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# Characterisation of the porcine cytokines which activate the CD131$_{\beta c}$ common sub-unit, for potential immune-augmentation

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ABSTRACT

Early acting cytokines and growth factors such as those of the CD131 $\beta_c$ subunit, may offer an alternative method to the current use of antibiotics and chemicals such as anthelmintics in maintaining Porcine (Po) health. Thus far, the recombinant Po (rPo) Granulocyte-macrophage colony-stimulating factor (GM-CSF), rPo interleukin-3 (IL-3) and rPo interleukin-5 (IL-5) proteins have been identified and cloned and the biological activity of each cytokine has been confirmed *in vitro,* however, *in vivo* immune system regulation and hematopoietic stem cell (HSC) augmentation are regulated by numerous cytokines and cellular signals within the bone marrow (BM) niche. In order to quantify the use of recombinant cytokines in augmenting the immune response, it is necessary to determine the stages of hematopoiesis induced by each cytokine and possible areas of synergy requiring further investigation. Here we used the chemotherapeutic agent 5-fluorouracil (5-FU), to chemically induce a 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. The recombinant cytokines PoGM-CSF, PoIL-3 and PoIL-5 were administered by intramuscular injections (i.m.) following confirmation of 5-FU induced leukocytopenia. Blood and BM samples were collected and then 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 basophils for animals treated with rPoIL-3. BM analysis of $CD90^+$ and $CD172a^+$ cells confirmed myelosuppression in week one with significant results observed between rPoIL-3 and the 5-FU control group in week two and for the rPoGM-CSF group in week three. These results have demonstrated the effects of each of these rPo cytokines within the hematopoietic processes of the pig and may demonstrate similar outcomes in other mammalian models including human.

## 1. 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 [5] and that increases in meat consumption by as much as 73% could be experienced by 2050 [5]. The demand for increase animal numbers in the past has led farmers to adopt methods of husbandry, which are no longer accepted, by health professionals and consumers alike, as being the safest for human consumption. These practices often include the prophylactic use of antibiotics and antimicrobials such as anthelmintics [26,46]. The prophylactic use of antibiotics is believed to lead to the increased risk in developing antibiotic resistant bacteria capable of infecting humans [46]. There is therefore a growing demand for alternative treatments for the maintenance of animal health.

Although there is already much known about hematopoiesis there is still little known about the microenvironment niche of the BM [45,32]. The generation of hematopoietic cells from the single self-renewable HSC, requires the involvement of cellular interactions, cytokines, chemokines, macromolecules and the extra cellular matrix which drive the proliferation and differentiation of HSC and resulting precursor cells to develop into a mature immune system [4,32]. The regulation of these processes is highly controlled and crucial to not only combat infection and disease but also regulate the body's thrombolytic state and gas exchange essential for energy production and homeostasis [42]. Cytokines, the immune systems signalling and regulatory proteins, control the activation of immune responses, cell proliferation, differentiation,

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and apoptosis [47]. As such, it is believed that cytokines may provide an alternative method of maintaining pig health. To this end, an investigation of the roles played by individual cytokines on HSC and precursor cells is a necessary step in the understanding of hematopoiesis in pigs, and could provide us with a unique insight into the pathways of individual cell development and maturation.

CD131- the common β Subunit, is a member of 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 [47]. These three classes include those which use gp130, those which use γc and those which use the common β subunit, β c), CD131 [47]. 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 [47,26]. Moreover, even though each of these cytokines have their own specific α subunit, the co-utilisation of CD131 suggests a common intra-cellular process of both unique and/or overlapping activities on expressing cells [26]. The cellular response elicited is therefore dependant on the specific cell targeted, the cytokine initiating the response, and to whether the cytokine is acting alone or in synergy with other cytokines [7]. Furthermore, the CD131 cytokines can compete for the CD131 subunit indicating cytokine concentrations and combinations are also relevant to the cellular pathway activated [13]. Previously, it was shown that the exogenous recombinant cytokines IL-3, IL-5 and GM-CSF are biologically active *in vivo* [2,1]. The activity of each of these cytokines on $CD34^+$ hematopoietic cells was demonstrated in a porcine model and levels of eosinophils and basophils noted. It was further demonstrated that no adverse effects were observed and peripheral granulocyte levels elevated by exogenous cytokines returned to basal levels 11 days post administration [23].

In order to adequately study the function of each 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 [16]. The antimetabolite 5-FU is a widely used cytoreductive cancer chemotherapeutic agent and thymidylate synthetase inhibitor which incorporates into RNA [31,16] and results in apoptosis of the incorporated cell by eliminating available thymine [9]. 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 which is involved in regulating apoptosis either by blocking or inducing it [9]. As HSC lie dormant in the G0 phase of the cell cycle unless stimulated [42], this procedure gives the advantage of removing linage 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 role of each of the cytokines in hematopoiesis [17].

In this study, a comparison of the effects on hematopoiesis of the rPo cytokines IL-3, IL-5 and GM-CSF were analysed *in vivo* both within the BM matrix and within 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.

## 2. Materials and methods

### 2.1. Animal Ethics

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

#### 2.1.1. Expression and purification of recombinant proteins

**Recombinant PoIL-3** was prepared and relative activity tested on PoBM as per [25] (relative activity results not shown).

**Recombinant PoIL-5** was prepared and relative activity tested in Mo BAF cells as per *Andrew M, 2006–7* [1] (relative activity results not shown).

**In brief:** cDNA fragments produced by PCR encoding mature porcine IL-3 and IL-5 were extracted from low-melting point agarose gels and sub-cloned 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 media and grown until the $OD_{600}$ reached 0.6–0.8. Isopropyl β-D-1-thiogalactopyranoside (IPTG), to a final concentration of 1 mM, was added and growth continued for an additional 3–4 h at 37 °C. Cells were harvested by centrifugation and solubilisation and purification of each recombinant protein was carried out as follows:

#### 2.1.2. Solubilisation and purification of pQE30 expressed rPoIL-3

Cells were resuspended in sonication buffer and lysed by mild sonication (Heat Systems sonicator, XL2020) then centrifuged 12,00$g_{max}$ 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 stained with 0.1% w/v Coomassie brilliant blue R-250 (Sigma, USA). 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 × 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.

#### 2.1.3. Solubilisation and purification of pQE30 expressed rPoIL-5

Cells were washed with 20 mM Tris–Cl, pH 8.0, 100 mM NaCl and pelleted by centrifugation. Cell pellets were resuspended in 8 mL of 20 mM Tris–Cl, pH 8.0, 100 mM NaCl, 10 mg/mL lysozyme and incubated for 30 min on ice. Lysis was achieved by the addition of Sarkosyl (1.5% final concentration) and mild sonication (Heat Systems sonicator, XL2020). The resulting lysate was clarified by centrifugation and the supernatant adjusted to 2% Triton X-100 (Sigma–Aldrich, USA) and incubated on ice for at least 2 h.

Soluble IL-5 was diluted 1:4 with 20 mM Tris–Cl, pH 8.0, 100 mM NaCl and purified using Talon™ metal affinity resin (Clontech, USA) previously equilibrated with 20 mM Tris–Cl, pH 8.0, 100 mM NaCl, 10 mM imidazole. Resin and diluted lysate were incubated for 30 min at RT on an orbital mixer. The unbound fraction was collected by centrifugation and the resin washed once with seven bed volumes of 20 mM Tris–Cl, pH 8.0, 100 mM NaCl, 0.1% Triton X-100, once with seven bed volumes of 20 mM Tris–Cl, pH 8.0, 100 mM NaCl and once with seven bed volumes of 20 mM Tris–Cl, pH 8.0, 100 mM NaCl, 5 mM imidazole prior to loading the slurry into a low pressure gravity flow column (Bio-Rad, USA) (column size 1.0 · 20 cm). Recombinant IL-5 was competitively eluted from the column by adding increasing amounts of imidazole in 20 mM Tris–Cl, pH 8.0, 100 mM NaCl (50–200 mM). Eluates were analysed for purified IL-5 by SDS–PAGE using 12% gels stained with 0.1% w/v Coomassie brilliant blue R250 (Sigma–Aldrich, USA). The protein concentration in purified samples