Immunophenotyping Pig Immune Cells by Flow Cytometry: A Powerful Tool for Pharmacology and Toxicology Studies

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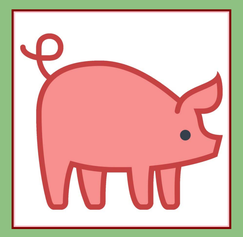
ABSTRACT

Pigs are increasingly used in biomedical research because they share many physiological and anatomical similarities to human. Although dogs and non-human primates have traditionally been used for this purpose, ethical concerns have increased the demand for alternatives (Swindle 2012). Because the pig has been a focus of vaccine research for many years, pig-specific immunology reagents exist as research tools. Pigs have some unique aspects to their immunology: in general pigs have higher circulating leukocytes, a lower ratio of CD4:CD8 T cells, and express more double positive (DP) CD4+/CD8+ cells than other species. These DP cells increase with age and after vaccine and/or infection, and are thought to have memory phenotype. Pigs also have a higher percentage of γδ T cells than other species. γδ T cells are highest in younger animals, express SLA-DR (Swine Leukocyte Antigen, MHC II), and CD8, and are thought to have cytolytic activity and memory. Unlike other species, SLA-DR is preferentially expressed on CD8+ T cells and is up- regulated on activated cells.

We describe Immunophenotyping panels that were designed to identify pig immune cells using flow cytometry. Using whole blood samples from naïve pigs, we identified pig T-cell populations including T-helper cells (CD3+CD4+CD8-), Cytotoxic T cells (CD3+CD4-CD8+), Double positive (CD3+CD4+CD8+) T cells, and γδ T cells (CD3+TCRγδ+). Consistent with the literature, we confirmed SLA-DR expression on CD8+ T cells, but not on CD4+ T cells. In addition we identified Pig Natural Killer Cells (NK, CD3-CD335+), Pig B cells (CD3-CD21+) and Pig Monocytes (CD3-CD14+CD163+). We conclude that we successfully identified pig adaptive (T and B cells), and innate (NK cells and Monocytes), immune cells using multi-parameter flow cytometry. We are currently working to optimize our method to identify Regulatory T cells (CD4+CD25+ FoxP3+) in the pig.

Immunophenotyping in the pig has many potential applications, including monitoring immune cells in pharmacology models and vaccine development. Also, in recent years the use of the pig has markedly increased as an alternative to the use of canines and non-human primates in Toxicology studies and research. With the current focus on immune-modulating reagents in drug development, monitoring pig immune cells in Toxicology studies is especially relevant.



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INTRODUCTION

Pigs are increasingly used for biomedical research, both in pharmacology and toxicology studies. Their immune system is mostly known from vaccine research and shows some commonalities, but also some differences from other large animals. The immune cells of the lymphoid and myeloid lineages of the pig are shown in Figure 1.
Flow cytometry is a powerful tool with many applications in the drug development process. Typically Complete Blood Counts (CBC) are included as standard measures on toxicology and pharmacology studies. Flow has the added advantage of providing a deeper analysis of cell phenotype and function. A CBC can provide general information on the major blood cell populations (lymphocytes, monocytes & neutrophils), but it is limited. In contrast, flow cytometry can identify Lymphocytes as T, B and NK cells, and further identify subsets of these and of all immune cells. In addition, flow can determine the ***activation*** status of these cell populations. Immunophenotyping of cells is easily done throughout a study because it requires a very small blood sample (typically 100 µL). Flow has the additional benefit of sampling thousands of cells at a time, and can be adapted to high throughput formats.

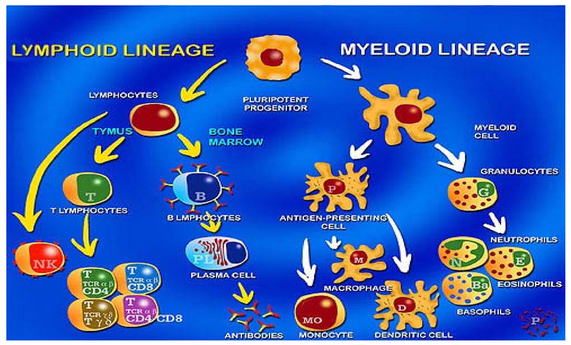

Figure 1. Cells of the Pig Immune System from Sanchez-Vizcaino Rodriguez 2015

MATERIALS AND METHODS

EDTA-treated blood samples were collected from 3 Yucatan pigs and used to optimize immunophenotyping panels. Reagents are listed below. Briefly, 100 µl of blood from each pig was incubated with antibody panels at room temp (ART). Red blood cells were lysed using BD PharmLyse™ in the dark, and samples washed twice, with BD Stain Buffer. Blood cells were brought up to a final volume of 200 µl in BD Stain Buffer prior to data acquisition on a FACSCanto™ II flow cytometer. The instrument was set to collect 20,000 mononuclear events. Data was analyzed using FlowJo (v10.2 TreeStar, Ashland, OR) software. The following Immunophenotyping panels were used to identify T-cell subsets and SLA-DR expression on those subsets:

Antigen	Fluorochome
SLA-DR	FITC
CD8b	PE
CD3ε	PerCP Cy5.5
CD4a	PE-Cy7
CD8a	AF647
TCRγδ	BV421™

A second flow panel was used to identify B cells and NK (Natural Killer) cells:

Antigen	Fluorochrome
CD335 (NKp46)	FITC
CD3ε	PerCP Cy5.5
CD21	APC

A third flow panel was used to identify Monocytes and their activation status:

Antigen	Fluorochrome
CD3ε	PerCP Cy5.5
CD163	PE
CD14	APC

RESULTS

Pig leukocytes (Figure 2) were gated and displayed by FSC (size) on the x-axis, and light scatter (SSC) on the y-axis. Neutrophils, lymphocytes and all mononuclear cells are gated based on light scatter properties (2A). From the mononuclear cell gate, Non-T cells populations were gated as follows: B cells are identified as CD3-CD21+ cells (Figure 2B), while NK cells are indentified as CD3-CD335+ cells (Figure 2C). Finally, subsets of pig monocytes are identified as CD3-CD14+ cells with varying CD163 expression (Figure 2D).

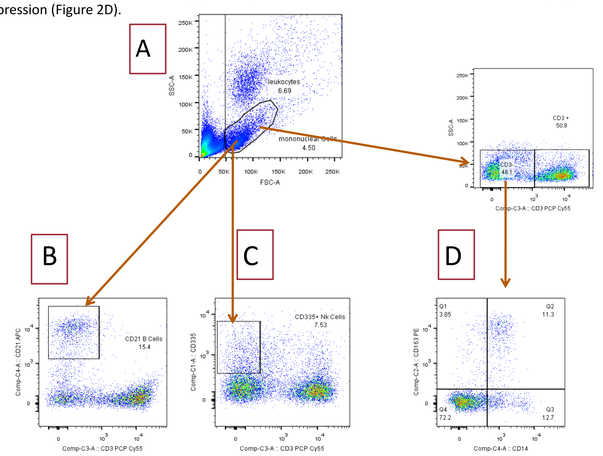

Figure 2. Porcine B cells (2B), NK cells (Natural Killer, 2C) and Monocytes (2D)

Porcine T cell populations and their activation status were gated as follows: using the mononuclear cell gate, T cells were indentified as CD3 + cells (Figure 3 A) . T cell subsets were gated from the CD3 gate: including Gamma Delta T cells (GDT, Figure 3B) and CD4+CD8- T helper cells (**TH**), CD4-CD8+ Cytoxic T cells (**TC**) and the unique porcine double positive CD4+CD8+ cells (**TDP,** 3C). SLA-DR (Activation status) expression was restricted to Cytotoxic T CD4-CD8+ (**TC**) and Double Positive (**TDP** CD4+CD8+ cells), but is not found on CD4+/CD8- T helper cells (**TH,** Figure 3D**).** The MFI (median fluorescent Intensity) of SLA-DR is shown graphically for 3 pigs (Figure 4).

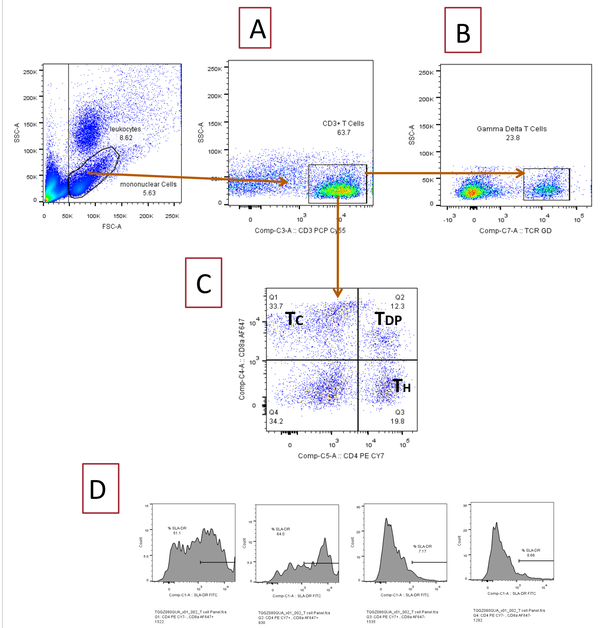

Figure 3. Porcine T cell subsets and their activation status (SLA-DR expression). Pigs have more Gamma Delta T cells than other large animals (3B), and also have more double positive CD4+CD8+ T cells (**TDP,** 3C) compared to other species. Figure 3D. SLA-DR is found only on CD8+ cells (**TC , TDP,** Q1, Q2 Figure 3C).

RESULTS (cont.)

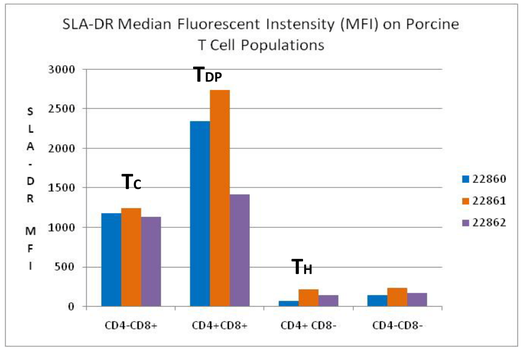

Figure 4. Summary SLA-DR MFI on T cell populations: SLA-DR is only expressed on CD8+ populations: **TC** and **TDP.**

CONCLUSIONS

•We successfully identified pig adaptive immune cells (T and B cells) and innate immune cells (Monocytes, NK cells) using flow cytometry Immunophenotyping panels.

• We demonstrated our ability to identify the following T-cell subsets: T helper (**TH**), cells (CD4+CD8-), Cytotoxic T (**TC**) cells (CD4-CD8+), double positive (**TDP**) T cells (CD4+CD8+), and Gamma Delta T cells (GDT, TCRγδ) T cells.

•In addition, we confirmed the expression of SLA-DR on Cytotoxic (CD4-CD8+) T cells and Double positive (DP) CD4+CD8+ cells. SLA-DR can be used as an activation marker on these cells.

•We are currently working to optimize our method to identify regulatory T cells (**T**regs) in the pig (CD4+/CD25+/FoxP+), and intracellular Interferon-gamma (INFγ), in Effector T cells.

•Monitoring immune cells in the pig has important applications in research, eg non-clinical safety assessment of investigational and/or immune-modulating therapies, transplant, and vaccine development.

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2019 is the year of the pig. If you were born in the year of the Pig, your characteristics include **diligence**, **compassion** and **generosity**.