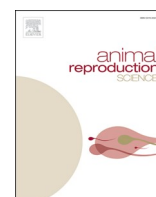Contents lists available at ScienceDirect

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Lymphocyte soluble factors from pregnant cows modulate mRNA transcript abundances encoding for proteins associated with trophoblast growth and development

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ABSTRACT

This study was conducted to determine whether T cell populations are responsible for modulating placental development during gestation in cattle. It was hypothesized that CD4+CD25+ and γ/δ+ T cells modulate gene expression, based on mRNA transcript abundances, and promote proliferation and survival of trophoblast cells. Peripheral blood was collected from cows at 160 to 180 days of gestation and non-pregnant cows, T cell populations CD8+, CD4+, CD4+CD25+, CD24+CD25-, and γ/δ+ T cells were isolated, cultured for 48 h, and supernatant was collected. Placental samples were digested, and trophoblast cells were cultured for 24 h. Trophoblast cells were cultured with 50 μL of T cell-conditioned media and 50 μL of fresh culture media for an additional 48 h. Samples in control wells were treated with unconditioned media. Trophoblast cell proliferation, apoptosis, and mRNA transcript assays were conducted. There was no effect of T cell population on trophoblast apoptosis rate, proliferation, and relative mRNA transcript abundances. The T cell supernatant from pregnant and non-pregnant cows induced greater apoptosis rates in trophoblast cells than unconditioned media. Trophoblast cells proliferated less when treated with T cell supernatant from pregnant compared to unconditioned medium and non-pregnant cows. Treatment with the T cell supernatant from pregnant cows resulted in larger abundances of *BMP5, IGF1R, PAG10, FGF2, RSPO3* and *TMED2* and also a lesser abundance of *FGF2* mRNA transcript than non-pregnant group and unconditioned media treatments. Supernatant from T cell derived from pregnant cows modulates trophoblast mRNA transcript abundances differently from T cell supernatant of non-pregnant cows.

1. Introduction

Among the multitude of reasons pregnancy loss occurs, the involvement of the maternal immune system in establishing pregnancy has recently received increased attention. There are many studies investigating the role of immune cells during gestation and in early pregnancy loss in humans and mice (reviewed by Schumacher et al., 2018; Yang et al., 2019). In few studies has there been investigation of the effects of the immune system in placental development of cattle (reviewed by Fair, 2015; Ott, 2019).

In the pregnant uterus, while a state of immunological tolerance needs to be established, host defenses against potential pathogens need to be preserved. Immune cells at the maternal-fetal interface have important functions in maintaining this balance between immune tolerance and protection. In humans and mice, it is well established that an aberrant maternal immune response is associated with pregnancy loss and infertility. Immune cells contribute to placental development and trophoblast invasion. Among the most prominent cells in the pregnant endometrium are natural killer cells, T and B lymphocytes, macrophages and dendritic cells (Schumacher et al., 2018). Regulatory T cells are responsible for suppressing T cell activity and are often defined as CD4+CD25+ and FoxP3+ (Richards et al., 2015). An increase in the number of CD4+CD25+ T cells in the endometrium as well as in the circulation has been associated with normal pregnancies (Aluvihare et al., 2004; Somerset et al., 2004). In addition, γ/δ+ T cells are in markedly larger abundances at the maternal-fetal interface relative to the non-pregnant uterus in mice (Heyborne et al., 1992).

In livestock species with epitheliochorial placentas, it is not clear which immune cell populations are responsible for supporting trophoblast growth and survival. Macrophages, CD4+, CD8+, γ/δ+ T, and B cells are present in the endometrium of pregnant cattle, sheep, goats, pigs and horses in large numbers and the recruitment of these cells is affected by the presence of the conceptus in the uterine lumen (Cobb and Watson, 1995; Dimova et al., 2007; Grünig et al., 1995; Koroghli et al., 2018; Oliveira and Hansen, 2009; Rutigliano et al., 2016; Vasudevan et al., 2017). In the second trimester of gestation in cattle, macrophages are in a state that supports immune regulation and tissue remodeling (Oliveira et al., 2010). The function of T cells during this period, however, is still unknown. The T cells produce cytokines that have actions at target cells in an autocrine, paracrine, or endocrine fashion to elicit changes in cell function. At the maternal-fetal interface, T cells likely interact with trophoblast cells through paracrine signaling. Cytokines and growth factors produced by immune cells at the maternal-fetal interface may contribute to promoting trophoblast cell growth and viability.

Unlike humans, in cattle CD4+CD25+ T cells may not function as T regulatory cells *in vitro* while the γ/δ+ T cells have regulatory functions (Hoek et al., 2009). A major difference between CD4+, CD8+ as compared with γ/δ+ T cells is that CD4+, CD8+ recognize antigens in an MHC restricted way while γ/δ+ T cells do not. It still is unknown how γ/δ+ T cells recognize antigens in a non-MHC-restricted way. The suggested functions for these cells include cytotoxicity, immunomodulation and immunosuppression. In pigs, γ/δ+ T cells decrease in number during the attachment phase and increase again at mid-gestation (Dimova et al., 2007) and in sheep these cells increase in numbers at mid-gestation (Liu et al., 1997).

During mid-gestation, after the placenta has been established, the endometrial epithelium is in direct contact with trophoblast cells. Results from studies in horses, humans and mice indicate there are selective expression of MHC class I antigens on invasive trophoblast cells at the beginning of gestation (Donaldson et al., 1990; Madeja et al., 2011). These processes have been suggested to result in the recruitment of T cells to the endometrium and in induction of maternal tolerance to fetal antigens because T regulatory cells have been observed around trophoblasts expressing MHC-I (Antczak et al., 2013). Conversely, close to the time of parturition, MHC-I compatibility between the dam and the fetus increases the risk of occurrence of retained placentas (Benedictus et al., 2012) suggesting that the actions of MHC-I molecules at this stage of gestation induce inflammation rather than immune tolerance.

The present study was conducted with the aim to determine whether the secretome of circulatory T cell cells support trophoblast growth and development. It was hypothesized that conditioned media from the CD4+CD25+ and γ/δ+ T cell populations of cattle would result in greater rates of trophoblast cell growth and survival compared to other T cell populations by modulating the expression of genes, based on mRNA transcript abundances, related to trophoblast cell development. It was also hypothesized that T cell supernatant from pregnant animals would modulate trophoblast growth, apoptosis, and mRNA transcript abundances differently as compared with T cell supernatant from non-pregnant cows.

2. Materials and methods

2.1. Animals

Animals were housed at the Utah State University Caine Dairy Teaching and Research Center. Veterinarians and staff with the Department of Animal, Dairy, and Veterinary Sciences are skilled at cattle husbandry, routine care, and surgical operations. All animal procedures were reviewed and approved by the Utah State University Institutional Animal Care and Use Committee (protocol # 10035). The Animal Welfare Act and the USDA humane animal care and use policies and procedures were followed.

2.2. Isolation of lymphocyte cell populations

All laboratory supplies were purchased from Thermo Fisher Scientific (Waltham, NY, USA) unless otherwise stated. To isolate the immune cell populations, 6 mL of blood were collected in Acid Citrate Dextrose vacutainer tubes (Beckton, Dickinson and Company, Franklin Lakes, NJ) *via* venipuncture of the caudal tail vein from multiparous Holstein-Friesian cows. Peripheral blood was obtained from three cows at 184, 154, and 172 days of gestation and from three non-pregnant cows in diestrus at approximately 65 days into lactation. Pregnant cows were in mid-gestation so as to be age-matched to a similar stage of gestation when placental samples were collected. Non-pregnant cows were at least 65 days in lactation to ensure the immune cell profile had returned to that of a non-pregnant cow. Blood samples were centrifuged at 800×*g* at 20 °C for 10 min to isolate the buffy coat. Buffy coat was re-suspended to 20 mL with Spinner Modification Minimum Essential Medium Eagle (SMEM, 5% fetal bovine serum [FBS], 4 μg/mL of penicillin/streptomycin), gently layered over 10 mL of sterile Accu-Paque (Accurate Chemical and Scientific Corporation, Carle Place, NY) density gradient and centrifuged at 1,600×*g* at 20 °C for 20 min with the brake off. The interface containing peripheral blood mononuclear cells (PBMCs) was collected and transferred to sterile 15 mL conical tubes. The PBMCs were washed in SMEM, vortexed,

and centrifuged at $300 \times g$ at 20 °C for 10 min. After removing the supernatant, the remaining red blood cells were eliminated by adding 2 mL of erythrocyte shock lysis solution. The cells were incubated for 2 min and then 8 mL of SMEM were added. The solution was centrifuged at $300 \times g$ at 20 °C for 6 min. The cell pellet was washed twice by bringing the volume to 10 mL with fluorescence buffer (SMEM with 0.1 % bovine serum albumin) and centrifuged at $300 \times g$ at 4 °C for 6 min. Cells were stored on ice for the remainder of the procedure. Peripheral blood mononuclear cells were stained with trypan blue and cell count and viability were assessed in a hemocytometer chamber. As depicted in Table 1, primary antibodies against CD4, CD8, CD4/CD25, γ/δ-TCR, and an isotype-matched negative control were purchased from the Washington State University Monoclonal Antibody Center (Pullman, WA). Cells were pelleted and primary antibody stock solutions were diluted to 15 μg/mL. Pellets were re-suspended using 100 μL of antibody solution per 2×10^6 cells and incubated on ice for 15 min. After primary antibody incubation, 4 mL of fluorescence buffer was added to each population and centrifuged at $800 \times g$ at 4 °C for 3 min. Cells were re-suspended in 3 mL of fluorescence buffer, lightly mixed on a flat top vortex mixer, and centrifuged again. The cells were re-suspended in secondary antibody solutions at 1:100 dilution and 1 μL of Live/Dead Fixable Blue Dead Stain Kit and incubated for 15 min on ice. The secondary antibody used for CD4, CD8, γ/δ-TCR, and controls was goat anti-mouse IgG conjugated with R-phycoerythrin (R-PE; Jackson ImmunoResearch Laboratories, West Grove, PA). The secondary antibodies used for CD4/CD25 were goat anti-mouse IgG2a with R-PE and goat anti-mouse IgG1 conjugated with allophycocyanin (APC). After incubation, cells were washed twice with 4 mL of fluorescence buffer. Stained cells were resuspended in 1 mL of fluorescence buffer and transferred to a sterile 12×75 mm Falcon tube through a cell strainer cap. Cells were sorted *via* flow cytometry (FACS Aria II equipped with FACS Diva software, Beckton, Dickinson and Company, Franklin Lakes, NJ) into T cell populations $CD8^+$, $CD4^+$, $CD4^+CD25^+$, $CD4^+CD25^-$, and $γ/δ-TCR^+$ based on their forward and side scatter characteristics and the fluorescence intensity after staining for the surface markers listed above. The proportions of the immune cell subpopulations previously described in this manuscript in peripheral blood of pregnant and non-pregnant cows was quantified.

After sorting, 3 mL of RPMI (5 % FBS, 1 μg/1 mL of penicillin/streptomycin, and 2.5 μg/mL of Amphotericin-B) were added to tube containing sorted cells and then centrifuged at $800 \times g$ and 20 °C for 3 min. Lymphocytes were re-suspended in 110 μL of RPMI (5 % FBS, 1 μg/mL of penicillin/streptomycin, and 2.5 μg/mL of Amphotericin-B) and 10 μL of the suspension was used to determine cell concentration. Each T cell population was seeded into four wells of a 96-well flat-bottomed cell culture plate at a concentration of 10,000 cells/200 μL RPMI medium. Unsorted PBMCs were cultured, and no-cell medium was used as a control. The T cells were cultured for 72 h at 37 °C and 5% CO_2. The conditioned media was removed and frozen at −20 °C until trophoblast cells were ready for treatment.

2.3. Isolation of trophoblast cells

Trophoblast cells were isolated from the placentas of four cows collected at a local abattoir. Based on the crown-rump length, stage of gestational periods were estimated to be 187 (52 cm), 207 (61 cm), 205 (60 cm), and 164 (43 cm) days. Pregnancies were collected randomly, but all contained a single, male fetus. Intact placentomes of healthy appearance were collected and transported to the laboratory in a glass jar containing 200 mL of 0.05 % chlorhexidine in PBS. The caruncle and cotyledon of the placentome were then separated while the caruncular and connective tissues surrounding the cotyledon were discarded. The cotyledon was minced using scissors into small pieces. Minced cotyledonary tissue was weighed in a beaker to ensure 100−120 grams of tissue was used for digestion.

Minced cotyledonary tissue was washed twice in 100 mL of 0.05 % chlorhexidine in water. Tissue was removed with a strainer. The washing process was repeated with sterile PBS (4 μg/mL penicillin/streptomycin and 10 μg/mL amphotericin B) and DMEM (4 μg/mL penicillin/streptomycin and 10 μg/mL amphotericin B). Tissue was transferred to an Erlenmeyer flask with 100 mL of warm digestion medium composed of 0.25 % trypsin in Hank's Balanced Salt Solution (MilliporeSigma, Saint Louis, MO) containing 1 % DNase (MilliporeSigma, Saint Louis, MO) at 1 mg/mL. The flask was placed on a medium speed shaker in an incubator at 37 °C for 30 min. Following digestion, the contents were run through a strainer to allow the supernatant to be collected into a new sterile flask with 20 mL of FBS. Supernatant with FBS was divided into four sterile 50 mL conical tubes through a disposable 100-micron nylon gauze cell strainer. The volume was brought to 45 mL with DMEM (5 % FBS, 4 μg/mL penicillin/ streptomycin, and 10 μg/mL amphotericin B). Tubes were centrifuged at $100 \times g$ at 20 °C for 10 min. Cells were re-suspended in 10 mL of DMEM and the solution layered over 10 mL

Table 1
Antibodies used for lymphocyte isolation through flow cytometry.

Marker	Antibody	Supplier	Isotype	Primary or Secondary
CD8	BAT82A	WSU[a]	IgG1	Primary
CD4	ILA11A1	WSU	IgG2a	Primary
CD25	CACT116A	WSU	IgG1	Primary
γ/δ-TCR	GB21A	WSU	IgG2b	Primary
PE_1	115-116-146	Jackson[b]	Goat anti-mouse IgG	Secondary
PE_2	P21139	Invitrogen[c]	Goat anti-mouse IgG2a	Secondary
APC	A10441	Invitrogen	Goat anti-mouse IgG1	Secondary
Control	S69A	WSU		Primary

[a] Washington State University Monoclonal Antibody Center (Pullman, WA).
[b] Jackson ImmunoResearch (West Grove, PA).
[c] Invitrogen (Camarillo, CA).

Table 2
Primers for gene expression analysis.

Functional Category	Abbreviation	Gene Name	Forward and Reverse Primer
House Keeping	GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase	F: GGGTCTTCACTACCATGGAGAA R: GTTCACGCCCATCACAAACA
Apoptosis	BCL2	BCL2 Apoptosis Regulator	F: ATGTGTGTGGAGAGCGTCAA R: GGTTCAGGTACTCGGTCATCC
	BCL2A1	BCL2 Related Protein A1	F: GCGGAGTTCATCACCGAAAA R: GCCAGCCAGATTTGGTTTCA
	BMP5	Bone Morphogenic Protein 5	F: GCCCATATGAATGCCACCAA R: CGCAGCAAGGCTTTGGTA
	BMP7	Bone Morphogenic Protein 7	F: GCCTTCCCTCTGAATTCCTACA R: ACCGTTTCCGGGTTGATGAA
Growth Factor Activity	EGF	Epidermal Growth Factor	F: ATGCAGTGGCTGTTCATCAC R: GCAGCCACATTTCCAGGATAC
	EGFR	Epidermal Growth Factor Receptor	F: ATGCATTTGCCAAGCCCTAC R: AGCATCCACAACATCCTCCA
	FGF2	Fibroblast Growth Factor 2	F: GCTTCTTCCTGCGCATCC R: CCCCTCTCTCTTCTGCTTGAA
	FGFR2	Fibroblast Growth Factor Receptor 2	F: AGCTGCCAGAAGATCCCAAA R: GCCCAAAGCAACCTTCTCC
	IGF1R	Insulin Like Growth Factor 1 Receptor	F: ACGGATCCCGTGTTCTTCTAC R: AATGGGCAGAGCGATCATCA
	IGFBP2	Insulin Like Growth Factor Binding Protein 2	F: ACAAGCATGGCCTGTACAAC R: GTTCACACACCAGCACTCC
	VEGFA	Vascular Endothelial Growth Factor A	F: TGACACAGAACTACCCATAGCC R: GCCTCCTCTTCCTTCATGTCA
Immune Response	IFNG	Interferon Gamma	F: CAGCTCTGAGAAACTGGAGGA R: TTATGGCTTTGCGCTGGATC
	IL-2	Interleukin 2	F: CTCTTGCACTCGTTGCAAAC R: TCCAGCAGCAATGACTTCAC
	IL-5	Interleukin 5	F: TTGACACTGCTCTCCTCTCA R: TGTATGCTGAGGAGTAGGAATCA
	IL-15	Interleukin 15	F: CCATGCTAGCAAACAGCAA R: CCTCACATTCTTTGCATCCC
	IL-23	Interleukin 23	F: CTGGAGTGCACACCTACCAA R: AGCCATCCTCACACTGGATAC
	LIFR	LIF Receptor Subunit Alpha	F: CCCACGCAGTACAAGTTACA R: TTTGCGGACACTTCATCTCC
	TNF	Tumor Necrosis Factor	F: CAAGTAACAAGCCGGTAGCC R: GGCATTGGCATACGAGTCC
Placental Development	ESRRB	Estrogen Related Receptor Beta	F: CCAAGCATGCTGCTGAACA R: ACGGCTCGGTCTTGATGAA
	GATA2	GATA Binding Protein 2	F: ACAAGATGAACGGGCAGAACC R: CAACAGGTGCCTGCTCTCC
	GATA3	GATA Binding Protein 3	F: AATGCCTGTGGGCTCTACTA R: TTCTGGTCTGGATCCCTTCC
	GCM1	Glial Cells Missing Transcription Factor 1	F: CGGCATCTTCCTCAGTCTCC R: TGGACAGCTTCCTGGAAAGAC
	GDNF	Glial Cell Derived Neurotrophic Factor	F: TGCCCGCCGGTAAGAG R: ATCAGGGTAGTCCTCTGGCATA
	GJB3	Gap Junction Protein Beta 3	F: GGACTTTGACTGCAACACCAA R: CGGATGTTGGAGATGGGGAA
	GJB5	Gap Junction Protein5 Beta	F: TGGAGTGATGACCACAAGGAC R: GAAGAACTCGTCGAAGCACAC
	HSPA1A	Heat Shock Protein Family A (Hsp70) Member 1A	F: GACGGCATCTTCGAGGTGAA R: TTCACCAGCCTGTTGTCGAA
	HSPD1	Heat Shock Protein D (Hsp60) Member 1	F: TTTTAGCCGATGCTGTAGCC R: TTTGGGACTTCCCCAACTCT
	MX1	MX Dynamin Like GTPase 1	F: CAGCCACCCGACATTGAATA R: AGTTGATGGTCTCCTGCCTA
	PAG1	Pregnancy Associated Glycoprotein 1	F: TCCACTTTCCGGCTTACCAA R: TGGTCAGTACTTACAAGGTTCCC
	PAG2	Pregnancy Associated Glycoprotein 2	F: CAGGGATTTCTTGGCTCTGAC R: CCCGTATTCCTCCAGGCTTA
	PAG10	Pregnancy Associated Glycoprotein 10	F: AAGTGGGCAGCTGGCATATA R: AGCCACGTTTACAAGCAACC
	PAG11	Pregnancy Associated Glycoprotein 11	F: AGGCCCTTCCACATCCTCTA R: GTCAGTGCTGACAAGTTTCCC
	PAG12	Pregnancy Associated Glycoprotein 12	F: GCCTTCCATCAGCTGTGTCA R: TTTGCCCGAAGCTGGAAGAA

Table 2 (*continued*)

Functional Category	Abbreviation	Gene Name	Forward and Reverse Primer
	PLAC1	Placenta Specific Protein 1	F: TTCAGCCTGTTCCGGACAA R: ATGAAGGGGTGTACCGTGAC
	PLET1	Placenta Expressed Transcript 1 Protein	F: TCAATTCCCTGAGAAATGCTGAAC R: TTGGTTGAGACGTGCTTGAC
	RSPO3	R-Spondin 3	F: ATTGCCCAGAAGGGTTGGAA R: CTCCATTCACTGGCCTCACA
	SOS1	SOS Ras/Rac Guanine Nucleotide Exchange Factor 1	F: GCTCCCTAGTGCTGACGTTTA R: GGGCTGCACGTTTTCTTCAA
	TMED2	Transmembrane P24 Trafficking Protein 2	F: CCCAAAGGACAAGACATGGAA R: GCCACTGCGAGCTCATTAA

of 40 % Percoll (MilliporeSigma, Saint Louis, MO) in PBS. The tube was centrifuged at $800 \times g$ at $20\ ^{\circ}C$ for 10 min with the brake off. The cell interface was harvested to a new tube, the volume brought to 45 mL with DMEM, and centrifuged at $100 \times g$ at $20\ ^{\circ}C$ for 10 min. Cells were washed with DME/F-12 culture medium and centrifuged as previously described in this manuscript. Following centrifugation, the cells were resuspended in $5-10$ mL of DMEM/F-12 and counted using a hemocytometer. Isolated trophoblast cells were seeded in 96-well flat-bottomed sterile culture plates at a concentration of 2,500 cells/100 μL of DMEM/F-12 culture medium (5 % FBS, 1 μg/mL penicillin/ streptomycin). Four plates of trophoblast cells were seeded from each placental sample and used for apoptosis, proliferation, and gene expression analyses. Trophoblast cells were cultured for 24 h after isolation at $37\ ^{\circ}C$ and 5 % CO_2 to allow the cells to equilibrate.

2.4. Trophoblast cell treatment with lymphocyte conditioned media

After 24 h of culture, media was removed from trophoblast cells. Trophoblast cells in each 96-well plate received isolated T cell-conditioned media as treatment (50 μL of lymphocyte-conditioned media and 50 μL of fresh media). Control wells were treated with 100 μL of unconditioned media. The trophoblast cells were cultured for an additional 48 h with treatments at $37\ ^{\circ}C$ and 5 % CO_2. After culture, one plate was used for proliferation assay and one plate was used for apoptosis assay. In the third and fourth plates, supernatant was removed by spinning at 800 x g for 6 min. Six μl of 2x reaction mix from Invitrogen™ Cell Direct™ One-Step qRT-PCR kit was added to each well and plates were frozen at $-80\ ^{\circ}C$ for later use in determination of abundances of mRNA transcripts.

2.5. Apoptosis assay

The eBioscience™ Annexin V-FITC Apoptosis Detection Kit was used according to manufacturer's instructions to determine trophoblast cell apoptosis after culture. Annexin and propidium iodide stained cells were run through a FACS Aria II to assess the number of dead trophoblast cells and trophoblast cells undergoing apoptosis. Annexin staining represented cells that were beginning to or were undergoing apoptosis. Propidium iodide positive cells indicated those that were dead.

2.6. Proliferation assay

Trophoblast cell proliferation was analyzed at 72 h of culture using the Cell Proliferation Kit II (XTT; MilliporeSigma, Saint Louis, MO) following manufacturer's instructions. This colorimetric assay quantifies proliferation, viability and cytotoxicity in cells cultured in 96-well plates (Roehm et al., 1991; Scudiero et al., 1988). Briefly, 50 μL of XTT labelling mixture was added to each cell culture well and cells were incubated for 2 h at $37\ ^{\circ}C$ and 5% CO_2. The plates were read at 450 nm and 650 nm on a Synergy H1 microplate reader (BioTek, Winooski, VT). To assess proliferation in response to treatment with T cell supernatant, absorbance measurements were taken at 72 h of trophoblast culture.

2.7. mRNA transcript abundance determinations

Relative abundances of mRNA transcript determinations were conducted using Fluidigm's 96.96 quantitative reverse transcriptase PCR (qPCR) Dynamic Array Integrated Fluidic Circuits (Fluidigm, South San Francisco, CA) according to Yang et al., 2016. The panel of 38 genes related to the following functional categories was assessed: placental development, apoptosis, immune response, and growth factor activity (Table 2). The reference genes used were glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta (*YWHAZ*), and actin beta (*ACTB*). All primers were designed and produced by Fluidigm (Fluidigm, San Francisco, CA). The single cell gene expression protocol was used with Fluidigm 96.96 Dynamic Array Integrated Fluidic Circuits (Fluidigm, San Francisco, CA) and included steps for reverse transcription, target amplification, and sample dilution prior to being loaded into the Fluidigm chip. Manufacturer instructions for priming and loading the 96.96 Fluidigm chip were followed, and the loaded chip was placed into the Biomark real-time PCR machine for qPCR (Fluidigm, San Francisco, CA). Relative abundance of mRNA transcripts was determined using the $2^{-\Delta\Delta Ct}$ method utilizing the average of the CT values of the reference genes. Values presented in this study are the fold change in abundances of mRNA transcripts for each treatment group

compared with the unconditioned medium (control group).

To verify the purity of the trophoblast cell culture the mRNA transcript profiles for trophoblast-specific genes, pregnancy-associated glycoprotein-10 (*PAG10*), and placenta-specific 1 (*PLAC1*) of trophoblast cells were assessed using qPCR procedures as previously described in this manuscript. A subset of trophoblast cells isolated for conducting the present study was harvested before culture and after 2 weeks of culture and the mRNA transcript abundance was assessed along with placental cotyledonary tissue and fibroblast cells as a means of comparison. Relative mRNA transcript abundance was calculated as described previously in this manuscript using the average of the CT values of the reference genes. The values presented for cotyledonary tissue, trophoblast cells harvested before culture and after culture are the fold change in mRNA transcript abundances compared with the abundances in fibroblast cells.

Two samples were used as standards to calculate intra and inter-assay repeatability. Intra-assay coefficient of variation (CV) was determined from results in individual procedures. Inter-assay CV was determined from results in three different chips conducted on three different days.

2.8. Statistical analysis

The difference of specific T cell subpopulations (CD4+, CD8+, γ/δ+, CD4+CD25-, CD4+CD25+ T cells) between pregnant and non-pregnant cows was analyzed using a Student's *t*-test using SPSS Statistics (version 26.0 IBM Corporation). The proliferation, apoptosis and mRNA transcript abundance data were analyzed using the mixed command of SPSS Statistics (version 26.0 IBM Corporation). The interactions between T cell population, pregnancy status (T cells originating from pregnant compared with non-pregnant cows), and placenta from which trophoblast cells were isolated were included in the model. Because there were no interactions that were significant, these were removed from the model. The T cell population, pregnancy status, and placenta were considered fixed factors while cow (donor of T cells) was considered a random factor in the model. Pairwise comparisons with Tukey's correction were used to determine which factors held significance. There were considered to be mean differences when there was a *P*-value equal to or less than 0.05. For the mRNA transcript abundance data, there were considered to be mean differences when the fold-change was greater than two and *P*-value was less than 0.05.

3. Results

### 3.1. Proportions of immune cell subpopulations in peripheral blood of pregnant and non-pregnant cows

As depicted in Fig. 1, the percentages of CD4+, CD8+, γ/δ+, and CD4+CD25- T cell populations in circulating blood of pregnant and non-pregnant cows did not differ. The number of CD4+CD25+ cells, however, was greater (*P* = 0.003) in pregnant compared with non-pregnant cows.

3.2. Apoptosis assay

Results from apoptosis assays indicated that at the end of the 72 h of trophoblast culture there were 57 %–75 % of live cells undergoing apoptosis (Fig. 2). There was no effect of T cell population on trophoblast cell apoptosis (Fig. 2A). Trophoblast cells treated with T cell supernatant from pregnant and non-pregnant cows had greater apoptosis rates than cells treated with unconditioned medium (Fig. 2B; *P* < 0.05).
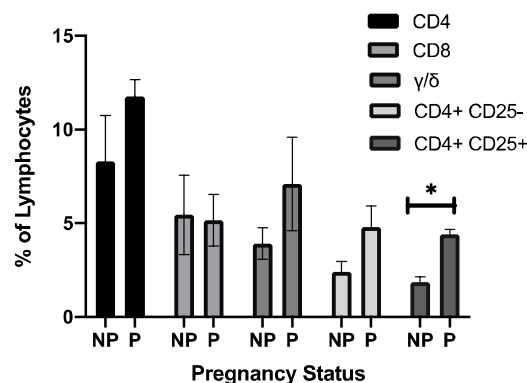
Fig. 1. Percentage of CD4+, CD8+, γ/δ+, CD4+CD25-, and CD4+CD25+T cells in circulating blood of non-pregnant (NP) and pregnant (P) cows; Data are the mean +/− SEM; *Indicate *P* ≤ 0.05 between pregnant and non-pregnant.
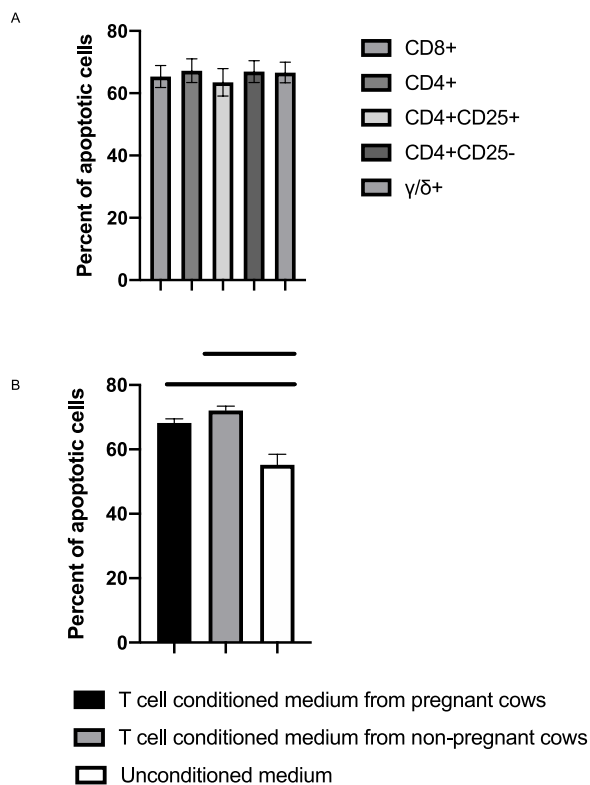
Fig. 2. (a) Percentage of apoptotic trophoblast cells treated with supernatant from T cell subpopulations CD8+, CD4+, CD4+CD25+, CD4+CD25−, and γ/δ+ at 72 h of culture; (b) Percentage of apoptotic trophoblast cells treated with T cell supernatant from pregnant, non-pregnant cows, and unconditioned medium; Data are the mean +/− SEM; *Indicate $P < 0.05$.

3.3. Proliferation assay

Treatment of trophoblast cells with supernatant from different T cell populations did not affect trophoblast proliferation (Fig. 3A). Treatment of trophoblast cells with T cell supernatant from pregnant cows decreased the extent of proliferation compared to unconditioned medium ($P < 0.001$). Trophoblast proliferation rates when there was treatment with the T cell supernatant from non-pregnant cows were greater than when there was treatment with T cell supernatant from pregnant cows ($P = 0.02$; Fig. 3B).

3.4. Trophoblast cell mRNA transcript abundances

There were lesser relative abundances of mRNA transcripts for trophoblast-specific genes in fibroblast cells compared with in placental tissue and trophoblast cells before and after culture. Relative abundances of *PAG10* and *PLAC1* mRNA transcripts in trophoblast cells before and after culture were markedly greater compared to the transcript profiles of fibroblast cells (Fig. 4). Overall, these results indicate the culture is composed of predominantly trophoblast cells and these cells are able to maintain placental-specific mRNA transcriptional functions.
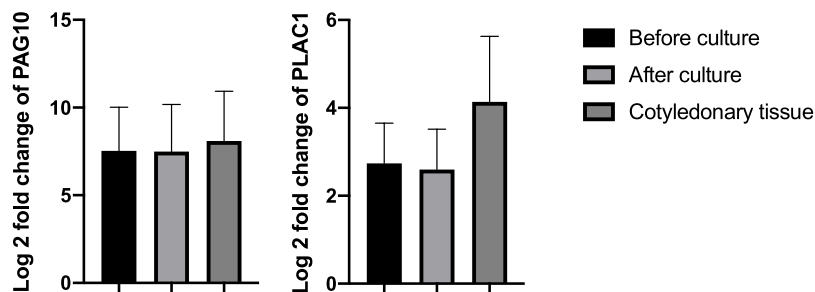
Trophoblast relative mRNA transcript abundances were not affected by the T cell population or placenta. Of the 38 genes investigated, pregnancy status had a significant effect on three growth factor associated genes (*BMP5, FGF2,* and *IGF1R*) and three genes associated with placental development (*PAG10, RSPO3,* and *TMED2*; Fig. 5). The log$_2$ fold change in values for mRNA transcript abundances are depicted in Fig. S1 for the 38 genes.

The T cell supernatant from pregnant and non-pregnant cows induced a 469-fold and an 8.2-fold increase in relative abundance of *BMP5* transcript compared with unconditioned medium ($P < 0.001$). The relative abundances of *PAG10* and *IGF1R* mRNA transcripts were increased by 10- ($P = 0.032$) and 45- ($P = 0.007$) fold, respectively, when trophoblast cells were treated with T cell supernatant from pregnant cows compared to unconditioned medium. Similarly, relative abundance of *TMED2* mRNA transcript was larger by 3.1-fold when trophoblast cells were treated with T cell supernatant from pregnant cows compared with unconditioned medium ($P = 0.018$). The relative abundances of mRNA transcripts for these four genes was also larger in trophoblast cells treated with pregnant compared with non-pregnant cow T cell supernatant.

There was a lesser relative abundance of *FGF2* mRNA transcript when T cell supernatant from non-pregnant cows was used to treat trophoblast cells compared to supernatants from the control ($P = 0.01$) and pregnant cows ($P = 0.003$), while treatment with the T cell supernatant from pregnant cows induced an increase in relative abundance of *FGF2* mRNA transcript compared to the control group (P

A

B
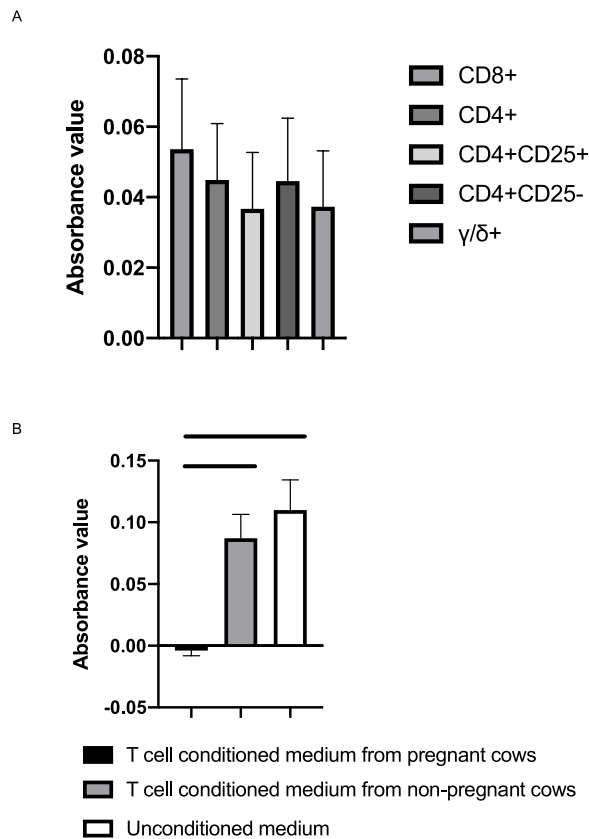
Fig. 3. Proliferation of trophoblast cells at 72 h of culture; (a) There was no statistical difference in the trophoblast proliferation rates induced by T cell supernatant from CD8+, CD4+, CD4+CD25+, CD4+CD25−, and γ/δ+ cells; (b) Proliferation of trophoblast cells treated with T cell supernatant from pregnant animals, non-pregnant cows and unconditioned medium; Data are the mean +/− SEM; *Indicate $P < 0.05$.

Fig. 4. Fold change in relative abundance of trophoblast specific mRNA transcripts, pregnancy associated glycoprotein-10 (*PAG10*) and placenta-specific 1 (*PLAC1*) mRNA transcripts in the cell culture system used in this study; Relative mRNA transcript abundance pattern of trophoblast cells collected before culture and after 2 weeks of culture was assessed along with cotyledonary tissue and fibroblast cells; Graphs depict $\log_2$ of ΔΔCt fold change in relative mRNA transcript abundances determined by quantitative polymerase chain reaction; Values for mRNA transcript abundances were normalized to reference mRNA transcript abundance for glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta (*YWHAZ*), and actin beta (*ACTB*) and values from fibroblast cells; Data are the mean +/− SEM.

= 0.002). The treatments with T cell supernatant from pregnant and non-pregnant cows resulted in a relatively larger abundance of *RSPO3* mRNA transcript by 3.5- ($P = 0.017$) and 12-fold ($P = 0.003$), respectively. The intra-assay coefficient of variation (CV) of each sample used as standards ranged between 0.3 % and 1.2 %. The inter-assay CV for relative abundance of mRNA transcript for these two samples ranged between 0.3 % and 1.4 % (Table S1).
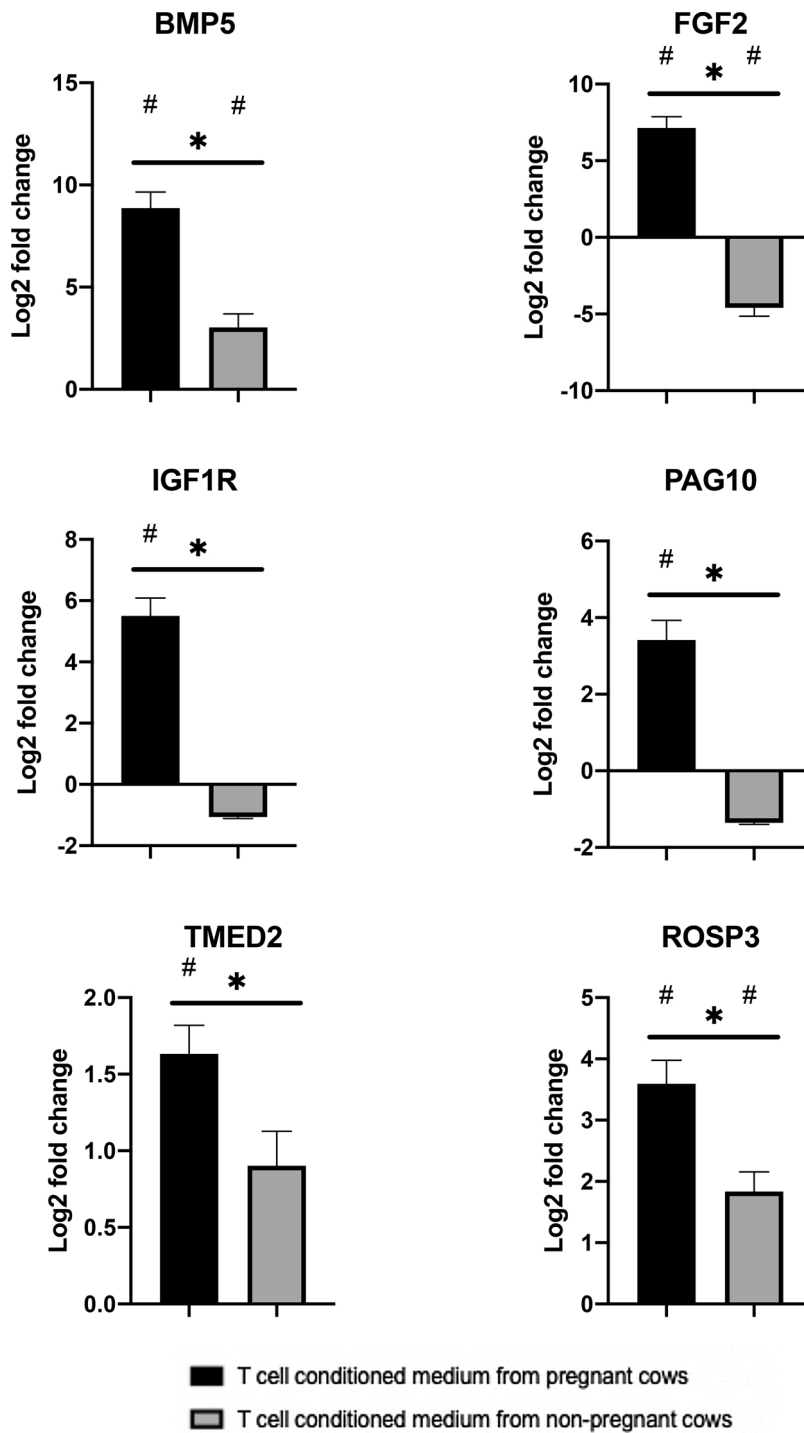
(caption on next page)

Fig. 5. Relative mRNA transcript abundances in trophoblast cells treated with T cell supernatant from non-pregnant and pregnant cows; Graphs depict $\log_2$ of $\Delta\Delta$Ct fold change in relative mRNA transcript abundances detected using the quantitative polymerase chain reaction. Values for mRNA transcript abundances were normalized to mRNA transcript abundance for glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta (*YWHAZ*), and actin beta (*ACTB*) and to treatment with unconditioned medium. Of a panel of 38 genes related to trophoblast apoptosis, growth factor activity, immune response, and placental development, the mRNA transcript abundances for six genes were changed: bone morphogenic protein 5 (*BMP5*), fibroblast growth factor 2 (*FGF2*), insulin growth factor (*IGF1R*), pregnancy-associated glycoprotein 10 (*PAG10*), transmembrane P24 trafficking protein 2 (*TMED2*), and R-spondin 3 (*RSPO3*); Data are the mean +/- SEM.

\#Indicate $P \leq 0.05$ compared with unconditioned medium.

*Indicate $P \leq 0.05$ between pregnant and non-pregnant supernatant.

4. Discussion

The present study is the first in which there was investigation of the paracrine effects of T cell populations on trophoblast survival, proliferation, and gene expression, as estimated based on abundances of mRNA transcripts, in cattle. Trophoblast cell apoptosis, proliferation, and profiles for relative abundances of mRNA transcripts were assessed after treatment with supernatant from CD8+, CD4+, CD4+CD25+, CD24+CD25-, and γ/δ+ T cells obtained from pregnant and non-pregnant cows.

Many immune cells are present at this interface during the pregnancy in cattle. These cells include macrophages, B cells, CD4+, CD8+, γ/δ+, and FoxP3+ T cells (Cobb and Watson, 1995; Koroghli et al., 2018; Oliveira and Hansen, 2009; Rutigliano et al., 2016; Vasudevan et al., 2017). Studies in humans and mice support the idea that the presence of CD4+CD25+ T regulatory cells in the endometrium is essential for the adequate modulation of uterine T cells and the establishment of pregnancy in species with hemochorial placentas (Rowe et al., 2011; Sasaki et al., 2004). The specific functions of CD4+CD25+ T and γ/δ+ T cells during pregnancy in cattle are still not well understood. In cattle, γ/δ+ T cells have immunomodulatory functions *in vitro* (Hoek et al., 2009; Rhodes et al., 2001). Determining whether or not CD4+CD25+ and γ/δ+ T cells support placental development in the endometrium of pregnant cattle is necessary to understand functions of these cells at the fetal-maternal interface.

In the present study, there was not a difference in the numbers of CD4+, CD8+, γ/δ+ and CD4+CD25- T cells between peripheral blood of pregnant and non-pregnant cows. There, however, was an increase in numbers of CD4+CD25+ cells in the blood of pregnant compared to non-pregnant cows. Results from the present study are consistent with those of Oliveira and Hansen (2008) where CD4+CD25+ cells were more abundant in circulation at 33–34 days of the gestational period in cattle.

Inconsistent with the initial hypothesis for the present study, there was not a T cell population effect on trophoblast proliferation, rate of apoptosis, or gene expression. There are a few possibilities as to why there was not this effect. First, the T cells used in the present study were not stimulated during culture. It is possible, therefore, that these cells did not secrete sufficient amounts of growth factors and cytokines. Second, it is possible that circulating T cells are substantially different from uterine counterparts. Although there has been detection of all circulating T cell populations used in this study in the pregnant uterus (Cobb and Watson, 1995; Koroghli et al., 2018; Oliveira and Hansen, 2009; Rutigliano et al., 2016; Vasudevan et al., 2017), these cells are present in different proportions in pregnant uterine tissue and circulating blood. Third, the gestational age of the fetal tissues used in the experiments (second trimester) may have affected results in the present study. At the timepoint tissues were collected in the present study, placentation and implantation will have occurred, trophoblast cells are fully differentiated and may not be as responsive to paracrine factors. Lastly, the present study was limited to investigating the direct effects of T cells in supporting trophoblast development rather than indirect effects by suppressing the function of effector T cells. Further experiments are needed to examine these possibilities.

The rate of apoptosis decreased in trophoblast cells treated with unconditioned medium which suggests that T cell soluble factors may have affected trophoblast cell viability. The T cells could not only have secreted factors into the cell culture medium that were harmful to trophoblast cells but also, these cells could have taken up soluble factors (extracellular vesicles and other molecules) from the medium that could promote cell function. This would cause the unconditioned medium to be more enriched with certain factors than the T cell conditioned medium and that may explain why the apoptosis rate of trophoblast cells treated with conditioned medium was greater than the proliferation of cells treated with unconditioned medium.

In the present study, trophoblast cells cultured with T cell supernatant from pregnant cows had lesser proliferation rates compared with trophoblast cells treated with T cell supernatant from non-pregnant cows and with unconditioned medium. Factors such as cytokines present in T cell supernatant could have suppressed trophoblast proliferation. These results are inconsistent with the thought that there is a shift from pro-inflammatory Th1 to anti-inflammatory Th2 cytokines during the gestational period with T helper 2 cytokines promoting growth of trophoblast cells (Lin et al., 1993; Wegmann et al., 1993), and T helper 1 cytokines contributing to placental toxicity and damage (Arck et al., 1999; Lim et al., 2000). Although the Th1 to Th2 shift has been well established in animals with hemochorial placentas such a shift has not been clearly documented in circulating T cells in cattle.

In the present study, there was an effect of pregnancy status on three placental development associated genes (*PAG10, RSPO3,* and *TMED2*) and three growth factor associated genes (*BMP5, FGF2,* and *IGF1R*) based on relative abundances of mRNA transcripts. Trophoblast *BMP5* mRNA was markedly increased in response to T cell supernatant from pregnant cows. Results from a study with bone morphogenic protein receptor-2 (*BMPR2*) in a mutant mouse model indicated the early embryonic development was arrested in these animals (Beppu et al., 2000). Results from another study indicated embryos with a *BMPR2* conditional knockout had abnormal implantation sites and trophoblast tissue, and fetal demise occurred at mid-gestation (Nagashima et al., 2013). The results from these studies, among others, indicate that BMPR2 in response to BMP5 and BMP7 binding is essential for decidualization and placental blood vessel formation.

There was a marked increase in *IGF1R* mRNA transcript abundance in response to T cell supernatant from pregnant cows. In the human placenta, the mRNA abundance of this transcript is greater in cases of intrauterine growth retardation compared to normal term placentas (Abu-Amero et al., 1998). Insulin-like growth factor-1 enhances proliferation of trophoblast cells collected during the first-trimester of the gestational period (Maruo et al., 1995), and rescues cytotrophoblast cells from apoptosis (Forbes et al., 2008). Contrary to results from these previous studies, in the present study there was an association between increased *IGF1R* mRNA abundance and decreased proliferation and increased rates of apoptosis in trophoblast cells treated with pregnant cow T cells supernatant. It is possible that the relative larger abundance of *IGF1R* mRNA transcript occurred to rescue trophoblast cells from apoptosis.

Pregnant cow T cell supernatant induced an increase in abundance of *PAG10* mRNA transcript in trophoblast cells. The function of PAGs has not been clearly elucidated but there is evidence that these proteins have functions in trophoblast development, placental formation, and, potentially, immune modulation (Mathialagan and Hansen, 1996; Telugu et al., 2010; Thompson et al., 2012; Wooding et al., 2005). Similarly, the relative abundance of *FGF2* mRNA transcript was greater in response to T cell supernatant from pregnant cows. In the placenta, *FGF2* induces angiogenesis (Reynolds et al., 2005; Zygmunt et al., 2003) and proliferation of endothelial cells (Wang et al., 2008). Collectively, the increase in relative abundances of *BMP5*, *IGF1R*, *PAG10,* and *FGF2* mRNA transcripts in trophoblast cells treated with T cell supernatant of pregnant cows indicates that peripheral T cells from pregnant cows secrete pregnancy-induced substances that enhance these protein abundances to promote trophoblast growth, proliferation, and angiogenesis.

In the present study, there were larger abundances of *TMED2* and *RSPO3* mRNA transcript in supernatant from pregnant animals. The TMED2 is essential for transporting cargo proteins between the endoplasmic reticulum and the Golgi apparatus. The *TMED2* gene expression has recently been reported to be required for chorion and allantois formation (Hou and Jerome-Majewska, 2018). The R-spondin 3 (*RSPO3*) is required for placental development in mice. When the *RSPO3* gene was modified, placental vascularization was abnormal causing embryonic death (Aoki et al., 2007). This leads to the conclusion that T cells from pregnant animals secrete substances that maintain the expression of genes important for cellular metabolism and trophoblast development.

Collectively, results from the present study indicate treatment with T cell supernatant from pregnant cows enhanced the expression of genes associated with placental development and growth factor activity based on abundances of mRNA transcripts, suggesting that T cell paracrine factors may modulate placental growth and development. It is possible that treatment of trophoblast cells for 48 h was sufficient to alter mRNA transcript abundance profiles but not for a long enough duration to induce changes in cell proliferation and apoptosis. Future studies, therefore, are needed to investigate the long-term paracrine effects of T cells on trophoblast development and identify specific factors responsible for this response. Studies are also warranted to determine the functional differences between T cells from pregnant and non-pregnant cows. Progesterone regulates the abundance of uterine serpins which function as immunomodulators (Padua and Hansen, 2010). In the present study, non-pregnant T cell donor cows were in diestrus which eliminates the confounding effect of progesterone on T cell function.

The reduced capacity of trophoblast cells treated with T cell supernatant to proliferate compared to unconditioned media may have induced an upregulation of gene transcripts related to growth and trophoblast development as a compensatory mechanism. Additionally, although apoptosis rates were increased in trophoblast cells treated with both pregnant and non-pregnant T cell supernatant, only pregnant T cell supernatant induced an increase in the abundance of mRNA transcripts that encode for proteins related to placental development and growth factor activity. It is possible that factors present in the T cell supernatant of pregnant cows caused a change in expression of genes related to cell growth and metabolism as a response to rescue trophoblast cell growth.

5. Conclusions

Results from the present study indicate there are effects of T cell culture supernatant on trophoblast cell apoptosis, proliferation and gene expression profiles as indicated by relative abundances of mRNA transcripts based on the pregnancy status of cows at the time of T cell collection. Considered together, the results from the present study are the first where T cells from pregnant cows may support trophoblast development in a paracrine fashion in cattle. This explains, in part, the important functions of maternal immune cells in the endometrium supporting the development of the fetus.

Author's contributions

Experimental design and conception were created by H.M.R. All laboratory procedures were conducted by K.A.L. and K.M. Data collection was carried out by K.A.L., P.A.C., K.M. and A.C.S. Statistical analyses and interpretation were performed by H.M.R. and K.A.L. Manuscript writing and proof reading K.A.L., H.M.R., and A.T. All authors have approved the final manuscript.

Declaration of Competing Interest

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

Acknowledgements

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.anireprosci.2021.106747.

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