**SUPPLEMENTARY MATERIAL**

**A. Protocols for assessing granulocyte migration and bactericidal activity**

1. *Culture media, media supplements and other reagents*

Cell culture media Roswell Park Memorial Institute (RPMI) 1640, RPMI 1640 with Glutamax, Dulbecco`s Modified Eagle Medium with F12 nutrient mixture (DMEM/F12), antibiotics Penicillin/Streptomycin and Amphotericine B, the buffers phosphate buffered saline (PBS), Dulbecco`s PBS (DPBS) and Hank`s Balanced Salt Solution (HBSS), enzymes Trypsin and Type II Collagenase, and LB Lenox L agar were all purchased from Thermo Fisher Scientific. Irradiated and heat-inactivated Foetal Calf Serum (FCS), the antibiotic Gentamycin, reagents Trypan Blue, EDTA, Triton X100, molecular biology grade glycerol and bacterial culture medium LB (Lennox) broth were ordered from Sigma-Aldrich. Distilled water was collected from onsite reverse osmosis filtration system. Absolute ethanol was bought from VWR. Bovine serum albumin was produced by PAA. Ficoll density gradient was obtained from Merck. Vacutainer™ Hemogard Closure Plastic K2-EDTA and plain tubes were purchased from Beckton Dickinson. FastRead chamber slides from Immune Systems were used for cell counting. Bambanker freezing medium was purchased from Alpha Laboratories. Cell culture plates, centrifuge tubes and transwell inserts were ordered from Greiner Bio One.

Digestive Solution: 1X HBSS in sterile-filtered distilled water, 10% Trypsin without phenol red (10%), 0.1% Type II Collagenase (100 IU/μL), 0.1% bovine serum albumin, 25 µg/mL DNAse I.

Culture medium for endometrial cell differentiation: RPMI 1640 with Glutamax, 5% FCS, 1% Penicillin/Streptomycin (10,000 units/mL of penicillin and 10,000 µg/mL of streptomycin), 1% Amphotericine B (250 µg/mL).

Culture medium for endometrial epithelium: DMEM/F12 without phenol red, 10% FCS, 1% Penicillin/Streptomycin, 1% Amphotericine B.

Granulocyte culture medium: RPMI 1640 formulation without phenol red, 10 % FCS

Cell lysis buffer: 0.1% Triton X100 prepared in PBS

*E. coli* maintenance: LB (Lennox L) Agar, cryogenic storage in 25% glycerol in LB broth.

1. *Granulocyte isolation*

24 mL of whole blood was collected using 4 x 6 mL Vacutainer tubes containing anticoagulant EDTA and transported to the laboratory for cell isolation. Twenty mL of whole blood was diluted with 20 mL of PBS containing EDTA (2 mM), this mixture was gently layered above 12 mL of Ficoll density gradient in a 50 mL tube and centrifuged at 800 ×g (without brake) for 45 min at room temperature. Plasma and mononuclear cells were aspirated and discarded from the top layers. Granulocytes and red blood cells remained in the bottom of the tube. Red blood cells were lysed by addition of 25 mL of distilled water and gentle shaking for maximum 40 sec. Three millilitres of 10X HBSS were added to regain isotonic strength and cells were pelleted again at 600 ×g for 10 min. Supernatants were discarded. The cells were lysed/washed as above three times, or until the pellet was white. The remaining granulocytes were suspended in 5 mL RPMI 1640 medium without phenol red and counted using a FastRead chamber slide. If the cell suspension was too dense to be counted accurately, the cells were suspended in 20 mL RPMI media without phenol red and recounted. The cell concentration was adjusted to 2x106/mL and passed on to the scientist performing in vitro assays.

1. *Endometrium epithelial cell isolation and culture*

Complete uteruses were collected in pre- to mid-luteal phase from clinically healthy and bovine viral diarrhoea virus free Holstein-Friesian cows at an abattoir (Dawn Cardington, Meadow Ln, Bedford, MK443SB), and transferred to the laboratory on ice. Whole organs were washed once in sterile-filtered PBS and once in 70% ethanol. After this point, work was continued in a laminar flow hood. The uterus was opened, and strips of endometrial tissue were cut out using autoclaved curved scissors, avoiding the underlying myometrium layer. The separated tissue was washed three times in HBSS with Mg2+ and Ca2+, then cut to approximately 10 mm3 pieces using sterile scalpel and Petri-dish. The tissue was transferred into 50 mL centrifuge tubes followed by the addition of “Digestive Solution” in a 1:5 w/v ratio. The mixture was incubated at 37 °C for 45 min with 50 rpm constant shaking, followed by thorough vortexing three times, with additional 15 min incubation steps between mixing. Lysates were filtered through 70 µm-pore-size centrifuge cell strainers to obtain single cells. Cell pellets were resuspended in HBSS containing 10 % foetal calf serum, pelleted at 300 ×g acceleration for 10 min, and resuspended for 20 sec in distilled water to lyse red blood cells. Lysates were topped up with 30 mL RPMI 1640 medium and pelleted again at 300 ×g for 10 min. The cells were resuspended in 1 mL “Culture medium for endometrial cell differentiation” and viability was determined using Trypan Blue exclusion.

Differential attachment plating method for epithelial cell enrichment: The isolated bovine endometrium cells were seeded at 5x105/mL density into 24-well cell culture plates and incubated overnight in a 37 °C, 5% CO2 in a humidified incubator. In 18 hours, stromal cells attached, and epithelial cells remained in suspension. After 18 h, cells in suspension were removed, pelleted at 300 ×g for 10 min and counted, then re-plated at 5x105/mL density into a new 24-well plate in “Culture medium for endometrial epithelium”. Medium was changed every 48 hours. After six days of culture, cells were stored cryogenically in Bambanker serum-free freezing medium at 106 cells/mL density.

1. *Migration assay*

General migratory function of granulocytes was assessed by placing 1x106 cells into the upper chamber of a transwell system (24 well plate) with 3.0 µm pore size and *E. coli* strain MS499 at a multiplicity of infection (MOI) of 10, or 10 ng/mL recombinant bovine IL-8, or RPMI 1640 introduced into the lower chambers of the transwell plate. Cells were cultured at 37 °C, in 5% CO2. The granulocytes migrating through the transwell pores were counted from the lower chamber using a FastRead chamber slide after 3 and 24 hours. Two replicate wells were used for each condition and samples for counting were pipetted in duplicates into the counting chambers (a total of 4 counts for each condition were performed). On a separate 24-well plate, migratory function of granulocytes towards compromised epithelial cells were assessed as above with the modification of cultured endometrial epithelial cells in the lower chamber of the transwell plate, grown to a confluent monolayer. Epithelial cells were thawed, maintained and cultured in advance ready for study animals reaching study day 2. On each culture plate, 4 wells from epithelial cells isolated from at least two different uteruses were grown, with the exception of one migration assay, where four wells of only one set of epithelial cells reached the required confluency. One day before each migration assay, cell monolayers were rinsed with PBS and antibiotic-free culture medium was added, then replaced with fresh antibiotic-free medium in advance of each assay setup. Before addition of granulocytes, MS499 *E. coli* at MOI 10 were inoculated into the bottom compartment of two wells from each set of epithelial cells. This layout resulted in two infected and two non-infected replicate wells for each set of epithelial cells. Migrated granulocytes were counted in the lower chambers as described above.

1. *Bacterial killing assay*

The isolated granulocytes were co-cultured with *E. coli* strain MS499 at a MOI of 10 (using 106 granulocytes and 107 bacterial cells in a well). In 4 wells of a 24-well plate, granulocytes and *E. coli* cells were cultured together. At the 3 hours timepoint, supernatants from all wells were collected and plated to determine remaining CFU counts. To assess phagocytosed and still living bacteria granulocytes were washed twice with 50 µg/mL Gentamycin at this timepoint and lysed for back-plating in two out of the four co-culture wells. The other two co-culture wells were washed, and the granulocytes were maintained in media containing 50µg/mL Gentamycin for additional 21 hours, when they were lysed and back-plated (24 hours timepoint). At this timepoint, bacteria taken up would have replicated if not killed intracellularly. Cells were lysed using a lysis buffer consisting of 0.1% Triton X100 prepared in PBS. To calculate CFU numbers, tenfold dilution series of bacterial suspensions were prepared and dilutions 2 to 7 (in case of culture supernatants) or 1 to 6 (in case of lysed granulocytes) were plated onto LB plates and grown at 37 °C for maximum 12 hours. Distinguishable colonies were counted in at least two dilutions for each replicate and CFUs were calculated according to (Miles et al., 2009).

1. *Culture of MS499 E. coli*

MS499 cells were freshly plated onto LB agar plates from glycerol stock every week. On days when bactericidal and migration assay setup was required, one colony was lifted off the plate and inoculated into 5 mL DMEM/F12 medium, incubated at 37 °C in an aerated tube and without shaking. Optical density at 600 nm was measured until reaching approximately 0.4, then bacterial cell concentrations were estimated based on the exact measured OD. The formula for bacterial cell estimation was calculated as a result of two preliminary experiments, describing the growth curve specific to the MS499 strain and the culture conditions. Cell concentration was adjusted to contain 109 cells/mL suspension and 10 µL of this (107 cells) was added to each inoculated well in bactericidal and migration assay plates.

**B. Results of ex vivo measured parameters**

Fatty acid levels measured in study groups nine days before estimated parturition:

Treatment group: mean±SD = 171.5±87.44 μmol/L

Placebo: mean±SD = 138.2±70.41 μmol/L.

BHBA levels measured at three timepoints (values shown as mean±SD):

|  |  |  |  |
| --- | --- | --- | --- |
|  | Day -9 | Day -5 | Day 2 |
| Treatment | 0.50±0.89 mmol/L | 0.45±0.12 mmol/L | 0.60±0.14 mmol/L |
| Placebo | 0.46±0.13 mmol/L | 0.46±0.09 mmol/L | 0.68±0.18 mmol/L |

WBC concentrations measured at all three timepoints (values (mean±SD of groups) are shown in x109 cells/L:

|  |  |  |  |
| --- | --- | --- | --- |
|  | Day -9 | Day -5 | Day 2 |
| Treatment | 7.83±1.7 | 20.27±6.8 | 43.01±21.2 |
| Placebo | 8.46±2.2 | 8.63±1.8 | 14.34±12.8 |

Neutrophil granulocyte concentrations measured at all three timepoints (values (mean±SD of groups) are shown in x109 cells/L:

|  |  |  |  |
| --- | --- | --- | --- |
|  | Day -9 | Day -5 | Day 2 |
| Treatment | 3.64±1.0 | 13.77±5.5 | 33.25±22.5 |
| Placebo | 3.82±1.5 | 4.60±1.2 | 9.62±10.3 |

Lymphocyte concentrations measured at all three timepoints (values (mean±SD of groups) are shown in x109 cells/L:

|  |  |  |  |
| --- | --- | --- | --- |
|  | Day -9 | Day -5 | Day 2 |
| Treatment | 3.10±0.9 | 3.33±1.2 | 3.65±1.6 |
| Placebo | 3.39±0.9 | 2.87±0.7 | 2.94±0.7 |

Monocyte concentrations measured at all three timepoints (values (mean±SD of groups) are shown in x109 cells/L:

|  |  |  |  |
| --- | --- | --- | --- |
|  | Day -9 | Day -5 | Day 2 |
| Treatment | 0.30±0.1 | 1.40±0.8 | 3.56±1.4 |
| Placebo | 0.58±0.3 | 0.57±0.2 | 0.68±0.5 |

Band neutrophil concentrations measured at all three timepoints (values (mean±SD of groups) are shown in x109 cells/L:

|  |  |  |  |
| --- | --- | --- | --- |
|  | Day -9 | Day -5 | Day 2 |
| Treatment | 0 | 0.96±0.8 | 2.02±0.8 |
| Placebo | 0 | 0 | 0.445±1.0 |

**C. Results of in vitro measured parameters**

Bactericidal assay: Geometric means of colony forming units (CFU+1)/mL of *E. coli* MS499 surviving intracellular killing after 3 and 24 hours of incubation: treatment group at 3 hours: 31.69, 24 hours: 200.3, placebo-treated group at 3 hours: 34.22 and at 24 hours: 12.02

Migration of granulocytes from pegbovigrastim and placebo treated animals towards endometrium: treatment: mean±SD=22.75±18.4x104 cells/mL at 3h, 42.35±22.9 x104 cells/mL at 24 h; placebo: 20.89±9.2x104 cells/mL at 3h, 34.0±28.5 x104 cells/mL at 24 h, infected endometrium: treatment: mean±SD=13.03±9.6x104 cells/mL at 3h, 51.02±54.4 x104 cells/mL at 24 h; placebo: 26.57±11.5x104 cells/mL at 3h, 36.42±18.4x104 cells/mL at 24 h.

Migration assay controls: migrated cells towards MS499 *E. coli*: treatment: mean±SD=7.11±4.7x104 cells/mL at 3h, 19.40±8.3 x104 cells/mL at 24 h; placebo: 11.0±5.9x104 cells/mL at 3h, 25.56±14.9 x104 cells/mL at 24 h, 10 ng/mL CXCL8: treatment: mean±SD=15.9±17.3x104 cells/mL at 3h, 39.54±37.5 x104 cells/mL at 24 h; placebo: 16.54±7.7x104 cells/mL at 3h, 20.67±16.3 x104 cells/mL at 24 h) and media only control (treatment: mean±SD=0.37±0.3x104 cells/mL at 3h, 0.93±1.0 x104 cells/mL at 24 h; placebo: 0.25±0.2x104 cells/mL at 3h, 0.64±0.6x104 cells/mL at 24 h.