Characterisation of cytokines involved in porcine hematopoiesis

Garth Stephenson
BSc Biotechnology (Hons)

Submitted in total fulfilment of the requirements of the degree of
Doctor of Philosophy

Department of Biochemistry and Molecular Biology
The University of Melbourne
ABSTRACT

The prophylactic of use in-feed antibiotics and chemical based medicines such as anthelmintic are commonly used in order to maintain health and promote growth in food production animals. The inclusion of low regular or sub-therapeutic doses of antimicrobials in animal feed increases their efficiency to digest food by suppressing sensitive gut flora and reducing infection caused by opportunistic pathogenic organisms which could be detrimental to both the animal’s health and productivity.

This practice has been linked to the emergence of drug resistant organisms, many of which are resistant to antibiotics commonly used in human medicine, posing a threat to both animals and humans (Hu). To reduce this threat, an alternative method of therapeutic treatment in food production animals must be developed. One suggestion is to enhance the animal’s own immune system.

Hematopoiesis is the process of developing mature WBC from hematopoietic stem cells (HSC) within the bone marrow (BM). Therefore an understanding of hematopoiesis in pigs may uncover new therapeutic treatments. Cytokines are signalling molecules released by stimulated white blood cells (WBC), which activate essential cells and pathways essential in an innate or adaptive immune response to infection. These proteins initiate proliferation, differentiation and maturation of immature immune cells and precursors within the BM. Cytokines may therefore provide a viable alternative to current therapeutic practices.

Although, many cytokines have been identified in Hu and mice (Mo), few have been studied in pigs. The cytokines of the common β subunit (CD131), interleukin-3 (IL-3), interleukin-5 (IL-5) and granulocyte, macrophage colony stimulating factor (GM-CSF) may prove to be valuable therapeutics. This unique group of cytokines not only share the same receptor subunit, but are involved in the production of several cells types. With this in mind, these cytokines, along with CD131, were analysed in silico, in vitro and in vivo.

In silico phylogenetic analysis confirmed these cytokines in pigs developed similarly to other mammals. Molecular modelling suggests that structural changes due to altered genetic sequences, along with differences in CD131 binding residues, may result in a lack of cross species activity. The recombinant (rec) form of each porcine (Po) cytokine was produced and biological activity confirmed. In vitro analysis for rPoGM-CSF and rPoIL-3 showed maturation of immature CD90+ and CD172a+ BM cells.
resulting in monocyte and macrophage development. Moreover, rPoIL-5 induced proliferation and maturation for eosinophils.

To assess the activity of each cytokine in vivo, pigs were administered the chemotherapeutic drug, 5-flurouricil (5-FU) to produce a state of myelosuppression. The in vivo activity was examined on BM and peripheral white blood cells (WBC). rPoGM-CSF induced proliferation of CD90+ and CD172a+ cells within the BM resulting in increased platelets whilst rPoIL-3 was also shown to significantly increase basophil levels. More importantly, all 3 cytokines increased levels of eosinophils at different stages throughout the trial, with rPoIL-5 inducing the highest eosinophil production. These results suggest that these Po cytokines may have potential as therapeutics to enhance immunity in pigs, specifically in parasite infections.

This report has analysed the activity of rPoIL-3, rPoIL-5 and rPoGM-CSF. In doing so, we have identified some of the roles of these cytokines in hematopoiesis. Furthermore, we have demonstrated the involvement of all three cytokines in the regulation of eosinophils and confirmed the role of PoIL-5 as the major eosinophilopoietin in the production of eosinophils in pigs.
DECLARATION

This is to certify that:

(i) This thesis comprises original work except where indicated;

(ii) Due acknowledgement has been made in the text to all other material used;

(iii) This thesis is less than 100 000 words in length, exclusive of figures, tables, bibliographies and appendices.

Garth Stephenson
ACKNOWLEDGEMENTS

The work in this thesis was completed on a part time basis at the CSIRO Australian Animal Health Laboratory (AAHL) Geelong, Australia in collaboration with the Melbourne University department of Medicine and Dentistry, Biochemistry.

I would like to begin by thanking my supervisors Andrew Bean and David Strom. Their continued assistance and knowledge along with their guidance and drive have made the completion of this research possible. I would especially like to thank John Lowenthal for the continued support of this work and his understanding of my demanding home life situation.

I would especially like to thank the many researchers and professionals within the CSIRO network who assisted me in my research and provided me with the training and techniques necessary to complete this work. Many thanks to all the people on level 6 for all your help and guidance. Of course there are many other people involved in such an organisation as CSIRO AAHL whom should never be taken for granted. I would like to give a special thank you to the staff of the Small Animal and Werribee Animal Facilities. My many thanks go out to the staff from administration to dispatch, from engineering to glass washing and from IT to the library. It is your ongoing contribution to the everyday running of AAHL which makes life so much easier for those of us involved in research.

I would like to thank my wife Natalie, whose presence in my life has reminded me why I began this journey and has rekindled my drive to complete this work.

Lastly I would like to thank my son Matthew. Although he does not understand the reasons for me undertaking such a task, it is his everyday presence in my life which has ultimately driven me to change the course of my life which has resulted in me completing this body of work. Thanks Matty.

So to all I say, “Thank you!”
Publications

A portion of this work has been previously published or presented at conferences as follows:


Conference presentations


### List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>aa</td>
<td>amino acid(s)</td>
</tr>
<tr>
<td>Amp&lt;sup&gt;R&lt;/sup&gt;</td>
<td>ampicillin resistance</td>
</tr>
<tr>
<td>APC</td>
<td>Allophycocyanin</td>
</tr>
<tr>
<td>BAF</td>
<td>B-cell activating factor</td>
</tr>
<tr>
<td>β&lt;sub&gt;c&lt;/sub&gt;</td>
<td>common β subunit</td>
</tr>
<tr>
<td>bp</td>
<td>base pair(s)</td>
</tr>
<tr>
<td>BM</td>
<td>bone marrow cells</td>
</tr>
<tr>
<td>BMHC</td>
<td>BM hematopoietic cells</td>
</tr>
<tr>
<td>BMHSC</td>
<td>bone marrow hematopoietic stem cells</td>
</tr>
<tr>
<td>BMM</td>
<td>bone marrow microenvironment</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>BrdU</td>
<td>5-bromo-2-deoxyuridine</td>
</tr>
<tr>
<td>BTY</td>
<td>brevibactillus trypone yeast</td>
</tr>
<tr>
<td>cDNA</td>
<td>DNA complementary to mRNA</td>
</tr>
<tr>
<td>CFU</td>
<td>colony forming units</td>
</tr>
<tr>
<td>CFU-Bas/Eo</td>
<td>CFU-basophil/eosinophil</td>
</tr>
<tr>
<td>CFU-G/M</td>
<td>CFU-granulocyte/monocyte</td>
</tr>
<tr>
<td>CFU-GEMM</td>
<td>CFU-granulocytes, erythrocytes, monocytes and megakaryocytes</td>
</tr>
<tr>
<td>CFU-ME</td>
<td>CFU-megakaryocyte/erythrocyte</td>
</tr>
<tr>
<td>CFU-mix</td>
<td>colony forming units mix</td>
</tr>
<tr>
<td>CHR</td>
<td>cytokine binding homology region</td>
</tr>
<tr>
<td>CLP</td>
<td>common lymphoid progenitor</td>
</tr>
<tr>
<td>CMP</td>
<td>common myeloid progenitor</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>CRM</td>
<td>cytokine receptor modules</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>CSF</td>
<td>Colony stimulating factors</td>
</tr>
<tr>
<td>DC</td>
<td>dendritic cell</td>
</tr>
<tr>
<td>dH₂O</td>
<td>distilled water</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's modified Eagle’s medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl-sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>ECL</td>
<td>enhanced chemiluminescence</td>
</tr>
<tr>
<td>E. coli</td>
<td><em>Escherichia coli</em></td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme linked immunosorbent assay</td>
</tr>
<tr>
<td>EPO</td>
<td>erythropoietin</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular signal-regulated protein kinases</td>
</tr>
<tr>
<td>EpoR-Lig-Bind</td>
<td>EPO Receptor Ligand Binding Domain</td>
</tr>
<tr>
<td>EU</td>
<td>European Union</td>
</tr>
<tr>
<td>FACS</td>
<td>fluorescence activated cell sorting</td>
</tr>
<tr>
<td>FCS</td>
<td>foetal calf serum</td>
</tr>
<tr>
<td>FDC</td>
<td>follicular DC</td>
</tr>
<tr>
<td>FL</td>
<td>flt3 ligand</td>
</tr>
<tr>
<td>FNIII</td>
<td>fibronectin type III</td>
</tr>
<tr>
<td>FSC</td>
<td>forward scatter channel</td>
</tr>
<tr>
<td>G-CSF</td>
<td>granulocyte colony stimulating factor</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>granulocyte macrophage colony stimulating factor</td>
</tr>
<tr>
<td>GPI</td>
<td>glycosylphosphatidylinositol</td>
</tr>
<tr>
<td>h</td>
<td>hour(s)</td>
</tr>
<tr>
<td>HAT</td>
<td>hypoxanthine-aminopterin-thymidine</td>
</tr>
<tr>
<td>hi</td>
<td>high</td>
</tr>
<tr>
<td>HRP</td>
<td>horse radish peroxidase</td>
</tr>
<tr>
<td>HSC</td>
<td>hematopoietic stem cells</td>
</tr>
<tr>
<td>HSS</td>
<td>hematopoietic supportive stroma</td>
</tr>
</tbody>
</table>
HT  hypoxanthine-thymidine
Hu  human
IFN  interferon
IgSF  immunoglobulin supergene family
IL  interleukin
IMAC  immobilised affinity chromatography
i.m.  intramuscularly
IPTG  isopropyl-β-D-thiogalactopyranoside
ITIM  immunoreceptor tyrosine-based inhibitory motifs
JAK  Janus kinase
JNK  c-Jun NH2-terminal kinases
kb  kilobase(s)
KL  c-kit ligand
LB broth  Luria Bertani broth
LMP  low melting point
lo  low
LPS  lipopolysaccharide
LTC4  leukotriene C4
LTCIC  long-term culture initiating cells
LTRC  long-termed repopulating HSC
MBTYNm  modified BTY neomycin
M-CSF  macrophage-CSF
MSC  mesenchymal stem cells
mAb  monoclonal antibody
MAP  mitogen-activated protein
MAPK  MAP kinases
MGDF  megakaryocyte growth and development factor
MHC  major histocompatibility complex
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>MHCI</td>
<td>major histocompatibility complex class I</td>
</tr>
<tr>
<td>MHCII</td>
<td>major histocompatibility complex class II</td>
</tr>
<tr>
<td>MIF</td>
<td>macrophage inhibitory factor</td>
</tr>
<tr>
<td>min</td>
<td>minute(s)</td>
</tr>
<tr>
<td>MRSA</td>
<td>Methicillin-resistant Staphylococcus aureus</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>Mo</td>
<td>mouse</td>
</tr>
<tr>
<td>MW</td>
<td>molecular weight</td>
</tr>
<tr>
<td>NiNTA</td>
<td>nickel nitrilotriacetate</td>
</tr>
<tr>
<td>NK</td>
<td>natural killer cell</td>
</tr>
<tr>
<td>NKP</td>
<td>natural killer cell progenitor</td>
</tr>
<tr>
<td>nt</td>
<td>nucleotide(s)</td>
</tr>
<tr>
<td>N-terminus</td>
<td>amino terminus</td>
</tr>
<tr>
<td>OD</td>
<td>optical density</td>
</tr>
<tr>
<td>O/N</td>
<td>overnight</td>
</tr>
<tr>
<td>pAb</td>
<td>polyclonal antibodies</td>
</tr>
<tr>
<td>PBL</td>
<td>peripheral blood leukocytes(s)</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PEG</td>
<td>polyethylene glycol</td>
</tr>
<tr>
<td>PGH</td>
<td>PEG/glycerol/HEPES</td>
</tr>
<tr>
<td>PHSC</td>
<td>primitive HSC</td>
</tr>
<tr>
<td>PI3K</td>
<td>phosphatidylinositol-3-kinase</td>
</tr>
<tr>
<td>Po</td>
<td>Porcine</td>
</tr>
<tr>
<td>PoBM</td>
<td>Porcine bone marrow</td>
</tr>
<tr>
<td>Rec</td>
<td>recombinant</td>
</tr>
<tr>
<td>RF</td>
<td>relative fold</td>
</tr>
<tr>
<td>RFC</td>
<td>relative fold change</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>RFD</td>
<td>relative fold decrease</td>
</tr>
<tr>
<td>RFI</td>
<td>relative fold increase</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>rPo</td>
<td>recombinant porcine</td>
</tr>
<tr>
<td>RT</td>
<td>room temperature</td>
</tr>
<tr>
<td>s.c.</td>
<td>subcutaneously</td>
</tr>
<tr>
<td>SCF</td>
<td>stem cell factor</td>
</tr>
<tr>
<td>SCOP</td>
<td>Structural Classification of Proteins</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulphate-polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SE</td>
<td>standard error</td>
</tr>
<tr>
<td>SF</td>
<td>steel factor</td>
</tr>
<tr>
<td>SH2</td>
<td>src homology</td>
</tr>
<tr>
<td>SHP-1</td>
<td>SH2 domain-containing tyrosine phophatase 1</td>
</tr>
<tr>
<td>SLA</td>
<td>swine leukocyte antigen</td>
</tr>
<tr>
<td>SMART</td>
<td>Simple Modular Architecture Research Tool</td>
</tr>
<tr>
<td>sp</td>
<td>signal peptide</td>
</tr>
<tr>
<td>SCC</td>
<td>side scatter channel</td>
</tr>
<tr>
<td>STAT</td>
<td>signal transducer and activator of transcription</td>
</tr>
<tr>
<td>TSA</td>
<td>trypticase soy agar</td>
</tr>
<tr>
<td>TSB</td>
<td>trypticase soy broth</td>
</tr>
<tr>
<td>TPO</td>
<td>thrombopoietin</td>
</tr>
<tr>
<td>WBC</td>
<td>white blood cells</td>
</tr>
<tr>
<td>WSXWS</td>
<td>Trp-Ser-Xaa-Trp-Ser</td>
</tr>
<tr>
<td>U</td>
<td>unit(s)</td>
</tr>
<tr>
<td>2ME</td>
<td>2-Mercaptopoethanol</td>
</tr>
<tr>
<td>6x His</td>
<td>6x Histidine tag</td>
</tr>
<tr>
<td>5-FU</td>
<td>5-fluorouracil</td>
</tr>
</tbody>
</table>
TABLE OF CONTENTS

ABSTRACT ............................................................................................................................... iii

ACKNOWLEDGEMENTS .......................................................................................................... vi

Publications .............................................................................................................................. vii

Conference presentations ........................................................................................................ vii

LIST OF ABBREVIATIONS .................................................................................................. IX

LIST OF FIGURES .................................................................................................................. XX

LIST OF TABLES .................................................................................................................... XXIII

CHAPTER 1 ............................................................................................................................... 1

Introduction ............................................................................................................................... 1

1.1 Cytokines and the immune system ................................................................. 1

1.2 The mammalian immune system ..................................................................... 3

  1.2.1 BM ontogenesis ................................................................................................. 3

  1.2.2 BM microenvironment (BMM) ......................................................................... 4

  1.2.3 HSC ...................................................................................................................... 5

  1.2.4 Hematopoiesis .................................................................................................... 5

  1.2.5 Cells of the immune system .............................................................................. 6

    1.2.5.1 Lymphocytes ................................................................................................ 7

    1.2.5.2 Myelocytes .................................................................................................. 8

1.3 Models for studying BM development .......................................................... 9

  1.3.1 Chemotherapeutic ablation and myelosuppression ......................................... 9

1.4 BM cell surface markers .................................................................................... 10

  1.4.1 CD90 .................................................................................................................. 10

  1.4.2 CD172a ............................................................................................................. 12

1.5 Cytokine receptors ............................................................................................. 14

  1.5.1 Type I cytokine receptors ................................................................................. 14

  1.5.2 CD131- the Common β Subunit .................................................................... 16

1.6 The cytokines associated with the CD131 receptor .................................... 18
1.6.1 IL-3 and the IL-3α subunit, CD123 ................................................. 18
1.6.2 IL-5 and the IL-5α subunit, CD125 ............................................... 21
1.6.3 GM-CSF and the GM-CSFα subunit, CD116 ................................. 22
1.7 Therapeutic use of hematopoietic cytokines ..................................... 24
1.8 Objectives ......................................................................................... 26

CHAPTER 2 ............................................................................................... 28
Characterization of GM-CSF, IL-3, and IL-5 CD131 in pigs ...................... 28
2.1 Abstract ............................................................................................. 28
2.2 Introduction ......................................................................................... 29
2.3 Methods ............................................................................................. 30
  2.3.1 Sequence alignments ................................................................. 30
  2.3.2 Phylogenetics ............................................................................. 30
  2.3.3 Predicted molecular structures of proteins ................................. 30
  2.3.4 Chromosomal gene locations ................................................... 30
  2.3.5 Common protein structure domains ........................................... 31
2.4 Results ............................................................................................... 31
  2.4.1 Sequence analysis of the hematopoietic cytokines ....................... 31
  2.4.2 GM-CSF is highly conserved across the mammalian species ....... 31
  2.4.3 Molecular structure of GM-CSF shows conserved conformation compared with HuGM-CSF .......................................................... 32
  2.4.4 Pig IL-3 shows low sequence homology to other species ............ 33
  2.4.5 Molecular structure of PoIL-3 suggests altered conformation compared to HuIL-3 ................................................................. 34
  2.4.6 PoIL-5 shows high sequence homology and conserved residues across mammalian species ................................................................. 34
  2.4.7 The predicted molecular structure of PoIL-5 shows similar conformation to HuIL-5 ................................................................. 36
  2.4.8 Identification of a predicted gene sequence for the pig CD131 subunit ................................................................. 36
2.4.9 Analysis of PoCD131 subunit shows sequence identity and conserved residues across all mammalian species ........................................37
2.4.9.1 Comparative predicted domain organization of PoCD131 and other mammalian species ..........................................................37
2.4.9.2 Phylogenetic analysis of the PoCD131 subunit shows three distinct groups between mammalian species ........................................38
2.4.9.3 Molecular structure of the CD131 subunit shows similar conformation to the Hu form ..............................................................38
2.5 Discussion ....................................................................................38
2.6 Conclusion ....................................................................................43

CHAPTER 3 ........................................................................................44

In vitro characterisation of pig GM-CSF, IL-3 and IL-5 ................................44

3.1 Abstract ........................................................................................44
3.2 Introduction ....................................................................................44
3.3 Materials and Methods .................................................................46
3.3.1 Cell collection techniques ..............................................................46
3.3.1.1 Animals ..................................................................................46
3.3.1.2 Extraction of BM from pigs .........................................................46
3.3.1.3 Freezing of BM .......................................................................46
3.3.1.4 Thawing BM cells .....................................................................47
3.3.1.5 Separation of peripheral blood lymphocytes (PBLC) from whole pig blood .. 47
3.3.2 Gene cloning ...............................................................................47
3.3.2.1 RNA isolation ...........................................................................47
3.3.2.2 cDNA synthesis .......................................................................47
3.3.2.3 Oligonucleotide synthesis and sequences ......................................48
3.3.2.4 Polymerase chain reaction .........................................................48
3.3.2.5 Agarose Gel Electrophoresis ......................................................48
3.3.3 Plasmid construction .................................................................49
3.3.3.1 Plasmid vectors ........................................................................49
3.3.3.2 Restriction endonuclease digestion of DNA ..................................49
3.3.3.3 Ligation of PCR products .......................................................... 49
3.3.3.4 Bacterial strains and techniques .................................................. 49
3.3.3.5 Media ....................................................................................... 49
3.3.3.6 Preparation of electro-competent *Escherichia coli* (*E. coli*) cells .......... 50
3.3.3.7 Preparation of electrocompetent *B. choshinensis* cells ......................... 50

3.3.4 **Transformation, isolation and sequencing** ................................. 50
3.3.4.1 *E. coli* transformation .................................................................. 50
3.3.4.2 *B. choshinensis* transformation .................................................... 51
3.3.4.3 DNA Extraction and concentrations ............................................ 51
3.3.4.4 Sequencing of plasmid DNA ......................................................... 51

3.3.5 **Recombinant protein procedures** ............................................... 52
3.3.5.1 Rapid screen expression of recombinant proteins .......................... 52
3.3.5.2 Expression of pQE30 expressed rPoIL-5 ..................................... 52
3.3.5.3 Solubilisation of pQE30 expressed rPoIL-5 ................................. 52
3.3.5.4 Purification of pQE30 expressed rPoIL-5 ...................................... 53
3.3.5.5 Expression of rPoIL-3 in *E. coli* ............................................... 53
3.3.5.6 Solubilisation of pQE30 expressed rPoIL-3 ................................. 53
3.3.5.7 Purification of rPoIL-3 ............................................................... 53
3.3.5.8 *B. choshinensis* expression of rPoGM-CSF protein ..................... 54
3.3.5.9 Purification of rPoGM-CSF protein ........................................... 54
3.3.5.10 SDS PAGE ............................................................................... 54
3.3.5.11 Western blot analysis of protein ................................................. 55
3.3.5.12 Detoxification and filtering of purified proteins ............................ 55

3.3.6 **Cell culture and bioassays** ....................................................... 56
3.3.6.1 Thawing of B-cell activating factor (BAF) cells ............................ 56
3.3.6.2 rPoIL-5 bioassay using BAF Mo cell line .................................... 56
3.3.6.3 Assay for the detection of GM-CSF .............................................. 57
3.3.6.4 Po BM proliferation assay ........................................................ 58

3.3.7 **Flow cytometry and cell analysis** ............................................. 58
3.3.7.1 Antibody staining and flow cytometry ......................................... 58
3.3.7.2 Characterisation of CD90+ and CD172a+ PoBM .......................... 59
3.3.7.3 BrdU ............................................................................................................ 59
3.3.8 Cell Differential Staining................................................................................. 59
3.3.9 Statistical analysis........................................................................................... 60
3.4 Results.................................................................................................................. 60
3.4.1 Cloning and expression of rec Po cytokines in a prokaryotic system produces biologically active proteins.................................................................60
3.4.2 BM proliferation assay with recombinant cytokines produces significant differences between pig and Hu .................................................................................61
  3.4.2.1 GM-CSF assay ............................................................................................... 61
  3.4.2.2 IL-3 assay .................................................................................................... 61
  3.4.2.3 IL-5 assay .................................................................................................... 61
3.4.3 FACS characterisation of CD90^+ and CD172a^+ BM. .................................62
3.4.4 FACS analysis of recombinant cytokine treated BM cells *in vitro* indicates cell differentiation and proliferation ..........................................................62
  3.4.4.1 BrdU/CD90 .................................................................................................. 63
  3.4.4.2 BrdU/CD72a ............................................................................................... 63
3.4.5 Morphology of cytokine treated cells..............................................................63
3.5 Discussion.............................................................................................................. 64
  3.5.1 Characterization of CD90 and CD172a BM ..................................................... 66
3.6 Conclusion............................................................................................................. 71
CHAPTER 4.................................................................................................................. 72
*In vivo* analysis of Po GM-CSF, IL-3 and IL-5 ......................................................... 72
4.1 Abstract ................................................................................................................ 72
4.2 Introduction.......................................................................................................... 72
4.3 Methods............................................................................................................... 74
  4.3.1 Animal Ethics.................................................................................................. 74
  4.3.2 Production of Po specific cytokines ................................................................. 74
  4.3.3 Myelosuppression ......................................................................................... 74
  4.3.4 Hematological analysis .................................................................................. 75
LIST OF FIGURES

CHAPTER 1

Figure 1.1 Mammalian hematopoiesis.
Figure 1.2 Activation of type 1 cytokine receptor.
Figure 1.3 Schematic of CD131 intracellular pathways.

CHAPTER 2

Figure 2.1 Nucleotide alignment of PoGM-CSF, HuGM-CSF and Mo GM-CSF.
Figure 2.2 Loci location of Po GM-CSF, PoIL-3 and PoIL-5 on Ch 2.
Figure 2.3 Multiple alignment of Po GM-CSF with GM-CSF of various species.
Figure 2.4 Unrooted phylogenetic tree of known GM-CSF.
Figure 2.5 Predicted molecular structure of Po GM-CSF.
Figure 2.6 Predicted surface structure of PoGM-CSF.
Figure 2.7 Nucleotide alignment of IL-3 derived from large white landrace cross pigs and CHEF1.
Figure 2.8 Nucleotide alignment of PoIL-3, HuIL-3 and MoIL-3.
Figure 2.9 Multiple alignment of PoIL-3 with IL-3 of various mammalian species.
Figure 2.10 Unrooted phylogenetic tree of known mammalian IL-3.
Figure 2.11 Predicted molecular structure of PoIL-3.
Figure 2.12 Predicted surface structure of PoIL-3.
Figure 2.13 Nucleotide alignment of PoIL-5, HuIL-5 and MoIL-5.
Figure 2.14 Multiple alignment of PoIL-5 with IL-5 of various mammalian species.
Figure 2.15 Unrooted phylogenetic tree of known IL-5.
Figure 2.16 Predicted molecular structure of PoIL-5 monomer.
Figure 2.17 Predicted surface structure of PoIL-5.
Figure 2.18 Predicted molecular structure of the PoIL-5 homodimer.
Figure 2.19 Location of Po.βc receptor subunit CD131 on Ch 5.
Figure 2.20 Nucleotide alignment of the Po CD131 subunit against the Hu and Mo CD131 sequences.
Figure 2.21  Multiple alignment of Po CD131 with the CD131 subunit of various mammalian species.

Figure 2.22  Comparative predicted domain organisation of the Po CD131 subunit and selected CD131 subunits from other mammalian species.

Figure 2.23  Unrooted phylogenetic tree of known CD131 subunits.

Figure 2.24  Predicted molecular structure of extracellular region of monomer 1 of CD131 subunit.

Figure 2.25  Predicted surface structure of extracellular region of monomer 1 of CD131 subunit.

CHAPTER 3

Figure 3.1  Agarose gels of PCR of Po cytokines.

Figure 3.2  SDS PAGE gels of rPo cytokines.

Figure 3.3  Western blot of rPo cytokines.

Figure 3.4  rPoGM-CSF shows biologically activity in Hu TF1 cell assay.

Figure 3.5  rPoIL-3 shows biological activity in PoBM proliferation assay.

Figure 3.6  rPoIL-5 shows biological activity in IL-5 dependent BAF Mo cell bioassay.

Figure 3.7  rPoGM-CSF produces PoBM proliferation in a dose dependant manner.

Figure 3.8  rPoIL-3 produces PoBM proliferation in a dose dependant manner.

Figure 3.9  rPoIL-5 stimulates PoBM proliferation in a dose dependant manner.

Figure 3.10  Characterization of CD90+ and CD172a+ PoBM cells.

Figure 3.11  PoBM treated with rPoIL-3 or rPoGM-CSF induces differentiation of CD90+ and CD172a+ cells.

Figure 3.12  PoBM treated with rPoIL-3 or rPoGM-CSF induces proliferation of CD90+ and CD172a+ cells.

Figure 3.13  PoBM cell morphology following cytokine treatment.

Figure 3.14  PoBM cell types following cytokine treatment.
CHAPTER 4

Figure 4.1  WBC count for myelosuppressed pigs treated with rPo cytokines.
Figure 4.2  Lymphocyte difference for myelosuppressed pigs treated with rPo cytokines.
Figure 4.3  Monocyte differential count for myelosuppressed pigs treated with rPo cytokines.
Figure 4.4  Neutrophil differential count for myelosuppressed pigs treated with rPo cytokines.
Figure 4.5  Eosinophil differential count for myelosuppressed pigs treated with rPo cytokines.
Figure 4.6  Basophil differential count for myelosuppressed pigs treated with rPo cytokines.
Figure 4.7  Platelet count for myelosuppressed pigs treated with rPo cytokines.
Figure 4.8  RBC count for myelosuppressed pigs treated with rPo cytokines.
Figure 4.9  In vivo response of CD90^+ PoBM cells to rec cytokine treatment.
Figure 4.10 In vivo response of CD172a^+ PoBM cells to rec cytokine treatment.
Figure 4.11 In vivo response of CD172a^{hi} PoBM cells to rec cytokine treatment.
Figure 4.12 In vivo response of CD172a^{lo} PoBM cells to rec cytokine treatment.

CHAPTER 5

Figure 5.1  Mammalian hematopoiesis showing suggested pig CD markers.
Figure 5.2  Mammalian hematopoiesis showing suggested pig cytokine interactions.
List of Tables

CHAPTER 2
Table 2.1  Intron and exon boundaries of PoIL-3, PoGM-CSF and PoIL-5 on chromosome 2.
Table 2.2  Comparison of PoGM-CSF aa sequence against GM-CSF of other species.
Table 2.3  Comparison of PoIL-3 aa sequence against IL-3 of other mammalian species.
Table 2.4  Comparison of PoIL-5 aa sequence against IL-5 of other mammalian species.
Table 2.5  Intron and exon boundaries of the PoCD131 subunit on chromosome 5.
Table 2.6  Comparison of PoCD131 subunit aa sequence against the CD131 subunit of other species.

CHAPTER 3
Table 3.1  DNA primer sequences for rPo cytokines.

CHAPTER 4
Table 4.1  Po cytokine activity animal trial time table.
CHAPTER 1

Introduction

1.1 Cytokines and the immune system

Intensive livestock systems, such as swine and poultry, often involve crowded and sometimes unsanitary conditions where animal health may be repeatedly challenged (Moser, et al. 1985, Foreyt 1990, Donham 2010). Each year, significant economic loss is experienced worldwide due to health related problems in young farm animals. Wearing animals are more susceptible to disease due to an immature immune system (Juul-Madsen, et al. 2009, Kyriakis 1989, Madec, et al. 1998), resulting in reduced growth and an increase in morbidity and mortality. In order to fight disease and to enhance animal growth and weight gain under these conditions, some farmers have adopted the practice of prophylactic or the non-therapeutic use of antibiotics (Walsh and Fanning 2008, Hamer and Gill 2002). Animals are routinely fed low doses of antibiotics in feed and water to maintain health (Walsh and Fanning 2008). Estimates in the USA in 2001 indicated that as high as 70% of all antibiotics sold were used in the food animal market (Mellon, et al. 2001, Mellon and Benbrook 2001). This equates to 11,158 tonnes of antimicrobials of which approximately 4,575 tonnes were consumed by swine (Mellon, et al. 2001, Mellon and Benbrook 2001). Unfortunately these quantities have failed to reduce over time. The latest figures obtained show that in 2011, the total mass of antimicrobials used in food-producing animals had increased to 13,743 tonnes and the quantity consumed by hogs had risen to 5,634 tonnes (Food and Drug Administration 2011). This represents an increase of 1059 tonnes of antimicrobials used within the pig industry within the USA alone. The repeated low doses of antibiotics coupled with natural selection acting upon random mutation, enable bacterial populations to adapt and develop antibiotic resistant strains (Walsh and Fanning 2008). The development of resistant bacteria associated with these animals consequently leads to increased risk of cross species contamination via the food chain and environmental contact, therefore, increasing the risk of infection to Hu (Walsh and Fanning 2008, Teuber 1999, White, et al. 2002). It has been suggested that as a consequence of this practice, several antibiotic resistant bacteria infections in Hu have been traced back to animal stocks and the non-discriminate use of antimicrobials (Mathew, et al. 2007). Furthermore, as many of the antibiotics given to food animals are the same as those used in Hu medicine, the development of resistant bacteria poses a serious Hu health threat (McDermott, et al. 2002). Additionally, these...
resistant bacteria have the ability to pass the resistance genes on to other bacterial strains (Levy 1998, Gilchrist, et al. 2007). To date, increases in antibiotic resistant strains have been detected amongst Hu pathogens with some strains of more virulent microorganisms being resistant to multiple antibiotics (Gilchrist, et al. 2007, Molbak, et al. 1999). For example, Methicillin-resistant Staphylococcus aureus (MRSA) in pigs, which originated in France in 2005 (Vanderhaeghen, et al. 2010) has spread to other European countries, in addition to the USA, Canada and Singapore (Vanderhaeghen, et al. 2010, Barton 2010). This migration, although also often associated with overcrowded farms and poor conditions, demonstrates the ease of which microorganisms can be transmitted between species. Consequently, alternative methods of animal husbandry and therapeutic control of disease in food animals are essential. One possible alternative is to enhance the pig’s own immune system to better respond to the threat of disease.

The development of HSC and the subsequent process of hematopoiesis are the processes responsible for producing the immune system within the body. Although, to date much research has investigated this process in both Hu and Mo contrastingly little has been done in pigs. A better understanding of each stage of these fundamental processes, which include proliferation, differentiation and maturation of primitive HSC, may contribute significantly to the therapeutic enhancement of the immune system. One major area associated with hematopoiesis is the regulation of immune cell development directed by cytokine signalling (Zhu and Emerson 2002). Cytokines are unique proteins which are involved in all aspects of immune cell development and activation (Geijsen, et al. 2001). In order to fully understand hematopoiesis, we must understand the signalling processes and specialized roles played by each of the hematopoietic cytokines, both within the BM and the periphery. Consequently, as cytokines are involved in the control of hematopoiesis, it is envisioned that an understanding of this process may identify specific cytokines as having therapeutic benefits.

Although the idea of using recombinant cytokines as therapeutics as an alternative to antibiotics is not new, it is currently on the increase (Hubel, et al. 2002, Lowenthal, et al. 2000). Previous work with chickens has shown that some cytokines can act as growth promoters stimulating the immune system to ward off pathogens (Lowenthal, et al. 2000, Doyle 2001). Furthermore, as cytokines are the body’s own mediators of the immune system, cytokines would be an ideal choice for immune system enhancement. Thus, the use of cytokines may aid in producing healthier livestock for the consumer market and relieve consumer concerns over the generation
of antibiotic resistant bacteria. Interestingly, changes have already been reported where resistance levels in pigs have declined due to better management and reduced or discontinued use of antibiotics (Barton 2010). *E.coli* isolates from pigs, collected in South Australian in 1998, which showed close to 100% resistance to tetracycline, declined to just over 80% in 2005 (Barton 2010, Malik, *et al.* 2005). Furthermore, incidence of apramycin resistance declined from 90% to 0%, and neomycin resistance from 80% to 10% between 1998 and 2005 (Barton 2010). Although, studies have found that multiple resistances in chicken and pig isolates are most prevalent in *E.coli* and salmonella, it has been shown that prevalence can change over time and that this may be in response to changing antibiotic use (Barton 2010). Currently there is legislation in place to ban in-feed antibiotics by the European Union (EU). This decision has prompted other countries to review their current practices including South Korea (Flynn 2011, Johnson 2011), New Zealand (Johnson 2011), China and Thailand, and it is envisioned that other countries including Australia and USA will also change their legislations governing the use of in-feed antimicrobials (Johnson 2011). It is envisioned that this reduced use of antibiotics will therefore lead to a reduced incidence of resistance, however, although it has been shown that a reduction in the use of antibiotics can reverse the prevalence of antibiotic resistant bacteria, the question of therapeutic care of these animals still lingers and the need for alternative therapeutic management is paramount.

Although there have been some studies into Po hematopoiesis and the ontogeny of the Po immune system, there is still much more we need to understand. Of particular interest to this research are the early acting cytokines and growth factors associated with the type one cytokine receptor common β subunit, CD131. It is highly anticipated that the correct usage of these proteins, PoIL-3, PoIL-5 and PoGM-CSF, may aid in the therapeutic regulation of the Po immune system.

### 1.2 The mammalian immune system

#### 1.2.1 BM ontogenesis

Initial investigations into the process of HSC in mice suggested that HSC first develop in the yolk sack (Auerbach, *et al.* 1996, Gunsilius 2001, Jordan and Van Zant 1998, Jose, *et al.* 1994, Jose, *et al.* 1994). Indeed, yolk sac cells have been shown to produce HSC which can progress to either erythroid colonies or colony forming units mix (CFU-mix)(Gunsilius 2001). More recent research has revealed that HSC are first developed in the embryo in a region that neighbours the dorsal aorta referred to as the
para-aortic splanchnopleura (Gunsilius 2001, Choi 1998, Orkin and Zon 2008). *In vitro* analysis has shown that HSC that develop in this area are pluripotent and, therefore, able to generate the entire hematopoietic cell lineage as well as endothelial cells (Auerbach, *et al.* 1996, Gunsilius 2001). The development of these cells is referred to as primitive hematopoiesis, as it occurs prior to the development of the foetal liver, which is the site of hematopoiesis during early embryonic development, known as definitive hematopoiesis. During foetal development, the various hematopoietic tissues are seeded with HSC (Choi 1998). In Hu this begins at around weeks 6 and 7 with the seeding of the liver and the spleen with HSC originating from the para-aortic splanchnopleura and aortagonad-mesonephros (Gunsilius 2001, Orkin and Zon 2008). This stage is followed by the seeding of the BM in weeks 14 to 20 with HSC deriving from the foetal liver during the definitive hematopoiesis. These HSC are intermingled within fat cells and connective tissue cells (stromal cells), (Chen, *et al.* 1999) in the intersinusoidal spaces (Gunsilius 2001) which provide a supporting meshwork of collagen fibres and extracellular matrix components (Chen, *et al.* 1999). The development of blood cells is generated from this site during the complete postnatal phase (Gunsilius 2001). In the pig however, gestation lasts for only 114 days (Sinkora and Butler 2009) as opposed to the 259–294 days of gestation in Hu (Hollis 2002). 

**1.2.2 BM microenvironment (BMM)**

The BMM is a complex system of cellular and non-cellular components (Frisch, *et al.* 2008). Within this space are cells which biologically support HSC proliferation, differentiation and maturation (Travlos 2006). The BMM consists of cells that maintain bone structure; mesenchymal stem cells (MSC), stromal cells, osteoblasts and adipocytes, and cells of the hematopoietic lineage, HSC, myeloid and lymphoid
progenitors, early precursors and immature immune cells (Yamamoto-Yamaguchi, et al. 1983). Within the BMM, support cells secrete tissue specific macromolecules which form a complex network that functions as a supporting framework for the organ (Dominici, et al. 2001, Kuo and Tuan 2003). In addition, the BMM provides support for cell to cell interactions, which along with specific cytokines, are necessary for the self-renewal, proliferation and differentiation of HSC (Dominici, et al. 2001).

1.2.3 HSC

The HSC are located in a region of the BM known as the subendosteum, adjacent to the inner bone surface where HSC keep in contact with BM stromal cells (Than, et al. 1992). HSC have the ability to self-renew upon differentiation producing both an identical daughter cell and a progenitor cell for either the lymphoid or myeloid cell line (Yan, et al. 2003). This ability to reproduce an identical daughter cell ensures that numbers of HSC are not greatly diminished over the life of the host (Bryder, et al. 2006). These somatic cells proliferate throughout the hosts entire life and are capable of producing at least eight to ten distinct lineages of mature cells (Chen, et al. 1999).

HSC are similar to lymphocytes in morphology as they are small undifferentiated progenitors (Donnelly and Krause 2001). In addition, HSC are only present in small numbers throughout the BM stromal where the cells represent approximately 1.5 to 4% of BM cells (Donnelly and Krause 2001). Identification of HSC therefore lies solely upon cellular staining and the detection of specific cell surface markers (Bryder, et al. 2006). These markers include receptors and glycoproteins which interact with other cell types and regulatory elements within the BMM including stromal cells, extracellular matrix molecules and cytokines (Gunsilius 2001). These extracellular proteins and cytokine receptors are required by HSC to initiate and maintain the process of hematopoiesis.

1.2.4 Hematopoiesis

The process of hematopoiesis is a multiple step continuum involving the commitment of HSC to differentiation and proliferation in order to regulate the required levels of mature blood cells within the periphery (Yan, et al. 2003). In the pig, the total number of blood leukocytes is approximately \(20.6 \pm 4.4 \times 10^9\) cells/L blood (Edfors-Lilja, et al. 1998). These blood cells are responsible for the constant maintenance and immunological protection of all cell types and tissues within the body. As the cells of the blood system have a short life span, on average 12 days for WBC and up to 120 days for red blood cells (RBC) (Neu, et al. 2003), the system requires constant
maintenance and renewal (Wu, et al. 2009). In Hu, this results in the continual turnover of HSC to supply short lived precursors that maintain all WBC and RBC in the order of $1 \times 10^{10}$ white cells, $2 \times 10^{11}$ red cells, and $4 \times 10^{11}$ platelets per day (Gunsilius 2001).

The complex organisation of hematopoiesis is controlled by a number of factors including cytokines and chemokines which promote the survival, proliferation and differentiation of HSC and progenitor cells (Broudy 1997, Metcalf 1993). Early acting cytokines are released and taken up by stromal cells within the BM matrix which initiates the first stage of hematopoiesis (Gulati, et al. 1988, Zipori 1981). In addition, cytokines are also released by activated leukocytes within the periphery, initiating a cascade of events which often involves HSC differentiation (Szabo and Hrabak 1978).

The BM stroma provides the support for developing cells within the BM and are involved in hematopoiesis through cell to cell contact (Roberts, et al. 1988). These cells are also referred to as hematopoietic supportive stroma (HSS) (Figure 1.1). Contact between HSC and HSS stimulates these stromal cells to express Stem Cell Factor (SCF), which is a membrane bound cytokine (Toksoz, et al. 1992, Cherry, et al. 1994). SCF is required to activate the c-Kit receptor on HSC efficiently. C-Kit acts as a cofactor for other cytokines such as IL-3 which must bind to its receptor on HSC at the same time as c-Kit is activated in order to stimulate hematopoiesis (Breems, et al. 1998).

The early proliferation and differentiation of HSC leads to one of two common progenitors or immature immune cells. These are the common lymphoid progenitor (CLP) and the common myeloid progenitor (CMP) (Zhu and Emerson 2002). The further differentiation of these cells leads to the development of multiple lineages of immature immune cells (Schmidt, et al. 2005)(Figure 1.1).

1.2.5 Cells of the immune system

Immature immune cells incorporate all precursor cells of the immune system which develop in the BM and originate from the HSC. These cells include the CLP and CMP as well as their downstream precursors. There are four major lineages that develop from HSCs. These are the lymphocytes (B-cells, T-cells, and natural killer (NK) cells), which develop from the CLP (Izon, et al. 2001, Allman and Miller 2003, Barker and Verfaillie 2000, Sanchez, et al. 1993, Palacios, et al. 1989) and the myelocytes (granulocytes and mononuclear phagocytes), erythrocytes and megakaryocytes, which all develop from the CMP (Figure 1.1)(Inaba, et al. 1993, Yunis, et al. 1980, Prchal, et al. 1978). As with HSC, each of these lineages develops
Figure 1.1 Mammalian hematopoiesis. The suggested process of mammalian hematopoiesis showing HSC self renewal and cell types for each of the lymphoid and myeloid cell lines. Cell surface markers of interest are indicated at several stages. Migration pathways and organs of maturation are also indicated.
under the influence of the BM matrix and microenvironment including cell to cell interactions and both soluble and membrane bound cytokines (Roberts, et al. 1988, Allman and Miller 2003, Cook, et al. 1989).

1.2.5.1 Lymphocytes

The development of B-cells, T-cells and NK cells, a process referred to as lymphopoiesis, is initiated in the BM but for B-cells and T-cells, is completed in other lymphoid organs (Loder, et al. 1999, Schwarz and Bhandoola 2006).

In Hu adults, early B-cells develop within the BM in contact with stromal cells (Kierney and Dorshkind 1987) within the subendosteal (Hermans and Opstelten 1991). Here, B-cells undergo multiple stages of differentiation and maturation under the influence of the microenvironment and cytokines. B-cell development involves a series of complex splicing and rearranging of genes which form B-cell immunoglobulins (Liu, et al. 1980, Singer, et al. 1980) and receptors for T cell interaction (Behlke and Loh 1986, Evans and Engleman 1985). Immature B-cells undergo as many as 7 stages of development and by as early as stage 4, the large pre-B-cells are expressing the surface immunoglobulin-associated molecules Igα and Igβ. Following further differential steps, the immature IgM+ B-cells undergo selection for release into the peripheral blood stream where they migrate to the primary lymphoid organs for final selection and maturity (King and Monroe 2000).

The development of T-cells within the foetal thymus has been detected as early as day 40, however, the development of T-cells within the BM has not been identified until day 45. This would therefore imply the pre-BM development of T-cells occurs within the foetal thymus (Sinkora, et al. 2000, Butler, et al. 2006, Sinkora, et al. 2003). As with B-cells, all adult T-cell initially develop from the CLP within the BM (Bhandoola and Sambandam 2006), however, only the first stages of the cell’s development take place here as generation of both α/β and γ/δ T cells arise intrathymically from the BM produced precursor (Fehling, et al. 1995, Kang, et al. 1998).

In adult Hu, natural killer cells (NK) are produced from HSC within the BM where NK precursors (NKP) mature in the presence of stromal cells, however, the exact environment required for NK development is currently unknown (Colucci, et al. 2003). Even less is known regarding the development of NK cells in pigs. What is currently known is NKP, as with other lymphocytes, developed from the CLP under the direction of cytokines produced from BM stromal cells (Miller, et al. 1994, Di Santo 2006). NK cells have been detected in Po ontogeny as early as day 45 in both the spleen and umbilical cord where they represent around 1-10% of immature immune
cells (Sinkora and Butler 2009). Furthermore, it appears that these immature cells are non-functional until 6 months post gestation (Sinkora and Butler 2009).

1.2.5.2 Myelocytes

During hematopoiesis, the CMP differentiates into early myeloid progenitors and colony forming units (CFU) of several phenotypes which eventually mature into the various monocytic lineages (Summerfield and McCullough 1997) In Hu, the CFU-basophil/eosinophil (CFU-Bas/Eo), CFU-granulocyte/monocyte (CFU-GM) and the CFU-megakaryocyte/erythrocyte (CFU-ME) are all generated from the CMP (Ogawa, et al. 1983, Kawamoto and Katsura 2009) (Figure 1.1).

Neutrophils develop during a process termed granulopoiesis, differentiating several times before finally maturing as a segmented neutrophil (Figure 1.1) (Egesten, et al. 1994, da Silva, et al. 1994, Witko-Sarsat, et al. 2000, Perez, et al. 2007). Evaluation of neutrophil numbers in piglets have reported neutrophils as being the most dominant myeloid cell in the periphery representing 60% to 40% of WBC at week 1 to week 15, respectively (Juul-Madsen, et al. 2009). Neutrophils are however, very short-lived cells with an average survival time in vivo of less than 2 days, although it has been suggested that cytokines found at inflammatory sites such as GM-CSF can extend their life for several days (Scheel-Toellner, et al. 2004).

Despite their vast numbers and importance in the innate immune response, surprisingly little study into neutrophil ontogeny has been reported in a non-stimulated Po model with the majority of research centred on the granulocytic involvement in classical swine fever virus. Similarly, little research has focused on eosinophils in pigs even though parasites are a common concern to the pig industry.

Research on Hu basophils has revealed that IL-3 is one of the most important cytokines in the survival and secretion mechanism of basophils thus these cells maintain a high level of CD123 expression (Dzinek, et al. 2000, Sarmiento, et al. 1995, Schroeder 2009). Furthermore, it has been shown that IL-3, IL-5 and GM-CSF all have significant effects on basophil mediator release during an innate immune response, including the anti-inflammatories leukotriene C4 (LTC4) and histamine (Sarmiento, et al. 1995, Miadonna, et al. 1993, Hirai, et al. 1990, Bischoff, et al. 1990). LTC4 is an agonist for the contraction of smooth muscle and has been shown to induce microvascular permeability. Histamine is vasodilator which promotes blood flow to tissues. These two mediators therefore act together to change the physiology of infected tissue and aid in the recruitment of immune cells (Newton and Dixit 2012).

As both eosinophils and basophils are involved in inflammatory response, a better understanding of eosinophil and basophil development and the effects of IL-3, IL-5 and GM-CSF on CFU-Bas/Eo proliferation may be of benefit to the pig industry (Munitz and Levi-Schaffer 2004).

1.3 Models for studying BM development

In order to identify the physiological mechanism initiated by GM-CSF, IL-3 and IL-5 in pigs, it is first necessary to develop a model for studying in vivo hematopoiesis.

1.3.1 Chemotherapeutic ablation and myelosuppression.

Chemotherapy involves the administration of cytotoxic chemicals (Ciordia, et al. 2000) which target and kill cells that divide rapidly (Marchini, et al. 2004). Although used to kill cancer cells, these chemicals also destroy cells of the BM, digestive tract and hair follicles (Marchini, et al. 2004). A chemotherapeutic attack on the BM can therefore result in myelosuppression (decreased production of blood cells) (Laurenz, et al. 1997).

The chemical 5-FU is a cytoreductive cancer chemotherapeutic which causes inhibition of thymidylate synthetase resulting in attenuation of deoxyribonucleic acid (DNA) synthesis and the incorporation of 5-FU into ribonucleic acid (RNA) (Laurenz, et al. 1997, Parker and Cheng 1990). The administration of high doses of 5-FU selectively kills cells in the cycling phase while sparing more dormant cells (Hodgson and Bradley 1979, Hodgson, et al. 1982, Nishio, et al. 1996). These cells have been shown to include pluripotent HSC (Nishio, et al. 1996). When administered to pigs, 5-FU causes myelosuppression of the immune system depleting WBC, RBC and
thrombocytes, resulting in rapid leukocytopenia (decreased WBC) which is proceeded by a period of leukocytosis (increased WBC) (Laurenz, et al. 1997, Hodgson, et al. 1982, Radley, et al. 1980, Yeager, et al. 1983). Therefore, the period of time between leukocytopenia and leukocytosis may provide an opportune interval for both monitoring and manipulating the process of hematopoiesis. Furthermore, it is endeavoured that a better understanding the roles of cytokines and chemical mediators, involved in hematopoiesis, will be gained from this process.

1.4 BM cell surface markers

All cells of the BM including HSC, monocytes, lymphocytes and precursors cells express a number of different molecules on the cell surfaces which can be used to isolate cell subsets (Negro Alvarez 1980). These molecules can be identified by monoclonal antibodies (mAb) and polyclonal antibodies (pAb) which have been developed specifically against these molecules (Bernard and Boumsell 1984). At the time of this work there was no singular mAb available for the isolation of HSC in pigs. Other cellular surface markers useful for identifying stages of hematopoiesis were therefore identified. CD90, a cell surface marker that is expressed on Hu HSC and early progenitors (Majeti, et al. 2007, Thornley, et al. 2002) and CD172a which has been identified on both HSC (Seiffert, et al. 2001) and on myeloid lineage cells in pigs, were selected for identifying and monitoring hematopoietic cell proliferation and differentiation (Pescovitz, et al. 1984).

1.4.1 CD90

The cell surface marker CD90, also known as Thy-1, is a glycosylphosphatidylinositol (GPI) anchored member of the immunoglobulin supergene family (IgSF) (Crawford and Barton 1986, Mason, et al. 1996, Henniker 2001). Originally named θ-AKR, the CD90 antigen was first located in the thymus of C57BL/6 leukaemia mice using isoantisera preparations (Gorer and Amos 1956, Reif and Allen 1964). Early studies into the cross reactivity of CD90 demonstrated remarkably varied tissue distribution across different species (Dalchau and Fabre 1979). Although CD90 appears to be expressed evenly on brain tissue across various species (Dalchau and Fabre 1979, Dalchau and Fabre 1979), the expression of CD90 in thymus and BM differs considerably.

Indeed, CD90 was identified in large quantities in the thymus of Mo and rats and small quantities in the thymus of dogs, however, these early studies indicated CD90 was absent in the thymus of Hu (Dalchau and Fabre 1979, Dalchau and Fabre
Furthermore, although peripheral T lymphocytes of both mice and dogs were positive for CD90 (Dalchau and Fabre 1979, Dalchau and Fabre 1979), rats and Hu were negative for the antigen (Dalchau and Fabre 1979). In addition, rat and dog BM were CD90+ and Mo and Hu BM CD90- (Dalchau and Fabre 1979).

Identification of restrictions in the use of isoantisera preparations therefore prompted the generation of a monoclonal antibody to CD90 (McKenzie and Fabre 1981). Following this and in contrast to earlier investigations, fluorescence activated cell sorting (FACS) analysis of Hu thymus cells using Mo anti-HuCD90 detected 7% of cells as CD90+ (McKenzie and Fabre 1981). Furthermore, frozen sections of Hu thymus tested highly positive for fluorescent CD90 mainly in the periphery of the thymus lobule (McKenzie and Fabre 1981). In addition, fluorescence was also detected in the marginal zone and on several periarteriolar lymphocytes within the spleen, and the post-capillary venules of lymph nodes (McKenzie and Fabre 1981). These results prompted the hypothesis that CD90 may be present on early T Lymphocytes (McKenzie and Fabre 1981).

Further investigation revealed two isoforms of CD90, Thy-1.1 and Thy 1.2 (Dalchau and Fabre 1979, Snell and Cherry.M 1972), and following the isolation and sequencing of CD90 from rat, Mo and Hu, it was revealed that one major difference between the Hu and rodent form of the gene was the presence of two N-linked glycosylation sites in the Hu CD90 as opposed to three N-linked glycosylation sites in the rodent gene (Seki, et al. 1985). It has been speculated that this difference may be partially responsible for the early varied results for CD90 expression between species (McKenzie and Fabre 1981). Further analysis isolated a primitive line of CD34+CD90+ Hu foetal BM cells which were further identified as HSC (Baum, et al. 1992). Immunofluorescence studies have now identified CD90 expression on both early pre- and immature B-lymphocytes and prothymocytes during T- lymphocyte development (Ritter, et al. 1983). Further studies using a newly developed monoclonal anti-HuCD90, revealed 25% of CD34+ HuBM to be CD90+ which were subsequently determined to be long-term culture initiating cells (LTCIC) (Craig, et al. 1993). In addition, a small population of CD90+ Hu peripheral blood cells were identified as being CD3+CD4+lymphocytes (Craig, et al. 1993).

In Hu, CD90 is a 161aa polypeptide with a molecular weight (MW) of 25-35 kDa (Mason, et al. 1996, Henniker 2001, Seki, et al. 1985, Craig, et al. 1993). The CD90 protein consists of a 19aa N-terminal signal sequence, an 111aa core and a 31aa C-terminus. During synthesis, the signal peptide is removed and the C-terminus
is replaced by a GPI membrane anchor (Crawford and Barton 1986, Mason, et al. 1996, Henniker 2001). The 111 aa core is then inserted into the cellular membrane via the GPI anchor (Crawford and Barton 1986).

The function of Hu CD90 is still not defined, however, there is much speculation. One hypothesis is that Hu endothelial CD90 may interact with leukocytes during the adhesion and migration process associated with inflammatory response (Mason, et al. 1996, Henniker 2001) and that the CD90 antigen may play a role in the permeability of endothelia (Mason, et al. 1996, Henniker 2001). Other possible roles include cell to cell recognition and neural differentiation (Henniker 2001, Clark and Springer 1997) as well as inhibition of immature HSC proliferation (Henniker 2001, Seeger, et al. 1982, Tiveron, et al. 1992, Mayani and Lansdorp 1994) and in the development of neuron memory formation in the central nervous system (CNS) (Kisselbach, et al. 2009, Clark 2004). Nevertheless, as CD90 has been isolated on early HSC in addition to both B-cell and T-cell precursors, CD90 may be an ideal marker for monitoring lymphoid cell development in the BM.

To date, very little research has been undertaken into the expression of anti-HuCD90 mAb in pigs, although previously work has shown CD90 to be expressed on PoBM early CD34+ progenitor cells (Layton, et al. 2007), as well as Po MSC (Moscoso, et al. 2005). This identification may lead to a similar expression in pigs as seen in Hu. However, in order to differentiate between lymphocyte and myelocyte lineages, a suitable myeloid cellular marker is also needed.

1.4.2 CD172a

Myeloid cells, granulocytes and monocytes/macrophages play an important role in inflammation and host defence (Dadfar, et al. 2004, Ezquerra, et al. 2009). CD172a is a pan-myeloid marker originally identified in pigs as a 230kDa antigen which is abundantly expressed on monocytes, granulocytes, dendritic cells (DC) and tissue macrophages, thus suggesting CD172a may be an early stage myeloid cell marker, possibly before lineage divergence (Summerfield and McCullough 1997). CD172a has homology to the signal regulatory proteins (SIRPα) family, which is a transmembrane glycoprotein involved the receptor tyrosine kinase coupling signalling pathway and is associated with src homology (SH2) domain-containing tyrosine phosphatase 1 (SHP-1) (Alvarez, et al. 2000). SHP-1 is a phosphatase involved in the negative regulation of signal transduction pathways (Ezquerra, et al. 2009). Previous work suggests that CD172a may function to control proliferation, differentiation and activation of cells, therefore, explaining its presence from early immature cells to

The low and high florescence intensity associated with CD172a gives an indication of its maturation dependant expression on BM hematopoietic cells (BMHC). As reported by Summerfield et al (2001), cells with CD172a<sup>lo</sup> expression are located in forward scatter channel (FSC) high (FSC<sup>hi</sup>)/ side scatter channel (SCC) high (SSC<sup>hi</sup>) and are mainly granulocytic precursors whilst those which are CD172a<sup>hi</sup> and are found in the FSC<sup>hi</sup> SCC<sup>lo</sup> region are mainly monocytic precursors. In contrast, those cells which are high in CD172a expression tend to be predominately mature granulocytes and are found in the FSC<sup>lo</sup> SCC<sup>hi</sup> region, where as mature monocyctic cells were found to be CD172a<sup>hi</sup> with only a moderate FSC signal (Summerfield, et al. 2001, Summerfield, et al. 2001). The properties of this surface marker therefore provide the ability to monitor increases and decreases in levels of precursor and mature cells monocytes. Early immune system analysis has identified CD172a<sup>+</sup> cells in the yolk sack at day 17 (Summerfield and McCullough 1997) and foetal liver at day 20 (Sinkora and Butler 2009) confirming monocytes as being amongst the first cells of the Po immune system to develop in the embryo (Sinkora and Butler 2009). This also confirms the presence of CD172a during the development of the Po immune system.


As CD172a is expressed intensely on early monocytes and hematopoietic cells of the myeloid lineages (Alvarez, et al. 2000, Alvarez, et al. 2007), and has been identified on CD34<sup>+</sup> PoHSC (Layton, et al. 2007), CD172a is an ideal marker for monitoring myeloid cell development and BM re-population in pigs.
In addition to CD90 and CD172a, there are numerous cell surface antigens and receptors present on BM cells. One type of receptor, the cytokine receptor, is required by immune cells for the cytokine signalling cascades initiated in the co-ordination of homeostasis of blood cells (Miyajima, et al. 1992). An appreciation of expression and function of cytokine receptors may lead to a better understanding of cytokine function in the immune system.

1.5 Cytokine receptors

Cytokines are a unique family of polypeptide growth factors and immunomodulating agents which are used extensively in cellular communication as signalling molecules to stimulate both the humoral and cellular immune responses (Watson, et al. 1979). They are soluble proteins which can act either in an autocrine or paracrine process to induce either a hematopoietic or immunological reaction (Martinez-Moczygemba and Huston 2003, Wang, et al. 2009). Cytokines are low molecular weight molecules ranging in size from 15-70 kDa (Kaushansky 2001) and are primarily secreted by leukocytes (Dumonde, et al. 1969). They can be categorized as either growth factors, or when released by leukocytes, are referred to as interleukins. HSC, progenitors and other hematopoietic cells respond to signalling from both hematopoietic growth factors and interleukins (Zanjani, et al. 1999, Evans 1997, Muller-Sieburg, et al. 2004).

The biological activities of cytokines are often pleiotropic, acting on multiple cell types to elicit a range of different responses including stimulating differentiation and growth, promoting cell survival or inhibiting proliferation of lineage-committed or multipotent HSC (Geijsen, et al. 2001, Clutterbuck, et al. 2000). Furthermore, it has been noted that cytokines may be redundant, in that several different cytokines may act on the same cell to elicit the same response (Geijsen, et al. 2001).

Cytokine receptors are cell surface receptors which bind specific cytokines and are categorised into one of six subtypes depending on their three dimensional structure.

1.5.1 Type I cytokine receptors

The type I cytokine receptors represent the largest group amongst the cytokine receptor families. This group includes:

- Interleukin receptors and receptors expressing the WSXWS motif, including,
- Interleukins 3 and 5
- Colony stimulating factor receptors including,
  - The GM-CSF receptor

The binding of a type 1 cytokine to a type 1 cytokine receptor regulates development, differentiation and activation of immune and inflammatory cells (Boulay, et al. 2003, Paul and Seder 1994). Each of the cytokine receptor types have specific structural formations that are unique to the particular group. The receptors of the type 1 interleukins include receptors expressing the common cytokine receptor superfamily motif, Trp-Ser-Xaa-Trp-Ser (WSXWS). This is a unique common amino acid (aa) motif or “Trp-Arg zipper”, located in the extracellular portion of the receptor adjacent to the cell membrane (Carr, et al. 2001) and is involved in maintaining tertiary structure of the receptor (Wang et al. 2009). The type 1 cytokine receptor also contains a cytokine binding homology region (CHR) (Bagley, et al. 1997). The CHR is made up of two cytokine receptor modules (CRM), each consisting of two 100aa fibronectin type III (FNIII) domains (Bagley, et al. 1997). Each of the FNIII domains is arranged in a 7ß strand formation which is connected by a linker representing a signature recognition module for helical cytokines (Wang, et al. 2009). This module is present in every type 1 cytokine receptor (Wang, et al. 2009, de Vos, et al. 1992) and is responsible for the recognition of type 1 cytokines with a four-helix bundle motif (Wang, et al. 2009, Bazan 1990, Bazan 1990, Sprang and Bazan 1993). The binding of these cytokines to their specific type 1 receptor leads to receptor oligomerization followed by the juxtaposition of the intracellular domains of the signalling subunits (Wang, et al. 2009).

Figure 1.2 Activation of type 1 cytokine receptor. Schematic showing cytokine / receptor interaction and activation for type 1 cytokine receptor. Upon binding of the cytokine to the receptor complex, the receptor is phosphorylated by JAK. This attracts STAT to bind to the receptor, which in turn is also phosphorylated. STAT-P then detaches from the receptor complex and initiates transcription within the nucleus.
The type 1 cytokine receptor pathway also encompasses the RAS-RAF-Ras/mitogen-activated protein (MAP) kinase pathways (Leonard 1999, Dong, et al. 2002) which leads to activation of one or more of the three major MAP kinases (MAPK), the extracellular signal-regulated protein kinases (ERK), the p38 MAP kinases and the c-Jun NH2-terminal kinases (JNK) (Figure 1.3)(Dong, et al. 2002). It has been shown that growth factors are associated with the ERK pathway and both cytokines and growth factors are linked to p38 and JNK (Dong, et al. 2002, Roberts and Der 2007).

The activation of these kinases is dependent on the upstream signal received which in turn relates to the specific cytokine / type 1 receptor complex being activated, in addition to the cell type the receptor is expressed on (Dong, et al. 2002). Moreover, the specific pathway activated becomes more complex by the availability of 4 different JAKs and 8 different STATs all with unique DNA binding and trans-activation properties which provides cellular specificity to these pathways (de Groot, et al. 1998). One type 1 cytokine receptor which has been linked to several cytoplasmic tyrosine kinases, in particular JAK2, is CD131 (de Groot, et al. 1998).

1.5.2 CD131- the Common β Subunit

Within the type 1 cytokine receptor family there are three major classes of heteroreceptor complexes, where the receptor complex utilises a common shared receptor subunit as a signal-transducing chain along with a cytokine specific subunit (Wang, et al. 2009). These three classes include those which use gp130, those which use γc and those which use the common β subunit, (βc), CD131 (Wang, et al. 2009). CD131, is shared by three of the “four-helical bundle” family of cytokines; IL-3, IL-5 and GM-CSF, and forms a receptor complex along with a cytokine specific α chain for each of these cytokines (Figure 1.2) (Wang, et al. 2009, Murphy and Young 2006). Moreover, even though all three of these cytokines have their own specific α subunit, the co-utilisation of CD131 suggests a common intra-cellular process or overlapping activities on expressing cells (Murphy and Young 2006).

Although there has been recent interest in the CD131 subunit in various species, little information is available in regards to the PoCD131 subunit. The majority of information on CD131 has, therefore, been collected in respect to other species including the Hu and Mo isotypes.
Hu CD131 is a type 1 transmembrane protein cytokine consisting of two monomers of approximately 881 aa, each with four domains, (Sato, et al. 1993) which form an intertwined strand-swapped anti-parallel homodimer (Carr, et al. 2006). It has been reported that CD131 is not actually bound to the cytokines IL-3, IL-5 and GM-CSF, but instead forms a high-affinity complex with the $\alpha$ subunit once the $\alpha$ subunit is bound with low-affinity to the $\alpha$ subunit specific cytokine (Bagley, et al. 1997, de Groot, et al. 1998, Miyajima, et al. 1992, Mui, et al. 1994). Furthermore, it has been suggested that the $\alpha$ subunit may be for recognition purposes only as there is no JAK kinase or tyrosine phosphorylation activity associated with the $\alpha$ subunit, therefore, signal transduction may lie solely with CD131 (Wang, et al. 2009). The activation and subsequent phosphorylation of STAT by JAK2 on the intracellular portion of CD131 leads to an extensive array of signalling pathways which include the MAPK and phosphotidylinositol-3-kinase (PI3K) pathways (Figure 1.3) (de Groot, et al. 1998, Murphy and Young 2006, Sato, et al. 1993). In addition, CD131 has been reported as being linked to the STAT5 signalling pathway (including STAT5a, STAT5b, and truncated STAT5 activation), although some STAT1, 3, and 6 activity has also been observed in some myeloid cells (de Groot, et al. 1998). Furthermore, STAT5 has been identified as a major cytokine induced pathway within the immune system associated with cellular proliferation, differentiation and survival (Wittig and Groner 2005, Hennighausen and Robinson 2008). To date, STAT5 activity in relation to IL-3, IL-5 and GM-CSF has been identified in monocytes, CD34$^+$ cells, DC, eosinophils and neutrophils (de Groot, et al. 1998) therefore indicating CD131 expression is mainly restricted to myeloid cells (Sato, et al. 1993). This would suggest that the therapeutic manipulation of CD131 by IL-3, IL-5 and GM-CSF would have a direct effect on the myeloid lineage of cells and when associated with the STAT5 pathway, would result in an increase survival and differentiation of specific myeloid cells as seen in an inflammatory response to pathogens.

Much work is currently underway in identifying molecular mechanisms and pathways elicited by each of the CD131 cytokines in the hope of using these cytokines for modulation of immune responses in Hu (Martinez-Moczygemba and Huston 2003). These results may therefore be beneficial to pigs due to the high degree of homology between these two species.

Crystallography results for both Hu and Mo CD131 has providing a detailed structure and surface topography of the receptor. The arrangement of the intertwined
Figure 1.3 Schematic of CD131 intracellular pathways. Schematic representation showing the formation of the ligand receptor complex for each of the cytokines associated with CD131. Complex formation initiates JAK2 phosphorylation and STAT pathway activation followed by activation of the PI3K and RAS/MAPK pathways resulting in proliferation/survival, differentiation and cell function. (Sato. N, et al 1993)
strands and the presence of the two fibronectin type III domains in domains 2 and 4 along with disulfide bridges present in domains 1 and 3 and the presence of the Trp-Arg zipper in domain 4 have all been confirmed (Carr, et al. 2001). Furthermore, these analyses have located possible ligand interaction sites for the α subunit/cytokine complex at His349 (Carr, et al. 2006, Lock, et al. 1994, Woodcock, et al. 1994) and Try347 (Carr, et al. 2006, Woodcock, et al. 1994) as proposed from mutation studies. The identification of these domains and binding sites may aid in the structural arrangement and recognition of cytokine binding sites for Po CD131.

1.6 The cytokines associated with the CD131 receptor.

The cytokines of CD131, being IL-3, IL-5 and GM-CSF, show a combination of both unique and overlapping characteristics and pathways (Murphy and Young 2006). The cellular response elicited is therefore dependent on the specific cell targeted, the cytokine initiating the response, and to whether the cytokine is acting alone or in synergy with other cytokines (Geijsen, et al. 2001). Furthermore, the CD131 cytokines can compete for the CD131 subunit indicating cytokine concentrations and combinations are also relevant to the cellular pathway activated (Kitamura, et al. 1991).

IL-3, IL-5 and GM-CSF bind their respective α subunit via their first and third loops and binds to CD131 via a glutamine residue in the first α helical bundle (Martinez-Moczygemba and Huston 2003). Comparisons of Po IL-3, IL-5 and GM-CSF protein sequences to Hu and other species indicate conserved aa sequences and the presence of α binding site and glutamine residues which in turn may indicate possible cross species activity.

Although to date IL-3, IL-5 and GM-CSF, have all been studied intensely in a broad range of species including Hu and Mo, little research has been initiated in pigs. A broader understanding of the roles of IL-3, IL-5 and GM-CSF in pig hematopoiesis, may indicate these cytokines as possible therapeutics for pig health.

1.6.1 IL-3 and the IL-3α subunit, CD123

IL-3 is a cytokine that acts early on in the HSC development and is commonly referred to as a multi-colony stimulating factor due to the cytokine’s ability to stimulate hematopoietic progenitors to produce several forms of hematopoietic cells (Le Bousse-Kerdiles, et al. 1986). IL-3 has an important role in the production of all myeloid cells beginning with the proliferation and differentiation of early CD34+ progenitor cells
Together with other cytokines, IL-3 is directly involved in the production of mast cells, basophils, dendritic cells, eosinophils, monocytes and macrophages (Martinez-Moczygemba and Huston 2003). Studies have shown that IL-3, when used in synergy with other early acting cytokines and growth and developmental factors enhances proliferation of long-term repopulating HSC (LTRC) in vitro and is involved with HSC self-renewal in vivo (Bryder and Jacobsen 2000).

IL-3 is a short-chain 4-α-helix bundle cytokine with a common steric structure pattern of a up-up–down–down style, which has been deemed very important to the functionality of the cytokine (Bagley, et al. 1997, Bazan 1990, Ouyang and He 2003, Miyajima 1992). This 4-α-helix bundle structural arrangement is also shared with IL-5 and GM-CSF (Miyajima 1992). Hull-3 is primarily produced by activated CD4 effector T cells (Mosmann, et al. 1986, Culpepper and Lee 1985) however, it is also released by macrophages, in addition to stromal cells during stromal cell/HSC interactions (Martinez-Moczygemba and Huston 2003). In a physiological role, IL-3 is involved in allergic inflammation in Hu (Kuna 2003) and parasite immunity in Mo (Martinez-Moczygemba and Huston 2003, Lantz, et al. 1998). Furthermore, Hull-3 administration increases the cellularity and cycling of BM progenitor cell populations and when used along with GM-CSF has been shown to stimulate multilineage hematopoiesis (Sosman, et al. 1995). Moreover, IL-3 together with GM-CSF stimulates HSC to promote the production of macrophages and granulocytes which are important effector cells in humoral and cell-mediated immunity (Turk 1969). In Hu, these cytokines stimulate BM precursors to produce type 1 and type 2 DC, respectively (Martinez-Moczygemba and Huston 2003). Furthermore, it has been shown in Hu in vitro studies, that these type 1 DC derived using IL-3 induced a T-cell type 2 helper cell, (T\(_{H2}\)) response as opposed to those derived from GM-CSF which induces a T-cell type 1 helper cell, (T\(_{H1}\)) response (Martinez-Moczygemba and Huston 2003, Ebner, et al. 2002). It has therefore been suggested, that IL-3 may become an important mediator in T\(_{H1}\) and T\(_{H2}\) immune responses (Martinez-Moczygemba and Huston 2003). Other research has established PoIL-3 as a major factor in the in vitro culturing of HSC derived mast cells as a first step into the possible therapeutic enrichment of mast cell populations in fighting Po intestinal parasites such as Ascaris suum and Trichinella Spiralis (Ashraf, et al. 1988, Frieling, et al. 1994, Urban, et al. 1988, Femenia, et al. 2005). In addition, PoIL-3 has been shown to be an effective adjuvant in DNA vaccination against classical swine fever and may...
play a similar role in other DNA vaccinations (Andrew, et al. 2006). As IL-3 is involved in the production or augmentation of a broad array of cells, its administration may prove to be a viable tool for therapeutic enhancement of the Po immune system.

The initiation of cellular response to IL-3 begins with the binding of IL-3 to the IL-3Rα subunit, CD123. Within the BM, CD123 expression is first seen on committed HSC and progenitor cells. Here IL-3 acts as a colony stimulating factor by regulating the survival, proliferation and differentiation of HSC (Moretti, et al. 2001). In addition to precursors, CD123 is also expressed predominantly on myeloid cells within the hematopoietic system including: monocytes, neutrophils, basophils, eosinophils, megakaryocyte and erythroid precursors, mast cells and macrophages (Moretti, et al. 2001). CD123 activity has previously been observed on Hu B-cells where IL-3 initiated B-cell growth thus implying IL-3 may act as an early growth factor on B-cells (Xia, et al. 1992). Receptor activation occurs upon the low affinity binding of IL-3 to CD123 which in turn binds with high affinity to CD131 (Moretti, et al. 2001). CD131 is then phosphorylated at the JAK2 site on the membrane proximal region of the β chain, which in turn activates STAT5 as previously mentioned (Moretti, et al. 2001, Hara and Miyajima 1996). When driven by IL-3, this chain of events results in intracellular signals which regulate gene expression, DNA synthesis, cell proliferation, differentiation and prevention of apoptosis (Moretti, et al. 2001, Barry, et al. 1997).

In Hu, CD123, is reported as being a type I transmembrane glycoprotein which incorporates a 200aa conserved region homologous to the FNIII domain which includes the membrane proximal WSXWS motif and membrane distal pair of cysteine residues both characteristic of the cytokine receptor superfamily (Martinez-Moczygemba and Huston 2003, Moretti, et al. 2001). The molecular weight of CD123 is approximately 41kDa or 70kDa when glycosylated as the protein contains 5 N-glycosylation sites (Moretti, et al. 2001). The CD123 protein is made up of 378aa residues of which 18aa make up the signal peptide, 288aa form the extracellular domain, 20aa span the transmembrane domain and 52aa produce the cytoplasmic domain (Moretti, et al. 2001).

Thus far, Po CD123 has not been reported as being isolated or analysed. Likewise, there is no available information regarding the PoIL-5 α subunit CD125 although several cases of PoIL-5 expression have been examined.
1.6.2 IL-5 and the IL-5α subunit, CD125

Interleukin-5, also member of the four α helical bundle motif cytokines, differs from IL-3 and GM-CSF due to the glycoproteins intertwined homodimeric conformation (Takatsu 1998). IL-5 is primarily released by T_{H}2 and mast cells in response to protection against parasites (Swain, et al. 1988) and in allergic disease (Shen, et al. 2003). Moreover, mRNA coding for IL-5 has been detected in eosinophils involved in some chronic Hu inflammatory diseases including Crohn’s, Celiac, and Asthma (Dubucquoi, et al. 1994). It has been speculated that this may be part of a self-priming and recruitment process initiated by eosinophils (Dubucquoi, et al. 1994). Furthermore, it has been shown that IL-5 in mice promotes B-cell activation and differentiation (McKenzie, et al. 1987) and induces the B-cell secretion of IgA (Matsumoto, et al. 1989). In Hu, IL-5 has been shown to stimulate B-cells under specific conditions (Huston, et al. 1996, Adachi and Alam 1998) and has been shown to increase expression of IL-2Rα on T-cells (Adachi and Alam 1998, Noma, et al. 1987) in addition to augmenting the generation of cytotoxic T-cells (Adachi and Alam 1998, Nagasawa, et al. 1991), however, these reports have been labelled controversial (Adachi and Alam 1998). In an opposing report, IL-5 was shown to produce no activity on Hu B-cells using a conventional B-cell assay (Clutterbuck, et al. 1987, Murata, et al. 1992). At the time of this report, no B-cell or T-cell activity directed by IL-5 has been reported in pigs.

Primarily, IL-5 is the major cytokine required for the production of eosinophils and basophils by CD34^+ precursor cells in the BM and subsequent maturation and release into the circulatory system (Clutterbuck, et al. 1987, Lopez, et al. 1988). In addition, studies have shown that IL-5 is an important mediator necessary for recruitment of eosinophils to areas of allergic inflammation (Sylvin, et al. 2000) and parasitic infection (Culley, et al. 2002). In fact, it has been shown that mice infected with the hookworm, Necator americanus, demonstrated chemokine attraction of eosinophils to the larvae infected lung tissue and the eotaxin mediated release of IL-5 from T_{H}2 cells. This resulted in the increase of eosinophils production and release from the BM in addition to recruitment of eosinophils from neighbouring tissues to the infected area (Culley, et al. 2002, Collins, et al. 1995, Palframan, et al. 1998).

In order to initiate a response, IL-5 must first bind to the IL-5αR, (CD125), of the IL-5 receptor complex, with low affinity. CD125 then binds to CD131 resulting in a high affinity complex between the two receptor subunits.
CD125 is a glycoprotein which, like CD123, contains a 200aa conserved region homologous to the fibronectin type III domain and a membrane proximal WSXWS motif in addition to a membrane distal pair of cysteine residues (Martinez-Moczygemba and Huston 2003). The CD125 protein is made up of 420aa residues of which 20aa construct a signal peptide, 324aa form the extracellular domain, 21aa span the transmembrane domain and 55aa produce the cytoplasmic domain (Murata, et al. 1992). The molecular weight of CD125 is approximately 46kDa when un-glycosylated (Murata, et al. 1992) and approximately 80kDa when glycosylated at the 6 N-glycosylation sites required for protein folding (Murata, et al. 1992, Zaks-Zilberman, et al. 2008). In addition to membrane bound CD125, a soluble form of CD125 is also expressed and secreted by cells. It has been suggested that this activity may act as a possible antagonist thus regulating IL-5 activity and preventing over expression (Tavernier, et al. 1991).

As pigs are highly susceptible to opportunistic parasites such as Ascaris Suum (Urban, et al. 1989) and Toxoplasma gondii (Dubey 2009), the presence of parasitic infection in pig stocks can compromise the health and growth of these animals in addition to increasing the risk of Hu contamination via the food chain (Dubey 2009). The use of IL-5 in pigs as a possible therapeutic alternative to antihelminthic drug treatments may therefore exist, indicating the need to investigate this possibility (Meeusen 1999).

In addition to the suggested function of IL-3 and IL-5, it has been shown that GM-CSF is also required for the production of inflammatory cells (Martinez-Moczygemba and Huston 2003). The early acting properties of GM-CSF, as with IL-3, imply that GM-CSF may be required to initiate proliferation and/or differentiation of early acting progenitors before the actions of cytokines such as IL-5, which are required to produce end stage cells (Ogawa 1993). In order to test this hypothesis, we need to further understand the actions of GM-CSF in pigs.

1.6.3 GM-CSF and the GM-CSFα subunit, CD116

Colony stimulating factors (CSF) are cytokines that stimulate the proliferation of specific BM pluripotent stem cells (Aymard 1983). These CSFs produce progenitor cells known as CFU from HSC which give rise to granulocytes, erythrocytes, monocytes and megakaryocytes (CFU-GEMM) (Greenberger, et al. 1984) Granulocyte-CSF (G-CSF) is specific for proliferative effects on cells of the granulocyte lineage (Nomura, et al. 1986), macrophage-CSF (M-CSF) is specific for cells of the
macrophage lineage (Yamamoto-Yamaguchi, et al. 1983) however, GM-CSF has proliferative effects on both classes of myeloid cells (Yamamoto-Yamaguchi, et al. 1983). GM-CSF is extensively reportedly in studies involving therapeutic and diagnostic use in addition to vaccine development and adjuvant technology (Lanzavecchia 1993, Tao and Levy 1993, Inumaru and Takamatsu 1995). Although a plethora of information has been reported for the actions of GM-CSF in Hu and Mo, extremely little has been reported in pigs.

GM-CSF is a soluble cytokine which is structurally related to IL-3 and IL-5 due to the cytokine’s four α helical bundle formation in addition to being closely co-located in the genome (McKenzie, et al. 1993). GM-CSF is produced by several cell types in the immune system including activated Th2 cells, eosinophils, mast cells, basophils, macrophages and BM stromal cells (Martinez-Moczygemba and Huston 2003). In addition, GM-CSF is also produced by Th1 cells and some cytotoxic lymphocytes (Carson and Vignali 1999). GM-CSF has a pleiotropic effect on hematopoietic cells in addition to having an overlapping activity on hematopoietic progenitor cells with other cytokines including IL-3 and IL-5 (Barreda, et al. 2004). GM-CSF is an essential cytokine in the production of monocytes and granulocytes and enhances both proliferation of myeloid precursor cells and the function of mature peripheral blood neutrophils and mononuclear phagocytes (Antman 1990) (Figure 1.4). These results indicate that GM-CSF is produced by a large number of immune cells and plays a vital role in the development and co-ordination of the immune response. For these reasons, it is suggested that GM-CSF may be a potential therapeutic for the Po industry.

In a synergistic role with IL-3 and IL-5, GM-CSF is involved in several different immune system responses and specialised cell production. In a delayed hypersensitivity reaction, GM-CSF, when released by T cells, acts in synergy with IL-3 within the BM to stimulate monocyte production from HSC which in turn increases peripheral macrophage numbers which are then recruited to the site of inflammation (Hamilos, et al. 1995). Other roles played by GM-CSF include the up regulation of mucosal mast cells and eosinophils in response to T cell activation which also requires IL-3 and IL-5 to be secreted by the T cell (Del Prete 1992). An understanding of these processes in the pigs may contribute to an alternative to current health practices in these animals.

As with both IL-3 and IL-5, GM-CSF has its own specific α subunit, (CD116), required for the GM-CSF receptor complex with CD131 (Lanza, et al. 1997).
Figure 1.4 Cytokine driven hematopoiesis. The suggested process of mammalian hematopoiesis showing HSC self renewal and cell types for each of the lymphoid and myeloid cell lines. IL-3, IL-5 and GM-CSF involvement in hematopoiesis are indicated at several stages. Migration paths and organs of maturation are also indicated.
CD116, is also part of the FNIII family (Nakagawa, et al. 1994) and is highly homologous to the IL-3 α chain, CD123 (Moretti, et al. 2001). The full length HuCD116 molecule encodes for a 400aa, 80kDa type I transmembrane protein (Barreda, et al. 2004, Muto, et al. 1995). A 22aa signal peptide is attached to a 378aa extracellular domain, 26aa transmembrane region and a 54aa cytoplasmic domain (Nicola 1991). In addition to the WSXWS motif and four conserved cysteine residues (Nicola 1991), HuCD116 also contains 11 potential N-linked glycosylation sites reported as being essential for GM-CSF binding and signal transduction (Barreda, et al. 2004, Ding, et al. 1995), which has significantly more glycosylation sites than CD123 (5 sites) and CD125 (6 sites). The number of N-linked glycosylation sites may therefore prove important in the affinity binding of each of these cytokines to their respective α subunit and subsequent binding to CD131 (Nettleton and Kochan 1995).

Although the genetic structure and molecular pathways of the α subunits CD116, CD123 and CD125 are not the primary focus of this research, the understanding of the protein structure of these molecules may contribute to a broader understanding of the binding, control and preference of CD131 for each of these cytokine specific subunits.

Therefore, as each of the cytokines, IL-3, IL-5 and GM-CSF, are involved in the regulatory control of immune cells both in the BM and the circulatory system, they are of particular interest as possible therapeutics in the pig industry.

1.7 Therapeutic use of hematopoietic cytokines

Hematopoietic cytokines and growth factors are a family of proteins responsible for the survival, differentiation and proliferation of hematopoietic progenitor cells and downstream progeny (Wadhwa and Thorpe 2008). Although many of these cytokines share a number properties including redundancy, pleiotropy, autocrine and paracrine effects and signal transduction mechanisms, individual cytokines also have unique specialized activity (Wadhwa and Thorpe 2008). There is therefore a significant interest attached to the understanding of the role played in hematopoiesis and augmentation of the immune system by individual cytokines. Therefore, it is anticipated that cytokines may play an important role in the therapeutic and prophylactic treatment of animal health in the near future (Hubel, et al. 2002).

In 1989, Bielefeldt et al. (1989), stated that “the use of recombinant cytokines for treatment of livestock disease has not progressed very far” (Bielefeldt Ohmann, et
Indeed, since the discovery of interferon (IFN), in 1957, the notion of using a cytokine in treating disease has been contemplated, however, progression during this time was limited (Isaacs and Lindenman 1957, Vilcek and Feldmann 2004). Early trials involving the use of IFN showed some promise, however, due to the lack of modern advanced expression and purification methods, no uncontaminated preparations of a single isotype of IFN were obtained (Strander, et al. 1973). Several years later in 1996, Wood and Seow (1996), added that the "Success in the application of cytokines as therapeutics will require a better understanding of the regulation of cytokine expression and cytokine networks" (Wood and Seow 1996). It was also concluded that further studies into the mechanisms of action of cytokines on the immune system would aid in achieving better and more complete vaccines in livestock and in domestic animals as large scale models for Hu research (Wood and Seow 1996). During this period, interest in cytokines as possible therapeutics did in fact grow and by 1998 the field was reported as having undergone a “tremendous growth” (Gillis and Williams 1998). In fact by 1998, several cytokines were approved for clinical use in Hu medicine and many more were being researched (Gillis and Williams 1998), nevertheless, the use of cytokines in livestock health still seemed to be lacking.

Since 1989, expression and purification methods for recombinant proteins have advanced considerably resulting in a large number of cytokines, having successfully been demonstrated as therapeutics (Andrew, et al. 2006, Vilcek and Feldmann 2004). Indeed, numerous cytokines such as growth factors, colony stimulating factors, interleukins, SCF, erythropoietin and thrombopoietin, have been characterised and several of these have been utilised in treating Hu patients with conditions such as anaemia, malignancy and cancer (Wadhwa and Thorpe 2008).

It has therefore been demonstrated that recombinant cytokines have an application in the health market. In considering the innate immune system and the cytokines at the centre of this research, IL-3, IL-5 and GM-CSF, it is duly noted that GM-CSF is currently used in clinical practice for Hu patients (Vilcek and Feldmann 2004). In the USA glycosylated GM-CSF expressed in a yeast system and labelled as Leukine® (sargramostim), is used for the accelerated recovery of myeloid cells following chemotherapy and BM transplantation (Vilcek and Feldmann 2004). Equally, the EU has an E. Coli expressed GM-CSF, Molgramostim-Leucomax, which has been available since 1991(Vilcek and Feldmann 2004). As GM-CSF promotes the increased regulation of myeloid cells in Hu, it is anticipated that a similar response may be seen in pigs. However, it is important that the correct dosage is analysed as adverse side
effects associated with high dosage have been reported including bone pain, weight gain oedema, and inflammatory problems (Vilcek and Feldmann 2004, Lieschke, et al. 1989).

Another possible therapeutic use of hematopoietic cytokines is as an adjuvant to promote the effectiveness of vaccines (Andrew, et al. 2006, Tovey and Lallemand 2010). Research in Mo has shown that recombinant cytokines enhance the effects of adjuvants such as Freund's, although the short half-life of cytokines may limit their use as vaccine adjuvants (Tovey and Lallemand 2010). These difficulties, however, have been overcome by new administration methods including cytokine encapsulation within liposomes (Tovey and Lallemand 2010). This method has been trialled on several cytokines including GM-CSF resulting in potentiation of the immune response to vaccination in various experimental models (Tovey and Lallemand 2010). In addition, recent studies in pigs have shown the benefits of IL-3 DNA when used in promoting DNA vaccination (Andrew, et al. 2006).

Consequently, as pigs have an anatomy that is physiologically similar to Hu, it is anticipated that Po cytokines may produce similar responses in pigs as in Hu. Moreover, pigs are of growing interest as a large animal model for Hu biomedical research (Rehakova, et al. 1998, Alvarez, et al. 2000), therefore, any research into the pig immune system and its associated cytokines may also be useful in Hu health. In fact, similarities between Hu and Po adhesion molecule expression have already been identified (Summerfield and McCullough 1997, Summerfield and McCullough 1997). This suggests common functionalities in hematopoietic regulation between the two species and therefore strengthens the potential of the pig as a model for hematopoietic study.

1.8 Objectives

Currently, little is known in relation to cytokine control of hematopoiesis in pigs. A better understanding into this process may lead to improved management of pig health. Moreover, the knowledge of these processes may also provide the pig industry with an alternative treatment for immune enhancement other than the current method of in-food antibiotics. As a high degree of physiological homology has been previously determined to exist between Po and Hu, there is the possibility of cross species activity for cytokines and similar actions between mammalian species. In order to determine these similarities a detailed in silico comparison of the DNA and protein sequences as well as the phylogenetic and structural formations of each of the proteins investigated
will be assessed. Protein expression technique for each of the recombinant Po cytokines will be optimised and the biological activity resulting in cell proliferation and differentiation will be analysed both *in vitro* and *in vivo*. The cell surface markers for lymphoid and myeloid cells, CD90 and CD172a, respectively, will be utilised in flow cytometric analyses to more closely examine the complex mechanism of lymphocyte production from HSC in pigs including both the proliferation and differentiation of cells from HSC in a myelosuppressed model obtained using 5-FU. The results of this research may help to appreciate more closely the complex microenvironment of Po BM and to further understand the similarities between Hu and Po hematopoietic systems. Furthermore, this work may contribute to our perception of the innate immunity in pigs and offer an alternative method for maintaining pig health other than current antibiotic treatments.
CHAPTER 2

Characterization of GM-CSF, IL-3, and IL-5 CD131 in pigs.

2.1 Abstract

Recombinant cytokines as immunomediators and hematopoietic signalling molecules may provide an alternative means of therapeutics in swine health with the potential to reduce the current prophylactic use of antimicrobials. The cluster of cytokines, GM-CSF, IL-3 and IL-5, which interact with the CD131 subunit, are induced during immune response to infection. This makes this family of cytokines ideal candidates as potential therapeutics. We have analysed each of these cytokines of the PoCD131 subunit in the pig. DNA and protein sequences were aligned across a range of mammalian species in order to support the identification and assess cross species identity. PoGM-CSF and PoIL-5 were identified as being highly conserved between species in contrast to PoIL-3 which appeared to have a lower cross species sequence identity. In addition, DNA information was used to determine chromosomal location for these cytokines identifying the location of these three genes in close proximity to each other on Ch 2. Phylogenetic analyses indicated a close inheritance for each of these cytokines. Predicted surface conformation suggested conservation between species. Similarly, we have identified, analysed and confirmed the DNA and aa sequences, predicted structural conformation and chromosomal location of the PoCD131 subunit. In summary, we have established the identity of the Po cytokines GM-CSF, IL-3 and IL-5 along with the predicted surface conformation, chromosomal location and phylogenetics for each cytokine. It is envisioned that this information will assist in the development of new therapeutic procedures for Po cytokines. In addition we have identified the sequence, Ch location, domain organisation, predicted molecular conformation and phylogenetics for the PoCD131 subunit. This information may provide background for further studies into the binding of each of the three cytokines, along with their respective α subunits, to the CD131 subunit and the resulting cellular responses initiated.
2.2 Introduction

Previously we discussed the concerns about the use of antimicrobials in the livestock industry and the need for alternative treatments for the food animal industry. Currently, there have been a number of suggestions with regard to alternative practices such as better housing and health management (Camerlink, et al. 2010), new vaccines (Beard and Mason 1998) and even probiotics and homeopathic remedies (Gilchrist, et al. 2007). Nonetheless, the recent identification of a number of cytokines with beneficial immune enhancing effects has raised the question as to the potential to use these cytokines as possible therapeutics.

The use of selected rec cytokines which regulate a desired immune response may provide a means of boosting the immunity of animals against opportunistic pathogens. GM-CSF, IL-3 and IL-5 are cytokines which are involved in multiple signalling cascades including inflammation and differentiation of early progenitors, indicating the possible use of these cytokines in several therapeutic treatments including the up regulation and expression of specific cell types. However, as with the development of any new therapeutic approach, an in depth analysis of the suggested therapeutic needs to firstly be accomplished, so that a rational decision can be made about its potential use.

Here, multiple in silico procedures were used to compare each of the cytokines GM-CSF, IL-3 and IL-5, across a broad range of mammalian species. Each protein has been analysed for identity at both the DNA and aa levels comparing genetic features including gene length, conserved cysteine bond formation, potential glycosylation sites and predicted receptor subunit binding residues. Predicted protein folding and surface conformations have projected both similarities and differences in morphology of the molecules raising the question of possible cross species activity for each of the cytokines. Phylogenetics has been used to reveal homologous species and give an insight into a predicted direction of divergent development from the ancestral gene. In addition, identification and analysis of the PoCD131 subunit was also achieved. Sequence homology and predicted backbone and surface conformation in addition to the predicted domain organisation would suggest this molecule is highly conserved between species although differences were noted in the predicted cytokine binding sights. This suggests that CD131 maybe species specific for cytokine activation.
2.3 Methods

2.3.1 Sequence alignments

Cytokine and cytokine receptor DNA and aa sequences from various species were obtained from the National Centre for Biotechnology Information (NCBI), PubMed database. DNA and aa sequence data for PoGM-CSF, PoIL-3 and PoIL-5 were obtained from their respected publications and compared to sequence data published on the NCBI Pig Genome Resources data bank (http://www.ncbi.nlm.nih.gov/genome/guide/pig/) and on the Pre-Ensembl Pig genome database (http://pre.ensembl.org/Sus_scrofa/Info/Index). DNA sequence data for HuCD131 was obtained from the published sequence and compared to the pig data bases in order to obtain the pig sequence for this gene. All sequences collected were compared using ClustalW2, via the European Bioinformatics Institute website (http://www.ebi.ac.uk/Tools/clustalw2/index.html), and Clone Manager 9 for Microsoft windows.

2.3.2 Phylogenetics

Phylogenetic trees for each protein were generated from the aa sequence attained for each reported species. Phylogenetic trees were generated using the following sequence of sub-programs using the Australian National Genomic Information Service (ANGIS) online Bioinformatics software BioManager (http://www.angis.org.au/). ClustalW sequences were analysed by seqboot, ProDist, neighbour and consense followed by phylogenetic tree generation. This sequence of analysis was used to produce the most probably evolutionary relationship across all species investigated.

2.3.3 Predicted molecular structures of proteins

The predicted molecular structures of each of the proteins investigated was modelled from the published aa sequence obtained using SWISS-MODEL and DeepView online Workspaces (Arnold K.) and PyMol (DeLano Scientific LLC).

2.3.4 Chromosomal gene locations

Chromosomal loci locations were mapped according to locations published on the NCBI Pig Genome Resources data bank and on Pre Ensembl Pig genome database. Genes were located and intron and exon boundaries identified using published DNA sequences for comparison.
2.3.5 Common protein structure domains

The predicted common structure domains for CD131 were obtained using the online Simple Modular Architecture Research Tool (SMART) (http://smart.embl-heidelberg.de/). The aa sequences for each of the species compared were individually analysed by the SMART program for common protein domains. The resulting locations were aligned and domains and repeats compared.

2.4 Results

2.4.1 Sequence analysis of the hematopoietic cytokines

The recent release of the pig genome allows the location and identification of sequences of specific genes of interest. With this in mind, in order to confirm the identification of the Po hematopoietic cytokines GM-CSF, IL-3, IL-5 and the CD131 subunit, an in-depth in silico analysis of each of these genes was undertaken.

2.4.2 GM-CSF is highly conserved across the mammalian species

Previous work involving the cloning of PoGM-CSF has been well reported, however, since then the GM-CSF gene sequence from many of other species have been published. This new information now allows a broader investigation into the sequence homology of GM-CSF across a wider range of species. The full PoGM-CSF gene consists of 435bp and the mature portion 384bp. Alignment of the full DNA sequence against the Hu (HuGM-CSF, genebank accession number NM_000758) and mouse (Mo) (MoGM-CSF, genebank accession number NM_009969) sequences showed corresponding nucleotide regions from 33-467 for HuGM-CSF and 290-715 for MoGM-CSF representing an 81% and 70% identity, respectively (Figure 2.1). A blast search of this sequence against the Po genomic data base resulted in a match of 99.9% identity to PoGM-CSF located on Ch 2 between bp 50619629 and 50621697 (Figure 2.2). The Hu and Mo genes are located on Ch 5p31.1 (UCSC Genome Browser on Human) and 11qB1.3 (UCSC Genome Browser on Mouse), respectively. Furthermore, the predicted gene sequence was determined to be 2068bp in length and consisted of 4 exons and 3 introns (Table 2.1). Both the HuGM-CSF and MoGM-CSF also consist of 4 exons and 3 introns, however, are much longer in terms of genomic DNA being 2377bp and 2379bp in length, respectively.

The putative PoGM-CSF protein sequence when aligned against currently available mammalian sequences showed the protein to be highly conserved (Figure 2.3), with identical residues ranging from 81.2% for the red deer to 56.7% for the Mo
Porcine: ATGTGGCTGCAGAACCTGCTTCTCCTGGGCACTGTGGTCTGCAGCATCTCCGCTCCCACC
Human: ATGTGGCTGCAGAGCCTGCTGCTCTTGGGCACTGTGGCCTGCAGCATCTCTGCACCCGCC
Mouse: ATGTGGCTGCAGAATTTACTTTTCCTGGGCATTGTGGTCTACAGCCTCTCAGCACCCACC

****************   * **  ** ****** ***** ** **** **** ** *** **

Porcine: CGCCCACCCAGCCCTGTCACCCGGCCCTGGCAGCATGTGGATGCCATCAAAGAAGCCCTG
Human: CGCTCGCCCAGCCCCAGCACGCAGCCCTGGGAGCATGTGAATGCCATCCAGGAGGCCCGG
Mouse: CGCTCACCCATCACTGTCACCCGGCCTTGGAA

***********   * **  ** ****** ***** ** **** **** ** *** **

Porcine: AGCCTTCTAAACAACAGTAATGACACAGCGGCTGTGATGAATGAAACCGTAGACGTCGTC
Human: CGTCTCCTGAACCTGAGTAGAGACACTGCTGCTGAGATGAATGAAACAGTAGAAGTCATC
Mouse: AACCTCCTGGATGACATGCCTGTCAC

-------------  GTTGAATGAAGAGGTAGAAGTCGTC

**********  *    *     * ***         * ********   ***** *** **

Porcine: TGTGAAATGTTTGACCCCCAGGAGCCGACATGCGTGCAGACTCGCCTGAACCTGTACAAG
Human: TCAGAAATGTTTGACCTCCAGGAGCCGACCTGCCTACAGACCCGC
Mouse: TCTAACGAGTTCTCCTTCAAGAAGCTAACATGTGTGCAGACCCGCCTGAAGATATTCGAG

*   *   ***   *  * ** ***  ** **  * ***** ****** *  * * * **

Porcine: CAGGGCCTGCAGGGGCAGCCTCACTAGGCTCAAGCACGTCTTCTGGCAGCCACAC
Human: CAGGGCCTGCAGGGGCAAGCCTCACCAAGCTCAAGGGCCCCTTGACCATGATGGCCAGCCAC
Mouse: CAGGGTCTACGGGGCAATTTCACCAAACTCAAGGGCGCCTTGAACATGACAGCCAGCTAC

***** ** *******   **** *  ****** ** ******   **   ****   **

Porcine: TATGAGCAGCACTGCCCCCTCACCGAGAATCTTCCTGTGAAACCCAGTCTATCACCTTT
Human: TACAAGACAGCACTGCCCCCTCACCGAGAATCTTCCTGTGAAACCCAGTCTATCACCTTT
Mouse: TACCAGCATACTGGCCCTCCACATTGGAAACCCAGTCTATCACCTAT

**  **    ******* *  **   ******   ***** *** **   ** *****

Porcine: AAAAGTTTCAAAGACAGTCTGAACAAATTTCTTTTTACCATCCCCTTCTGACTGCTGGGGG
Human: GAAACTTCTAAAGAAGAACCTGAGAGGTCTTTCTGACTGCTGGGGAG
Mouse: GCGGATTTCTAAGACAGCTTAAAACTTTCTGACTGATATCCCCTTCTGACTGCTGGGGAG

***********   * **  ** ****** ***** ** **** **** ** *** **

Porcine: CCAGTCAAAAAAGTAA
Human: CCAGTCCAGGAGTGA
Mouse: CCAGGCCCCAAATGA

**** * * **  ** **

Figure 2.1 Nucleotide alignment of PoGM-CSF, HuGM-CSF and MoGM-CSF. The mature portion of PoGM-CSF (genebank accession U67175) was sequenced and its length determined to be 435bp. The sequence was compared to HuGM-CSF (genebank accession NM_000758) and MoGM-CSF (genebank accession NM_009969) and was found to correspond to the nucleotide regions of 33-467 for HuGM-CSF and 290-715 for MoGM-CSF. These regions were found to have an 81% and 70% identity, respectively, to PoGM-CSF. * refers to an identical nucleotide. Dashes (-) represent gaps introduced to optimise alignment.
Figure 2.2 Loci location of Po GM-CSF, PoIL-3 and PoIL-5 on Ch 2.
The mRNA sequences for Po GM-CSF (accession no. U67318), IL-3 (CHEF1) and IL-5 (similar to NM_214205) were compared to the Po genome and sequences with 99.9%, 100% and 99.65% homology respectfully were located. All 3 loci were identified on chromosome 2 in succession to each other. Interleukin 3 was located between Mbp 50.599179 – 50.600943, GM-CSF 50.619629 – 50.621697 and IL-5 51.095611 – 51.097285. Figure shows exons (black) and introns (white) for each gene as listed in table 2.4.
Table 2.1. Intron and exon boundaries of PoIL-3, PoGM-CSF and PoIL-5 on chromosome 2

<table>
<thead>
<tr>
<th>Cytokine number</th>
<th>Start (bp)</th>
<th>End (bp)</th>
<th>Start (bp)</th>
<th>End (bp)</th>
<th>exon length</th>
<th>intron length</th>
</tr>
</thead>
<tbody>
<tr>
<td>GM-CSF</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td>159</td>
<td>50619629</td>
<td>50619787</td>
<td>158</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>159</td>
<td>202</td>
<td>50619887</td>
<td>50619930</td>
<td>43</td>
<td>100</td>
</tr>
<tr>
<td>3</td>
<td>199</td>
<td>328</td>
<td>50620654</td>
<td>50620783</td>
<td>129</td>
<td>724</td>
</tr>
<tr>
<td>4</td>
<td>326</td>
<td>435</td>
<td>50621588</td>
<td>50621697</td>
<td>109</td>
<td>805</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>439</strong></td>
<td></td>
<td></td>
<td></td>
<td><strong>439</strong></td>
<td><strong>1629</strong></td>
</tr>
<tr>
<td>IL-3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td>159</td>
<td>50599179</td>
<td>50599337</td>
<td>158</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>160</td>
<td>195</td>
<td>50599436</td>
<td>50599471</td>
<td>35</td>
<td>99</td>
</tr>
<tr>
<td>3</td>
<td>195</td>
<td>288</td>
<td>50600470</td>
<td>50600563</td>
<td>93</td>
<td>999</td>
</tr>
<tr>
<td>4</td>
<td>287</td>
<td>325</td>
<td>50600678</td>
<td>50600716</td>
<td>38</td>
<td>115</td>
</tr>
<tr>
<td>5</td>
<td>324</td>
<td>435</td>
<td>50600832</td>
<td>50600943</td>
<td>111</td>
<td>116</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>435</strong></td>
<td></td>
<td></td>
<td></td>
<td><strong>435</strong></td>
<td><strong>1329</strong></td>
</tr>
<tr>
<td>IL-5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td>144</td>
<td>51097428</td>
<td>51097285</td>
<td>143</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>144</td>
<td>177</td>
<td>51097082</td>
<td>51097049</td>
<td>33</td>
<td>203</td>
</tr>
<tr>
<td>3</td>
<td>178</td>
<td>301</td>
<td>51096032</td>
<td>51095909</td>
<td>123</td>
<td>1017</td>
</tr>
<tr>
<td>4</td>
<td>316</td>
<td>484</td>
<td>51095790</td>
<td>51095620</td>
<td>168</td>
<td>119</td>
</tr>
<tr>
<td>5</td>
<td>493</td>
<td>529</td>
<td>51095611</td>
<td>51095575</td>
<td>36</td>
<td>9</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>503</strong></td>
<td></td>
<td></td>
<td></td>
<td><strong>503</strong></td>
<td><strong>1348</strong></td>
</tr>
</tbody>
</table>
Figure 2.3. Multiple alignment of porcine GM-CSF with GM-CSF of various species. Porcine GM-CSF amino acid sequence was aligned with known GM-CSF sequences from other species using CLUSTALW program and shows identical (*), conserved (:) and semi-conserved residues (.) residues. Boxes show conserved cysteine (C) residues. N represents possible glycosylation sites. E represents conserved glutamic acid involved in βc subunit binding. Grey shaded areas represent α subunit binding sites. ▼represents start of mature protein. Numbers represent residue number starting from the first residue of the mature protein. Dashes (-) represent gaps introduced to optimise alignment. Latin names and genebank accession numbers as follows: pig (Sus Scota, AAB06854); mouse (Mus musculus, NP_034099); cattle (Bos taurus, NP_776452); water buffalo (Bubalus bubalis, AAS59070); human (Homo sapiens, NP_000749); horse (Equus caballus, AAL41017); sheep (Ovis aries, CAA39463); goat (Capra hircus, AAY16326); rhesus monkey (R. monkey)(Macaca mulatta, NP_001028121); cat (Felis catus, AAX63391); red-deer (Cervus elaphus, AAA21439); rat (Rattus norvegicus, AAH98642); dog (Canine lupus familiaris, NP_001003245); olive baboon (O. baboon)(Papio anubis, NP_001106121); chimpanzee (Pan troglodytes, XP_527005); african green monkey (A.G. Monkey) (Chlorocebus aethiops, ABH100803).
(genebank accession number NP_034099) (Table 2.2). Identical residues for the HuGM-CSF and MoGM-CSF are relatively high at 72.2% and 56.7%, respectively, with conserved aa being 85.3% and 73.7% respectively (Table 2.2). All species GM-CSF molecules appear to be of a similar length ranging from 141 to 146aa (Table 2.2).

Cysteine residues reportedly involved in disulphide bonds are conserved in all species and are located at positions Cys$^{54}$, Cys$^{88}$, Cys$^{96}$ and Cys$^{121}$ (Figure 2.3). A further cysteine is present at position Cys$^{3}$ for 13 of the 16 species, with exception of the horse, rat and the Mo. Conserved asparagines were also identified at residues Asn$^{27}$ and Asn$^{37}$ and are probable N-glycosylation sites. The glutamic acid residue reportedly involved in βc subunit binding in Hu (Woodcock, et al. 1994) is located at Glu$^{21}$ and is conserved in all species, however, residues reported to be involved in α subunit binding in Hu (Glu$^{104}$ and Asp$^{112}$) (Hercus, et al. 1994) are not conserved amongst all species. In the pig these have been substituted by 2 Lys residues, (Lys$^{104}$ and Lys$^{112}$). Although 9 of the 16 species have a Lys$^{104}$ residue only the pig has a Lys at position 112 (Figure 2.3). This may suggest an independent divergent development from other species. A look at the predicted phylogenetic organization of all species shows three distinct branches resulting in four predicted clusters or groups (Figure 2.4). The pig is observed as being closely grouped to the cat and dog and branched away from Hu which is clustered with the primates (olive baboon, chimpanzee, African green monkey) (Figure 2.4). This suggests that PoGM-CSF has evolved along with cat and dog GM-CSF along a divergent tangent and not with HuGM-CSF.

2.4.3 Molecular structure of GM-CSF shows conserved conformation compared with HuGM-CSF

A PyMOL (DeLano Scientific LLC) interpretation of the predicted molecular configuration of the PoGM-CSF molecule however, shows a secondary structure similar in conformation to the Hu molecule with no apparent visible changes to structure (Figure 2.5). The molecule appears to consist of an arrangement of 4 α helices (labeled A-D, Figure 2.5C) and two β sheets in a tight bundle similar to the reported Hu configuration. Furthermore there appears to be two disulphide bonds formed between the Cys residues situated at positions, Cys$^{54}$ and Cys$^{96}$ and between Cys$^{88}$ and Cys$^{121}$ (Figure 2.5A). Furthermore, as with HuGM-CSF, residues predicted to be involved in α subunit binding are located on helices B and C whereas Glu$^{21}$ implicated in CD131 subunit binding, is located on helices A indicating aa substitution and not relocation (Figure 2.5C). An overlay of the surface conformation of the two molecules shows only minor differences (Figure 2.6).
<table>
<thead>
<tr>
<th>Organism</th>
<th>Common name</th>
<th>Size (aa)</th>
<th>(%)^a</th>
<th>(%)^b</th>
<th>(%)^c</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Sus Scrofa</em></td>
<td>Pig</td>
<td>144</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Cervus elaphus</em></td>
<td>Red deer</td>
<td>144</td>
<td>81.2</td>
<td>9.0</td>
<td>90.2</td>
</tr>
<tr>
<td><em>Ovis aries</em></td>
<td>Sheep</td>
<td>144</td>
<td>79.1</td>
<td>9.7</td>
<td>88.8</td>
</tr>
<tr>
<td><em>Capra hircus</em></td>
<td>Goat</td>
<td>144</td>
<td>79.1</td>
<td>9.7</td>
<td>88.8</td>
</tr>
<tr>
<td><em>Felis catus</em></td>
<td>Domestic cat</td>
<td>144</td>
<td>74.3</td>
<td>12.5</td>
<td>86.8</td>
</tr>
<tr>
<td><em>Canine lupus familiaris</em></td>
<td>Dog</td>
<td>144</td>
<td>72.9</td>
<td>13.8</td>
<td>86.7</td>
</tr>
<tr>
<td><em>Equus caballus</em></td>
<td>Horse</td>
<td>146</td>
<td>76.7</td>
<td>8.9</td>
<td>85.6</td>
</tr>
<tr>
<td><em>Homo sapiens</em></td>
<td>Human</td>
<td>144</td>
<td>72.2</td>
<td>13.1</td>
<td>85.3</td>
</tr>
<tr>
<td><em>Pan troglodytes</em></td>
<td>Chimpanzee</td>
<td>144</td>
<td>72.2</td>
<td>13.1</td>
<td>85.3</td>
</tr>
<tr>
<td><em>Macaca mulatta</em></td>
<td>Rhesus monkey</td>
<td>144</td>
<td>70.8</td>
<td>14.5</td>
<td>85.3</td>
</tr>
<tr>
<td><em>Chlorocebus aethiops</em></td>
<td>African green monkey</td>
<td>144</td>
<td>70.8</td>
<td>14.5</td>
<td>85.3</td>
</tr>
<tr>
<td><em>Bubalus bubalis</em></td>
<td>Water buffalo</td>
<td>143</td>
<td>74.3</td>
<td>10.4</td>
<td>84.7</td>
</tr>
<tr>
<td><em>Papio anubis</em></td>
<td>Olive baboon</td>
<td>144</td>
<td>70.8</td>
<td>13.8</td>
<td>84.6</td>
</tr>
<tr>
<td><em>Bos taurus</em></td>
<td>Cattle</td>
<td>143</td>
<td>73.4</td>
<td>11.1</td>
<td>84.5</td>
</tr>
<tr>
<td><em>Rattus norvegicus</em></td>
<td>Rat</td>
<td>144</td>
<td>65.2</td>
<td>12.7</td>
<td>77.9</td>
</tr>
<tr>
<td><em>Mus Musculus</em></td>
<td>Mouse</td>
<td>141</td>
<td>56.7</td>
<td>17.0</td>
<td>73.7</td>
</tr>
</tbody>
</table>

^a^ - percentage of aa identical to PoGM-CSF; ^b^ - percentage of conserved substitutions; ^c^ - total percentage of identical and conserved aa to PoGM-CSF. Species listed in order of highest total percentage of identical and conserved aa.
Figure 2.4. **Unrooted phylogenetic tree of known GM-CSF.** An unrooted phylogenetic tree was constructed based on 100 bootstrapped amino acid alignments of GM-CSF from known species using the neighbour-joining method. Shaded areas represent closely related groups highly homologous to each other.
Figure 2.5 Predicted molecular structure of PoGM-CSF. Comparison of backbone conformation of A PoGM-CSF and B HuGM-CSF as determined by PyMOL (DeLano Scientific LLC). Figures show N and C terminal residues, S-S bonds and residues predicted to be involved in receptor interactions. Figure C shows a schematical representation of the protein. Shaded areas indicate proposed areas of α and CD131 subunit interaction. Protein consists of a four α helices bundle and two S-S bonds.
Figure 2.6 Predicted surface structure of PoGM-CSF. Comparison of predicted surface conformation of (A) PoGM-CSF and (B) HuGM-CSF as determined by PyMOL (DeLano Scientific LLC). Figure C shows an overlay of the two molecules with HuGM-CSF being shown in mesh form to show conformational differences between the two proteins. Figure D shows an overlay of the two molecules with PoGM-CSF being shown in mesh form to show conformational differences between the two proteins. Arrows indicate predicted differences in conformation between GM-CSF molecules. PoGM-CSF is shown in red, HuGM-CSF is shown in green.
2.4.4 Pig IL-3 shows low sequence homology to other species

The sequence for Po IL-3 was originally designated chimeric enhancement factor 1 (CHEF1) (ATCC accession number 75567). The cloning and sequencing of the mature portion of the PoIL-3 gene from cDNA from large white landcross pigs allowed the sequence alignment and identification of CHEF1 as IL-3 (Figure 2.7).

This sequence, when aligned against the Hu (genebank accession number NM_000588) and Mo (genebank accession number NM_010556) sequences, corresponded with the regions of 54-503bp and 51-473bp respectively. Furthermore, IL-3 was found to have only 55% and 33% nucleotide identity to HuIL-3 and MoIL-3 which is considered low (Figure 2.8). A blast search of this sequence against the Po genomic data base resulted in a matching sequence of 100% identity to IL-3 located on Ch two between bp 50599179 and 50600943. This loci is located downstream of the GM-CSF gene (Figure 2.2). Similarly, the HuIL-3 gene is also located downstream from the HuGM-CSF gene, however, is located on Chs 5 (UCSC Hu Genome Browser).

Furthermore, the predicted gene sequence was determined to have 5 exons and 4 introns and spans 1764bp in length (Table 2.1). Likewise, the gene structure for HuIL-3 is similar, however, the HuIL-3 covers a length of 2,676 bp.

The deduced protein sequence of 144aa when aligned against other species shows a large variance with only 14 residues being conserved across all species (Figure 2.9). The percentage of identical residues for IL-3 ranged from 47.9% for the water buffalo to 27.1% for western gorilla, with those for the Hu and Mo being only 36.8% and 27.8%, respectively, (Table 2.3). The N-glycosylation sites previously reported for HuIL-3 (Goulder, et al. 2000) are located at positions Asn36 and Asn94 for the Hu, however, in the pig it is located at Asn37 and Asn94 (Figure 2.9). Neither of these residues are conserved in all species, however, a conserved Asn residue was present at position Asn79 in all species and may also be a possible N-glycosylation site. An additional Asn residue was situated at position Asn101 in the pig, sheep, cattle, buffalo and dog sequences and may also be a possible N-glycosylation site. Furthermore, the two reported cysteine residues involved in S-S bond formation in HuIL-3, Cys38 and Cys108, (Yang, et al. 1986) were not present in the pig, sheep, cattle, water buffalo or dog although these appear to be conserved in the primates, Mo and rat (Figure 2.9). In addition, the Glutamic acid residue, reportedly involved in CD131 subunit binding in Hu (Glu22) (Barry, et al. 1994) was located at position 44 and was conserved in all species. Residues reported to be involved in CD123 binding in Hu (Asp21, Glu43, Asp84, Arg94 and Lys110)(Olins, et al. 1995) align with pig residues Glu22,
Figure 2.7 Nucleotide alignment of PoIL-3 derived from large white landrace cross pigs and CHEF1. The mature portion of PoIL-3 cloned from Large White Landrace Cross pigs was sequence analysed and its length observed at 435bp. The sequence was then compared to IL-3 derived from miniature swine (Chimeric Enhancement Factor 1, CHEF1, ATCC Accession number 75567). The corresponding nucleotide regions were found to have a 100% identity. * refers to an identical nucleotide. Numbers represent sequence length.
**Figure 2.8** Nucleotide alignment of PoIL-3, HuIL-3 and MoIL-3. The mature portion of PoIL-3 was sequenced and its length observed at 435bp. The sequence was then compared to HuIL-3, (genebank accession NM_000588) and MoIL-3, (genebank accession NM_010556). The corresponding nucleotide regions from 54-503 for HuIL-3 and 51-473 for MuIL-3 were found to have a 55% and 33% identity, respectively, to PoIL-3. * refers to an identical nucleotide. Numbers represent sequence length. Dashes (-) represent gaps introduced to optimise alignment.
Figure 2.9. Multiple alignment of PoIL-3 with IL-3 of various mammalian species.

Porcine IL-3 amino acid sequence was aligned with known mammalian IL-3 sequences from other species using CLUSTALW program and shows identical (*), conserved (:), and semi-conserved residues (.). The boxed sections represent possible cysteine residues involved in s-s bonds. N represents conserved asparagine residues and possible N-glycosylation sites. E represents conserved glutamic acid involved in βc subunit binding. Grey shade areas represent possible residues involved in α subunit binding. ▼ represents start residue. Numbers represent residue positions within the sequence. Dashes (-) represent gaps introduced to optimise alignment. Accession numbers: Pig (Sus scrofa, AAB06854); Sheep (Ovis aries, NP_001009420); Cattle (Bovis taurus, NP_776345); Buffalo (water buffalo, Bubalus bubalis, AAS59069); Dog (Canine lupus familiaris, AAK37958); Human (Homo sapiens, NP_000749); Gorilla (western gorilla, Gorilla gorilla, AAM76638); Orangutun (bornean orangutun, Pongo pygmaeus, AAM76639); Monkey (rhesus monkey, Macaca mulatta, NP_001095204); Baboon (olive baboon, Papio anubis, ABW96793); Tamarin (cotton-top tamarin, Saguinus oedipus, CAA52865); Marmoset (white-tufted-ear marmoset, Callithrix jacchus, CAA52864); Titi (red-bellied titi, Callicebus moloch, ABC21276); Mouse (Mus musculus, NP_034686); Rat (Rattus norvegicus, NP_113701).
Table 2.3  Comparison of PoIL-3 aa sequence against IL-3 of other mammalian species.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Common name</th>
<th>Size (aa)</th>
<th>(%)^a</th>
<th>(%)^b</th>
<th>(%)^c</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sus Scofa</td>
<td>Pig</td>
<td>144</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Bubalus bubalis</td>
<td>Water buffalo</td>
<td>144</td>
<td>47.9</td>
<td>22.9</td>
<td>70.8</td>
</tr>
<tr>
<td>Ovis aries</td>
<td>Sheep</td>
<td>146</td>
<td>46.5</td>
<td>24.3</td>
<td>70.8</td>
</tr>
<tr>
<td>Canine lupus familiaris</td>
<td>Dog</td>
<td>143</td>
<td>46.5</td>
<td>21.5</td>
<td>68.0</td>
</tr>
<tr>
<td>Bos taurus</td>
<td>Cattle</td>
<td>146</td>
<td>39.6</td>
<td>24.3</td>
<td>63.9</td>
</tr>
<tr>
<td>Callithrix jacchus</td>
<td>White-tufted-ear marmoset</td>
<td>142</td>
<td>34.0</td>
<td>20.8</td>
<td>54.8</td>
</tr>
<tr>
<td>Homo sapiens</td>
<td>Human</td>
<td>152</td>
<td>36.8</td>
<td>17.7</td>
<td>54.5</td>
</tr>
<tr>
<td>Macaca mulatta</td>
<td>Rhesus monkey</td>
<td>143</td>
<td>37.5</td>
<td>15.3</td>
<td>52.8</td>
</tr>
<tr>
<td>Papio anubis</td>
<td>Olive baboon</td>
<td>143</td>
<td>36.8</td>
<td>15.9</td>
<td>52.7</td>
</tr>
<tr>
<td>Callicebus moloch</td>
<td>Red-bellied titi</td>
<td>144</td>
<td>36.8</td>
<td>15.9</td>
<td>52.7</td>
</tr>
<tr>
<td>Rattus norvegicus</td>
<td>Norway rat</td>
<td>169</td>
<td>26.1</td>
<td>23.6</td>
<td>49.7</td>
</tr>
<tr>
<td>Mus Musculus</td>
<td>Mouse</td>
<td>166</td>
<td>27.8</td>
<td>20.8</td>
<td>48.6</td>
</tr>
<tr>
<td>Saguinus oedipus</td>
<td>Cotton-top tamarin</td>
<td>142</td>
<td>31.2</td>
<td>15.3</td>
<td>46.5</td>
</tr>
<tr>
<td>Pongo pygmaeus</td>
<td>Bornean orangatun</td>
<td>104</td>
<td>25.7</td>
<td>15.3</td>
<td>41.0</td>
</tr>
<tr>
<td>Gorilla gorilla</td>
<td>Western gorilla</td>
<td>104</td>
<td>27.1</td>
<td>13.8</td>
<td>40.9</td>
</tr>
</tbody>
</table>

^a - percentage of aa identical to PoIL-3; ^b - percentage of conserved substitutions; ^c - total percentage of identical and conserved aa to PoIL-3. Species listed in order of highest total percentage of identical and conserved aa.
Asn\textsuperscript{41}, Glu\textsuperscript{42}, Glu\textsuperscript{91} and Lys\textsuperscript{107}, respectively, however, these residues are not conserved between species although Lys\textsuperscript{107} is conserved amongst all species (Figure 2.9). The predicted phylogenetic organization of IL-3 for all available species shows 5 distinct branches resulting in 5 predicted groups (Figure 2.10). PoIL-3 is grouped with cattle, water buffalo, sheep and dog and appears to have developed separately from huIL-3 which is grouped with the larger of the primates (orang-utan and gorilla) (Figure 2.10). The collective differences in residues and low cross species homology suggest PoIL-3 maybe an orthologue which has developed divergently from Hu and primates.

### 2.4.5 Molecular structure of PoIL-3 suggests altered conformation compared to HuIL-3

Using PyMol to analyse the predicted secondary structure of PoIL-3 compared to that of HuIL-3 appears to suggest visible changes to the confirmation including a possible additional α helix situated between Thr\textsuperscript{104} and Glu\textsuperscript{109} (Figure 2.11A). The HuIL-3 molecule consists of five α helices which have been labelled A-E (Figure 2.9C). A shorter C helic is also present in the PoIL-3 protein which extends from residues Arg\textsuperscript{71} to Phe\textsuperscript{80} being only 10aa long (Figure 2.11A) compared to 13aa long in the Hu which extends from Arg\textsuperscript{74} to Ser\textsuperscript{86} (Figure 2.11B). This S-S bond seen in the Hu molecule at Cys\textsuperscript{38} and Cys\textsuperscript{108} is not present. There are also visible differences in the backbone conformation of the protein. An overlay of the predicted surface conformation of the two molecules shows distinctive differences in the surface area and configuration (Figure 2.12). In addition, PoIL-3 appears to be more compact or tightly arranged as to produce a smaller molecule (Figure 2.12C).

### 2.4.6 PoIL-5 shows high sequence homology and conserved residues across mammalian species

The PoIL-5 gene was cloned and sequence from Large Landrace white pigs and compared to the HuIL-5 and MoIL-5 DNA sequences. The sequence was found to correspond to the nucleotide regions from 45-449 for HuIL-5 and 44-445 for MoIL-5 and had a sequence identity of 81% and 74%, respectively, (Figure 2.13). A search against the Po genomic data base resulted in a matching sequence of 99.65% identity to IL-5 located on Ch 2 between bp 51097428 and 51095575 situated upstream of the GM-CSF and IL-3 genes (Figure 2. 2). In contrast, the HuIL-5 and MoIL-5 genes are located on Chs 5p31.1 and 11qB1.3, respectively. Furthermore, the predicted gene sequence was determined to have 5 exons and 4 introns and span 1851bp in length.
Figure 2.10 Unrooted phylogenetic tree of known mammalian IL-3. An unrooted phylogenetic tree was constructed based on 100 bootstrapped amino acid alignments from IL-3 from known species using the neighbour-joining method. Shaded areas represent closely related groups highly homologous to each other.
Figure 2.11 Predicted molecular structure of PoIL-3. Comparison of backbone conformation of (A) PoIL-3 and (B) HuIL-3 as determined by PyMOL (DeLano Scientific LLC). Figure shows N and C terminal residues, proposed conformational changes and predicted residues involved in receptor interactions. Figure (C) shows a schematical representation of the human protein colored by secondary structure, including proposed areas of receptor subunit interaction.
Figure 2.12 Predicted surface structure of PoIL-3. Comparison of predicted surface conformation of (A) PoIL-3 and (B) HuIL-3 as determined by PyMOL (DeLano Scientific LLC). Figure C shows an overlay of the two molecules with HuIL-3 being shown in mesh form to show conformational differences between the two proteins. Figure D shows an overlay of the two molecules with PoIL-3 being shown in mesh form to show conformational differences between the two proteins. Arrows indicate predicted differences in conformation between IL-3 molecules. PoIL-3 is shown in red, HuIL-3 is shown in green.
Pig
---
ATGAGAATGCTTCTGCATTGGCTTTGCTAGGTCTTGGAGCTGCCTACGTTAGTGCC
Human
---
ATGAGAAAGGATGCTTCTGCATTGGCTTTGCTAGCTCTTGGAGCTGCCTACGTGTATGCC
Mouse
ATGAGAAGGATGCTTCTGCATTGGCTTTGCTACGTTAGTGCC

Mouse
CTGTGTCTGGGCC

Pig
ATTGCTGTAGAAAATACCATGAATAGACTGGTGGCAGAGACCTTGACACTGCTCTCCATT
Human
ATCCCCACAGAAATTCCCAACAGTTGGAATTGCTTGGAAGAAGAGACCTTGACACTGCTCTCCATT
Mouse
ACGGACCTTGCTTGGCACTGGAAGTTGGAGCTGCCTACGCTTTCTACT

Mouse
CTGTGTCTGGGCC

Pig
CATCGAACTCTGCTGATAGGCGATGGGAACTTGATGATTTCAACTCCTGTACATACAAAT
Human
CATCGAACTCTGCTGATAGCCAATGAGACTCTGAGGATTCCTGTTCCTGTACATACAAAT
Mouse
CACCGAGCTCTGTTGCAACAGCAATGAGACGATGAGGCTTCCTGTCCCTACTCATAAAAT

Mouse
CTGTGTCTGGGCC

Pig
CATCGAACTCTGCTGATAGGCGATGGGAACTTGATGATTTCAACTCCTGTACATACAAAT
Human
CATCGAACTCTGCTGATAGCCAATGAGACTCTGAGGATTCCTGTTCCTGTACATACAAAT
Mouse
CACCGAGCTCTGTTGCAACAGCAATGAGACGATGAGGCTTCCTGTCCCTACTCATAAAAT

Mouse
CTGTGTCTGGGCC

Pig
CGAGGGGATGCCGTGGAAAAACTATTCAAAAACTTGTCCTTAATAAAAGAATATATAGAC
Human
CAAGGGGGTACTGTGGAAAGACTATTCAAAAACTTGTCCTTAATAAAAGAATATATAGAC
Mouse
CGTGGGGGTACTGTGGAAATGCTATTCAAAAACTTGTCCTTAATAAAAGAATATATAGAC

Mouse
CTGTGTCTGGGCC

Pig
CGCCAAAAAAAAATTGTGGAGGGAAAGA
Human
GGCCAAAAAAAAATTGTGGAGGGAAAGA
Mouse
CGCCAAAAAAAAATTGTGGAGGGAAAGA

Figure 2.13  Nucleotide alignment of PoIL-5, HuIL-5 and MoIL-5. The mature portion of PoIL-5 (similar to genebank accession NM_214205) was sequence analysed and its length observed at 405bp. The sequence was then compared to HuIL-5 (genebank accession NM_000879) and MoIL-5 (genebank accession NM_010558). The corresponding nucleotide regions from bp 45-449 for HuIL-5 and bp 44-445 for MuIL-5 were found to have 81% and 74% identity respectively to PoIL-5 as shown. * refers to an identical nucleotide.
(Table 2.1), as opposed to the Hu and Mo genes which both consist of 4 exons and 3 introns and are 2079bp and 4310bp in length, respectively.

Alignment with current available IL-5 sequences from other mammalian species revealed that the protein was highly conserved (Figure 2.14). Cysteine residues reportedly involved in homodimer disulphide bonds were located in all species at position Cys$^{65}$ and Cys$^{107}$. Several asparagine residues are conserved amongst the different species but only one was located in all species, Asn$^{92}$, suggesting a probable N-linked glycosylation site. Other possible N-linked glycosylation sites within the pig protein are Asn$^{29}$ (present in 7/16 species), Asn$^{51}$ (present in 7/16 species), Asn$^{61}$ (present in 15/16 species with exception of the mangabey) and Asn$^{129}$ (present in 12/16 species). In addition, a further site not found in the pig but present in the Hu sequence was Asn$^{492}$. This residue was present in the 9/16 species which do not have the Asn$^{51}$ residue (Figure 2.14). The mature protein started with an alanine residue at position 22. The glutamic acid residue reported in the Hu protein as being involved in CD131 subunit binding (Glu$^{12}$) (Hercus, et al. 1994, Lopez, et al. 1992) was located at position 34 and was conserved over all species. Residues reported in HuIL-5 to be involved in CD125 subunit binding (His$^{37}$, Lys$^{38}$, His$^{40}$, Glu$^{88}$, Arg$^{90}$, Thr$^{108}$, Glu$^{109}$, Trp$^{110}$ and Ile$^{112}$) (Graber, et al. 1995, Tavernier, et al. 1995) were located at positions 59, 60, 62, 110 112, 130, 131, 132 and 134 and correspond to the Po residues His$^{37}$, Thr$^{38}$, His$^{40}$, Glu$^{88}$, Trp$^{90}$, Thr$^{108}$, Glu$^{109}$, Trp$^{110}$ and Met$^{112}$, respectively (Figure 2.14, 2.15). From these residues only; His$^{40}$, Glu$^{88}$, Thr$^{108}$, Glu$^{109}$ and Trp$^{110}$ were identically conserved across all species, however, His$^{37}$ was conserved in 15/16 species with only the dog residue differing (substituted by an asparagine). Furthermore the arginine residue located at position 112 is conserved in Hu, primates and the rodents but has been replaced by a tryptophan residue in the pig and remaining species (Figure 2.14). A comparison of identical residues between animals showed the conserved identities of HuIL-5 (genebank accession number NP_000870) and the MoIL-5 (genebank accession number NP_034688) were 78.3% and 73.6%, respectively, when compared to PoIL-5, however, cattle (genebank accession number P52173) and cat (genebank accession number AAC64505) were closest in identity with each being 87.3% identical to PoIL-5 (Table 2.4). Furthermore, the predicted phylogenetic organization, which like IL-3 produced 5 possible groups, suggests that PoIL-5 was closely related to the cat and dog in development (Figure 2.15).
<table>
<thead>
<tr>
<th>Pig</th>
<th>MR-MLHLSLGLGLAGAVYSIAAVQ-PHNLVAETTLLSIHTLIGCGLIGLLMSTPVHTEGFLVGTMD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffalo</td>
<td>MR-MLHLTLVALGAAYVCANAVES-PHNLVAETTLLSIHTLIGCGLIGLLMPSPQHT</td>
</tr>
<tr>
<td>Cattle</td>
<td>MR-MLHLTLVALGAAYVCANAVES-PHNLVAETTLLSIHTLIGCGLIGLLMPTPEHNN</td>
</tr>
<tr>
<td>Sheep</td>
<td>---MLHLTLVALGAAYVCANAVES-PHNLVAETTLLSIHTLIGCGLIGLLMPFRQHT</td>
</tr>
<tr>
<td>Cat</td>
<td>MR-MLHLSLALGALGAAYSAFAVEN-PHNLVAFTTLTLSTHRWLGGLIGLMPTPEHK</td>
</tr>
<tr>
<td>Horse</td>
<td>MR-MLHLSVLALGALGAAYSAFAVEN-PHNLVAETTLLSIHTLIGCGLIGLLMPTPEH</td>
</tr>
<tr>
<td>Guinea</td>
<td>MR-MLHLSLALGALGAAYSAFAVEN-PHNLVAETTLLSIHTLIGCGLIGLLMPFRQHT</td>
</tr>
<tr>
<td>Rhesus</td>
<td>MR-MLHLLSIHALGAAYSAFAVEN-PHNLVAFTTLTLSTHRWLGGLIGLMPTPEHNN</td>
</tr>
<tr>
<td>Human</td>
<td>MR-MLHLSLALGALGAAYSAFAVEN-PHNLVAFTTLTLSTHRWLGGLIGLMPTPEHNN</td>
</tr>
<tr>
<td>Mangabey</td>
<td>MR-MLHLSLALGALGAAYSAFAVEN-PHNLVAFTTLTLSTHRWLGGLIGLMPTPEHNN</td>
</tr>
<tr>
<td>C.s. Monkey</td>
<td>MR-MLHLSLALGALGAAYSAFAVEN-PHNLVAFTTLTLSTHRWLGGLIGLMPTPEHNN</td>
</tr>
<tr>
<td>Rat</td>
<td>MR-MLCLNLVTLTSCVWAAMTEIPMSTVKEETLQLSTHALLLSETMTRLVPVTTH</td>
</tr>
<tr>
<td>Mouse</td>
<td>MR-MLHLGLVLTLSCVWAAMTEIPMSTVKEETLQLSRAHLLLSETMTRLVPVTTH</td>
</tr>
<tr>
<td>Hispid Rat</td>
<td>MR-MLHLGLVLTLSCVWAAMTEIPMSTVKEETLQLSRAHLLLSETMTRLVPVTTH</td>
</tr>
<tr>
<td>Gerbil</td>
<td>MR-LPLQLSILTLAWVNAVAEEIMSAVKEETLQLSRAHLLLSETMTRLVPVTTH</td>
</tr>
</tbody>
</table>

### Comparison Alignments

<table>
<thead>
<tr>
<th>10</th>
<th>20 ▼</th>
<th>30</th>
<th>40</th>
<th>50</th>
<th>60</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pig</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Buffalo</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cattle</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sheep</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cat</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Horse</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Guinea</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rhesus</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mangabey</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C.s. Monkey</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rat</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mouse</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hispid Rat</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gerbil</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Sequence Alignments

<table>
<thead>
<tr>
<th>Pig</th>
<th>LQVFGLVINTNHVTEES</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffalo</td>
<td>LQVFGLVINTNHVTEES</td>
</tr>
<tr>
<td>Cattle</td>
<td>LQVFGLVINTNHVTEES</td>
</tr>
<tr>
<td>Sheep</td>
<td>LQVFGLVINTNHVTEES</td>
</tr>
<tr>
<td>Cat</td>
<td>LQVFGLVINTNHVTEES</td>
</tr>
<tr>
<td>Dog</td>
<td>LQVFGLVINTEHHTEG</td>
</tr>
<tr>
<td>Horse</td>
<td>LQVFGLVINTNHHIEG</td>
</tr>
<tr>
<td>Guinea</td>
<td>LQEFGLVMINTNHIEG</td>
</tr>
<tr>
<td>Rhesus</td>
<td>LQEFGLVMINTNHIEE</td>
</tr>
<tr>
<td>Human</td>
<td>LQEFGLVMINTNHIEE</td>
</tr>
<tr>
<td>Mangabey</td>
<td>LQEFGLVMINTNHIEE</td>
</tr>
<tr>
<td>C.s. Monkey</td>
<td>LQEFGLVMINTNHIEE</td>
</tr>
<tr>
<td>Rat</td>
<td>LQEFGLVSTENHMV</td>
</tr>
<tr>
<td>Mouse</td>
<td>LQEFGLVSTENMEG</td>
</tr>
<tr>
<td>Hispid Rat</td>
<td>LQEFGLVSTENMEH</td>
</tr>
<tr>
<td>Gerbil</td>
<td>LQEFGLVSTENMEG</td>
</tr>
</tbody>
</table>

### Amino Acid Alignments

<table>
<thead>
<tr>
<th>Pig</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffalo</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cattle</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sheep</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cat</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dog</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Horse</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Guinea</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rhesus</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mangabey</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C.s. Monkey</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rat</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mouse</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hispid Rat</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gerbil</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 2.14 Multiple alignment of Po IL-5 with IL-5 of various mammalian species.

Porcine IL-5 amino acid sequence was aligned with known mammalian IL-5 sequences from other species using CLUSTALW program and shows identical (*), conserved (:) and semi-conserved residues (.). Boxes show conserved C residues. N refers to conserved asparagine residue. E represents conserved glutamic acid residue involved in \(\beta_c\) subunit binding. Grey shaded amino acids represent residues involved in \(\alpha\) subunit binding. ▼ indicates the start of the mature protein. Dashes (-) represent gaps introduced to optimise alignment. Numbers represent residue number. Latin names are in brackets; genebank accession no: pig (S. Scofa, NP_999370); water buffalo (B. bubalis, ABO16236); cattle (B. taurus, P52173); sheep (O. aries, Q28586); domestic cat (F. catus, AAC64505); dog (C. lupus familiaris, Q95J76); Horse (E. caballus, NP_001075968); guinea pig (Cavia porcellus, AAB61357); rhesus monkey (M. mulatta, AAA86710); human (H. sapiens, NP_000870); sooty mangabey (Cercocebus. torquatus atys, P46685); common squirrel monkey (c.s.monkey) (Saimiri sciureus, AAK92043); rat (R. norvegicus, NP_034688); mouse (M. musculus, NP_034688); hispid cotton rat (Sigmodon hispidus, AAG16722); Mongolian gerbil (Meriones unguiculatus, Q62575).
Figure 2.15 Unrooted phylogenetic tree of known IL-5. An unrooted phylogenetic tree was constructed based on 100 bootstrapped amino acid alignments of IL-5 from known species using the neighbour-joining method. Shaded areas represent closely related groups highly homologous to each other.
Table 2.4 Comparison of PoIL-5 aa sequence against IL-5 of other mammalian species.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Common name</th>
<th>Size (aa)</th>
<th>(%)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>(%)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>(%)&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sus Scofa (landrace)</td>
<td>Pig</td>
<td>134</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Sus Scofa (yorkshire)</td>
<td>Pig</td>
<td>134</td>
<td>100</td>
<td>-</td>
<td>100</td>
</tr>
<tr>
<td>Bos taurus</td>
<td>Cattle</td>
<td>134</td>
<td>87.3</td>
<td>5.2</td>
<td>92.5</td>
</tr>
<tr>
<td>Felis catus</td>
<td>Cat</td>
<td>134</td>
<td>87.3</td>
<td>5.2</td>
<td>92.5</td>
</tr>
<tr>
<td>Bubalus bubalis</td>
<td>Water buffalo</td>
<td>134</td>
<td>86.5</td>
<td>5.9</td>
<td>92.4</td>
</tr>
<tr>
<td>Canine lupus familiaris</td>
<td>Dog</td>
<td>134</td>
<td>85.0</td>
<td>7.4</td>
<td>92.4</td>
</tr>
<tr>
<td>Ovis aries</td>
<td>Sheep</td>
<td>132</td>
<td>84.1</td>
<td>7.5</td>
<td>91.6</td>
</tr>
<tr>
<td>Equus caballus</td>
<td>Horse</td>
<td>134</td>
<td>85.0</td>
<td>3.7</td>
<td>88.7</td>
</tr>
<tr>
<td>Cercocebus torquatus atys</td>
<td>Sooty mangabey</td>
<td>134</td>
<td>68.6</td>
<td>10.4</td>
<td>79.0</td>
</tr>
<tr>
<td>Cavia porcellus</td>
<td>Guinea pig</td>
<td>135</td>
<td>67.4</td>
<td>11.1</td>
<td>78.5</td>
</tr>
<tr>
<td>Homo sapiens</td>
<td>Human</td>
<td>134</td>
<td>69.4</td>
<td>8.9</td>
<td>78.3</td>
</tr>
<tr>
<td>Macaca mulatta</td>
<td>Rhesus monkey</td>
<td>134</td>
<td>68.6</td>
<td>8.9</td>
<td>77.5</td>
</tr>
<tr>
<td>Saimiri sciureus</td>
<td>Common squirrel monkey</td>
<td>134</td>
<td>69.4</td>
<td>7.4</td>
<td>76.8</td>
</tr>
<tr>
<td>Sigmodon hispidus</td>
<td>Hispid cotton rat</td>
<td>132</td>
<td>62.1</td>
<td>12.8</td>
<td>74.9</td>
</tr>
<tr>
<td>Meriones unguiculatus</td>
<td>Mongolian gerbil</td>
<td>132</td>
<td>62.1</td>
<td>12.8</td>
<td>74.9</td>
</tr>
<tr>
<td>Mus Musculus</td>
<td>Mouse</td>
<td>133</td>
<td>60.1</td>
<td>13.5</td>
<td>73.6</td>
</tr>
<tr>
<td>Rattus norvegicus</td>
<td>Rat</td>
<td>132</td>
<td>58.3</td>
<td>13.6</td>
<td>71.9</td>
</tr>
</tbody>
</table>

\(\text{\%}^a\) - percentage of aa identical to IL-5; \(\text{\%}^b\) - percentage of conserved substitutions; \(\text{\%}^c\) - total percentage of identical and conserved aa to PoIL-5. Species listed in order of highest total percentage of identical and conserved aa.
2.4.7 The predicted molecular structure of PoIL-5 shows similar conformation to HuIL-5

As with the HuIL-5 monomer, the predicted PoIL-5 monomer consists of a 4 α helices bundle with the C and N terminals finishing central to the protein (Figure 2.16). Only minor visible difference are noted including a shorter α helix A at the N-terminal of the molecule of 4 residues compared to the Hu form (Figure 2.16). There were no visible disulphide bonds present in the monomer formed by the two conserved cysteine residues indicating these were not required for maintaining monomer configuration suggesting the tertiary structure may depend on hydrophobic and hydrophilic charges. An overlay of the PoIL-5 monomer against HuIL-5 shows the two proteins were similar in conformational arrangement with only minor differences in surface conformation noted (figure 2.17)

The predicted configuration of the PoIL-5 homodimer, when modelled, had the C-terminal of monomer 1 in close proximity to the N-terminal of the monomer 2. Disulphide bonds reported in the HuIL-5 homodimer (Goulder, et al. 2000) appeared to be present between Cys\textsuperscript{63} from monomer 1 and Cys\textsuperscript{105} from monomer 2 and from Cys\textsuperscript{105} from monomer 1 with Cys\textsuperscript{63} from monomer 2 (Figure 2.18A). These bonds appear to be formed from the N-terminal end of α helices b to the C-terminal end of α helices c for each molecule therefore placing the monomers in opposite directions bringing the predicted residues involved in CD125 subunit interactions into close proximity (Figure 2.18B). The conserved residues predicted in CD131 subunit binding were located on α helix a of each monomer, therefore, suggesting the molecule may interact with the receptor in one of two directions.

2.4.8 Identification of a predicted gene sequence for the pig CD131 subunit

The class 1 cytokine receptor CD131, complexes with GM-CSF, IL-3 and IL-5, along with their respective α subunits. The published Hu CD131 molecule, a mRNA of 2670 bp, was blast searched against the pig genomic data base, in order to locate a possible gene sequence for the pig CD131 subunit. A homologous genomic sequence was located on Ch 5 between bp 5208502-5222721 (Figure 2.19), which was determined to be 14219bp in length. Although this is shorter than the Hu gene of 16597bp, which was located at Ch 22q13.1, a sequence comparison of this gene against both the Hu and Mo coding regions, for the CD131 subunit, revealed a suggested mRNA measuring 2376bp in length with an identity of 77% and 55%, to the
Figure 2.16 Predicted molecular structure of PoIL-5 monomer. Comparison of backbone conformation of (A) PoIL-5 monomer and (B) HuIL-5 monomer, colored by secondary structure as determined by PyMOL (DeLano Scientific LLC). Figures A and B shows start and stop residues and residues proposed to be involved in receptor subunit interaction. Figure C shows a schematical representation of the IL-5 protein, colored by secondary structure, including delegated helices labels. Shaded areas indicate residues proposed to be involved in receptor subunit interaction.
Figure 2.17 Predicted surface structure of PoIL-5. Comparison of predicted surface conformation of (A) PoIL-5 and (B) HuIL-5 as determined by PyMOL (DeLano Scientific LLC). Figure C shows an overlay of the two molecules with HuIL-5 being shown in mesh form to show conformational differences between the two proteins. Figure D shows an overlay of the two molecules with PoIL-5 being shown in mesh form to show conformational differences between the two proteins. Arrows indicate predicted differences in conformation between IL-5 molecules. PoIL-5 is shown in red, HuIL-5 is shown in green.
Figure 2.18 Predicted molecular structure of the PolL-5 homodimer. Conformation of (A) IL-5 homodimer, as determined by Pymol. Figure shows N and C terminals, disulphide bonds (yellow) and residues involved in receptor subunit interaction. Each monomer has been given a solid color to determine configuration. aa involved in Cys bond formation and subunit interaction are labelled by aa single letter abbreviation. (B) Schematical representation of IL-5 homodimer. Diagram shows labelled α helices (a- d), disulphide bonds (S-S), and N and C terminals. Shaded areas represent proposed receptor subunit interaction sites as labelled.
The mRNA sequence of the human colony stimulating factor 2 receptor, β, (The pig CD131 subunit sequence was located on pig chromosome 5 between Mbp 5.208502-5.222721. The gene measured 2376bp in length and was determined to consist of 15 exons (black) and 14 introns (white) as listed in table 2.5. The sequence was found to have 77% identity to human CD131 (genebank accession number BC070085) and 55% to the mouse CD131 (genebank accession number NM_007780).
Hu and Mo sequences, respectively (Figure 2.20). Furthermore, the gene was determined to consist of 15 exons and 14 introns (Table 2.5) which was similar to the Hu gene.

As the conserved identities between Hu and pig for this sequence are considered high, the gene sequence was subjected to further bioformatical analysis to confirm the likelihood of this gene as being the pig CD131 subunit orthologue.

2.4.9 Analysis of PoCD131 subunit shows sequence identity and conserved residues across all mammalian species

The predicted ORF of the PoCD131 subunit revealed a protein sequence consisting of a total of 791 aa with a non-glycosylated molecular mass of 86.9 kDa (Figure 2.21). This was smaller than the 889 aa HuCD131 subunit of 96.1 kDa and the 896 aa Mo CD131 subunit of 98.9 kDa. It was also considerably shorter than the dog CD131 subunit being the longest at 945 aa (Table 2.6). Furthermore, the aa sequence when aligned against other known species, shows a varied level of identity ranging from 75% for cattle to 54.1% for the rat (Figure 2.21, Table 2.6). Residue alignment with the Hu CD131 subunit sequence results in a predicted extracellular domain of 409 aa which proceeds a 24 aa signal peptide. The WSXWS common motif, which is conserved in class 1 cytokine receptors, is present at position 493-497 and includes a lysine residue as the variable aa differing to from the Hu molecule which expresses a glutamic acid. The presence of this motif confirms the protein as a class 1 cytokine receptor. There are also 15 conserved cysteine residues between species however, only 14 of these are in the pig sequence. Ten cysteine residues were identified in the extracellular region and maybe involved in S-S bond formation (Figure 2.21).

2.4.10 Comparative predicted domain organization of PoCD131 and other mammalian species

SMART software analysis of the predicted PoCD131 subunit molecule shows the presence of two fibronectin type III domains from Pro\textsuperscript{133} - Gly\textsuperscript{219} and from Gln\textsuperscript{337} and Tyr\textsuperscript{419} (Figure 2.22). The presence of a SCOP (Structural Classification of Proteins Database) domain consisting of an immunoglobulin like 7 strand 2 sheet $\beta$ sandwich was present between Gln\textsuperscript{25} and Leu\textsuperscript{127} as well as a Erythropoietin (EPO) Receptor Ligand Binding Domain, (EpoR-Lig-Bind), between residues Ser\textsuperscript{235} and Arg\textsuperscript{322}. The common motif, WSXWS, was situated prior to the transmembrane domain which was then followed by an Extensin-2 like region extending between residues 445 - 785, and was present in all species except the Mo. A signal peptide was present for all
Pig
Human
Mouse

GAGGAGAAGATCCCTAACCCCAGCAAGAGCCACCTGTTCCAGAACGGGAGCGCCGGGCTG
GAGGAGAAGATCCCCAACCCCAGCAAGAGCCACCTGTTCCAGAACGGGAGCGCAGAGCTT
AAGGAAAAGATCCCCAACCCCAGCAAGAGCCTCCTGTTCCAGGATGGAGGTAAAGGTCTC
**** ******** **************** ********** * ** *
* **

Pig
Human
Mouse

CGGCTCCCAGGCAACATGTCCATCCTCAGCAGCGGGAGCCGCCCACACAAGGGGCCCTGG
TGGCCCCCAGGCAGCATGTCGGCCTTCACTAGCGGGAGTCCCCCACACCAGGGGCCGTGG
TGGCCTCCTGGCAGCATGGCAGCCTTCGCCACTAAGAACCCCGCTCTCCAGGGGCCACAG
*** ** **** **** *
* **
*
** * * * * * *******
*

Pig
Human
Mouse

GGTGGCGGCTACCCTGAGCTGGAGGGGGCGTCCCCTGTAGACTTCGGGCACAGTGAGGTG
GGCAGCCGCTTCCCTGAGCTGGAGGGGGTGTTCCCTGTAGGATTCGGGGACAGCGAGGTG
AGCAGGCTTCTTGCTGAGCAACAGGGGGAGTCATATGCACATTTGGAAGACAACAACGTG
* *
******
****** **
** *
** *
***
* ***

Pig
Human
Mouse

TCACCTCTCACCACGGAGGACCCTAAAGAAGCCTGCGACTTGTCATCTGAGTCTGGAGTG
TCACCTCTCACCATAGAGGACCCCAAGCATGTCTGTGATCCACCATCTGGGCCTGACACG
TCACCTCTCACTATAGAGGACCCTAATATAATTCGAGTTCCACCATCCGGGCCTGATACA
*********** * ******** **
* *
**** * * ***

Pig
Human
Mouse

ACTCTGGGTGTCTTGAACCTCCCCACGGAGCCACCCCCCGAGCCCCCACCGGGGCTGGCA
ACTCCAGCTGCCTCAGATCTACCCACAGAGCAGCCCCCCAGCCCCCAGCCAGGCCCGCCT
ACCCCAGCTGCCTCATCCGAATCCACAGAGCAACTTCCCAATGTTCAAGTAGAGGGACCA
** * * ** **
**** **** * ***
*
*
*

Pig
Human
Mouse

GCCTCCTCGGGTGGGCCTGAGAGCCGGGTTTCTGGCTTTGACTTCAATGGCCCCTACCTG
GCCGCCTCCCACACACCTGAGAAACAGGCTTCCAGCTTTGACTTCAATGGGCCCTACCTG
ACTCCT---AACAGACCTAGGAAGCAATTACCCAGCTTTGACTTCAATGGGCCCTACCTG
* *
*** ** *
* **************** *********

Pig
Human
Mouse

GGGCCGCCCCACAGCCGCTCCCTGCCTGACATCGTGGGCCAGCAGGGGCCCCCGCAGGCA
GGGCCGCCCCACAGCCGCTCCCTACCTGACATCCTGGGCCAGCCGGAGCCCCCACAGGAG
GGGCCTCCCCAATCCCACTCTCTGCCTGATCTCCCAGACCAGCTGGGTTCCCCCCAGGTG
***** *****
** *** ** ***** **
* ***** **
**** ****

Pig
Human
Mouse

GGTGGGAGCAGCAAGCCACAGCCCCCAGGGTCCCTGGAATACCTGTGTCTGCCCGCAGGG
GGTGGGAGCCAGAAGTCCCCACCTCCAGGGTCCCTGGAGTACCTGTGTCTGCCTGCTGGG
GGTGGGAGCCTGAAGCCAGCACTGCCAGGCTCCTTGGAGTACATGTGTCTGCCCCCTGGA
*********
*** *
* ***** *** **** *** ********** * **

Pig
Human
Mouse

GGGCGGGTGCAGCTGGTGCCACTGGCCCAGGC---------------------------GGGCAGGTGCAACTGGTCCCTCTGGCCCAGGCGATGGGACCGGGACAGGCCGTGGAAGTG
GGTCAAGCGCAACTGGTTCCATTGTCCCAGGTGATGGGGCAGGGCCAGGCTATGGATGTG
** * * *** ***** ** ** ******

Pig
Human
Mouse

-----------------------------------------------------------GAGAGAAGGCCGAGCCAGGGGGCTGCAGGGAGTCCCTCCCTGGAGTCCGGGGGAGGCCCT
CAGTGTGGGTCCAGCCTGGAGACCTCAGGGAGCCCTTCTGTGGAGCCAAAGGAGAACCC-

Pig
Human
Mouse

-----------------------------------------------------------GCCCCTCCTGCTCTTGGGCCAAGGGTGGGAGGACAGGACCAAAAGGACAGCCCTGTGGCT
--------TCCAGTTGAGCTGAGCATGGAGGAACAGGAGGCACGGGACAACCCAGTGACT

Pig
Human
Mouse

-----------------------------------------------------------ATACCCATGAGCTCTGGGGACACTGAGGACCCTGGAGTGGCCTCTGGTTATGTCTCCTCT
CTGCCCATAAGCTCTGGGGGCCCTGAGGGCAGTATGATGGCCTCTGATTATGTCACTCCT


Figure 2.20 Nucleotide alignment of the Po CD131 subunit against the Hu and Mo CD131 sequences. This sequence was compared to both the human sequence and mouse sequence (genebank accession NM_007780: region 259-2949). These sequences were found to have 77% and 55% identity to the human and mouse homologues, respectively. * refers to an identical nucleotide. Dashes (-) represent gaps introduced to optimise alignment.
Table 2.5. Intron and exon boundaries of the PoCD131 subunit on chromosome 5

<table>
<thead>
<tr>
<th>Exon number</th>
<th>Start</th>
<th>End</th>
<th>Start bp</th>
<th>End bp</th>
<th>exon length bp</th>
<th>intron length bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>76</td>
<td>5222646</td>
<td>5222721</td>
<td>76</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>77</td>
<td>200</td>
<td>5221299</td>
<td>5221422</td>
<td>124</td>
<td>1224</td>
</tr>
<tr>
<td>3</td>
<td>201</td>
<td>391</td>
<td>5219935</td>
<td>5220125</td>
<td>191</td>
<td>1174</td>
</tr>
<tr>
<td>4</td>
<td>392</td>
<td>543</td>
<td>5216311</td>
<td>5216462</td>
<td>152</td>
<td>3473</td>
</tr>
<tr>
<td>5</td>
<td>544</td>
<td>712</td>
<td>5216059</td>
<td>5216227</td>
<td>169</td>
<td>84</td>
</tr>
<tr>
<td>6</td>
<td>713</td>
<td>848</td>
<td>5215365</td>
<td>5215500</td>
<td>136</td>
<td>559</td>
</tr>
<tr>
<td>7</td>
<td>849</td>
<td>1006</td>
<td>5215056</td>
<td>5215213</td>
<td>158</td>
<td>152</td>
</tr>
<tr>
<td>8</td>
<td>1007</td>
<td>1146</td>
<td>5213532</td>
<td>5213671</td>
<td>140</td>
<td>1385</td>
</tr>
<tr>
<td>9</td>
<td>1147</td>
<td>1309</td>
<td>5212860</td>
<td>5213022</td>
<td>163</td>
<td>510</td>
</tr>
<tr>
<td>10</td>
<td>1310</td>
<td>1400</td>
<td>5211385</td>
<td>5211475</td>
<td>91</td>
<td>1385</td>
</tr>
<tr>
<td>11</td>
<td>1401</td>
<td>1458</td>
<td>5211106</td>
<td>5211163</td>
<td>58</td>
<td>222</td>
</tr>
<tr>
<td>12</td>
<td>1459</td>
<td>1562</td>
<td>5210132</td>
<td>5210235</td>
<td>104</td>
<td>871</td>
</tr>
<tr>
<td>13</td>
<td>1563</td>
<td>1926</td>
<td>5209076</td>
<td>5209439</td>
<td>364</td>
<td>693</td>
</tr>
<tr>
<td>14</td>
<td>1927</td>
<td>2190</td>
<td>5208557</td>
<td>5208820</td>
<td>264</td>
<td>256</td>
</tr>
<tr>
<td>15</td>
<td>2191</td>
<td>2376</td>
<td>5208317</td>
<td>5208502</td>
<td>186</td>
<td>55</td>
</tr>
</tbody>
</table>

Total 2376 12043

bp – base pair
<table>
<thead>
<tr>
<th></th>
<th>Pig</th>
<th>Cattle</th>
<th>Dog</th>
<th>Human</th>
<th>Chimpanzee</th>
<th>Rhesus</th>
<th>Guinea Pig</th>
<th>Mouse</th>
<th>Rat</th>
</tr>
</thead>
<tbody>
<tr>
<td>600</td>
<td>HSEVSPLITTEDPKEACDSLSESGVTLGLINLPTPEEPFPF-P-GGSRPEGSRVSRPGDF</td>
<td>HSEVSPLITTEDPKCVDLPEFDPTEPTDTPATSDVYSGQAPGSQGSRSAATGPEQSPQGDF</td>
<td>HSEVSPLITTEDPKDVECDLPEFDPTEPTDTPATSDVYSGQAPGSQGSRSAATGPEQSPQGDF</td>
<td>DSEVSPLITTEDPKDVCDSLPEFDPTEPTDTPATSDVYSGQAPGSQGSRSAATGPEQSPQGDF</td>
<td>DSEVSPLITTEDPKDVCDSLPEFDPTEPTDTPATSDVYSGQAPGSQGSRSAATGPEQSPQGDF</td>
<td>DSEVSPLITTEDPKDVCDSLPEFDPTEPTDTPATSDVYSGQAPGSQGSRSAATGPEQSPQGDF</td>
<td>DSEVSPLITTEDPKDVCDSLPEFDPTEPTDTPATSDVYSGQAPGSQGSRSAATGPEQSPQGDF</td>
<td>DSEVSPLITTEDPKDVCDSLPEFDPTEPTDTPATSDVYSGQAPGSQGSRSAATGPEQSPQGDF</td>
<td>DSEVSPLITTEDPKDVCDSLPEFDPTEPTDTPATSDVYSGQAPGSQGSRSAATGPEQSPQGDF</td>
</tr>
<tr>
<td>610</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>620</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>630</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>640</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>650</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Pig</th>
<th>Cattle</th>
<th>Dog</th>
<th>Human</th>
<th>Chimpanzee</th>
<th>Rhesus</th>
<th>Guinea Pig</th>
<th>Mouse</th>
<th>Rat</th>
</tr>
</thead>
<tbody>
<tr>
<td>660</td>
<td>NGPYLGPPHSRSLPDIVGQQGPPQAG</td>
<td>NGPYLGPPHSRSLPDIVGQQGPPQAG</td>
<td>NGPYLGPPHSRSLPDIVGQQGPPQAG</td>
<td>NGPYLGPPHSRSLPDIVGQQGPPQAG</td>
<td>NGPYLGPPHSRSLPDIVGQQGPPQAG</td>
<td>NGPYLGPPHSRSLPDIVGQQGPPQAG</td>
<td>NGPYLGPPHSRSLPDIVGQQGPPQAG</td>
<td>NGPYLGPPHSRSLPDIVGQQGPPQAG</td>
<td>NGPYLGPPHSRSLPDIVGQQGPPQAG</td>
</tr>
<tr>
<td>670</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>680</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>690</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>700</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>710</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Pig</th>
<th>Cattle</th>
<th>Dog</th>
<th>Human</th>
<th>Chimpanzee</th>
<th>Rhesus</th>
<th>Guinea Pig</th>
<th>Mouse</th>
<th>Rat</th>
</tr>
</thead>
<tbody>
<tr>
<td>720</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>730</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>740</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>750</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>760</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>770</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Pig</th>
<th>Cattle</th>
<th>Dog</th>
<th>Human</th>
<th>Chimpanzee</th>
<th>Rhesus</th>
<th>Guinea Pig</th>
<th>Mouse</th>
<th>Rat</th>
</tr>
</thead>
<tbody>
<tr>
<td>780</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>790</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>800</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>810</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>820</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>830</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Pig</th>
<th>Cattle</th>
<th>Dog</th>
<th>Human</th>
<th>Chimpanzee</th>
<th>Rhesus</th>
<th>Guinea Pig</th>
<th>Mouse</th>
<th>Rat</th>
</tr>
</thead>
<tbody>
<tr>
<td>840</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>850</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>860</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>870</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>880</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>890</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 2.21 Multiple alignment of Po CD131 subunit with the CD131 subunit of various mammalian species. PoCD131 subunit aa sequence was aligned with known mammalian CD131 subunit sequences from other species using CLUSTALW program and shows identical (*), conserved (:) and semi-conserved residues (:). Boxes show conserved C residues. Grey shaded area represent WSXWS common motif. Blue arrow indicates predicted signal peptide. Red arrow indicates Fibronectin type III domains (FN3). Green arrow indicates predicted erythropoietin receptor ligand binding domain (EpoR_Lig-Bind). Light blue arrow indicates transmembrane domain. Grey arrow indicates extension-2 like region. Yellow shading represents suggested α subunit/cytokine binding site, ▼ indicated the start of the mature protein. ● indicates end of extracellular domain. N indicates reported N-linked glycosylation sites. Dashes (-) represent gaps introduced to optimise alignment. Numbers represent residue number. Latin names are in brackets; genebank accession no: pig (S. Scofa, predicted); cattle (B. taurus, XP_606956); dog (C. lupus familiaris, XP_538397); domestic guinea pig (C. porcellus, AAC77520); rhesus monkey (M. mulatta, XP_001086084); human (H. sapiens, AAH70085); rat (R. norvegicus, EDM15880); mouse (M. musculus, NP_031806); chimpanzee (P. troglodytes, XP_001145357)
Table 2.6 Comparison of PoCD131 subunit aa sequence against the CD131 subunit of other species.

<table>
<thead>
<tr>
<th>Organism</th>
<th>common name</th>
<th>Size (aa)</th>
<th>(%)^a</th>
<th>(%)^b</th>
<th>(%)^c</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sus. Scofa</td>
<td>Pig</td>
<td>791</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Bos taurus</td>
<td>Cattle</td>
<td>890</td>
<td>75.0</td>
<td>10.8</td>
<td>85.8</td>
</tr>
<tr>
<td>Canine lupus familiaris</td>
<td>Dog</td>
<td>945</td>
<td>67.0</td>
<td>12.6</td>
<td>79.6</td>
</tr>
<tr>
<td>Pan troglodytes</td>
<td>Chimpanzee</td>
<td>924</td>
<td>67.0</td>
<td>10.6</td>
<td>77.6</td>
</tr>
<tr>
<td>Homo sapiens</td>
<td>Human</td>
<td>889</td>
<td>66.2</td>
<td>10.6</td>
<td>76.8</td>
</tr>
<tr>
<td>Macaca mulatta</td>
<td>Rhesus monkey</td>
<td>932</td>
<td>65.1</td>
<td>11.3</td>
<td>76.4</td>
</tr>
<tr>
<td>Cavia porcellus</td>
<td>Guinea pig</td>
<td>890</td>
<td>59.0</td>
<td>13.5</td>
<td>72.5</td>
</tr>
<tr>
<td>Mus Musculus</td>
<td>Mouse</td>
<td>896</td>
<td>54.1</td>
<td>14.1</td>
<td>68.2</td>
</tr>
<tr>
<td>Rattus norvegicus</td>
<td>Rat</td>
<td>896</td>
<td>54.1</td>
<td>13.2</td>
<td>67.3</td>
</tr>
</tbody>
</table>

(\%)^a - percentage of aa identical to PoCD131 subunit; (\%)^b - percentage of conserved aa substitutions; (\%)^c - total percentage of identical and conserved aa to PoCD131 subunit. Species listed in order of highest total percentage of identical and conserved aa.
Figure 2.22 Comparative predicted domain organisation of the Po CD131 subunit and selected CD131 subunits from other mammalian species. SMART analysis software was used to predict domain structure of pig βc subunit peptide sequences and structure of βc subunit from other species. Abbreviations: FN3 - Fibronectin type III domain, Epo R-Ling-Bind - Erythropoietin Receptor Ligand Binding Domain, SCOP - Structural Classification of Proteins database. Species arranges by structure homology.
molecules except for the rhesus monkey and the chimpanzee. All species appear to consist of similar structural organization.

2.4.11 Phylogenetic analysis of the PoCD131 subunit shows three distinct groups between mammalian species

An un-rooted phylogenetic analysis of the CD131 subunit sequence from all known species produces three evolutionary groups. The pig was grouped with the dog and cattle whilst the Hu, primates and the rodents made up the other two groups (Figure 2.23). This was similar to the phylogenetic organization of each of the βc subunit cytokines.

2.4.12 Molecular structure of the CD131 subunit shows similar conformation to the Hu form

Secondary unfolding of the first monomer of the CD131 subunit protein by PyMOL software shows a conformation similar to that of the Hu molecule. The molecule appears to be a group of 4 domains made up of β sheets (Figure 2.24). There are 5 S-S bonds in the Hu molecule but only 4 suggested bonds in the pig. Although cysteine residues are present at cys\textsuperscript{88} and cys\textsuperscript{91} there was no apparent sulfide bond formation modelled. This may be due to the residues being closer together in the pig sequence (separated by 3 residues), thus, preventing the residues from coming into contact and forming the S-S bond (Figure 2.24). In other species, as in the Hu, these cys residues are separated by 5 residues. Four other sulfide bonds appear to be present between residues, cys\textsuperscript{45}-cys\textsuperscript{35}, cys\textsuperscript{75}-cys\textsuperscript{96}, cys\textsuperscript{248}-cys\textsuperscript{258} and cys\textsuperscript{287}-cys\textsuperscript{304} which correspond to bonds in the Hu molecule at residues cys\textsuperscript{45}-cys\textsuperscript{35}, cys\textsuperscript{75}-cys\textsuperscript{96}, cys\textsuperscript{250}-cys\textsuperscript{260} and cys\textsuperscript{289}-cys\textsuperscript{306}. Moreover, there are slight differences present in the conformation of the proteins backbone due to residue substitution and S-S bond location (Figure 2.24). A comparison of the predicted surface conformation of PoCD131 and HuCD131 shows minor difference in surface topography related to expression of differing aa residues (Figure 2.25).

2.5 Discussion

The cytokines of the CD131 subunit, IL-3, IL-5 and GM-CSF are growth factors involved in hematopoietic cell regulation and the induction of hematopoiesis in response to infections and the pathogenesis of allergic and inflammatory diseases (Murphy and Young 2006). These cytokines are unique as they share the CD131 subunit as part of their receptor ligand complex (D’Andrea and Gonda 2000).
An unrooted phylogenetic tree was constructed based on 100 bootstrapped amino acid alignments of CD131 subunit of known species using the neighbour-joining method. Shaded areas represent closely related groups highly homologous to each other.
Figure 2.24 Predicted molecular structure of extracellular region of monomer 1 of CD131 subunit. Comparison of backbone conformation of (A) PoCD131 and (B) HuCD131. Molecular conformation was determined using PyMOL (DeLano Scientific LLC). Figures shows N and C terminal residues, disulphide bonds (S-S) and predicted conformational differences between molecules.
Figure 2.25 Predicted surface structure of extracellular region of monomer 1 of CD131 subunit. Comparison of predicted surface conformation of PoCD131 and HuCD131. Molecular conformation was determined using PyMOL (DeLano Scientific LLC). Figure A shows an overlay of the two molecules with HuIL-5 being shown in mesh form to show conformational differences between the two proteins. Figure B shows an overlay of the two molecules with PoIL-5 being shown in mesh form to show conformational differences between the two proteins. Arrows indicate some of the predicted differences in conformation between molecules. PoCD131 is shown in red, HuCD131 is shown in green.
An in depth understanding of the action of these early acting cytokines both in pigs and other species may prove beneficial to the future of animal health. For Livestock Industries, these things are of great interest. Of equal importance are the similarities in these processes across species. In-depth analysis of genetic similarities between homologous species may bring us closer to understand cross species interactions of cytokines and similarities in hematopoiesis and immune responses between species.

A comparison of genetic information for the PoCD131 associated cytokines against other mammalian species revealed varied results both between species and cytokines. PoGM-CSF and PoIL-5 were found to be highly conserved across all species investigated at both the nucleotide and aa levels; however, PoIL-3 was determined to have low identity between species. This is more apparent when comparing identical aa for PoIL-3 and IL-3 for other species, where identity ranged from 47.9% to 27.1%. In comparison, PoGM-CSF and PoIL-5 had identities of 81.2 - 56.7% and 87.3% – 58.3%, respectively. Phylogenetic studies indicated that all three PoCD131 cytokines were closely related to the dog and cat with the addition of sheep, cattle and water buffalo for PoIL-3. This would suggest that each of these molecules is an orthologue which has developed divergent of the Hu and primate forms which were shown to be closely related.

The predicted molecular structure for each of PoGM-CSF, PoIL-3 and PoIL-5 when compared to the Hu forms for each molecule further confirmed the identity of each of these proteins. The 4 α helix bundle and common steric up – up – down - down arrangement were observed. The predicted secondary structures of PoGM-CSF and PoIL-5 show only minor conformational changes when compared to HuGM-CSF (Walter, et al. 1992) and HuIL-5 (Milburn, et al. 1993). Likewise, predicted surface structure overlays for each of the proteins show only slight differences in predicted surface conformation between pig and Hu molecules. These differences may nonetheless prevent or hinder cross species activity between these two species, however, other species with higher identity are anticipated as having lower conformational differences. Conformation differences observed for the predicted surface structure overlay for PoIL-3 and HuIL-3 (Lyne, et al. 1995) were considered higher and more pronounced and are expected to result in low or no cross species activity between pig IL-3 and Hu or primate cells. In addition, differences in residues reportedly involved in receptor interactions, were observed for all cytokines compared to other species indicating species specific receptor binding. Low identity conservation for these residues is expected to feature significantly in cross species activity,
although, these differences were observed mainly for residues involved in α subunit interactions and not CD131 subunit interactions. Glutamic acid (GLU) residues essential for CD131 subunit binding (Lopez, et al. 1992) are conserved and may result in CD131 subunit interaction and activation between species but are expected to fail to bind or activate α subunits. Single aa substitute mutagenesis previously performed for these residues in HuIL-3 receptor interaction studies confirmed that residues interacting with the CD123 subunit were essential for cytokine receptor biological activity (Barry, et al. 1994). Conversely, predicted receptor interaction residues for PoIL-5 are highly conserved between species and may result in adequate ligand receptor functionality between species. Nonetheless, each of these cytokines appears to have maintained structural inheritance comprising similar α helix and β sheet formation to the Hu orthologues. The predicted PoIL-5 homodimer appears to have a similar conformational structure to the HuIL-5 orthologue including S-S bond formation between monomers and head to tail arrangement (Murphy and Young 2006) although, this would need to be confirmed by crystal x-ray modelling. In addition to sequence and structural similarities, it was discovered that all three cytokine genes are coded in sequence on the same Ch. This synteny of cytokine genes, which also includes the gene for IL-4, is referred to in humans as the proximal cluster of cytokines and is located along with a distal cluster within a larger group comprising cytokines and immune function genes called the cytokine gene cluster (van Leeuwen, et al. 1989). To date this cluster has been mapped in the Hu (van Leeuwen, et al. 1989), cattle (Buitkamp, et al. 1995), Mo, rat, and sheep (Hawken, et al. 1996). The relative positions of the proximal and distal clusters have been shown to be conserved in the Hu, cattle and sheep being located on Ch 5, however, they are located on different Ch in the Mo and the rat (Hawken, et al. 1996). Here we add to the comparative map for the proximal region by showing that the proximal cluster in the pig is located on Ch 2. In addition, as it has been postulated that cytokines which portray similar tertiary structures and receptor-binding characteristics may have evolved from one primordial gene, by gene duplication (Bazan 1990, van Leeuwen, et al. 1989, Hawken, et al. 1996, Cosman, et al. 1990, Boulay and Paul 1992), and as all three of these cytokines share a common receptor subunit, IL-3, IL-5 and GM-CSF may also have evolved from the same proximal gene.

To date, some cross species studies of the CD131 subunit cytokines have been reported, however, very few of these studies have included the Po cytokines with the majority of Po studies relating to xenografting. Cross species activity for GM-CSF in Hu, Mo, cattle (Maliszewski, et al. 1988) and sheep (Stevenson and Jones 1994)
have produced varied results. Weak biological activity was shown for HuGM-CSF on bovine cells and for bovine GM-CSF on Hu cells in BM proliferation studies. In addition, HuGM-CSF did not induce proliferation of sheep or Mo BM cells, and Mo did not support Hu or bovine cells. This would indicate no cross species interaction of GM-CSF between phylogenetic populations. Cross species studies into IL-3 have shown no activity for PoIL-3 in up regulating circulating CFUs in baboons (Down, et al. 2000). HuIL-3 produced proliferation of monkey cells (Dvorak, et al. 1989) but not sheep cells (Stevenson and Jones 1994) and activity was detected for rhesus monkey IL-3 and chimpanzee IL-3 on Hu cells in proliferation studies (Burger, et al. 1994). However, no activity was observed on Hu cells for marmoset IL-3 or tamarin IL-3 (Burger, et al. 1994) and oddly enough, little activity was observed between Mo and rat for IL-3 (Cohen, et al. 1986). These results differ slightly from those reported for GM-CSF in that there appears to be communication between IL-3 from the phylogenetic populations containing Hu and primates but still no activity between other groups. This would suggest a closer relationship between these two groups. Finally cross species studies on IL-5 have shown activity for Hu IL-5 on both Mo (McNamee, et al. 1991) and sheep cells (Stevenson and Jones 1994) and demonstrated rat IL-5 (Bosward, et al. 1999) and sheep IL-5 (Pierrot, et al. 1998) activity on Mo cells. In addition, guinea pig IL-5 was shown to promote activity on Hu cells but is nearly inactive on Mo cells (Scott, et al. 2000). Unlike the cross species activity demonstrated for GM-CSF and IL-3, we suggest that IL-5 is more highly conserved between species. With these results in mind we hypothesis that cross species activity for PoGM-CSF and PoIL-3 would be very weak or nonexistent and cross species activity for PoIL-5 may exist. Furthermore, activity between species for each cytokine may be most viable within each of the predicted phylogenetic groups, however, these hypothesis need to be analysed. In addition to differences in each of the CD131 cytokines, there may also be differences in their associated receptors. This may also explain lack of cross species activity. We therefore conducted an in-depth genetic analysis of the PoCD131 subunit.

The HuCD131 subunit has been reported as being a stable intertwined homodimer consisting of fibronectin type III domains (Murphy and Young 2006). It is suggested that the formation of the CD131 dimer is the result of a domain swapping of G strands between domain 1 of one protein chain and domain 3 of the second chain. The results of this positions domain 1 of one protein chain adjacent to the membrane proximal domain 4 of the symmetry related protein chain therefore resulting in the existence of the CD131 receptor as a stable homodimer (Schlunegger, et al. 1997). The expression of the Hu form of the CD131 ectodomain from insect cells has
demonstrated that CD131 is exclusively dimeric. Furthermore, no monomer was detected confirming that CD131 is only present in the form of a homodimer (Murphy, et al. 2004). As it is not known at this stage which residues are involved in domain swapping for PoCD131 to produce the final homodimer formation that is of course assuming that the molecule undergoes similar translational and molecular arrangement to the Hu molecule, only the first monomer of the homodimer was identified and analysed. The high level of identity between HuCD131 and PoCD131, which was determined to be 77% at the DNA level, enabled the identification and location of the genetic sequence for PoCD131 when integrating the pig genome. The proposed gene was located on Ch 5 and consisted of the same number of exons and introns as HuCD131 although of different size. The protein sequences varied amongst species, however, appears to be relatively conserved amongst the 9 species examined. Phylogenetic arrangement confirmed the PoCD131 molecule as being closely related to the cattle and dog molecules. This follows the trend seen for each of the CD131 cytokines which all showed close phylogenetic development in addition to high sequence homology to the dog analogue. The predicted surface and backbone conformation showed only minor differences between monomer 1 of the PoCD131 subunit compared to monomer 1 of the HuCD131 subunit (Carr, et al. 2006) and appears to be composed of 4 domains of β sheets. Similarly to the HuCD131 molecule, the PoCD131 molecule has ten Cys residues which are predicted to from Cys-Cys bonds apart from the Cys pair at positions Cys^{88}-Cys^{91}. It is thought that due to the close proximity of these two residues in the Po molecule compared to the Hu form, this sulphide bridge may not form. This may therefore have an effect on the conformation of the molecule in this region thus differing from the HuCD131. Although the predicted conformation appears the same it is worth noting that this is a predicted conformation of the molecule based on computer generated parameters. In addition to the predicted conformation, the molecule appears to have maintained its fibronectin type-III domain structure (Bazan 1990) between species in addition to domain arrangement despite phylogenetic separation. Moreover, the WSXWS motif which is synonymously recognized as the hallmark of class 1 cytokine superfamily receptors (Bazan 1990), is present and conserved over all species examined. Also of interest is the highly conserved suggested α subunit binding site. It appears from aa sequence alignment that these residues are highly conserved across species with the only deviation being for the Mo and rat. This observation is supported by the equally conserved CD131 α subunits aa binding sites visualized for each of the species examined. This would suggest that cross species binding of each of the CD131 α
subunit may be viable, whereas cross species cytokine activity is indicated as being restricted. This also confirms that changes resulting in reduced cross species activity have occurred at the cytokine / α subunit interface. Finally, it was observed that the CD131 protein lengths differ considerably amongst species, ranging from the pig sequence as smallest at 791aa and dog at 945aa, structural homology appears to have been maintained across species. We believe that the genetic and structural similarities between CD131 from each of the species examined and the molecule proposed here confirms classification of this molecule as PoCD131.

2.6 Conclusion

In summary, it is reported here that the identities of the three PoCD131 common subunit cytokines PoIL-3, PoIL-5 and PoGM-CSF have been confirmed and that it has been suggested that these proteins have developed as orthologues of the ancestral genes. The suggested changes observed including those for recommended cytokine receptor binding sites and predicted cytokine molecular surface conformational changes may result in reduced or non-biological activity in cross species studies. However, it is our hypothesis that as changes to IL-5 appear to be less pronounced than those observed for GM-CSF and IL-3, and as previous cross species studies resulted in activity between phylogenetic groups for IL-5, cross species activity for PoIL-5 may indeed exist. In addition, the location for the proximal cluster in the pig has been identified on Ch 2. Additionally, the location and identity of PoCD131 has been confirmed and compared between species. As a result the gene for the PoCD131 subunit was shown to be highly conserved between species. Furthermore, the identity of the α subunit binding site on the CD131 subunit has also been determined as being highly conserved between species. This observation could be useful in developing therapies or pharmaceuticals where the activity of CD131 needs to be reduced or enhanced. For example, an antagonist capable of binding to a CD131 α subunit binding site would prevent receptor activation by all of the associated cytokines thus reducing proliferation, differentiation, maturation or activation of CD131 specific cells. Moreover, as the binding sites appear to be highly conserved, the same antagonist may uniformly work between species.
CHAPTER 3

*In vitro* characterisation of pig GM-CSF, IL-3 and IL-5

3.1 Abstract

The use of recombinant cytokines as a means of enhancing the immune system against infection may provide an alternative therapeutic treatment for livestock as opposed to the current use of antibiotics (Lowenthal, *et al.* 2000). However, in order to assess the therapeutic properties of cytokines in pigs, specifically, PoIL-3, PoIL-5 and PoGM-CSF, it is firstly necessary to produce these rec Po cytokines and then characterise the activity of each cytokine *in vitro*. By using the genetic sequences for these cytokines as previously identified, a rec protein for each cytokine was developed in a bacterial expression system. Following identification and purification, each rec protein was shown to induce biological activity on both cytokine dependant cell lines and PoBM proliferation assays. FACS analysis was used for characterisation of cytokine induced cell population’s while light microscopy and differential staining were used to confirm differentiation of PoBM and identify dominant cell populations produced. These cytokines will now be used to analyse their actions on hematopoiesis *in vivo* and to subsequently assess their potential use as future therapeutics in pigs.

3.2 Introduction

The early isolation and acquisition of cytokines and colony-stimulating factors involved the extracting of the cytokines from serum, medium conditioned by cultured cells or from mononuclear cells (Greenberger 2000). The development of new techniques of molecular cloning in the 1980s inevitably led to the expression of biologically active cytokines in bacteria, yeast, and mammalian cellular systems (Greenberger 2000). Since then, an enormous number of proteins have been identified, expressed and characterised in numerous species. With the isolation and expression of cytokines no longer being a barrier, the characterisation of specific proteins has taken on a larger role. As previously shown (chapter 2), the cytokines GM-CSF, IL-3 and IL-5 have been identified in multiple species, however the exact role of each of these cytokines in each of these species is yet to be established. Furthermore, it is yet to be established if each of these cytokines retains the same activity across species.
As the name would suggest, GM-CSF has been demonstrated to stimulate the production of granulocytic and macrophage cells in both Hu and mice (Metcalf, et al. 1986, Metcalf 1986). However, the activity of GM-CSF is far more complex than just this. In fact, GM-CSF has also been shown to cause the proliferation and differentiation of early progenitor cells, including the CMP and committed granulocyte/macrophage progenitor cells, in addition to the regulation, survival and function of mature neutrophils, macrophages and DC (Wadhwa and Thorpe 2008, Metcalf, et al. 1986, Ruef and Coleman 1990, Caux, et al. 1992, Jones 1996). Moreover, GM-CSF is active in myeloid cell regeneration (Armitage 1998), and has been identified as an inhibitor of mast cell terminal differentiation (Martinez-Moczygemba and Huston 2003, Zuberbier, et al. 2001, Iwasaki-Arai, et al. 2003). If we were to assume that cytokines act the same way between species then we could assume that similar results to these would be observed in pigs. However, although the genetic identity of GM-CSF between species is high, the cytokine appears to be species specific in its activity (Cantrell, et al. 1985, Inumaru, et al. 1998). Therefore, in order to compare the role of GM-CSF in pigs to that observed in other species it is necessary to firstly express rPoGM-CSF. Previous studies of glycosylated rPoGM-CSF expressed in baculovirus demonstrated proliferation and differentiation activity of PoBM cells, in addition to the culturing of monocyte/macrophage cells and monocyte/DC (Inumaru, et al. 1998). However, in vivo studies in Hu where glycosylated and non-glycosylated GM-CSF were compared, produced pharmacokinetic results indicating non-glycosylated GM-CSF produced a faster response in changes of leukocyte numbers (Denzlinger, et al. 1993).

Unlike GM-CSF, IL-3 is not highly conserved amongst species. Conversely, IL-3 appears to be species specific, again prompting the need to express and analyse the activity of rPoIL-3 (Stevenson and Jones 1994). In both Hu and Mo, IL-3 plays an important role in the growth and differentiation of CD34+ progenitor cells into basophils and mast cells, myeloid and non-myeloid derived DC, eosinophils and monocytes-macrophages (Martinez-Moczygemba and Huston 2003, Bazan 1990, Lantz, et al. 1998, Arai, et al. 1990, Yamada, et al. 1998, Munoz, et al. 2001). In in vitro PoBM cultures, rPoIL-3 has been shown to produce myeloid line cells, whereas rPoIL-3 in vivo has been demonstrated to be a major cytokine involved in the process of eosinophilopoiesis (Morris, et al. 2008). As IL-3 is involved in multiple processes of hematopoiesis in other species, we were interested in the activity of IL-3 in hematopoiesis in pigs. Unlike, IL-3, IL-5 has also been shown to be highly conserved across species, however, differences in binding sites of the cytokine across species
may hinder cross reactivity resulting in little to no response as reported for GM-CSF and IL-3.

Previous studies for PoIL-5 have confirmed direct involvement of IL-5 in eosinophil development and suggest that IL-5 may be the dominant cytokine involved in eosinophilopoiesis (Andrew, et al. 2007). Nonetheless, in other species IL-5 has shown multiple roles, including the promotion of B-cell activation and/or differentiation in Mo (McKenzie, et al.) and the proliferation and/or differentiation of basophils in Hu (Gauvreau, et al. 2009).

In this study, the rec Po cytokines GM-CSF, IL-3 and IL-5 were expressed in bacterial systems and purified. Biological activity was demonstrated in cytokine specific assays, and proliferation and differentiation properties of each cytokine were demonstrated on PoBM. In addition, FACS analysis incorporating anti-CD90 and anti-CD172a antibodies, in addition to other known cell markers, were used to characterise cytokine cultured hematopoietic cell lines whilst anti-BrdU was used to determine cell proliferation from cytokine/PoBM incubations. Finally, cell differential staining and light microscopy were utilized in visualizing cell morphology.

3.3 Materials and Methods

3.3.1 Cell collection techniques

3.3.1.1 Animals

One week old, Large White Landrace Cross pigs (Department of Primary Industries, Werribee, Australia), were used for tissue collection.

3.3.1.2 Extraction of BM from pigs

Pigs were euthanized before tissue was collected and processed into a single cell suspension. To extract BM, femurs were removed and cleaned of muscle tissue. The bones were then dissected longitudinally and compressed to excise BM cells. BM cells were recovered and washed with Dulbecco’s modified Eagles medium (DMEM) containing 1% (v/v) foetal calf serum (FCS) and 5 U/mL heparin.

3.3.1.3 Freezing of BM

Cold DMEM containing dimethyl sulfoxide (DMSO) freezing solution (Appendix 1) was added to the BM at a one to one ratio under gently agitation. The cells were then stored in a microcentrifuge tube (Eppendorf, Germany) in cotton wool at -80°C for 24 h then transferred to liquid nitrogen storage.
3.3.1.4 Thawing BM cells

The cells were removed from liquid nitrogen storage and thawed by shaking in 37°C Thermomix 1420 water bath, model 850024 (B. Braun, Germany). Cells transferred to a sterile 15 mL Falcon tube (Falcon). DMEM (Appendix 1) at 37°C was added and cells were centrifuged at 300 x $g_{\text{max}}$ for 8 min, Rotina 48R centrifuge (Hettich Zentrifugen). The supernatant was discarded and pellet resuspended in DMEM.

3.3.1.5 Separation of peripheral blood lymphocytes (PBLC) from whole pig blood

Peripheral blood was taken from the jugular vein and clotting inhibited by EDTA. Twenty-five mL of blood was layered on top of 25 mL of Ficoll-Paque (Pharmacia Biotech, Sweden d=1.077g/mL) pre-warmed to 37°C in a 50mL Falcon Blue Max™ tube (Falcon). The blood was then centrifuged at 1100 x $g_{\text{max}}$ for 20 min with no brake in a Rotina 48R centrifuge (Hettich Zentrifugen). The buffer containing cells of interest was pipetted off and washed with DMEM supplemented with 20 mM HEPES, 9 mM sodium bicarbonate, 1 mM L-glutamine, 50 µM 2-mercaptoethanol, 100 IU/mL penicillin and streptomycin and 10% foetal bovine serum (FCS/DMEM). Lymphocytes were counted by trypan blue exclusion and resuspended at 5x10⁶ cells/mL in FCS/DMEM.

3.3.2 Gene cloning

3.3.2.1 RNA isolation

BM cells were adjusted for cell density to 5x10⁶ cell/mL in 5 mL of DMEM. PBLC were used as previously diluted. RNA was extracted using using RNeasy® (QIAGEN, Germany) total RNA isolation kit as per manufacturer’s instructions. The isolated RNA was eluted with 50μl RNAse free H₂O. RNA was stored at -80°C.

3.3.2.2 cDNA synthesis

cDNA was synthesised using the first stand synthesis method. Four µL of 10x amplification buffer (Appendix 1), 2 µL Rnasin (Promega, USA), 4 µL 10x dNTPs, 2 µL random primers (Promega), 4 µL MgCl₂, 23 µL heated RNA sample and 1 µL reverse transcriptase (Promega) were mixed in a sterile microcentrifuge tube (Eppendorf, Germany) and incubated at 42°C for 1 h, then at 95°C for 5 min in a Thermomix water bath (B. Braun Biotech), prior to storage at -20°C.
### 3.3.2.3 Oligonucleotide synthesis and sequences

The different primers that were utilized throughout this project are listed in Table 3.1 (Geneworks, Australia). Mature refers to the extracellular region of cDNA without the signal peptide (sp).

### 3.3.2.4 Polymerase chain reaction

PCR amplification was carried out in a DNA Thermocycler 480, model number P15332 (Perkin Elmer Applied Biosystems, USA) in sterile 0.5mL thin walled microcentifuge tubes (Eppendorf) using pre-determined primers for each cytokine as listed in Table 3.1. PCR was carried out as follows:

**EasyStart™ 50 PCR Mix in a Tube**

Each 0.5mL thin walled tube contained: 2µL 50mM MgCl₂, 5µL 10X PCR buffer, 4µL 2.5 mM dNTP mix and 14µL double distilled water (ddH₂O). To each tube was added 20µL dH₂O, 1µL of each primer, 2 µL template DNA and 1µL of RedTaq™ DNA Polymerase (Sigma USA). PCR was performed according to the following cycling conditions.

**PCR Program**

- **Step 1** 94°C for 1 min
- **Step 2** 55°C for 1 min 36 cycles
- **Step 3** 72°C for 1 min
- **Step 4** 94°C for 1 min, 55°C for 1 min, 72°C for 15 min for extension.

### 3.3.2.5 Agarose Gel Electrophoresis

DNA samples were size separated and analysed on 1% or 1.2% (w/v) low melting point (LMP) agarose/TAE gels (Appendix 1) as described by Sambrook et al., (1989). Briefly, 1% (w/v) agarose (Sigma, Germany) was dissolved in 1 x TAE buffer (Appendix 1) and ethidium bromide was added at 0.25 µg/mL. DNA samples (10 µL) were loaded in 6 x gel loading buffer and run with 123 bp markers (Gibco, USA). The loaded samples were electrophoresed at 90V for 45 min using BioRad Power Supply power packs, model number 200-2.0 (BioRad). DNA bands were visualised under ultraviolet light, using a UV transilluminator model number UVM-19 (Hoefer Scientific Instruments, USA). Gels were photographed using a UVP Darkroom GDS7500 fitted to a UVP transilluminator model TM-40E (AGP Technologies, Upland, CA, USA) and processed via UVP Grab-it® Annotating Grabber Version 2.04.7 (Ultra Violet Products)
<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Primer direction</th>
<th>Primer sequence</th>
<th>Product length-nt</th>
<th>R.E.</th>
<th>Tm°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>PoGM-CSF mature</td>
<td>fwd</td>
<td>ACTGGATCCGCTCCCCACCCGCCACCCAGCA 5’end</td>
<td>402</td>
<td>BamHI</td>
<td>73</td>
</tr>
<tr>
<td>PoGM-CSF</td>
<td>rev</td>
<td>ACTAAGCTTTTACTTTTTTTGA CTGGCCCCAGCA 3’end</td>
<td>-</td>
<td>HindIII</td>
<td>63</td>
</tr>
<tr>
<td>PolL-3 full</td>
<td>fwd</td>
<td>ACTGGATCCATGAGCAGCCCTCCCTTTATG 5’end</td>
<td>454</td>
<td>BamHI</td>
<td>66</td>
</tr>
<tr>
<td>PolL-3 mature</td>
<td>fwd</td>
<td>ACTGATCCACAGCAGATGCCTACC 5’end</td>
<td>395</td>
<td>BamHI</td>
<td>68</td>
</tr>
<tr>
<td>PolL-3 mature</td>
<td>rev</td>
<td>ACTAAGCTTTTAGGGCTCTG TGATCATGGG 3’end</td>
<td>-</td>
<td>HindIII</td>
<td>62</td>
</tr>
<tr>
<td>PolL-5 mature</td>
<td>fwd</td>
<td>ACTGGATCCGTAGAAAAATACCATGAATAGAC 5’end</td>
<td>360</td>
<td>BamHI</td>
<td>59</td>
</tr>
<tr>
<td>PolL-5 mature</td>
<td>rev</td>
<td>ACTAAGCTTTAATTTTCCA TTGTCCACTCGGT 3’end</td>
<td>-</td>
<td>HindIII</td>
<td>61</td>
</tr>
</tbody>
</table>

**Table 3.1 DNA primer sequences for rPo cytokines.** Primer sequences used for the PCR of PoGM-CSF, PolL-3 and PolL-5 indicating restriction enzymes (R.E), melting temperatures (Tm°C) and product total length in nucleotides. Underlined sequence indicates the restriction enzyme location.
Ltd (UVP), Cambridge UK). A 123 bp ladder (Gibco) or \( \lambda \)HindIII ladder, lambda DNA (Promega) cut with the HindIII restriction enzyme, were run with each gel. Bands of interest were excised from the gel and the PCR product was removed and cleaned using the Wizard\textsuperscript{\textregistered} PCR preps DNA purification system (Promega, USA).

### 3.3.3 Plasmid construction

#### 3.3.3.1 Plasmid vectors

The map for each of the following plasmid vectors used in this study are outlined in Appendix 2, pGEM-T, pQE30, and pNC-H3.

#### 3.3.3.2 Restriction endonuclease digestion of DNA

To set up the restriction digest on DNA samples, 10 \( \mu \)L DNA, 2 \( \mu \)L 10 x restriction buffer (Promega), 10\% (v/v) restriction enzymes (10 U/\( \mu \)L) appropriate restriction enzymes (Table 3.1) and 6 \( \mu \)L ddH\textsubscript{2}O were mixed in a sterile 1.5mL microcentrifuge tubes (Eppendorf). The tubes were incubated for 90 min at 37\(^{\circ}\)C. Five microlitres of 6 x loading dye (Appendix 1) was added to the contents of the tube and separated using low melt agarose gel electrophoresis.

#### 3.3.3.3 Ligation of PCR products

Ligation reactions were performed in a final volume of 15 \( \mu \)L and the relevant DNA inserts and vectors were used at a 4:1 ratio. One and a half microlitres of ligation buffer (Promega) and 0.5 \( \mu \)L T4-DNA ligase (Promega) were added and the ligation mix was incubated at 15\(^{\circ}\) C overnight (O/N).

#### 3.3.3.4 Bacterial strains and techniques

Bacterial strains used in this study are listed in Appendix 2, Table A2.1. Bacterial cultures were stored at -80\(^{\circ}\)C in 30\% (v/v) glycerol.

#### 3.3.3.5 Media

All strains of \textit{E. coli} bacteria were routinely grown on trypticase soy agar (TSA; Appendix 1) or in trypticase soy broth (TSB, Appendix 1) (Becton Dickinson, USA). Ampicillin (amp, Sigma) was added to broth and solid media at a final concentration of 100 \( \mu \)g/mL to allow plasmid selection. \textit{Brevibacillus choshinensis} (\textit{B. choshinensis}) were grown in brevibactillus trypone yeast (BTY) buffer and on modified BTY neomycin (MBTYNm) agar plates (Appendix 1).
3.3.3.6 Preparation of electro-competent *Escherichia coli* (*E. coli*) cells

Electro-competent *E. coli* strain DH5α and Topp10F were prepared as follows. Ten mL of fresh O/N culture of *E.coli* was used to inoculate 1 L of Luria Bertani (LB) broth (Appendix 1) in a sterile 3 L flask. The bacterial cells were grown in a shaking incubator (Forma Scientific, USA) at 37°C until the absorbance at 600 nm was between 0.8-1.0. The cells were chilled on ice for 30 min, and then centrifuged in a pre-cooled (4°C) Beckman Avanti centrifuge, model number J-251 (Beckman, USA) at 2500 x *g* max for 10 min. The cell pellets were resuspended gently in 1 L of 10% (v/v) chilled glycerol in ddH₂O water and re-centrifuged at 2500 x *g* max for 10 min. The pellets were then resuspended in 500 mL 10% (v/v) chilled glycerol, re-centrifuged at 2500 x *g* max for 10 min, and resuspended in 20 mL 10% (v/v) glycerol. Following a final centrifugation step, the weight of the pellet was determined and the pellet resuspended in an equal weight (usually 2-3 mL) of 10% (v/v) glycerol. Cells were stored in 40 μL aliquots at -80°C in microcentrifuge tubes.

3.3.3.7 Preparation of electrocompetent *B. choshinensis* cells

Electrocompetent HPD31 *B. choshinensis*, (Appendix 2) was prepared as follows: 1 mL of fresh overnight culture of *B. choshinensis* was used to inoculate 100 mL of BTY media (Appendix 1.16) containing 50 μg/mL neomycin (Sigma, Germany), in a 1 L flask. The bacterial cells were grown in a Rajek orbital mixer incubator (Ratek Instruments, Australia) at 30°C until the A₆₀₀ was between 3.0-3.25. The cells were then centrifuged in a Beckman Avanti J-251 at 5000 x *g* max for 10 min at 4°C. The cell pellet was resuspended gently in 20 mL of PGH buffer (Appendix 1.33) and then centrifuged again at 6000 x *g* max for 20 min at 4°C. Following the final centrifugation, the pellet was resuspended in 2 mL of PEG/glycerol/HEPES (PGH) buffer and 100 μL aliquots of the cells were stored in eppendorf tubes at -80°C.

3.3.4 Transformation, isolation and sequencing

3.3.4.1 *E. coli* transformation

Electro-competent cells (40 μL) were thawed on ice and 2 μL of the ligation mix was added. The mixture was then transferred to a cold 2mm gap Gene Pulser cuvette (Bio-Rad, USA). The cells were electroporated in a Bio-Rad Gene Pulser, model 200-2.0 (Bio-Rad) set at 2.2 kV and the Bio-Rad Pulse controller was set at 200 Ω resistance. After electroporation, the cells were transferred to an sterile microcentrifuge tube containing 1 mL of TSB media (Appendix 1), followed by
incubation at 37°C for 45 min. Aliquots (5 µL, 100µL and 700µL) of transformed cells were plated on TSB plates (Appendix 1.47) containing 100 µg/mL amp and incubated at 37°C O/N.

3.3.4.2 B. choshinensis transformation

pNC-H3 plasmid (Appendix 2) DNA containing the gene of interest was extracted from E. coli cells using a Wizard® PCR preps DNA purification system (Promega, USA). Four microlitres of pNC-H3 plasmid DNA and 1µL salmon sperm DNA was electroporated with 100 µL of B. choshinensis competent cells using a Gene Pulser, model 200-2.0 (Bio-Rad) set at 1.5kV, 25μF and 1000ohm. Cells were recovered in 0.9 mL MBTY buffer (Appendix 1) and incubation at 37°C for 45 min in an orbital mixer incubator (Ratek, Australia). Clones were then selected by growth on MBTY agar plates (Appendix 1.17) containing 50 µg/mL neomycin (Sigma, Germany) overnight at 30°C in a humidified atmosphere.

3.3.4.3 DNA Extraction and concentrations

Small-scale plasmid DNA extraction was performed using the Wizard® PCR preps DNA purification system (Promega, USA) according to the manufacturer’s instructions. The plasmid DNA integrity was examined by restriction enzyme digestion followed by agarose gel electrophoresis. DNA concentrations were analysed using a NANOdrops® ND-1000 spectrophotometer (BioLab, USA).

3.3.4.4 Sequencing of plasmid DNA

Sequencing of plasmid DNA pQE30 vector containing the IL-3 and IL-5 cDNA and pNC-H3 vector containing PoGM-CSF cDNA were completed by Monash University DNA Sequencing Facility (Monash University, Australia), using PRISM DyeDeoxy Terminator Cycle Sequencing Ready Reaction Kits (Applied Biosystems, USA) with Perkin Elmer Dye Terminator FS Chemistry (Perkin Elmer) in a Corbett Research Cycle Sequencer, model number FTS320 (Corbett Research, Australia), and Applied Biosystems DNA Sequencer, model number 373 (Applied Biosystems). DNAsis version 7 and PROSIS version 7 were used to generate aa sequence and estimate expected protein weight (Hitachi Software engineering Co. Ltd, Japan).
3.3.5 Recombinant protein procedures

3.3.5.1 Rapid screen expression of recombinant proteins

Small scale 10 mL cultures were set up with TSB containing 100 μg/mL ampicillin and inoculated with a 1 in 5 volume of fresh overnight culture of *E. coli* containing pQE30 and the gene of interest. Cultures were grown at 37°C for 1 h in an orbital mixer incubator (Ratek, Australia) before being induced with 2 mM isopropyl-β-D-thiogalactopyranoside (IPTG) (Progen, Australia) for a further 2 h. One mL aliquots were taken and centrifuged at 13,000 x rpm for 1 min in a Biofuge pico centrifuge (Heraeus, Germany). The pellets were resuspended in 50 μL 2x SDS gel-loading buffer, heated at 100°C for 5 min and expression of protein determined by running on an SDS PAGE gel.

3.3.5.2 Expression of pQE30 expressed rPoIL-5

pQE30 IL-5 was plated onto a TSBA100 plate and incubated overnight at 37°C. Two 10 mL aliquots of TSB containing 100μg/mL ampicillin were each inoculated with a single colony containing pQE30 IL-5 and incubated overnight at 250-300 rpm on an orbital mixer incubator (Ratek, Australia) at 37°C. Each of 2 x 500 mL pre-warmed TSB and 100μg/mL amp was seeded with 10 mL of overnight culture and incubated at 300 rpm and 37°C on an orbital mixer incubator for 2.5 to 3h until OD₆₀₀ reached 0.7-0.9. Protein expression was induced with 2mM IPTG and cells were incubated for a further 3h. Cells were harvested by pelleting at 6,000 g max for 15 min at 4°C in a Beckman Avanti JA10.5. The media was decanted off and the cell pellet stored at -20°C O/N.

3.3.5.3 Solubilisation of pQE30 expressed rPoIL-5

Cell pellets were resuspended and washed in 10mL TALON™ (Clontech Laboratories Inc, USA) buffer and pelleted at 7000 g_max for 10 min at 4°C in a Beckman Avanti JA10.5. Supernatant was collected as pellet wash. Cells were resuspended and pooled in 8.4 mL of TALON buffer containing lysozyme (Sigma, Germany) and vortexed to mix then incubated for 15 min on ice. 1.5 mL of 10% Sarkosyl (Sigma, Germany) was then added and suspension sonicated for 6 x 10sec (x2) using an ultrasonic liquid processor/sonicator (Heat Systems, USA) on setting 5 with the medium probe. Suspension was then spun at 10,000 g_max for 10 min at 4°C and the supernatant carefully decanted off into a 50 mL Falcon tube (Falcon, USA). Triton X-100 (Sigma, Germany) was added to a final concentration of 2% and solution was refrigerated overnight at 4°C. A sample was saved as pre-bind.
3.3.5.4 Purification of pQE30 expressed rPoIL-5

Recombinant PoIL-5 was purified using TALON™ Metal Affinity Resin (Clontech Laboratories Inc, USA). Solubilised protein samples were mixed with TALON™ resin which had been washed and equilibrated with TALON Tris buffer containing 10mM imidazole as instructed. Samples were allowed to bind for 30 min at RT on a rotating wheel and the matrix sedimented by centrifugation in a Rotina 48R centrifuge (Hettich Zentrifugen, Germany) at 300 x g\text{max} for 5 min. The unbound fraction was collected and the beads washed in 20 mL TALON buffer containing 0.1% Triton X-100 (Appendix 1), then 20 mL of TALON buffer followed by 20 mL of TALON buffer containing 5mM imidazole (Sigma, Germany). The rPoIL-5 was then eluted from the beads by placing resin into a 1 cm diameter Econo-Column®, and eluting with 3 x 5 ml TALON elution buffer containing increased concentrations of imidazole of 50mM, 100mM and 200mM (Appendix 1). Protein concentrations in purified preparations were estimated using the Bradford dye assay (Biorad, USA) using BSA as a standard.

3.3.5.5 Expression of rPoIL-3 in E. coli

cDNA fragments produced by PCR encoding mature rPoIL-3 were extracted from low melting point agarose gels and subcloned into the BamHI/HindIII sites of the pQE30 (Qiagen, Germany) expression vector and electroporated into E. coli TOPP F10 cells. Recombinant colonies were grown overnight in Trypticase Soy broth (Becton Dickinson, USA) supplemented with ampicillin at 37°C, then diluted into fresh broth and grown until the OD\text{600} reached 0.7-0.9. IPTG to a final concentration of 2 mM was added and growth continued for an additional 3.5 h at 37°C. Cells were pelleted by centrifugation and stored for a short term at -20°C.

3.3.5.6 Solubilisation of pQE30 expressed rPoIL-3

Cells were thawed on ice and resuspended in sonication buffer (Appendix 1), lysed by mild sonication (Heat Systems sonicator, XL2020) then centrifuged 12,000 x g\text{max} for 7 min. The supernatant was removed and the process repeated using increasing concentrations (0.1% - 0.25%) of Zwittergent 3-14 (Calbiochem, USA) in sonication buffer. The supernatants were analysed for soluble 6xHis tagged rPoIL-3 by SDS-PAGE using 12% gels (Appendix 1) stained with 0.1% w/v Coomassie brilliant blue R-250 (Sigma, USA).

3.3.5.7 Purification of rPoIL-3

Pooled rPoIL-3 supernatants were purified using TALON™ metal affinity resin (Clontech, USA), previously equilibrated with sonication buffer and 10 mM imidazole
(Sigma, USA) and mixed for 30 min at RT on a rotating platform. The unbound fraction was collected and the resin washed once with 5 bed volumes of Talon wash buffer and then once with 5 bed volumes of Talon wash buffer containing 5 mM imidazole prior to loading the slurry into a low pressure gravity flow column (Biorad, USA) (column size 1.0 x 10.0 cm). rPoIL-3 was competitively eluted from the column at pH 8.0 by adding 100 mM imidazole in sonication buffer. The supernatants were analysed for purified 6xHis tagged rPoIL-3 by SDS-PAGE using 12% gels and stained with 0.1% w/v Coomassie brilliant blue R-250 and by Western Blot. Protein concentrations in purified preparations were estimated using the Bradford dye assay (Biorad, USA) using BSA as a standard.

3.3.5.8 **B. choshinensis expression of rPoGM-CSF protein**

Expression of rPoGM-CSF protein by *B. choshinensis* was performed using the method developed by the company Higeta Shoyu (Chiba, Japan). Bacteria containing the pNC-H3-GM-CSF clone 2.1 (MB2.4) vector were cultured overnight in 3 mL BTY media (Appendix 1) containing 50 μg/mL neomycin at 30°C in a shaking incubator (Ratek, Australia). A one mL aliquot was then transferred to 100 mL BTY media in a 1 L flask and grown at 30°C for a further 48 h before aspirating secreted protein. The crude supernatant was dialysed against three changes of Ni-NTA phosphate buffer.

3.3.5.9 **Purification of rPoGM-CSF protein**

Recombinant PoGM-CSF was purified using Ni-NTA resin (Qiagen, Germany). 20 mL of solubilised protein samples mixed with 4 mL Ni-NTA resin were allowed to bind for 1 h at RT on a rotating wheel. The resin was applied to a 1 cm diameter Econo-Column® and unbound fractions allowed to flow through. The resin was washed twice with 20 mL of Ni-NTA wash buffer (Appendix 1) before rPoGM-CSF protein was eluted in 4 x 1 mL Ni-NTA elution buffer (Appendix 1). The resin was resuspended in an equal volume of Ni-NTA buffer. Aliquots of the unbound, washed and eluted samples were analysed by SDS-PAGE and Western blot. Protein concentrations in purified preparations were estimated using the Bradford dye assay using BSA as a standard.

3.3.5.10 **SDS PAGE**

Polyacrylamide gels, 12% (w/v), were assembled following the methods of Sambrook *et al.* (1989) (Appendix 1). Protein samples and relative molecular weight (M_r) markers (Bio-rad) were diluted in 2 x SDS gel-loading buffer (Appendix 1) and were denatured prior to loading by heating at 100°C for 3 min. The protein samples
were then electrophoresed at 200 V using an electrophoresis power supply box, model number 300XI (Bio-rad) under reducing conditions in running buffer (Appendix 1). Protein bands in the gels were fixed and detected with 0.1% (w/v) Coomassie Brilliant Blue stain (Appendix 1) and excess dye was removed from the gel using destaining solution (Appendix 1). The concentration of proteins was routinely determined using the Bio-Rad protein assay (Bio-Rad) and standard concentrations of BSA protein according to the manufacturer’s instructions and read at 620 nm using a Titertek Multiscan Plus plate reader (Titertek, USA).

### 3.3.5.11 Western blot analysis of protein

Proteins were electrophoresed on SDS-PAGE gels under reducing conditions in running buffer (Appendix 1). The method used to transfer the protein to nitrocellulose membranes (Schleicher and Schuell, Germany) was a modified version from Tong et al. 1993 (Tong, et al. 1993). The gels and 3M filter paper (Schleicher and Schuell, Germany) were equilibrated in Western transfer buffer (Appendix 1) for 15-30 min. The gel holder cassette was then assembled forming a sandwich of gel, membrane, filter paper and fibre pads so that the protein would be transferred to the nitrocellulose membrane. Following transfer at 200 mA for 1 h using an electrophoresis power supply box (Bio-rad, USA) the membrane was incubated overnight in Western blocking buffer (Appendix 1).

The membrane was washed twice for 5 min in TBS-Tween/Triton buffer (Appendix 1) at RT followed by a 10 min wash in TBS buffer (Appendix 1). Antibodies were diluted in Western blocking buffer and incubated at RT for 1 h. The membrane was subsequently washed twice in TBS-Tween/Triton buffer for 5 min followed by one wash in TBS buffer for 10 min. Secondary antibodies were diluted in 5% skim milk powder made up in TBS buffer and incubated for another 1 h at RT. The membrane was finally washed 4 x 5 min each in TBS-Tween/Triton.

ECL™ Western blotting detection reagents were used for chemiluminescent detection according to the manufacturer’s instructions (Amersham, UK). The membranes were subjected to autoradiography film (Fuji, Japan) then developed using a Fuji X-Ray Film Processor RGII (Fuji, Japan).

### 3.3.5.12 Detoxification and filtering of purified proteins

Recombinant proteins were detoxified to remove any endotoxins which may be present. 1 mL of packed Detoxi-gel™ (Pierce, USA) was applied to a 1 cm diameter Econo-Column® and the storage buffer was allowed to elute through. Five mL of
Deoxycholate 17 in ddH₂O was added to the resin and allowed to equilibrate for 1 min before draining off. Resin was washed three times with 10 mL of ddH₂O. Recombinant proteins were passed through the resin twice. Endotoxin free proteins were then filter sterilised through a 0.22μm Cameo 25AS filter (Micron Separations Inc MSI, USA). Filtered proteins were refrigerated at 4°C.

3.3.6 Cell culture and bioassays

3.3.6.1 Thawing of B-cell activating factor (BAF) cells

The BAF cell line is an immortalized IL-5-dependent Mo BM derived pro-B-cell line (Ba/F3) (Palacios and Steinmetz 1985) which was derived by stable transfection with the Hu IL-5 receptor α chain followed by selection with recombinant Hu IL-5 (Sylvin, et al. 2000, Palacios and Steinmetz 1985, Coombe, et al. 1998). These BAF cells specifically detect Hu IL-5 (Sylvin, et al. 2000, Coombe, et al. 1998). Here we report that BAF cells also detect recombinant PoIL-5. BAF cells were removed from liquid nitrogen storage and defrosted as per BM thawing. After first wash the cells were re-suspended up to 5 mLs with 5% X63 supernatant then aliquoted into the first well of a 6 well tissue culture test plate (TPP, Switzerland). A 1:5 dilution was made into the next well. The cells were incubated at 37°C and 5% CO₂ until well grown. Cells were then passaged into a 75cm Corning® culture flasks. Well grown cells were decanted into a 50 mL falcon tube and centrifuged at 1200 g max for 7 min in a Rotina 48R centrifuge, model 4370. The cell pellet was washed 3 times by re-suspending with 30 mL DMEM containing 10% FCS and centrifuging at 1200 g max for 7 mins. A trypan blue stained cell count was performed and cells adjusted to 5 x 10⁴ cells/mL.

3.3.6.2 rPoIL-5 bioassay using BAF Mo cell line

Mo BAF cells were grown in the presence of X63 media (Appendix 1) as an IL-5 source. Test cytokine samples are positive when BAF cells deprived of IL-5 continue to grow and proliferate. X63 is a Mo myeloma cell line which produces IL-5 (Karasuyama, et al. 1988).

The assay was performed using MoIL-5 as a positive control. All samples were titrated across a 96 well Nunclon™ surface plates (Nunc, Denmark) plate in three fold dilutions. One hundred μl of DMEM was added to each well. rPoIL-5 to be tested was added to wells in the first column and titrated across the plate. One hundred μL of cells at the above concentration were added last, giving a final well volume of 200μL. The plates were incubated in a 5% CO₂ Forma Scientific water jacketed incubator (Forma Scientific, USA) at 37°C for 2 days. On day 3 the cultures were pulsed with 1.0 μCi per
well of methyl $^3$H-thymidine ($^3$HT, Amersham, UK) for an additional 6 h before being harvested onto glass fibre filters (Wallac, Finland) by a manual cell harvester (Tomtec, USA). The fibre filter papers were dried and radioactivity measured using Betaplate Scint Liquid Scintillation (Wallac, Finland) and luminescence counter and represented as counts per minute (cpm).

3.3.6.3 Assay for the detection of GM-CSF

TF1 cells are a non-adherent cell line of Hu origin, derived from erythroleukemia BM. Cells have complete dependency on GM-CSF or HuIL-3 for their long-term growth (Hintz-Obertreis, et al. 1991).

TF1 cells were removed from liquid nitrogen and rapidly thawed by shaking in a 37°C water bath. Two mL of warmed RPMI 1640 medium (Appendix 1) was added drop wise, whilst gently shaking the tube. A further 7 mL of media was added whilst gently mixing. Cells were centrifuged for 8 min at 1,000 g$_{max}$ in a Rotina 48R centrifuge, model 4370. The supernatant was decanted off and the cells gently re-suspended in 5-10 mL of TF1 growth medium (appendix 1) and cultured in a 25cm$^2$ flask until log growth phase was reached. Cells were decanted into a 50 mL falcon tube and centrifuged at 1200 g$_{max}$ for 7 min in a Rotina 48R centrifuge. The cell pellet was resuspended and topped up to 40 mL with PBS. Cells were centrifuged at 1200 g$_{max}$ for 7 min in a Rotina 48R centrifuge. This procedure was repeated twice more (3 washes). A trypan blue stained cell count was performed and cells were adjusted to 5 x $10^4$ cells/mL in RPMI 1640 medium (without the addition of GM-CSF).

The assay was performed using recombinant bovine GM-CSF as a positive control. Recombinant PoIL-3 was used as a negative control. All samples were titrated across a 96 well Nunclon™ surface plates in three fold dilutions. One hundred $\mu$L of RPMI 1640 medium (without the addition of GM-CSF) was added to each well of the 96 well plates. One hundred $\mu$L of RPMI 1640 medium containing recombinant $B.\ choshinensis$ expressed PoGM-CSF was added to three wells in the first column and titrated across the plate. One hundred $\mu$L of cells at the above concentration (final concentration 5x10$^3$ cells/well) were added last to each well, giving a final well volume of 200$\mu$L. Three replicate wells per dilution were plated. The plates were incubated in a 5% CO$_2$ Forma Scientific water jacketed incubator for 2 days. On day 3 the cultures were pulsed with 1.0 $\mu$Ci per well of methyl $^3$HT for an additional 6 h before being harvested onto glass fibre filters by a Tomtec manual cell harvester. The fiber filter papers were dried and radioactivity measured using Betaplate Scint Liquid Scintillation (Wallac, Finland) and luminescence counter and represented as cpm.
3.3.6.4 Po BM proliferation assay

Po BM cells were seeded at $2 \times 10^6$ cells/mL into a 96 well Nunclon™ surface plate and incubated with serially diluted rec PoGM-CSF, PoIL-3 or PoL-5 and relevant controls. Plates were incubated for 4 days at 37°C in 5% CO$_2$. Proliferating cells were counted at 24h intervals and compare to both cells alone (media) and cells grown with negative controls. Cells were pulsed with 1.0 $\mu$Ci per well of methyl $^3$HT and incubated for 6 h before harvesting and counting. Incorporated thymidine was counted using a Wallac 1450 Microbeta liquid scintillation and luminescence counter and presented as counts per minute (cpm). Results of proliferation assays were then presented as concentration verses stimulation index and significant differences analysed. The stimulation index was calculated by dividing the mean result for the cytokine treated cells by the mean result of the cells in media alone. This presents the cells in media alone as one. This method provides a ratio of the number of proliferating cells present in a cell culture after exposure to cytokine to the total number of cells.

3.3.7 Flow cytometry and cell analysis

3.3.7.1 Antibody staining and flow cytometry

PoBM previously collected was removed from -80°C storage and thawed. After resuspension, approximately $2 \times 10^5$ cells/well were aliquoted into Dynex round bottom plates (DYNE, Technologies, USA). Plates were centrifuged in a Rotina 48R centrifuge (Rotina) at $400 \times g_{\text{max}}$ for 1 min and supernatant was discarded. Thirty $\mu$L of the primary antibody solution containing CD90-PE-Cy5 (clone 5E10) and CD172a-FITC (clone 74-12-4), diluted in FACS wash, was added to wells and plates were incubated on a cold Delfia Plateshaker, model 1296-001 (Perkin Elmer) for at least 15 min. One hundred and seventy $\mu$L of FACS wash was added to each well and samples washed by centrifuging again at $400 \times g_{\text{max}}$ for 1 min. Suspensions were acquired using either a FACSCalibur™ or a BD LSR II™ (Becton Dickinson, USA), with a total of $3-5 \times 10^4$ cells analysed for each sample. Analysis of data was performed using CELLQuest software for FACSCalibur samples and by FACSDiva Version 6.1.1 for BD LSR II samples (Becton Dickinson, USA). Dead cells were excluded from data acquisition by first staining with 7-Aminoactinomycin D (7AAD) (Becton Dickinson, USA) then back gating on the resulting histogram against the live cell population. Chicken anti Mo CD25-FITC was used as a negative isotype control for each cell population stained to measure non-specific background levels of antibody binding. The
commercial premixed isotype control MIF2bP2aT containing IgG₁-FITC (MOPC-21), IgG₂b-R-PE (MOPC-195) and IgG₂a-TC (UPC-10), Caltag Laboratories, USA, was used to establish fluorescent parameters.

### 3.3.7.2 Characterisation of CD90+ and CD172a⁺ PoBM

In order to characterize CD90+ and CD172a⁺ PoBM cells, BM from 1 week old Large White Landrace Cross pigs was prepared as previously explained in section 3.3.7.1 and stained with either CD90 APC (clone 5E10) and CD172a PE (clone 74-12-4) antibodies. In addition, preparations were each stained with one of CD14 FITC, CD16 FITC, CD21 FITC, MHC I PE-Cy5 and MHCII PE-Cy5. A total of 3-5x10⁴ cells were analysed for each sample. Acquisition and analysis of data and isotype and negative controls were as previously stated.

### 3.3.7.3 BrdU

To measure BrdU incorporation by cells, BM from 1 week old Large White Landrace Cross pigs was prepared as previously explained. Cells were washed once in SF DMEM and cultured in DMEM containing 1%FCS and recombinant Po cytokines for 96h. During the final 6h of incubation, BrdU (Becton Dickinson, USA) to a final concentration of 10 μM was added to the cell culture. Cells were examined for BrdU incorporation according to the manufacturer’s instructions (Becton Dickinson, USA). Briefly, cells were stained with cell surface antibodies against HuCD90 and PoCD172a then fixed and permeabilized using Cytofix/Cytoperm buffers. BrdU/DNase (Becton Dickinson, USA) was used to expose incorporated BrdU and then labelled with anti-BrdU-Allophycocyanin (APC) antibody. Finally, cell suspensions were acquired using a FACS Calibar™ and analysed with CELLQuest software.

### 3.3.8 Cell Differential Staining

Cells to be stained were first counted and numbers were adjusted to 1 x 10⁶ cells/mL. A 200μL aliquot of cells was then adhered to a microscope slide by centrifugation at 1000 rpm for 3 min in a SHANDON CYTOSPIN 2 (Shandon Southern Products LTD, England). Cells were then air dried for 1 h before differential staining using a DIFF-QUIK stain set (Lab Aids P/L, Australia). Three populations of cells were chosen from each slide at random and observed at 40 x magnification on a Leica DMLB bright field microscope (Leica Microsystems P/L, Australia). The mean results were calculated and each of the populations from each cytokine was compared to both the cells alone and negative controls.
3.3.9 Statistical analysis

GraphPad Prism (GraphPad Software, USA) was used to determine whether results were statistically significant. A Kruskal-Wallis test was used to compare multiple groups and where differences were found, a Dunn’s multiple comparison test was used to compare 2 groups of distributed data. Experimental values differing significantly from control values are indicated by significance p<0.05.

3.4 Results

3.4.1 Cloning and expression of rec Po cytokines in a prokaryotic system produces biologically active proteins

BM cDNA was amplified using Po specific primers for GM-CSF, IL-3 and IL-5 (Table 3.1). The resulting DNA strands were isolated by agarose gel and were shown to produce products of the pre-determined sizes according to DNA sequence analysis (Table 3.1). DNA strand sizes were as follows: PoGM-CSF - 402bp, whole PoIL-3 - 454bp, mature PoIL-3 - 395bp and PoIL-5 - 360bp (Figure 3.1). Recombinant protein for each of the cytokines were then produced in the most appropriate expression systems which labelled the recombinant cytokine with a 6 x His tag for isolation and purification. For rPoIL-3 and rPoIL-5, this tag was located at the N-terminal end of the multi cloning site of the pQE-30 vector. In the case of rPoGM-SCF, the His tag was located at the C-terminal end of the multi-cloning site of the pNCM02 vector. The proteins where then purified by affinity chromatograph and identified by SDS-PAGE (Figure 3.2). Protein sizes were observed at 18kDa, 15kDa and 14kDa for GM-CSF, IL-3 and IL-5 respectively. Western blot analysis was performed and each protein was identified by an anti-His antibody (Figure 3.3). Expression of each cytokine was then optimised for maximum quantity by adjustments to IPTG concentrations and incubation and expression durations. The resulting expression levels of 40mg/L, 1.0g/L and 20mg/L were achieved for GM-CSF, IL-3 and IL-5, respectively.

To determine the bioactivity of the rPo proteins, each of the cytokines was tested in an appropriate cell assay. rPoGM-CSF was tested for biologically activity using Hu TF1 cells which require GM-CSF for proliferation (Hintz-Obertreis, et al. 1991). The assay showed activity for rPoGM-CSF for concentrations of 0.15 to 3.70μg/mL (Figure 3.4). As IL-3 is a known inducer of proliferation for HSC and precursor cells, rPoIL-3 activity was assessed on Po BM cells. Proliferation of cells was determined from 1.0 to 33ng/mL (Figure 3.5). Similarly, rPoIL-5 activity was
**Figure 3.1  Agarose gels of PCR of Po cytokines.** Figure shows 1% agarose gel with PCR products of rec Po cytokines used in these experiments. A. GM-CSF (mature), B. IL-3 (full length and mature) and C. IL-5 (mature). Each cDNA stand was run along with a 100 bp molecular weight marker. PCR strands were further amplified for sequence analysis and confirmed positive for individual cytokines. Arrows indicate the amplified DNA and corresponding size.
Figure 3.2  SDS PAGE gels of rPo cytokines. rec Po cytokines were run on separate SDS PAGE gels A. B. choshinensis expressed GM-CSF, B. E. coli expressed IL-3, and C. E. coli expressed IL-5. Rec cytokines were detected by Coomassie Brilliant blue. Each protein was run along with a broad range molecular weight markers with sizes indicated in kDa, as indicated in A. Arrows indicate the location of the purified proteins and the molecular weight in kDa.
Figure 3.3 Western blot of rPo cytokines. Rec Po cytokines were detected on individual Western blots as shown, A. *B. choshinensis* expressed GM-CSF, B. *E. coli* expressed IL-3, and C. *E. coli* expressed IL-5. Each protein was detected by anti-His antibody. Molecular weight markers show relative size in kDa. Arrows indicate the location of the purified proteins and the molecular weight in kDa.
Figure. 3.4 rPoGM-CSF shows biologically activity in Hu TF1 cell assay. Data showing TF1 proliferation assay in the presence of *B. choshinensis* expressed rPoGM-CSF. PoIL-3 was used as negative control. Data shows the mean result for proliferating cell numbers. n=3. Similar results were obtained in three independent assays. mean ± SEM
Figure 3.5 rPoIL-3 shows biological activity in PoBM proliferation assay. Data showing 96h PoBM proliferative assay in the presence of rPoIL-3. PoIFN was used as a negative control. n=3 Similar results were obtained in three independent assays. mean ± SEM.
determined in a Mo BAF cells which require IL-5 for proliferation. Biological activity was observed when BAF cells were incubated with rPoIL-5 for concentrations of 0.8 to 200ng/mL (Figure 3.6). All three cytokines where observed to produce notably higher levels of cell proliferation compared to growth with negative controls and cells grown in media alone. It was therefore established that each cytokine was bioactive. In addition, it was shown that Hu TF1 cells are responsive to rPoGM-CSF and Mo BAF cells are responsive to rPoIL-5.

3.4.2 BM proliferation assay with recombinant cytokines produces significant differences between pig and Hu

Each Po cytokine was compared to the Hu isotype in a 96h BM proliferation assay. Proliferating cells were counted at 24h intervals and compare to both cells alone (media) and cells grown with negative controls and significant differences were analysed.

3.4.2.1 GM-CSF assay

Proliferating cells numbers for rPoGM-CSF treated cultures were considerably low for the first 72h following administration of cytokines (data not shown). Cell numbers were however significantly increased by 96h with significant differences observed at between rPoGM-CSF and cells alone for 250, 84, 28, 9, 1 and 0.3ng (Figure 3.7). It was established that the onset of activity of rPoGM-CSF on BM was observed over 96h with the increase in proliferation from 72h. No activity was observed for HuGM-CSF.

3.4.2.2 IL-3 assay

As with rPoGM-CSF, there was notably little proliferation of BM cells for rPoIL-3 for the first 48h of incubation, however, some increase was evident by 72h (data not shown). By 96h an increase in proliferating BM cells for rPoIL-3 cultures was clearly noted. Significant differences were observed between rPoIL-3 and cells alone for 50, 16.6, 5.55, 1.85 and 0.20ng. In addition, a significant difference was detected between HuIL-3 and cells alone at 96h for 0.007ng (Figure 3.8). It was established that the onset of activity of rPoIL-3 on BM was observed over 96h with the increase in proliferation observed from 72h.

3.4.2.3 IL-5 assay

There was little change in proliferation detected for rPoIL-5 cultures within the first 48h (data not shown). Proliferation increased more notably at the 72h before
Figure 3.6 rPoIL-5 shows biological activity in IL-5 dependent BAF mouse cell bioassay. Data showing the results of a Mo BAF cell bioassay after 48h in the presence of rPoIL-5 in. n=3. PoIFN was used as a negative control. Similar results were obtained in three independent assays. mean ± SEM
Figure 3.7 PoGM-CSF produces PoBM proliferation in a dose dependant manner. Data showing the stimulation index of the proliferative effects of rPoGM-CSF and HuGM-CSF on PoBM after 96h. Significant differences were observed between rPoGM-CSF and cells alone for 250, 84, 28, 9, 1 and 0.3ng/mL. All measurements report mean ± SEM where n=3. All measurements display error bars. * indicates P<0.05.
Figure 3.8 rPoIL-3 produces PoBM proliferation in a dose dependant manner. Data showing the stimulation index of the proliferative effects of rPoIL-3 and HuIL-3 on PoBM after 96h. Significant differences were observed between rPoIL-3 and cells alone * at 50, 16.6, 5.55, 1.85 and 0.2 ng/mL. A significant difference was also noted between HuIL-3 and cells alone, ° for 0.007ng/mL. All measurements report mean ± SEM where n=3. All measurements display error bars. */° indicates P<0.05.
reducing again by 96h (data not shown). Significant differences were detected between rPoIL-5 and cells alone at 72h for 16.6ng, 5.55ng, 1.85ng, 0.06ng and 0.002ng (Figure 3.9). It was established that the onset of activity of rPoIL-5 on BM was observed over 96h with the maximum increase in proliferation observed at 72h. There were no significant differences observed for HuIL-5 induced proliferation on PoBM.

### 3.4.3 FACS characterisation of CD90+ and CD172a+ BM.

Linage characterisation of CD90+ BM was established by FACS using available antibodies to known cell surface markers and was plotted on dot plots. Staining of CD90+ cells produced positive populations of total BM as follows, CD90+CD14- -1%, CD90+CD16- -4%, CD90+CD21- -3%, CD90+MHCI -20%, CD90+MHCII -<1% (Figure 3.10).

Linage characterisation of CD172a+ BM was established by FACS using available antibodies to known cell surface markers and was plotted on dot plots. Staining of CD172a+ cells produced positive populations of total BM as follows, CD172a+CD14- -6%, CD172a +CD16- -31%, CD172a +CD21- -4%, CD172a +MHCI - 34%, CD172a +MHCII -10% (Figure 3.10).

### 3.4.4 FACS analysis of recombinant cytokine treated BM cells in vitro indicates cell differentiation and proliferation

Antibodies against cluster of differentiation molecules for Thy1 (CD90) and SIRPα (CD172a, SWC3) were first used to establish a profile for both non and cytokine incubated BM cells (Figure 3.11). Histograms developed on CD90 expression indicated CD90+ expression for 62% and 56% of BM cells for cells alone and for negative control, respectively. A slight increase in CD90+ cells was seen in rPoGM-CSF and rPoIL-3 preparations being 68% and 66%, respectively, whereas rPoIL-5 treated cells present with a similar profile to cells alone and negative control cells with only 60%. CD172a+ cells are divided into CD172a+, CD172a^lo and CD172a^hi. Histogram profiles for cells alone and negative control were only slightly different and may provide a range for each level of expression. Cells alone and negative control for each level of CD172a were as follows. CD172a^lo cells 28% and 33%, CD172a^lo 60% and 56%, and for CD172a^hi 12% and 11% respectively. CD172a expression for cytokine treated cells were as follows. CD172a^lo cells 26%, 28% and 30%, CD172a^lo 21%, 17% and 58%, and for CD172a^hi 56%, 55% and 12%, respectively for rPoGM-CSF, rPoIL-3 and rPoIL-5.
Figure 3.9 rPoIL-5 stimulates PoBM proliferation in a dose dependant manner. Data showing the stimulation index of the proliferative effects of rPoIL-5 and HuIL-5 on PoBM after 72h. Significant differences were observed between PoIL-5 and cells alone for 16.6, 5.55, 1.85, 0.06 and 0.002ng/mL. All measurements report mean ± SEM where n=3. All measurements display error bars. * indicates P<0.05.
Figure 3.10 Characterization of CD90\(^+\) and CD172a\(^+\) PoBM cells.
Dot plots showing the flow cytometric characterization of CD90\(^+\) and CD172a\(^+\) PoBM co-stained with lineage specific antibodies. Numbers in each quadrant of each dot plot represent the percentage positive cells in that quadrant. Controls included isotype matched mAb.
Figure 3.11 PoBM treated with rPoIL-3 or rPoGM-CSF induces differentiation of CD90+ and CD172a+ cells. BM cells were incubated for 90 h with or without cytokines (rPoIL-3, 10ng/mL; rPoGM-CSF, 5μg/mL; rPoIL-5, 10ng/mL; or negative control). Cells were then analyzed for CD90 and CD172a expression. n=3, numbers represent percentage of cells.
Both CD90 and CD172a monoclonal antibodies were used in conjunction with anti-BrdU antibody in flow cytometric analysis to determine proliferating BM cells after incubation with recombinant cytokines.

### 3.4.4.1 BrdU/CD90

Cells gated on BrdU/CD90 were analysed for each cytokine in addition to cells alone and negative control. Both cells alone and negative controls produced the equivalent histogram profiles and similar cell percentages indicating 18% proliferation of CD90+ cells. Similarly, cells treated with rPoIL-5 produced the same profile and 18% proliferation of CD90+ cells lymphoid cells. The results for rPoGM-CSF and rPoIL-3 however, indicate cytokine enhanced proliferation of BM cells. Cells treated with rPoGM-CSF showed an increase in proliferation from 31% to 49% and rPoIL-3 increased proliferation from 26% to 44% over both cells alone and negative control cells (Figure 3.12).

### 3.4.4.2 BrdU/CD72a

As CD172a produces a multiple peaked profile we are able to view CD172a- CD172a<sup>lo</sup> and CD172a<sup>hi</sup> cells each representing a different cell population. As with CD90, CD172a labelled BrdU+ cells showed similar profiles for both cells alone and negative control cells although slightly less CD172a<sup>hi</sup> cells were observed for negative control (21%) than for cells alone (18%). Likewise, as with CD90 labelled cells, rPoIL-5 treated cells show no increase in proliferation for myeloid cells over both cells alone and negative control. rPoGM-CSF and rPoIL-3 treated cells however, show a considerable increase in proliferating cells. rPoGM-CSF treated cells had a decrease in CD172a<sup>-</sup> and CD172a<sup>lo</sup> cells of 24% and 21%, respectively, and an increase in CD172a<sup>hi</sup> cells of 45% compared to cells alone. Similarly, rPoIL-3 treated cells had a decrease in CD172a<sup>-</sup> and CD172a<sup>lo</sup> cells of 20% and 14%, respectively, and an increase in CD172a<sup>hi</sup> cells of 34% over cells alone (Figure 3.12).

### 3.4.5 Morphology of cytokine treated cells

Wright Giemsa stains of BM cells incubated for 96 h with or without cytokines were scored to determine numbers of cell types which had developed between groups. Cells were morphologically identified as being lymphocytes, monocytes, granulocytes, eosinophils and macrophages. Results for rPoGM-CSF show a significant decrease in lymphocytes and neutrophils compared to cells alone and neutrophils compared to the negative control. In addition, a significant increases in monocytes was observed and an increase in macrophages over cells alone and negative control, however, not
Figure 3.12 PoBM treated with PoIL-3 or PoGM-CSF induces proliferation of CD90+ and CD172a+ cells. BM cells were incubated for 90 h with or without cytokines (IL-3, 10ng/mL; GM-CSF, 5μg/mL; IL-5, 10ng/mL; or negative control), followed by 6 h with Bromodeoxyuridine (BrdU). Cells were then analyzed for proliferation. n=3, numbers represent percentage of Brdu+ gated cells.
significant (Figure 3.13). There were no significant differences for rPoIL-3 treatment although a slight increase in macrophages compared to control cultures and a decrease in neutrophils were noted (Figure 3.14). rPoIL-5 treatment of cells produced a significant increase in eosinophils but had no effect on other cell types.

3.5 Discussion

The control of disease in food production animals is an ongoing concern. The uncontrolled prophylactic use of antibiotics has been linked to the production of antibiotic resistant strains of bacteria (Lowenthal, et al. 1999). The use of cytokines as a substitute for antibiotics may be one way to combat this problem (Lowenthal, et al. 2000). The recent development and refinement of cloning techniques has allowed us to not only isolate specific cytokines from their biological source, but to also reproduce these proteins in their recombinant form (Greenberger 2000). These cytokines can be tested for biological activity and then characterised to assess their role in hematopoiesis and immune response. Here we cloned the rPo cytokines, GM-CSF, IL-3 and IL-5, and then tested their biological activity and characterised specific cells produced under their influence.

Initial stages of cloning for each of the cytokines produced cDNA strands of the predetermined sizes of 402bp, 395bp and 360bp for PoGM-CSF, PoIL-3 and PoIL-5 respectively. All three cytokine PCR products (the mature PoIL-3 sequence was selected for use as the PoIL-3 clone) were isolated by agarose gel electrophoresis prior to ligation into their appropriate expression vectors before transformation into the selected bacterial strains. Following bacterial culturing and chemically induced expression, each of the rec cytokines were visually observed on SDS PAGE. The protein bands were identified to be of the appropriate sizes of 18kDa, 15kDa and 14kDa for rPoGM-CSF, rPoIL-3 and rPoIL-5, respectively, and were later confirmed by Western blot transfer and anti-HIS antibody detection. Biological activity for each cytokine was assessed in vitro using cytokine dependant bioassays. Hu TF1 cells showed optimal proliferation in the presence of rPoGM-CSF at 1μg/mL with activity detected down to 5ng/mL. This appeared to be considerably higher than the activity recorded for HuGM-CSF on TF1 cells which is optimum at 3ng/mL (Kitamura, et al. 1989). This reduced activity is possibly due to cross species differences in the GM-CSF molecule. As previously shown, PoGM-CSF is 85.3% identical to HuGM-CSF, however, the Po molecule has appeared to have developed phylogenetically separately to Hu resulting in changes to the genetic code between species. Although both Hu and pig GM-CSF express the Glu21 residue which is required for CD131
Figure 3.13 PoBM cell morphology following cytokine treatment. Wright Giemsa stain of BM cells incubated for 96 hours with A - media alone, B - negative control, C - rPoGM-CSF, D - rPoIL-3 and E - rPoIL-5. Slides show predominant cell morphology for each cytokine incubation. M- monocytic cell, L- lymphocyte, N- neutrophil, E- eosinophil, Ø- macrophage. Cells were observed at 40x magnification.
**Figure 3.14 PoBM cell types following cytokine treatment.** Data showing resulting cell populations of 96h PoBM cell incubation both with or without cytokines. Cells were Wright Giemsa stained then visualized by light microscopy. Cell populations were randomly selected and counted and numbers were graphed showing cell type and the mean percentage of population ± SEM, n=10. Significant differences where observed for lymphocytes between cells alone and rPoGM-CSF*, monocytes between cells alone and rPoGM-CSF**, neutrophils between ChINFγ and rPoGM-CSF*, cells alone and rPoGM-CSF**, and for eosinophils between cells alone and PoIL-5*. where */♦ = P<0.05 and ** = P<0.01.
binding, PoGM-CSF appears to have alternative residues to the Hu molecule required for CD116 binding. This may reduce to some degree, the ability of rPoGM-CSF to adequately bind to and activate HuCD116. This may also explain the results when comparing the activity of rPoGM-CSF and HuGM-CSF in the PoBM proliferation assay. Four day PoBM cell cultures showed little to no proliferation in the presence of up to 250ng/mL of HuGM-CSF, whereas rPoGM-CSF had detectable activity for as low as 0.1ng/mL. Furthermore, it has been shown that rPoGM-CSF has no effect on primate BM cells (cynomolgus monkey, Macaca fascicularis) which showed normal activity when incubated with HuGM-CSF (Hawley, et al. 1997). This confirms the lack of cross species activity between HuGM-CSF and PoGM-CSF and supports the necessity to produce PoGM-CSF in order to affect a response for this cytokine in pigs.

Recombinant PoIL-3 activity was first measured in a BM proliferation assay. Biological activity showed an optimum activity of 3.7ng/mL, similar to TF1 cell response to rHuIL-3 which is optimal at 3ng/mL (Kitamura, et al. 1989). This is also similar to the results seen for the cross species activity of GM-CSF. A 96h BM assay showed an activity for rPoIL-3 of >4SI compare to <2SI for the Hu rIL-3 which equated to no response on PoBM. Low identity between Hu and pig IL-3 of only 54.5%, phylogenetic differences and alternative residues involved in CD123 binding may all be contributing to the reduced cross species activity. Previous studies by Emery et al (1999), produced a similar result where it was shown that there was no response to rHuIL-3 by swine BM cells. It was also shown that there was no response to rPoIL-3 by Hu BM cells (Emery, et al. 1999). This therefore reinforces the necessity of producing species specific IL-3.

The IL-5 dependent BAF cell line was used as a bioassay. rPoIL-5 showed a clear dose response with optimum activity recorded at approximately 7ng/mL. Results for the 96h BM assay for rPoIL-5 showed that rPoIL-5 was less effective at inducing proliferation then IL-3 and GM-CSF. A maximum of 2 SI was noted for rPoIL-5 in addition there were also several significant differences observed. This result is however more favourable than first noted. This is due to the percentage of cells which are affected by each of these cytokines. If it is first taken into consideration that GM-CSF and IL-3 act on a broad range of myeloid cells and that IL-5 is dominantly specific to eosinophils, and then compare the large populations affected by GM-CSF and IL-3 to the small population of eosinophils present, it can be conceived that a two fold increase in overall cell numbers is a positive result.
3.5.1 Characterization of CD90 and CD172a BM

FACS analysis of BM cells stained with CD90 and CD172a antibodies along with antibodies to the known cell surface markers CD14, CD16, CD21, MHCI and MHCII, produced a varying level of characterisation for both myeloid and lymphoid cell lines. The plasma membrane protein, CD14, activates monocytes when bound to bacterial and fungal components particularly and most importantly lipopolysaccharide (LPS). Subsequently, CD14 has been defined as a central pattern recognition molecule involved in innate immunity (Antal-Szalmas 2000, Pugin, et al. 1994). The presence of CD14 in pigs has thus far been reported on mature myeloid cells, specifically monocytes, macrophages, DC and granulocytes (Summerfield and McCullough 1997, Kapetanovic, et al. 2012), although it has been suggested that subsets of CD14+ mature monocytes may be down regulated during maturation and differentiation (Chamorro, et al. 2005). Staining of CD172a cells with CD14 produced a positive population of 10% of cells. As previous characterization of CD172a+ BM cells has determined CD172a as being only present on myeloid cells, and that lymphoid cells were only present in the CD172a- population (Summerfield and McCullough 1997), we can determine that the CD172a+ cells are of myeloid origin. Furthermore, as CD14+ has been previously shown to be present only on mature myeloid cells and not precursor cells, we can determine the 10% population of CD172a+CD14+ cells identified as being mature myeloid cells. Also of Interest is the 3.7% population of CD90+CD14+ cells observed. Although a population of CD90+CD172a+ cells has previously been identified as being HSC (Layton, et al. 2007), the addition of CD14+CD90+ cells has not previously been identified in the pig, however, work by Ziegler-Heitbrock HW et al. 1994, identified a population of mature B-lymphocytes in Hu as being positive for CD14 (Ziegler-Heitbrock, et al. 1994). In that instance, the CD90+CD14+ cells were shown to respond to LPS stimulation and subsequently produce both IL-6 and IL10 (Ziegler-Heitbrock, et al. 1994). Although identification of this population has currently not been confirmed in the pig, further characterizion of this CD14+CD90+ population may be warranted and may demonstrate similarities with the Hu cell population. In addition to CD14, both the CD172a+ and CD90+ populations tested positive for CD16. CD16 is cell membrane receptor which binds to the Fc portion of IgG antibodies. In the pig, CD16 has been identified on all peripheral blood monocytes and NK cells (Piriou-Guzylack and Salmon 2008, Sanchez, et al. 1999, Wierda, et al. 1993), both mature monocytic DC and blood DC (Piriou-Guzylack and Salmon 2008, Carrasco, et al. 2001), macrophages (Kapetanovic, et al. 2012) and polymophonuclear cells (Halloran, et al. 1994, Graziano and Fanger 1987). Staining of
BM with CD16 indicated a population of 51.6% of CD172a+ cells. This level was considerably higher than the population observed to be CD14+. Although a population of pro-inflammatory monocytes have been identified as being positive for both CD14 and CD16 (Chamorro, et al. 2005, Ziegler-Heitbrock 2007), the increase in staining here between CD14 and CD16 may be contributed by the expression of CD16 on neutrophils, which account for between 40 and 50% of blood cells. Furthermore, if we consider the differences in cells identified between CD14 and CD16 for the myeloid line and include the only differing cell line between the two markers as being PMN then we can clearly see that 25% of BM or approximately 42% of monocytes contribute to the PMN cell line. As this is the largest population of WBC type in the blood stream it is understandable that a high percentage of the BM population would be required for maintenance of this cell population. In addition, it is speculated that the presence of CD16 on NK cells would account for the 15.4% of CD90+ CD16+ cells, or 4% of BM observed. Furthermore, as 4% of BM is CD16+ CD90+ and only 1% of BM is CD16+ CD172a+, it appears a small population of cells may exist to be CD16+ CD90+ CD172a+. As HSC have been identified as CD90+ CD172a+, this population may represent a small number of transient precursor cells expressing CD16. Moreover, it has been shown in Hu and Mo that DC may indeed develop from either the myeloid and lymphoid cell lines (Takeuchi and Furue 2007, Martin, et al. 2000, Traver, et al. 2000). If both these pathways also exist in the pig, then we could suggest a portion of the CD16+ populations as being either lymphoid or myeloid derived DC provided both lines of DC express CD16. Further characterization of BM by CD21 selected for approximately 6% of cells of which 4% was CD172a+ and 3% CD90+ which equated to 6.8% of CD172a+ cells and 11.5% of CD90+ cells. In Hu, CD21 (complement receptor 2) is expressed on mature B cells (Suryani, et al. 2010), follicular DC (FDC) (Braun, et al. 1998) and T cell subsets (Braun, et al. 1998, Fischer, et al. 1999) however thus far in the pigs, CD21 has only been identified on mature B-cells (Kaleczyc, et al. 2010, Denham, et al. 1998) and FDC (Denham, et al. 1998). This would therefore indicate the probable cell type associated with the CD172a+CD21+ population identified as being a myeloid cell line derived FDC. Similarly, the CD90+CD21+ identified cells here are believed to be of lymphoid derived FDC. As CD90 is expressed on early B cells and CD21 is expressed on mature B cells we can determine without further characterization that the cell population identified here may not be a B cell population. This would conversely indicate that the 2% of CD90+CD21+ cells identified are most likely mature B cells.
Finally, we looked at the major histocompatibility complex (MHC) expression of both CD90+ and CD172a+ cells in the form of both MHC class I (MHCI) and class II (MHCII). MHCI expression was determined as being present on 61.8% of CD172a+ cells and 71.4% of CD90+ for cells. These results are considerably lower than the expected values of MHCI as MHCI is expressed on all nucleated cells in the body, however, as we are characterizing BM, a high degree of immature and precursors cells which have low expression of MHCI, were expected to be present. Nevertheless, expression of MHCI during immune cell development from BM was observed. The CD172a MHCI population followed the signatory pattern of CD172a staining where three main populations are indicated. These populations indicate the level of development for monocytes and granulocytes. As the population shifts to the right from CD172a− to CD172a(lo) we get a shift from non-myeloid cells to immature monocytes and granulocytes (Summerfield, et al. 2001). Further shifting to the right from CD172a(lo) to CD172a(hi) selects for mature monocytes and granulocytes (Summerfield, et al. 2001). These populations therefore allow the recognition of when MHCI is present on both immature and mature myeloid cells and that expression appears to increase with cell development and maturity. Furthermore, as neutrophils account for the highest population of WBC (Summerfield, et al. 2001) and are also one of the most short lived WBC, we can hypothesis that these positive populations are high in PMN. This would conversely indicate that a large portion of myeloid cells, 38.2%, are negative or low for the cell surface expression of MHCI whilst undergoing differentiation within the BM matrix. MHCI expression on CD90+ cells was positive on a higher percentage of cells than on the CD172a population, however, this may simple be due to the fewer number of cell lines which develop within the lymphoid cell line. Nevertheless, there was still a population of approximately 29% of CD90+ cells which were negative for MHCI. As with the myeloid cell line, it is hypothesised that these are most likely immature cells which are yet to express MHCI on the cell surface. Further characterisation of these populations would give a clearer timeline for MHCI expression on WBC within the BM matrix. Expression of MHCII was analysed for both cell lines. CD90+ MHCII+ cells accounted for only 2.5 of CD90 cells. Although this is only a minor population, it allows for an easy identification. B-cell expression of MHC II occurs early in the B cell development as the presence of this marker is required for long term mature B cell selection (Sproul, et al. 2000). As CD90 is also expressed on early progenitors we can hypothesis that the population of CD90+ MHCII+ observed may relate to early B cells. Subsequently, as CD90+ expression is down regulated for B-cells during maturity, we can determine the 11% of CD90− MHCII+ BM cells would contain a population of
mature B-cells. Furthermore, as this population would also include the population of CD172a⁺MHCII⁺ BM cells, which selected for 10% of BM and 13.7% of CD172a cells, we can hypothesize that approximately 1% of BM could be characterized as CD90⁻MHCII⁺ mature B-cells. In addition, as T cells express CD90 throughout development, we can determine that the population observed for CD90⁺ MHCII⁺, are most likely made up of developing T cells. MHCII expression on CD172a⁺ BM was observed for 13.7% of cells or 10% of total BM. As MHCII is expressed solely by antigen presenting cells it is safe to suggest that the population of CD172a⁺ MHCII⁺ BM observed would be mostly comprised of both DC and macrophages. In addition, as the majority of this population of cells is CD172a⁺, we can determine these cells to be mature APC. This may indicate that MHCII expression is low on immature APC and more highly expressed on mature APC prior to release into the periphery.

Further FACS analysis of cultured BM cells stained with CD90 and CD172a antibodies appears to indicate that a portion of BM cells were positive for both of these surface markers. This may suggest that a large portion of the cells are precursor cells, which includes B-cell and T-cell precursors, CLP, CMP, and HSC (Baum, et al. 1992, Ritter, et al. 1983). Cells cultured with rPoGM-CSF or rPoIL-3 produced higher percentages of CD90⁺ cells than the cells alone group. As both rPoGM-CSF and rPoIL-3 act on precursor cells to promote proliferation and differentiation (Martinez-Moczygemba and Huston 2003, Moretti, et al. 2001, Barreda, et al. 2004), these differences may indeed denote increases in replication of B-cell and T-cell precursors and common progenitors (Baum, et al. 1992, Ritter, et al. 1983) in addition to CD34⁺ HSC (Craig, et al. 1993). Furthermore, IL-3 has been confirmed as a growth factor for B-cells in Hu, and as CD90 is expressed on early B-cells, the high number of CD90 for the rPoIL-3 treated cells may also consist of increased B-cell precursors. When analysing these groups for proliferation we see a large increase in the number of proliferating cells when compared to cells alone and the negative control. This supports the role of both rPoGM-CSF and rPoIL-3 as being potent early acting cytokines which induce proliferation. When analysing the morphology of these cells, we note that there is a decrease in lymphocytes for the rPoGM-CSF treated cells, whereas the rPoIL-3 treated cells appear to be unchanged for lymphocyte numbers. This may be explained by the confirmation in Hu that GM-CSF acts as a potent colony stimulating factor on CD34⁺ progenitors but at the same time acts as a potent inhibitor for the differentiation of CD34⁺ progenitor cells into lymphoid progenitors (Martinez-Moczygemba and Huston 2003, Iwasaki-Arai, et al. 2003). Furthermore, it was suggested that a majority of CLPs and a fraction of pro-T cells possess plasticity for
myelomonocytic differentiation which can be activated by the GM-CSF signal (Iwasaki-Arai, et al. 2003). Therefore, expression of both CD90 and CD172a on precursors may account for a higher reading of CD90+ lymphoid cells by FACS, which may have, under the influence of GM-CSF, contributed to the myelocyte populations. Therefore, it is suggested that the increases in CD90+ cells for the rPoGM-CSF and rPoIL-3 treated groups may include early progenitor cells which are possibly positive for both CD90 and CD172a but retain morphology similar to myeloid cells. CD172a staining on rPoGM-CSF and rPoIL-3 treated cells showed definite changes in cell line composition. Although there were only a minor differences observed for CD172a- cells for rPoGM-CSF (2%), and no change of CD172a- cells for rPoIL-3 treated cells when compared to non-treated cells, there was a definite shift in maturity of monocytes and granulocytes with an increase in mature CD172a hi cells of 44% and 43% for rPoGM-CSF and rPoIL-3 respectively. Furthermore, proliferation staining with BrdU indicated not only a decrease in proliferation of CD172a- and CD172a lo cells for both rPoGM-CSF and rPoIL-3 treated cells but also a large increase in proliferation of CD172a hi mature myeloid cells. This may indicate that both rPoGM-CSF and rPoIL-3 are acting as CSF in promoting proliferation of precursor cells in addition to directing maturity of immature myeloid cells. These results confirm findings in Hu and mice where GM-CSF was demonstrated to act on HSC and early progenitors to initiate proliferation and produce CFU of myeloid lineage (Yamamoto-Yamaguchi, et al. 1983). Similarly, IL-3 was found to act directly on HSC and early progenitors resulting in the proliferation and differentiation of myeloid cells (Martinez-Moczygemba and Huston 2003, Le Bousse-Kerdiles, et al. 1986). Morphology of these samples as previously mentioned include significant increases in monocytes and macrophages for each of these groups in addition to a drop in neutrophils for the rPoGM-CSF treated group. Previous in vitro studies of the effects of rHuGM-CSF on huBM cells after 7 days incubation produced a mean colony count of 8.5% neutrophilic granulocyte colonies, 53.5% neutrophilic-granulocyte-monocyte colonies and 35.5% monocyte-macrophage colonies (Metcalf 1986). The results here may very well be similar as monocytes observed maybe in their pre-macrophage stage due to the differences in incubation time. Staining of rPoIL-5 treated BM cells with CD90 and CD172a selected for populations of cells which were slightly lower than the cells alone sample. Furthermore, when compared to the BrdU stained samples we can see that there is no increase in proliferation over the cells alone group. It is therefore suggested that either little proliferation to this group has occurred or, more likely, differentiation of eosinophil precursors had occurred thus retaining the same sample size. This hypothesis is supported when analysing cell
morphology which clearly shows a significant increase in eosinophil numbers for BM cells incubated for 96h with IL-5 whilst a moderate decrease in monocytes and macrophages is also noted for this population.

3.6 Conclusion

In summary, the successful cloning, expression and biological activity of the rPo cytokines GM-CSF, IL-3 and IL-5 has been confirmed. Furthermore, each of these cytokines was confirmed to initiate proliferation of PoBM cells in vitro. Consequently, the effect of these cytokines on cell differentiation and maturity was demonstrated. Finally, dominant cells types produced from PoBM treated with each of these cytokines in vitro was also indicated. These cytokine can now be examined for activity in an in vivo biological system and outcomes can be compared to the results obtained here to further educate our understanding of the actions of these cytokines in Po hematopoiesis.
CHAPTER 4

In vivo analysis of Po GM-CSF, IL-3 and IL-5

4.1 Abstract

Early acting cytokines and growth factors, such as those of the CD131 βc subunit may offer an alternative method to the current use of antibiotics and chemicals such as anthelmintics in maintaining Po health. Thus far the rPoGM-CSF, rPo IL-3 and rPo IL-5 proteins have been identified and cloned and the biological activity of each cytokine has been confirmed in vitro. The function of these cytokines must now be confirmed in vivo. 5-FU was used to chemically induce a hematopoietic state of myelosuppression in young pigs. This allowed for the monitoring of both the autologous BM reconstitution and recombinant cytokine induced BM repopulation, precursor cell proliferation and cellular differentiation. rPoGM-CSF, rPoIL-3 and rPoIL-5 were administered by intramuscular injections (i.m.) following 5-FU induced leukocytopenia. Blood and BM samples were analysed for cell composition. Statistically significant results were observed in several blood cell populations including eosinophils for animals treated with rPoIL-5, rPoGM-CSF and rPoIL-3 and basophils for animals treated with rPoIL-3. BM analysis of CD90+ and CD172a+ cells confirmed myelosuppression in week one. Significant results were observed between rPoIL-3 and the 5-FU control group in week two where positive cell numbers were significantly lower than the 5-FU control group. BM cell numbers appeared to return to normal levels by week three, although significant differences were recorded within each bone marrow subset for rPoGM-CSF treated animals. These results have demonstrated the effects of each of these rec cytokines within the hematopoietic processes of the pigs.

4.2 Introduction

The ever expanding world population is constantly intensifying the need for a subsequent increased global livestock population in order to maintain adequate food stocks. The Food and Agriculture Organization of the UN (FAO) recently reported on the strain that the growing world population and increased consumption of animal protein will place on natural resources (FAO 2011). The FAO projected that increases in meat consumption by as much as 73% could be experienced by 2050 (FAO 2011). This demand for increases in animal numbers in the past has led farmers to adopt methods of husbandry which are no longer accepted by health professionals and
consumers as being the safest for human consumption. These practices, which often include the prophylactic use of antibiotics and chemicals treatments such as anthelmintics, (Walsh and Fanning 2008, Morris, et al. 2008), are believed to lead to the increased risk of developing antibiotic resistant bacteria capable of infecting humans (Walsh and Fanning 2008). There is therefore a growing demand for alternative treatments for the maintenance of animal health.

Hematopoiesis is the complex process of white and red blood cell generation which takes place in adults within the microenvironment niche of the BM (Walkley, et al. 2008, Renstrom, et al. 2010). The generation of these cells from the single self-renewable HSC, requires the involvement of cellular interactions, cytokines, chemokines and macromolecules which drive the proliferation and differentiation of HSC and resulting precursor cells to develop into a mature immune system (Dominici, et al. 2001, Renstrom, et al. 2010). The regulation of these processes is highly controlled and crucial to not only combat infection and disease but also regulate the bodies thrombolytic state and supply oxygen necessary for maintaining life (Thomas, et al. 2004). Cytokines, the immune systems signalling and regulatory proteins, are produced by activated leukocytes. By binding to specific cell surface receptors, they initiate signals that are critical to a diverse spectrum of functions. These functions include the activation of immune responses, cell proliferation, differentiation, and apoptosis (Wang, et al. 2009). As cytokines are the body's natural immune control molecules, it is believed that they may provide an alternative method of maintaining pig health. An understanding of the role played by individual cytokines on HSC and precursor cells is therefore a necessary step in the knowledge of hematopoiesis in pigs and could provide us with a unique insight into the pathways of individual cell development and maturation.

In order to study the function of these cytokines in vivo, a method of observing changes to the subpopulations of individual cell types within the immune system first needs to be established. Laurenz et al developed a procedure where pigs are myelosupressed with 5-FU prior to cytokine inoculation (Laurenz, et al. 1997). The antimetabolite 5-FU is a widely used cytoreductive cancer chemotherapeutic agent and thymidylate synthetase inhibitor which incorporates into RNA (Laurenz, et al. 1997, Parker and Cheng 1990) and results in apoptosis of the incorporated cell by eliminating available thymine (Goulder, et al. 2000). Previous work has shown that HSC and precursor cells are resistant to the effects of 5-FU due to their ability to increase expression of the bcl-2 gene. Bcl-2 is a member of a gene family which is involved in regulating apoptosis, either by blocking or inducing it (Goulder, et al. 2000).
HSC also lie dormant in the G0 phase of the cell cycle unless stimulated (Thomas, et al. 2004). This procedure therefore gives the advantage of removing lineage committed cells in the BM and blood stream whilst sparing HSC and precursor cells. BM and circulatory system immune cell reconstitution can then be observed with and without cytokines to determine the each cytokines role in hematopoiesis (Laurenz, et al. 1997).

In this study, a comparison of the effects on hematopoiesis of the rPo cytokines IL-3, IL-5 and GM-CSF was analysed in vivo both within the BM matrix and the peripheral circulation. BM derived hematopoietic cells from myelosupressed pigs were monitored for proliferation, maturation and mobilization into the periphery. In addition, changes to the composition of the WBC population of the peripheral immune system were observed.

4.3 Methods

4.3.1 Animal Ethics

This work was carried out and completed under CSIRO Livestock Industries Australian Animal Health Laboratory Animal Ethics Committee approval to use animals, Application number AEC1088.

4.3.2 Production of Po specific cytokines

Recombinant Po IL-3, IL-5 and GM-CSF cytokines were produced as previously described (chapter 3).

4.3.3 Myelosuppression

Four week old large White Landrace Cross pigs which had previously been weaned onto solid food were weighed and placed into 5 groups of 10 as follows:

1. Non-5-FU Control (saline), n=10  
2. 5-FU treated with IL-3, n=10  
3. 5-FU treated with IL-5, n=10  
4. 5-FU treated with GM-CSF, n=10  
5. 5-FU control (saline), n=10

Total population = 50

5-FU (Sigma, Australia) was dissolved in 0.697mg of NaOH per 2.5g 5-FU and diluted to concentration followed by adjusting pH by CO₂ aspiration to pH 8.5. All animals except control group 1, were administered 37.5mg/kg total cumulative dose of 5-FU in solution over 2 days by i.v. injection (days 0 and 1) as per AEC1-1088 and established by Laurenz et al (1997) (Laurenz, et al. 1997).
4.3.4 Hematological analysis

Pigs were routinely bled every 48h (week days) or 72h (week end) starting from day 0, prior to 5-FU inoculation, to establish a base line for cell populations (see table 4.1). Four mL of blood was collected via the external jugular vein using a 21g needle and Vacutainer® containing EDTA. The proportion of each pig leukocyte population was determined using a Cell Dyn hematology analyser Model 3700 (Abbott Diagnostics, USA).

4.3.5 Cytokine inoculation

All animals were inoculated from day 5 to day 9. Control animals were administered 1 mL of 0.9% saline per day. Non control animals were administered 100µg of recombinant Po cytokine suspended in 1 mL of 0.9% saline per day by i.m. injection into the semitendinosus muscle as pre-determined by Andrews et al 2006 (Andrew, et al. 2006, Andrew, et al. 2007). Groups 1 and 5 were selected as being the non 5-FU control group and 5-FU control group, respectively, receiving 1 mL saline. Test groups 2-4 received 1 mL (100ug/mL) recombinant Po cytokine per day as follows, group 2 received rPoIL-3, group 3 received rPoIL-5 and group 4 received rPoGM-CSF (Table 4.1).

4.3.6 BM collection

On days 7, 14 and 21 three animals from each group were euthanized by an overdose of the barbiturate anaesthetic Lethabarb, administered intravenously at a rate of 150mg/kg bodyweight (Table 4.1). BM was harvested as previously described (section 3.3.1.2). Cells were resuspended in DMEM with 1% (v/v) FCS and viable cells were counted by trypan blue exclusion. The cell number was adjusted to 5 X 10⁶ viable cells/mL for FACS analysis.

4.3.7 Flow cytometry

The cell surface markers CD90 (clone 5E10) and CD172a (clone 74-12-4) were used in flow cytometry for identifying specific BM cells. Three samples of approximately 2 x 10⁵ cells per animal were analysed. Non-viable cells were excluded from data acquisition by first staining with 7AAD (Becton Dickinson, USA) then back gating on the resulting histogram against the live cell population. A commercial isotype antibody was used to establish fluorescent parameters.
Table 4.1  Porcine cytokine animal trial time table.
Table showing times frame of 5-FU administration, blood collection, cytokine administration and BM harvest for cytokine pig trial. Animals received 37.5 mg/kg of 5-FU by i.v. injection over 2 days. Blood was collected via the external jugular vein and analysed for individual cell types. Cytokines were administered each day for five days by i.m. injection. Groups 1 and 5 received 1mL saline per day. Groups 2-4 received 1mL x 100µg/mL per day IL-3, IL-5 or GM-CSF respectively. BM was collected from euthanized animals on days 7, 14 and 21.
4.3.8 Statistical Analysis

GraphPad Prism (GraphPad Software, USA) was used to determine whether results were statistically significant. A two-tailed Mann Whitney T test was used to determine differences between two groups of data. A Kruskal-Wallis test was used to compare multiple groups and where differences were found, a Dunn’s multiple comparison test was used to compare two groups of distributed data. Experimental values differing significantly from control values are indicated by different levels of significance: *p<0.05, **p<0.01, ***p<0.001.

4.4 Results

4.4.1 The effects of Po cytokines on WBC following myelosuppression with 5-FU

In general, there was no significant change in WBC levels for pigs treated with cytokines over the 5-FU control group (Figure 4.1). Leukocytopenia was not evident in 5-FU treated pigs from the WBC profile although a reduction in cell numbers was observed for the rPoIL-3 and rPoIL-5 groups. All groups showed recovery from the suppressive phase by day 5 increasing above the control baseline by day 12 indicating a state of leukocytosis (Figure 4.1). Cytokine treated groups produced a similar profile to that of the 5-FU control pigs which received saline only.

Individual cell types were then examined in order to detect any underlying differences in cell population.

Blood lymphocyte levels were analysed, however, no significant differences were observed (Figure 4.2). Likewise, mean blood monocyte levels show a similar pattern for cytokine treated groups as for the 5-FU control indicating no influence by the cytokines (Figure 4.3).

Peripheral blood neutrophil levels were maintained for a longer period than monocyte levels. A decrease was observed for the 5-FU control group on day 7-9 followed by recovery on day 12. All three cytokines showed a similar profile although cytokine levels for all three groups remained slightly lower than the 5-FU control on day 9 (Figure 4.4).

Mean eosinophil levels did not appear to be affected by 5-FU treatment, however, there were significant differences between cytokine treated animals and those of control groups. Pigs treated with IL-5 had up to 6 fold increase in eosinophil levels for up to 11 days post inoculation, (P<0.05) (Figure 4.5). Cells levels for this
Figure 4.1  WBC count for myelosuppressed pigs treated with rPo cytokines. Data shows mean WBC difference in peripheral blood of pigs following chemical myelosuppression with 5-FU followed by treatment with the rPo cytokines IL-3, IL-5, GM-CSF or saline alone (controls both 5-FU treated and non 5-FU treated). Red box indicates period of 5-FU treatment d0-d1. Blue box indicates period of cytokine inoculation (d5-d9). Yellow shaded area indicates the mean value of non 5-FU treated control +/- SEM (18.95 +/- 0.74). All values represent mean values +/- SEM (day 0 – 7 n=10, day 8-14 n=7, day 14-21 n=4). No significant differences observed p>0.05
**Figure 4.2** Lymphocyte differential count for myelosuppressed pigs treated with rPo cytokines. Data shows mean lymphocyte difference in peripheral blood of pigs following chemical myelosuppression with 5-FU followed by treatment with the rPo cytokines IL-3, IL-5, GM-CSF or saline alone (controls, both 5-FU treated and non 5-FU treated). Red box indicates period of 5-FU treatment d0-d1. Blue box indicates period of cytokine inoculation (d5-d9). Yellow shaded area indicates the mean value of non 5-FU treated control +/- SEM (44.66 +/- 2.20). All values represent mean values +/- SEM (day 0 – 7 n=10, day 8-14 n=7, day 14-21 n=4). No significant differences observed p>0.05
Figure 4.3  Monocyte differential count for myelosuppressed pigs treated with rPo cytokines. Data shows mean monocyte differential count in peripheral blood of pigs following chemical myelosuppression with 5-FU followed by treatment with the rPo cytokines IL-3, IL-5, GM-CSF or saline alone (controls, both 5-FU treated and non 5-FU treated). Red box indicates period of 5-FU treatment d0-d1. Blue box indicates period of cytokine inoculation (d5-d9). Yellow shaded area indicates the mean value of non 5-FU treated control +/- SEM (11.12 +/- 064). All values represent mean values +/- SEM (day 0 – 7 n=10, day 8-14 n=7, day 14-21 n=4). Significant differences observed day 0: IL-5 vs Control, where * = P<0.05. * = significant difference to non 5-FU treated control.
Figure 4.4  Neutrophil differential count for myelosuppressed pigs treated with rPo cytokines. Data shows mean neutrophil differential count in peripheral blood of pigs following chemical myelosuppression with 5-FU followed by treatment with the rPo cytokines IL-3, IL-5, GM-CSF or saline alone (controls, both 5-FU treated and non 5-FU treated). Red box indicates period of 5-FU treatment d0-d1. Blue box indicates period of cytokine inoculation (d5-d9). Yellow shaded area indicates the mean value of non 5-FU treated control +/- SEM (41.43 +/- 2.04). All values represent mean values +/- SEM (day 0 – 7 n=10, day 8-14 n=7, day 14-21 n=4). Significant differences observed Day 7 Control vs IL-3*, Control vs IL-5*, Control vs 5-FU*, Control vs GM-CSF**, Day 9: Control vs IL-3** where * = P<0.05 and ** = P<0.01, * = significant difference when compared to the non 5-FU treated control.
Figure 4.5 Eosinophil differential count for myelosuppressed pigs treated with rPo cytokines. Data shows mean eosinophil differential count in peripheral blood of pigs following chemical myelosuppression with 5-FU followed by treatment with the rPo cytokines IL-3, IL-5, GM-CSF or saline alone (controls, both 5-FU treated and non 5-FU treated). Red box indicates period of 5-FU treatment d0-d1. Blue box indicates period of cytokine inoculation (d5-d9). Yellow shaded area indicates the mean value of non 5-FU treated control +/- SEM (1.45 +/- 0.12). All values represent mean values +/- SEM (day 0 – 7 n=10, day 8-14 n=7, day 14-21 n=4). Significant differences observed Control vs GM-CSF * day 9, IL-5 vs 5-FU* days 9, 12, 14 and 16,, Control vs GM-CSF ** day 7, Control vs IL-5 ** day 9 and Control vs IL-5 *** day 7 where */* = P<0.05, ** = P<0.01 and *** = P<0.001. * = significant difference to non 5-FU treated control, ♦ = significant difference to 5-FU treated control.
group continued to increase to a peak of $12 \times 10^6$ cells/mL on D14 compared to $1.74 \times 10^6$ cells/mL for the 5-FU control group. Eosinophil levels for the IL-5 group remained high until day 21 although they had significantly reduced in number by this time being only 60% higher than the 5-FU control group (Figure 4.5). Pigs treated with rPoGM-CSF showed increased eosinophil numbers from day 5-12 increasing to $3.89 \times 10^6$ cells/mL (P<0.01) (Figure 4.5). IL-3 treated animals showed increased in eosinophil numbers from day 14 to 21, however, these were not significant.

Basophil levels were the most varied amongst all treated groups (Figure 4.6). This is not unusual to expect as basophil numbers make up such a low percentage of the overall WBC population. All 5-FU treated groups demonstrated a decrease in basophil numbers post treatment (Figure 4.6). The rPoIL-3 treated group indicated an increase in basophils from day 7-21 peaking at day 12-16 (P<0.05) (Figure 4.6). Levels for the rPoIL-5 and rPoGM-CSF groups fluctuated slightly however appear to follow the same pattern as the 5-FU control group. (Figure 4.6).

Platelet levels dropped for all groups on day 7 with the 5-FU control group having the lowest levels (Figure 4.7). All cytokine treated groups followed a similar pattern; however, they dipped slightly less than the 5-FU control group on day 7 with the rPoGM-CSF group reducing the least and not significantly when compared to the non-treated control. There were no significant differences recorded between the cytokine treated groups and the 5-FU control group (Figures 4.7).

There were no significant changes observed for peripheral red blood cell (RBC) numbers caused by 5-FU treatment, however, a decrease in RBC numbers was observed for the rPoIL-3 group day 7-14 (Figure 4.8). RBC numbers for the rPoIL-3 group decreased from day 0-9, $(5.65 \times 10^6$ cells/mL to $4.8 \times 10^6$ cell/mL) on day 9 before increasing to around $5.1 \times 10^6$ cell/mL by day 21 being below the base level of $5.35-5.45 \times 10^6$ cells/mL.

4.4.2 *In vivo* BM response to recombinant cytokines

To better understand the effects of specific cytokines during hematopoiesis and the regulation of cell release from the bone matrix into the periphery, BM was collected from animals from each group on days 7, 14 and 21. Cells were analysed by FACS for surface markers CD90 and CD172a. Statistical analysis was performed between each cytokine treated group and both of the control group and 5-FU control groups.
Figure 4.6  Basophil differential count for myelosuppressed pigs treated with rPo cytokines. Data shows mean basophil differential count in peripheral blood of pigs following chemical myelosuppression with 5-FU followed by treatment with the rPo cytokines IL-3, IL-5, GM-CSF or saline alone (controls, both 5-FU treated and non 5-FU treated). Red box indicates period of 5-FU treatment d0-d1. Blue box indicates period of cytokine inoculation (d5-d9). Yellow shaded area indicates the mean value of non 5-FU treated +/- SEM (1.76 +/- 0.20). All values represent mean values +/- SEM (day 0 – 7 n=10, day 8-14 n=7, day 14-21 n=4). Significant differences observed day 5; Control vs 5-FU* and Control vs GM-CSF*** and day 12 Control vs IL-3*, where * = P<0.05, and *** = P<0.001. * = significant difference to non 5-FU treated control.
Figure 4.7 Platelet count for myelosuppressed pigs treated with rPo cytokines. Data shows mean platelet count in peripheral blood of pigs following chemical myelosuppression with 5-FU followed by treatment with the rPo cytokines IL-3, IL-5, GM-CSF or saline alone (controls, both 5-FU treated and non 5-FU treated). Red box indicates period of 5-FU treatment d0-d1. Blue box indicates period of cytokine inoculation (d5-d9). Yellow shaded area indicates the mean value of non 5-FU treated control +/- SEM (739.43 +/- 23.26). All values represent mean values +/- SEM (day 0 – 7 n=10, day 8-14 n=7, day 14-21 n=4). Significant differences observed Control vs IL-3*, Control vs IL-5* day 7, Control vs 5-FU*** day 7 where * = P<0.05, and *** = P<0.001. * = significant difference to non 5-FU treated control.
Figure 4.8  RBC count for myelosuppressed pigs treated with rPo cytokines. Data shows mean RBC count in peripheral blood of pigs following chemical myelosuppression with 5-FU followed by treatment with the rPo cytokines IL-3, IL-5, GM-CSF or saline alone (controls, both 5-FU treated and non 5-FU treated). Red box indicates period of 5-FU treatment d0-d1. Blue box indicates period of cytokine inoculation (d5-d9). Yellow shaded area indicates the mean value of non 5-FU treated control +/- SEM (5.39 +/- 0.06). All values represent mean values +/- SEM (day 0 – 7 n=10, day 8-14 n=7, day 14-21 n=4). No significant differences were observed p>0.05
4.4.3 The effects of cytokines on CD90+ BM cells in vivo

Initial response for the 5-FU control group was a significant decrease in CD90+ cells with a 0.54 relative fold (RF) reduction of CD90+ cells when compared to the non-treated control group (Figure 4.9, week 1). Cell numbers increased in week 2 by 0.67 fold to 1.13 relative fold change (RFC) (Figure 4.9, week 2). There was no significant difference observed for CD90+ cells for the 5-FU control group in week 3 (Figure 4.9, week 3).

Levels of CD90+ BM cells for the rPoIL-3 group were equivalent to the 5-FU control for week 1 (Figure 4.9, week 1), however, only a 0.15 RF increase (RFI) was observed in week 2 resulting in a significant difference in CD90+ cell number between the rPoIL-3 and 5-FU groups (p<0.01) (Figure 4.9, week 2). CD90+ BM cells returned to basal level in week 3 (Figure 4.9, week 3).

Similarly, rPoIL-5 treated animals indicated a decline in CD90+ BM cells in week 1. This was followed by a 0.23 RFI in CD90+ cell numbers in week 2, although not significant (Figure 4.9, week 1). In week 3, the rPoIL-5 treated group had a 0.60 RFI in CD90+ cells to above the non-treated control, however, there was no significant difference observed.

A decrease in CD90+ BM cells was also observed for the rPoGM-CSF group in week 1 (Figure 4.9 week 1), followed by a significant increase in week 2 by 0.73 RF. A further 0.20 RFI in CD90+ BM cells by in week 3 resulted in 0.35 RF more CD90+ BM cells for the rPoGM-CSF treated group over the 5-FU control group (p<0.05) (Figure 4.9, week 3).

4.4.4 The effects of Po cytokines on CD172a+ expression on BM cells in vivo

CD172a+ BM cells were also monitored throughout the trial (Figure 4.10). As expected, CD172a+ BM cells for the 5-FU control were lower than the untreated control in week 1, (P<0.001), with a 0.57 RFD (Figure 4.10, week 1). A significant increase in cells was observed in week 2 with a 0.77 RFI to 1.20 RFC and a 0.20 RFI over the non-treated control group (Figure 4.10, week 2). This level was maintained in week 3. (Figure 4.10, week 3).

The rPoIL-3 group indicated a 0.41 RFD in CD172a+ cells in week 1 compared to basal level, (P<0.05) (Figure 4.10, week 1), however, only increased in week 2 by 0.06 RF being significantly less than the 5-FU control (P<0.01) (Figure 4.10, week 2). CD172a+ BM cells levels increased in week 3 to a RFC of 1.15 and approximate with the 5-FU control (Figure 4.10, week 3).
Figure 4.9  *In vivo* response of CD90*+* PoBM cells to rec cytokine treatment. Bar graph of CD90*+* PoBM cells, as determined by flow cytometry, for pig groups administered with the rPo cytokines IL-3, IL-5, GM-CSF or saline (both controls) following myeloablation with 5FU. Bars represent relative fold change of CD90*+* Po BM and +/- standard error of the mean (week 1 n=3, week 2 n=3, week 3 n=4). Significant differences were as follows: week 1: Control vs GM-CSF *, Control vs IL-5 **, Control vs IL-3 ***, Control vs 5-FU ***, week 2: IL-3 vs 5-FU **, week 3: 5-FU vs GM-CSF *, Control vs GM-CSF ***, where */* = P<0.05, **/♦♦ = P<0.01 and *** = P<0.001, * = significant difference to non 5-FU treated control, ♦ = significant difference to 5-FU treated control.
Figure 4.10  *In vivo* response of CD172a+ PoBM cells to rec cytokine treatment. Bar graph of CD172a+ PoBM cells, as determined by flow cytometry, for pig groups administered with the rPo cytokines IL-3, IL-5, GM-CSF or saline (both controls) following myeloablation with 5FU. Bars represent relative fold change of CD172a+ PoBM and +/- standard error of the mean (week 1 n=3, week 2 n=3, week 3 n=4). Significant differences were as follows: week 1: Control vs IL-5 *, Control vs IL-3 *, Control vs GM-CSF **, Control vs 5-FU***, week 2: IL-3 vs 5-FU***, week 3: Control vs GM-CSF ***, where * = P<0.05, ***/*** = P<0.01 and *** = P<0.001, * = significant difference to non 5-FU treated control, ∗ = significant difference to 5-FU treated control.
The rPoIL-5 treated group showed a 0.38 RFD in CD172a+ BM cells in week 1 compared to the non-treated control (P<0.05) (Figure 4.10, week 1). Cell numbers increased by in week 2 to 0.69 RFC (Figure 4.10, week 2), returning to basal level in week 3 (Figure 4.10, week 3). No significant differences were detected between the rPoIL-5 treated group and the 5-FU control for CD172a+ BM cells.

The CD172a+ levels for the rPoGM-CSF treated group also decreased in week 1 being significantly lower than the non-treated control, (P<0.01)(Figure 4.10, week 1). Similarly to the 5-FU control, CD172a+ BM cells for this group increased significantly in week 2 from 0.52 to 1.23 RF (Figure 4.10, week 2). This level was then maintained in week 3 similar to the 5-FU control and significantly higher that the non-treated control, (P<0.001) (Figure 4.10, week 3)

4.4.5 The effects of Po cytokines on CD172a^lo expression on BM cells in vivo

As CD172a^lo expression represent immature granulocytes and monocytes, we analysed the levels of these cells to better understand the changes in proliferation of precursor cells within the BM (Figure 4.11).

As expected, initial expression of CD172a^lo cells for week 1 for the 5-FU control group demonstrated a significant RF decrease (RFD) in cells of 0.45 compared to the non-treated control, (P<0.001) (Figure 4.11, week 1). A 0.64 RFI in cells from 0.55 to 1.19 RFC compared to the non-treated group was observed in week 2 (Figure 4.11, week 2). This level was maintained in week 3 (Figure 4.11, week 3).

CD172a^lo cells for the rPoIL-3 treated group decreased in week 1 with a 0.70 RFC observed and no significant difference recorded (Figure 4.11, week 1). Cell numbers in week 2 increased by 0.07 RF in being significantly less than the 5-FU control group, (p<0.01) (Figure 4.11, week 2). A 0.44 RFI in cells above the non-treated control group, (p<0.05), was observed in week 3 (Figure 4.11, week 3).

A 0.21 RFD in CD172a^lo cells was observed for the rPoIL-5 in week 1, although not significant (Figure 4.11, week 1). Cell numbers increased in week 2 from 0.79 RF to 0.86 RF, (Figure 4.11, week 2). Levels for this group increase in week 3 to be similar to the 5-FU control group (Figure 4.11, week 3).

A 0.21 RFD in CD172a^lo cells was observed for the rPoIL-5 in week 1, although not significant (Figure 4.11, week 1). Cell numbers increased in week 2 from 0.79 RF to 0.86 RF, (Figure 4.11, week 2). Levels for this group increase in week 3 to be similar to the 5-FU control group (Figure 4.11, week 3).

The rPoGM-CSF group results for CD172a^lo cells were similar the 5-FU control group in week 1, 0.62 RFC, being significantly lower than the non-treated control, (P<0.01) (Figure 4.11, week 1). RFI were observed for the rPoGM-CSF group for both weeks 2 and three to similar levels of the rPoIL-5 group (Figure 4.11, week 2&3).
Figure 4.11  *In vivo* response of CD172a<sup>lo</sup> PoBM cells to rec cytokine treatment. Bar graph of CD172a<sup>lo</sup> PoBM cells, as determined by flow cytometry, for pig groups administered with the rPo cytokines IL-3, IL-5, GM-CSF or saline (both controls) following myeloablation with 5FU. Bars represent relative fold change of CD172a<sup>lo</sup> Po BM and +/- standard error of the mean (week 1 n=3, week 2 n=3, week 3 n=4). Significant differences were as follows: week 1: Control vs GM-CSF **, Control vs 5-FU ***, week 2: IL-3 vs 5-FU **, week 3: Control vs IL-3 *, where * = P<0.05, **/** = P<0.01 and *** = P<0.001, * = significant difference to non 5-FU treated control, ♦ = significant difference to 5-FU treated control.
4.4.6 The effects of Po cytokines on CD172a<sup>hi</sup> expression on BM cells in vivo

As CD172a<sup>hi</sup> expressing cells represent mature granulocytes and monocytes, we analysed the levels of these cells present in order to better understand the reconstitution and regeneration of a mature immune cells population within the BM (Figure 4.12).

The level of expression of CD172a<sup>hi</sup> cells for the 5-FU control group in week 1 indicated a significant reduction in cells of 0.69 RFD to 0.31 RFC when compared to the non-treated control, (P<0.001) (Figure 4.12, week 1). A significant increase of 0.91 RF in CD172a<sup>hi</sup> cells was observed in week 2 bringing the level to 1.22 RFC and above the non-treated control (Figure 4.12, week 2). A further 0.12 RFI was observed in week 3 producing a significant difference above the non-treated control, (P<0.05) (Figure 4.12, week 3).

Expression of CD172a<sup>hi</sup> for rPoIL-3 in week 1 was 0.46 RFC, being significantly below the non-treated control, P<0.01 (Figure 4.12, week 1). A 0.06 RFI to 0.52 RFC was observed in week 2, resulting in a significant difference compared to the 5-FU control group, (P<0.05) (Figure 4.12, week 2). A 0.54 RFI in CD172a<sup>hi</sup> cells in week 3 increased cells above the basal level, however, this was still determined to be significantly below the 5-FU control group, (P<0.05) (Figure 4.12, week 3).

The rPoIL-5 treated group produced a significant decrease in CD172a<sup>hi</sup> cells in week 1 when compared to the non-treated control, (p<0.05) (Figure 4.12, week 1). This decrease was less than the 5-FU control. A 0.26 RFI in cells occurred in week 2 increasing levels to 0.70 RFC. This was below both controls (Figure 4.12, week 2). Cell numbers increased in week 3 by 0.62 RF to be similar to the 5-FU control (Figure 4.12, week 3).

Levels of CD172a<sup>hi</sup> expression for the rPoGM-CSF group were again similar to the other 5-FU treated subjects in week 1 with a 0.62 RFD in cells to 0.38 RFC when compared to the non-treated group, ( p<0.01), however, there was no significant difference indicated against the 5-FU control group (Figure 4.12, week 1). A 4 fold increase in CD172a<sup>hi</sup> cells for the rPoGM-CSF group was observed in week 2 bring levels to 1.62 RFC (Figure 4.12, week 2). Although the level of CD172a<sup>hi</sup> cells for the rPoGM-SCF group was 0.40 RF above the 5-FU control group, no statistical significant difference was recorded (Figure 4.12, week 2). The level of CD172<sup>hi</sup> cells for the rPoGM-CSF group remained significantly above the non-treated group in week three,
Figure 4.12  *In vivo* response of CD172a\textsuperscript{hi} PoBM cells to rec cytokine treatment. Bar graph of CD172a\textsuperscript{hi} PoBM cells, as determined by flow cytometry, for pig groups administered with the rPo cytokines IL-3, IL-5, GM-CSF or saline (both controls) following myeloablation with 5FU. Bars represent relative fold change of CD172a\textsuperscript{hi} Po BM and +/- standard error of the mean (week 1 n=3, week 2 n=3, week 3 n=4). Significant differences were as follows: week 1: Control vs IL-5 *, Control vs GM-CSF **, Control vs IL-3 **, Control vs 5-FU ***, week 2: IL-3 vs 5-FU ♦, week 3: Control vs 5-FU * and IL-3 vs 5-FU ♦ Control vs GM-CSF ***, where */* = P<0.05, ** = P<0.01 and *** = P<0.001, * = significant difference to non 5-FU treated control, ♦ = significant difference to 5-FU treated control.
(P<0.001), however, there was no significant difference recorded between the rPoGM-CSF and 5-FU control groups (Figure 4.12, week 3).

4.5 Discussion

To date, much work has been conducted in order to understand the complex pathways associated with hematopoiesis and immune function in mammals. Advances in molecular biology, and techniques for identifying specific cell types, have allowed the ability to track the migration of primitive HSC during early oncology from yolk sac to foetal liver to BM. Recent work by Kissa et al (2008) using CD41-green fluorescent protein zebrafish embryos, has demonstrated in real time, the migration patterns of early HSC from the aorta-gonad-mesonephros to the fetal liver via the axial vein (Kissa, et al. 2008). In the porcine embryo, the aorta-gonad-mesonephros, also referred to as the para-aortic-slanchnopleure, has been demonstrated to give rise to the mesoblast (Sinkora and Butler 2009). This is the earliest multipotent HSC located in the embryo and is the source of HSC which seed primary hematopoietic organs (Sinkora and Butler 2009). Although much has been learned in regards to embryologic development and the origin of the immune system, there is still much to learn about the development of individual cell types of the immune system. Differentiation and maturation of precursor cells, is dependent on the environment of the extracellular matrix present within the bone cavity where adult hematopoiesis takes place. Within the matrix, cell to cell interactions coupled with complicated signalling systems facilitated by cytokines, control and regulate the population of individual cell types of the immune system. An understanding of these systems may provide alternative pathways in order to maintain and sustained healthy animals in the Po industry. Therefore, in order to identify the actions of the cytokines IL-3, IL-5 and GM-CSF, myelosupressed pigs were administered the rPo form of each of these cytokines prior to monitoring for individual immune cell development and amplification in addition to BM reconstitution.

4.5.1 5-FU control

Initial results obtained for the 5-FU control group were unexpected as no apparent leukocytopenia was observed in total WBC counts. Previous trials using the cumulative dose of 37.5mg/kg of 5-FU in piglets produced significant leukocytopenia with a residual WBC count of approximately 33% remaining at day 8 post 5-FU infusion (Laurenz, et al. 1997). Conversely, an earlier report showed that mice treated with 5-FU two days apart showed 75% residual WBC in the periphery, and only 25%
and 14% for treatments of 3 and 5 days apart, respectively (Harrison and Lerner 1991). The presence of leukocytopenia was however, more evident when examining individual blood cell types. Although there was no decrease in lymphocyte numbers within the first week, a small increase in numbers on days 2 and 7 was noted and quickly followed on days 12 – 19 with a decrease in cell numbers. Decreases in cell numbers by 5-FU was evident in monocytes day 2-5 (38%), neutrophils days 7 (61.5%), p<0.05, and day 9 (54.5%), basophils D9 (66.5%), p<0.05 and platelets day 5 (32.8%), p<0.001. Furthermore, evidence of leukocytosis, which again was not observed in full WBC counts, was present for lymphocytes on day 7, monocytes on days 7-9, neutrophils from days 12-19, basophils on days 7 and 16. These increases were masked by decreases in other cell types and may indicate a preferred order for cell type reconstitution.

Monocyte levels demonstrated leukocytopenia from day 2-5, as a result of 5-FU treatment, with decreased cell numbers. Elevated levels were observed from days 7 to 9 which were then followed by a decrease back to day 2 levels at day 14. Cell numbers then returned to just below basal level. This profile was seen across all treated groups with no significant differences noted, indicating there was no observable influence being directed on total monocytes cell numbers by the rec cytokines. As with lymphocytes, neutrophil numbers appear to increase slightly post 5-FU treatment. This may indicate an innate response with up regulation of release of immature neutrophils into the periphery. Neutrocytopenia is apparent from day 7 with significant differences recorded on day 7 for the decrease in neutrophils numbers. This drop in neutrophils corresponds with reported data on the treatment of cancer patients where 5-FU was indicated in chemotherapy-induced neutrocytopenia (Gilmore, et al. 1999). Neutrocytopenia at this time point was also similar to previous reports by Laurenz et al. (1997), however, these did not include a rise in cell numbers prior to nadir (Laurenz, et al. 1997, Laurenz, et al. 1997). This data suggests an up regulation of neutrophil cell numbers as part of the immune response to cellular insult prior to the death of a large proportion of cells. Neutrophil numbers quickly recover for the 5-FU group with a 48% increase in neutrophils between day 7 and day 9 prior to full recovery by day 12.

Eosinophil numbers appeared to be unremarkable in response to 5-FU treatment although may be due to cell migration from within the tissues. Conversely, a significant decrease was observed for basophil levels by day 5, P<0.05, which was quickly followed up by a 4 fold increase in basophils by day 7. In addition to this, a second decrease at day 14 was followed up on day 16 by a 4.5 fold increase. This was
again followed up by a decrease and further recovery. Although this profile seems erratic when viewed as a whole, it may simply be an interrupted view of the lifespan of a basophil. A previous report by Didichenko, S.A et al (2008) found that the half-life of basophils in culture was approximately 3 days in the absence of pro-survival cytokines (Didichenko, et al. 2008). If we were to take this into consideration, the duration of time which cells increased on day 5 would be around 7 days which is what we are seeing here. We could therefore hypothesise that the effects of 5-FU on basophils is to induce apoptosis in the mature circulating cells which is then followed up immediately by a release of new cells into the periphery. These cells then die off after the 6 to 7 day life span if not supported by the pro-survival cytokines, which levels may be affected by the death of supporting cells, and are therefore replaced by a new compliment of cells. The surge of cell release may indicate the effects of 5-FU on the precursor cells causing an interruption in cell production, although this is purely speculative. Additionally, as basophils represent the smallest population of immune cells circulating in the periphery, minor fluctuation in numbers are easily recognised. What’s more, these low levels should allow for clearer observation into cytokine directed influence on basophil numbers.

Platelet levels appear to have followed the same trend as for neutrophils, with the onset of mild thrombocytopenia observed on day 7. This is similar to previous reports showing 5-FU induced thrombocytopenia by day 7 being 5 days post treatment(Laurenz, et al. 1997). Interestingly, it appears that 5-FU does not reduce platelet numbers directly but most probably by the flow on effect of reducing megakaryocyte numbers by the 5-FU treatment. In fact similar results have previously been hypothesized by Warren et al (1996), who suggest that low platelet numbers and delayed platelet recovery post 5-FU treatment may be associated with the suppressive effects of recovery of the megakaryocyte precursors (Warren, et al. 1996). Furthermore, the actions of 5-FU upon platelets has also been demonstrated to effect the aggregation function of the cells, thus reducing the effectiveness of treated platelets, more so than reducing the cell number directly (Kumar, et al. 1999). Platelet numbers are primarily controlled in the periphery by the uptake of free thrombopoietin (TPO) by platelets (Wendling 1999, De Gabriele and Penington 1967). As platelet numbers drop, less TPO is taken up in the periphery which then leads to an excess of TPO. This increase then promotes several actions within the BM niche. Firstly, the binding of TPO to the TPO receptor, c-MPL, on precursors cells thus promoting an increase in megakaryocytes numbers and the maturation of these cells, and secondly, the binding of TPO to pre-existing mature megakaryocytes triggering the up regulation
of platelets production and their subsequent release into the periphery (de Sauvage, et al. 1994). A decrease in platelet numbers due to the natural lifespan of platelets and the lack of support in maintaining cell numbers by megakaryocytes effected by 5-FU would therefore see an increase in free TPO in the periphery. If this were the case, the results observed would therefore indicate that free TPO would be at its highest concentration in the blood at around day 7, when platelets are at their lowest. As an increase is noted on day 9, we can surmise that functional megakaryocytes are present at this time. This would also indicate a time span of 7 days from total infusing of 5-FU to functional platelet production. This may also indicate a time period of 7 days to produce new functional megakaryocytes. Furthermore, as the decrease in platelet cells was noted at around D5 we can hypothesise a period of around 4 days for the up regulation from megakaryocyte precursor to mature platelet producing cells. In fact, previous investigation has confirmed that the development of the megakaryocyte from the megakaryoblast takes around 4-5 days thus supporting this hypothesis (Garcia, et al. 2004).

RBC levels for the 5-FU group were unremarkable with no significant differences observed. Although there were minor fluctuations in cell numbers, it is believed these were controlled and maintained by the pig’s autonomic and homeostatic control mechanism. This would therefore indicate that any fluctuations in cell numbers in other groups, differing to those portrayed by the control group, are of a direct effect of the cytokine used upon the production of RBC.

A significant difference was detected for CD90+ BM cells in week one, p<0.001, when comparing the 5-FU control group to the non-treated control group. Previous studies into the expression of CD90 has confirmed that CD90 is expressed on early CD34+ PoBM progenitor cells (Layton, et al. 2007) and that CD34+CD90+ Hu BM cells have been identified as HSC (Baum, et al. 1992). In addition, BM depletion with 5-FU, which essentially attacks fast proliferating cells, has been demonstrated to coincides with a reduction in both T-cell and B-cells, immature B-lymphocytes and common hematopoietic progenitors (Kakiuchi, et al. 2004). As these cells are all implicated as expressing CD90, it is suggest that a large portion of CD90+ cells remaining post 5-FU treatment may be slow proliferating cells. These would include both fully differentiated mature lymphocytes and HSC (Nishio, et al. 1996). Furthermore, primitive HSC (PHSC), have been observed to proliferate rapidly at 3 to 5 days post 5-FU treatment (Harrison and Lerner 1991). Consequently, as the first BM sample from this current trial was obtained 5 days post 5-FU infusion, we can deduce that HSC proliferation had been initiated thus increased production of immature progenitor cells had
commenced in order to repopulate the peripheral white blood cells pool. This would, therefore, suggest a portion of the CD90+ population observed would now include newly produced precursor cells. Continued investigation showed an increase in CD90+ BM cells on day 14 for the 5-FU control group with an increase of 1.5 fold. This increase brought the level of CD90+ BM cells for the 5-FU control group above the non-treated control by 0.13 RF. BM CD90+ cells in week 3 were maintained above the non-treated control group by approximately 0.09 RF. This would suggest that homeostatic mechanisms controlling levels of BM cells, once initiated by the myelobalation, continue to produce excess CD90+ cells 21 days post 5-FU treatment. This confirms the ability of the BM to maintain adequate precursor cells which are necessary for maintain a functional immune system, even following a direct assault upon the BM environment.

The total CD172a+ BM cell profile was very similar to the CD90+ profile. The control group indicated leukocytopenia with a RFD of 0.57 on day 7 and a significant difference observed, p<0.001, when compared to the non-treated control group. This decrease in CD172a+ cells corresponds to the observed reduction in mean monocytes on day 2-7. This was consistent to previous work where leukocytopenia for monocytes was achieved by day 4 using the same concentration of 5-FU, however, in this current case the level of monocyte myelobalation was far less intense than previous studies which showed almost complete loss of peripheral monocytes (Laurenz, et al. 1997, Laurenz, et al. 1997). A significant surge in CD172a+ cells was observed in week 2 where the number of CD172a+ cells increases above the non-treated control by 0.20 RF. Furthermore this increase in cells was maintained in week 3 where the 5-FU control group remained 0.21 RF above the basal level. Again, although the levels are slightly higher, this is a similar profile to that observed for CD90+ cells. It is also worth noting that a portion of the CD172a+ cells would represent HSC and precursor cells which would also be positive for CD90 (Layton, et al. 2007). This would therefore indicate a common link for a portion of these cells for equal increases and decreases under each profile.

A breakdown of the levels of CD172a+ cells into CD172a-lo and CD172a-hi cells was initiated in order to differentiate between immature and mature cells respectively.

On first observation of the CD172a-lo cells population, it was revealed that the level of CD172a-lo cells in the non-treated control group represented 50% of the CD172a+ cell population (data not shown). This therefore suggests that 50% of the CD172a+ cell population represents immature cells, incorporating HSC, precursor cells and
immature monocytes and granulocytes. This also suggests that the remaining 50% of CD172a<sup>+</sup> cells represents mature monocytes and granulocytes. Therefore, this indicates a larger portion of cells in each of the 5-FU treated groups for most time points were CD172a<sup>lo</sup> immature cells. This would be expected as an up regulation of precursor cells would be required for the reconstitution of the BM following myelobalation. Furthermore, it is noted that increases in each level of CD172a<sup>lo</sup> and CD172a<sup>hi</sup> cells were similar, with RFI above the basal level of 0.19 and 0.22 for CD172a<sup>lo</sup> and CD172a<sup>hi</sup> cells, respectively, in week two. This suggests an overall up regulation of CD172a cells resulting in an increase in proliferation of both immature and mature levels of myeloid cell. This simultaneous up regulation would ensure recovery of both the immune cell population in the BM matrix and the reconstitution of the periphery with WBC. A similar profile was observed in week three indicating continued proliferation of immature cells.

4.5.2 Cytokines

Initial blood results for total WBC numbers showed no significant differences between cytokine treated groups and either the 5-FU or non-treated controls. However, a closer look at individual cell types treated with each cytokine revealed that mediated differences in WBC numbers had been detected for each of the 5-FU treated groups. These differences were not made apparent when examining the total WBC counts as increases in some cell populations coincide with decreases in others demonstrating a shift in the composition of the total WBC pool.

4.5.2.1 rPoIL-3

Results obtained indicate there were no significant differences detected between the rPoIL-3 group and the 5-FU control for Lymphocytes, monocytes or neutrophils numbers.

IL-3 has been well documented as a potent stimulator of eosinophil and basophil differentiation in BM suspension cultures (Saito, et al. 1988, Ottmann, et al. 1989, Gillio, et al. 1990). Here, the rPoIL-3 group showed a trend towards increasing eosinophils from day 14 to day 19, however, no significant differences were observed. Nonetheless, it is suggested here that IL-3 may contribute to the late induction of eosinophils.

The rPoIL-3 group showed a significant increase in basophils on day 12, although the increase was constant from day 9-16, before a steady decline back to basal levels on day 21. This corresponds to previous reports which state that IL-3,
although not a specific basophilopoietin, does produce basophil expansion during incubation of normal BM cells (Lantz, et al. 1998, Schneider, et al. 2010). Furthermore, it has already been shown that basophils express a higher level of CD123 than is found on eosinophils thus indicating a higher affinity for IL-3 to bind to basophils (Zheng, et al. 2002). The maintenance of these increased cell numbers may therefore be the result of the action of rPoIL-3 on the eosinophil/basophil precursor (Ohmori, et al. 2009). Increases in these precursors would produce cells which could then be differentiated into either eosinophils or basophils. The maintenance of high rPoIL-3 levels would therefore select for continued differentiation of the basophil cell line thus increasing numbers of basophils (Arock, et al. 2002).

Although a significant difference was observed for the rPoIL-3 group platelet levels on day 7, p<0.05, compared to the non-treated control, there was no difference when compared to the 5-FU control. There was however, a slightly less decline in platelet numbers when compared to the 5-FU control. This slight difference may be due to the influence of rPoIL-3 on the differentiation of megakaryocyte/erythrocyte precursor cells. Previous work has demonstrated the influence of rPoIL-3 on Mo megakaryocytes in vivo, where rPoIL-3 was shown to promote maturation of these cells (Ishibashi and Burststein 1986).

RBC numbers for the rPoIL-3 group, appear not to be altered by 5-FU treatment, rather, they appear to be reduced by the action of the cytokine although no significant differences were observed. RBC numbers for the rPoIL-3 group appear to be lowest on day 9 with only $4.8 \times 10^6$ cells per/mL. The number of RBC for this group appears to have remained below the basal level for the remainder of the trial. This reduction may be partially due to the up regulation of eosinophils and basophils for this period giving preference for these cell types by the cytokine. This would indicate a selective drive in the direction of the CFU-Bas/E precursor for these cells over the CFU-ME precursor. This hypothesis corresponds to the increases in basophils/eosinophils (CFU-Bas/E) and a decrease in RBC (CFU-ME) seen for the rPoIL-3 group.

CD90<sup>+</sup> BM cells for the rPoIL-3 group were reduced in week 1 to a similar level as the control group indicating no apparent influence on CD90<sup>+</sup> cells by rPoIL-3 in the first three days of treatment. The level of CD90<sup>+</sup> in week 2, however, was only slightly higher and significantly less than the 5-FU control, indicating the action of rPoIL-3 on these cells appears to have maintained the level of CD90<sup>+</sup> cells to a low level. Furthermore, this period does not appear to correspond with any statistically significant
increase in any specific cell type within the periphery; although, there was an 11% increase in lymphocytes for this group at this time point when compared to day 12. As we already know, IL-3 is a potent promoter of proliferation and cell survival for both committed myeloid cells lines and uncommitted progenitor cells when acting in synergy with other early acting cytokines. For example, SF along with IL-3 has been demonstrated to support formation of GEMM colonies (Crooks, et al. 2000, Tsuji, et al. 1992). IL-3 plus IL-6 enriches levels of HSC (Okada, et al. 1991). When added to cultures of BM cells harvested 2 days post 5-FU treatment, IL-11 enhanced the dependent colony formation of IL-3 in mice and shorten the G0 period of early progenitors (Musashi, et al. 1991). In cultures of day 2 post 5-FU BM cells, Granulocyte colony stimulating factor (G-CSF) revealed synergism with interleukin 3 in supporting the proliferation of multipotent progenitors (Crooks, et al. 2000, Ikebuchi, et al. 1988). TPO when used along with IL-3 has been shown to support the formation of multiple types of hematopoietic colonies including multilineage colonies (Crooks, et al. 2000, Ku, et al. 1996). These are but a few of the synergistic actions of IL-3. As rPoIL-3 alone has been administered in this case, it is unknown which other cytokines are up regulated by the immune system and BM matrix. Therefore, it may be necessary to examine small changes in cell expression also to determine the overall effects of each cytokine. Nonetheless, it has previously been demonstrated that when acting alone, HuIL-3 can promote the production of CD19+ B cell progenitors from CD34+ CD38+ HSC hence increasing the production of B cells (Crooks, et al. 2000). As we have previously shown, the level of CD90 expression on B-cells is down regulated as B-cells mature. It could therefore be suggested that as there is a reduction in CD90+ cells in the BM and an increase in lymphocytes in the periphery that the action of rPoIL-3 in this case has been to increase production of B-cell progenitors leading to the increase in mature B-cells and the down regulation of CD90+ cells (Suryani, et al. 2010).

A similar result for the rPoIL-3 group was observed for the CD172a+ population in week 1 as that of the CD90+ population. Although there was no significant difference in cell numbers observed between the IL-3 group and the 5-FU control for this time, it was noted that there was a less significant value observed for cells numbers when compared to the basal level. This would, suggests an increase in the myeloid cell lineage at this point. As with the CD90+ population, we observed a significant difference in CD172a+ cells in week 2 when compared to the 5-FU control. This difference is believed to be contributed to by the proposed increase in B-cells. Furthermore, as CD172a is not expressed on mature B-cells, an increase in mature B-
cells would represent a reduced population in CD172a+ cells as observed here. As with the CD90+ cell population we observed an increase in cells back to basal levels in week 3. A closer look at the underlying CD172a+ expression revealed a higher proportion of the CD172a+ cells were of CD172aLO expression representing immature granulocytes and monocyte therefore indicating an up regulation of these cells initiated by rPoIL-3. This was again the case in week 2 with more immature cells being produced. This would also support our hypothesis that IL-3 may up regulate B-cell progenitors as the rPoIL-3 population in week 2 was significantly less than the 5-FU control. It is well documented that IL-3 is a potent stimulator of proliferation of HSC and myeloid progenitor cells (Mohle and Kanz 2007). This would therefore support the increase in CD172aLO population in week 3, where cell numbers significantly increased above the basal level, whereas the CD172aHI population, which represents the mature monocytes and granulocytes, was significantly less than the 5-FU control. This also implies a build-up of immature myeloid cells in the BM and a lack of maturation at this point which may be due to the absence of sufficient cytokines necessary for maturation at this point. This result can be supported by previous work by Ogawa, M. (1993), where it was stated that the hematopoietic effects of IL3 were restricted to progenitors at the intermediate stages of hematopoietic development (Le Bousse-Kerdiles, et al. 1986, Ogawa 1993) and that IL-3 did not independently support the lineage-restricted processes with the possible exception of mast cells/basophils (Ogawa 1993). This also supports our previous observations for the increases in basophils and eosinophils for the IL-3 treated group.

4.5.2.2 rPoIL-5

An analysis of individual cell types for the rPoIL-5 group demonstrated lymphocytopenia, monocytopenia and neutrocytopenia, followed by lymphocytosis, monocytosis and neutrocytosis post 5-FU treatment. These results indicate the effects of 5-FU on these cell types was not altered or influenced by the actions of IL-5. Conversely, the effect of rPoIL-5 upon the eosinophil population was significant. rPoIL-5 produced an almost immediate effect on increasing eosinophil numbers from day 7 after only two inoculations. Significant differences were noted on several days with levels increasing by as much as 8 fold over control basal level, from 1.43 cells/mL to 12 cells/mL on day 14 and over the 5-FU control group of 1.53 cells/mL. Levels peaked on day 14 before reducing back to near basal levels by day 21. It was anticipated that levels would have returned to near basal level by day 24 based on the extrapolated decline in the level of expression. Eosinophils, therefore, reached their maximum level by 9 days from the beginning of the rPoIL-5 treatment and 5 days from
the final inoculation. If we were to determine that the peak titre of cells was due to the accumulative dosing then we can suggest that maximum titre of cells was achieved 5 days post inoculation with rPoIL-5 from day 9. This result therefore confirms the role of IL-5 in pigs as being a potent stimulator of eosinophil production and maturation, being similar to results observed in other species including mice and Hu. IL-5 has been well documented in recent years as being a potent eosinophilopoietin along with IL-3 and GM-CSF, which together regulate the proliferation and differentiation of eosinophils (Lopez, et al. 1988, Rothenberg, et al. 1988, Lopez, et al. 1991, Rothenberg and Hogan 2006, Lopez, et al. 1986). Moreover, IL-5 has been well established as the most important of these cytokines in the development of eosinophils as it is the most specific to the eosinophil lineage (Sanderson 1990). This is due to the high number of IL-5α receptors present on eosinophils in comparison to basophils and mast cells (Martinez-Moczygemba and Huston 2003, Yamada, et al. 1998, Sehmi, et al. 1997). Several other factors have previously been reported which support the observed results, including the identification of IL-5 as the main maturation factor responsible for the stimulation of eosinophils for release from the BM into the periphery (Collins, et al. 1995, Rothenberg and Hogan 2006). This fact maybe the most relevant when comparing the actions of IL-5 on eosinophilopoiesis to those of IL-3 and GM-CSF. IL-5 appeared to act similarly to GM-CSF from first inoculation to increase eosinophil numbers, however, numbers for the GM-CSF group reduce back to basal levels by day 12 whereas the IL-5 group continuously increased in number until day 14. However, this may be solely due to the different actions of each of these cytokines in the eosinophilopoietic pathway. As IL-5 is predominantly an eosinophilopoietin it was expected that the major action of IL-5 on BM would be in the production of eosinophils (Schrezenmeier, et al. 1993). Conversely, GM-SCF is involved in the production of several cell types of granulocytes and monocytes, therefore, acting in several levels of different linages. Interestingly, eosinophils have been confirmed to produce, store and release GM-CSF and IL-3 when stimulated by inflammatory cytokines such as TNF-α and IFN-γ (Curfs, et al. 1997, Lampinen, et al. 2004). Recent work by Sakai,H.et al.,(2013) demonstrated the increased levels of inflammatory cytokines such as TNF-α and IFN-γ in the gastrointestinal tract following intraperitoneal injections of 5-FU in mice (Sakai, et al. 2013). This may therefore suggest a contributory role of GM-CSF and IL-3 in the up regulation of eosinophil production in this case. The inflammatory state induced by 5-FU treatment may cause an up regulation of TNF-α and IFN-γ therefore initiating the release of GM-CSF and IL-3 from eosinophils in the site of inflammation. Although the pharmacokinetics of eosinophils in the pig have not as yet
been reported, results in mice have been described by Ohnmacht, C. et al. (2007) where eosinophil migrating was monitored with BrdU. Here they reported that the eosinophils population in the BM diminished by 50% over 15h suggesting a 15h period from early differentiation to maturity (Ohnmacht, et al. 2007). Furthermore, it was determined that the peripheral eosinophil pool was nearly fully replaced in approximately 36h (Ohnmacht, et al. 2007) indicating a short life span for these cells in the peripheral circulation. In fact, it has previously been determined that mature eosinophils only reside in the peripheral circulation for up to 25h before migration into the target tissues (Lampinen, et al. 2004). Furthermore, it was reported that up to 500 times more eosinophils reside within the tissues than in the peripheral circulation (Lampinen, et al. 2004). This would suggest that although eosinophil levels appear to decline from day 14, that migration into tissues may have initiated as early as day 6-7.

Under normal conditions, eosinophils mainly traffic into the thymus, mammary gland, uterus (under the influence from oestrogen), and most prominently into the gastrointestinal tract where they reside within the lamina propria (Rothenberg and Hogan 2006). In these tissues eosinophils have been observed to survive for up to two weeks (Rothenberg and Hogan 2006, Rothenberg, et al. 1987). As, eosinophils travel to the site of inflammation under the chemotactic control of IL-5 and eotaxins released from inflammatory cells (Rothenberg and Hogan 2006, Lampinen, et al. 2004, Rankin, et al. 2000, Kroegel, et al. 1994) it is suggested that administration of rPoIL-5 by i.m. injection may have initiated the migration of a large number of eosinophils into the tissue of inoculation in addition to sites of increased inflammation derived from the myelosupressed condition attenuated by 5-FU including the gastrointestinal tract. Nonetheless, it is suggested that the action of IL-5 to increase the survival of eosinophils has aided the increased cell numbers in the periphery as observed here before distribution of cells between target tissues. These results therefore confirm the role of IL-5 in the proliferation and maturation in eosinophilopoiesis as previously observed in other species (Sanderson 1990, Matthaei, et al. 1997).

BM results CD90+ cells for the first week for rPoIL-5 treated pigs were unsurprising and followed the same pattern at the 5-FU control, however, the second week result was unexpected with a similar result observed to that of rPoIL-3. As CD90+ cells for the 5-FU group recovered to basal levels in week 2, it was hypothesised that IL-5 would also follow this same pattern. Therefore, although not significant, the lower than expected level of CD90+ cells is of interest. IL-5 has previously been demonstrated to contribute to the proliferation of B-cells as seen with mice and rats (McKenzie, et al. 1987, Ishihara, et al. 1999), however, this pathway has so far not
been demonstrated in pigs. Furthermore, the confirmation of this pathway in Hu has demonstrated to be controversial, although, Hu B-cells have been shown to express mRNA for the IL-5R and can respond to IL-5 if appropriately stimulated to undergo terminal differentiation (Huston, et al. 1996). Our previous studies of PoBM cells incubated with rPoIL-5 in vitro demonstrated no increase in total lymphoid cell numbers (Figure 3.13), and there was no change in the expression of CD90+ on BM cells incubated with rPoIL-5 when compared to controls (Figure 3.11). This result is not completely surprising as there are clearly differences between the conditions of cells incubated in vitro and in vivo with in vitro cultures commonly requiring multiple cytokines to mimic the BM matrix and elicit the proliferation of one specific cell type (Peters, et al. 1996). These would include other endogenous factors released within the BM matrix which could act in synergy with rPoIL-5 to stimulate B-cell production. Nevertheless, as there were no changes in the peripheral levels of lymphocytes for this group it is difficult to confirm this hypothesis, although a shift in the composition of the blood lymphocytes may also have occurred. The CD172a+ results for rPoIL-5 were again similar to those of rPoIL-3 although, again, no significant difference was observed when compared to the 5-FU control. The low reading recorded for week 2 could again be contributed to by the hypotheses that IL-5 is implementing a B-cell lineage response, thus accounting for lower percentages of myeloid cells. The levels of CD172alo immature monocyte/granulocyte cells for this group were at the highest level of the groups tested in week one with no significant difference recorded when compared to the basal control. This would suggest an increase in precursor and immature cells for rPoIL-5 after just two treatments over the controls. CD172ahi levels were again similar to the rPoIL-3 group. The considerable lower level of mature monocytes/granulocytes recorded suggesting either low production of these cells or completed maturation and release into the periphery. It is believed that the later has occurred in this situation. This can be confirmed by first observing the slightly higher WBC count for this day which may be due to the large eosinophil count, suggesting a major shift of mature eosinophils into the periphery. This would again confirm the action of rPoIL-5 on the maturation of immature eosinophils in pigs as demonstrate in other animal models (Molfino, et al. 2012).

4.5.2.3 rPoGM-CSF

As with the 5-FU control there was no leukocytopenia evident in the total WBC results for this group. Furthermore, there was no indication of any effect of rPoGM-CSF upon the total WBC numbers. This result differs to that reported by Laurenz et al. (1997), where GM-CSF appeared to enhance the rate of suppression. However, it is
worth noting the difference in treatment with GM-CSF in the trial held by Laurenz, which began with GM-CSF being administered on day 1 and ran to day 10 and comprised 2 x i.m. injections daily of bovine GM-CSF (Laurenz, et al. 1997). Although treatment with rPoGM-CSF had little effect in this trial on total WBC numbers, changes in populations of individual cell types were noted with the main differences noted being for eosinophils, basophils and platelets. There was no difference noted in lymphoid cells numbers which correlates to previous research where GM-CSF was defined as a potent inhibitor of CD34+ progenitor cell differentiation into lymphoid progenitors or type 2 DC (Martinez-Moczygemba and Huston 2003, Iwasaki-Arai, et al. 2003).

An increase in eosinophil cell numbers in the rPoGM-CSF group resulted in differences present on day 7 and day 9 when compared to the non-treated control, however, there was no difference recorded when compared to the 5-FU control. Nevertheless, although not significant, the result indicates rPoGM-CSF involvement in increasing eosinophil cell numbers as previously reported (Ruef and Coleman 1991, Tomioka and Kagami 1993). It was felt, however, that as the increased level of eosinophils was only low, that it was unlikely that rPoGM-SCF played a significant direct role in eosinophilopoiesis. In fact, it has been previously shown that large doses of rGM-CSF are required in vitro to produce a small increase in eosinophil numbers and that equally, large doses are required in vivo, to produce the same response (Sanderson 1992). Nonetheless, low numbers of eosinophils have been identified as being present in mice engineered for deletion of either IL-5 or IL-5Rα suggesting that rGM-CSF may indeed stimulate eosinophil production under adverse conditions (Martinez-Moczygemba and Huston 2003, Kopf, et al. 1996, Yoshida, et al. 1996). Despite this, it was determined that the role of rPoGM-CSF here was to first stimulate HSC differentiation thus up regulating production of the CMP followed by an increase to the granulocyte precursor cells (Sanderson 1992). However, in order for rPoGM-CSF to induce proliferation, fully differentiated, mature and release eosinophils into the periphery, it would also require the assistance of IL-5. This would therefore suggest the release of endogenous IL-5 was orchestrated by either activated TH2 cells or eosinophils due to the inflammatory state established by myelosuppression with 5-FU. Nevertheless, eosinophil numbers quickly returned to basal levels immediately post cytokine treatment thus indicating rPoGM-CSF impact on the production of eosinophils may be short termed when compared to the actions of rPoIL-5.

Basophil numbers decrease post 5-FU and were significantly lower than the control groups on day 5, however, as this reduction occurred prior to the administering
of rPoGM-CSF it was determined to be due to the myelosuppressive effects of 5-FU on specific subjects rather than being caused by rPoGM-CSF.

The effect of rPoGM-CSF on platelets was not noted by the presence of a significant difference on platelet numbers, moreover, in this case the fact that there was no significant difference may indicate activity by GM-CSF. This would be due to the increase in megakaryocytes by rPoGM-CSF and subsequently, platelets on day 7. As platelet numbers remained slightly higher for the rPoGM-CSF group from day 7-12 when compared to the 5-FU control (although not significantly), it is suggest that rPoGM-CSF may have contributed to early platelet recovery. Moreover, it is noted that platelet numbers for all treated groups decline to end up below basal levels on day 21. The reason for this is not known, however, we could hypothesise that this is merely due to normal control of platelet levels during repopulation. As with all blood cells, the steady state number of platelets is a balance between their production and breakdown (Mason, et al. 2007). As platelets only survive for 8-10 days (Mason, et al. 2007, Lindsten, et al. 2000), a timeline of 10 days post 5-FU treatment would put the replacement of those platelets not destroyed by 5-FU (those released from day 3) at around day 12 –14. In addition, platelets replaced after thrombocytopenia would be removed from the periphery at around day 15-17 thus bringing numbers back down. This result corresponds to previous reports which showed that platelet levels peaked at day 15 for GM-CSF treated pigs and day 17 for untreated (Laurenz, et al. 1997). Furthermore, it was expected that platelet numbers would recover to basal level by around day 25 –30 due to homeostatic control as previously reported (Laurenz, et al. 1997, Stewart, et al. 1993).

Levels of expression of CD90+ for the rPoGM-CSF treated group was similar to the 5-FU control, and was also significantly different to the non-treated control, p<0.05, as the rPoGM-CSF group was slightly higher in number. This maybe an indication of early recovery of cell numbers due to the influence of rPoGM-CSF on HSC to increase proliferation. The percentage of CD90+ cells for the rPoGM-CSF treated group had recovered to above basal levels in week two, being a 1.5 fold increase over the week 1 level. As the recovery of the control group and the rPoGM-CSF group were similar, it was suggested that rPoGM-CSF may indeed be a contributing cytokine released by WBC during the 5-FU insult to initiate hematopoiesis within the BM. This release would also explain the RFI of CD90+ cells for the GM-CSF treated group above the non-treated control by 0.24 RF. Furthermore, this could also explain the continually increasing CD90+ cell numbers in week 3 post rPoGM-CSF treatment. This continued increase in CD90+ cells by GM-CSF produced a RFC over the non-treated control and
5-FU groups of 0.44 RF and 0.33 RF, respectively. This increase resulted in a significant difference to the non-treated group, p<0.001, and more importantly a significant difference to the control group, P<0.05. As we previously mentioned, it is believed that a large portion of these cells in weeks two and three would contain HSC and precursor cells. In fact, previous studies have shown that primitive HSC in 5-FU treated mice were enriched when treated with GM-CSF (Stewart, et al. 1993). This, therefore, suggests that rPoGM-CSF works within the BM of pigs to increase precursor cell numbers.

The total CD172a+ BM cell profile for the rPoGM-CSF group was very similar to the CD90+ profile establish by the non-treated control indicating BM leukocytopenia was achieved by day 7. This was supported by the reduction in peripheral monocytes on day 2-7. This result corresponds to previous work where leukocytopenia for monocytes was achieved by D4 using the same concentration of 5-FU however, in this current situation the level of monocyte myelobalation was far less intense than with previous studies which showed almost complete loss of peripheral monocytes (Laurenz, et al. 1997). The difference in results here may again be due to the difference in timelines for rPoGM-CSF treatment between the two trials. Week two results also mimicked those of the CD90+ cells with the rPoGM-CSF group recovering above the non-control group to a similar level as the 5-FU control. Initial observation of this result suggests that there is no influence on the CD172a cell population by GM-CSF. Week 3 GM-CSF CD172a+ cells were also high as seen for the CD90+ cells for this time point. A closer look at the subpopulations of CD172a+ cells shows an increase in the CD172a hi population for both weeks 2 and week 3 thus indicating an increase in mature BM cells. This therefore indicates a direct effect of rPoGM-CSF on the maturation of immature monocytes within the BM cavity.

4.6 Conclusion

This study was conducted to determine the processes initiated within hematopoiesis by the rPo cytokines, IL-3, IL-5 and GM-CSF. To monitor these roles, each cytokine was administered to a chemically immunosuppressed animal. The observed results for BM and blood samples indicated myelosuppression of individual WBC types but not total WBC numbers. Reduced cells numbers were observed for neutrophils, basophils, platelets and RBC in various groups which were masked by increased monocyte and eosinophil levels.
PoIL-3 was shown to act early on precursor cells by increasing CD172a+ BM cells in week 1. Furthermore, IL-3 was shown to induce a RFD in both CD90+ and CD172a+ BM cells in week 2 in addition to a RFI of immature CD172a hi BM cells over mature CD172a lo BM cells in week 3. These changes corresponded to an increase in basophils in weeks 2 and 3 and a late increase in eosinophils in week 3. Furthermore, it is suggested that rPoIL-3 may also have contributed to stimulation the B-cell lineage thus inducing a reduced RFC in CD90+ and CD172a+ cells.

It was confirmed that the actions of rPoIL-5 in the pig is to act in all levels of eosinophilopoiesis from the increased proliferation of eosinophil precursor cells to the maturation and release of these cells into the periphery as mature eosinophils. The increase in eosinophils was observed within two days of commencement of rPoIL-5 treatment and remained above basal levels until completion of the trial.

Finally, significant differences within both the BM and peripheral WBC indicated that rPoGM-CSF contributed to increases in BM precursor cells resulting in the early increase in levels of eosinophils and an indirect increase in platelet levels.

As a result of this work, it is believed that rPoIL-5 may be a suitable therapeutic for the treatment of parasitic worms in pig. Future work may include the combined synergistic effects of rPoIL-3, rPoIL-5 and rPoGM-SCF on eosinophil enhancement and the therapeutic protection against parasitic worms such as ascaris suum, in pigs.
CHAPTER 5

General Discussion

In recent years, there has been a growing concern amongst consumers and researchers alike, over the prophylactic use of antibiotics and chemicals in the management of health in livestock (Baker 2006). The widespread and increased use of non-therapeutic antimicrobial growth promoters has been suggested as a contributing factor in the emergence of antibiotic resistant microorganisms in food animals (Gilchrist, et al. 2007). These practices may now pose a threat to the livestock industry and Hu alike. (Gilchrist, et al. 2007). Although the inappropriate and over use of antibiotics can also be traced to Hu medicine, the sheer magnitude of overuse in animals when compared to Hu indicates that closer monitoring in Hu alone will not solve this problem. Consequently, it is believed that this threat is very real and therefore, alternative practices need to be adopted (Gilchrist, et al. 2007). We therefore, require alternative therapeutic treatments for pig health and one alternative currently being contemplated is the use of cytokines.

In mammals, the cluster of cytokines which utilize the common cell surface receptor β subunit CD131, namely GM-CSF, IL-3 and IL-5, have previously been demonstrated to show both early and synergistic activity in hematopoiesis in Hu and Mo (Martinez-Moczygemba and Huston 2003, Bazan 1990, Murphy and Young 2006, Rozwarski, et al. 1994). The ability of these cytokines to promote and differentiate specific myeloid cells required for both the innate and adaptive inflammatory responses, raised the question of whether they would behave similarly in pigs and more importantly, whether they could be successful therapeutics. Although each of these cytokines has previously been investigated in other mammalian species, particularly Hu and Mo, little research has been done in the pig model. Moreover, very little is known in regards to the cytokines which drive hematopoiesis in pigs. The complex microenvironment of the BM matrix and continuous regulation of the immune system by signalling molecules released by activated WBC is an area we are only beginning to understand in pigs. However, despite the lack of research, several trials have been reported involving the use of cytokines, including those which have used cytokines therapeutically as adjuvants. These studies have shown that the inclusion of cytokine DNA as adjuvants have improved the overall effects of the accompanied vaccine (Tovey and Lallemand 2010). Indeed, Tovey.M and Lallemand.C, (2010), reported how liposome encapsulated cytokine expression vectors co-administered with
DNA vaccines were able to potentiate the immune response to vaccination in various experimental models (Tovey and Lallemand 2010). Furthermore, it has been repeatedly demonstrated in Mo models, that the use of GM-CSF DNA as an adjuvant for DNA vaccines, consistently enhances antibody and CD4 T-cell responses (Geissler, et al. 1997, Pasquini, et al. 1997, Sin, et al. 1998, Barouch, et al. 2002, Robinson, et al. 2006). Although there are some mechanisms for enhancing antibody responses currently understood, there are many that still are not. It is apparent, however, that the co-administration of GM-CSF DNA along with a DNA vaccine recruits and stimulates the maturation of antigen-presenting cells (Haddad, et al. 2000, McKay, et al. 2004). As we have previously mentioned, IL-3 has been shown as an effective adjuvant in DNA vaccination against classical swine fever (Andrew, et al. 2006). Furthermore, IL-5 DNA has been shown to markedly increase IgA reactivity to co-expressed heterogenous antigen (Stevceva and Ferrari 2005). While these results demonstrate the role of cytokines as prospective therapeutics, before we can employ the use of cytokines in pig health, a full understanding of the involvement of these cytokines in pig hematopoiesis and its effect on the immune system must be obtained. Therefore, a comprehension of cellular responses associated with each cytokine, in addition to the understanding of any potentially harmful contraindications which may result, must first be appreciated (Tagawa 2000).

When considering previous findings for each of these cytokines, it is expected that results seen in the pig may be similar to other mammalian species. In short, IL-3 is referred to as being a hematopoietic growth factor or early acting cytokine involved in the proliferation of HSC (Mroczko and Szmitkowski 2004) and the early development of granulocytes, macrophages, megakaryocytes, eosinophils, basophils, mast cells, and erythroblasts (Kusumi and Kumagai 1994). GM-CSF, as its name suggests is involved in the production of granulocytes and macrophages but is also involved in the production of eosinophils, megakaryocytes and red blood cells (Metcalf 2008). The third cytokine of interest, IL-5, is a growth and differentiation factor for B- cells in mice (Takatsu 1998, Moon, et al. 2004), however, it is mainly involved in the process of eosinophilopoiesis promoting the differentiation of eosinophil precursor cells to maturation and then activation (Hansel, et al. 1993). In addition, IL-5 has also been linked to the development of basophils and mast cells (Martinez-Moczygemba and Huston 2003). All 3 of these cytokines are secreted by activated CD4+ and CD8+ T cells (Bruserud, et al. 1993, Harada, et al. 1996). However, as we have discovered, not all proteins are structured the same between species and although differences are sometimes subtle, they may result in activation of altered pathways. This is clearly
seen when comparing the role of IL-5 upon B-cells in mice and Hu. Therefore, to
determine whether any genetic or structural differences or similarities exist for each of
these proteins between pigs and other mammal species, an in depth *in silico* analysis
of the Po isotypes for each of the cytokines, in addition to the CD131 subunit, was
firstly conducted.

At first it was anticipated that due to the physiological and immunological
similarities between pigs and Hu, that molecularly, we would see a similar trend. On
the contrary, phylogenetic analysis revealed that the pig isotypes of these cytokines
were more closely related to those of the cat and dog and that the Hu homologues
were more closely related to the primates. This pattern was also seen for the CD131
subunit. Further aa sequence analysis showed identity between pigs and Hu for these
cytokines was relatively low with GM-CSF and IL-5 being around 70% and IL-3 only
36%. Similarly, CD131 had only at 66% aa identity to the Hu molecule. This raised the
suggestion of whether these cytokines played a similar role in the pig as that of other
mammals and, furthermore, do these molecules share the same conformation thus
raising the possibility of cross species reactivity. Comparing predicted molecular
models of the protein backbone of each of the Po and Hu molecules of these cytokines
revealed similar folding formation, however, minor predicted conformational
differences were observed for GM-CSF, IL-3 and CD131. More importantly, was the
differences in crucial α subunit binding residues, which would probably affect the
cross-species binding of each of these cytokines. Furthermore, although all predicted
cysteine bonds were present for both GM-CSF and IL-5, this was not the case for IL-3
and CD131. The presence of cysteine residues expressed at positions 38 and 108 in
Hu IL-3 were confirmed in all species analyzed except for the pig and all those species
categorized within the pig’s phylogenic group. In addition, it was predicted that one of
the cysteine bonds in CD131 may be incomplete in the mature protein due to the
position of one cys residue. The lack of these cys bonds may further alter the final
conformational shape of these molecules. Further modeling of the predicted tertiary
structure of each molecule revealed additional conformational changes in the
molecules surface topography associated with variance in aa residues between the Hu
and Po molecules, particularly for IL-3, which again would imply possible difficulty in
cross species binding and activity.

Although these results have revealed similarities for each of these proteins
between species, it has also confirmed species specific orthologues which may have
developed independently during natural selection. It is therefore crucial to identify if
these differences equate to differences in the role of these cytokines, and the CD131
receptor, between species. In addition identifying any cross species activity which may be present would reduce the need to produce species specific recombinant proteins. In order to confirm their function, each of the cytokines needed to be cloned and expressed in their biologically active form. This has been made possible by advances in prokaryotic expression systems, which permit the expression of mammalian proteins in large amounts (Georgiou and Segatori 2005). However, there are problems associated with these methods including the lack of post-transitional modification (Sakaguchi 1997), non-specific disulfide bridges and the non-glycosylation of proteins (Fischer, et al. 1992). It is often necessary to trial several expression systems before the most appropriate system for optimum expression levels is determined. Furthermore, the integration of recombinant genes into plasmids, although the most popular vector for gene expression due to their easy manipulation, can often increase unwanted variables in optimization due to metabolic load (Palomares, et al. 2004). Metabolic load can be increased when the insert size is too large, or due to expression level, yield, temperature, or protein toxicity (Palomares, et al. 2004, Corchero and Villaverde 1998, Summers 1998). All these factors can adversely affect plasmid bearing cells thus reducing growth rates and subsequently protein yield. This will often result in the overgrowing of non-transformed cells (Palomares, et al. 2004, Summers 1998). Nonetheless, these barriers can be overcome and often involve the testing of several different expression systems and the careful manipulation and optimization of growth conditions and parameters. Although rPoIL-3 and rPoIL-5 were expressed in E. coli with little difficulty, rPoGM-CSF proved to be more difficult and was best expressed in B. choshinensis (Mizukami, et al. 2010). This expression system, although slower growing than E. coli, produced sufficient quantities of purified rPoGM-CSF yield for investigation and has previously been used in expressing other cytokines including chicken interferon γ (Yashiro, et al. 2001).

The biological activity and effectiveness for each cytokine was demonstrated firstly in individual assays on cytokine dependent cells where all 3 cytokines were shown to promote cell proliferation, as seen in previous studies (Morris, et al. 2008, Andrew, et al. 2007, Zon 2001). However, it is also important for these cytokines to be able to control differentiation from the progenitor to end stage cells. This can be done by varying the concentration of cytokines administered therefore manipulating the outcome. Simply, IL-3 stimulates HSC to proliferate and subsequently increases myeloid progenitor cells which lead to increased production of monocytes and granulocytes, therefore demonstrating a dose dependant response (Wadhwa and Thorpe 2008, Metcalf 1991, Clark and Kamen 1987). In order to determine the
optimum dosage required for this process, assays were carried out using serial dilutions of cytokines. Once optimal activity was established, each cytokine was compared with those of other mammalian species. Investigations therefore expanded to compare the activity of each of the cytokines along with their Hu homologue, in a PoBM proliferation assay. These results were able to show that all 3 pig cytokines initiated proliferation of BM cells, however, there was no significant cross species activity on the pig cells for the HuGM-CSF and HuIL-3. Interestingly, some activity was detected in rHuIL-5 stimulated PoBM cultures at 96h, a time at which activity for rPoIL-5 had dissipated. This activity may therefore indicate a difference in duration of activity for these two IL-5 homologues. Currently there appears to be little research into the cross species activity of IL-5 between Po and Hu, however, some interspecies studies have been reported between HuIL-5 and other mammal species. As the aa residues required for α subunit binding for the pig appears to be conserved across buffalo, cattle, sheep, cat and dog, any cross species activity with these species may produce similar results in the pig. In fact, investigations into the cross reactivity of both rHuIL-5 and rMoIL-5 on the survival or proliferation (or both) of sheep BM eosinophils in vitro, showed that these cytokines along with rOvIL-5 stimulated sheep cells. This may therefore support the possibility of cross species activity of IL-5 between Hu and Po and warrants further investigation. In a similar study, it has been shown that although rOvGM-CSF and rOvIL-3 stimulated sheep BM cells, rHuGM-CSF and rMoGM-CSF in addition to rHuIL-3 and rMoIL-3 failed to have any effect on the cells (Stevenson and Jones 1994). The significance of these results may be due to the differences in the α subunit binding residues between each of these species. Therefore, a hypothesis can be postulated that the lack of activity for the rHuGM-CSF and rHuIL-3 cytokines on PoBM may simply be due to the differences in α subunit binding aa residues in addition to differences in the predicted 3D tertiary structure of the molecules, as previously described. This would therefore indicate that these proteins have developed to be specific for their species, although, cross species activity may still occur within each of the phylogenetic groups. Conversely, the glu residues expressed on these cytokines required for CD131 binding appear to be conserved amongst all species therefore indicating that only the α subunit binding sites maybe species specific. Additionally, although phylogenetically separated, the predicted tertiary molecular structure of the PoCD131 and HuCD131 appear similar with only minor predicted conformational differences when compared to the cytokines themselves. This would suggest that these cytokines, in addition to their associated α subunit, may have evolved over time to differ for each species whereas the CD131 molecule seems to
have evolved with little changes. Previous studies into the variation which exists within the sequence of similar proteins amongst different species have given rise to several theories including the neutral theory of molecular evolution (Fay and Wu 2003, Kimura 1968). This theory provides an essential framework in which functional DNA sequences can be defined and therefore functional changes can be identified (Fay and Wu 2003, King and Jukes 1969). This hypothesis suggests that “the vast majority of DNA polymorphism within a species and divergence between species is neutral or non-functional with respect to fitness” (Fay and Wu 2003, Kimura 1968). Therefore, positively selected mutations bestow a fitness advantage resulting in their rapid fixation, whereas neutral mutations follow a stochastic process of genetic drift through a population. Subsequent work thus showed that functionally important sites evolve more slowly than average (Fay and Wu 2003, King and Jukes 1969). In addition, aa comprising similar physicochemical properties are more readily substituted than those with dissimilar aa (Fay and Wu 2003). This theory would therefore dictate that functionally important aa positions within a protein, as in the case of the glu residues necessary for CD131 binding present within these particular cytokines, would remain constrained and that neutral substitutions constitute the bulk of protein evolution. However, if this hypothesis was totally true, then one would expect the α subunit binding residues present here to also remain constant between species. Furthermore, the changes observed here for the α subunit binding residues between PoGM-CSF and HuGM-CSF indicate changes of different physicochemical properties have occurred with aa, differing between basic and acidic properties. Although to totally evaluate the changes which have occurred it would also be necessary to observe the sequence data for the α subunit, which unfortunately was not within the scope of this work. Nonetheless, as mutation is the cornerstone of evolution, changes in the aa composition of proteins may simply be a case of adaptive evolution. This implies that the substitutions are functional and benefit the organism. Recent studies into adaptive evolution have revealed that some of the most rapidly evolving genes in this comparison involve genes that function in reproduction, immune response and olfaction (Swanson 2003). One example of this is the difference in the MHC I locus between Hu and Mo in which adaptive evolution has been found to promote diversity of the antigen recognition site thus supporting the hypothesis of over dominant selection and heterozygote advantage (Swanson 2003, Hughes and Nei 1988). A similar evolutionary change may indeed have occurred here within the IL-3 and GM-CSF cytokines.
The roles of cytokines in pigs when compared to other species have revealed varied roles which have been highlighted in previous studies. For example, IL-4 in mice plays a central role as a regulatory molecule in the development of the TH2 phenotype and is a soluble diagnostic marker of the TH2 cell type, in addition to being an important cytokine essential for antibody production (Mosmann, et al. 1986, Mosmann and Coffman 1989, Murtaugh, et al. 2009). However, Murtaugh, M.P et al, reported that in the pig, IL-4 actually blocks antibody production and IL-6 secretion and suppresses antigen-stimulated proliferation of B cells. It was furthermore found that IL-4 did not stimulate T lymphocyte proliferation, but rather induced cell growth in lymphoblasts in a dose-dependent fashion (Murtaugh, et al. 2009). This further illustrates that not all cytokines are consistent in their activity between species. Previous work has demonstrated that cytokine receptors, in addition to other cell surface antigens, can be expressed on different cell types between species. For example, it has been demonstrated that in pigs, the MHCII molecule (Pescovitz, et al. 1984) as with dogs (Holmes and Lunn 1994) and horses (Lunn, et al. 1993), is constitutively expressed on resting T cells and granulocytes, which is in contrast to Hu and Mo (Davis and Hamilton 1998). In fact, the presence of MHCII on both resting T cells and granulocytes would suggest these cells may be involved in MHCII mediated immune responses which are not found in Hu or rodents (Davis and Hamilton 1998). Therefore, this suggests that the cell surface expression of molecules within the immune system, as with cytokines, may not be conserved across mammalian species. Keeping this in mind, it was necessary to identify which cells were affected by each of these cytokines and in doing so, identify which cells are expressing CD131 and each of the α subunits. Therefore by using flow cytometric analysis of cells incorporating BrdU, CD90 and CD172a, along with other available lineage markers; the proliferation of PoBM induced by each of the CD131 cytokines in addition to identifying the cell lineage promoted was confirmed.

As there was no anti-pig CD131 antibody available at the time of this research, alternative CD markers were used to observe both the myeloid and lymphoid cell lines in addition to progenitor cells and HSC. The results confirmed rPoGM-CSF and rPoIL-3 proliferation of the myeloid cell line as observed by CD172a staining, however, there was no indication any proliferation initiated by rPoIL-5. Although, as previously shown, rPoIL-5 activity appears to diminish after 72h in vitro and as these results were analysed at 90h incubation, we speculate that any proliferation initiated by IL-5 may have completed by this time point. Nonetheless, results have shown that both rPoGM-CSF and rPoIL-3 initiated stimulation of CD172a⁺ cells in addition to stimulating the
proliferation of CD90 cells. As CD90 is also expressed on HSC and some precursors, this may also suggest proliferation of these progenitors (Kisselbach, et al. 2009). Identification of cell morphology following cytokine incubation confirmed no increases in lymphocytes, implying these CD90+ cells may indeed be precursor cells. This of course would confirm the early acting activity of both of these cytokines on HSC as previously seen in Hu and Mo BM (Mohle and Kanz 2007, Ikuta, et al. 1991). Clear shifts in cell populations were observed for each cytokine, thus, providing a profile of the early acting capabilities of each cytokine. Increases in monocytes and macrophages induced by rPoGM-CSF and rPoIL-3 which corresponded to decreases in granulocytes for both of these cytokines, whereas, rPoIL-5 alone resulted in an increase in eosinophils numbers. These results give a clear indication of cell types directed by each of these cytokines. However, whilst these in vitro assays provide some information of both the bioactivity and the impact of these cytokines on PoBM cells, the more complex system of the micro environment of the BM and full body systems, found only in vivo, were required to investigate the full potential of these cytokines in enhancing animal health.

To date, little work has been carried out in the pig, where it has been used as a large animal model for therapeutic research. The size, cost and daily requirements for these animals requires a large specialized facility not regularly available. However, in order to determine the effects of each of these cytokines upon the immune system, we needed to develop a whole animal model in the pig where the effects of these cytokines could be monitored. In a previous study Laurenz, J. C et al, (1997), were able to establish the therapeutic dosage of the cytoreductive cancer chemotherapeutic drug 5-FU in pigs, so as to cause myelosuppression. In this study Laurenz et al, were essentially investigating the ability of uteroferrin and GM-CSF to modulate the myelosuppressive effects of 5-FU (Laurenz, et al. 1997, Laurenz, et al. 1997, Davis and Hamilton 1998). In contrast to that model, here both bloods and BM were examined for the actions of rPo cytokines post myelosuppression with 5-FU. This approach not only provided valuable information on the activity of these cytokines on differentiation, proliferation and cell modulation of precursor cells in the BM, but also provided information on the therapeutic effects on peripheral WBC repopulation. In addition, in this study, cytokines were administered post 5-FU treatment as opposed to during treatment as in the previous study led by Laurenz et al. In doing this, we were able to identify changes in cell differentiation and proliferation as opposed to cell protection. Therefore, in order to determine the effects of each of these cytokines in
vivo, an in depth trial was initiated where both blood and BM were analysed following myelosuppression with 5-FU and treatment with recombinant cytokines.

The overall results of the in vivo activity of the cytokines, when first examined differed somewhat to the in vitro results previously obtained. Firstly, there were no significant differences recorded for WBC numbers between groups. Although there were no expectation for significant changes to WBC numbers following treatment with rPoIL-5, it was anticipated that there would be significant differences for rPoGM-CSF and/or rPoIL-3. Previous reports indicated an increase of 22% in WBC numbers for pigs treated with GM-CSF (Laurenz, et al. 1997) and as IL-3 is an early acting growth factor which initiates proliferation of HSC (Martinez-Moczygemba and Huston 2003), a similar result was also expected for this group. Furthermore, there were no significant increases in either monocyte or lymphoid cell numbers for any of the groups. This, however, does not contradict results seen in vitro, where both rPoGM-CSF and rPoIL-3 treated BM cell cultures showed increases in monocytes, but rather confirms that both rPoGM-CSF and rPoIL-3 may act early on in hematopoiesis to increase cell populations. This notion was verified by analysing activity in the BM where on week one there is not only a significant difference in CD172\textsuperscript{+} cells for all 5-FU treated groups confirming myelosuppression within the BM cavity, but more importantly, a less significant increase for the cytokine treated groups than the 5-FU control group, indicating an increase in cells for these groups. At this point, subjects analysed for BM activity had only received 2 doses of cytokine. Interestingly, we also observed an increase in CD172\textsuperscript{+} cells for the rPoIL-5 group which was not expected (Figure 4.10). This may confirm our earlier suggestion that IL-5 proliferation occurs within 72h of administration. On examination of the CD172a sub-groups we saw that there was no significant difference for the CD172\textsuperscript{lo} cells for the rPoIL-3 and rPoIL-5 groups indicating these groups have had an increase in precursor cells over the rPoGM-CSF and 5-FU control groups, although the rPoGM-SCF group (P<0.05) was slightly less significant to the 5-FU control group (P<0.001) (Figure 4.11). A similar result for the CD172a\textsuperscript{hi} mature cells indicated increases to the cytokine treated cells over the 5-FU control group, however to a less extent than the precursor cells (Figure 4.12), therefore indicating an increase in both immature and mature myeloid cells had occurred in the BM for the cytokine treated groups. This time point also coincides for the period in which eosinophil numbers began to increase for the rPoIL-5 group therefore indicating that the increases in monocytic precursors may have been committed to the eosinophil linage. In addition, it is worth noting the time of response to the cytokines in vivo when compared to in vitro in regard to BM proliferation. The predicted time of cell
proliferation in bioassays occurred after 72h incubation whereas in vivo BM proliferation occurred after just 48h. This is most likely due to additional activity by support cells within the BM cavity and the availability of endogenous factors required.

By week two, both the rPoGM-CSF and 5-FU control groups had returned to above basal level for CD90+ and CD172a+ cell numbers. In the case of rPoGM-CSF, although there were no significant differences, this group showed a considerably large increase in CD172ahi cells over the non-treated control group of 0.62 RFI, and a 0.40 RFI over the 5-FU control group. This implied an increase in the mature monocytes and granulocytes for the rPoGM-CSF group of 4.2 fold over the 7 day period from confirmation of myelosuppression from D7 to D14. Furthermore, a 2.4 fold increase in CD90+ cells was also observed. There were, however, no significant increases in peripheral cells for this group for this time period, other than eosinophils which were twice the basal level. Nevertheless, as this is a small population of cells, there appears to be no large increase in cells in the periphery differing from the other 5-FU treated groups. This, therefore, indicates a large number of myeloid lineage cells, monocytes and metamonocytes were still retained within the BM matrix. As only the rPoGM-CSF and 5-FU groups returned to above normal at this time, we hypothesize that rPoGM-CSF may be one of the key cytokines released into the BM matrix during or after a major assault of this type. Consequently, this would provide the BM with a large population of myeloid cells, ready to be released into the periphery if needed. This may therefore support the use of rPoGM-CSF as a useful tool in up regulating myeloid cell numbers in association with other lineage specific cytokines. Further investigation revealed that this increase was not seen for either the rPoIL-3 or rPoIL-5 groups. In fact, the IL-3 group was significantly lower than the 5-FU control group in both the CD90+ and CD172a+ cell populations for the week 2 subjects, although the CD172a+ population was only slightly lower than the rPoGM-CSF and rPoIL-5 level for this time point. Nonetheless, this result indicated that there was a reduction in CD90+ and CD172a+ cell populations compared to the 5-FU control and rPoGM-CSF groups at this point. In addition, it was noted that proliferation of precursor cells had increased from week 1, although, this was less than the 5-FU control. What was more notable was the low level of mature CD172a+ cells for these two groups. As each of these cytokines was responsible for increases to specific cells, most notably eosinophils for rPoIL-5 and basophils for rPoIL-3, it could be suggested that these BM levels were low due to the increases in these cell populations in the periphery. Although rPoGM-CSF also increased both of these two cell lineages at this time point, the levels obtained with rPoIL-3 and rPoIL-5 were much higher. It could also suggest the actions of rPoIL-
3 in the BM may also have increased the B-cell line thus contributing to reduced CD90+ and CD172a+ cells in the BM and an increase in lymphocyte cells in the periphery. Additionally, it could be proposed that levels of mature CD172a hi cells within the BM may have been low during week two due to the maturation of eosinophilic and basophilic precursors. In supporting this theory, levels of eosinophils for the IL-3 group increased around this period which may have contributed to the lower level of CD172a hi cells in the BM for this group compared to the other groups. IL-3 has been well documented as a potent stimulator of eosinophil and basophil differentiation in BM suspension cultures (Saito, et al. 1988, Ottmann, et al. 1989, Gillio, et al. 1990). In fact, IL-3 has been identified as the most potent growth factor for basophils although both GM-CSF and IL-5 also promote basophil production in Hu (Valent and Dahinden 2010). This may suggest a higher number of IL-3α receptors on basophils that those for IL-5 and GM-CSF. It was also noted that IL-3 increased eosinophil levels from D14 to D19 before reducing to basal levels. Although there were no significant differences observed, it is suggested that rPoIL-3 had indeed contributed to the up regulation of eosinophils although the delayed increase in cells suggests that rPoIL-3 regulation of eosinophils may be due to the increased number of CFU-Bas eos precursor cells by rPoIL-3. Furthermore, the lack of increase in basophils by GM-SCF and IL-5 dictates their specificity to other areas of granulocyte maturation.

Further results for rPoIL-3, concentrating upon progenitor cells and HSC, also appear to be inconsistent between in vitro and in vivo cultures. Proliferation studies with rPoIL-3 clearly showed increases in both CD172a+ and CD90+ BM cells, however, these results were not mirrored in vivo. In fact, a reduction both CD90+ and CD172a+ cells was observed in vivo. As rPoIL-3 treated pigs show increased peripheral WBC in correlation with rPoGM-CSF and rPoIL-5, with the exception of significant findings, we can confirm that IL-3 is not inhibiting proliferation. Previous results in Mo demonstrated IL-3 not only increased and hastened the development of nucleated cells from HSC in in vitro cultures but also suppressed the production of CFU and possibly reduced the self-renewal capabilities of HSC (Yonemura, et al. 1996). Moreover, it was shown that IL-3, when added to methylcellulose culture assays for Mo lympho-hematopoietic cultures, abrogated the T-cell and B-cell potential of the progenitors (Ogawa, et al. 1997). However, if this action was occurring here, then a reduced population of CD90+ proliferating cells would be expected opposed to an increased level as observed. Although, one must also appreciate that in these Mo studies, IL-3 was added to other growth factors including SCF, IL-6, IL-11 and EPO (Ogawa, et al. 1997). In fact, it is hypothesized that the addition of IL-3 to varied combinations of growth factors may
indeed result in differing results in HSC renewal. This has previously been shown by introducing IL-3 into Mo HSC cultures containing KL, FL and megakaryocyte growth and development factor (MGDF), which resulted in the enhancement HSC self-renewal and proliferation capabilities (Bryder and Jacobsen 2000). Furthermore, the continued culturing of these cells over 5 cell divisions demonstrated that there was no inhibition of long term repopulating HSC induced by IL-3 (Bryder and Jacobsen 2000). Nonetheless, we believe that here, rPoIL-3 is not acting as an inhibitory factor and that early proliferation and maturation of progenitor cells and precursors maybe responsible for the resulting profile. It is therefore suggested, that the reduction in CD90+ and CD172a+ cells may be due to the increase in B-cell precursors by rPoIL-3. This would explain the absence of these markers. By week three, all groups had returned to above basal control levels for BM cells with few exceptions. A significant increase in CD172ahi for both rPoGM-CSF and the 5-FU control groups again suggest the action of the 5-FU control group to reconstitute the BM may include the action of rPoGM-CSF even though rPoGM-CSF alone resulted in a more significant result. This of course may be reflective of the quantity of rPoGM-CSF administered in addition to the endogenous release of PoGM-CSF. In addition, the significant increase in both CD90+ and CD172a+ cells for rPoGM-CSF for this period indicates the proliferation of HSC and progenitor cells may still be active for this group over the other groups. This would also indicate that rPoGM-CSF may be longer acting on HSC and pre-cursor cells than the other cytokines tested. Nevertheless, apart from the actions of rPoGM-CSF in week three, only rPoIL-3 produced a significant difference within the BM. Firstly, an increase in CD172alo BM cells when compared to the untreated control was noted. This increase in immature myeloid cells was also coupled with an increase in CD90+ for this group however, was not significant and there was no significant difference compared to the 5-FU control group. Nevertheless, this result suggests rPoIL-3’s ability to initiate proliferation of progenitor cells and immature monocytes and granulocytes within the BM. Finally, a significant difference between the rPoIL-3 and 5-FU control group for CD172ahi cells indicates more mature monocytes and granulocyte within the BM for the 5-FU control group when compared to the rPoIL-3 group although at this point the rPoIL-3 group had recovered back to basal levels.
Summary

Analysis of the effects of these cytokines on peripheral WBCs demonstrated that rPoGM-CSF initially contributed to a decrease in neutrophils and basophils whilst increasing eosinophils and platelets. rPoIL-3 appears to have increased basophil numbers although eosinophil numbers did increase late within the trial whilst rPoIL-5 has distinctly and significantly increased eosinophil numbers.

From the results shown here, further conclusions as to the expression of CD131 and its corresponding α subunits, CD116, CD123 and CD125, can be made. In addition, suggestions as to the actions of each of the cytokines upon the process of hematopoiesis can be determined. As results from both in vitro and in vivo studies have been observed, the combined data has been used to produce both a predicted CD marker expression map and cytokine activity map in the pig (Figure 5.1, 5.2).

As both rPoGM-CSF and rPoIL-3 have been shown to up regulate monocyte numbers in vitro and are not directly implicated in granulocyte regulation, it is suggested that CD131, in addition to CD116 and CD123, are expressed on the CMP in addition to the precursor cell CFU-GM (Figure 5.1). Furthermore it can also be implied that these clusters are expressed on the monocyte lineage cells deriving from these precursors. This can be proposed as it was shown that both GM-CSF and IL-3 acted to increase macrophage numbers, therefore, implying that these receptor sub-units are expressed continually throughout the monocyte lineage, however, the expression of these receptor sub-units on macrophage cells is unable to be confirmed from this information (Figure 5.2). Similarly, the involvement of these cytokines and receptors in the regulation of mast cells and DC is also unable to be confirmed, although, as these cells share the same precursor cells, it is not presumptuous to suggest the early involvement of these cytokines in up regulation of these cells. This notion is supported by previous in vitro investigations which confirmed the production of immature Po DC cells from BM cells in the presence of rPoGM-CSF and rPoIL-4 (Carrasco, et al. 2001, Foss, et al. 2003). In addition, both GM-CSF and IL-3 have been previously implicated as survival factors for CD172a expressing myeloid and plasmacytoid DC (Summerfield, et al. 2003). This suggests that although DC were not specifically analyzed in this study, DC precursor cells would indeed be present amongst CD172a+ cells within the BM. Furthermore, in addition to the monocyte lineage, it is suggested that rPoGM-CSF has assisted in the increase of platelet numbers. This has possibly been as a result of an increase in megakaryocytes rather than a direct stimulation of platelet production. This result is similar to that observed for rhesus monkeys after
Figure 5.1 Mammalian hematopoiesis showing suggested pig CD markers. The suggested process of mammalian hematopoiesis showing HSC self renewal and cell types for each of the lymphoid and myeloid cell lines. Pig cell surface markers, including CD131, CD116, CD123 and CD125, are indicated at several stages as predicted from in vitro and in vivo studies. CD markers in red indicate lineage end cell produced via these receptors. CD markers in green suggest possible interactions.
Figure 5.2 Mammalian hematopoiesis showing suggested pig cytokine interactions. The suggested process of mammalian hematopoiesis showing HSC self renewal and cell types for each of the lymphoid and myeloid cell lines. IL-3, IL-5 and GM-CSF involvement in pig hematopoiesis are indicated at several stages, as suggested from in vitro and in vivo research. Cytokines in green suggest possible interactions.
sequential treatment with IL-3 and GM-CSF and suggests the involvement of endogenous PoIL-3 produced by the pigs (Stahl, et al. 1992). It is, therefore, suggested that the action of rPoGM-CSF is on the precursor cell, CFU-ME, thus directing proliferation and differentiation towards the megakaryocyte lineage and not the erythrocyte lineage. This is also confirmed by the lack of increase in RBC following treatment with rPoGM-CSF. We can therefore suggest that CD131 and CD116 are expressed on the CFU-ME megakaryocyte precursor (Figure 5.1). Furthermore, both the in vitro and in vivo results indicated reduced neutrophil levels resulting from the treatment with rPoGM-CSF. In addition, in vitro levels of monocytes were significantly increased, although, in vivo levels of monocytes seem unchanged. Nevertheless, these results would suggest in this case that rPoGM-CSF has acted on the CFU-GM precursor resulting in the reduction of neutrophils and increase in monocytes. The reason for this is not understood, however, it is suggested that CD131 and CD116 are expressed on the CFU-GM in the pig (Figure 5.1). Furthermore, it is suggested that treatment with rPoIL-3 also resulted in the increase in basophils and eosinophils. This would suggest that the CFU BAS/Eo cell lineage has been stimulated rather than the CFU-GM in this case, therefore, reducing the proliferation of monocytes and neutrophils and subsequently increasing numbers of basophils and eosinophils. This was determined due to the following results. Firstly, increases in basophil levels for rPoI3-3 treated pigs associates rPoIL-3 involvement in basophilopoiesis (Figure 4.6). Secondly, increased eosinophil levels in rPoIL-3 treated pigs were noted late in the in vivo trial. Although this was not significant, it is proposed that as levels of eosinophils for the rPoIL-3 group were 3 fold higher than the basal level at D16-19, that rPoIL-3 has contributed to this increase (Figure 4.5). Adding to this, the significant increases in eosinophils observed in vivo for both rPoIL-5 and rPoGM-CSF (Figure 4.5) suggests that each of these cytokines contributed to the production and regulation of eosinophils and basophils. Therefore, it is suggested that the surface receptors CD131, CD116, CD123 and CD125 are expressed on the CFU-Bas/Eo precursor (Figure 5.1). Since rPoIL-3 increased basophil numbers it is suggested that the IL-3 receptors are present throughout the differentiation and maturation of basophils and that equally, the receptors for rPoIL-5 and rPoGM-CSF are present throughout differentiation and maturation of eosinophils.
Conclusion

This research has produced the biologically active CD131 cytokines, rPoIL-3, rPoIL-5 and rPoGM-CSF. Furthermore, the in vitro and in vivo activity of these cytokines has been determined. In addition, the common β subunit, CD131, has also been investigated in silico. This work was an important step towards the understanding of the process of hematopoiesis in the pig, however, this is by no means an exhaustive investigation of the process. Phylogenetic ancestry has been assessed and the basis of the lack of cross-species activity of IL-3 and GM-CSF was discussed. In addition, the suggested preserved cross-species activity for IL-5, has been addressed. Recombinant PoGM-CSF has been demonstrated to increase CD90+ and CD172a+ cells in the BM in addition to increasing macrophages in vitro and eosinophils and platelets via increased megakaryocytes in vivo. Furthermore, it is suggested that rPoIL-3 acts to increase precursor cells in the BM, in addition to basophils and eosinophils levels in the periphery. Finally and most importantly, it was demonstrated that rPoIL-5 is a simulator of eosinophilopoiesis by acting on eosinophil precursor cells within the BM resulting in increased eosinophils in the peripheral bloodstream. This result was demonstrated both in in vitro BM studies and in vivo studies.

This work may contribute to the further development of therapeutic management and disease control within the pig industry. The use of cytokines such as rPoIL-5, rPoIL-3 and rPoGM-CSF may prove to be useful tools in the reduced reliance on prophylactic antibiotic treatment in the pig industry. Furthermore, the eosinophilopoietin properties of these cytokines, especially rPoIL-5, may provide a more host friendly method of reducing and controlling parasitic infections in pigs other than the use of helminthics. In addition, the pig has been determined to be an ideal large scale model for human research. These results may therefore provide the basis for translational studies in Hu which may see the pig as an ideal a large scale model for immune deficiency and enhancement studies. For example, the confirmation of IL-5 as the major contributor to the product of eosinophils in pigs, as it is in Hu, may contribute to studies into Hu conditions which involve eosinophilia, such as asthma.
Reference list


146. Clark, R. A. S., Timothy A. 2004. CD90. In *Protein reviews on the web*. 120


APPENDIX 1

Preparation of buffers, gels and reagents

A1.1 Phosphate-buffered saline (PBS) pH 7.3
PBS tablets (Oxoid, UK) were dissolved in milli Q water (MILLI-RO®/Milli-Q®UPLUS, Millipore Corporation, Massachusetts, USA) according to the instructions.

A1.2 Complete Dulbecco's Modified Eagle Medium (DMEM)
Sterile DMEM is diluted from a 5X concentrated stock (Trace Biosciences, Australia) in milli Q water to a final concentration of 1X, with the following additions:
- 20 mM HEPES (Gibco, USA)
- 0.75 mg/mL NaHCO₃
- 1 mM L-glutamine
- 100U/mL penicillin
- 100μg/mL streptomycin sulfate
- 50μM 2-mercaptoethanol

A1.3 Inactivation of foetal calf serum (FCS)
FCS was inactivated at 56°C for 1hr and cooled to room temperature.

A1.4 Iscove's Modified Dulbecco's Medium (IMDM)
IMDM is made from a powdered medium containing L-glutamine and 25mM HEPES according to the manufacturer's instructions, to which is added
- 36 mM NaHCO₃
- 100 units (U)mL penicillin
- 100 μg/mL streptomycin sulfate
- 5 0μM 2-mercaptoethanol

The medium was sterilised through a 0.22μm filter.

A1.5 10X cDNA Synthesis Buffer
- 100 mM Tris-HCl pH 8.3
- 500 mM KCl
15 mM MgCl₂
100 μg/mL BSA

**A1.6 10X PCR Amplification Buffer**
100 mM Tris-HCl pH 8.4
500 mM KCl
10-40 mM MgCl₂

**A1.7 50X TAE Buffer (Sambrook et al, 1989)**
2 M Tris base
1 M glacial acetic acid
0.05 M EDTA pH 8.0

**A1.8 TE Buffer pH 8.0**
10 mM Tris-HCl
1 mM EDTA pH 8.0

**A1.9 (SDS-PAGE) gels**

<table>
<thead>
<tr>
<th>For 2X 12% polyacrylamide gels</th>
<th>Component volumes</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Solution components</strong></td>
<td><strong>Resolving gel</strong></td>
</tr>
<tr>
<td>Milli Q water</td>
<td>1.95 mL</td>
</tr>
<tr>
<td>30% Acrylamide/Bis sol 29:1 (Bio-Rad, USA)</td>
<td>4.18 mL</td>
</tr>
<tr>
<td>1 M Tris-HCl pH 8.0</td>
<td>3.75 mL</td>
</tr>
<tr>
<td>0.5 M Tris-HCl pH 6.8</td>
<td>-</td>
</tr>
<tr>
<td>10% SDS</td>
<td>100 µl</td>
</tr>
<tr>
<td>TEMED (Bio-Rad, USA)</td>
<td>5 µl</td>
</tr>
<tr>
<td>10% ammonium persulphate (APS)</td>
<td>100 µl</td>
</tr>
</tbody>
</table>
A1.10 2X SDS – gel loading buffer
(current Protocols in Immunology, 1991)
125 mM Tris-HCl pH 6.8
200 mM dithiothreitol (DTT)
4% SDS
1% bromophenol blue
20% glycerol

A1.11 Reagents: for BAF cells
X63 supernatant used at 5%
DMEM with 10% FCS
75cm³ flask
96 well tissue culture plates
Tritiated thymidine (1:50 dilution)

A1.12 TF1 reagents Culture medium
RPMI 1640 medium
2mM L-glutamine
10 mM HEPES
1 mM sodium pyruvate
4.5 g/L glucose
1.5 g/L sodium bicarbonate
Supplemented with: 10% Foetal Calf Serum (FCS)

A1.13 TF1 Complete Growth Medium
RPMI 1640 medium
2mM L-glutamine
10 mM HEPES
1 mM sodium pyruvate
4.5 g/L glucose
1.5 g/L sodium bicarbonate
Supplemented with: 10% Foetal Calf Serum (FCS)
5% ovine GM-CSF expressed from vaccinia virus
(or 1ng/mL of rec Hu IL-3 or recombinant Hu GM-CSF)

A1.14 Sonication buffer
(20 mM Tris-HCl, pH 8.0, 100 mM NaCl)
A1.15 Talon Wash buffer
20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 0.1% Zwittergent 3-14,

A1.16 BTY media
1% glucose (BDH, Australia)
2% tryptone (Oxoid, UK)
0.5% yeast extract (Difco, USA)
0.001% FeSO₄ (Sigma, Germany)
0.001% MnSO₄ (BDH, Australia)
0.0001% ZnSO₄ (Sigma, Germany)

A1.17 MBTY plates
1% glucose
2% tryptone
0.5% yeast extract
0.001% FeSO₄
0.001% MnSO₄
0.0001% ZnSO₄
1.5% Bacto Agar (Difco, USA)

A1.18 Carbonate buffer
15 mM Na₂CO₃ (Ajax Chemicals, Australia)
35 mM NaHCO₃ (Prolabo, France)
pH 9.6

A1.19 Destaining solution
5% v/v methanol (BDH, Australia)
10% v/v acetic acid (Ajax Chemicals, Australia)

A1.20 Dulbecco’s Modified Eagle Medium (DMEM)
DMEM (Trace Biosciences, Australia) was diluted 1:5 in MilliQ water with the following additions:

A1.21 20 mM HEPES (ICN, USA)
0.75 mg/mL NaHCO₃ (Prolabo, France)
1 mM L-glutamine (Sigma, Germany)
A1.22 100 U/mL penicillin (CSL, Australia)
   100 μg/mL streptomycin sulfate (CSL, Australia)
   50 μM 2-mercaptoethanol (Sigma, Germany)

A1.23 FACS permeabilising solution
   Perm/Wash™ buffer (Becton Dickinson, USA) diluted 1:10 in distilled water

A1.24 FACS wash
   2% FCS (Trace Biosciences, Australia)
   0.01% NaN₃ (BDH, UK)

A1.25 HAT
   HAT media supplement (Sigma, Germany) diluted 1:50 in media

A1.26 H₂SO₄ stop solution
   0.5 M H₂SO₄ (BDH, Australia)

A1.27 Inactivation of foetal calf serum (FCS)
   FCS (Trace Biosciences, Australia) was inactivated at 56°C for 1 h and cooled to room temperature before use

A1.28 Luria broth
   1% tryptone
   0.5% yeast extract
   1% NaCl (BDH, Australia)

A1.29 Ni-NTA phosphate buffer
   (Qiagen QIAexpressionist protocol booklet)
   50 mM NaH₂PO₄, pH 8.0 (APS, Australia)
   300 mM NaCl
   10 mM Imidazole (Sigma, Germany)

A1.30 Ni-NTA wash buffer
   50 mM NaH₂PO₄, pH 8.0
   300 mM NaCl
   20 mM Imidazole
A1.31 Ni-NTA elution buffer
50 mM NaH$_2$PO$_4$, pH 8.0
300 mM NaCl
250 mM Imidazole

A1.32 PBS-Tween
0.1% v/v Tween-20 in PBS

A1.33 PGH buffer
1 mM HEPES, pH 7.0
15% glycerol (BDH, Australia)
15% PEG 6000 (Merck, Australia)

A1.34 Quillaja saponaria (QuilA) cocktail
Per injection:
20 µg QuilA (Superfos Biosector, Denmark)
200 µg DEAE-dextran (Pharmacia, Sweden)
100 µl montamide emulsify (Tall-Bennett, Australia)

A1.35 Red cell lysis buffer
1 mL 170 mM Tris hydroxymethyl methane (BDH, Australia), pH 7.6
9 mL 0.83% NH$_4$Cl (BDH, Australia)

A1.36 RT PEG
50% PEG 4000 (Merck, Australia)

A1.37 Running buffer
(Sambrook et al., 1989)
25 mM Tris hydroxymethyl methane
192 mM glycine (APS, Australia)
170 mM sodium dodecyl sulphate (SDS; BDH, Australia)
pH 8.3

A1.38 Sodium casein
1% Casein sodium salt (Sigma, Germany) made up in PBS
A1.39 TAE buffer (50x)
(Sambrook *et al.*, 1989)
2 M Tris hydroxymethyl methane
1 M acetic acid
0.05 M EDTA, pH 8.0 (Bio-rad, USA)

A1.40 Talon tris buffer
(Clontech laboratories protocol booklet)
20 mM Tris-Cl, pH 8.0
100 mM NaCl

A1.41 Talon wash buffer
20 mM Tris-Cl, pH 8.0
100 mM NaCl
5 mM Imidazole

A1.42 Talon elution buffer
20 mM Tris-Cl, pH 8.0
100 mM NaCl
100 mM Imidazole

A1.43 TBS buffer
10 mM Tris-Cl, pH 7.5
150 mM NaCl

A1.44 TBS-Tween/Triton buffer
20 mM Tris-Cl, pH 7.5
500 mM NaCl
0.05% Tween-20 (Sigma, Germany)
0.2% TritonX-100 (Sigma, Germany)

A1.45 TMB substrate solution
15 mL MilliQ H$_2$O
15 mL 200mM sodium acetate (BDH, Australia)
450 µl citric acid (BDH, Australia)
3.6 µl H$_2$O$_2$ (Sigma, Germany)
300 μl tetramethyl benzidine (TMB; Boehringer Mannheim, Germany)

A1.46 **Trypticase™ Soy Broth (TSB)**
(Becton Dickinson, USA)
1.7% Pancreatic Digest of Casein
0.3% Papaic Digest of Soybean Meal
0.5% Sodium chloride
0.25% Dipotassium phosphate
0.25% Dextrose

A1.47 **TSB™ agar plates**
1.5% Bacto Agar
Trypticase™ Soy Broth

A1.48 **Western transfer buffer**
25 mM Tris hydroxymethyl methane
192 mM glycine
20% ethanol (v/v)

A1.49 **Western blocking buffer**
3% bovine albumin (BSA; Sigma, Germany)
10 mM Tris-Cl, pH 7.5
150 mM NaCl

A1.50 **0.1% Coomassie brilliant blue stain**
0.1% Brilliant blue R250 (Sigma, Germany)
45% methanol
9% acetic acid

A1.51 **2x SDS gel-loading buffer**
125 mM Tris-Cl, pH 6.8
20% glycerol
4% SDS
200 mM Dithiothreitol (DTT; Progen, Australia)
1% Bromophenol Blue (Sigma, Germany)
### APPENDIX 2

#### BACTERIAL STRAINS AND PLASMIDS

---

**A2.1 Bacterial strains and genotypes used in this study**

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Strain</th>
<th>Genotype</th>
<th>Source/Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Escherichia coli</em></td>
<td>DH5α</td>
<td>supE44 ΔlacU169 (φ80 lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1</td>
<td>Bethesda Research Laboratories</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>TOPP 10F</td>
<td>F’(lacIq, Tn10(TetR)) mcrA Δ(mrr-hsdRMS-mcrBC) Φ80lacZΔM15 ΔlacX74 recA1 araD139 Δ(ara leu) 7697 galU galK rpsL (StrR) endA1 nupG</td>
<td>Invitrogen Australia</td>
</tr>
</tbody>
</table>
A2.2 Plasmid map of the pGEM-t easy plasmid

The pGEM t easy plasmid incorporates an ampicillin resistance gene (Amp\(^\text{R}\)), origin of replication, ColE1 origin of replication (ColE1 ori), HWP P2 promoter and a multiple cloning site (MCS) followed by a transcriptional terminator (T\(_\text{o}\)).
A2.3 Plasmid map of the pQE30 vector

The pQE30 plasmid (Qiagen, Germany) incorporates a β-lactamase gene (AmpR) for ampicillin resistance, the Col E1 origin of replication (Ori), an E. coli phage T5 promoter, a poly histidine tag (6x His), a multiple cloning site (MCS) immediately followed by stop codons in all three reading frames, a lambda phage transcriptional terminator (T₀), and an rrnB t1 transcription terminator (t1).
A2.4 Plasmid map of the pNCMO2 vector

The pNCMO2 plasmid incorporates a pUC derived ampicillin resistance gene (Amp<sup>R</sup>), a neomycin resistance gene (Nm<sup>R</sup>), a pUB110 origin of replication (pUB110 ori), a ColE1 origin of replication (ColE1 ori), a HWP P2 promoter and a multiple cloning site (MCS) followed by a transcriptional terminator (T<sub>0</sub>).
Minerva Access is the Institutional Repository of The University of Melbourne

Author/s:
STEPHENSON, GARTH

Title:
Characterisation of cytokines involved in porcine hematopoiesis

Date:
2014

Persistent Link:
http://hdl.handle.net/11343/42056

File Description:
Characterisation of cytokines involved in porcine hematopoiesis