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—Original Article—

Downregulation of Lymphatic Vessel Formation Factors in $PGF_{2\alpha}$-induced Luteolysis in the Cow

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Abstract. Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) induces luteolysis in cows and causes infiltration of immune cells, which resembles inflammatory immune response. Since the general immune response is mediated by the lymphatic system, we hypothesized that luteolysis is associated with generation of an immune response that involves lymphatic vessels in the bovine corpus luteum (CL). The CL was obtained from Holstein cows at the mid-luteal phase (days 10–12, ovulation = day 0) by ovariectomy at various time points after $PGF_{2\alpha}$ injection. Lymphatic endothelial cell (LyEC) marker, endothelial hyaluronan receptor 1 (LYVE1), levels decreased significantly 12 h after $PGF_{2\alpha}$ injection. Podoplanin, another LyEC marker, decreased from 15 min after $PGF_{2\alpha}$ injection. $PGF_{2\alpha}$ also diminished mRNA expression of lymphangiogenic factors, such as vascular endothelial growth factor (VEGF) C, VEGFD and VEGF receptor 3 (VEGFR3). During $PGF_{2\alpha}$-induced luteolysis, the levels of mRNA expression of tumor necrosis factor α (TNFα; the major pro-inflammatory cytokine) and chemokine (C-X-C motif) ligand 1 (neutrophil chemokine) were increased. On the other hand, chemokine (C-C motif) ligand 21, which regulates outflow of immune cells from tissues via the lymphatic vessels during an immune response, was decreased. This study demonstrated that the lymphatic network in the CL is disrupted during luteolysis and suggests that during luteolysis, immune cells can induce a local immune response in the CL without using the lymphatic vessels.

Key words: Corpus luteum, Cow, Luteolysis, Lymphatic vessel, Tumor necrosis factor α

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The corpus luteum (CL) is a complex ovarian organ consisting of vascular endothelial, steroidogenic and immune cells. The luteolytic cascade of the bovine CL is primed by the pulsatile release of uterine prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$). The decrease in progesterone concentrations is closely followed by a structural degeneration of vasculature and apoptosis of steroidogenic cells [1, 2]. In the CL, the expression of vascular endothelial growth factor (VEGF) A, the most common angiogenic factor, was shown to be downregulated by $PGF_{2\alpha}$ injection [3, 4], suggesting that $PGF_{2\alpha}$ inhibits the angiogenic process in the regressing CL. Additionally, endothelin-1 (EDN1) and angiotensin (Ang) II, strong vasoconstrictive factors, were shown to be associated with the process of luteal regression in ruminants [5–8]. Furthermore, $PGF_{2\alpha}$ upregulated the expression of EDN1 and Ang II *in vivo* and *in vitro* [5–8], resulting in intensive vasoconstriction and disruption of oxygen and nutrient supply during luteolysis.

The immune response also plays an essential role in luteolysis [9–11]. Leukocytes such as T lymphocytes, macrophages and neutrophils infiltrate the CL and produce different cytokines including tumor necrosis factor (TNF)-α, interleukin (IL)-1β, interferon (IFN)-γ and monocyte chemoattractant protein 1 (MCP1) during CL regression [10, 12–21, 22]. TNFα is secreted by activated macrophages and has been implicated in neutrophil and monocyte recruitment to inflammatory sites [23–27]. Spontaneous or $PGF_{2\alpha}$-induced luteolysis are associated with a significant rise in intraluteal TNFα as shown in previous studies by using a CL microdialysis system [28]. Moreover, TNFα induces apoptotic death of steroidogenic and endothelial cells *in vitro* [11, 29, 30]. In short, the luteolytic phenomenon is an inflammatory-like immune response characterized by a massive recruitment of leukocytes and high production of cytokines.

The cardiovascular circulatory system consists of a tree-like hierarchy of vessels formed from a primitive vascular network. The lymphatic system is a distinct type of vascular system present in most organs of the body. Lymphatic endothelial cells (LyECs) form the vessels that drain the interstitial fluid from the tissues back into venous circulation after passage through the lymph node network [30, 31]. The lymphatic system also plays a crucial role in an immune response to infectious agents. LyECs act as a gatekeeper that controls immune cells, such as dendritic cells and macrophages, during migration from tissues to lymph nodes. Lymphatic drainage is essential for the recirculation of lymphocytes, allowing access of professional antigen-presenting cells to lymph nodes [32, 33]. During inflammation and infection, the number of dendritic cells reaching the

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lymph nodes drastically increases [34–36]. We recently reported the expression of lymphatic vessel markers, such as lymphatic endothelial hyaluronan receptor 1 (LYVE1) and podoplanin, in the bovine CL [37], suggesting that luteolysis involves the immune response by increasing the number of immune cells that traverse the lymphatic vessels into the CL.

This study aimed to evaluate the lymphatic system, including lymphatic vessel-related factors and cytokines in the CL, at different time points following $PGF_{2\alpha}$-induced luteolysis.

Materials and Methods

CL collection was conducted at the Clinic for Cattle of the University of Veterinary Medicine Hannover, Germany. The experimental procedures complied with the guidelines of the Ethics Committee on Animal Rights Protection of Oldenburg, Germany, in accordance with the German legislation on animal rights and welfare. The protocol was approved by the committee on the Ethics of Animal Experiments of the University of Veterinary Medicine Hannover (permit number: 33.9-42502-04-07/1275).

$PGF_{2\alpha}$-induced luteolysis

For collecting CLs during luteolysis, 29 normal cyclic German Holstein cows were used in this study. The day of estrus was designated Day 0. Cows (n = 4–5 for each time point) at the mid-luteal phase (days, 10–12) were injected with $PGF_{2\alpha}$ via the intramuscular route (0 min; 0.5 mg of cloprostenol, 2.0 ml Estrumate™, Essex Tierarznei, Munich, Germany), and ovaries were collected by ovariectomy [3] through the vagina before $PGF_{2\alpha}$ injection (0 min), and at 15 min, 30 min, 2 h and 12 h after injection.

Processing of the corpus luteum

The CL was enucleated from the ovary and dissected, free of connective tissues, as described previously [38]. The CL tissue samples were then minced, immediately placed into a 1.5-ml microcentrifuge tube with or without 0.4 ml of TRIzol reagent (Invitrogen, Karlsruhe, Germany) and stored at –80 C until analysis.

RNA extraction, cDNA synthesis and reverse-transcription quantitative PCR

Total RNA was extracted from the CL following the protocol of Chomczynski and Sacchi [39] using TRIzol reagent, treated with DNase using a commercial kit (Promega, Madison, WI, USA) and frozen at –20 C in THE RNA Storage Solution (Ambion, Austin, TX, USA). The cDNA was synthesized as previously described [40]. The levels of mRNA expression of *LYVE1, podoplanin, VEGFR3, VEGFC, VEGFD, TNFα, CXCL1, CCL21* and *β-actin* were quantified by reverse-transcription quantitative polymerase chain reaction (RT-qPCR) as previously described [40]. RT-qPCR reactions were performed in duplicate in a final volume of 10 µl containing 5 µl of QuantiTect™ SYBR Green PCR Buffer (QIAGEN GmbH, Hilden, Germany), 2.8 µl of H_2O (Sigma, St. Louis, MO, USA), 0.1 µl of 50 µM forward and reverse primers (Table 1 lists primer sequences and accession numbers) and 2 µl of cDNA template or water (as a non-template negative control). RT-qPCR conditions were 10 min at 95 C, followed by 40 cycles of 95 C for 15 sec, 56 C for 30 sec and 72 C for 30 sec using a LightCycler (Roche Diagnostics, Mannheim, Germany). The PCR products were resolved by electrophoresis, and the target bands were cut out and purified using a DNA purification kit (SUPRECTM-01, Takara Bio, Otsu, Japan). The mRNA expression levels in the CL were normalized using β-actin as an internal standard. Each PCR amplification product was sequenced using an Applied Biosystems 3730 × l DNA Analyzer (Applied Biosystems, Foster City, CA, USA).

Western blotting

The CL tissue samples were homogenized in lysis buffer containing 25 mM Tris-HCl (pH 7.4), 0.3 M sucrose, 2 mM Na_2EDTA and cOmplete Protease Inhibitor Cocktail (Roche Diagnostics, Mannheim, Germany), and then filtered with a 70-µm filter (Cell Strainer, REF 352350, BD Falcon, Franklin Lakes, NJ, USA). The proteins were dissolved in sample buffer (0.5 M Tris-HCl [pH 6.8], glycerol, 10% SDS and 0.5% bromophenol blue) and steamed for 5 min. The entire samples were subjected to electrophoresis on 10% SDS-PAGE gels for 50 min at 200 V. The proteins were transferred to PVDF membranes (Bio-Rad Laboratories, Hercules, CA, USA) for 2 h at 60 V. The membranes were blocked with 4% Block Ace Powder (DS Pharma Biomedical, Osaka, Japan) in TBS with 0.5% Tween-20 (Sigma) for 1 h at room temperature. The membranes were next incubated with a rabbit anti-mouse-LYVE1 polyclonal antibody (1:500 dilution, Abcam, Cambridge, UK) and a mouse anti-β-actin monoclonal clone AC-15 antibody (1:10,000 dilution, Sigma) at 4 C overnight. The membranes were then washed 3 times in TBS with 0.5% Tween-20, incubated with HRP-conjugated anti-rabbit (1:10,000 dilution, Rockland Immunochemicals, Gilbertsville, PA, USA) or anti-mouse (1:10,000 dilution, GE Healthcare, Buckinghamshire, UK) IgG antibodies for 1 h at room temperature, and washed 3 times with TBS with 0.5% Tween-20. The signals were detected using an ECL Western Blotting Detection System (GE Healthcare). The optical densities of the immunospecific bands were quantified using an NIH Image computer-assisted analysis system.

Statistical analysis

All data are presented as means ± standard error of the mean (SEM). The statistical significance of differences was assessed by one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison. A P value less than 0.05 was considered significant.

Results

mRNA expression of LYVE1 and podoplanin and LYVE1 protein expression in the bovine CL during $PGF_{2\alpha}$-induced luteolysis

Figure 1 shows the mRNA expression of *LYVE1* and *podoplanin* (markers of LyECs) and LYVE1 protein expression in the bovine CL during $PGF_{2\alpha}$-induced luteolysis (15 min, 30 min, 2 h and 12 h after $PGF_{2\alpha}$ injection). The level of *LYVE1* mRNA expression did not change from 0 min to 2 h, but decreased at 12 h compared with those at 15 min and 2 h after $PGF_{2\alpha}$ injection (Fig. 1A; $P<0.05$). Lowered expression of LYVE1 protein was found for the first 2 h ($P<0.1$), and the expression was decreased 12 h after $PGF_{2\alpha}$ injection (Fig. 1B; $P<0.05$). Lowered *Podoplanin* mRNA expression was

Table 1. Primer sequences for the investigated genes

Gene	Primer sequence		Accession No.	Product size (bp)
LYVE1	FWD	AGG TTG AAG AAG CAC GGA AA	NM_205815	231
	REV	AGG GAT CAT CGG TGG TGA TA		
Podoplanin	FWD	TGG CTA CGG AGC TTT TTC AT	ENSBTAT	291
	REV	CAC ACC CAG GGT TGT TTT CT	0000002341	
VEGFR3	FWD	TGA GGA TAA AGG CAG CAT GGA	AF030379	66
	REV	CCC AGA AAA AGA CAG CGA TGA		
VEGFC	FWD	CTC AAG GCC CCA AAC CAG T	NM_174488	71
	REV	CAT CCA GCT TAG ACA TGC ATC G		
VEGFD	FWD	GGA GAA TGC CTT TTG AAC CA	NM_001101043	272
	REV	CCA GTC CTC GAA GTG TGT GA	XM_590821	
TNFα	FWD	TAA CAA GCC GGT AGC CCA CG	K_00622	221
	REV	GCA AGG GCT CTT GAT GGC AGA		
CXCL1	FWD	CTA TTT TTG GGG AGA GGG TAT TCC	U66096	94
	REV	CGT GAC CTA TCT GTT TGC TTG AAAC C		
CCL21	FWD	AGT TGC GCT ATG CCA GCT AT	NM_001038076.2	184
	REV	TTC CCT TCT TGC CAG ACT TG		
β-actin	FWD	CCA AGG CCA ACC GTG AGA AGA T	K00622	256
	REV	CCA CGT TCC GTG AGG ATC TTC A		

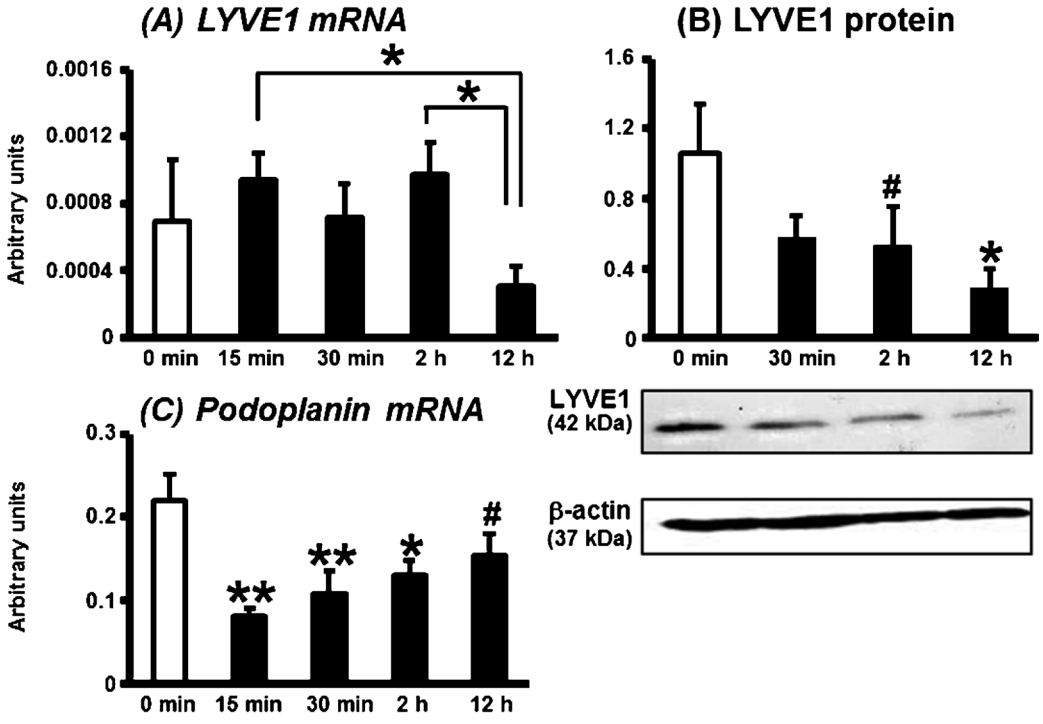

Fig. 1. mRNA expression of *LYVE1* and *podoplanin* and LYVE1 protein in the bovine CL during $PGF_{2\alpha}$-induced luteolysis. *LYVE1* mRNA expression decreased in the CL at 12 h as compared with 15 min and 2 h after $PGF_{2\alpha}$ injection (A). LYVE1 protein expression also decreased at 12 h compared with that at 0 min after $PGF_{2\alpha}$ injection (B). *Podoplanin* mRNA expression was decreased in the CL at all points after $PGF_{2\alpha}$ injection (C). All values are shown as means ± standard error of the mean (SEM; n = 4–5 in each time). *·** Significant difference ($P<0.05$ or $P<0.01$ compared with control) as determined by Bonferroni's multiple comparison test. # Tendency for a difference ($P<0.1$) as determined by Bonferroni's multiple comparison test.

found at 15 min, but the expression tended to recover after that up until 12 h after $PGF_{2\alpha}$ injection (Fig. 1C; P<0.01, P<0.05 or P<0.1).

mRNA expression of lymphangiogenic factors in the bovine CL during $PGF_{2\alpha}$-induced luteolysis

Changes in the transcripts of lymphangiogenic factors (*VEGFC*, *VEGFD* and *VEGFR3*) in the CL during $PGF_{2\alpha}$-induced luteolysis are shown in Fig. 2. The levels of *VEGFC* and *VEGFR3* mRNA expression were continuously suppressed after $PGF_{2\alpha}$ injection (Figs. 2A and C; P<0.01 or 0.05). *VEGFD* mRNA expression decreased at 15 min to 2 h as compared with 0 min after $PGF_{2\alpha}$ injection (P<0.1) and decreased significantly 12 h after $PGF_{2\alpha}$ injection (Fig. 2B; P<0.05).

mRNA expression of TNFα, CXCL1 and CCL21 in the bovine CL during $PGF_{2\alpha}$-induced luteolysis

TNFα mRNA expression increased significantly in the CL at 15 and 30 min and 2 h as compared with 0 min after $PGF_{2\alpha}$ injection (Fig. 3A; P<0.01 or 0.05). Chemokine (C-X-C motif) ligand 1 (CXCL1) enhances the recruitment of neutrophils and acts as a mediator of inflammation during the early wound healing process [41, 42]. *CXCL1* mRNA expression also increased at 15 min (P<0.1) and was higher at 30 min and 2 h (P<0.01 or 0.05) as compared with 0 min after $PGF_{2\alpha}$ injection (Fig. 3B).

Chemokine (C-C motif) ligand 21 (CCL21) is involved in modulation of inflammatory responses and may play a role in the migration of leukocytes from peripheral tissues through afferent lymphatic vessels. *CCL21* mRNA expression decreased at 15 min, 2 h and 12 h after $PGF_{2\alpha}$ injection compared with the expression level at 0 min (Fig. 3C; P<0.1 or P<0.05).

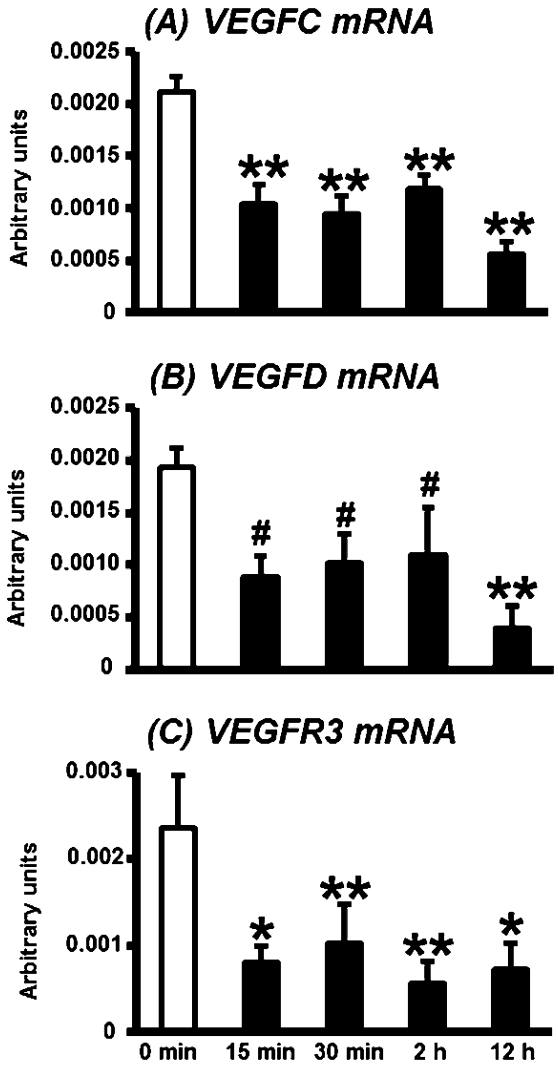

Fig. 2. mRNA expression of *VEGFC*, *VEGFD* and *VEGFR3* in the bovine CL during $PGF_{2\alpha}$-induced luteolysis. mRNA expressions of *VEGFC* (A), *VEGFD* (B) and *VEGFR3* (C) started to decrease at 15 min after $PGF_{2\alpha}$ injection and then remained at, then kept low levels. All values are shown as means ± standard error of the mean (SEM; n = 4–5 in each time). *,**Significant difference (P<0.05 or P<0.01 compared with control) as determined by Bonferroni's multiple comparison test. #Tendency for a difference (P<0.1) as determined by Bonferroni's multiple comparison test.

Discussion

It is well known that cell death of luteal endothelial cells is induced during luteolysis, which is called structural luteolysis. The treatment with $PGF_{2\alpha}$ resulted in a downregulation of *fibroblast growth factor* (*FGF*)*-2* mRNA expression and mRNA and protein expression of VEGFA, which are potent angiogenic factors in the CL [3, 4]. In the CL, $PGF_{2\alpha}$ decreased *angiopoietin (ANPT) 1* mRNA expression [43] and stimulated a high level of angiopoietin ANPT 2 in relation to ANPT1, inducing the destabilization of blood vessels [3]. The presence of VEGFA may also define the fate of destabilized blood vessels [44], and thus a deficiency in VEGFA may result in the disruptive destabilization of blood vessels after $PGF_{2\alpha}$ injection. The lymphatic vascular system has a role in the body's circulation system together with blood vessels, but there have been no studies about the changes of the lymphatic network in the CL during luteolysis. This study showed for the first time that lymphatic vessel markers, such as LYVE1 and podoplanin, and lymphangiogenic factors, such as VEGFC, VEGFD and VEGFR3, were downregulated in luteolysis. Interestingly, VEGFA and FGF2 have potent lymphangiogenic activity [45, 46]. Additionally, ANPT-1 resulted in lymphatic endothelial cell proliferation, lymphatic vessel enlargement, sprouting and branching *in vivo* [47] and promoted survival and proliferation of LyECs *in vitro* [48]. Thus, these findings suggest that luteolytic $PGF_{2\alpha}$ downregulates the production of vascular-related factors, resulting in destruction of the vascular system through angiolysis and lymphangiolysis in the CL during luteolysis. Berisha *et al.* [49] showed that the number of VEGFR-3-immunostained large luteal cells significantly decreased in the bovine CL during regression (day>18). Immunohistochemical observation of lymphatic vessels markers in the CL after luteolysis is required to clarify how the luteal lymphatic structures disintegrate during $PGF_{2\alpha}$-induced luteolysis.

During luteolysis, leukocytes, especially neutrophils, macrophages and T lymphocytes, significantly increase in number in the CL [10, 14, 21, 22, 50]. Pro-inflammatory cytokines such as TNFα, IL-1β

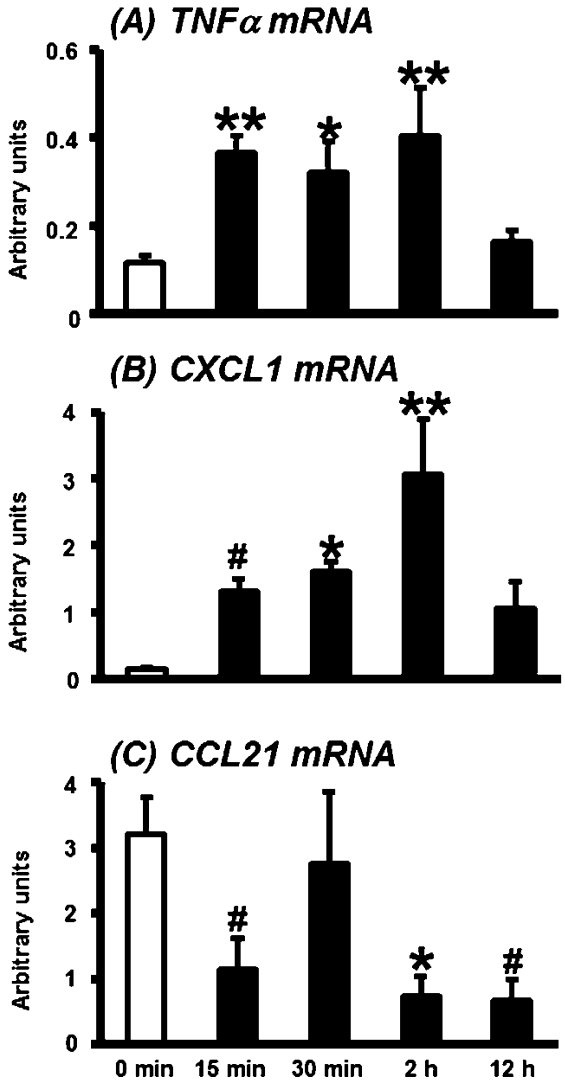

Fig. 3. mRNA expression of *TNFα*, *CXCL1* and *CCL21* in the bovine CL during $PGF_{2\alpha}$-induced luteolysis. mRNA expression of *TNFα* (A) and *CXCL1* (B) increased in the CL after $PGF_{2\alpha}$ injection, whereas *CCL21* mRNA expression decreased (C, mean ± standard error of the mean [SEM; n = 4–5 in each time]). *,** Significant difference ($P<0.05$ or $P<0.01$ compared with control) as determined by Bonferroni's multiple comparison test. #Tendency for a difference ($P<0.1$) as determined by Bonferroni's multiple comparison test.

and IFNγ and chemokines such as MCP1 and IL-8 are associated with luteal regression [3, 13, 14, 51, 52]. These findings suggest that the luteolytic phenomenon is an inflammatory-like immune response. Accordingly, we hypothesized that various immune cells promote an immune response involving the lymphatic vessels during luteolysis in the CL. The immune cells that enter sites of inflammation, such as neutrophils, dendritic cells and macrophages, migrate from tissues and travel to lymph nodes through peripheral afferent lymphatic vessels [33, 34, 36, 53–55]. In the lymph nodes, dendritic cells present antigens to T cells, and in the case of immune response, this leads to the clonal expansion and differentiation of antigen-specific T cells. These T cells recirculate from the lymph nodes to inflammatory peripheral tissues through the blood vessels, resulting in an effective immune response through the lymphatic vessels and lymph nodes, which is called lymphocyte homing. In this process, the homeostatic chemokine CCL21 plays an important role of regulating outflow of immune cells from tissue. With regard to the exit of leukocytes from peripheral tissues through afferent lymphatic vessels, the expression of homeostatic chemokine CCL21 by dermal afferent lymphatic vessels is essential in guiding naïve T cells, dendritic cells and neutrophils [32, 56, 57]. In the CL during luteolysis, *CCL21* mRNA expression was decreased. Additionally, the decrease in the expression of lymphatic vessel-related factors suggests the loss of lymphatic vessels in the CL during luteolysis. Thus, luteolysis may be a local, not systemic, inflammatory-immune response that does not utilize lymphatic vessels and lymph nodes.

In summary, expression of lymphatic cell markers and lymphangiogenic factors dramatically decreased in the CL during luteolysis, suggesting that the lymphatic network is disrupted in the CL during luteolysis, as well as the vascular structure.

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