Short communication

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

SHORT COMMUNICATION: EFFECTS OF PEGBOVIGRASTIM ON GRANULOCYTES IN VITRO

Short communication: Pegbovigrastim treatment in vivo does not impact on granulocyte capability to migrate to endometrial cells and kill bacteria in vitro in healthy cows

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ABSTRACT

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

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

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

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

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

Calving dates (study day 0) were estimated based on service date records. Nine days before anticipated calving (study day -9), whole blood and serum samples were collected, using the Vacutainer system with EDTA-treated and plain tubes (Becton Dickinson). EDTA blood was submitted to the Diagnostic Laboratories of the Royal Veterinary College for complete blood count (CBC) using an automated Advia 2120i system (Siemens) and microscopic blood smear analysis with manual differential count. The serum sample was submitted for measurement of non-esterified fatty acid (NEFA) to the Animal and Plant Health Agency laboratory (Shrewsbury, Shropshire, UK). Animals identified to have NEFA levels elevated above 400 μmol/L (Oetzel, 2003) were to be removed from the study. On study day -7, the animals received their first treatment. Health observations were made once daily from here on to completion of the animal. Two days later, on day -5, an additional EDTA blood sample was collected and submitted for haematological analysis as described above. Within 24 hours of actual 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 (1×10^6) 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

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

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

RESULTS AND DISCUSSION

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

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

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

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

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

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

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

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

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

developed corpus luteum. Differences in the number of migrating granulocytes were assessed between the results obtained for cells generated from treated or untreated cows to either endometrial epithelium cells alone, or endometrial cells infected with *E. coli* strain MS499, at 3 as well as 24 hours, in a T-test paired for each condition. There was no significant difference detectable between the results of the two endometrium cultures ($p=0.7114$, mean of differences=$1.6x10^5$ migrated cells/mL, not shown). The source of tissue did not affect the outcome of the results obtained, in line with observations that in vitro cultures of endometrial epithelial and stromal cells have been described to mount innate immune responses to E. coli independently of the stage of oestrus cycle (Saut et al., 2014).

Pegbovigrastim is described currently to have its most beneficial effects during the period of negative energy balance, where neutrophil numbers are decreased. It may be possible that a negative energy balance causes oxidative stress, inhibiting granulocyte function (Kuwabara et al., 2015), which could be exacerbated by the presence of ketone bodies during this period (Hoeben et al., 1997). However, in general, our observations indicate that granulocytes generated from both study groups are readily able to migrate to endometrial cells in a transwell system. Furthermore, we observed no differences in granulocyte function between negative controls (cells incubated with media alone) and cells exposed to treatment. Our results do not indicate decreased or enhanced function of granulocytes isolated from pegbovigrastim-treated cows compared to granulocytes isolated from control-treated cows on a per-cell base. In our study, placebo-treated animals maintained WBC concentrations throughout the experiment, and granulocytes remained functional for the parameters tested. As our study was performed using healthy cows, it remains to be seen whether effects of pegbovigrastim treatment on neutrophil functionality may have been different in animals with negative energy balance, hypocalcemia, any other form of stress, or in animals with reduced neutrophil concentrations in future studies.

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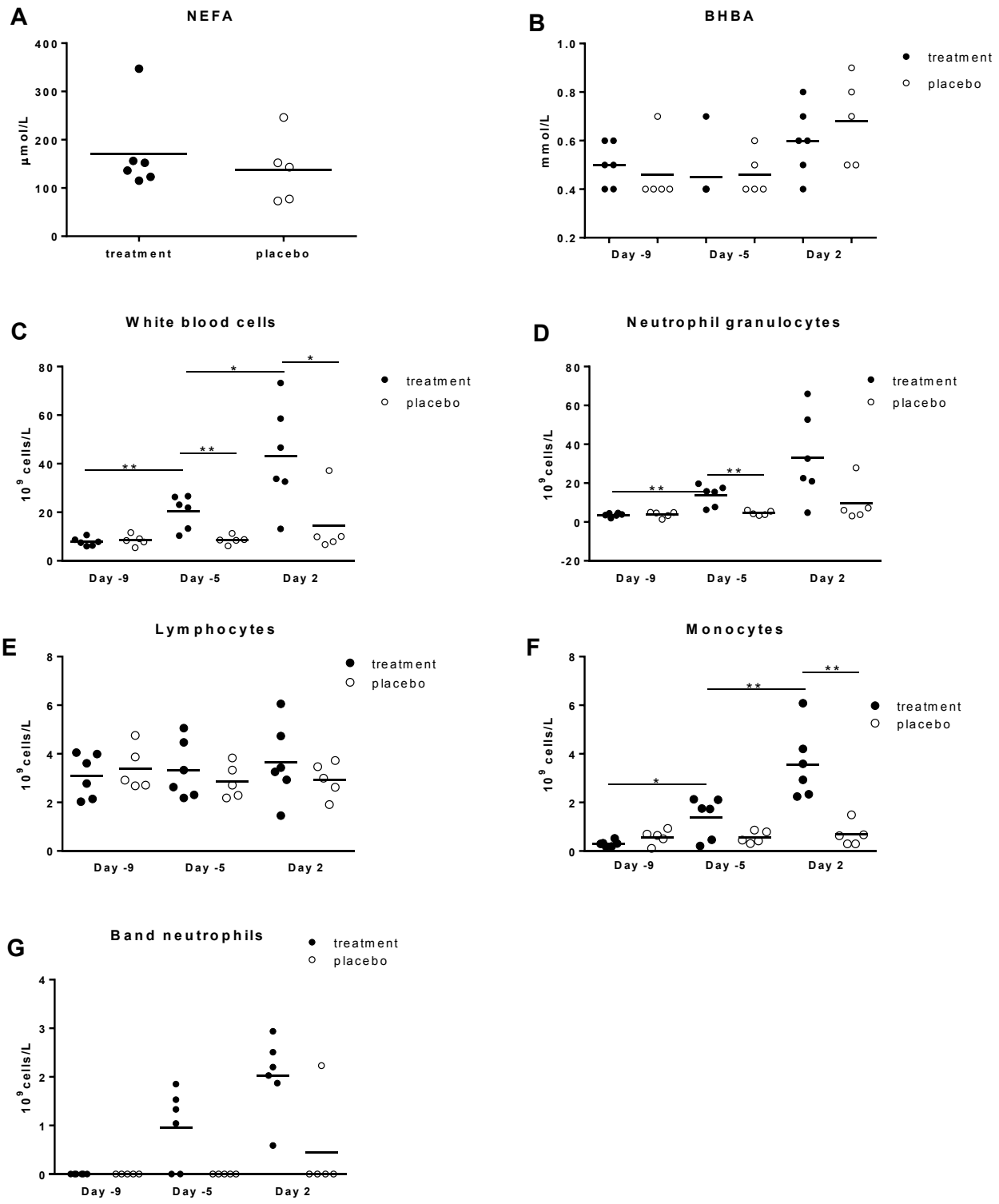

383

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.
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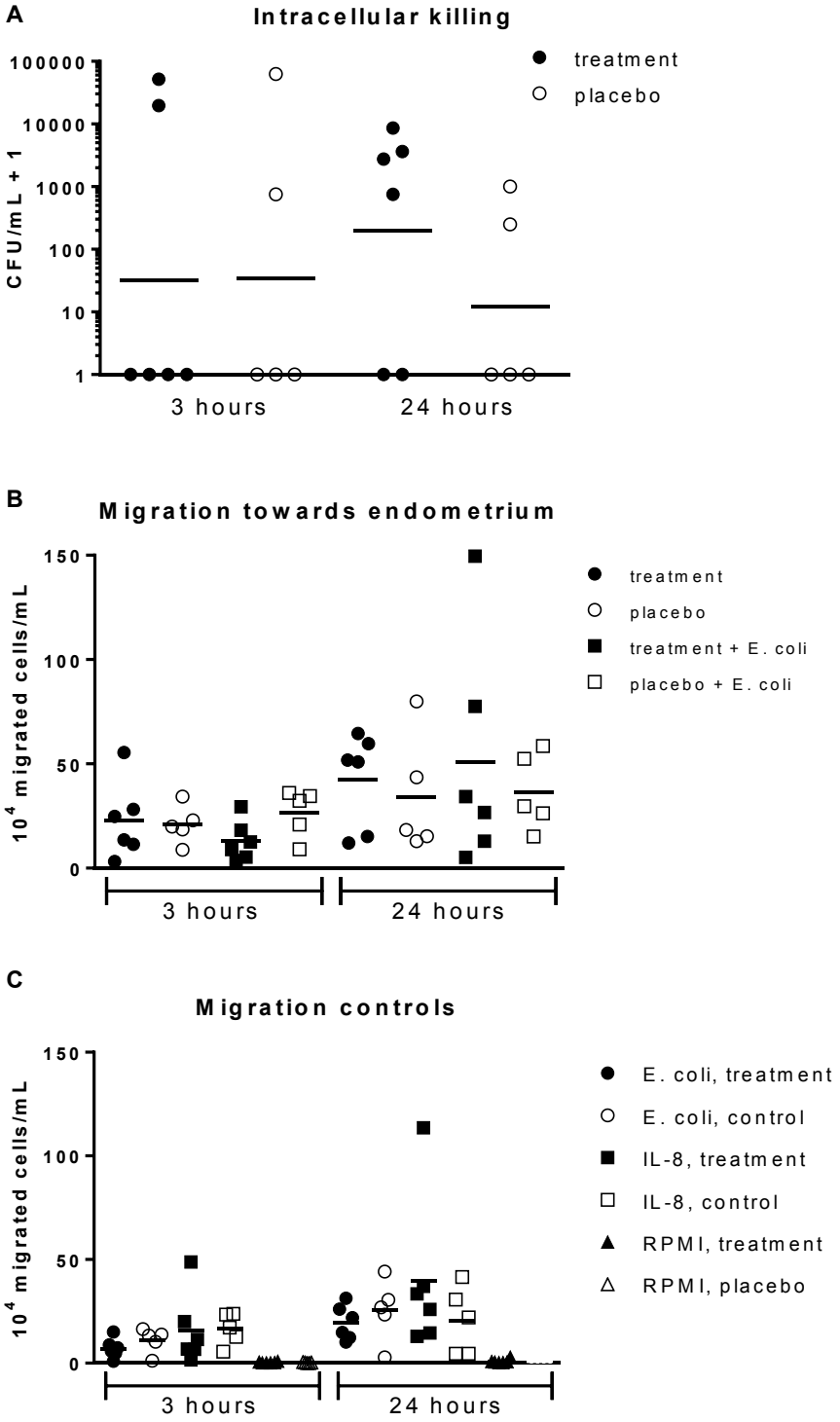

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392 Tombacz, Figure 2.

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