REPRODUCTION

Characterization of local and peripheral immune system in pregnant and nonpregnant ewes

Laurel D. Quirke,[†] Paul H. Maclean,[‡] Neville A. Haack,[‖] Sara J. Edwards,[†] Axel Heiser,[‖] and Jennifer L. Juengel[†,1]

[†]Reproduction, Animal Science, Invermay Agricultural Centre, AgResearch Ltd, Mosgiel, 9092, New Zealand, [‡]Bioinformatics and Statistics, AgResearch Ltd, Grasslands Research Centre, Private Bag 11008, Palmerston North, New Zealand, [‖]Infectious Diseases, Hopkirk Research Institute, AgResearch Ltd, Private Bag 11008, Palmerston North, 4442, New Zealand

[1]Corresponding author: jenny.juengel@agresearch.co.nz

ORCiD number: 0000-0002-2717-7311 (J. L. Juengel).

Abstract

Modulation of the immune system is known to be important for successful pregnancy but how immune function might differ between the lymph nodes draining the reproductive tract and peripheral lymph nodes is not well understood. Additionally, if immune system changes in response to the presence of an embryo during early pregnancy, and if this response differs in local versus peripheral immune tissue, has not been well characterized. To address these questions, we examined expression of genes important for immune function using NanoString technology in the ampulla and isthmus of the oviduct, endometrium, lymph nodes draining the reproductive tract (lumbo-aortic and medial iliac) as well as a peripheral lymph node (axillary), the spleen, and circulating immune cells from ewes on day 5 of the estrous cycle or pregnancy. Concentrations of estradiol and progesterone in plasma were also determined. Principal component analysis revealed separation of the local from the peripheral lymph nodes (MANOVA $P = 3.245e-08$, $R^2 = 0.3$) as well as separation of tissues from pregnant and nonpregnant animals [lymph nodes (MANOVA $P = 2.337e-09$, $R^2 = 0.5$), reproductive tissues (MANOVA $P = 2.417e-14$, $R^2 = 0.47$)]. Nine genes were differentially ($FDR < 0.10$) expressed between lymph node types, with clear difference in expression of these genes between the lumbo-aortic and axillary lymph nodes. Expression of these genes in the medial iliac lymph node was not consistently different to either the axillary or the lumbo-aortic lymph node. Expression of *IL10RB* was increased (FDR < 0.05) by 24% in the reproductive tissue of the pregnant animals compared to nonpregnant animals. Analysis of gene categories revealed that expression of genes of the T-cell receptor pathway in reproductive tract tissues was associated ($P < 0.05$) with pregnancy status. In conclusion, assessment of gene expression of reproductive and immune tissue provides evidence for a specialization of the local immune system around the reproductive tract potentially important for successful establishment of pregnancy. Additionally, differences in gene expression patterns in reproductive tissue from pregnant and nonpregnant animals could be discerned as early as day 5 of pregnancy. This was found to be associated with expression of genes important for T-cell function and thus highlights the important role of these cells in early pregnancy.

Key words: early pregnancy, immune system tissues, inflammation, reproductive tissues

Abbreviations

JAK/STAT	Janus kinase/signal transducer and activator of transcription
PCA	principal component analyses
RCC	reporter code count
SEB	Staphylococcal enterotoxin B
WBC	white blood cells

Introduction

Comparisons between events occurring during early embryo development in marsupials and eutherians have led to the theory that, in placental mammals, pregnancy is successfully established through co-opting the genes involved in normal immune response for its benefit, in essence "turning a foe into a friend" (Chavan et al., 2017; Griffith et al., 2017, 2018). Genes normally associated with immune functions such as *CSF2*, *IL1B*, and *LIF* are also expressed in the embryo and regulate early embryo development (Hansen, 2014). Several genes linked to immune functions such as *DBQ* (MCH class II antigen), *PTGS2*, *ALOX5*, *ITGA6*, and *IL2* are differentially expressed in reproductive tissues and immune cells in models of greater and lesser fertility (Wathes et al., 2009; Killeen et al., 2016; Dickinson et al., 2018). Furthermore, inflammatory disease and immune cell trafficking pathways are differentially expressed in animal models of differing fertility (Valour et al., 2013; Killeen et al., 2016).

The immune system is, however, presented with the unique challenge of providing a localized environment in the reproductive tract that is physiologically distinct from the rest of the animal. This is because cells that would normally be recognized as damaged or foreign (i.e., the embryo) must be protected and supported. In particular, the reproductive immune system needs to accept the developing embryo and fetus, which will express antigens foreign to the mother driven from paternal genes. While it is known that many genes classically recognized as regulating immune function are important in early embryo development (Hansen, 2014; Brown et al., 2017), there is limited information regarding regulation of immune system function during early pregnancy. How this may differ between tissues directly in contact with the embryo or lymph nodes draining this tissue compared to the peripheral immune system remains to be determined. Initial transcriptomic analysis of endometrium from pregnant and nonpregnant cows did not observe a difference in gene expression until around day 13 of pregnancy (Forde et al., 2011; Forde et al., 2012), thus questioning if there was a local response to pregnancy. However, other studies have been able to observe changes in gene expression of oviductal cells in response to the presence of early stage embryos using more targeted techniques to isolate the reproductive tissue in intimate contact with the embryo (Schmaltz-Panneau et al., 2014; Hamdi et al., 2019; Passaro et al., 2019).

The hypothesis tested is that expression of genes associated with immune function would differ between the lymph nodes draining the reproductive tract and peripheral lymph nodes. Furthermore, we hypothesized that gene expression in both reproductive tract and immune tissues would differ between pregnant ewes and those that were at a similar stage of the estrous cycle but were not pregnant. To test these hypotheses, we examined gene expression in the ampulla and isthmus of the oviduct, the endometrium of the uterus, lymph nodes draining the reproductive tract [lumbo-aortic (drains the ovary, oviduct and parts of the uterus) and medial iliac (drains some of the uterus)] as well as a peripheral lymph node (axillary), the spleen, and circulating immune cells from ewes on day 5 of the estrous cycle or pregnancy. Target genes for gene expression analysis were selected based on their general relevance for immunity (Murphy et al., 2007) or because they had been reported as being expressed by the oviduct and are potentially affected by insemination (Fazeli et al., 2004), early pregnancy (Maillo et al., 2015; Martyniak et al., 2018), and/or in context of local inflammation (Paik et al., 2012; Hess et al., 2013; Talukder et al., 2018) or immune responses (Yang et al., 2019a; Yang et al., 2019b).

Materials and Methods

Experimental design

In accordance with the Animal Welfare Act regulations of New Zealand, prior approval for the experiment was granted by the Invermay Animal Ethics Committee (application number 14544). The experiment was carried out at the Invermay Agricultural Center, located in the South Island of New Zealand (45°51′ S, 170°23′ E) in Autumn 2019.

All animals were drenched approximately every 4 wk or as needed to control internal parasites (e.g., Converge, MSD Animal Health, Upper Hutt, New Zealand), or as required for the control of flystrike and lice (e.g., Cyrex Liquid, Elanco Animal Heath, Auckland, New Zealand). All animals were supplemented with vitamin B12 at weaning (SmartShot B12 Prime Lamb, Virbac, Hamilton, New Zealand) and with iodine (Flexidine, Bayer New Zealand Limited, Auckland, New Zealand). All animals received vaccinations against toxoplasmosis, campylobacter, leptospirosis, and clostridial diseases (Toxovax, MULTINE 5-in-1 Selenised, Campyvax4, MSD Animal Health, Upper Hutt, New Zealand; Ultravac 7in1, Leptoshield, Zoetis New Zealand Limited, Auckland, New Zealand). Campyvax4 vaccination and iodine supplementation were administered approximately 4 wk prior to the introduction of the rams.

A total of 140 composite ewes (3.5 to 4.5 yr old; dominant breeds Coopworth, Romney and Texel) were equally divided into two groups (based on weight and body condition score) and joined with either three adult intact rams fitted with a marking harness (pregnant group), or three adult intact rams fitted with a marking harness and a NOMATE teaser harness (Rurtec Limited, Hamilton, New Zealand; nonpregnant group). A teaser harness fitted to the intact ram was used to exclude seminal fluid being a source of possible immune response in the reproductive tract.

The average weight of ewes assigned to each group at the time of joining was 73 kg (range: 59–93 kg) with body condition scores ranging from 3 to 4. Body condition was scored on a 0-to-5 (very thin to very fat) scale by tactile evaluation of body fat deposition (Beef+Lamb, 2019). Ewes were inspected for mating marks at 9:00 a.m. and 4:00 p.m. daily. Ewes that showed a new mating mark between the hours of 9:00 a.m. and 4:00 p.m. (considered as day 0) were assigned to tissue collection on day 5. Day 5 was chosen for tissues collection as this represents the time when the embryo would just have left the oviduct and entered the uterus. This allows for the maximum exposure of the oviduct to the embryo while also allowing examination of endometrial differences that might occur around the time the embryo initially enters the uterus around day 4 (Murray et al., 1995; Spencer et al., 2004).

Sample and tissue collection

At day 5, a 20 mL blood sample was collected by venipuncture into tubes containing lithium heparin anti-coagulant (Becton Dickinson, New Jersey, USA). Ten milliliters of whole blood were centrifuged at 1300 × g for 20 min, and the plasma stored at

–80 °C until quantification of progesterone and estradiol was performed. Six milliliters of whole blood were processed for in vitro stimulation of leukocytes.

At day 5, a total of 28 animals were euthanized by captive bolt and exsanguination to obtain ovulation rate number, collect embryo and/or unfertilized oocytes, and collect tissue samples from a final number of 10 pregnant animals and 10 nonpregnant animals. The tissue samples collected included the spleen; axillary, medial iliac, and lumbo-aortic lymph nodes; oviduct (isthmus and ampulla); and endometrium. Although tissue was collected from both the left and right sides of the animal (except spleen), the criteria for the inclusion of an animal in the final number were the presence of a corpus luteum and exposure of the oviduct to an embryo (as established by recovery) or unfertilized oocyte (established by presence of corpus luteum and confirmed by recovery of the unfertilized oocyte in the majority of animals).

The ovulation rate for each ovary was determined by the number of corpora lutea with visible ovulation stigmata. To recover embryos and/or unfertilized oocytes, a 10 cm long polyethylene tube (OD 2.5 mm, ID 1.5 mm, Tyco Electronics, Biocorp Aust Pty Ltd, Huntingdale, Victoria, Australia) was inserted into the fimbria of the oviduct. Using a syringe and blunt needle, 5 mL of compound sodium lactate (Hartmann's) solution (Baxter Healthcare Pty. Ltd, Toongabbie, Australia) supplemented with 1 mM 3-(N-morpholino) propanesulfonic acid (MOPS) were introduced into the uterine horn, with the flush collected from the polyethylene tube. Each side of the reproductive tract was flushed twice. The recovered embryos and/or unfertilized oocytes were assessed and graded as previously described (O'Connell et al., 2016). The effectiveness of the NOMATE teaser harness was confirmed by the absence of embryos in the nonpregnant group.

Reproductive tissue was collected from the oviduct/uterine horn ipsilateral to the corpus luteum. A 1 cm piece of the isthmus of the oviduct adjacent to the utero-tubal junction and a 2 cm piece of the ampulla of the oviduct adjacent to the junction of the ampulla and isthmus were collected. A piece of uterus adjacent to the utero-tubal junction was also collected, dissected open, and the endometrium layer separated and collected. All reproductive tissues were frozen in liquid nitrogen and stored at –80 °C.

An axillary lymph node, located on the medial surface of the teres major muscle, was identified (Yen et al., 2019), and collected from both the left and right sides of each animal. Based on schematic diagrams as described in Staples et al. (1982), the medial iliac and lumbo-aortic lymph nodes were identified and collected from both the left and right sides of each animal. The medial iliac lymph node is situated near the dorsal aorta, between the internal and external iliac arteries. The lumbo-aortic lymph node is situated near the junction of the uterine artery and the dorsal aorta. Nodes were removed by dissection and the visible fat removed. A piece of the spleen was also collected and along with the lymph nodes, all were frozen in dry ice and stored at –80 °C. The lymph nodes used for further analysis were ipsilateral to the recovered embryo/corpus luteum. For one pregnant animal, the medial iliac lymph node ipsilateral to the recovered embryo provided low-quality RNA and thus the other medial iliac lymph node was used for RNA isolation. Additionally, for one nonpregnant animal, high-quality RNA was not isolated from the axillary lymph node from the side chosen for isolation and thus the other axillary lymph node was used for analysis. Both ewes had ovulated from both ovaries.

Analysis of progesterone and estradiol in plasma

Concentrations of progesterone were quantified in plasma samples using a Coat-a-Count radioimmunoassay (IBL, Hamburg, Germany) as described previously (Smith et al., 2019). The sensitivity of the assay was 0.2 ng/mL, and the intra-assay CV of three quality control samples with average progesterone concentrations of between 9.02 and 1.79 ng/mL was <13%. Concentrations of estradiol were quantified in plasma samples using an Ultra-sensitive Estradiol radioimmunoassay kit (Beckman Coulter, California, USA) as described previously (Juengel et al., 2020). Recovery of 25 pg estradiol spiked into plasma averaged 103.3 ± 5.46%. The corrected sensitivity of the assay was 2 pg/mL, and the intra- and inter-assay CV of three quality control samples with average estradiol concentrations of between 25.8 and 80.8 pg/mL included in each assay was all <16 %.

In vitro stimulation of whole blood

Whole blood was processed for in vitro stimulation with *Staphylococcal enterotoxin* B (SEB) as described previously (Lange et al., 2019), with modifications. SEB is a so-called superantigen, which causes nonspecific activation of T-cells by binding to the T-cell receptor. SEB is commonly used for T-cell stimulation in cell culture assays. Briefly, 3 mL of whole blood were stimulated with SEB (Sigma Aldrich, Missouri, USA) to a final concentration of 1 µg/mL. For each blood sample, another 3 mL was processed without stimulation. Samples were incubated for 23 to 24 h at 37 °C. For lysis of the red blood cells, 42 mL of sterile H_2O were added to each sample, gently mixed, and incubated at room temperature for 1 min. Five milliliters of 10× phosphate buffered saline (1.37 M NaCl, 27 mM KCl, 100 mM Na_2HPO_4, 18 mM KH_2PO_4) were added to each sample, followed by centrifugation at 1000 × g for 10 min. Following centrifugation, the supernatant was discarded, and the white blood cell pellet was dried for 5 min at room temperature aided by inversion of the tubes. Each cell pellet was resuspended in 200 µL RLT Buffer (Qiagen, Hilden, Germany) and mixed gently for 30 s to disperse the cells. Lysates were stored at –80 °C.

RNA isolation procedure

Total RNA was extracted and isolated from the frozen tissue using a Nucleospin miRNA kit (Macherey-Nagel, Duren, Germany) as per the manufacturer's instructions. Samples were homogenized using an automated tissue homogenizer (SPEX SamplePrep 2010 Geno/Grinder, New Jersey, USA) with 3 × 5 mm stainless steel beads for 3 min at 1500 rpm. Homogenates were incubated for 5 min at room temperature and then cleared by centrifugation at 11,000 × g for 1 min. RNA isolation was then performed on the cleared lysate according to the manufacturer's instructions. The total cellular RNA was eluted in 40 µL RNase-free water and stored at –80 °C.

RNA quantity and quality assessment

RNA quantity was measured using a Qubit BR RNA kit with a Qubit 4.0 Fluorometer (Thermo Fisher Scientific, Massachusetts, USA), following the manufacturer's instructions. The integrity of the RNA was determined using an Agilent RNA 6000 Pico kit with an Agilent 2100 Bioanalyzer (Agilent Technologies, California, USA), following the manufacturer's instructions.

Gene expression analysis

Gene expression analysis was performed using the nCounter Analysis System (NanoString Technologies Inc., Seattle, WA). The

use of NanoString technology enables RNA expression analysis from either purified RNA or directly from cell lysates without further RNA purification (Malkov et al., 2009) or amplification. The method uses molecular barcodes on gene-sequence-specific probes and single molecule imaging to count RNA copies (Geiss et al., 2008). Briefly, multiplexed probes were designed with two sequence-specific probes for each gene of interest. The capture probe was coupled to biotin as affinity tag. The reporter probe was coupled to a color-coded tag. Each target molecule of interest is identified by the unique color code generated by the ordered fluorescent tags on the reporter probe. The level of expression was measured by counting the number of codes for each mRNA using digital imaging. This allows the analysis of multiple genes from the same sample (multiplexing) using a customized set of probes with distinct bar codes, called a ProbeSet.

The RNA samples prepared from tissues and cell lysates from unstimulated and in vitro stimulated whole blood were analyzed using a ProbeSet consisting of probes specific for 131 genes from *Ovis aries*. Additionally, nine reference genes were included based on published recommendations (Zaros et al., 2010; Vorachek et al., 2013; Mahakapuge et al., 2016; Sahu et al., 2018). A complete list of genes and probe sequences is provided in Supplementary File, Probe Summary. Cell lysates or RNA were hybridized with the ProbeSets according to the manufacturer's instructions (nCounter Elements XT Reagents User Manual; NanoString MAN-10086-01 June 2018). Briefly, RNA (tissues) and cell lysates (WBC) were thawed on ice. Samples were hybridized by adding 13.5 µL of MasterMix and 1.5 µL of cell lysate or RNA per each tube of a 12-tube strip immediately before placing the strip at 67 °C for 22 h. After hybridization, samples were transferred to the nCounter Prep Station which automatically removed excess probe and aligned and immobilized the probe-target complexes in the nCounter cartridge. Sample cartridges were placed in the nCounter Digital Analyzer which counted and tabulated color codes on the surface of the cartridge for each target molecule. Data were retrieved from the analyzer as raw data (Reporter Code Count, RCC) files.

For analysis, RCC files were imported into nSolver Analysis Software v4.0 (https://www.nanostring.com/products/analysis-software/nsolver) and underwent the software's sample quality control routine set to the following criteria: 1) imaging: fields of view registration < 75%; 2) binding density outside the 0.05 to 2.25 range; 3) positive control linearity: positive control R^2 value < 0.95; and 4) positive control limit of detection: 0.5 fM positive control ≤ 2 standard deviations above the mean of the negative controls. All samples used for statistical analysis passed the quality control routine.

Background subtraction was performed by subtracting the geometric mean of 8 internal negative controls from each sample. Positive control normalization was performed using the geometric mean of 6 internal positive controls to compute the normalization factor. The normalization factor of all samples was inside the 0.15 to 15 range.

Reference gene normalization was performed using the geometric mean of counts for the nine reference genes included in the ProbeSet. The average of these geometric means across all lanes was used as the reference against which each lane is normalized. A normalization factor was then calculated for each of the lanes based on the geometric mean of counts for the reference genes in each lane relative to the average geometric mean of counts for the reference genes across all lanes. This normalization factor was then used to adjust the counts for each gene target and controls in the associated lane. The normalization factor of all samples was inside the 0.1 to 30 range.

For the WBC lysates, in each of the pregnant and nonpregnant groups, for one ewe both the unstimulated and stimulated samples failed NanoString quality control analysis. Additionally, one other stimulated WBC lysate from the nonpregnant group failed quality control analysis.

Statistical analysis

The normalized NanoString counts were saved into a text file and loaded into R version 4.0.2 (R Core Team, 2020). Principal component analysis (PCA) was used to understand relationship of gene expression among tissue types. Partial least squares discriminant analyses (PLS-DA) were undertaken to explore overall differences in gene expression within tissue groups and between pregnant and non-pregnant animals within those tissue groups. The "pca" function within the mixOmics R package version 6.12.1 (Rohart et al., 2017) was used to perform PCA and the "plsDA" function in the DiscriMiner R package version 0.1-29 (Sanchez et al., 2013) was used to perform PLS-DA. To evaluate separation of the treatments by the PLS-DA procedure, MANOVAs were performed on the PLS-DA scores using the "Manova" function in the car R package version 3.0-10 (Fox and Weisberg, 2019) in addition to the cumulative R^2 value (percent of variance explained in the dependent variate). To evaluate differences in expression of single genes among tissue types and between pregnant and nonpregnant animals, the "aovp" function in the lmPerm R package version 2.1.0 (Wheeler et al., 2016) was used to perform permutation ANOVAs with 1,000,000 iterations and the "independence_test" function in the coin R package version 1.3-1 was used to perform pairwise permutation tests (Zeileis et al., 2008). Multiple testing correction was performed using the Benjamini–Hochberg (FDR) method (Benjamini and Hochberg, 1995) using the "p.adjust" function in R. Outside of a pathway, individual genes were explored further only when their FDR-corrected P-value was below 0.1. When exploring pathways and gene categories, this was relaxed to genes with uncorrected P-values below 0.05 as not all genes are expected to be highly differentially expressed at once, and transcriptomics only provides a "snapshot" of gene expression at the time of sampling in a complex system. Gene categories were assigned using the KEGG and UniProt pathway database tools within DAVID version 6.8 (Sherman and Lempicki, 2009). Significance of gene categories was evaluated by Fisher exact test (Fisher, 1922) in R. The input to the exact test was a table containing four numbers for each gene and each factor (and the interaction): 1) the number of differentially expressed ($P < 0.05$) genes in the category; 2) the number of non-differentially expressed genes in the category; 3) the number of genes on the Nanostring panel that were differentially expressed ($P < 0.05$) but not in the category; and 4) the number of genes on the Nanostring panel that were neither differentially expressed nor in the category. Correlation tests between counts and the hormone concentrations were performed using the "cor.test" function in R using the default of Pearson correlation.

Results

Characterization of animals used for tissue collection

Average live weight and body condition score as well as concentrations of progesterone and estradiol 17β at the time

of tissue collection are presented in Table 1. No differences (*P* > 0.05) were observed between nonpregnant and pregnant animals for live weight, body condition score, or concentrations of estradiol-17β or progesterone.

Multivariate analyses for tissue types and pregnancy status

Principal component analysis of gene expression patterns highlighted three main tissue clusters, namely, 1) the reproductive tissues (ampulla and isthmus of the oviduct, and uterine horn endometrium), 2) lymph nodes and spleen, and 3) the stimulated and unstimulated white blood cells (WBC; Fig. 1). Close examination of the lymph node and spleen group reveals that the gene expression of the spleen is separate from that of the lymph nodes (Supplementary Fig. 1, see online supplementary material for a color version of this figure). Therefore, further analysis focused on reproductive tissues and lymph nodes, which were analyzed separately to further understand tissue grouping and to explore the effects of pregnancy status on gene expression in these tissues.

Analysis restricted to the lymph nodes revealed moderate separation (MANOVA *P* = 3.245e-08, R^2 = 0.3) of the medial iliac (drains uterus) and lumbo-aortic (drains ovary and some of the uterus) from the axillary (systemic) lymph node, with the first two components accounting for approximately 32% of the variation in gene expression (Fig. 2A). Separation according to pregnancy status was also observed (MANOVA *P* = 2.337e-09, R^2 = 0.5), with the first two components accounting for 27% of the variation in gene expression (Fig. 2B).

Analysis of the reproductive tissues alone revealed clear separation (MANOVA *P* < 2.2e-16, R^2 = 0.96) of the gene expression patterns between these tissues (accounting for 13-22% of gene expression variation), with many differentially expressed genes (Fig. 3A). Additionally, good (MANOVA *P* = 2.417e-14, R^2 = 0.47) separation was observed according to pregnancy status (Fig. 3B), although only 16% of gene expression variation was observed in the first two components.

Permutation ANOVA analysis of the effects of pregnancy and tissue type on expression of individual genes

The tissues were analyzed according to their grouping within the PLS-DA analysis with reproductive tissues, lymph nodes, spleen, and WBC being the final groupings analyzed. There was no effect of pregnancy status on expression of any of the genes analyzed in the spleen (Supplementary File, Spleen statistics tab).

White blood cells

Stimulation of WBC with staphylococcal enterotoxin B (SEB) resulted in both up- and down-regulation of multiple genes (Supplementary File, WBC tab). Genes highly up-regulated (>4 fold) by stimulation with SEB included *IFNG*, *IL2RA*, and *PDL1*. Highly down-regulated genes (<0.26) included *ALOX15* and *SPP1*.

Expression of any individual gene was not affected by pregnancy status when accounting for multiple comparisons, nor were there any significant interactions between SEB treatment and pregnancy status.

Reproductive tissues

There were no significant interactions observed between tissue type and pregnancy status when accounting for multiple comparisons for expression of any of the measured genes. As would be expected, many genes were differentially expressed between tissue type (Supplementary File, Reproductive tissues statistics tab) but there was no consistent pattern of differential gene expression. Some genes were more strongly expressed in the endometrium than the oviduct (either isthmus or ampulla), for example, *PAQR8*, *PTGS2*, and *ALOX12*. Some had a more graded expression when moving from the ampulla to the isthmus to the endometrium [for example *MUC4*, *MUC12*, *LOC11411 (WFDC18)*, *ATF3*, and *EPHX1*], whereas others had reverse gradation (i.e., strongest in endometrium: *ESR2*, *HAND2*, *IHH*, *GADD45B*, and *NOS1*). The ampulla had very strong expression of *SSP1*, with reduced expression in the endometrium and the lowest expression in the isthmus. Genes such as *NOS3*, *NR2F2*, and *ITGA1* were most strongly expressed in the isthmus, followed by the endometrium and then ampulla, with genes such as *CSF2RB* and *AKR1B1*, being more strongly expressed in the isthmus/endometrium than the ampulla. Only one gene was identified as being differentially expressed between pregnant and nonpregnant animals when taking into account corrections for multiple comparisons, namely, *IL10RB*. Expression of *IL10RB* was increased by 24% in pregnant compared to nonpregnant animals. As previously highlighted, differences between tissue type and pregnancy status were further explored using PLS-DA (Fig. 3), which mirrored the permutation test results, showing clear differences between tissue types and less pronounced separation by pregnancy status. Many of the genes associated with differences in pregnancy status were related to the T-cell receptor pathway (see below).

Lymph nodes

No interactions were observed between tissue type and pregnancy status when correcting for multiple comparisons (Supplementary File, Lymph node tissue statistics tab). Additionally, expression differences between pregnant and nonpregnant animals were not significant for any individual gene when accounting for multiple testing. Nine genes were differentially expressed (FDR < 0.10) between lymph node types (Fig. 4), with expression of most genes being decreased in the axillary lymph node compared with the lumbo-aortic lymph node. Expression in the medial iliac lymph nodes was intermediary, with gene expression pattern not consistently different than that observed in the axillary or lumbo-aortic lymph nodes (Fig. 4).

Table 1. Average (± SEM) of the weight, BCS, estradiol-17β, and progesterone concentrations in ewes that were nonpregnant or pregnant at the time of tissue collection

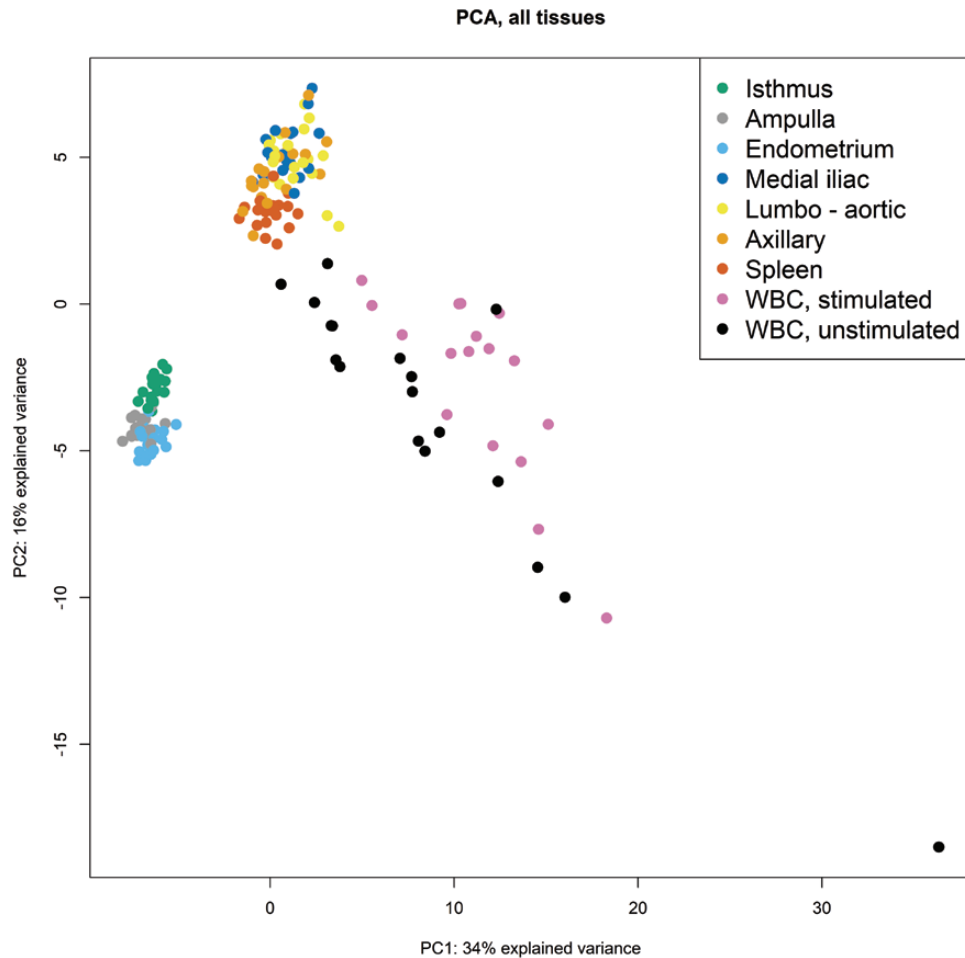| Group	Weight, kg	BCS, 1-5 scale	Estradiol-17β, pg/mL	Progesterone, ng/mL
Nonpregnant	72.6 ± 2.3	3.5 ± 0.1	4.09 ± 0.51	2.82 ± 0.21
Pregnant	74.0 ± 1.8	3.3 ± 0.1	3.60 ± 0.27	2.65 ± 0.26
P-value	0.62	0.09	0.40	0.62

Figure 1. Scores plot for the PCA of the normalized and scaled gene expression data for all tissues for all animals. The point color indicates tissue of the sample. The first and second components explain 34% and 16% of the variation, respectively. Clear separation into three tissue types with similarity in gene expression, namely, reproductive tissues, lymph tissues, and white blood cells, can be observed.

Association between concentrations of steroid hormones and gene expression

Correlations between concentrations of steroid hormones and gene expression profiles were also examined. After adjusting for false discovery rate, no significant correlations were consistently observed between progesterone or estradiol-17β and the various genes measured. Correlations between progesterone and estradiol-17β were rarely observed even when individual tissues were considered (Supplementary File, Gene steroid correlations tab).

Effects of pregnancy on the T-cell receptor signaling pathway

Analysis of the gene categories revealed that expression of genes of the T-cell receptor pathway (Fig. 5; Acuto and Michel, 2003; Smith-Garvin et al., 2009) was associated with pregnancy status ($P = 0.0401$) in tissues of the reproductive tract (isthmus, ampulla, and endometrium combined). Expression of *PTPRC* was not different between the two statuses. PTPRC is also known as CD45 antigen and is expressed exclusively by leukocytes. Although expression of its isoforms is regulated (Zhang et al., 2020), expression of the mRNA is very stable in leukocytes (Zikherman et al., 2008). Hence, its equal expression indicates that equal

numbers of leukocytes were present in tissues from both groups. Expression of *CD40*, *CD40LG*, *CD86*, and *PDL1* was not different between cycling and pregnant animals. However, several genes involved in T-cell receptor signaling were expressed at lower RNA copy numbers in pregnant animals, significantly (without multiple testing correction) for *CTLA4* ($P = 0.0239$) and *PDCD1* ($P = 0.0397$), and tending for *CD28* ($P = 0.0884$). Of the cytokines, expression of *IL4* ($P = 0.0186$), *IL5* ($P = 0.0378$), *IL10* ($P = 0.0221$), and *IL17A* ($P = 0.0342$) was significantly (without multiple testing correction) reduced in pregnant animals. *PRF1* ($P = 0.0763$) followed as a tendency. In contrast, the MHC class II molecule *DQB* was more highly expressed in pregnant vs non-pregnant animals ($P = 0.0366$, without multiple testing correction).

Discussion

The pattern of gene expression in the peripheral lymph node was different to lymph nodes draining reproductive tissue, thus supporting our hypothesis that expression of genes associated with immune function would differ between the lymph nodes draining the reproductive tract and peripheral lymph nodes. Furthermore, pregnancy affected the gene expression pattern in both the lymph nodes and reproductive tissue very early during pregnancy. This supports our second hypothesis that gene
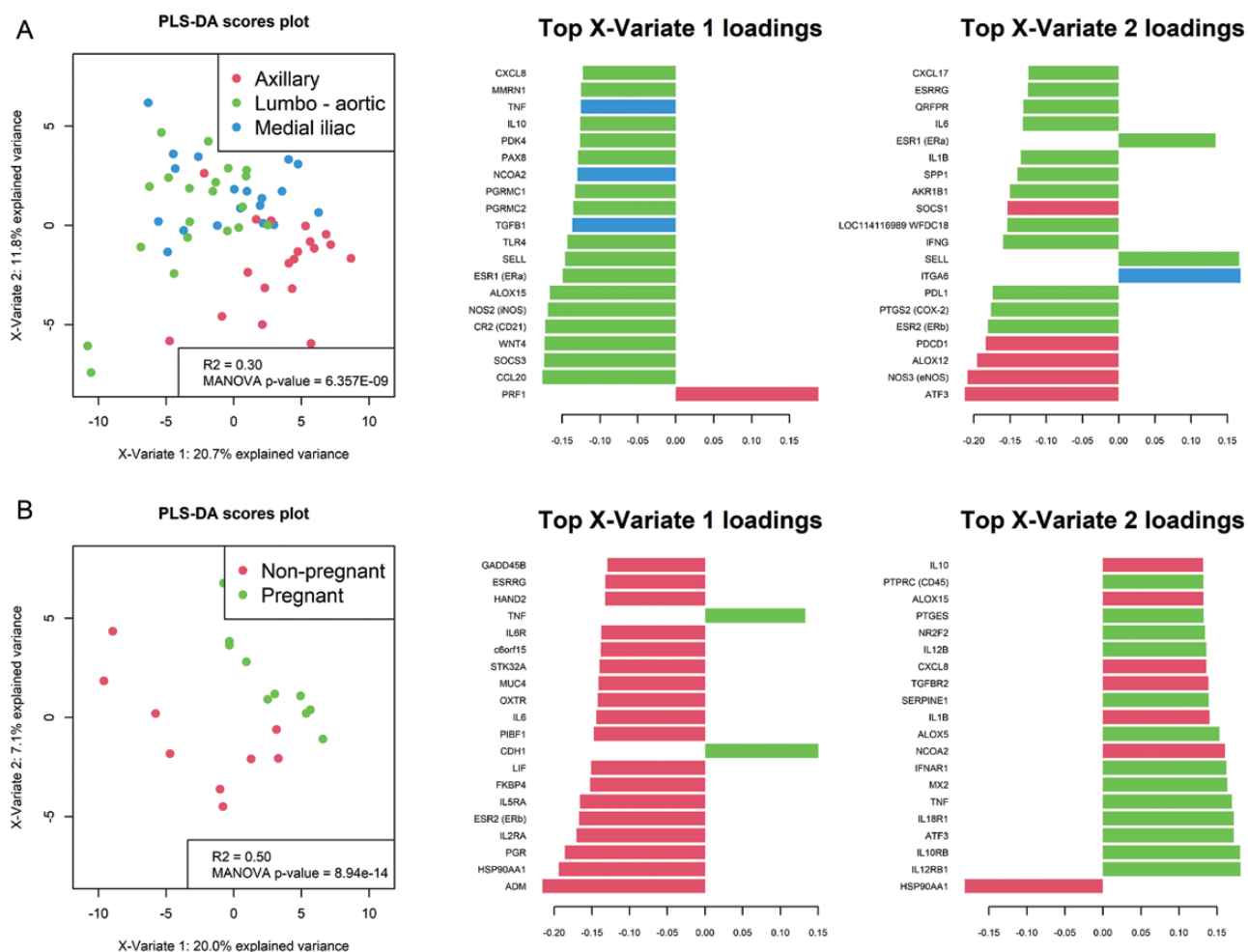
Figure 2. Scores plots and loading plots of the first two X-Variates for PLS-DA of the normalized and scaled gene expression data for lymph node tissues where the categorical variable was (A) tissue and (B) reproductive status. In panel A, separation of the lumbo-aortic and medial-iliac lymph nodes from the axillary lymph node is clearly observable. When examining expression of all genes measured collectively, clear separation of gene expression patterns of tissues from pregnant animals from those from nonpregnant animals can be observed. The key genes driving the separations of the groups are given on the right-hand side of the panel with the color of the bars in the loading plots corresponding to the group with the maximal expression levels with the mean criterion to assess the contribution.

expression would differ between the reproductive tract tissues and immune tissues of pregnant ewes and those that were at a similar stage of the estrus cycle but were not pregnant. However, it is important to note that changes during pregnancy could have been driven by either the presence of the embryo and/or presence of seminal plasma/sperm as those animals that were not pregnant had not been exposed to seminal plasma/sperm since the previous breeding season (approximately 12 mo).

Examining the genes that were differentially expressed between lymph nodes draining the reproductive tract and the systemic (axillary) lymph node, the lumbo-aortic lymph node showed the greatest difference. This lymph node drains the ovary, oviduct, and uterus. It is known that steroids produced by the ovary are found in much higher concentrations in lymph fluid from lymph vessels found alongside the utero-ovarian pedicle than in peripheral plasma (10-1000 fold higher; Staples et al., 1982). The increased expression of the estrogen receptor ESR1 in the lumbo-aortic and medial iliac lymph nodes in the current study supports a role for increased estrogen action in driving differences in gene expression. Estradiol or BPA, which has estrogenic properties, has been shown to increase expression of *Nos2* mRNA in macrophages (Kim et al., 2019) and

uterine mast cells (Hunt et al., 1997). Estrogen has also been shown to accelerate resolution of inflammation in macrophage cell lines through *Socs3* (Villa et al., 2015). Expression of *Ccl20* in murine lymph nodes has been shown to be stimulated by 17β-estradiol (Andersson et al., 2015). The authors postulated that the increased expression of *Ccl20* by 17β-estradiol in this mouse model of experimental autoimmune arthritis could result in attracting T-helper 17 phenotype cells, preventing them from migrating to the joints to cause arthritis (Andersson et al., 2015). Therefore, it could be postulated that the increased expression of *CCL20* observed in lymph nodes of the reproductive tract maintains the interleukin 17 producing T-helper (Th17) cells within the lymph node, reducing their presence in the reproductive tract tissues. In support of this, in the current study, expression of *IL17A* expression was decreased ($P < 0.05$ not adjusted for multiple testing) by approximately 25% in reproductive tissue of pregnant animals when compared with nonpregnant animals. The ability to downregulate the T-cell pathway may be important for pregnancy as a reduced expression of the T-cell receptor signaling pathway was observed in the reproductive tissues during pregnancy in the current study. Pregnancy loss in women with recurrent
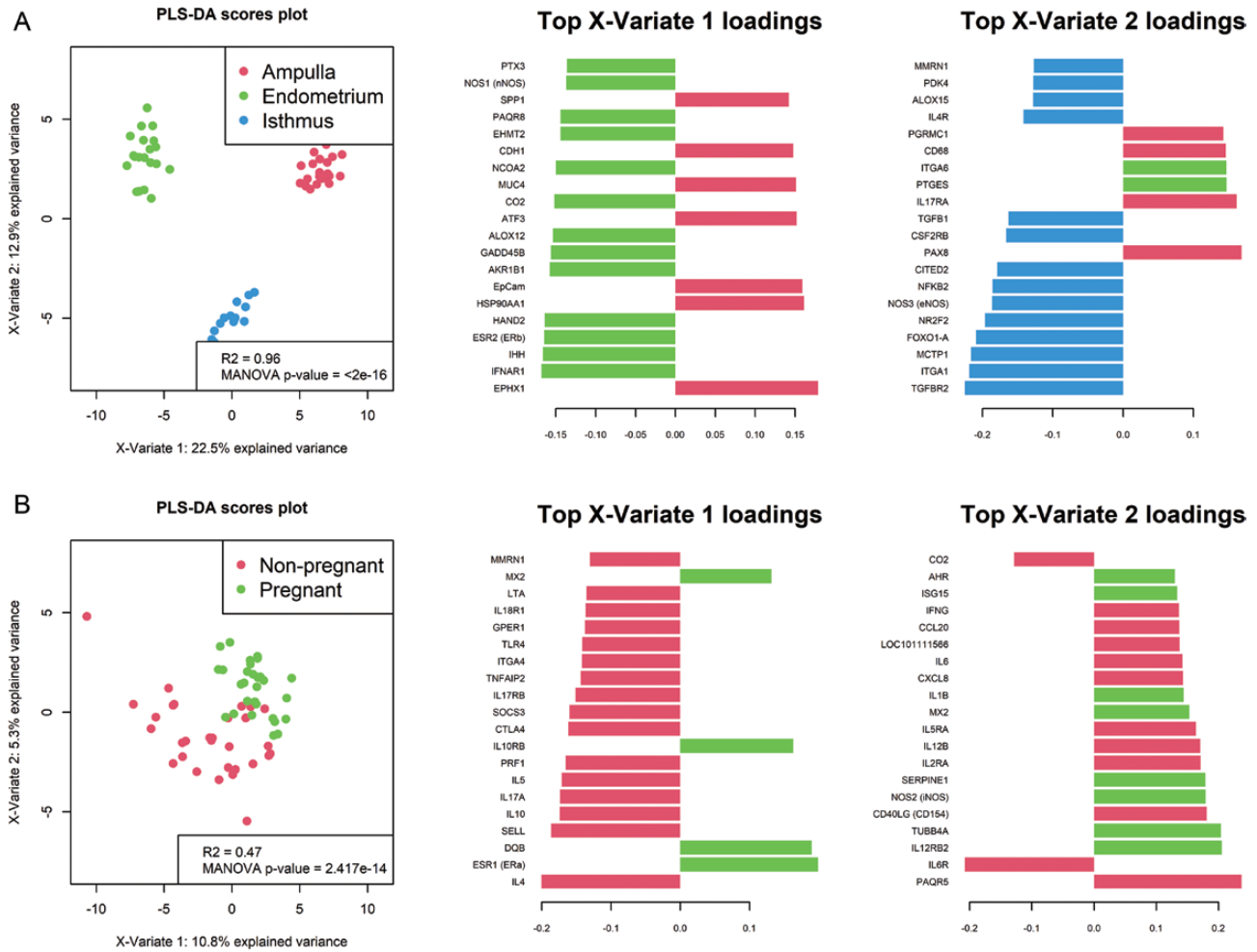
Figure 3. Scores plots and loading plots of the first two X-Variates for PLS-DA of the normalized and scaled gene expression data for reproductive tissues where the categorical variable was (A) tissue and (B) reproductive status. In panel A, clear separation of the ampulla, isthmus, and endometrium is observable. In panel B, when examining expression of all genes measured collectively, separation of gene expression patterns of tissues from pregnant animals from those from nonpregnant animals can be observed. The key genes driving the separations of the groups are given on the right-hand side of the panel with the color of the bars in the loading plots corresponding to the group with the maximal expression levels with the mean criterion to assess the contribution.

miscarriage undergoing immunocytotherapy is associated with upregulation of IL-17 production at weeks 5 and 6 of pregnancy (Krechetova et al., 2020).

Expression of *IL10RB* was increased in the reproductive tissues of pregnant compared to nonpregnant animals, mainly driven by high expression in the endometrium. *IL10RB* is constitutively expressed in most human cells and tissues with the highest expression found in placenta, which is three times higher than expression in endometrium (Fagerberg et al., 2014). The IL-10RB chain is used for signaling by several class II cytokines, including IL-10, IL-22, IL-26, and the IFN-λ proteins (Donnelly et al., 2004). Signaling of IL-10 requires 2 receptors, with the ligand binding to a specific alpha receptor (IL-10RA) and a common beta receptor (IL-10RB) (Donnelly et al., 2004). Expression of *IL10RA* was not affected by pregnancy and thus signaling of IL-10 was likely not affected. None of the alternative ligands (IL-22, IL-26, and the IFN-λ proteins) nor their specific receptors were examined in the current study but there is evidence that at least some of these ligands are produced by the reproductive tract and differentially expressed in response to infection (Xu et al., 2019). These findings indicate that IL-10RB signaling plays a role in early

embryo development and for the response of the reproductive tract to infections, and warrant further study if the signaling is induced by IL-22, IL-26, or IFN-λ.

Many genes were differentially expressed between the ampulla and isthmus of the oviduct as well as the endometrium. Given the specialist functions of these different tissues, with fertilization occurring at the junction of the ampulla and isthmus, the embryo undergoing the first few cell divisions in the isthmus and then moving into the uterine horn for blastocyst hatching and implantation, it is not surprising that their gene expression patterns differed. On day 5 of gestation, the embryo would be expected to have recently left the isthmus and entered the uterine horn (Murray et al., 1995). Differential gene expression between oviduct tissues and the uterus could also be related to the inclusion of the whole oviduct for gene analysis, whereas endometrial tissue was specifically collected from the uterus.

Seminal fluid transmits signaling agents that induce female reproductive tissues to facilitate conception and pregnancy. One important mechanism is the induction and expansion of regulatory T cells, which mediate tolerance to paternal antigens
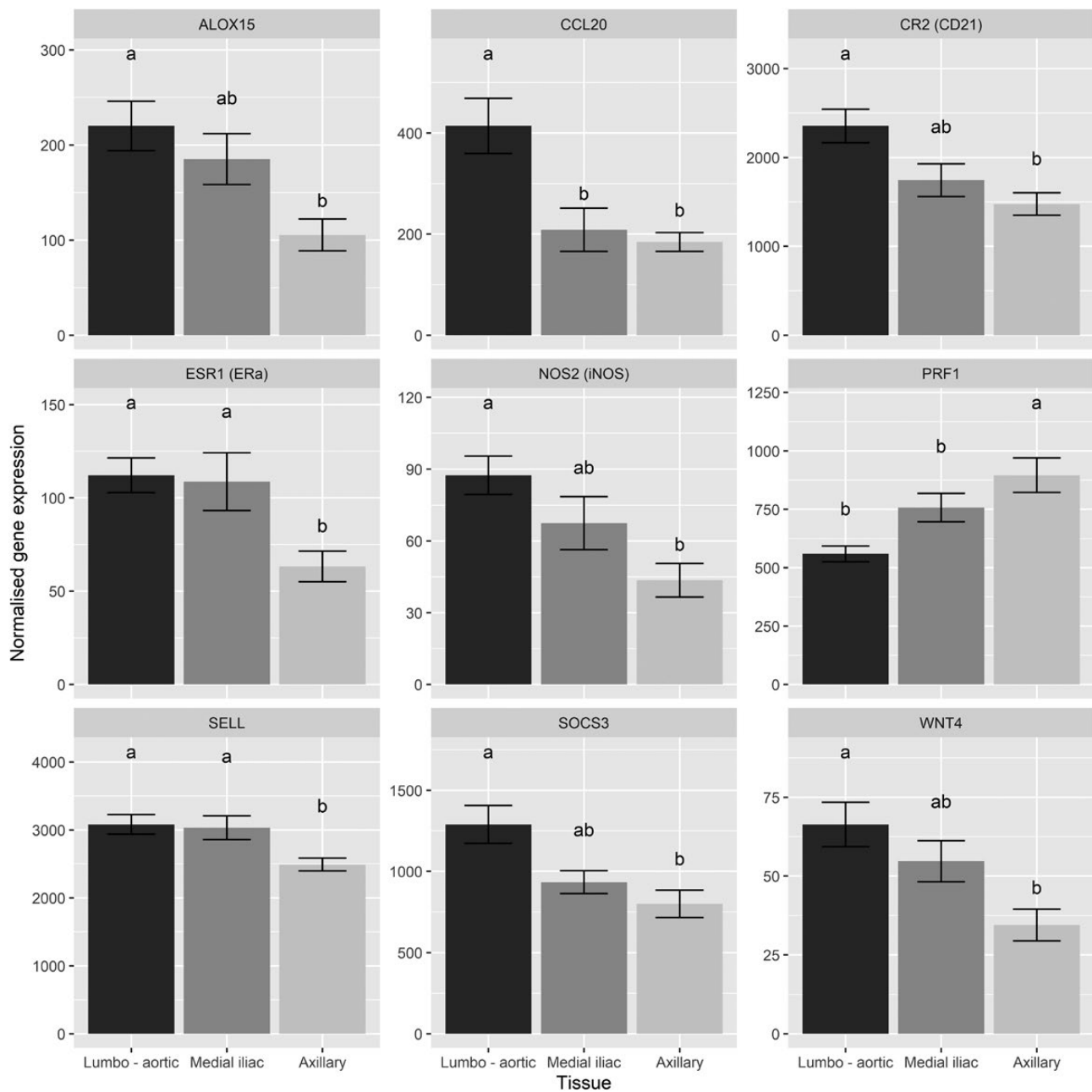
Figure 4. Bar plots of the means of *ALOX15, CCL20, CR2 (CD21)*, *ESR1 (Era)*, *NOS2 (iNOS)*, *PRF1*, *SELL*, *SOCS3*, and *WNT4* for lymph node tissues. Error bars indicate standard errors of the means. Within each gene, gene expression values of tissues without common letters are different (*FDR < 0.05*).

(Robertson and Sharkey, 2016; Schjenken and Robertson, 2020). These cells need to be locally recruited and induced based on antigen specificity and activation during the first days of pregnancy. During this time, it is likely that active T-cell responses are induced by paternal and other antigens that were carried by the seminal fluid and the sperm.

The first step in the induction of T-cell responses is the presentation of antigen by antigen-presenting cells in context of the major histocompatibility complex. The ovine major histocompatibility complex (OLA) class II DQ genes encode cell-surface glycoproteins that play a central role in the regulation of the immune response through their ability to bind peptides and present those peptides to T-cell receptors. In a cattle study, RNA-sequencing of endometrial samples collected 24 h after mating with an intact bull revealed >20 differentially expressed genes when comparing to unmated animals or those mated with a vasectomized bull (Recuero et al., 2020). In that study, DQB expression was decreased in endometrium 24 h after mating. In the current study, we found increased expression of DQB in pregnant animals at day 5 of pregnancy. This higher expression of *DQB* in the reproductive tract of pregnant compared to nonpregnant animals indicates an influx and/or activation of antigen presenting cells during this time period. On the other hand, expression of *CD28* tended to be lower in pregnant animals, indicating that the activation of local T cells was already waning by day 5 of pregnancy.

Both CTLA4 and PDCD1 are immune checkpoints that regulate activation of T cells (Greenwald et al., 2005). CTLA-4
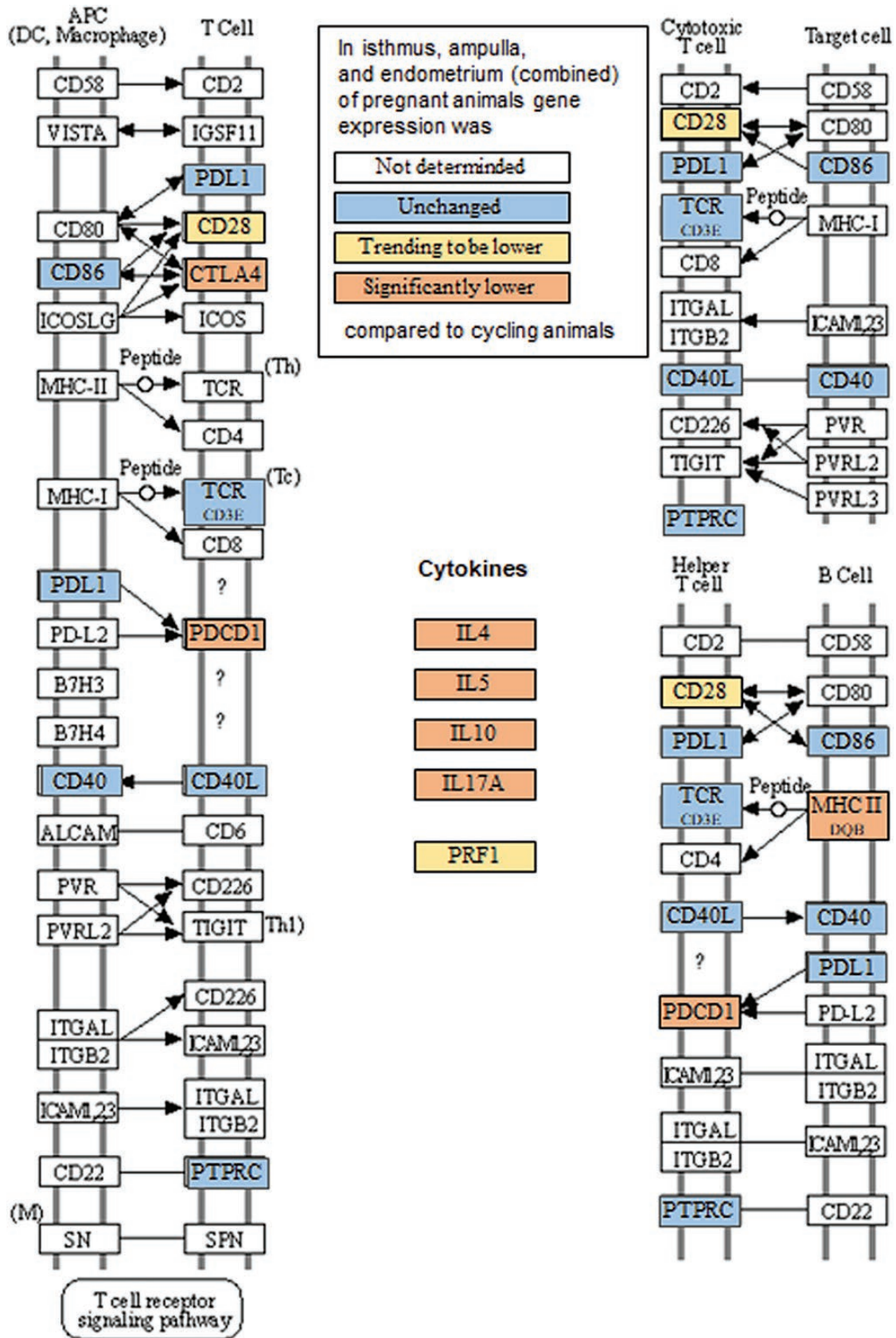
Figure 5. Schematic view of T-cell receptor signaling pathways between antigen presenting cells (APC) and T cells (left), T cells and target cells (right), and of cytokines affected by pregnancy (center). The color corresponds to the significance of changes in expression in pregnant versus cycling animals (modified from "KEGG map04514 Cell adhesion molecules"; Smith-Garvin et al., 2009).

stops potentially autoreactive T cells at the initial stage of naive T-cell activation (Krummel and Allison, 1995; Fife and Bluestone, 2008). The PDCD1/PDL1 pathway regulates previously activated T cells at the later stages of an immune response (Fife and Bluestone, 2008). The lower expression of CTLA4 and PDCD1 in pregnant animals observed here indicates the relative absence of immunosuppressed T lymphocytes at this stage of pregnancy. This is agreement with Moldenhauer et al. (2009), who showed that at conception, seminal fluid induced an activation and expansion of paternal Ag-reactive CD4+ and CD8+ T cell populations, and that female antigen presenting cells had an essential role in cross-presenting Ag to CD8+ T cells.

As highlighted with the pathway analysis, cytokine expression in the oviduct of pregnant animals follows a similar pattern. The stimulatory cytokines IL-4, which induces differentiation of naive helper T cells to Th2 cells and up-regulates MHC class II, IL-5, which stimulates B cells, and IL-17, which links T cell and neutrophil activation, were expressed at lower levels, supporting the notion of a waning immune response, whereas the lower expression of the anti-inflammatory cytokine IL-10 suggests that immunoregulatory pathways had not yet been activated.

In sheep, the endometrium is first exposed to paternal antigens expressed by the hatching blastocyst 8 d after ovulation (Spencer et al., 2004). This is preceded by oviduct epithelium inducing interferon-tau in bovine day 4 embryos, which supports an anti-inflammatory response (Talukder et al., 2018).

Taken together, these findings are consistent with the immune response against paternal and other antigens beginning to wane, but tolerance inducing pathways not yet being activated in the oviduct at day 5 of pregnancy.

In summary, differentiation of immune function of the local compared with the peripheral lymph nodes was observed, consistent with specialization of immune function localized to the reproductive tract to facilitate establishment of pregnancy. Furthermore, differences in immune function by day 5 of pregnancy were observed in pregnant compared with nonmated ewes. This was associated with genes important for T-cell function and thus highlights the important role of these cells in early pregnancy.

Supplementary Material

Supplementary data are available at *Journal of Animal Science* online.

Acknowledgments

Conflict of interest statement

The authors declare no real or perceived conflicts of interest.

Literature Cited

Acuto, O., and F. Michel. 2003. CD28-mediated co-stimulation: a quantitative support for TCR signalling. *Nat. Rev. Immunol.* **3**:939–951. doi:10.1038/nri1248.

Andersson, A., A. Stubelius, M. N. Karlsson, C. Engdahl, M. Erlandsson, L. Grahnemo, M. K. Lagerquist, and U. Islander. 2015. Estrogen regulates T helper 17 phenotype and localization in experimental autoimmune arthritis. *Arthritis Res. Ther.* **17**:32. doi:10.1186/s13075-015-0548-y.

Beef+Lamb, N. Z. 2019. Ewe Body Condition Scoring (BCS) fact sheet. Available from https://beeflambnz.com/knowledge-hub/PDF/ewe-bcs-fact-sheet.

Benjamini, Y., and Y. Hochberg. 1995. Controlling the false discovery rate: a practical and pwerful approach to multiple testing. *J. Royal Stat. Soc. Ser. B. (Stat. Method.)* **57**(1):289–300.

Brown, H. M., K. R. Dunning, M. Sutton-McDowall, R. B. Gilchrist, J. G. Thompson, and D. L. Russell. 2017. Failure to launch: aberrant cumulus gene expression during oocyte in vitro maturation. *Reproduction* **153**:R109–R120. doi:10.1530/REP-16-0426.

Chavan, A. R., O. W. Griffith, and G. P. Wagner. 2017. The inflammation paradox in the evolution of mammalian pregnancy: turning a foe into a friend. *Curr. Opin. Genet. Dev.* **47**:24–32. doi:10.1016/j.gde.2017.08.004.

Dickinson, S. E., B. A. Griffin, M. F. Elmore, L. Kriese-Anderson, J. B. Elmore, P. W. Dyce, S. P. Rodning, and F. H. Biase. 2018. Transcriptome profiles in peripheral white blood cells at the time of artificial insemination discriminate beef heifers with different fertility potential. *BMC Genomics* **19**:129. doi:10.1186/s12864-018-4505-4.

Donnelly, R. P., F. Sheikh, S. V. Kotenko, and H. Dickensheets. 2004. The expanded family of class II cytokines that share the IL-10 receptor-2 (IL-10R2) chain. *J. Leukoc. Biol.* **76**:314–321. doi:10.1189/jlb.0204117.

Fagerberg, L., B. M. Hallström, P. Oksvold, C. Kampf, D. Djureinovic, J. Odeberg, M. habuka, s. tahmasebpoor, a. danielsson, k. edlund, *et al*. 2014. analysis of the human tissue-specific expression by genome-wide integration of transcriptomics and antibody-based proteomics. *Mol. Cell. Proteomics* **13**:397–406. doi:10.1074/mcp.M113.035600.

Fazeli, A., N. A. Affara, M. Hubank, and W. V. Holt. 2004. Sperm-induced modification of the oviductal gene expression profile after natural insemination in mice. *Biol. Reprod.* **71**:60–65. doi:10.1095/biolreprod.103.026815.

Fife, B. T., and J. A. Bluestone. 2008. Control of peripheral T-cell tolerance and autoimmunity via the CTLA-4 and PD-1 pathways. *Immunol. Rev.* **224**:166–182. doi:10.1111/j.1600-065X.2008.00662.x.

Fisher, R. A. 1922. On the interpretation of χ 2 from contingency tables, and the calculation of P. *J. Royal Stat. Soc.* **85**(1):87–94. doi:10.2307/2340521

Forde, N., F. Carter, T. E. Spencer, F. W. Bazer, O. Sandra, N. Mansouri-Attia, L. A. Okumu, P. A. McGettigan, J. P. Mehta, R. McBride, *et al*. 2011. Conceptus-induced changes in the endometrial transcriptome: how soon does the cow know she is pregnant? *Biol. Reprod.* **85**:144–156. doi:10.1095/biolreprod.110.090019.

Forde, N., G. B. Duffy, P. A. McGettigan, J. A. Browne, J. P. Mehta, A. K. Kelly, N. Mansouri-Attia, O. Sandra, B. J. Loftus, M. A. Crowe, *et al*. 2012. Evidence for an early endometrial response to pregnancy in cattle: both dependent upon and independent of interferon tau. *Physiol. Genomics* **44**:799–810. doi:10.1152/physiolgenomics.00067.2012.

Fox, J., and S. Weisberg. 2019. The car package: an {R} companion to applied regression. R package version 3.0–10. https://cran.r-project.org/package=car

Geiss, G. K., R. E. Bumgarner, B. Birditt, T. Dahl, N. Dowidar, D. L. Dunaway, H. P. Fell, S. Ferree, R. D. George, T. Grogan, *et al*. 2008. Direct multiplexed measurement of gene expression with color-coded probe pairs. *Nat. Biotechnol.* **26**:317–325. doi:10.1038/nbt1385.

Greenwald, R. J., G. J. Freeman, and A. H. Sharpe. 2005. The B7 family revisited. *Annu. Rev. Immunol.* **23**:515–548. doi:10.1146/annurev.immunol.23.021704.115611.

Griffith, O. W., A. R. Chavan, S. Protopapas, J. Maziarz, R. Romero, and G. P. Wagner. 2017. Embryo implantation evolved from an ancestral inflammatory attachment reaction. *Proc. Natl. Acad. Sci. U. S. A.* **114**:E6566–E6575. doi:10.1073/pnas.1701129114.

Griffith, O. W., A. R. Chavan, S. Protopapas, J. Maziarz, R. Romero, and G. P. Wagner. 2018. Reply to liu: inflammation before implantation both in evolution and development. *Proc. Natl. Acad. Sci. U. S. A.* **115**:E3–E4. doi:10.1073/pnas.1717001115.

Hamdi, M., M. J. Sánchez-Calabuig, B. Rodríguez-Alonso, S. Bagés Arnal, K. Roussi, R. Sturmey, A. Gutiérrez-Adán, P. Lonergan, and D. Rizos. 2019. Gene expression and metabolic response of bovine oviduct epithelial cells to the early embryo. *Reproduction* **158**:85–94. doi:10.1530/REP-18-0561.

Hansen, P. J. 2014. Consequences of interations between the maternal immune system and the preimplantation embryo in cattle. In: J. L. Juengel, A. Miyamoto, C. Price, L. P. Reynolds, M. F. Smith, and R. Webb, editors. *Reproduction in domestic ruminants VIII*. Ashby de la Zouch: Context Products Ltd. p. 141–152.

Hess, A. P., S. Talbi, A. E. Hamilton, D. M. Baston-Buest, M. Nyegaard, J. C. Irwin, F. Barragan, J. S. Kruessel, A. Germeyer, and L. C. Giudice. 2013. The human oviduct transcriptome reveals an anti-inflammatory, anti-angiogenic, secretory and matrix-stable environment during embryo transit. *Reprod. Biomed. Online* **27**:423–435. doi:10.1016/j.rbmo.2013.06.013.

Hunt, J. S., L. Miller, K. F. Roby, J. Huang, J. S. Platt, and B. L. DeBrot. 1997. Female steroid hormones regulate production of pro-inflammatory molecules in uterine leukocytes. *J. Reprod. Immunol.* **35**:87–99. doi:10.1016/s0165-0378(97)00060-0.

Juengel, J. L., L. D. Quirke, K. Hyslop, E. D. Meenken, J. Peers-Adams, P. Smith, and S. J. Edwards. 2020. Association of fertility with group mating behavior in ewes. *Anim. Reprod. Sci.* **216**:106359. doi:10.1016/j.anireprosci.2020.106359.

Killeen, A. P., M. G. Diskin, D. G. Morris, D. A. Kenny, and S. M. Waters. 2016. Endometrial gene expression in high- and low-fertility heifers in the late luteal phase of the estrous cycle and a comparison with midluteal gene expression. *Physiol. Genomics* **48**:306–319. doi:10.1152/physiolgenomics.00042.2015.

Kim, H., H. S. Kim, Y. J. Piao, and W. K. Moon. 2019. Bisphenol a promotes the invasive and metastatic potential of ductal carcinoma in situ and protumorigenic polarization of macrophages. *Toxicol. Sci.* **170**:283–295. doi:10.1093/toxsci/kfz119.

Krechetova, L. V., L. V. Vanko, V. V. Vtorushina, M. A. Nikolaeva, E. V. Inviyaeva, and N. K. Tetruashvili. 2020. Lymphocyte activation in the development of immune tolerance in women with recurrent pregnancy loss. *Biochemistry. (Mosc).* **85**:583–593. doi:10.1134/S0006297920050077.

Krummel, M. F., and J. P. Allison. 1995. CD28 and CTLA-4 have opposing effects on the response of T cells to stimulation. *J. Exp. Med.* **182**:459–465. doi:10.1084/jem.182.2.459.

Lange, J., S. Ganesh, S. Meier, J. K. Kay, M. A. Crookenden, C. G. Walker, M. D. Mitchell, J. J. Loor, J. R. Roche, and A. Heiser. 2019. Far-off and close-up feeding levels affect immunological performance in grazing dairy cows during the transition period. *J. Anim. Sci.* **97**:192–207. doi:10.1093/jas/sky427.

Mahakapuge, T. A. N., J. P. Y. Scheerlinck, C. A. A. Rojas, A. L. Every, and J. Hagen. 2016. Assessment of reference genes for reliable analysis of gene transcription by RT-qPCR in ovine leukocytes. *Vet. Immunol. Immunopathol.* **171**:1–6. doi:10.1016/j.vetimm.2015.10.010

Maillo, V., P. Ó. Gaora, N. Forde, U. Besenfelder, V. Havlicek, G. W. Burns, T. E. Spencer, A. Gutierrez-Adan, P. Lonergan, and D. Rizos. 2015. Oviduct-Embryo interactions in cattle: two-way traffic or a one-way street? *Biol. Reprod.* **92**:144. doi:10.1095/biolreprod.115.127969.

Malkov, V. A., K. A. Serikawa, N. Balantac, J. Watters, G. Geiss, A. Mashadi-Hossein, and T. Fare. 2009. Multiplexed measurements of gene signatures in different analytes using the Nanostring nCounter™ Assay System. *BMC Res. Notes* **2**:80. doi:10.1186/1756–0500–2–80.

Martyniak, M., K. Zglejc-Waszak, A. Franczak, and G. Kotwica. 2018. Transcriptomic analysis of the oviduct of pigs during the peri-conceptional period. *Anim. Reprod. Sci.* **197**:278–289. doi:10.1016/j.anireprosci.2018.08.040.

Moldenhauer, L. M., K. R. Diener, D. M. Thring, M. P. Brown, J. D. Hayball, and S. A. Robertson. 2009. Cross-presentation of male seminal fluid antigens elicits T cell activation to initiate the female immune response to pregnancy. *J. Immunol.* **182**:8080–8093. doi:10.4049/jimmunol.0804018.

Murphy, K. M., P. Travers, and M. Walport. 2007. *Janeway's immunobiology*. 7 ed. New York: Garland Science.

Murray, M. K., M. M. DeSouza, and S. M. Messinger. 1995. Oviduct during early pregnancy: hormonal regulation and interactions with the fertilized ovum. *Microsc. Res. Tech.* **31**:497–506. doi:10.1002/jemt.1070310606.

O'Connell, A. R., K. J. Demmers, B. Smaill, K. L. Reader, and J. L. Juengel. 2016. Early embryo loss, morphology, and effect of previous immunization against androstenedione in the ewe. *Theriogenology* **86**:1285–1293. doi:10.1016/j.theriogenology.2016.04.069.

Paik, D. Y., D. M. Janzen, A. M. Schafenacker, V. S. Velasco, M. S. Shung, D. Cheng, J. Huang, O. N. Witte, and S. Memarzadeh. 2012. Stem-like epithelial cells are concentrated in the distal end of the fallopian tube: a site for injury and serous cancer initiation. *Stem Cells* **30**:2487–2497. doi:10.1002/stem.1207.

Passaro, C., D. Tutt, S. Bagés-Arnal, C. Maicas, R. Laguna-Barraza, A. Gutierrez-Adán, J. A. Browne, D. Rath, S. K. Behura, T. E. Spencer, *et al.* 2019. Global transcriptomic response of bovine endometrium to blastocyst-stage embryos. *Reproduction* **158**:223–235. doi:10.1530/REP-19-0064.

R Core Team. 2020. R: a language and environment for statistical computing. Team, R. C. 2020. Available from https://www.R-project.org/.

Recuero, S., J. M. Sánchez, Y. Mateo-Otero, S. Bagés-Arnal, M. McDonald, S. K. Behura, T. E. Spencer, D. A. Kenny, M. Yeste, P. Lonergan, *et al.* 2020. Mating to intact, but not vasectomized, males elicits changes in the endometrial transcriptome: insights from the bovine model. *Front. Cell Dev. Biol.* **8**:547. doi:10.3389/fcell.2020.00547.

Robertson, S. A., and D. J. Sharkey. 2016. Seminal fluid and fertility in women. *Fertil. Steril.* **106**:511–519. doi:10.1016/j.fertnstert.2016.07.1101.

Rohart, F., B. Gautier, A. Singh, and K. A. Lê Cao. 2017. mixOmics: an R package for 'omics feature selection and multiple data integration. *Plos Comput. Biol.* **13**:e1005752. doi:10.1371/journal.pcbi.1005752.

Sahu, A. R., S. A. Wani, S. Saxena, K. K. Rajak, D. Chaudhary, A. P. Sahoo, A. Khanduri, A. Pandey, P. Mondal, W. A. Malla, *et al.* 2018. Selection and validation of suitable reference genes for qPCR gene expression analysis in goats and sheep under Peste des petits ruminants virus (PPRV), lineage IV infection. *Sci. Rep.* **8**:15969. doi:10.1038/s41598-018-34236-7.

Sanchez, G., C. Determan, and M. G. Sanchez. 2013. R package discriminer. Version 0.1–29. https://cran.r-project.org/package=DiscriMiner

Schjenken, J. E., and S. A. Robertson. 2020. The female response to seminal fluid. *Physiol. Rev.* **100**:1077–1117. doi:10.1152/physrev.00013.2018.

Schmaltz-Panneau, B., A. Cordova, S. Dhorne-Pollet, C. Hennequet-Antier, S. Uzbekova, E. Martinot, S. Doret, P. Martin, P. Mermillod, and Y. Locatelli. 2014. Early bovine embryos regulate oviduct epithelial cell gene expression during in vitro co-culture. *Anim. Reprod. Sci.* **149**:103–116. doi:10.1016/j.anireprosci.2014.06.022.

Sherman, B. T., and R. A. Lempicki. 2009. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat. Protoc.* **4**(1):44.

Smith, P., J. L. Stanton, L. Quirke, and J. L. Juengel. 2019. Gestational nutrition 1: alterations to gestational nutrition can increase indicators of fertility in sheep. *Reproduction* **157**:199–213. doi:10.1530/REP-18-0357.

Smith-Garvin, J. E., G. A. Koretzky, and M. S. Jordan. 2009. T cell activation. *Annu. Rev. Immunol.* **27**:591–619. doi:10.1146/annurev.immunol.021908.132706.

Spencer, T. E., G. A. Johnson, F. W. Bazer, and R. C. Burghardt. 2004. Implantation mechanisms: insights from the sheep. *Reproduction* **128**:657–668. doi:10.1530/rep.1.00398.

Staples, L. D., I. R. Fleet, and R. B. Heap. 1982. Anatomy of the utero-ovarian lymphatic network and the composition of afferent lymph in relation to the establishment of pregnancy in the sheep and goat. *J. Reprod. Fertil.* **64**:409–420. doi:10.1530/jrf.0.0640409.

Talukder, A. K., M. B. Rashid, M. S. Yousef, K. Kusama, T. Shimizu, M. Shimada, S. S. Suarez, K. Imakawa, and A. Miyamoto. 2018. Oviduct epithelium induces interferon-tau in bovine Day-4 embryos, which generates an anti-inflammatory response in immune cells. *Sci. Rep.* **8**:7850. doi:10.1038/s41598-018-26224-8.

Valour, D., I. Hue, S. A. Degrelle, S. Déjean, G. Marot, O. Dubois, G. Germain, P. Humblot, A. A. Ponter, G. Charpigny, *et al.* 2013. Pre- and post-partum mild underfeeding influences gene expression in the reproductive tract of cyclic dairy cows. *Reprod. Domest. Anim.* **48**:484–499. doi:10.1111/rda.12113.

Villa, A., N. Rizzi, E. Vegeto, P. Ciana, and A. Maggi. 2015. Estrogen accelerates the resolution of inflammation in macrophagic cells. *Sci. Rep.* **5**:15224. doi:10.1038/srep15224.

Vorachek, W. R., G. B. Hugejiletu, and J. A. Hall. 2013. Reference gene selection for quantitative PCR studies in sheep neutrophils. *Int. J. Mol. Sci.* **14**:11484–11495. doi:10.3390/ijms140611484.

Wathes, D. C., Z. Cheng, W. Chowdhury, M. A. Fenwick, R. Fitzpatrick, D. G. Morris, J. Patton, and J. J. Murphy. 2009. Negative energy balance alters global gene expression and immune responses in the uterus of postpartum dairy cows. *Physiol. Genomics* **39**:1–13. doi:10.1152/physiolgenomics.00064.2009.

Wheeler, B., M. Torchiano, and M. M. Torchiano. 2016. Package 'lmPerm'. R package version, 2.1.0. https://cran.r-project.org/package=lmPerm

Xu, J. Z., R. Kumar, H. Gong, L. Liu, N. Ramos-Solis, Y. Li, and W. A. Derbigny. 2019. Toll-like receptor 3 deficiency leads to altered immune responses to chlamydia trachomatis infection in human oviduct epithelial cells. *Infect. Immun.* **87**(10):e00483-19. doi: 10.1128/IAI.00483–19

Yang, L., Q. Wang, Y. Liu, L. Zhang, W. Lv, and B. Liu. 2019. Expression profiles of interferon-stimulated gene 15 and prostaglandin synthases in the ovine lymph nodes during early pregnancy. *Mol. Reprod. Dev.* **86**:100–108. doi:10.1002/mrd.23085.

Yang, L., P. Wang, H. Mi, W. Lv, B. Liu, J. Du, and L. Zhang. 2019a. Comparison of Th1 and Th2 cytokines production in ovine lymph nodes during early pregnancy. *Theriogenology* **123**:177–184. doi:10.1016/j.theriogenology.2018.10.004.

Yen, H. H., C. M. Murray, E. A. Washington, W. G. Kimpton, and H. M. S. Davies. 2019. Characterisation of ovine lymphatic vessels in fresh specimens. *PLoS One* **14**:e0209414. doi:10.1371/journal.pone.0209414.

Zaros, L. G., L. L. Coutinho, L. H. Sider, H. R. de Medeiros, M. R. Neves, C. L. Benvenuti, A. M. do Carmo Navarro, and L. da Silva Vieira. 2010. Evaluation of reference genes for real-time PCR studies of Brazilian Somalis sheep infected by gastrointestinal nematodes. *Genet. Mol. Biol.* **33**:486–490. doi:10.1590/S1415-47572010000300018.

Zeileis, A., M. A. Wiel, K. Hornik, and T. Horthorn. 2008. Implementing a class of permutation tests: the coin package. *J. Stat. Software* **28**(8):1–23.

Zhang, T., Z. Dong, H. Cai, J. Rong, and Z. Su. 2020. Estradiol regulates the expression of CD45 splicing isoforms in lymphocytes. *Mol. Biol. Rep.* **47**:3025–3030. doi:10.1007/s11033-020-05373-y.

Zikherman, J., and A. Weiss. 2008. Alternative splicing of CD45: the tip of the iceberg. *Immunity* **29**:839–841. doi:10.1016/j.immuni.2008.12.005.