct effects on PMs. Macdonald *et al.* (2010) demonstrated that in the mouse the two monocyte subsets are almost certainly different maturation stages of the same cell and that CSF1 was essential for maturation of resident $Ly6C^{hi}$ cells into the more mature $Ly6C^{lo}$ subset and thence to tissue macrophages but not for generation of monocytes (Macdonald et al., 2010). The development of porcine specific anti-CSF1R antibodies would allow similar experiments in the pig to fully understand the role of CSF1 in monocyte/macrophage differentiation and the relationship between mononuclear phagocytes from different locations.

6.4 Species-specific differences in CSF-1 action

Previous research has shown that the set of CSF1-induced genes in human and mouse monocytes differ. Mouse macrophages exposed to CSF1 induced a set of pro-inflammatory genes while human macrophages treated in a similar manner instead switched on genes associated with atherogenesis and cholesterol biosynthesis (Irvine et al., 2009). The data generated in **Chapter 5** partly addresses the position of the pig in this spectrum of species-specificity, although the effect of CSF1 deprivation and re-stimulation was not explicitly tested. The mRNA levels of the pro-atherogenic chemokine *CCL2* were expressed at high levels by the rhCSF1-matured pig BMDM. Similarly many genes involved in cholesterol biosynthesis (*HMGCR, MVD, IDI1, FDPS, SQLE, CYP51A1, EBP, NSDHL, DHCR7*) were constitutively expressed by CSF1 treated porcine BMDM. Unlike the human macrophages studied by Irvine *et al.* (2009) CSF1-matured porcine BMDM also expressed high mRNA levels of the anti-atherogenic chemokine receptor *CXCR4*. Although further experiments are needed to assess if CSF1 induces similar genes in pigs and humans, CSF1 did not prime pig macrophages for production of TNFα (Kapetanovic *et al.*, under review) suggesting that CSF1 did not support a pro-inflammatory response as has been described in the mouse (Irvine et al., 2009). Given the ubiquitous use in many laboratories of CSF1 to differentiate immature monocytes into macrophages further data on its effects on transcription in the pig are required.

Monocytes isolated from the blood or bone marrow and cultured in rhCSF1 increased expression of CD14, CD16 and CD172a. Perhaps surprisingly expression of CD163 did not increase as monocytes from either blood or bone marrow matured into macrophages. The level of CD163 expression was an important difference between the *in vitro* CSF1-matured macrophages and freshly isolated mature macrophages which did express CD163. CD163 is commonly used as a marker of monocyte maturation in the pig (Chamorro et al., 2005; Chamorro et al., 2004; Chamorro et al., 2000) and rhCSF1 has been shown to increase levels of CD163 on human monocytes (Tippett et al., 2011). Differences in culture conditions may be

responsible for the variation in CD163 expression reported between the CSF1 matured macrophages described in this study and previous experiments which examined CD163 expression on human CSF1 matured macrophages (Tippett et al., 2011). The macrophages described in this thesis were adherent and grown on bacteriological plastic. Tippett *et al.* (2011) differentiated macrophages in suspension cultures in either Teflon pots or polypropylene tubes. Unsurprisingly these different culture conditions may have affected the expression of surface molecules. Tippett *et al.* also permeabilised the monocytes before staining with anti-CD163 antibodies and found greater increases in CD163 expression after culture in CSF1 when comparing total (intracellular and surface) to surface CD163 expression alone. The data presented in this thesis relates purely to surface expression of CD163. Tippett *et al.* showed peak CD163 expression occurring after 10 days culture in CSF1 with little increase being observed before 5 days. This thesis only measured expression of CD163 in monocytes cultured for up to 7 days culture in CSF1. In addition to using a wider panel of antibodies, future experiments would need to examine cells matured in CSF1 over a longer time course.

6.5 Conservation of gene expression in CD14^{hi} monocyte populations in humans and pigs

Chapter 4 investigated gene expression in porcine monocyte subsets in the context of similar expression data from mice and humans. This was the first analysis of differential gene expression in porcine monocyte populations and provided interesting data on possible roles for the populations isolated. The results also raised questions on whether the previously identified porcine subsets could be considered as orthologous to human monocyte subsets. Re-analysis of previous array data from human monocyte subsets in **Chapter 4** (Ingersoll et al., 2010; Zhao et al., 2009; Ancuta et al., 2009) identified a list of genes which were consistently differentially expressed between monocyte subsets in man. Expression of many of these genes was conserved in pig monocyte subsets. As discussed above, the main surface marker used to distinguish porcine monocytes subsets, CD163, has been shown to be

differentially expressed by human monocyte subsets albeit more highly expressed by human $CD14^{hi}CD16^{-}$ monocytes (Tippett et al., 2011; Ingersoll et al., 2010). Porcine $CD14^{hi}CD163^{lo}$ monocytes expressed higher levels of pro-inflammatory genes such as those belonging to the S100 family and genes involved in control and regulation of the immune system such as IL8 and IL18. Many of these genes were also expressed more highly by human $CD14^{hi}$ monocytes suggestive of a specific role for $CD14^{hi}$ monocytes in both species in regulating the immune response. Similarly $CD14^{lo}$ monocytes from both species expressed higher levels of genes associated with antigen presentation but generally did not appear as closely related as the $CD14^{hi}$ subsets. $CD163^{hi}$ porcine monocytes are commonly referred to as expressing low levels of CD14. Analysis of FACs plots show they actually express only marginally lower levels of CD14 than the so called porcine $CD14^{hi}$ population and comparison with human FACS plots of CD14 vs CD16 staining suggests the level of CD14 expression on porcine $CD163^{hi}$ monocytes (**Figure 6.1A population A3)** may be closer to the human intermediate monocyte subset (**Figure 6.1C population P3**). There appeared to be no true $CD14^{lo}$ monocyte population when looking at $CD163^{+}$ monocytes in the pig. Interestingly a population of porcine monocytes which expressed very low levels of CD14 and did not express either CD16 or CD163 was also be seen by FACS analysis (**Figure 6.1A, population A1**). The study of monocyte subsets in the pig is still in its infancy compared to the vast number of experiments carried out on similar monocyte populations in humans and mice. These $CD14^{lo}CD163^{-}$ monocytes have never been examined in any previous publications and may warrant future investigation as an alternative $CD14^{lo}$ population. Further analysis by FACS and gene expression studies could identify possible functional roles or similarities to already characterised human and mouse monocyte populations.

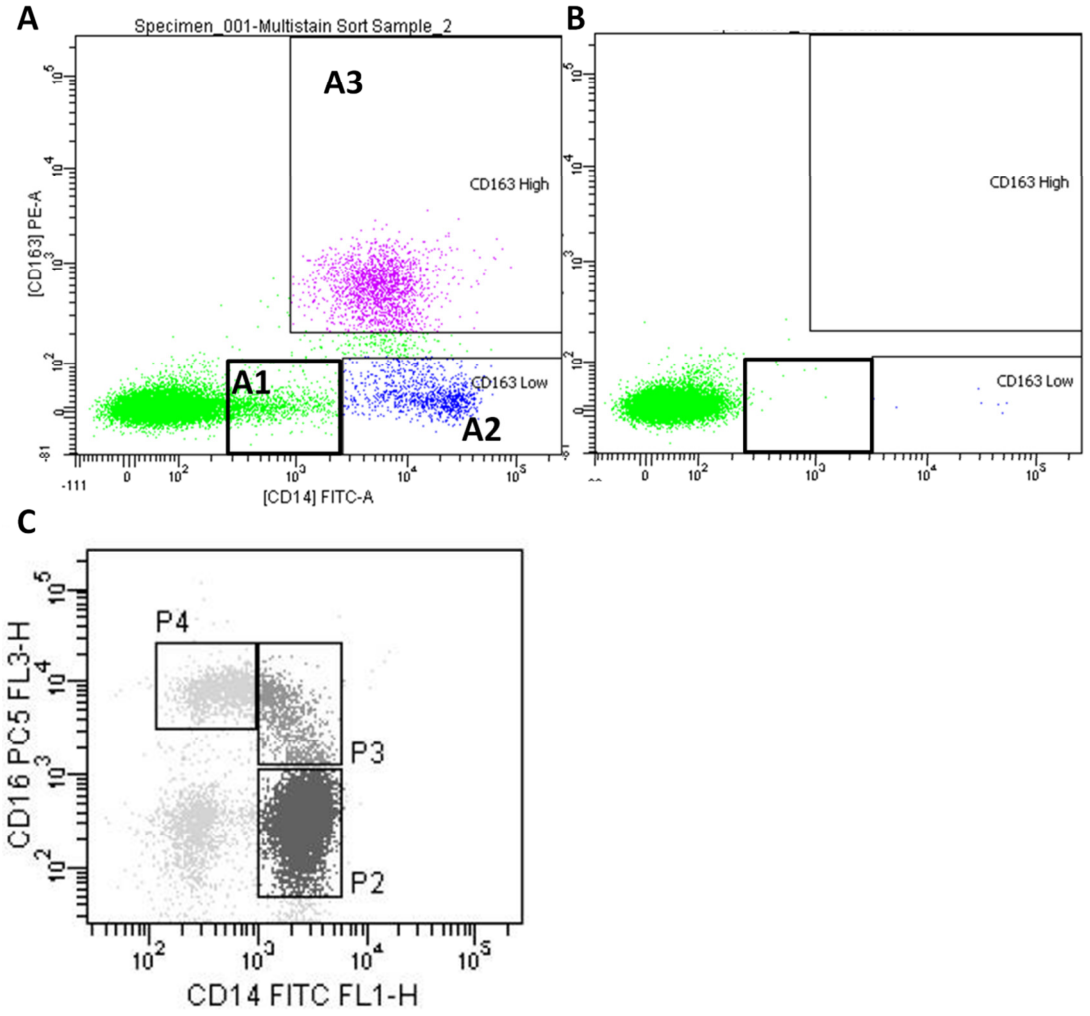

Figure 6-1 comparison of previously identified monocyte subsets in pig and human

Pig monocytes stained with CD14 and CD163 have been separated into two subsets (**A**), **A2** $CD14^{hi}CD163^{lo}$ and **A3** $CD14^{lo}CD163^{hi}$. There was also a population of $CD14^{lo}$ monocytes which did not express CD163(**A1**). Isotype control is also shown (**B**). Human monocytes stained with CD14 and CD16 have been separated into three subsets, **P2** $CD14^{hi}CD16^{-}$, **P3** $CD14^{hi}CD16^{+}$, **P4** $CD14^{lo}CD16^{hi}$.

6.6 Relating gene expression to function

Perhaps the most unexpected finding from this chapter was the higher expression of *CCR2* by $CD14^{lo}CD163^{hi}$ monocytes. This is in contrast to what was observed in human and mouse monocytes (Ingersoll et al., 2010) and previous reports in the pig (Moreno et al., 2010). There were two probesets specific for *CCR2* in the array used to determine relative gene expression in the monocyte subsets. Although average expression of both probesets showed higher expression by $CD14^{lo}CD163^{hi}$ monocytes, one probeset was more highly expressed by $CD163^{hi}$ monocytes and one by $CD163^{lo}$ monocytes meaning the relative levels of *CCR2* so the data are equivocal, and need to be resolved with qRT-PCR or FACS. Unfortunately the porcine *CCR2* gene has not been fully annotated and porcine specific anti-CCR2 antibodies are not commercially available making further investigation of CCR2 expression at the protein or mRNA level difficult. Previous studies have relied on indirect methods to assess expression of CCR2 (Moreno et al., 2010). Development of porcine specific anti-CCR2 antibodies will allow quantitative determination of relative expression levels on the cell surface of different cell populations. Similarly expression of CX3CR1 at the protein level has not been reported, porcine specific antibodies to CX3CR1 would allow analysis of surface expression of this molecule which is differentially expressed on human and mouse monocytes. Expression of CCR2 and CX3CR1 is functionally important for recruitment of monocyte subsets to sites of inflammation by CCL2 or CX3CL1 expressing cells. Porcine anti-CCR2 or CX3CR1 antibodies would help identify likely *in vivo* functions of the populations of monocytes which expressed these receptors.

In addition to the subset-specific expression of inflammatory and immune regulatory genes by $CD14^{hi}CD163^{lo}$ and antigen presentation related genes by $CD14^{lo}CD163^{hi}$ monocytes, one of the most differentially-expressed genes in the monocyte subsets was *Clec7A* (Dectin1). Dectin1 has been shown to function as a co-receptor with TLR2 to mediate recognition of mycobacteria (Yadav and Schorey, 2006), zymosan (Dillon et al., 2006) and *Candida albicans* (Ferwerda et al., 2008b). Dectin1 and

CD163 were among the most differentially expressed genes by CD163^{hi} monocytes. CD163 is well characterised as a virus receptor for PRRSV and ASF, based upon previous studies describing dectin1 working in concert with TLRs in the recognition of a range of PAMPS, a role in virus recognition is at last plausible. Use of anti-dectin1 antibodies prior to infection with PRRSV would enable determination of the role of dectin1 in PRRSV to be assessed. In addition stimulation of porcine macrophages with a specific dectin ligand such as curdlan (beta-1,3-glucan) in combination with a range of TLR ligands such as the TLR4 ligand LPS, the TLR3 ligand dsRNA or the TLR7/8 ligand ssRNA would highlight possible synergistic relationships with other immune receptors in porcine macrophages.

The expectation with studies such as described in **Chapter 4** is that differing expression of genes will relate to specific functions of each monocyte subset. The increase of a particular monocyte subset in disease, after exercise or in the elderly has been described in humans (Allen et al., 1991; Baeten et al., 2000; Blumenstein et al., 1997; Fingerle et al., 1993; Kawanaka et al., 2002; Sadeghi et al., 1999; Saleh et al., 1995; Seidler et al., 2010; Zimmermann et al., 2010; Steppich et al., 2000). Identification of genes which are preferentially expressed by particular subsets is desirable in order to either track the relative numbers of that population as a measure of disease progression or to identify possible targets to manipulate in order to affect disease outcome. Targeting of specific monocyte subsets to affect disease outcome is a promising area for future research (Leuschner et al., 2011) therefore the identification of possible target genes shared by multiple species is desirable to allow more accurate modelling of human pathologies by animal models. Analysis of porcine monocyte subsets is still in its infancy compared to the knowledge accumulated on these cell populations in humans and mice. The pig is already used as a model for many inflammatory diseases (discussed in **Chapter 1** and (Fairbairn et al., 2011) and the investigation of relative numbers of each monocyte subset in already established models could provide important information on the role of monocyte subsets in disease in the pig. Additionally the discovery that mouse Gr1^{-} (Auffray et al., 2007) and human CD14^{dim} (Cros et al., 2010) monocytes perform a

patrolling function in the blood vasculature raises the question of whether a specific monocyte subsets performs this function in the pig. The large size of pigs already provides many benefits over the smaller rodent in allowing easier intervention and study of the animals. Transgenic pigs, for example porcine versions of the MacGreen (Sasmono et al., 2003) or CX3CR1^{gfp} (Jung et al., 2000) mice could be highly illuminating! Previous studies of monocyte subsets in the pig have largely focused on investigating surface marker expression alone. The work presented in **Chapter 4** detailed a fuller examination of the proposed similarities between mouse, human and pig monocyte subsets than has been possible previously. Nevertheless examination of expression of differentially expressed genes at the protein level is required to validate the gene expression data presented here. The data presented in **Chapter 4** identified specific functions likely to be preferentially carried out by CD163^{lo} monocytes and it is hoped future experiments will confirm the specific functions proposed in this thesis for the CD163^{lo} monocyte subset for instance possible interactions between CD14^{hi}CD163^{lo} monocytes and neutrophils or an antigen presentation role for CD14^{lo}CD163^{hi}monocytes. Whether the markers presently used to delineate monocyte subsets in the pig are adequate will require further investigation as well.

6.7 Cross-species comparisons of the macrophage response to LPS

Chapter 5 detailed the analysis of the porcine BMDM response to LPS, which allowed a comparison to similar data for mouse and human macrophages. As expected a large number of genes were regulated by LPS including those involved in negative regulation and feedback control (Wells et al., 2005). For instance a number of inhibitory cytokines such as IL1RN were up-regulated by LPS as shown previously in other species (Coll and O'Neill, 2010). The macrophage response to LPS in humans and mice is a well studied temporal cascade of inducible transcription factors followed by induction of their target genes (Nilsson et al., 2006; Gilchrist et al., 2006; Ghisletti et al., 2010). The data presented in this chapter extended that

knowledge to the pig, and identified many inducible transcription factors. Murine BMDMs are one of the most commonly utilised cells in the laboratory but they may respond to infectious stimuli rather differently than human macrophage populations (Gordon et al., 2001; Fairbairn et al., 2011; Schroder et al., 2012). Furthermore *in vitro* experiments on human macrophages most often use MDM meaning comparisons of the same macrophage populations across species have not previously been possible. Comparison of LPS regulated genes in pigs, humans and mice identified conservation of some pathways in the pig that were known to be differentially expressed between human and mouse macrophages e.g. the Vitamin D3 pathway and the IDO pathway. Comparison of LPS-inducible genes, which were divergently regulated in human and mice, showed that these genes were induced in both pig BMDM and MDM showing the differences previously noted between human and mouse macrophages were most likely attributable to the species rather than cell type. Analysis also showed the human and pig response to LPS appeared to involve the adaptive immune system in a way that the mouse response did not with induction of a number of lymphocyte regulating cytokines and receptors e.g. *CCL20*, *CXCL9, CXCL11, CXCL13*, *IL7R, IL27R*. Alignment of the promoters of some of these genes suggested that divergent regulation most probably occurred at the transcription level, the promoters of pig and human genes were much more conserved than mouse and human. Pigs, rodents and humans diverged at approximately the same time (Springer et al., 2003; Jorgensen et al., 2005) but because of short generation time and large effective population size, the mouse branch is considerably longer when one compares DNA sequences. At the sequence level pigs and humans are more alike, especially in 5' and 3' untranslated and flanking sequences (Wernersson et al., 2005).

A striking example of the differing human and mouse response to LPS is the induction of NO by murine macrophages. Human macrophages stimulated in vitro do not produce NO nor do they induce transcription of arginase or the arginase transporter (*CAT2*) (Schroder et al., 2012). This thesis also demonstrated that the NO pathway was not activated by LPS in pig BMDM; in addition to an absence of

measurable NO, there was no detectable induction of the rate limiting enzyme *CAT2*. The lack of induction in human and pig macrophages of NO raised the interesting question of whether biopterin production is an alternative pathway in both species utilising the products of the inducible GTP cyclohydrolase, which is induced in both species. There is some evidence biopterin is produced by pigs (Breinekova et al., 2007; Myers et al., 2003) and the array data showed that the other enzymes needed to make tetrahydrobiopterin, the cofactor for NOS2, are expressed at very low levels in pig macrophages as they are in humans (biogps.gnf.org, (The FANTOM Consortium et al., 2005) and FANTOM5 published data (DAH Personal communication)). In summary, like human macrophages, pig macrophages did not produce NO or induce transcription of any of the rate limiting steps in the NO pathway when stimulated with LPS. There remains a debate as to whether NOS2 has any function in human innate immunity. The human and pig NOS2 promoter regions are reasonably well-conserved (Kapetanovic *et al* manuscript under review). The pig may therefore provide a model to study the function of NOS2, if any, in human immunity, there is some evidence that NOS2 may have a role in the macrophage response to *Mycobacterium tuberculosis* in man (Ehrt et al., 2001; Nicholson et al., 1996) and some experiments have suggested pigs may produce NO in response to live organisms (Benga et al., 2009).

6.8 Future technologies

Cross-species comparisons always pose difficulties. When using microarray technology the comparative specificity of primers used in each species may not always be equal. This can be overcome by the design of custom arrays which target the same part of the gene for each species although newer technologies such as CAGE and RNAseq may provide a better solution. Microarrays limit the search to what is known while CAGE and RNAseq detect what is expressed whether it has been fully identified on the genome or not. They can also show expression of genes across the species more clearly, for example mouse and human TNFα are not strict one-to-one orthologs so would be excluded from a custom designed orthologous

array despite fulfilling similar functions and having similar actions in both species. Future investigations using CAGE or RNAseq could be used to fully examine key differences in the response of murine macrophages to LPS compared to human and pig macrophages. Pig macrophage libraries have been prepared and sequenced in our laboratory, and the data will allow comparative analysis of the massive datasets of CAGE for humans previously published (Carninci et al., 2006), and currently being generated in the FANTOM5 project. Identifying such differences could lead to the discovery of why humans are more susceptible to LPS and provide targets for the therapy of sepsis in man, an area where little success has been seen with rodent models.

At present one of the major benefits of working on rodents is the large number of transgenic animals available. There is a huge genetic diversity already present in the porcine population, as in humans, therefore it is likely that some pigs will possess natural mutations that make them more or less susceptible to a disease. Genetic diversity among pig breeds may therefore do away with some of the need for genetically engineered animals and could allow identification of genes which are involved in disease in both men and pigs. Identification of such naturally occurring mutations may lead to animal models which are closer to what occurs in man. Large numbers of apparent null mutations have been seen in pig macrophage RNAseq data from Large-White-Landrace F1 animals (unpublished, Dario Beraldi). Because pigs are multiparous, such null mutants identified in an individual animal, could readily be bred to homozygosity through brother-sister matings of the progeny. Hence, it may be possible to generate “knockouts” in pigs without resorting to transgenic technology.

6.9 Conclusion

Overall the aim of this thesis was to increase our knowledge of transcriptional control of macrophage function in the pig and its relationship to infectious disease

susceptibility. It is hoped that the studies described here show some of the ways that pigs may address the shortcomings of commonly used mouse models for the study of macrophage differentiation and activation *in vitro*, and the biology of sepsis and other pathologies *in vivo*. Gene expression analysis studies, such as presented in this thesis, provide possible targets for future studies rather than an end in their own right. The regulation of genes in porcine BMDMs must now be studied in more detail and these results carried over to functional studies. For instance IDO inhibitors did not show promising results in the pig despite early indication of success in mice models. Induction of IDO is a relatively late response, and might conceivably be part of the feedback control rather than an antimicrobial effector. Additionally the high induction of genes regulating the adaptive immune system in response to LPS in both humans and pigs (e.g. *CCL20*, *CXCL9, CXCL11, CXCL13*, *IL7R, IL27R*) provides targets for future experiments to elucidate their role in the immune response. The data presented in this thesis could provide the basis for future i*n vivo* work by identifying genes differentially expressed between monocyte subsets in the pig or which are important in the pig response to LPS. Gene studies in pigs with sepsis showed that some of the genes which were highly LPS-inducible in pig and human macrophages were also up-regulated in sick animals. Further analysis could provide possible targets to manipulate in pursuit of a treatment for sepsis. Furthermore the easy availability of different cell populations from within the pig will allow the analysis of genes which are specific to particular cell types and may perhaps provide an answer to why some populations are more susceptible to certain pathogens. The completion of the pig genome and the characterisation of many key immune regulators and markers has ensured the value of the pig as a model of human immune responses is being increasingly recognised.

Chapter 7: Supplementary Information

7.1 Appendix 1

Antibody	Concentration	Source
CD14	1:50	MCA1218F (AbD Serotec)
CD16	1:200	MCA1971PE (AbD Serotec)
CD163	1:200	MCA2311PE (AbD Serotec)
CD172a	1:400	4525-09 (Southern Biotech)

Table 7.1-1 Antibodies used in FACS anlaysis

Name	Breed	Age
LWL1	Large White X Landrace F1 cross	8 weeks
LWL4	Large White X Landrace F1 cross	11 weeks
LWL5	Large White X Landrace F1 cross	11 weeks
LAN1	Landrace	7 weeks
LWLxPIE	Large White - Landrace F1 cross X Pietrain	11 weeks
LWL2	Large White X Landrace F1 cross	9 weeks
LW2	Large White	6 weeks
LW3	Large White	7 weeks
LAN1	Landrace	7 weeks

Table 7.1-2 Animals used

7.2 Appendix 2

Target cDNA	Forward primer	Reverse primer	Amplicon size (bp)
HPRT	ACACTGGCAAAACAATGCAA	ACACTTCGAGGGGTCCTTTT	103
CCL20	GGTGCTGCTGCTCTACCTCT	GCTGTGTGAAGCCCATGATA	113
IDO	GGTTTCGCTATTGGTGGAAA	CTTTTGCAAAGCATCCAGGT	100
STAT4	GAAAGCCACCTTGGAGGAAT	ACAACCGGCCTTTGTTGTAG	100
LCN2	TCGTGCGTGTGGTGTCTACT	ACCTTGGTCCTCCCGTAAAG	109
NOS2A	AGAGCCTCTGGACCTCAACA	CTCACAGCAGAGTTCCACCA	136
GTP CH1	AAGTTCTTGGCCTCAGCAAA	TACTCCGACTCCAGCAGGTC	135
AS	CCCTCTACAACGAGGAGCTG	TCTGGAGGCGATGATATTCC	117
CAT2	GCCTCGCAGAGTATCCTGAC	ACCCAGCCACCATAACAAAG	150
AL	GCCTGGACAAGGTAGCTGAG	GTTCCGACTTCGTCCTGTGT	146
ARG2	CACCCCTCACCACTTCATCT	CAAGGCTTGATCCAGGAAAA	112
OAT	TGCAAAATCGCTCGTAGATG	GACAACGTTCTTCCCCAAAA	101
JAG1	CCCGACTGCAGGATAAACAT	ACACACCGGTACCCATTGAT	
IL8	CTTCGATGCCAGTGCATAAA	GGTCCAGGCAGACCTCTTTT	
TNFA	CCCAAGGACTCAGATCATCG	ATACCCACTCTGCCATTGGA	

Table 7.2-1 Oligonucleotides used in RT qPCR anlaysis

7.3 Appendix 3

Molecule	Function	Expression in mice	Expression in man	References
CD11a	Involved in cellular adhesion through its interaction with ICAMS1-3. CD11a and CD18 together form LFA1 which binds to ICAM1. Required for patrolling behaviour of monocytes	T cell, B cell, DC, NK cell, macrophage, granulocyte	T cell, B cell, NK cell, macrophage, granulocyte	(Marlin and Springer, 1987; Auffray et al., 2007)
CD11c	Forms complement receptor with CD18, often used as a marker for DCs	T cell, B cell, DC, NK cell, macrophage, granulocyte	T cell, B cell, DC, NK cell, macrophage, granulocyte	
CX3CR1	Fractalkine receptor, required for patrolling behaviour of monocytes	Mast cells, macrophages,	NK cells, DC, T cells, macrophages	(Auffray et al., 2007)
CD43	Sialogycoprotein. Pro and anti adhesion, involved in signalling and cytoskleetal intercation	T cell, NK cell, stem cell precursor, macrophages, granulocytes, platelets	T cell, NK cell, stem cell precursor, macrophage, granulocyte, platelet	(Ostberg et al., 1998)

Table 7.3-1 Surface markers commonly expressed by human CD14+CD16+ and Murine Gr1- monocytes

Molecule	Function	Expression in mice	Expression in man	References
CD62L	Leukocyte specific cell adhesion molecule which regulates entry of leukocytes to secondary lymphoid tissue	T cell, B cell, NK cell, macrophage, granulocyte	T cell, B cell, NK cell, macrophage, granulocyte	(Spertini et al., 1991; Smith et al., 1991)
CCR1	G protein coupled receptor. Ligands include MIP1α, RANTES, MCP3 and MPIF1	Granulocytes, mast cells, macrophages	eosinophils, monocytes, neutrophils	(Kaufmann et al., 2001)
CCR2	Ligands include MCP1, involved in monocyte recruitment from bone marrow to blood	NK cells, granulocytes, macrophages	Monocytes, DC	(Kaufmann et al., 2001; Serbina and Pamer, 2006)

Table 7.3-2 Surface markers commonly expressed by human CD14++CD16- and Murine Gr1+ monocytes

LFA1: Lymphocyte function associated antigen 1. ICAM: Intracellular adhesion molecules. MIP1α: Macrophage inflammatory protein 1 α. RANTES: regulated on activation normal T expressed and secreted protein. MCP3: Monocyte chemoattractant protein 3. MPIF1: myeloid progenitor inhibitory factor 1. DC: Dendritic cell. NK cell: natural Killer cell

7.4 Appendix 4

Breed	sex	CD14lo CD16hi (%)	CD14hi CD16lo (%)	CD14- CD16+ (%)	CD14lo CD163hi (%)	CD14hi CD163lo (%)	CD14+ CD172a+ (%)	CD14-CD172a+ (%)
LWL1	M	3.80	3.00	22.00	2.90	2.70	7.20	9.80
LWL2	M	2.90	0.90	19.00	3.40	3.50	4.20	15.50
LWL4	F	3.40	2.70	11.00	2.60	1.50	7.90	7.60
LWL5	M	4.20	3.90	12.00	3.70	4.20	8.00	10.00
HAM1	M	7.70	4.60	11.90	6.60	5.40	11.40	8.10
HAM2	F	5.50	4.50	10.30	5.30	4.30	9.80	7.60
HAM5	F	2.60	2.20	19.20	1.70	1.70	4.50	4.90
DUR3	F	2.00	3.00	22.60	2.00	3.50	5.50	13.30
DUR4	M	5.50	2.80	42.70	5.00	3.00	9.80	8.00
LW2	F	8.40	4.20	22.10	5.40	5.00	13.14	12.20
LW3	M	2.40	1.70	28.70	1.90	1.90	5.00	9.30
LW4	M	2.20	1.90	19.00	1.70	2.00	5.30	5.40
LW5	F	3.00	1.20	31.30	2.60	1.10	4.20	3.70
LAN2	F	3.60	2.50	26.00	3.20	2.20	6.80	7.30
LAN3	F	1.10	1.20	12.50	1.10	0.80	2.70	3.90
PIE2	F	0.90	0.30	19.30	1.00	0.54	1.50	2.60
PIE3	M	10.70	8.40	12.80	13.40	5.20	15.40	13.70
	Average	4.11	2.88	20.14	3.74	2.86	7.20	8.41
	Average for all M	4.93	3.40	21.01	4.83	3.49		
	Average for all F	3.39	2.42	19.37	2.77	2.29	6.23	7.01

Table 7.4-1 Animals used for study of monocyte populations LWL; Large White x Landrace F1 X, HAM; Hampshire, DUR; Duroc, LW; Large White, LAN; Landrace, PIE; Pietrain

7.5 Appendix 5

Table 7.5-1 Go-terms enriched in human CD16+ monocytes

Category	Term	Count	%	PValue
Annotation Cluster 1				
GOTERM_BP_FAT	GO:0002683~negative regulation of immune system process	7	5.00	9.73E-05
GOTERM_BP_FAT	GO:0002695~negative regulation of leukocyte activation	6	4.29	1.52E-04
GOTERM_BP_FAT	GO:0050866~negative regulation of cell activation	6	4.29	2.10E-04
GOTERM_BP_FAT	GO:0051250~negative regulation of lymphocyte activation	5	3.57	1.36E-03
GOTERM_BP_FAT	GO:0070664~negative regulation of leukocyte proliferation	4	2.86	2.87E-03
GOTERM_BP_FAT	GO:0050672~negative regulation of lymphocyte proliferation	4	2.86	2.87E-03
GOTERM_BP_FAT	GO:0032945~negative regulation of mononuclear cell proliferation	4	2.86	2.87E-03
GOTERM_BP_FAT	GO:0002694~regulation of leukocyte activation	7	5.00	3.75E-03
GOTERM_BP_FAT	GO:0050865~regulation of cell activation	7	5.00	4.85E-03
GOTERM_BP_FAT	GO:0050868~negative regulation of T cell activation	4	2.86	6.22E-03
GOTERM_BP_FAT	GO:0050670~regulation of lymphocyte proliferation	5	3.57	6.50E-03
GOTERM_BP_FAT	GO:0070663~regulation of leukocyte proliferation	5	3.57	6.78E-03
GOTERM_BP_FAT	GO:0032944~regulation of mononuclear cell proliferation	5	3.57	6.78E-03
GOTERM_BP_FAT	GO:0051249~regulation of lymphocyte activation	6	4.29	1.06E-02

GOTERM_BP_FAT	GO:0050863~regulation of T cell activation	5	3.57	2.08E-02
GOTERM_BP_FAT	GO:0042130~negative regulation of T cell proliferation	3	2.14	2.41E-02

Annotation Cluster 2

GOTERM_BP_FAT	GO:0042981~regulation of apoptosis	20	14.29	9.07E-05
GOTERM_BP_FAT	GO:0043067~regulation of programmed cell death	20	14.29	1.03E-04
GOTERM_BP_FAT	GO:0010941~regulation of cell death	20	14.29	1.08E-04
GOTERM_BP_FAT	GO:0042035~regulation of cytokine biosynthetic process	6	4.29	5.19E-04
GOTERM_BP_FAT	GO:0006917~induction of apoptosis	11	7.86	5.93E-04
GOTERM_BP_FAT	GO:0012502~induction of programmed cell death	11	7.86	6.07E-04
GOTERM_BP_FAT	GO:0042108~positive regulation of cytokine biosynthetic process	5	3.57	8.74E-04
GOTERM_BP_FAT	GO:0043065~positive regulation of apoptosis	12	8.57	1.63E-03
GOTERM_BP_FAT	GO:0043068~positive regulation of programmed cell death	12	8.57	1.72E-03
GOTERM_BP_FAT	GO:0010942~positive regulation of cell death	12	8.57	1.79E-03
GOTERM_BP_FAT	GO:0010557~positive regulation of macromolecule biosynthetic process	13	9.29	1.41E-02
GOTERM_BP_FAT	GO:0043066~negative regulation of apoptosis	9	6.43	1.42E-02
GOTERM_BP_FAT	GO:0043069~negative regulation of programmed cell death	9	6.43	1.53E-02
GOTERM_BP_FAT	GO:0060548~negative regulation of cell death	9	6.43	1.55E-02
GOTERM_BP_FAT	GO:0001817~regulation of cytokine	6	4.29	2.32E-02

production

Annotation Cluster 3

Category	Term	Count	%	PValue
GOTERM_CC_FAT	GO:0044459~plasma membrane part	33	23.57	1.64E-03
GOTERM_CC_FAT	GO:0005887~integral to plasma membrane	20	14.29	6.47E-03
GOTERM_CC_FAT	GO:0031226~intrinsic to plasma membrane	20	14.29	8.18E-03
GOTERM_BP_FAT	GO:0007166~cell surface receptor linked signal transduction	25	17.86	3.83E-02

Annotation Cluster 4

Category	Term	Count	%	PValue
GOTERM_BP_FAT	GO:0006952~defense response	14	10.00	3.38E-03
GOTERM_BP_FAT	GO:0009611~response to wounding	12	8.57	7.89E-03
GOTERM_BP_FAT	GO:0006954~inflammatory response	9	6.43	8.77E-03

Annotation Cluster 5

Category	Term	Count	%	PValue
GOTERM_CC_FAT	GO:0000267~cell fraction	20	14.29	2.35E-03
GOTERM_CC_FAT	GO:0019717~synaptosome	5	3.57	6.36E-03
GOTERM_CC_FAT	GO:0045202~synapse	8	5.71	3.50E-02
GOTERM_CC_FAT	GO:0005626~insoluble fraction	13	9.29	5.99E-02
GOTERM_CC_FAT	GO:0005624~membrane fraction	12	8.57	9.24E-02

Annotation Cluster 6

Category	Term	Count	%	PValue

GOTERM_BP_FAT	GO:0042035~regulation of cytokine biosynthetic process	6	4.29	5.19E-04
GOTERM_BP_FAT	GO:0042108~positive regulation of cytokine biosynthetic process	5	3.57	8.74E-04
GOTERM_BP_FAT	GO:0009967~positive regulation of signal transduction	9	6.43	4.99E-03
GOTERM_BP_FAT	GO:0010647~positive regulation of cell communication	9	6.43	9.40E-03
GOTERM_BP_FAT	GO:0043123~positive regulation of I-kappaB kinase/NF-kappaB cascade	5	3.57	1.11E-02
GOTERM_BP_FAT	GO:0051048~negative regulation of secretion	4	2.86	1.31E-02
GOTERM_BP_FAT	GO:0043122~regulation of I-kappaB kinase/NF-kappaB cascade	5	3.57	1.55E-02
GOTERM_BP_FAT	GO:0010740~positive regulation of protein kinase cascade	6	4.29	1.71E-02
GOTERM_BP_FAT	GO:0001817~regulation of cytokine production	6	4.29	2.32E-02
GOTERM_BP_FAT	GO:0010627~regulation of protein kinase cascade	7	5.00	2.44E-02
GOTERM_BP_FAT	GO:0051051~negative regulation of transport	5	3.57	3.29E-02

Annotation Cluster 7

Category	Term	Count	%	PValue
GOTERM_BP_FAT	GO:0045087~innate immune response	6	4.29	7.95E-03
GOTERM_BP_FAT	GO:0006954~inflammatory response	9	6.43	8.77E-03
GOTERM_BP_FAT	GO:0002449~lymphocyte mediated immunity	4	2.86	2.48E-02
GOTERM_BP_FAT	GO:0006958~complement activation, classical pathway	3	2.14	2.75E-02
GOTERM_BP_FAT	GO:0002455~humoral immune response mediated by circulating	3	2.14	3.12E-02

	immunoglobulin			
GOTERM_BP_FAT	GO:0006959~humoral immune response	4	2.86	3.38E-02
GOTERM_BP_FAT	GO:0002443~leukocyte mediated immunity	4	2.86	4.18E-02
GOTERM_BP_FAT	GO:0006956~complement activation	3	2.14	5.42E-02
GOTERM_BP_FAT	GO:0002541~activation of plasma proteins involved in acute inflammatory response	3	2.14	5.65E-02
GOTERM_BP_FAT	GO:0002526~acute inflammatory response	4	2.86	5.76E-02
GOTERM_BP_FAT	GO:0016064~immunoglobulin mediated immune response	3	2.14	8.41E-02
GOTERM_BP_FAT	GO:0019724~B cell mediated immunity	3	2.14	8.95E-02

Annotation Cluster 8

Category	Term	Count	%	PValue
GOTERM_MF_FAT	GO:0019955~cytokine binding	5	3.57	1.29E-02
GOTERM_MF_FAT	GO:0004896~cytokine receptor activity	3	2.14	7.66E-02

Annotation Cluster 9

Category	Term	Count	%	PValue
GOTERM_CC_FAT	GO:0005615~extracellular space	14	10.00	6.36E-03
GOTERM_BP_FAT	GO:0048534~hemopoietic or lymphoid organ development	8	5.71	8.82E-03
GOTERM_BP_FAT	GO:0002520~immune system development	8	5.71	1.20E-02
GOTERM_CC_FAT	GO:0044421~extracellular region part	15	10.71	3.81E-02

Annotation Cluster 10				
Category	**Term**	**Count**	**%**	**PValue**
GOTERM_BP_FAT	GO:0008284~positive regulation of cell proliferation	11	7.86	3.99E-03
GOTERM_BP_FAT	GO:0048534~hemopoietic or lymphoid organ development	8	5.71	8.82E-03

Table 7.5-2 Go-terms enriched in human CD16+ monocytes

Category	Term	Count	%	PValue
Annotation Cluster 1				
GOTERM_CC_FAT	GO:0031224~intrinsic to membrane	66	51.16	6.21E-04
GOTERM_CC_FAT	GO:0016021~integral to membrane	64	49.61	7.74E-04
Annotation Cluster 2				
GOTERM_CC_FAT	GO:0005576~extracellular region	35	27.13	5.81E-05
GOTERM_CC_FAT	GO:0005615~extracellular space	18	13.95	8.19E-05
GOTERM_CC_FAT	GO:0044421~extracellular region part	21	16.28	2.03E-04
Annotation Cluster 3				
GOTERM_CC_FAT	GO:0005887~integral to plasma membrane	25	19.38	6.76E-05
GOTERM_CC_FAT	GO:0031226~intrinsic to plasma membrane	25	19.38	9.63E-05
GOTERM_CC_FAT	GO:0005886~plasma membrane	51	39.53	3.72E-04
GOTERM_CC_FAT	GO:0044459~plasma membrane part	32	24.81	3.29E-03
Annotation Cluster 4				

GOTERM_MF_FAT	GO:0030246~carbohydrate binding	13	10.08	1.89E-05
GOTERM_MF_FAT	GO:0005529~sugar binding	8	6.20	8.10E-04

Annotation Cluster 5

GOTERM_BP_FAT	GO:0042060~wound healing	13	10.08	5.72E-08
GOTERM_BP_FAT	GO:0007599~hemostasis	7	5.43	2.71E-04
GOTERM_CC_FAT	GO:0030141~secretory granule	8	6.20	9.70E-04
GOTERM_BP_FAT	GO:0050878~regulation of body fluid levels	7	5.43	1.12E-03
GOTERM_CC_FAT	GO:0031091~platelet alpha granule	5	3.88	1.40E-03
GOTERM_BP_FAT	GO:0007596~blood coagulation	6	4.65	1.57E-03
GOTERM_BP_FAT	GO:0050817~coagulation	6	4.65	1.57E-03
GOTERM_CC_FAT	GO:0044433~cytoplasmic vesicle part	5	3.88	7.99E-02

Annotation Cluster 6

GOTERM_BP_FAT	GO:0006928~cell motion	15	11.63	3.68E-05
GOTERM_BP_FAT	GO:0042330~taxis	9	6.98	5.39E-05
GOTERM_BP_FAT	GO:0006935~chemotaxis	9	6.98	5.39E-05
GOTERM_BP_FAT	GO:0007626~locomotory behavior	11	8.53	9.07E-05
GOTERM_BP_FAT	GO:0007610~behavior	14	10.85	1.32E-04
GOTERM_BP_FAT	GO:0001525~angiogenesis	8	6.20	2.26E-04
GOTERM_BP_FAT	GO:0051674~localization of cell	11	8.53	2.29E-04
GOTERM_BP_FAT	GO:0048870~cell motility	11	8.53	2.29E-04
GOTERM_BP_FAT	GO:0030593~neutrophil chemotaxis	4	3.10	4.12E-04
GOTERM_BP_FAT	GO:0016477~cell migration	10	7.75	4.72E-04
GOTERM_BP_FAT	GO:0048514~blood vessel morphogenesis	8	6.20	1.85E-03

GOTERM_BP_FAT	GO:0030595~leukocyte chemotaxis	4	3.10	3.50E-03
GOTERM_BP_FAT	GO:0060326~cell chemotaxis	4	3.10	4.06E-03
GOTERM_MF_FAT	GO:0005125~cytokine activity	7	5.43	4.14E-03
GOTERM_BP_FAT	GO:0001568~blood vessel development	8	6.20	4.24E-03
GOTERM_BP_FAT	GO:0001944~vasculature development	8	6.20	4.84E-03
GOTERM_BP_FAT	GO:0050900~leukocyte migration	4	3.10	1.17E-02
GOTERM_BP_FAT	GO:0045765~regulation of angiogenesis	3	2.33	9.58E-02
Annotation Cluster 7				
GOTERM_CC_FAT	GO:0030141~secretory granule	8	6.20	9.70E-04
GOTERM_CC_FAT	GO:0031988~membrane-bounded vesicle	14	10.85	1.27E-03
GOTERM_CC_FAT	GO:0031091~platelet alpha granule	5	3.88	1.40E-03
GOTERM_CC_FAT	GO:0031982~vesicle	15	11.63	1.95E-03
GOTERM_CC_FAT	GO:0016023~cytoplasmic membrane-bounded vesicle	13	10.08	2.91E-03
GOTERM_CC_FAT	GO:0031410~cytoplasmic vesicle	14	10.85	3.71E-03
GOTERM_CC_FAT	GO:0031983~vesicle lumen	4	3.10	7.38E-03
GOTERM_CC_FAT	GO:0031093~platelet alpha granule lumen	3	2.33	4.92E-02
GOTERM_CC_FAT	GO:0060205~cytoplasmic membrane-bounded vesicle lumen	3	2.33	5.58E-02
GOTERM_CC_FAT	GO:0044433~cytoplasmic vesicle part	5	3.88	7.99E-02
Annotation Cluster 8				
GOTERM_MF_FAT	GO:0030246~carbohydrate binding	13	10.08	1.89E-05

GOTERM_MF_FAT	GO:0005539~glycosaminoglycan binding	5	3.88	2.37E-02
GOTERM_MF_FAT	GO:0030247~polysaccharide binding	5	3.88	3.21E-02
GOTERM_MF_FAT	GO:0001871~pattern binding	5	3.88	3.21E-02
GOTERM_MF_FAT	GO:0008201~heparin binding	4	3.10	4.62E-02

Annotation Cluster 9

GOTERM_CC_FAT	GO:0043235~receptor complex	6	4.65	3.37E-03
GOTERM_BP_FAT	GO:0007229~integrin-mediated signaling pathway	4	3.10	2.02E-02
GOTERM_CC_FAT	GO:0008305~integrin complex	3	2.33	2.60E-02
GOTERM_CC_FAT	GO:0009986~cell surface	7	5.43	8.25E-02

Annotation Cluster 10

GOTERM_BP_FAT	GO:0048878~chemical homeostasis	12	9.30	3.42E-03
GOTERM_BP_FAT	GO:0042592~homeostatic process	15	11.63	3.58E-03
GOTERM_BP_FAT	GO:0019725~cellular homeostasis	11	8.53	5.26E-03

7.6 Appendix 6

Table 7.6-1 Go-terms enriched in porcine CD163+ monocytes

Category	Term	Count	%	PValue
Annotation Cluster 1				
GOTERM_CC_FAT	GO:0005739~mitochondrion	189	13.52	3.94E-26
GOTERM_CC_FAT	GO:0044429~mitochondrial part	115	8.23	2.36E-19
GOTERM_CC_FAT	GO:0031980~mitochondrial lumen	46	3.29	9.80E-09
GOTERM_CC_FAT	GO:0005759~mitochondrial matrix	46	3.29	9.80E-09
Annotation Cluster 2				
GOTERM_BP_FAT	GO:0006412~translation	85	6.08	1.77E-23
GOTERM_CC_FAT	GO:0030529~ribonucleoprotein complex	112	8.01	4.91E-23
GOTERM_CC_FAT	GO:0005840~ribosome	61	4.36	1.76E-18
GOTERM_MF_FAT	GO:0003735~structural constituent of ribosome	51	3.65	8.97E-18
GOTERM_BP_FAT	GO:0006414~translational elongation	39	2.79	1.86E-17
GOTERM_CC_FAT	GO:0033279~ribosomal subunit	41	2.93	1.78E-14
GOTERM_CC_FAT	GO:0044445~cytosolic part	45	3.22	1.93E-14
GOTERM_CC_FAT	GO:0022626~cytosolic ribosome	28	2.00	6.15E-11
GOTERM_CC_FAT	GO:0015935~small ribosomal subunit	21	1.50	4.78E-08

GOTERM_CC_FAT	GO:0015934~large ribosomal subunit	20	1.43	7.44E-07
GOTERM_CC_FAT	GO:0022625~cytosolic large ribosomal subunit	14	1.00	4.22E-06
GOTERM_CC_FAT	GO:0022627~cytosolic small ribosomal subunit	14	1.00	8.07E-06
GOTERM_MF_FAT	GO:0005198~structural molecule activity	73	5.22	3.77E-04
Annotation Cluster 3				
GOTERM_CC_FAT	GO:0005739~mitochondrion	189	13.52	3.94E-26
GOTERM_CC_FAT	GO:0044429~mitochondrial part	115	8.23	2.36E-19
GOTERM_CC_FAT	GO:0031090~organelle membrane	166	11.87	2.17E-16
GOTERM_CC_FAT	GO:0044455~mitochondrial membrane part	41	2.93	7.19E-15
GOTERM_CC_FAT	GO:0031966~mitochondrial membrane	80	5.72	1.14E-14
GOTERM_CC_FAT	GO:0005740~mitochondrial envelope	83	5.94	1.45E-14
GOTERM_CC_FAT	GO:0019866~organelle inner membrane	71	5.08	1.83E-14
GOTERM_BP_FAT	GO:0006091~generation of precursor metabolites and energy	67	4.79	2.82E-14
GOTERM_CC_FAT	GO:0005743~mitochondrial inner membrane	67	4.79	5.34E-14
GOTERM_CC_FAT	GO:0031967~organelle envelope	105	7.51	1.59E-13
GOTERM_CC_FAT	GO:0031975~envelope	105	7.51	1.97E-13
GOTERM_BP_FAT	GO:0045333~cellular respiration	31	2.22	1.77E-

				11
SP_PIR_KEYWORDS	mitochondrion inner membrane	42	3.00	7.49E-11
GOTERM_BP_FAT	GO:0006119~oxidative phosphorylation	30	2.15	1.27E-10
GOTERM_BP_FAT	GO:0022900~electron transport chain	31	2.22	1.43E-09
GOTERM_BP_FAT	GO:0015980~energy derivation by oxidation of organic compounds	35	2.50	2.59E-09
GOTERM_MF_FAT	GO:0016651~oxidoreductase activity, acting on NADH or NADPH	23	1.65	1.55E-07
GOTERM_BP_FAT	GO:0022904~respiratory electron transport chain	20	1.43	1.87E-07
GOTERM_CC_FAT	GO:0005746~mitochondrial respiratory chain	20	1.43	3.38E-07
GOTERM_BP_FAT	GO:0006120~mitochondrial electron transport, NADH to ubiquinone	15	1.07	1.64E-06
GOTERM_BP_FAT	GO:0042773~ATP synthesis coupled electron transport	17	1.22	3.04E-06
GOTERM_BP_FAT	GO:0042775~mitochondrial ATP synthesis coupled electron transport	17	1.22	3.04E-06
GOTERM_CC_FAT	GO:0070469~respiratory chain	20	1.43	4.77E-06
GOTERM_MF_FAT	GO:0016655~oxidoreductase activity, acting on NADH or NADPH, quinone or similar compound as acceptor	15	1.07	1.09E-05
GOTERM_CC_FAT	GO:0030964~NADH dehydrogenase complex	14	1.00	1.47E-05
GOTERM_CC_FAT	GO:0045271~respiratory chain complex I	14	1.00	1.47E-05
GOTERM_CC_FAT	GO:0005747~mitochondrial	14	1.00	1.47E-

	respiratory chain complex I			05
SP_PIR_KEYWORDS	ubiquinone	11	0.79	1.99E-05
GOTERM_MF_FAT	GO:0008137~NADH dehydrogenase (ubiquinone) activity	13	0.93	5.98E-05
GOTERM_MF_FAT	GO:0050136~NADH dehydrogenase (quinone) activity	13	0.93	5.98E-05
GOTERM_MF_FAT	GO:0003954~NADH dehydrogenase activity	13	0.93	5.98E-05
Annotation Cluster 4				
GOTERM_BP_FAT	GO:0046907~intracellular transport	102	7.30	8.86E-12
GOTERM_BP_FAT	GO:0015031~protein transport	113	8.08	1.12E-11
GOTERM_BP_FAT	GO:0006886~intracellular protein transport	69	4.94	1.73E-11
GOTERM_BP_FAT	GO:0045184~establishment of protein localization	113	8.08	2.01E-11
GOTERM_BP_FAT	GO:0070727~cellular macromolecule localization	70	5.01	6.59E-10
GOTERM_BP_FAT	GO:0034613~cellular protein localization	69	4.94	1.21E-09
GOTERM_BP_FAT	GO:0008104~protein localization	115	8.23	1.77E-08
GOTERM_BP_FAT	GO:0006605~protein targeting	37	2.65	7.56E-06
Annotation Cluster 5				
GOTERM_CC_FAT	GO:0031974~membrane-enclosed lumen	232	16.60	4.28E-13
GOTERM_CC_FAT	GO:0043233~organelle lumen	225	16.09	3.49E-

				12
GOTERM_CC_FAT	GO:0070013~intracellular organelle lumen	219	15.67	1.21E-11
GOTERM_CC_FAT	GO:0005730~nucleolus	86	6.15	5.50E-05
GOTERM_CC_FAT	GO:0031981~nuclear lumen	155	11.09	1.06E-04
GOTERM_CC_FAT	GO:0005654~nucleoplasm	83	5.94	8.44E-02
Annotation Cluster 6				
GOTERM_CC_FAT	GO:0048770~pigment granule	30	2.15	2.27E-11
GOTERM_CC_FAT	GO:0042470~melanosome	30	2.15	2.27E-11
GOTERM_CC_FAT	GO:0031988~membrane-bounded vesicle	77	5.51	5.42E-06
GOTERM_CC_FAT	GO:0016023~cytoplasmic membrane-bounded vesicle	75	5.36	5.85E-06
GOTERM_CC_FAT	GO:0031410~cytoplasmic vesicle	84	6.01	7.56E-06
GOTERM_CC_FAT	GO:0031982~vesicle	86	6.15	1.22E-05
Annotation Cluster 7				
GOTERM_BP_FAT	GO:0006396~RNA processing	84	6.01	1.33E-09
GOTERM_BP_FAT	GO:0008380~RNA splicing	52	3.72	9.46E-09
GOTERM_BP_FAT	GO:0006397~mRNA processing	54	3.86	9.02E-08
GOTERM_BP_FAT	GO:0016071~mRNA metabolic process	59	4.22	1.43E-07

GOTERM_BP_FAT	GO:0000398~nuclear mRNA splicing, via spliceosome	32	2.29	5.26E-07
GOTERM_BP_FAT	GO:0000377~RNA splicing, via transesterification reactions with bulged adenosine as nucleophile	32	2.29	5.26E-07
GOTERM_BP_FAT	GO:0000375~RNA splicing, via transesterification reactions	32	2.29	5.26E-07
GOTERM_CC_FAT	GO:0005681~spliceosome	27	1.93	1.44E-05
Annotation Cluster 8				
GOTERM_BP_FAT	GO:0006457~protein folding	35	2.50	5.62E-07
GOTERM_MF_FAT	GO:0051082~unfolded protein binding	26	1.86	1.36E-06
Annotation Cluster 9				
GOTERM_BP_FAT	GO:0045333~cellular respiration	31	2.22	1.77E-11
GOTERM_BP_FAT	GO:0015980~energy derivation by oxidation of organic compounds	35	2.50	2.59E-09
GOTERM_BP_FAT	GO:0009060~aerobic respiration	15	1.07	1.20E-07
GOTERM_BP_FAT	GO:0009109~coenzyme catabolic process	13	0.93	1.45E-07
GOTERM_BP_FAT	GO:0051187~cofactor catabolic process	14	1.00	1.75E-07
GOTERM_BP_FAT	GO:0046356~acetyl-CoA catabolic process	12	0.86	3.09E-07
GOTERM_BP_FAT	GO:0006099~tricarboxylic acid cycle	12	0.86	3.09E-07
GOTERM_BP_FAT	GO:0006084~acetyl-CoA metabolic process	13	0.93	1.47E-06

GOTERM_BP_FAT	GO:0006732~coenzyme metabolic process	29	2.07	1.43E-05
GOTERM_BP_FAT	GO:0051186~cofactor metabolic process	33	2.36	3.60E-05
GOTERM_BP_FAT	GO:0043648~dicarboxylic acid metabolic process	10	0.72	8.36E-04
Annotation Cluster 10				
GOTERM_BP_FAT	GO:0022613~ribonucleoprotein complex biogenesis	42	3.00	1.97E-10
GOTERM_BP_FAT	GO:0042254~ribosome biogenesis	32	2.29	1.94E-09
GOTERM_BP_FAT	GO:0016072~rRNA metabolic process	22	1.57	1.02E-05
GOTERM_BP_FAT	GO:0006364~rRNA processing	21	1.50	1.82E-05
GOTERM_BP_FAT	GO:0034660~ncRNA metabolic process	36	2.58	7.93E-05
GOTERM_BP_FAT	GO:0034470~ncRNA processing	28	2.00	1.13E-03

Table 7.6-2 Go-terms enriched in porcine CD163- monocytes

Category	Term	Count	%	PValue
Annotation Cluster 1				
GOTERM_BP_FAT	GO:0045321~leukocyte activation	45	4.46	5.64E-13
GOTERM_BP_FAT	GO:0001775~cell activation	48	4.75	4.57E-12
GOTERM_BP_FAT	GO:0046649~lymphocyte activation	35	3.47	1.41E-09

GOTERM_BP_FAT	GO:0042110~T cell activation	27	2.67	2.05E-09
GOTERM_BP_FAT	GO:0048534~hemopoietic or lymphoid organ development	39	3.86	1.39E-08
GOTERM_BP_FAT	GO:0002520~immune system development	40	3.96	2.32E-08
GOTERM_BP_FAT	GO:0030097~hemopoiesis	36	3.56	3.58E-08
GOTERM_BP_FAT	GO:0002521~leukocyte differentiation	25	2.48	9.58E-08
GOTERM_BP_FAT	GO:0030217~T cell differentiation	15	1.49	6.46E-06
GOTERM_BP_FAT	GO:0030098~lymphocyte differentiation	19	1.88	7.62E-06

Annotation Cluster 2	Enrichment Score: 5.44368698644761			
GOTERM_MF_FAT	GO:0030528~transcription regulator activity	130	12.87	6.35E-09
GOTERM_BP_FAT	GO:0045449~regulation of transcription	195	19.31	1.67E-07
GOTERM_BP_FAT	GO:0006357~regulation of transcription from RNA polymerase II promoter	69	6.83	3.88E-06
GOTERM_BP_FAT	GO:0045941~positive regulation of transcription	57	5.64	4.97E-06
GOTERM_BP_FAT	GO:0010628~positive regulation of gene expression	58	5.74	5.89E-06
GOTERM_BP_FAT	GO:0045935~positive regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	61	6.04	6.21E-06
GOTERM_BP_FAT	GO:0051173~positive regulation of nitrogen compound metabolic process	62	6.14	8.35E-06
GOTERM_BP_FAT	GO:0006350~transcription	152	15.05	4.63E-05

GOTERM_MF_FAT	GO:0003700~transcription factor activity	78	7.72	1.51E-04
GOTERM_BP_FAT	GO:0006355~regulation of transcription, DNA-dependent	126	12.48	5.50E-04
GOTERM_MF_FAT	GO:0003677~DNA binding	155	15.35	7.40E-04
GOTERM_BP_FAT	GO:0051252~regulation of RNA metabolic process	127	12.57	8.94E-04

Annotation Cluster 3	Enrichment Score: 5.428658183492806			
GOTERM_BP_FAT	GO:0010557~positive regulation of macromolecule biosynthetic process	70	6.93	3.63E-08
GOTERM_BP_FAT	GO:0010604~positive regulation of macromolecule metabolic process	84	8.32	6.63E-08
GOTERM_BP_FAT	GO:0009891~positive regulation of biosynthetic process	72	7.13	8.26E-08
GOTERM_BP_FAT	GO:0031328~positive regulation of cellular biosynthetic process	71	7.03	9.99E-08
GOTERM_BP_FAT	GO:0006357~regulation of transcription from RNA polymerase II promoter	69	6.83	3.88E-06
GOTERM_BP_FAT	GO:0045941~positive regulation of transcription	57	5.64	4.97E-06
GOTERM_BP_FAT	GO:0010628~positive regulation of gene expression	58	5.74	5.89E-06
GOTERM_BP_FAT	GO:0045935~positive regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	61	6.04	6.21E-06
GOTERM_BP_FAT	GO:0051173~positive regulation of nitrogen compound metabolic process	62	6.14	8.35E-06
GOTERM_BP_FAT	GO:0045944~positive regulation of transcription from RNA polymerase II promoter	38	3.76	1.84E-04
GOTERM_BP_FAT	GO:0045893~positive regulation of transcription, DNA-dependent	44	4.36	5.31E-04

GOTERM_BP_FAT	GO:0051254~positive regulation of RNA metabolic process	44	4.36	6.27E-04
Annotation Cluster 4	Enrichment Score: 5.269221617369486			
GOTERM_BP_FAT	GO:0006954~inflammatory response	41	4.06	6.69E-07
GOTERM_BP_FAT	GO:0006952~defense response	62	6.14	1.94E-06
GOTERM_BP_FAT	GO:0009611~response to wounding	50	4.95	1.20E-04
Annotation Cluster 5	Enrichment Score: 4.793189227096972			
GOTERM_BP_FAT	GO:0043067~regulation of programmed cell death	81	8.02	5.60E-08
GOTERM_BP_FAT	GO:0010941~regulation of cell death	81	8.02	6.67E-08
GOTERM_BP_FAT	GO:0042981~regulation of apoptosis	80	7.92	7.75E-08
GOTERM_BP_FAT	GO:0043065~positive regulation of apoptosis	49	4.85	9.58E-07
GOTERM_BP_FAT	GO:0043068~positive regulation of programmed cell death	49	4.85	1.16E-06
GOTERM_BP_FAT	GO:0010942~positive regulation of cell death	49	4.85	1.33E-06
GOTERM_BP_FAT	GO:0006917~induction of apoptosis	36	3.56	4.37E-05
GOTERM_BP_FAT	GO:0012502~induction of programmed cell death	36	3.56	4.64E-05
GOTERM_BP_FAT	GO:0043069~negative regulation of programmed cell death	34	3.37	1.65E-03
GOTERM_BP_FAT	GO:0060548~negative regulation of cell death	34	3.37	1.72E-03
GOTERM_BP_FAT	GO:0043066~negative regulation of	33	3.27	2.50E-

	apoptosis			03
GOTERM_BP_FAT	GO:0006916~anti-apoptosis	18	1.78	4.94E-02
Annotation Cluster 6	Enrichment Score: 4.362997933144974			
GOTERM_MF_FAT	GO:0016564~transcription repressor activity	38	3.76	3.43E-06
GOTERM_BP_FAT	GO:0031327~negative regulation of cellular biosynthetic process	56	5.54	8.95E-06
GOTERM_BP_FAT	GO:0009890~negative regulation of biosynthetic process	56	5.54	1.62E-05
GOTERM_BP_FAT	GO:0051172~negative regulation of nitrogen compound metabolic process	52	5.15	1.76E-05
GOTERM_BP_FAT	GO:0010558~negative regulation of macromolecule biosynthetic process	54	5.35	1.79E-05
GOTERM_BP_FAT	GO:0045934~negative regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	51	5.05	2.53E-05
GOTERM_BP_FAT	GO:0016481~negative regulation of transcription	47	4.65	2.81E-05
GOTERM_BP_FAT	GO:0010605~negative regulation of macromolecule metabolic process	66	6.53	3.53E-05
GOTERM_BP_FAT	GO:0051253~negative regulation of RNA metabolic process	38	3.76	1.12E-04
GOTERM_BP_FAT	GO:0010629~negative regulation of gene expression	48	4.75	1.36E-04
GOTERM_BP_FAT	GO:0045892~negative regulation of transcription, DNA-dependent	37	3.66	1.71E-04
GOTERM_BP_FAT	GO:0000122~negative regulation of transcription from RNA polymerase II promoter	26	2.57	4.31E-03
Annotation Cluster 7	Enrichment Score: 4.068026061967488			

GOTERM_CC_FAT	GO:0044459~plasma membrane part	147	14.55	1.21E-05
GOTERM_CC_FAT	GO:0005887~integral to plasma membrane	85	8.42	1.57E-04
GOTERM_CC_FAT	GO:0031226~intrinsic to plasma membrane	85	8.42	3.27E-04

Annotation Cluster 8	Enrichment Score: 3.9848846149456407			
GOTERM_BP_FAT	GO:0050778~positive regulation of immune response	23	2.28	8.52E-06
GOTERM_BP_FAT	GO:0048584~positive regulation of response to stimulus	31	3.07	8.75E-06
GOTERM_BP_FAT	GO:0002429~immune response-activating cell surface receptor signaling pathway	11	1.09	2.73E-05
GOTERM_BP_FAT	GO:0002768~immune response-regulating cell surface receptor signaling pathway	11	1.09	5.46E-05
GOTERM_BP_FAT	GO:0002757~immune response-activating signal transduction	12	1.19	7.51E-05
GOTERM_BP_FAT	GO:0002253~activation of immune response	16	1.58	1.27E-04
GOTERM_BP_FAT	GO:0002764~immune response-regulating signal transduction	12	1.19	1.52E-04
GOTERM_BP_FAT	GO:0050851~antigen receptor-mediated signaling pathway	9	0.89	2.67E-04
GOTERM_BP_FAT	GO:0050852~T cell receptor signaling pathway	5	0.50	3.17E-02

Annotation Cluster 9	Enrichment Score: 3.3241600390928516			
GOTERM_BP_FAT	GO:0002684~positive regulation of immune system process	40	3.96	3.09E-10
GOTERM_BP_FAT	GO:0050865~regulation of cell activation	29	2.87	1.68E-07

GOTERM_BP_FAT	GO:0002694~regulation of leukocyte activation	27	2.67	7.08E-07
GOTERM_BP_FAT	GO:0050863~regulation of T cell activation	21	2.08	3.43E-06
GOTERM_BP_FAT	GO:0051249~regulation of lymphocyte activation	24	2.38	3.53E-06
GOTERM_BP_FAT	GO:0050867~positive regulation of cell activation	20	1.98	5.87E-06
GOTERM_BP_FAT	GO:0002696~positive regulation of leukocyte activation	18	1.78	4.30E-05
GOTERM_BP_FAT	GO:0046634~regulation of alpha-beta T cell activation	10	0.99	8.56E-05
GOTERM_BP_FAT	GO:0050870~positive regulation of T cell activation	14	1.39	1.73E-04
GOTERM_BP_FAT	GO:0051251~positive regulation of lymphocyte activation	16	1.58	1.82E-04
GOTERM_BP_FAT	GO:0046637~regulation of alpha-beta T cell differentiation	7	0.69	1.67E-03
GOTERM_BP_FAT	GO:0046635~positive regulation of alpha-beta T cell activation	7	0.69	2.08E-03
GOTERM_BP_FAT	GO:0046638~positive regulation of alpha-beta T cell differentiation	6	0.59	3.39E-03
GOTERM_BP_FAT	GO:0050670~regulation of lymphocyte proliferation	12	1.19	4.48E-03
GOTERM_BP_FAT	GO:0032944~regulation of mononuclear cell proliferation	12	1.19	4.91E-03
GOTERM_BP_FAT	GO:0070663~regulation of leukocyte proliferation	12	1.19	4.91E-03
GOTERM_BP_FAT	GO:0042129~regulation of T cell proliferation	10	0.99	5.35E-03
GOTERM_BP_FAT	GO:0045582~positive regulation of T cell differentiation	7	0.69	6.23E-03
GOTERM_BP_FAT	GO:0045621~positive regulation of	7	0.69	9.75E-

	lymphocyte differentiation			03
GOTERM_BP_FAT	GO:0042102~positive regulation of T cell proliferation	7	0.69	1.64E-02
GOTERM_BP_FAT	GO:0045580~regulation of T cell differentiation	8	0.79	1.79E-02
GOTERM_BP_FAT	GO:0045619~regulation of lymphocyte differentiation	9	0.89	1.83E-02
GOTERM_BP_FAT	GO:0050671~positive regulation of lymphocyte proliferation	8	0.79	2.62E-02
GOTERM_BP_FAT	GO:0032946~positive regulation of mononuclear cell proliferation	8	0.79	2.86E-02
GOTERM_BP_FAT	GO:0070665~positive regulation of leukocyte proliferation	8	0.79	2.86E-02
Annotation Cluster 10	Enrichment Score: 3.3236251917740094			
GOTERM_MF_FAT	GO:0046983~protein dimerization activity	58	5.74	3.13E-07
GOTERM_MF_FAT	GO:0042803~protein homodimerization activity	29	2.87	9.19E-03
GOTERM_MF_FAT	GO:0042802~identical protein binding	45	4.46	3.72E-02

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