1 *Short communication*: **Pegbovigrastim treatment in vivo does not impact on granulocyte**
2 **capability to migrate to endometrial cells and kill bacteria in vitro.** *By Tombácz et al*. The in vivo
3 effects of pegylated bovine granulocyte colony stimulating factor (pegbovigrastim, Imrestor™ Elanco
4 Animal Health, Greenfield, IN) are well described, however, its effects on granulocyte function on a
5 per cell basis are not yet fully elucidated. We applied a recently developed co-culture and bactericidal
6 assay to assess migratory and bacterial killing activities of granulocytes isolated from animals treated
7 with pegbovigrastim (n=6) or placebo (n=5). While treatment increased circulating neutrophil
8 granulocyte and monocyte concentrations in treated animals, it did not affect granulocyte function in
9 vitro. We suggest that the benefits of treatment are due to increased production of functional
10 granulocytes.
11
12 **SHORT COMMUNICATION: EFFECTS OF PEGBOVIGRASTIM ON GRANULOCYTES IN**
13 **VITRO**
14
15 Short communication: Pegbovigrastim treatment in vivo does not impact on granulocyte 16 capability to migrate to endometrial cells and kill bacteria in vitro in healthy cows

17 K. Tombácz, [*] L. M. Peters, [*] Y.-M. Chang, [*] M. Steele,[†, 2] D. Werling, [*, 1] A.J. Gibson [*]
18 *Department of Pathobiology and Population Sciences, Royal Veterinary College, North Mymms,
19 Hatfield, AL9 7TA, UK
20 [†]Elanco Animal Health, Eli Lilly and Company Ltd, Lilly House, Priestley Road, Basingstoke, RG24
21 9NL, UK; [2] present address: Inspire Cattle Solutions, Winslow, UK.
22 [1] Corresponding author: Dirk Werling, Royal Veterinary College, Department of Pathobiology and
23 Population Sciences, Hawkshead Lane, Hatfield, AL9 7TA; Phone: **44 (0)1707 666358; e-mail:
24 dwerling@rvc.ac.uk
25
26

27 **ABSTRACT**

28 In periparturient dairy cows, immune suppression, resulting in decreased neutrophil numbers and
29 function, leads to increased susceptibility to postpartum conditions such as mastitis, retained placenta
30 and metritis. The administration of polyethylene glycol-conjugated bovine granulocyte colony
31 stimulating factor (pegbovigrastim, Imrestor™ Elanco Animal Health, Greenfield, IN) 7 days before and
32 within 24 hours of calving, effectively improves granulocyte production and function in vivo as well as
33 in the milk. A recently developed co-culture assay was adapted for use with endometrial epithelial cells
34 to assess the effects of pegbovigrastim application on directed granulocyte migration and bactericidal
35 activity in vitro on a per cell basis in endometrial cell cultures. Granulocytes from treated and untreated
36 periparturient cows (6 and 5 per group, respectively) were evaluated for their ability to migrate to and
37 kill bacteria after treatment, in context of the infected endometrium. We hypothesized that in addition to
38 increasing the absolute concentration of circulating neutrophil granulocytes, pegbovigrastim treatment
39 in vivo alters the ability of granulocytes to migrate to endometrial cells in vitro. The results clearly show
40 a significant increase in the total concentration of granulocytes and monocytes between the two treatment
41 groups as early as two days after the first injection, and this increased between the samples taken two
42 days after calving. No migratory or killing differences were identified between granulocytes of both
43 groups, suggesting that pegbovigrastim-induced granulocytes were as effective as non-induced cells.
44 This may also be due to the absence of negative energy balance in the study animals and leads us to
45 conclude that the positive effects seen in vivo are most likely based on the larger number of granulocytes
46 being present rather than a direct effect of pegbovigrastim treatment on the functionality of cells for the
47 parameters tested in this study.

48

49 Keywords: granulocyte, pegbovigrastim, endometrial cell, bacterial killing, periparturient period

50

51

52

ABBREVIATIONS

BHBA: β-hydroxy-butyric acid

CBC: Complete blood count

CFU: Colony forming unit

CXCL8: Chemokine (C-X-C motif) ligand 8

G-CSF: Granulocyte colony-stimulating factor

MOI: Multiplicity of Infection

NEFA: Non-esterified fatty acid

PEG: Polyethylene-glycol

WBC: White blood cell

Short communication

INTRODUCTION

Puerperal metritis and postpartum endometritis are prevalent conditions in dairy cattle, compromising animal welfare, leading to economic damage by reducing milk production, and causing delayed fertility or infertility. In most cases, these conditions are caused by bacterial infections, facilitated by the presence of tissue damage caused by retained placenta, stillbirth, twins, caesarean section, and aggravated by the innate immune response of the host to bacteria and endotoxin (Carneiro et al., 2016). The treatment of puerperal uterine disease relies heavily on antibiotics and although in some cases necessary for the welfare of the animal, the results are unreliable (Pyorala et al., 2014). Given the increasing drive to reduce the use of antibiotics in food producing animals, new treatment possibilities are currently being investigated that impact on immune cell subsets. The first immune cells responding to damage signals from compromised tissue and infection are polymorphonuclear cells. These cells are recruited predominantly by the chemokine CXCL (IL-)8, produced as a direct response

78 to innate recognition of Gram-negative bacterial infection by a variety of cells, including endothelial
79 cells (Cronin et al., 2016).

80 Periparturient dairy cows experience a fluctuation in neutrophil count and decrease in function,
81 starting before calving, remaining low and slowly reaching normal levels again by the fourth week of
82 lactation (Kehrli et al., 1989). This phenomenon is associated with increased susceptibility to postpartum
83 conditions, including retained placenta, acute puerperal metritis, and chronic endometritis (Kehrli et al.,
84 1991, Detilleux et al., 1995, Hammon et al., 2006). The immunosuppression results from both
85 physiological (maintaining pregnancy) and pathophysiological events (negative energy balance).
86 Elevated ketone bodies can directly impair some neutrophil functions, increasing disease susceptibility
87 (Hoeben et al., 1997, Hoeben et al., 2000, Grinberg et al., 2008).

88 Prophylactic use of bovine granulocyte-colony stimulating factor (G-CSF) has the potential to pre-
89 emptively increase neutrophil numbers and modulate their function ahead of parturition (Kehrli et al.,
90 1991). Pegbovigrastim (Imrestor™ Elanco Animal Health, Greenfield, IN), a form of G-CSF covalently
91 bound to polyethylene-glycol to increase its half-life, is effective in maintaining increased neutrophil
92 granulocyte levels, compensating for decreased bacterial killing (Kimura et al., 2014). In addition,
93 pegbovigrastim has been shown to have an impact on gene expression in neutrophils, affecting gene
94 families related to neutrophil function, migration, interaction with pathogens, and cellular survival
95 (Heiser et al., 2018).

96 As well as reducing the incidence of clinical and experimental mastitis (Powell et al., 2018),
97 pegbovigrastim treatment decreased the risk of failure to return to oestrus within 80 days of calving
98 (Canning et al., 2017). A recent study found a reduction in the incidence of retained placenta and mastitis
99 as a result of label use of pegbovigrastim, however, metritis was reported to occur more frequently in
100 treated animals (Ruiz et al., 2017). In a different study, pegbovigrastim treatment was shown to reduce
101 the incidence of acute puerperal metritis in primiparous dairy cows, as well as the number of antibiotic
102 doses required for treatment (Freick et al., 2018). An increasing body of clinical data are available on

the in vivo effects of pegbovigrastim use (Zinicola et al., 2018), however, its action on granulocytes on a per-cell base is yet to be determined.

The objective of this study was to investigate the effects of Imrestor™ (pegbovigrastim) on bacterial clearance in the context of the endometrium of cows on a per-cell basis. Blood-derived granulocytes collected from pegbovigrastim or placebo treated periparturient animals were examined in vitro in a newly developed co-culture assay to observe their migration and bactericidal activity towards infected endometrial epithelial cells.

MATERIALS AND METHODS

Our study was designed to compare functional differences between granulocytes isolated from periparturient cattle treated with pegbovigrastim and placebo. In our experience, a minimum of 6 biological repeats is necessary in order to overcome the natural variation in animal responsiveness to obtain significant p-values (with significance set at $p = 0.05$) with regards to cytokine production and bacterial killing (Conejeros et al., 2015, Joekel et al., 2015, Gibson et al., 2016, Jensen et al., 2016). The study was conducted at the Royal Veterinary College's Bolton Park (Potter Bar, UK) research and teaching farm, under the authority of the UK Animal Scientific Procedures Act (ASPA, 1986). Although no formal quality standard is claimed, the study was conducted in line with the principles of Good Clinical Practice Guidelines and laboratory work was conducted in accordance with the Research Councils UK Policy and Guidelines on the Governance of Good Research Conduct.

Animals (n=12) enrolled in the study (autumn 2016 to spring 2017) were pregnant, multiparous Holstein-Friesian cows from the herd at the study site. Individual animals were identified by the unique number on their official primary ear tag and were also marked as study participants using coloured tail tape. All cows included received a physical examination, including assessment of the respiratory, cardiovascular, gastrointestinal, musculoskeletal and reproductive systems as well as skin, udder and teats. The physical examination included body condition score, pulse rate, respiration rate and a rectal temperature conducted by a licensed veterinarian or trained designee approximately 7 days prior to their

129 anticipated calving date. All animals had body condition scores between 2.5 and 3.5 (Wildman et al.,
130 1982). Animals exhibiting abnormal clinical signs that could be anticipated to have an impact on the
131 expected calving or uterine health and cattle undergoing any surgical or medical treatment 30 days before
132 the trial, as well as animals carrying more than one calf, were not enrolled in the study.

133 The experimental unit for all variables was the individual animal. A treatment administrator
134 allocated the animals to treatment or placebo groups using a random selection program written in R and
135 delivered the assigned treatments. Cows in the treatment group received two doses of Imrestor™ (Elanco
136 Animal Health, Basingstoke, UK) as indicated on the product leaflet. Animals in the placebo group were
137 injected with the same volume of sterile saline (Steripod, Mölnlycke Health Care) subcutaneously. The
138 treatment data were stored in a secure location and the scientists taking the blood sample as well as
139 conducting the in vitro assessments were kept blinded until the completion of the statistical analysis.
140 After excluding one cow in the placebo group due to calving outside of the prescribed treatment window
141 of 17 days, 11 animals finished the study. One animal gave birth overnight between days 17 and 18 and
142 was included in the analysis described in this paper.

143 Calving dates (study day 0) were estimated based on service date records. Nine days before
144 anticipated calving (study day -9), whole blood and serum samples were collected, using the Vacutainer
145 system with EDTA-treated and plain tubes (Becton Dickinson). EDTA blood was submitted to the
146 Diagnostic Laboratories of the Royal Veterinary College for complete blood count (CBC) using an
147 automated Advia 2120i system (Siemens) and microscopic blood smear analysis with manual
148 differential count. The serum sample was submitted for measurement of non-esterified fatty acid (NEFA)
149 to the Animal and Plant Health Agency laboratory (Shrewsbury, Shropshire, UK). Animals identified to
150 have NEFA levels elevated above 400 μmol/L (Oetzel, 2003) were to be removed from the study. On
151 study day -7, the animals received their first treatment. Health observations were made once daily from
152 here on to completion of the animal. Two days later, on day -5, an additional EDTA blood sample was
153 collected and submitted for haematological analysis as described above. Within 24 hours of actual
154 calving (study day 0), the second treatment was administered and two days later (study day +2), 24 mL

155 EDTA blood was collected for haematological analysis and to isolate granulocytes for setting up in vitro
156 functional assays. B-hydroxy-butyric acid (BHBA) testing was also performed using fresh whole blood
157 on all sampling days using the Precision Xtra Blood Glucose and Ketone Monitoring System (Precision)
158 with Precision Xtra Blood Ketone Test Strips (Abbott).

159 The in vitro assessments of granulocyte function were split into two sections measuring
160 bactericidal activity and directed migratory function of granulocytes. Granulocytes were isolated by
161 density centrifugation of EDTA blood (Munoz-Caro et al., 2015a, Munoz-Caro et al., 2015b) collected
162 by venepuncture of jugular vein from study animals two days after calving. Granulocyte isolation was
163 started within one hour of sampling in all cases. After cell separation and counting, the cell
164 concentrations were set to $2x10^6$/mL for each sample, and cells passed on to another scientist blinded to
165 the original cell counts, to set up the migration and bactericidal assays.

166 The bacterial strain used for in vitro functional assays was *Escherichia coli* strain MS499, which
167 has recently been described as a prototypic endometrial pathogenic *E. coli* strain (Goldstone et al., 2014a,
168 Goldstone et al., 2014b). *E. coli* MS499 was freshly plated from cryopreserved stock every week. For
169 each migration and bactericidal assay, single colonies were selected and cultured, then diluted
170 appropriately to ensure a multiplicity of infection (MOI) of ten with regards to granulocyte numbers.

171 Primary bovine endometrium epithelial cells were cultured using uteruses collected from two
172 clinically healthy Holstein-Friesian cows post mortem at an abattoir (Dawn Cardington, Meadow Ln,
173 Bedford, UK). Endometrial cultures, consisting of epithelial (>95%) and stromal cells were isolated
174 using the differential attachment plating method after trypsin/collagenase digestion of endometrium
175 tissue, as described elsewhere (Cheng et al., 2013). Cells were cultured to at least 75% confluency in
176 anticipation of calving. Primary endometrium cultures from at least two animals were used in duplicates
177 for each assay condition.

178 General migratory function and migration towards compromised epithelial cells were assessed by
179 placing granulocytes ($1 x 10^6$) into the upper chamber of a transwell system (24 well plate) with a pore
180 size of 3.0 µm (Greiner Bio One). Granulocytes migrated through these pores towards stimuli in the

181 lower chambers of the plates, where the following conditions were present: i) a monolayer of primary
182 endometrium culture ii) a monolayer of primary endometrium culture, infected with *E. coli* MS499 at
183 an MOI of 10 relative to the number of granulocytes, iii) *E. coli* MS499 bacteria only, iv) positive
184 migration control (10 ng/mL recombinant bovine CXCL8) and v) spontaneous migration control
185 (Roswell Park Memorial Institute medium (RPMI) only). Each condition was set up in duplicate.
186 Migrating granulocytes were counted from the lower chamber using a FastRead chamber slide (Immune
187 Systems) after 3 and 24 hours, by counting in 4 grids per replicate. In the bactericidal assays,
188 granulocytes were co-cultured with MS499 at a MOI of 10 for 3 hours at 37 °C before removing cell
189 culture media. One set of cells (3 hour-time point) were washed twice with Gentamycin (50 mg/mL,
190 Sigma Aldrich) and lysed with Triton X100 (0.1%). Supernatants were plated for subsequent CFU counts
191 by serial dilution on LB Agar to assess the number of viable phagocytosed bacteria. Another set of cells
192 (24 hour-time point) were washed twice, and Gentamycin was added to granulocyte media. These cells
193 were cultured for further 21 hours, followed by lysis. Lysates were plated for CFU counts as described
194 above.

195 The full reproducible protocols of in vitro migration and bactericidal assays are detailed in
196 Supplementary material A. WBC data were assessed for normal distribution, and biologically relevant
197 comparisons were made between subsequent timepoints of data from one treatment group and between
198 treatment groups at the same timepoints, using T-test in Prism v. 5 (GraphPad Software).

199

200 RESULTS AND DISCUSSION

201 The blood tests performed at day -9, two days before the first treatment, confirmed that all animals
202 met the inclusion criteria regarding NEFA levels, and no significant difference in NEFA values were
203 observed between animals enrolled in either study group (Figure 1A).

204 Pegbovigrastim treatment increased the overall WBC concentration as well as the concentration
205 of circulating neutrophil granulocytes and monocytes, but did not affect the concentration of circulating
206 lymphocytes. At day -9, two days before the first treatment, there were no significant differences for the

207 total concentration of WBC, neutrophil granulocytes, monocytes or lymphocytes (Figure 1C-F, Day -9).
208 Two days after the first treatment, at day-5, WBC concentration, the concentration of circulating
209 neutrophil granulocytes and monocytes, but not that of lymphocytes was increased in treated animals.
210 These concentrations increased even further by the last sampling (day +2). In pegbovigrastim-treated
211 animals, the increase in total WBC concentration was significant between all timepoints ($p<0.01$ day -9
212 to -5 and $p=0.03$ day -5 to 2). Differences were also significant between treated and placebo groups at
213 both timepoints after the first treatment ($p<0.01$ on day -5 and $p=0.03$ on day 2). The elevation of total
214 WBC concentration was reflected in neutrophil and monocyte concentration, with significant increase
215 in neutrophil granulocyte concentrations compared to before treatment (day -5 $p<0.01$) and placebo
216 counts (day -5, $p<0.01$). Monocyte concentration showed significant responses to treatment ($p=0.01$ by
217 day -5 and $p<0.01$ by day 2), reaching a significant difference to cells from placebo-treated animals on
218 day 2 ($p<0.01$). In contrast, no significant increases in the total concentration of monocytes and
219 granulocytes were seen in the control group at any timepoint.

220 Interestingly, our results demonstrated a clear increase in absolute numbers of both myeloid cell
221 subsets (and therefore also WBC) in pegbovigrastim-treated animals as early as two days after the first
222 treatment, even in this small cohort of animals. However, it is noted that this effect is not homogenous
223 in all animals in our study, as one animal did not respond to the same extent.

224 Contrary to results in other studies (Kimura et al., 2014), using our study protocol, we did not see
225 a decrease in WBC and neutrophil granulocyte concentration in placebo-treated control animals, or an
226 increase in circulating lymphocytes in pegbovigrastim treated animals, as described by (Powell et al.,
227 2018). Since genetically all animals used on the study were high-producing Holstein-Friesian dairy
228 cattle, the maintenance of leukocyte numbers throughout parturition is probably due to a less marked
229 negative energy balance, caused by the management practices on the smaller-scale study farm where the
230 trial was conducted. This is in line with the result that none of the animals had NEFA levels above 400
231 µmol/L (Figure 1A), nor BHBA levels elevated above 1.4 mmol/L (Figure 1B), indicating the absence
232 of subclinical ketosis (Oetzel, 2003).

233 Alongside fully mature granulocytes, band neutrophils were also released into circulation in four
234 out of six animals after the first, and in all cows after the second pegbovigrastim treatment (Figure 1G).
235 In one treated animal, metamyelocytes appeared in peripheral blood on day 2. In contrast, band
236 neutrophils were measured in only one animal in the placebo control group after the second injection.
237 This is a known effect of G-CSF treatment in cattle (Kehrli et al., 1991, Detilleux et al., 1995).

238 Bacterial survival was not impacted by pegbovigrastim treatment on a per cell base (Figure 2A).
239 As granulocytes from pegbovigrastim treated animals have been described to show a higher
240 myeloperoxidase activity, which could increase bacterial killing, we were interested to assess differences
241 in bactericidal activity of granulocytes exposed to *E. coli* bacteria from either treatment group. Neither
242 at 3h incubation nor at 24h did the amounts of surviving bacteria recovered from lysed granulocytes
243 show a significant difference, indicating similar bactericidal rates between both groups.

244 Granulocytes from either treated or untreated cows were also assessed in their ability to migrate
245 towards isolated endometrial cells, *E. coli* infected endometrial cells, *E. coli* alone or towards CXCL8.
246 Overall, the number of granulocytes that migrated under any condition was numerically greater (no
247 significant differences) after 24h compared to those values obtained in the same condition after 3h
248 (Figures 2B, 2C). The number of migrated granulocytes was greater under all conditions tested compared
249 to the negative (medium alone) control, however, no significant differences between granulocytes
250 isolated from pegbovigrastim treated or untreated animals were seen at 3h or 24h. The number of
251 granulocytes showing random migration (negative control, RPMI only) was lower than in any other
252 condition. It was recently described that pegbovigrastim treatment increased the expression of genes
253 involved in granulocyte migration/function (Heiser et al., 2018). However, as these changes were not
254 confirmed by flow cytometry or ex vivo functional assays, our data may not be regarded as contradictory
255 to these observations.

256 To determine whether cells generated from uteruses of different animals affected migration,
257 primary cells from two animals were used. These animals were in slightly different stages of their oestrus
258 cycles, one just after ovulation with corpus haemorrhagicum present and the other one having a well-

259 developed corpus luteum. Differences in the number of migrating granulocytes were assessed between
260 the results obtained for cells generated from treated or untreated cows to either endometrial epithelium
261 cells alone, or endometrial cells infected with *E. coli* strain MS499, at 3 as well as 24 hours, in a T-test
262 paired for each condition. There was no significant difference detectable between the results of the two
263 endometrium cultures ($p=0.7114$, mean of differences=$1.6x10^5$ migrated cells/mL, not shown). The
264 source of tissue did not affect the outcome of the results obtained, in line with observations that in vitro
265 cultures of endometrial epithelial and stromal cells have been described to mount innate immune
266 responses to E. coli independently of the stage of oestrus cycle (Saut et al., 2014).
267 Pegbovigrastim is described currently to have its most beneficial effects during the period of negative
268 energy balance, where neutrophil numbers are decreased. It may be possible that a negative energy
269 balance causes oxidative stress, inhibiting granulocyte function (Kuwabara et al., 2015), which could be
270 exacerbated by the presence of ketone bodies during this period (Hoeben et al., 1997). However, in
271 general, our observations indicate that granulocytes generated from both study groups are readily able
272 to migrate to endometrial cells in a transwell system. Furthermore, we observed no differences in
273 granulocyte function between negative controls (cells incubated with media alone) and cells exposed to
274 treatment. Our results do not indicate decreased or enhanced function of granulocytes isolated from
275 pegbovigrastim-treated cows compared to granulocytes isolated from control-treated cows on a per-cell
276 base. In our study, placebo-treated animals maintained WBC concentrations throughout the experiment,
277 and granulocytes remained functional for the parameters tested. As our study was performed using
278 healthy cows, it remains to be seen whether effects of pegbovigrastim treatment on neutrophil
279 functionality may have been different in animals with negative energy balance, hypocalcemia, any other
280 form of stress, or in animals with reduced neutrophil concentrations in future studies.

281

282 **ACKNOWLEDGEMENTS**

283 We would like to thank Dr. Daniel Hampshire, Marie-Christine Bartens, and Dawn Meats for
284 technical assistance. Furthermore, we would like to thank the staff of RVC Bolton Park farm for

collaboration and help, especially Ms Charlotte Verity. The study was funded by a grant to DW from Elanco Animal Health UK, covering salary for KT and costs of consumables. This manuscript represents publication PPS_01959 of the RVC.

REFERENCES

Canning, P., R. Hassfurther, T. TerHune, K. Rogers, S. Abbott, and D. Kolb. 2017. Efficacy and clinical safety of pegbovigrastim for preventing naturally occurring clinical mastitis in periparturient primiparous and multiparous cows on US commercial dairies. Journal of Dairy Science 100(8):6504-6515.

Carneiro, L. C., J. G. Cronin, and I. M. Sheldon. 2016. Mechanisms linking bacterial infections of the bovine endometrium to disease and infertility. Reprod Biol 16(1):1-7.

Cheng, Z., D. R. Abayasekara, F. Ward, D. M. Preece, K. A. Raheem, and D. C. Wathes. 2013. Altering n-3 to n-6 polyunsaturated fatty acid ratios affects prostaglandin production by ovine uterine endometrium. Anim Reprod Sci 143(1-4):38-47.

Conejeros, I., A. J. Gibson, D. Werling, T. Munoz-Caro, C. Hermosilla, A. Taubert, and R. A. Burgos. 2015. Effect of the synthetic Toll-like receptor ligands LPS, Pam3CSK4, HKLM and FSL-1 in the function of bovine polymorphonuclear neutrophils. Dev Comp Immunol 52(2):215-225.

Cronin, J. G., V. Kanamarlapudi, C. A. Thornton, and I. M. Sheldon. 2016. Signal transducer and activator of transcription-3 licenses Toll-like receptor 4-dependent interleukin (IL)-6 and IL-8 production via IL-6 receptor-positive feedback in endometrial cells. Mucosal Immunol 9(5):1125-1136.

Detilleux, J. C., M. E. Kehrli, Jr., J. R. Stabel, A. E. Freeman, and D. H. Kelley. 1995. Study of immunological dysfunction in periparturient Holstein cattle selected for high and average milk production. Vet Immunol Immunopathol 44(3-4):251-267.

Freick, M., M. Zenker, O. Passarge, and J. Weber. 2018. Reducing the incidence of acute puerperal metritis in primiparous cows by application of pegbovigrastim in a Holstein dairy herd. Veterinarni Medicina 63(04):151-160.

Gibson, A. J., S. Woodman, C. Pennelegion, R. Patterson, E. Stuart, N. Hosker, P. Siviter, C. Douglas, J. Whitehouse, W. Wilkinson, S. A. Pegg, B. Villarreal-Ramos, and D. Werling. 2016. Differential macrophage function in Brown Swiss and Holstein Friesian cattle. Vet Immunol Immunopathol 181:15-23.

Goldstone, R. J., R. Popat, H. J. Schuberth, O. Sandra, I. M. Sheldon, and D. G. Smith. 2014a. Genomic characterisation of an endometrial pathogenic Escherichia coli strain reveals the acquisition of genetic elements associated with extra-intestinal pathogenicity. BMC Genomics 15:1075.

Goldstone, R. J., R. Talbot, H. J. Schuberth, O. Sandra, I. M. Sheldon, and D. G. Smith. 2014b. Draft Genome Sequence of Escherichia coli MS499, Isolated from the Infected Uterus of a Postpartum Cow with Metritis. Genome Announc 2(4).

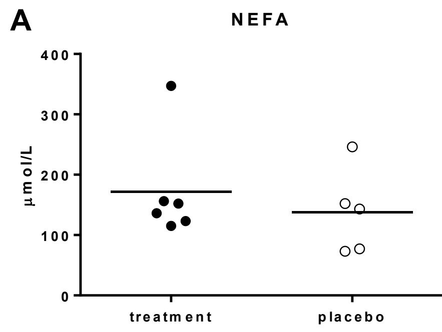
Grinberg, N., S. Elazar, I. Rosenshine, and N. Y. Shpigel. 2008. Beta-hydroxybutyrate abrogates formation of bovine neutrophil extracellular traps and bactericidal activity against mammary pathogenic Escherichia coli. Infection and immunity 76(6):2802-2807.

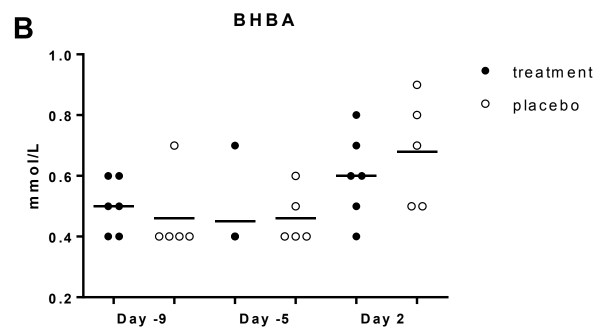
Hammon, D. S., I. M. Evjen, T. R. Dhiman, J. P. Goff, and J. L. Walters. 2006. Neutrophil function and energy status in Holstein cows with uterine health disorders. Vet Immunol Immunopathol 113(1-2):21-29.

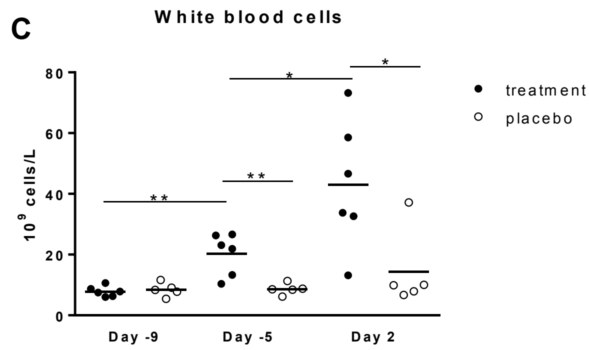
Heiser, A., S. J. LeBlanc, and S. McDougall. 2018. Pegbovigrastim treatment affects gene expression in neutrophils of pasture-fed, periparturient cows. J Dairy Sci 101(9):8194-8207.

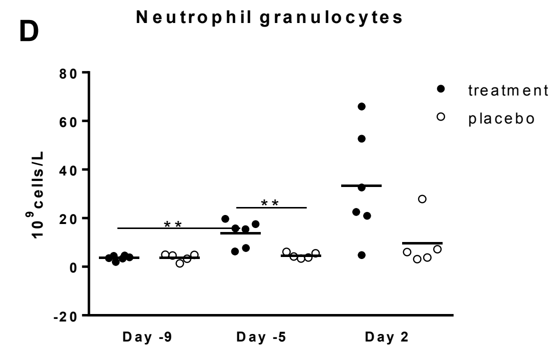
330 Hoeben, D., R. Heyneman, and C. Burvenich. 1997. Elevated levels of beta-hydroxybutyric acid in
331 periparturient cows and in vitro effect on respiratory burst activity of bovine neutrophils. Vet Immunol
332 Immunopathol 58(2):165-170.
333 Hoeben, D., E. Monfardini, G. Opsomer, C. Burvenich, H. Dosogne, A. De Kruif, and J. F. Beckers.
334 2000. Chemiluminescence of bovine polymorphonuclear leucocytes during the periparturient period
335 and relation with metabolic markers and bovine pregnancy-associated glycoprotein. J Dairy Res
336 67(2):249-259.
337 Jensen, K., I. J. Gallagher, A. Kaliszewska, C. Zhang, O. Abejide, M. P. Gallagher, D. Werling, and E.
338 J. Glass. 2016. Live and inactivated Salmonella enterica serovar Typhimurium stimulate similar but
339 distinct transcriptome profiles in bovine macrophages and dendritic cells. Vet Res 47:46.
340 Joekel, D., P. Hinse, M. K. Raulf, S. Schicht, W. Baumer, D. Werling, E. Kremmer, and C. Strube.
341 2015. Vaccination of calves with yeast- and bacterial-expressed paramyosin from the bovine
342 lungworm Dictyocaulus viviparus. Parasite Immunol 37(12):614-623.
343 Kehrli, M. E., Jr., J. P. Goff, M. G. Stevens, and T. C. Boone. 1991. Effects of granulocyte colony-
344 stimulating factor administration to periparturient cows on neutrophils and bacterial shedding. J Dairy
345 Sci 74(8):2448-2458.
346 Kehrli, M. E., Jr., B. J. Nonnecke, and J. A. Roth. 1989. Alterations in bovine neutrophil function
347 during the periparturient period. Am J Vet Res 50(2):207-214.
348 Kimura, K., J. P. Goff, P. Canning, C. Wang, and J. A. Roth. 2014. Effect of recombinant bovine
349 granulocyte colony-stimulating factor covalently bound to polyethylene glycol injection on neutrophil
350 number and function in periparturient dairy cows. Journal of Dairy Science 97(8):4842-4851.
351 Kuwabara, W. M., L. Zhang, I. Schuiki, R. Curi, A. Volchuk, and T. C. Alba-Loureiro. 2015. NADPH
352 oxidase-dependent production of reactive oxygen species induces endoplasmatic reticulum stress in
353 neutrophil-like HL60 cells. PLoS One 10(2):e0116410.
354 Munoz-Caro, T., M. Lendner, A. Daugschies, C. Hermosilla, and A. Taubert. 2015a. NADPH oxidase,
355 MPO, NE, ERK1/2, p38 MAPK and Ca2+ influx are essential for Cryptosporidium parvum-induced
356 NET formation. Developmental and comparative immunology 52(2):245-254.
357 Munoz-Caro, T., S. J. Mena Huertas, I. Conejeros, P. Alarcon, M. A. Hidalgo, R. A. Burgos, C.
358 Hermosilla, and A. Taubert. 2015b. Eimeria bovis-triggered neutrophil extracellular trap formation is
359 CD11b-, ERK 1/2-, p38 MAP kinase- and SOCE-dependent. Veterinary research 46:23.
360 Nicola, N. A. and D. Metcalf. 1986. Specificity of action of colony-stimulating factors in the
361 differentiation of granulocytes and macrophages. Ciba Found Symp 118:7-28.
362 Oetzel, G. R. 2003. Herd-Based Biological Testing For Metabolic Disorders. in American Association
363 of Bovine Practitioners 36th Annual Conference. Columbus, Ohio, USA.
364 Powell, E. J., T. A. Reinhardt, E. Casas, and J. D. Lippolis. 2018. The effect of pegylated granulocyte
365 colony-stimulating factor treatment prior to experimental mastitis in lactating Holsteins. J Dairy Sci
366 101(9):8182-8193.
367 Pyorala, S., J. Taponen, and T. Katila. 2014. Use of antimicrobials in the treatment of reproductive
368 diseases in cattle and horses. Reprod Domest Anim 49 Suppl 3:16-26.
369 Ruiz, R., L. O. Tedeschi, and A. Sepúlveda. 2017. Investigation of the effect of pegbovigrastim on
370 some periparturient immune disorders and performance in Mexican dairy herds. Journal of Dairy
371 Science 100(4):3305-3317.
372 Saut, J. P., G. D. Healey, A. M. Borges, and I. M. Sheldon. 2014. Ovarian steroids do not affect bovine
373 endometrial cytokine or chemokine responses to Escherichia coli or LPS in vitro. Reproduction
374 148(6):593-606.
375 Wildman, E. E., G. M. Jones, P. E. Wagner, R. L. Boman, H. F. Troutt, Jr., and T. N. Lesch. 1982. A
376 Dairy Cow Body Condition Scoring System and Its Relationship to Selected Production
377 Characteristics. Journal of Dairy Science 65(3):495-501.
378 Zinicola, M., H. Korzec, A. G. V. Teixeira, E. K. Ganda, L. Bringhenti, A. Tomazi, R. O. Gilbert, and
379 R. C. Bicalho. 2018. Effects of pegbovigrastim administration on periparturient diseases, milk
380 production, and reproductive performance of Holstein cows. J Dairy Sci 101(12):11199-11217.

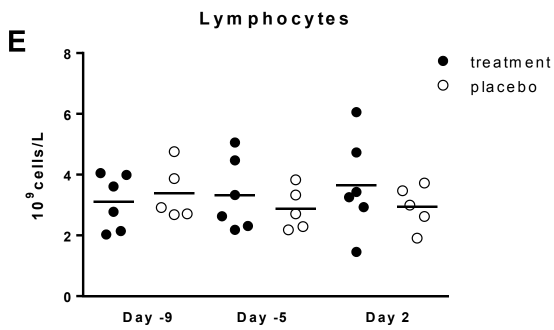
381

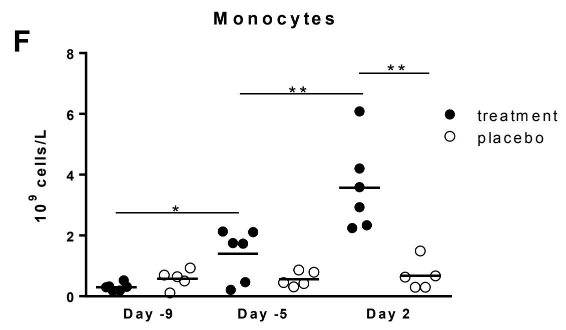


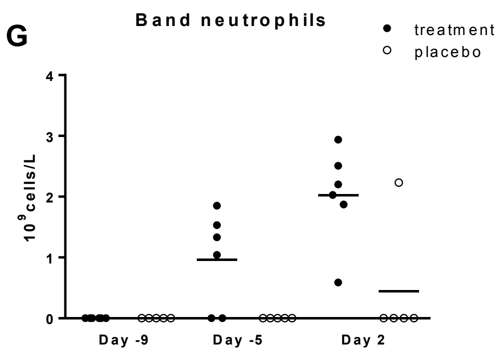








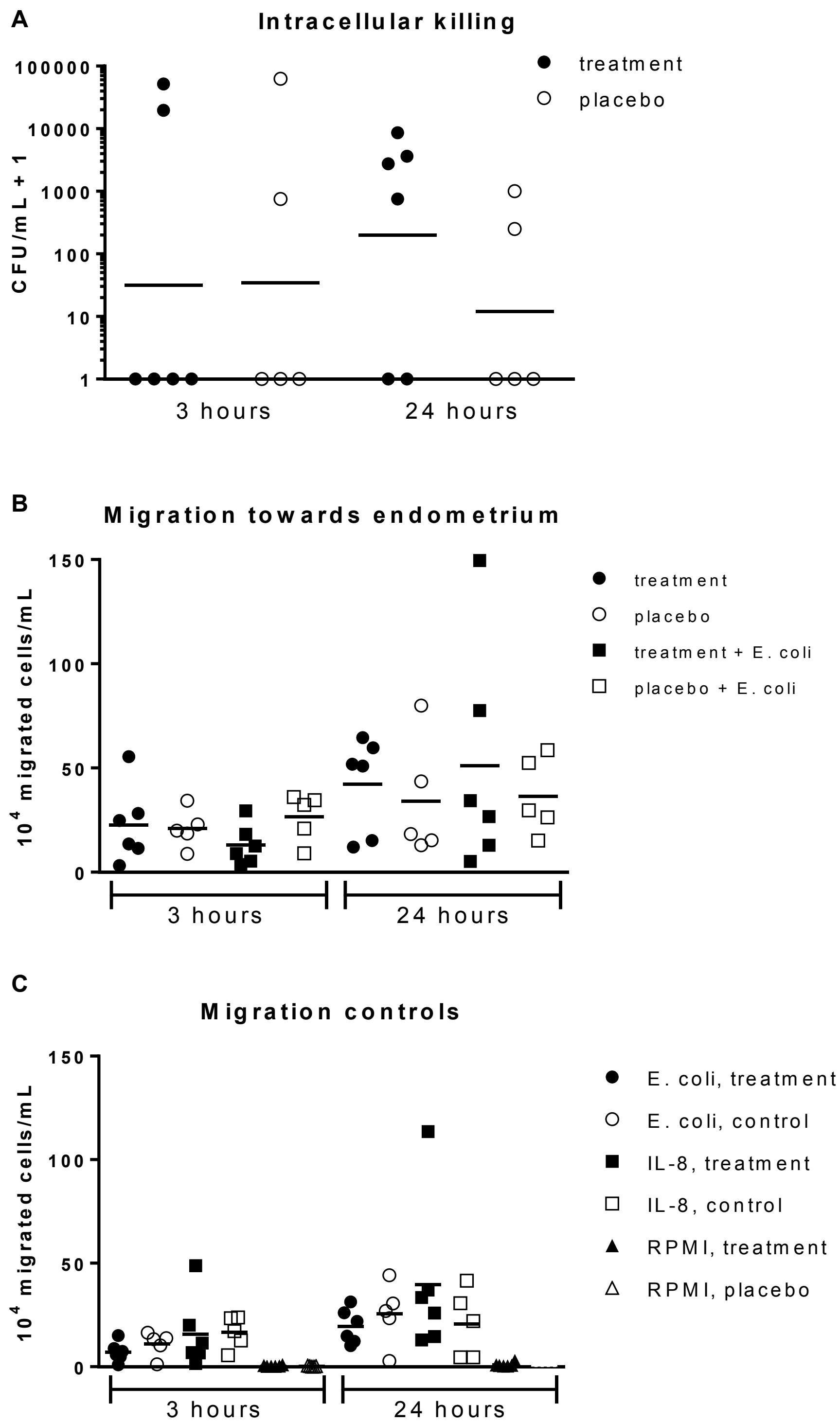




383

384 Tombacz, Figure 1.

385 Figure 1. Ex vivo parameters measured in study groups. Dot plot diagram showing A) NEFA levels
386 measured in study groups nine days before estimated parturition, B) BHBA levels measured at three
387 timepoints, C) WBC, D) Neutrophil granulocyte, E) Lymphocyte, F) Monocyte concentrations measured
388 at three timepoints. G) Dot plot of band neutrophil concentrations measured in study groups. Group
389 mean and SD values are listed in Supplementary material B for each parameter.
390



391

392 Tombacz, Figure 2.

393 Figure 2. In vitro assay results. A) Bactericidal assay. Dot plots showing individual values and geometric
394 means of colony forming units (CFU+1)/mL of *E. coli* MS499 surviving intracellular killing after 3 and
395 24 hours of incubation. For the purpose of representing zero values on a logarithmic scale, 1 was added
396 to all mean CFU counts. B) Dot plot: migration of granulocytes from pegbovigrastim and placebo treated
397 animals towards endometrium and infected endometrium. C) Results of migration assay controls. Dot
398 plot of migrated cells towards MS499 *E. coli*, 10 ng/mL CXCL8 and media only control. Group mean
399 and SD values are listed in Supplementary material C for each parameter.