Pegbovigrastim treatment affects gene expression in neutrophils of pasture-fed, periparturient cows

A. Heiser,^{*1} S. J. LeBlanc,[†] and S. McDougall[‡]

^{*}AgResearch, Hopkirk Research Institute, Palmerston North, 4442, New Zealand

[†]Ontario Veterinary College, University of Guelph, Guelph, ON, Canada N1G 2W1

[‡]Cognosco, AnexaFVC, Morrinsville, 3300, New Zealand

ABSTRACT

Treatment with granulocyte colony-stimulating factor has been reported to increase circulating neutrophil count and enhance neutrophil function in the periparturient cow. It was hypothesized that a commercially available recombinant bovine granulocyte colony-stimulating factor product (pegbovigrastim) affects gene expression profiles of neutrophils and supports neutrophil function in periparturient cows. Hence this study was undertaken to analyze expression of genes involved in neutrophil functions, including migration, interaction with pathogens, and cell survival. It also assessed the hypothesis that gene expression profiles in neutrophils are modulated by negative energy balance in the peripartum period. Holstein-Friesian, Jersey, and mixed-breed cows on pasture were blocked by expected calving date and body condition score and randomly assigned in a 2 × 2 factorial design. Cows were fed to exceed energy requirements prepartum (122%) or restricted to approximately 85% of prepartum energy requirements. At approximately 7 d before expected calving date, half the cows in each feed group were randomly assigned to be injected with pegbovigrastim or saline. Treatments were repeated within 24 h after calving. Blood samples were collected pretreatment approximately 7 d before calving (d −7). Blood, uterine flush, and milk samples were collected at 4 (d 4) and 7 d in milk (d 7) to measure the expression of a panel of 20 genes representing cell adhesion, pattern recognition, inflammation and cytokine response, antimicrobial capacity, and apoptosis functions in neutrophils using NanoString technology (NanoString Technologies Inc., Seattle, WA) to quantify RNA copy numbers. No effects were observed of prepartum feeding group or a feeding group × treatment interaction for any of the investigated genes. An effect was observed of time on

expression of several genes in blood neutrophils. After calving, expression of 2 of 4 cell adhesion-related genes, 3 of 4 pattern recognition receptors, 2 of 4 inflammatory genes, 2 antimicrobial genes, and 2 of 4 cell survival genes was significantly greater at d 4 or 7 or both compared with before calving (d −7). Expression of *ICAM1*, *TLR2*, and *PTGS2* was significantly higher in blood neutrophils from animals treated with pegbovigrastim compared with untreated controls, suggesting greater migration, pattern recognition, and inflammatory response ability. Pegbovigrastim also affected RNA expression in uterine cells with *ICAM1*, *NOD1*, *CLEC6A*, *PTGS2*, *MPO*, *DEFB5*, and *CATHL6* being expressed at higher levels and *SELL*, *ITGB8*, *IL8RB*, and *IL10* at lower levels. Milk somatic cells showed a similar pattern but with fewer significant changes. In contrast to the reported decline in neutrophil function in the transition period, neutrophil gene expression was increased for many of the genes studied, an apparent attempt to compensate for reduced neutrophil function. Treatment with pegbovigrastim further increased expression of several genes involved in these processes in blood neutrophils and changed uterine cells to a phenotype with increased antimicrobial capacity, typical for neutrophils that have migrated into their target tissue.

Key words: transition cow, neutrophil, gene expression, pegbovigrastim

INTRODUCTION

During the peripartum period, dairy cows are at an increased risk of disease (Ingvarsen et al., 2003). A decrease in circulating neutrophil number and function has been reported during this time, which contributes to increased susceptibility to uterine and mammary infections (Kehrli et al., 1989; Gilbert et al., 1993; Mallard et al., 1998). Decreased neutrophil function has been linked to the incidence and outcome of peripartum-related diseases, including mastitis, displaced abomasum, and retained placenta (Goff and Horst, 1997; Kimura

Received November 12, 2017.

Accepted May 17, 2018.

¹Corresponding author: axel.heiser@agresearch.co.nz

et al., 2002; Burton et al., 2005). These studies indicate that neutrophil function is affected by peripartum nutrition and by metabolic adaptation to negative energy balance (Hammon et al., 2006; Graugnard et al., 2012; Zhou et al., 2015) and the need to counteract these effects.

The production of neutrophils is regulated by granulocyte colony-stimulating factor (**G-CSF**; Semerad et al., 2002; Martin et al., 2003; Bendall and Bradstock, 2014). A long acting analog of bovine G-CSF (pegbovigrastim injection, Imrestor, Elanco Animal Health, Greenfield, IN) is commercially available. It has been reported that pegbovigrastim injection increased neutrophil count in peripheral circulation and increased neutrophil function in dairy cattle (Kimura et al., 2014; Hassfurth et al., 2015; Canning et al., 2017; McDougall et al., 2017).

A previous report of the current experiment confirmed that pegbovigrastim treatment resulted in significant increases in neutrophil count and enhanced neutrophil function as indicated by increased myeloperoxidase release (McDougall et al., 2017). The objective of the present study was to gain further insight into the effects of pegbovigrastim on neutrophils by analyzing gene expression profiles in neutrophils from blood, uterus, and milk from periparturient dairy cows. The study also aimed to assess the hypothesis that gene expression profiles in neutrophils are modulated by negative energy balance in the peripartum period. Therefore, genes were selected to represent key neutrophil functions, such as their ability to be recruited to sites of infection (cell adhesion), to recognize (pattern recognition) and phagocytose microbes, to alert other cells of the immune system (inflammation and cytokine response), to kill pathogens (antimicrobial capacity), and eventually to undergo programmed cell death (apoptosis).

MATERIALS AND METHODS

Animals and Treatment

All animal manipulations reported in this study were approved by the animal ethics committee of AgResearch, Ruakura, New Zealand. Multiparous cows ($n = 99$; 48 Holstein Friesians, 13 Jerseys, and 38 Holstein Friesian $\times$ Jersey) on pasture housed at a research dairy facility (Massey University, Palmerston North, New Zealand) were enrolled in the study. Cows were blocked by week of expected calving, ranked on BCS, and randomly assigned to treatment within block in a 2×2 factorial design, regardless of their breed.

As reported previously (McDougall et al., 2017), of the 99 cows that were originally enrolled, 17 were removed from the study for various reasons. For the 82

cows remaining in the study, no difference was present in the calving date or parity between the feeding or treatment groups. With 41 (50%) Friesian, 33 (40.2%) crossbred, and 8 (9.8%) Jersey cows no difference was present in the breed distribution between treatment groups. The actual day of enrolment (i.e., d -28) and the day of first treatment (i.e., d -7) did not differ by feed or treatment group. The timing of scheduled sampling was actually -10.9 ± 5.5 , 0.0 ± 0.0 , 3.7 ± 0.6 , and 6.8 ± 0.7 d relative to calving for visits scheduled for d -7 , 0, 4, and 7 relative to calving, respectively. The actual day of sampling did not vary between treatment ($P = 0.72$) or feed groups ($P = 0.64$).

On 3 occasions, at weekly intervals, cows that were 4 to 5 wk before anticipated calving were assessed by a veterinarian. Cows were assigned to either a full ration (**FR**) or restricted ration (**RR**) at 31.4 (SD = 5.5) and 30.9 (SD = 4.9) d before calving, respectively (details of the feeding treatment were reported previously; McDougall et al., 2017). The FR cows were offered a ration to provide approximately 120 MJ of ME per cow per day, or 122% of prepartum energy requirements. The RR group was offered a ration providing approximately 82 MJ of ME per cow per day, or 85% of prepartum energy requirements. Postpartum, the 2 precalving feed groups were together on the same pastures and were offered the same ration.

At approximately 7 d before anticipated calving, cows were injected subcutaneously with 15 mg in 2.7-mL injection volume of the pegylated form of recombinant bovine G-CSF (pegbovigrastim injection, Imrestor, Elanco Animal Health) or 3 mL of 0.9% NaCl (control). On d 0 (the day of calving), cows were milked for the first time between approximately 1500 and 1700 h and a second injection of the same treatment was performed.

Blood, Milk, and Uterine Samples

Blood samples (7 mL) were drawn from the tail vessels 7 (± 3) d before calving (d -7) and 4 (± 1) d (d 4) and 7 (± 1) d (d 7) after calving into evacuated glass containers containing EDTA (Becton Dickinson and Company, Franklin Lakes, NJ). Blood samples were drawn in the morning before supplements were fed and before Imrestor or saline treatment (d -7) held and transported at ambient temperature, and processed at the laboratory within 2 h of collection. At 4 (± 1) and 7 (± 1) d postpartum, composite milk samples (~ 100 mL) were collected during morning milking. On the same days in the following morning milking, uterine fluid (~ 20 mL) was collected. The vulva of the cow was wiped with a paper towel if gross contamination with fecal material was present. A 75-cm flexible catheter

(Insemination Catheter Deep Equivet, Shoof International Ltd., Cambridge, New Zealand) was passed through the cervix and 50 mL of 0.9% sodium chloride (Sodium Chloride 0.9% Intravenous Infusion, Baxter International Inc., Toongabbie, NSW, Australia) was infused. The uterus was then gently massaged and negative pressure was applied to the syringe until at least (10 mL) of fluid was retrieved. If fluid was not immediately retrieved, the catheter was repositioned within the uterus until sufficient fluid was obtained for the minimum sample size. The sample was then immediately transferred into a sterile sample container for transport at ambient temperature to the laboratory.

Milk microbiology was performed by Cognosco (An-exaFVC, Morrinsville, New Zealand).

The number of neutrophils per liter (as part of complete blood count) was determined using an automated hemocytometer (Sysmex XT2000i, Roche Diagnostics), and cell morphology was assessed by microscopic examination of blood smears. The counts have been reported previously by the same authors (McDougall et al., 2017), and Figure 1 has been redrawn from those results.

Preparation of Cells for Gene Expression Analysis

Neutrophils were prepared from whole blood samples using a method modified from Roth and Kaeberle (1981). For ease and speed of the procedure, the separation by density gradient was omitted. Briefly, blood was transferred into 50-mL conical tubes and centrifuged at $1,500 \times g$ for 30 min at room temperature. After centrifugation, the plasma, buffy coat layer, and top layer (approximately 1/3) of packed red blood cells were removed. To the remaining cells, 40 mL/tube of red blood cell lysis buffer (Tris-buffered ammonium chloride) was added, and tubes incubated for 10 min at 37°C in a water bath. Subsequently, cells were centrifuged at $600 \times g$ for 10 min at room temperature, washed once in PBS (10 mM PBS, pH 7.4), and resuspended in HBSS (Hanks' buffered salt solution, Thermo Fisher Scientific, Waltham, MA) at 2.5×10^7 cells/mL. Cell suspensions prepared using this procedure contained $88.3 \pm 6.7\%$ (all $\geq 80\%$) neutrophils as confirmed by Kwik-Diff staining (Thermo Fisher Scientific).

For the preparation of cells from milk, 20 mL of milk was transferred into 50-mL conical tubes and PBS added to a final volume of 50 mL. After centrifugation at $1,500 \times g$ for 30 min at room temperature the fat and liquid layer were carefully removed, the cell pellet resuspended in 50 mL of PBS, and centrifuged at $600 \times g$ for 10 min at room temperature and resuspended in 200 μ L of PBS. The cell preparation was not further

Figure 1. Effect of time and treatment on blood neutrophil counts. Mean counts of neutrophils ($\times 10^9$ /L; $\pm$ SEM) for cows treated with pegbovigrastim (black bars) on d -7 and 0 compared with cows treated with saline (gray bars). Asterisks indicate differences ($P < 0.05$) between pegbovigrastim and saline-treated cows on that day. This figure is redrawn from a previous report by the same authors (McDougall et al., 2017).

characterized. Generally, milk somatic cells consist of several cell types, including neutrophils, macrophages, lymphocytes, and a smaller percentage of epithelial cells (Sordillo et al., 1997).

For the preparation of cells from uterine flushes, the flushed samples were transferred into 50-mL conical tubes and PBS added to a final volume of 50 mL. After centrifugation at $1,500 \times g$ for 30 min at room temperature, the supernatant was carefully removed and the cell pellet resuspended in 200 μ L of PBS. An analysis of the cellular composition of these preparations was not undertaken.

The cell suspensions from blood, milk, and uterine flushes were transferred to 1.5-mL tubes and centrifuged at $600 \times g$ for 5 min at room temperature. Supernatant was completely removed and cells lysed in 200 μ L of a commercial lysis buffer for RNA preparation (RLT buffer, Qiagen, Hilden, Germany). Lysates were stored at -80°C for later gene expression analysis.

Gene Expression Analysis

Gene expression analysis from cell lysates was performed using the nCounter Analysis System (NanoString Technologies Inc., Seattle, WA). The use of NanoString technology enables direct RNA expression analysis from cell lysates without further RNA purification (Malkov et al., 2009) or amplification. This system uses molecular barcodes on gene-sequence-specific probes and single molecule imaging to count RNA copies (Geiss et al., 2008). Briefly, multiplexed probes are designed with 2 sequence-specific probes for each gene of interest. The capture probe is coupled to biotin as affinity tag. The second probe, the reporter probe, is coupled to a color-coded tag. Each target molecule of interest is identified by the unique color code generated by the ordered fluorescent tags on the reporter probe. The level of expression is measured by counting the number of codes for each mRNA using digital imaging. This allows the analysis of multiple genes from the same sample (multiplexing) using a customized set of probes with distinct bar codes, called a CodeSet. Cell lysates were analyzed using a CodeSet consisting of probes specific for 20 genes from *Bos taurus* (see Table 1). Additionally, 3 reference genes (**RG**; i.e., *YWHAZ*, *RPL15*, and *GUSB*), were included based on published recommendations (Anstaett et al., 2010; Macabelli et al., 2014). The 20 genes of interest were selected based on their key role in 5 neutrophil functions: (1) cell adhesion (*ICAM1*, *ITGB2*, *SELL*, *TLN1*); (2) pattern recognition (*NOD1*, *CLEC6A*, *TLR2*, *TLR4*); (3) inflammation, including cytokine release (*PTGS2*, *IL8RB*, *IL10*, *ALOX5*); (4) antimicrobial capacity (*MPO*, *DEFB5*, *CATHL6*, *LTF*); and (5) apoptosis and cell survival (*FAS*, *CASP9*, *CASP2*, *BAK1*).

Cell lysates were hybridized with the CodeSets according to the manufacturer's instructions (nCounter Gene Expression Assay User Manual; MAN-C0003-03). Briefly, cell lysates were thawed on ice. MasterMix was prepared by adding 130 μ L of hybridization buffer and 13 μ L of RNase-free water to Reporter CodeSet tubes. Twenty-one microliters of MasterMix, 4 μ L of cell lysate, and 5 μ L of Capture ProbeSet were added to each tube of a 12-tube strip immediately before placing the strip at 65°C for 18 h.

After hybridization, excess probe was removed and the probe-target complexes aligned and immobilized in the nCounter cartridge. These steps were performed automatically by the nCounter Prep Station.

Sample cartridges were placed in the nCounter Digital Analyzer for data collection. Color codes on the surface of the cartridge are counted and tabulated for each target molecule. Data were retrieved from the Analyzer as RCC files.

For analysis, the raw data (RCC files) were imported into nSolver Analysis Software v2.5 (<http://www.nanostring.com/products/nSolver>) and underwent the software's sample quality control routine set to the following criteria: (1) imaging: fields of view registration <75%; (2) binding density outside the 0.05 to 2.25 range; (3) positive control linearity: positive control R^2 value <0.95; and (4) positive control limit of detection: 0.5 fM positive control ≤ 2 standard deviations above the mean of the negative controls. All samples passed the quality control routine.

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

Reference gene normalization was performed using the geometric mean of counts for the RG *RPL15*, *GUSB*, and *YWHAZ*. The average of these geometric means across all lanes was used as the reference against which each lane is normalized. A normalization factor was then calculated for each of the lanes based on the geometric mean of counts for the RG in each lane relative to the average geometric mean of counts for the RG across all lanes. This normalization factor was then used to adjust the counts for each gene target and controls in the associated lane. The normalization factor of all samples was inside the 0.1 to 30 range.

Statistical Analysis

The statistical analysis was performed using R software version 3.3.1 (R Core Team, 2015, R Foundation for Statistical Computing, Vienna, Austria). Because repeated measurements were made on the same experimental unit (cows), a repeated measures ANOVA with 2 experimental factors: feed group, with 2 levels (FR or RR), and treatment, with 2 levels (control or pegbovigrastrim) and 1 repeated measures factor (2 or 3 time points), was used. Linear mixed effects models were used with an appropriate variance function for modeling heteroscedasticity and with cow as the random effect. None of the response variables met the assumptions for ANOVA. All response variables met (at least approximately) the ANOVA assumptions with a natural logarithm transformation. For those genes meeting the ANOVA assumptions only marginally, we evaluated permutation tests (with 5,000 randomizations), and the resulting significance of the various effects was very similar to those of the usual ANOVA; thus, the results of the ANOVA were retained. *P*-values <0.05 were considered statistically significant.

Table 1. Gene expression analysis results: mean RNA copy numbers ($\pm$ SD) for genes selected as indicators of categories of neutrophil functions (cell adhesion, pattern recognition, inflammation/cytokine response, antimicrobial capacity, and apoptosis/cell survival)¹

Function	Gene	Day	Blood		Uterus		Milk	
			CTR	PEG	CTR	PEG	CTR	PEG
Cell adhesion	<i>ICAM1</i>	-7	1,510 $\pm$ 344	1,705 $\pm$ 687	2,343 $\pm$ 521	4,266 $\pm$ 890§	535 $\pm$ 97	547 $\pm$ 110
		4	1,962 $\pm$ 1,484	3,423 $\pm$ 1,67#§	2,168 $\pm$ 512	3,998 $\pm$ 1,076§	508 $\pm$ 103	499 $\pm$ 112
	<i>ITGB2</i>	7	2,241 $\pm$ 1,830#	2,771 $\pm$ 1,256#				
		-7	1,656 $\pm$ 407	1,625 $\pm$ 377				
Pattern recognition	<i>SELL</i>	4	1,805 $\pm$ 649	1,795 $\pm$ 577	2,119 $\pm$ 407	1,693 $\pm$ 321§	1,087 $\pm$ 166	878 $\pm$ 169§
		7	2,054 $\pm$ 1,053#	1,802 $\pm$ 582	1,938 $\pm$ 431	1,629 $\pm$ 382§	1,027 $\pm$ 158	797 $\pm$ 152§
	<i>TLN1</i>	-7	435 $\pm$ 106	422 $\pm$ 98				
		4	462 $\pm$ 90	478 $\pm$ 92	488 $\pm$ 104	440 $\pm$ 95§	66 $\pm$ 23	68 $\pm$ 19
Inflammation/cytokine response	<i>NOD1</i>	7	462 $\pm$ 91	456 $\pm$ 85	448 $\pm$ 111	433 $\pm$ 115	75 $\pm$ 19	71 $\pm$ 22
		-7	4,749 $\pm$ 1,113	4,752 $\pm$ 1,170				
	<i>CLEC6A</i>	4	5,106 $\pm$ 1,079	5,199 $\pm$ 839	1,876 $\pm$ 344	1,751 $\pm$ 356	85 $\pm$ 18	87 $\pm$ 20
		7	5,121 $\pm$ 1,059	5,290 $\pm$ 1,173	1,761 $\pm$ 371	1,707 $\pm$ 513	82 $\pm$ 19	79 $\pm$ 17
Antimicrobial capacity	<i>TLR2</i>	-7	168 $\pm$ 60	199 $\pm$ 66§	212 $\pm$ 70	398 $\pm$ 108§	216 $\pm$ 55	197 $\pm$ 63
		4	204 $\pm$ 60#	199 $\pm$ 60	193 $\pm$ 64	378 $\pm$ 116§	192 $\pm$ 49	176 $\pm$ 56
	<i>TLR4</i>	7	201 $\pm$ 59#	207 $\pm$ 69				
		-7	96 $\pm$ 20	98 $\pm$ 19	110 $\pm$ 21	188 $\pm$ 42 §	109 $\pm$ 17	112 $\pm$ 22
Antimicrobial capacity	<i>PTGS2</i>	4	104 $\pm$ 16	99 $\pm$ 16	99 $\pm$ 23	173 $\pm$ 42 §	102 $\pm$ 17	103 $\pm$ 21
		7	109 $\pm$ 21#	103 $\pm$ 19				
	<i>IL8RB</i>	-7	1,292 $\pm$ 256	1,337 $\pm$ 446	2,619 $\pm$ 627	2,531 $\pm$ 625	95 $\pm$ 18	96 $\pm$ 19
		4	1,723 $\pm$ 1,035	2,220 $\pm$ 837#§	2,315 $\pm$ 580	2,363 $\pm$ 756	89 $\pm$ 16	90 $\pm$ 17
Antimicrobial capacity	<i>IL10</i>	7	1,807 $\pm$ 1,258#	2,209 $\pm$ 741#				
		-7	1,837 $\pm$ 682	1,799 $\pm$ 561	5,286 $\pm$ 1,248	5,245 $\pm$ 1,247	96 $\pm$ 19	100 $\pm$ 22
	<i>ALOX5</i>	4	1,884 $\pm$ 801	2,167 $\pm$ 1,206	4,790 $\pm$ 1,191	4,653 $\pm$ 1,615	87 $\pm$ 17	88 $\pm$ 19
		7	2,132 $\pm$ 896	2,059 $\pm$ 752				
Antimicrobial capacity	<i>DEFB5</i>	-7	1,062 $\pm$ 360	1,152 $\pm$ 360	3,040 $\pm$ 586	3,784 $\pm$ 743§	195 $\pm$ 54	190 $\pm$ 71
		4	1,256 $\pm$ 501	2,169 $\pm$ 423#§	2,788 $\pm$ 619	3,563 $\pm$ 974§	184 $\pm$ 51	171 $\pm$ 61
	<i>CATHL6</i>	7	1,373 $\pm$ 615#	2,277 $\pm$ 430#§				
		-7	9,212 $\pm$ 1,794	9,419 $\pm$ 1,954	17,186 $\pm$ 3,255	10,010 $\pm$ 1,821§	1,779 $\pm$ 272	777 $\pm$ 151§
Antimicrobial capacity	<i>LTF</i>	4	9,852 $\pm$ 1,600	9,976 $\pm$ 2,129	15,745 $\pm$ 3,494	9,423 $\pm$ 2,534§	1,681 $\pm$ 263	719 $\pm$ 162§
		7	10,413 $\pm$ 1,891#	9,835 $\pm$ 1,824				
	<i>MPO</i>	-7	347 $\pm$ 113	322 $\pm$ 116	455 $\pm$ 130	344 $\pm$ 109§	290 $\pm$ 99	284 $\pm$ 90
		4	368 $\pm$ 105	360 $\pm$ 89	340 $\pm$ 103	364 $\pm$ 104	271 $\pm$ 96	234 $\pm$ 97
Antimicrobial capacity	<i>DEFB5</i>	7	388 $\pm$ 132	384 $\pm$ 101#				
		-7	223 $\pm$ 76	218 $\pm$ 86	260 $\pm$ 79	223 $\pm$ 75§	258 $\pm$ 90	242 $\pm$ 77
	<i>ITGB2</i>	4	231 $\pm$ 69	226 $\pm$ 83	222 $\pm$ 82	233 $\pm$ 77	239 $\pm$ 78	249 $\pm$ 72
		7	261 $\pm$ 86	228 $\pm$ 91				
Antimicrobial capacity	<i>DEFB5</i>	-7	133 $\pm$ 50	149 $\pm$ 49	185 $\pm$ 47	366 $\pm$ 74§	136 $\pm$ 62	140 $\pm$ 55
		4	155 $\pm$ 52	150 $\pm$ 41	174 $\pm$ 45	321 $\pm$ 106§	147 $\pm$ 56	136 $\pm$ 59
	<i>CATHL6</i>	7	157 $\pm$ 56	162 $\pm$ 48				
		-7	258 $\pm$ 57	269 $\pm$ 56	940 $\pm$ 195	1,037 $\pm$ 211§	300 $\pm$ 55	303 $\pm$ 61
Antimicrobial capacity	<i>DEFB5</i>	4	310 $\pm$ 124	313 $\pm$ 152	866 $\pm$ 188	1,003 $\pm$ 293§	275 $\pm$ 45	276 $\pm$ 62
		7	329 $\pm$ 142#	307 $\pm$ 130				
	<i>ITGB2</i>	-7	139 $\pm$ 27	142 $\pm$ 30	159 $\pm$ 30	254 $\pm$ 60§	156 $\pm$ 24	163 $\pm$ 31
		4	151 $\pm$ 26	145 $\pm$ 23	144 $\pm$ 31	145 $\pm$ 32	146 $\pm$ 23	149 $\pm$ 30
Antimicrobial capacity	<i>DEFB5</i>	7	157 $\pm$ 30#	150 $\pm$ 29	222 $\pm$ 130	221 $\pm$ 111	254 $\pm$ 131	257 $\pm$ 145
		-7	223 $\pm$ 110	242 $\pm$ 142	208 $\pm$ 132	230 $\pm$ 151	259 $\pm$ 140	234 $\pm$ 121
	<i>ITGB2</i>	4	216 $\pm$ 130	219 $\pm$ 121				
		7	256 $\pm$ 138	266 $\pm$ 126				

Continued

Table 1 (Continued). Gene expression analysis results: mean RNA copy numbers ($\pm$ SD) for genes selected as indicators of categories of neutrophil functions (cell adhesion, pattern recognition, inflammation/cytokine response, antimicrobial capacity, and apoptosis/cell survival)¹

Function	Gene	Day	Blood		Uterus		Milk	
			CTR	PEG	CTR	PEG	CTR	PEG
Apoptosis/cell survival	<i>FAS</i>	-7	6,072 $\pm$ 1,148	6,254 $\pm$ 1,312				
		4	6,744 $\pm$ 1,472	6,761 $\pm$ 1,702	885 $\pm$ 165	1,193 $\pm$ 217§	180 $\pm$ 28	190 $\pm$ 37
		7	7,119 $\pm$ 1,793#	6,761 $\pm$ 1,541	793 $\pm$ 169	1,128 $\pm$ 302§	170 $\pm$ 26	171 $\pm$ 36
	<i>CASP2</i>	-7	90 $\pm$ 32	89 $\pm$ 27	172 $\pm$ 36	160 $\pm$ 35	151 $\pm$ 33	155 $\pm$ 36
		4	103 $\pm$ 30	88 $\pm$ 23§	155 $\pm$ 38	155 $\pm$ 36	139 $\pm$ 23	144 $\pm$ 33
		7	100 $\pm$ 31	88 $\pm$ 26				
	<i>CASP9</i>	-7	139 $\pm$ 28	142 $\pm$ 28	159 $\pm$ 29	152 $\pm$ 29	156 $\pm$ 24	162 $\pm$ 31
		4	149 $\pm$ 25	144 $\pm$ 23	144 $\pm$ 34	146 $\pm$ 35	150 $\pm$ 24	149 $\pm$ 30
		7	157 $\pm$ 30#	149 $\pm$ 27				
	<i>BAK1</i>	-7	264 $\pm$ 147	249 $\pm$ 62	394 $\pm$ 181	458 $\pm$ 317	344 $\pm$ 54	414 $\pm$ 151§
		4	263 $\pm$ 76	271 $\pm$ 90	357 $\pm$ 124	373 $\pm$ 141	338 $\pm$ 48	352 $\pm$ 59
		7	283 $\pm$ 101	285 $\pm$ 119				

¹Analyses were performed with neutrophils from blood samples (blood) and cells from uterine flushes (uterus) and milk (milk). Blood samples were taken at d -7, 4, and 7; uterus and milk samples on d 4 and 7. Samples were either from untreated control cows (CTR) or pegbovigrastim-treated cows (PEG).

indicates significant differences ($P < 0.05$) between postcalving d 4 and 7 compared with precalving (d -7).

§ indicates significant differences ($P < 0.05$) between pegbovigrastim-treated and control animals.

RESULTS AND DISCUSSION

Neutrophil Count

To allow putting the gene expression differences into context, neutrophil counts are presented in Figure 1, which has been redrawn from a previous report by the same authors (McDougall et al., 2017). As reported there, neutrophil count was affected by pegbovigrastim treatment, varied with time, and a treatment by time interaction was present whereby the neutrophil count was higher in pegbovigrastim-treated than control cows from d -6 onward (Figure 1). Feed group was not significant and no interactions were observed between feed group and treatment or feed group and time ($P > 0.2$). More pegbovigrastim-treated than control cows had a very high neutrophil count ($>4.9 \times 10^9$ cells/mL) on d -6, 4, 7, and 21. These results align well with the outcome previously reported (Kehrli et al., 1991; Cullor et al., 1992; Kimura et al., 2014; Hassfurth et al., 2015; Canning et al., 2017). Detailed hematology results from the present study have been reported elsewhere (McDougall et al., 2017).

Milk Microbiology

Milk samples had a very low incidence of IMI in both control and pegbovigrastim-treated cows. There were 9 (3.0%) and 5 (1.7%) glands and 8/82 (9.8%) and 6/82 (7.3%) cows with minor (i.e., CNS or *Corynebacterium* spp.) and major (i.e., *Staphylococcus aureus*, *Streptococcus uberis*, *Escherichia coli*) pathogen IMI on d 4, respectively. No difference was observed in prevalence between treatment groups (4/144 and 2/144 minor and major for the Inrestor-treated group, respectively, versus 5/152 and 4/152 in the control group; $P = 0.60$), feed groups ($P = 0.85$), breed category ($P = 0.72$), or parity ($P = 0.42$).

The observed low incidence of IMI indicated that only few neutrophils would be attracted to migrate into the mammary gland and activation levels were to be expected very low. These expectation were confirmed by the gene expression results. The gene expressions profiles from milk somatic cells had generally much lower RNA copy numbers than those from blood and uterine flushes. Consequently, these results cannot be interpreted with the same confidence.

Gene Expression in Neutrophils

Gene expression profiles for genes involved in various neutrophil functions were obtained from neutrophil preparations from blood (on d -7, 4, 7), and uterine flushes and milk (on d 4 and 7). Gene expression, ex-

pressed as RNA copy number in samples from all tissues and all time points, is presented in Table 1.

Expression profiling is a well-established technique for identifying expression patterns within cells. The biological samples used here contain mixtures of cell types, and neutrophils were a large subset of the cells in the sample. Normalization of gene expression as applied here accounts for variability in regard to the total cell number analyzed per samples. However, it does not provide reliable and accurate way to account for variability of the cellular composition of a samples, particularly an increase of the relative number of neutrophils. Hence, it is important to note that measured changes in gene expression might have been caused by a change in relative number of neutrophils in the sample or up-/downregulation of gene expression or both.

No significant effect of feed group on gene expression was observed nor was any significant effect observed for any of the interactions of feed group (feed group $\times$ treatment, feed group $\times$ time, feed group $\times$ treatment $\times$ time; all $P > 0.2$). This confirms other findings from this study where feed group had little effect on basal and stimulated neutrophil function (McDougall et al., 2017).

There was an effect of time on the expression of several genes in blood neutrophils, such that after calving expression of several genes was significantly greater at d 4 or 7 or both compared with before calving (d -7 ;

Tables 1 and 2). Figure 2 illustrates these changes over time in the control group, to isolate the effect of time from the effect of treatment.

Accounting for the effect of time, expression of several genes was significantly higher in neutrophils from animals that had received treatment with pegbovigrastim compared with untreated controls (Tables 1 and 2, and Figure 3), indicating effects in the categories of cellular functions that they represent.

Cell Adhesion

Two of the 4 cell adhesion-related genes were expressed at higher RNA copy numbers after calving. Expression of *ICAM1* was significantly greater in blood neutrophils from pegbovigrastim-treated animals compared with controls. In pegbovigrastim-treated cows *ICAM1* was also expressed at higher copy number in uterine cells but not in milk somatic cells. In pegbovigrastim-treated cows *ITGB8* RNA expression was lower in uterine cells and milk somatic cells but only nonsignificantly lower in blood neutrophils. Expression of *SELL* and *TLN1* was not significantly affected by pegbovigrastim treatment except for a slight but significant lower expression of *SELL* in uterine cells on d 4.

As part of the early response to infection, neutrophils migrate to the site of infection, while switching their gene expression to expression of inflammatory and

Table 2. Effects of time and pegbovigrastim treatment on gene expression¹

Function	Gene	Name	Time effect	Pegbovigrastim effect	
			Blood	Blood	Uterus
Cell adhesion	<i>ICAM1</i>	Intercellular adhesion molecule 1 (CD54)	↑	↑	↑
	<i>ITGB2</i>	Integrin, β 2 (CD18)	↑		↓
	<i>SELL</i>	L-Selectin (CD62)			↓
	<i>TLN1</i>	Talin 1			
Pattern recognition	<i>NOD1</i>	Nucleotide-binding oligomerization domain containing 1	↑		↑
	<i>CLEC6A</i>	C-type lectin domain family 6, member A (dectin-2)	↑		↑
	<i>TLR2</i>	Toll-like receptor 2	↑	↑	
	<i>TLR4</i>	Toll-like receptor 4			
Inflammation/cytokine response	<i>PTGS2</i>	Prostaglandin-endoperoxide synthase 2 (COX2)	↑	↑	↑
	<i>IL8RB</i>	IL-8 receptor β	↑		↓
	<i>IL10</i>	IL-10			↓
Antimicrobial capacity	<i>ALOX5</i>	Arachidonate 5-lipoxygenase			↓
	<i>MPO</i>	Myeloperoxidase			↑
	<i>DEFB5</i>	β -Defensin 5	↑		↑
	<i>CATHL6</i>	Cathelicidin 6	↑		↑
	<i>LTF</i>	Lactotransferrin			
Apoptosis/cell survival	<i>FAS</i>	Tumor necrosis factor receptor superfamily, member 6	↑		↑
	<i>CASP2</i>	Caspase 2		↓	
	<i>CASP9</i>	Caspase 9	↑		
	<i>BAK1</i>	BCL2-antagonist/killer 1			

¹The effects of time (pooled data from d 4 and 7 postcalving vs. d -7 precalving) or pegbovigrastim treatment (vs. control animals) for genes selected as indicators of categories of neutrophil functions: cell adhesion, pattern recognition, inflammation/cytokine response, antimicrobial capacity, and apoptosis/cell survival. ↑ indicates higher and ↓ indicates lower RNA copy number ($P < 0.05$).

antimicrobial pathways. Leukocyte migration has been demonstrated to increase with increased infection challenge in dairy cows (Hill, 1981; Burvenich et al., 1994; Van Werven et al., 1997). Migration of neutrophils is dependent on adhesion molecules, including ICAM-1 (*ICAM1*), L-selectin (*SELL*), talin-1 (*TLN1*), and CD18 (*ITGB2*). In this study, blood neutrophils had higher expression of *ICAM1* and *ITGB2* postpartum relative to the prepartum sample; expression of *ICAM1* was incrementally greater at d 4 in cows that received pegbovigrastim. *ICAM1* usually mediates migration via its expression on endothelial cells but *ICAM1* expression has also been described on a subset of blood neutrophils in context of systemic inflammation and reverse migration (Buckley et al., 2006). The higher expression of *ITGB2* postpartum confirms previous findings (Crookenden et al., 2016). Cross-linking of surface *ITGB2* molecules on bovine neutrophils has

been shown to result in degranulation (Hussen et al., 2016). No effects of time on *SELL* were observed, which confirms Diez-Fraile et al. (2004) who reported comparable basal L-selectin expression on bovine neutrophils at all stages of lactation. For an earlier time point (24 h postpartum), Lee and Kehrli (1998) demonstrated decreased L-selectin and CD18 expression using flow cytometry. Crookenden et al. (2016) observed that expression of *SELL* was lower from 1 wk after calving relative to precalving and the day of calving.

Pegbovigrastim increased expression of *ICAM1* in blood neutrophils. Higher expression of *ICAM1* and other adhesion molecule can be expected to increase trans-epithelial migration of the neutrophils (Gorlino et al., 2014). Indirectly, this has been confirmed when Fiuza et al. (2002) showed that G-CSF significantly enhanced neutrophil adhesion to microvascular endothelial cells in healthy humans.

Figure 2. Effects of time on blood neutrophil gene expression. Mean RNA copy numbers ($\pm$ SEM) for neutrophils from untreated control cows. For all variables with no letter or the same letter, the difference between the means is not statistically significant. Variables with different letters are significantly different ($P < 0.05$).

Neutrophils that have migrated into the uterus or the mammary gland lumen (milk) can be expected to then downregulate the molecules involved in migration. Pegbovigrastim-treated cows had significantly lower expression of *ITGB2* and *SELL* in uterine cells than untreated cows. Milk somatic cells showed a similar but nonsignificant difference. This could be a sign that pegbovigrastim supports a faster switch of neutrophils from migratory to effector functions. In contrast, uterine cells from pegbovigrastim-treated cows had greater expression of *ICAM1*, which likely helps attracting further neutrophils into the uterus or can be attributed to *ICAM1*'s nonmigratory functions, particularly their contribution to inflammatory processes observed during leukocyte infiltration (Etienne-Manneville et al., 1999).

Pattern Recognition

Of the 4 pattern recognition receptors investigated here, 3 (*NOD1*, *CLEC6A*, and *TLR2*) had higher expression at the RNA level after calving. On d 4 blood neutrophils had increased expression of *TLR2* RNA in pegbovigrastim-treated cows. In blood neutrophils the other pattern recognition markers were not significantly affected by pegbovigrastim. In uterine cells *NOD1* was expressed at higher copy number in pegbovigrastim-treated animals both on d 4 and 7, as was *CLEC6A* RNA.

Neutrophils are no longer considered to be transcriptionally inert and neutrophil pattern recognition receptor-mediated signaling has been identified as im-

Figure 3. Effect of treatment on neutrophil gene expression. Mean RNA copy numbers (combined from d 4 and 7 samples; $\pm$ SEM) for neutrophils from blood and cells from uterus from cows treated with pegbovigrastim (on d -7 and 0; PEG) compared with cows treated with saline (CTR). An asterisk indicates a significant change ($P < 0.05$).

portant for orchestrating innate and adaptive immunity via cytokine/chemokine production and the release of antimicrobials (Thomas and Schroder, 2013). Pattern recognition receptors can be membrane bound, such as *TLR2*, *TLR4*, and C-type lectin-like receptors (e.g., *CLEC6A*) or cytoplasmic, such as *NOD1*. In this study, *TLR4*, *CLEC6A*, and *NOD1* genes were increasingly expressed postpartum. These findings confirm previous observations (Crookenden et al., 2016) that furthermore showed a decrease of *TLR2* expression at later time points. During a *Streptococcus uberis* intramammary challenge lactating cows with a negative energy balance had *TLR2* and other genes upregulated (Moyes et al., 2010). Chapwanya et al. (2009) reported that *TLR4* and other genes were increased in uterine lavages from dairy cows 1 wk postcalving.

Pegbovigrastim treatment increased the expression of *TLR2* in blood neutrophils and *NOD1* and *CLEC6A* in uterine cells. Expressing an increased number of pattern recognition receptors is likely to be beneficial for the cow because their signaling rapidly induces various inflammatory cytokines, chemokines, and type I interferons, consequently triggering an array of antimicrobial immune responses (Kumar et al., 2011).

Inflammation and Cytokine Response

Two of the 4 genes selected for their role in inflammation and cytokine response (*PTGS2* and *IL8RB*) had significantly higher expression after calving. Blood neutrophils had increased expression of *PTGS2* in pegbovigrastim-treated cows. The other genes involved in inflammation and cytokine response were not significantly affected by treatment in blood neutrophils. In uterine cells *PTGS2* was expressed at higher copy number in pegbovigrastim-treated animals both on d 4 and 7, whereas *IL8RB* RNA was expressed at lower copy number on d 4 and 7. A reduced expression of *IL8RB* RNA was also observed in milk somatic cells on d 4 and 7. Expression of RNA for *IL10* was reduced in uterine cells from pegbovigrastim-treated cows on d 4.

After migrating into infected tissue and recognizing patterns on the infecting pathogens, neutrophils are involved in maintaining or resolving inflammation (Soehnlein et al., 2017). In blood neutrophils, *PTGS2* and *IL8RB* were upregulated postpartum. Pegbovigrastim upregulated expression of *PTGS2* in blood and uterine cells. Similarly, dietary CLA supplementation has been reported to increase the uterine endometrial expression of *PTGS2* (Abolghasemi et al., 2016). In most cells *PTGS2* is normally not expressed, but elevated levels are found during inflammation. COX-2 (encoded by *PTGS2*) is primarily responsible for the synthesis of prostanoids, which contribute to the patho-

logical processes of several inflammatory conditions (Rumzhum and Ammit, 2016).

In this study, *IL10* and *ALOX5* were not affected by time, but Crookenden et al. (2016) have reported a higher expression of *IL10* and *ALOX5* for the day of calving compared with 1 wk before and 1 wk after calving. Interleukin-8 expression is not affected by calving (Crookenden et al., 2016), but negative energy balance has been reported to upregulate *IL8RB* expression in postpartum cows (Wathes et al., 2009). Such enhanced availability of IL-8 receptor is likely to increase the effects of the available IL-8, which plays a causative role in acute inflammation by recruiting and activating neutrophils (Harada et al., 1994). In this study pegbovigrastim decreased the expression of *IL8RB*, *IL10*, and *ALOX5* in uterine cells compared with untreated cows, which might have the benefit to avoid chronic inflammation of the uterus.

Antimicrobial Capacity

After calving blood neutrophils expressed higher levels of 2 genes (*DEFB5* and *CATHL6*) encoding for proteins with antimicrobial activity. Expression of genes encoding antimicrobials in blood neutrophils was not significantly affected by pegbovigrastim treatment. In contrast, uterine cells had higher copy numbers of *MPO* on d 4 and 7, *DEFB5* on d 4 and 7, and *CATHL6* on d 4. A treatment effect on gene expression in milk somatic cells was not observed.

In addition to recruiting and activating other cells of the immune system, one of the main functions of neutrophils is to directly kill invading pathogens by phagocytosis (via release of *MPO*) or by release of antimicrobials, such as defensins (e.g., *DEFB5*), cathelicidins (e.g., *CATHL6*), or lactoferrin (*LTF*; Amulic et al., 2012).

Myeloperoxidase synthesis is mainly restricted to myeloid cells in the bone marrow (Tobler et al., 1988; Hansson et al., 2006). In neutrophils the myeloperoxidase protein is stored in azurophilic granules and released following priming and activation by inflammatory mediators (Dang et al., 1993; Holle et al., 2013). Consequently, the very low number of *MPO* RNA copies in blood neutrophils was expected. However, in this study pegbovigrastim stimulated a small but significant increase of *MPO* RNA in uterine cells. Because no such increase was observed in blood neutrophils, this might indicate that *MPO* transcription was reinitiated during activation of uterine cells, which has been reported before (Sugiyama et al., 2001).

Similar to *MPO*, the constitutive expression of defensins in neutrophils occurs during myelopoiesis and mature peptides are packaged into neutrophilic gran-

ules (Yount et al., 1995). A re-initiation of defensin synthesis has been reported for the epithelium of mammary gland alveoli (Diamond et al., 1996, 2000; Russell et al., 1996), and β -defensins in uterine tissue (Walker et al., 2015). Furthermore, β -defensin expression can be stimulated with LPS in bovine monocytes (Merriman et al., 2015). In our study *DEFB5* and *CATHL6* were expressed at higher copy numbers in blood neutrophils of control cows after calving. A higher expression of *DEFB5* and *CATHL6* was found in uterine cells of pegbovigrastim-treated cows compared with control animals. This is in line with previous reports of higher expression of genes encoding antimicrobial peptides (*DEFB1*, *DEFB10*, *BNBD4*, and *LAP*) in circulating neutrophils on the day of calving (Crookenden et al., 2016) and increased expression of defensin genes, including *DEFB5*, in neutrophils from uterine lavages in cows with severe inflammation (Chapwanya et al., 2009). These transcription profiles during the calving period likely reflect the cow's response to inflammatory signals from increased exposure of damaged internal epithelial surfaces to potentially pathogenic microorganisms.

Cell Survival

Of the 4 genes related to cell survival/apoptosis, 2 (*FAS* and *CASP9*) had significantly higher expression after calving. Expression profiles for genes involved in cell survival were somewhat inconsistent. Pegbovigrastim-treated cows had lower expression of *CASP2* in blood neutrophils on d 4, higher expression of *FAS* in uterine cells on d 4 and 7, and higher expression of *BAK1* in milk somatic cells on d 4.

The severity of inflammatory processes is at least partly controlled by regulation of cell death and survival of neutrophils (Geering et al., 2013). The 2 best-described apoptotic pathways are the extrinsic or (death-)receptor-induced pathway and the intrinsic or mitochondrial stress-induced pathway; both pathways include executioner proteases known as caspases (Taylor et al., 2008). Fas receptor signaling mediates apoptosis via FADD and Caspase 8 (Simon, 2001), whereas the intrinsic pathway involves caspase-9 (Luo and Loison, 2008; Li et al., 2017). Chang et al. (2004) reported a drop in *FAS* expression at calving, probably due to the peripartum rise in glucocorticoids, but a recovery thereafter, which is consistent with our finding that *FAS* and *CASP9* RNA expression increased in blood neutrophils after calving. Pegbovigrastim might increase apoptosis in neutrophils that have reached their target location as indicated by our finding that

FAS expression was increased in uterine cells from pegbovigrastim-treated cows compared with controls. In contrast, pegbovigrastim reduced the expression of *CASP2* in blood neutrophils.

Similar to previous reports, the results presented here are inconclusive. Reduced expression of *CASP2* indicates a potentially increased lifespan of blood neutrophils, which would be considered advantageous at the beginning of an infection, as the onset of apoptosis leads to functional impairment (Whyte et al., 1993). Increased *FAS* and *CASP9* mediated apoptosis after calving and increased *FAS* expression in uterine cells from pegbovigrastim-treated cows might pose a benefit because phagocytic removal of intact, apoptotic neutrophils by macrophages is an important mechanism preventing neutrophils from releasing their cytotoxic content into the extracellular milieu that would occur if the cells died by necrosis (Akgul et al., 2001).

Collectively, the data indicate that within days after calving, neutrophils showed greater expression of genes supporting innate immune defense through cellular adhesion, pattern recognition, inflammatory response, and antimicrobial capacity, and changed in their regulation of cell survival (Table 2). The data also indicate that treatment with pegbovigrastim reinforced these functions in blood neutrophils (Table 2). The effects of pegbovigrastim on uterine cells were different in that their migratory and inflammatory capacity was reduced while pattern recognition and antimicrobial capacity were strengthened (Table 2). It is worth underscoring that in contrast to blood neutrophils, those recovered from the uterine lumen and endometrial surface by flushing have already been activated and undergone migration and presumably one or more elements of interaction with damaged cells or pathogens.

CONCLUSIONS

The observed changes in gene expression in neutrophils in transition cows indicate altered cell functions, including cell adhesion/migration, pattern recognition, inflammation/cytokine response, antimicrobial capacity, and cell survival. Postpartum cows regulate neutrophil gene expression attempting to overcome the immunosuppression at calving by enhancing neutrophil function, which likely decreases susceptibility of dairy cows to infection postpartum. Treatment with pegbovigrastim further increased expression of several genes involved in these processes in blood neutrophils. Uterine cells changed to a phenotype with increased antimicrobial capacity typical for neutrophils that have migrated into their target tissue.

ACKNOWLEDGMENTS

The assistance of the Massey University farm staff including Hamish Doohan and Fiona Brown, Jenny Nixey and the technical staff of the Institute of Veterinary, Animal and Biomedical Sciences (Massey University), the laboratory staff from New Zealand Veterinary Pathology at Palmerston North, and the technicians from Cognosco (Cathy Yanez, Elizabeth Blythe) is gratefully acknowledged. Clinical examination and sampling of cattle was undertaken by veterinarians at the Institute of Veterinary, Animal and Biomedical Sciences including Richard Laven, Kevin Lawrence, Kristina Mueller, and Lisa Whitfield. Funding was provided by Elanco Animal Health.

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