Protocol for isolation of 8 subpopulation of porcine peripheral blood mononuclear cells (PBMCs) (from Dr. Kristen Byrne USDA)	PI	Dr. Chris Tuggle
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Mononuclear cell isolation

Up to 120ml of blood was drawn into BD Vacutainer mononuclear Cell Preparation Tubes – Sodium Citrate (CPT^{TM}, Becton Dickson) or into 2 - 60cc syringes containing 6ml of acid citrate dextrose (ACD). CPTs were centrifugated for 25 minutes at 1800 relative centrifugal force (RCF) with no brake, after which the PBMC containing interface was collected following the manufacturer's instructions. Whole blood collected in ACD syringes was diluted with twice the volume of Hanks Balanced Salt Solution (HBSS, ThermoFisher and PBMCs were isolated from the interface following density gradient centrifugation with Histopaque 1.077. Isolated PBMCs were washed once in HBSS and remaining RBCs were lysed by incubating cells for 3 mins in 20 ml ACK lysing buffer (ThermoFisher). The cells were washed twice in HBSS, filtered between washes using a 40µm cell strainer (Fisher), and resuspended in supplemented HBSS (HBSS with 0.5% BSA (Sigma-Aldrich) and 2 mM ethylenediaminetetraacetic acid (EDTA; ThermoFisher)). Viable cells were enumerated using the Count and Viability Assay Kit on the MUSE® detection system (Merck Millipore).

MACS isolation of CD3-positive and CD3-negative cells

PBMCs were separated in to CD3-positive ($CD3^+$) and CD3-negative ($CD3^-$) fractions by magnetic cell activation sorting (MACS®). Briefly, PBMCs were incubated with biotin labeled anti-porcine CD3 (PPT3, Washington State University Monoclonal Antibody Center) for 15 minutes at 4ºC with continuous mixing. The incubated cells were washed with supplemented HBSS and then incubated with anti-biotin microbeads (Miltenyi Biotec) according to manufactures recommendations. Labeled cells were placed on LS columns (Miltenyi Biotec) and separated in to $CD3^+$ (CD3 microbead labeled cells retained on column) and $CD3^-$ fractions (flow through containing non-CD3 labeled cells). Cells were enumerated as described above.

Separation of CD3-positive and CD3-negative subpopulation by FACS

Sorting of specific cell populations was based on phenotyping previously described (Gerner et al., 2009). $CD3^+$ and $CD3^-$ fractions were each further separated into 4 subpopulations based on extracellular markers by FACS to yield a total of 8 distinct peripheral blood monocellular cell populations. In brief, isolated subpopulations from the CD3+ fraction include SWC6gdT (CD3e+SWC6+), CD4T (CD3e+SWC6-CD4+CD8a-), CD4CD8T (CD3e+SWC6-CD4+CD8a+), CD8T (CD3e+SWC6-CD4-CD8a+) and from the CD3- fraction: Myeloid (CD3e-CD172a+CD8a-), NK cells (CD3e-CD172a-CD8a+), CD21pB (CD3e-CD172a-CD8a-CD21+), CD21nB (CD3e-CD172a-CD8a-CD21-). Additionally, each fraction was confirmed $CD3^+$ or $CD3^-$ by labeling with an anti-mouse IgG1 PECy7 antibody to bind to the anti-porcine CD3 antibody used for the MACS sort. Only cells that were PE-Cy7 positive were further isolated into the $CD3^+$ subpopulation and only cells that were PE-Cy7 negative were further isolated into the

$CD3^-$ subpopulation. Cells of interested were FACS sorted into supplemented HBSS using the BD FACSAria II with a 70um nozzle. After the respective sorts, each of the populations were pelleted and enumerated on the MUSE detection system® as described above. The purity of the sorted cells was confirmed to be greater than 98% percent positive for the respective populations using the BD FACSymphony™ flow cytometer. All cells were stained and sorted within 10 hours after blood collection and kept on ice between processing steps.