RECENT ADVANCES IN VETERINARY IMMUNOLOGY CONCEPTS AND METHODOLOGY

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RECENT ADVANCES IN VETERINARY IMMUNOLOGY CONCEPTS AND METHODOLOGY

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Table of Contents

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Novel Engraftment and T Cell Differentiation of Human Hematopoietic Cells in *ART*$^{-/-}$ *IL2RG*$^{-/Y}$ SCID Pigs

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Pigs with severe combined immunodeficiency (SCID) are an emerging biomedical animal model. Swine are anatomically and physiologically more similar to humans than mice, making them an invaluable tool for preclinical regenerative medicine and cancer research. One essential step in further developing this model is the immunological humanization of SCID pigs. In this work we have generated T$^-$ B$^-$ NK$^-$ SCID pigs through site directed CRISPR/Cas9 mutagenesis of *IL2RG* within a naturally occurring *DCLRE1C (ARTEMIS)* $^{-/-}$ genetic background. We confirmed *ART*$^{-/-}$ *IL2RG*$^{-/Y}$ pigs lacked T, B, and NK cells in both peripheral blood and lymphoid tissues. Additionally, we successfully performed a bone marrow transplant on one *ART*$^{-/-}$ *IL2RG*$^{-/Y}$ male SCID pig with bone marrow from a complete swine leukocyte antigen (SLA) matched donor without conditioning to reconstitute porcine T and NK cells. Next, we performed *in utero* injections of cultured human CD34$^+$ selected cord blood cells into the fetal *ART*$^{-/-}$ *IL2RG*$^{-/Y}$ SCID pigs. At birth, human CD45$^+$ CD3ε$^+$ cells were detected in cord and peripheral blood of *in utero* injected SCID piglets. Human leukocytes were also detected within the bone marrow, spleen, liver, thymus, and mesenteric lymph nodes of these animals. Taken together, we describe critical steps forwards the development of an immunologically humanized SCID pig model.

Keywords: severe combined immunodeficiency, SCID, swine, biomedical model, humanization

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INTRODUCTION

Animals with severe combined immunodeficiency (SCID) are invaluable to biomedical researchers because they are permissive to engraftment of human cells, allowing one to study developmental processes within an *in vivo* environment. In 2012, we discovered the first naturally occurring SCID pigs (1, 2), caused by mutations within the *ARTEMIS* gene, resulting in a T^- B^- NK^+ SCID phenotype (3, 4). Since then, pigs with mutations in *RAG1* (5, 6), *RAG2* (7, 8), *IL2RG* (9–11), and *RAG2/IL2RG* (12) have also been generated through different mutagenic approaches. Within the past few years, such SCID pigs are now being utilized by cancer (13), disease model (12), and stem cell therapy (7) researchers. Biocontainment facilities (14), isolators (12), and Cesarean section (15) techniques have allowed survival of animals, enabling longer term studies. An important step in further developing the SCID pig model is to immunologically humanize these animals through the introduction of human $CD34^+$ hematopoietic stem cells. Similarities between human and porcine immune genes (16) suggest that human immune development would be supported *in vivo* within the pig (17). Development of such a model could provide researchers with a larger humanized animal for use in cancer (13, 17), HIV, and vaccine development research.

The first SCID mouse, described in 1983 (18), is capable of being humanized by either injection of human peripheral blood leukocytes (19) or by implantation of human fetal liver, thymus, and/or lymph node tissue (20). Reconstitution of human immune cell subsets in SCID mice often requires addition of human cytokine genes, humanization of resident mouse immune genes, or administration of developmental cytokines to the mice (21–24). However, limitations of mouse models include differences in size, drug metabolism, and disease pathology compared to humans (25, 26). Thus, one major goal of the SCID pig community is to create an immunologically humanized SCID pig, which would provide a valuable and unique tool for preclinical research, in a more anatomically and/or physiologically relevant animal model.

The most commonly used strain for humanization is the non-obese diabetic (NOD)-SCID- *IL2RG* (NSG) mouse (27). The NOD mouse background contains polymorphisms within the *SIRPA* (signal regulatory protein alpha) gene, allowing it to bind to human CD47 to transduce a "don't eat me" signal in mouse myeloid cells to inhibit phagocytosis (28–30). We have demonstrated that porcine SIRPA also binds to human CD47 to inhibit phagocytosis of human cells (31), indicating pigs may be permissive to human xenografts, similar to NOD mice. In addition to the SIRPA polymorphism, NSG mice also have a T^- B^- NK^- cellular phenotype. This cellular phenotype can be generated through mutagenesis of genes required for VDJ recombination (i.e., *ARTEMIS or RAG1/2)*, in addition to *IL2RG*. Previous reports show that mouse NK cells negatively impact human cell engraftment in SCID mice (27). NK cells in $ART^{-/-}$ SCID pigs are functional *in vitro* (4), and thus we anticipated swine NK cells could also negatively impact human cell engraftment. To deplete NK cells in our current $ART^{-/-}$ SCID pig model, we mutagenized *IL2RG* in an $ART^{-/-}$ mutant cell line. The resulting pigs are similar to NSG mice in cellular phenotype and are expected to be similar in SIRPA/CD47 dependent phagocytic tolerance (31).

Here we describe the generation of $ART^{-/-}$ $IL2RG^{-/Y}$ SCID pigs derived by site-directed CRISPR/Cas9 mutagenesis of *IL2RG* in an $ART^{-/-}$ fetal fibroblast cell line. Modified $ART^{-/-}$ $IL2RG^{-/Y}$ embryos, derived from somatic cell nuclear transfer, were implanted in gilts via surgical embryo transfer. Piglets were born at full term and confirmed to have the expected T^- B^- NK^- cellular phenotype based on flow cytometry and immunohistochemical (IHC) analysis of blood and lymphoid organs. We next determined if these double mutant pigs could be humanized via the introduction of human $CD34^+$ cord blood stem cells. Gestational day 41 $ART^{-/-}$ $IL2RG^{-/Y}$ fetuses were injected with human $CD34^+$ cells within the intraperitoneal space by ultrasound guidance and piglets were delivered via Cesarean section at gestational day 119. We probed for human myeloid, lymphoid, and erythroid cells in peripheral blood and lymphoid organs in piglets for up to 7 days of age. We found evidence of human $CD45^+$ cell engraftment in several tissues in the $ART^{-/-}$ $IL2RG^{-/-}$ pigs. Specifically, we detected $CD3\varepsilon^+$ T and $Pax5^+$ B lymphocytes in blood and lymphoid organs. Taken together, we successfully established the first steps toward the generation of a humanized SCID pig model.

MATERIALS AND METHODS

Study Design

Our study was designed to develop a T^- B^- NK^- SCID pig model by generating $ART^{-/-}$ $IL2RG^{-/Y}$ pigs by CRISPR/Cas9 site directed mutagenesis of our existing $ART^{-/-}$ pig line, which was discovered in 2012 (1, 2, 32). We aimed to generate these pigs as a large animal biomedical model for human cell and tissue xenotransplantation. Once we successfully created the $ART^{-/-}$ $IL2RG^{-/Y}$ fibroblast cell line, we performed a total of eight embryo transfer surgeries to generate piglets. Of these transfers, five females became pregnant, and a total of three litters were born; one of which we performed *in utero* injections of human $CD34^+$ cells. Once piglets were born, we confirmed their T^- B^- NK^- phenotype. We performed a pig to pig bone marrow transplant on one $ART^{-/-}$ $IL2RG^{-/Y}$ boar, which would allow us to eventually collect semen for future breeding and use of this genetic line. We performed *in utero* injections of human cord blood selected $CD34^+$ cells on $ART^{-/-}$ $IL2RG^{-/Y}$ fetuses from one pregnant female. Three piglets were born from this litter, with two piglets showing evidence of human immune cell engraftment. The low number of animals in this study are a result of small litter sizes of cloned piglets, as well as low pregnancy rates of embryo transfer procedures.

Ethics Statement

All animal protocols were approved by Iowa State University's Institutional Animal Care and Use Committee. All animals were utilized in accordance with the Animal Welfare Act and the Guide for the Care and Use of Laboratory Animals. All human sample collection protocols were approved by Iowa State University's Institutional Review Board.

Establishment of Porcine Fetal Fibroblast Cell Lines With *ART*$^{-/-}$ Genetic Background

Our population of SCID pigs has two natural mutations in two separate *ARTEMIS* alleles, termed *ART12* and *ART16*. The *ART12* allele contains a nonsense mutation in exon 10, while the *ART16* allele contains a splice site mutation in intron 8(3). Frozen semen from a bone marrow transplanted (BMT) rescued *ART12/16* (33) boar was utilized to artificially inseminate two *ART*$^{+/-}$ carrier sows. The sows were sacrificed at day 35 of gestation and fetuses were collected in a sterile manner to obtain fetal fibroblasts (pFF), as described previously (34). Briefly, minced tissue from each fetus was digested in 20 mL of digestion media (Dulbecco-modified Eagle medium [DMEM] containing L-glutamine and 1 g/L D-glucose [Cellgro] supplemented with 200 units/mL collagenase and 25 Kunitz units/mL DNaseI) for 5 h at 38.5°C. After digestion, pFF cells were washed in sterile PBS and cultured in DMEM supplemented with 15% fetal bovine serum (FBS) and 40 μg/mL gentamicin (Sigma Aldrich). Upon reaching 100% confluence, the pFF cells were trypsinized, frozen in FBS with 10% dimethyl sulfoxide (DMSO) and stored long-term in liquid nitrogen. Simultaneously, cellular DNA was sent for swine leukocyte antigen (SLA) typing, as described in Powell et al. (33). *SRY* (sex determining region Y) and *ARTEMIS* primers (**Supplemental Table 1**) were utilized to identify pFF sex and *ARTEMIS* genotype (3).

CRISPR/Cas Plasmid and sgRNA Product

Guide RNAs targeting exon 5 of *IL2RG* were designed utilizing software available from Zhang Lab (https://zlab.bio/guide-design-resources). The sequence of the designed sgRNA was:

5′-GGCCACTATCTATTCTCTGA**AGG**-3′; the bold font identifies the PAM site. The sgRNA oligos were annealed and ligated into the human codon-optimized SpCas9 expression plasmid (pX330; Addgene plasmid # 42230), as described previously (35). We only transfected male *ART12/12* cell lines (herein referred to as *ART*$^{-/-}$).

Identification of Off-Target Sequences

To identify putative off-target sequences for the CRISPR/Cas9 mutagenesis used in *ART*$^{-/-}$ *IL2RG*$^{-/Y}$ piglets, bioinformatics tools (http://www.rgenome.net/cas-offinder/) were used. Ten potential off-target sites were identified and primers for the off-target positions were designed. Genomic DNA samples obtained from ear notches of *ART*$^{-/-}$ *IL2RG*$^{-/Y}$ pigs were used as templates in PCR amplification of potential off-target regions. Primers and gene information for this purpose are in **Supplemental Table 2**. DNA sequencing results revealed no mutations had occurred in any of the potential off-target positions.

Establishment of Transfected Clonal Colonies and Identification of IL2RG Mutagenesis

Male *ART*$^{-/-}$ pFFs were used for cell transfection, as described in Whitworth et al. (36). Briefly, pFFs were cultured in 75 cm^2 flasks to reach 90% confluency, trypsinized, resuspended at a concentration of 1.0 × 10^6 cells/mL in Electroporation Buffer medium (25% Opti-MEM [Gibco, 319850070] and 75% cytosalts [120 mM KCl, 0.15 mM CaCl2, 10 mM K2HPO4; pH 7.6, 5 mM MgCl2]), and prepared for transfection. A mixture of 1 μg sgRNA ligated vector and 200 μL of cell suspension in electroporation buffer was then transferred into 2 mm gap cuvettes (Fisher Sci, 9104-6050) and exposed to three- 1 ms square-wave pulses at 250 V, using the BTX Electro Cell Manipulator (Harvard Apparatus). Transfected cells were then diluted, 80–200 cells were plated in 100 mm culture dishes to obtain distinct clonal cell colonies and maintained at 38.5°C in 5% CO_2. After 10–12 days in culture, cell colonies were delineated using cloning cylinders and picked for clonal colony propagation and DNA sequencing. Primers (**Supplemental Table 1**) flanking the *IL2RG* exon 5 target region were utilized to test clonal colonies and PCR products thus obtained were purified by ExoSAP-IT PCR product Cleanup kit (Affymetrix Inc, Thermo Fisher Scientific). Mutant PCR products were cloned into PCR2.1 vectors (Life Technologies) and transformed into *E.coli* DH5-α maximum competent cells (Life Technologies). Ten colonies were chosen and DNA from these samples were sent to the Iowa State University DNA Facility for sequencing. Sequences were aligned by Bio-Edit software (Ibis Biosciences, Carlsbad, CA, USA) for comparison with wild-type alleles, to identify cell lines with the appropriate mutations in *IL2RG* on the *ART*$^{-/-}$ background.

Double Mutant Embryo Production and Surgical Embryo Transfer

Purchased pig oocytes (DeSoto Biosciences, Inc.) or those derived from aspirating ovaries collected from a local abattoir were utilized for *in vitro* maturation (IVM), as previously described (37, 38). Briefly, oocytes were matured *in vitro* with maturation medium (TCM-199 with 2.9 mM HEPES, 5 μg/mL insulin, 10 ng/mL epidermal growth factor, 0.5 μg/mL follicle stimulating hormone, 0.5 μg/mL luteinizing hormone, 0.91 mM pyruvate, 0.5 mM cysteine, 10% porcine follicular fluid, and 25 ng/mL gentamicin) (Sigma Aldrich), and transferred into fresh medium after 22 h. Following IVM, cumulus-oocyte-complexes (COC) were vortexed for 3 min in 0.1% hyaluronidase in TCM199 with HEPES to obtain denuded oocytes. Metaphase II (MII) oocytes, identified by the presence of an extruded polar body, were placed in manipulation medium (TCM199 with HEPES supplemented with 7 μg/mL cytochalasin B) and used thereafter for somatic cell nuclear transfer (SCNT). The extruded polar body, along with a portion of the adjacent cytoplasm, presumably containing the M II plate, were removed, and a donor nucleus of the appropriate *ART*$^{-/-}$ *IL2RG*$^{-/Y}$ genotype was placed in the perivitelline space by using a thin glass capillary. The reconstructed embryos were then placed in a fusion medium (0.3 M mannitol, 0.1 mM $CaCl_2$, 0.1 mM $MgCl_2$, and 0.5 mM HEPES) (Sigma Aldrich) and exposed to two DC pulses (1-s interval) at 1.2 kV/cm for 30 μs using a BTX Electro Cell Manipulator (Harvard Apparatus). After fusion, these embryos were activated in embryo activation medium (10 μg/mL cytochalasin B) for 4 h. After chemical activation, cloned zygotes were treated with 500 mM Scriptaid for 12–14 h and then cultured in porcine zygote medium 3 (PZM-3) (recipe per 100 mL: 0.6312 g NaCl, 0.2106 g $NaHCO_3$, 0.0746 g KCl, 0.0048 g KH_2PO_4, 0.0022 g Na-pyruvate,

0.0146 g L-glutamate, 0.0546 g hypotaurine, 0.0617 g Ca-lactate, 0.001 g gentamicin, 2.0 mL BME essential amino acid, 1.0 mL MEM non-essential amino acid, and 0.3 g BSA). until embryo transfer. Embryos produced over 2 days were then surgically transferred into the ampullary-isthmic junction of the oviduct of the surrogate on day 1–2 post estrus. Pregnancies were confirmed by ultrasound ∼30 days following embryo transfer.

Cesarean Section and Rearing of *ART*$^{-/-}$ *IL2RG*$^{-/Y}$ SCID Pigs

At gestational day 119, pregnant gilts underwent Cesarean sections. We chose gestational day 119 instead of 114 (normal gestation) because piglets derived from somatic cell nuclear transfer typically requiring longer gestational period. Initial anesthesia was induced with either a lumbar epidural of propofol (0.83–1.66 mg/kg) (Zoetis) or intravenous injection of Ketamine (1–2 mg/kg) (Akorn) and Xylazine (1–2 mg/kg) (Akorn), and anesthesia was maintained on oxygen and isoflurane (Phoenix). An abdominal incision was made to expose and remove the uterus. After removal, the uterus was immediately rinsed in chlorohexidine and then surgically opened to remove the piglets. All piglets had their cords clamped before being immediately placed into sterile polystyrene boxes and delivered into biocontainment facilities (14). All piglets were fed ∼250 mL of pasteurized porcine colostrum within the first 24 h of life. A total of eight *Art*$^{-/-}$ *IL2RG*$^{-/Y}$ SCID pigs were created and assessed within this study (animal IDs: 6401, 6402, 6403, 6701, 6702, 6901, 6902, and 6903). Piglets derived from the gilt that underwent laparotomy procedures for human stem cell injection (see below) did not receive colostrum. After birth, DNA was isolated from ear notch tissues and subjected to genotyping for *ART* and *IL2RG* status using primers and protocols described in **Supplemental Table 1**.

Flow Cytometry Staining for *ART*$^{-/-}$ *IL2RG*$^{-/Y}$ Pig Characterization and Bone Marrow Engraftment Monitoring

Whole blood or cord blood from newborn *ART*$^{-/-}$ *IL2RG*$^{-/Y}$ piglets was collected into an EDTA blood collection tube. Whole blood was stained for porcine CD3ε, CD8α, and CD172α to assess the presence of porcine T and NK cells. Cells were additionally stained for porcine CD21 to assess the presence of B cells. Blood from the *ART*$^{-/-}$ *IL2RG*$^{-/Y}$ BMT was also stained for CD79α to assess B cell reconstitution. Additional information about the antibodies used can be found in **Table 1**.

Blood was collected from the bone marrow transplanted boar approximately once a month after the BMT and subjected to either a complete blood count (CBC) at Iowa State University's Veterinary Diagnostic lab or by flow cytometry analysis using the above listed antibodies. All samples were run on a custom BD LSR II (BD Biosciences) and data were analyzed using Flowjo (Tree Star).

Pig Bone Marrow Isolation and Bone Marrow Transplantation

A complete SLA-matched female sow of ∼4 years of age was euthanized and used as a bone marrow donor for one *ART*$^{-/-}$ *IL2RG*$^{-/Y}$ piglet. Briefly, sternum and ribs collected from the animal were dipped in 70% ethanol after collection. A sterilized Dremel tool was used to make holes halfway through the bone to expose bone marrow. Sterilized Spratt Brun bone curettes were used to scrape marrow from the bone. HBSS (without phenol red) was used to flush the bone marrow to collect cells; any other loosened marrow was also placed in HBSS. After marrow isolation, the suspension was washed in HBSS. The cells were resuspended in ACK (ammonium chloride potassium) lysing solution (Lonza) for 10 min at room temperature. The suspension was washed in HBSS and filtered through a 70 μm cell strainer. A total of 2.27×10^8 million unfractionated cells were isolated and resuspended in ∼3 mL of HBSS for infusion.

To infuse bone marrow-derived cells into the *ART*$^{-/-}$ *IL2RG*$^{-/Y}$ recipient, the 5-day old SCID piglet was anesthetized with and maintained on isoflurane gas during the procedure. A catheter was placed in an ear vein and the cell suspension slowly infused. After infusion, personnel monitored the piglet until fully recovered.

Immunohistochemistry of Human and Porcine Immune Markers

Lymphoid organs were collected into 3.7% formaldehyde in 1X PBS for 24 hours. Tissues were then moved to 70% ethanol until processing. IHC staining for T and B lymphocyte markers (for *ART*$^{-/-}$ *IL2RG*$^{-/Y}$ immune characterization) was performed in paraffin-embedded tissue thin sections at the Kansas State Veterinary Diagnostic Laboratory (KSVDL). Briefly, deparaffinized slide-mounted thin sections were pre-treated for 5 min with a peroxide block, followed by incubation with primary antibody. A mouse monoclonal anti-CD3ε (clone LN10, Leica Biosystems) was used to stain for pig T cells, while a mouse monoclonal anti-CD79α (clone HM57, Abcam) was used to stain for B cells. Primary antibodies were incubated with PowerVision Poly-HRP anti-mouse IgG at room temperature for 25 min with DAB chromagen, and then counterstained with hematoxylin.

Lymphoid tissues from SCID pigs engrafted with human cells were treated similarly as above. Staining was performed at Michigan State University's Department of Pathobiology and Diagnostic Investigation. Tissues were stained for anti-CD3ε (Dako #A0452) and anti-Pax5 (Ventana clone 24) to assess for the presence of human T and B cells, respectively. Briefly, CD3ε staining was performed by antigen retrieval with standard ER1 retrieval for 20 min, and tissues were analyzed on a BondMax (Leica Biosystems) for the detection of DAB chromagen. For Pax5 staining, antigen retrieval was performed with standard CC1 retrieval for 64 min, and tissues were analyzed on a Discovery Ultra AP (Roche) for ultrared detection.

Human Hematopoietic Stem Cell Isolation From Cord Blood

Human cord blood was collected at the Mary Greeley Medical Center in Ames, Iowa, into 50 mL conical tubes containing 8 mL of anticoagulant citrate dextrose solution (38 mM citric acid, 85.25 mM sodium citrate, 136 mM dextrose). Mononuclear cells (MNCs) were isolated from cord blood by diluting blood 1:2 in HBSS and then layering over Ficoll-Paque (GE

TABLE 1 | Flow cytometry antibodies used for assessing pig and human cell subsets.

Purpose	Marker	Clone	Fluorophore	Company
Porcine immunophenotyping	pCD3ε	BB23-8E6-8C8	PE-Cy7	BD Bioscience
	pCD8α	76-2-11	PE	BD Bioscience
	pCD172α	72-22-15A	FITC	BioRad
	pCD16	G7	X	BioRad
	anti ms IgG1	RMG-1-1	BV421	BioLegend
	pCD21	BB6-11C9.6	Alexa Fluor 647	Southern Biotech
	hCD79α	HM47	PE	Invitrogen
	pCD45	LS-C127705	FITC	LSBio
Human immunophenotyping	hCD45-biotin	HI30	x	eBioscience
	SA-PeCy5	x	PE-Cy5	BD Bioscience
	hCD20	2H7	PE	BioLegend
	hCD3ε	UCHT1	X	BioLegend
	Anti ms IgG1/IgG2a		BV421	BD Bioscience
	hCD56	B159	PE-Cy7	BD Bioscience
	hCD33	WM53	PE	BioLegend
	hCD11b	ICRF44	PE-Cy7	BioLegend
	hCD4α	RPA-T4	PE-Cy7	BioLegend
	hCD8α	HIT8a	PE	BioLegend
	hCD15	H198	PE	Invitrogen
	hCD34	AC136	APC	Miltenyi
	hCD47	B6H12	x	eBioscience
	Anti ms IgG1	Cat #1072-09	PE	Southern Biotech

Healthcare). Buffy coats were collected and washed in HBSS. Prior to stem cell isolation, MNCs were resuspended in an isolation HBSS (iHBSS) consisting of 0.5% FBS and 2 mM EDTA in HBSS.

To isolate human $CD34^+$ cells, we used a CD34 MicroBead kit from Miltenyi Biotech. Briefly, MNCs were incubated in iHBSS with CD34 microbeads and FcR blocking reagent for 30 min at 4°C on a rocker. After the incubation period, cells were washed in iHBSS and then passed through a LS column in a Miltenyi magnet (Miltenyi Biotech) to capture human $CD34^+$ cells. The column was then removed from the magnet and cells were flushed, washed once more in HBSS, and then frozen in 10% DMSO and 90% FBS at −80°C until use.

Human HSC Thawing, Culturing, and Preparation for Fetal Injection

Human $CD34^+$ cells were thawed by diluting into complete RPMI media (10% FBS, 2 mM glutamine, 50 μg/mL gentamicin, and 10 mM HEPES) (Gibco). Cells were cultured in Miltenyi StemMACS media containing Thrombopoietin (TPO), Stem Cell Factor (SCF), and Flt3-Ligand (FLT-3L), at starting concentrations of 38-42 $\times$ 10^3 cells/mL. Cells were left in culture for 7 days and expanded 184.5-fold. Cells were prepared for injection by washing three times in phosphate buffered saline and resuspended at a concentration of 26.6 $\times$ 10^6 cells/mL. A total of 150 μL of cell suspension with either 2 or 4 $\times$ 10^6 cells were administered to the fetuses in 0.9% saline.

Laparotomy Procedure for Fetal Injection of Human Stem Cells

Laparotomy procedures were performed as previously described in Boettcher et al. (39). Briefly, the gilt was started on 15 mg of Matrix (Merck Animal Health) orally 1 day before surgery and maintained on Matrix until gestational day 118. Immediately prior to sedation, the gilt was given 0.01 mg/kg Glycopyrrolate (West-Ward Pharmaceuticals) by intramuscular injection and then anesthetized with 2 mg/kg Xylazine (Akorn) and 5 mg/kg Telazol (Zoetis) by intramuscular injection. The gilt was then placed in dorsal recumbency, intubated, and started on isoflurane (Phoenix, St. Joseph, MO) (3–5%) and oxygen (2.5 L/min). Lactated Ringer's solution (Hospira) was given in an ear catheter at a constant rate infusion within 10 min of the first incision. The abdominal area was scrubbed with chlorohexidine and the surgical field was covered with sterile drapes and Ioban drapes.

A ventral midline incision was made from the caudal most nipple extending to the caudal aspect of the umbilicus through the *linea alba* into the peritoneal cavity. The left uterine horn was exposed and visualized with a ZONARE ultrasound with an L14-5sp intraoperative linear array transducer (10 MHz). Two live and one nonviable fetus were visualized in the left horn; one was injected with 4 million cultured human stem cells in a volume of 0.15 mL within the intraperitoneal space. The left horn was placed back into the abdominal cavity and the right horn was exposed and visualized, and three fetuses were observed. One fetus was injected with 2 million and another fetus with 4 million human stem cells within the intraperitoneal space.

In total, three out of the five viable fetuses were injected. The abdominal cavity was lavaged with 500 mL of Lactated Ringer's solution and sutured closed. The gilt then received 0.18 mg/kg Buprenophine- Sustained Release (ZooPharm) subcutaneously, 5 mg/kg Ceftiofur Crystalline Free Acid (Excede) (Zoetis), and 0.3 mg/kg of meloxicam (Norbrook) and monitored by personnel until recovered from anesthesia. The gilt recovered from the laparotomy surgery with no issues and underwent a Cesarean section at day 119 of gestation, as described above.

At the time of Cesarean section, we observed two live piglets within the left horn and one live piglet in the right horn of the uterus. During the laparotomy, we observed two viable fetuses in the left horn, of which one was injected, and their relative position was recorded. After Cesarean delivery, by position in the uterus we were able to identify the two developed piglets in the left horn as injected (6901) and non-injected (6902). The right horn originally had three viable fetuses but only one piglet survived to term. After flow cytometric analysis, we confirmed that the one piglet on the right horn (6903) had been injected based on presence of human cells.

Isolation of MNC From Tissue for Flow Cytometric Analysis

To collect lymphoid MNCs, tissue was collected and placed into Hanks Balanced Salt Solution (HBSS) with 10 μg/mL gentamicin. Tissues were minced in a digestion solution of HBSS with 300 mg/mL of collagenase, 3% FBS, and 2 mM HEPES (referred to as d-HBSS). The tissue incubated for 1 h at 37°C with vortexing every 15 min, and then strained over a 70 μm cell strainer. Cells were washed once and counted with a BD counting kit, and then were stained as described below.

Flow Cytometry Analysis for Human Cells in *ART*$^{-/-}$ *IL2RG*$^{-/Y}$ Pig Blood

Peripheral or cord blood was collected from piglets into EDTA blood containers (BD Biosciences). Whole blood or MNC from tissues were stained with antibodies against human and pig cell subset markers (**Table 1**). Each staining step was incubated for 15 min at 4°C and washed with 1X PBS with 0.1% sodium azide. Red blood cells were lysed with ammonium chloride lysing solution. Cells were fixed in 2% formaldehyde in 1X PBS and data were acquired on a FACs Canto II flow cytometer (BD Biosciences) and analyzed using FlowJo (Treestar).

RESULTS

Generation of *ART*$^{-/-}$ *IL2RG*$^{-/Y}$ SCID Pigs by Site Directed CRISPR/Cas9 Mutagenesis of *ART*$^{-/-}$ Fetal Fibroblasts

We previously described the discovery of naturally occurring *ART*$^{-/-}$ SCID pigs (3), which we have been able to raise and breed (14, 33) for research purposes. We started with this genetic background for *IL2RG* site-directed mutagenesis. One goal of producing *ART*$^{-/-}$ *IL2RG*$^{-/Y}$ SCID pigs is to generate a breeding colony, such that *IL2RG* knockout piglets can be derived through natural birth rather than cloning procedures. In our breeding protocol, this would require bone marrow transplantation of a SCID boar, so he could be raised to sexual maturity and bred to *ART*$^{-/+}$ carrier females. Mutagenizing our existing *ART*$^{-/-}$ line would facilitate producing SCID pigs with matching SLA to carrier animals within our colony. Therefore, we decided to utilize cloned *ART*$^{-/-}$ fibroblasts for *IL2RG* mutagenesis for somatic cell nuclear transfer (SCNT) to generate pigs with these desired genetics. **Figure 1** shows a schematic for the process of generating *ART*$^{-/-}$ *IL2RG*$^{-/Y}$ piglets.

ART$^{-/-}$ pFFs for gene editing were derived from an *ART*$^{-/-}$ male by *ART*$^{-/+}$ female mating (see Materials and Methods for information on *ARTEMIS* genotypes) (**Figure 1A**). Gestational day 35 fibroblasts were collected and underwent SLA typing and were genotyped for *ART* status. Genotyping revealed that two male and two female cell lines were *ART*$^{-/-}$ mutants, while the other six male and three female cell lines were *ART*$^{-/+}$ carriers (**Supplemental Table 3A**).

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To mutagenize *IL2RG*, a single guide RNA (sgRNA) was designed to target exon 5 of *IL2RG* (**Figure 1B.**) We selected a male *ART*$^{-/-}$ cell line (7707-FB1) that had a complete SLA-match (haplotype 26.6/68.19a) to a male carrier fibroblast line (7709-FB6) to transfect with a vector to express sgRNA and Cas9 protein (**Supplemental Tables 3A,B**). After transfection, a total of 202 individual clonal colonies from the 7707-FB1 cell line were screened using PCR and Sanger sequencing. Five (2.5%) clonal colonies were confirmed to be *IL2RG*$^{-/Y}$ (hemizygous) (**Supplemental Table 4**), with one cell line (7707-FB1-U23) carrying a 120 bp deletion in intron 4 and exon 5 of the *IL2RG* locus, which was expected to cause a frameshift leading to an premature stop codon (**Figure 1B**). A total of 920 *IL2RG*$^{-/Y}$ *ART*$^{-/-}$ (7707-FB1-U23) and 512 non-modified (7709-FB6) SCNT derived embryos were transferred surgically into seven recipient gilts. Both cell lines shared the same SLA haplotype of 26.6/68.19a. Among those transferred, four gilts were confirmed pregnant and two carried their piglets to full term and produced five live male piglets via Cesarean section that were reared in biocontainment facilities (14) (**Supplemental Table 5**). All five piglets were confirmed to have the 120 bp loss in *IL2RG* and the established mutation in exon 10 of *ARTEMIS* (3) *(ART12* allele information found in Materials and Methods). None of the live born piglets were derived from non-modified, carrier embryos (7709-FB6) that would have been used as a bone marrow donor.

ART$^{-/-}$ *IL2RG*$^{-/Y}$ SCID Pigs Lack T, B, and NK Cells in Blood and Lymphoid Organs

Once *ART*$^{-/-}$ *IL2RG*$^{-/Y}$ piglets were delivered, blood was collected and analyzed by flow cytometry to confirm the expected T$^-$ B$^-$ NK$^-$ cellular phenotype of these animals. A total of three *ART*$^{-/-}$ *IL2RG*$^{-/Y}$ pigs were born (6401, 6402, 6403) in this litter and were analyzed with a wildtype and *ART*$^{-/-}$ pigs. We assessed forward and side scatter (FSC/SSC) and stained cells for CD172α, CD16, CD3ε, and CD8α to assess for myeloid, T, and B cells (**Figure 2**). FSC/SSC plots show that the lymphocyte population

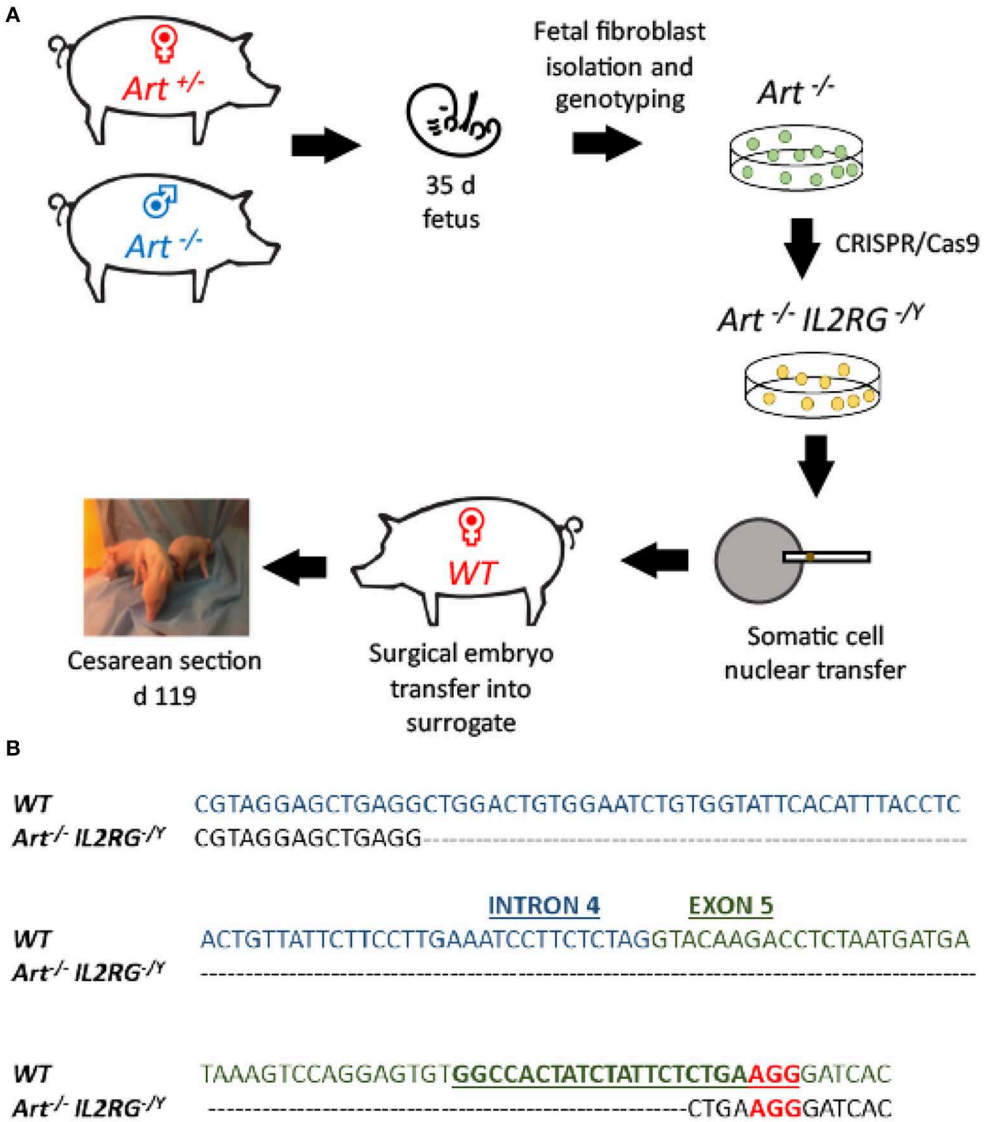

FIGURE 1 | Use of CRISPR/Cas9 system to generate *ART*$^{-/-}$ *IL2RG*$^{-/Y}$ SCID pigs. **(A)** Semen from an *ART*$^{-/-}$ boar was used to inseminate *ART*$^{-/+}$ carrier sows. Two pregnant sows were euthanized at 35 days of gestation and fetal fibroblasts were collected. Collected *ART*$^{-/-}$ fetal fibroblasts were transfected with PX330 plasmid with Cas9 and sgRNA against IL2RG target site, generating *ART*$^{-/-}$ *IL2RG*$^{-/Y}$ mutant fetal fibroblasts (pFF). Once the mutant pFFs lines were established, somatic cell nuclear transfer was performed, and embryos were transferred into surrogate gilts. Piglets born from these litters were delivered via Cesarean section at gestational day 119 into biocontainment facilities. **(B)** Sequence of IL2RG from wildtype and mutated line. Blue sequence is intron 4, and green sequence is exon 5. Mutation spans through intron 4 and exon 5. Underlined sequence indicates the designed sRNA with the letters in red showing the protospacer adjacent motif (PAM) sequence (NGG).

is nearly absent in *ART*$^{-/-}$ *IL2RG*$^{-/Y}$ SCID pigs compared to *ART*$^{-/-}$ and wild type pigs. We measured CD172α and CD16 staining on monocytes as a staining positive control and show CD172α^{+} CD16^{+} monocytes. Compared to wildtype, T cells (CD3ε^{+}) and NK cells (CD3ε^{-} CD8α^{+}) were absent in *ART*$^{-/-}$ *IL2RG*$^{-/Y}$ piglets. We also stained for pCD21 in a second litter (6701 and 6702) and confirmed there were no B cells in the circulation of *ART*$^{-/-}$ *IL2RG*$^{-/Y}$ pigs (**Supplemental Figure 1**). Lack of B cells in these pigs is consistent with our previous findings in *ART*$^{-/-}$ SCID pigs, the genetic background used to generate *ART*$^{-/-}$ *IL2RG*$^{-/Y}$ pigs (1, 32). Furthermore, *ART*$^{-/-}$ *IL2RG*$^{-/Y}$ pigs had atrophic and smaller thymus, spleen, and lymphoid tissue within the intestines compared to wildtype pigs (**Supplemental Figure 2**).

We then confirmed that T and B cells were absent from lymphoid tissues. Thymus, spleen, and Peyer's patches were collected from a wildtype and two *ART*$^{-/-}$ *IL2RG*$^{-/Y}$ pigs (6401 and 6402) and stained for CD3ε and CD79α to assess for the presence of T and B cells, respectively (**Figure 3**). Thymic tissue was assessed from only one *ART*$^{-/-}$ *IL2RG*$^{-/Y}$ pig. The *ART*$^{-/-}$ *IL2RG*$^{-/Y}$ pigs lacked normal T and B cells in all lymphoid tissues assessed.

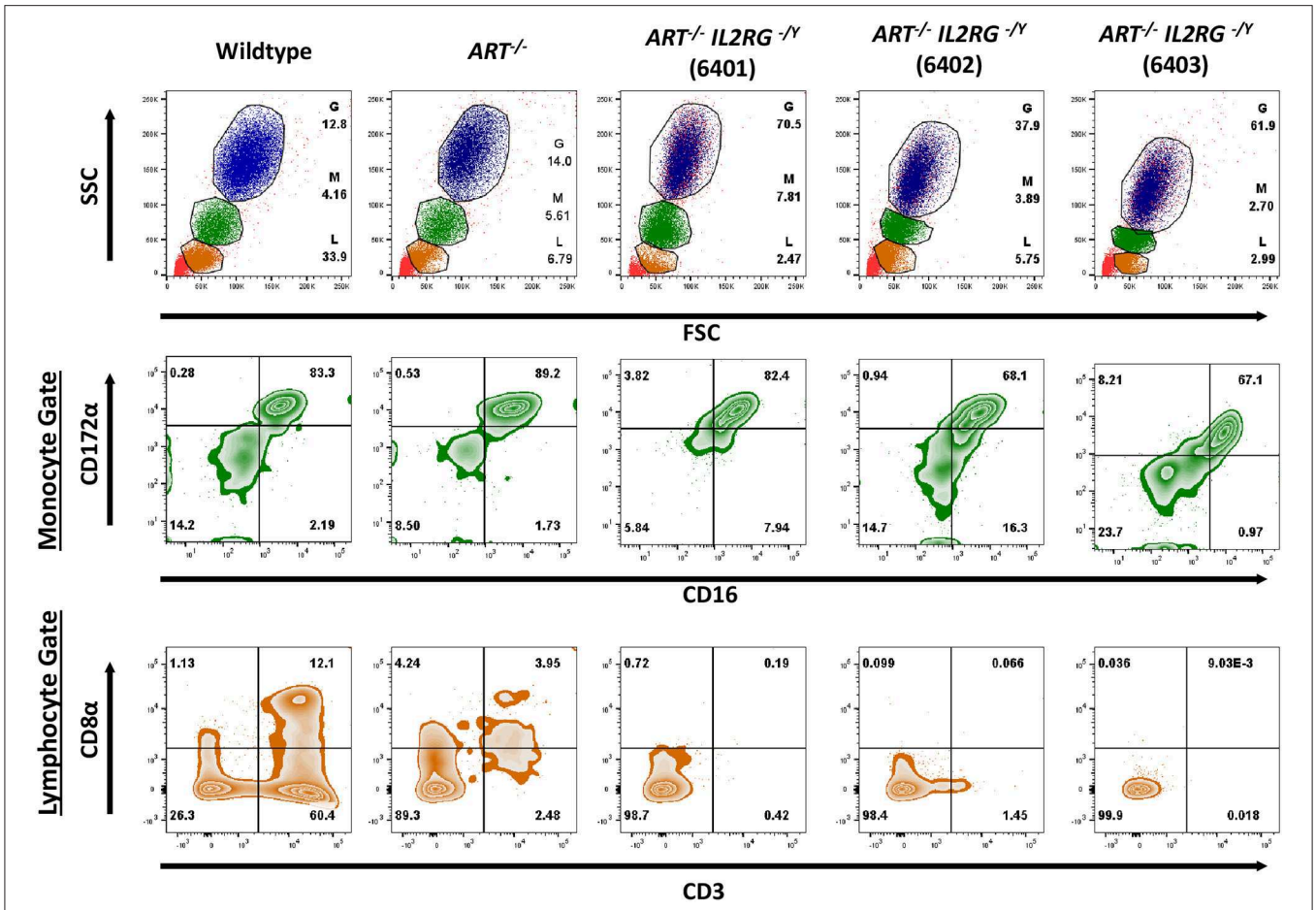

FIGURE 2 | *ART*$^{-/-}$ *IL2RG*$^{-/Y}$ SCID pigs lack T and NK cells in peripheral blood. Flow cytometric analysis of major leukocyte populations from peripheral blood of wildtype, *ART*$^{-/-}$, and *ART*$^{-/-}$ *IL2RG*$^{-/Y}$ pigs. Percentages for granulocytes (G, blue), monocytes (M, green), and lymphocytes (L, orange) are shown for each type of pig. The monocyte population was assessed for CD172α and CD16 expression. The lymphocyte population was assessed for CD3ε and CD8 for T cells (CD3ε^{+}, CD8α$^{-/+}$) and NK cells (CD3ε^{-} CD8α^{+}).

T and NK Cell Reconstitution in an *ART*$^{-/-}$ *IL2RG*$^{-/Y}$ SCID Pig After Pig Bone Marrow Transplantation

An additional goal for generation of *ART*$^{-/-}$ *IL2RG*$^{-/Y}$ SCID pigs was to establish a male breeding population to maintain this line. Carrier *ART*$^{-/+}$ females bred with an *ART*$^{-/-}$ *IL2RG*$^{-/Y}$ SCID boar would generate litters with a mix of males and females with different *ART* and *IL2RG* genotypes for future studies and breeding. The original intent of performing embryo transfers with non-modified carrier embryos was for them to provide a source of bone marrow for the *ART*$^{-/-}$ *IL2RG*$^{-/Y}$ pigs. In our two full-term pregnancies, one gilt was transferred with carrier and *ART*$^{-/-}$ *IL2RG*$^{-/Y}$ embryos (7707-FB1-U23 and 7709-FB6), while the other was transferred with only *ART*$^{-/-}$ *IL2RG*$^{-/Y}$ embryos. From both litters, only *ART*$^{-/-}$ *IL2RG*$^{-/Y}$ piglets were born, thus requiring an alternative source of bone marrow for a BMT.

We therefore performed a BMT on one *ART*$^{-/-}$ *IL2RG*$^{-/Y}$ male piglet using complete SLA matched (26.6/68.19a) bone marrow from a 4-year-old sow, which was raised on a conventional farm. A total of 2.27 × 10^{8} million unfractionated bone marrow cells from the ribs and sternum were collected and administered to one *ART*$^{-/-}$ *IL2RG*$^{-/Y}$ SCID pig. The piglet was not conditioned prior to the BMT, based on our previous success with porcine T and B cell reconstitution without conditioning in *ART*$^{-/-}$ SCID pigs (33).

Peripheral blood was collected approximately once a month after the BMT and analyzed by either flow cytometric analysis (FACS) or a complete blood count (CBC) (**Figure 4**). FACS analysis using antibodies against CD3ε, CD172α, CD16, CD79α, and CD21 revealed circulating T (CD3ε^{+}) and NK cells (CD3ε^{-} CD172α^{-} CD16^{+}) (4), but very few B cells (CD79α^{+} CD21$^{+/-}$) Of note, B cell reconstitution is variable in human SCIDs with mutations in *ARTEMIS* post BMT (40–42), which may be a function of conditioning regimens. CBC analysis showed that white blood cells and lymphocytes increased monthly after the transplant, to near normal levels by 4 months post BMT. Importantly, the *ART*$^{-/-}$ *IL2RG*$^{-/Y}$ boar post BMT has been maintained in the biocontainment bubble since birth and is sexually mature and healthy as of 1.5 years of age.

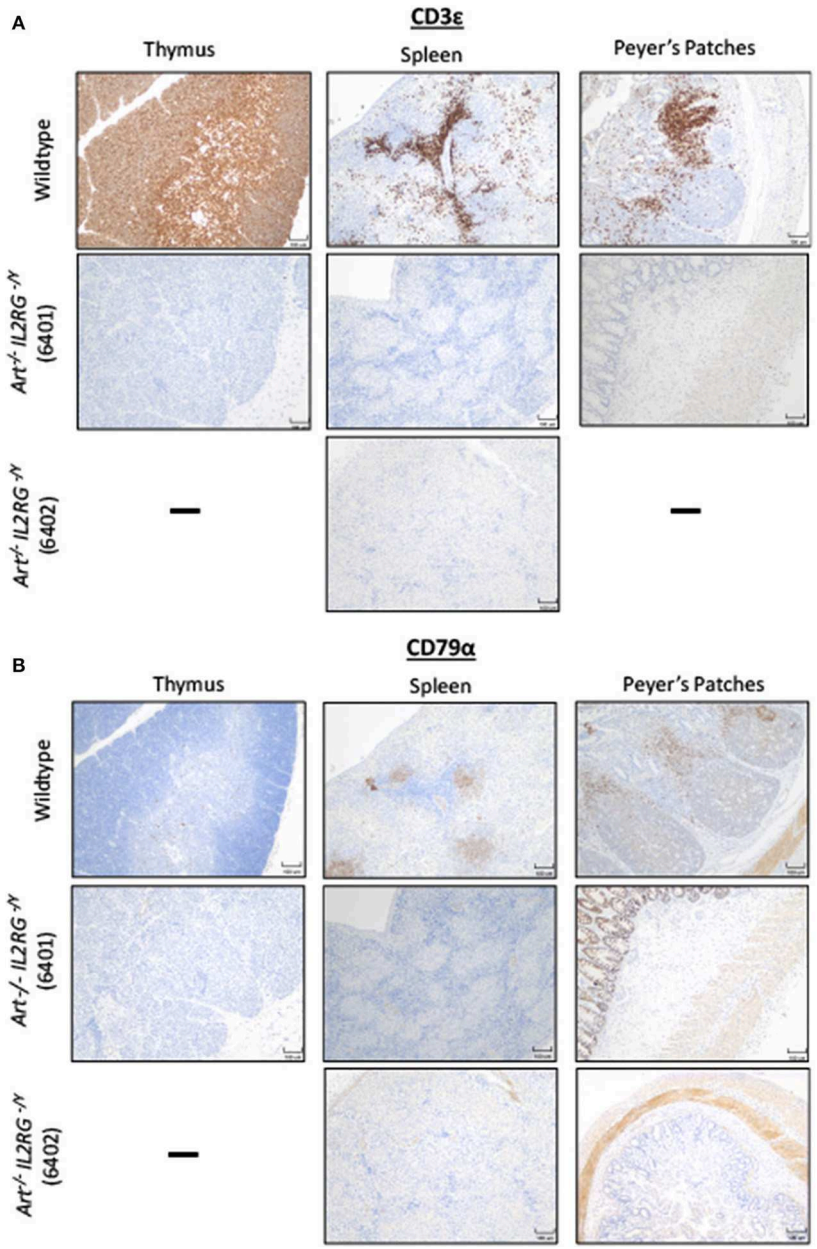

FIGURE 3 | *ART*$^{-/-}$ *IL2RG*$^{-/Y}$ SCID pigs lack T and B cells in lymphoid organs. Thymus, spleen, and Peyer's Patches were collected and assessed for **(A)** T cells (CD3ε) and **(B)** B cells in a wildtype and two *ART*$^{-/-}$ *IL2RG*$^{-/Y}$ SCID pigs.

Circulating Human T Cells in Neonatal *ART*$^{-/-}$ *IL2RG*$^{-/Y}$ Piglets After *in utero* Injection of Human Hematopoietic Stem Cells

Once we confirmed the cellular phenotype of *ART*$^{-/-}$ *IL2RG*$^{-/Y}$ pigs, we investigated whether these pigs were capable of engrafting human CD34^{+} hematopoietic stem cells. We had previously attempted intravenous or intraosseous injection of human CD34^{+} cells into single mutant *ART*$^{-/-}$ piglets (within 1 week of age), with various cell doses and busulfan conditioning (**Supplemental Table 6**). We did not detect any evidence of engraftment in peripheral blood or lymphoid organs 15 weeks post-transplant. As an alternative approach, *in utero* injection of human stem cells into the intraperitoneal space of *ART*$^{-/-}$ *IL2RG*$^{-/Y}$ SCID pig fetuses was performed.

During mid-gestation (30–45 days), the fetal liver is the major site for hematopoiesis for many species (43–46), including swine (47). Previous reports show that injection of human CD34^{+} cells into the intraperitoneal space of fetal piglets and sheep leads to differentiation and engraftment

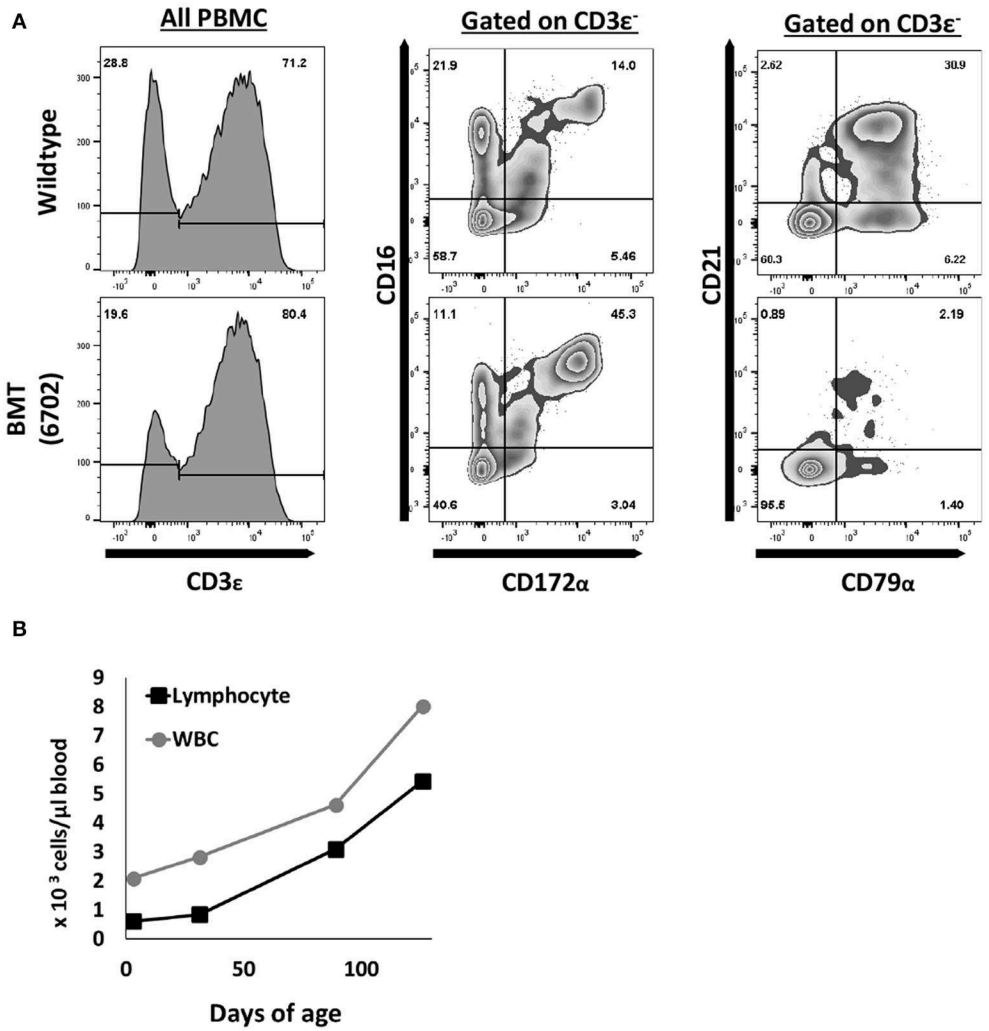

FIGURE 4 | T and NK cells are reconstituted after pig to pig bone marrow transplantation. **(A)** One *ART*$^{-/-}$ *IL2RG*$^{-/Y}$ SCID piglet underwent a bone marrow transplant at 5 days of age. At five months post transplantation, mononuclear cells were stained for CD3ε, CD172α, CD16, CD79α, and CD21 to assess for donor T (CD3ε^{+}), NK (CD3ε^{-} CD16^{+} CD172α^{-}), and B cell (CD79α^{+} CD21$^{+/-}$) development. **(B)** Complete blood counts were also performed routinely, and white blood cell counts increased upon every collection point. CBC data shown for up to 4 months post BMT.

of human immune cells (48–51). Engraftment of human cells into pig and sheep fetuses is facilitated by the fact that the cellular environment is immunologically privileged early in gestation. However, such injections have not been reported in SCID pigs. Approaches to inject fetal piglets require laparotomy procedures to expose the uterus to visualize fetuses via ultrasound imaging. Thus, laparotomy surgery with ultrasound guidance was used to inject human CD34^{+} cells into the intraperitoneal space of *ART*$^{-/-}$ *IL2RG*$^{-/Y}$ fetuses (39) (**Figure 5A**). Positively selected human CD34^{+} cells isolated from cord blood were cultured with thrombopoietin (TPO), FLT-3L, and stem cell factor (SCF) for the 7 days prior to injection to enhance expansion for the appropriate cell dose delivery. After culturing, cells were either CD45^{+}CD34^{+} (23-28%) or CD45^{+} CD34^{-} (71–76%) (**Supplemental Figure 3**).

To create fetuses for human cell injections, one surrogate gilt was transplanted with 320 *ART*$^{-/-}$ *IL2RG*$^{-/Y}$ mutant embryos (**Supplemental Table 7**). Pregnancy was confirmed at 28 days of gestation and laparotomy surgery for *in utero* injection was performed on gestational day 41. We injected half of the viable fetuses ($n = 5$ total; 3 injected) with 2–4 $\times$ 10^{6} cultured human CD34^{+} stem cells. The position in the uterine horn was recorded for all fetuses. The dam fully recovered from the laparotomy surgery and three piglets (6901, 6902, 6903) were delivered via Cesarean section at gestational day 119. The uterine positions at Cesarean section were noted and allowed identification of injected (6901, 6903) and un-injected (6902) piglets (see details in Methods). Cord blood was immediately collected from the three piglets to assess for the presence of human immune cells. We have tested and determined panels of anti-human antibodies that are not cross reactive to swine leukocytes (**Supplemental Figure 4**).

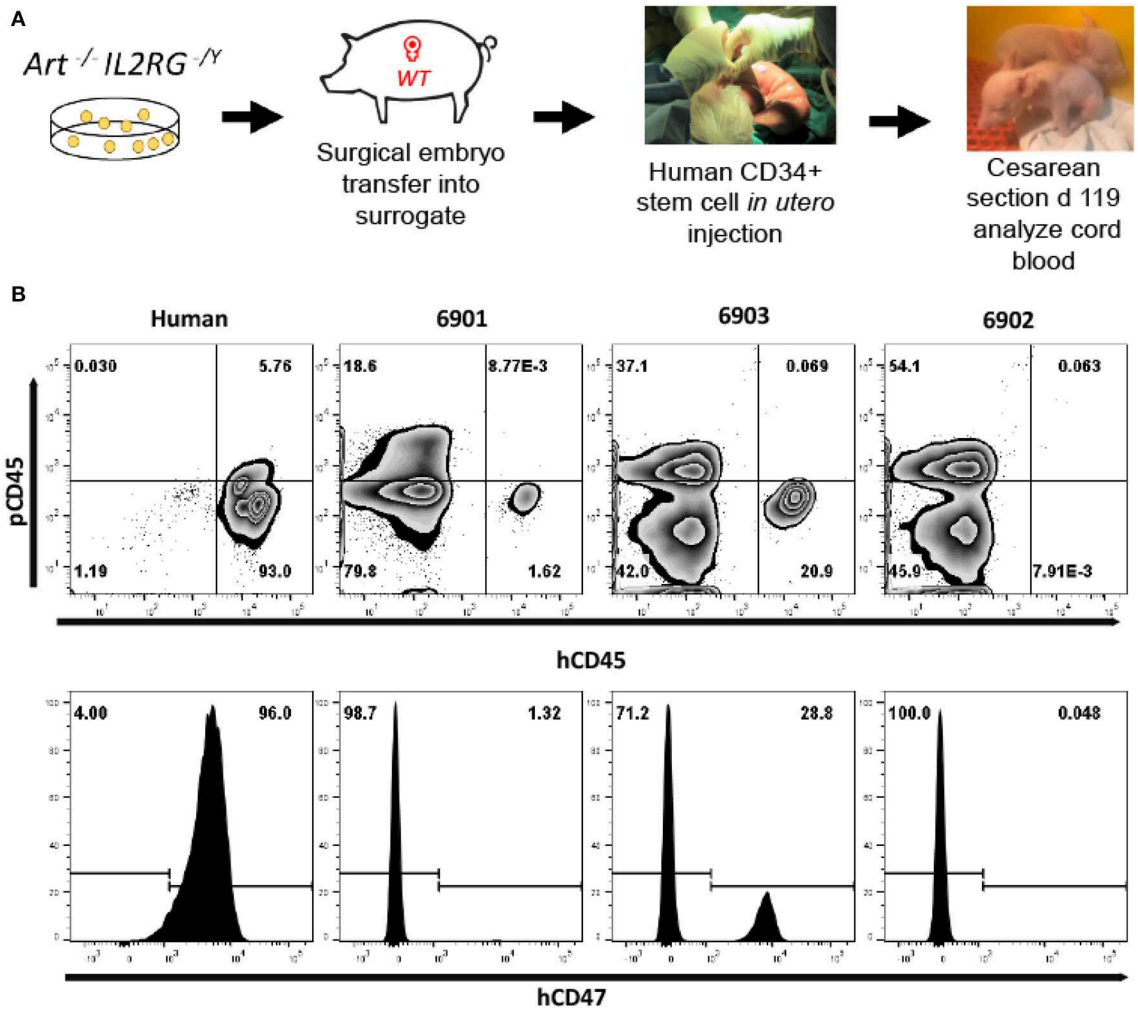

FIGURE 5 | Human leukocytes in *ART*$^{-/-}$ *IL2RG*$^{-/Y}$ SCID pig cord blood. **(A)** Somatic cell nuclear transfer derived *ART*$^{-/-}$ *IL2RG*$^{-/Y}$ embryos were surgically transferred into a surrogate gilt. At gestational day 41, the pregnant gilt underwent a laparotomy procedure to expose the uterus and ultrasound guidance was utilized to inject human CD34^{+} stem cells into the intraperitoneal space of fetal piglets. After surgery, piglets were delivered via Cesarean section at gestational day 119 into biocontainment facilities. **(B)** Blood was collected from a human (peripheral blood) and three neonatal (6901, 6902, and 6903) SCID pigs (cord blood) and stained for human and pig CD45, as well as human CD47. All cells in circulation of cord blood were gated from SCID pigs. Pigs 6901 and 6903 both had human cells in cord blood, as shown by presence of hCD45^{+} and hCD47^{+} cells.

Whole cord blood was stained for pig and human CD45, as well as for human CD47. The two CD34^{+} cell injected pigs (6901 and 6903) were found to have human CD45^{+}, as well as human CD47^{+} cells in cord blood (**Figure 5B**).

We next assessed if human cells were circulating in peripheral blood. One piglet (6901) was euthanized on day 0 due to a severe cleft palate. Peripheral blood was collected from 6901 at day 0 and at 1 day of age for 6902 and 6903. These blood samples were stained for human T cells (hCD3ε, hCD4α, and hCD8α) (**Figure 6A**), B cells (hCD20) (**Figure 6B**), and myeloid cells (hCD33) (**Figure 6C**). Red blood cells were also stained for human CD47 (**Figure 6D**). Nearly all human CD45^{+} cells circulating in 6901 and 6903 consisted of human CD3^{+} cells that were positive for hCD4α or hCD8α (**Figure 6A**).

Human CD45^{+} Cell Engraftment in *ART*$^{-/-}$ *IL2RG*$^{-/Y}$ Bone Marrow, Liver, Spleen, and Thymic Tissue

Since we observed human cells in peripheral blood, we next evaluated whether human immune cells were present within lymphoid organs. Interestingly, during necropsy, we observed grossly visible mesenteric lymph nodes in 6901 and 6903, but not in 6902. Additionally, all three pigs had some remnant, immature thymic tissue present over the heart (**Supplemental Figure 5**), which was collected for analysis. Of the tissues collected, cells were isolated from bone marrow, liver, spleen, and thymic tissue for flow cytometric analysis. Whole cell suspensions from bone marrow and thymus were stained, while mononuclear cells were stained from liver and spleen. Human CD45^{+} cells were found

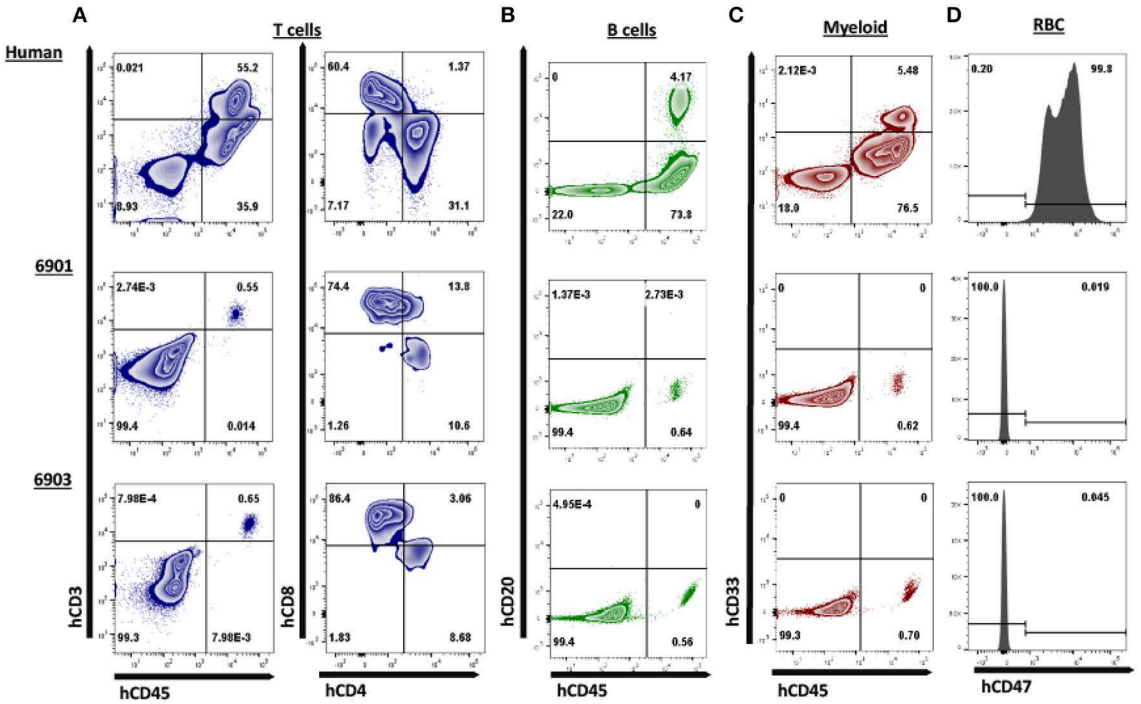

FIGURE 6 | Human CD3ε$^+$ cells are the primary cell type in peripheral blood of *ART*$^{-/-}$ *IL2RG*$^{-/Y}$ SCID pigs. Peripheral blood was collected from 6901 (day 0), 6902, and 6903 (Day 1) and stained for various human immune cell markers. Whole human blood was stained as a control to show gating strategy. All cells in circulation were gated from SCID pigs, while only lymphocytes and monocytes were gated from the human sample for the analysis of T, B, and myeloid cells. RBC were also stained separately for human CD47. **(A)** Presence of human T cells were assessed by staining for human CD45, CD3ε, CD4α, and CD8α. Cells that were CD3ε$^+$ (left) were then assessed for CD4α and CD8α expression (right). Human T cells were present in both 6901 and 6903. Single positive CD4α$^+$ and CD8α$^+$ cells were also present. **(B)** Human B cells were assessed by staining for hCD45 and hCD20. B cells were not present in any pigs. **(C)** Human myeloid cells were assessed by staining for hCD45 and hCD33. Myeloid cells were not present in any pigs. **(D)** RBCs were stained for human CD47 expression to determine if human erythroid lineage differentiated in SCID pigs. No RBCs from SCID pigs expressed hCD47.

in all four tissues assessed in 6901 and 6903, which both had human CD45$^+$ cells in circulation (**Figure 7**). At least half of the isolated thymic tissue cells from these two animals were human cells. Animal 6902 did not have any human CD45$^+$ cells in any lymphoid organs.

Since a majority of the human cells we observed in these pigs were hCD3ε$^+$, we assessed the expression of hCD4α and hCD8α within the thymic tissue cells. Early in development, T cells express both CD4α and CD8α. In animal 6901, we observed that the thymic hCD45$^+$ hCD3ε$^+$ cells were either hCD4α$^+$ hCD8α$^-$ or hCD4α$^-$ hCD8α$^-$, while in 6903 they appeared to be hCD4α$^-$ hCD8α$^-$ (**Figure 7B**).

In addition to flow cytometric analysis of cells within lymphoid tissues, we also analyzed tissues by IHC. Lymphoid tissues were collected and assessed for the presence of human T and B cells in the *in utero* injected piglets. Thymic tissue, spleen, ileum, and mesenteric lymph nodes from both 6901 and 6903 had CD3ε$^+$ cells (**Figure 8A**). Spleens from both 6901 and 6903 also had punctate Pax5$^+$ cells present, which is a marker for B cell development. A mesenteric lymph node from 6901 also had Pax5$^+$ cells (**Figure 8B**). Tissues from 6902 did not stain positively for either CD3ε or Pax5, which is consistent with the lack of human CD45$^+$ cells in blood. These histology results are consistent with the flow cytometric analyses demonstrating human leukocyte engraftment within *ART*$^{-/-}$ *IL2RG*$^{-/Y}$ SCID pigs injected with human CD34$^+$ cells.

DISCUSSION

Herein we have described foundational steps toward the development of an immunologically humanized large animal SCID model. To create the model, we introduced a mutation into the *IL2RG* gene using the CRISPR/Cas9 system in a naturally occurring *ART*$^{-/-}$ SCID background to generate *ART*$^{-/-}$ *IL2RG*$^{-/Y}$ pigs that lacked T, B, and NK cells. We performed a pig to pig BMT procedure on one male *ART*$^{-/-}$ *IL2RG*$^{-/Y}$ pig, which led to successful reconstitution of graft T and NK cells, but very few B cells. Next, we utilized *in utero* injection procedures to introduce human hematopoietic stem cells into the intraperitoneal space of SCID pig fetuses. We observed that human CD3ε$^+$ cells were present in bone marrow, spleen, liver, and thymic tissue in injected pigs after birth. Human Pax5$^+$ cells were also present within the spleen and mesenteric lymph nodes of injected pigs. Intrahepatic injection of human CD34+ cells in newborn *NOD Rag*$^{-/-}$ *IL2RG*$^{-/-}$ mice has resulted in similar patterns of reconstitution (T and B cell development) as our *ART*$^{-/-}$ *IL2RG*$^{-/Y}$ pig (52).

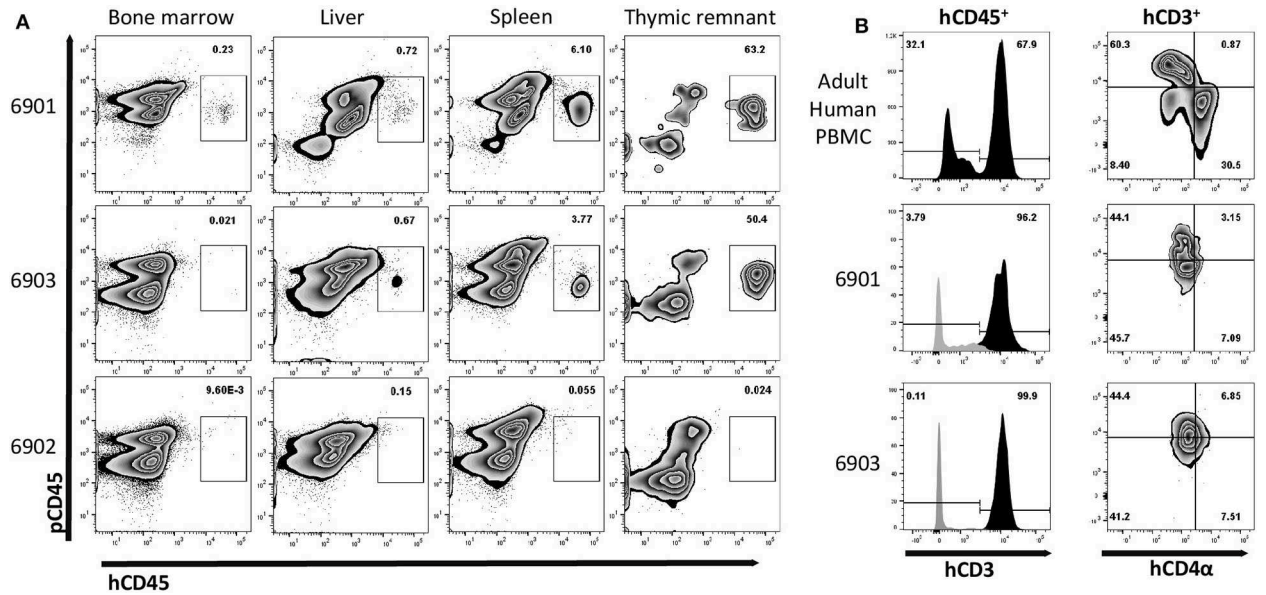

FIGURE 7 | Human leukocyte engraftment in bone marrow, liver, spleen, and thymic tissue of *in utero* injected *ART* $^{-/-}$ *IL2RG* $^{-/Y}$ SCID pigs. **(A)** Lymphoid organs from 0-day old (6901) or 7-day old (6902 and 6903) SCID pigs were analyzed for the presence of human leukocytes by staining with human and pig CD45. Bone marrow liver, spleen, and thymic tissue from both 6901 and 6903 contained human CD45$^+$ cells. All cells from isolated bone marrow and thymic tissue were stained, while mononuclear cells from spleen and liver were stained. **(B)** Human CD45$^+$ cells in isolated cells from thymic tissue expressed human CD3ε. PBMCs from an adult human were stained as a gating control. Black histogram is gated on hCD45$^+$ cells, while gray is hCD45$^-$ cells. Human CD4α and CD8α expression was assessed on human CD3ε$^+$ cells within the *Art*$^{-/-}$ *IL2RG*$^{-/Y}$ thymic tissue and human PBMC.

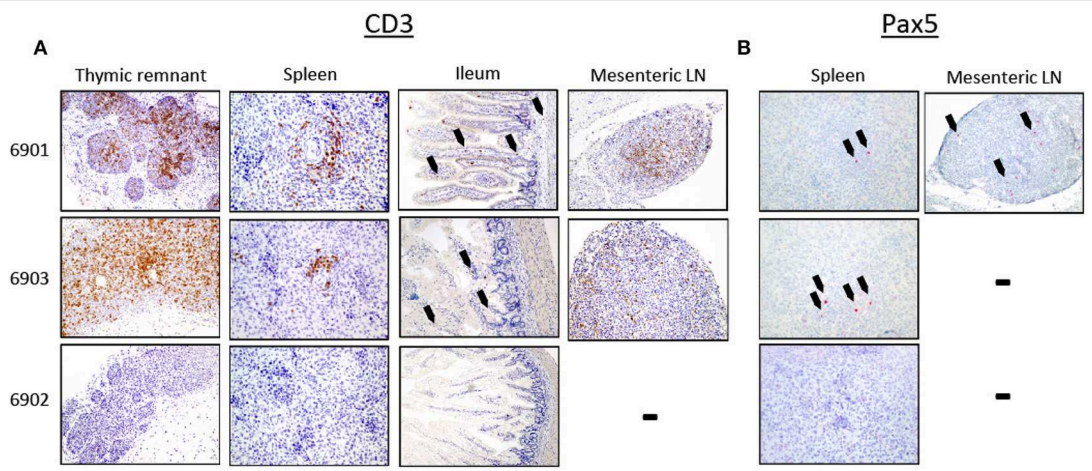

FIGURE 8 | Human CD3ε$^+$ and Pax5$^+$ cells in lymphoid organs of *in utero* injected *ART* $^{-/-}$ *IL2RG* $^{-/Y}$ SCID pigs. Lymphoid tissues were collected and stained for **(A)** CD3ε and **(B)** Pax5. **(A)** Thymic tissues from 6901 and 6903 were robustly populated with CD3ε$^+$ cells, and CD3ε$^+$ cells were also present in periarteriolar sheaths within the spleen. The ileum tissue from both animals had punctate CD3ε$^+$ cells (black arrows); CD3ε$^+$ cells were also present within mesenteric lymph nodes (LN). Lymphoid tissues from 6902 did not contain CD3ε$^+$ cells. **(B)** Spleen from both 6901 and 6903 had punctate Pax5$^+$ cells. Only the mesenteric LN in 6901 had Pax5$^+$ cells. No Pax5$^+$ cells were found in tissues from 6902.

Improving Human Cell Engraftment in SCID Pigs

One surprising finding was that we did not detect human myeloid cells, as has previously been reported in past *in utero* injections of human CD34$^+$ cells in immunocompetent pig fetuses (48). In this initial humanization model, we had a pre-determined end point of 7 days to assess human cells in blood and tissues, and therefore we only probed for myeloid cells during this period. In previous studies, myeloid lineage development had been assessed at 40 days post injection (80 days of gestation) (48). It may be that human myeloid cells are transient during gestation in this fetal injection model. Further investigation is needed to improve human myeloid reconstitution in neonatal *ART*$^{-/-}$ *IL2RG*$^{-/Y}$ SCID pigs.

In the process of *in vitro* $CD34^+$ cell culture with SCF, TPO, and FLT-3L, some cells may lose their stemness, which likely contributed lack of a variety of cells that differentiated (i.e., only T and B cells) within our SCID pig model. In mouse models genetic modifications have been required to attain human myeloid and NK cell engraftment, including human CSF-1, IL-15, GM-CSF, Flt-3L, IL-3, TPO, as human cells do not recognize mouse cytokines (21, 23, 53–56). An area to be investigated is how human cells respond to swine cytokines and if the swine bone marrow niche is supportive of human myeloid cells. Humanization of certain cytokine genes may be required in future humanization attempts in our pig model. *In vitro* culturing assays with human hematopoietic stem cells and porcine cytokines can be a first-line screen for assessing porcine cytokine cross-reactivity. Additionally, in future studies, we can assess cytokine secretion by developed human cells within the pigs.

In our model, we observed that a majority of human cells that developed were $CD3\varepsilon^+$. We assessed the thymic tissue to better understand the development of human T cells. We interestingly did not observe $CD4\alpha^+CD8\alpha^+$ double positive cells within the thymus, which is an expected normal stage of T cell development. In future studies, it will be imperative that we perform deeper phenotyping of human cells that have differentiated within the pig. Previous reports by Kalscheuer et al. (57) and Ogle et al. (49) show that the swine thymus can support engraftment and differentiation of human T cells. The lack thymic development in a SCID pig fetus may negatively impact the ability of human cells to develop, which may warrant transplantation of human thymic tissue after birth.

Another potential method to increase engraftment is to condition the fetuses prior to human cell injection. Plerixafor, a drug that mobilizes stem cells out of bone marrow (58), has previously been utilized for *in utero* injections of human cells into sheep fetuses to improve engraftment of human cells (59). Plerixafor is an agonist for CXCL12 on stromal cells, which binds to CXCR4 on hematopoietic stem cells (HSC) (58). Administration of plerixafor mobilizes sheep HSC out of bone marrow, providing more available niches for human HSC to engraft. Goodrich et al. (59) described that administration of plerixafor along with injection of $CD34^+$ $CXCR4^+$ human stem cells and mesenchymal stem cells improved chimerism (in peripheral blood) 5 weeks after transplantation from 2.80 to 8.77%. Now that T^- B^- NK^- SCID pigs and biocontainment facilities are available for extended postnatal follow-up, a similar regimen could be administered to SCID pig fetuses prior to *in utero* injection with human stem cells.

Further Characterization of *de novo* Differentiated Human Immune Cells

We show that human T cells differentiated and homed to lymphoid tissues in the $ART^{-/-}$ $ILR2G^{-/Y}$ SCID pigs. Human B cells also differentiated, but to a much lower extent. While the human cells that differentiated within the SCID pigs were not extensively characterized, the major aims of this study were to develop methodologies to humanize SCID pigs and to determine the feasibility of performing these methodologies on $ART^{-/-}$ $ILR2G^{-/Y}$ SCID fetuses. Moving forward, we expect that optimization of humanization methods would lead to increased levels of engraftment, and thus a higher number of human cells in any given tissue which could be used for different types of analyses.

Performing single cell RNAseq on isolated human cells form thymic and bone marrow tissues would be of particular interest to understand how human cells differentiate within the SCID pig primary lymphoid organs and the composition of the differentiating cell population within the graft. A comparison could be made between human cells that differentiated in NSG mice compared to $ART^{-/-}$ $ILR2G^{-/Y}$ SCID pigs. Additionally, since we have now established that human T and B cells differentiate and home to swine lymphoid organs, the functionality of these cells could be assessed either through *in vivo* vaccination studies or *in vitro* stimulation assays. Understanding the full extent of human cell differentiation and functionality will be critical as this model is developed further.

Outlook on B Cell Reconstitution in $ART^{-/-}$ $ILR2G^{-/Y}$ SCID Pigs

One issue in both pig to pig BMT, as well as *in utero* injection of human HSCs, was the failure of pig or human B cells to robustly develop. Historically, some human SCID patients that underwent BMT have also failed to develop graft derived B cells (40). In some cases, significant B cell reconstitution in human BMT can require up to 2 years (60, 61). One leading hypothesis regarding B cell development issues is due to differences in the B cell niche in the bone marrow. Single mutant *IL2RG* knock out pigs are capable of developing B cells, which can be detected in circulation (9, 10), however they are non-functional due to the absence of T helper cells. Mutations in *ARTEMIS* lead to a B cell block of differentiation at the pre-B cell phase (62). Together, an $ART^{-/-}$ $Il2RG^{-/Y}$ pig likely still has premature B cells present in the bone marrow, which would prevent further engraftment and differentiation of graft stem cells in this niche. Conditioning prior to stem cell transplantation has helped improve B cell reconstitution in some cases (63), although such conditioning procedures would be difficult prior to *in utero* cell transplantation. To our knowledge this is the first time a bone marrow transplantation has been performed on a double mutant SCID pig. Thus, further assessment of the bone marrow niches of $ART^{-/-}$ $IL2RG^{-/Y}$ pigs may be required to better understand conditioning regimens that may be needed for engraftment.

CONCLUDING REMARKS

As the field of biomedical SCID pig research expands, new techniques will arise to optimize human cell engraftment within SCID pig models. We can draw from previous large animal *in utero* injection protocols (48–51, 59, 64), as well as humanization techniques performed in immunocompromised mice (65, 66). In

our SCID pig model, we show that human T and B cells can develop. As we improve reconstitution of human cell subsets, the humanized SCID pig will be a critical alternative large animal model for researchers preclinical or co-clinical trials.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Iowa State University Institutional Review Board. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by the Iowa State University Institutional Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

AB designed humanization experiments, isolated human stem cells, performed fetal injections, human immune cell flow cytometry, and wrote the draft of the manuscript. YL isolated fetal fibroblasts, performed CRISPR/Cas9 genome modification, performed somatic cell nuclear transfer and embryo transfers to generate double mutant piglets, and wrote the draft of the manuscript. BS was involved with double mutant pig generation. AA performed fetal injection laparotomy procedures on pregnant gilt. MK performed immunohistochemistry for human immune cells in lymphoid tissues. KB and CL performed flow cytometry for $ART^{-/-}$ $IL2RG^{-/Y}$ immunophenotyping and bone marrow transplantation monitoring. AC-O and JW performed immunohistochemistry for porcine immune cells in lymphoid tissues. EP performed IV injections of human stem cells in $ART^{-/-}$ pigs. JS collected cord blood for stem cell isolations that were used in fetal injections of $ART^{-/-}$ $IL2RG^{-/Y}$ fetuses. ES performed pig bone marrow isolation for bone marrow transplantation. C-SH performed MHC PCR analysis for bone marrow donors. JR performed bone marrow transplantation. SC genotyped fetal fibroblast cell lines for *ART* status. SC, ZK, and MA were involved in experimental planning for all facets of this project. JC was involved in experimental flow cytometry planning. SS performed numerous ultrasound pregnancy checks on surgically transferred gilts. GD'A and JJ assisted with fetal injection procedures. FG and EW provided human stem cells for IV injection procedures in $ART^{-/-}$ pigs. JD provided guidance in pig breeding to generate the litters for pFF collection and assisted in project planning discussions. JR and CT were involved in all aspects of procedures and experiments performed in this study and edited the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.00100/full#supplementary-material

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REVIEW
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From Beef to Bees: High-Throughput Kinome Analysis to Understand Host Responses of Livestock Species to Infectious Diseases and Industry-Associated Stress

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Within human health research, the remarkable utility of kinase inhibitors as therapeutics has motivated efforts to understand biology at the level of global cellular kinase activity (the kinome). In contrast, the diminished potential for using kinase inhibitors in food animals has dampened efforts to translate this research approach to livestock species. This, in our opinion, was a lost opportunity for livestock researchers given the unique potential of kinome analysis to offer insight into complex biology. To remedy this situation, our lab developed user-friendly, cost-effective approaches for kinome analysis that can be readily incorporated into most research programs but with a specific priority to enable the technology to livestock researchers. These contributions include the development of custom software programs for the creation of species-specific kinome arrays as well as comprehensive deconvolution and analysis of kinome array data. Presented in this review are examples of the application of kinome analysis to highlight the utility of the technology to further our understanding of two key complex biological events of priority to the livestock industry: host immune responses to infectious diseases and animal stress responses. These advances and examples of application aim to provide both mechanisms and motivation for researchers, particularly livestock researchers, to incorporate kinome analysis into their research programs.

Keywords: kinome, kinase, phosphorylation, peptide array, stress, infectious disease

INTRODUCTION

Human and animal health research have each been revolutionized by technologies that enable global perspectives on cell biology. Omic approaches, an example of such technologies, conducted at a variety of biological levels have opened new frontiers for understanding biology as well as for diagnosis and treatment of disease. Ideally, the same omic approaches within human and animal health research fields can be applied with minimal barriers to translation allowing researchers to benefit from the advances made within each realm. This is true, to varying degrees, for the different omic disciplines. The effort required for the successful translation of the omic technologies does

differ depending on characteristics of the biomolecule under consideration. That is to say, some omic technologies are more amenable to translation across species.

The opportunities enabled by broadly applicable omic technologies are particularly evident within nucleic acid-based investigations: DNA for genomics and RNA for transcriptomics. These approaches are based on research platforms that are largely species-independent such that identical omic technologies can be applied to virtually any organism. For example, the same basic approaches can be applied to either sequence the genome or define the transcriptome largely independent of the specific species. With that, technological advances within nucleic acid-based omics have great potential to offer immediate benefit to livestock researchers. For example, by serving as a catalyst for development of higher-throughput sequencing technologies, the Human Genome Project enabled determination of the genomes of livestock species including cattle (1), pigs (2), chickens (3), and turkeys (4). Likewise, within transcriptomics, there is a similar pattern of the development of technologies for traditional species of research priority (human and mouse) which are subsequently adopted by livestock researchers. This includes the use of transcriptional arrays (5), RNA-Seq (6), and single-cell RNA sequencing (7) to define transcriptional responses in livestock species.

The translation of technologies from human to animal health applications is of mutual benefit in that livestock researchers are empowered with cutting-edge technologies to advance their fields of study and the emerging data adds value and dimension to the human data by enabling species-comparative perspectives for human models of physiology and disease. Furthermore, as large animal models are representing an essential foundation for our understanding of human health and disease it is imperative that these species are investigated using advanced technologies (8). However, not all omic approaches share the same technological versatility for application across species, nor is there always the same level of motivation for their translation.

Within human health research, the priority for investigations of global cellular kinase (kinome) activity has been heavily motivated by the fact that kinases are intimately associated with many diseases and represent excellent drug targets (9). The "druggability" of kinases reflects both structural features of this class of enzymes that enables design of inhibitors as well as the central role of kinases as regulators of cellular responses and phenotypes (10, 11). In humans, many small-molecule protein kinase inhibitors have been approved or are advancing through clinical trials for the treatment of a diverse array of diseases (12). However, in animals the use of kinase inhibitor treatment has been limited. Select tyrosine kinase inhibitors have been approved for the treatment of cancer in companion animals (13, 14) as well as preliminary investigations of similar applications in horses (15). A category of kinase inhibitors, referred to as bumped kinase inhibitors (BKIs), target calcium-dependent protein kinases belonging to parasites of human and veterinary importance, including *Toxoplasma gondii*, *Plasmodium falciparum*, and *Cryptosporidium parvum* (16). BKIs have shown promising results as anti-parasitic drugs within food-animal species, including cattle (17) and pigs (18). From a safety perspective, BKIs represent the most likely usage of kinase inhibitors in livestock as the BKIs target non-mammalian kinases. Overall, however, the cost of these treatments relative to the value of the animals, as well as safety considerations (real, perceived, and regulatory) of such treatments, has prevented the use of kinase inhibitors as therapeutics in livestock animals.

The opportunities to employ kinase inhibitors as therapeutics is not, however, the sole benefit of kinome profiling. Kinome analysis also offers the unique advantage to understand the molecular basis of complex phenotypes. In part, this reflects the fact that kinase-mediated phosphorylation events succeed the transcriptional and post-transcriptional regulatory events that complicate the extraction of meaningful biological data from genomic and transcriptomic approaches. As kinase-mediated phosphorylation events often initiate cellular responses and phenotypes, defining host responses at the level of the kinome provides an opportunity for an unobstructed perspective of cellular events that anticipate, and are responsible for, organismal phenotypes. These same features also position kinases to serve as biomarkers of important phenotypes. Therefore, in spite of the somewhat restricted potential to the use of kinase inhibitor therapeutics in livestock, the other benefits of kinome analysis warrant effort to address the technological barriers that restrict the application of these approaches to livestock.

EXPERIMENTAL APPROACHES TO DEFINE KINASE-MEDIATED PROTEIN PHOSPHORYLATION

There are two primary methodologies that are employed to define kinase-mediated protein phosphorylation: phosphoproteome analysis, which characterizes the targets of the kinases, and kinome analysis, which quantifies the activities of the kinases. The different philosophical and technological basis of these approaches have been reviewed elsewhere (19). Each approach is associated with unique challenges and opportunities for application to livestock species (20).

Phosphoproteome Analysis

Phosphoproteome investigations typically employ mass spectrometry to determine the phosphorylation status of proteins based on changes in molecular mass corresponding to the addition of a phosphoryl group (21). These types of phosphoproteomic characterizations can be performed in a largely species-independent manner as the basis for mass spectrometry analysis reflects changes to peptide characteristics (independent of their biological source) and that detailed predicted proteomes and their proteolytic peptide libraries are readily available for most species. Indeed, phosphoproteome characterizations have been applied to livestock to explore biological questions such as host-pathogen interactions (22), meat quality (23) and regulation of metabolism (24). The major technical limitations are the prohibitive costs and requirement for specialized equipment and personnel. The primary biological limitations are the challenges of defining dynamic patterns of phosphorylation within low abundance proteins, in particular

those that reflect relatively small changes in the extent of phosphorylation of these proteins, a situation that often occurs within the context of signal transduction.

The phosphoproteome can be interrogated using antibodies that exclusively react with phosphorylated amino acids (i.e., serine, threonine, and tyrosine) or more specifically investigated using site-specific antibodies that only react with the protein in its phosphorylated state. This offers advantages of more quantitative assessment of priority phosphorylation events but it is ultimately limited by the availability and specificity of the antibody reagents. The availability of phosphorylation-specific antibodies is particularly problematic for livestock. While some commercially available phosphorylation-specific antibodies include information on reactivity across a range of species, some of which include livestock, this information is often unavailable or, in our experience, unreliable. Secondary challenges to this approach include technological obstacles to applying the antibodies in a high-throughput fashion; this is particularly challenging when using site-specific phosphorylation antibodies.

Kinome Analysis

In contrast to phosphoproteome approaches, kinome analysis capitalizes on the fact that post-translational modifications represent enzymatic reactions. By providing an appropriate substrate, it is possible to quantify the activity of a particular kinase within the context of an enzymatic assay. As the specificity of many kinases is determined by the residues adjacent to the phosphorylation site (within 4 amino acid residues) (25, 26) it is theoretically possible to use short peptides as surrogate substrates for kinases. As short peptides are easily synthesized, relatively inexpensive and amenable to presentation in array formats this offers tremendous potential to develop peptide arrays that enable high-throughput analysis of cellular kinase activity. Early applications of kinase peptide arrays were performed to define phosphorylation sites and target-site specificity based on the ability of the kinases to modify peptides with shared sequence similarity. Once the utility and specificity of the arrays was established, these investigations evolved into applications to define cellular signaling responses within cellular lysates using kinome arrays. In this regard, the first true global kinome profiling experiment with peptide arrays defined signaling responses of human peripheral blood mononuclear cells (PBMCs) following stimulation of the innate immune receptor Toll-like receptor (TLR) 4 with its ligand lipopolysaccharide (LPS) (27); a major cell-wall constituent of Gram-negative bacteria. Notably, this pioneering investigation interpreted the emerging data from the perspective that each peptide represented a specific phosphorylation event of a particular protein rather than each peptide representing a general substrate for a specific kinase. That is to say, the data was interpreted from a phosphoproteome, rather than a kinome, perspective.

One of the considerable advantages of peptide arrays is that they are readily customized to represent the phosphorylation events that are of highest priority to the individual researcher. Designing the peptide arrays consists of selecting an appropriate number of phosphorylation sites from public phosphoproteome databases, such as PhosphoSite (28) and Phospho ELM (29). Within these databases, the phosphorylation events are typically presented as sequences of fifteen amino acids in length with the phosphoacceptor site in the central position. This format matches the design for most peptide arrays such that the information from these databases can be rapidly translated into a customized array.

One of the major hurdles with kinome analysis of livestock was that the commercially available arrays represented sequences derived from the human or mouse proteome and that information available within the phosphorylation databases was heavily biased toward those same species. The desire to perform kinome analysis of livestock, coupled with the scarcity of available experimental phosphoproteome information for these species, motivated alternate approaches for peptide array design. In particular, observation of the extent of conservation of the regions immediately surrounding phosphoacceptor sites indicated the potential to apply bioinformatic approaches to predict the phosphoproteome of species of interest.

PLATFORM TECHNOLOGIES FOR GENERATING SPECIES-SPECIFIC KINOME ARRAYS

Species-Specific Peptide Arrays

As a first exploration of the extent of conservation of phosphorylation sites across species nearly one thousand experimentally determined human phosphorylation sites (represented by a sequence of 15 amino acids with a centered phosphoacceptor site) were investigated within the bovine proteome (30). Of these phosphorylation sites, half were perfectly conserved across these two species (the same sequence within the context of a homologous protein), a quarter showed minor (less than three amino acids) sequence differences, and the final quarter had no identifiable protein homologs within the bovine proteome (**Figure 1**) (30). These results demonstrated both the potential, as well as the need, to construct species-specific peptides arrays.

The relatively high degree of conservation of phosphorylation sites across these species encouraged the potential to create species-specific peptide arrays. As such, in this example, it is possible to generate a bovine-specific peptide array by simply selecting phosphorylation sites whose peptide sequences are either absolutely conserved, or can be accommodated through minor species-dependent sequence adjustments. For example, a researcher could opt to limit the array to peptides whose sequences were absolutely conserved across the two species. In the described example, absolute conservation of sequence would represent half of the experimentally defined human phosphorylation sites as possible candidates for inclusion on the bovine peptide array (**Figure 1**). Notably, selecting phosphorylation sites whose surrounding sequences are absolutely conserved across the two species results in a tool that is equally appropriate for either humans or cattle use. Such "dual-species" arrays provide a common tool that facilitates direct comparison of results from each species and are therefore of particular value for species-comparative investigations. For

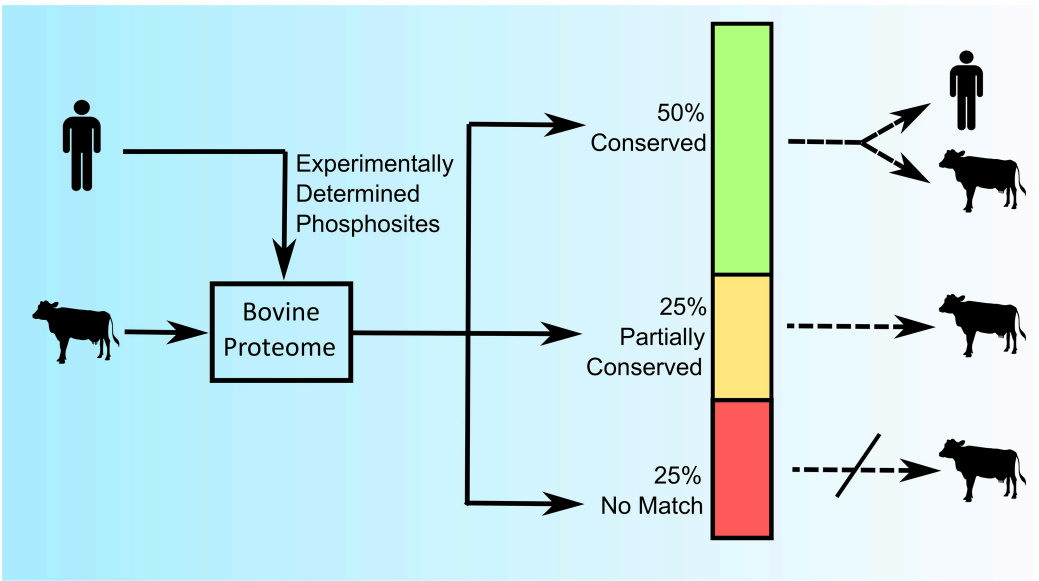

FIGURE 1 | Comparative analysis of conserved and species-specific phosphorylation sites in the human and bovine proteome. Nearly 1,000 experimentally determined human phosphorylation sites (15 amino acids in length) from PhosphoSite were queried against the *Bos taurus* proteome using BLASTp (version 2.2.13) to identify protein homologs. "Conserved" represents 100% amino acid sequence identity; "Partially Conserved" represents less than 3 mismatches in the amino acid sequence; and "No Match" represents no identified homolog in the bovine proteome (30).

example, the use of a pig/human dual-species peptide array demonstrated the ability to directly compare datasets from both human and porcine samples (31).

The list of potential peptides for a customized array can also be expanded through the inclusion, with appropriate modification, of peptides with minor species-specific sequence variations. In the previous example, the inclusion of the sequence-corrected peptides of this category would expand the list of peptides to include another quarter of the initial library of experimentally determined phosphorylation events but would also limit the application of that array to cattle (**Figure 1**).

The half of the peptides which were a perfect match, and the quarter which can be easily adopted through minor species-specific sequence variations, highlight the potential to create species-specific peptides arrays. The remaining quarter of human phosphorylation events which had no counterpart within the bovine proteome, speak to the need to create customized arrays for specific species. Consider, for example, if one were to utilize a peptide array representing human phosphorylation events to define kinome responses in bovine samples. In a best-case scenario the fraction of peptides (approximately 25%) representing human phosphorylation sites for which there is no functional equivalent within the bovine proteome would simply not be recognized by bovine kinases. While this would limit the efficiency of the array, there would be minimal consequences to the overall quality of the emerging data. Peptide arrays are typically applied to investigate relative differences in phosphorylation under different conditions rather than absolute levels of phosphorylation in a single condition; an unmodified peptide would appear as having no response to the stimulus under investigation. It would be more problematic if peptides representing phosphorylation events that do not exist within the bovine proteome were recognized and modified by bovine kinases, as this would imply the occurrence of phosphorylation events which, in reality, have no biological significance.

A recent investigation into the patterns of conservation of kinases and phosphorylation sites indicated there was greater evolutionary stability within the kinases as opposed to their phosphorylation sites (32). That is to say, a relatively stable infrastructure of kinases serves to modify a more malleable proteome. This would seem to support the potential for the presence of kinases with the ability to modify peptides that represent phosphorylation sites absent from within the proteome of that species. For these reasons, it is not recommended to utilize peptide arrays designed for a particular species to define the kinome of another species.

Design Array for Phosphorylation Experiment (DAPPLE)

A software platform called Design Array for Phosphorylation Experiment (DAPPLE) was created to streamline and automate the process of developing species-specific peptide arrays. DAPPLE utilizes BLAST to analyze the sequence similarity between experimentally determined phosphorylation sites in other organisms against the proteome of the species of interest (33). DAPPLE returns to the user a list of peptides containing a putative phosphoacceptor site for inclusion on a species-specific array. This peptide library typically ranges in size from thousands to tens of thousands. DAPPLE was later expanded into DAPPLE2 (34), to improve on the original program by surveying a greater number of phosphorylation

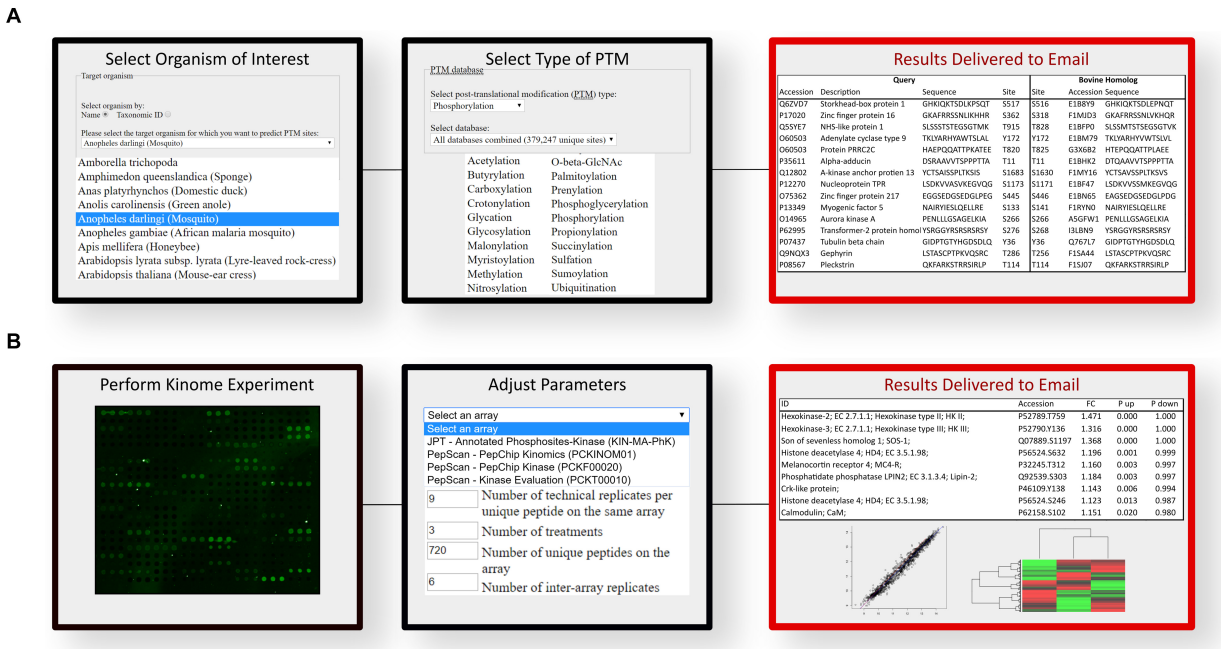

FIGURE 2 | Computational workflow for the prediction of phosphopeptides and analysis of kinome array data. **(A)** DAPPLE2 facilitates the prediction of post-translational modification sites in proteins. Specific to kinome technology, DAPPLE2 can predict and generate a list of all putative phosphopeptides from the selected species of interest. **(B)** PIIKA2 software was custom designed for the transformation, deconvolution, statistical analysis and visualization of kinome array data. PIIKA2 results returned to the user include various statistical analyses and data visualization such as principal component analysis plots, scatter plots, and hierarchical clustering heat maps. Both software platforms are freely accessible to all researchers at http://saphire.usask.ca/saphire/.

site datasets as well as enabling consideration of other forms of post-translational modification. As current arrays typically contain approximately a thousand unique peptides, it can be a daunting task to manually select these from the tens of thousands that are typically present within the DAPPLE output files. DAPPLE2 facilitates this process by providing the user with gene ontology terms, signaling pathways, and indicators of the confidence of the predicted phosphorylation site including sequence identity within the phosphorylation region and the homology of the protein in which the phosphorylation site is contained. DAPPLE2 also provides information on the number and nature of supporting publications for the phosphorylation event. The nature of the supporting papers being further defined on the basis of whether the source publication represents high- or low-throughput approaches. Greater confidence and priority are assigned to phosphorylation events characterized using low-throughput approaches (like site-directed mutagenesis) as opposed to high-throughput global characterizations of the phosphoproteome. This information also allows researchers to simplify peptide selection based on clear and rational criteria relating to both the biological function and confidence in the predicted phosphorylation site. For example, a user could specify the selection of phosphorylation events which are involved in metabolism, which are absolutely conserved within the target species, and are supported by at least three publications, one of which representing a low-throughput study. Based on the number of peptides meeting these criteria, the user can choose to alter the selection criteria until a suitable number of peptides are identified. Using this approach, it is possible for a biologist with minimal background in bioinformatics to design a species-customized array, with or without an emphasis on specific biological processes, in a matter of hours. The workflow interface of DAPPLE2 is illustrated (**Figure 2A**).

It is also worthy to note that peptide arrays designed on the basis of predicted phosphoproteomes are inherently less reliable than those reflecting phosphorylation sites characterized through low-throughput experimental approaches. The conservation of a matching sequence, in a homologous protein, as an experimentally determined phosphorylation site is not absolute assurance that the same phosphorylation, and by extension the same biological outcome, will occur in the target species. With that appreciation, our central philosophy is that kinome analysis is a tool, similar to other omic technologies, employed to generate data that lead to novel hypotheses that are then further substantiated by independent approaches.

Platform for Integrated, Intelligent Kinome Analysis (PIIKA)

Interpreting the results of high-throughput analyses that involves high-level statistics on thousands of data points can be extremely difficult. As such, extraction of meaningful biological information is a significant challenge to any omic approach, with kinome analysis being no exception. To this end, a software tool, Platform for Intelligent, Integrated, Kinome Analysis (PIIKA), and our latest version PIIKA2, was created with specific

consideration of the technical and biological characteristics of kinome peptide arrays (35). An easy to use web-based interface allows biologists lacking a strong background in data science to upload kinome datasets to perform various analyses and tests: data normalization, evaluation of how well different experimental groups cluster together, identification of peptides with consistent phosphorylation patterns amongst experimental groups, view false negative probabilities, positive and negative predictive values for t-tests between pairs of samples, and readily quantify experimental reproducibility (36). PIIKA2 includes various statistical analyses such as fold-change analysis, principal component analysis, determination of Euclidean distance between groups, and hierarchical clustering (35). Visualization tools within PIIKA2, such as volcano plots, scatterplots and heat maps aid in the selection process and statistical interpretation as well as being readily presentable and easy to understand, in comparison to the raw output. The output files of PIIKA2 are compatible with software platforms for higher-level analysis, such as pathway analysis. The workflow interface of PIIKA2 is illustrated (**Figure 2B**).

APPLICATIONS OF KINOME ANALYSIS

In the decade following the development of the species-specific peptide arrays there has been a wealth of publications that highlight the utility of kinome analysis of livestock species (**Table 1**). These investigations explore a variety of species, biological questions, and sample matrices. In the subsequent sections we present examples to highlight the diversity within each of these. Species-specific peptide arrays have been created and applied for the primary food-associated livestock animals (i.e., cattle, pigs, and chickens). In terms of biological questions, the application of kinome analysis to livestock has focused on two issues of greatest significance to the industry, infectious diseases, and response of animals to stresses associated with modern management practices. Within these investigations, a variety of biological samples have been considered which range in complexity from immortalized cell lines and highly purified primary cells to complex cell populations like PBMCs and tissue samples (i.e., muscle and intestine), and even whole organism kinome profiling. These examples collectively highlight the utility and robustness of the technology. The workflow for the design, application, and interpretation of peptide arrays for kinome analysis is illustrated (**Figure 3**).

TABLE 1 | Applications of peptide array kinome analysis to livestock species.

		Sample			
Species	**Challenge**	**Cell culture**	**PBMCs**	**Muscle**	**Intestine**
Bovine (cattle)	Infectious disease	(57, 58, 61, 109)	(74)		(70)
	Stress		(90)		
Swine (pig)	Infectious disease				(93)
	Stress				
	Other		(31)		
Avian (chicken)	Infectious disease	(64, 66, 110, 111)		(75)	(76–78, 112, 113)
	Stress			(92)	
	Other				(103, 114)
Ovine (sheep)	Infectious disease	(94, 115)			(94)

Kinome Analysis of Infectious Disease

As the activation of innate immune responses relies heavily on phosphorylation-mediated signal transduction, kinome analysis is a particularly appropriate approach for defining host responses to microbial pathogens (37). Given the importance of kinase-mediated signaling in the activation of immune responses, it is not surprising that many pathogens, in particular those that result in chronic infections, can subvert protective host immune responses using their own effector kinases, and phosphatases in addition to utilizing other virulence factors that function to manipulate host signaling either directly or indirectly (38–40). These tactics can represent a critical obstacle in the development of effective vaccines and/or immunotherapeutics. These limitations are potentially addressed through a more detailed understanding of the host-pathogen interaction; understanding the molecular mechanisms of these interactions can guide rationale development of vaccines and/or therapeutics (particularly, in the form of kinase inhibitors), as well as facilitating the identification of biomarkers that anticipate the susceptibility, resistance, severity, or outcome of infection. With this, it is not surprising that early examples of kinome analysis through peptide arrays were performed in the context of investigating the host-pathogen interaction.

KINOME ANALYSIS OF *EX VIVO* INFECTION MODELS

To enable greater opportunity for insight into host immune responses to pathogenic challenge, the earliest kinome investigations of host-pathogen interactions were often conducted within simplified infection models, like cell lines or highly purified primary cell populations. The biological significance of the findings of these investigations were then typically validated within *in vivo* infection models through either direct confirmation of biological responses or through the effective use of therapeutics informed by the *ex vivo* investigation.

Mycobacterium avium subsp. *paratuberculosis* (*ex vivo*)

Johne's disease, a chronic inflammatory disorder of the small intestine of ruminants, is caused by *Mycobacterium avium* subsp. *paratuberculosis* (41). *M. paratuberculosis* is an intracellular pathogen that achieves chronic infection through subversion of the host immune response (42, 43). In host macrophages, *M. paratuberculosis* inhibits phagosome maturation (44) to promote its intracellular survival and alters cellular signaling to inhibit the normal bactericidal activity of the host cell (45). Inhibiting interferon gamma (IFNγ) expression and signaling

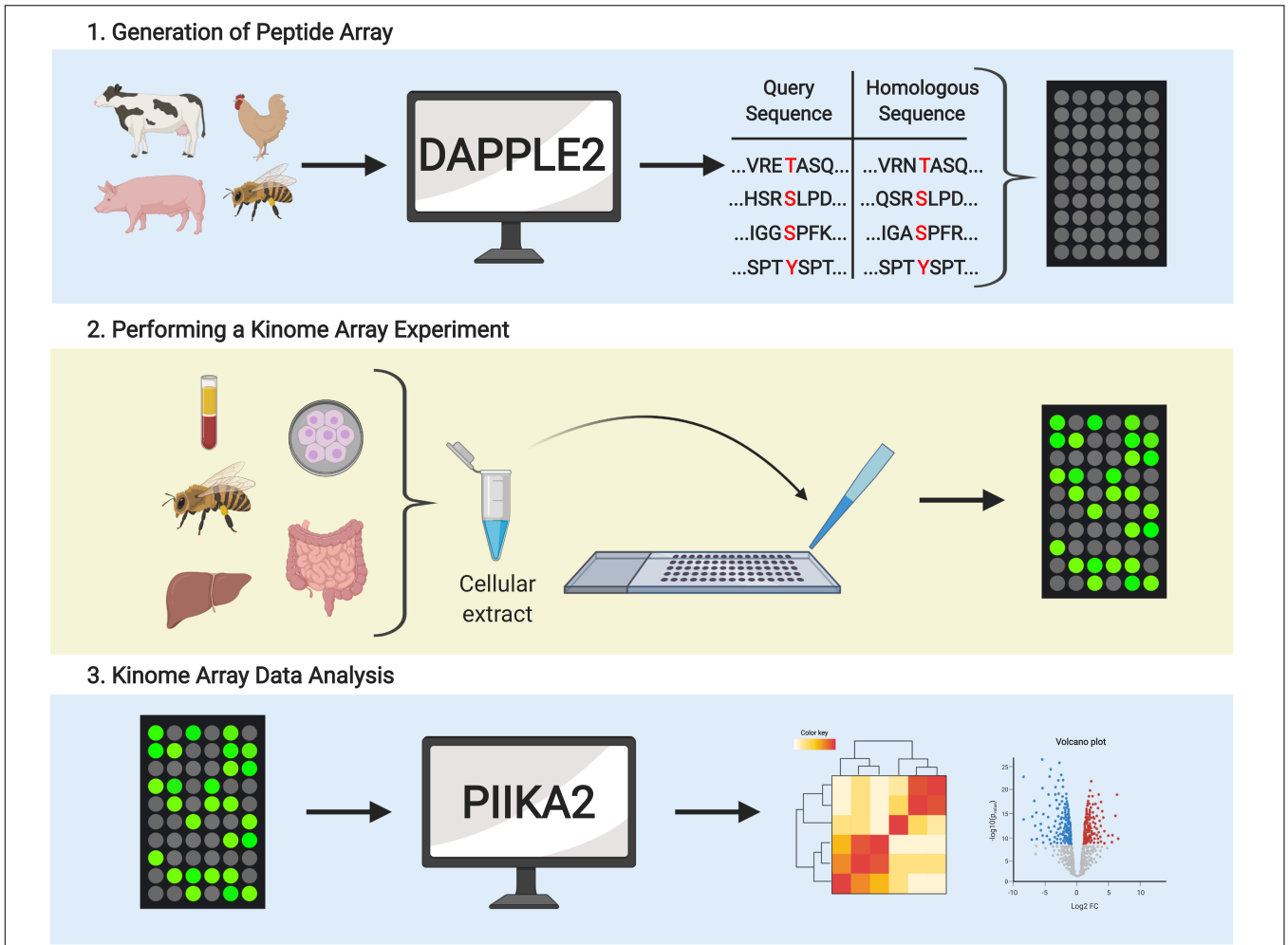

FIGURE 3 | Overview of the kinome array workflow. Schematic representation of the three main components of the kinome array pipeline. **(1)** Computational workflow (DAPPLE2) for predicting putative phosphoacceptor peptides for spot printing on peptide microarrays. **(2)** Representation of the various cells, tissues and whole organisms in which cellular extracts can be prepared to quantify kinase-mediated phosphorylation using peptide arrays. **(3)** Computational workflow (PIIKA2) for transforming, statistical analysis and graphical visualization of kinome array data.

is of central importance to intracellular pathogens, including *M. paratuberculosis*, to evade cell-mediated immunity (43, 46). A number of pathogens including *Trypanosoma cruzi* (47), *Leishmania donovani* (48), and *Mycobacterium avium* (49) block IFNγ responsiveness by dampening the expression of the IFNγ receptor. Induced expression of suppressor of cytokine signaling (SOCS), a key regulator in the IFNγ signaling pathway, has also been observed following infection with various pathogens including *Toxoplasma gondii* (50), *Burkholderia pseudomallei* (51), and Group A *Streptococcus* (52). IFNγ treatment of macrophages prior to *M. paratuberculosis* infection promotes their ability to clear infection, but the same treatment is ineffective post-infection (53, 54). This suggests that *M. paratuberculosis* infection desensitizes infected cells to IFNγ stimulation. Highly analogous to the situation with IFNγ, prophylactic stimulation of TLRs on macrophages prior to infection enhanced bactericidal activity against *M. tuberculosis*, but was ineffective post-infection (55); *in vivo*, *M. paratuberculosis* infected sheep show differential expression of TLRs suggesting this pathogen also targets these innate immune pathogen-recognition receptors to evade protective host responses (56). Among the earliest applications of the species-specific peptide arrays were two investigations to determine the extent and mechanisms by which *M. paratuberculosis* influences the responsiveness of bovine macrophages to both endogenous and exogenous activators of the innate immune response: IFNγ (57) and CpG-ODN (a TLR9 agonist) (58), respectively. Both investigations employed an infection model of primary bovine monocytes that enabled a homogeneous and biologically relevant cell population.

The responsiveness of uninfected and *M. paratuberculosis* infected monocytes was measured by induction of released cytokines: TNFα in response to IFNγ, and IL-10 in response to CpG-ODN. IFNγ stimulation of uninfected monocytes caused a dramatic release of TNFα while CpG-ODN stimulation induced the release of IL-10. By contrast, *M. paratuberculosis* infection

TABLE 2 | Bovine monocytes differentially respond to IFNγ and CpG-ODN depending on *M. avium* subsp. *paratuberculosis* infection status.

		Uninfected monocytes				*M. paratuberculosis*-infected monocytes			
		Up		Down		Up		Down	
	↕	#	*p* value	#	*p* value	#	*p* value	#	*p* value
IFNγ stimulation									
JAK-STAT signaling pathway	16	15	0.002	1	1	4	0.8	7	0.3
Gene expression of SOCS	6	6	0.03	0	1	1	0.9	3	0.3
CpG-ODN stimulation									
TLR signaling	18	14	0.02	4	1	2	1	8	0.06
Pyk2 signaling	10	3	1.0	7	0.07	9	0.01	1	1

Pathway analysis was completed using InnateDB (116). Functional enrichment and pathway prediction (p value) is based on the number of differentially phosphorylated proteins from the experimental dataset represented within the annotated pathway in the database. Reported in the table are the total number of differentially phosphorylated peptides (↕), when compared to uninfected control cells, belonging to each pathway including the total number of peptides (#) within that pathway that show increased (Up) and decreased (Down) phosphorylation (58).

of monocytes significantly diminished responsiveness to IFNγ and CpG-ODN. Kinome profiling of the uninfected monocytes indicated activation of signaling pathways classically associated with each ligand; JAK-STAT signaling in response to IFNγ and TLR signaling in response to CpG-ODN (**Table 2**) (57, 58). Infected monocytes, however, failed to induce JAK-STAT signaling responses indicating that *M. paratuberculosis* blocks IFNγ responsiveness at, or near, the IFNγ receptor (**Figure 4**) (57), and CpG-ODN induced signaling was redirected away from traditional TLR pathway into Pyk2-mediated signaling (**Table 2**). Further investigation revealed that as early as 1-h post-infection SOCS1 and SOCS3 expression significantly increased with subsequent decreased expression of the IFNγ receptor by 18 h post-infection. These data suggest that each of these events desensitized *M. paratuberculosis*-infected cells to IFNγ stimulation. In contrast to the complete repression of IFNγ-induced signaling, *M. paratuberculosis* infection redirected CpG-ODN signaling to an early intermediate of TLR signaling, Pyk2 (59). This redirection was confirmed through phosphorylation-specific antibodies as well as functional assays (58). As Pyk2 signaling had not been previously implicated in *M. paratuberculosis* infection this highlights the power of kinome technology for novel discovery.

Mycoplasma bovis (*ex vivo*)

Mycoplasma bovis (*M. bovis*) is responsible for a number of diseases of cattle including pneumonia, mastitis, arthritis, and abortion (60). *M. bovis* typically functions as a respiratory pathogen entering the host through lung epithelial cells and subsequently establishing residence within blood monocytes. Persistence of *M. bovis* within the monocytes affords the opportunity for protected dissemination throughout the host.

The mechanisms by which *M. bovis* establishes persistent infection of host immune cells had yet to be fully described. Given the success of kinome analysis in determining the mechanisms by which *M. paratuberculosis* achieves persistent infection of bovine monocytes, a similar *ex vivo* investigation was performed for *M. bovis*. One of the key findings of the kinome analysis was the implication by the kinome data that *M. bovis* sought to influence apoptosis through manipulation of the caspase system. Specifically, the signaling events induced by *M. bovis* were consistent with an anti-apoptotic outcome. Functional assays of both spontaneous and induced apoptosis confirmed the kinome results in that *M. bovis*-infected cells had decreased rates of spontaneous apoptosis as well as lower levels of induced apoptosis in response to pro-apoptotic stimuli (61). The influence of *M. bovis* on apoptosis was suggested as a mechanism to prolong bacterial survival as well as to enable dissemination of the pathogen throughout the host.

Salmonella (*ex vivo*)

Poultry is the most significant contributor to food-borne Salmonellosis in humans (62). Colonization of chickens with Salmonella results in a rapid (less than 4 h) inflammatory response that evolves into an asymptomatic, persistent infection during which time the bacterium is continuously shed in feces (63). This underscores the capacity of Salmonella to rapidly evade host innate immune defenses and persistently colonize the avian host without eliciting an active immune response. Understanding this host-pathogen interaction is essential for developing novel intervention strategies to eradicate infection especially as antibiotic-resistance and the restricted use of antibiotics in the poultry industry both continue to grow.

Kinome analysis has provided substantial contributions in understanding how Salmonella evades innate immune defenses and perturbs host cell signaling *in vitro* to gain the advantage. In one particular study, chicken macrophages were infected with *Salmonella* Enteritidis and *S.* Heidelberg for 1.5, 3, and 7 h to identify species-specific host responses (64). Kinome analysis indicated that phosphorylation events associated with lysosome and phagosome processes were significantly different between these two serovars, specifically suggesting that *S.* Enteritidis more effectively alters these signaling pathways to evade host innate defenses. This finding is consistent with the greater intracellular survival of *S.* Enteritidis in chicken macrophages *in vitro* compared to *S.* Heidelberg (65). Pathway analysis of the differentially phosphorylated peptides in Salmonella infected macrophages also identified a number of common pathways upregulated by both serovars that are potentially involved in pathogen response and control including: increased dephosphorylation (i.e., activation) of inducible nitric oxide; activation of TLR4 and TLR5 pathways including many of the adaptor and intermediate signaling proteins involved in the signal cascade leading to NF-κB activation (**Figure 5**); and reduced phosphorylation (i.e., activation) of NLRP3 – a major hub in the inflammasome. Salmonella evasion of innate responses

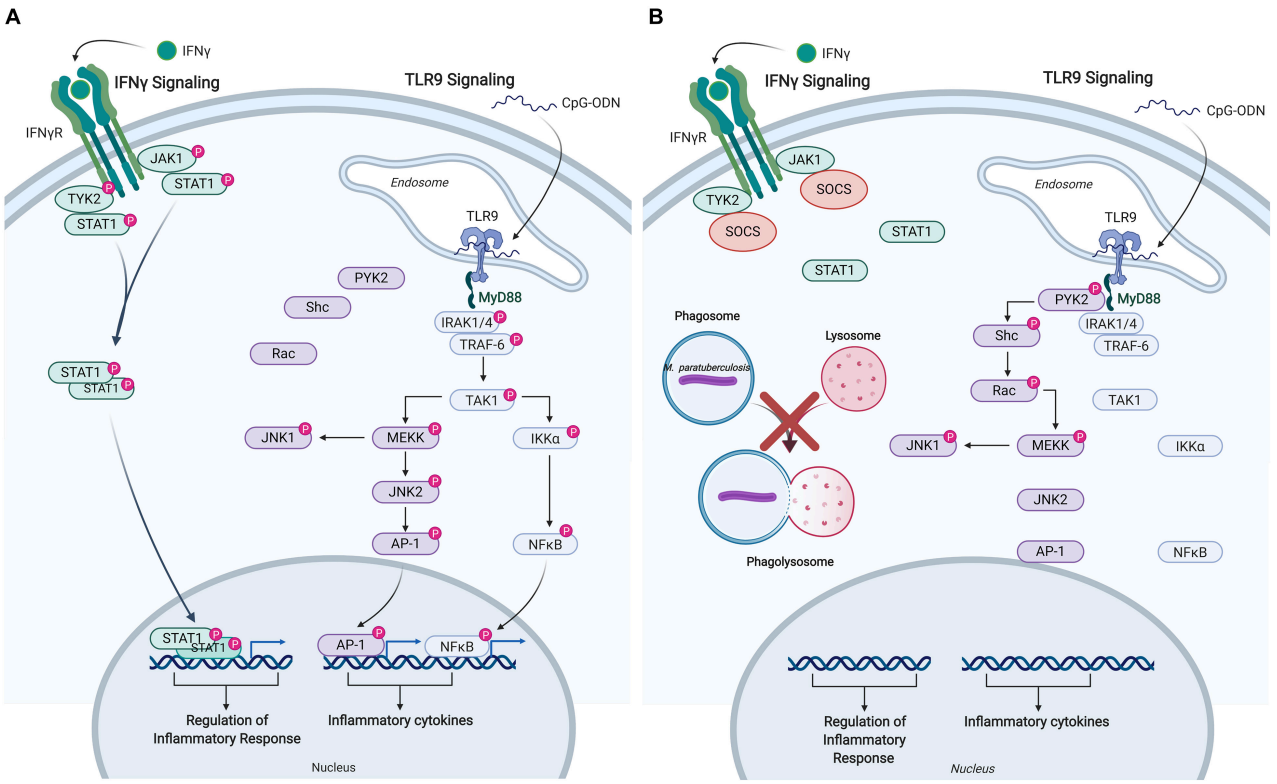

FIGURE 4 | Immune signaling pathways in bovine monocytes. Schematic representation of the IFNγ and TLR9 signaling pathways in response to exogenous stimuli and *M. avium* subsp. *paratuberculosis* infection. **(A)** Signaling pathways activated in uninfected bovine monocyte following stimulation with IFNγ, and TLR9 agonist CpG-ODN. Both pathways lead to the downstream phosphorylation of unique intermediates terminating with the translocation of distinct transcriptional activators into the nucleus to induce the expression of pro-inflammatory responses and cytokines. **(B)** *M. paratuberculosis* infection of bovine monocytes interferes with IFNγ signaling near the receptor by inducing the expression of SOCS, which disrupts JAK-STAT signaling. *M. paratuberculosis* infection also dysregulates TLR9 signaling by shunting the response toward the PYK2 pathway effectively blocking the induction of pro-inflammatory responses via MYD88. "P" designates protein in its phosphorylated state. Figure is adapted from Arsenault et al. (57, 58) and generated using BioRender. AP-1, adaptor protein complex 1; CpG-ODN, cytosine triphosphate-guanine triphosphate oligodeoxynucleotide; IFN, interferon; IKKα, inhibitor of nuclear factor kappa-B kinase subunit alpha; IRAK, interleukin-1 receptor-associated kinase 1; JAK, janus kinase; JNK, c-Jun N-terminal kinase; MEKK, MAPK/ERK kinase; MYD88, myeloid differentiation primary response protein; NFκB, nuclear factor kappa B; PYK, protein-tyrosine kinase; RAC, Ras-related C3 botulinum toxin substrate; SHC, Src homology 2 domain-containing-transforming protein; SOCS, suppressor of cytokine signaling; STAT, signal transducer and activator of transcription; TAK, TGF-beta-activated kinase; TRAF, TNF receptor-associated factor; TYK, non-receptor tyrosine protein kinase; TLR, Toll-like receptor.

in macrophages was most evident in the dephosphorylation (i.e., inactivation) of the adaptor protein caspase recruitment domain-containing protein 9 involved in mediating NOD-like receptor signaling leading to activation of cell apoptosis and the production of NF-κB, and the continued perturbation of mitogen-activated protein (MAP) kinases at all time points (**Figure 5A**). These findings provide the basis for developing and testing novel therapeutics that target these pathways and gene products to potentially boost innate immune defenses that restrict Salmonella intracellular persistence.

A follow up *in vitro* study by the same authors focused their analysis on phosphorylation targets associated with proteins in the calcium/calmodulin signaling pathway (66). Both *S.* Enteritidis and *S.* Heidelberg infection of chicken macrophages resulted in differential phosphorylation of peptides associated with calcium/calmodulin signaling pathway suggesting Salmonella dysregulates this pathway to promote its intracellular survival. This conclusion was further supported by the observation that treatment of chicken macrophages with a calmodulin inhibitor both inhibited nitric oxide production and promoted the intracellular survival of Salmonella. Taken together, these *in vitro* studies illustrate how kinome technology can be applied to investigate global changes in host cell signaling pathways to specifically identify mechanisms exploited by pathogens to evade host innate immune defenses, and those in which the host activates in an attempt to control infection.

KINOME ANALYSIS OF *IN VIVO* INFECTION MODELS

While the early kinome investigations of host-pathogen interactions prioritized *ex vivo* infection models, there was early evidence of the opportunity for consideration of samples of greater biological complexity. Specifically, that the pioneering investigation of peptide arrays described signaling responses

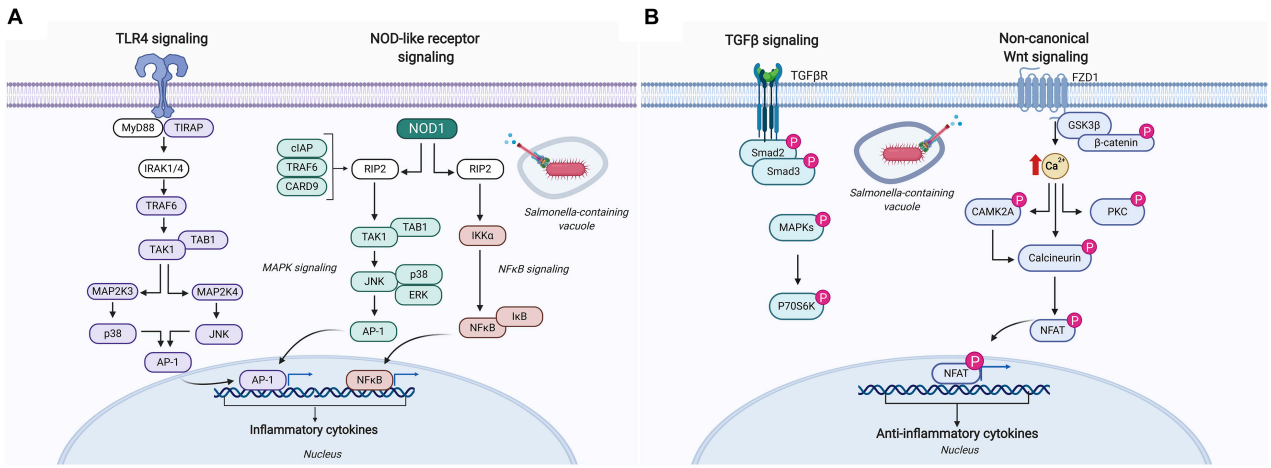

FIGURE 5 | Schematic representation of the immune signaling responses occurring in chickens infected with Salmonella. **(A)** Salmonella infection of chicken macrophages *in vitro* activates TLR4 and NOD-like receptor signaling pathways leading to the activation of unique transcriptional activators involved in inducing pro-inflammatory responses (64). **(B)** Kinome analysis of cecal tissue from Salmonella-infected chickens revealed differential phosphorylation of TGF-β and non-canonical Wnt signaling pathways suggesting a dampening of pro-inflammatory responses that supports Salmonella evading host immune defenses and establishing a persistent local infection (76). Figure is adapted from He et al. (64) and Kogut et al. (76); additional signaling responses have been characterized through biochemical analyses of TLR4, CARD9, and the NLRP3 inflammasome in bone marrow-derived macrophages and are described elsewhere (117–119). Protein identities in the figure that are colored (i.e., purple, blue, and pink) were identified as differentially phosphorylated ($p < 0.05$) (64, 76). Figure is adapted from KEGG Pathway Mapper (https://www.genome.jp/kegg/mapper.html), and generated using BioRender. "P" designates protein in its phosphorylated state. AP-1, adaptor protein complex 1; CAMK2A, calcium/calmodulin-dependent protein kinase type II subunit alpha; CARD, caspase recruitment domain-containing protein; cIAP, cellular inhibitor of apoptosis; ERK, extracellular signal-regulated kinase; FZD1, Frizzled-1; GSK3β, glycogen synthase kinase-3 beta; IκB, NF-κB inhibitor alpha; IKKα, inhibitor of nuclear factor kappa-B kinase subunit alpha; IRAK, interleukin-1 receptor-associated kinase; JNK, c-Jun N-terminal kinase; MAP, mitogen-activated protein kinase; MYD88, myeloid differentiation primary response protein; NFAT, nuclear factor of activated T-cells; NFκB, nuclear factor kappa B; NOD, nucleotide-binding oligomerization domain-containing protein; P70S6K, ribosomal protein S6 kinase beta-1; PKC, protein kinase C alpha type; RIP, receptor-interacting serine/threonine-protein kinase; Smad, mothers against decapentaplegic homolog; TAB, TAK1-binding protein; TAK, mitogen-activated protein kinase; TGFβ, transforming growth factor beta; TGFβR, TGFβ receptor; TIRAP, toll/interleukin-1 receptor domain-containing adaptor protein; TRAF, TNF receptor-associated factor.

within PBMCs (27). Encouraged by the results of the kinome analysis within *ex vivo* models, and emboldened by the results of the characterization in PBMCs, the technology was translated to *in vivo* infection models. The following examples represent kinome analysis of different biological matrices from *in vivo* infection models: PBMCs, intestinal biopsies, and muscle samples. Additionally, in the unique situation of investigating host responses of an insect to pathogenic challenge, whole organism kinome profiling is also described.

Mycobacterium avium subsp. *paratuberculosis* (Intestinal Samples)

The majority of cattle infected with *M. paratuberculosis* do not develop clinical Johne's disease. This indicates the ability of animals to mount a local immune response that controls the infection. The specific mechanisms by which these animals resist infection are not clearly defined. Understanding the molecular basis of a protective response would provide valuable guidance in the efforts to develop vaccines and therapeutics. It was hypothesized that differences in this host-pathogen interaction in the early stages of infection at the local site of infection in the small intestine determine the nature and efficiency of the induced immune response. To study the host-*M. paratuberculosis* interaction at the local site of infection in the bovine host, a novel intestinal segment model was employed (67). This intestinal segment model enables targeted delivery of a defined dose of a pathogen contained to a specific region of the gut (68, 69). In this study, intestinal segments were surgically isolated in the ileum, the site of persistent *M. paratuberculosis* infection. Additionally, uninfected intestinal segments were prepared proximal to the *M. paratuberculosis*-infected segments of the same animal serving as valuable intra-animal, syngeneic controls.

Kinome analysis was performed on samples from the uninfected and *M. paratuberculosis*-infected segments at 1-month post-infection. The datasets emerging from kinome analysis on these ileal intestinal samples clustered into two distinct groups, indicative of the occurrence of distinct cellular responses to *M. paratuberculosis*. These differences in signaling corresponded to innate immune and interleukin (IL-1, IL-4, IL-6, and TGF-β) signaling pathways as well as differences in the Wnt/β-catenin pathway. The distinct signaling responses to *M. paratuberculosis* at the site of infection were also reflected at the level of the organismal *M. paratuberculosis*-specific immune responses where the experimental animals could be classified into two distinct groups based on distinct antibody, T cell proliferation, and IFNγ responses (70). Most significantly, the distinct patterns of cell signaling anticipated the differences in the *M. paratuberculosis*-specific immune responses. Understanding the cellular mechanisms that determine

the balance between cell-mediated and antibody responses could be of considerable importance in the development of treatments for *M. paratuberculosis* as well as providing a novel method for rationale selection and/or design of mucosal vaccines and adjuvants.

In addition to the biological insight that was provided into *M. paratuberculosis* infection, this investigation represented a key step in the evolution of the application of kinome analysis for understanding host-pathogen interactions. By demonstrating that critical differences in signaling could be detected in response to stimuli of the intact animal motivated subsequent efforts to apply kinome to define responses occurring within the context of the intact host.

Bovine Viral Diarrhea Virus (PBMCs)

As previously described, many pathogens, in particular those that result in persistent infections, utilize immunosuppression as a significant component of their pathogenic mechanism. Within this, a common theme is to limit the ability of the infected host to produce, or respond to, interferons. Bovine viral diarrhea virus (BVDV) is responsible for some of the most significant losses to the global cattle industry (71). While BVDV causes persistent infection of cattle, there is debate of the extent and mechanisms by which the pathogen impacts host immune responses.

Bovine viral diarrhea virus strains cluster into two genotypically distinct clades, BVDV1 and BVDV2, with further sub-division of each genotype into cytopathic (cp) and non-cytopathic (ncp) phenotypic biotypes on the basis of their lytic activity to tissue culture epithelial cells (72). In general, cpBVDV strains are associated with the activation of IFN responses while there is less consensus on whether this is also true for ncp-BVDV strains (72–74). It is also important to keep perspective that manipulating the induction of these cytokines is just one possible mechanism by which ncp-BVDV could manipulate this aspect of the host immune response, that blocking the ability of the infected cells to respond to these signals, as observed for *M. paratuberculosis* and other pathogens, is another option to functionally negate this host immune response.

An investigation was conducted to determine the occurrence and functionality of interferon responses following the challenge of cattle with ncp-BVDV. There were three aspects to this characterization: (1) defining levels of interferon in response to challenge with ncp-BVDV, (2) kinome analysis of PBMCs from infected calves to investigate interferon-associated signaling, and (3) transcriptional analysis of interferon-regulated genes at time points corresponding to the IFNγ and IFNα responsive phases of acute BVDV infection. This collectively covers the induction of interferon release, the ability of these cytokines to induce signaling events within immune cells, and the functional consequences of these signaling events.

In response to the infection of cattle with ncpBVDV2-1373 there were significant increases in serum levels of both IFNγ and IFNα. The functionality of these responses was dually supported at the levels of both signal transduction and gene expression; there was clear evidence for activation of classic IFN-activated signaling pathways, as well as induced expression of IFNγ and IFNα regulated genes, within the PBMCs of the infected animals relative to the age-matched controls (74). Dampening of the IFNγ responsiveness of peripheral blood immune cells had also been proposed as an element of the pathogenic mechanism of BVDV (72) but kinome analysis of PBMCs from BVDV-infected cattle indicated activation of IFNγ induced signaling which was further confirmed through induced-expression of IFNγ regulated genes (74).

This paper was highly significant in demonstrating the ability to monitor host responses to pathogens within a cell population that is readily available for repeated sampling in a non-lethal fashion. As will be discussed later, PBMCs seem to hold tremendous potential for kinome investigations to determine responses to a number of stimuli as well as for the identification of phosphorylation-associated biomarkers.

Salmonella Infection of Chickens (Muscle Samples)

Salmonella enterica serovar Typhimurium (*Salmonella* Typhimurium) infection of young chickens results in asymptomatic colonization of the cecum accompanied by persistent fecal shedding. Despite the apparent disease-free state of these infected birds, it was hypothesized that the local colonization of the cecum has systemic effects influencing the physiology of the avian host. To address this, kinome analysis was completed on breast muscle collected from Salmonella challenged and uninfected broiler chickens to identify differentially phosphorylated peptides (75). Biological pathway analysis of the differentially phosphorylated peptides revealed that host metabolic pathways were significantly dysregulated in breast muscle during the early stages of infection (<3 weeks post-infection). Specifically, pathways associated with decreased energy currency (i.e., glucose metabolism, and intermediates shared between insulin and mTOR pathways), fatty acid metabolism (via AMPKα signaling) and immune-related pathways (i.e., Fc receptor and TLR signaling). Despite the apparent lack of clinical signs associated with Salmonella infection in these chickens, kinome analysis identified profound systemic effects of infection on skeletal muscle suggesting colonization negatively affects the physiology of the avian host with specific ramifications on meat quality.

A number of studies have used kinome technology to identify the cell signaling pathways exploited by Salmonella to support its persistence in the intestines of broiler chickens. In one particular study, cecal tissue collected from Salmonella-challenged broiler chickens revealed an up-regulation of the pro-inflammatory cytokine gene *IL-6* early in infection (48 h post-infection) that quickly regressed by 4 days post-infection as the anti-inflammatory cytokine gene *TGF-β4* was up-regulated and remained significantly higher at 7, 10, and 14 days post-infection (76). Kinome analysis was used to identify the signaling pathways and mechanisms responsible for this persistent local anti-inflammatory state. Cecal tissue extracts from Salmonella-infected and control chickens applied to chicken species-specific kinome arrays revealed significant kinase-mediated phosphorylation of peptides associated with

canonical Wnt/β-catenin, non-canonical Wnt/Ca^{2+}, and TGF-β signaling pathways at 4 days post-infection (**Figure 5B**). Closer investigation into individual phosphorylation events on the kinome array showed increased phosphorylation of nuclear factor of activated T cell (NFAT) peptides in addition to dephosphorylation of IKK and NF-κB suggesting Salmonella targets key host proteins to suppress the activation of pro-inflammatory cytokine responses thus promoting an anti-inflammatory microenvironment to support its persistence within this tissue (**Figure 5B**).

To further elucidate host immune signaling pathways elicited by the avian host following *Salmonella* Enteritidis infection, cecal tissue was collected from Salmonella challenged broiler chickens and extracts applied to chicken species-specific kinome peptide arrays (77). Differentially phosphorylated peptides at 4 days post-infection belonged predominantly to two immune-related pathways: T cell signaling and JAK-STAT pathways. Further characterization of these differential phosphorylated peptides led to the proposed mechanism whereby dephosphorylation of phospholipase c-G1 fails to activate either NF-κB or NFAT leading to the inhibition of local pro-inflammatory responses providing Salmonella with an immune privileged site to establish persistent infection. In addition to elucidating proposed mechanisms associated with immune evasion, kinome analysis has also helped identify host cell signaling responses associated with increased natural resistance to Salmonella infection. Intestinal tissue extracts from chickens categorized as high and low bacterial burden were applied to kinome peptide arrays and differentially phosphorylated peptides were comparatively analyzed. Pathway analysis revealed that intestinal tissue from chickens with low bacterial burden, when compared to high bacterial burden, up-regulated pathways associated chemokine signaling, FcεRI signaling, focal adhesion, insulin signaling, JAK-STAT signaling pathway, MAP kinase signaling, neurotrophin signaling, and T cell receptor signaling (78). These analyses suggest that early activation of these pathways at the local site of infection are associated with increased natural resistance to Salmonella infection in the avian host. Continued investigation and validation into these gene products and pathways will further advance our understanding of intracellular Salmonella infection in an effort to improve animal health and meat quality, and provide greater food safety to consumers.

Varroa Mite Infestation of Honeybees

While not a traditional livestock species, honeybees are key contributors to food production with approximately a third of food crops depending on them for pollination. As such, there is considerable concern over the trend of worldwide declines in honeybee populations and health (79). Infestation by Varroa mites is typically regarded as the most detrimental threat to honeybee health. A current priority of the honeybee industry is to identify mechanisms and biomarkers of Varroa mite tolerance to inform breeding efforts toward this phenotype. To this end, a honeybee-specific peptide array was developed to enable investigation of kinome responses to Varroa mite challenge. The development of a peptide array for an insect represented a significant advancement in the prediction of phosphorylation sites in a species of interest. This was achieved through consideration of experimentally determined phosphorylation sites from a variety of species but perhaps most importantly, from *Drosophila melanogaster* which was the closest relative for which phosphorylation sites had been defined.

The application of this array to uninfested bees representing colonies of defined, differentially sensitivities to Varroa mite infestation revealed unique signaling profiles between bees of the two phenotypes (80). That is to say, the differences in phenotypes were reflected at the level of whole organism signaling profiles, supportive of the potential to use these differences as biomarkers to guide breeding efforts. Furthermore, bees of the different phenotypes demonstrated distinct signaling responses to Varroa mite challenge. Gene ontology analysis of the peptides which were differentially phosphorylated between the bees of the two phenotypes indicated that the distinct susceptibilities to Varroa mite infestation did not reflect compromised immunity within the uninfested Varroa mite susceptible bees. Instead, there was evidence that mite infestation results in immune suppression specifically within bees of the susceptible phenotype. This immunosuppression increases the susceptibility of these bees to secondary viral infections, including those carried by the Varroa mites. The demonstration of more diverse viral infections in mite-infested, susceptible adult bees would seem to support this hypothesis (80).

KINOME ANALYSIS FOR IDENTIFICATION OF ANTIMICROBIAL THERAPEUTICS

While many kinase inhibitors used in the context of cancer chemotherapies, there is emerging appreciation of the potential to repurpose licensed kinase inhibitors, including as antibiotics and antivirals (81). This includes, but is not limited to, pathogens that directly impact host signaling through the use of kinase effector molecules. In these instances, there is the opportunity to use kinase inhibitors designed for bacterial kinases as antimicrobials (82). As previously mentioned, the BKIs which are specific for parasitic targets are under active investigation for the treatment of a number of human and veterinary infections. It is also possible to use kinase inhibitors to impact host signaling to promote the clearance of a pathogen. For example, imatinib, an FDA approved chemotherapeutic kinase inhibitor, facilitates clearance of *Mycobacterium tuberculosis*, the causative agent of tuberculosis, from a human fibroblast cell line (83).

Kinome analysis of host-pathogen interactions also has the potential to identify signaling pathways impacted by infection which, when acted upon through kinase inhibitors, have the potential to promote more effective clearance of the pathogen. For example, kinome analysis of *M. paratuberculosis*-infected monocytes indicated that the pathogen redirects TLR signaling through the Pyk2 pathway, an action that would seem to serve the benefit of the pathogen. Based on this hypothesis, Pyk2

inhibitors were investigated as potential therapeutics of this chronic infection. Consistent with the hypothesis, treatment of *M. paratuberculosis*-infected cells with Pyk2 inhibitors promoted clearance of the pathogen (58).

In a pair of investigations of high consequence pathogens, kinome analysis was applied in an effort to identify therapeutic targets. Kinome analysis of human hepatocytes to Ebola infection identified VEGF signaling as a critical component of the pathogenic mechanism. Treatment of cells with VEGF inhibitors reduced viral loads in tissue culture models as well as reducing lethality in a mouse model of Ebola infection. In this model, the VEGF inhibitors were more effective as prophylactics than as treatments with 50 and 20% reductions in lethality respectively (84). In a second kinome study, investigations performed on human monocytes in response to Monkeypox infection identified phosphorylation events associated with Akt specific to a more pathogenic clade of the virus (Congo Basin MPXV) as compared to Western African MPXV, which has lower rates of lethality. The use of kinase inhibitors to Akt phosphorylation resulted in a significant reduction in viral titres of Congo Basin MPXV but, as predicted by the kinome data, did not impact viral replication of Western African MPXV (85). Given the potential for kinome analysis to rapidly translate into potential therapeutics, including the repurposing of licensed therapeutics, it is not surprising that the technology has been incorporated for characterizing high consequence pathogens (86).

While kinase inhibitors are unlikely to be used in the treatment of livestock, the identification of host responses associated with effective clearance of the pathogen may enable the development of alternative therapeutic approaches, such as vaccines, which are more amenable to livestock. These examples also highlight the potential to apply kinome analysis to animal models of human diseases, or diseases causing co-infection of animals and humans, to the identification of potential human therapies.

KINOME ANALYSIS OF RESPONSES OF LIVESTOCK TO STRESS

Livestock are exposed to a multitude of stressors during routine industry practices like weaning, shipping, and restraint. There is a growing appreciation within the livestock industry of the negative consequences of these stresses. Stress decreases milk production, decreases weight gain, and compromises meat quality and increases susceptibility to, and severity of, infectious disease (87). With implications for animal health, well-being and productivity, minimizing animal stress through improved animal management procedures and/or selective breeding is becoming a priority to the livestock industry. Effective management of stress, however, depends on the ability to identify and quantify the effects of various stressors and determine if individual or combined stressors have distinct biological effects.

Responses of Cattle to Restraint Stress

Cattle are commonly restrained during routine handling practices such as vaccination, therapeutic intervention, and transport. Restraint can elevate plasma cortisol (88), heart rate, and breathing rate (89). As not all animals respond equally to restraint stress, there is a desire to better understand the molecular basis of the stress responses as well as to identify biomarkers that anticipate maladaptive responses to stress.

In an effort to fully describe the range of response to restraint stress, cattle were subject to repeated episodes of brief (5 min) restraint and evaluated for behavioral (chute entry order, chute behavior, and exit velocity), physiological (serum cortisol), and biochemical (kinome) responses. Based on serum cortisol levels (the traditional biomarker of stress responses) subgroups of animals representing the extremes of stress response were identified. Kinome profiling of PBMCs collected from these animals following a restraint episode revealed distinct signaling events between the high and low cortisol responders. These signaling patterns anticipated differences in apoptosis and carbohydrate metabolism between the two phenotypes, biological differences which were validated through independent techniques (90). In particular, the kinome data anticipated a shift toward the anabolic stage of glycogen metabolism in the high stress responding animals, a finding that was verified by elevated serum glucose levels as well as depleted glycogen stores in the animals of this phenotype. Most importantly, serum glucose provided a reliable, inexpensive indicator of serum cortisol levels and often had greater predictive value than cortisol for stress-related behavioral responses (90).

Responses of Chicken to Heat Stress

Livestock are often exposed to environmental conditions that impact their health and well-being. For example, during mass transport poultry are subject to conditions that can result in significant fatalities as well as compromising the health and meat quality of the surviving animals (91). Much of this reflects extremes of temperature that can exist within shipping containers, in particular during transport in harsh climates. As a consequence of the positioning of the heating and cooling systems the front of the container often results in temperature extremes at the front and back of the container. Each of these extremes of temperature can have negative consequences on animal health.

To identify the molecular mechanisms underlying these changes, a species-customized peptide array was created for kinome analysis of chickens. This array was designed with a specific priority to include representation of phosphorylation events with central roles in regulation of metabolism. As different regions of the body have unique responses and susceptibilities to thermal stress, kinome analysis was performed on breast and thigh muscle in response to both hot (+35°C) and cold (−15°C) stress temperatures that mimic those which are often experienced during transport. Initial evaluation of meat quality following stress treatments revealed cold stress, compared to heat stress, was more detrimental to meat quality as evident by increased pH_u, water binding capacity, darker color and lower glycolytic potential – these effects were more pronounced in the thigh than the breast muscle. Subsequent kinome analysis revealed tissue-specific phosphorylation events occurring in breast and thigh muscle (92). Specific to breast muscle, pathway analysis revealed the activation of ErbB signaling pathway in response to cold

stress, a pathway implicated as having cytoprotective effects in various animal models and associated with muscle repair and cell survival. This finding was consistent with the moderate effect of thermal stress observed in breast tissue. Conversely, thigh muscle, which showed extensive changes in meat quality following cold stress, resulted in the activation of innate immune response and TGF-β signaling, pathways commonly associated with tissue damage and repair responses. Collectively, this study offers insight into the unique susceptibilities, as well as functional consequences, of thermal stress of these tissues.

A further important outcome of this analysis was the observation that samples from different muscle types had distinct signaling profiles, even in the absence of thermal stress. This contributes to, and adds depth to, the emerging hypothesis that distinct phenotypes are often reflected at the level of the kinome; that distinct signaling profiles (kinotypes) exist across species, between individuals of the same species, and within different tissues of the same individual (93). This was later supported by an investigation of cattle which demonstrated distinct patterns of kinome activity within adjacent, but functionally distinct, regions of the intestine (94). Further to this, comparative kinome analysis of $CD21^{+}$ B cells obtained from two anatomically distinct sites (i.e., intestinal Peyer's patches and blood) revealed significant differences in signaling profiles as well as offering mechanistic insight into critical functional differences between these populations (94).

FUTURE DIRECTIONS

Technological Limitations (Software)

Even with the development of computational tools customized specifically for kinome data analysis, a few technical challenges still need to be addressed. Kinome arrays can create a large amount of statistical noise, due to a low signal-to-noise ratio, that may interfere with the results. Currently, this is mitigated through the use of normalization techniques such as variance stabilizing normalization, however no technique can remove all statistical noise. Advancements in normalization methods can be integrated into the kinome analysis pipeline but many features need to be specifically tailored to the unique characteristics of kinome data. A number of key sources of noise that affect kinome arrays have been identified.

A large degree of variance can be seen across regions of a peptide microarray. One source of such variance causes increased foreground and background signal at the bottom of the array resulting in spatial bias. This is a systemic bias as a result of uneven distribution of biological sample applied to the array and/or the stain used to bind and detect phosphorylated residues. There are methods that sharply reduce this variance that have been implemented in PIIKA. However, there is location-based variance between the edges and the center of the array, and variance between replicate blocks that currently cannot be corrected for as easily.

While the development of PIIKA has been a large improvement from using DNA microarray technology for kinome data analysis, there remains the necessity for using DNA microarray software for image analysis as no kinome-specific technology has been created to date. This leads to issues regarding the alignment of the spots on the microarray. For now, a large degree of manual alignment is required for kinome arrays that is done relatively automatically for DNA microarrays. This is not only a large investment of time, especially in experiments with a large number of arrays and replicates, it is also subject to error. The outcome of this type of error can be categorized by the shift in the foreground mean relative to the foreground median. This indicates the mislabeling of pixels of the spots as foreground rather than background. This is then improperly corrected and causes the foreground pixels to be asymmetrically distributed as the majority of pixels in an improperly aligned spot are mislabeled, and ultimately results in a foreground mean that is much lower than the actual value. It is very possible that the mean values of many of the top hits are significantly lower than they should be and thereby not appearing in the results as a top hit.

The selection of the top hits in a kinome array must be done carefully, and with the amount of noise often present in the output it is necessary to apply statistical methods to normalize the data and remove background noise. However, different methods have vastly different outcomes, and with a plethora of possible tools, algorithms and techniques to choose from, it is unknown which methods translate more accurately to biological truth. This may not be consistent among different experiments and array conditions. There may be specific characteristics of arrays that favor different statistical methods. Further investigation into which techniques are best applied situationally is needed.

Technological Limitations (Hardware)

Currently, the majority of peptide array kinome efforts utilize either radioactivity or phosphorylation-specific stains for the detection of the signal on the arrays; neither of these options are ideal. Radioactive approaches are challenged by issues relating to safety and regulation while the stains can be problematic due to lack of specificity, including background staining of the arrays which complicates evaluation of the signal-to-noise ratios. Other detection methods should be investigated. This could include a variety of antibodies with general reactivity toward phosphorylated residues; either reactivity with modified serine and threonine residues, or serine, threonine, and tyrosine, or a combination of such antibodies. There are modified forms of ATP, such as gamma-modified ATP analogs, which are functionally analogous to the radioactive derivatives but with a basis of detection in fluorescent labeling (95). These ATP analogs might be effective for peptide array investigations, although the efficiency by which they are recognized and utilized by the various kinases would need to be carefully defined. At the very least, these alternatives for detection should be evaluated within a comparative experiment.

Realms of Application

Within the upcoming years there are a number of fields of investigation which would seem logical and strategy directions to apply the kinome, both in terms of organisms to be

considered, applications to understanding the mechanisms and efficiencies of treatments, and philosophies of application. While the information provided by DAPPLE2 enables the creation of customized peptide arrays for virtually any species, there are a couple of areas where there seems to particularly promising and strategic opportunities.

Kinome Analysis for Insects and Other Small Eukaryotes

The use of peptide arrays to define signaling events within the honeybee highlights the potential for the application of this approach to other insect species which are of environmental, economic, and scientific importance. In particular, the opportunity to conduct whole-organism kinome profiling to identify biomarkers and mechanisms of phenotypes could enable high-throughput screening efforts of insects. For example, this additional layer of information could be particularly useful for species, such as *D. melanogaster*, which have been extensively characterized through genomic approaches. As the phosphoproteome of Drosophila has been experimentally defined, creation of a highly reliable Drosophila-specific array would be a straightforward endeavor. The defined phosphoproteome of Drosophila also serves as an important resource for the creation of peptide arrays for other insect species of undefined phosphoproteomes, as was the case for honeybees (96).

In contrast to the distinct, phenotype-specific signaling profiles that were observed in whole organism kinome profiling of honeybees, within higher organisms there is clear evidence for distinct patterns of signaling within different regions of the body, and even within specialized compartments of the same region of the body. However, the lower levels of tissue specialization within smaller organisms may translate into a more homogenous signaling response throughout the organism. It would be interesting to investigate the extent to which whole organism kinome profiling can be effectively applied to other small eukaryotes, in particular those that serve as important research models, such as nematodes, would seem logical targets for future investigations.

Plants

Considerable efforts have been expended to define both the kinase complement (in terms of number and identities of kinases) and the phosphoproteomes of many plant species (97–99). As with animal species used for food production, infectious diseases and stress are also two major priorities affecting crop production. Kinome analysis could similarly be applied to define phenotypic traits associated with increased stress tolerance and susceptibility, in addition to better understanding plant immune defenses against the diverse range of pathogens they encounter. There has been considerably less effort toward global kinome profiling in plants, in particular through peptide arrays. There are two publications describing the application of peptide arrays to define kinome activity in the model organism *Arabidopsis thaliana* (100, 101). The peptide arrays utilized in these investigations were not customized to reflect the Arabidopsis phosphoproteome. While these investigations supply high-level proof-of-principle evidence of the opportunity to apply the technology to plants, it is very difficult to extract specific biology from this type of cross-species application of peptide arrays. Given the efforts expended to define plant responses through other omic approaches, as well as the success achieved in translating this approach to livestock, it would seem timely and appropriate to extend this approach to plants. In particular, the available plant phosphoproteomes databases can enable the creation of peptide arrays based on experimentally defined phosphorylation sites as well as serving as an effective starting point for the creation of arrays for plant species whose phosphoproteomes have yet to be defined.

MECHANISMS OF THERAPEUTICS

In addition to the potential to use kinome analysis to inform rational selection of therapeutics, there may also be opportunities to decipher the mechanisms of action of potential therapeutics. Such an approach could be applied to inform the rational selection, application, and refinement of these treatments. The ability of kinome analysis to offer nuanced information about host responses could also provide valuable correlates of protection to facilitate high-throughput screening of libraries of potential therapeutic agents and/or treatments. Specifically, there is an opportunity to apply kinome to investigate the modes of action for prebiotics, probiotics and postbiotics. There is rapidly growing appreciation of the importance of the microbiome to human and animal health. With this, there is emerging priority to manipulate the commensal bacterial environment through the use of prebiotics, probiotics, and postbiotics. This is not a trivial task. The contributions of the microbiome reflect complex, dynamic interactions between the host and associated microbes. More specific information on the mechanisms and consequences of action of these interactions would enable rational application and refinements of these treatments, including indicators to define therapeutic benefits.

Prebiotics

Prebiotics are non-digestible sugars that are utilized to promote the establishment of a healthy microbiome. The traditional view is that prebiotics function by promoting the growth of beneficial gut microbes, independent of any direct effects on the host. An investigation was conducted to determine the occurrence and consequences of direct impacts of prebiotics on the intestinal mucosa. Treatment with two commercial prebiotics, inulin and short-chain fructo-oligosaccharide, in the absence of microbes, had beneficial responses by promoting intestinal epithelial integrity to limit barrier disruptions by pathogenic intestinal microbes. These outcomes were achieved through the induction of select tight junction proteins via a mechanism involving activation of protein kinase C signaling (102). In addition to specific information of the action, mechanisms, and consequences by which these prebiotics exert barrier protective effects on the intestinal epithelium, this study also challenged the paradigm that the action of prebiotics was limited to, and dependent on, microbial influence. Shifting this

perspective will enable new opportunities for the selection and refinement of prebiotics.

Postbiotics

A major contributor to the evolution of antibiotic resistance is their overuse as growth-promoters in livestock species such as poultry. Probiotics present an attractive alternative to populate the alimentary tract with beneficial microbes to improve overall health. Postbiotics provide products derived from probiotics to elicit and prime beneficial host immune responses. Kinome analysis was recently used to understand how postbiotics administered to *Clostridium perfringens*-challenged broiler chickens altered the intestinal microenvironment to contribute to reduced lesion scoring, lower bacterial loads and mortality (103). Comparative analysis of intestinal tissue extracts from chickens administered the postbiotic alone showed very few peptide phosphorylation events on chicken species-specific kinome arrays when reacted with duodenal tissue extracts, but extensive kinase-mediated phosphorylation events when reacted with jejunal tissue extracts. Further analysis of jejunal tissue revealed postbiotic treatment alone impacted peptides associated with innate immune pathways. By contrast, jejunal tissue extracts from chickens challenged with *C. perfringens* alone revealed kinase-mediated phosphorylation of peptides associated with T cell receptor signaling, natural killer cell mediated cytotoxicity, and the Fc epsilon receptor I signaling suggesting the induction of adaptive immune pathways. In chickens challenged with *C. perfringens* after receiving the postbiotic treatment pathway analysis revealed, unexpectedly, activation of overall fewer immune-related pathways in jejunal tissue as compared to either postbiotic administration alone or *C. perfringens* challenge alone.

These data suggest that postbiotics induced an immune-modulating effect in the jejunum of broiler chickens resulting in an altered intestinal microenvironment capable of controlling *C. perfringens* infection while, as importantly, maintaining gut barrier and function. Further investigation into this balanced immune response will provide critical information on understanding how mucosal surfaces can control infection while maintaining a homeostatic (i.e., anti-inflammatory) state. Moreover, this study shows that kinome technology can provide a novel, and complementary, approach in understanding the mode of action for these alternative, antibiotic-independent therapeutics.

Correlates of Immune Protection

Researchers typically focus on a very limited number of correlates of immune protection for screening vaccine antigens. However, chronic infectious diseases, in particular, would benefit from a more comprehensive assessment of immune responses both during infection and in response to vaccines. As an example, there is clear evidence that IFNγ is a key cytokine contributing to control and clearance of mycobacterial infection. However, *in vivo*, IFNγ has not been a reliable correlate of immune protection for either *M. paratuberculosis* in bovine Johne's disease (104, 105) or *Mycobacterium bovis* in bovine tuberculosis (106). Thus, employing IFNγ as the sole readout of immune protection when screening for potential vaccine candidates could negate a number of promising candidates. Understanding the processes leading to IFNγ induction, and the subsequent consequences, would create a better understanding of immune protection and lead to better selection of immune correlates when screening vaccine antigens.

Phenotype-Driven Kinome Profiling

Thus far, kinome profiling has typically been applied to describe signaling events in response to a defined stimulus. There is, however, the opportunity to adapt a "phenotype first" approach that has been highly successful for other omic approaches. That is, to identify and investigate phenotypically distinct sub-groups of a population in an effort to identify molecular biomarkers and mechanistic insight into those differences. The example of describing mechanisms of Varroa mite tolerance within colonies of honeybees of distinct phenotypes highlights the potential of this approach for kinome investigations. Within the context of livestock applications such biomarkers could function to guide breeding efforts while in the context of human health applications to differentiate signaling events within healthy and disease-associated individuals.

PERIPHERAL BLOOD MONONUCLEAR CELLS

The first use of peptide arrays for global kinome profiling was to describe signaling events within human PBMCs in response to *ex vivo* stimulation with LPS (27). Since this foundational work PBMCs have remained a convenient and informative cellular population for describing responses to a variety of stimuli but with a shift from *ex vivo* to *in vivo* stimulations. A critical advantage of PBMCs is the opportunity to collect samples pre- and post-treatment which provides a valuable control to minimize the contributions of the individual-specific kinome profiles. For example, recent efforts for kinome profiling of human PBMCs have investigated responses to acute stress in the form of bungee jumping (107) while another investigation of human PBMCs facilitated investigation of signaling responses activated by consumption of marijuana (108). Important phenotypic differences between individuals, in particular with respect to immune function, may also manifest in unique signaling profiles of PBMCs.

INTEGRATION WITH OTHER OMICS

As the respective omic disciplines evolve and refine there is the overarching priority for more systems biology perspectives that integrate cellular responses across a range of biomolecular levels. To date there has been minimal effort to attempt to integrate peptide array kinome data with the outputs of other omic approaches, at least on a global scale.

From a more focused perspective, gene expression data has often been used to verify the results of kinome studies. For example, the observation of activation of interferon-associated signaling pathways was shown to coincide with elevated expression of interferon-regulated genes, highlighting

the potential for cohesion between transcriptional and kinomic responses. Other examples can highlight a seeming disconnect between transcriptional and kinomic responses; *M. paratuberculosis*-infected bovine monocytes had elevated levels of expression of a receptor but decreased signaling through the associated pathway. The information from each of these techniques is not actually contradictory and these differences instead highlight a key biological mechanism that occurs within the functional realms that separate gene expression and signal transduction. Efforts to merge kinome datasets with those from other omics should look for not only supporting evidence where the same biology is suggested at each level of investigation but also where the results would appear in contradiction with each other may suggest important points of regulation. The implication of the same biology, independent of the implied direction of change, activation or inhibition, nevertheless still implicates the involvement of that process.

CONCLUDING REMARKS

In the decade since the development of the first species-specific peptide array there have been a wealth of publications that demonstrate the value of kinome analysis for investigations of livestock species. There has been a consistent evolution in the tools supporting and enabling kinome analysis which have enabled a consistent progression of the technology and the complexity of the biology which is addressed. The technology has not, however, achieved a point where it has been widely incorporated into research programs. Instead, there is the trend toward relatively small groups of labs applying the technology through collaboration, organized research groups within large organizations, and as fee-for-service opportunities, both by commercial companies as well as units within academia (University of Delaware Kinome Center).

The coming years will likely see the expansion of the peptide arrays into new spheres of application including the characterization of insect and plant species. Within these realms, there will be greater opportunities to apply the technology to samples of defined genetic and phenotypic diversity that will strengthen the relationship between kinotypes and phenotypes.

Ironically, while the initial applications of peptide arrays to livestock were inspired by advances in kinome science within human health realms, the advances which have been made during efforts to apply the technology to livestock are now increasingly being applied within human health applications. This reflects both the advances in the software for analysis of kinome data but also by facilitating the development of peptide arrays that are customized for consideration of specific biology. This is a healthy and desired evolution of the technology as the emergence of kinome data from a variety of species will enable greater opportunities to consider phosphorylation-mediated signal transduction from species-comparative, evolutionary perspectives. This will undoubtedly include emphasis on how the presence and absence of specific kinases and phosphorylation sites across species impact the phenotypic characteristics of the organism that may serve as an important evolutionary selection pressure.

AUTHOR CONTRIBUTIONS

All authors contributed to writing and preparing the manuscript.

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Assessment of Immunological Response and Impacts on Fertility Following Intrauterine Vaccination Delivered to Swine in an Artificial Insemination Dose

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To protect the health of sows and gilts, significant investments are directed toward the development of vaccines against infectious agents that impact reproduction. We developed an intrauterine vaccine that can be delivered with semen during artificial insemination to induce mucosal immunity in the reproductive tract. An *in vitro* culture of uterine epithelial cells was used to select an adjuvant combination capable of recruiting antigen-presenting cells into the uterus. Adjuvant polyinosinic:polycytidylic acid (poly I:C), alone or in combination, induced expression of interferon gamma, tumor necrosis factor alpha, and select chemokines. A combination adjuvant consisting of poly I:C, host defense peptide and polyphosphazene (Triple Adjuvant; TriAdj), which previously was shown to induce robust mucosal and systemic humoral immunity when administered to the uterus in rabbits, was combined with boar semen to evaluate changes in localized gene expression and cellular recruitment, *in vivo*. Sows bred with semen plus TriAdj had decreased γδ T cells and monocytes in blood, however, no corresponding increase in the number of monocytes and macrophages was detected in the endometrium. Compared to sows bred with semen alone, sows bred with semen plus TriAdj showed increased CCL2 gene expression in the epithelial layer. These data suggest that the adjuvants may further augment a local immune response and, therefore, may be suitable for use in an intrauterine vaccine. When inactivated porcine parvovirus (PPV) formulated with the TriAdj was administered to the pig uterus during estrus along with semen, we observed induction of PPV antibodies in serum but only when the pigs were already primed with parenteral PPV vaccines. Recombinant protein vaccines and inactivated PPV vaccines administered to the pig uterus during breeding as a primary vaccine alone failed to induce significant humoral immunity. More trials need to be performed to clarify whether repeated intrauterine vaccination can trigger strong humoral immunity or whether the primary vaccine needs to be administered via a systemic route to promote a mucosal and systemic immune response.

Keywords: breeding, pigs, estrus, mucosal vaccine, uterus, semen, adjuvant

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INTRODUCTION

Mucosal vaccination of livestock has the potential for several benefits over classical parenteral vaccinations, including the initiation of a strong mucosal and systemic immune response (1, 2) while reducing the incidence of common needle-stick injuries by veterinarians (3). However, several challenges need to be overcome in order to generate a successful mucosal immune response including avoiding vaccine elimination by the flow of mucosal fluids across mucosal surfaces, recruitment of antigen presenting cells (APCs), and targeting of the vaccine toward APCs (4). Mucosal surfaces are primed to induce a tolerogenic response toward antigens thereby limiting the reaction to microflora, food, and environmental particles (5). Currently, no studies have identified a commensal flora in the upper reproductive tract of pigs, which may mean that the porcine uterus may be less predisposed to a tolerogenic bias to antigens encountered at its surface. In fact, studies in rats and rabbits have shown that the uterus may be a suitable immunization site as vaccines delivered to the uterus triggered a measurable antigen-specific systemic and local humoral immunity (6–8). Because a number of economically important diseases in pigs such as porcine parvovirus (PPV) and porcine reproductive and respiratory syndrome virus (PRRSV) impact reproduction, it may be very beneficial to have a mode of vaccine delivery that triggers a strong mucosal immune response in the uterus to protect growing fetuses (9). For livestock systems that use natural breeding, the uterus is not readily accessible for immunization. However, because the majority of commercial pigs are bred by artificial insemination (AI) (10), current husbandry practices allow routine access to the uterus during each reproductive cycle.

Adjuvant facilitate uptake of the antigen across the epithelial barrier, recruitment of APCs, activation of APCs, and they protect the antigen from degradation (1). One or several of these mechanisms of action may be required to generate a successful mucosal vaccine response and, therefore, the inclusion of multiple adjuvants may be necessary for an effective vaccine formulation (11). Certain mucosal surfaces have specialized epithelial cells such as M cells, which are efficient at sampling and delivering antigens to underlying immune cells and these cells can be targeted by adjuvants (4, 12). Although the uterine epithelia has no known specialized epithelial cells or canonically organized lymphoid tissue, it contains a multitude of epithelial cells and both luminal (13) and subepithelial lymphocytes (14). Thus, vaccine formulation and delivery need to be directed toward normal epithelial cells or at immune cells recruited to the uterine lumen or tissue.

The following study aims to determine which adjuvant components and combinations can generate an immune response in uterine epithelial cells (UECs). Additionally, we seek to determine if the inclusion of adjuvants in a semen dose modulates the uterine immune response to sperm and what role, if any, the UECs play in this response. Finally, we investigate whether delivering a vaccine during AI triggers an effective immune response in pigs. It is critical that any intrauterine vaccine administered during breeding does not have a negative effect on fertility or piglet growth kinetics.

MATERIALS AND METHODS

The majority of these methods are previously described in the thesis by Hamonic, University of Saskatchewan (15) and are presented here with permission.

Animal Ethics

All experimental procedures were conducted in accordance with the guidelines of the Canadian Council on Animal Care (CCAC) under approval from the Animal Research Ethics Board at the University of Saskatchewan. Pigs were Landrace/Large White from Prairie Swine Centre, Inc. (PSC), a High Health herd that is free from porcine reproductive and respiratory syndrome virus, *Mycoplasma hyopneumoniae* and swine influenza virus. Pigs were housed in stalls for the duration of the experiments.

Animal Trials and Sample Collection

Adjuvant Trial

Single parity sows were synchronized following a fixed-time AI protocol (16) prior to post-cervical insemination (**Supplementary Figure 1**). In brief, pigs were synchronized by oral progestin (Regu-mate; Merck Animal Health, USA) (17). Twenty-four hours after the final dose of oral progestin, pigs received 800 international units of pregnant mare serum gonadotrophin (Folligon; Merck Animal Health, USA) by intramuscular (i.m.) injection. Eighty hours later, pigs were given 5 mg porcine pituitary luteinizing hormone (Lutropin-V; Bioniche Animal Health, Belleville, ON) by i.m. injection (16). Thirty-two hours post-Lutropin-V injection, pigs were bred using post-cervical insemination catheters (Megapor) with a semen dose mixed with 3.2 ml of phosphate-buffered saline (PBS; Sigma Aldrich, Oakville, ON, Canada) (mock control sows, $n = 3$) or a standard semen dose containing 4 mg poly I:C (Invivogen, San Diego, CA, USA), 8 mg Host defense peptide 1002 (HDP, Genscript, Piscataway, NJ, USA), and 4 mg polyphosphazene (PCEP; Idaho National Laboratory, Idaho Falls, ID, USA) in 3.2 ml of PBS (TriAdj sows, $n = 4$). Adjuvants were administered into the opened semen bag then mixed by gentle inversion prior to being attached to the catheter for breeding. Sows were euthanized by captive bolt 24 h post-breeding and exsanguinated to allow necropsy of the reproductive tract and collection of uterine lavage. Small sections of tissue were collected from the cervix, lower uterine horn, mid uterine horn, upper uterine horn, ampulla, isthmus and ovaries for histology. Sections of the uterine horns were flash frozen in liquid nitrogen for RNA isolation and a duplicate section was frozen in Shandon cryomatrix (Thermofisher) for laser-capture microdissection collection.

Vaccine Trial 1

Sows used in this trial had previously received Porcine ParvoShield vaccine (Elanco Animal Health) by the i.m. route at each parity. The period between the last vaccination and the current intrauterine (i.u.) or i.m. immunization was at least 120 days. Sows were bred with semen alone or semen plus the vaccine (see below) using post-cervical catheters. Control sows ($n = 3$) received i.m. ParvoShield vaccine as they entered

into farrowing crates (day 100 gestation) and they remained at PSC. Sows that were subjected to i.u. immunization (n = 4) were brought to VIDO-InterVac (Saskatoon, SK, Canada) prior to the start of the trial. The i.u. vaccine was comprised of 1×10^7 $TCID_{50}$ BEI-inactivated PPV (NADL-7; American Type Culture Collection) along with 400 μg poly I:C, 800 μg HDP, and 400 μg PCEP adjuvants (TriAdj) in 1 ml total volume, which were administered to the semen bag immediately prior to breeding. Sows were heat-checked twice daily after weaning by experienced personnel looking for a standard lordosis response following exposure to 5-α-androstenone (Hog-Mate; Reproduction Provisions, Inc., Walworth, WI, USA). Sows were inseminated with the AI dose alone or plus the vaccine 12 h after the first detection of lordosis (day 0) and then bred every 24 h with semen alone for the duration of the standing estrus. Blood was collected at day 0, 15, and 30 and then the i.u. vaccinated sows were humanly euthanized by captive bolt and exsanguination at day 30 post-vaccination. Reproductive tracts were externalized, the number of viable embryos in each uterine horn was recorded, and *corpus luteum* (CL) were counted as a measure of ovulation. Each fetus was visually inspected to establish whether they appeared viable to time of sow death.

Vaccine Trial 2

Gilts were administered oral progestin (Regu-Mate) for 14 days and then heat checked by experienced personnel using mature boars. Gilts were bred at the first sign of standing estrus by conventional AI with a standard semen dose with or without the vaccine and then every 12 h after with semen dose alone. The i.u. vaccine was comprised of 400 μg recombinant (r)VP2-Trx protein [cloned, expressed, and purified in *E. coli* as detailed in (6)] plus 400 μg poly I:C, 800 μg HDP, and 400 μg PCEP in 1 ml total volume ($n = 7$ gilts). Mock-vaccinated gilts ($n = 9$) received the standard semen dose and they were administered ParvoShield vaccine i.m. when they entered into farrowing crates at day 100 gestation. Blood serum was obtained at day 0, 15, 30, 70, 90, and at weaning. Piglet weights were obtained at day 3 and at day 21 from 6 randomly reselected gilts per group.

Vaccine Trial 3

Gilts were bred by cervical AI with a standard semen dose alone (control gilts, n = 5) or semen mixed with a combination of 3 separate vaccines (treatment gilts, n = 8). The i.u. vaccines were formulated with a consistent adjuvant dose of 266 μg poly I:C, 533 μg HDP and 266 μg PCEP combined with either 400 μg recombinant porcine epidemic diarrheal virus (PEDV) Spike protein, 200 μg recombinant *Lawsonia intracellularis* (LI) FliC protein or 1×10^7 BEI-inactivated PPV. Recombinant FliC was purified from *E. coli* and rSpike protein was purified from HEK293 cells as detailed in Obradovic et al. (18) and Makadiya et al. (19), respectively. The control animals received i.m. injection with FarrowSure B Gold (Zoetis, Canada) to compare the anti-PPV vaccine response. Gilts were humanely euthanized after 30 days. The fetus viability relative CL numbers was presented as a ratio. The crown-rump ratio was measured using Image J and the average weight of the fetuses per litter was recorded.

PBMC and Luminal Cell Processing

PBMCs were isolated from blood collected using EDTA Vacutainers (BD Biosciences) then centrifuged at 1,100 × g for 30 min. The buffy coats were collected and layered onto Ficol-Paque plus (GE life sciences) and centrifuged at 400 × g for 40 min. The PBMC layer was collected, washed in PBS 3 times with centrifugation at 250 × g for 10 min and stained for immunotyping by flow cytometry (described below) or stained with CFSE and restimulated with vaccine antigens (described below). The uterine horns were removed from the sows and flushed with 25 ml PBS + 1% BSA (Sigma-Aldrich) per horn to collect luminal cell populations, which were counted and stained for immunotyping by flow cytometry analysis and to quantify CCL2 (see below).

Isolation, Culture, and Stimulation of Primary Uterine Epithelial Cells

Primary UECs were isolated from uterine tissue of gilts/sows collected from a local abattoir (n = 4) as described in detail in a previous study (20). Cells were polarized for 7–10 days as determined by stable 10x increase in transepithelial electrical resistance (TEER) with media changes taking place every second day. After cells achieved stable TEER, they were stimulated with 50 μg/ml poly I:C (Invivogen), 50 μg/ml lipopolysaccharide (LPS; *Salmonella enterica* serovar Minnesota from Sigma-Aldrich), 50 μg/ml CpG oligodeoxynucleotides (CpG 2395; Merial), 50 μg/ml muramyl dipeptide (MDP; Sigma-Aldrich), 100 μg/ml HDP (Genscript), 50 μg/ml PCEP (Idaho National Laboratory) or combined together in various combinations at the stated concentrations including as the triple combination adjuvant (TriAdj; poly I:C, HDP, PCEP). Six hours post-stimulation, cells were collected in Trizol (Invitrogen) for RNA extraction (described below).

Sperm Abnormality and Mobility

Sperm abnormality assessment was performed on extended semen (PIC, Kipling, SK) alone or including the vaccine components from Trial 2 (individually or combined), which includes 1×10^7 $TCID_{50}$ binary ethylenimine (BEI)-inactivated PPV, 400 μg Poly I:C, 800 μg HDP 1002 and 400 μg PCEP. Extended semen alone or with the vaccine components was stored for 1, 3, 5, and 7 days at 17°C to mimic industry standard conditions. Alternatively, semen and components were warmed to 39°C with periodic readings for up to 360 min to assess how the extended semen alone or with the vaccine components were impacted at sow body temperature for a period of time after breeding. Sperm abnormality was assessed using multi-color flow cytometry to identify acrosome-reacted sperm by binding with peanut agglutinin (PNA) conjugated to Alexa-647 (Life Technologies). Sperm were stained with propidium iodide (BioVision, Milpitas, CA, USA) at a concentration of 5 mg/mL and PNA-Alexa647 at a concentration of 30 ng/mL, at room temperature for 5 min. Samples were then diluted 1:4 with Beltsville thawing solution (PIC) and 1×10^5 events were

collected using a FACSCalibur (BD Bioscience Franklin Lakes, NJ, USA) with analysis performed using FlowJo (Tree Star, Ashland, OR, USA). Dead sperm were identified if they were stained with propidium iodide. Experiments were repeated with three separate batches of semen.

Sperm motility was assessed for semen extended with Beltsville thawing solution alone or combined with 400 μg rPEDV spike protein, 200 μg rFliC protein, 1×10^7 BEI-inactivated PPV and 800 μg poly I:C, 1,600 μg HDP and 800 μg PCEP (i.e., the cumulative components of Trial 4 vaccine). Sperm motility was evaluated following incubation for 30 min at 37°C and average motility across 5 unique fields of view were performed using an SCA CASA system for automatic sperm analysis.

Porcine Parvovirus Propagation and Inactivation

PPV was propagated on fetal porcine testicular fibroblast testis (ST; CRL-1746) from American Type Culture Collection (Cedarlane, Burlington, Ontario, Canada). ST cells were cultured in Eagles minimal essential medium (Sigma) with the addition of 5% FBS (Gibco) and Antibiotic/Antimicotic (Life Technologies). Cells were hypotonically lysed in 0.01 M PBSA and free-thawed twice before removal of cell debris by centrifugation at 2,500 × g for 15 min. Viral particles were isolated from the resulting supernatant by centrifugation on top of a 25% sucrose cushion at 210,000 g for 2 h. Purification of the virus from the resulting pellet was carried out on a discontinuous gradient consisting of 1.2 and 1.4 M CsCl, centrifuged at 210,000 g for 1.5 h. Finally, the lower of the two resulting bands was collected and dialyzed against 3 changes of 10 mM Tris-HCl. The identity of the virus was confirmed by qPCR and $TCID_{50}$ by serial infection of ST cells.

Inactivation of PPV was carried out with binary ethylenimine (BEI) following this published methodology (21). In short, BEI was prepared through the reaction of 0.1 M 2-bromo-ethylamine hydrobromide with 0.175 N NaOH at 37°C for 1 h with reaction validated colorimetrically with the addition of 0.0005% β-naphthol violet. Viral stock at 1×10^8 $TCID_{50}$/ml was inactivated with 1.5 mM BEI for 30 h at 37°C, before BEI was neutralized with 10 mM sodium thiosulfate. To confirm virus neutralization, inactivated PPV was passaged on ST cells for 5 passages with no evidence of CPE carried out both in house and by Prairie Diagnostic Services, Inc. (Saskatoon, Saskatchewan).

Laser-Capture Microdissection Sample Collection

Cryoblocks were sectioned at 14 μm thickness onto polyethylene naphthalate membrane slides and immediately fixed in 70% ethanol. Residual cryomatrix was removed by submersion in DEPC treated water (Invitrogen), and slides were stained in cresyl violet (Sigma-Aldrich) for 30 s. Excess stain was removed by submersion in 70% and then 100% ethanol. Epithelial cells were captured within 45 min of staining using a PALM-Microbeam System (Zeiss), removing the basolateral third of the epithelial cell prior to capture to eliminate contamination of samples from sub-epithelial lymphocytes.

RNA Isolation and Gene Expression Analysis

RNA analysis was carried out on both *uterine tissue (UTE) and laser captured uterine epithelia (LC-UE)* from gilts in Trial 1. Uterine tissue collected from the animal trial were ground at −80°C by mortar and pestle until the entire tissue section was reduced to a fine powder. Up to 100 mg of tissue was dissolved in 1 ml of Trizol (Invitrogen) for RNA extraction as detailed in Pasternak et al. (22). DNAse treatment was carried using the Turbo DNAse kit (Thermofisher) following the manufacturer's specifications and the inclusion of 10 units RNase inhibitor (Thermofisher). RNA quantity was determined by Nanodrop (Thermofisher) and RNA quality was validated by denaturing agarose gel. cDNA was generated from 2 μg of RNA using the high capacity cDNA kit (Thermofisher) following the manufacturer's specifications. Gene expression analysis was carried out on a StepOne Plus (Thermofisher) using KAPA SYBR mix (Sigma-Aldrich), containing 0.2 mM primer concentrations [primer sequences and annealing temperature used in **Supplementary Table 1**; (23–27)] and 10 ng/sample cDNA in 15 μl reactions run in duplicate.

For gene expression analysis from laser-captured uterine epithelial cells (LC-UE), RNA was isolated using the Picopure RNA isolation kit (Thermofisher) following the manufacturer's specifications including an on-column DNase treatment (Qiagen). RNA quantity and integrity were confirmed using the Bioanalyzer (Agilent) and 200 ng RNA per sample was converted to cDNA using the High-Capacity cDNA Reverse transcription kit as described above. Gene expression analysis was carried out as described above using 4 ng/sample in each reaction. Primer amplification efficiency was measured at the optimal annealing temperature and in all instances was found to be >90%. Gene expression was normalized to the geometric mean of multiple stable reference genes, RPL19, YWHAZ and GAPDH for the *in vitro* analysis, and GAPDH and β-Actin for *in vivo* analysis (**Supplementary Table 1**).

Immunotyping of PBMCs and Cells Obtained by Uterine Flush

Cells collected from uterine flush were washed 2x in PBS + 0.1% EDTA at 400 × g for 15 min and counted by a coulter counter (Beckman Coulter). Both PBMCs and cells flushed from the uterine tissues (from Trial 1) were stained for flow cytometry (FCM) analysis in 96 well plates with 1×10^6 cells/wells. All FCM stains were incubated in stains diluted in PBS + 2% FBS for 10 min at room temperature followed by 3x washes in PBS + 2% FBS centrifuging at 500 × g for 3 min. All antibody concentrations and details are available in **Supplementary Table 2**. PBMC and flushed T cells were stained in a four-step staining procedure beginning with anti-CD4, anti-CD8α and anti-TCRγδ, followed by the secondary antibodies anti-IgG2b-FITC, anti-IgG2a-Alexa 647, and anti-IgG1-biotin. Next, IgG and Streptavidin (SA)-PerCP-Cy5.5 was added,

followed by the directly labeled anti-CD3-PE antibody. PBMCs and flushed B cells were stained with anti-CD21 followed by anti-IgG1-APC. PBMC monocytes were stained with anti-CD172 and anti-CD14, followed by anti-IgG1-PE and anti-IgG2b-APC. Flushed myeloid cells were stained with anti-CD172, anti-MHCII, anti-SWC9, and anti-CD16, followed by anti-IgG2b-FITC, anti-IgG2a-PE, and anti-SA-PerCP-Cy5.5. FCM samples had 60,000 events for PBMCs and 250,000 events for flushed cells, all of which were immediately collected on a FacsCalibur (BD) with appropriate fluorescence minus one (FMO), single stains, and isotype stains. FCM analysis was carried out using FlowJo (FlowJo LLC). A representative flow cytometry gating scheme for blood analysis (and luminal cell lymphocytes only) is shown in **Supplementary Figure 2**, such that $CD3^{-}CD8\alpha^{+}$ represent natural killer (NK) cells, $CD3^{+}TCR\gamma\delta^{-}CD4^{+}CD8\alpha^{-}$ represent $CD4^{+}$ T cells, $CD3^{+}TCR\gamma\delta^{-}CD4^{-}CD8\alpha^{+}$ represent $CD8^{+}$ T cells, $CD3^{+}TCR\gamma\delta^{-}CD4^{+}CD8\alpha^{+}$ represent $CD4^{+}CD8^{+}$ T cells, and $CD21^{+}$ represent B cells. A representative gating scheme for the flushed myeloid cells is shown in **Supplementary Figure 3**, such that $CD172^{+}MHCII^{-}CD16^{+}$ cells represent neutrophils, and $CD172^{+}MHCII^{+}SWC9^{-}$ cells represent APCs.

CCL2 ELISA

Uterine horn luminal CCL2 was quantified by sandwich ELISA against porcine CCL2 (Kingfisher Biotech) following manufacturer's instructions. In short, 96 well high binding plates (Immulon II, VWR) were coated with a polyclonal anti-swine CCL2 (Kingfisher Biotech) at 1 μg/ml in PBS overnight at RT. Plates were then blocked by 4% BSA in PBS for 2 h at RT prior to a 1 h RT incubation with CCL2 standard (1 in 2 dilutions from 10 ng/ml to 10 pg/ml) and undiluted flush samples. Plates were washed with TBST and biotinylated anti-swine CCL2 antibody was incubated at 0.5 μg/ml in PBS + 4% BSA for 1 h at RT followed by washing and a 30 min RT incubation with streptavidin-HRP. Plates were developed with TBS for ∼30 min in the dark before stopping with a 2N sulfuric acid and absorbance was read at 450 nm.

Antibody ELISAs

Antibody ELISAs were performed on serum and on supernatants from uterine tissue finely minced then incubated in AIM-V media for 48 and 120 h. To measure antibody response to BEI-inactivated PPV, rVP2-TRx, and/or rFliC, Immulon II plates (VWR) were coated over night at with 0.6 μg/ml rVP2-TRx (6) or 2 μg/ml rFliC protein in coating buffer. Plates were washed with tris-buffered saline with 2% Tween-20 (TBST). When detecting antibodies against rPEDV protein, Immulon plates were coated with 0.5 μg/ml purified rSpike S1 protein in coating buffer. Plates were washed with TBST + 0.1% Tween 20.

For all ELISAs, sera and supernatants from minced tissues were serially diluted in assay diluent buffer TBST (+ 1% fish gelatin for the rSpike S1 protein ELISA only). After 2 h incubation, the plates were washed in TBST then incubated for 1 h with 1/5,000 Alkaline phosphatase-conjugated Goat anti-Pig IgG (H+L) (KPL catalog #151-14-06). ELISAs were then developed with 1 mg/ml p-nitrophenyl phosphate in DE buffer (1 M diethanolamine, 0.5 M magnesium chloride) and absorbance at λ405 nm was measured on a SpectraMax plus microplate reader (Molecular Devices). All end-point titers were determined using 4-fold serial dilutions with initial dilutions of serum and culture supernatants performed at 1:4.

Histology and Immunohistofluoresence

Small sections of tissue were collected from the gilts (Trial 1) cervix, lower uterine horn, mid uterine horn, upper uterine horn, ampulla, isthmus, and ovaries and fixed in formalin for 36 h. Formalin-fixed tissue was processed and embedded into paraffin blocks that were sectioned at 4 μm and floated onto superfrost plus slide (Thermofisher). Tissue blocks were deparaffinized by xylene and rehydrated by decreasing concentrations of ethanol prior to Haemotoxylin and Eosin (H&E) staining.

Duplicate slides were deparaffinized and rehydrated from the middle uterine tissue for anti-CD163 immunohistofluoresence (IHF) wherein the slides underwent heat-mediated antigen retrieval in 10 mM Na-Citrate, pH 6 for 30 min at 90°C before being blocked in 5% skim milk in TBS for 1 h at room temperature. Primary antibody staining with mouse anti-human CD163 (EdHu-1; Bio-Rad) at 10 μg/ml in dilution buffer (PBS with 1% BSA, 1% horse serum, 0.3% triton-X, and 0.01% sodium azide) overnight at 4°C. Slides were washed 3x in TBS + 0.05% Tween 20 and incubated in 5 μg/ml donkey anti-mouse IgG Al555 (Invitrogen) for 90 min at room temperature. Slides were again washed 3x in TBS + 0.05% Tween 20 and then stained with 4′,6-diamidino-2-phenylindole (DAPI; Invitrogen) in methanol for 10 min before being cover slipped and imaged on Axiovert 200M (Zeiss) at 20x magnification with appropriate isotype controls. CD163 positive cells were counted in ImageJ by analyze particles, selecting particles between 100 and 1,000 pixels and identified cells were confirmed manually.

Statistical Analysis

All statistical analysis was carried out using GraphPad Prism 7 (GraphPad Software). Gene expression analysis of *in vitro* UEC stimulations were evaluated by one-way ANOVA and significant differences between mock-treated cells and individual treatments were determined by Holm-Sidak's multiple comparisons test. Gene expression and blood immunotyping from *in vivo* experiments and weights of newborn and weaners, fetus to CL ratios, average length of crown/rump ratio per litter were evaluated by unpaired *t*-test with Welch's correction. Uterine flush immunotyping was evaluated by Mann Whitney test. CD163 recruitment analysis was evaluated by unpaired *t*-test with Welch's correction. In all cases, significant differences were reported by $^{*}p < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

RESULTS

Cytokine and Chemokine Gene Expression Changes in Uterine Epithelial Cell in Response to Stimulation With Adjuvants

We first evaluated the potential impact of vaccine adjuvants on the uterus through *in vitro* culture with primary epithelial cells. Following stimulation of UECs with adjuvants alone or in combination, the cells stimulated with poly I:C-HDP and poly I:C-HDP-PCEP showed TEER values that dropped significantly at 6 h (**Supplementary Figure 4A**).

TEER values returned to initial levels by 24 h post-stimulation (**Supplementary Figure 4B**) which suggest that these combinations of adjuvants may transiently impact tight-junction integrity.

Compared to mock-stimulated UECs, poly I:C significantly increased UEC expression of IFNβ (4.5-fold increase, $p < 0.0005$), TNFα (3.18-fold increase, $p < 0.03$), CCL2 (3.81-fold increase, $p < 0.005$), and CCL4 (3.56-fold increase, $p < 0.007$) but poly I:C did not significantly increase expression of GM-CSF, IL-6, IL-8, CCL3, CCL20, or CCL28 (**Figure 1**). Stimulation of UECs with LPS, MDP, PCEP, HDP alone, or MDP-HDP-PCEP in combination did not significantly impact the expression of any of the evaluated immune response genes. When poly I:C was co-incubated with other adjuvants, there was a significant change in gene expression relative to the mock-stimulated cells, but no differences relative to poly I:C alone. For example, poly I:C-HDP stimulated UECs showed significantly induced IFNβ (4.44-fold increase, $p < 0.0004$), CCL2 (4.18-fold increase, $p < 0.01$), and CCL4 (3.25-fold increase, $p < 0.006$) gene expression relative to the mock-stimulated cells. The poly I:C-HDP-PCEP and poly I:C-MDP stimulated UECs showed significantly induced expression of IFNβ gene (4.31, $p < 0.003$ and 3.31-fold increase respectively, $p < 0.002$), TNFα (2.99, $p < 0.04$ and 3.14-fold increase, $p < 0.04$, respectively), and CCL2 (4.34, $p < 0.02$ and 3.3-fold, $p < 0.02$ increase, respectively). Stimulation of UECs with poly I:C-MDP in combination significantly induced CCL4 (2.81-fold increase, $p < 0.04$) and was the only treatment able to significantly induce CCL3 expression (3.3-fold increase, $p < 0.05$) relative to the mock-stimulated UECs, although non-significant, equivalent numerical changes were noted in all other treatments which included poly I:C. No adjuvants significantly induced the expression of GM-CSF, IL6, and CCL28 when compared to the mock stimulation. SLA-DRA gene expression was not detected in any UEC stimulation sample (data not shown) indicating porcine UECs do not express MHC class II.

Impact of Semen and Adjuvants on Uterine Luminal Cell Populations and PBMC Composition After Breeding

Because we are interested in understanding how adjuvants administered with semen impacts the pig uterus, our next steps were to measure changes in luminal cell population 24 h post-breeding with semen alone or semen plus adjuvants. We selected three adjuvants (4 mg poly I:C, 4 mg PCEP, and 8 mg HDP; TriAdj) to use in combination. Sows were administered semen +/− TriAdj and we observed that the semen spiked with TriAdj (STA) triggered a non-significant trend in increased luminal cells ($p = 0.057$) compared to the number of luminal cells in sows administered semen only (SO) (**Figure 2A**). To determine whether the changes in CCL2 gene expression analysis observed in polarized UECs stimulated with TriAdj (**Figure 1**) correlates to increased CCL2 secretion 24 h after breeding with STA relative to SO, we quantified CCL2 secretion from uterine flushes and saw no significant differences (**Figure 2B**). STA did not significantly impact CCL2 luminal secretion by luminal cells which could indicate a lack of protein translation or that secretion of CCL2 was directed into the tissue as opposed to into the lumen.

To determine whether inclusion of TriAdj with the semen dose impacted cell recruitment to the uterus, we enumerated total cells collected from the uterine lumen 24 h after breeding with SO or STA. The most predominant cell populations in the uterine lumen following breeding were neutrophils with mean population percentages at 45% total events in response to SO and 53% of total events in response to STA, followed by non-macrophage APCs at 0.79% total events in response to SO and 1.19% total events in response to STA, respectively (**Figure 2C**). All other cell populations were below 1% of total events, regardless of treatment with the exception of one animal bred with STA which had higher total events for NK (3%), γδ T cells (8.4%), and CD8 T cells (6.98%). Overall, the inclusion of TriAdj in semen did not appear to significantly impact the proportions of immune cell populations in the uterine, although there was a trending increase in the total number of cells collected ($p = 0.0571$).

We performed immunotyping on PBMCs to discern whether the number of T cell subsets, B cells, and monocytes were impacted by either breeding (i.e., pre-semen vs. post-semen; pre-semen + TriAdj vs. post-semen + TriAdj) or by the adjuvants administered to the uterus during breeding (SO vs. STA). Before and after breeding with SO or STA, there was no significant change in the percentages of the blood cell population of $CD3^{-}CD8^{+}$ NK cells, CD4 T cells, CD8 T cells, $CD4^{+}CD8^{+}$ co-positive T cells, or $CD21^{+}$ B cells (**Figure 2D**). After animals were bred with STA, there was a significant drop in the percentage of γδ T cells (10.5% decrease) and monocytes (4.7% decrease) in the PBMC mixed cell populations relative to the percentages present in PBMCs prior to STA immunization suggesting that the TriAdj may have impacted blood cell composition. However, when we compared the blood cell populations in sows bred with semen vs. sows bred with semen plus TriAdj, we did not observe significant differences in any of the population percentages.

CD163 Positive Cell Recruitment to Uterine Tissue Following Breeding

To determine if the decreased monocytes in blood in response to STA (shown in **Figure 2D**) shows a corresponding influx of CD163 positive monocytes into uterine tissue, immunohistofluorescence was carried out on sections from the middle of the uterine horn (representative staining in **Supplementary Figure 5A**). CD163 positive cells were enumerated per 100 μm^2 section. No significant differences in the number of $CD163^{+}$ cells were found in the uterine tissue from sows bred with SO (1.23 cells per 100 μm^2) or sows bred with STA (2.03 cells per 100 μm^2; **Supplementary Figure 5B**).

Impact of Semen Alone or Semen Plus Adjuvants on Uterine Tissue and Laser-Captured Uterine Epithelial Cell Gene Expression

Twenty-four hours after sows were bred with semen alone or semen plus Triadj, the uterine tissue (UT) from lower to

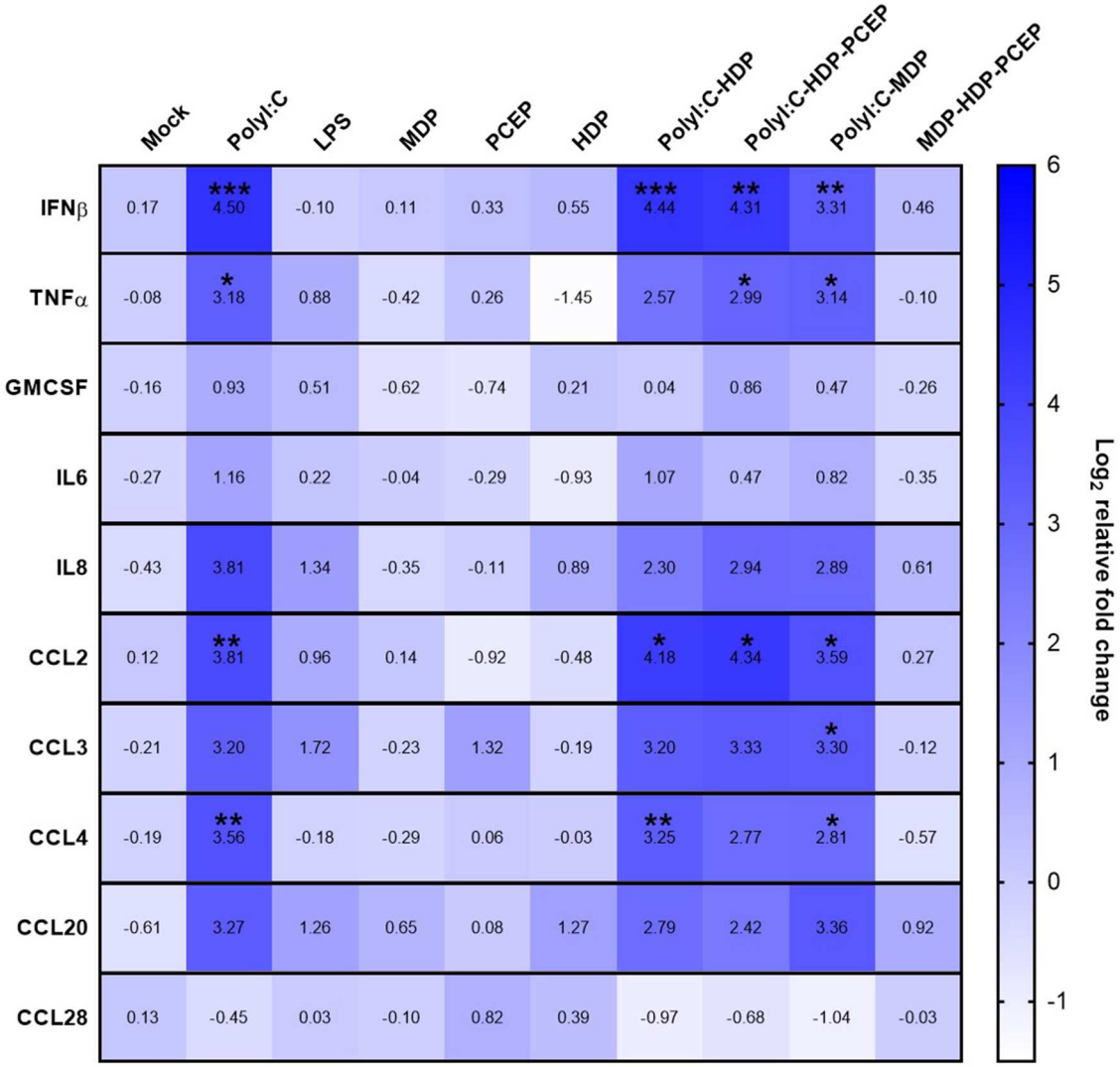

FIGURE 1 | Gene expression heat map of polarized uterine epithelial cells (UECs) stimulated with multiple adjuvant components alone and in combination. UECs were cultured until polarized and stimulated by adjuvant components (horizontal axis) for 6 h before cells were collected, RNA was isolated and gene expression was analyzed by qPCR. Median $\log_2$ increases are presented in the heat map with significant differences were evaluated by one-way ANOVA and significant differences between mock-treated cells and individual treatments were determined by Holm-Sidak's multiple comparisons tests (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$).

upper uterine horns were subjected to gene expression analysis. Relative to the UT exposed to SO, UT exposed to STA did not result in significant differences in expression of TNFα, IFNβ, GM-CSF, IL-6, IL-8, CCL2, CCL3, CCL4, or CCL28 genes (**Supplementary Figure 6**).

We speculated that we may not be able to discern whether gene expression profiles of the uterine epithelial cells were being masked by the expression profiles of the multiple cell populations present in UT. Therefore, we performed laser-capture microdissection (LCM) such that we captured only the uterine epithelial cells (LC-UEs). LCM was performed on cryoblocks from only the middle of the uterine horn as no significant differences in gene expression were observed between lower, middle and upper uterine horn UT. LC-UE cells from animals bred with SO or STA also showed no changes in expression of TNFα, IFNβ, GM-CSF, IL-6, IL-8, CCL3, CCL4, or CCL28 (**Supplementary Figure 6**). However, the LC-UE cells isolated from sows bred with STA showed significantly induced expression of CCL2 (2.4-fold increase; $p < 0.0274$) relative to the expression profile observed in LC-UE cells from sows bred with SO. Lastly, SLA-DRA gene expression was not detected in the LC-UE samples and had no significant differences when observed in tissue (data not shown). Collectively, these data suggest that TriAdj administered with semen during breeding had an impact on select uterine epithelial cell chemokine expression. Our next steps were to determine whether i.u. vaccination with the TriAdj triggered an immune response.

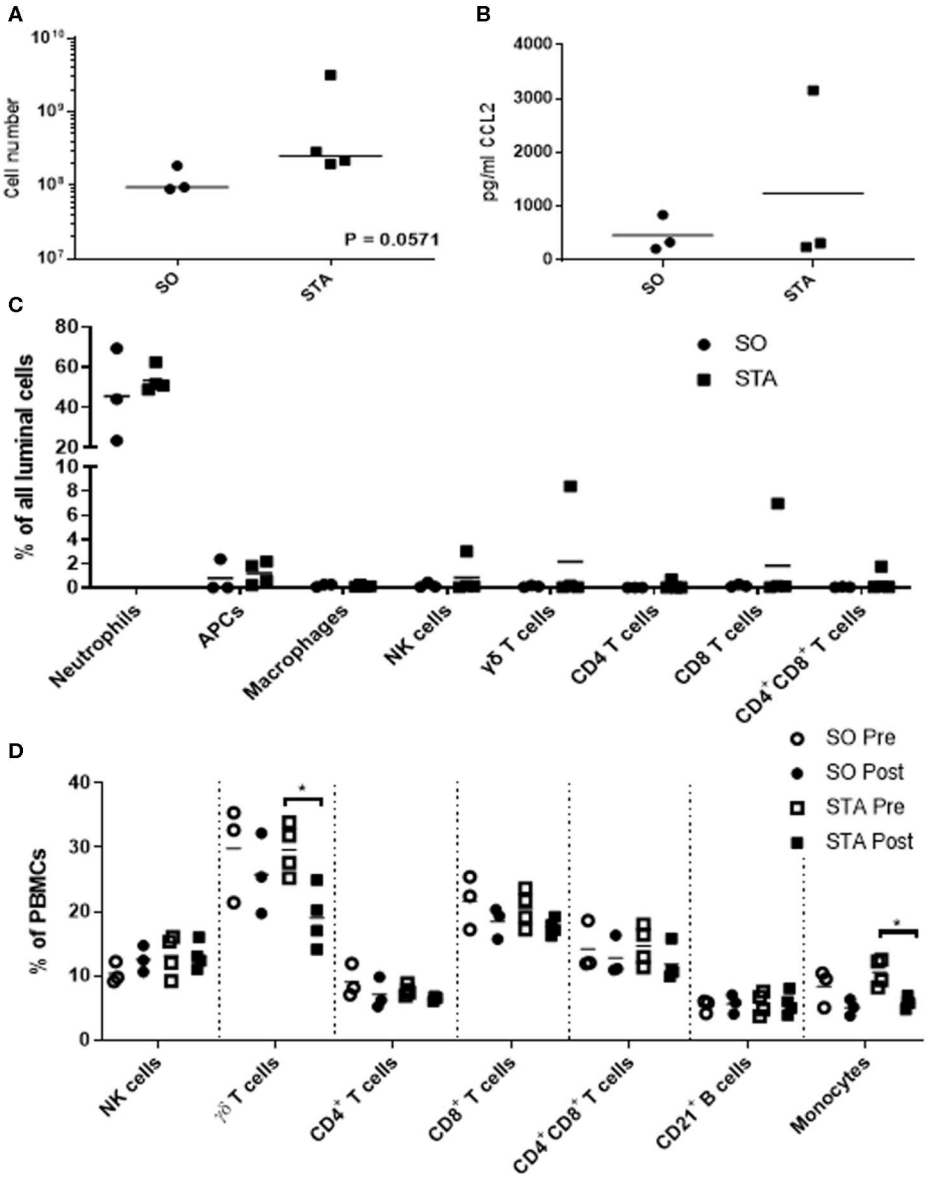

FIGURE 2 | Uterine flush cell counts and immunotyping of luminal cell populations of sows 24 h after breeding with semen only (SO) or semen containing a triple adjuvant combination (STA) in addition to PBMC immunotyping before or 24 h after breeding. Flushed cells were counted by coulter counter **(A)**. Luminal CCL2 was quantified by sandwich ELISA **(B)** and significant differences between treatments were determined by Mann Whitney test. Immunotyped cells in the uterine flush were stained with CD3, CD4, CD8α, γδ T cells, CD172, MHCII, SWC9, and CD16 **(C)**. PBMCs were isolated from blood and stained for CD3, CD4, CD8α, γδ T cells, CD21, CD172, and CD14 **(D)**. Stained cells were analyzed on a FACScalibur and significant differences between treatments determined by Mann Whitney test. Each circle or square represents a unique biological replicate and the line represents mean data. *$p > 0.05$.

Response to Intrauterine Vaccine Administered With Semen at the Time of Breeding

For our first animal trial, the i.u. vaccine was comprised of 1 × 10^7 $TCID_{50}$ BEI-inactivated PPV vaccine formulated with 400 μg Poly I:C, 800 μg HDP and 400 μg PCEP. Prior to vaccination we evaluated the impact of this formulation on sperm and found no significant effect on either acrosome reaction or viability during storage for 7 days (**Figure 3A**) or at physiological temperatures over 360 min incubation (**Figure 3B**). Flow cytometric analysis showed that the vaccine components alone or in combination had no significant impact on the percentage of abnormal semen. Next, treatment sows ($n = 4$) were bred with semen combined with the vaccine immediately prior to breeding. Control sows ($n = 3$) were immunized with ParvoShield vaccine by i.m. route when they entered into farrowing crates. All sows had previously been vaccinated i.m. with ParvoShield at each breeding cycle when they entered

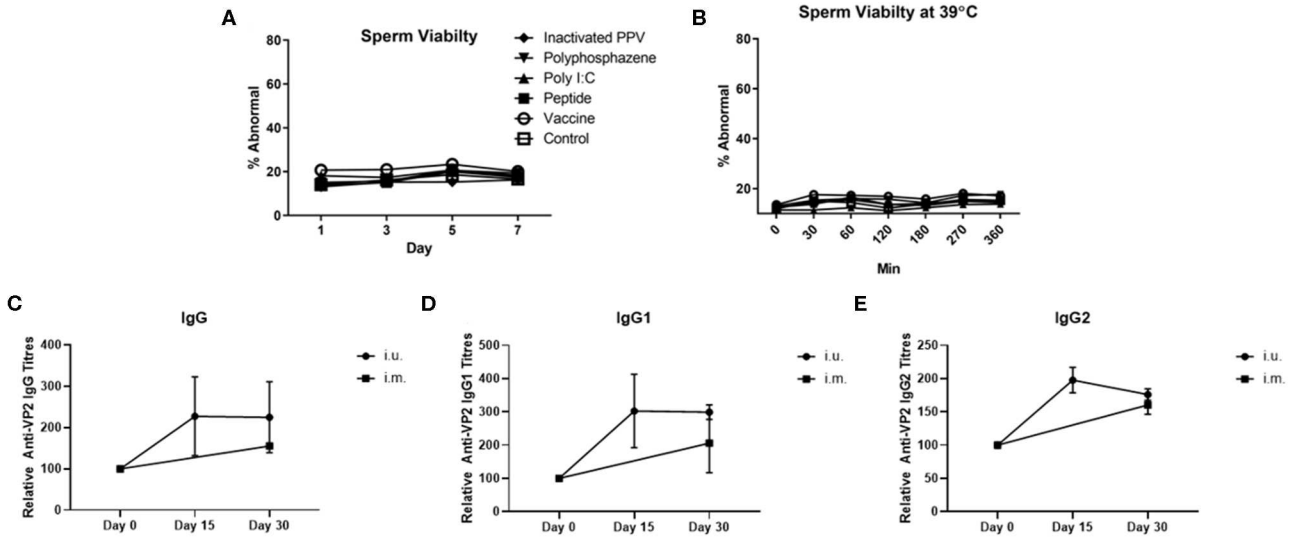

FIGURE 3 | Sperm abnormality assessment of extended semen incubated with intrauterine (i.u.) vaccine components and fetus viability after i.u. immunization and serum antibody titers from animals vaccinated through the i.u. or intramuscular routes (i.m.). Commercially extended semen was incubated alone or in the presence of 1×10^7 $TCID_{50}$ BEI-inactivated PPV, 400 μg Poly I:C, 800 μg HDP, 400 μg PCEP (i.u. vaccine) at 17°C for 7 days **(A)** or 39°C **(B)** with periodic readings for up to 360 min. Acrosome-reacted sperm was bound by peanut agglutinin (PNA) conjugated to Alexa-647 and identified on a FACScalibur. Dead sperm were identified if they were stained with propidium iodide (PI). Experiments were repeated with three separate batches of extended semen. **(C–E)** Animals were bred with extended semen alone or with i.u. vaccine. Control sows ($n = 3$) were immunized with ParvoShield vaccine by i.m. route. All sows had previously been vaccinated i.m. with ParvoShield at each breeding cycle when they entered into the farrowing crates (∼120 days previously). Serum anti-VP2 IgG **(C)**, IgG1 **(D)** and IgG2 **(E)** antibody titres over time were quantified relative to each sow's anti-VP2 titres at day 0 to give relative anti-VP2 titres for i.u.-vaccinated (black circle) and i.m.-vaccinated (black square) sows. Data are presented as means [horizontal bars; **(A,B)**] and mean with standard deviation in **(C–E)**.

into the farrowing crates (∼120 days previously) so we are measuring a booster vaccine response. Serum was tested for anti-VP2 antibodies up to 30 days later. Results showed that sows responded to the i.u. vaccine with anti-VP2 IgG (**Figure 3C**), IgG1 (**Figure 3D**), and IgG2 (**Figure 3E**) titres that were comparable to the titres from sows immunized with the commercial i.m. PPV vaccine. The individual antibody titres for each animal is shown in **Supplementary Figures 7A–C** and the data shown as percentage change from the zero time point is shown in **Supplementary Figures 7D–F**. Together these results show that the i.u. vaccine did not negatively affect sperm function or embryo viability and that sows responded to an inactivated PPV vaccine administered with the semen dose with elevated serum anti-VP2 titres if the sows had previously received an i.m. porcine parvovirus vaccine.

For our second trial, we immunized gilts via the i.u. route ($n = 7$) with 800 μg rVP2 antigen with 400 μg Poly I:C, 800 μg HDP and 400 μg PCEP. Mock-control sows ($n = 9$) were administered a comparable volume of saline with the semen dose. Serum was obtained throughout gestation and continued until weaning (21 days after birth). Piglets born from i.u. vaccinated gilts ($n = 6$ randomly selected) had comparable weights at 3 days of age (**Supplementary Figure 8A**) and at weaning (**Supplementary Figure 8B**) relative to the piglets born from mock-vaccinated dams ($n = 6$ randomly selected) suggesting that the i.u. vaccine components did not negatively affect piglet development. Serum anti-VP2 IgG titres were at comparable low levels across all time points with no significant differences between the 2 groups (**Supplementary Figure 8C**) suggesting that either rVP2 was a poor antigen or that the i.u. vaccine was not effective as a primary vaccine.

For our third trial, we combined semen with TriAdj and one of three antigens including rPEDV Spike protein, rFliC, and BEI-inactivated PPV. We performed CASA analysis to assess sperm motility and we observed no difference in the percent motile sperm between semen alone or semen incubated with the vaccines (**Figure 4A**). The two vaccine groups consisted of i.u.-vaccinated sows ($n = 8$) and control sows ($n = 5$) which were immunized with parvovirus vaccine FarrowSure B Gold i.m. at breeding. After 30 days, fetuses were visually inspected and the CL were counted. There was no difference in the viable fetus/CL ratio between both groups of sows (**Figure 4B**). The length of the fetus from the crown to the rump (mm) was measured for each fetus and the average crown-rump length was comparable across both groups of sows (**Figure 4C**). There was no significant difference in the average fetus weight born to either groups of sows (**Figure 4D**). Collectively, these data indicate that the vaccines comprised of recombinant proteins or inactivated PPV vaccine each formulated with TriAdj did not negatively affect sperm function or fetus viability, fetal crown-rump length, or birth weight in the i.u. vaccinated sows relative to the control sows. Finally, we assessed the impact of the anti-VP2 response in the sow sera and uterine tissue immune responses (**Figure 5**). Thirty days post-immunization, serum anti-VP2 IgG were assessed and we observed that the animal immunized with Farrowsure B Gold vaccine i.m. had a significant increase in antibody titres relative to the i.u. vaccinated gilts after 30 days (**Figure 5A**). Similarly, when the

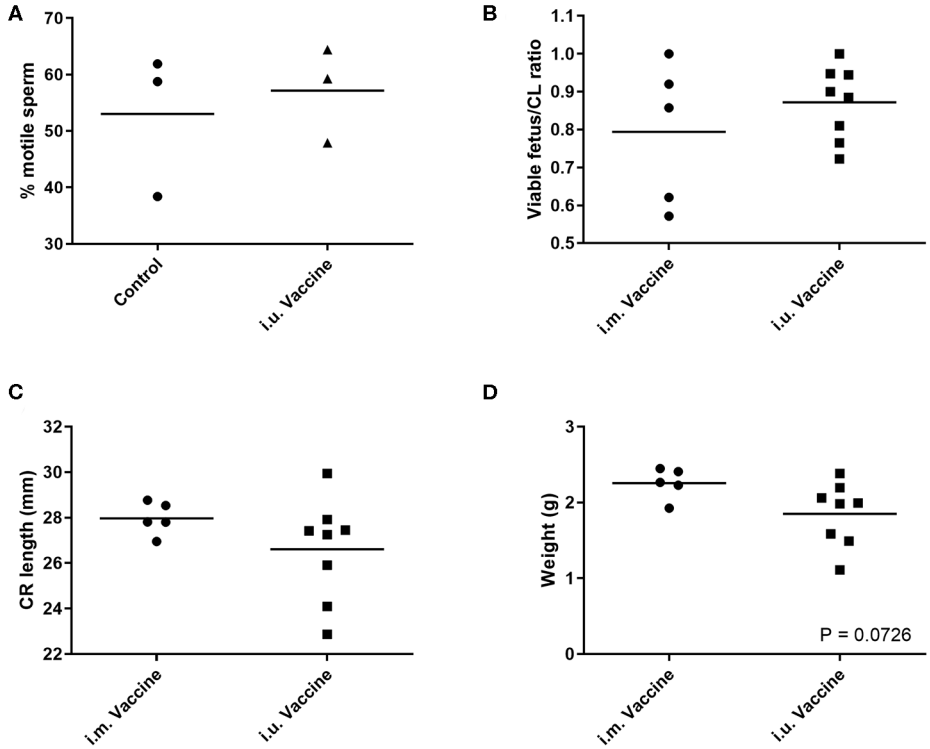

FIGURE 4 | Sperm motility measurements of extended semen incubated with vaccine and fetal morphometrics from animals vaccinated through the intrauterine or intramuscular routes. **(A)** Sperm motility was evaluated in the presence of inactivated PPV, Spike and LI FliC (i.u. vaccine) and TriAdj using SCA CASA system for automatic sperm analysis and the average motility across 5 unique fields of view. **(B–D)** Fertilization rates and fetal morphometrics were measured 30 days after breeding following i.m. vaccination with Farrowsure B Gold vaccine (which contains PPV antigens) or i.u. vaccination with 3 vaccines each consisting of 400 μg recombinant PEDV spike protein, 200 μg recombinant LI FliC protein, and 1×10^7 BEI-inactivated PPV each formulated with 266 μg poly I:C, 533 μg HDP and 266 μg PCEP. **(B)** The ratio of viable fetuses divided by the CL per sow are presented. **(C)** The distance in mm between the crown and rump was measured for each fetus and the ratio are presented. Each data point represents the average length for the fetuses born to each gilt. **(D)** The average weight of the fetuses (g) from each litter are presented. Statistical analysis carried out by Kruskal-Wallis test and Dunns multiple comparisons test. Horizontal bars represent mean values.

uterine tissues were minced and incubated in media for 48 and 120 h to allow measurement of local antibody production, only the i.m. vaccinated animals showed a statistically not-significant ($P < 0.063$) increase in anti-VP2 IgG titres (**Figure 5B**). The serum and mucosal antibody titres for i.u. vaccinated gilts were also calculated for the other two antigens included in the i.u. vaccine, rPEDV Spike and rFliC protein (which are absent in Farrowsure B Gold vaccine). There was no significant increase in anti-PEDV Spike IgG in serum (**Figure 5C**) or uterine tissue (**Figure 5D**) or anti-FliC IgG in serum (**Figure 5E**) or uterine tissue (**Figure 5F**). These data suggest that a primary vaccine comprised of BEI-inactivated PPV or recombinant proteins formulated with TriAdj administered to the uterus at breeding failed to promote a systemic or mucosal humoral immune response.

DISCUSSION

Initiating a strong mucosal immune response to inactivated virus or subunit vaccines requires potent adjuvants that overcome the mucosal barriers and initiate recruitment of APCs to the mucosal surface. As the uterine epithelial layer is the first cellular contact for an i.u. vaccine, generating a strong chemoattractive response that leads to APC recruitment to the uterine tissue or the uterine lumen may increase i.u. vaccine efficacy. Immunostimulatory adjuvants frequently considered for use in mucosal vaccines are TLR agonists and other pattern recognition receptor ligands that act through the inflammasome. Although porcine UECs express the necessary receptors for all the ligands evaluated [TLR3 bound by poly I:C, TLR4 bound by LPS, TLR9 bound by CpG, NOD2 bound by MDP (28)], our study showed that these cells only induced expression of the pro-inflammatory cytokine IFNβ and TNFα and chemokine genes CCL2 and CCL4 in response to poly I:C suggesting that TLR3 was a viable adjuvant target. This analysis shows agreement with our previous research which also showed that pig uterine epithelial cells express functional TLR3 which is targeted by poly I:C (20). *In vitro* experiments have shown poly I:C and LPS stimulation of murine UECs significantly induced secretion of CCL2, while CpG stimulation was unable to induce CCL2 expression (29). In contrast, LPS stimulated Human UECs showed suppressed CCL2 expression whereas poly I:C induced secretion of TNF-α, GM-CSF, IL-6, G-CSF, CCL2, and CCL4 (30, 31). Lastly, although

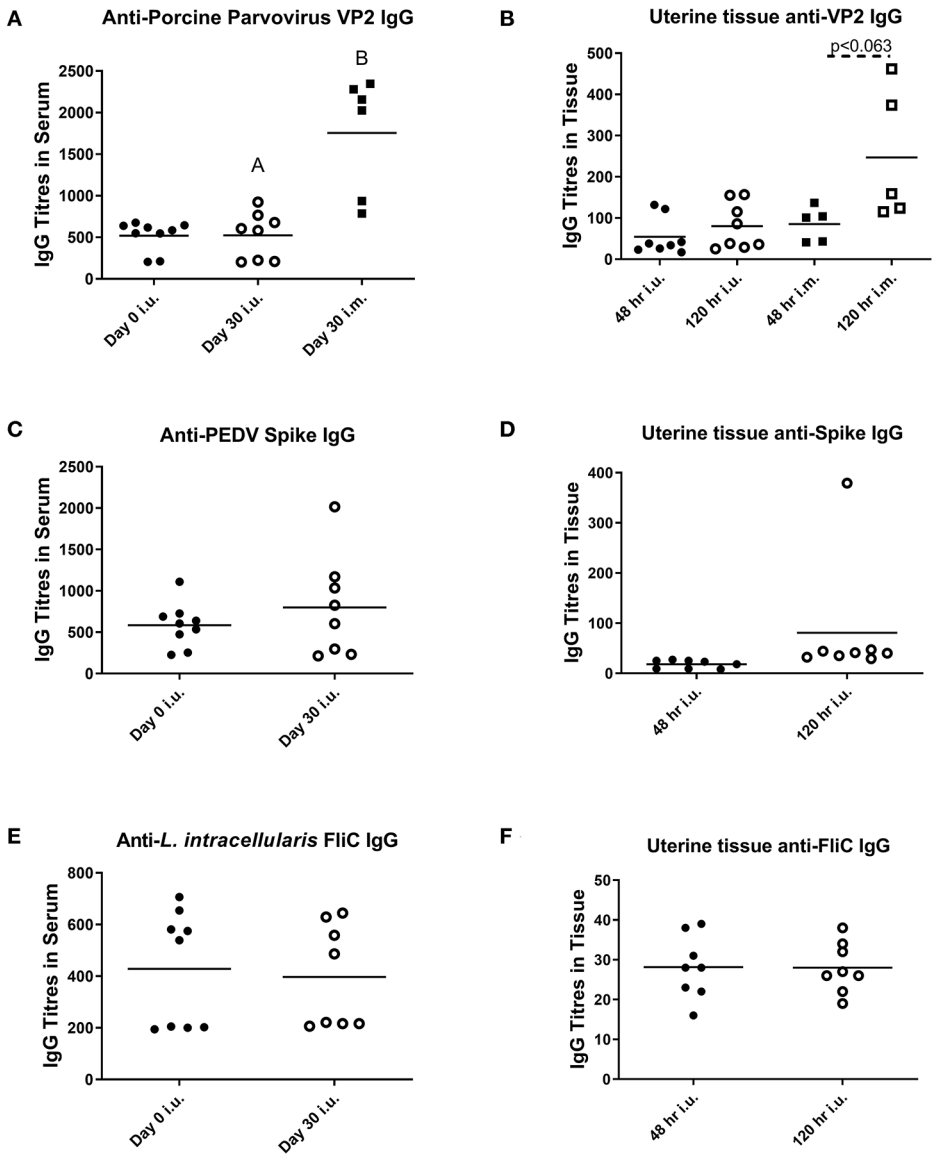

FIGURE 5 | Serum and mucosal antibody titers from animals vaccinated through the intrauterine or intramuscular routes. Serum **(A,C,E)** and mucosal antibody titers **(B,D,F)** were measured after breeding animals with semen alone then immunizing them through the i.m. route with Farrowsure B Gold vaccine (which contains PPV antigens) or after breeding animals with semen combined with 3 vaccines consisting of 400 μg recombinant PEDV spike protein, 200 μg recombinant LI FliC protein, and 1×10^7 BEI-inactivated PPV each formulated with 266 μg poly I:C, 533 μg HDP and 266 μg PCEP. Serum was collected at day 0 and 30 days later and uterine tissue was collected at day 30 after gilts were humanely euthanized. The supernatants from the minced uterine tissues was collected after 48 and 120 h to establish mucosal antibody production. Data are presented as mean values. Statistical analysis carried out by Kruskal-Wallis test and Dunns multiple comparisons test. Significantly different groups are denoted by differing letters.

porcine UECs showed induced expression of pro-inflammatory cytokine and chemokine genes in response to poly I:C, LPS stimulation had no observable impact on the assayed genes. These results suggest that poly I:C alone or in combination may be a suitable adjuvant to use to target uterine epithelial cells innate immune responses. The notable discrepancies of responses between species supports the concept that although TLR expression in UECs is relatively conserved across species, the response upon TLR ligand stimulation between species can vary significantly and caution should be taken in attempting to extrapolate results across species.

Non-TLR ligands are less regularly evaluated as adjuvants, however, porcine UECs express the receptors for several potential adjuvants such as NOD2, the receptor for MDP which may indicate that NOD2 may be a suitable adjuvant (28). Although there are no studies showing significant *in vitro* stimulation of UECs with MDP, *in vitro* studies with mouse APCs showed minimal NF-κβ activation unless MDP was combined with other

ligands such as CpG (32). Our results show that pig UECs did not induce expression of any assayed genes in response to MDP alone nor did MDP amplify the response generated toward poly I:C. Therefore, we do not anticipate that it will be an effective adjuvant in inducing APC recruitment or activation in an i.u. vaccine. HDP, which has no known receptor, has been implicated in modulating the immune response in several cell types including monocytes where *in vitro* stimulation resulted in increased CCR5 expression and enhanced recruitment to CCL3 and CCL5 (33). Although there has been observed HDP modulated activity in other cells, both when alone and combined with other adjuvant components, HDP showed no significant impact on the capacity for porcine UECs to respond to poly I:C. Lastly, there have been studies evaluating polyphosphazene in both mucosal and parenteral vaccine formulations where PCEP alone induced protective immune responses (34). Intramuscular injection of mice with PCEP triggered local production of CCL2 and pro-inflammatory cytokines as IL-1beta, and IL-18 cytokines and when injected intradermally into pigs, PCEP induced the expression of chemokine CCL2 and pro-inflammatory cytokine IL-6 suggesting that it has immunostimulatory potential (35, 36). These observations suggest that PCEP can act as an immunostimulatory adjuvant and it may potentiate immune responses to antigens. Despite these results in mice and pigs after parenteral injection/vaccination, porcine UECs stimulated with PCEP did not induce expression of cytokine or chemokine genes and may not be an effective i.u. vaccine adjuvant alone.

TriAdj as a vaccine adjuvant has been evaluated in multiple vaccine formulations, in multiple species, and delivered via several routes. Primarily it has been evaluated for use as an i.m. vaccine adjuvant where it has been used in mice, rats, cattle, sheep, and pigs generating strong systemic immunity against human parainfluenza type 3 (in mice and rats), bovine viral diarrhea virus (in cattle and sheep) and porcine epidemic diarrhea virus (in pigs) (11, 19, 37). TriAdj has also been used to generate a strong single dose humoral and cell-mediated immune response when delivered subcutaneously in koalas as a subunit chlamydia vaccine (38). When TriAdj was used in conjunction with mucosal vaccine studies, there was increased mucosal immunity and protection generated to an intranasal vaccine to respiratory syncytial virus in mice (39). A promising use for the TriAdj as a mucosal adjuvant was shown when it was administered as part of a subunit vaccine in the rabbit uterus as it induced strong systemic and mucosal humoral immune responses even after a single dose (6). Although there have been limited studies on the initial innate immune response generated to TriAdj, an *in vitro* study with mouse macrophages found that they induced significant expression of several chemokines including CCL2, CCL3, and CCL4 in addition to upregulation of the co-stimulatory molecules CD80/86 and MHC class II (40) in the presence of TriAdj.

Because i.u. vaccination in commercial sows would only be used during AI, it is important to take into account the immune response generated during breeding. Breeding in swine elicits an inflammatory immune response and neutrophil infiltration into the uterine lumen (41, 42). However, with the exception of a widely accepted IL-8 induction and corresponding polymorphonuclear cell recruitment to the lumen (43, 44), there are limited studies examining the exact cytokine and chemokine genes induced following breeding. Interestingly one previous study showed that the semen extender Androhep and seminal plasma alone induced IL-10, TGF-β, IL-8, and TNF-α, however when combined with spermatozoa, these values returned to baseline expression levels (45). The possible suppression of cytokine and chemokine expression by spermatozoa may contribute to the discrepancy in the magnitude of expression observed *in vivo* that was lower than what was observed in the *in vitro* experiments. However, studies evaluating immune cell recruitment into the endometrium following breeding remain somewhat unclear whether spermatozoa, seminal plasma, or semen extender is the primary inducer of this response (43). We speculate that this inflammatory response may reduce the requirement of an i.u. vaccine to induce an inflammatory response itself, and may instead require the adjuvants to modulate the inflammatory response toward a higher proportion of recruited APCs in the uterine mucosa, possibly through the induction of chemokines that will preferentially recruit APCs, such as CCL2 and CCL3. In pigs bred with semen alone or semen plus TriAdj, we observed increased expression of CCL2 and CCL4 genes but no detectable increase in luminal CCL2 protein. While it is possible that CCL2 is secreted by the uterine epithelia basolaterally, we would anticipate observing a greater degree of APC recruitment into the endometrium if this were the case. Further, despite the increased expression of chemokines known to promote monocyte and macrophage recruitment chemokines as well as decreased levels of monocytes in the blood, we did not observe a significant increase in the numbers of monocytes/macrophages (CD163 positive cells) in the uterine tissue when compared to the response to extended semen. Although there are numerous studies characterizing the polymorphonuclear cell recruitment into the lumen following breeding and the inflammatory response following breeding with extended semen (41, 42, 45), data on APC recruitment in swine is limited. However, a single study observed increased MHCII expression on uterine macrophages and DCs following breeding, indicative of APC maturation (44). These data and the non-significant decrease of blood monocytes after breeding in our study may be indicative of a certain degree of APC engagement to extended semen alone and inclusion of TriAdj in semen although more research is required to understand this.

Previous studies have described that the lumen of the porcine uterus, in a native state, has a relatively low-level complement of T cells (13) which is consistent with our observations. Further, our data shows that not only does semen plus TriAdj not impact T cell recruitment to the uterine lumen, we also show that breeding appeared to have minimal effect on luminal T cell numbers. It remains to be clarified why blood γδ T cells were reduced after animals bred with semen plus TriAdj but not in animals bred with semen alone and why there is no evidence that the γδ T cells were recruited to the uterine lumen. Current data indicate that circulating porcine γδ T cells are primarily pro-inflammatory (46) and therefore further research should be carried out to determine if the inflammatory response induced by TriAdj plus semen is specifically recruiting these cells. Based

on the limited data available for γδ T cells and their subtypes in pigs, we currently do not know the impact these cells may have in mounting a response to the i.u. vaccination.

To establish combining vaccines with semen during breeding as a viable alternative method of immunization, it is critical that we establish not only an effective immune response, but we also must ensure that sperm function and fertility are not negatively affected. Our results show that vaccinating gilts or sows via the i.u. route with recombinant proteins and/or inactivated PPV formulated with TriAdj did not negatively impact sperm function or motility, fetal viability, CR length or fetal weight suggesting that a properly formulated i.u. vaccine does not negatively impact fertility. Piglet weight at birth and weaning also did not appear to be negatively affected by i.u. vaccination. However, i.u. vaccines with inactivated virus or recombinant proteins did not promote a significant humoral response in gilts or sows when the i.u. vaccine was a primary immunization. Only sows that had previously been vaccinated with an i.m inactivated PPV vaccine produced a humoral anti-VP2 IgG, -IgG1, and -IgG2 immune response that was comparable to the i.m control sows. These results contrast with what has been observed in rats and rabbits which showed that a single i.u. vaccine triggered a measurable antigen-specific systemic and local humoral immunity (6–8). The reasons why the i.u. vaccine may have been effective in rodents or rats after a single dose may be due to the fact that they were administered without semen. Because we observed increased humoral immunity to a booster i.u. vaccine in sows that had previously received a primary systemic vaccine, it is possible for an i.u. vaccine to be effective under still undefined conditions. More trials need to be performed to clarify whether repeated i.u. vaccination can trigger strong humoral immunity or whether the primary response needs to occur via a systemic route. Of additional concern is the possibility of generating an immune response to sperm that results in infertility or reduced fertility in future pregnancies, as has been observed in in humans, mice and rabbits following immunization with sperm specific proteins (47). We hypothesize that by delivering the sperm through its conventional route, the mechanisms for prevention of infertility inducing immune responses to sperm will be maintained (41), however further studies will be required to determine if immunization utilizing an artificial insemination dose impacts future pregnancies. Lastly, we could establish whether the semen dose itself interferes with the efficacy of a primary immunization by administering the first i.u. dose in gilts during their first-heat detection.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/ **Supplementary Material**.

ETHICS STATEMENT

This animal study was reviewed and approved by University Animal Care Committee (UACC) University of Saskatchewan Animal Research Ethics Board (AREB).

AUTHOR'S NOTE

Portions of this manuscript are derived from the Ph.D. thesis of GH with permission from Dr. Janet Hill, the Department Head of Veterinary Microbiology, University of Saskatchewan. This manuscript was published with permission by the Director of VIDO-InterVac as journal series #893.

AUTHOR CONTRIBUTIONS

GH, JP, and HW conceived of and designed the experiments. GH carried out qPCR experiments, optimized and carried out the UEC isolation experiments as well as the laser-capture and stimulation experiments, analyzed all adjuvant alone data, and performed the CASA analysis. GH and JP developed and optimized all staining protocols, performed the vaccine trials, and processed the results. JP performed the flow cytometric analysis to measure sperm abnormality. JP, GH and SN performed the serum antibody analysis. KF, OS, and BD performed tissue processing and fetal measurements in the final trial. HW and GH drafted the manuscript. All authors read and approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.01015/full#supplementary-material

Supplementary Figure 1 | Schematic timeline of hormonal synchronization method for fixed time artificial insemination of sows.

Supplementary Figure 2 | Gating strategy used for T cell, B cell, and monocyte immunotyping stains from the blood.

Supplementary Figure 3 | Gating strategy used for myeloid cell immunotyping stain used for luminal cell populations.

Supplementary Figure 4 | Changes in the primary uterine epithelial cell (UEC) transepithelial electrical resistance TEER stimulated with multiple adjuvant components alone and in combination. UECs were cultured until polarized and stimulated by adjuvant components (horizontal axis) and had the TEER measured prior to the addition of stimulants at 6 h **(A)** and again at 24 h **(B)**. Statistical analysis was done by Kruskal-Wallis test and significant differences between mock and individual stimulations were determined by Dunn's multiple comparison tests ($^*p < 0.05$). Each circle, square, etc. represents a unique biological replicate and mean values are represented by a horizontal line.

Supplementary Figure 5 | Representative immunohistofluorescence of $CD163^+$ cells in uterine tissue after breeding with semen only (SO) or with a triple adjuvant combination (STA). **(A)** Twenty-four hours after breeding with semen alone or with TriAdj, uterine tissue was processed for immunohistofluorescence. Stained slides were imaged in 10 random fields of view and CD163 positive cells were counted by Image J **(B)** and significant differences were determined by unpaired *t*-test with Welch's correction. Each circle or square represents a unique biological replicate and the line represents mean data.

Supplementary Figure 6 | Gene expression of uterine tissue and laser captured uterine epithelia (LC-UE) of sows 24 h following breeding with semen only (SO) or semen containing a triple adjuvant combination (STA). Gene expression analysis was performed for the following genes: TNFα, IFNβ, GM-CSF, IL6, IL8, CCL2, CCL3, CCL4, and CCL28. UTE expression shows averaged gene expression profiles across the lower, middle and upper uterine horn and LC-UE samples were collected from samples in the middle of the uterine horn. Significant differences within sample types were determined by unpaired *t*-test with Welch's correction ($^*p < 0.05$). Each circle or square represents a unique biological replicate and the line represents mean data.

Supplementary Figure 7 | Serum antibody titers from animals vaccinated through the i.u. or intramuscular routes (i.m.). Animals were bred with extended semen alone or with i.u. vaccine comprised of 1×10^7 $TCID_{50}$ BEI-inactivated PPV, 400 μg Poly I:C, 800 μg HDP, 400 μg PCEP (i.u. vaccine) and control sows ($n = 3$) were immunized with ParvoShield vaccine by i.m. route. All sows had previously been vaccinated i.m. with ParvoShield at each breeding cycle ∼120 days previously. Serum anti-VP2 IgG **(A)**, IgG1 **(B)**, and IgG2 **(C)** antibody titres for i.u.-vaccinated (closed symbols) and i.m.-vaccinated (open symbols) sows. Percent change of serum anti-VP2 IgG **(D)**, IgG1 **(E)**, and IgG2 **(F)** antibody titres for i.u.-vaccinated (closed symbols) and i.m.-vaccinated (open symbols) sows are also shown.

Supplementary Figure 8 | Weight of piglets born from IU-vaccinated and control gilts and anti-VP2 serum antibody titres over time. Intrauterine-vaccinated animals were bred with standard extended semen dose plus 800 μg recombinant VP2-Trx formulated with 400 μg Poly I:C, 800 μg HDP, and 400 μg PCEP. Control animals received the standard semen dose. Blood was obtained for the gilts day 0, 15, 30, 70, 90, and at wean (21 days after piglet birth). Piglet weights were measured on day 3 after birth **(A)** and at weaning **(B)** and the average weight of the piglets born to each gilt is shown. **(C)** Serum anti-VP2 IgG antibody titres were quantified relative to each gilt's anti-VP2 titres at day 0 to give relative anti-VP2 IgG titres for i.u.-vaccinated (orange circle) and i.m.-vaccinated (blue triangles) gilts. Horizontal bars present mean values.

Supplementary Table 1 | Primer names, sequences, annealing temperature, and target sequence used in all qPCR experiments.

Supplementary Table 2 | Antibodies used in FCM analysis, final concentrations, and suppliers.

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Identification of Altered miRNAs in Cerumen of Dogs Affected by Otitis Externa

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Otitis externa is one of the most common diseases in dogs. It is associated with bacteria and yeast, which are regarded as secondary causes. Cerumen is a biological substance playing an important role in the protection of ear skin. The involvement of cerumen in immune defense is poorly understood. MicroRNAs can modulate the host immune response and can provide promising biomarkers for several inflammatory and infectious disorder diagnosis. The aims of this study were to profile the cerumen miRNA signature associated with otitis externa in dogs, integrate miRNAs to their target genes related to immune functions, and investigate their potential use as biomarkers. Cerumen was collected from healthy and otitis affected dogs and the expression of miRNAs was profiled by Next Generation Sequencing; the validation of the altered miRNAs was performed using RT-qPCR. The potential ability of miRNAs to modulate immune-related genes was investigated using bioinformatics tools. The results pointed out that 32 miRNAs, of which 14 were up- and 18 down-regulated, were differentially expressed in healthy vs. otitis-affected dogs. These results were verified by RT-qPCR. To assess the diagnostic value of miRNAs, ROC analysis was carried out, highlighting that 4 miRNAs are potential biomarkers to discriminate otitis-affected dogs. Bioinformatics showed that cerumen miRNAs may be involved in the modulation of host immune response. In conclusion, we have demonstrated for the first time that miRNAs can be efficiently extracted and quantified from cerumen, that their profile changes between healthy and otitis affected dogs, and that they may serve as potential biomarkers. Further studies are necessary to confirm their diagnostic value and to investigate their interaction with immune-related genes.

Keywords: otitis externa, dogs, miRNA, sequencing, biomarkers

INTRODUCTION

Otitis externa is defined as the inflammation of the external ear canal and represents one of the most prevalent skin disorders in dogs (1–4). The causes of otitis externa can be divided into primary and secondary (5, 6). Primary causes of otitis include inflammatory conditions, such as autoimmune or immune-mediated diseases, keratinization and glandular disorders, and ectoparasites. Onset of secondary causes such as bacterial and *Malassezia* spp. infections is generally associated by the emergence of primary diseases such as canine atopic dermatitis or combined with several predisposing factors (7). The host immune response to microorganisms in the external ear canal likely plays a pivotal role, but few data are available in dogs, except for studies on the immune reaction against *Malassezia* (8–10). Cerumen, or earwax, is a biological substance composed of lipids, proteins, amino acids, and carbohydrates produced by the combination of the excretions of ceruminous and sebaceous glands in the auditory canal of the external ear of mammalians. Cerumen is believed to protect the epithelial lining of the ear canal against pathogens. Besides its importance as a physical barrier, the involvement of cerumen in other functions, including specific immune defense, remains largely unexplored. Cerumen is supposedly involved in antimicrobial defense as demonstrated by the presence of lysozyme and immunoglobulins (11), as well as of additional proteins with antimicrobial functions, as recently shown by proteomics (12). In human cerumen, proteins belonging to β-defensin families were also detected (13), suggesting a possible role in the local innate immune response.

In addition to its biological function, cerumen has gained interest in the clinical setting as a potential source of biomarkers (14). Cerumen composition indeed reflects the pathophysiological status of the patient, containing lipids, proteins, and metabolites derived from blood (14). Although the amount and the variation of texture and color of cerumen during ear diseases have been accurately described in dogs (15), the active protective role of cerumen in the development of immunity during otitis externa is yet to be determined.

MicroRNAs (miRNAs) are short (∼22 nucleotides), single-stranded non-coding RNAs that modulate gene expression by binding to complementary target mRNA. MiRNAs down-regulate gene expression by silencing or degrading their mRNA target (16). Extensive research over the last years demonstrated that miRNAs fulfill a fundamental role in pathogen recognition and inflammatory responses (17). The profile of miRNAs is tissue-dependent and relative stable during several disorders and pathological alterations. Therefore, besides their importance as regulators of immune defenses and inflammation, miRNAs also provide promising targets and biomarkers for molecular-based diagnostics and therapies in both humans (18) and animals (19–21). Changes in miRNAs expression pattern have been observed in association with skin diseases (22) and in otitis media, where they were located in middle ear fluid exosomes (23, 24).

Since no information on miRNAs derangements in canine otitis externa is available, and that cerumen might provide a source of biomarkers, the present study aimed to assess miRNAs expression profiles in the cerumen of dogs affected by otitis externa. This study tested the hypothesis that (a) cerumen microRNA could be differentially abundant between healthy and otitis affected dogs; (b) cerumen microRNA could provide a source of biomarkers to discriminate between healthy and otitis-affected dogs, and (c) cerumen could be a source of microRNAs involved in immune reaction, and as such participates to the regulation of ear innate immunity. A next-generation sequencing pilot study was carried out to identify a list of potential differentially expressed (DE)-miRNAs extracted from cerumen. Results were validated and quantified by RT-qPCR, and functional enrichment analysis of target genes and functional interaction network analysis was finally carried out to identify pathways potentially affected by DE-miRNAs.

MATERIALS AND METHODS

Subjects and Sample Collection

The study was prospective, randomized, and blinded. Twenty client-owned dogs, of which 16 with bilateral and 4 with unilateral bacterial otitis externa, were included. Written informed consent was secured from dog owners prior to enrolment. Diagnosis of otitis externa was based on history, clinical signs such as head shaking, pruritus, local pain, otorrhea, erythema, or swelling of at least one ear canal, visible debris and discharge in the ear canal upon otoscopic examination, and cytological confirmation of bacterial overgrowth and/or bacterial infection by microscopic examination of the exudate. To collect the ear exudate, the external ear canal of the right and left ear was swabbed and the non-sterile cotton-tipped swabs obtained were streaked onto two glass slides, which were then heat-fixed and stained with a modified Wright's stain (Quick Panoptic Kit; Pokler Italia). At least 10 fields per slide were examined under optical microscopy and a number of bacteria $\geq$25 per high power microscopy field (400×), with or without bacterial phagocytosis by neutrophil granulocytes, were considered positive (infection) as previously described (25). Dogs with any topical or ongoing treatments for otitis externa were excluded. In the control group, 28 dogs deemed healthy based on history, physical, and otoscopic examination and on the absence of neutrophil granulocytes, bacteria <25 and yeast <5 per high power microscopical field (400x) on ear cytology (25) were included. **Supplementary Table 1** summarizes the characteristics of dogs.

After inclusion, the skin of each vertical ear canal was sampled by rubbing (I) a tubed sterile dry swab™ rayon [ref MW1028; MWE Co (Bath) LTD—England] for small RNA extraction; (II) a Transystem AMIES w/o charcoal plastic applicator rayon tipped swab (Copan Italia SPA—Brescia—Italy) for microbiological test; and (III) a non-sterile cotton-tipped swab for cytology for 10 s.

Microbiological Analysis

Microbiological analyses were performed as previously reported (26). Each swab was plated on Blood Agar plates with 5% sheep blood (Thermo Fisher Scientific), Mannitol Salt Agar (Thermo Fisher Scientific), and Mac Conkey agar (Thermo Fisher Scientific); the plates were aerobically incubated at 37°C for 24–48 h.

The same samples were plated on Sabouraud's dextrose agar with chloramphenicol and incubated at 30°C for 7 days and were used to identify the fungal flora.

The isolated bacteria were identified according to standard laboratory procedures (morphology, Gram staining, catalase, oxidase, etc.) and subjected to biochemical identification using the API system (bioMèerieux SA, Marcy L'Etoile, France). The species identification by miniaturized biochemical tests was accepted when the probability was >90%.

Cytology

The ear swabs were air-dried and stained with May-Grünwald Giemsa. The following parameters were evaluated at the microscope: cellularity, presence of epithelial cells, inflammatory cells, bacteria, and *Malassezia* spp. A semi-quantitative scoring system to evaluate all the parameters was designed. A five-point scale scoring was proposed as follows: 0, absent; 1, very rare presence; 2, mild presence (scarce number in some microscopic fields); 3, moderate presence (variable number in almost all microscopic fields); or 4, good presence (good number in all microscopic fields). When bacteria were present, their morphology was recorded.

Small RNA Extraction and Sequencing

Total RNA was extracted using miRNeasy Serum/Plasma Kit (Qiagen, Cat. No 217184) following the manufacturer's instruction. The RNA quality and quantity were verified according to MIQE guidelines (27). For all samples, RNA concentration was quantified by Qubit® 2.0 Fluorometer with Qubit® microRNA Assay Kit (Invitrogen, Cat. No. Q32880).

A pilot sequencing was performed on 3 healthy (**Supplementary Table 1**, no. 15 right, 19 right, and 20 right) and 3 otitis-affected samples (**Supplementary Table 1**, no. 32 left, 35 left, and 41 right). Small RNA transcripts were converted into barcoded cDNA libraries. Library preparation was performed as previously reported (28) using the NEBNext Multiplex small RNA Library Prep Set (Cat. No. NEB#E7560) for Illumina and run on the NextSeq500 (Illumina Inc., USA).

Computational Analyses

The output of NextSeq500 Illumina sequencer was demultiplexed using bcl2fastq Illumina software embedded in docker4seq package (29). miRNA expression quantification was performed using the workflow previously described (30), using the implementation as previously described (31). In brief, after adapter trimming with cutadapt (32), sequences were mapped using SHRIMP (33) to *Canis familiaris* precursors miRNAs available in miRBase 22.0—March 2018 (http://www.mirbase.org/). Counts table and cpm tables were used.

Validation by RT-qPCR

Total RNA was extracted from all samples included in the study using miRNeasy Serum/Plasma Kit (Qiagen, Cat. No. 217184). One ml of Qiazol (Qiagen) was added and, after incubation at room temperature for 5 min, 3.75 μl (25 fmol final concentration) of the exogenous synthetic spike-in control *Caenorhabditis elegans* miRNA cel-miR-39 (Qiagen, Cat. No. 219610) was spiked into samples. The reverse transcription was performed using the TaqMan Advanced miRNA cDNA Synthesis Kit (Applied Biosystems, Cat. No. A28007) as per manufacturer's instruction. The qPCR experiments were designed following MIQE guidelines (27). The small RNA TaqMan assays were performed according to the manufacturer's instructions using the selected primer/probe assays (ThermoFisher Scientific), including: cel-miR-39-3p (assay ID 478293_mir); cfa-miR-21-5p (assay ID rno481342_mir); cfa-miR-26a-5p (assay ID mmu481013_mir); cfa-miR-27b-3p (assay ID rno478270_mir); cfa-miR-320a-3p (assay ID 478594_mir); cfa-miR-342-3p (assay ID 478043_mir); cfa-miR-146a-5p (assay ID 478399_mir); cfa-miR-378a-3p (assay ID 478349_mir); cfa-miR-375-3p (assay ID mmu481141_mir); cfa-miR-423-5p (assay ID mmu481834_mir); miR-125b (assay ID rno480907_mir); and miR-199 (custom-designed). miRNAs were selected among those with the highest read counts. Quantitative reactions were performed in duplicate in scaled down (15 μl) reaction volumes using the TaqMan Fast Advanced Master Mix (Applied Biosystems, Cat. No. 4444558) on CFX96 Real-Time PCR detection system (BioRad Laboratories). The standard cycling program was 50°C for 2 min, 95°C for 3 min, and 40 cycles of 95°C for 10 s and 60°C for 30 s. Endogenous control for qPCR normalization was identified adapting the pipeline developed by Eisenberg and Levanon (34). Briefly, reference miRNAs were selected considering the individual raw count and with at least 50 reads for each sample; a standard error of the $\log_2$ fold change value <0.75 and a $\log_2$ fold change ranging between −0.074 and 0.46. Three reference miRNAs (cfa-miR-21, cfa-miR-26a, and cfa-miR27b) have been selected. No-RT controls and no-template controls were performed. The geometric mean of reference miRNA abundance was used for normalization. The relative quantification of target miRNAs was carried out after normalization of the sample using the geometric mean of reference miRNAs.

miRNA Target Prioritization

The target genes of DE-miRNAs were predicted using MiRWalk 3.0 (35), which includes 3 miRNA-target prediction programs [miRDB (36), miRTarBase (37), and Targetscan (38)]. The analysis was performed targeting the entire gene sequence (including 5′UTR, CDS, and 3′UTR). The list of target genes predicted by the three tools was included in further analysis and functional mRNA enrichment was performed using DAVID (Database for Annotation, Visualization and Integrated Discovery) bioinformatic resource (39, 40) and biological pathways in the KEGG (Kyoto Encyclopedia of Genes and Genomes) (41) were examined for enrichment. To visualize the interaction between immune-related genes and up- and down-regulated miRNAs, miRNet (42) software was employed to construct the miRNA-hub gene networks.

Computational and Statistical Analysis

Raw reads quality-check, adapter clipping, and mapping were performed as previously reported (30). After reads mapping, a matrix of integer values was created. The value in the *i*-th row and the *j*-th column of the matrix reported how many

reads have been unambiguously assigned to mature miRNA *i* in the sample *j*. The unwanted variation present in the data was estimated using the functions implemented in the SVA package (43). The differential expression analysis was run using DESeq2 (44), setting a thresholds adjusted $p < 0.1$ and $|\log_2 FC| > 1$. The differential expressed miRNAs (DE-miRNAs) were those associated with adjusted False Discovery Rate (FDR) ≤ 0.05 and the mean read count ≥ 300.

Statistical analysis was carried out using XLStat for Windows (Addinsoft, New York, U.S.A.), IBM SPSS Statistics 25 software (IBM Corp., 2017) and MedCalc 14.0 (MedCalc Software bvba, Ostend, Belgium). Statistical significance was accepted at $p < 0.05$. Data were tested for normality and homogeneity of variance using the Kolmogorov-Smirnov and Levene tests, respectively. As data were not normally distributed, non-parametric statistical tests were applied. The Kruskal-Wallis test was used to assess differences in miRNAs concentrations. *P*-values were adjusted using the Bonferroni correction.

A multivariate statistical analysis (Principal Component Analysis—PCA, correlation matrix, no rotation) was used for miR-320a, miR-342, miR-146a, miR-378a, miR-375, miR-423a, miR-125b, miR-199 as an exploratory analysis to detect the underlying relationships among miRNAs and to identify cases clusters. Data assumptions were checked, KMO (Keiser Meyer Olkin) and Bartlett's test of sphericity were applied to test the suitability of the data for structure detection. Factor scores were calculated for dogs when the component's Eigen value was greater than one, to evaluate the distribution of the subjects according to the considered variables and classed using the categories healthy dogs and dogs with otitis.

To determine the diagnostic accuracy of targets differing statistically between healthy and otitis affected dogs, receiver operating characteristic (ROC) analysis was performed as previously reported (45). The diagnostic values were calculated for miRNAs that showed significant differential expression in the buffalo blood.

RESULTS

Demographics and Characteristics of Study Subjects

A total of 95 samples, 59 from healthy and 36 from ears affected by otitis externa, were collected. The median age in the control and otitis affected groups was 9 (ranging from 6 months to 15 years) and 8 (ranging from 1 to 14 years) years, respectively. The male-to-female ratio was 11:17 in the healthy group and 11:10 in the otitis-affected group. A total of 19 different breeds was included in the list, with an over-representation of Labrador Retrievers (8) and German shepherds (5). The list of samples including the diagnosis and cytological and bacteriological data are listed in **Supplementary Table 1**.

Cytology and Bacteriology

Cytological findings and yeast and bacterial isolation results are listed in **Supplementary Table 1**. Mites were not observed in any of the cytological specimens. Cytology and culture evidenced bacterial organisms (both coccoid and rod-shaped) and yeast in clinically healthy and otitis affected ears independently of the clinical presentation. As it could be assessed by cytology, bacteria were higher in diseased ears. Yeast numbers did not correlate with otitis. In three dogs with a clinical diagnosis of otitis, neutrophils phagocytizing bacteria were observed in high numbers and were consistent with the clinical finding of severe otitis. *Malassezia* and bacterial organisms were isolated from healthy and diseased ears and no association with otitis externa was observed between number and type of yeast or bacteria.

RNA Extraction From Cerumen and Determination of miRNome Profile

To characterize miRNA expression profiles of cerumen, a pilot study small RNA-seq was performed on RNA extracted from the cerumen samples of three healthy and three otitis-affected dogs. After RNA extraction, small RNAs were selected according with their size (≈146 bp band) and sequenced on the NextSeq500 sequencer (Illumina). Multiple reads per sample, varying from 349,000 to 11,000,000, were obtained. Counts table was used to detect differentially expressed miRNAs via DESeq2 analysis (44). Furthermore, the analysis revealed the expression of 102 *Canis familiaris* (cfa) miRNAs, discarding lowly expressed miRNAs (≤ 1 raw count across 6 samples).

MiRNAs Are Modulated in Cerumen of Otitis-Affected Dogs

A cluster analysis based on the expression profiles of the six sequenced samples was performed. The results allowed to differentiate the samples in two clusters, namely cluster of otitis-affected and cluster of healthy control group (**Figure 1A**). To determine whether there were differences in the miRNAs expression profile of healthy and otitis-affected samples, a differential expression (DE) analysis applying using DESeq2 (44), with a threshold adjusted $P < 0.1$ and $|\log_2 FC| > 1$, was performed. A difference in miRNA profiles was observed, suggesting molecular changes due to otitis externa. Thirty-two miRNAs were significantly altered in otitis-affected dogs, of which 14 resulted upregulated (1.5- to 3.9- fold) and 18 down-regulated (1.6- to 5.5- fold) (**Figure 1B**).

Validation of Differentially Expressed miRNAs in Otitis-Affected and Healthy Dogs

RT-qPCR validation was performed on the 6 sequenced samples and on a separate independent set of 89 samples, collected from 56 from healthy and 33 from otitis-affected ears. To validate the sequencing results, eight differentially expressed (DE)-miRNAs were selected following their potential involvement in regulating the immune system. Their relative abundance was quantified using RT-qPCR. MiR-21-5p, miR-26a-5p, and miR-27-3p were analyzed as endogenous controls for normalization. Cel-miR-39, an artificial spike-in, was used as an internal control. The results are presented in **Figure 2**. The selected miRNA targets were detected in all samples. In accordance with the sequencing data, RT-qPCR results demonstrated that the levels of five miRNAs (miR-320a: $P \leq 0.0001$, $\text{ratio}_{\text{healthy/Otitis}}$

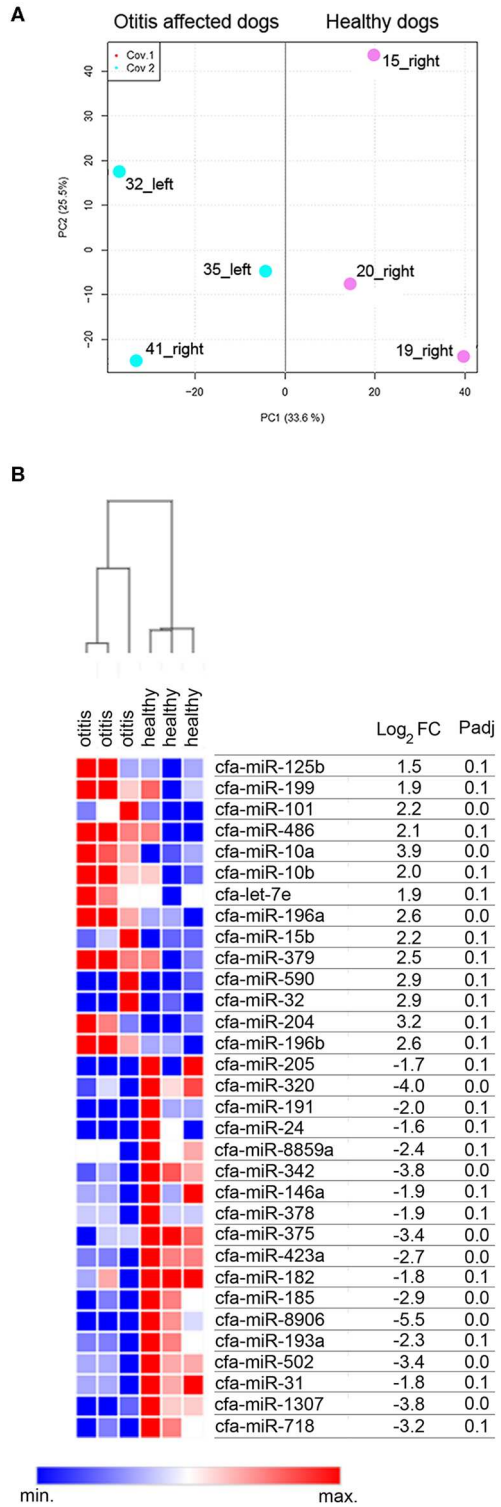

FIGURE 1 | Cerumen sequencing results. **(A)** Principal Component Analysis (PCA) of six sequenced samples. Two-dimensional PCA was used to determine whether otitis affected (blue point) could be distinguished from healthy (pink points) subjects. **(B)** Identification of DE-miRNAs between otitis affected and healthy dogs. Heat-map and table displaying the fold change and Padj of DE-miRNAs.

= 17.5; miR-342: $P \leq 0.0001$, $ratio_{healthy/Otitis} = 15$; miR-146a: $P \leq 0.0001$, $ratio_{healthy/Otitis=} 2.1$; miR-378a: $P = 0.0035$, $ratio_{healthy/Otitis} = 2.9$; miR-375: $P \leq 0.0001$, $ratio_{healthy/Otitis} = 11$) were significantly down-regulated in otitis-affected dogs. Remarkably, the RT-qPCR validation for miR-125b ($P \leq 0.0001$, $ratio_{healthy/Otitis} = 12.3$) did not confirm the sequencing results, presenting the evidence that this miRNA is down-regulated. MiR-199 and miR-423a did not exhibit statistically significant differences between otitis affected and healthy dogs.

The miR-320a, miR-342, miR-146a, miR-378a, miR-375, miR-423a, miR-125b, and miR-199 abundance for the 95 samples were also analyzed together using Principal Component Analysis (PCA, correlation matrix, no rotation), which is an exploratory analysis tool used to explain the structure of a set of variables through linear combinations. Good suitability of data for PCA analysis was valued (KMO = 0.795 and Bartlett's test $P \leq 0.001$). The PCA revealed two main factors with Eigenvectors greater than one, which together explains 75.9% of the variation between dogs. As shown in **Figure 3A**, the first factor (PC1-Component 1; Eigenvalue = 3.927; Explained variance = 49.092%) shows positive loadings for miR-320a, miR-342, miR-375, miR-423a, and miR-125b. The second factor (PC2-Component 2; Eigenvalue = 2.143; Explained variance = 26.791%; Cumulative explained variance = 75.883%) shows positive loadings for miR-146a, miR-378a, and miR-199. To test whether there were any significant effects of the dog condition, the PC miRNAs scores attributed to the samples on the first two main components of the PCA (explaining 75.883% of total variance) were analyzed through a Kruskal-Wallis test. Based on the category healthy and otitis-affected, dogs did not cluster homogeneously but were significantly ($P \leq 0.001$) sorted into two groups on PC1 (**Figure 3B**): one group with higher variable values associated with healthy dogs and the second group identified by a lower variability for otitis-affected dogs.

Assessment of the Diagnostic Value of DE-miRNAs

To investigate the diagnostic value and the diagnostic potency of DE-miRNAs in the cerumen, ROC curves and the area under the curve (AUC) were calculated. The diagnostic performance is reported in **Table 1**. The AUC was fair for miR-146a and miR-378a, good for miR-342 and miR-375, and excellent for miR-320a and miR-125b (**Figure 4**). Discriminant analysis was carried out to investigate the potential for improving diagnostic performance by analyzing multiple DE-miRNAs. The weighted average relative quantification (RQ) values of the miRNAs with an AUC>0.9 (miR-let-320a and miR-125b) and with AUC>0.8 (miR-let-320a, miR-125b, miR-342, and miR-375) were analyzed (**Supplementary Figure 1**). Median expression levels including the RQ of 2 DE-miRNAs were 26 (range, 17.07–703.13) and 3.2 (range, 0.74–17.74) in healthy and otitis affected dogs, respectively (**Supplementary Figure 1A**). Median expression levels including the RQ of 4 DE-miRNAs were 13.3 (range, 3.31–367.7) and 1.69 (range, 0.24–9.1) in healthy and otitis affected dogs, respectively (**Supplementary Figure 1C**). The predicted probability of being discriminated as infected

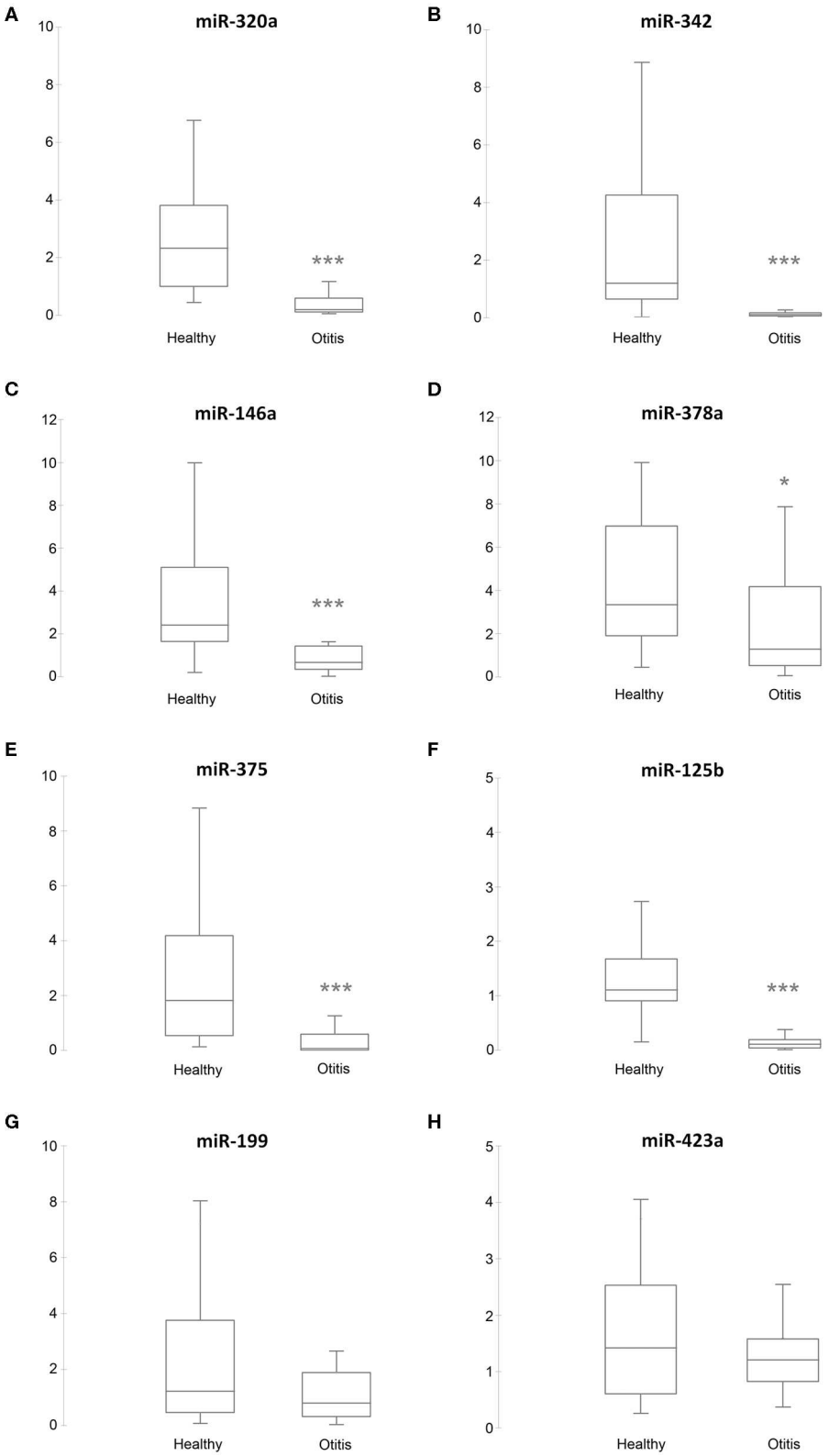

FIGURE 2 | Box plots of DE-miRNAs in otitis affected compared with healthy dogs. Significance was declared at $^{*}P < 0.05$, and $^{***}P < 0.001$. Black lines inside the boxes mark the medians. Whiskers indicate variability outside the upper and lower quartiles. **(A)** miR-320a, **(B)** miR-342, **(C)** miR-146a, **(D)** miR-378a, **(E)** miR-375, **(F)** miR-125b, **(G)** miR-199, and **(H)** miR-432a.

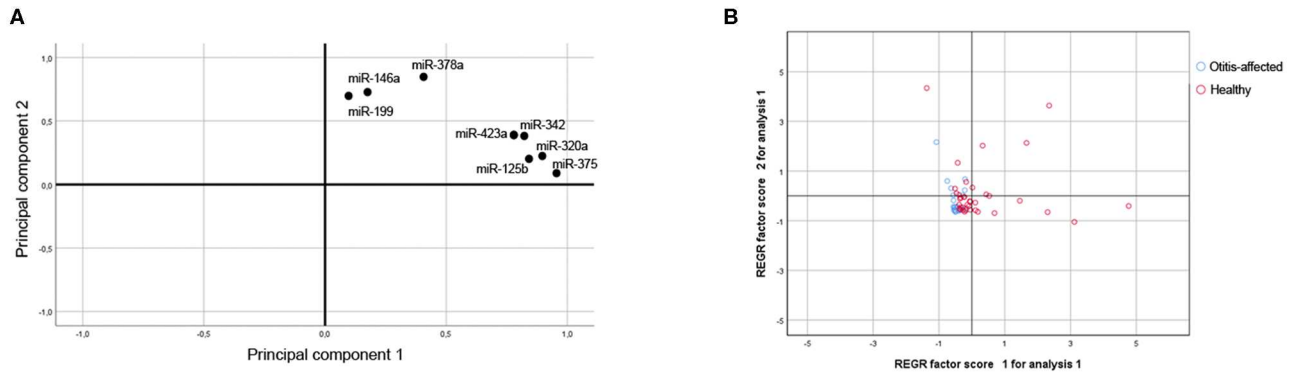

FIGURE 3 | Multivariate statistical analysis. **(A)** Projection for the loadings of the miRNAs considered for the First and Second Principal Component. **(B)** Score plot of dogs in terms of clinical outcome.

TABLE 1 | Area under the curve (AUC), sensitivity, specificity, and accuracy for DE-miRNAs in the cerumen.

miRNA	AUC	95% CI	*P*-value	Cut-off	Sensitivity	Specificity	Accuracy
miR-320a	0.9202	0.8656–0.9748	<0.0001	0.9084	0.8636	0.9748	0.8254
miR-342	0.8758	0.79–0.9616	<0.0001	0.2308	0.7273	0.9268	0.8571
miR-146a	0.7749	0.6487–0.9012	<0.0001	1.7416	0.8636	0.7317	0.7778
miR-378a	0.7129	0.5732–0.8525	0.0028	1.5993	0.5909	0.8537	0.7619
miR-375	0.8703	0.7823–0.9583	<0.0001	0.1464	0.6364	1	0.8730
miR-125b	0.9834	0.9834–0.9834	<0.0001	0.4365	0.9545	0.9512	0.9524
Av_2	0.9607	0.9349–0.9865	<0.0001	10.28	0.8696	0.9286	0.9077
Av_4	0.9762	0.9762–0.9762	<0.0001	4.32	0.8696	0.9762	0.9385

Av_2, weighted average relative quantification of miR-320a and miR-125b; Av_4, weighted average relative quantification of miR-320a, miR-125b, miR-342, and miR-375.

from the logit model based on the two [logit = (19.5 × expression level of miR-320a) + (10.4 × expression level of miR-125a)] or the four cerumen DE-miRNAs [logit = (19.5 × expression level of miR-320a) + (10.4 × expression level of miR-125a) + (5.26 × expression level of miR-342) + (-4.55 × expression level of miR-375)] was used to construct the ROC curves (**Supplementary Figures 1B,D**). The results of ROC curves analysis are reported in **Table 1**.

miRNA Localization, Target Prediction, and Pathway Enrichment

To investigate the immune relevance, predicted targets of DE-miRNAs were computationally retrieved from miRWalk resources. The mRNA enrichment was performed using DAVID bioinformatic tool. Since little information on alterations in immune response contributing to the onset and progression of otitis externa are available, an enrichment of mRNA targets that encode for immunologically relevant genes was performed comparing the target genes obtained from miRWalk with the Gene List of ImmPort (46). The predicted mRNA targets of up-regulated miRNAs were 270 [164 at 3′ untranslated region (UTR), 21 at 5′UTR, and 85 at codon sequence (CDS)], of which 21 were immune-related. The predicted mRNA targets of down-regulated miRNAs were 133 (78 at 3′UTR, 10 at 5′UTR, and 45 at CDS), of which 15 were involved in immunity. The list of immunologically relevant genes is reported in **Table 2**. KEGG pathway analysis was performed on the enriched immune-related targets of up- and down-regulated miRNAs using DAVID. The top 10 significantly enriched KEGG pathways are reported in **Figure 5**. The up-regulated miRNAs (**Figure 5A**) were identified to be predominantly involved in the following pathways: HIF1 (Hypoxia Inducible Factor 1) and FoxO (Forkhead box O3) signaling pathways. The down-regulated miRNAs (**Figure 5B**) were revealed to be involved in the T cell receptor signaling pathway, MAPK (Mitogen-Activated Protein Kinase) signaling pathway, Focal adhesion, and RAP1 (Ras-proximate-1) signaling pathway. Aiming for further understanding the associated functions of the DE-miRNAs, Gene Ontology (GO) analysis was performed. GO enrichment analysis included the categories molecular function (MF), cellular component (CC), biological process (BP) (**Figure 6**). For down-regulated miRNAs, most MF items mainly included genes involved in the regulation of MAPK activity, growth factor activity, and heparin-binding; the enriched CC converged on genes associated with the nucleoplasm and extracellular exosomes, while BP on ROS (Reactive oxygen species) metabolic species, signal transduction in response to DNA damage, and VEGF (Vascular-Endothelial Growth Factor) receptor signaling pathway. For up-regulated miRNAs, MF items focused on steroid hormone receptor activity and insulin receptor substrate binding; CC converged on receptor complex, phosphatidylinositol 3-kinase complex and

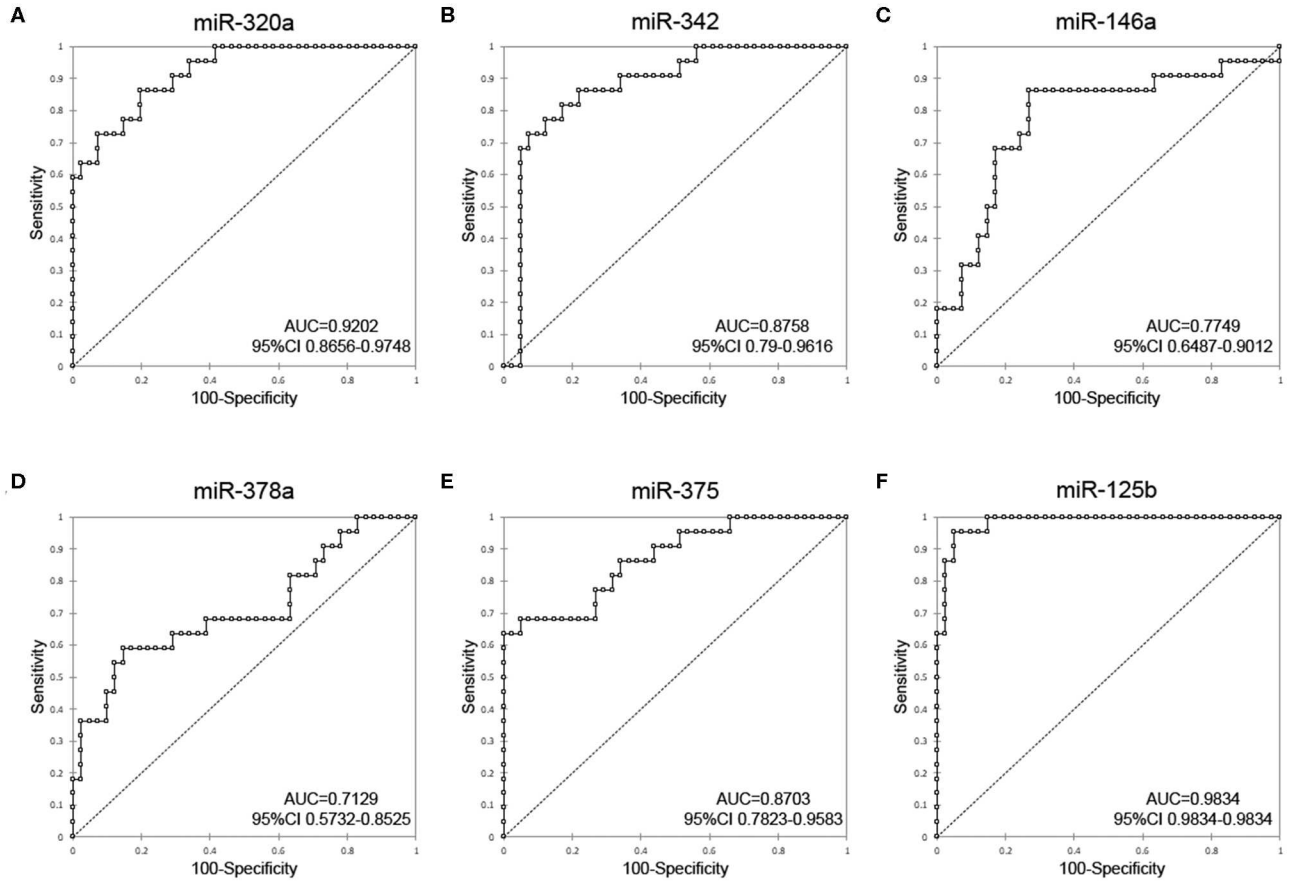

FIGURE 4 | Receiver-operator characteristic (ROC) curve analysis of DE-miRNAs in the cerumen. AUC, area under the curve; CI, confidence interval. **(A)** miR-320a, **(B)** miR-342, **(C)** miR-146a, **(D)** miR-378a, **(E)** miR-375, and **(F)** miR-125b.

integral component of plasma membrane, and BP on positive regulation of transcription from RNA polymerase II promoter, steroid hormone-mediated signaling pathway, and positive regulation of cell migration. To identify which type of cells in cerumen express DE-miRNAs, the atlas of miRNA expression (FANTOM5) in immune cells and keratinocytes was explored. The radar chart reported in **Figure 7A** presents the contribution of cells to the production of up- and down-regulated miRNAs. In detail, down-regulated miRNAs are produced mainly by keratinocytes, monocytes, dendritic cells, and T cells, while up-regulated miRNAs by B cells and mast cells. The miRNA–mRNA networks determined using miRNet database are presented in **Figures 7B,C**.

TABLE 2 | Immune-related target genes of differentially expressed miRNAs.

Immune-related genes targeted by up-regulated miRNAs	Immune-related genes targeted by down-regulated miRNAs
FASLG, IGF1R, TGFBR3, SOCS1, CRLF3, NR2C2, RORA, NR4A3, CREB1, STAT3, VDR, INSR, ACVR2A, KDR, NR3C1, PTGER4, EDN1, PIK3R1, NR6A1, PIK3CA, PPP3R1	*BDNF, THBS1, CDC42, VEGFA, IFNG, PDGFRB, MAPK14, PAK4, CMTM4, GRB2, LRP1, NFATC3, NFAT5, SP1, MAPK1*

DISCUSSION

The findings of this study provided for the first-time evidence that (a) miRNAs can be efficiently extracted, sequenced, and quantified by RT-qPCR from canine cerumen and (b) cerumen microRNAs quantities change during otitis externa. In the first part of the investigation, a pilot sequencing study was performed to profile the miRNome of cerumen, showing that otitis externa changed the expression of 32 miRNAs, of which 14 were more abundant and 18 were less abundant compared to healthy dogs. In the second step, 8 differentially expressed miRNAs were validated on a larger cohort using a RT-qPCR approach. It was found that miR-320a, miR-342, miR-146a, miR-378a, miR-375, and miR-125b were down-regulated in cerumen from otitis affected dogs. The results are supported by PCA analysis, of which the first principal component accounts for as much of the variability in the data as possible. miRNAs on this component are the most important in explaining the differences between healthy and otitis-affected dogs. Moreover, heat map, hierarchical

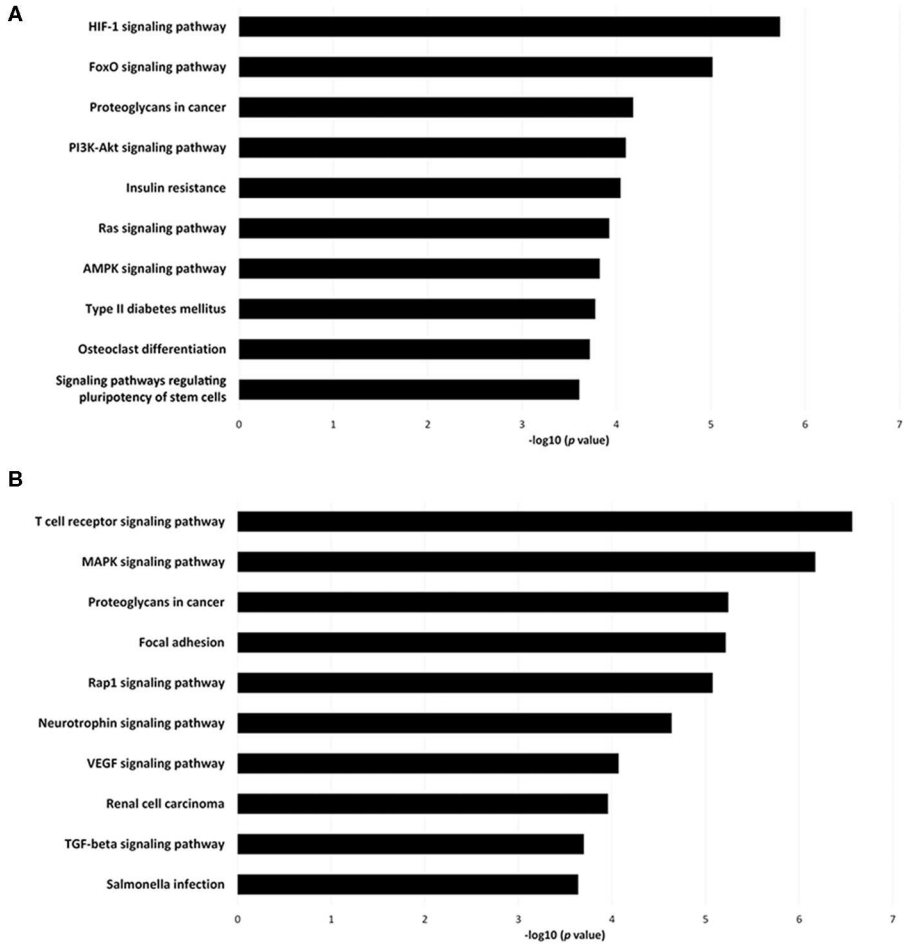

FIGURE 5 | Pathway enrichment analysis for genes regulated by **(A)** up- and **(B)** down-regulated miRNAs. Genes regulated by DE-miRNAs were retrieved and enriched in KEGG using DAVID. The *P*-value was negative 10-base log transformed. The top 10 enriched KEGG pathways are reported.

clustering, and PCA revealed that dogs with otitis showed increased variability in miRNA levels compared to healthy ones.

Given the functions of the target genes regulated by DE-miRNAs, the current findings demonstrated that miRNAs contained in cerumen might interact with several pathways involved in the host innate immunity, including modulation the inflammatory reaction, the regulation of M1/M2 monocyte lineage polarization, the resolution of inflammation, and reparation of damaged tissues.

All DE-miRNAs are involved in pathways that regulate the inflammatory reaction. Therefore, their down-regulation provides cerumen with a potential pro-inflammatory activity, following a mechanism different for each DE-miRNAs. For example, the downregulation of miR-320a induced the overexpression of pro-inflammatory cytokines through promoting *COX-2* (*Cyclooxygenase-2*) expression by targeting *MAPK-1* (47). In macrophages, decreasing miR-125b-5p has a dual, apparently opposite, effect of increasing secretion of the pro-inflammatory chemokine *MCP-1* (*Monocyte chemoattractant protein-1*) (48) and upregulating *B7-H4* in macrophages, which induces an anti-inflammatory effect (49). Decreasing miR-378a through targeting CD47-*SIRPα* (Signal Regulatory Protein Alpha) inhibits phagocytosis in macrophages, and promotes the secretion of *TNFα* (*Tumor Necrosis Factor-alpha*) and *IL-6* (*Interleukin-6*) (50). MiR-375 regulates the expression of pro-inflammatory cytokines such as *IL1-β, TNFα,* and *IL-6*: therefore, miR-375 decrease also reduces cytokine expression, as shown in a myocardial infarction model (51). Finally, miR-146 is involved in the regulation of inflammation via negative feedback of toll-like receptor signaling (*TLR*) (52), as already described in otitis media in humans (24): consequently, down-regulation of miR-146 induces a pro-inflammatory effect. Moreover, miR-125b and miR-146, which are also reduced after TLR activation, can promote tolerance to endotoxin (53). We found that all these miRNAs are less abundant in cerumen of dogs affected by otitis, confirming what has been already reported in other diseases such as for miR-342 (54) and miR-375 in sepsis, or during C5a (complement component C5a) activation for miR-320a (55).

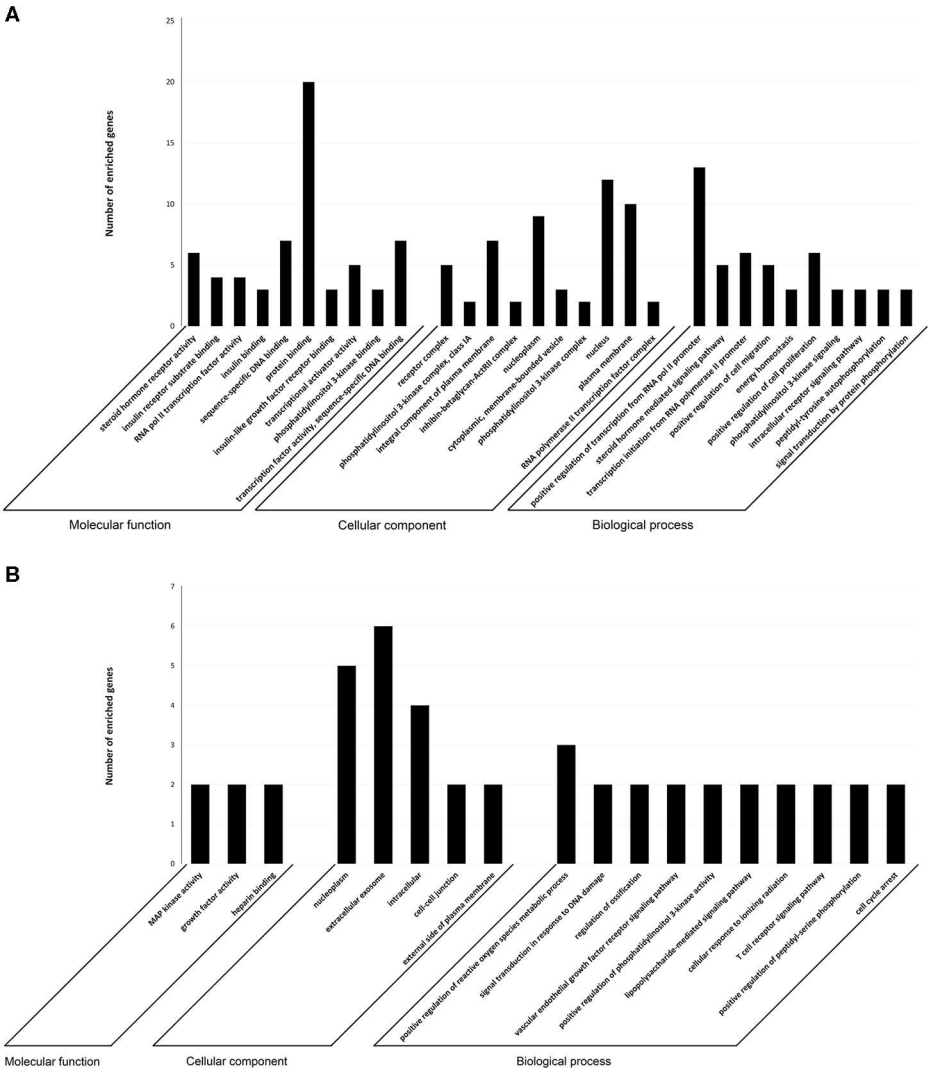

FIGURE 6 | Target prediction. GO annotation of genes regulated by **(A)** up- and **(B)** down-regulated miRNAs. The target genes were annotated by DAVID at three levels: molecular function, cellular component, and biological process. The top 10 significantly enriched items are shown.

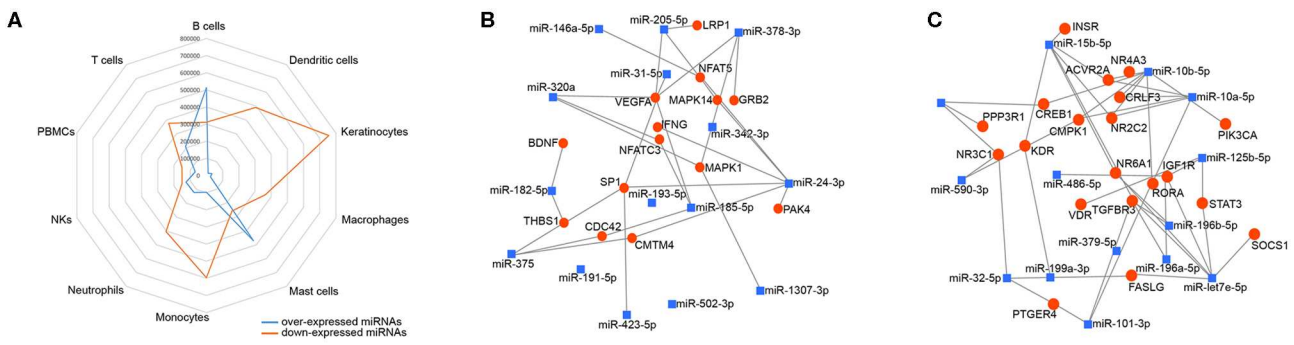

FIGURE 7 | Localization of DE-miRNAs and network analysis of miRNAs-immune-related target genes in the cerumen. **(A)** FANTOM5 miRNAS atlas was analyzed for the expression of DE-miRNAs in immune cells and keratinocytes. Expression (counts per million) was plotted for these miRNAs in the radar graph; **(B)** network of over-expressed miRNAs constructed using miRNet; **(C)** network of down-regulated miRNAs constructed using miRNet. The blue squares represent miRNAs, the red dots mRNA.

All DE-miRNAs are also involved in the modulation of monocyte/macrophage polarization. Specifically, miR-125b, miR-378, miR-375, and miR-372 are involved in polarization to M1 lineage whereas miR-320a and miR-146a are involved in polarization toward M2 lineage. MiR-320a, in particular, was found in epithelial-derived microvesicles, and could activate macrophage pro-inflammatory effects (56). The effect of miR-320a may be even more complex since a more recent study demonstrated that miR-320a promotes the polarization toward immunosuppressive M2 macrophages meanwhile inducing polarization toward M1 lineage (57). Moreover, the effects of miR-375 require a more in-depth investigation since the inhibition of miR-375 represses M1 macrophage polarization and promotes M2 macrophage polarization, targeting *PDK-1* (*Pyruvate Dehydrogenase Kinase 1*) (51).

Following their inflammatory functions, classical M1 macrophages feature higher capabilities of phagocytosis and, more in general, a pro-inflammatory phenotype. On the contrary, non-classical M2 monocytes share a lower pro-inflammatory activity, although their precise physiological roles remain still poorly defined (58). Given the background that miRNAs play pivotal roles in macrophage activation and polarization (59), the finding that miRNAs involved in monocyte/macrophage polarization were detected in cerumen was not surprising and suggests that during inflammatory responses, monocytes are attracted to cerumen, and become activated on site and modulated by miRNAs.

The DE-miRNAs are involved in a third mechanism represented by the regulation of repair pathways after inflammation. For example, miR-320a is involved in intestinal mucosal reconstitution and repair after inflammation (60), and miR-125b inhibits proliferation and promotes differentiation of keratinocytes in the skin (61). Therefore, the capability of cerumen to down-regulate miR-320a and miR-125b may result in keratinocyte proliferation, which in turn may accelerate wound healing and homeostasis restitution.

Although neutrophils were observed in high numbers only in three dogs, cytological findings associated always with otitis paralleling the observations of Angus (62). According to our results, cytology should be considered a specific diagnostic technique assisting in the diagnosis of otitis in dogs although bearing lower sensitivity. Isolation of yeasts and bacteria species did not correspond to a specific condition; however, increased numbers of bacteria were evidenced in cases of otitis as previously reported (62). These findings suggested that isolation of organisms should be assessed in the context of clinical presentation and cytological findings as an adjunctive tool to support diagnostic and therapeutic protocols. Noteworthy, cytology and microbiology did not always result in sensitive techniques to distinguish healthy vs. diseased ears. On the contrary, ROC analysis highlighted that two miRNAs, namely miR-125b and miR-320a, can discriminate otitis-affected from healthy dogs with high sensitivity (>86%) and specificity (>97%), confirming that these miRNAs may be excellent candidate biomarkers. These findings are more relevant if considered that differentiation among diseased and normal ears occurred independently from the presence of elevated numbers of neutrophils thus independently of morphological features of inflammation.

Currently, one of the main issues in human as well as in veterinary medicine is the overuse of antibiotics, which promotes the selection of resistant commensal flora. Careful use of antibiotics for treatment of cutaneous infections, including otitis externa in dogs, is recommended (63). We believe that molecular biomarkers, such as the miRNAs identified in this work, may assist the clinical monitoring of drug effectiveness during otitis externa treatment. To support this hypothesis, further studies will be performed on cerumen collected from dogs affected by otitis and treated with antibiotics. Moreover, as allergic dermatitis is the most frequently recognized primary cause of canine otitis externa (64), further studies evaluating the change of biomarkers expression in allergic dogs, without symptomatic otitis externa, compared to healthy subjects could provide the clinician with a valuable screening method to monitor the ear canal inflammatory status. This could, in turn, support the proactive use of targeted anti-inflammatory treatments aimed to prevent the development of secondary infections decreasing further the use of antibiotics and the risk of bacterial resistance.

In conclusion, to the best of the authors' knowledge, this is the first report demonstrating the presence of miRNAs in cerumen and their changes in dogs with acute otitis externa. These findings provided insights on the role of miRNAs in modulating immune defenses in cerumen, a biological fluid whose importance has been almost completely neglected so far, meanwhile highlighting the potential role of cerumen as a source of biomarkers. In this work, the finding of miRNAs differential expression is relevant for a better understanding of the pathogenic mechanism leading to otitis and tampering of external ear damage and provides a novel technique able to discriminate healthy vs. otitis-affected ears representing a more specific and sensitive diagnostic tool compared to cytology and microbiology. Thus, the abnormal expression of miRNAs may lead to an early diagnosis of otitis and timely treatment.

Further studies are necessary to confirm their diagnostic values by increasing the number of clinical samples, associating their abundance with specific pathogens and antibiotic treatment, and to investigate the direct interaction between these miRNAs and their target genes.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. Samples were collected during routine health checks or clinical evaluation of affected dogs, under informed consent of

the owners and out of the scope of Directive 2010/63/EU (art. 1.5.f practices not likely to cause pain, suffering, distress or lasting harm equivalent to, or higher than, that caused by the introduction of a needle in accordance with good veterinary practice). This study design was approved by the Italian Ministry of Health, project n. IZS ME 13_15 RC.

AUTHOR CONTRIBUTIONS

CL and FC conceived and designed the experiments and provided the original idea of the study. PR, RP, SL, and ED'U enrolled patients and performed the clinical diagnosis. MC and GGr carried out the cytology and microbiological analysis, respectively. MA performed the sequencing experiment. RC, MM, and CL performed bioinformatics and statistical analysis. CL and VZ performed the RT-qPCR experiments. FC, GGa, and GB funded the project. CL wrote the first draft of the manuscript. All authors read and approved the final draft of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.00914/full#supplementary-material

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REVIEW
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Innate Immunomodulation in Food Animals: Evidence for Trained Immunity?

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Antimicrobial resistance (AMR) is a significant problem in health care, animal health, and food safety. To limit AMR, there is a need for alternatives to antibiotics to enhance disease resistance and support judicious antibiotic usage in animals and humans. Immunomodulation is a promising strategy to enhance disease resistance without antibiotics in food animals. One rapidly evolving field of immunomodulation is innate memory in which innate immune cells undergo epigenetic changes of chromatin remodeling and metabolic reprogramming upon a priming event that results in either enhanced or suppressed responsiveness to secondary stimuli (training or tolerance, respectively). Exposure to live agents such as bacille Calmette-Guerin (BCG) or microbe-derived products such as LPS or yeast cell wall ß-glucans can reprogram or "train" the innate immune system. Over the last decade, significant advancements increased our understanding of innate training in humans and rodent models, and strategies are being developed to specifically target or regulate innate memory. In veterinary species, the concept of enhancing the innate immune system is not new; however, there are few available studies which have purposefully investigated innate training as it has been defined in human literature. The development of targeted approaches to engage innate training in food animals, with the practical goal of enhancing the capacity to limit disease without the use of antibiotics, is an area which deserves attention. In this review, we provide an overview of innate immunomodulation and memory, and the mechanisms which regulate this long-term functional reprogramming in other animals (e.g., humans, rodents). We focus on studies describing innate training, or similar phenomenon (often referred to as heterologous or non-specific protection), in cattle, sheep, goats, swine, poultry, and fish species; and discuss the potential benefits and shortcomings of engaging innate training for enhancing disease resistance.

Keywords: trained innate immunity, veterinary species, disease resistance, beta-glucans, innate memory

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INNATE MODULATION

While various approaches are used to limit disease and antibiotic usage in agricultural animals, efficacious intervention strategies remain unavailable for many diseases. Immunomodulation is one approach to engage or prime (1) the host's own immune system to defend against infectious disease. Vaccines are effective immunomodulators, priming the adaptive immune system, and

BOX 1 | Defining immunomodulation.

Immunomodulation

Changes to the immune system after exposure to a substance or compound (i.e., agonist) that stimulates or suppresses the immune response. This review is focused on immunomodulation that alters the immune response to subsequent exposure with non-related (heterologous) immune agonist, not the priming agonist.

Immunomodulation of adaptive immunity: altered vaccination or natural exposure of an animal to pathogens or other foreign agents induces the generation of effector and memory T- and B-cells to provide long-term (multi-year to lifetime) protection against the foreign agent.

Exposure of an animal to a priming substance or compound, often a protein or protein-polysaccharide, such that subsequent exposure to similar compound results in cross-reactive response. dependent on B- and T-cells. anergy and tolerance are functions of non-repsonsiveness or suppressive responses by B- or T-cells

Immunomodulation of innate immunity (innate memory): exposure of an animal or cells to a priming substance or compound (i.e, agonist), often a microbial-associated molecular pattern (MAMP), such that exposure to non-related immune agonist results in heightened (trained) or suppressed (tolerant) response. Observed in non-T, non-B cells, and primarily in myeloid lineage and NK cells. Mechanism includes epigenetic and metabolic reprograming of innate immune cells and progenitor cells. Duration of effect is not yet determined, but there is evidence for months to a few years.

Training: *enhanced* response to heterologous agonists

Tolerance: *decreased* response to heterologous agonists

well-understood by infectious disease experts. However, less common or at least less frequently discussed is immunomodulation of the innate arm of the immune system for enhanced disease protection. A related, rapidly evolving field of immunomodulation is innate training, which relies on memory of the innate immune system (see **Text Box**).

While adaptive immune memory is well-understood at the cellular and molecular level, the innate system was not known to have memory, and concordantly disease prevention strategies primarily targeted the adaptive immune system (e.g., vaccination). However, the paradigm on innate memory has recently shifted, with substantial evidence indicating that innate immune cells functionally adapt after stimulation or microbial exposure. More specifically, circulating monocytes, monocyte-derived macrophages, and NK cells have altered secondary responses to various pathogens or microbe-associated molecular patterns (MAMPs) after an initial priming event with the same or different MAMP (1–4). *In vitro*, purified monocytes stimulated with specific innate agonists, such as β-glucan or live-attenuated tuberculosis vaccine [*Mycobacterium bovis* bacillus Calmette-Guerin (BCG)], and restimulated days later with a heterologous MAMP, had heightened responses compared to cells that were not primed with β-glucan or BCG [reviewed in (3)]. Thus, the adaptive immune system may not be the only consideration for development of disease intervention strategies for enhancing food animal health.

The sustained effect of trained innate immunity is dependent on epigenetic changes, chromatin remodeling, and basal metabolic shifts that occur in the cell after primary stimulation, with effects that can be long-lasting. Primary MAMP (5, 6) exposure leaves the cell in a "poised" state, or a state in which the cell is ready to respond to secondary insult or exposure. In a trained response, cells respond with increased production of effector molecules, including proinflammatory cytokines, upon secondary stimulation or exposure (**Figure 1**). The trained response differs from innate tolerance, in which poised cells respond with reduced production of effector molecules (**Figure 1**). While circulating immune cells such as monocytes and NK cells may exhibit a trained response, the lifespan of circulating cells is relatively short-lived (7) and the length of time cells exhibit a trained phenotype may concordantly be short-lived. However, epigenetic modification of bone marrow progenitor cells that become circulating effector cells (including monocytes and NK cells), underlies innate training *in vivo* and contributes to the longevity of innate training (8, 9). Experimental vaccination of humans with BCG enhances *in vitro* PBMC pro-inflammatory cytokine production upon stimulation with the MAMP lipopolysaccharide (LPS), even 12 months after BCG administration (1). Furthermore, epidemiological studies with human infants found that BCG vaccination is associated with non-specific (i.e., not related to BCG) protection. In other words, BCG vaccinated children had enhanced resistance to other diseases (5, 6), leading to increased overall survival and decreased incidences of morbidity. Importantly, the protective benefits were noted months to years after vaccination (10). Food animals are relatively short lived, and changes to innate immunity in early life may afford a protective effect against disease for the animals' entire lifespan.

In this paper, we briefly review important concepts from mouse, rabbit, and human literature, including mechanisms of innate memory, training, and tolerizing agents, and evidence for innate training in various cells types. However, the primary focus of this review is to summarize the available evidence for innate immunomodulation and memory in agricultural species including cattle, pigs, poultry, fish, and small ruminants.

REVIEW OF HUMAN AND ANIMAL MODEL LITERATURE

Immunomodulator Molecules

Bacterial endotoxin, or LPS, is the earliest described and best-known agonists capable of modulating the innate immune system (11). Priming of innate cells, most notably monocytes, with low doses of LPS, followed by homologous LPS restimulation resulted in a depressed inflammatory immune response (i.e., tolerance) (12, 13). However, Gregory Shwartzman described a phenomenon whereby rabbits intradermally injected with super low doses of gram-negative sterile culture filtrate had dermal necrosis at the site of injection following intravenous rechallenge with the same filtrate (14), suggesting the cells primed at the initial injection site responded to the secondary stimulation with a heightened response. The discovery of the "Shwartzman

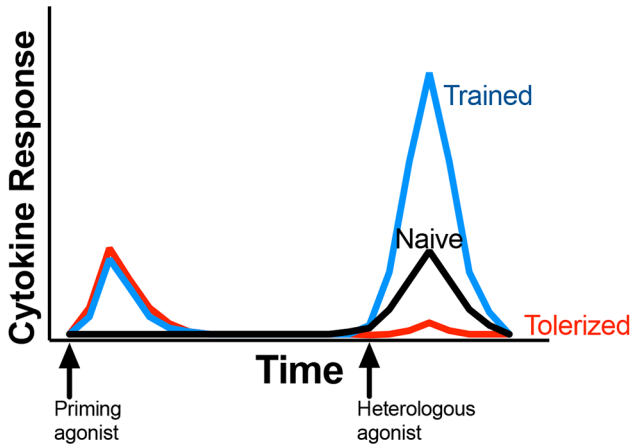

FIGURE 1 | General outline of immune response associated with innate memory. Initial exposure to a priming agonist (red and blue lines) induces an innate response, typically measured as induction of proinflammatory cytokine production. After a rest period, subsequent stimulation of the same cells or animal with a heterologous agonist (commonly LPS or Pam_3CSK_4) results in tolerance or trained response, marked by reduced (red line) or enhanced (blue line) cytokine production when compared to naïve response (black line). Proinflammatory cytokines, including IL-1β, TNF-α, or IL-6 are the most common cytokines measured for innate memory. Priming and heterologous stimulation may all occur *in vitro* with primary cells; *in vivo*; or a combinantion of *in vivo* with *ex vivo* cell stimulation.

phenomenon" was followed by multiple studies highlighting the importance of dose, as low doses of LPS induced tolerance while super low doses resulted in heightened immune responses (15–18). Compounds besides LPS can induce a tolerant state in myeloid cells when restimulated with the same molecule (homologous tolerance). Additionally, priming with one agonist could induce tolerance in response to restimulation with a heterologous agonist, a process termed cross-tolerance (13, 19, 20). Zymosan, a particulate preparation of β-glucans, mannans, and proteins from *Saccharomyces cerevisiae* was one of the earliest known inducers of cross-tolerance (21, 22). Collectively, various molecules have been implicated in tolerance, and the dose upon primary exposure impacts the induction of tolerance.

As LPS is known as a classic tolerizing agent, the BCG vaccine is a highly described inducer of innate training. There are numerous reports on the non-specific effects of BCG vaccination of infants in countries that actively administer neonatal BCG, with significant reductions in non-tuberculosis diseases (5, 6, 23). In a set of particularly compelling studies, BCG vaccination of low-birth weight infants in Guinea-Bissau was associated with a nearly 50% reduction in mortality rates, primarily due to reductions in sepsis and respiratory infections (5, 6). Monocytes and NK cells from BCG-vaccinated adults and infants, compared to non-vaccinated cohorts, display increased expression of toll-like receptors and increased cytokine production in response to various pathogens and their products (e.g., *M. tuberculosis*, *Candida albicans*, *Staphylcoccus aureus*, LPS, and Pam_3CSK_4) (17–19). Mice vaccinated with BCG are protected from lethal *C. albicans* challenge via a mechanism that requires macrophages (24) and humans vaccinated with BCG show reduced viral titer when experimentally infected with the attenuated yellow fever virus vaccine strain (25). However, BCG-induced trained immunity did not protect mice against experimental influenza A infection (26). Early studies with *Mycobacterium tuberculosis* or BCG indicate enhanced resistance against subsequent disease may not be a universal phenomomen. While protection in mice against *Bacillus anthracis, Brucella suis*, *Staphylococcus aureus, Pasteurella pestis, Listeria monocytogenes,* and *Klebsiella pneumonia* has been noted (27–30), BCG treated mice are more sensitive to endotoxin and had similar mortality to untreated mice when challenged intravenously with a low dose of *Salmonella enteritis* (1×10^4 CFU) (31). While not all encompassing, numerous reports indicate BCG administration in humans or rodent animals models enhances resistance to subsequent disease with increased immune responses to the secondary agent, a hallmark of innate training.

β-glucans, which activate innate cells via the Dectin-1 receptor or CR3, can also induce innate training. However, the source and type of β-glucan impacts the primary immune response and subsequent induction of innate memory. Human myeloid-lineage cells require cross-linkage of the Dectin-1 receptor and formation of a phagocytic synapse for downstream signaling (32). Soluble β-glucans, such as laminarin with (1–3)(1–6) linkages, can bind to the Dectin-1 receptor but are incapable of cross-linking multiple receptors and thereby fail to initiate an immune response (32, 33). As discussed above, zymosan induces tolerance in monocytes. It is a complex compound of β-glucan with highly branched (1–3)(1–6) linkages that, along with mannan and proteins, forms the cell wall. However, human and mouse monocytes primed with β-glucan from *C. albicans* exhibited a trained phenotype upon heterologous restimulation (17, 34–36). In fact, heat-inactivated *C. albicans* alone is sufficient to induce a trained state in human monocytes (34). β-glucans primarily induce innate training, but there are instances in which tolerance is induced, though the reason may not be related to dose, but receptor binding or signaling by additional cell wall components contained within the product.

There are many documented and perceived benefits associated with innate memory, but some potential drawbacks. While β-glucans and BCG are the most studied innate priming agonists other MAMP molecules, such as flagellin, muramyl dipeptide (MDP), polyinosinic-polycytidylic [Poly(I:C)] can induce innate training (1, 17). Innate training is the proposed mechanism for the non-specific benefits observed with certain vaccines, including the yellow fever vaccine, measles vaccine, vaccinia, and the influenza vaccine (37, 38). However, a heightened immune response to subsequent infections may result in enhanced pathology, an undesired effect. Indeed, trained immunity is hypothesized to contribute to autoimmune diseases (39, 40) and in a controlled experiment, patients vaccinated with BCG and later infected with malaria experienced earlier and more clinically severe symptoms than those not vaccinated with BCG (41). Thus, a deeper understanding of the implications associated with harnessing innate memory are warranted.

Mechanisms and Cells in Trained Immunity

Trained immunity is based on epigenetic reprogramming in innate immune cells, which has been documented primarily in monocytes (35, 42). The epigenetic modifications lead to changes in gene expression and consequent protein production upon secondary stimulation. Chromatin modifications and changes in DNA accessibility are the central processes of epigenetic reprogramming associated with trained immunity. Histone modifications, such as increased methylation of the latent enhancer histone H3 at K4 (H3K4me1), reduced methylation of the repressor histone marks such as histone H3 at K9 (H3K9me3) and the most informative histone marker, increased acetylation at the poised/active enhancer mark (H3K27ac), are associated with a trained phenotype (1, 2, 35, 36). Other posttranscriptional regulatory mechanisms, such as microRNA (miRNA) modulation of mRNA levels, are involved in the regulation of the immune response (43). MicroRNA genes, as well as protein coding genes, can be regulated by histone modifications, and at the same time, miRNAs can directly and indirectly target effectors of the epigenetic machinery (44) and immune system mRNAs.

Changes to metabolic state are also noted in trained cells, and likely the result of epigenetic reprogramming whereby cells are primed to respond to secondary stimulation. Metabolic state is important for rapid release of intermediate substrates, such as nucleic and amino acids, necessary for the production of effector molecules (45). Innate training by *C. albicans* β-glucan is evidenced by an increase in basal glycolysis and a decrease in basal mitochondrial respiration, a measure of oxidative phosphorylation (Warburg effect) (46). The importance of glycolysis in β-glucan mediated innate training was noted in a clinical trial wherein β-glucan injection was administered to human volunteers, with a cohort also receiving metformin (a drug that prevents gluconeogenesis). Individuals on metformin did not exhibit enhanced *ex vivo* cytokine production following heterologous restimulation, which was in contrast to volunteers who received the β-glucan injection without prior metformin treatment (47). Thus, the availability and capacity to utilize glucose is critical in trained innate cells.

In addition to evidence of metabolic state impacting β-glucan induced training, BCG impacts cellular metabolism. BCG-treated cells have an increase in basal glycolysis as well as oxidative phosphorylation (48). Activation of the metabolic Akt/mTOR/HIF1 pathway is a critical feature of BCG-mediated trained immunity. Inhibition of glutamine or mTOR/glycolysis metabolism during *in vitro* training with BCG inhibited mRNA expression, and also prevented the epigenetic changes (H3K4me3 and H3K9me3) normally associated with BCG trained immunity (48). Collectively, the epigenetic and metabolic changes associated with BCG-induced training, or lack thereof, are linked. Understanding the mechanisms associated with training can provide a more targeted approach to modulate immune status.

Innate training and tolerance are well-described in monocytes and monocyte-derived macrophages, and to a lesser extent in NK cells (19, 34, 46, 49, 50). *In vitro* studies with purified monocytes and *in vivo* studies with severe combined immunodeficient (SCID) mice indicate T and B cells are not required for development of trained immunity (1). Epigenetic reprograming of myeloid progenitor cells leads to long lasting changes to emigrating monocytes, contributing to the longevity of innate training (8, 9). Tissue resident macrophages (e.g., Kupffer cells in the liver) are terminally-differentiated and do not rely on circulating monocytes for regeneration (51). It is unclear if tissue-resident macrophages can be trained, or if presence of trained myeloid lineage cells in tissue is the result of circulating monocytes migrating into a tissue. A study identifying alveolar macrophages with a trained phenotype showed CD8 T cells were required for induction of a trained state following a viral infection (52). In another study, reprogramming of tissue-resident macrophages occurred upon placement in new microenvironments, and cells may be driven into a trained or tolerant phenotype (53). Innate training is noted in other cell types, including dendritic cells (54, 55), non-immune cells such as mesenchymal and epithelial stem cells, and intestinal stromal cells (56). Additional research will be required to understand how alterations in the innate responsiveness of various cell types may contribute to disease resistance at the level of the organism.

INNATE MEMORY IN FOOD ANIMALS

Innate memory, defined as both training and tolerance, is well-described in human and rodent literature, and a mechanistic model of innate training as described above is beginning to be defined. However, there exists a paucity of information regarding innate memory in agriculture animals. Although broadly similar to human and rodent immune systems, there are important species-specific differences in the innate immune systems of individual food animals which can significantly impact the development of innate memory. For example, LPS dose plays a critical role in the induction of tolerance or training. However, it is difficult to draw parallels across species because of differences in LPS sensitivities. A very low dose of LPS can induce cellular and physiological changes in sheep, while a much higher dose is needed for a similar effect in chickens (57, 58). Thus, in the future, it will be critically important to assess the induction and effects of innate training in each individual species, ensuring that species-specific differences in innate immune function are fully acknowledged. As an impetus to encourage futher research, this review is focused on evidence for innate training in individual commercially important agricultural species and the potentiall benefits and limitations of innate training to enhance disease resistance.

Cattle

To date, there are only a handful of reports detailing innate training as described by Netea et al. (3) in cattle. In one report, vaccination of 3- to 6- months old beef calves with heat-killed *M. bovis* resulted in an enhanced capacity for monocyte-derived macrophages from these animals to phagocytose and kill *M. bovis in vitro*. This effect was independent of cellular or humoral adaptive immune responses, and lasted up to 6 months

after vaccination (59). Recent data from our group has shown that aerosol BCG vaccination induces a trained phenotype in circulating bovine monocytes. Specifically, monocytes isolated from BCG-vaccinated calves produce more proinflammatory cytokines in response to stimulation with LPS or Pam_3CSK_4 compared to cells from non-vaccinated, control calves (60). Thus, it is clear that the bovine innate immune system can be trained in a similar fashion as that of humans and rodents. Further analysis of the literature suggests other instances in which innate training may occur in cattle, although without experiments designed to specifically address the duration or mechanisms of innate memory, one must infer based upon the nature of the stimuli or resulting phenotype. In one report, immunization of cattle with an ultrasonicated lysate of *Corynebacterium cutis* had positive effects on morbidity and mortality in three different age groups of animals (61). Ten days old calves receiving the *C. cutis* lysate demonstrated a nearly 50% reduction in morbidity compared to controls due to enteric and respiratory diseases in the first 6 months of life. When pregnant cows were given *C. cutis* lysate in the final month of pregnancy, the resultant calves had a higher birth weight and greater weight gain in the first 3 months of life. Of the 23 control calves that were born, only 15 calves survived to 3 months of age, while 25 of 25 calves from the *C. cutis* immunized dams survived to the study endpoint (61). In a number of early studies, oral vaccination of calves using attenuated, live auxotrophic mutants of *Salmonella enteritidis* serovar Typhimirium (*S.* Typhimurium) resulted in homologous and heterologous protection against *S.* Typhimurium and *S.* Dublin (62–64). Protection was non-specific and T cell-independent, and endured for about 1 month after vaccination. Similar results were subsequently recapitulated in mouse models (65); and in fact, more recent results have shown that oral vaccination with live, attenuated *S.* Typhimurium induces sufficient non-specific protection to prevent lethal influenza virus infection in a mouse model (66). Thus, while the authors did not investigate the mechanisms of non-specific resistance in the calves, we speculate that the live *Salmonella* vaccine may have induced some form of innate memory.

Several recent commercial therapies have emerged with potential to enhance the bovine innate immune response during times of stress. One such DNA-based immunostimulant, marketed as the commercial product Zelnate™, can reduce lung-pathology scores in cattle experimentally challenged with *M. haemolytica* (67), and significantly reduce mortality in high-risk cattle after feedlot placement (68, 69). While the product's exact mechanism(s) of action is not well-defined, it is likely stimulating the immune system through pattern-recognition receptors such as TLR9 or the innate cytosolic DNA sensing c-GAS-STING pathway (70). It is unclear, however, if Zelnate™'s mechanism of action can be classified as innate memory. Product literature encourages the use of Zelnate immediately prior or within 24 h of a perceived stressful event. Given that a critical aspect of innate training is the duration of the effect, this form of immunomodulation may not fit the definition. Another immunomodulatory product, marketed as Amplimune™, is a mycobacterial cell wall fraction derived from the non-pathogenic *Mycobacterium phlei*. Amplimune™ non-specifically activates the innate immune system and can significantly reduce the incidence and severity of K99 *Escherichia coli* infection in newborn calves (71). It is currently marketed in the United States and Canada for this use. A recent study revealed that Amplimune™ also had significant beneficial effects in reducing the incidence and mortality associated with bovine respiratory disease in newly received, light-weight beef calves (72), suggesting it may have broader applications for ruminant health. Another commercial immunomodulator, Baypamun™, an inactivated preparation of Orf virus (Parapoxvirus ovis), was sold in Europe for several years for use in food animals and horses. Treatment with Baypamun™ immediately prior to, or in the early stages of infectious bovine rhinotracheitis infection was shown to significantly reduce clinical disease and virus shedding (73–76). Again, while it is evident that these commercial products have enhancing effects on the innate immune system, it is unclear if the immune system remains in a poised state for prolonged periods following treatment. More research will be required to determine if these products have the capacity to induce the long-term effects of innate memory, or simply a transient increase in innate activation.

The use of immunomodulatory feed compounds has grown with the increasing interest in alternatives to antibiotics. Many of these compounds are comprised of a mixture of whole yeast or yeast cell wall components. Several can promote innate immune functions, such as increasing phagocytic activity, increasing the generation of reactive oxygen species or restoring proinflammatory cytokine secretion to leukocytes from transition cows (77–82). The use of particular immunomodulatory feed additives can increase disease resistance in bovine. For example, supplementing with a *S. cerevisiae* fermentation product improves outcome of experimental *Salmonella* or *Cryptosporidium* challenges in preweaned calves (83–85); and reduces the size and number of liver abscesses in finishing beef steers, with efficacy comparable to standard in-feed antibiotic regimens (86). Yeast-supplemented cattle have reduced incidences of bovine respiratory disease during the receiving period (87, 88); while preweaned dairy calves receiving a yeast-based supplement have improved fecal scores and overall reductions in morbidity and mortality during the first 70 days of life. Given the capacity of the yeast cell wall component, β-glucan, to train the innate immune system in rodents and humans (34, 35, 46), it seems likely that at least some of the positive effects of such yeast-based additives on bovine health may be attributed to the induction of innate memory. More in-depth analyses of innate cell function and the specific epigenetic and metabolic alterations accompanying these changes will be required to determine if innate memory is a mechanism contributing to enhanced disease resistance.

Sheep and Goats

Similar to the other species in this review, β-glucans are the most common immunomodulatory compounds investigated in sheep. Oral supplementation of β-(1-3)(1-6)-glucans to ewes has positive effects on reproductive performance, and on growth rate and body composition of the resultant lambs (89), potentially due to the positive effects of β-glucan supplementation on

milk yield and milk composition in lactating ewes (89, 90). Monocytes and neutrophils isolated from lambs fed β-glucans have increased phagocytic and respiratory burst activities, and increased lysozyme activity (90, 91). Lactating ewes fed β-glucans have reduced somatic cell counts in milk (89), while an intramammary infusion of β-glucans resulted in selective recruitment of $CD14^+$ monocytes/macrophages to the udder (92), potentially priming the animal to be more resistant to mastitis.

A recent series of studies has shown that the marine yeast, *Debaryomyces hansenii* and its cell wall, has the potential to train the innate immune system in newborn goats (93–96). *In vivo* supplementation of newborn goats with live *D. hansenii* induced upregulation of the genes encoding for TLR2, 4, and 6, IL-1β and TNF-α in circulating leukocytes, and resulted in increased respiratory burst, catalase and superoxide dismutase activity (94–96). The cell wall of *D. hansenii* is comprised primarily of (1-6)-branched (1-3)-β-D-glucan (96). *In vitro* training of goat monocytes with purified *D. hansenii* β-glucans results in increased expression of CD11b and the macrophage-associated gene F4/80, increased viability upon LPS challenge and increased phagocytic activity (93). *In vivo,* newborn kid goats supplemented with purified β-glucans from *D. hansenii* and subsequently challenged with LPS demonstrate increased plasma concentrations of IL-6, IL-1β, and TNF-α, and isolated leukocytes show increased respiratory burst activity and nitric oxide production (93).

BCG has not been widely studied in sheep. However, a few early studies showed that vaccination with BCG affords some resistance to infection with rift valley fever virus (97) and resistance to caseous lymphadenitis caused by *Corynebacterium pseudotuberculosis* (98). In the former study, a fraction of the BCG immunized sheep developed short fevers and viremia for only 24–48 h, while control sheep were viremic and febrile for up to 8 days after challenge. Sheep receiving two doses of BCG were completely protected from liver involvement due to rift valley fever infection (97). The latter study followed more than 500 head of sheep and used a model of natural *C. pseudotuberculosis* infection by seeding the herd with clinically infected animals. Over a period of 4 years, 99% of the lambs vaccinated with BCG were protected from development of caseious lymphadenitis (98). Thus, it appears that the innate immune systems of sheep and goats have the capacity to be trained, and the strategy holds significant potential for promoting disease resistance in small ruminants.

Swine

β-glucans are readily used in pig production systems across the world with noted health benefits [reviewed in (99)]. Commerical in-feed products may be formulated with purified β-glucan from various sources (yeast, algae, fungi), or contain live yeast, yeast with fermentation products, or a semi-purified mix of cell-wall polysaccharides (mannans). Each product includes some amount of β-glucan, either purified or in the cell wall, which is presumably the ingredient responsible for noted changes in health status. Oral supplementation with β-glucans can improve weight gain, though not every trial indicates improved performance (100–103). Oral β-glucan can improve performance when low levels of aflatoxin are also present (104). Post-weaning diarrhea in pigs is caused by enterotoxigenic *E. coli* (ETEC), and antibiotics are commonly administered to limit ETEC. Oral β-glucan supplementation for the 2 weeks post-weaning decreased susceptibility to ETEC (105). Addition of yeast fermentation product to the diet decreases ETEC attachment to mucosa (106). However, frequency of diarrhea after ETEC challenge increases with a yeast-whole cell supplemented diet, although *E. coli* levels in feces do not increase (107). Interestingly, dietary β-glucan improves piglet health after rotavirus infection (108). However, inclusion of β-glucan in the diet increases susceptibility to intravenous *Streptococcus suis* challenge, even with increased performance measures (100). The authors hypothesize dietary β-glucan increases expression of IL-1R antagonist, which may enhance feed uptake by blocking IL-1R signaling, but enhances susceptibility to disease due to lack of necessary IL-1R signaling. Following LPS injection, pigs on a β-glucan diet have less TNF-α and IL-6 in the plasma (103), suggesting reduced responsiveness upon secondary stimulation. While β-glucan and related products are used in pig production systems, the mechanisms of improved health are not completely understood and various factors, including microbiota, age, and pathogen insult may impact outcomes.

Though β-glucan is most commonly administered by the oral route, changes in peripheral, as opposed to intestinal, immune status is often assessed. Multiple cell types have receptors for β-glucans in pigs, including myeloid progenitor cells in the bone marrow (22, 109, 110); and in mice, intravenous injected fluorescent labeled β-glucan is located in bone marrow macrophages and neutrophils (111). Thus, β-glucans may translocate from the intestine into the periphery to modulate progenitor cells, with peripheral impact. As noted above, levels of proflammatory cytokines in the sera are lower in response to LPS injection when pigs are fed a β-glucan supplemented diet (102, 103). Inclusion of whole yeast cells in the diet leads to shifts in circulating leukocyte populations (107, 112), though the impact of changes on disease resistance are unclear. Peripheral blood mononuclear cells produce less proinflammatory cytokine following LPS stimulation if pig diet is supplemented with β-glucan (103). While Dectin-1 and CR3 are expressed by different pig leukocytes (109), and intestinal dendritic cells may be the first to encounter dietary β-glucan (113), it's unclear how dietary supplementation alters responsiveness of peripheral immune cells to heterologous stimulation. Overall, dietary products containining yeast and/or β-glucan can modulate peripheral immune responses, but the longevity of the shifts and translation to innate memory warrant further investigation.

While BCG is a classically defined training agonist across multiple species, there are few reports on the impact of BCG administration on pig innate immunity. Coe et al. report BCG administration in pigs did not alter neutrophil function (114). In a recent study, pigs administered inactivated *Mycobacterium paratuberculosis* vaccine had enhanced pathology and inflammatory responses following *Actinobacillus pleuropneumoniae* challenge (115), suggesting a heightened secondary response indicative of innate training. BCG readily

interacts with cells of the innate immune system; however, in pigs it's unclear if changes result in protection against heterologous infections. Pigs are a proposed model for tuberculosis research (116) and BCG vaccination in wild boar to limit *M. bovis* infection is proposed (117, 118). Given the conserved aspects of innate immunity across species, BCG is anticipated to induce innate memory in pigs, but has yet to be adequately demonstrated.

Poultry

Of the food animals, commercial meat poultry (including chickens, turkeys, ducks, and quail) have some of the shortest times to market, with broiler chickens reaching market weights at an average of 47 days post hatch (119). While poultry breeds raised for egg production are longer-lived (1–2 years) (120), they have early life disease challenges and adaptive immune system limitations similar to meat poultry. Disease prevention via the adaptive immune system is controlled through vaccination against specific organisms and reaches full potential at around 3 weeks after vaccination (almost half a commercial broilers life span) (119, 121). Durning that 3 weeks span, maternal antibodies provide additional protection to the chick, but are dependent on multiple factors such as individual antibody titer levels and time post vaccination (122). Vaccination is relatively expensive with each vaccine providing protection against few pathogens. Contrasted to the adaptive immune response, the fast induction and breadth of innate memory presents a prime mechanism to reduce disease and foodborne organisms in poultry.

β-glucans are the most well-studied of the known innate memory immunostimulants in poultry (123, 124). However, due to the practical limitation of inoculating thousands of birds with β-glucans, stimulation with β-glucan occurs almost exclusively through the oral route via feed supplementation (125–129). Yeast are the most common form of dietary β-glucan as cereal β-glucans with (1–4)(1–6) linkages (i.e., barley and oat) are detrimental to poultry production due to reduced nutrient digestion and adsorption (130). In chicks, dietary supplementation with yeast β-glucans reduces *Salmonella* colonization of the cecum (131) and visceral organs (131, 132). Intermittent feeding of a β-glucan containing yeast product decreased the effects of transportation stress in turkey poults and tended to decrease colonization of the ceca with the foodborne pathogens, *Salmonella* and *Campylobacter* (129, 133). Interestingly, the same positive effect was not observed with continuous feeding of the yeast β-glucan product (129). No benefit of a β-glucan diet was observed when broiler chicks were challenged with *Eimeria* oocysts (128). While no studies in poultry have directly addressed the ability of β-glucans or other immunostimulants to induce trained immunity, β-glucan can alter the chicken immune system both *in vitro* and *in vivo*. Nitric oxide and IL-1, but not IL-6, production was increased in a chicken macrophage cell line following β-(1-3)(1-6)-glucan stimulation (134). The same study also detected increases in *ex vivo* macrophage phagocytic activity. Heterophil leukocyte function (phagocytosis, bactericidal killing, oxidative burst) is altered in yeast and β-glucan fed broiler chicks and turkey poults (125, 132, 133).

The production benefits (body weight, feed:gain ratios, feed consumption) of dietary β-glucans in poultry are less clear, as β-glucans and yeast products can enhance, reduce, or not change production parameters in chickens, turkey, or ducks (125, 127, 135). It is unclear if the conflicting results are due to different products (whole yeast, mannan oligosaccharides, purified β-glucan, etc.), source of yeast product (*Saccharomyces cerevisiae*, *Aureobasidium pullulan*, or other), relative dose of β-glucan, age of the animals, or some other factor. Huff et al. (135) suggest a potential mechanism for differences in production parameters, as they found in absence of *E. coli* challenge, chicks on control diets had higher body weights and feed:gain ratios than β-glucan fed chicks, but with challenge, the β-glucan chicks had higher production parameters. Dietary β-glucan is also associated with enhanced intestinal barrier functions (increased villus height/crypt depth ratio, number of goblet cells, and secretary IgA levels), but the authors did not determine if the effect was due to direct β-glucan stimulation of host immune cells or alterations in the gut microbial populations (131, 136).

In ovo injection of vaccines or immunostimulants represents an interesting way to alter the immune system of poultry before hatch and environmental exposure to pathogens. For the past 25 years, poultry producers have utilized *in ovo* technologies to safety and effectively vaccinate chicken, turkey, and quail embryos for common poultry diseases such as Marek's disease, infectious bursal disease (IBD), and coccidiosis (137). Recently, immunostimulants have come to the forefront of *in ovo* applications as a mechanism to non-specifically enhance the immune system of poultry before hatch. *In ovo* injection of resiquimod, a TLR7/8 agonist, at embryo day 18 increased MCR1L-B positive macrophages in the trachea, lungs, duodenum, and large intestine of chicks at hatch (138). Furthermore, the authors show that following infection with infectious laryngotracheitis virus (ILTV) 1 day post hatch, cloacal shedding of ILTV at 7 d post infection was significantly reduced in resiquimod injected embryos and that resiquimod treatment induced type 1 IFN activity in macrophages. Of the *in ovo* immunostimulants, CpG DNA is perhaps the most well-studied (139–141). Abdul-Cader et al. (140) show that CpG DNA delivered *in ovo* upregulates IL-1β expression and macrophage proportions in the lungs and these changes are associated with reduced ILTV induced mortality and weight loss in chicks. A 2018 study (139) with *in ovo* administration of CpG DNA reported reduced mortality and clinical scores from experimental *E. coli* infection of yolk sacs in day old chicks. Indeed, a commercial product Victrio®, is an *in ovo* DNA immunostimulant marketed to reduce mortality in embryonated eggs and chicks from *E. coli* and is shown to activate TLR21 on chicken macrophages and increase nitric oxide production (70). Overall, *in ovo* exposure to innate agonists altered immune responses to disease; however, it is unclear if the impact is the result of ongoing immune activation, or was the result of innate memory. The length of time from agonist exposure to challenge testing suggests innate memory may be at play, but targeted studies are warranted to clearly define the mechanism of protection.

Fish

Unlike most other food animal species, review articles have been published summarizing the evidence for trained immunity in fish (142, 143) and we would direct readers to those sources for an in-depth review of innate training in various fish species. Since the 1990's, β-glucan from a variety of sources was studied or fed in commercial fisheries for its growth promoting and immunomodulating effects (144, 145). However, detailed analysis suggests the observed benefits are dependent on β-glucan source and dose, fish species, and age (145). Heterologous protection against bacterial challenge occurs after intraperitoneal or oral administration of β-glucans in multiple fish species ranging from Zebrafish (*Danio rerio*) (146) to Yellowtail (*Seriola quinqueradiata*) (147) to Orange spotted grouper (*Epinephelus coioides*) (148). Researchers observed lower mortality; increased oxidative burst, cytokine production, and lysozyme activity (143, 146, 147). The decreased mortality was observed up to 30 days after β-glucan feeding ceased (148). The length of effect after withdrawl suggests, similar to mammalian studies (34, 36, 123), dietary β-glucan treatment in fish induces epigenetic changes at the progenitor level allowing for sustained changes to innate immune cells. Fish express a higher diversity and variation of innate receptors that are both similar to and distinct from mammalian receptors (149). Dectin-1, a C-type lectin, is the primary myeloid receptor for β-glucans in mammals (32, 150–152); however, no corresponding β-glucan receptor has been identified in fish. Recently, Petit et al. (153) identified several potential candidate receptors for β-glucan in European common carp (*Cyprinus carpio carpio*). They also showed that, as with mammals, the C-type lectin pathway is involved in detection and signaling in response to β-glucan (153). This and other studies lay a foundation for mechanistic work in fish to determine the direct and long-lasting effect of β-glucan on the fish immune system.

Of the molecules known to stimulate innate memory (1, 123, 154), β-glucans are those most commonly used in aquaculture, but others, including mycobacteria, have been studied. Reviewed in Petit and Wiegertjes (142), studies show intraperitoneal injection with *Mycobacterium butyricum* enhances bactericidal activity up to 33 d post injection (155). A series of studies by Kato et al. (156–158) indicate injection with BCG enhances innate immune responses in multiple fish species and induces protection against challenge with *Nocardia seriolae* in Japanese flounder. Yellowtail (*Seriola quinqueradiata*) first exposed to one of several immunostimulants were protected against *Pasteurella piscicada* disease. Specifically, pre-exposure to Freund's complete adjuvant (CFA), which contains inactivated *M. bovis*, was found to be the most protective (147). Indeed, while enhanced survival and immune markers were observed with glucan pretreatment, the effect was markedly heightened with CFA treatment. The observed cross protection observed with these last studies are hallmarks of innate training and is strong evidence for innate memory in fish.

When reviewing literature published before innate memory was well-described, and in the absence of studies specifically designed to investigate the induction of heterologous protection independent of adaptive immune system, it can be difficult to assign innate memory as the mechanism for enhanced disease resistance. For example, Lorenzen et al. (159) observed cross-protection against viral hemorrhagic septicemia virus (VHSV) in rainbow trout inoculated with a plasmid DNA encoding the viral glycoprotein from an unrelated virus. Mortality was decreased in plasmid inoculated fish when challenged with virus either 4 and 7 days after plasmid inoculation, but not at 60 or 84 days post inoculation. The limited window of protection suggests a mechanism independent of adaptive immunity. A later study of juvinal turbot (*Scophthalmus maximus*) inoculated with DNA plasmid encoding the VHSV envelope glycoprotein and challenged with unrelated virus also observed reduced mortailities in the plasmid inoculated fish (160). It's unclear if the protection was due to innate training, or just non-specific protection due to the primary reponse to plasmid DNA. Regarless, some immunomodulation occurred to enhance disease resistance. Mechanistic evidence of contemporary trained immunity (as described by human and rodent literature) remains to be described. As demand for fish increases, methods to enhance disease resistance in farmed fish without high cost and antibiotics is desired.

IMPLEMENTING INNATE MODULATION IN FOOD ANIMAL AGRICULTURE

The primary objective of innate immunomodulation in food animals is to enhance the immune status of the animal, thus resisting disease to enhance animal welfare and production efficiency. Enhancing the animal's ability to resist disease could reduce the need for antibiotics and amount of feed required to get an animal to market weight. In most production systems there are clearly defined periods in which animals are known to be at high risk for infection. Universal to all production systems is the susceptibility of the neonatal or very young animal to disease (161). As maternal immunity wanes, and an infant's own adaptive immune system is inexperienced, there is a window of heightened vulnerability to disease. The adaptive immune system may not be fully matured in neonates, but the innate immune system is active and provides a key role in immune responses at this age [reviewed in (162)] making it a good target for enhanced protection. In humans, innate training mediated by BCG is effective for more than a year (124). While longevity of protection by innate training is important, it may also be important to initiate protection early in life. The time from birth to market for a particular species can range from weeks to years, for example broilers go to market at around 6 weeks of age, pigs at 6 months, and beef cattle at 2 years. Thus, an important consideration for harnessing innate training is length of protection but also how quickly innate training protection is evident in the animal. One recent review of antibiotic usage by pig producers in Belgium reported that more than 80% of all antibiotics are administered to piglets <10 weeks of age (163); and similar results were reported for North America and several other countries in Europe (164). An important consideration for harnessing innate training is length of protection but also how

quickly innate training protection is induced to minimize disease during a high-risk period.

Calves are most susceptible to diarrheal diseases in the first 4 weeks of life, and then the risk for respiratory diseases increases as they mature to 2–6 months of age. Induction of innate training in the neonatal period would be expected to promote improved disease resistance through at least 6 months of age, when the "window of susceptibility" to infection is the greatest (165). Subsequent high-risk periods for beef cattle include the shipping and receiving period (first 50 days on feed), when cattle are weaned, trucked, co-mingled, and placed in the feedlot (166). Administration of BCG, β-glucan, or other known immunomodulator in the period prior to weaning and shipping could protect the animal through the receiving period. In support of this supposition, the commercial immunomodulators Zelnate™ and Amplimune™ have both shown some benefit when administered to calves immediately prior to or following placement in the feedlot (67–69, 72). Dairy cattle are known to go through a period of immunosuppression during the transition period (the 3 weeks prior to calving, through the 3 weeks after calving) leading to a sharp increase in the prevalence of infectious and metabolic diseases (167, 168). Exploiting the effects of innate training or tolerance during the periparturient period could have beneficial effects on cow health and performance.

As with pigs and cattle, fish and poultry are most susceptible to disease while they are very young. In fish, immunostimulants have been administered to newly hatched larvae directly via feed pellets, or indirectly via bath treatment (143, 144, 169–171). In poultry, as described in detail above, *in ovo* vaccination is industry standard and there exists both experimental and commercial (Victrio®) evidence of protection from disease with non-specific immunostimulants (70, 139, 140). As egg laying animals, immunomodulation can occur before hatch and before the fry or chick is exposed to a broader range of pathogens. Additionally, the passive transfer of antibodies from the mother to the progeny via the yolk in egg-laying species is well-described, but in fish, innate immune components have been shown to transfer from the dams to the oocytes and direct passage of immunostimulants from mother to young have also been described (172–174). One last benefit of innate modulation in both poultry and fish is the evidence of passage of transgenerational epigenetic changes to innate immune phenotypes and genes for both broiler chickens (175) and fish (176, 177). Combined with a dam's ability to produce large numbers of eggs, changes in epigenetic phenotypes (the hallmark of innate memory) could be inherited by the embroyos allowing for animals to hatch in a primed state to face bacterial or viral challenge. In some ways fish and poultry producers have an advantage beyond that of mammalian species to harness innate memory to prevent disease, and both industries have made strides to study and utilize these evolutionary advantages.

Potential Pitfalls of Innate Training

Although harnessing innate training for enhancing disease resistance is appealing, particularly in the context of the known periods of susceptibility or immunosuppression described above, there are still potential pitfalls that warrant consideration. In humans and rodents, innate training can have deleterious effects in the context of chronic inflammatory conditions such as autoimmunity, atherosclerosis, and diabetes (3). In food producing animals, while heightened immune responses may be beneficial for pathogen clearance, an increased inflammatory response may lead to tissue damage. In the case of respiratory diseases, for example, dysregulated inflammatory responses are often implicated as causing more damage to the host than the pathogen itself (178–181). Further enhancing this innate inflammatory response may not be ideal. However, if innate training has the capacity to reduce shedding of the organism, its use may still provide significant benefit to the health of the herd by reducing the risk of disease transmission. The impact of innate training on tissue pathology, pathogen burden and overall outcome of disease will need to be carefully evaluated in the context of particular disease settings in order to determine the risk vs. reward of engaging innate memory.

In addition to the potential of enhancing pathology of a disease, harnessing the immune system for disease resistance may negatively impact production parameters. Activation of the immune system comes at a considerable metabolic cost to an organism (182–185) and becomes energy not spent on production of muscle or milk. Activation of cells for the synthesis and secretion of cytokines and acute phase proteins, and cellular proliferation all require glucose, amino acids, and energy. To ensure survival of the host, the integrity of the immune system is maintained above nearly all other biological functions, partitioning nutrients away from growth, reproduction, and lactation (182). Although there is currently little supporting evidence available, it is almost certain that induction and maintenance of the innate immune cells in a trained state comes at some catabolic cost to the animal. Glycolysis, the metabolic pathway favored by trained monocytes and macrophages (46, 47) is less efficient than oxidative phosphorylation; thus increasing the cost of per-cell energy use. In addition, the initial activation of the immune system to induce a trained state utilizes metabolic resources. Although there is significant benefit to the animal in limiting disease upon pathogen exposure, it is currently unknown if the energy costs of a trained immune system will negatively affect performance, or if this possible performance loss will outweigh the benefit of increased disease resistance. Additional studies focused on the efficacy of innate training for preventing disease in food animals, as well as the impacts of training on animal performance and growth, will be required to unravel these possibilities. Regardless, strategies that enhance disease resistance without antibiotics warrant consideration to limit the impacts of antimicrobial resistance.

SUMMARY AND CONCLUSIONS

Few studies have directly examined trained immunity in food animal species. However, as discussed here, a plethora of evidence exists for a variety of immunostimulants to enhance non-specific, heterologous protection against bacterial and viral disease in cattle, swine, poultry, fish, and small ruminants. Innate memory presents an exciting opportunity to prevent or limit disease as well as reduce antibiotic use and AMR in agricultural animals. A number of opportunities exist for mechanistic studies to elucidate the cell types, pathways, and molecules involved

in innate memory in food animals. Until innate training and tolerance are better understood, caution is warranted to determine the immunological and metabolic costs and efficacy of protection to specific diseases. Within innate memory is the potential to reduce disease burden and antibiotic use in animal agriculture, and we feel this area of investigation represents one of the most exciting fields of study for a new generation of scientists.

AUTHOR CONTRIBUTIONS

KB, CL, and JM wrote and reviewed the manuscript.

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REVIEW
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Molecular Dissection of the Antibody Response: Opportunities and Needs for Application in Cattle

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Improving understanding of the bovine adaptive immune response would equip researchers to more efficiently design interventions against pathogens that impact upon food security and animal welfare. There are features of the bovine antibody response that differ substantially from other mammalian species, including the best understood models in the human and mouse. These include the ability to generate a functionally diverse immunoglobulin response despite having a fraction of the germline gene diversity that underpins this process in humans and mice, and the unique structure of a subset of immunoglobulins with "ultralong" HCDR3 domains, which are of significant interest with respect to potential therapeutics, including against human pathogens. However, a more detailed understanding of the B cell response and the production of an effective antibody response in the bovine is currently hampered by the lack of reagents for the B cell lineage. In this article we outline the current state of knowledge and capabilities with regard to B cell and antibody responses in cattle, highlight resource gaps, and summarize recent advances that have the potential to fundamentally advance our understanding of this process in the bovine host.

Keywords: B cell, immunoglobulin, bovine, single cell analysis, ultralong HCDR3 domain

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INTRODUCTION

The molecular basis of how antibody repertoires are generated is broadly similar between mammalian species. Rearrangement of genes encoding immunoglobulin heavy and light chains during B cell development, from a pre-existing library of variable gene segments, results in each B cell expressing a unique immunoglobulin specificity. Immunoglobulin diversity is further refined by somatic mutation of the immunoglobulin genes during development of an immune response, which enables selection of B cells expressing antibodies with enhanced affinity for the immunogen. Studies of immunoglobulins in domestic animal species have highlighted certain unique features. One such example is that Camelids produce a subset of immunoglobulins composed only of a heavy chain, in which antigen recognition involves only one variable region. This has allowed isolation of these single heavy chains and their expression as recombinant antibodies, referred to as nanobodies, for various practical applications (1). Compared to humans and mice, cattle and sheep have a more restricted repertoire of immunoglobulin variable gene segments, but they compensate for this by utilizing antigen-independent somatic mutation of their rearranged immunoglobulin genes to generate further sequence diversity. Another distinct feature in cattle is that ∼10–15% of immunoglobulins possess an ultralong heavy chain CDR3 domain. In contrast to conventional antibodies where antigen recognition involves interaction with six hypervariable

loops (or complementarity determining regions–CDRs)–three on the heavy chain and three on the light chain—recognition of antigen by these ultralong antibodies is determined predominantly by the HCDR3, which has an extended stalk-knob like structure (2–5).

The capacity to analyse antibody responses against infectious agents at the single B cell level provides a powerful means to identify the biological properties of individual antibody specificities, including their potential role in immunity. Until recently, such analyses have proved difficult in outbred species. Techniques developed in the 1970s for generating monoclonal antibodies in mice and rats were not readily applicable to other species, because of the absence of suitable myeloma cell lines for use as fusion partners. Attempts to use murine myeloma cell lines with bovine B cells to generate heterohybridomas had some success in producing bovine monoclonal antibodies, but these systems were not sufficiently efficient to allow their routine use (6–8). A further factor that constrained the ability to analyse antibody responses at the clonal level was the limited capacity of antibody-producing cells to proliferate, as they undergo terminal differentiation as plasma cells. This is in marked contrast to antigen-specific T cells, which can be propagated and cloned *in vitro*, allowing analyses of their specificity at the clonal level. In the last few years, advances in the sensitivity of methods to examine gene expression at the single cell level have opened up new opportunities to analyse B cell responses, including the isolation of expressed immunoglobulin genes from individual B cells.

This paper aims to provide a brief review of new and emerging approaches to interrogating bovine antibody responses, focusing particularly on analyses of responses at the single B cell level.

ADVANCES IN CLONAL ANALYSES OF ANTIBODY RESPONSES IN OUTBRED SPECIES

In the last few years, methods have been established for generating antigen-specific human monoclonal antibodies from B cells isolated *ex vivo* from humans mounting an antibody response. These methods are based on the ability to enrich for specifically reactive B cells and the capacity to isolate and express immunoglobulin genes from single responding B cells. Enrichment for antigen-specific B cells has relied either on use of fluorescently labeled antigen tetramers to identify and isolate antigen-specific B cells or isolation of plasmablasts and plasma cells using surface markers expressed specifically on these activated B cell populations. Rapid methods for isolation and expression of immunoglobulin heavy and light chain genes from single B cells have allowed analyses of the antibody specificities. Such approaches have proved to be highly successful in generating novel data on the fine specificity of human antibody responses to a number of pathogens, most notably influenza and Ebola viruses (9, 10).

The ability to conduct similar analyses of antibody responses in cattle would represent a major advance, particularly with respect to identification of antibody targets for use in vaccination. Many pathogens induce antibody responses to multiple antigens, only some of which play an important role in immune protection. The capacity to screen the biological activities of monoclonal antibodies induced in the target species, provides a direct means of identifying antigens that are likely to be immunogenic. In some diseases, immune responses are dominated by antibodies against antigens that vary between pathogen strains, leading to strain-specific immunity (e.g., foot and mouth disease virus). In such cases, interrogation of the fine specificity of the response at the clonal level, offers the means of identifying subdominant cross-reactive antigenic specificities with potential for vaccination.

REAGENTS FOR STUDYING B CELL RESPONSES IN CATTLE

The ability to apply these new technologies to studies of bovine B cell responses has been constrained by a paucity of reagents for studying B cell differentiation. Studies of human B cell responses are able to utilize a suite of reagents developed against surface markers, which enables relatively precise characterization and placement of B cells within the differentiation cascade. Identification of particular stages of differentiation frequently relies on the use of combinations of several markers, and in some instances consideration of their levels of expression.

Two distinct lineages of B cells, B-1 and B-2, have been identified in humans and mice. In contrast to conventional B-2 cells, which cooperate with helper T cells and undergo Ig isotype switching and affinity maturation within germinal centers, B-1 B cells have minimal requirement for auxiliary signals and respond rapidly by producing predominantly IgM (11, 12). The majority of B-1 cells are $CD5^+$ (referred to as the B-1a subset), with a minor subset being $CD5^-$ (B-1b subset). In cattle, expression of surface CD5 has been used as a marker for B-1a B cells, which represent ∼20–25% of B cells in PBMC (13). $CD5^+$ B cells play a prominent role in bovine immune responses to a number of pathogens, including *Trypanosoma congolense* (14), foot and mouth disease virus (15) and Bovine Leukosis Virus (16). In the case of *T. congolense*, the percentage of $CD5^+$ B cells in peripheral blood approximately doubles during the first 3–4 weeks of infection.

Of greater relevance to the present discussion are B-2 B cells, which undergo a complex series of differentiation events during generation of an antibody response. Interaction of mature-naïve B cells with antigen via the B cell receptor (BCR), which is associated with a complex of proteins (CD19, CD21 and CD79) that are responsible for co-stimulation, promotes B cell activation and differentiation (17, 18). Multiple changes in cell surface phenotype occur once the B cells have been activated, including increased levels of CD40, CD69, CD80 and CD86. Up-regulation of CD40, coupled with antigen uptake by specific B cells, enables them to interact with antigen-specific T cells in the follicles of secondary lymphoid organs, leading to germinal center formation and further B cell differentiation, including Ig isotype switching and affinity maturation. The latter involves a clonal selection process, in which antigen-specific B cells with the highest affinity are selected for survival and clonal expansion.

Finally, B cells either differentiate into long-lived memory cells or develop to plasmablasts and antibody-secreting plasma cells. Increased expression of surface CD27 is an important marker for memory cells, although they show considerable heterogeneity in phenotype and function (19, 20). Among the phenotypic changes that occur during differentiation to plasma cells is increased levels of expression of CD38, which is frequently exploited for identifying antibody-secreting cells (21).

In contrast to human B cells, there is a distinct lack of antibody reagents that enable discrimination between the different states of differentiation of B-2 B cells in cattle. Apart from surface immunoglobulin and CD21, there are no well-defined pan-B cell markers in cattle. Although IgD is used as one of the surface markers of naive human B cells, its existence in cattle was only demonstrated in 2006 (22) and there is only one report of expression of the protein on a minor subset of bovine B cells (23). There are also no monoclonal antibody reagents that can be used to identify plasmablasts and plasma cells. Similarly, memory B cell markers are not well-developed for cattle. Therefore, the ability to resolve and understand the intricacies of the bovine B cell response is substantially hampered at present, and requires investment to generate the tools required to fill this gap; this is starting to be addressed by initiatives such as the Veterinary Immunological Toolbox (https://www.immunologicaltoolbox.co.uk/).

GENOMIC ORGANIZATION OF IMMUNOGLOBULIN GENES IN THE COW AND DIVERSITY GENERATION

It is now known that bovine B cells express five isotypes of immunoglobulin: IgM, IgD, IgG, IgE and IgA, with the IgG isotype differentiated into three sub-isotypes (IgG1, IgG2 and IgG3), and IgM into two sub-isotypes (24, 25). Until relatively recently, annotation of the bovine heavy and light chain genomic loci was incomplete. Of the genes that encode the immunoglobulin antigen binding domains [heavy and light chain variable (V), diversity (D) and joining (J) segments], which are generated by VDJ recombination, cattle differ substantially from humans and mice, in particular with respect to the comparative paucity of variable gene content. Cattle have only twelve genes encoding functional heavy chain variable gene segments (IGHV—located on chromosome 21), and all belong to one subgroup, IGHV1 (compared to seven diverse subgroups in humans), with a number of pseudogenes also described in both IGHV1 and two further subgroups, IGHV2 and IGHV3. Only four of the twelve documented heavy chain joining gene segments (IGHJ) and sixteen of the twenty-three diversity gene segments (IGHD) found in cattle appear to be functional (25). Additionally, compared with humans and mice, available data suggest that cattle have a more restricted set of putatively functional light chain genes (26). Most vertebrates express two light chain isotypes: kappa (κ) and lambda (λ). However, the bovine light chain repertoire is dominated by the expression of λ genes [κ usage represents ∼5% of the expressed antibody repertoire (27)], and predominantly by one subfamily, $V_\lambda 1$. V_λ genes are clustered close to the J_λ and C_λ cluster on chromosome 17 (28) and V_κ genes on chromosome 11 (26). In cattle, the limited data available suggest the light chain may have a subsidiary role in antigen recognition, with most antigen binding being driven by the heavy chain variable region. Recent x-ray crystallography data on the structure of two bovine IgG antibodies support this assertion by showing that the heavy chain predominantly contributes to the antigen-combining site (29, 30). When the light chains were exchanged between these two antibodies, antigen recognition by one of the antibodies (but not the other) was substantially reduced and structurally this was associated with a subtle change in the orientation of the associated heavy chain. An earlier study of a poly-specific IgM long-CDR3 antibody had also demonstrated a predominant role of the heavy chain in antigen recognition, although interaction with some antigenic ligands was influenced by the light chain (29).

The information on the genomic architecture of the bovine immunoglobulin loci has been derived from work on European *Bos taurus* breeds, with the most complete genome assembly and associated resources deriving from a Hereford cow (31–34). Immune gene loci tend to be highly repetitive by nature, and therefore difficult to accurately assemble without the use of resource-intensive sequencing technologies that enable accurate construction across large stretches of multiple and similar gene members—for example, long-read or chromatin-linking sequencing approaches. While such genomic resources are being developed for other breeds [e.g., Brahman *Bos indicus* (35)], there are still too few genomes sequenced to a sufficient depth across diverse cattle breeds and lineages to enable assessment of the degree of immunoglobulin locus polymorphism, and how that may impact upon antibody expression and function. This is the focus of increasing effort (e.g., the Bovine Pan Genome Consortium), and increasing the genomic resources across breeds and lineages will be important in functionally linking genomic diversity to phenotypic diversity with respect to the bovine antibody response.

The limited repertoire of germline variable gene segments in cattle has been proposed to be offset by the occurrence of somatic hypermutation in rearranged B cells prior to exposure to antigen, thus generating greater diversity and expanding the B cell repertoire (36, 37). There is evidence from studies in both sheep and cattle (36, 38) that the ileal Peyer's patch is a major site of this antigen-independent somatic mutation. This organ, which differs histologically from conventional Peyer's patches, develops with the kinetics of a primary lymphoid organ (i.e., similar to the thymus). In sheep, the ileal Peyer's patch undergoes significant development during the latter half of gestation, with further enlargement in the first few months of life, and gradual involution from about 3 months onwards (39).

Bovine HCDR3 length on average is longer than in other vertebrates such as humans or mice (bovine HCDR3 ranging from <10 to at least 67 amino acids in length, in contrast to 4–36 amino acids in humans) (2, 5, 22, 24, 40, 41). It has been known for many years that a proportion (∼10%) of bovine immunoglobulin transcripts contain unusually long

HCDR3 domains up to and beyond 60 amino acids long (40, 42–45)—often termed "ultralong" HCDR3 domains. Resolution of the structure of these antibodies identified an unusual and relatively conserved stalk-knob protrusion, which comprised the HCDR3 antigen-binding domain (2, 3, 5). Formation of the stalk structure is facilitated by the presence of several disulphide bonds. The ultralong antibodies described thus far all utilize a single variable gene (IGHV1-7) and diversity gene (IGHD8-2) donor (2, 41), and the few paired heavy and light chain data available also suggest utilization of a limited number of λ light chain V genes (46). Based on analyses of the sequences of multiple long HCDR3 antibodies, a recent study by Deiss et al. (41) has identified a number of key features of the rearranged genes encoding these antibodies. Firstly, they confirmed the almost exclusive use of the IGHV1-7 gene segment and showed that this variable gene contains an internal 8-nucleotide duplication (which contributes to formation of the elongated stalk structure). They also found that, in contrast to other IGHV gene rearrangements, the IGHV1-7 CDR1 and CDR2 regions contain a low frequency of mutations, whereas the CDR3 regions of the same genes show very high levels of mutation compared to the germline sequence (41). This relative conservation of CDR1 and CDR2 sequences is consistent with evidence that these regions have little involvement in antigen binding but rather play a structural role in the long HCDR3 antibodies, whereas the knob-like structure formed by the CDR3 region is the primary antigen-binding site. Direct evidence for the latter was provided by the demonstration that removal of the "knob" sequence ablated antigen binding by the modified antibody (2). Deiss et al. also identified an unusually high degree of deletion events in the HCDR3 domains of long antibodies (predominantly in the IGHD8-2 segment), including deletions that alter the reading frame, thus contributing to ediversity in both the length and sequences of the CDR3 segments and hence structural diversity of these antibodies (41). They hypothesized that this may also be mediated by the enzymatic driver of somatic hypermutation, activation induced cytosine deaminase (AID). This mechanism has been proposed to be a means of generating structural diversification through modification of the pattern of disulfide bond formation (41). This is facilitated by an unusual codon bias in HCDR3, which predicates mutation to cysteine (particularly in the IGHD8-2 segment codons) during bovine VDJ recombination, resulting in the generation of diversity in structure due to the making and breaking of di-sulfide bonds between paired/unpaired cysteines (2, 47). This diversification mechanism has also been shown to operate in conventional length bovine antibodies (47). Analyses of the sequences of re-arranged bovine Ig genes has additionally indicated evidence of a low frequency of gene conversion events in both light and heavy chains that involves short nucleotide segments from light and heavy chain pseudogenes (48, 49), potentially providing a further means of generating sequence diversity–although current data are limited and its importance has yet to be fully determined. In summary, the long HCDR3 antibodies exemplify the bovine host's adaptations to generating antibody diversity from a limited germline repertoire–the combination of codon bias and (possible AID-mediated) targeted deletions resulting in changing of the pattern of cysteine pairs, generating a remarkable ability to create structural diversity in epitope-binding domains, despite being restricted to the use of a single V and D segment.

While the function of these ultralong antibodies remains unclear [interestingly the proportion of ultralong antibodies is significantly higher in neonatal calves (50)], their unusual structure quickly raised the hypothesis that such antibodies could bind to antigen epitopes that were not accessible to conventionally structured immunoglobulins. For example, sites on bacterial pore proteins or proteins embedded within the complex surface coat of parasitic pathogens, which are hidden from conventional antibodies, may be potential targets. There has also been significant interest in application of ultralong antibodies to non-bovine pathogens and their exploitation for development as potential therapeutics, in particular for human pathogens such as HIV (46)—the potential for therapeutics of relevance to veterinary pathogens is also clearly a possibility that is currently underexploited. However, the exact roles that these antibodies play during natural immune responses in cattle, or, for example, whether they may be an important factor in the efficacy of immune responses induced by vaccines, are unclear. Additionally, all studies analyzing long HCDR3 antibodies have examined European *Bos taurus* cattle—although the long HCDR3 antibody expression levels been shown to be consistent across several European *B. taurus* breeds, current data on, for example, long HCDR3 antibody expression data in *Bos indicus* or African *B. taurus* breeds, and any role they may play across the very different infectious disease contexts that such breeds are exposed to, are all currently unknown. These are all areas that clearly merit further research.

IN VITRO CULTURE OF ACTIVATED B CELLS

Although most studies of antibody responses at the single cell level have focused on analyses of actively responding B cells harvested *ex vivo*, in recent years there have been attempts to analyse the antibody repertoire of memory B cells (51). Since memory B cells are normally present at low frequencies, this approach is dependent on use of culture systems to activate and expand the memory B cells. This in turn requires precise phenotyping reagents (52). A number of studies have reported successful establishment of *in vitro* culture systems that allow expansion of human memory B cell populations and differentiation to immunoglobulin secretion (53). These studies have used combinations of factors that stimulate activation, proliferation, and differentiation, coupled with inhibition of apoptosis, of B cells to maintain growth *in vitro*, albeit for a limited period of time.

Systems for culturing B cells have attempted to mimic the events that drive B cell development during antibody responses *in vivo*. The stimulation of B cells by crosslinking of the BCR with anti-IgM antibodies is well-established as a means of mimicking antigen stimulation (54). Uptake and processing of antigen by specific B cells enables them to present the antigen to T cells, which provide co-stimulatory signals by interaction of CD40

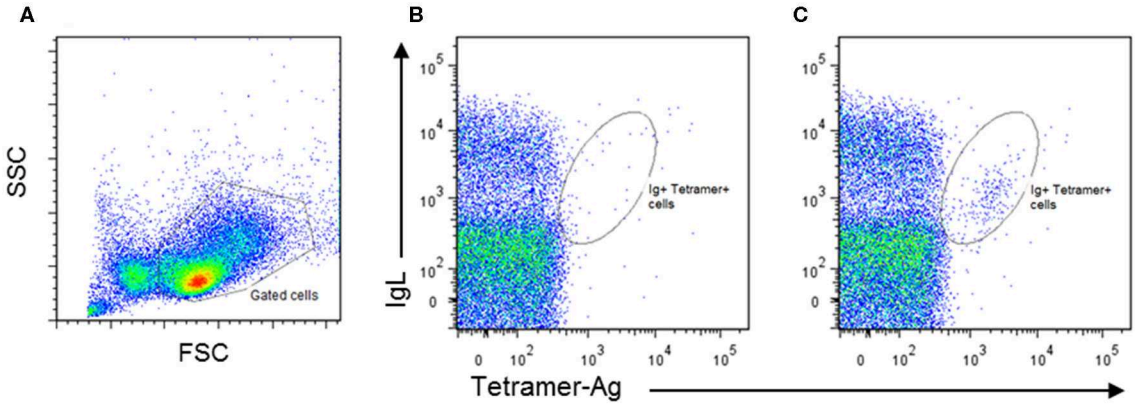

FIGURE 1 | Identification of bovine antigen-specific B cells in peripheral blood mononuclear cells (PBMCs) of an antigen-immunized calf by staining with Phycoerythrin-labeled antigen tetramers: **(A)** PBMCs isolated from naïve and immunized calves were stained by two-color immunofluorescence with a monoclonal antibody (IL-A58) specific for bovine immunoglobulin light chain (IgL) and antigen tetramer. Representative plots of stained cells from a naïve animal **(B)** and an immunized animal **(C)** are presented, showing the presence of an IgL^+ Tetramer-Ag^+ population in the immunized animal. Approximately 1% of IgL^+Tetramer-Ag^+ B cells were detected in the immunized calve, compared to <0.2% in the unimmunized control.

on the activated B cells with CD40 ligand (CD40L) on the T cells. This process can be mimicked *in vitro* by stimulation of B cells with soluble CD40L (55, 56). Activation *in vitro* via CD40 promotes an increase of levels of IL-21 receptor on the B cell surface (57). Among the cytokines that also contribute to B cell activation, IL-21, induced by T cells upon interaction with B cells is a key stimulus for B cell proliferation and differentiation (58). One of the main surface ligands involved in B cell survival is the BAFF receptor (BAFFR) that binds BAFF (B cell activating factor of the TNF family). Other similar related receptors that bind BAFF, are TACI and BCMA, which can also bind APRIL (α proliferation-inducing ligand), and these play a key role in preventing cell death and increasing plasma cell survival (59).

This knowledge has been applied to successfully culture activated porcine B cells, taking advantage of the cross-reactivity of the human reagents with porcine B cells (60). Addition of IL-21 plus CD40L to purified pig B cells resulted in activation and proliferation over a 4-day period, and inclusion of BAFF and APRIL maintained the viability of the cells for 7 days. Secretion of low levels of both IgM and IgG by these cultures was detected on day 7 of culture indicating differentiation of some of the activated B cells. The reagents used in this study also cross-react with bovine B cells and we have been able to obtain similar activation, proliferation and maintenance of bovine B cells similar to that reported by Rahe and Murtaugh (60). The development of phenotyping reagents that allow identification of bovine memory B cells will enable these culture systems to be used to amplify memory cell populations prior to clonal analyses of their specificities.

ISOLATION OF ANTIGEN-SPECIFIC B CELLS

The isolation of antigen-specific B cells is a critical step in the ability to evaluate and analyze the bovine humoral immune response, particularly responses to either specific pathogens or vaccination. In addition, due to the unique properties of bovine antibodies as described above, the isolation of antigen-specific cells is a route to explore their potential relevance as novel molecular tools for research or therapeutic use.

Populations of human B cells enriched for antibody-producing cells have been isolated from blood by flow cytometry using a combination of cell surface markers, including CD19, CD20, CD38, and CD71 (61). In the absence of such markers for cattle, the ability of antibody-producing cells to bind fluorescently labeled tetramerised antigen offers an alternative. This approach is challenging because of the low frequencies of antigen-producing B cells in peripheral blood, and the short time window during which these cells are present at sufficient frequency for detection by flow cytometry. Moreover, antigen tetramers do not detect all antibody-producing cells, as mature plasma cells lose expression of surface Ig upon transition from plasmablasts (62). We have employed established methods (63) to produce streptavidin-labeled tetramers incorporating a recombinant protein from the major cattle pathogen, *Trypanosoma congolense* and used these tetramers to monitor the blood of calves immunized with this antigen. These experiments revealed the presence of a small population of surface Ig^+ $tetramer^+$ cells, detectable for several days after the third dose of antigen administered in adjuvant (**Figure 1**). In Giemsa–stained cytospin preparations the positive cells exhibited a plasmablast morphology. Further studies are underway to isolate and analyse the immunoglobulin genes expressed by these B cells.

IMMORTALISATION OF B CELLS BY INFECTION WITH *THEILERIA ANNULATA*

One of the potential uses of isolated antigen-specific B cells is to transform the cells to allow clonal expansion of the populations and potentially examine antibody secretion. Some species of

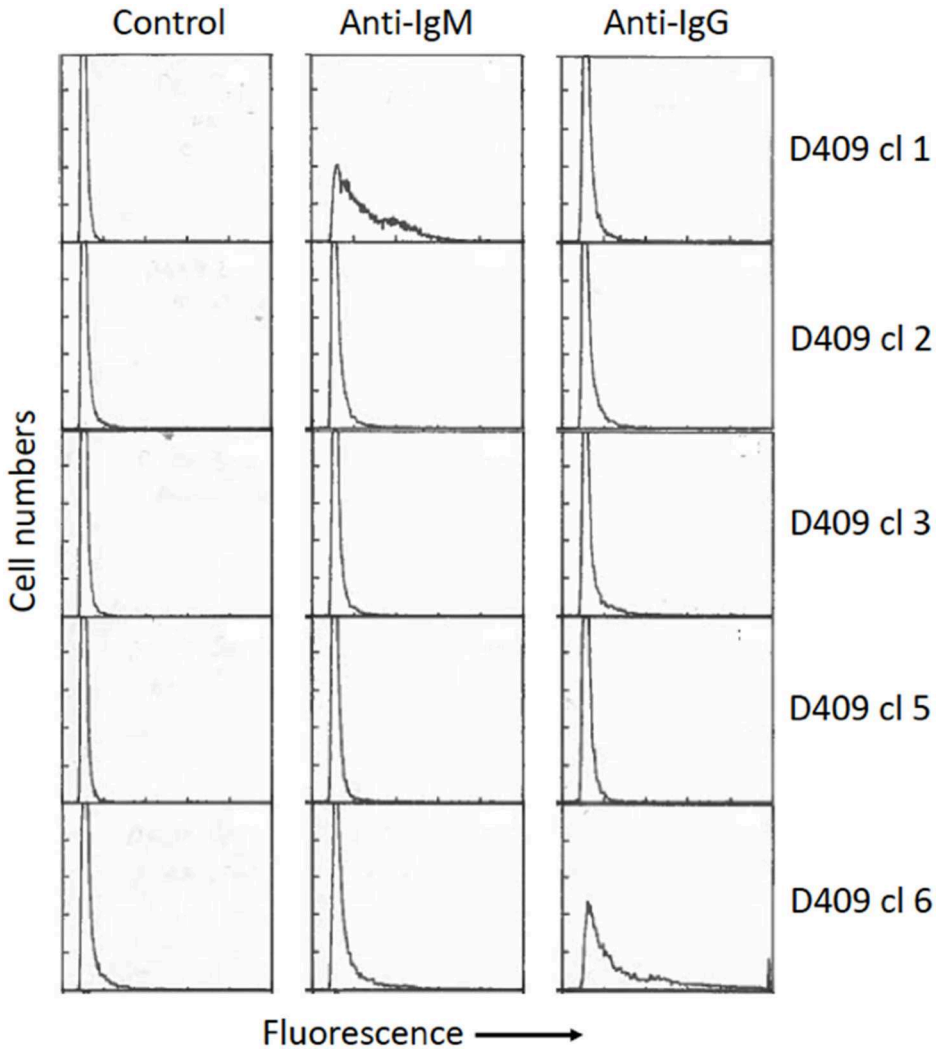

FIGURE 2 | Surface phenotype of five cloned B cell lines infected with *Theileria parva,* as described by Baldwin et al. (65). Briefly, surface Ig+ B cells (>98% purity) were isolated by cell sorting from healthy resting peripheral blood mononuclear cells and infected *in vitro* by incubation with *T. parva* sporozoites, followed by cloning at limiting dilution in 96-well round-bottom plates. The cells were phenotyped 8 weeks after infection by staining with monoclonal antibodies specific for bovine IgM and IgG (Mab B5/4 and IL-A2, respectively) followed by fluorescein-labeled anti-mouse Ig. Significant levels of IgM or IgG expression were detected only on two of the clones (clone 1–IgM; clone 6–IgG). The percentages of positive gated cells for each clone are (Clone 1: Control−1%, IgM−54%, IgG−6%. Clone 2: Control−3%, IgM−6%, IgG−6%. Clone 3: Control−2%, IgM−3%, IgG−10%. Clone 5: Control−2, IgM−4%, IgG−4%. Clone 6: Control 5%, IgM−2%, IgG−47%). Controls were incubated with secondary antibody only. All clones were negative for T cell markers (CD2, CD4, CD8)–data not shown.

Theileria parasites are able to infect and transform bovine lymphocytes. *Theileria* are tick-borne apicomplexan protozoa found in tropical and subtropical regions of the world. The most important species in cattle are *Theileria annulata* and *Theileria parva* (64). Both species infect leukocytes: *T. parva* infects T and B lymphocytes, while *T. annulata* infects monocytes and B cells (65, 66). A characteristic feature of infection with both parasites is that they induce activation and proliferation of the cells they infect (67), during which the parasites divide synchronously with the host cells (68). This relationship, coupled with inhibition of apoptosis of the host cells by the parasite (69) results in clonal expansion of the cells initially infected by the parasite. These properties enable the infected cells to be maintained as continuously growing cell lines *in vitro*, and such cell lines can be initiated by *in vitro* infection of leukocytes with the tick-derived infective stage of the parasite, the sporozoite. In previous studies, we examined the phenotype of purified resting Ig^+ B cells several weeks after infection *in vitro* with *T. parva*. Most infected cells were found to gradually lose surface expression of immunoglobulin, although analyses of cloned populations revealed continued Ig expression, either IgM or IgG, by some clones (70) (**Figure 2**). Similar gradual loss of Ig expression has also been observed in B cells infected with *T. annulata.* However, expression of Ig by B cells in the early stages after infection by *Theileria* was not studied, nor was the susceptibility of activated B cells to infection examined in these

experiments. In recent studies, we have shown that purified tetramer$^+$ B cells are similarly susceptible to infection with *T. annulata*. However, similar to previous findings with infected resting B cells, following cloning and expansion of the cloned populations over a 3–4 week period, only a subset of the clones secreted antibody.

These preliminary finding suggest that this system could be used to obtain cells secreting antibody with particular antigenic specificities, but may not be suitable for direct large-scale clonal analyses of antibody responses at the level of Ig secretion. Nevertheless, the generation of cloned transformed B cells from antigen-specific B cells could prove to be a valuable resource of immortalized cells from which the rearranged Ig heavy and light chain pairs can be retrieved for further analyses. However, further studies are required to explore the full potential of this system.

IMMUNOGLOBULIN GENES EXPRESSED BY SINGLE B CELLS

While advances have been made in our understanding of the genomic repertoire of bovine immunoglobulin loci, we still have only limited data on the usage of these gene families in generating functional, effective antibody proteins. This partly stems from the difficulty in deconvoluting data generated from cell pools or populations into that relevant at the single cell level—this is in most cases an insurmountable bioinformatic challenge, whether short or long read sequencing approaches are used. An obvious route to gaining data at this level is to analyse gene expression of multiple single cells, rather than averaging gene expression across RNA extracted from populations. There are several factors required to do this—one being an ability (within the context of a response to a particular immunogen) to identify and isolate multiple single antigen specific cells (current limitations and challenges around this are outlined above). Despite this challenge, data on single cells, yielding paired heavy and light chain sequences, are emerging in bovine studies, although still only from small numbers of cells.

The ability to analyse single cell data at scale from humans has significantly advanced in recent years. Single cells can be isolated by various routes [micro-dissection, flow cytometry, microfluidics and droplet-based methods (71)], each of which have their advantages and limitations. Droplet-based methods in particular have led to a step change in terms of scale, providing the ability to potentially analyse thousands of single cells (72–74). The challenge of analyzing the VH and VL sequences of B cells has been to some extent overcome in human studies by isolating single cells within emulsion droplets, in which the cells are lysed and mRNA captured by poly-dT beads. From this substrate physically linked VH and VL transcripts are generated through overlap-extension reverse-transcription PCR (OE RT-PCR), effectively splicing the VH and VL amplicons together, which can then be resolved into paired VH and VL chain data (75). This has resulted in novel insights in terms of VL and VH pairing and use, and the identification of broadly virus neutralizing antibodies (76). However, while providing a substantial improvement on individual VH and VL PCRs from isolated cells, the OE RT-PCR approach is still technically indirect, with the full-length variable sequence inferred from assembly of several partial sequences because of the limitations of sequencing technologies. A recent development, termed sc-BCRseq, applied barcodes to fragmented VH and VL sequences from single B cells within droplets, importantly then providing confident downstream assembly into full length paired VH and VL sequences (77). This approach also can be employed in a high throughput manner, and was successfully applied to 250,000 B cells and enabled high resolution analysis of antibody lineages in response to immunization (77). All of these approaches still have their challenges, one particular issue with B cells (if an aim is to analyse antibody response development through the B cell lineage) being sensitivity bias—the increased Ig transcript levels in plasmablasts meaning they are over-represented in expression data.

While the advances in single cell technologies present exciting future possibilities when applied to the bovine antibody response, in the context of analyzing antibody responses to a specific antigen, the initial step in identifying and isolating antigen-specific B cells is still necessary. Thus, the generation of B cell reagents that allow more precise analysis of single cells remains a priority in order to fully realize our ability to analyse the antibody response in cattle.

SUMMARY AND CONCLUSIONS

Our understanding of the bovine B cell and antibody response has advanced significantly in recent years, with genomic and experimental data resolving the unique manner in which the cow generates immunoglobulin diversity from a restricted germline VH repertoire. This has included the characterization of ultralong HCDR3 domain antibodies and their structure, and consequent interest in their potential application to novel therapeutics. However, our ability to advance understanding of many aspects of the antibody response is restricted by a lack of reagents for bovine B cells, in particular those that allow identification and characterization of B cells at different stages of differentiation along the B cell lineage. Such tools would enable more detailed analysis of the initiation, progression and maturation of an effective antibody response, as well as the ability to address specific questions such as the role of HCDR3 antibodies during infection or in response to vaccination. The ability to isolate antigen-specific B cells is also key to facilitating analyses within the context of infection or vaccination, and we have outlined potential routes to how this could be achieved–the development of better reagents for bovine plasma cells/plasmablasts would certainly significantly enhance this capability. Finally, application of single cell sequencing technologies has the potential to revolutionize the analysis of B cell responses, enabling the isolation of paired heavy and light chain data from hundreds of thousands of B cells. Therefore, with investment in the development of key reagents combined with single cell sequencing at scale, we are poised to enter

an era that can transform our understanding of the bovine antibody response.

AUTHOR CONTRIBUTIONS

RB, LM and WM wrote the manuscript.

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Intraepithelial T Cells Diverge by Intestinal Location as Pigs Age

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T cells resident within the intestinal epithelium play a central role in barrier integrity and provide a first line of immune defense. Intraepithelial T cells (IETs) are among the earliest immune cells to populate and protect intestinal tissues, thereby giving them an important role in shaping gut health early in life. In pigs, IETs are poorly defined, and their maturation in young pigs has not been well-studied. Given the importance of IETs in contributing to early life and long-term intestinal health through interactions with epithelial cells, the microbiota, and additional environmental factors, a deeper characterization of IETs in pigs is warranted. The objective of this study was to analyze age- and intestinal location-dependent changes in IETs across multiple sites of the small and large intestine in pigs between 4- and 8-weeks of age. IETs increased in abundance over time and belonged to both γδ and αβ T cell lineages. Similar compositions of IETs were identified across intestinal sites in 4-week-old pigs, but compositions diverged between intestinal sites as pigs aged. $CD2^+CD8\alpha^+$ γδ T cells and $CD4^-CD8\alpha^+$ αβ T cells comprised >78% of total IETs at all intestinal locations and ages examined. Greater percentages of γδ IETs were present in large intestine compared to small intestine in older pigs. Small intestinal tissues had greater percentages of $CD2^+CD8\alpha^-$ γδ IETs, while $CD2^+CD8\alpha^+$ γδ IET percentages were greater in the large intestine. Percentages of $CD4^-CD8\alpha^+$ αβ IETs increased over time across all intestinal sites. Moreover, percentages of $CD27^+$ cells decreased in ileum and large intestine over time, indicating increased IET activation as pigs aged. Percentages of $CD27^+$ cells were also higher in small intestine compared to large intestine at later timepoints. Results herein emphasize 4- to 8-weeks of age as a critical window of IET maturation and suggest strong associations between intestinal location and age with IET heterogeneity in pigs.

Keywords: intraepithelial T cells, intraepithelial lymphocytes, intestinal T cells, porcine T cells, pig T cells, pig intestine, intestinal development, T cell maturation

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INTRODUCTION

The intestinal tract contains the single largest and most diverse compartment of immune cells in the body and is a highly versatile organ system, with different regions performing various physiological and immune functions (1). Furthermore, the intestinal epithelium performs a key function by serving as a selective physical barrier between the intestinal lumen and the body. Intraepithelial

T cells (IETs) are T cells located within the epithelial layer throughout the intestinal tract. In neonates, IETs are among the earliest immune cells to populate and protect intestinal tissues (2). IETs are tissue-resident cells that respond to foreign antigen from the intestinal lumen and to self-derived, stress-induced molecules, positioning IETs as first responders to enteric pathogens or as mediators of epithelial stress, respectively (2–5). IETs are also important in regulating metabolism; mice lacking IETs are resistant to weight gain, even when fed a high-fat diet, due to a hyperactive metabolic profile, indicating IETs have an important role in promoting weight gain efficiency (6). In the context of immunity, IETs can release cytotoxic molecules, cytokines, and/or antimicrobial peptides upon activation, inducing intestinal inflammation and/or antimicrobial activity associated with immune protection (7–9). Conversely, to avoid unwarranted inflammation, IETs are tightly regulated and exercise regulatory functions, giving them a primary role in maintaining epithelial integrity and immune quiescence (7, 9). In the absence of such regulation, inflammation induced or exacerbated by IETs can threaten epithelial barrier integrity and promote immunopathology (2). Hence, IETs are critical in balancing immune tolerance and protection within the intestinal tract. Moreover, microbial and dietary antigen exposure majorly influence the development, specificity, reactivity, and homeostasis of IETs (6, 10–14), largely contributing to their fate in promoting or deteriorating intestinal health (2).

In humans and rodents, IET prevalence, phenotype, and function varies by anatomical location along the intestinal tract, indicating regional specialization of intestinal IETs, especially between small and large intestinal locations (15–20). Additionally, age is a primary driver of changes to intestinal IETs, indicating time-dependent changes to the cells occur due to further immune maturation and continual antigen exposure (16, 20–22). In pigs, information pertaining to IETs across intestinal locations or in regards to the impact of age during intestinal immune maturation is limited. While multiple studies have analyzed porcine T cells across different intestinal locations or across ages (23–29), the focus was primarily the small intestine and included combined cell fractions from epithelial and lamina propria compartments. Given the specialized role of intestinal IETs, it is important to understand whether previous findings can be applied specifically to IETs or generalized to additional intestinal locations. In general, we know porcine small intestinal IETs are located primarily within the apical and middle portions of the villi, and the number of IETs increases with age, primarily in the first 3 months post-parturition, in a microbiota-dependent fashion (30–33). Similar to humans and rodents, the majority of IETs in the porcine small intestine are $CD4^{-}CD8\alpha^{+}$ (34); however, whether IETs belong to αβ or γδ T cell lineages is unknown and could have further implications into cell function. Meanwhile, studies analyzing IETs within the porcine large intestine are lacking.

In pigs, the window of ∼3- to 8-weeks of age (often referred to as the weaning and nursery period in pig production) is a critical time during which porcine intestinal T cell communities are still developing (23, 35–37). Stress from the weaning process, as pigs are moved from the dam and a milk-based diet to new surroundings, pen mates, social structure, and solid food, can result in intestinal inflammation, increased epithelial permeability, diarrhea, increased susceptibility to disease, decreased nutritional absorption, and weight loss with life-long effects (38, 39). A better understanding of age- and location-dependent characteristics of intestinal IETs during stages of major immune maturation and increased stress, such as that of the nursery phase, may prove useful in developing strategies to improve pig health, improve market performance, and/or reduce antibiotic usage during the nursery phase. To our knowledge, an analysis of age- and intestinal location-dependent changes in porcine αβ and γδ IET abundance, phenotype, and distribution throughout multiple compartments of both small and large intestine during intestinal T cell maturation in nursery-age pigs has not been completed. Hence, the objective of this study was to quantify IET numbers, assess presence and proportional phenotypes of both αβ and γδ IET populations, and assess expression of the T cell activation marker CD27 between jejunal, ileal, cecal, and colonic tissues across multiple weeks of age in pigs during the nursery period.

MATERIALS AND METHODS

Study Overview

Conventional, mixed-breed pigs [Camborough (1050) × 337 (Pig Improvement Company, Hendersonville, TN)] were weaned from dams at ∼19–21 days of age and transported to the Iowa State University Swine Nutrition Facility. Upon arrival, pigs were randomly selected, weighed, and placed into individual pens with shared horizontal bar gating. Pens allowed for nose-to-nose contact and sight lines between pigs in adjacent pens to facilitate visual and some physical contact, thereby minimizing any social deprivation and undue stress (40). Moreover, pigs could move freely within respective pens, which exceeded minimum space guidelines. All pigs had free access to water and feed at all times. Pigs were fed a corn-soybean meal-based diet that met or exceeded nutrient and energy requirements for this size pig (NRC, 2012 #146). The diet was free of antibiotics and therapeutic concentrations of minerals. At ∼4-, 6-, and 8-weeks of age (7, 21, and 35 days post-weaning, respectively), pigs were randomly chosen and humanely euthanized via captive bolt and exsanguination. Immediately thereafter, intestinal tissue samples were collected. Average weights, feed intakes, and weight gain values for each timepoint are available in **Supplementary Table 1**. The study was completed in 2 identical replicates, with 4 pigs necropsied at each timepoint per replicate ($n = 8$ pigs per timepoint; $n = 24$ total).

Sample Collection

Sections of jejunum, ileum, cecum, and colon were collected for tissue fixation and flow cytometric (FCM) staining. Jejunal sections were collected ∼95 cm distal to the pylorus. The most proximal ∼7.5 cm jejunal section was collected for tissue fixation, and the next ∼7.5 cm jejunal section was collected for FCM staining. Ileal sections were collected starting ∼7.5 cm proximal to the ileocecal valve. The more distal ∼7.5 cm ileal section was

used for FCM staining, and the next ∼7.5 cm ileal section was collected for tissue fixation. Cecal sections were collected as two adjacent ∼5 cm by ∼10 cm sections located in the middle of the cecal pouch, one section for FCM staining and one section for tissue fixation. Colonic sections were collected from the apex of the spiral colon as two adjacent ∼7.5 cm colonic sections for FCM staining and tissue fixation.

Immunohistochemistry (IHC)

Intestinal tissues were fixed in a 10% neutral-buffered formalin solution (3.7% formaldehyde) for ∼24 h at room temperature (RT). Tissues were then cut to appropriate size, placed in cassettes, transferred to 70% ethanol, and embedded in paraffin blocks. Formalin-fixed, paraffin-embedded (FFPE) tissues were cut into 4-micron thick sections and adhered to Superfrost-Plus charged microscope slides (Thermo Fisher Scientific). Immunohistochemical staining was performed for detection of CD3 protein as described previously (41). Briefly, slides were baked, deparaffinized, and rehydrated for IHC staining. Antigen retrieval was carried out by incubating slides in 1X sodium citrate buffer, pH 6.0 at 95°C for 20 min in a pressurized Decloaking Chamber NxGen (Biocare Medical, LLC) and then allowing slides to cool down in antigen retrieval solution for ∼10 min outside of the decloaking chamber. Next, slides were sequentially incubated with endogenous enzyme blocker (Dako S2003) for 10 min at RT; protein block (Dako X0909) for 20 min at RT; 0.006 g/L polyclonal rabbit anti-human CD3 antibody (Dako A0452, stock concentration 0.60 g/L diluted 1:100 in 1% bovine serum albumin [BSA] phosphate-buffered saline [PBS]) for 60 min at RT; horseradish peroxidase (HRP)-labeled anti-rabbit antibody (Dako K4003) for 30 min at RT; and 3,3′-diaminobenzidine (DAB) substrate (Dako K3468) for 3 min at RT. Volumes used for each incubation varied between slides but was enough to fully cover all tissue sections. Between each incubation, slides were washed with 0.05% PBS-Tween (PBS-T), pH 7.35 ± 0.02. Slides were then counterstained with Gill's Hematoxylin I (American Mastertech) for 1 min, rinsed with distilled water, dehydrated, and coverslipped.

Dual Chromogenic IHC and RNA *in-situ* Hybridization (IHC/ISH)

Dual chromogenic IHC/ISH staining was performed to simultaneously detect T receptor delta constant (*TRDC*) mRNA and CD3 protein. FFPE intestinal tissues were fixed, processed, and sectioned as described in IHC methods. Slides were first stained for *TRDC* mRNA using the RNAscope 2.5 HD Reagent Kit-RED (Advanced Cell Diagnostics, ACD) and custom-designed probe complementary to *Sus scrofa TRDC* mRNA (ACD 553141). A probe targeting *Bacillus subtilis DAPB* (ACD 310043) was used as a negative control. Slides were baked at 60 °C in a dry oven for 1 h, followed by deparaffinization and rehydration using incubations in xylenes (2 × 5 min), 100% ethanol (2 × 1 min), and air drying at RT. Slides were incubated with Hydrogen Peroxide (ACD) for 10 min at RT, rinsed with water, incubated in 1X Target Retrieval Solution (ACD) for 15 min at 95°C in a pressurized Decloaking Chamber NxGen (Biocare), rinsed with distilled water, incubated in 100% ethanol for 2 min, and air dried at RT. Once dry, a hydrophobic barrier was drawn around each tissue using an ImmEdge PAP pen (Vector Laboratories, Inc.).

ISH staining for *TRDC* RNA was completed by incubating slides in a humidifying tray either at at 40°C in a HybEZ Hybridization System oven (ACD) or at RT on the benchtop for all steps. Protein digestion was performed by incubating slides with Protease Plus (ACD) for 15 min at 40°C, followed by rinsing with distilled water. Next, slides were sequentially incubated with the following reagents and washed with 1X Wash Buffer (ACD) 2 × 2 min between each incubation: undiluted *TRDC* probe (ACD) 2 h at 40°C; 5X saline-sodium citrate (SSC) buffer overnight at RT; AMP1 (ACD) at 40°C for 30 min; AMP2 (ACD) at 40°C for 15 min; AMP3 (ACD) at 40°C for 30 min; AMP4 (ACD) at 40°C for 15 min; AMP5 (ACD) at RT for 30 min; AMP6 (ACD) at RT for 15 min; and prepared RED detection solution (diluted according to manufacturer's instructions; ACD) at RT for 10 min.

Following RNA ISH, IHC was performed for CD3 protein staining. Slides were washed with 0.05% PBS-T, pH 7.35 ± 0.02 (2 × 2 min) following RNA ISH and following incubations with protein block, primary antibody, and secondary antibody as outlined in the CD3 IHC method. Next, slides were incubated with HIGHDEF Yellow HRP chromogen (diluted according to manufacturer's instructions; Enzo Life Sciences) for 10 min at RT followed by washing again with PBS-T. To counterstain, slides were placed into 25% Gill's Hematoxylin I (American Mastertech) for 30 s. Following counterstaining, slides were rinsed well with distilled water, dried for 20 min at 60°C, and mounted with VectaMount Permanent Mounting Media (Vector) and #1 thickness coverslips.

IHC Stain Quantification

Quantification of CD3 IHC staining within the intestinal epithelium of tissues was performed using the HALO image analysis platform (Indica Labs). Regions of interest were manually annotated around epithelium of 3 villi of jejunal and ileal tissues and 3 crypts of cecal and colonic tissues per sample. Only crypts or villi non-adjacent to mucosal-associated lymphoid tissue (e.g., Peyer's patches) were annotated for analysis. Due to the inability to accurately define individual cell borders of tightly-packed cells using the software, CD3 staining was quantified as a percentage of CD3-stained surface area over the total surface area of the annotated regions with user-defined parameters for stain detection from the Area Quantification (v2.1.3) package. The percentage of CD3-stained surface area for each of the 3 annotated villi or crypts per sample were averaged together to obtain a single value for each sample. Software quantification of dually-stained CD3 and *TRDC* in tissues was not performed due to cross-detection between the two chromogenic stains. Values did not vary statistically between the 2 study replicates, as determined using the Mann-Whitney non-parametric test between time-matched data from each study replicate. Correlation between data and animal necropsy weight were not noted but would be difficult to discern due to small sample size and low statistical power.

Analysis of Variance (ANOVA) Statistical Analyses of IHC Data

One-way ANOVA analyses of percentages obtained from IHC staining quantification were performed using Prism 8 (version 8.1.2; GraphPad Software). A Gaussian distribution could not be assumed based on small sample size; therefore, non-parametric tests were used to analyze data. The rank-based Kruskal-Wallis test was performed on sets of data within a single tissue across timepoints. All combinations of multiple comparisons between tissues or timepoints were analyzed within each dataset for both analyses. $P < 0.05$ were considered significant (* <0.05, ** <0.01, *** <0.001). Input data can be found in **Supplementary Materials**.

Intestinal Epithelial Isolation

Sections of jejunum, ileum, cecum, and colon were collected to obtain single-cell suspensions for cell phenotype labeling and analysis by FCM. Intestinal sections were cut open to expose the lumen, and the epithelium was gently rinsed with PBS to remove intestinal contents. Sections were placed in RT stabilization buffer of Hank's balanced salt solution (HBSS; Gibco 14175) containing 2 mM ethylenediaminetetraacetic acid (EDTA; Invitrogen AM9261), 2 mM L-glutamine (Gibco 25030), and 0.5% BSA (Sigma A9418) for transport back to the lab. In the lab, ∼1.5 g sections of tissues were processed for single-cell isolates, and all subsequent incubations were performed in a shaking incubator (200 rpm, 37°C). Mucus dissociation was performed by incubating tissues in 30 mL of HBSS containing 5 mM dithiothretol (DTT; Invitrogen 15508) and 2% heat-inactivated fetal calf serum (FCS; Gibco A38401) for 20 min. Epithelial cell removal was carried out by transferring tissue into 30 mL of HBSS containing 5 mM EDTA and 2% FCS. A total of 3 sequential incubations in fresh epithelial removal solution were carried out for 25 min each, transferring the tissue to fresh solution for each incubation. Tissues were then washed in 20 mL of HBSS containing 10 mM HEPES (Fisher Scientific BP299) for 10 min before transferring to 10% neutral buffered formalin to confirm epithelial cell removal. Liberated cells from the epithelial removal and wash solutions were retained, pooled, passed through a 100-micron nylon filter, and washed with HBSS containing 2 mM L-glutamine and 2% FCS. Isolated cells were centrifuged 8 min at 450 × g at RT, and the pellet was resuspended in HBSS/L-glutamine/FCS solution. Viability and quantity of the final epithelial-enriched cell suspensions were determined with the Muse Cell Analyzer with the Muse Count & Viability Assay Kit (Luminex).

Peripheral Blood Mononuclear Cell (PBMC) Isolation

Immediately prior to euthanasia, ∼8 mL of whole blood was collected from each animal into a sodium citrate cell-preparation tube (CPT; BD Biosciences). Samples were transported to the lab at RT and processed using manufacturer's recommendations as previously described (42). HBSS was used to wash and resuspend cells. Cell enumeration and viability was determined as described for epithelial cell fractions. PBMCs were stored on ice for all steps unless temperature was noted otherwise.

Cell Phenotype Labeling and Data Acquisition by Flow Cytometry

For each sample, 5×10^5 live cells were seeded into a single well of a 96-well round bottom plate, pelleted, resuspended, and stained with Fixable Viability Dye eFluor 780 (eBioscience) diluted 1:1,000 in PBS for 30 min according to manufacturer's recommendations. Next, sequential incubations with unconjugated primary antibodies, secondary fluorophore-conjugated antibodies, and primary antibodies directly-conjugated to fluorophores were carried out for 15 min each at RT. Unconjugated primary antibodies included anti-γδ T cell receptor (α-γδTCR; PGBL22A, mouse IgG_1) and α-CD2 (MSA4, mouse IgG_{2a}) from Washington State University. Secondary antibodies included rat α-mouse IgG_1-BUV395 (A85-1; BD) and rat α-mouse IgG_{2a}-BV605 (R19-15; BD). Directly conjugated antibodies included α-CD3ε-PE-Cy7 (BB23-8E6-8C8, mouse IgG_{2a}; BD), α-CD4-PerCP-Cy5.5 (74-12-4, mouse IgG_{2b}; BD), α-CD8α-PE (76-2-11, mouse IgG_{2a}; BD), and α-CD27-FITC (b30c7, mouse IgG_1; BioRad). Between incubations, cells were washed with PBS. After antibody staining, cells were fixed with BD Stabilizing Fixative (BD) and stored at 4 °C overnight. The following day, fixed cells were resuspended, passed through a 35-micron nylon filter to remove aggregates, and data were acquired using a BD FACSymphony A5 flow cytometer (BD). The instrument was set up according to manufacturer's recommendations using bead capture reagents to set compensation controls.

Flow Cytometry Gating Analysis

FCM data were analyzed with FlowJo (FlowJo, LLC). Single stains and fluorescence-minus-one antibody combinations were used to set appropriate gates for each fluorochrome and respective sample type (43, 44). Data were quantified as frequencies of specified parent populations or total cell counts. Samples with low event yields were analyzed for outlier data using box-and-whisker plot outlier analysis for frequency measurements collected. If no outliers were detected, samples were included in further analysis. Values did not vary statistically between the 2 study replicates, as determined using the Mann-Whitney non-parametric test between time-matched data from each study replicate. Correlation between data and animal necropsy weight were not noted but would be difficult to discern due to small sample size and low statistical power.

t-Distributed Stochastic Neighbor Embedding (t-SNE) Visualization of Flow Cytometry Data

Dimensionality reduction using t-SNE visualization was performed on FCM data within FlowJo using the t-SNE and DownSample plug-ins available from FlowJo Exchange. Prior to t-SNE visualization, similar fluorescence intensities and gating for flow cytometry markers in each tissue at each

timepoint were confirmed. Cells from the CD3ε$^+$ gate of each sample were down-sampled ($n = 990$ cells per sample) using the DownSample plug-in to obtain equal numbers of cells for each sample type (based on combination of 4 tissues and 3 timepoints; 12 sample types total), and subsequent gates were reapplied to down samples. Next, the 8 gated IET populations were concatenated between all down samples within each tissue/timepoint combination to create a total of 96 concatenated files (8 files belonging to each IET population × 12 belonging to each tissue/timepoint combination). Keyword value series were applied based on IET population and sample type. Compensation was reapplied to the concatenated files, and the 96 files were again concatenated into a single file, including the keyword value series as additional parameters. Compensation was reapplied again to the final concatenation, and t-SNE analysis was completed with input parameters for fluorescence intensities of compensated CD3ε, γδTCR, CD2, CD4, CD8α, and CD27 being considered for visualization. Default options for the opt-SNE learning configuration, exact KNN algorithm, and Barnes-Hut gradient algorithm were used with 1,000 iterations and a perplexity of 150. To identify αβ and γδ IET populations within the final concatenation, gates were drawn based on the keyword series values assigned to IET populations and tissue/timepoint variables. CD27$^+$ and CD27$^-$ expression was assessed by redrawing gates used previously.

Non-metric Multidimensional Scaling (NMDS) Visualization and Permutational Multivariate Analysis of Variance (PERMANOVA) Statistical Analyses of Flow Cytometry Data

For a multivariate comparison of sample similarity, the IET compositions of each sample were considered. IET communities were composed of 16 discrete subpopulations defined by expression of CD27 (positive or negative) for each of the 8 T cell populations defined by flow cytometry gating analysis. Frequencies of the 16 subpopulations were calculated from cell counts exported from FlowJo and were considered as discrete, non-overlapping groups comprising the total CD3ε$^+$ IET community within each sample. From the frequency data, a dissimilarity matrix was calculated using the Bray-Curtis dissimilarity metric and this dissimilarity matrix was used for visualization and statistical testing of sample similarity. NMDS visualization of Bray-Curtis dissimilarities was performed in R with the vegan (version 2.5-5) (45), and tidyverse (version 1.2.1) (46) packages. PERMANOVA testing was completed in R using vegan's 'adonis' function. Tissue, age, and a combination of tissue and age were used as variables. *Post-hoc* pairwise PERMANOVA tests were completed on comparisons of interest (single tissue type between timepoints or within a single timepoint between tissues), correcting *p*-values with the false discovery rate (FDR) method. Corrected $p < 0.05$ were considered significant (* <0.05, ** <0.01, *** <0.001). Input data and R scripts can be found at https://github.com/jwiarda/Intraepithelial_T_cells.

ANOVA Statistical Analyses of Flow Cytometry Data

One-way ANOVA using percentages obtained from individual gates of flow cytometry data were performed using Prism 8. Because a Gaussian distribution could not be assumed based on small sample size, non-parametric tests were used to analyze data. The rank-based Kruskal-Wallis test was performed on sets of data within a single tissue across timepoints, while the paired, rank-based Friedman test was performed on sets of data within a single timepoint across tissues, pairing samples derived from the same animal. All combinations of multiple comparisons between tissues or timepoints were analyzed within each data set for both analyses. $P < 0.05$ were considered significant (* <0.05, ** <0.01, *** <0.001). Input data can be found in **Supplementary Materials**.

RESULTS

Intestinal IET Abundance Increased With Age and Was Composed of Both γδ and αβ T Cells

Jejunum, ileum, cecum, and colon were collected from 4-, 6-, and 8-week-old pigs (**Figure 1A**). To assess the presence of T cells in the intestine, IHC staining of CD3 protein was completed using the collected tissues, and any CD3 stain-positive cells were considered T cells. T cells were found in both the epithelial layer and the lamina propria of all intestinal tissues, as well as within the Peyer's patch areas of the ileum. CD3 staining within the epithelium appeared to be more frequent in the villi compared to the crypts of small intestinal tissues (jejunum and ileum) and within the apical portions of crypts in large intestinal tissues (cecum and colon). Moreover, T cell staining within the epithelium appeared more frequent overall in small intestinal compared to large intestinal tissues (**Figure 1B**). Within a respective intestinal tissue, T cell staining both throughout the entire tissue (including epithelium, lamina propria, and submucosa compartments) and specifically within the epithelial layer appeared to increase as animals aged (**Figure 1B**). To confirm the latter observation, CD3 staining was quantified within villus epithelium of jejunal and ileal tissues or crypt epithelium of cecal and colonic tissues and compared across time (**Supplementary Figure 1** and **Supplementary Table 2**). CD3 staining within the epithelium increased significantly at all intestinal sites as age increased (**Figure 1C**), suggesting IETs became more abundant across the 4- to 8-weeks of age time frame.

To further characterize intestinal IETs, dual staining of CD3 protein and T receptor delta constant (*TRDC*) mRNA was completed in a subset of jejunal, ileal, cecal, and colonic tissues. Using the rationale that CD3 protein (yellow staining) would be expressed by all T cells, *TRDC* mRNA (red staining) would only be expressed by γδ T cells, and that *TRDC*-specific red staining would mask co-localizing CD3-specific yellow staining, cells staining red were presumably γδ T cells (*TRDC*$^+$), whereas cells staining yellow were presumably αβ T cells (CD3$^+$*TRDC*$^-$). Staining revealed the presence of both

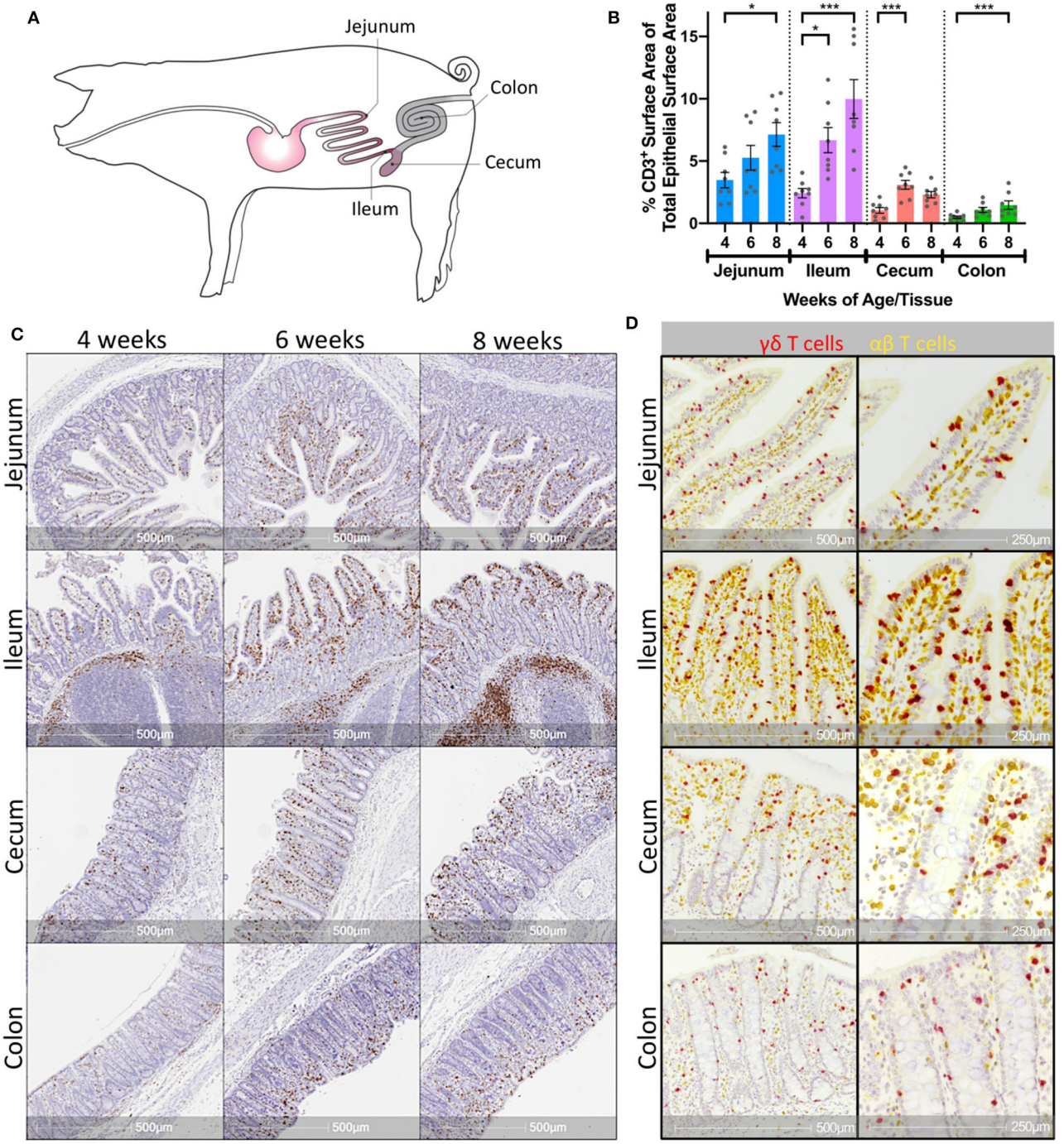

FIGURE 1 | Porcine intestinal IETs increased with age and were composed of γδ and αβ T cells. **(A)** Anatomical diagram of tissue collection sites from jejunum, ileum, cecum, and colon within pig intestinal tract. **(B)** Representative images of T cell-specific CD3 protein staining (brown) within multiple intestinal tissues at different ages in pigs. **(C)** Percentages of epithelial surface area stained for CD3 protein within a single tissue over multiple timepoints. Statistical significance was determined within a single tissue across timepoints by the rank-based Kruskal-Wallis test using all combinations of multiple comparisons within a tissue. $P < 0.05$ were considered significant (* < 0.05, *** < 0.001). **(D)** Representative images of T cell-specific CD3 protein staining (yellow) and γδ T cell-specific *TRDC* transcript staining (red) in pig intestinal tissues at 8-weeks of age. Distance scales on the bottom of each image are included for size comparisons. Samples were taken from 4 animals per timepoint in each of two separate experiments ($n = 8$ per timepoint; $n = 24$ total).

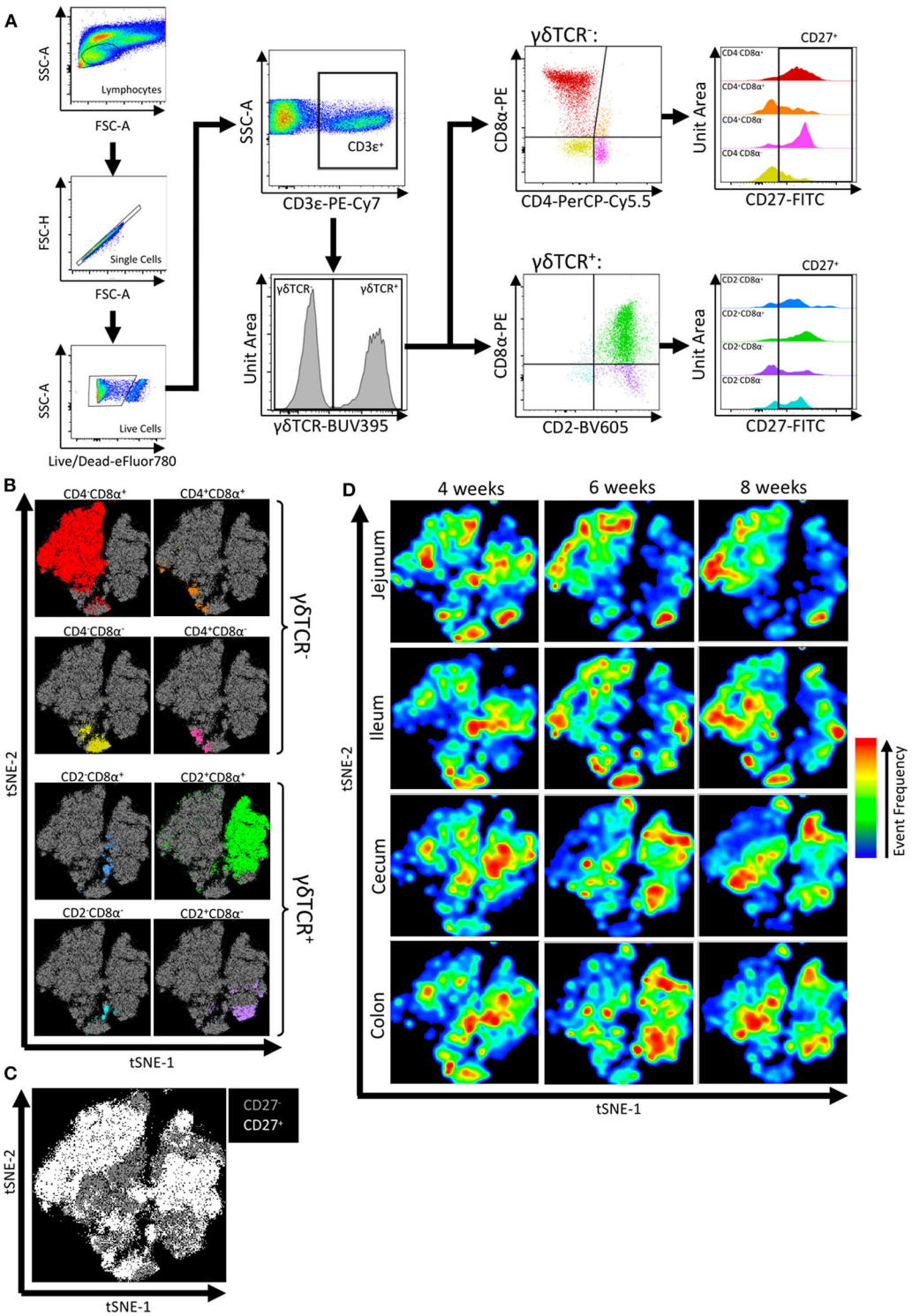

FIGURE 2 | Flow cytometry gating revealed IET populations and CD27 expression varied by intestinal location and age. **(A)** Gating strategy used to identify IET populations and subpopulations from flow cytometry data of epithelial-enriched samples. **(B–D)** t-SNE dimensional reduction of gated flow cytometry data from all samples to reveal clustering of αβ and γδ IET populations **(B)**, $CD27^+$ and $CD27^-$ expression **(C)**, and tissue and age-specific cell frequency distributions **(D)**. In **(B,C)**, individual points represent single cells. Plot axes indicate t-SNE dimensions. Cells highlighted in non-gray colors coordinate with corresponding αβ and γδ IET populations **(B)** or $CD27^+$ classification **(C)**. In **(D)**, areas with more cells present are indicated by red, whereas areas with less cells present are indicated by blue. Subsets of total IETs were taken from each sample ($n = 990$) to obtain equal cell numbers for each individual sample; an equal number of cells are present for each animal and combination of intestinal tissue and timepoint. Samples were taken from four animals per timepoint in each of two separate experiments ($n = 8$ per timepoint; $n = 24$ total).

TRDC^{+} and CD3^{+}*TRDC*$^{-}$ cells, corresponding to presumable γδ and αβ T cells, respectively, within the epithelium of all intestinal tissues analyzed (**Figure 1D**). T cells in the lamina propria and submucosa were primarily CD3^{+}*TRDC*$^{-}$, with *TRDC*$^{+}$ cells noted infrequently outside of the epithelium (**Supplementary Figure 2**). These findings suggest both αβ and γδ T cells are located within the intestinal epithelium of both small and large intestine in pigs.

IET Compositions Diverged by Intestinal Location as Pigs Aged

To further phenotype intestinal IETs, cell staining and FCM analysis was performed on epithelial-enriched fractions of the jejunum, ileum, cecum, and colon collected at 4-, 6-, and 8-weeks of age. The protocol used to liberate epithelial cells from the intestinal tissues resulted in isolation of primarily epithelial cells, though some lamina propria cells may have been released during processing and included in the analysis (**Supplementary Figure 3**). CD3ε was used to identify T cells, and further characterization of total T cells by expression of cell surface markers γδTCR, CD2, CD4, CD8α, and CD27 was assessed. CD2 and CD8α expression are commonly used to identify functional porcine γδ T cell populations (47, 48); CD4 and CD8α expression identify functional porcine αβ T cell populations (49, 50); and CD27 expression is indicative of T cell activation and memory states (51–54). In total, this gating strategy yielded 16 discrete, non-overlapping IET subpopulations based on CD27$^{+/-}$ expression within each of the 8 defined IET populations (**Figure 2A**).

Interpretation of flow cytometry data by t-SNE analysis allows users to obtain an overall impression of marker-specific characteristics by simultaneously considering all marker expression patterns to spatially resolve cellular identities and visually identify cell populations of interest. Hence, fluorescence intensities of cell surface markers within the total CD3ε^{+} T cell community were utilized for implementation of t-SNE visualization (**Supplementary Figure 4**). Visualization revealed close proximities of cells assigned to each of the 8 IET populations, as defined by γδTCR, CD2, CD4, and/or CD8α expression in **Figure 2A**, and variability in frequencies of cells belonging to the 8 populations (**Figure 2B**). Within each of the 8 IET populations, expression of CD27 was variable based on CD27^{+} or CD27^{-} classification, but some populations were largely CD27^{+} while others were largely CD27^{-} (**Figure 2C**). Thus, heterogeneity existed within IETs based on the cell surface markers assessed here. We next analyzed distribution of different IETs by observing cell distribution frequencies within the t-SNE plot across the multiple intestinal tissues and ages. Biases toward different IET populations or different CD27 expression within an IET population were observed between different intestinal tissues at a single timepoint or within a tissue across time, indicating both age- and intestinal location-dependent changes to IET distributions occurred (**Figure 2D**). Therefore, t-SNE analysis demonstrated heterogeneity amongst and variability between IETs, as related to both age and intestinal site.

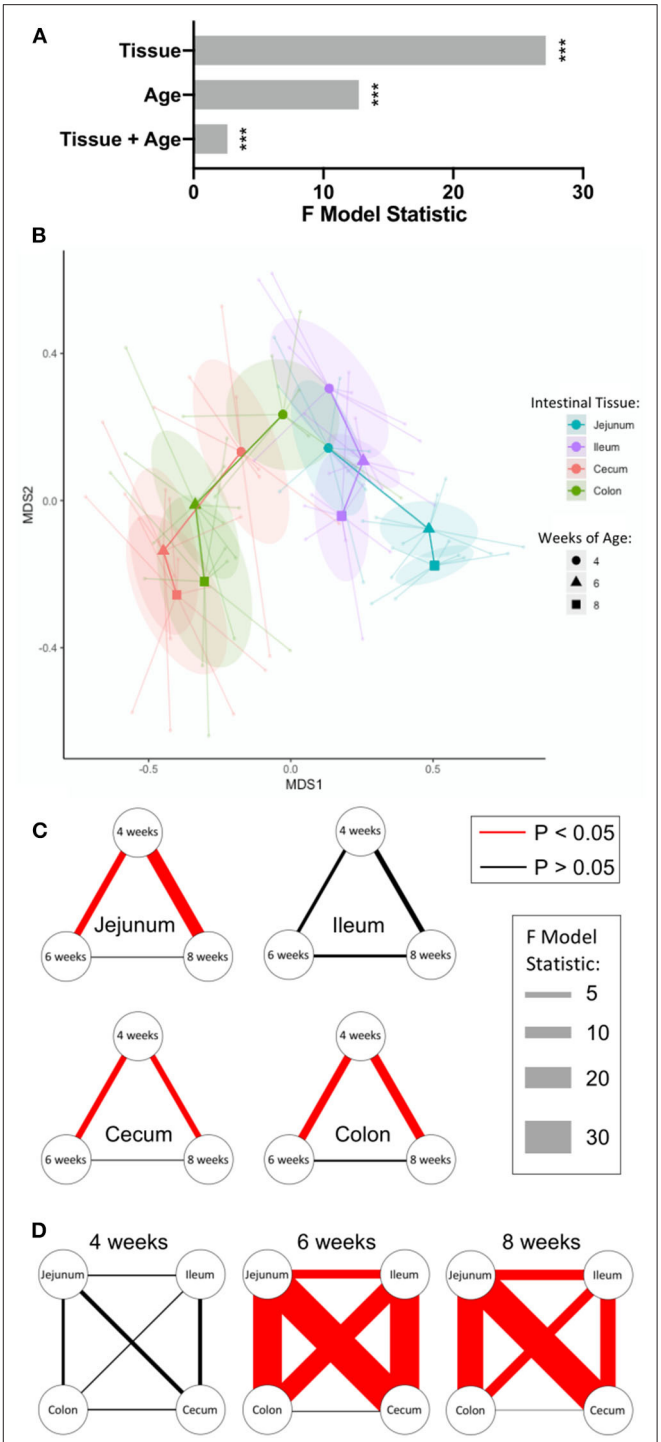

FIGURE 3 | IETs diverged by intestinal location over time. **(A)** Multivariate analysis of flow cytometry data to assess tissue- and age-dependent effects on compositions of IET communities. Sample frequencies of the 16 discrete cell subpopulations defined by flow cytometry gating of αβ or γδ T cell populations and CD27 expression were analyzed for their overall tissue and age-dependent effects using PERMANOVA analysis. Greater F model statistic values represent greater magnitude of differences, while $p < 0.05$ were considered significant (*** < 0.001). **(B)** NMDS visualization of IET communities based on sample frequencies of the 16 discrete cell subpopulations

(Continued)

FIGURE 3 | defined by flow cytometry gating of αβ or γδ T cell populations and CD27 expression. Shorter distances between points represent greater similarities between samples. Centroids are plotted at the center of each 95% confidence ellipse for each sample type based on both intestinal tissue and pig age during sample collection. Centroids from each particular timepoint of a single tissue are sequentially connected chronologically. Point, ellipse, and line color is tissue-specific, while centroid shape is age-specific. **(C,D)** Visualization of pairwise PERMANOVA test results comparing IET compositions from samples between timepoints within a tissue **(C)** or between tissues within a single timepoint **(D)**. Relationships between variables are represented by connecting lines. Magnitudes of the F model statistics demonstrate the influence of a comparison and are represented by the width of the lines, while color of the line represents statistical significance (red = significant; black = not significant). *P*-values were corrected for multiple comparisons using the FDR method considering all tests displayed here, $p < 0.05$ were considered significant. Samples were taken from four animals per timepoint in each of two separate experiments ($n = 8$ per timepoint; $n = 24$ total).

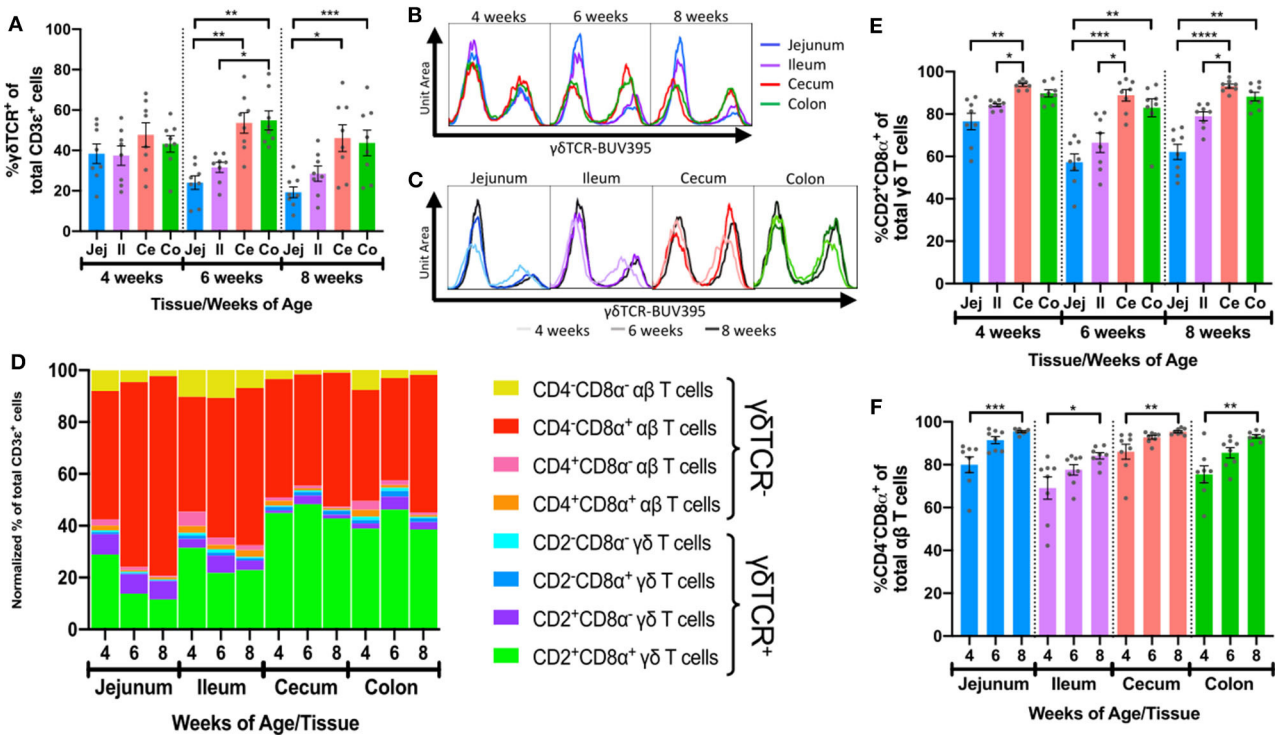

FIGURE 4 | Intestinal location and age affected frequencies and phenotypes of αβ and γδ IETs. **(A)** Comparison of percentages of γδTCR$^+$ cells from the total IET communities across intestinal tissues within a single timepoint. **(B,C)** Fluorescence intensities of γδTCR from the total IET communities. Frequency is proportional to unit area under the curve, and all samples are represented by equal total unit area under the curve. For fluorescence intensity curves **(B,C)**, data are acquired from merging the subsetted data of 8 samples from each age/tissue combination used for t-SNE analysis. The same data are presented in B and C to show tissue-dependent **(B)** or age-dependent **(C)** changes. **(D)** Mean normalized frequencies of αβ and γδ IET populations across intestinal tissues and timepoints. Each IET population frequency is represented by a different color, and percentages of all IET populations within a single tissue and timepoint total 100%. **(E)** Comparison of percentages of CD2$^+$CD8α$^+$ cells from the total γδ IET communities across intestinal tissues within a single timepoint. **(F)** Comparison of percentages of CD4$^-$CD8α$^+$ cells from the total αβ IET communities across timepoints within a single intestinal tissue. In **(A,E)**, statistical significance was determined within a single timepoint across tissues by the paired, rank-based Friedman test using all combinations of multiple comparisons within timepoint. In **(F)**, statistical significance was determined within a single tissue across timepoints by the rank-based Kruskal-Wallis test using all combinations of multiple comparisons within a tissue. $P < 0.05$ were considered significant (* < 0.05, ** < 0.01, *** < 0.001, **** < 0.0001). Samples were taken from four animals per timepoint in each of two separate experiments ($n = 8$ per timepoint; $n = 24$ total).

The overall effects of intestinal tissue, pig age, and a combination of tissue and pig age on intestinal IET community compositions were analyzed statistically by PERMANOVA, a multivariate comparison of the frequencies of the 16 discrete IET subpopulations of each sample identified in **Figure 2A**. This analysis revealed heterogeneity in the compositions of IET communities was significantly affected by tissue and age, and tissue exerted a greater influence on IET compositions than did age. A significant combinatorial influence of both tissue and age could also be observed, suggesting the effect of age was not consistent across all tissues (**Figure 3A**).

NMDS visualization and pairwise *post-hoc* tests were next employed to extract results for tissue and age-specific comparisons of interest. Compositions of IET communities in jejunum, ileum, cecum, and colon were relatively similar in pigs at 4-weeks of age but diverged as age increased, with the greatest differences occurring between small and large intestinal locations (**Figure 3B**). The largest changes in IET compositions

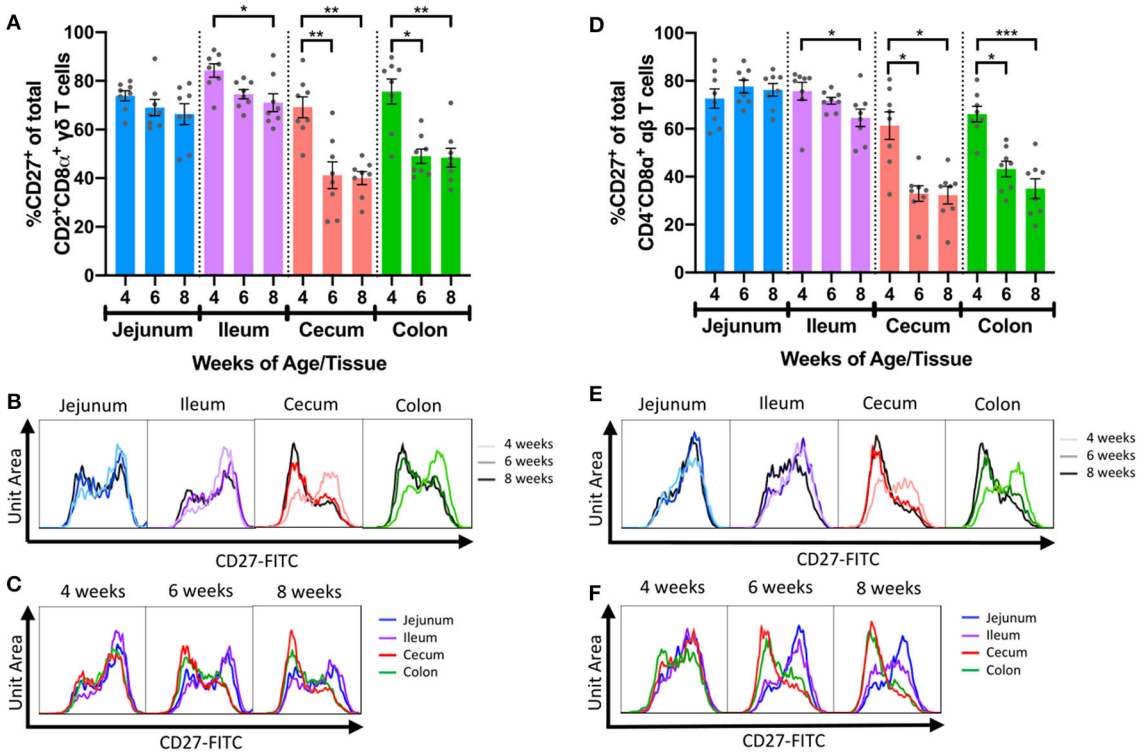

FIGURE 5 | Frequencies of IETs expressing CD27 decreased with age in distal intestinal tract. **(A)** Comparisons of percentages of $CD27^+$ cells within $CD2^+CD8\alpha^+$ γδ IETs across timepoints within a single intestinal tissue. **(B,C)** Fluorescence intensities of CD27 from total $CD2^+CD8\alpha^+$ γδ IETs within a single intestinal tissue across time **(B)** or within a single timepoint across intestinal tissues **(C)**. **(D)** Comparisons of percentages of $CD27^+$ cells within $CD4^-CD8\alpha^+$ αβ IETs across timepoints within a single intestinal tissue. **(E,F)** Fluorescence intensities of CD27 from $CD4^-CD8\alpha^+$ αβ IETs within a single intestinal tissue across time **(E)** or within a single timepoint across intestinal tissues **(F)**. In (**B,C**) and **(D,E)**, frequency is proportional to unit area under the curve, and all samples are represented by equal total unit area under the curve. For fluorescence intensity curves, data are from merging the subsetted data of 8 samples from each timepoint/tissue combination used for t-SNE analysis. The same data are presented in B and C or D and E to show age-dependent **(B,D)** or tissue-dependent **(C,E)** changes. Statistical significance was determined for percentages of total $CD27^+$ cells within a single tissue across timepoints by the rank-based Kruskal-Wallis test using all combinations of multiple comparisons within a tissue. $P < 0.05$ were considered significant (* < 0.05, ** < 0.01, *** < 0.001). Samples were taken from four animals per timepoint in each of two separate experiments ($n = 8$ per timepoint; $n = 24$ total).

within tissues over time occurred between 4- and 6-weeks of age: IET compositions in the jejunum, cecum, and colon but not ileum at 4-weeks of age were significantly different from the same sites at 6- and 8-weeks of age, while 6- and 8-week IET communities were not significantly different from each other (**Figure 3C**). Divergence in IET compositions at 6- and 8-weeks of age contributed to jejunum and ileum of the small intestine becoming increasingly divergent from large intestinal tissues and, to a lesser extent, from each other (**Figure 3D**). Hence, within the age span analyzed, IET communities changed most drastically within a single tissue between 4- and 6-weeks of age, and were largely defined by small or large intestinal location, although significant differences between locations within the small intestine were also detected.

Frequencies and Phenotypes of Intestinal αβ and γδ IETs Differed With Age and Intestinal Location

We next compared frequencies of different IET populations defined by FCM gating to discover the manners by which IETs differed between intestinal tissues and as pigs aged. To determine the composition of γδ vs. αβ T cells within the total IET community, percentages of $\gamma\delta TCR^+$ cells from total $CD3\varepsilon^+$ T cells obtained from our epithelial-enriched cell fractions (**Supplementary Table 3**) were compared across age and tissues. As no antibody reactive to the porcine αβTCR is currently available (55), $CD3\varepsilon^+\gamma\delta TCR^-$ cells were inferred to be αβ T cells, while γδ T cells were identified as $CD3\varepsilon^+\gamma\delta TCR^+$ (47, 56, 57). Percentages of $\gamma\delta TCR^+$ IETs were similar between intestinal sites at 4-weeks of age but differed between small and large intestinal tissues at both 6 and 8-weeks of age, by which times $\gamma\delta TCR^+$ percentages were greater in large intestinal compared to small intestinal tissues (**Figures 4A,B**). Percentages of $\gamma\delta TCR^+$ cells decreased in jejunum between 4- and 8-weeks ($p = 0.0157$), whereas percentages did not change significantly in ileum, cecum, and colon over time (**Figure 4C**). Thus, as animals aged, γδ T cells constituted a smaller proportion of the total IETs in small intestine compared to large intestine.

Frequencies of the γδ and αβ IET populations defined by CD2 and CD8α or CD4 and CD8α expression, respectively,

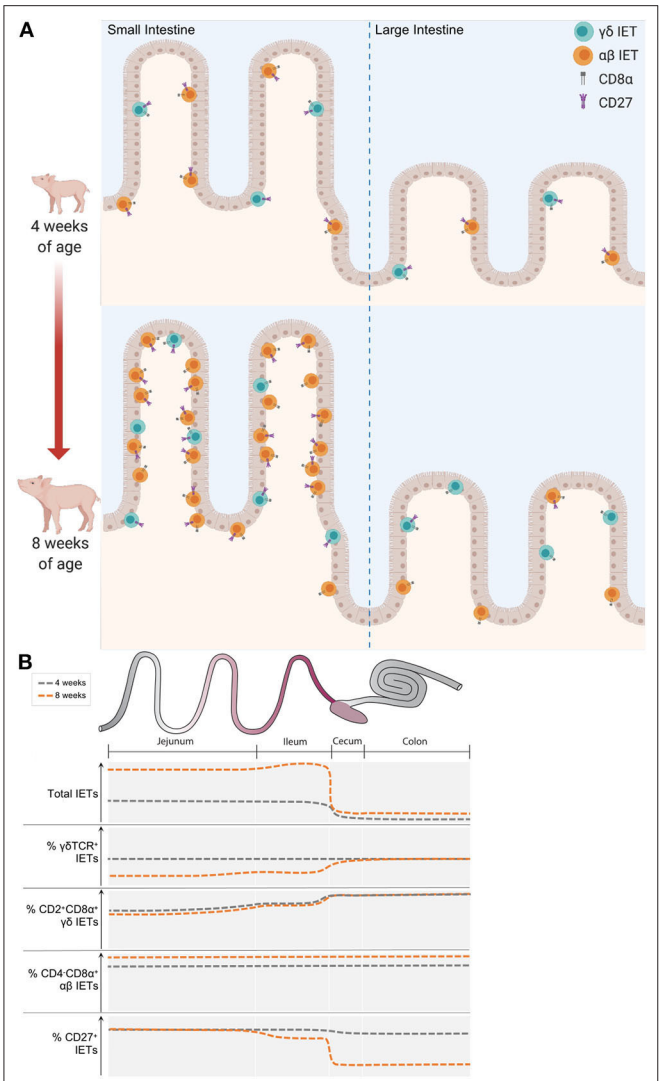

FIGURE 6 | IET community landscape was altered by intestinal location and pig age. **(A)** Representation of IET community landscapes in small and large intestine of 4- vs. 8-week-old pigs. γδ IETs are shown in teal, while αβ IETs are shown in orange. Expression of CD27 and CD8α receptors are also indicated. Whether CD8α is expressed in conjunction with another CD8α molecule or a CD8β molecule is unknown. **(B)** Summary of key changes in IET populations observed across intestinal locations in 4- and 8-week-old pigs. Y-axes show qualitative increases in measurements specified for each plot. Representative approximations in 4-week-old pigs are illustrated with dark gray dotted lines, while orange dotted lines represent approximations in 8-week-old pigs.

varied within our total dataset (**Figure 2B**). In support of this observation, the normalized frequencies of the 8 discrete γδ and αβ IET populations to the entire $CD3\varepsilon^+$ IET community were calculated for samples based on intestinal tissue and age (**Supplementary Table 4**). Two major populations comprised a combined normalized frequency of between 78.4 and 91.7% of the total IET communities within the 12 sample groups: $CD2^+CD8\alpha^+$ γδ T cells and $CD4^-CD8\alpha^+$ αβ T cells (**Figure 4D**).

Not only did frequencies vary between αβ and γδ IET populations, but populations of γδ or αβ IETs also appeared to vary between tissues and with age. To investigate, percentages of each of 4 IET populations within total γδ or αβ IETs (**Supplementary Tables 5**, **6**) were compared across tissues and age. γδ IET populations were defined by expression of CD2 and CD8α (**Figure 4E** and **Supplementary Figures 5A–C**). $CD2^+CD8\alpha^+$ γδ T cells, which have been proposed to be a terminally differentiated cell population (28, 48, 54), comprised the largest fraction of γδ IETs in all samples, while $CD2^+CD8\alpha^-$ γδ T cells, which are proposed to be a naïve or memory population (28, 48, 54), were the second most abundant γδ IET population (**Figure 4D**). Increased percentages of $CD2^+CD8\alpha^-$ γδ IETs within total γδ IETs (**Supplementary Figure 5A**) coincided with complementary decreases in percentages of $CD2^+CD8\alpha^+$ γδ IETs (**Figure 4E**) in small intestine compared to large intestine. Moreover, differences in compositions of γδ IET populations were tissue- but not age-dependent, and, overall, $CD2^+CD8\alpha^+$ cells were still the predominating γδ IET population within the intestinal epithelium, making up an average of between 57.3 and 93.8% of all γδ IETs, regardless of intestinal location or timepoint analyzed. Thus, the majority of γδ IETs were associated with a terminally differentiated cell phenotype ($CD2^+CD8\alpha^+$), and γδ IETs associated with a naïve or memory phenotype ($CD2^+CD8\alpha^-$) were more frequent in the small intestine compared to the large intestine.

Porcine αβ T cell populations were defined by CD4 and CD8α expression as presumable cytotoxic T cells ($CD4^-CD8\alpha^+$) (49, 58), naïve helper T cells ($CD4^+CD8\alpha^-$) (49, 50, 58), activated or memory T helper cells ($CD4^+CD8\alpha^+$) (50, 58), and $CD4^-CD8\alpha^-$ T cells that were potentially mucosal-associated invariant T (MAIT) cells (59), invariant natural killer T (iNKT) cells (60), or immature αβ T cells (9) (**Figure 4F** and **Supplementary Figures 5D–F**). The majority of intestinal αβ IETs were $CD4^-CD8\alpha^+$ (average of 69.1 to 95.4%), followed in frequency by $CD4^-CD8\alpha^-$ (1.9 to 17.3%) and then $CD4^+CD8\alpha^{+/-}$ populations (0.8 to 5.0% and 0.7 to 9.5%, respectively) (**Figure 4D**). As pigs aged, significant increases in $CD4^-CD8\alpha^+$ αβ IET percentages (**Figure 4F**) and concomitant decreases in all other αβ IET population percentages ($CD4^+CD8\alpha^+$, $CD4^+CD8\alpha^-$, and $CD4^-CD8\alpha^-$; **Supplementary Figures 5D–F**) occurred across all tissues. Findings indicate a potential increase in $CD4^-CD8\alpha^+$ αβ IETs, decrease in other αβ IET populations ($CD4^+CD8\alpha^+$, $CD4^+CD8\alpha^-$, and $CD4^-CD8\alpha^-$), or combination of both scenarios occurred at all intestinal locations as pigs aged.

Fewer IETs Expressed CD27 in Distal Intestinal Tract as Pigs Aged

CD27 is commonly used as a phenotypic marker for functional classification of porcine T cells and is downregulated on activated/effector T cells (51–54, 61, 62). CD27 expression within intestinal IET populations was assessed to compare activation phenotypes across intestinal tissues with age (**Supplementary Table 7**). The 2 predominating IET

populations, $CD2^+CD8\alpha^+$ γδ IETs and $CD4^-CD8\alpha^+$ αβ IETs, had similar patterns of CD27 expression: as pigs aged, percentages of $CD27^+$ IETs decreased significantly in ileum, cecum, and colon but not jejunum (**Figures 5A,B,D,E**). Decreases in percentages of $CD27^+$ cells also occurred in $CD2^+CD8\alpha^-$ γδ IETs but not any other γδ or αβ IET populations (**Supplementary Figures 6A–F**). Although percentages of $CD27^+$ cells decreased in ileum, decreases in percentages of $CD27^+$ cells in large intestine between 4- and 6-weeks of age were more drastic (**Figures 5C,F**). Consequently, average percentages of $CD27^+CD2^+CD8\alpha^+$ γδ IETs and $CD27^+CD4^-CD8\alpha^+$ αβ IETs were no >49.0% in large intestine compared to a minimum of 64.6% in small intestine at 6- and 8-weeks of age. The data suggest the majority of IETs were not activated in 4-week-old pigs, but, as pigs continued to age, IETs of the more distal intestinal tract became activated, especially those of the large intestine.

Furthermore, proportions of $CD27^+$ $CD2^+CD8\alpha^+$ γδ and $CD27^+$ $CD4^-CD8\alpha^+$ αβ IETs in 6- and 8-week-old pigs were compared to corresponding populations in peripheral blood (PB) (**Supplementary Tables 7, 8**). Direct comparison revealed significantly higher $CD27^+$ percentages in PBMCs compared to cecum and colon IETs for $CD2^+CD8\alpha^+$ γδ T cells at 6-weeks of age (**Supplementary Figure 7A**) and for $CD4^-CD8\alpha^+$ αβ T cells at both 6- and 8-weeks of age (**Supplementary Figure 7B**). Neither $CD27^+$ $CD2^+CD8\alpha^+$ γδ nor $CD4^-CD8\alpha^+$ αβ IET percentages in jejunum or ileum were statistically different from that of PBMC populations at either timepoint (**Supplementary Figures 7A,B**). Collectively, the data suggest a similar fraction of small intestine IETs are in an activated state compared to peripheral T cells, while more large intestine IETs are in an activated state compared to peripheral T cells in 6- and 8-week-old pigs, as evidenced by expression of CD27.

IET Community Landscape Is Altered by Both Intestinal Location and Pig Age

In summary, IET abundance increased with age at all intestinal locations analyzed, and compositional differences were largely noted between small and large intestine in older pigs (**Figure 6**). In older pigs, lower proportions of γδ IETs from the total IET community were present in the small compared to the large intestine. Of these γδ IETs, nearly all were $CD2^+CD8\alpha^+$ in the large intestine, while expression of CD8α on γδ IETs was more variable in the small intestine. Percentages of $CD4^-CD8\alpha^+$ αβ IETs from total αβ IETs were similar between intestinal locations and uniformly increased with age. Lastly, CD27 expression was lost by IETs in the ileum and especially in the large intestine as pigs aged. In total, these alterations contributed to an overall divergence in IET abundances and compositions as pigs aged within the 4- to 8-week-old timespan.

DISCUSSION

Understanding changes in IET quantities, proportional phenotypes of αβ and γδ IET populations, and expression of T cell activation marker CD27 between intestinal locations over the nursery period is important for establishing a paradigm of porcine IET maturation that may contribute to developing strategies to improve pig health and/or market performance. Herein, we demonstrate dynamics of IET maturation within the porcine intestinal epithelium arise in an age- and intestinal location-dependent manner, and our findings recapitulate and expand upon results of previous studies of porcine IETs. In line with previous work, we report IETs increased in abundance within the small intestine as pigs aged (31) and further demonstrated increased abundances of IETs within the large intestine as pigs aged. The majority of porcine IETs are $CD4^-CD8\alpha^+$ (34), and we further show that porcine IETs belonged to both αβ and γδ T cell lineages. Overall, IETs were primarily $CD2^+CD8\alpha^+$ γδ T cells and $CD4^-CD8\alpha^+$ αβ T cells. Early in the nursery period (4-weeks of age), IET communities were similar throughout the intestinal tract. As pigs aged, not only did IET numbers increase, but communities became regionally specialized by 6-weeks of age, corresponding to previous findings denoting intestinal T cell communities do not resemble adult populations until between 5- to 8-weeks of age in conventional pigs (36). Moreover, shifts in IET compositions were largely due to several alterations to IET populations. First, small intestinal tissues had lower percentages of γδ IETs (and presumably greater percentages of αβ IETs) but higher proportions of $CD2^+CD8\alpha^-$ cells (complemented by lower percentages of $CD2^+CD8\alpha^+$ cells) within total γδ IETs than did large intestinal tissues at the later nursery stages (6- and 8-weeks of age). Second, as pigs aged, the percentages of $CD4^-CD8\alpha^+$ αβ IETs increased in all tissues. Third, the percentages of IETs expressing CD27 decreased with age in the major IET populations ($CD2^+CD8\alpha^+$ γδ IETs and $CD4^-CD8\alpha^+$ αβ IETs) of the ileum and large intestine. Fourth, at later nursery stages (6- and 8-weeks of age), lower percentages of cells from the major IET populations were $CD27^+$ in the large compared to small intestine. Moreover, percentages of $CD27^+$ IETs in large intestine were significantly lower than corresponding T cell populations in the periphery, while percentages between small intestine IETs and peripheral T cells were similar at later nursery stages (6- and 8-weeks of age). To our knowledge, this work comprises the most comprehensive and detailed analysis of porcine IETs in regards to intestinal location and nursery pig age and highlights important age- and intestinal location-associated dynamics of IET maturation to consider in future work.

While interactions between IETs and microbiota were not directly observed in this study, associations exist between intestinal IET phenotype data obtained from our study and previous reports of intestinal microbial diversity in the developing pig. The largest microbial differences in the porcine intestinal tract correlate to intestinal location, but age also correlates to microbial differences (63). Drawing parallels to the current study, intestinal location gave greater dictation to IET community composition than age, though age still had significant influence. In pigs, distinct microbial communities exist between the small and large intestine and become more prominent with age (64). Distinct microbial communities

also exist between the jejunum and the ileum of the small intestine (65). Similarly, we detected significant differences in overall IET community compositions not only between the small and large intestine but also between jejunum and ileum. Microbial abundance and diversity increase going from proximal to distal end of the intestinal tract and as pigs age (1, 66, 67), resulting in exposure to a larger and more diverse microbially-derived antigenic repertoire in distal intestinal regions and in older pigs. Correspondingly, percentages of $CD27^+$ IETs were reduced at the distal but not proximal end of the intestinal tract as pigs aged, indicating distally-located intestinal IETs may be activated at higher frequencies, and the frequency of activation accrues with age (and presumably, antigen exposure). Future studies investigating how parallel development of microbial and IET communities influence one another would greatly enhance our understanding of both components. Hence, antigenic stimulation from the microbiota should be strongly considered for potential influences on variability, maturation, and/or activation of intestinal IETs.

Ultimately, functional specialization of different intestinal segments may contribute directly or indirectly to variability in IET numbers, compositions, and activation. Variability in microbiota, dietary constituents, and immune-modulating molecules have all correlated to physiological functions at distinct intestinal locations (1). IETs were less abundant in intestinal crypts than in villi and in large compared to small intestine. In the small intestine, villi are important for increasing surface area to maximize nutrient absorption (1, 68). Hence, the small intestinal villi provide a large surface area with close contact with luminal contents, and interactions at the luminal-epithelial interface may attract greater numbers of IETs. Compared to the single mucus layer of the small intestine, the large intestine has two mucus layers, providing an additional degree of physical separation between the epithelium and luminal contents (69), and the large intestine also lacks villi. Thus, IETs located within large intestinal crypts may have less exposure to luminal contents, resulting in fewer IETs present there. Microbes in the large intestine are also important bioreactors that ferment indigestible components into metabolites that serve as fuel or signaling molecules for the host. To maintain beneficial microbes, a symbiotic relationship is established, and microbes are tolerated yet tightly regulated by the host (70, 71), perhaps through IETs in close proximity with these microbes being primed for activation. It's unclear why lower percentages of IETs expressed CD27 in large intestine compared to small intestine as pigs aged, indicating greater percentages of IETs were activated in the large intestine compared to the small intestine. However, differing compositions and abundances of microbial species present in the large intestine compared to the small intestine may play a role in IET activation. Moreover, large intestinal IETs may be exposed to a different repertoire of soluble factors than IETs of the small intestine, such as microbially-derived components including lipopolysaccharide (LPS) or microbially-constructed metabolite products including short-chain fatty acids (SCFAs).

In peripheral blood and non-intestinal tissues, the presence or absence of CD8α expression within porcine $CD2^+$ γδ T cells is associated with different functional states. $CD2^+CD8\alpha^+$ γδ T cells exhibit greater expression of the effector T cell transcription factor T-bet (54), greater cytokine production (48), and expression patterns of cell surface molecules indicative of T cell activation (28, 51) when compared to $CD2^+CD8\alpha^-$ γδ T cells. Resultingly, $CD2^+CD8\alpha^-$ γδ T cells are a proposed naïve or memory cell population, while $CD2^+CD8\alpha^+$ γδ T cells are proposed to be a terminally differentiated population (48, 54). Moreover, porcine $CD2^+CD8\alpha^-$ γδ T cells can gain expression of CD8α following *in vitro* IL-2 stimulation (47). In humans and rodents, IETs may gain surface expression of the CD8αα homodimer upon activation (9), supporting the notion that porcine $CD2^+CD8\alpha^+$ γδ T cells are a terminally differentiated cell population arising from $CD2^+CD8\alpha^-$ γδ T cells. In our study, greater percentages of γδ IETs expressed CD8α in the distal compared to proximal intestinal tract. Expression of the CD8αα homodimer on T cells is implicated in increasing the threshold for T cell receptor-mediated activation (72–74). Hence, CD8α expression in our proposed terminally differentiated $CD2^+CD8\alpha^+$ γδ IETs may indicate an increased threshold for TCR-mediated activation, suggesting a more regulatory or tolerant phenotype for $CD2^+CD8\alpha^+$ γδ IETs compared to $CD2^+CD8\alpha^-$ counterparts. Whether similar phenomena occur in pigs is unknown but supported by previous *in vitro* and *in vivo* work in gnotobiotic pigs (27, 75). Wen et al. demonstrate ileal $CD2^+CD8\alpha^-$ γδ IETs secreted higher levels of IFN-γ, secreted lower levels of IL-10, and expressed lower levels of regulatory transcription factor Foxp3 compared to $CD2^+CD8\alpha^+$ γδ IETs (75). Moreover, frequencies of $CD2^+CD8\alpha^+$ γδ T cells increased in ileum of gnotobiotic pigs following colonization with probiotic *Lactobacilli* or infection with human rotavirus, while $CD2^+CD8\alpha^-$ frequencies decreased (27). Therefore, it is plausible that γδ IETs in the more distal intestinal tract have an increased threshold for TCR-mediated activation, giving way to a more tolerogenic profile. The same might also hold true for αβ IETs; however, whether $CD4^-CD8\alpha^+$ αβ IETs expressed CD8αα, CD8αβ, or a combination of both cannot be determined from our data.

In the United States, pigs are typically weaned at ∼21 days of age and immediately proceed to the nursery period (3- to 10-weeks of age) of production thereafter. The weaning period is considered to be one of the most stressful life events for pigs. Weaning involves cessation of passive immune transfer of milk-derived immunoglobulins by abrupt removal of piglets from the sow; introduction of new social and environmental stressors from inter-litter mixing and transport to a new facility; and introduction to a solid-food diet (38, 39). In addition, animals are exposed to a plethora of new environmental, microbial, and dietary antigens that may induce age-associated changes to intestinal IETs. In humans and rodents, major naturally-occurring IET populations include $CD4^-CD8\alpha^+CD8\beta^-$ αβ T cells ($CD8\alpha\alpha^+$ αβ IETs), which express the CD8αα homodimer rather than the CD8αβ heterodimer, and γδ T cells (2, 9). Natural IETs are recruited to the epithelium in an antigen-independent

manner and do not show increased recruitment with antigen exposure or age (2). Induced IETs, on the other hand, are predominately $CD4^{-}CD8\alpha^{+}CD8\beta^{+}$ αβ T cells ($CD8\alpha\beta^{+}$ αβ IETs) expressing the CD8αβ coreceptor (2, 9). Induced IETs encounter their cognate antigen, then are recruited to the intestinal epithelium and reside within the epithelial layer as antigen-experienced effector or memory cells (7, 76). Induced IETs increase with age in association with increased antigen exposure (2). Hence, increased IET numbers observed in our study may be related to the recruitment of induced IETs associated with exposure to increased antigenic load and/or diversity as pigs aged. Increases in induced IET recruitment as antigen is experienced could account for overall increases in IET numbers observed as pigs aged, as well as increased percentages of $CD4^{-}CD8\alpha^{+}$ αβ IETs observed across time at all intestinal sites. Moreover, decreased percentages of γδ IETs in the small intestine compared to large intestine may be attributed to greater recruitment and abundance of induced $CD8\alpha\beta^{+}$ αβ IETs in the small intestine. To our knowledge, natural and induced IETs have not been characterized in the pig intestinal tract, but knowledge of CD8β expression for αβ T cells required to further investigate presumable induced or natural IET phenotypes based on human and rodent data is lacking from our data.

CONCLUSIONS

Overall, we demonstrated heterogeneity in IET numbers, compositions, and activation phenotypes between small and large intestinal tissues and across age in nursery pigs. IET communities were largely similar between intestinal sites early in the nursery phase; however, tissue-specific divergence occurred as pigs aged, indicating the nursery period is a critical time of intestinal IET maturation in conventional pigs. Divergence in IET communities was evident by variation in cell numbers, γδ vs. αβ T cell compositions, frequencies of γδ and αβ IET populations, and CD27 expression. Due to the uniqueness of IETs by intestinal location and pig age, results pertaining to IETs should not be generalized, but rather the variables of age and intestinal location should be strongly considered. Our findings are based on cellular phenotypes, and functional significance remains to be shown. In this regard, caution should be taken when applying functional characteristics based from research performed using T cells that are not of intestinal epithelial origin. Though not addressed by our study, additional factors such as intestinal microbiota, weaning age, antibiotic usage, environmental stress, diet, animal market performance, and disease, should be strongly considered for their correlations with or impacts on the age- and intestinal location-dependent IET communities defined herein.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**. Supporting code for analysis in R can be found at https://github.com/jwiarda/Intraepithelial_T_cells.

ETHICS STATEMENT

The animal study was reviewed and approved by Iowa State University Institutional Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

JW, NG, and CL designed experiments. JW, ZB, NG, and CL collected samples. JW, ZB, KB, and CL performed experiments. JW and JT analyzed data. JW wrote the manuscript. All authors reviewed and edited the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.01139/full#supplementary-material

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REVIEW
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The Veterinary Immunological Toolbox: Past, Present, and Future

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It is well-recognized that research capability in veterinary species is restricted by a lack of immunological reagents relative to the extensive toolboxes for small rodent biomedical model species and humans. This creates a barrier to the strategic development of disease control solutions for livestock, companion animals and wildlife that not only affects animal health but can affect human health by increasing the risk of transmission of zoonotic pathogens. There have been a number of projects aimed at reducing the capability gaps in the veterinary immunological toolbox, the majority of these focusing on livestock species. Various approaches have been taken to veterinary immunological reagent development across the globe and technological advances in molecular biology and protein biochemistry have accelerated toolbox development. While short-term funding initiatives can address specific gaps in capability, they do not account for long-term sustainability of reagents and databases that requires a different funding model. We review the past, present and future of the veterinary immunological toolbox with specific reference to recent developments discussed at the International Union of Immunological Societies (IUIS) Veterinary Immunology Committee (VIC) Immune Toolkit Workshop at the 12th International Veterinary Immunology Symposium (IVIS) in Seattle, USA, 16–19 August 2019. The future availability of these reagents is critical to research for improving animal health, responses to infectious pathogens and vaccine design as well as for important analyses of zoonotic pathogens and the animal /human interface for One Health initiatives.

Keywords: immunological toolbox, veterinary, reagents, technologies, databases, monoclonal antibodies

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INTRODUCTION

The development of novel tools and technologies has been fundamental to the advancement of basic and applied immunology across species. The rate of progress of immunological reagent development for veterinary species has been much slower than that for humans and small rodent biomedical model species, and has impacted research capability in those species (1). Historically, however, innovations in surgical procedures in veterinary species have resulted in major step-changes in our understanding of the ontogeny, compartmentalization and function of the immune system. For example, bursectomy in chickens shed new light on mechanisms of B cell development and immunoglobulin production (2), *in utero* thymectomy of lambs revealed the importance of the thymus for lymphocyte development (3) and lymphatic cannulation of sheep revealed that lymphocyte subsets differ between blood, afferent and efferent lymph (4). These ground-breaking

experiments were feasible, in part, due to the size of the species under investigation, particularly for the technique of lymphatic cannulation due to the diameter of lymphatic vessels in ruminants (5).

However, this momentum in veterinary immunological studies was not maintained; the vast majority of technological innovations and discoveries in immunology in the past 50 years have been made in mice. The development of congenic mice, differing at a single histocompatibility locus, was a fundamental technological innovation in immunology that led to mice being the primary species of choice for research. That pioneering work of George Snell and the later capability of genetically manipulating congenic mice has allowed immunologists to ascribe functions to genes, molecules and cells with high precision (6). The development of monoclonal antibody (mAb) technology using congenic mice subsequently created almost boundless opportunities for research in basic and translational immunology (7).

The availability of mAbs that could phenotype cells and detect cytokines by ELISA underpinned the discovery of two distinct $CD4^{+ve}$ T-cell subsets in congenic mice (8). The subsequent Th1/Th2 paradigm provided a fundamental framework for investigating immune activation and regulation that has expanded far beyond those original two subsets. Current capability now extends to multi-parametric analyses such as simultaneous fourteen-color flow cytometry that can identify 89 functionally-relevant $CD4^{+ve}$ T-cell subsets in human blood (9). Mass cytometry (CyTOF) methods using panels of well over 40 conjugated antibodies are now allowing for even deeper analysis of single cell expression, offering new insights into cellular subsets and their differentiation (10, 11).

Such technologies cannot usually be applied directly to different species since molecular differences in immunological orthologs result in low cross-reactivity of reagents across species (12) as affirmed by a recent comparison of reactivity of immune protein reagents for other species with swine orthologs (13). Thus, reagent development needs to be evaluated on a case-by-case basis. Gaps in capability for veterinary species are often prioritized based on the extensive mouse and human immunological toolboxes. The expansion of the toolboxes has revealed substantial differences in the ways that humans, mice and veterinary species respond to disease and highlighted to need for studying different species in their own right (14, 15). There have been coordinated efforts to evaluate species cross-reactivity of anti-human CD antigen mAbs through the animal homologs section of the human leukocyte differentiation antigen (HLDA) workshops: for horses (16), dogs (17), pigs (18), and ruminants (19).

In an effort to generate greater international co-ordination for immune reagent characterization activities, the International Union of Immunological Societies (IUIS) Veterinary Immunology Committee (VIC) supported a Toolkit Workshop at the 6th International Veterinary Immunology Symposium (IVIS) in Upsala, Sweden in 2001. This set the scene for a series of VIC Toolkit Workshops (20). It is almost 10 years since the last published review of the veterinary immunology toolbox from the IUIS VIC Toolkit Workshop at the 9th IVIS in Tokyo, Japan (1). Here, we review progress over the past decade by reporting on the IUIS VIC Toolkit Workshop at the 12th IVIS in Seattle, USA in 2019 and take a forward look to the future of the veterinary immunology toolbox.

THE PAST

The success of the HLDA workshops was based on good co-ordination, high-quality work and collective effort by the veterinary immunology community, as well as results from past species-specific CD workshops supported by IUIS VIC. Common standards were applied to the distribution and evaluation of anti-human CD reagents being assessed in different laboratories and the collective generated data being reviewed centrally. The outcome was an evidence-based assessment for the activity of species cross-reactive mAbs, with affirmation that only a limited number of mAb directed against human CD antigens actually cross-react with other animal species (21). These results instilled confidence in the performance of those reagents and promoted their uptake by the research community and industry, including companies that market and sell veterinary immunological reagents.

Although the HLDA workshops were primarily focused on evaluation of species cross-reactive antibodies, they played an important role in informing of capability gaps and therefore the prioritization of reagents for future development. A major step-change in the way veterinary immunological reagent development was supported came with the inception of a UK Immunological Toolbox funded by the Biotechnology and Biological Sciences Research Council (BBSRC) and the Scottish Executive Environment and Rural Affairs Department (SEERAD) in 2003. This was unique as it united several laboratories within a single project to take a collective multi-species approach to immunological reagent development. This was followed by the Veterinary Immune Reagent Network (VIRN) funded by United States Department of Agriculture (USDA)/National Institute of Food and Agriculture (NIFA) in the US in 2005. Both projects included the creation of databases listing available veterinary immunological reagents, which will be discussed later. They also expanded the emphasis from mAb anti-CD antigens to expression of immune proteins (cytokines and chemokines) and protein reactive mAbs. The US project included direct collaboration with commercial partners to express these immune proteins. The US and UK projects worked together under a Memorandum of Understanding (MOU) to avoid duplication of effort. This MOU was created in the absence of a mechanism for joint international funding by the respective national agencies. The structure, priorities and achievements of these projects has been published previously (1). A key output from these initiatives was an increased recognition of the importance of coordinated, complementary approaches to

Abbreviations: BMGF, Bill & Melinda Gates Foundation; BBSRC, Biotechnology and Biological Sciences Research Council; NIFA, National Institute of Food and Agriculture; REEIS, Research, Education and Economics Information System; SG, Scottish Government; USDA, United States Department of Agriculture.

reagent prioritization and development. Their success has also been reflected by continued support for reagent development initiatives by funders seeking to build on the significant benefits from their original investments, with the assertion that long-term sustainability is essential.

The funding for veterinary immunology reagent development has changed over the past 10 years, moving from the multi-species models of the UK Immunological Toolbox and US VIRN, to single-species projects. With the exception of ruminants, there is very little species cross-reactivity of veterinary reagents, highlighting that the genes involved in immune responses are amongst the most rapidly evolving in vertebrate genomes (22, 23). However, this does not diminish their potential as disease models. BBSRC and USDA/NIFA have supported reagent development projects for ruminants, swine, horses, aquaculture species and poultry in the past 10 years (**Box 1**). A barrier to formal international collaboration was lifted in 2013 when USDA/NIFA and BBSRC launched a pilot call to support animal disease research of strategic importance to both the US and UK which included the development of veterinary immunological reagents for agriculturally-relevant animal species. The swine toolkit was a landmark first transatlantic veterinary immunology reagent project funded under this initiative in 2015 (**Box 1**).

Although we have focused here on projects funded specifically to develop reagents and supporting technologies, this is not intended to ignore the veterinary immunological reagent development that is conducted within disease-driven projects, networks and within strategic programmes of government research institutes across the globe. The challenge is in capturing the outputs of these diverse activities. The websites of commercial reagent suppliers and peer-reviewed publications are sources of validated information on reagent activity. However, they do not capture everything, a particular gap being the paucity of "negative" data when reagents are found to be non-functional or where repeated attempts fail to generate specific antibodies. These are very valuable data as they can potentially prevent the duplication of wasted effort. The solution lies in community engagement for the sharing of knowledge on reagent availability and performance. Workshops such as those hosted by IUIS VIC Toolkit are a focal point for international information exchange, but they do not have the facility to capture, store and disseminate information at a detailed level. It has been recognized for many years that a major unmet need in veterinary immunology is the lack of centralized, non-commercial, searchable reagent databases (20). The original UK Immunological Toolbox (2003–2009) and the US VIRN (2005–2015) both created lists of reagents but the databases were not sustainable beyond the term of funding. This is not surprising as curation is time-consuming, requiring expert knowledge of immunology and information technology input to create web-based interfaces. This also highlighted the problem of sustainability when there is reliance on short-term funding for reagent development projects. Finding solutions to these problems has been the focus of several recent workshops as discussed below. One exception to this has been the USDA Agricultural Research Service (ARS) supported Porcine Translational Research database (PTRD, http://tinyurl.com/hxxq3ur) (15).

THE PRESENT

The current landscape of the veterinary immunology toolbox has been shaped by new funding approaches to facilitate reagent development while also addressing the complex issues of database construction, collection and validation of data, and sustainability of the database and biobanks of the reagents listed therein. This report summarizes the outcomes of several international workshops where these various elements have been considered.

Before summarizing those outcomes, it is worth reviewing the scope of the toolbox in terms of species coverage and knowledge of immunological capability within those species. In the broadest sense, the concept of a veterinary immunological toolbox encompasses a broad range of livestock, companion animal, biomedical model and wildlife species. There has been progress in reagent development across all of those species in the past 10 years which has been presented at various meetings and workshops. We have identified a number of published articles where reagent availability for different species have been reviewed. For the purposes of the toolbox, livestock species can largely be regarded as belonging to one of four major groupings, namely swine (24, 25), ruminants (22, 26), poultry (27–29), and aquaculture (30, 31). Companion animals include horses (32, 33), cats (34), and dogs (35). As previously discussed, mice are the most common small-animal biomedical model for human (12). However, rabbits (36) and ferrets (37) are also popular small-animal biomedical models for human disease. There is interest in expanding the immunological toolboxes for wildlife species, for example buffalo (38) and badgers (39) due to their potential to act as reservoirs for economically-important livestock diseases. There is also interest in developing immunological reagents for marine mammals such as dolphins (40). In addition, although camelid species are not often regarded as a major target host species for disease studies, they have come to the fore with heightened awareness of MERS-CoV and the potential to reduce zoonotic transmission by investigating vaccine-induced responses in camels (41). Importantly, camels make a unique technological contribution to the immunological toolbox via the production of nanobodies (42, 43).

To date, the concept of the veterinary immunological toolbox has largely (but not exclusively) focused on reagent development for livestock species due to their strategic relevance for funders with a stake in livestock health, food safety and global food security. In the period between the last published review of the IUIS VIC Toolkit Workshop at the 9th IVIS in Tokyo (1) and the IUIS VIC Toolkit Workshop at the 12th IVIS in Seattle, there have been several key meetings whose outcomes are directly relevant to the current status and future directions of the toolbox and merit discussion here. The first was at the 10th IVIS in Milan, Italy in 2013 when BBSRC and The Global Strategic Alliances for the Coordination of Research on the major Infectious Diseases of Animals and Zoonoses (STAR-IDAZ) supported a vaccinology

BOX 1 | Veterinary immunological reagent and technology projects first funded in the period 2010–2020.

BMGF: Livestock Antibody Hub: Cattle, swine, poultry (2019–2024):

https://www.pirbright.ac.uk/news/2019/11/bill-melinda-gates-foundation-funds-development-pirbright%E2%80%99s-livestock-antibody-hub. To study cattle, pig and poultry antibody responses at high resolution to expand the understanding of protective immunity in those species and that can also be used as models for a range of human infectious diseases.

USDA/NIFA: Cattle (2019–2022):

https://portal.nifa.usda.gov/web/crisprojectpages/1016686-immune-reagents-for-ruminants-with-primary-focus-on-bovine-specific-reagents.html. To develop, and make commercially available, mAb reagents needed to elucidate cattle immune mechanisms by focusing on CD antigens, cytokines, and chemokines and relevant assays.

USDA/NIFA: Swine (2019–2022):

https://portal.nifa.usda.gov/web/crisprojectpages/1019192-development-of-new-swine-reagents-to-broaden-our-understanding-of-immune-correlates-of-protection-and-microbial-pathogenesis.html. To generate priority reagents for swine immune proteins and pipeline them for marketing. Develop SLA class I tetramers and new assays for important swine immune markers.

USDA/NIFA/BBSRC (US-UK Collaborative): Swine (2015–2019):

https://gtr.ukri.org/projects?ref=BB%2FM028232%2F1

https://portal.nifa.usda.gov/web/crisprojectpages/1005670-us-uk-collaborative-swine-immune-toolkit-development-of-new-immune-reagents-for-swine-health-vaccine-and-disease-studies.html. To develop panels of mAb reactive with swine targets (cytokine, chemokines and their receptors) using conventional and phage-display methods. Use resultant mAbs to develop new assays for swine immunity and make the reagents commercially available.

USDA/NIFA: Horse (2015–2019):

https://portal.nifa.usda.gov/web/crisprojectpages/1005524-equine-immune-reagents-development-of-monoclonal-antibodies-to-improve-the-analysis-of-immunity-in-horses.html. To develop and characterize mAbs for the analysis of horse immunity and distribute these to the scientific community for immunological research.

USDA/NIFA: Aquaculture (2016–2020):

https://portal.nifa.usda.gov/web/crisprojectpages/1009003-collaborative-immune-reagent-network-for-aquacultured-species.html. To develop and provide immunological tools and assays to the aquaculture community to advance health for four fish species: rainbow trout, Atlantic salmon, channel catfish and Nile tilapia.

USDA/NIFA: Poultry (2017–2022):

https://reeis.usda.gov/web/crisprojectpages/1012306-development-of-poultry-immune-reagents.html. To identify chicken immune molecules, particularly cytokines, chemokines and cell surface markers, express them as recombinant proteins, and characterize their function. Develop mAbs to the target molecules and use these for multiplexed detection assays.

BBSRC/SG/BioRad: Cattle and Sheep (2012–2015):

https://bbsrc.ukri.org/research/grants-search/AwardDetails/?FundingReference=BB%2FI019863%2F1. To develop reagents and techniques to enable the investigation of the activation and regulation of the immune systems of cattle and sheep with specific reference to cell-surface molecules, intracellular transcription factors and cytokines that can define phenotypically-distinct macrophage, dendritic cell (DC) and T cell subsets.

USDA/NIFA: US Veterinary Immune Reagent Network (2010–2015):

https://portal.nifa.usda.gov/web/crisprojectpages/0221344-us-veterinary-immune-reagent-network.html. To clone, express, develop mAb reagents specific for ruminants, swine, poultry, equine and aquaculture species, sharing methods across species. Work with commercial partner to market expressed proteins for use by veterinary immunology community.

workshop. The lack of immunological tools and reagents was recognized as a major barrier to progress. This can be seen in the subsequent BBSRC Veterinary Vaccinology Strategy (https://bbsrc.ukri.org/about/reviews/scientific-areas/1506-veterinary-vaccinology-strategy/) and the creation of the BBSRC UK Veterinary Vaccinology Network (VVN).

In 2017, BBSRC VVN hosted a workshop to discuss the toolbox initiatives in the UK and US with specific relevance to the aims and objectives of the newly-formed Global Challenges Research Fund (GCRF) International Veterinary Vaccinology Network (IVVN). A full report is available on the BBSRC VVN website (http://www.vetvaccnet.ac.uk/publications/veterinary-immunology-toolbox-meeting-uk-veterinary-vaccinology-network). At this workshop, The Pirbright Institute and The Roslin Institute at the University of Edinburgh announced plans for a new UK Immunological Toolbox project. The combined project would be underpinned by core Institute funding from the BBSRC, with additional support from the BBSRC GCRF Tools and Resources (https://www.immunologicaltoolbox.co.uk/about/funders). This project is addressing major gaps in capability and sustainability. The first of these is the creation of a publicly accessible, searchable database of veterinary immunological reagents to be accessed via a dedicated website. A follow up meeting was held at the VVN Conference in Stirling in early 2018 (https://www.vetvaccnet.ac.uk/news/2018/01/uk-veterinary-vaccinology-network-conference-2018-report) to discuss in more detail the focus of the website and new reagent development. It was agreed by the community that a key driver for the website would be the facility for researchers to submit information on reagent performance and request reagent production where gaps exist. It was discussed that the primary focus of new reagent development should be around T cell and B cell subsets to help dissect in more detail pathogen and vaccine responses. As well as new reagent development the toolbox aims to exploit new technologies to translate current hybridoma stocks into gene blocks via sequencing and create a recombinant antibody pipeline, express recombinant proteins (including cytokines and chemokines), build multiplex platforms and develop high-throughput screening systems for new antibodies. These sequences act as the template from which

the constant region can be switched between different species while maintaining target specificity.

A toolkit workshop was held at the 6th European Veterinary Immunology Workshop (EVIW) conference in Utrecht, Netherlands in 2018. Although this conference was organized under the auspices of the European Veterinary Immunology Group (EVIG), as opposed to IUIS VIC, the IUIS VIC Toolkit Committee took a leading role in the organization of the toolkit workshop. Notably, the toolkit workshop was structured to reflect four newly-formed major livestock groupings (swine, ruminants, poultry, aquaculture) of IUIS VIC Toolkit which were announced for the first time at this meeting. The leaders of the species groups represented their respective areas at the workshop. They are listed on the IUIS VIC webpage and can be contacted by members of the community who are seeking information or looking to engage in reagent development for each of those areas (https://iuis.org/committees/vic/). The workshop covered the major projects in Europe and the US on reagent development, including a presentation on the plans for the new UK Immunological Toolbox. In the panel discussion, there was broad international support for the approaches being taken within the new toolbox project and recognition of the complementary work being supported by USDA/NIFA in all of the target species (**Box 1**). This meeting cemented the requirement for community engagement in the website to provide and maximize information exchange about the availability and performance of reagents and the focus on the generation of novel antibodies and methods to distinguish T and B cell subsets. This particular area will be advanced by the development of a new Livestock Antibody Hub centered at The Pirbright Institute which aims to improve both animal and human health globally by translating research outcomes in livestock diseases (**Box 1**). A core aim of this Antibody Hub is to develop tools, techniques and reagents for livestock research that bring the research capability to the same level as that for humans and mice.

The IUIS VIC Toolkit workshop at the 12th IVIS in Seattle was the forum for the international launch of the Pirbright/Roslin UK Immunological Toolbox website and the associated database (http://www.immunologicaltoolbox.co.uk). This database was built around the original information collated during the 2003–2009 BBSRC SEERAD-funded UK Immunological Toolbox and is therefore skewed toward three of the four major livestock groupings (swine, ruminants and poultry). However, aquaculture species, companion animals and now major animal pathogens are also included, and as the community engages the amount of information will increase. The main aim of the website is to collate reagent information and act as a centralized source to increase information exchange but is not the only source for any particular species. For example, the USDA Porcine Translational Research Database (http://tinyurl.com/hxxq3ur) is considered a very wide ranging and valuable community resource and cannot be duplicated but information is shared with the UK Immunological Toolbox via mutual awareness and direct communication.

The UK Immunological Toolbox database contains data on reagents that are held in research laboratories, and also those available commercially, which immediately raises questions on the quality and reproducibility of reagents from different sources. The standardized production, evaluation and storage of commercially-available reagents would be expected to reduce batch-to-batch variation, whereas the same reagent produced and stored in different research laboratories is likely to have more variability due to the different conditions. When reagents are listed on the UK Immunological Toolbox website there will be information on their specificity and performance, preferably supported by peer-review publication wherever possible.

There is also a facility for registered users to provide feedback on performance to add to the available information. Such information will be checked before posting against the user's identification. It was emphasized that such a database can be as complete and useful as the community wants it to be. The website and database will be curated centrally, but the community has to take collective ownership by submitting reagents and information on their performance. It is pleasing to see that this is already happening. The toolbox website also serves as a reference point for non-veterinary immunologists looking to expand their choice of biomedical models and facilitate comparative immunology research (44).

Finally, several new opportunities were identified during the open discussion at the IUIS VIC Toolkit Workshop in Seattle. These included the unique opportunity to salvage and store "orphan" mAbs via the sequencing technology within the UK Immunological Toolbox. The preservation of sequences does not incur the high costs associated with maintaining hybridoma cells in liquid nitrogen. In addition, the sharing of sequences circumvents many of the logistical and financial issues involved in the shipment of live cells, particularly across international borders.

THE FUTURE

As we enter the third decade of the 21st century, the "One Health" agenda has never been more important. The development of solutions for controlling infectious diseases in livestock, companion animals and wildlife not only has direct benefits for the target species but can reduce disease transmission across species, including zoonotic transmission, thereby reducing the wider global disease burden (45). Close contact between different animal species and between animals and humans is a risk for zoonotic disease, which can be difficult to manage in low and middle income countries (LMICs) (46). Given the importance of livestock to LMICs, the veterinary immunological toolbox provides economic and health benefits by underpinning animal vaccine development.

The quality of toolbox reagents and associated information in the UK Immunological Toolbox database are paramount. Evidence-based validation and standardization of new technologies is essential to generate confidence in performance and encourage uptake by the community. There remain major capability gaps in multi-analyte protein technologies for veterinary species. The development of such technologies is technically challenging, but entirely feasible with the appropriate

resources and effort. The key to success is in working together. The single-tube technology that simultaneously identifies 89 functionally-relevant $CD4^{+ve}$ T-cell subsets in human blood was developed and validated through the collective efforts of the multiple partners in the EuroFlow and PERISCOPE consortia (9). Multiparametric technologies are extremely powerful; one way of expanding the flexibility of the relatively limited range of antibodies in veterinary species is the ability to efficiently conjugate small amounts of antibodies with different labels for defining immune correlates. The identification and quantification of immunological correlates of protection are aspirational goals for the development of safe and effective vaccines (47, 48). However, with the exception of anti-virus neutralizing antibodies, immunological correlates of protection tend to be multifactorial rather than singular, particularly in the case of cell-mediated protective immunity requiring not only cell subset identification but appropriate cytokine co-expression. The solution to identifying such correlates lies in the application of a range of multi-plex technologies that all detect multiple analytes at the genetic, protein, and cellular level, so called "systems vaccinology" (49).

We are also moving into an era of high dependency on computational infrastructure as the data generated by such complex studies require specialized programmes for full analysis. Hence, collective approaches are becoming increasingly important if we are to maximize our potential to develop and adopt complex technologies in the future. The importance of genomic information and alternate expression systems such as *Pichia pastoris,* insect and mammalian cells has meant wider availability of species-specific immune proteins. The veterinary immunology community has a long history of working together for collective good, such as the HLDA workshops, international CD workshops, toolkit committees, collaborative funding initiatives and the immunological toolbox. In doing so we need to maintain a global perspective and consider technologies that create solutions for animal diseases across borders. One example is the antibody sequencing technologies of the new UK Immunological Toolbox. In addition to the advantages described earlier, this technology offers particular cost-effective and sustainability benefits for the transfer and storage of reagents to LMICs where veterinary immunology research is being conducted.

In parallel to sequencing, expressing and engineering mAbs, companies and research groups all over the world are adapting single B cell sequencing technologies to a range of host species. These technologies often rely to some extent on existing reagents to identify B cells (including antigen specific B cells) but are generally very adaptable to any given species and synergise well with existing mouse recombinant antibody expression methods. These methods are providing a completely new route to identifying antibodies against specific epitopes on pathogens as well as other foreign immunizing antigens. These antibodies can be used as reagents, including mapping complex epitope landscapes to inform structural vaccinology approaches to increase efficacy, and may also be used as therapeutics. Antibodies are now a primary therapeutic goal of many companies for a range of human diseases. Cats and dogs are not only a profitable target market for immunotherapeutics, they provide value data on *in vivo* mAb function (50). Although the cost of such treatments is currently prohibitive for food producing species, large animal models and species-specific reagents can have a very important role in testing manufacture, delivery and efficacy of mAbs as part of the One Health approach.

The impact of veterinary immunology research will ultimately be measured by the development, or contribution to the development, of disease-control solutions including diagnostic tests, vaccines and genetic-based strategies. The range of vaccine-delivery platforms is rapidly expanding, including improved adjuvants, vector-based delivery systems and genetic vaccination with DNA and RNA. Although viral-vectored vaccines are successfully deployed in humans and companion animals (51), public safety concerns remain regarding their use food animals (52). The immunological toolbox can be applied to safety and efficacy studies in livestock, thereby informing on the benefit-risk ratio that would be impossible to do at the same scale in humans or primates.

Animal genetics can provide insights into responses to infection and vaccination which can be translated into livestock breeding programmes (53, 54). Breeding programmes require several generations to observe population effects and conclusive proof for the effect of a specific genotype on immune status requires functional evidence, hence reliance on the toolbox. New gene-editing technologies such as CRISPR now allow very targeted approaches to livestock production (55). This is the future of livestock farming and the immunological toolbox not only has a role to play in the identification of genes to be targeted, but it will also be important for defining subsequent immune function, including potential off-target effects. Genome editing is also creating the opportunities to engineer species to act as better models for human diseases alongside or in addition to genetically defined and tailored breeds, such as SCID pigs and MHC homozygous pigs (56, 57). For example, pigs are emerging as a very powerful model to predict human influenza vaccine responses but to achieve the maximum benefit of such models a complete toolkit is required (58). Gene editing is already providing pig organs for future human xenotransplantation, a biomedical application that has helped drive reagent development in pigs (59, 60).

CONCLUSION

The veterinary immunological toolbox is very broad in its scope and has evolved from multiple efforts across the globe. In the broadest sense, the toolbox incorporates livestock, companion animals, wildlife and biomedical animal species. Each is important in its own right, but all are collectively important for the One Health agenda and for controlling existing and emerging diseases that infect different animal populations and have zoonotic potential. As human populations expand, there is a need to protect food security

without compromising food safety. Disease prevention and control results in improvements in animal health and welfare, which not only has economic and ethical benefits but can also address concerns for climate change by making food production more efficient. Basic immunology underpins these approaches, from vaccine design to understanding the effects of gene editing. The immunological toolbox website and associated searchable database provides a new focal point for information and knowledge exchange for the veterinary immunology community. The key to future success is global collective working facilitated by networks such as national immunological societies, EVIW, IVVN, American Association of Veterinary Immunologists (AAVI), and IUIS VIC Toolkit Committee.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REVIEW
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Current Understanding of Natural Antibodies and Exploring the Possibilities of Modulation Using Veterinary Models. A Review

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Natural antibodies (NAb) are defined as germline encoded immunoglobulins found in individuals without (known) prior antigenic experience. NAb bind exogenous (e.g., bacterial) and self-components and have been found in every vertebrate species tested. NAb likely act as a first-line immune defense against infections. A large part of NAb, so called natural autoantibodies (NAAb) bind to and clear (self) neo-epitopes, apoptotic, and necrotic cells. Such self-binding antibodies cannot, however, be considered as pathogenic autoantibodies in the classical sense. IgM and IgG NAb and NAAb and their implications in health and disease are relatively well-described in humans and mice. NAb are present in veterinary (and wildlife) species, but their relation with diseases and disorders in veterinary species are much less known. Also, there is little known of IgA NAb. IgA is the most abundant immunoglobulin with essential pro-inflammatory and homeostatic properties urging for more research on the importance of IgA NAb. Since NAb in humans were indicated to fulfill important functions in health and disease, their role in health of veterinary species should be investigated more often. Furthermore, it is unknown whether levels of NAb-isotypes and/or idiotypes can and should be modulated. Veterinary species as models of choice fill in a niche between mice and (non-human) primates, and the study of NAb in veterinary species may provide valuable new insights that will likely improve health management. Below, examples of the involvement of NAb in several diseases in mostly humans are shown. Possibilities of intravenous immunoglobulin administration, targeted immunotherapy, immunization, diet, and genetic modulation are discussed, all of which could be well-studied using animal models. Arguments are given why veterinary immunology should obtain inspiration from human studies and why human immunology would benefit from veterinary models. Within the One *Health* concept, findings from veterinary (and wildlife) studies can be related to human studies and *vice versa* so that both fields will mutually benefit. This will lead to a better understanding of NAb: their origin, activation mechanisms, and their implications in health and disease, and will lead to novel health management strategies for both human and veterinary species.

Keywords: natural (auto-)antibodies, disorders, food animals, modulation, homeostasis

INTRODUCTION

Natural antibodies (NAb) are defined as immunoglobulins found in individuals without (known) prior antigenic experience (1). Albeit a heterogeneous group, NAb are generally characterized as oligo-specific low affinity binding immunoglobulins which recognize exogenous and self-antigens (2). The majority of reported NAb are IgM and IgG, whereas IgA is much less studied and described. NAb have germline encoded VH and VL regions that restrict their binding capacity to phylogenetically conserved epitopes (3), in contrast to adaptive immunoglobulins that could theoretically recognize any epitope of an antigen. NAb have minimal N-nucleotide insertions and few or no somatic hypermutations and therefore are of low affinity (4). In comparison, low affinity NAb have a dissociation constant (K_d) ranging between 10^{-4} and 10^{-6} M, whereas high affinity conventional antibodies range between 10^{-6} and 10^{-10} M (5). With respect to their functions, NAb were reported to initiate apoptosis (6), enhance T cell proliferation (7), activate complement (8–10), opsonize antigen (11), enhance antigenicity (12), target antigen to lymph nodes (13), and are involved in FcR-mediated phagocytosis (10). They also act as broad neutralizing agents (6) and endogenous adjuvants for CD8+ T-cell responses (14), and they sustain differentiation and maturation of dendritic cells (15, 16) (**Table 1**). For extensive reviews of NAb functions see also references 4 and 17.

A substantial part of NAb can react with intracellular and membrane expressed autoantigens and circulating macromolecules and haptens that are conserved during evolution. Such antibodies are called natural autoantibodies (NAAb) (23, 24). NAAb can react with many autoantigens, and damaged and senescent cells. Damage or senescence of cells might be due to oxidative mechanisms resulting in the generation of neo-epitopes on or within the cell. Thereby, NAAb facilitate antigen-mediated removal of apoptotic cells by phagocytosis and display anti-inflammatory activity. This decreased exposure to intracellular autoantigens from apoptotic cells might also mitigate the development of autoimmune diseases (17, 25). On the other hand, NAb are indicated in the pathogenesis of autoimmunity, inflammatory bowel diseases, contact hypersensitivity, and sepsis (31), but only a minority of NAb and NAAb have pathogenic features (29). Moreover, many individuals possess antibodies directed against common epitopes in highly mutating viral infections, like influenza and HIV. These, so-called "broadly neutralizing antibodies" share some characteristics with NAb (20, 21). Antibodies binding previous versions of the viral strain consist of about 0.01% of the antibodies raised after infection or vaccination and react with all variants of the virus and thus appear to be multi-specific. Such antibodies might constitute passive vaccines against non-mutable common structures in otherwise highly mutating viruses.

Since their initial discovery early 1960s, NAb were found in every vertebrate species investigated: mammals (2), birds (32, 33), fish (34, 35), and reptiles (36). Nevertheless, NAb have been regarded as contradictive with established immunological dogmas, but gradually receive more attention in main stream immunology.

TABLE 1 | Involvement of natural antibodies in immune responses and immune status.

	References
Initiation of apoptosis	(6, 17)
Complement activation	(8–10, 18)
FcR-mediated phagocytosis	(10, 19)
Neutralization of infective agents	(6, 18, 20, 21)
Adjuvanting properties	(14, 22)
Maturation of dendritic cells	(15, 16)
Clearance of senescent/necrotic cells	(23, 24)
Prevention of autoimmunity	(17, 25–28)
Opsonization of antigens	(11)
Enhancement of antigenicity	(12)
Antigen targeting to lymph nodes	(13, 18)
T cell proliferation	(29)
Allograft rejection	(30)

B1-CELLS ARE THE PREDOMINANT SOURCE OF NATURAL ANTIBODIES

The origin of NAb has mostly been studied in mice, where they predominantly originate from B1-cells (B220low, CD19high, IgMhigh, CD23–, CD43+), which are further delineated in B1a-cells (CD5+) and B1b-cells (CD5–). B1-cells are present within peritoneal and pleural cavities and lymphoid tissues like spleen and lymph nodes (37). Such B1-cells were found to be long-lived and retain their self-renewing capacity and hence their suggested innate-like properties. Besides their reduced junctional diversity and their low somatic hypermutation, their IgH VH gene rearrangements favor usage of the V_H12 segment generating antibodies able to react with phosphatidylcholine. Phosphatidylcholine is a major lipid in general the protective mucus layer of the gastrointestinal tract and membranes of various bacterial species. These B1-cells maintain an active first line of defense against bacteria (37). The typical V_H12 containing B1 receptor is able to reprogram B2-cells into becoming B1-cells and thereby adopting the B1 receptor and other B1-cell surface markers and start to spontaneously produce antibodies. Therefore, apparently no distinct progenitor cells for B1-cells required. This shows that driving the generation of B1-cells is because of their special B-cell receptors (38).

Approximately 90% of NAb in mice are secreted by B1a-cells whereas B1b-cells and marginal zone (MZ) B-cells do so to a lesser extent (2). Approximately 80% of total murine serum IgM is derived from B1-cells under steady state conditions (17). Therefore, B1-cells were regarded as the main source of NAb whereas B2-cells (B220+, CD19+, IgMlow, CD23+, CD43–) are considered as the main source of conventional antibodies. In humans B1-cells were defined as CD20+CD27+CD43+CD70– (39), and CD19+CD20+CD27+CD38low/intCD43+. The latter cells were found to decrease with aging, probably because of

poor bone marrow production which might have an impact on the ability to fight infections and the development of age-related diseases (40). In all other species, B1-cells require identification and characterization, and their role in the release of NAb is unknown. For instance, B1-cells in cattle were defined by the originally used murine markers CD5 and CD11b and subdivided in CD5+CD11b- B1a-cells, CD5-CD11b+ B1b-cells and CD5-CD11b- conventional B2-cells (41). Flow cytometry analysis showed a distinct cell population of IgM+, pSYK+ cells, indicating B1-cells in dairy cattle (42). Phenotypical properties of NAb-secreting B-cells in other species remain enigmatic.

Although NAb B-cells are regarded as pre-defined, it is suggested that a NAb B-cell still requires antigenic selection and even T-cell help, remarkably by γδ T-cells (29), but the exact mechanisms are not known (2). One theory suggests that B1-cells are educated at mucosal (intestinal) sites under the influence of the microbiome. This is supported by the finding that NAb binding the carbohydrate Galα1-3Galβ1-4GlcNAc (α-Gal) in GALT$^{-/-}$ mice were influenced by the *Clostridiales*, *Bacteriodales*, *Lactobacillales,* and *Deferribacterales* orders (43). Anti-Gal NAb can block the entry and transmission of membrane-binding viruses as these cannot produce glycosylated proteins themselves (44).

Fetal and neonatal self-reactive B1-cells do not show clonal expansion upon B-cell receptor (BCR)-signaling because of the expression of the inhibitor CD5 and a lack of fully functional CD19. Consequently, these B1-cells are silenced and thereby prevented to induce autoimmunity. Nevertheless, B1-cells can respond rapidly to different infections by firstly migrate to secondary lymphoid tissues and subsequently differentiate into IgM-secreting cells (45). Thus, stimulation of murine B1-cells in peritoneal cavities does not directly lead to the secretion of NAb as these activated B1-cells migrate toward the spleen and lymph nodes before the secretion of natural IgM takes place (46, 47). However, by Toll like receptor (TLR)-mediated activation these B1-cells can respond and circumvent the BCR-induced signaling block (45). The restricted fetal preimmune repertoire in humans may contain potentially beneficial self-reactive innate-like B cell specificities that are involved in the removal of apoptotic cells and shaping of the gut microbiota after birth (48). Another hypothesis is that IgM NAb B-cells are educated by maternal IgG, which in humans is the only antibody isotype that passes the placental wall. This IgG pool represents the unique environment experienced by the mother and is passed into the neonate as a single passive immunization. This idea is supported by observations that human neonates share a similar IgM profile with each other, whereas the IgG profiles of neonates are similar with their respective mothers (49). During aging, the IgM and IgG profiles merge suggesting that the IgM repertoire is shaped by maternal IgG. Therefore, maternal IgG may act as the immunological homunculus (50) shaping or educating the neonatal immune system. Whether this is true for all species is currently unknown. Bovine calves that do not receive maternal antibodies prior to intake of colostrum showed both IgM and IgG self-binding antibodies (51), which are, however, dramatically increased after colostrum intake. Nevertheless, the exact origin of germline encoded NAb remains unknown and requires further investigation.

THE MECHANISMS LEADING TO NATURAL ANTIBODY SECRETION ARE NOT FULLY UNDERSTOOD

Little is known about the mechanisms that underlie the secretion of NAb, but Holodick et al. (2) propose some interesting models that may explain the activation routes of NAb B-cells. The first model states that a NAb B-cell is pre-existing, but in order to secrete NAb it must undergo classical maturation, activation and differentiation into plasma cells and memory B-cells. The existence of homeostatic self-binding NAb B-cells in this model could then be explained by the fact that IgM-BCRs have similar low affinity binding like IgM NAb and would therefore be able to escape negative selection. However, the model does not explain the necessity of structurally and functionally unique pre-existing immunoglobulins if editing and selection procedures will take place eventually.

The second model embraces the idea that a NAb B-cell is pre-existing and generates NAb at a constant rate without the need for antigenic activation. This is supported by the observation that NAb are universally present in many species without (known) antigenic stimulation and that IgM levels seem constant throughout life (52), suggesting that NAb are a tightly regulated pool of immunoglobulins. However, the model fails to explain the presence of IgG and IgA NAb (53, 54) as it does not allow hypermutation and class switching to occur. Instead, an antigenic overload would require compensation by adaptive IgG's that could lead to an excessive or irrelevant immune response.

The third model suggests that a NAb B-cell is pre-existing but that a slight antigenic push is required in order to secrete NAb. While the secretion of NAb has been implicated to be T-cell and antigen independent, there is a possibility that exogenous antigens are indeed involved in B-cell activation, but in a B-cell Receptor (BCR) independent manner instead of an antigen independent manner. Besides BCR, B-cells also express innate receptors (e.g., TLRs), and it was demonstrated that they are important mediators of B-cell activation, proliferation, and class-switching (45, 55). One example is the BCR independent secretion of natural and self-reactive immunoglobulins binding LPS (55), suggesting the involvement of the LPS recognizing TLR4. Moreover, B1-cells from naïve mice stimulated with IL-5 and TLR-agonists secreted IgM against oxidized lipids *ex vivo* (3, 56), further suggesting that the secretion of NAb is BCR independent and rather regulated by innate pathways. Recently, it was also demonstrated that TLRs are critical for regulating antibody production by B1a-cells (45, 57). Microbial-sensing TLR (e.g., TLR2 and TLR4) are required for anti-microbiota B1a-cell responses, whereas nucleic-acid binding TLR7 and TLR9 control B1a-cell responses to self-antigens like phosphorylcholine (the headgroup of oxidated phosphatedylcholine) and microbiota-derived antigens (57). Unfortunately, this model is not able to explain the constant

secretion of IgM as it will only provide IgM when there is an antigenic demand.

The fourth model tries to create a middle ground by stating that a NAb B-cell is pre-existing and secretes IgM NAb in steady state conditions. However, it is able to differentiate into IgG or IgA secreting plasma cells after antigenic stimulation that allows somatic hypermutation and class switching. This view is supported by the finding that IgG NAb against citrate synthase (CS) in the pericardial fluid (PF) correlated with antibody titers against pathogens associated with cardiovascular diseases, whereas anti-CS IgM NAb were not (58, 59). This also implies that only IgM antibodies could be defined as NAb according to the classical definition. As opposite to classical antigen-induced B-cell responses which are helped by αβ T-cells, NAb producing B cells were indicated not to require cognate T-cell help but depend on soluble mediators produced by γδ T-cells which should play a prominent role in their regulation through the fine-tuning of IL-4-levels (29). The earlier arising γδ T-cells during ontogeny should be better positioned than αβ T-cells to shape the developing repertoire of NAb. Since ligand specificities of NAb and γδ T-cell receptors appear to overlap, this may allow γδ T-cell help for certain NAb specificities (29). Lastly, since vertebrates share many macromolecules with the microbiome, "cross reactivity," and the role of the microbiome in shaping and maintaining the NAb-repertoires cannot be excluded. Many "classical" NAb may be initiated by the intestinal and oral cavity microflora (44, 60). In conclusion, further research regardless of species is required to fully understand the origin, induction and activation pathways of NAb B-cells and NAb.

NATURAL ANTIBODY REACTIVITY

Since their discovery in the early 1960s, NAb were neglected or denied within the immunological society because of their apparent contradiction with established immunological dogma's. Germline encoded immunoglobulins do not fit in the fundamentals of random VDJ-rearrangement, and the existence of self-binding NAb is incompatible with Burnet's clonal selection theory (61), stating that self-binding B-cells are selectively removed from the circulation. Furthermore, the properties of NAb could also be perceived as redundant because high-affinity binding and mono-specificity are regarded as key characteristics of relevant and effective immunoglobulins.

NATURAL ANTIBODIES BINDING TO SELF-ANTIGENS ACT AS HOMEOSTATIC AGENTS

On average, humans possess around 5 l of blood containing 4 x 10^9 white blood cells per liter of blood of which 5% is comprised of B-cells. In turn, ∼5% of the B-cell population are considered to be B1-cells, amounting to 5 x 10^7 B1-cells in an average human which suggests that NAb are a major part of the systemic antibody pool (5, 62).

Autoantibodies have a bad reputation in immunology as they are the primary mediators in many autoimmune diseases. The majority of these disorders are hallmarked by the presence of autoantibodies against specific target antigens (63). For example, Graves' disease is characterized by antibodies targeting the Thyroid Stimulating Hormone (TSH) receptor, which results in an unregulated secretion of thyroid hormones (63). Autoantibodies against Ro/SSa and La/SSb are hallmarks of Sjögrens Syndrome, which is an autoimmune disorder that mainly affects mucous membranes and moisture-secreting glands in the eyes and mouth (64). More than 180 unique autoantibodies were identified in Systemic Lupus Erythematosus (SLE), a systemic autoimmune disease that affects multiple organs (65). Despite these negative associations, self-binding immunoglobulins can already be detected in future patients with autoimmune diseases years before the onset of autoimmunity without showing any signs of pathology (66, 67). A large portion of B-cells are self-binding under steady state conditions and murine B1a-cells are positively selected for self-reactivity (37). Moreover, 75% of early immature naïve murine B-cells and 20% of mature naïve B-cells are self-binding and somatic hypermutation even restores self-reactivity back to approximately 45% (68, 69). Despite the immense pool of diverse antigens available, "only" 100 immune diseases are known, of which half of them have signature antigens for autoantibodies, which is a very small part of the total proteome (63) as already indicated above (29). This raises questions about the nature of these hallmark antigens and why the rest of the proteome is not a trigger for autoimmunity.

In all "normal" healthy individuals, in human cord blood and in "antigen-free" mice (1), self-binding antibodies are found of the IgM, IgG, and IgA classes, binding a variety of structurally different serum proteins, surface molecules, and intracellular structures like ubiquitin, collagen, hemoglobin-α, ss- and dsDNA, fibrin, the carbohydrate α-Gal, extracellular cytokines (54), nuclear membrane antigens (70) and cell membrane components such as oxidized lipoproteins (24, 71). Exposure of these kind of self-antigens in the wrong context, for example due to necrosis or aberrant apoptosis could lead to unwanted presentation to adaptive immunity and subsequent autoimmunity with severe consequences for the affected individual. Therefore, it can be hypothesized that NAb neutralize these antigens before an adaptive immune response or inflammation is initiated against them. For instance, protective natural IgM's binding phosphorylcholine were negatively correlated with IL-6 and TH17 responses in SLE patients and could be related to the intestinal microbiota (72).

The antigens that are targeted by self-binding NAb may in fact function as Damage/Danger Associated Molecular Patterns (DAMPs), which are endogenous compounds that are constitutively expressed in all tissues. When released into the periphery during degranulation, cell injury or necrosis, they induce chemotaxis and various forms of immune activation (73). Heat shock proteins (HSP), annexin, S100 proteins and galectins are considered as signature DAMPS (74), but were also found to be targets for NAb (54). It was demonstrated that a pool of IgM's inhibited TLR mediated cytokine expression and mitogen activated protein (MAP) kinase activation *in vitro* and specifically induced inhibitory signaling pathways in innate immune cells

(17). While this shows that NAb can have a direct inhibitory function on immune cells, it can be hypothesized that this is mediated by the formation of immune complexes, presumably with DAMPs. This would be an essential mechanism as decreased NAb-levels would leave DAMPs in the circulation and susceptible to be intercepted by adaptive immune cells, leading to a pro-inflammatory immune response. While this is beneficial in some cases, it could have severe consequences if inflammation occurred in the wrong context. Natural antibodies that are part of immune complexes can essentially be eliminated without the induction of inflammation, tissue repair and controlled catabolism.

Another mechanism that is suggested to be used by NAb against self-antigens is the regulation of B-cell development and selection by IgM's, as it was found that selective IgM deficient mice developed pathological autoimmunity (26). B cells, which express BCRs specific to hen egg lysozyme (HEL) were found to display diminished responsiveness to HEL stimulation in presence of soluble anti-HEL IgM antibodies suggesting IgM as negative regulator of BCR signaling. Soluble IgM antibodies may than act as decoy receptors for self-antigens that are recognized by membrane bound BCRs (75). Together with other data from FcμR$^{-/-}$ mice, it was demonstrated that IgM NAb most likely facilitate the healthy development of B-cells in an FcμR-dependent manner (76, 77). As IgM is not able to pass the placental wall, an IgM-dependent IgM secreting B1-cell subset must pre-exist to facilitate this process (26). Natural IgM deficiency does affect B-cell development and selection and induces tolerance that prevents development of primary auto-immune diseases (26).

It is most likely that NAb also bind self-antigens that are not considered as typical DAMPs. For instance, antibodies binding many self-antigen fragments were found in liver from mice (78), liver, brain, kidney, and muscle from humans (79–83), and liver from cows (84) and poultry (85), but the functions of NAb binding such to be defined self-tissue antigens is still unknown. Hartman et al. (86) found that hybridomas from unmanipulated adult murine spleen cells revealed a pattern of a diverse VH usage reflecting the germline repertoire. The majority of murine organ reactive IgM NAb were polyreactive, expressing a broad range of unique and not indiscriminate reactivity patterns for both self and foreign antigens, suggesting that many naturally activated adult B-cells are highly polyreactive and that autoreactivity is a consequence of polyreactivity. The population of NAb exhibiting organ reactivity overlaps the populations of other IgM autoantibodies, and all these derive from a pool of polyreactive IgM antibodies which are polyclonally activated in the early immune response. These polyreactive natural antibodies may then represent a first line of defense and offer protection for the host against a variety of foreign agents (86).

In summary, it is very likely that self-binding NAb are systemic surveillance molecules that maintain immune homeostasis by aiding in the clearance of dying cells and apoptotic debris, thereby preventing activation of the immune system against the self and the subsequent development of self-immunity (3, 27, 28). In this light, it is fitting to regard pathological autoimmunity as a dysregulated state of initial homeostatic autoimmunity, rather than onset of previously absent self-recognition (87).

NATURAL ANTIBODIES BINDING TO FOREIGN ANTIGENS ACT AS A FIRST LINE OF DEFENSE

Immunoglobulins in the absence of known immunization or vaccination against foreign antigens are persistently found in many species and have been isolated from various sources, including serum, milk, saliva, mucus, eggs, and feces. For an extensive review on NAb binding fungi, viruses and bacteria see also reference 17. NAb bind to foreign (microbial) antigens like lipopolysaccharide (LPS), lipoteichoic acid and peptidoglycans (88), which are present on many different types of bacteria. NAb were found to react with phosphorylcholine, which is present in the cell wall of *Streptococcus pneumoniae* (89), but also occurs on mammalian cell-membranes when phosphatidylcholine is oxidized. NAb are reactive with viruses and showed to bind to lymphocytic choriomeningitis virus (LCMV), vesicular stomatitis virus (VMV) (90), and various strains of Influenza (91). In addition, NAb also bind foreign (non-self) antigens that are not considered as pathological. Humans, rats, mice and alligators without previous immunization showed antibodies binding chicken red blood cells (92), whereas poultry (32), pigs (93), and cattle (94) all demonstrated to have NAb against Keyhole Limpet Hemocyanin (KLH). KLH is a large 390 kDa glycosylated protein from the gastropod *Megathura crenulata* which is found within the waters near California (95) and therefore an antigen that is highly unlikely to be experienced by non-marine individuals. To our knowledge, there is little evidence of cross reactivity with known infectious agents albeit the largeness of KLH does not completely exclude cross reactive antibodies. KLH is a potent immunogenic protein, but it does not cause adverse immune effects in humans and it is therefore a widely used vaccine carrier protein. Thus, KLH-binding NAb and likely NAb to other non-self-antigens appear to act as a vanguard of the immune system by protecting the host in an innate fashion during the relatively slow development of a specific antibody response.

IgM AND IgG NATURAL ANTIBODIES IN HEALTH AND DISEASE

IgM and IgG are the most extensively described classes of NAb in literature and were found to be implicated in many human infectious diseases and disorders, including neurological disorders, cancer, diabetes, and cardiovascular diseases. For an extensive review of the involvement of NAbs in health and various infectious-, tumor-, neurological-, and metabolic diseases see reference 94. Interestingly, lower levels of self-binding NAb are usually negatively correlated with disease onset and progress whereas high levels often correlate with protection or the absence of disease. In humans, profiles of NAb binding self-antigens were proposed as biomarkers or fingerprints for the physiological and health status of individuals (78, 96), including parasite infections such as malaria and schistosomiasis (97). The observed decline in the amount, or efficacy of homeostatic natural antibody levels were associated with a relative loss of protection against

molecules involved in diseases whose incidence rises in the older age population, and that those individuals experiencing the greatest loss are at the highest risk. Natural antibodies were thus proposed as rheostats for susceptibility to several age-related diseases (98).

In veterinary species, clear relations between NAb and diseases were hardly expected, and thus much less studied, and are therefore much less known. In pigs, significant associations with osteochondrosis (OC) were found for IgM levels binding chondroitin sulfate A at 6 weeks of age (odds ratio (OR) 1.4 and 1.5), actin at 6 weeks of age (OR 1.4 and 1.3), thyroglobulin at 24 weeks of age (OR 1.5 and 1.3), and levels of IgG binding at 6 weeks of age (OR 1.7 and 1.4). Additionally, significant associations with OC were also found with IgM levels binding albumin or KLH at 6 weeks of age (OR 2.3 and 1.4), and with IgM levels binding actin at 24 weeks of age (OR 1.3) suggesting associations between the presence and levels of NAb-idiotypes at a young age and development of OC at later age (99).

NATURAL ANTIBODIES AGAINST BRAIN EPITOPES AND THEIR RELATIONSHIP WITH NEURODEGENERATION

For decades, the central nervous system (CNS) has been considered as an immune privileged site with relatively low to no detectable immune activity. Microglia and astrocytes can present antigen, but Major Histocompatibility Complex (MHC) -I and MHC-II expression is low and classical lymphatic drainage is apparently absent (100). However, the concept of an immune privileged brain has been moved aside in favor of "*an immunologically unique environment*" as it becomes more apparent that the CNS is more accessible to the immune system than has previously been thought. This access probably also applies for NAb as self-binding NAb targeting brain epitopes were found in healthy subjects or in the context of neurodegenerative disorders as exemplified below.

Multiple sclerosis (MS) is a neurodegenerative disorder in which an immune-mediated degeneration of myelin and subsequent loss of cognition is observed in about 50% of the patients. A human natural IgM (hIgM22) bound to oligodendrocytes in fresh brain slices (101). hIgM22 is thought to bind sulphated molecules, especially the myelin essential component sulfatide (102). Sulfatide acts as a negative feedback regulator for oligodendrocyte survival (103–105), which is the major cell type to produce myelin. A decrease in hIgM22 could lead toward neurodegeneration due to an increased availability of inhibitory sulfatide whereas binding of hIgM22 to sulfatide retains oligodendrocyte survival and subsequent myelin synthesis (102, 104). Indeed, hIgM22 induced remyelination in Theiler's Murine Encephalomyelitis Virus (TMEV), which is commonly used as a murine model of human MS. Lastly, hIgM22 was also able to bind to gangliosides and was therapeutic in a murine model of human Amyotrophic Lateral Sclerosis (ALS) (106).

Alzheimer's Disease (AD) is a neurodegenerative disorder characterized by deposition of Amyloid βeta (Aβ) plaques and Tau rich neurofibrillary tangles (NFT) (107). Aβ originates from the cleavage of the Amyloid Precursor Protein (APP), which is thought to play a role in synapse formation although its function is not fully understood (108). Neuroprotective NAb binding to assemblies of amyloidogenic peptides were reported to decrease with normal aging and advancing AD (109), and AD patients had decreased amounts of natural IgM and IgG against Aβ compared to age matched healthy individuals (110). This proved to be a therapeutic target of interest as APP-transgenic mice maintained their initial cognition level while having decreased cerebral Aβ depositions after intravenous administration of anti-Aβ antibodies (19). An observed side effect in mice, however, was the occurrence of cerebral microhaemorrhages. This was explained by a lower specificity of IgG for Aβ compared to IgM and the ability of IgG to pass the blood-brain barrier (111), demonstrating a more protective role of natural IgM in contrast to natural IgG. The second major AD associated protein is tau which, in its native form acts as a mediator in the generation and stabilization of microtubules. In NFT however, it is present in a hyper phosphorylated form (112) making it an excellent target for homeostatic immunity due to its modifications. Indeed, anti-tau IgG was found in healthy controls and pooled commercial IgG, although no clear differences in concentrations were found between these groups and AD patients (113). Of note, the therapeutic efficacy of anti-Tau antibodies was epitope dependent (114).

Many mouse strains suffer from an age-related progressive clustering of Periodic acid-Schiff granules within the hippocampus, which are characterized by the expression of a not fully defined carbohydrate neo-epitope. It was found that ICR-CD1, BALB/c and SAMP8 mouse strains have natural IgM's against these carbohydrate structures at all ages and even under germfree conditions (115). Strikingly, the same study also found that serum of rats, rabbits, goats and even commercially available antibodies also reacted with pathological granules in hippocampal tissue of ICR-CD1 mice, suggesting that they are conserved and widespread across species. In summary, literature (**Table 2**) demonstrates that brain epitopes are targets for NAb and that decreased levels can be negatively associated (or correlated) with neurodegeneration, whereas protection to behavioral disorders such as schizophrenia were correlated with for instance protective platelet associated autoantibodies (117).

NAb could also influence behavior, depression or anxiety, as these mental states were demonstrated to show immune alterations in general (134). Decreased levels of IgM NAb against oxidative stress epitopes like malondialdehyde and azelaic acid were found in deficit schizophrenia (118).

Veterinary models on the relations between NAb and neurological disorders are scarce. Pigs that were housed for 9 weeks in a straw embedded environment showed higher levels of IgM NAb binding myelin basic protein (MBP) compared to pigs kept in a barren environment (119), suggesting that the straw embedded environment has an enriching effect on the brain and either results in higher NAb-levels or prevents a decrease of these antibodies in barren environment kept pigs. Interestingly, in the barren kept pigs increasing levels of IgM binding MBP positively correlated with a decrease in viral PPRSV RNA levels (135) suggesting that high NAb-levels to

TABLE 2 | Involvement of natural antibodies in disorders.

Species	Antigen(s)	Isotype	Disorder	Protection	References
Human, mice	Various self-antigens and neo-epitopes	IgM and IgG	Various: anti-inflammation, OxLDL, tumors, neurologic diseases, infections, clearance senescent cells and cytokines, passive protection to autoimmunity and tissue injury	Yes	(116)
Pig	Chondroitin sulfate, Actin, KLH Thyroglobulin	IgM, IgG	Osteochondrosis	Yes?	(99)
Mice	Myelin sulfatide	IgM	Multiple sclerosis	Yes	(101)
Human	Amyloid	IgM	Alzheimer	Yes	(109–111)
mice	Amyloid beta-peptide	Polyclonal	Alzheimer	Yes	(19)
Human	Platelets	IgG	Schizophrenia	Yes	(117)
Human	Malondialdehyde	IgM	Schizophrenia	Yes	(118)
Pig	Myelin basic protein	IgM	unknown		(119)
Chicken	PC-BSA	IgM	Non-aggressive behavior		(120)
Chicken	PC-BSA	IgG	Aggressive behavior		(120)
Human	oxLDL	IgM	Carotid atherosclerosis	Yes	(121)
Human	PC-KLH, PC-BSA	IgG	Cardiovascular diseases	Yes	(122, 123)
Human	Phosphorylcholine, Cardiolipin	IgM	Atherosclerosis, Stroke, Myocardial infections	Yes	(27, 124)
Mice	Malondialdehyde	IgM	Hepatic inflammation	Yes	(125)
Mice	Phosphoryl-enriched-Pneumococci	Not specified	Non-alcoholic Steatohepatitis	Yes	(126)
Mice	Oxidized phospholipids	IgM	Atherosclerosis	Yes	(127)
Mice	Phosphorylcholine, T15-idiotype	IgM	Vein graft atherosclerosis	Yes	(128)
Chicken	KLH	IgM	longevity		(129, 130)
Cow	KLH	IgM, IgG	Mastitis	Unknown	(131)
Human	Low density lipoprotein	IgM IgG	Atherosclerosis Pro-atherosclerosis	Yes	(132)
Mice	CNS-cells	IgM	Remyelination		(101)
Mice	Gangliosides	IgM	Amyotrophic Lateral sclerosis		(108)
Human	Galα1-3Galβ1-GlcNAc	IgM, IgG, IgA	Henoch-Schönlein purpura IgA nephropathy Crohn's disease		(43, 44, 54, 133)

a self-antigen enhanced resistance to PRRSV. The underlying mechanism remained unknown. Also recently, higher levels of IgG NAb were found in poultry strains bred for aggressive behavior, whereas the non-aggressive strain showed higher levels of IgM NAb (120). Further research is required to understand the relationship between NAb (isotypes) and behavior, but the current data suggest that self-binding antibodies protect against autoimmunity, chronic inflammation and necrosis which may underlie neurological disorders and misbehavior.

NATURAL ANTIBODIES AGAINST TUMOR-ASSOCIATED EPITOPES AND THEIR ANTI-TUMOR EFFECTS

Cellular transformation occurs in all types of cells and may lead to the development of tumors, albeit this is a relatively rare phenomenon compared to the high frequency of spontaneous mutations that occur in an individual (136). Immune processes are likely involved in clearing corrupted cells or components out of circulation. NAb may play an important part in this as nearly all monoclonal tumor targeting antibodies isolated from cancer patients so far were oligo-specific low affinity binding pentameric IgM's (137). Furthermore, natural IgM's to cancer associated autoantigens were detected up to 5 years before onset of breast cancer (138), suggesting their pre-existence but also providing diagnostic value as early biomarkers.

Carbohydrate structures are highly expressed on tumor cells and can be recognized by NAb (139). SC-1 is an isolated monoclonal IgM from a signet-ring cell carcinoma patient (140) and binds to a carbohydrate modified version of decay acceleration factor B (DAF/CD55), which is highly expressed on tumor cells and aids in immune evasion (140). SC-1 mediated crosslinking of DAF resulted in tumor-regression and apoptosis

of stomach cancers without showing cross-reactivity with healthy tissue (121). PAM-1, a natural IgM isolated from a gastric carcinoma patient, binds to a carbohydrate modified isoform of cysteine rich fibroblast growth factor receptor (CFR-1), which is expressed on malignant cells but not on healthy tissue (141). PAT-SM6 is a natural IgM isolated from a gastric cancer patient (142) and binds to a glycosylated form of glucose-regulated protein 78 (GRP78), which is found on malignant cells but not on healthy tissue (143). PAT-SM6 was also found to induce apoptosis in multiple myeloma cells binding to the glycosylated form of GRP78 while leaving healthy tissue unharmed (144). Another study found that activation of peritoneal B1-cells with the C-type lectin agonist monophosphoryl lipid A (MPL) and the TLR agonist trehalose-6,6'-dicorynomicolate (TDCM) lead to increased production of IgM NAb in mice. These IgM's were targeted at carbohydrate antigens and suppressed tumor growth of peritoneal metastasis via the classical complement pathway (145). In summary, NAb are able to challenge tumors by recognizing tumor specific antigens, specifically those with carbohydrate modifications. Circulating autoantibodies in cancer patients had high specificity for glycoxidation modified histone H2A suggesting that glycoxidation of proteins and related autoantibodies could act as early biomarkers of cancer (146).

NATURAL ANTIBODIES AGAINST OXIDIZED LIPIDS AND THEIR ROLE IN CARDIOVASCULAR DISEASE

Atherosclerosis is a chronic inflammatory disease that is characterized by the accumulation of apoptotic cells and oxidized lipids, specifically oxidized Low Density Lipoprotein (oxLDL) (147). It was demonstrated that oxLDL is an important target for NAb. In mice, around 30% of the IgM NAb bound to oxidized lipids, atherosclerotic lesions or apoptotic cells (71). Autoantibodies to oxLDL derived from "naïve" atherosclerotic mice shared complete genetic and structural identity with antibodies from the classic anti-phosphorylcholine B-cell clone, T15, which protects against common infectious pathogens, including pneumococci. S. pneumoniae immunized mice showed high circulating levels of oxLDL-specific IgM and persistent expansion of oxLDL-specific T15 IgM-secreting B cells, a decreased the extent of atherosclerosis (148) and blocked uptake of OxLDL by macrophages (127). High levels of IgM NAb against oxLDL were associated with protection against carotid atherosclerosis in hypertensive humans (149), but high levels of IgG binding LDL could be pro-artherosclerosis (132). NAb binding phosphorylcholine conjugated to BSA or KLH were decreased in patients with cardiovascular diseases and SLE and therefore proposed as potential protective factors (122, 123). NAb against other oxidation-specific epitopes have also been described, including those against malondialdehyde and 4-hydroxynonenal which were found in mice under pathogen free and germfree conditions (150). Immunoglobulins against phosphatidylserine and cardiolipin are generally associated with thrombosis, whereas immunoglobulins against their oxidized forms are associated with protection against atherosclerosis (27, 124). Natural IgM and IgG against citrate synthase (CS) were found in serum of healthy individuals and pericardial fluid (PF) of patients that went through open heart surgery (58, 59). CS is a highly conserved mitochondrial inner membrane enzyme involved in the citric acid cycle which occurs in nearly every cell, and especially in mitochondria-rich heart muscle cells. A relatively high number of B1-cells were present within PF and the prevalence of IgM NAb in PF was only half the amount of serum anti-CS IgM NAb in comparison to the total Ig levels that were four to eight times higher in serum (58). Together, the data suggests that NAb play an important role in the regulation or prevention of cardiovascular diseases (**Table 2**).

NATURAL ANTIBODIES AND THEIR ROLE IN TRANSPLANTATION IMMUNOLOGY

NAb play an important role in transplantation immunology and allograft rejection (30). NAb against the oligosaccharide moieties of the ABO blood group system have been well-described and a mismatched blood transfusion leads to hyper acute transplantation rejection with severe clinical consequences. Graft B-cells infiltrate coronary arteries resulting in cardiac allograft vasculopathy (CAV), an accelerated form of coronary artery disease (CAD) limiting the long-term survival after cardiac transplantation (151). It was found that half of 100 B-cell clones isolated from three CAV cases showed oligo-reactivity toward apoptotic cells, dsDNA, cardiolipin, LPS and insulin (152). Renal proximal tubular epithelial cells are considered relatively susceptible to ischemia reperfusion injury, and this was mediated by IgM NAb via the classical complement pathway (153). Higher levels of IgG NAb binding apoptotic cells prior to kidney transplantation negatively correlated with graft loss, which was mediated by C4b complement deposition (154). A subsequent study also found that polyreactive IgG clones from two kidney transplant recipients were able to bind to Human Leukocyte Antigen (HLA) class I, albeit non-native denatured HLA (155). It can be postulated that an incorrect collection and transplantation of the organ would induce stress and subsequent antigen modification, therefore allowing homeostatic NAb to attack the neo-epitopes within the graft. Together, these studies demonstrate that graft-rejection should not strictly be attributed to monospecific immunoglobulins but probably rather to NAb, although the threshold for initiating this antibody-mediated rejection is unknown.

Apart from studies on pig tissues for human transplantation, transplantation studies are not a main topic in veterinary species and therefore knowledge on the role of NAb in these models is completely lacking.

NATURAL ANTIBODIES AGAINST PATHOGENS AND INFECTIONS

NAb were acknowledged as a first line of defense to infectious agents (29). IgM NAb might be involved in tuberculosis as a decrease in serum IgM levels against phospholipids is observed after intensive phase treatment, probably due to a decrease in bacterial burden (156). However, a decrease in IgM contrasts with the observation in other models where a decrease in NAb usually

is a negative predictor for disease. An age-dependent decline in IgM NAb against pneumococcal capsular polysaccharides (PPS) and IgG NAb against a pool of virulence-associated proteins (VAP) of various *Streptococcus pneumoniae (S. pneumoniae)* strains was observed in humans, which could lead to increased susceptibility to *S. pneumoniae* infection (157).

In mice, NAb provide protection to viral infections (in an indirect fashion) by targeting virus-antibody complexes to the spleen and by contributing to the resolution of the acute phases of some viral diseases (18, 90). Infections are also prevented indirectly by NAb binding self-receptors such as CCR5, essential for the entry of the HIV virions (158). Maternal natural IgG antibodies protected neonatal mice from infection with enterotoxigenic *E. coli* infections when these antibodies were delivered across the placenta or through milk (159).

Protection to infections has been observed in veterinary species, but information is still scarce. High levels of NAb binding *Aeromonas salmonicida* protected goldfish against experimental infection (160), and high levels of NAb (and complement activity) correlated with fitness of wild boar when exposed to classical swine fever (161). Chickens bred for high levels of anti-KLH NAb showed improved resistance to avian pathogenic *E. coli* (162). The latter group also identified the existence of a single nucleotide polymorphism (SNP) variation in likely the TLR1A gene involved in determining the levels of natural antigen-specific IgM and total IgM antibodies in chickens (163). Heritability of natural IgM antibody levels was found which was absent or low for natural IgG or IgA antibodies (164).

In summary, NAb of the IgM and IgG class have been implicated in both health and diseases and are associated with protection against infections (**Table 3**) and disorders (**Table 2**) in humans and veterinary species. Future research should aim to expand this knowledge by further identifying more diseased states in which NAb are involved to further demonstrate their importance in maintaining health, and whether modulation of NAb-levels is feasible and desirable.

IgA NATURAL ANTIBODIES REQUIRE MORE INTENSIVE INVESTIGATION

IgA is the most abundant immunoglobulin, with a production in humans of about 66 $mg.kg^{-1}.day^{-1}$ (reflecting 3–5 g per day). In humans, monomeric IgA (at 2 $mg.ml^{-1}$) predominantly resides in serum where it functions as a potent pro-inflammatory agent by inducing rapid FcαRI mediated activation of neutrophils (166). In humans, two subclasses, IgA1 and IgA2, were identified in serum and secretions. These pro-inflammatory properties are not well-known as IgA has been perceived as a redundant non-inflammatory immunoglobulin in the intestinal lumen, which is true for secretory IgA (sIgA). sIgA originates at the basolateral side of mucosal areas where J-chain linked dimeric IgA (dIgA) is transported across the mucosal barrier into the lumen via the polymeric IgA receptor (pIgR). Upon its release into the lumen, dIgA retains a fraction of the pIgR, known as the secretory component (SC), which makes sIgA more robust and resistant against bacterial derived proteases. The SC also prevents association with the FcαRI which prevents interaction with immune cells, resulting in a homeostatic immunoglobulin that neutralizes microbiota and food antigens to prevent interactions with the host (167). Innate-like B1-cells can be stimulated by IL-5, IL-10, Toll-like receptor (TLR) agonists or whole bacteria to secrete IgM and IgA. As a pro-inflammatory immunoglobulin, serum IgA is crucial in the first line of defense against pathogens as a rapid activator of neutrophils. Meanwhile, homeostatic sIgA at mucosal sites most likely experiences the largest and most diverse amount of antigen interactions and is constantly challenged by this hostile environment.

IgA is perhaps the most important Ig-class, but the available literature on IgA NAb in humans and mice is lacking far behind in contrast to IgM and IgG NAb. Research in domesticated animals pointed to an important role for IgA NAb in binding larval antigens on mucosal tissues and aiding in the development of immunity to nematodes (168), and other parasites. Further studies into the role of IgA NAb in veterinary species are urgently needed.

IgA NATURAL ANTIBODIES IN SERUM BIND TO SELF-ANTIGENS

An antigen microarray screening of self-binding NAb in serum and cord blood of ten mothers and their infants found IgA NAb against myelin oligodendrocyte glycoprotein (MOG), Gelsolin, Low Density Lipoprotein (LDL), Factor X and Protease in all subjects (54). While the reactivity of IgM NAb was nearly always higher than the reactivity of IgA NAb to a specific antigen, this was not the case for High Density Lipoprotein (HDL) and α2-microglobulin. On other occasions, IgA NAb showed higher reactivity against HDL, α2-microglobulin, LDL, Factor X, and Gelsolin compared to IgG NAb. Additional research is required to understand why IgA specifically seems to favor these antigens. Other studies found IgA NAb against α-Gal, which is considered as one of the most abundant natural antibodies (169). Anti α-Gal IgA was found in healthy subjects (170) but was also associated with Henoch-Schönlein purpura, IgA nephropathy and Crohn's disease (133). As serum IgA is a potent pro-inflammatory immunoglobulin, a sufficient amount of IgA NAb in serum against foreign antigens could be very beneficial in the critical time period of adaptive immunoglobulin development, by rapidly recruiting neutrophils to the side of infection. A pro-inflammatory response against self-antigens is questionable, unless it concerns oxidized, or (carbohydrate) modified (neo-epitope) forms of these antigens.

IgA NATURAL ANTIBODIES AT MUCOSAL SITES LIKELY ORIGINATE FROM COMMITTED B1b-CELLS

Commensal gut bacteria are targeted in the small intestine by polyclonal oligo-specific B1b-cell derived IgA NAb whereas B1a-cells recognize restricted microbial regions (171). In contrast to B1a-cells, B1b-cells are able to switch to IgA+ plasma cells in a T-cell independent manner under the influence of TGF-β and Retinoic Acid (RA), which induces upregulation of α4β7+ and CCR9, providing a gut-homing phenotype (171, 172). A

TABLE 3 | Involvement of natural antibodies in prevention or combat of infection.

Antigen(s)	Isotype	Infection	Effect	References
Self- and microbiota	IgM, IgA	Microbiota	Protection	(37)
Glycans and microbiota	Not specified	Microbiota	Orchestration	(43)
Carbohydrates	IgM	Microbiota		(44)
LPS,KLH, Peptidoglycan	IgM, IgG, IgA	Unknown	Heritable	(88)
PC-rich *Streptococcus pneumonia*	IgM	*S. pneumonia*	Protection	(89, 148)
OxLDL, T15 idiotype				
VSV virus, Listeria	IgM	VSV virus, Listeria	Protection	(90)
Influenza strains	Not specified	Influenza	Protection	(91)
Malaria	IgM, IgG	Malaria	Protection	(97)
Schistosome		Schistosomiasis		
myelin basic protein	IgG	Porcine reproductive respiratory syndrome virus	Protection?	(119)
Phospholipids	IgM	Tuberculosis	Protection?	(156)
Virulence-associated Protein	IgM, IgG	*S. pneumonia*		(157)
self-antigens, Phospholipids, T-cell independent-antigens	IgM	Microbiota	Protective?	(18)
CCR5	Not specified	HIV	Protective?	(158)
Pantoea-1 microbes	IgG	*E. coli*	Protective	(159)
Aeromonas salmonicida	IgM	*A. salmonicida*	Protective	(160)
Chicken red blood cells	not specified	Classical swine fever	Protective?	(161)
KLH	IgM	*E. coli*	Protective	(162)
Phosphatidylcholine	IgG	*Plasmodium chabaudi*	Protective	(165)
Galα1-3Galβ1-GlcNAc	IgM, IgG, IgA	Block infections		(43, 44, 54, 155)

follow-up study found that naïve B-cells recirculated through Peyer's Patches to become IgA-secreting plasma cells in germfree and antigen-free mice (173). So it appears that B1b-cells are committed to eventually secrete IgA NAb at mucosal sites whereas activated B1a-cells migrate from the peritoneal cavities toward the spleen where they eventually secrete IgM (46, 47). Targeted modulation of the B1b-cell population might improve or diversify IgA NAb-responses at mucosal sites which could result in a better protection against exposure to microbes.

Chickens supplemented with probiotics showed higher levels of NAb (IgM and IgG) in their serum and intestines (IgA and IgG). These NAb also reacted with bacterial exotoxins (174). This implicates that studies on the role of microbes and hygiene in the formation of serum (IgM and IgG) and mucosal NAb (IgA) via dietary interventions could add in health management of both humans and veterinary species.

IgA NATURAL ANTIBODIES IN MILK MAY SHAPE NATURAL IMMUNITY OF THE INFANT

Human milk is highly saturated with sIgA in concentrations up to 12 g/l in colostrum and 1 g/l in mature milk (175). These IgA NAb bind to endogenous antigens like actin, myosin, tubulin, transferrin, thyroglobulin, spectrin, laminin, myoglobulin, and native DNA (176, 177). Human colostrum derived sIgA reacted *in vitro* with human Hep-2 cells and monkey ovary, pancreas and adrenal gland tissue while in a lesser extend to monkey liver, testes, salivary gland, muscle, and thyroid glands (175). IgA NAb in milk can also be directed against foreign antigens, like protein disulfide isomerase (PDI) of *Toxoplasma gondii* (178). There is probably an interesting link between IgA NAb in milk and the gut. One study phenotyped milk derived B-cells as CD38-high, complement receptor-low, indicating that the milk derived B-cell population predominantly contained plasma blasts and plasma cells that actively secreted immunoglobulins. Further phenotyping revealed that the majority of milk derived B-cells were α4β7+ CD62L–, which are migration patterns similar to Gut Associated Lymphoid Tissue (GALT) B-cells (179). These findings lead to the hypothesis that an IgA NAb-profile of the environment is created in the maternal gut, specifically by B1b-cells that locally switch to IgA to create a highly promiscuous pool of immunoglobulins that react to both foreign and self-antigens. Human breast milk or raw cow's milk-derived immunomodulatory cytokines, like TGF-β2 and (very low levels of) IL-10, might upon consumption induce a regulatory environment in the gut which induces Regulatory

T-cells and leading to the production of IgA and IgG4. Supplying sIgA NAb in breast milk can potentially enhance intestinal immunity in early life (180).

The exact effect and function of maternal sIgA for the infant is not known, but it fits in Jerne's idiotypic immune network theory where natural IgA would act as an educator of the infant's immune system (181). In this model, maternal sIgA (Ab1) is elicited against an environmental epitope in the mother and transferred toward the infant via the milk. In the infant, an anti-idiotypic immunoglobulin (Ab2) is generated against the maternally acquired Ab1. Subsequently, a third immunoglobulin mimicking the Ab1 BCR (Ab3) is generated against Ab2, which allows the infant to imprint this maternal immunoglobulin or BCR within its own repertoire. Previously mentioned findings in serum further support this idea (49), where it was observed that maternal IgG educated the neonatal independent IgM repertoire. However, the relationships between serum IgA levels and maternal IgG and/or neonatal IgM were not investigated. In mice, it was already shown that anti-idiotypic IgM antibodies specific for the IgA myeloma protein TEPC-15 (anti-phosphorylcholine) specificity, share similar or even identical idiotypes (182). In summary, natural IgA NAb or maternally derived natural antibodies may provide protection of the infant gut and be involved in maturation of the mucosal immune system.

In most veterinary species (e.g., cows and poultry) IgA is not the predominant maternal antibody as its role is fulfilled by IgG. Birds receive maternal IgG in the yolk, and are thus hatched with the maternal antibody repertoire, including self-binding antibodies (183). Calves, like most mammalian food animals, receive maternal IgG via colostrum including self-binding antibodies (51). Whether these maternal IgG antibodies shape the neonatal antibody repertoire as discussed above for man is currently unknown.

MODULATING NATURAL ANTIBODIES AND THERAPEUTIC OPPORTUNITIES

NAb are important as a first line of defense against pathogens and as homeostatic agents that inactivate or clean up potential dangerous self-antigens. Modulation or enhancement of NAb-levels and their diversity could lead to new therapeutic strategies and new insights into the usefulness of NAb. There is increasing knowledge of NAb in humans and their implications in health and disease, but studying intentional enhancement or decrease of NAb-levels in humans faces ethical objections because the effects and eventual risks are unknown, therefore urging the use of animal models. Mice are usually the first model of choice as they are economically affordable, easy to handle and share many parallels with human immunology. While mice have given many tremendous new insights into human immunology, there are also significant differences in immune development, activation mechanisms and immune response as mice and men are different in physiology, anatomy, size and lifespan (184, 185). Using non-human primates would be a logical alternative as they come closest to humans in genetics, physiology and behavior (186), but they are expensive and also require tight ethical regulations.

Alternative animal models that would fill a niche between mice and men are veterinary species like cattle, poultry, sheep and pigs which are not as tightly restricted by regulations and relatively economically affordable. In addition, contemporary agricultural practices require more knowledge on the maintenance or enhancement of health and welfare in veterinary species as well. Pigs are physiologically and anatomically close to humans, sharing similarities in cardiovascular systems, feeding (omnivorous) and skin composition (187, 188). Chickens being the most wide spread and most consumed veterinary species would also be interesting models as some major immunological breakthroughs in the past were achieved in chickens, including the principles of graft vs. host reactions and the delineation of the adaptive immune system into immunoglobulin secreting B-cells and cell-mediated immunity by T-cells (189).

Findings from veterinary species can be translated back to humans, but can also be applied within the field of veterinary immunology itself. Veterinary species are constantly challenged by bacteria, viruses, and parasites which not only has a major impact on animal welfare but also on the economy due to prevention and treatment costs, production losses and premature culling (190, 191). Diseases of bacterial nature are often treated with antibiotics, but the popularity of antibiotics has decreased due to risk of antibiotic resistance. Vaccination has received more popularity as it is preventive and actively stimulates the immune system, but vaccines are not always fully protective (192) or available. Therefore, there is a need for innovation in veterinary treatment strategies (193) and elucidating NAb and their functionality in veterinary species may provide new exciting opportunities. NAb have been described in veterinary species and it has been demonstrated that they are able to be modified, but the clinical relevance of NAb in veterinary species remains enigmatic. Humans and veterinary species would mutually benefit from the combined effort to study NAb and allow for the reciprocal exchange of findings from their respective fields.

INTRAVENOUS OR ORAL ADMINISTRATION OF IMMUNOGLOBULINS

Intravenous immunoglobulin (IVIg) preparations contain large amounts of immunoglobulins reactive with various constituents and a portion of these are most likely (self-binding) NAb. IVIg has been used in humans as a therapeutic in immunodeficiency to replace missing immunoglobulins (194). IVIg was used as a successful treatment for Kawasaki disease, which is a pediatric disorder that leads to inflammation of coronary arteries, and diminished coronary dilation and improved coronary flow (195). IVIg was also used as a therapeutic for unexplained recurrent spontaneous abortion, and is especially effective when repetitive miscarriage occurs after an initial live birth (196), suggesting that tolerance against the neonate is breached during first pregnancy and that IVIg, which likely includes NAb, might restore this. This inspires the investigation of NAb-exclusive IVIg

administration for the treatment of immune mediated diseases. Natural antibodies, present in IVIG, could be used to prevent autoimmune reactions and to enhance the immune response to vaccination.

Albeit no IVIg *sensu stricto*, intravenous administration of KLH binding NAb to chickens enhanced specific antibody responses to KLH after immunization indicating an "adjuvant" role of NAb (22). Oral administration of NAb could also be beneficial, as pigs fed with pig plasma-derived natural IgG showed a decrease in shedding of *Salmonella enterica diarizone* and three strains of *E. coli* (O138, O149:F4 and F18), and a restoration of microbiota diversity compared to untreated pigs (197). NAb binding glutamate dehydrogenase, carbonic anhydrase, myosin and transferrin were found in unborn calves prior to intake of colostrum, and were greatly enhanced by colostrum resident NAb against the same self-antigens (51). This is in line with previously mentioned findings on the presence of self-binding natural sIgA in colostrum and milk of humans (175–178), speculating that oral ingestion of NAb may lead to immune education and therefore adequate NAb-levels in the neonate. These findings suggest that NAb-levels in neonates, and immunity in general, heavily rely on these early maternal NAb and stresses the importance of breastfeeding or oral Ig-supplementation.

From a veterinary perspective: IVIg procedures are likely not useful, but providence of colostrum and allowing food animals such as calves and piglets to stay with their mothers for an extended period of time would give them a more extensive immune-education that would prevent disease later in life.

IDENTIFYING NATURAL ANTIBODIES AND TARGET EPITOPES TO DEVELOP THERAPEUTIC IMMUNOGLOBULINS

Several IgM NAb-clones were isolated from cancer patients and were able to bind carbohydrate structures on tumors and subsequently decrease tumor burden (136). Another example of an isolated NAb is the IgM clone "EO6," which was isolated from apolipoprotein E-deficient mice (198). EO6 bound to oxLDL, apoptotic cells, atherosclerotic lesions and oxidized phospholipids whereas it did not recognize native lipoproteins (199). Furthermore, EO6 administration in ApoE deficient mice lead to less oxLDL uptake by macrophages and thus decreased formation of foam cells (200). Intravenous administration of a specific MDA antibody *in vivo* neutralized endogenously generated MDA epitopes that resulted in decreased hepatic inflammation in low-density lipoprotein receptor-deficient mice on a Western-type diet (125).

There is an opportunity to isolate and develop monoclonal therapeutic NAb. This approach would have several benefits in comparison to monoclonal conventional antibodies: (i) NAb would be cost-efficient as they could be directly isolated from donor volunteers which would leave the immunization of mice and other laboratory animals unnecessary. (ii) NAb have been demonstrated to be oligo-specific, so by binding to multiple antigens a single therapeutic NAb could be applied in the treatment of multiple diseases. (iii) NAb that have been investigated so far did not show to bind to healthy tissue or native forms of their target antigens, suggesting less therapeutic side-effects. These therapeutic NAb-inspired immunoglobulins could also be administered to veterinary species to treat inflammatory diseases or prevent cancers, such as Marek's disease in poultry.

IMMUNIZATION OR ENVIRONMENTAL EXPOSURE AS TRIGGERS FOR NATURAL ANTIBODY SECRETION

NAb in neonates have not been positively associated with vaccinations due to maternal IgG. IgG in humans and apes is the only isotype that can pass the placental wall and serves as a single dose of immunoglobulins to the neonate which possess them post-natal up to 12 months. This single dose immunization helps to defend against pathogens in a critical window where the infant's immune system is under development, as demonstrated in agammaglobulinemia patients that were fully protected against bacterial infection up to 6 months after birth (201). While maternal antibodies are considered as essential in the critical window of neonatal immune development, it was demonstrated that maternal IgG may have a substantial inhibitory effect on many human and veterinary vaccines and could even lead to a partial or complete lack of protection in humans and cotton rats [reviewed in (202)].

It can be hypothesized that maternal IgG immunoglobulins are able to neutralize the antigen components from the vaccine and therefore prevent recognition by adaptive immunity. So, the ideal time-point for a vaccination would be when these maternal IgG's have disappeared, but this is highly variable and difficult to predict (202). These effects might be due to neutralization of live vaccines, epitope masking, elimination of antibody-coated vaccines by FcγR-mediated phagocytosis, and inhibition of B-cell activation by Fcγ-receptor mediated signaling. A strategy to evade this phenomenon could be to extend the protection of maternal IgG's and vaccinate with known NAb-epitopes (203), therefore stimulating the development of natural immunity itself and thus provide protection without conventional vaccines. Maternal NAb, likely initiated by the intestinal microbiota, protected neonatal mice in an antigen-non-specific fashion (203). Serum from mice immunized with KLH, DNP and peanut extract showed increased binding of immunoglobulins on brain, liver and spleen slices *in vitro*, demonstrating that NAb-levels can be regulated via (non-specific) immunization (204). This suggests that (non-specific) immunization can increase NAb-levels and therefore be utilized as prevention against autoimmune diseases. In mice solely expressing IgM NAb, approximately 30% of all NAb bound to model oxidation-specific epitopes, atherosclerotic lesions and apoptotic cells. It was hypothesized that these epitopes exert selective pressure to expand NAb, which in turn play an important role in mediating homeostatic functions consequent to inflammation and cell death, as demonstrated by their ability to facilitate apoptotic cell clearance thereby preventing chronic inflammatory diseases and atherosclerosis (71). Indeed, active immunization

with phosphorylcholine-enriched pneumococci protected mice against non-alcoholic steatohepatitis (126), whereas immunization with phosphatidylcholine, a component of red blood cells protected mice against Plasmodium infection (165). Also passive immunization of mice with monoclonal IgM against phosphorylcholine reduced vein graft atherosclerosis (128).

Higher IgM NAb-levels, but not IgG, have been found in wild rats compared to their laboratory counterparts (205), suggesting environmental antigens directly influence NAb-levels and diversity. Moreover, several bacterial orders were demonstrated to influence α-Gal NAb-levels (43), while chickens fed with probiotics showed enhanced levels of NAb binding to KLH (174). Recently, it was demonstrated that immunization of rats with model antigens (KLH-FITC or DNP-Ficoll) enhanced the level of antibodies binding various autologous organ extracts for both IgM and IgG, suggesting an enhanced network of NAb (204). Flynn et al. (206) found that domestic cats infected with feline immunodeficiency virus (FIV) showed enhanced levels of antibodies toward non-viral antigens: trinitrophenol (TNP), ovalbumin, beta-galactosidase, and DNA, which were not due to the presence of cross-reacting epitopes on recombinant FIV p17 or p24 antigens and suggesting that B-cell activation associated with infection was polyclonal rather than entirely virus specific (206).

Unpublished results from our lab revealed that chickens kept under high hygienic conditions had low levels of NAb to KLH and self-binding antibodies to liver as opposite to chickens kept under unhygienic conditions. NAb therefore would fit in the hygiene hypothesis, stating that a decreased incidence of infections, especially in the Western world, results in a higher incidence of autoimmunity and allergy (207). Here, microbes would educate natural immunity to peritoneal B1-cells that subsequently secrete homeostatic NAb to prevent autoimmune diseases. Almost by definition, this activity starts immediately after birth and is relevant in early life, precisely as implicated by the hygiene hypothesis. This also would suggest that dietary antigens could influence NAb levels, especially since the "Western Diet" that is rich in refined sugars, salt and saturated fat has been associated with immune alterations, including pathological autoimmunity (208).

Thus, altering antigenic experience by changes in diet or supplementation with probiotics or challenge by microbes might improve NAb-levels and diversity and thus enhance resistance to infection and decrease the incidence of pathological autoimmunity, or enhance the homeostatic function of NAb in preventing mal-behavior and metabolic disorders in veterinary species.

BREEDING OR GENETIC MODIFICATION OF NATURAL ANTIBODIES, USE OF VETERINARY SPECIES

NAb are often germline encoded, so there is a possibility to modify their levels and diversity on a genetic level. Additionally, it is also important that NAb generally have a restricted V_H gene usage, which can also be modified genetically. While performing genetic alterations in humans is obviously difficult due to ethical reasons, veterinary models could be used instead as they are less tightly regulated and experimental circumstances are much more controlled. In addition, breeding companies continuously search for new breeds with higher health status.

There is evidence that breeding for high levels of NAb and NAAb is possible. Different NAAb-levels were earlier determined in inbred mouse strains (78), but studying veterinary species also allows (unexpected) linkage of NAb-levels with various other physiological and important production and welfare traits. The advance of synthetic biology approaches relies on the use of omics information and these greatly improved insights provide opportunities to more closely monitor health conditions, modulate the genetic background, and thus improve animals on a pre-selected genetic background (209). High levels of anti-nuclear immunoglobulins were found to be heritable in sheep and were associated with higher longevity (70). Overall survival during a laying period was higher in chickens with high NAb-levels (129, 130), and life history: "fast" or "slow" correlated with constitutive immune defenses, i.e., that slower developing species showed higher NAb-levels, as was also true for solitary living bird species (210). NAb levels can thus be used to compare constitutive humoral immunity among and within species with respect to strain, age, sex, treatments, ecology, and "life span or history" (33).

Genetic regulation of NAb was demonstrated in poultry that were divergently bred for high levels of anti-KLH NAb (211). Divergent breeding of poultry for NAb also affected their self-antigen binding antibodies (212). A genome wide association study (GWAS) showed that the KLH NAb High line of chickens possessed a single nucleotide polymorphism (SNP) within the TLR1A gene significantly explaining levels of KLH binding IgM's, indicating that TLR1A has a major impact on NAb-levels and/or NAb B-cells. This TLR1A region was also significant for total levels of IgM in blood (163) and most likely levels of IgM antibodies binding self-antigens (213).

NAb-levels to KLH from pigs in high-health environments were proposed to be used as phenotypical predictors for resilience and mortality under a disease challenge, and higher NAb-levels at a young age correspond to increased resilience and decreased mortality in swine (214). NAb against KLH were also found to be heritable in cattle (88, 94). NAb-levels were associated with inflammatory diseases in cattle (215), and NAb-levels in milk and serum correlated both phenotypically and genetically with immune associated traits and diseases in cows (216) including mastitis (131, 217). It is suggested that breeding of cattle against diseases such as mastitis or uterus inflammation may benefit from specifically breeding for high NAb-levels. Different levels of NAb were also found in different genetic lines of common carp (*Cyprinus carpio)* independent of antigen, age and environment, further suggesting that NAb are for an important part under genetic control and could therefore be modulated genetically to improve disease resistance in fish (218) and food animals such as cattle and poultry.

Genetic modulation of NAb could give new insights in key genes that regulate NAb-levels and findings from these studies might be translated to humans in future gene therapies and

could possibly restore defected genes associated with decreased NAb-levels that are correlated with disease.

CONCLUDING REMARKS

While Burnet's forbidden clone paradigm still provides a barrier to many immunologists, others gradually accept the existence of NAb and their importance in health and disease. In humans and mice, various infectious, neurological-, tumor-, cardiovascular-, and metabolic diseases were related with (usually decreased) levels of (self-binding) NAb. Still, homeostatic NAb and their target antigens deserve more attention, especially in food animals as they most likely contribute to maintaining health by preventing development of disease in animals as well. While IgM and IgG have been thoroughly investigated in many species, the data on IgA NAb are lacking far behind and should be more intensively investigated. Importantly, NAb may not function completely in an antigen-non-specific manner as previously thought, since relations between diseases, specific antigenic epitopes and specific NAb-isotypes and idiotypes become more apparent. Genomics, proteomics, and quantitative Western blotting approaches will likely reveal many (un)expected self, non-self- and neo-antigens that contribute to the formation and maintenance of NAb. Understanding the functional relationship between NAb and their antigen will lead to intervention, such as vaccination and diet modulation in both humans and animals, or selective breeding and hygiene management strategies in animals. This may result in new health management strategies, such as vaccination and diet modulation in both humans and animals, or selective breeding and hygiene management strategies in animals. Albeit that the role of NAb in veterinary species in contrast to humans is largely unknown, veterinary animals would provide excellent models to investigate the possibilities of modulating NAb, allowing the reciprocal exchange of data that will mutually benefit both human and veterinary immunology.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REVIEW
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New Immunoinformatics Tools for Swine: Designing Epitope-Driven Vaccines, Predicting Vaccine Efficacy, and Making Vaccines on Demand

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Novel computational tools for swine vaccine development can expand the range of immunization approaches available to prevent economically devastating swine diseases and spillover events between pigs and humans. PigMatrix and EpiCC are two new tools for swine T cell epitope identification and vaccine efficacy analysis that have been integrated into an existing computational vaccine design platform named iVAX. The iVAX platform is already in use for the development of human vaccines, thus integration of these tools into iVAX improves and expands the utility of the platform overall by making previously validated immunoinformatics tools, developed for humans, available for use in the design and analysis of swine vaccines. PigMatrix predicts T cell epitopes for a broad array of class I and class II swine leukocyte antigen (SLA) using matrices that enable the scoring of sequences for likelihood of binding to SLA. PigMatrix facilitates the prospective selection of T cell epitopes from the sequences of swine pathogens for vaccines and permits the comparison of those predicted epitopes with "self" (the swine proteome) and with sequences from other strains. Use of PigMatrix with additional tools in the iVAX toolkit also enables the computational design of vaccines *in silico*, for testing *in vivo*. EpiCC uses PigMatrix to analyze existing or proposed vaccines for their potential to protect, based on a comparison between T cell epitopes in the vaccine and circulating strains of the same pathogen. Performing an analysis of T cell epitope relatedness analysis using EpiCC may facilitate vaccine selection when a novel strain emerges in a herd and also permits analysis of evolutionary drift as a means of immune escape. This review of novel computational immunology tools for swine describes the application of PigMatrix and EpiCC in case studies, such as the design of cross-conserved T cell epitopes for swine influenza vaccine or for African Swine Fever. We also describe the application of EpiCC for determination of the best vaccine strains to use against circulating viral variants of swine influenza, swine rotavirus, and porcine circovirus type 2. The availability of these computational tools accelerates infectious disease research for swine and enable swine vaccine developers to strategically advance their vaccines to market.

Keywords: vaccine, swine, immunoinformatics, T cell, epitope, SLA, infectious disease, immunity

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INTRODUCTION

Pigs are an important component of the agricultural economy worldwide and are an important contributor to protein intake for populations living in developed and developing world economies. Due to the concentration of pigs in industrial farming operations and concern about the overuse of antibiotics for food animals, the control and prevention of infectious diseases in swine has become an important topic that is not only relevant to animal health and wellbeing but also to global food security and economic stability. Vaccine development for swine is likely to be facilitated by the emergence of computational tools for vaccine design. These same tools may also contribute to research on the spread of swine pathogens within herds and across geographical borders. For example, influenza is more diverse in swine populations than in humans. Spillover of influenza strains from pigs to humans was observed in 2009, and efforts to predict the next such event may be improved by comparisons of circulating strains in different species, a process that can be enabled by computational tools. Such tools may also contribute to the development of novel vaccines for important pathogens of swine for which effective vaccines are not yet available, such as African Swine Fever Virus (ASFV), a pathogen that is affecting swine populations in Asia and Europe (1).

Veterinary vaccines are one of the more cost-effective means of controlling, eradicating diseases and protecting herd health. Nevertheless, culling infected animals and strict containment are, in many instances, the only method available to limit the spread of disease during outbreaks (2). In order to move away from culling and quarantining infected animals, new types of vaccines and new vaccine methodologies that reduce the susceptibility of swine to infections bear serious consideration. Given the emergence of new strains of influenza and diseases that become endemic in new locations such as ASF in swine populations, and ethical considerations related to the culling of animals in industrial farming operations, there is a critical need for tools that can enable novel vaccine design, accelerate vaccine design, and assess the efficacy of vaccines against circulating strains, *in silico*.

Most veterinary vaccines are developed using standard methods, such as inactivating the pathogen using chemical or physical methods and then injecting killed organism directly into the animals (a process that can be called "shake and bake"). Alternatively, molecular tools are used to selectively modify a pathogen so as to limit virulence, resulting in an attenuated version that can be used as a vaccine. These vaccine approaches do not adequately address strain variation, which is a significant problem for the development of swine vaccines, as many of the pathogens affecting swine are highly variable. Additionally, viral pathogens have been shown to modify T cell epitopes to evade host immune response (immune escape) and more recently, selected epitope sequences of pathogens have been shown to resemble epitopes found in their hosts (immune camouflage) (3). Research in the field of human immunology has contributed to the development of tools that permit the evaluation of pathogen variation and immune camouflage. Although no examples of immune camouflage have been demonstrated in pigs, evolution of pathogens in pigs and the close resemblance of human and swine immune systems, including the Th1/Th2/Th17/Treg paradigm, suggests immune camouflage may occur in pigs like in humans. The availability of tools that discover pathogen epitopes that resemble their host sequences may lead to improvement in the process of antigen selection and enabling researchers to improve the efficacy of vaccines for swine.

Computational tools for vaccine design usually start with T cell epitope prediction due to the important role of T cell epitopes in cell-mediated immunity (CMI). T cell epitope mapping algorithms enable the analysis of complete proteomes of any size to identify vaccine candidates for experimental validation. Despite the demonstrated utility of computational vaccinology in human vaccine development (4), computational tools for vaccine design are very limited for non-human species. This is mainly due to the limitations on available experimental data that is required to develop prediction models. However, methods for extracting similarities between human and swine immune system orthologs exist and have been applied to develop new epitope prediction tools for swine (5), and this makes it possible to imagine further improvements in epitope-prediction models and further expansion of computational vaccinology tools. The fact that swine are both "patient" and "experimental model" facilitates the testing of hypotheses and will enable the development of at least as many applications of immunoinformatics tools as for humans and the acceleration of porcine immunology research.

Here, we review new immunoinformatics tools for swine developed by a team of scientists at EpiVax in partnership with researchers based in academic settings (University of Rhode Island, University of Georgia), that have been integrated into an existing toolkit for human vaccine design. The hybrid toolkit has been applied to design and evaluation of novel vaccines for influenza and African Swine fever, and to the analysis of vaccine for protective efficacy against circulating strains of influenza and porcine circovirus. We also discuss current challenges and future perspective in the field.

THE iVAX TOOLKIT

Computational vaccinology is a term that incorporates epitope mapping, antigen selection and vaccine construct design using computational tools. *In silico* tools are at the core but validation is used to improve the efficacy of prediction and to measure the impact on immune responses to pathogens. A wide range of tools have been developed in the past 20 years that dramatically accelerate the design of novel and next generation vaccines. In a recent publication, we have described the utility of iVAX for human vaccine design and analysis (4). Here we will focus on the integration of PigMatrix into a pre-existing toolkit, and describe applications of the combined tools to swine vaccines.

The iVAX toolkit has been in development since 1998. It is an interactive internet-based platform that integrates user input, immunoinformatics algorithms and several sequence databases, enabling users to rapidly identify and triage candidate

antigens, select immunogenic T cell epitopes, eliminate potential regulatory T cell epitopes, and optimize antigens for immunogenicity and protection against disease. Detailed descriptions of the tools are published [see references (4, 6–8)]. While the tools were designed for humans, swapping out the tools used for epitope prediction from Human Leukocyte Antigen (HLA) to Swine Leukocyte Antigen (SLA) has enabled EpiVax vaccine developers to apply these advanced tools to infectious disease problems affecting swine.

Overview of the iVAX Toolkit

iVAX contains a compilation of tools that implement information derived from the T cell epitope mapping tool, EpiMatrix (9). This tool accepts sequence input for human, swine, and murine major hisotocompatability complex (MHC) class I and class II epitope prediction. The generated predictions can then be incorporated into further analysis using a variety of tools including the **Conservatrix, ClustiMer**, **EpiAssembler** (4) and **VaxCAD** algorithms (10). Conservatrix enables a search for sequences across variable pathogens, for example, swine influenza A, or Porcine Reproductive and Respiratory Syndrome Virus (PRRSV). ClustiMer finds regions of class II SLA-binding epitopes that cluster into a single longer sequence, and EpiAssembler is used for identifying epitopes that are conserved across several different strains of the same pathogen. Additional tools include **JanusMatrix**, a unique homology analysis tool that predicts the potential of a given peptide to contain epitopes exhibiting cross-reactivity between a pathogen and a host (such as swine) based on the conservation at the MHC-T cell receptor (TCR) interface. A list of the tools is provided in **Figure 1** with a short description of their function.

Immunogenicity Scale – Triaging Antigens

During the process of selecting candidate vaccine antigens, the overall immunogenic potential should be taken into consideration as it directly relates to the cytotoxic T cell (CTL) or T helper (Th) T cell epitope content. We have observed that the greater the concentration of HLA ligands and putative T cell

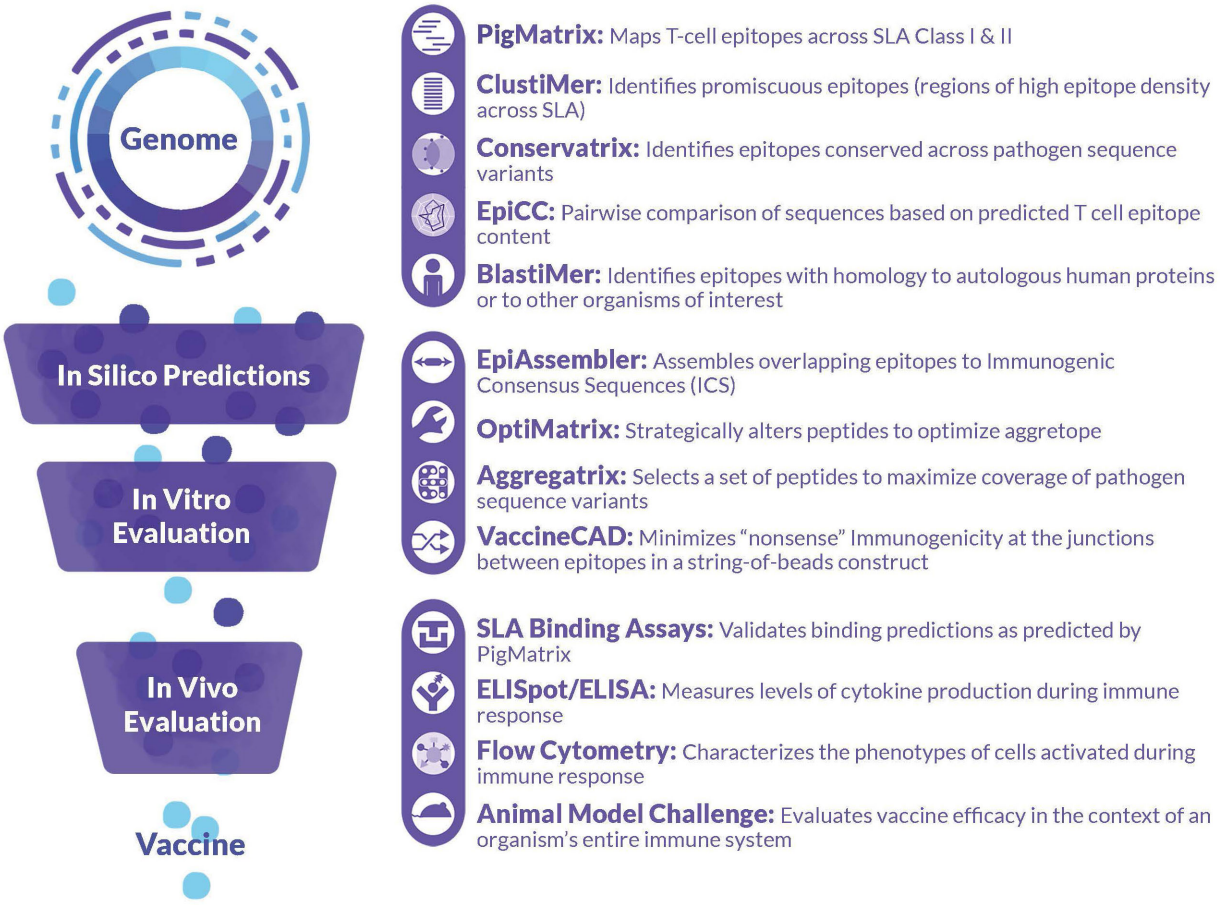

FIGURE 1 | Integration of PigMatrix and EpiCC into the iVAX Toolkit. The iVAX toolkit is a comprehensive set of tools for *in silico* analysis and computational vaccine design for humans. PigMatrix analyzes protein sequences for Class I and Class II SLA-restricted T cell epitopes. EpiCC defines the relatedness of sequences based on their T cell epitope content. The integration of PigMatrix and EpiCC into the existing iVAX toolkit allows them to be applied for the development of accelerated and improved swine vaccines.

epitopes that are contained in an antigen, the more likely it will induce an immune response.

T cell epitope concentration can be expressed as an overall EpiMatrix score called the **EpiMatrix Protein Score**, which is the difference between the number of T cell epitopes predicted in a given protein and the number of T cell epitopes expected to be found in a random protein sequence, normalized for length (per 1,000 amino acids). The average number of T cell epitopes contained in 10,000 randomly generated protein sequences is set to zero, proteins considered to have a significant immunogenic potential score above 20 on the normalized scale, on which several swine pathogen antigens included for comparison, in **Figure 2**.

Regional Immunogenicity

While the normalized **EpiMatrix Protein Score** provides an approximation of the overall protein immunogenicity, regional immunogenicity also plays a role in the immunogenic potential. T cell epitopes tend to cluster in regions of protein sequences. **ClustiMer** was developed to identify regions with unusually high densities of putative T cell epitopes. For a given region, ClustiMer calculates a T cell epitope cluster score. Clusters with scores above 10 are considered potentially immunogenic. The length of T cell epitope clusters ranges from nine to approximately twenty-five residues and can contain from four to forty HLA binding motifs. T cell epitope clusters usually contain one or more 9-mer frame sequences predicted to bind to four or more HLA alleles. This epitope bar feature (EpiBar) is highlighted in the iVAX report. T cell epitope clusters can be highly immunogenic. An example is given of a Swine Influenza A Hemagglutinin epitope cluster (**Figure 3**). Human T cell epitope clusters that have a similar EpiBar have been defined for Tetanus toxin 825–850, GAD65 557–567 and are often used as controls for T cell assays (11). In our experience, these clusters are recognized in outbred populations of humans (12, 13); however similar epitopes have not yet been defined for swine.

JanusMatrix and Self-Like T Cell Epitopes

Although T cells possessing anti-self TCRs were previously thought likely to be eliminated in the thymus, evidence emerged showing that anti-self immune response is also controlled by regulatory T cells recognizing the same antigens (14, 15). The phenotype of these regulatory T cells may be reinforced by repetitive re-exposure to their cognate self-antigens (16). Thus, immune response to new antigens is shaped by previous

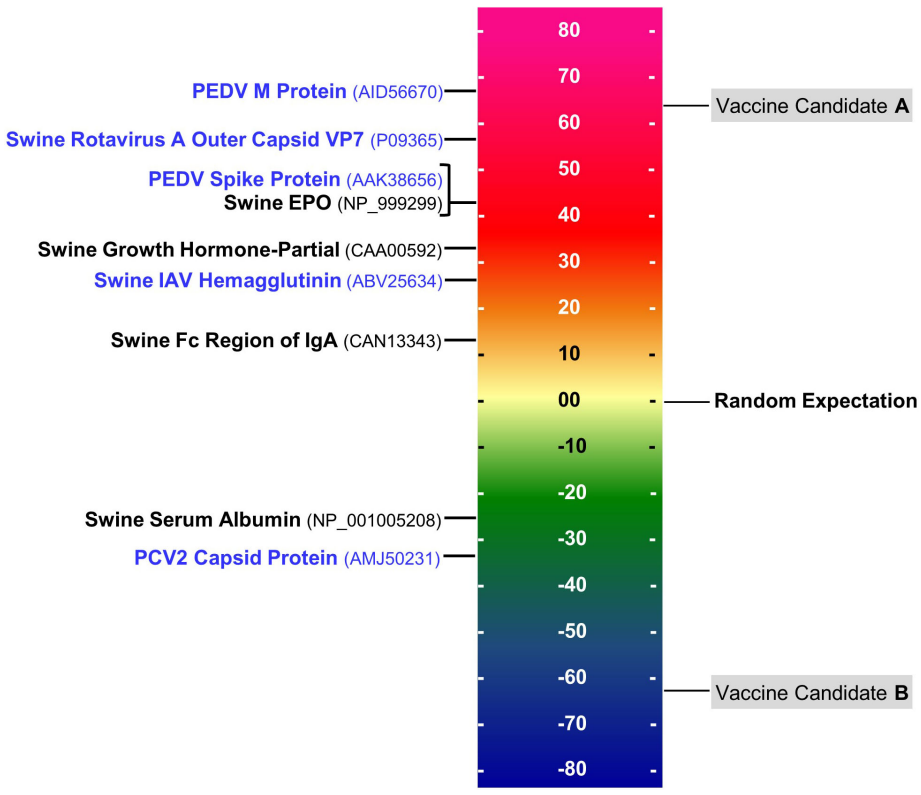

FIGURE 2 | EpiMatrix immunogenicity scale. The immunogenicity scale shows swine pathogen antigens that have been reported to be immunogenic, and non-immunogenic antigens. Sequence accession numbers in GenBank are provided in the parentheses. The EpiMatrix immunogenicity scale is set to zero based on the average epitope content in a randomly generated protein sequence. Normalization of SLA scoring enables the ranking and direct comparison of candidate antigens; for example, candidate vaccine antigen A would be preferred over candidate vaccine antigen B for inclusion in a vaccine designed to elicit T helper immune response and to drive humoral response.

experience in the thymus and by exposure-driven reinforcement in the course of immune system maturation.

We observed that certain pathogens contain critical antigens with T cell epitopes that are highly conserved with self-antigens. This is true for humans and consequently deserves attention in swine. We hypothesized that pathogens use these epitopes as a means of "immune camouflage"; thus, these epitopes might be tolerated or actively tolerogenic upon vaccination (17). In retrospective studies, we determined that peptide epitopes that have identical TCR-facing residues and similar MHC binding anchors can be potentially tolerogenic and/or activate T cells that have a regulatory T cell phenotype or induce immunosuppressive responses (3). To identify these self-like epitopes, we developed the JanusMatrix tool. Using this tool, we are studying the impact of mutating these epitopes to enhance vaccine immunogenicity in humans (18) and anticipate that we will extend this work in collaborations that will evaluate the impact of self-like epitopes for swine.

For any given putative T cell 9-mer epitope, JanusMatrix analyzes residues in contact with the MHC molecule, and those in contact with the T cell receptor (TCR). Positions 1, 4, 6, and 9 are assumed to interact with MHC class II molecules and positions 2, 3, 5, 7, and 8 are assumed to interact with TCRs (**Figure 4**). For class I epitopes, the TCR-facing residues vary from allele to allele.

The JanusMatrix algorithm then searches a reference database for similar epitopes, considering both MHC- and TCR-facing residues. The reference database (to which pathogen epitopes are compared) can be human, swine, murine, or any other organism (including other pathogens from the same, or similar species). JanusMatrix finds reference epitopes with identical TCR-facing residues that are predicted to bind to the same MHC molecule despite amino acid differences. JanusMatrix calculates a Homology Score as the average depth of coverage within the reference database for the putative MHC binding epitopes identified in the input peptide. JanusMatrix Homology Scores above two are considered to be significant, indicating

EpiMatrix Cluster Report

Accession: Swine Influenza Sequence: HA (Accession #ABU80287) Cluster 531

Frame Start	AA Sequence	Frame Stop	SLAII-DRB1_0101 Z-Score	SLAII-DRB1_0201 Z-Score	SLAII-DRB1_0401 Z-Score	SLAII-DRB1_0402 Z-Score	SLAII-DRB1_0601 Z-Score	SLAII-DRB1_0602 Z-Score	SLAII-DRB1_0701 Z-Score	SLAII-DRB1_1001 Z-Score	Hits
531	LAIYSTAAS	539	1.43	-0.09	1.41	1.3	0.77	0.64	1.02	-0.42	0
532	AIYSTAASS	540	0.87	-0.13	0.9	0.83	1.01	0.83	0.16	-0.41	0
533	IYSTAASSL	541	1.91	2.33	0.93	0.81	1.09	1.29	0.86	2.16	3
534	YSTAASSLV	542	2.08	2.43	1.4	1.84	2.52	2.18	1.69	3.03	7
535	STAASSLVL	543	1.75	1.78	0.9	0.71	1.56	1.47	0.66	1.78	3
536	TAASSLVLL	544	-0.23	-0.17	-0.89	-0.63	1.2	0.93	-0.65	0.16	0
537	AASSLVLLV	545	1.03	0.92	0.14	0.06	1.08	0.87	0.18	0.94	0

Total Assessments Performed: 56 **EpiMatrix Cluster Score (Deviation from Expectation): 21.71**

Z-score indicates the potential of a 9-mer frame to bind to a given SLA allele; the strength of the score is indicated by the blue shading. For example: Top 10% Top 5% Top 1%
All scores in the Top 5% (Z-Score ≥ 1.64) are considered "Hits". Scores in the top 10% are considered elevated, other scores are grayed out for simplicity.
Frames containing 4 or more alleles scoring above 1.64 are referred to as EpiBars and are highlighted in yellow.

FIGURE 3 | PigMatrix class II analysis. The cluster report of a swine influenza hemagglutinin sequence shows a 9-mer frame that contains three top 1% hits (strong binding likelihood) and four top 5% hits for SLA class II alleles. This feature is called an EpiBar and is characteristic of highly immunogenic epitopes.

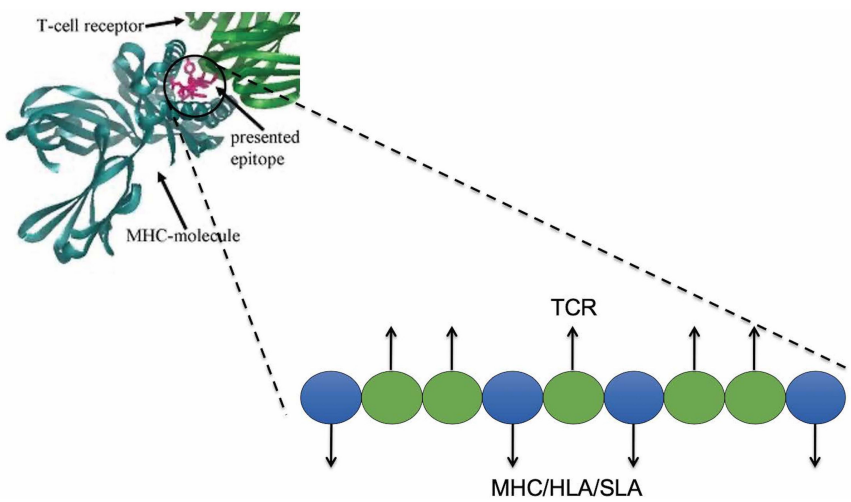

FIGURE 4 | JanusMatrix and self-like epitopes. Predicted SLA ligand with identical TCR-facing residues with the swine proteome (presented in blue) and variant SLA-binding residues (presented in green) may stimulate cross-reactive tolerizing or Treg responses, if both bind to the same SLA allele.

an elevated level of conservation between putative epitopes in the input peptide and epitopes in the reference database. Using this threshold, we identified epitopes that are more likely to be tolerated or actively regulatory (19). For a given EpiMatrix Score, a high JanusMatrix Homology Score suggests that T cells recognizing that epitope may exhibit a bias toward immune tolerance, which has been validated in retrospective and prospective studies (3) in the human context. More remains to be done to evaluate whether the same observation is true in swine.

PIGMATRIX

In general, the development of models for prediction of T cell epitopes requires a large amount of experimental data for training and testing. A variety of approaches provide data that can be used to define peptide:MHC binding rules and enable binding predictions, all of which have been applied to identification of SLA ligands. High throughput methods that define MHC binding peptides include biochemical assays that measure peptide:MHC binding affinity or a proteomics approach that uses immunoprecipitation of solubilized peptide:MHC complexes from the cell surface followed by peptide elution and liquid chromatography/mass spectrometry (20–22). Additionally, in a low throughput manner, epitope-specific T cell lines are used to define binding anchor residues by assaying epitope variants at anchor positions for T cell stimulation as measured by cytokine or chemokine release (23). While such binding data are abundantly available for HLA, they are limited for MHC of other species. Only one online tool algorithm has been trained and evaluated for prediction of SLA class I alleles (24). Prediction tools have not been available for SLA class II alleles. To overcome this lack of binding data for SLA, PigMatrix leverages similarities between the secondary structure of HLA and SLA molecules and predefined HLA binding preferences to generate SLA epitope predictors based on the pocket profile method (5, 25).

The crystallographic structure of HLA molecules reveals that the peptide-binding groove contains a number of pockets and that polymorphic residues in the HLA sequence are often involved in forming these pockets (26). Consequently, the residues in the pocket define allele-specific binding preferences for particular amino acid side chains of the antigenic peptides (27). Thus, for each MHC molecule, the profile of a given binding pocket can be defined by its residues and binding preferences. Sturniolo et al. demonstrated that each "pocket profile" was nearly independent of other pockets in the HLA-DR binding groove (25). The authors also showed that an MHC molecule could be defined in terms of its individual pocket profiles as a quantitative matrix of binding preferences. Therefore, once a pocket profile is determined experimentally, it can be shared with other HLA-DR molecules that have identical pocket residues.

A number of pan-specific algorithms for T cell epitope prediction based on the pocket profile method have been developed, including TEPITOPE (25), TEPITOPEpan (28), and PickPocket (29). The predictive performance of these methods for novel HLA alleles depends on the similarity of pocket residues; performance decreases as similarity decreases (29). For HLA alleles with limited quantitative data, algorithms based on the pocket profile method have demonstrated better or comparable performance when compared to methods, such as artificial neural networks, that require a large amount of training data (28, 29). NetMHCpan, an artificial neural network-based algorithm, has been used for prediction of SLA class I-restricted peptides (24, 30).

PigMatrix (5) is the first algorithm that was designed for the prediction of SLA class II T cell epitopes. Using the Sturniolo et al. approach described above, PigMatrix matrices were created by integrating the binding preferences of the best-matched HLA pocket for each SLA pocket, using SLA or HLA crystal structures as a basis for pocket selection. PigMatrix achieved a favorable predictive performance, comparable to or better than PickPocket and NetMHCpan for SLA class I alleles (5). PigMatrix class II epitope predictions were validated prospectively (see section "Swine Influenza A Virus Vaccine" below). Overall, using the pocket profile method for SLA, and defined binding preferences from HLA, shows promise for developing T cell epitope prediction tools for pigs.

Limitations of PigMatrix: Class I and II SLA Coverage

To effectively harness epitope immunoreactivity data, the identity of SLA alleles involved in peptide presentation to T cells is required. This information is needed to establish knowledge of the prevalence of allelic families on a population level, which is used in turn to ascribe immunological significance to epitope-specific T cell responses detected in infection and vaccine studies. Furthermore, knowledge of MHC allele sequences is required for T cell epitope prediction.

The diversity of SLA and the lack of information on SLA frequencies represent a significant challenge for the development of T cell epitope vaccines for swine (31). The problem of SLA coverage is illustrated by a small swine influenza vaccine immunogenicity study that was performed using PigMatrix-identified T cell epitopes, SLA alleles expressed by the pigs in the study cohort were different from those reported to be prevalent in the United States swine population. Information about SLA allele diversity in the United States swine population is critically important to develop a more comprehensive set of predictions that target the most prevalent SLA alleles. Once the prevalence and diversity of United States swine SLA are better understood, it may be possible to cluster SLA molecules into supertypes. The concept of supertypes has been applied to HLA for selection of few representative alleles from different clusters to cover a high percentage of the HLA diversity in the human population (32, 33). An epitope-based vaccine containing peptides predicted to bind SLA supertype alleles could induce immune responses in pigs expressing diverse alleles.

Fortunately, the importance of SLA diversity for vaccine development and studies to identify commonly expressed haplotypes has been recognized and new studies are expanding available information on prevalent SLA alleles in swine

poulations (34). Currently, the Immune Polymorphism Database lists 90 SLA-1, 96 SLA-2, 41 SLA-3, and 99 DRB1 alleles. Continuing efforts to expand the identification of specific alleles are needed, as are studies that will determine allelic frequencies on a population level for prediction of T cell epitope binding for vaccine development and analysis of epitope-specific T cell responses in infection and vaccination.

SLA typing is commonly performed using sequence-specific primers in PCR (PCR-SSP) (35, 36). This is a labor-intensive approach that yields low resolution results at the allele group level; e.g., SLA-1*08XX refers to a group of alleles that encode the SLA-1*08 antigen or sequence homology to other SLA-1*08 alleles. Improved resolution to four digits is needed to identify specific allele proteins (e.g., SLA-1*0801, SLA-1*0802). High-resolution and high-throughput methods have also been developed (37). Next generation sequencing is a widely used technology for HLA typing (38, 39) and has been used for SLA typing in a few studies (40, 41). A commercially available high-throughput method for high-resolution SLA-typing would improve the ability of researchers and producers to determine SLA diversity.

EPITOPE CONTENT COMPARISON (EpiCC)

Using PigMatrix, it is possible to identify potential T cell epitopes and rank proteins based on their immunogenic potential. In addition to immunogenicity, vaccines need to induce memory T cells that will recognize epitopes contained in circulating strains. In other words, the epitope content of a vaccine should be similar to that of the circulating strains to elicit broad immune recognition and protection.

To estimate the relationship between pathogen sequences based on their putative T cell epitope content and predict cross-protection potential, we developed the T cell Epitope Content Comparison tool (EpiCC) which facilitates sequence pairwise comparison based on epitope content rather than sequence identity (42). EpiCC assesses the relatedness of T cell epitopes contained in a protein sequence of one strain and those in another based on a comparison of the epitope sequences and their PigMatrix SLA binding score. T cell epitopes can be either shared (cross-conserved) between sequences, or unique to each strain. Thus, the EpiCC score for the comparison of two strains is based on the PigMatrix scores of shared and unique epitopes, which are defined using JanusMatrix. For a pair of protein sequences, the EpiCC score is high if the epitope content shared between both sequences is dense and similar. For comparison of a vaccine and outbreak strains, vaccine sequences that share more T cell epitope content with circulating strains have higher EpiCC scores.

EpiCC can be applied to estimate whether a given vaccine would protect against circulating or newly emerging strains of a pathogen. It can also potentially be used to assist in the selection of live or killed organism vaccine candidates by comparing one or multiple antigens and identifying the vaccine strain sequence that best represents the T cell epitope content of circulating strains and that may induce the broadest cross-reactive T cell response. See for example, the publication by Bandrick, M. et al., comparing monovalent and bivalent PCV2 vaccines to field strains (43). EpiCC also has applications for analysis of large-scale surveillance data to identify circulating or novel viruses distantly related to current vaccines for further experimental evaluation to determine potential risk of vaccine failure.

CASE STUDIES

Vaccine Development Against Swine Pathogens Using the iVAX Toolkit

Swine Influenza A Virus Vaccine

Influenza A virus (IAV) is considered one of the most important infectious disease agents affecting North American swine (44). The majority of currently licensed swine IAV vaccines consist of whole inactivated viruses administered with adjuvants by intramuscular injection (45). This platform primarily induces systemic IgG antibody responses to the surface glycoproteins, mainly HA (45, 46). However, antibody-mediated immunity does not typically provide protection against divergent strains of IAV (46, 47). In contrast, CMI can be broadly cross-reactive to a variety of IAV subtypes (48, 49). Moreover, CMI contributes to virus clearance, reduces symptom severity, and virus shedding (50). A vaccine that can induce CMI and reduce morbidity could prevent anorexia and weight loss in swine, which cause significant economic loss to pork producers. Therefore, the identification of T cell epitopes conserved in diverse strains of IAV represents the first step toward the development of a potentially broadly protective vaccine.

Using PigMatrix and Conservatrix, the complete proteomes of representative IAV strains in a United States swine population were screened for class I and II T cell epitopes (31). EpiAssembler was used to construct immunogenic consensus sequences - peptides of 16–25 amino acid containing SLA-DRB1-restricted epitopes that were highly conserved in IAV strains, predicted to bind to multiple alleles, and enriched for immunogenicity. Using VaxCAD, 28 class I and 20 class II predicted epitope sequences were concatenated into two multi-epitope genes (one for SLA class I and one for class II epitopes). Cleavage promoting spacers or binding inhibiting "breaker" sequences were introduced where VaxCAD reordering did not eliminate junctional immunogenicity. Vaccine genes were synthesized and subcloned into vectors containing signals for proteasome or secretory pathway targeting.

The immunogenicity of the 48 predicted T cell epitopes was determined by measuring IFNγ recall responses using PBMCs from pigs immunized intramuscularly with the prototype DNA vaccine. Positive responses were observed upon restimulation with pooled peptides as well as eleven individual peptides. Recall responses to peptides were not observed in pigs immunized with a tetravalent inactivated commercial vaccine, despite containing similar internal antigens. This result suggested that the epitope-based DNA vaccine promoted more efficient processing and presentation of its own epitopes as compared to whole-protein-based vaccines.

In a vaccine challenge study, intradermal immunization with the epitope-based DNA vaccine followed by an intramuscular

tetravalent inactivated vaccine boost was effective against H1N1 homosubtypic challenge. Pigs had reduced lung lesions and no detectable IAV antigen at necropsy. Moreover, IFNγ secreting cells, recognizing vaccine epitope-specific peptides and pH1N1 challenge virus were highest in PBMCs from pigs vaccinated using the prime-boost approach (51).

African Swine Fever Vaccine

African swine fever virus (ASFV) is the etiological agent of African swine fever (ASF), a highly contagious hemorrhagic disease of swine that affects domestic pigs and wild boars of all ages and breeds. Several clinical forms of ASF are presented in swine and include a hyper-acute or acute disease, a sub-acute disease and a chronic disease with mortality rates ranging from 100 to 3% depending on the virulence of the viral isolate, route of infection, and the host (52, 53). ASFV transmission to unexposed domestic pigs occurs by direct contact with an infected animal or the body fluids and carcasses of infected animals, or by indirect contact with contaminated materials or through the consumption of contaminated products (54). Wild pigs and soft ticks of the genus *Ornithodoros* are the natural reservoir for the ASF virus (55).

ASF poses a devastating threat to the global pig industry and has been spreading at an alarming rate in the past few years, affecting more than 55 countries in three different continents: Africa, Asia, and Europe (56). The introduction of ASF into these countries has dramatically impacted their socio-economics, pig production and status for international trade (57). Prevention, control, and eradication measures for ASF are mainly based on early detection and on the implementation of strict sanitary measures (58). However, successful control of ASF has proven to be challenging and the risk of introducing the virus into ASF-free countries is increasing. A vaccine against ASF is urgently needed to improve prevention and control strategies and mitigate major economic losses in endemic and non-endemic areas.

No licensed vaccine currently exists against ASF. The complexity of the virus and the large number of encoded proteins, with some involved in the modulation of host immune responses (59, 60), has made it challenging to identify immunogenic targets and hindered the development of an efficacious ASF vaccine. Another challenge is the genetic diversity of the ASFV and the limited knowledge of antigens involved in conferring cross-protection. Thus far, little to no cross-protection has been reported (61–63); however, pigs that survive ASFV infection generate protection against subsequent infections with a homologous ASFV (58). Several efforts have been made to develop an ASF vaccine with a current focus on the induction of both humoral and cellular immune responses due to their potential role in conferring ASF protection (64–67).

Using iVAX, we developed a T cell-directed ASF vaccine composed of swine MHC class I and class II epitopes conserved across 21 European, Asian and African isolates covering genotypes I, II, IX, and X. T cell epitopes identified by JanusMatrix as potentially regulatory (highly cross-conserved with the swine proteome) were excluded. Multi-epitope genes encoding class I and class II epitopes separately were each subcloned into plasmids to produce a DNA vaccine. The vaccine has undergone immunogenicity testing and is immunogenic (unpublished collaboration); further development is currently anticipated in collaboration with a commercial animal vaccine company.

Applications of EpiCC

Swine Influenza A Virus Vaccine Analysis

For influenza and other viruses, sequence data and antibody cross-reactivity are commonly used to predict vaccine-induced protection (45, 46). However, previous efficacy studies demonstrated that even in the absence of cross-reactive antibodies, a commercial swine IAV vaccine was capable of inducing protection or partial protection (reduced lung lesions, reduced viral titers in lungs and/or nasal swabs) against heterologous challenge strains (46, 68–72).

To determine the potential role of T cell epitope-driven CMI in vaccine-induced protection in the absence of cross-reactive antibodies, an EpiCC analysis was performed to compare the T cell epitope content of HA sequences from swine IAV strains representing the major H1 clusters circulating in the North American swine population and those of H1 viruses in a commercial vaccine. Using experimental data from previous vaccine efficacy studies testing one of the H1 viruses in the commercial vaccine against different challenge viruses (46, 68–70, 72), a threshold level of T cell epitope relatedness associated with protection was identified. The published results provided supportive evidence that T cell epitopes that are conserved between vaccine sequences and circulating strains contributed to vaccine efficacy. We have provided a typical EpiCC analysis, using example influenza vaccines and strains, for illustration purposes, in **Figure 5A**.

For the initial influenza study, EpiCC analysis was restricted to HA sequences from 23 viruses representing diverse clusters of field strains, assuming limited T cell epitope variation of other antigens. However, the same approach is currently being applied to multiple antigens or to complete proteomes of influenza strains, and to hundreds of variant strains representing other pathogens such as PCV2. We anticipate that EpiCC may complement existing methods for vaccine selection in outbreak situations and could be used by animal vaccine companies for strain selection during vaccine development.

Swine Rotavirus Vaccine Analysis

We have also applied EpiCC to understand vaccine strain selection for swine rotavirus. Swine rotavirus serogroups A and C (RVA and RVC, respectively) are a significant cause of piglet morbidity and mortality across the world. The outer capsid of the RV particle is composed of the viral proteins VP7 and VP4, both of which are targets for neutralizing immunity and they also determine the G and P genotypes of RV strains (73). Cross-protection between RVA and RVC is non-existent while heterotypic immunity across different G and P genotypes remains limited (74). Given the large genetic diversity of RV genotypes, vaccination efforts have been limited. There is one currently available commercial vaccine that only contains three strains of RVA (75, 76). RV vaccine strains with high T cell epitope conservation with circulating strains may induce broader cross-protective immunity. Using EpiCC and PigMatrix, we investigated the presence of SLA class II putative T cell epitopes

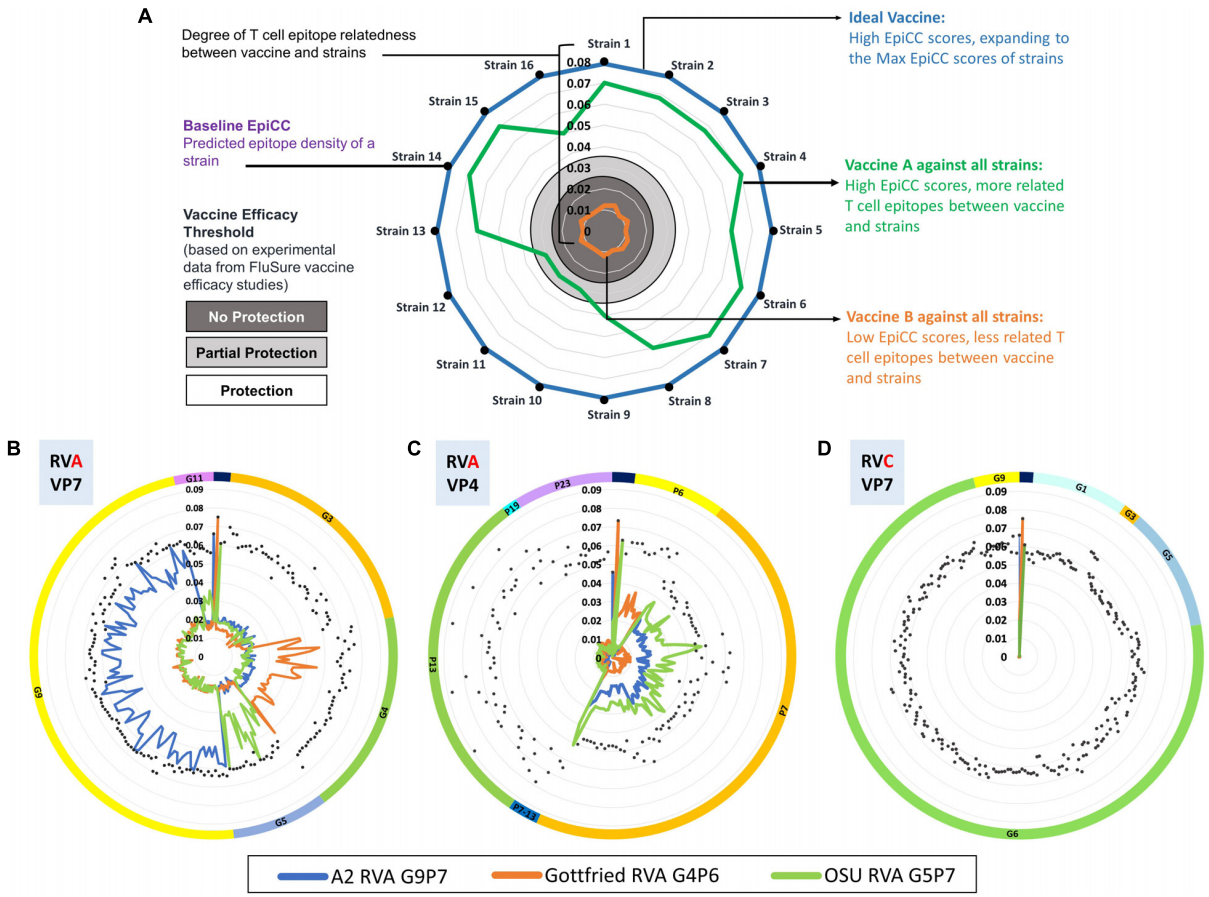

FIGURE 5 | EpiCC radar plot. Radar plots are used to visualize the relationship between vaccine strains and circulating strains. In the top plot, Panel **(A)** for illustration purposes, 16 typical circulating swine influenza A virus strains are presented on the perimeter of the chart. The EpiCC scores of their HA antigens (hemagglutinin) are indicated by their distance from the center to the perimeter. Vaccine efficacy thresholds for protection (white area), partial protection (light gray area) and no protection (dark gray area) have been based on experimental data from efficacy studies of swine Influenza A virus vaccines (17). The blue line represents an example of an ideal vaccine strain that contains T cell epitopes fully matched to all the circulating strains. The green line represents an example of an influenza vaccine strain HA protein that contains T cell epitopes well matched to the majority of circulating strains. The orange line represents an example of a vaccine strain HA protein that contains T cell epitopes not well-matched to any of the circulating strains. This example is intended to illustrate how EpiCC provides guidance on vaccine selection but does not provide data on any specific influenza strains. An example of how EpiCC can be used is provided for a set of swine rotavirus vaccines and circulating rotavirus strains. Rotaviruses (RVs) are among the most common causes of acute diarrheal disease in humans and swine. Speciation of RVs is based on sequencing of the viral protein (VP) 6, the middle capsid protein. Rotavirus group A (RVA) is the most prevalent and pathogenic species of RV. The VP7 and VP4 proteins stimulate neutralizing antibodies and are used as a binary classification system for genotypes (G and P genotypes, respectively). Due to the binary classification system, we have performed an EpiCC analysis based on comparisons of the VP7 and VP4 components of each strain and their equivalent viral protein-specific vaccine components VP7 and VP4. In Panel **(B)**, we compare RVA strain VP7 proteins to the VP7 component of the vaccine, and in Panel **(B)** we compare RVA strain VP4 proteins to the VP4 component of the vaccine. The viruses are sorted by genotype (by G for Panel **(B)** and by P for Panel **C**); the classification is highlighted by the color of the outermost circle (orange for G3 and green for G4 and so on). Each of the three RVA strains in the ProSystems vaccine is represented with a different colored line: the blue line represents the A2 RVA strain which contains viral proteins derived from genotypes G9 and P7), the orange line the Gottfried RVA strain (which contains G4 and P6) and the green line the OSU RVA strain (G5 and P7). In Panel **(B)**, the EpiCC scores of the A2 vaccine strain (G9P7) are highest against strains that fall into the same genotype (G9) and low for all other genotypes. The EpiCC scores of the Gottfried strain (G4P6) are highest for strains that are in genotype G4 but low against other strains. This suggests that vaccine strains are more related to homologous field strains than to other strains. Therefore, the T cell epitope content of circulating swine rotavirus strains is highly genotype specific explaining why it is necessary to use genotype-specific RVA vaccines to protect against field strains. Panel **(D)** illustrates the expected finding that swine RVA vaccine strain VP7 has no conservation against circulating strains from rotavirus group C (RVC) VP7. If T cell epitopes are protective against swine rotavirus, a 'universal' RV vaccine would need to include T cell epitopes representing all of the genotypes.

in the VP7 and VP4 of circulating porcine RVA and RVC strains and assessed the degree of their cross-conservation with the RVA strains in the ProSystems Rota vaccine (77). This data is shown in **Figures 5B,C**.

To perform this analysis, we first used PigMatrix to identify SLA class II-restricted T cell epitopes in a set of VP7 and VP4 proteins of RVA and RVC strains circulating in the United States as well as in the RVA strains Gottfried (G4P[6]), OSU (G5P[7]) and A2 G9P[7]) (76) that are used in the ProSystems Rota vaccine. We then performed an EpiCC analysis to assess the relationship between the T cell epitopes found in VP7 and VP4 of circulating RVA strains and the T cell epitope content of the

RVA vaccine. The analysis demonstrated that T cell epitope cross-conservation between circulating strains and the RVA vaccine is genotype-specific and is limited to homologous strains as seen in (**Figures 5B,C**). In other words, T cell epitopes from the vaccine's G9 genotype strain (called A2) are only conserved with field strains that belong to the G9 genotype, and this was also true for the G4 (Gottfried) and G5 (OSU) vaccine strains. There was very limited conservation between the T cell epitopes of the VP7 protein in the RVA vaccine with T cell epitopes found in non-homologous VP7 proteins in other genotypes of RVA (Panel B). This was also true when the VP4 protein is considered (Panel C). Thus, the existing RVA vaccine has genotype-specific T cell epitope content.

We then performed the same EpiCC analysis to assess the relationship between the VP7 of circulating RVC strains and RVA vaccine strains. The results again show that swine RVA VP7 T cell epitopes are serogroup specific and are not at all cross-conserved with the VP7 of RVC strains (**Figure 5D**). This study demonstrates that T cell epitopes found in circulating swine and vaccines are serogroup and genotype-specific, and may explain why vaccines to protect against swine rotavirus have to be multivalent.

Porcine Circovirus Type 2

Porcine circovirus type 2 (PCV2) is one of the top infectious agents in the porcine industry. Eight PCV2 genotypes have been described based on ORF2 phylogenetic analysis (78). Due to its remarkable evolutionary rate, further genetic variation of PCV2 is expected, limiting the usefulness of single vaccine strains. Currently, PCV2a, PCV2b, and PCV2d are considered to be clinically relevant causes of disease in swine populations, and PCV2d is currently the predominant genotype. However, most of the commercial vaccines available are based on the PCV2a genotype (79).

PCV2 vaccines were based on the 2a genotype because this was the first genotype that was discovered. Currently, eight genotypes of PCV2 viruses are known to circulate in swine populations, and further variation in PCV2 is expected. For these reasons, there is a need to determine how well existing and future vaccines cover field strains. We therefore used EpiCC to analyze the sequences of two major structural proteins, the replicase (encoded by ORF1) and the capsid (encoded by ORF2) from selected vaccines and compared the epitopes in the vaccines to those found in field strains. The two commercial vaccines that were analyzed in this study were based on PCV2a, PCV1-PCV2a chimeric virus (cPCV2a), an experimental PCV1-PCV2b chimeric virus (cPCV2b), and an experimental combination of cPCV2a and cPCV2b provided by the study's co-authors at Zoetis.

The putative T cell epitope content of these vaccines was compared to that of 161 field strains representing PCV2 genotypes a-f using EpiCC (43). The analysis, performed using EpiCC and PigMatrix, demonstrated that the combination cPCV2a-cPCV2b vaccine had, on average, the highest EpiCC score against circulating strains. EpiCC scores of this vaccine were higher than those of the monovalent vaccines not only for PCV2a and PCV2b, but also PCV2d, which suggested that developing the combination vaccine would be preferable to developing a monovalent vaccine against the predominant circulating strain. EpiCC analysis suggested that the combination of cPCV2a and cPCV2b would confer the broadest cross-reactive cell-mediated immunity and protection against field strains (43).

CONCLUSION

Recent developments in computation and genomics usher in new opportunities to address these unmet needs using immunoinformatic tools for accelerated design of safe and effective vaccines starting from sequence data. However, more research is needed. For example, further development of PigMatrix is necessary, to enable prediction for the broad range of SLA alleles that exist in global pig populations. Larger datasets of SLA-restricted peptides are required to further evaluate the PigMatrix approach and improve predictions. To generate quantitative binding data and test PigMatrix, binding assays for commonly expressed SLA molecules could be developed. Currently, these assays have been developed for a limited number of SLA class I and II alleles (24, 80–82). Binding assays provide valuable information to better define binding preferences and potentially develop predictions based on SLA specificities rather than pocket preferences. High-throughput binding assays using planar peptide microarrays have been applied to produce large amount of data (83). This technology could generate the data required to train and test SLA-specific models. One of the most significant interventions that would promote progress on new epitope-prediction models for additional SLA would be funding to carry out these studies.

Improvements to current methods of vaccine development are needed to protect swine from devastating pathogens and to stabilize the global food supply. Introduction of PigMatrix into the iVAX vaccine design platform has enabled demonstration of a heterologous prime-boost immunization strategy that protects against IAV and can be applied to other pathogens (51). Additionally, integrating PigMatrix into iVAX enables the comparison of related strains of highly variable pathogens to guide rational selection of candidate vaccine strains to advance to field trials and implementation. These novel computational tools are a valuable resource for countering pig-associated zoonotic disease to lower burden on pig production and human health.

In the context of epidemic outbreaks of infectious diseases, SLA-restricted epitopes can be identified and vaccines designed in under 48 h (84). Therefore, this computational "vaccines on demand" approach can be applied to other swine diseases of economic importance to accelerate vaccine development timelines by rapidly generating vaccine designs ready for production and testing. We note that requests for access to the tools for academic research can be directed to the University of Georgia technology transfer office, where two of the authors (ADG and LM) now have faculty appointments.

As illustrated here, vaccine design using the PigMatrix and the iVAX toolkit, may offer some advantages over standard approaches to developing vaccines for pathogens affecting the pork industry. PigMatrix and iVAX tools can be used to (i) accelerate vaccine design for new and emerging pathogens;

(ii) identify highly conserved epitopes from the sequences of diverse strains that are able to drive cross-protective immune responses, reducing the need for developing a vaccine for each new strain of a pathogen; (iii) identify potential regulatory T cell epitopes; (iv) improve existing vaccines by engineering in more T cell epitopes or removing regulatory T cell epitopes; and (v) to predict the efficacy of existing vaccines against newer circulating strains of pathogens.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Conflict of Interest: AD and WM are senior officers and shareholders, and LM, AG, SK, and MA are employees of EpiVax, Inc., a privately owned biotechnology company located in Providence, RI. These authors acknowledge that there is a potential conflict of interest related to their relationship with EpiVax and attest that the work contained in this research report is free of any bias that might be associated with the commercial goals of the company. The iVAX toolkit is currently available for use by commercial developers by subscription or for specific projects under a fee-for-service arrangement. Academic researchers are invited to contact the authors at University of Georgia (UGA), or the technology transfer office at the UGA School of Veterinary Medicine, for access to the iVAX Toolkit for research purposes.

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