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Interleukins' expression profile changes in granulosa cells of preovulatory follicles during the postpartum period in dairy cows

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ABSTRACT

The postpartum period in dairy cows is associated with a state of temporary negative energy balance and could induce functional changes into ovarian granulosa cells (GC) resulting in significant impact on the ovarian function and fertility. Yet, the regulation of interleukin receptors (ILRs) in GC as well as ILs expression profile during the postpartum period have not been fully investigated. We hypothesized that the postpartum period is associated with changes in ILs expression profile that could affect follicular development and ovulation rate. First, we aimed to investigate the expression and regulation of different IL and IL receptors in GC at different stages of follicular development and then analyse the changes in target ILs expression profile induced during the postpartum period. In the first objective, normal cycling cows were selected and GC were collected from small follicles (SF), dominant follicles at day 5 of the estrous cycle (DF), and ovulatory follicles, 24 h following hCG injection (OF). In the second objective, dairy cows between 50 and 70 days postpartum were randomly selected, and β-hydroxybutyrate (BHB) concentrations were measured in blood samples in order to assign cows to the BHB^+ group (> 1.4 mmol/L) or BHB^- group (< 1.2 mmol/L). GC were collected from preovulatory follicles by transvaginal aspiration. Total RNA was extracted from GC of all groups for analysis of target ILs and ILRs expression. Steady-state mRNA levels of *IL4R* was strongest in the DF, while *IL15R* expression was greatest in the OF, and *IL21R* showed increased steady-state mRNA levels in the corpus luteum as compared to the different groups of follicles. Overall, expression of *IL1A*, *IL1B*, *IL8*, *IL15*, *IL23* and *TNFα* was stronger in OF as compared to DF, while *IL4* and *IL10* expression was stronger in SF than in DF. Similarly, expression of *IL1A*, *IL1B*, *IL8*, *IL15*, *IL23*, and *TNFα* were significantly stronger in GC of BHB^+ cows than in the control, while *IL4* expression was significantly reduced in BHB^+ as compared to control cows. We have established an *IL* expression profile, which suggest a correlation with BHB levels during the postpartum period. Additionally, we have demonstrated a differential regulation of target *ILRs* in GC at different stages of follicular development. Overall, these data provide a better understanding of the changes that could affect follicular development and ovulation during the postpartum period and lay the ground for further investigations.

1. Introduction

It is documented that dairy cows' fertility has significantly declined over the past decades [1]. Although the causes associated with declining fertility are diverse, the deficiency or absence of ovulation during the postpartum period represents a major problem that could contributed to this decline. About 40% of dairy cows in large productions display anestrus at 60 days postpartum [2]. This could be attributed in part to intense milk production, which represents a very important energy investment for cows resulting in a state of negative energy balance [2,3]. The postpartum period has a significant impact on dairy cow fertility since it affects the microenvironment of the follicle and causes a delay in return to ovarian cyclicity, a reduction in the number of large follicles, and a high anovulation rate [4,5]. When follicular growth is disrupted and the follicular environment altered, the oocyte quality is compromised leading to negative effects on the conception rate and gestation. Thus, investigations of factors that affect the health of the ovarian follicle and the oocyte, specifically factors

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originating from granulosa cells, are crucial for improving fertility.

Granulosa cells are a particularly important component of the follicle because they play a critical role in reproductive functions. They contribute to steroid hormone synthesis [6], and are involved in the oocyte maturation and release [7], and in the corpus luteum formation after ovulation [8,9]. Therefore, the control of GC proliferation, differentiation, and function depends on precise regulation and activation of specific target genes. This regulation is essential for normal follicular development and timely production of paracrine factors as it affects the physiological state of a dominant preovulatory follicle. A common feature of most reproductive tissues and cells, including granulosa cells, is the presence of cytokines that are involved in the regulation of reproductive function [10–12]. Specifically, the ovarian follicular fluid contains a variety of proteins such as cytokines and other peptides, in addition to steroids [13].

Cytokines are small, soluble signaling proteins that are better known for their immunoregulatory properties but are also known for controlling cell proliferation, differentiation, and function [12,14]. Cytokines include interleukins (ILs), colony-stimulating factors (CSFs), Tumor necrosis factors (TNFs), Transforming growth factors (TGFs), and other peptides that are produced by a myriad of cell types. Interleukins are mainly synthesized by lymphocytes, monocytes, macrophages, and endothelial cells but also by granulosa cells of the ovarian follicle [11,13,15–17]. There are convincing evidences that cytokines, including interleukins (ILs), have functional effects on reproductive tissues affecting follicular development, ovulation, and the corpus luteum function [18–21]. They create an immuno-permissive and embryotropic environment that supports multiple aspects of reproduction [11,12]. However, the involvement of ILs in follicular development and fertility during the postpartum period hasn't been fully investigated. Stassi et al. have recently shown that altered expression of cytokines including IL1α, IL1β, IL4, IL6, IL8 and TNFα may contribute to follicular persistence and ovulation failure in cattle with follicular cysts [17,22]. In another study, it was shown that proinflammatory cytokines TNFα and IL6 suppress androgen production and downregulated the expression of markers for theca interna cells [16]. Based on these reports and others [23,24], we analyzed the presence of ILs and target IL receptors in bovine granulosa cells during follicular development. Moreover, we analyzed the regulation of selected proinflammatory interleukins (IL1A, IL1B, IL8, IL15, IL21, IL23 and TNFα) and anti-inflammatory interleukins (IL4, IL10) during the postpartum period. We report here the differential regulation of ILRs in GC through follicular development and the association between the postpartum period in dairy cows and significant changes in the expression profile of ILs in GC. The identification of target ILs most affected by the postpartum period may provide new tools and information for a better understanding of the pathways that control ovarian function.

2. Materials and methods

2.1. Animal model for analysis of IL and IL receptors expression in granulosa cells

The experimental protocol was reviewed and approved by the Animal Ethics Committee of the Faculty of Veterinary Medicine of the University of Montreal and the cows were cared for in accordance with the Canadian Council on Animal Care guidelines [25]. Expression of IL and target IL receptors were first analyzed in order to determine their regulation during follicular development and ovulation using an *in vivo* model previously characterized [26]. Following estrous synchronization with $PGF_{2\alpha}$, normal cycling crossbred heifers were randomly assigned to a dominant follicle group (DF, n = 4), or an ovulatory hCG-induced follicle group (OF, n = 4). The DF was collected at day 5 of the estrous cycle (day 0 = day of estrus) and was defined as ≥8 mm in diameter and growing while subordinate follicles were either static or regressing. The OF were obtained following an injection of 25 mg of $PGF_{2\alpha}$ on day 7 to induce luteolysis, thereby promoting the development of the DF of the first follicular wave into a preovulatory follicle. An ovulatory dose of hCG (3000 IU, iv; APL, Ayerst Lab, Montréal, QC) was injected 36 h after the induction of luteolysis, and ovaries bearing the hCG-induced OF were collected by ovariectomy 24 h post-hCG. Immediately following collection, follicles were dissected into separate isolates of GC [26] and stored at –70 °C. Granulosa cells and follicular fluid were collected from individual dominant follicles and pre-ovulatory hCG-induced follicles. Follicular fluid was first aspirated with a 21G needle, centrifuged (3000*g*, 2 min, 4 °C) and stored at −20 °C whereas granulosa cell pellets were immediately frozen at −70 °C. The granulosa cells still attached to the follicle wall were dislodged by gentle flushing with ice-cold HEPES-buffered DMEM into the follicle through a small incision in the follicle wall. The follicle washes were pooled and centrifuged (3000*g*, 2 min, 4 °C), the supernatant was discarded and the granulosa cells were immediately frozen at −70 °C. Additionally, GC were collected from 2 to 4 mm small follicles (SF) obtained from slaughterhouse ovaries, and a total of three pools of twenty SF were prepared. CL at day 5 of the estrous cycle were obtained by ovariectomy and were dissected from the ovarian stroma, frozen in liquid nitrogen and stored at −70 °C.

2.2. Animal model for analysis of ILs expression profile during the postpartum period

Lactating dairy cows between 50 and 70 days postpartum were selected and blood samples were collected from each cow and analyzed for BHB concentrations. Cows with BHB levels of at least 1.4 mmol/L were considered energy deficient and included in the BHB^+ group (n = 20 cows), while a group of low BHB cows (BHB^-; < 1.2 mmol/L) was used as control (n = 20). Selected animals were synchronized and follicular development was monitored by daily transrectal ultrasound. Using transvaginal aspiration, follicular fluid and GC from each cow were harvested from preovulatory follicles at ≥10 mm in diameter, and samples were collected in tubes containing RNAlater and processed for RNA extraction.

2.3. Total RNA extraction and RT-qPCR analysis

Total RNA was extracted from bovine GC collected from BHB^+ and control follicles and from follicles at different developmental stages (SF, DF, OF) and CL described above using the TRIzol Plus RNA purification kit (Invitrogen). Reverse transcription and PCR were performed using the SMART (Switching Mechanism At 5′-end of RNA Transcript) technology (Clontech). The same amount of total RNA (250 ng) from each sample was used to synthesize the first-strand cDNA with the SuperScript III transcriptase (Invitrogen) followed by cDNA amplification by long-distance (LD)-PCR using the Advantage 2 Polymerase Mix (Clontech). Expression and regulation of *IL* and *IL* receptors (*ILR*) mRNA in GC during follicular development and postpartum period were analyzed by RT-qPCR using the SsoAdvanced Universal SYBR Green supermix (Clontech) following the manufacturer's instruction manual. Specific PCR primers for *IL4R, IL15R,* and *IL21R* and for *IL1A, IL1B, IL4, IL8, IL10, IL15, IL21, IL23* and *TNFα* were used (Table 1). *ILs* and *ILRs* mRNA relative abundance were calculated using the $2^{-\Delta\Delta Ct}$ method [27] with *RPL19* as reference gene. RT-qPCR data were presented as normalized *ILR* or *IL* amounts relative to $2^{-\Delta\Delta Ct}$.

2.4. Total protein extraction and western blot analysis

Granulosa cells from BHB^+ and control follicles were obtained as described above and homogenized in M-PER buffer (Thermo Fisher Scientific) supplemented with complete protease inhibitors (Sigma Aldrich) as described by the manufacturer's protocol, and centrifuged at 16,000*g* for 10 min at 4 °C. Protein concentrations were determined from the recovered supernatant according to Bradford method [28]

Table 1
Primers used in the expression analyses of *Bos taurus* genes by RT-qPCR.

Gene Names		Primer sequence (5′–3′)[a]	Accession no.	AS (bp)
RPL19	Fwd	gaccaatgaaatcgccaatgc	NM_001040516	154
	Rv	acctataccatatgcctgcc		
IL4R	Fwd	tggctctgatggtcactctg	NM_001075142	164
	Rv	cagctgtgggtctgagtcaa		
IL15R	Fwd	aggctccggaacacacatac	XM_010822356	197
	Rv	cacactctccatgctctcca		
IL21R	Fwd	acccagtcgtctttcacacc	NM_001193179	155
	Rv	ggacacctgcaaccatacct		
IL1A	Fwd	atctggaggaggcagtgaaat	NM_174092	201
	Rv	gcttttcccagaagaagagga		
IL1B	Fwd	catggagaagctgaggaacag	NM_174093	169
	Rv	tctgtcctggagtttgcactt		
IL4	Fwd	ccccaaagaacacaactgaga	M77120	190
	Rv	tccaagaggtctttcagcgta		
IL8	Fwd	tgtgtgaagctgcagttctgt	BC103310	188
	Rv	cttggggtttaagcagacctc		
IL10	Fwd	gcactactctgttgcctggtc	NM_174088	180
	Rv	taagctgtgcagttggtcctt		
IL15	Fwd	gtgcaagtcttcccaaaacag	U42433	170
	Rv	tcgtaactccaggagaaagca		
IL21	Fwd	tgcctgatggtcatcttctct	NM_198832	182
	Rv	gaccgctcacagtgtctcttt		
IL23	Fwd	ctggagtgcacacctaccaat	NM_001205688	229
	Rv	gccatttgggagtagagaagg		
TNFα	Fwd	gtcaacatcctgtctgccatc	NM_173966.3	186
	Rv	aaagtagacctgcccagactc		

Abbreviations: AS, amplicon size (base pairs); Fwd, forward primer; Rv, reverse primer.

[a] All primers were designed and validated by the authors. Each primer was used at a final concentration of 600 nM.

(Bio-Rad Protein Assay, Bio-Rad Lab, Mississauga, ON, Canada). Western blot analyses were performed as previously described [29]. Samples (150 ng of proteins) were resolved by one-dimensional denaturing Novex Tris-glycine gels (Invitrogen, Burlington, ON, Canada) and electrophoretically transferred to polyvinylidene difluoride membranes (PVDF; Thermo Fisher Scientific). Membranes were incubated with anti-IL4 and anti-IL15 antibodies (Cedarlane Laboratories Ltd., Burlington, ON Canada) at concentrations of 1.5μg/ml. Immunoreactive proteins were visualized by incubation with horseradish peroxidase-linked anti-goat secondary antibody (1:10000 dilution) and the enhanced chemiluminescence system, ECL plus (Thermo Fisher Scientific) according to the manufacturer's protocol followed by revelation using the ChemiDoc XRS + system (Bio-Rad). β-actin was used as reference protein with anti-β-actin antibodies purchased from Immune Biosolutions (Sherbrooke, QC Canada) and used at a dilution of 1:1000.

2.5. Statistical analyses

Relative amounts of target genes mRNA were normalized with those of the reference gene *GAPDH*. Homogeneity of variance between groups was verified by O'Brien and Brown-Forsythe tests. Corrected values of gene specific mRNA levels were compared between follicular or CL groups by one-way analysis of variance (ANOVA). When ANOVA indicated a significant difference ($P < 0.05$), the Tukey-Kramer test was used for multiple comparison of individual means among SF, DF, OF and CL, whereas the Student *t*-test ($P < 0.05$) was used to compare the BHB^+ group of cows to the control group. Statistical analyses were performed using GraphPad prism 5.0 software.

3. Results and discussion

The dynamic nature of follicular development and the presence of the oocyte in the follicular fluid, surrounded by granulosa and internal theca cells, make the follicles an excellent model for uncovering changes in the IL and ILRs receptors expression profile based on the animal status, and provide better understanding of the IL roles in follicular development and oocyte maturation. In this regard, we demonstrated the expression of target IL receptors in GC with differential regulation throughout the follicular development and in the CL. For *IL4R*, the greatest steady-state mRNA amounts were observed in granulosa cells (GC) of the dominant follicle (DF) as compared to GC of small follicles (SF), ovulatory follicles (OF) and corpus luteum (CL) (Fig. 1A; $P < 0.05$). In contrast, *IL15R* showed significantly greater expression in the OF than in DF, SF and CL (Fig. 1B, $P < 0.001$). For IL21R, there was an increase within the CL as compared to the different groups of follicles (Fig. 1C; $P < 0.05$). Among the different groups of follicles, *IL21R* mRNA expression was greater in DF and OF as compared to SF (Fig. 1A; $P < 0.01$).

While steady-state mRNA levels of *IL4R* were stronger in the dominant follicle, *IL15R* expression was greatest in the ovulatory follicle, and *IL21R* showed increased steady-state mRNA levels in the CL as compared to the different groups of follicles, suggesting that these cytokine receptors might be hormonally-regulated. *IL15R* was significantly induced by hCG injection (Fig. 1B) while *IL4R* expression was reduced in GC post-hCG injection, and *IL21R* was more abundant in the CL. These differences in regulation of interleukin receptors could also be caused by different cell types within tissues specially in the CL, which contains a large proportion of leucocytes, in addition of luteinized granulosa and theca cells. Nevertheless, the results suggest that these receptors would activate different targets upon cytokines binding and they could play significant roles in signaling pathways controlling either granulosa cells (GC) proliferation and function, ovulation or formation of the CL. We have previously shown that the expression of several genes was dramatically reduced in the ovulatory follicle

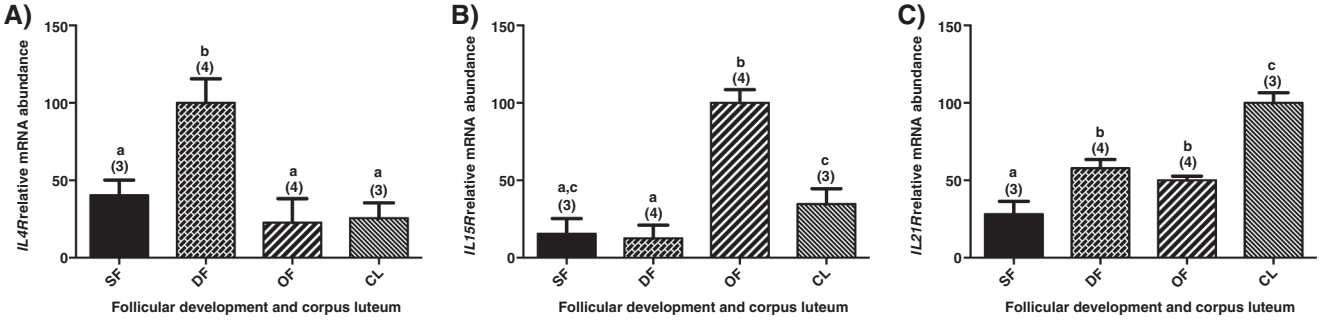

Fig. 1. Expression of target interleukin receptors in bovine granulosa cells during follicular development. Total RNA extracts of GC from SF, DF, OF, and CL were analyzed by RT-qPCR for different interleukin receptors mRNA expression. Expression of *IL4R* (A) was greatest in DF as compared to SF, OF and CL ($P < 0.05$). *IL15R* expression (B) was significantly greater in OF as compared to SF, DF and CL ($P < 0.001$), while *IL21R* (F) mRNA expression were greatest in the CL as compared to the different groups of follicles. Relative mRNA expression was calculated using the $2^{-\Delta\Delta Ct}$ method with *RPL19* as reference gene. Different letters denote samples that differ significantly ($P < 0.05$).

following the LH surge or hCG injection [30,31]. Cytokines whose receptors are induced by LH/hCG, such as the pro-inflammatory cytokine *IL15* shown in this study, may be involved in various biological processes such as the ovulation process but could also hinder GC proliferation and follicular growth. Conversely, *IL4R*, which is expressed at greater amounts in GC of dominant follicle would mediate IL4's possible actions in promoting GC proliferation and steroidogenic activity. Indeed, decreased *IL4* amounts during the postpartum period could indicate that IL4 promotes cell proliferation as previously shown in lymphocytes [32], or oocyte maturation and ovulation [33,34]. Interestingly, IL4 was found overexpressed during oocyte maturation along with other growth factors [33] supporting the assumption that IL4 plays a role in the production of a competent oocyte. Therefore, a significant decrease in *IL4* expression during the postpartum period as shown in our study could contribute to limited GC proliferation and delayed oocyte maturation resulting in a prolonged anovulation period. Moreover, our data agree with previous reports demonstrating that alteration of IL4 expression contributed to follicle persistence and ovulation failure in cows with ovarian cysts [17].

We analyzed the expression of different proinflammatory and anti-inflammatory cytokines in granulosa cells during follicular development and found a differential regulation for target cytokines. Of interest, steady-state mRNA expression of *IL1A* was strongest in the OF as compared to SF, DF and the CL (Fig. 2A; $P < 0.05$), while *IL1B* relative expression was strongest in OF and SF as compared to DF (Fig. 2B; $P < 0.05$). Expression of *IL8* (Fig. 2D) and *TNFα* (Fig. 2I) was strongest in the SF as compared to other groups of follicles and the CL. Moreover, *IL8* and *TNFα* expression was still stronger in the OF than in the DF (Fig. 2D and I, respectively; $P < 0.05$). Expression of *IL4* (Fig. 2C) and *IL10* (Fig. 2E) was strongest in SF as compared to DF. However, *IL10* expression was stronger in OF than in the DF (Fig. 2E, $P < 0.05$). As for *IL15*, relative mRNA expression was more abundant in the OF and the CL as compared to the DF (Fig. 2F). *IL21* relative expression was stronger in SF as compared to DF, OF and CL (Fig. 2G; $P < 0.05$), while *IL23* was strongest in SF and OF as compared to DF (Fig. 2H; $P < 0.05$). Overall, these results are in line with the status of the follicles since pro-inflammatory cytokines analyzed are more abundantly present in the ovulatory follicle as compared to the dominant follicle and that the ovulation process is similar to an inflammatory response [19,35,36]. The LH surge triggers several LH-induced signaling cascades associated with inflammation and leads to the release of the oocyte during ovulation. Granulosa cells are one of the first cells to respond to LH with a dramatic change in gene expression as previously reported (Lussier et al., 2017; Benoit et al., 2019). These changes also include induction of mediators of inflammatory processes such as cytokines as shown for most of the pro-inflammatory cytokines

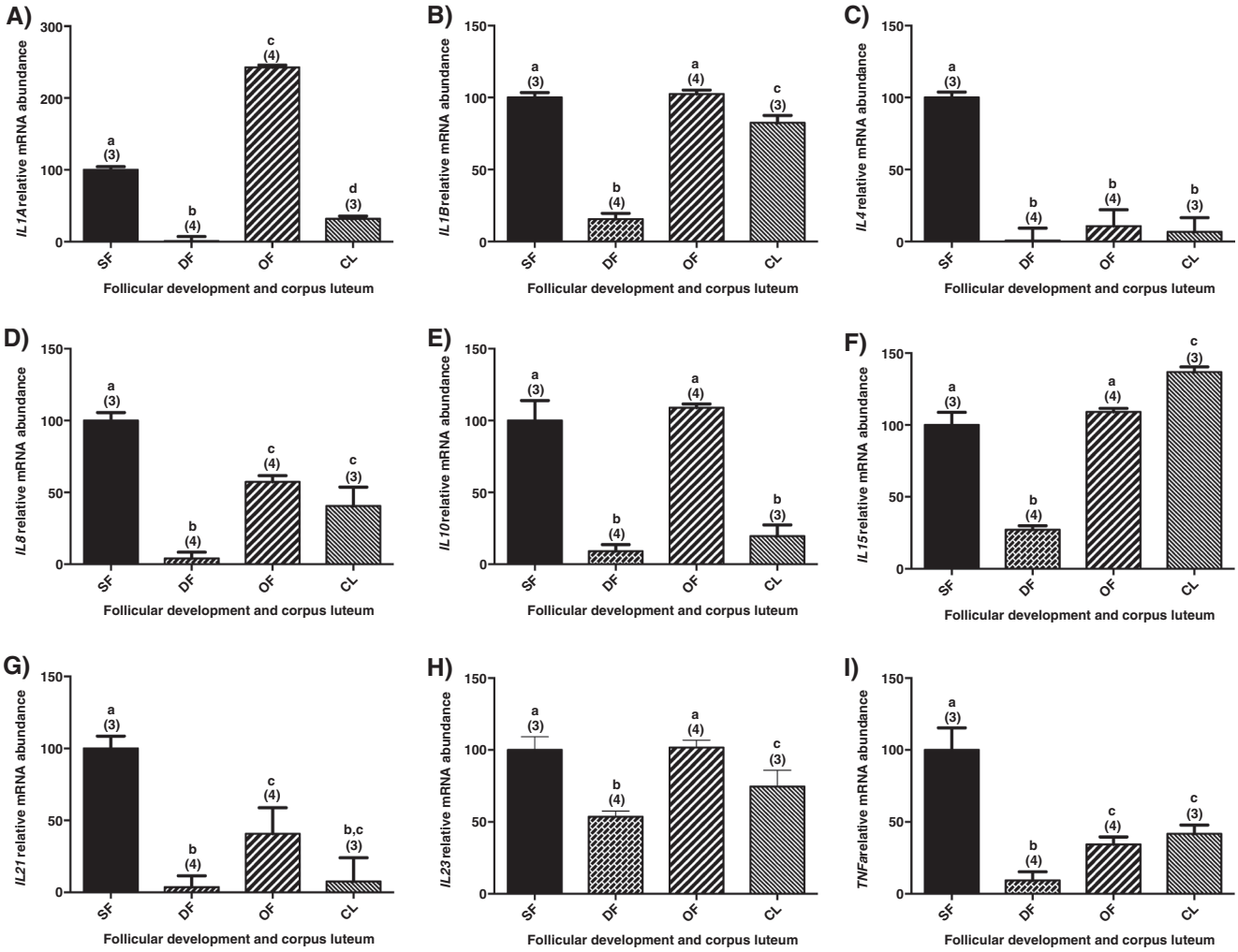

Fig. 2. Expression of target interleukins in bovine granulosa cells during follicular development. Total RNA extracts of GC from SF, DF, OF, and CL were analyzed by RT-qPCR for different interleukin mRNA expression. *IL1A* (A), *IL1B* (B), *IL8* (D), *IL15* (F), *IL21* (G), *IL23* (H) and *TNFα* (I) mRNA expression were greatest in the OF as compared to the DF. Expression of *IL4* (C) was greatest in SF as compared to DF, OF and CL ($P < 0.001$), while *IL10* expression (E) was significantly greater in SF and OF as compared to DF and CL ($P < 0.001$). Relative mRNA expression was calculated using the $2^{-\Delta\Delta Ct}$ method with *RPL19* as reference gene. Different letters denote samples that differ significantly ($P < 0.05$).

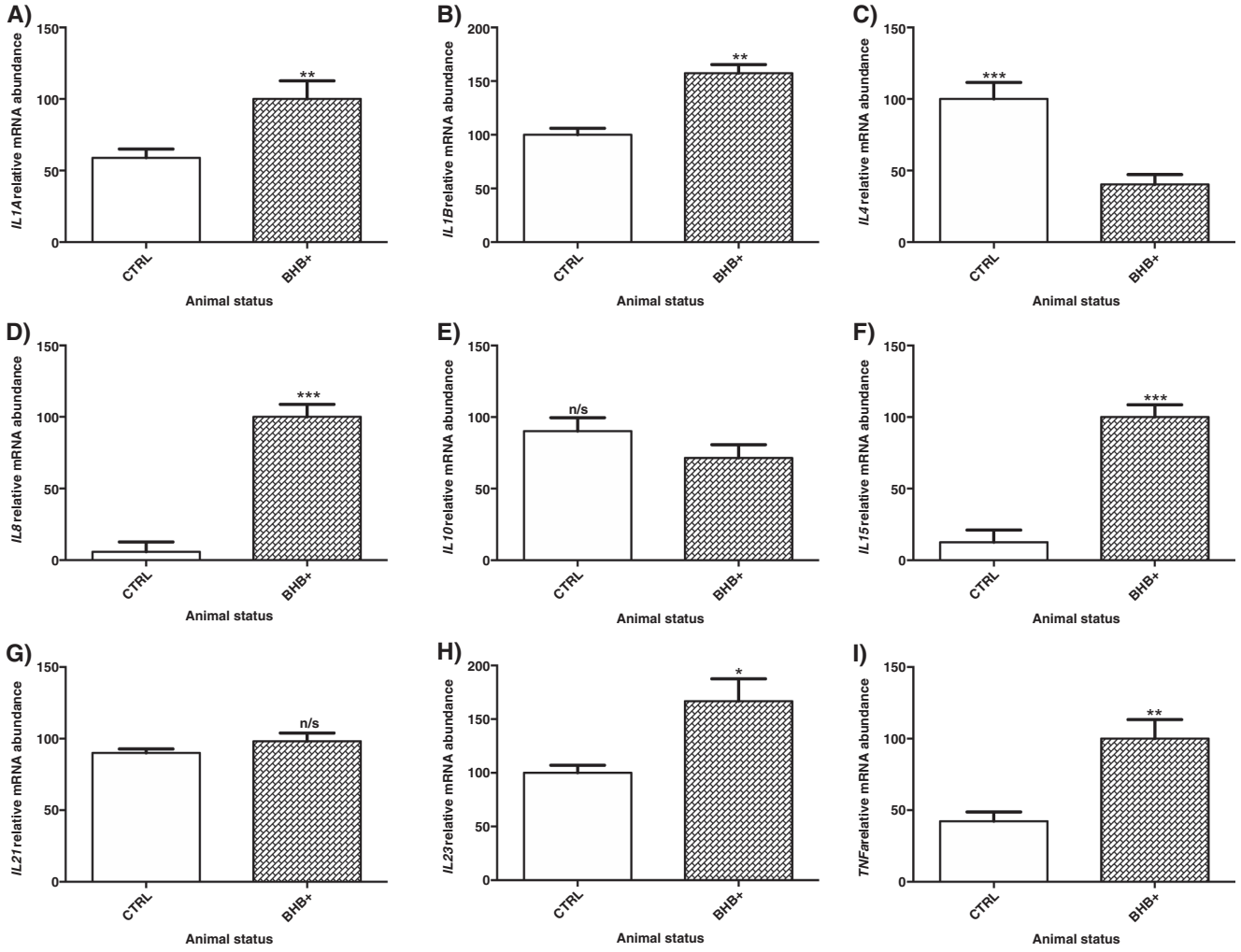

Fig. 3. Expression profile of target interleukins in bovine granulosa cells during the postpartum period. Total RNA was extracted from GC of either postpartum cows with high BHB levels (BHB^+) or low BHB (CTRL) and analyzed by RT-qPCR for different interleukin mRNA expression. *IL1A* (A), *IL1B* (B), *IL8* (D), *IL15* (F), *IL23* (H) and *TNFα* (I) were significantly induced during the postpartum period (BHB^+ group) as compared to the control group. There was no difference for *IL21* mRNA expression between BHB^+ and control groups (G). *IL4* (C) was significantly reduced during the postpartum period (BHB^+ group) as compared to the control group, while there was no difference in *IL10* expression between the two groups (E). mRNA relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method with *RPL19* as reference gene. Bars marked with asterisks are significantly different from the control group ($P < 0.05$). n/s, non-significant.

analyzed in this study.

In the second part of this study, our results show that the expression of pro-inflammatory interleukins such as *IL1A* (Fig. 3A; $P < 0.05$), *IL1B* (Fig. 3B; $P < 0.05$), *IL23* (Fig. 3H; $P < 0.05$), *TNFα* (Fig. 3I; $P < 0.05$), and most notably *IL8* (Fig. 3D; $P < 0.001$) and *IL15* (Fig. 3F; $P < 0.001$) was significantly induced during the postpartum period (BHB^+ group) as compared to the control group. However, steady-state mRNA levels for the other analyzed pro-inflammatory cytokines, such as *IL21* (Fig. 3G) did not change significantly between the two groups suggesting that these particular ILs might not affect bovine fertility during the postpartum period. Among the anti-inflammatory interleukins that were analyzed, *IL4* was significantly reduced during the postpartum period in BHB^+ cows as compared to the control group (Fig. 3C; $P < 0.01$), while the steady-state mRNA levels of *IL10* did not significantly change (Fig. 3E). Western blot analyses showed protein expression of IL4 and IL15 as differentially regulated similar to their mRNA expression. IL4 was relatively reduced in the postpartum period as compared to the control although there was no significant difference between the two groups, while IL15 tended to be induced in postpartum cows (Fig. 4).

Quantitative PCR analyses of *IL4R*, *IL15R* and *IL21R* during the postpartum period revealed greatest steady-state mRNA amounts in granulosa cells from BHB^+ cows as compared to controls cows for all three receptors (Fig. 5A–C; $P < 0.05$). Significant expression of IL15R and IL21R in BHB^+ granulosa cells correlates well with the regulation of the corresponding pro-inflammatory cytokines IL15 and IL21 in BHB^+ granulosa cells as shown in Fig. 3 where IL15 was significantly increased in BHB^+ as compared to control (Fig. 3F), while IL21 was expressed in BHB^+ similar to the control (Fig. 3G). Conversely, IL4R was significantly increased in BHB^+ granulosa cells while IL4 expression was stronger in the control as compared to BHB^+ (Fig. 3C). Although IL4 is considered an anti-inflammatory cytokine [37], it can regulate a variety of biological responses by binding to specific IL4Rs expressed by a wide range of cell types [38–40]. Additionally, IL4R can form two complexes consisting of two classes of IL4Rs with a type I IL4R and a type II IL4R [41], suggesting that increased IL4R in BHB^+ cows could mean that IL4 may induce different actions in granulosa cells or IL4R could serve for the signaling of other cytokines such as IL13 as previously shown [42].

Our study shows that the postpartum period is associated with changes in the expression profile of various ILs that could result in functional changes in steroidogenic cells, particularly granulosa cells, leading to negative effects on follicular development and ovulation rate. Because the health and maturation of the oocyte is closely

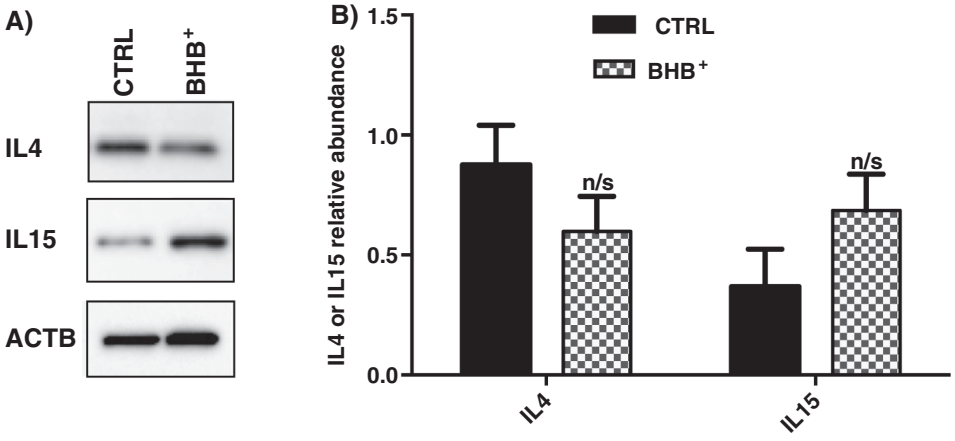

Fig. 4. Western blot analysis of IL4 and IL15 expression during the postpartum period. Total proteins were extracted from GC of BHB^+ and control cows and analyzed using anti-IL4 and anti-IL15 antibodies. Abundance of IL4 and IL15 were measured as ratios of IL4/ACTB and IL15/ACTB, respectively. IL4 was relatively less abundant in BHB^+ than in control GC while IL15 tended to be induced in BHB^+ compared to the control (N = two replicates). CTRL, control; ACTB, beta actin; n/s, non-significant as compared to CTRL.

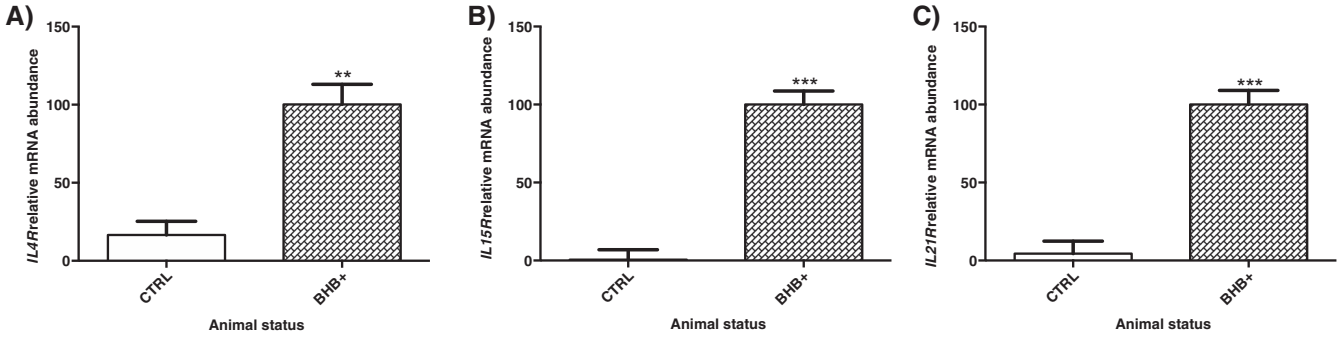

Fig. 5. Expression profile of target interleukins receptors in bovine granulosa cells during the postpartum period. Total RNA was extracted from GC of either postpartum cows with high BHB levels (BHB^+) or low BHB (CTRL) and analyzed by RT-qPCR for different interleukin receptors mRNA expression. *IL4R* (A), *IL15R* (B), and *IL21R* (C) were significantly induced during the postpartum period (BHB^+ group) as compared to the control group. mRNA relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method with *RPL19* as reference gene. Bars marked with asterisks are significantly different from the control group ($P < 0.05$).

dependent on the proper development of the follicle and the function of GC, the identification of target ILs directly induced during the postpartum period suggest that these ILs might affect the ovarian function and dairy cows' fertility. Of interest, among the ILs analyzed, *IL15* was one of the two most significantly induced interleukins during the postpartum period supporting the idea of a negative role for IL15 in follicular development and oocyte maturation. Similarly, it was shown in women that increased IL15 concentrations in the follicular fluid are adversely related with the size of follicles and oocyte maturity [43] and could also negatively affect IVF-embryo transfer outcome [44]. These data correlate well with our findings and may suggest that increased expression of IL15 during the postpartum period could delay oocyte maturation and ovulation, therefore contributing to a prolonged anovulation state. Our own data strongly support a correlation between increased expression of IL15/IL15R and the absence of ovulation in postpartum dairy cows. Alternately, IL15 could be considered as a potential marker for lactation performance [45], and its expression profile and regulation also support the idea that IL15 might be involved in other cellular mechanisms including inflammation as previously suggested [46].

The present observations provide an insight into the regulation of target cytokines during follicular development and during the postpartum period. Since one of the causes of decreased fertility during the post-partum period could be a prolonged anovulatory status, it is of interest to analyze and understand the exact regulation of these cytokines. It has been shown that cows with delayed ovulation or anovulation during post-partum period had greater concentrations of inflammatory cytokines such as TNFα as compared to ovulated cows [47]. In agreement with these data, we have shown that TNFα as well as IL8, IL15 and IL23, all inflammatory cytokines, were expressed more abundantly in BHB^+ cows than in control cows. Conversely, we have shown that expression of anti-inflammatory cytokines such as IL4 was altered in BHB^+ post-partum cows as compared to BHB^- cows suggesting that alteration of certain cytokines could be associated with prolonged anovulation. These findings are also consistent with previously reported data [17], which together are evidence that a different regulation or alteration of cytokines during the post-partum period could contribute to ovulation failure. Overall, these observations help better understand the effects of high BHB levels during the postpartum period on the ovarian function and granulosa cells activity, although the mechanisms underlying the changes in ILs expression profile and their possible effects on fertility require further investigation.

4. Conclusion

In this study, the expression profile of target ILs in GC was established during the postpartum period in dairy cows. Of interest, we have shown anti-inflammatory IL4 and pro-inflammatory IL8 and IL15 as the interleukins most significantly altered and induced, respectively, during the postpartum period. In addition, we showed ILRs to be differentially regulated in GC at different developmental stages of the ovarian follicles. Future investigations will determine whether these changes are correlated with reduced ovulation rate and whether they affect negatively dairy cows' fertility during the postpartum period.

CRediT authorship contribution statement

Aly Warma: Validation, Investigation, Formal analysis, Data curation, Writing - original draft, Visualization. **Marianne Descarreaux:** Investigation, Formal analysis, Data curation, Visualization. **Younes Chorfi:** Methodology, Writing - review & editing. **Raynald Dupras:** Methodology, Resources. **Roxane Rémillard:** Methodology, Resources.

Kalidou Ndiaye: Conceptualization, Methodology, Validation, Formal analysis, Resources, Writing - original draft, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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