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Peripheral blood leukocytes of cows with subclinical endometritis show an altered cellular composition and gene expression

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ABSTRACT

Subclinical endometritis (SCE) is an important postpartum disease in dairy cows, but conventional cytobrush diagnosis often gives imprecise results. The aim of this study was to analyze disease-associated changes in peripheral blood as potential diagnostic parameters. Cellular subpopulations of blood leukocytes from cows with or without SCE (45–55 days postpartum) were flow-cytometrically quantified. Gene expression of whole blood leukocytes was assessed by PAXgene analysis. Subclinical endometritis cows showed significantly higher number of blood mononuclear cells and neutrophils. Among mononuclear cells, numbers of B-cells, NK-cells, and CD172a-positive monocytes were significantly elevated. Compared with non-SCE cows, blood leukocytes of SCE cows significantly expressed higher copy numbers of *CXCL8*, *TNF*, and *IL12*. To test whether circulating plasma factors are responsible for these changes, leukocytes, polymorphonuclear cells, and monocyte subpopulations (classical, intermediate, nonclassical) of healthy cows were stimulated with plasma of SCE and non-SCE cows. Although gene expression of whole leukocytes and polymorphonuclear cells remained unaltered, plasma from SCE animals significantly elevated expressed messenger RNA copy numbers of *CXCL8, CXCL1*, and *IL1B* in intermediate monocytes. In conclusion, elevated number of selected mononuclear subpopulations in peripheral blood and enhanced expression of distinct genes encoding for inflammatory mediators in blood leukocytes reflect the subclinical uterine inflammatory process in cows. Whether the observed changes in the periphery of SCE cows are the consequence of the uterine inflammatory process, or whether they affect the pathogenesis of the disease is currently unknown.

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1. Introduction

Subclinical endometritis (SCE) is defined as endometrial inflammation of the uterus in the absence of purulent material in the vagina [1]. Because of the lack of clinical signs, the most convenient and powerful diagnostic method is still a matter of debate. Although endometrial biopsy may constitute the ideal method of diagnosis of endometritis, the procedure is invasive, expensive, and time-consuming [2–6]. Furthermore, it has been shown that an endometrial biopsy has a potential impairment on future fertility [7]. A standard technique to diagnose SCE is the quantification of polymorphonuclear cell (PMN) among uterine cells by the cytobrush technique [8]. Between 34 to 47 days postpartum (PP), the presence of more than 10% PMN defines the diagnosis SCE [8]. An altered cellular composition in cytobrush samples of cows with SCE is also reflected by a higher messenger RNA (mRNA) expression of lipocalin-type prostaglandin D synthase, *IL1A*, *IL1RN*, *IL6*, *TNF*, and *CXCL8* in uterine cells obtained by cytobrush technique compared with healthy cows [9].

Less invasive methods for the detection and diagnosis of SCE have involved the use of analysis of metabolic blood parameters, serum inflammatory mediators, antibodies, and the cellular composition of the blood: In cows with SCE, Heidarpour, et al. [10] found higher serum concentrations of β-hydroxybutyrate (BHB), haptoglobin, and total sialic acid than in healthy cows. Serum concentrations of nonesterified fatty acids (NEFAs), BHB, bilirubin, and urea at week (wk) −1, at wk +1, and at wk +5 relative to calving, were unsatisfactory for disease prediction [11]. Concentrations of nitric oxide, an inflammatory mediator, in both blood and uterine secretions were higher in animals with subclinical and clinical endometritis when compared with control cows [12].

Neutrophils of SCE cows had reduced glycogen content than healthy cows at Days 7, 28, and 42 in milk, suggesting a diminished phagocytosis activity of PMN in diseased animals [13]. Polymorphonuclear cell of cows with SCE also showed significantly lower PMN myeloperoxidase activity between Day 1 prepartum and Day 8 PP than uterine healthy cows [14].

Data on cellular composition and gene expression profiles of peripheral blood leukocytes of cows with and without SCE are limited. Galvao, et al. [15] analyzed blood monocyte gene expression and secretion of selected cytokines from calving to 42 days after calving in Holstein cows with or without uterine disease. They showed that *Escherichia coli*–stimulated monocytes from cows with metritis expressed fewer key proinflammatory cytokine genes (*TNF*, *IL1B*, and *IL6*) than healthy cows from calving to 14 days after calving.

Cellular subset analyses of peripheral blood leukocytes from cows with SCE are not yet available. Disease-associated changes in peripheral blood have been observed in human endometriosis, a pelvic inflammatory process associated with infertility. Changes involved the increase of selected monocyte subpopulations in blood of patients compared with healthy controls [16], and enhanced the serum concentrations of inflammatory mediators.

To avoid surgical interventions for the diagnosis of endometritis, identification of biomarkers in the periphery for diagnosis and treatment monitoring of bovine SCE would be of significant benefit. The objective of this study was, therefore, to analyze differences in peripheral blood cell composition and the expression of genes associated with inflammation by blood leukocytes between cows with histologically proven SCE and uterine healthy cows.

2. Materials and methods

2.1. Study design, groups of animals, and source of bovine endometrial tissue

The study involved 22 pluriparous Holstein-Friesian cows, housed at the Farm for Education and Research of the University of Veterinary Medicine, Hannover, Germany. Animals between 4 and 10 years were enrolled for this study. All cows were followed from calving (Day 0) to 55 days PP. For both blood sampling and collection of endometrial tissue, a complete general examination was carried out at Days 45 to 55 PP. The gynecologic examination was performed at the same time of sampling.

Only cows considered clinically healthy were included in this study. Cows with postparturient metritis, retained fetal membranes, or mastitis were excluded from the study. A general examination was performed followed by a gynecologic examination. Body condition was scored using a five-point (one = thin to five = fat) system (Table 1). Cows were first inspected for the presence of fresh discharge on the vulva, perineum, or tail. The vulva was wiped clean with damp paper towels. A transrectal palpation of the reproductive tract was performed, and findings were classified as follows: size and contractility of the uterus, symmetry of the uterine horns, size of the ovaries, and palpable ovarian structure (CL, follicle, cyst [>2.5 cm in diameter]). Afterward, all cows were examined by ultrasound to confirm the diagnostic findings followed by a vaginal examination. Collection of uterine specimen by biopsy was performed at 45 to 55 days PP.

Diagnosis and quantification of leukocytes in endometrial tissue was performed as a diagnostic service in the Institute of Pathology, Faculty of Veterinary Medicine, Leipzig, Germany. Based on the histologic diagnosis of the presence or absence of nonpurulent endometritis 45 to 55 days PP in addition to the absence of clinical signs of endometritis (Table 4), cows were divided retrospectively into subclinical (n = 8) and non-subclinical cows (n = 14). All procedures were carried out in accordance with German legislation on animal welfare (AZ 33.14-42502-04-12/0794).

2.2. Collection of cytobrush smears and bacteriologic examination

Cytobrush samples were collected using the modified technique described by Kasimanickam, et al. [8]. In brief, the cytobrush was cut to approximately 5 cm in length threaded onto a solid stainless steel rod, and placed in a stainless steel tube, for passage through the cervix. Inside the uterus, the catheter was retracted, and the brush was rolled along the

Table 1
Age, body condition scores, parity, and milk yield of SCE and non-SCE cows.

Cow	BCS	Age (y)	Parity	Milk yield (kg/y)
SCE cows				
1	3.25	8	5	10,215
2	3	6.6	5	12,995
3	2.75	5	4	9762
4	2.5	4.6	3	10,453
5	3	4	2	7533
6	2.75	5	3	11,911
7	3	4	2	8953
8	2.5	4	2	8106
Non-SCE cows				
1	3.25	5.2	3	9433
2	2.75	5	3	9238
3	3.25	4	2	9788
4	3.25	4	2	8229
5	3.5	8.8	7	11,007
6	3.25	4.8	3	8256
7	2.75	10	7	10,426
8	2.75	5	2	10,832
9	2.75	4.6	2	9413
10	3	4.6	3	6513
11	3	5	3	10,989
12	2.75	5.6	4	8066
13	2.5	6	4	10,261
14	3	6	4	9544

Abbreviations: BCS, body condition score; SCE, subclinical endometritis.

uterine wall of the right horn. If detectable, the postgravid horn was chosen. Thereafter, the brush was retracted into the catheter to protect it during the passage through the genital tract. The cytobrush was rolled onto a sterile glass slide and air dried. The slide was immediately fixed and stained (Diff-Quick, Medion Diagnostics, Düdingen, Suisse) according to the manufacturer's protocol. The cytobrush was conserved in Amies medium, and examined bacteriologically for the presence of *E. coli* and *Trueperella pyogenes* (performed as a diagnostic service at the Institute for Microbiology, University of Veterinary Medicine, Hannover).

2.3. Biopsy

Endometrial biopsy samples were collected transcervically using a Kevorkian biopsy forceps (Eickemeyer, Tuttlingen, Germany) under transrectal palpation control. One biopsy sample was taken from the base of one uterus horn. If detectable, the larger postgravid horn was chosen for sampling. The sample was macroscopically checked for caruncular or intercaruncular part, and cut into two pieces. One part was fixed in 4% neutral buffered formalin according to Lillie for 24 hours, and subsequently dehydrated in a graded ethanol series, and finally embedded in paraffin to differentiate clearly between caruncular and intercaruncular tissue. The second part was immediately transferred into liquid nitrogen (−169 °C), and stored at −80 °C for molecular analyses.

2.4. Cytologic evaluation of endometrial cells and leukocytes collected by cytobrush

Stained slides were examined microscopically (magnification: ×200). Cells were identified as PMN and uterine epithelial cells. Areas of each slide that contained equally dispersed epithelial cells were preferentially selected, and all intact identifiable nucleated cells were counted in those fields. A total of 300 cells were counted from each slide, and results were expressed as a percentage of total nucleated cells. All slides were read blinded by the same investigator.

Table 2
Primer sequences for real-time PCR analysis.

Gene	Forward (for) and reverse (rev) primer sequences (5′ → 3′) and concentrations (nM)	bp[a]	Reference
IL1B	for: TTCTCTCCAGCCAACCTTCATT (300) rev: ATCTGCAGCTGGATGTTTCCA T (300)	198	[32]
TNF	for: CTTCTGCCTGCTGCACTTCG (300) rev: GAGTTGATGTCGGCTACAACG (300)	156	[33]
CXCL8	for: CCTCTTGTTCAATATGACTTCCA (900) rev: GGCCCACTCTCAATAACTCTC (50)	170	[33]
CXCL1	for: CGCCTGTGGTCAACGAACT (300) rev: CACCTTCACGCTCTGGATGTT (300)	83	[34]
NOS2	for: GAGATAGAAACAACAGGAACCTAC (300) rev: AGGCCTGCTTGGTGGCGAA (50)	70	[35] (Modified)
ARG1	for: ATCTGGGTTGATGCCCATGC (300) rev: AGGAAAATCCTGGGAGCTGT (50)	100	Novel design, Acc. no[b]: NM_001017942
CCL3	for: CTCTGCAGCCAGGTCTTCTC (900) rev: TGGTCTGGAAGATGACACCA (50)	154	Novel design, Acc. no.: NM_174511
CCL5	for: CCTGCTGCTTTGCCTATATCT (300) rev: AGCACTTGCTGCTGGTGTAG (300)	78	[36]
IL10	for: CCTTGTCGGAAATGATCCAGTTTT (300) rev: TCAGGCCCGTGGTTCTCA (300)	67	[37]
IL12	for: TGGTCGTTTCCTGGTTTTCC (300) rev: GTTTTGCCAGAGCCCAAGAC (300)	205	Novel design, Acc. no.: NM_174356.1
CCL20	for: GACTGCTGTCTCCGATATACA (300) rev: GCCAGCTGCTGTGTGAAGC (300)	71	Novel design, Acc. no.: NM_174263

Abbreviation: PCR, polymerase chain reaction.
[a] Length of amplicons in base pairs (bp).
[b] GenBank accession number of nucleotide sequence used for primer generation.

Table 3
Antibodies for membrane immunofluorescence.

Specificity	Catalogue no.[a]	Isotype	Fluorochrome label
Bovine CD2	MCA833 F	Mouse IgG1	FITC
Bovine CD4	MCA1653PE	Mouse IgG2a	RPE
Bovine CD8	MCA837A647	Mouse IgG2a	ALEXA FLUOR 647
Bovine CD25	MCA2430 F	Mouse IgG1	FITC
Bovine CD172a	MCA2041P647	Mouse IgG2b	RPE- FLUOR 647
Bovine CD21	MCA1424PE	Mouse IgG1	RPE
Bovine WC1	MCA838 F	Mouse IgG2a	FITC
Bovine CD335	MCA2365PE	Mouse IgG1	RPE
Human CD14	MCA1568 F	Mouse IgG2a	FITC
Human CD14	MCA1568PE	Mouse IgG2a	RPE
Human CD16	MCA5665 F	Mouse IgG2a	FITC
Ovine MHCII	MCA2226 F	Mouse IgG2a	FITC

[a] All antibodies were purchased from AbD Serotec.

2.5. Blood sampling for PAXgene analysis

Blood collection was performed once between Day 45 and 55 PP. before carrying out the general and gynecologic examination to avoid the impact of cortisol on parameters obtained in the peripheral blood. Immediately after blood collection, the PAXgene Blood RNA Tubes (Becton Dickinson, Heidelberg, Germany) were inverted gently eight to 10 times. The tubes were stored between 2 and 6 hours at room temperature, frozen at −20 °C for 24 hours followed by storage at −80 °C.

Among 22 examined animals, five SCE and five non-SCE cows were randomly chosen for PAXgene analysis (Table 4). Blood was obtained by puncture of the vena jugularis externa into PAXgene Blood RNA Tubes using a Becton Dickinson Vacutainer System (Becton Dickinson) according to the manufacturer's instructions.

2.6. Blood plasma and cell separation

For plasma, blood was collected in EDTA-vacutainer tubes, and centrifuged at 4 °C for 15 minutes at 3000× *g*. Plasma was removed, pipetted into Eppendorf tubes, and stored at −20 °C until usage. For cell separation, blood was collected in heparinized vacutainer tubes (Becton Dickinson), layered on Ficoll-Isopaque (PAA, Pasching, Austria), and centrifuged at 4 °C for 30 minutes at 1000× *g*. Mononuclear cells (MNCs) at the interphase were washed three times in PBS (500× *g*, 250× *g*, and 100× *g*), and finally suspended in Dulbecco's Modified Eagle's Medium (DMEM, 10% fetal calf serum, 1% Penicillin/Streptomycin). Polymorphonuclear cell were separated by hypotonic lysis of the erythrocyte-containing pellet after discontinuous gradient separation as described [17]. Briefly, the blood was layered on Ficoll-Isopaque (PAA), and centrifuged at 4 °C for 30 minutes at 1000× *g*. The cell pellet below the Ficoll-Isopaque was lysed for 20 seconds with distilled water followed by the addition of double-concentrated PBS. This was repeated (usually twice) until complete erythrolysis. Finally, the cells were washed once in PBS (250× *g*, 100× *g*).

For separation of total leukocytes, blood was diluted 1:2 with PBS, and centrifuged at 100× *g* for 30 minutes at 4 °C. Erythrocytes were lysed with distilled water until complete erythrolysis.

Table 4
Histologic and bacteriologic findings of uterine tissue samples (stratum compactum).

Animal	NG	EG	Ly	MPh	PC	*E. coli*	*T. pyogenes*	PAXgene[a]
SCE cows								
1	(+)	−	+(+)	+	+	+	−	•
2	+	−	++	+	(+)	+	−	•
3	(+)	−	+	+	−	−	−	•
4	+	(+)	+	+	(+)	+	−	•
5	−	−	+	(+)	−	+	−	•
6	−	−	++	−	+	−	−	—
7	(+)	−	(+)	++	+	+	−	—
8	−	−	+	+	(+)	−	−	—
Non-SCE cows								
1	−	+	+	+	(+)	+	−	•
2	−	−	(+)	+	−	−	−	•
3	−	−	(+)	+	−	−	−	•
4	−	−	(+)	+	−	−	−	•
5	−	−	(+)	−	−	−	−	•
6	−	−	+	+	(+)	−	−	—
7	−	−	+	(+)	−	−	−	—
8	(+)	−	(+)	−	(+)	−	−	—
9	−	−	(+)	(+)	(+)	−	−	—
10	−	−	−	−	−	−	−	—
11	−	−	(+)	−	−	−	−	—
12	−	−	(+)	+	−	−	−	—
13	−	−	+	+(+)	(+)	+	−	—
14	+	−	(+)	++	−	−	−	—

Cell composition in the stratum compactum of eight subclinical and 14 non-subclinical animals. Tissue samples of 22 cows were taken between Days 44 to 45 PP. Grouping in cows with or without SCE was based on the histopathologic diagnosis 'nonpurulent endometritis' in addition to the absence of clinical signs for endometritis. The presence and abundance of immune cell types are indicated as follows: −, absent; (+), scarce; +, low abundance; +(+), medium abundance; ++, high abundance. *E. coli* and *T. pyogenes* were isolated from cytobrush smears.
Abbreviations: EG, eosinophilic granulocytes; Ly, lymphocytes; MPh, macrophages; NG, neutrophilic granulocytes; PC, plasma cells; SCE, Subclinical endometritis.
[a] From indicated animals (•) blood was taken for PAXgene analysis.

For separation of bovine monocyte subsets, MNCs of one healthy, midlactating donor animal were labeled with an fluorescein isothiocyanate (FITC)-conjugated CD16-specific antibody (AbD Serotec, Oxford, UK) for 15 minutes (4 °C). After incubation with anti-FITC MultiSort MicroBeads (Anti-FITC MultiSort Kit; Miltenyi Biotec), cells were subjected to magnetic-activated cell sorting. Enriched CD16++ monocytes and flow-through cells were incubated with CD14 MicroBeads (Miltenyi Biotec), and subjected to magnetic-activated cell sorting again according to the manufacturer's instructions. The two-step procedure resulted in three monocyte subpopulations (see below). All separation steps were performed at 4 °C. After each labeling step, cells were washed with PBS-EDTA (300× *g*, 4 °C, 10 minutes). Negatively and positively selected cells were checked flow cytometrically for their purity and viability after labeling the cells with a phycoerythrin (PE)-conjugated mouse antibovine CD14 monoclonal antibody (AbD Serotec) and adding propidium iodide (PI, 2 μg/mL) respectively. The separation procedure yielded a purity of 94.9 ± 5.4% for classical (CD14++ CD16−), 82.9 ± 4.5% for intermediate (CD14++ CD16+), and 90.3 ± 2.2% for nonclassical (CD14+ CD16++) monocytes. The mean viability was 92% for all subsets.

2.7. Blood plasma stimulation of cells

Separated classical, intermediate, and nonclassical monocyte subpopulations were adjusted to 2×10^5 cells/mL, PMNs were adjusted to 2.2×10^5 cells/mL, and total leukocytes were adjusted to 1.1×10^5 cells/mL in I10 F+ medium containing 50 units heparin. Before adding blood plasma, monocytes were seeded at 1 mL per well into a 24-well plate, and incubated for 24 hours (37 °C, 5% CO_2). Afterward, medium was removed, and substituted with 900 μL fresh medium (50 units heparin). Polymorphonuclear cell and total leukocyte suspension were seeded in duplicates at 900 μL per well into a 24-well plate. To parallel set ups, 100 μL blood plasma from an SCE (n = 5) or a non-SCE animal (n = 5) was added. Blood plasma was obtained from the same animals, which served for the PAXgene analysis. The cells were incubated at 37 °C (5% CO_2 in air) for 3 hours. Medium instead of blood plasma served as negative control. Afterward, supernatants were removed, and cells were lysed using the RNeasy Micro Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

2.8. RNA Preparation and Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

Total ribonucleic acid (RNA) of leukocytes was extracted using the PAXgene Blood RNA Kit (Qiagen) according to the manufacturer's instructions. For RNA extraction of monocyte subpopulations, the RNeasy Plus Micro Kit (Qiagen) was used according to the manufacturer's protocol including the use of QIAshredder (Qiagen) and a DNase digestion step. Integrity and concentration of isolated RNA of both peripheral blood leukocytes and separated cells were analyzed

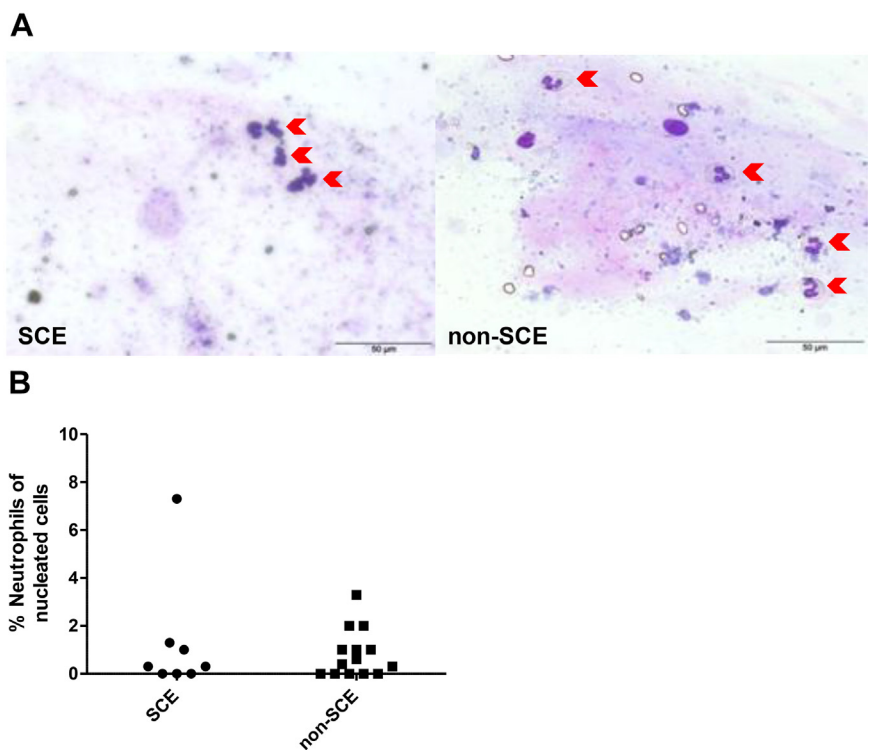

Fig. 1. Polymorphonuclear cells in cytobrush smears. (A) Identification of PMN (arrows) in cytobrush smears of one SCE cow and one non-SCE cow. (B) Semi-quantitative analysis of PMN content in cytobrush smears of SCE cows (n = 8) and non-SCE cows (n = 14). PMN, polymorphonuclear cell; SCE, subclinical endometritis.

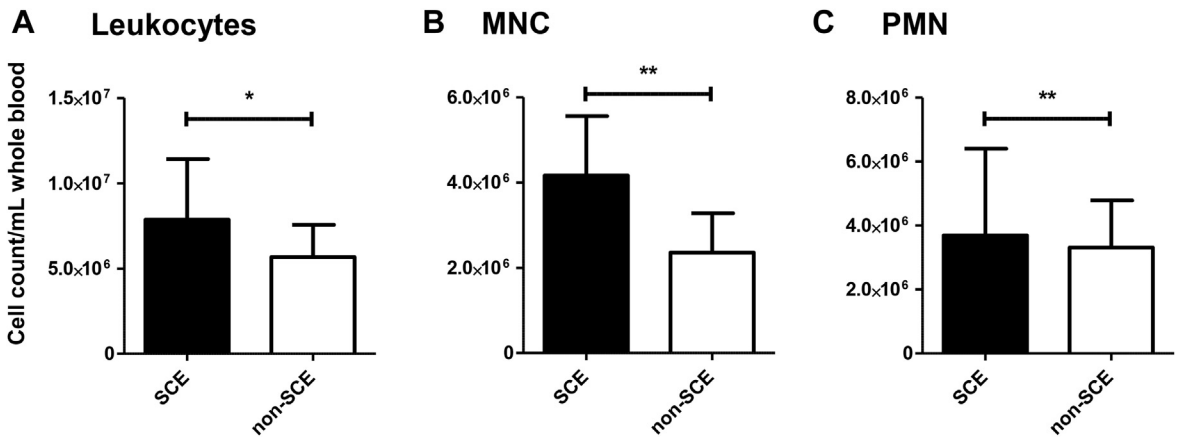

Fig. 2. Blood leukocyte composition. Number of leukocytes (A), mononuclear cells (B), and polymorphonuclear cells (C) were determined in peripheral blood of SCE cows (n = 8) and non-SCE cows (n = 14) at Days 45 to 55 PP. Mean values ± SD; * Denotes P less than 0.05. MNC, mononuclear cells; PMN, polymorphonuclear cells; PP, postpartum; SCE, subclinical endometritis; SD, standard deviation.

using a microfluidic chip-based automated electrophoresis system (Experion, Bio-Rad, Munich, Germany). Experion RNA Standard Sens Chips (peripheral blood leukocytes and monocyte subpopulations) and RNA HighSens Chips (PMN) were used according to the manufacturer's instructions.

With a total-RNA amount of each 140 ng (PAX-extracted peripheral blood leukocytes), 10 ng (RNA of monocyte subpopulations), 400 pg (PMN), and 4 ng (total leukocytes), complementary DNA synthesis was performed using the SuperScript II reverse transcriptase and oligo (dT) nucleotide primers (both Invitrogen, Karlsruhe, Germany) according to the manufacturer's protocol. For PMN, SuperScript III reverse transcriptase and random primers were used because of the low RNA concentration of PMN. RT-qPCR was described recently [18]. In brief, a StepOnePlus Real-Time PCR system (Applied Biosystems, Darmstadt, Germany) was used for thermocycling using the Power SYBR Green PCR Master Mix (Applied Biosystems). Primer sequences and concentrations used for amplification reactions are indicated in Table 2. The quality of amplification was verified by melt curve analysis. A dilution series (10^6–10^2 copies) of complementary DNA subclones was analyzed for each gene simultaneously with the samples, and used for the determination of the relative copy numbers of individual transcripts. The amplification efficiency was calculated from the slope of the standard curve by the formula: $E = 10^{-1}$/slope for each run, and ranged between 90% and 110%.

2.9. Membrane immunofluorescence

Blood from 22 cows was obtained once between Day 45 and 55 PP. Blood collection and separation of total leukocytes was performed as described above. Total leukocyte count was carried out using Türck's solution (diluted 1:10 with whole blood) in a Bürker counting chamber. After separation, cells were suspended in PBS containing 0.5% BSA and 0.01% sodium fluoride (membrane immunofluorescence (MIF) buffer), and finally adjusted at 4×10^6 cells/mL. A total of 100 μL of the diluted cell suspension was pipetted into a 96-well plate for each antibody combination. All experiments were performed in duplicates. The monoclonal antibodies used were bovine-specific or cross-reactive with bovine cells (Table 3).

To phenotype MNCs, the following combinations of antibodies were added to the cells (10 μL of each used antibody): (A) anti-CD2 (FL1) + anti-CD4 (FL2) + anti-CD8 (FL4); (B) anti-CD25 (FL1) + anti-CD4 (FL2); (C) anti-CD2 (FL1) + anti-CD335 (FL2); (D) anti-WC1 (FL1) + anti-CD14 (FL2); (E) anti-MHCII (FL1) + anti-CD21 (FL2) + anti-CD172a (FL3); (F) anti-MHCII (FL1) + anti-CD14 (FL2) + anti-CD172a (FL3); and (G) anti-CD16 (FL1) + anti-CD14 (FL2) + anti-CD172a (FL3). To exclude dead cells from the analysis, fluorochromes staining the DNA of membrane-damaged (dead) cells were added to the set ups. Propidium iodide (detected in the FL3 channel) was added to set ups (A) to (D), and To-Pro-3 (detected in the FL4 channel) was added to set ups (E) to (G).

Previous controls with isotype-matched, irrelevant monoclonal antibodies proved the specificity of the bovine-specific monoclonal antibodies (data not shown). For routine analysis, MIF-buffer instead of antibody dilutions served as negative control. Cells were incubated for 30 minutes on ice in the dark, and washed twice (200× *g*, 4 minutes, 4 °C) in MIF-buffer. Cells were pipetted in 1.5 mL tubes containing 100 μL To-Pro-3 (1 μmol/L) or 100 μL Sheath-PI (2 mg/mL final), and analyzed flow cytometrically (Accuri C6; Becton Dickinson) gating on PI- or To-Pro-3-negative cells.

2.10. Plasma NEFA, Ca^{2+}, BHB, and progesterone concentrations

Serum non-esterified fatty acids (NEFA) concentrations were determined uisng an automated clinical chemistry analyzer (ABX Pentra 400; Horiba, Montpellier, France) using colorimetric enzymatic reactions for NEFA concentration (intraassay coefficient of variation (CV): 6.2%). Analysis of Ca^{2+} was performed using an automated biochemistry analyzer (Cobas-Mira, Hoffmann-La Roche & Co. AG Diagnostics, Basel, Suisse). Serum BHB concentration was determined using spectrophotometric enzymatic analysis (Sigma-Aldrich Diagnostics, Munich, Germany; intraassay CV: 7.1%). Plasma progesterone concentrations were analyzed using a commercially

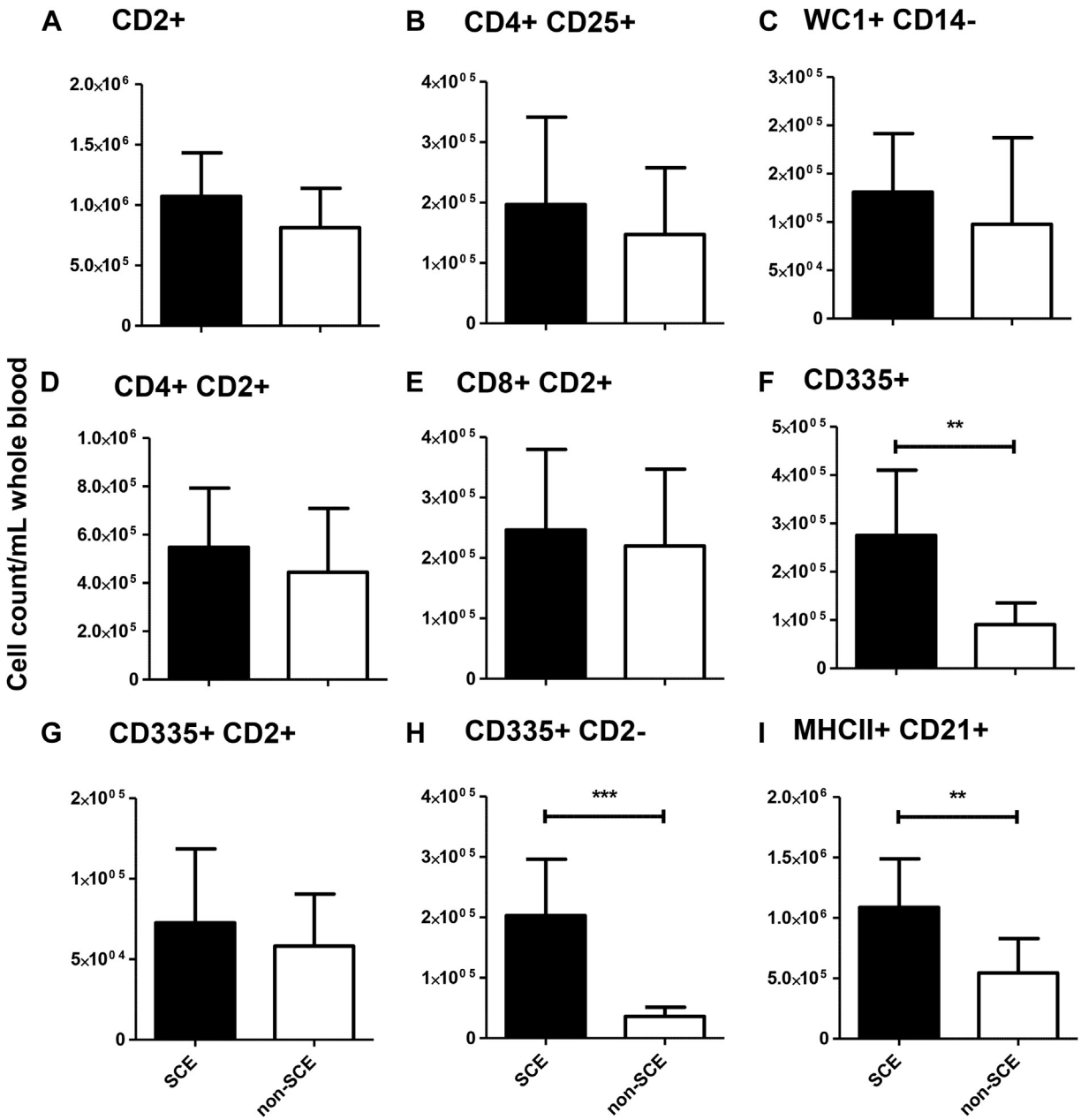

Fig. 3. Blood lymphoid cell composition. Number of T-cells (A), activated T-cells (B), γδ T-cells (C), CD4+ T-cells (D), CD8+ T-cells (E), NK-cells (F), CD2+ NK-cells (G), CD2- NK-cells (H), and B-cells (I) were determined in peripheral blood of SCE cows (n = 8) and non-SCE (n = 14) cows at Days 45 to 55 PP. Mean values ± SD; * Denotes P less than 0.05. PP, postpartum; SCE, subclinical endometritis; SD, standard deviation.

available competitive chemiluminescence immunoassay (IMMULITE, 2000; Siemens Diagnostic Products Corporation, Los Angeles, CA, USA) with an intraassay CV of 6.3% to 8.1% and a lower measuring range less than 0.09 ng/mL [19].

2.11. Statistical analyses

Statistical analysis was performed using the statistical software package SAS (version 9.1; SAS Institute Inc., Cary, NC, USA). Data were tested for normal distribution with the Shapiro–Wilk test. Differences between means were tested with one-factorial ANOVA for repeated measurements and Bonferroni's correction for normally distributed data. Results were considered statistically significant at a P-value of less than 0.05.

3. Results

3.1. Endometrial histology

According to the histologic diagnosis 'nonpurulent endometritis', animals were categorized as 'subclinical endometritis'

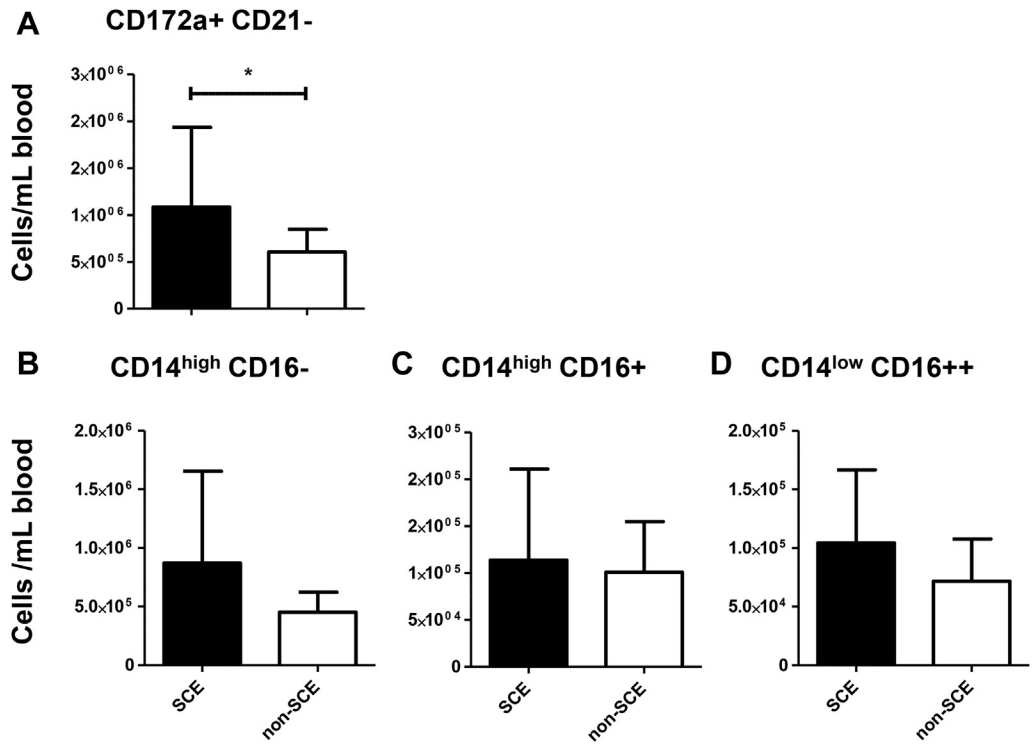

Fig. 4. Blood monocytic cell composition. Numbers of CD172a+ CD21− myeloid cells (A) and monocyte subsets within the CD172a+ cell population (B, C, and D) were determined in peripheral blood of SCE cows (n = 8) and non-SCE (n = 14) cows at Days 45 to 55 PP. (B) $CD14^{high}$ CD16− cells define classical monocytes. (C) $CD14^{high}$ CD16+ cells define intermediate monocytes. (D) $CD14^{low}$ CD16++ defines nonclassical monocytes. Mean values ± SD; * Denotes P less than 0.05. PP, postpartum; SCE, subclinical endometritis; SD, standard deviation.

cows. Nonpurulent endometritis was characterized by higher number of neutrophils in the stratum compactum compared with animals with inconspicuous histologic findings (non-SCE cows; Table 4). Subclinical endometritis cows tended to have more lymphocytes in the stratum compactum compared with non-subclinical animals, whereas the number of tissue macrophages did not differ between both animal groups.

3.2. Endometrial bacteriology

E. coli was isolated in five of eight SCE cows and in two of 14 non-SCE animals. *T. pyogenes* was not isolated from the uterine samples. None of the analyzed samples contained anaerobic bacteria.

3.3. Neutrophilic granulocytes in cytobrush samples

The percentage of neutrophilic granulocytes among nucleated cells in SCE cows ranged between 0% and 7%. Non-SCE animals showed a percentage up to 3.8% (Fig. 1). There was no difference between SCE and non-SCE animals (data not shown).

3.4. Composition of blood leukocytes

Subclinical endometritis cows showed significantly higher blood leukocyte numbers with significantly elevated MNC and PMN numbers (Fig. 2). Among MNCs, SCE cows had significantly ($P < 0.05$) higher numbers of B-cells (Fig. 3I), natural killer (NK) cells (Fig. 3F, H), and CD172a+ cells (Fig. 4A). There was a tendency in SCE cows for higher number of classical (Fig. 4B; $P = 0.07$) and nonclassical monocytes (Fig. 4D; $P = 0.05$), whereas number of intermediate monocytes (Fig. 4C) was similar between the animal groups.

3.5. Blood leukocyte mRNA expression of selected cytokines

Compared with non-SCE animals, leukocytes of SCE cows revealed significantly higher mRNA expression of *TNF, IL12,* and *CXCL8*. Messenger RNA expression of *IL1B, IL10*, and *CCL3* did not differ between the two groups. Peripheral blood leukocytes of both animal groups lacked the expression of *NOS2, ARG1, CXCL1,* and *CCL20* (Fig. 5).

To analyze whether blood plasma of SCE cows contains factors affecting the gene expression of leukocytes, separated cells of healthy cows were used. When separated monocyte subpopulations from healthy cows were incubated with blood plasma of both animal groups, classical and nonclassical monocytes did not differ in their expression (Fig. 6). In contrast, intermediate monocytes showed a significantly enhanced expression of *IL1B, CXCL8,* and *CXCL1* after incubation with plasma from SCE cows (Fig. 6).

Blood plasma of SCE and non-SCE cows did not differ significantly in BHB, calcium (Ca^{2+}), progesterone, and NEFA concentrations (Fig. 7A–D).

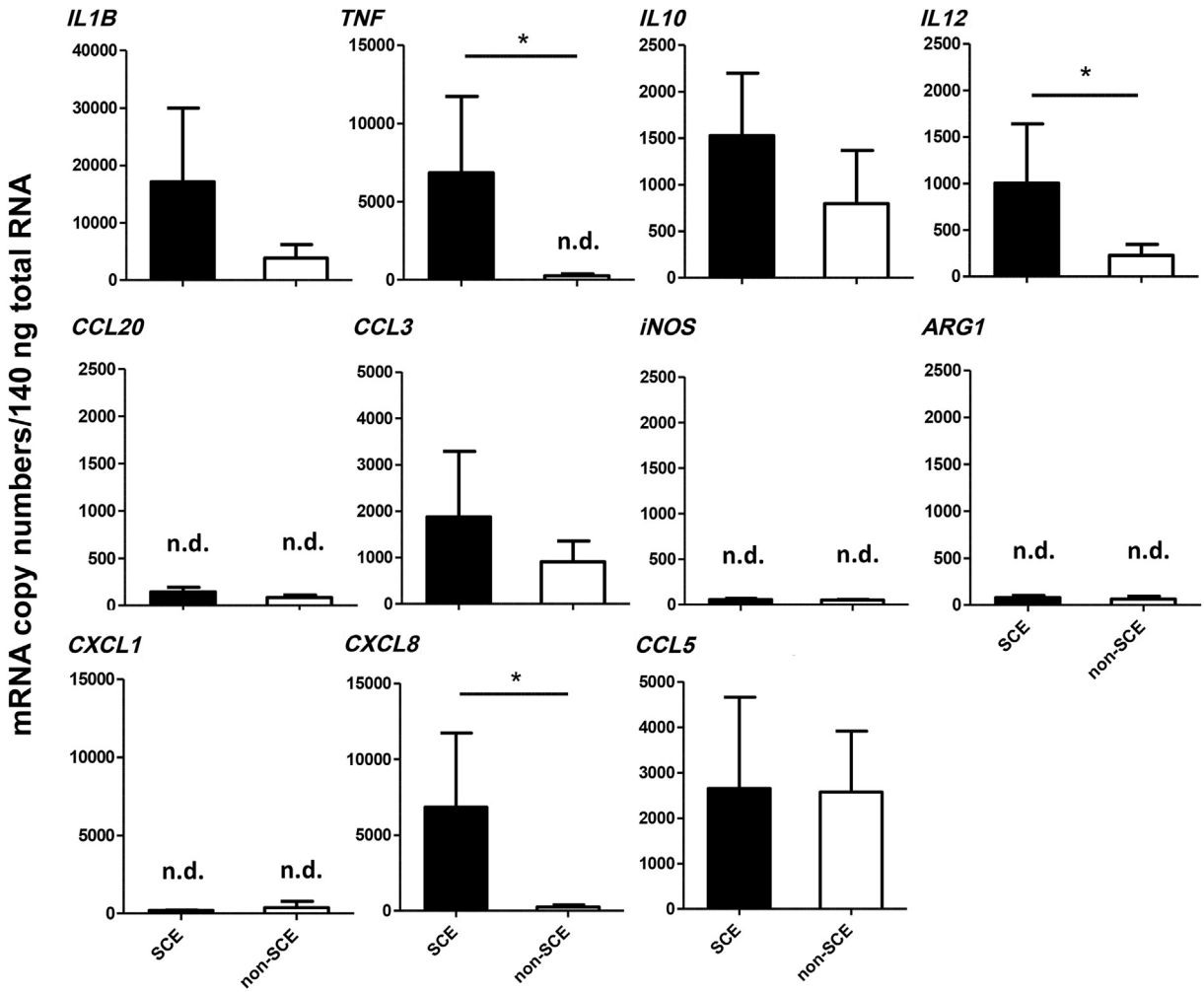

Fig. 5. mRNA expression of selected chemokine and cytokine genes in blood leukocytes. Gene expression was assessed by PAXgene analysis of blood samples from SCE animals (n = 5) and non-SCE animals (n = 6) at Days 45 to 55 PP. mRNA copy numbers were determined by RT-qPCR. Mean values ± SD; * Denotes P less than 0.05; mRNA, messenger RNA; ND, not detectable; PP, postpartum; SCE, subclinical endometritis; SD, standard deviation.

4. Discussion

The objective of this study was to investigate whether SCE is reflected in a changed blood cell composition and an altered gene expression profile of blood leukocytes. Although the cytobrush technique is routinely used to diagnose SCE [9,20–23], it became apparent that histologically proven SCE did not correlate with cytobrush results (Fig. 1A, B). In uterine tissue, diseased animals indeed showed higher number of neutrophils (Fig. 2A) especially in the stratum compactum, which, however, are not necessarily attracted into the uterine lumen, which may explain the negative cytobrush results in such cases. The higher frequency of bacteriologically positive samples (*E. coli*) of cows with SCE is in line with the general statement that uterine pathogens are correlated with inflammation involving the epithelium and stratum compactum, especially with segmented cells at Day 40 PP. [24]. However, some cases of SCE (where *E. coli* was not isolated) could have been driven by sterile inflammatory processes. Given that routine endometritis biopsies are time consuming, and may interfere with subsequent fertility of the animals [25], we raised the question whether the observed tissue differences are reflected in the periphery.

The higher number of total leukocytes, MNCs, and PMNs (Fig. 1A–C) in blood from SCE cows partially confirms previous observations which were obtained in an earlier time frame PP (1–4 weeks PP) [26]. A more detailed analysis of mononuclear cell composition revealed that the increase in MNC was because of a selected raise in several subpopulations, namely B-cells, NK-cells, and CD172a-positive cells (Figs. 2 and 3). Whether their increase is because of their enhanced release from primary or secondary immune organs, or whether their migration into tissues is inhibited and/or delayed is currently unknown. Increased B-cell numbers in case of bovine subclinical infections have not been described, whereas subclinical *Mycobacterium avium* subspecies *paratuberculosis* infection of cows resulted in a drop of blood B-cell percentages ante partum [27]. The increased NK-cell numbers may be because of a rapid

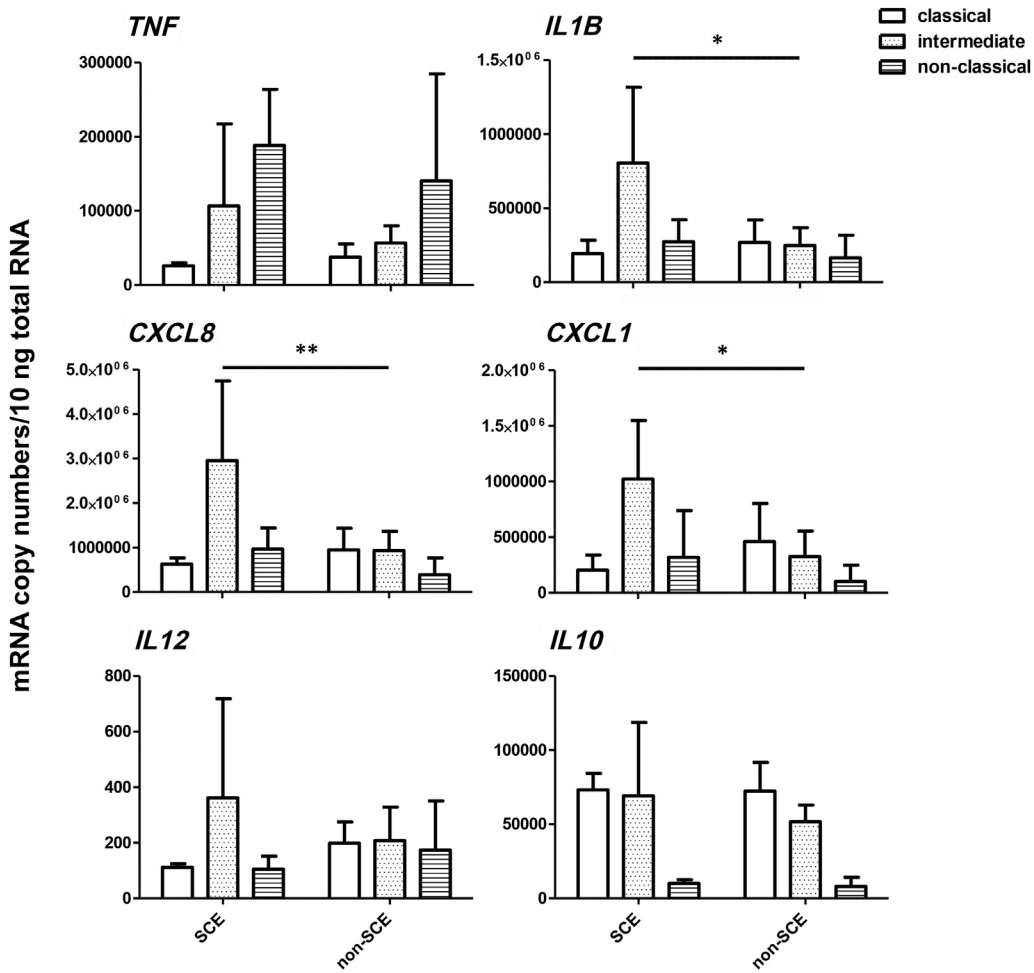

Fig. 6. mRNA expression of selected chemokine and cytokine genes in monocyte subpopulations. Separated classical, intermediate, and nonclassical monocytes of one donor cow were stimulated with blood plasma from SCE animals (n = 5) and non-SCE animals (n = 5). mRNA copy numbers in 10 ng RNA were determined by RT-qPCR. Mean values ± SD; * Denotes P less than 0.05. mRNA, messenger RNA; PP, postpartum; SCE, subclinical endometritis; SD, standard deviation.

mobilization from secondary immune organs as discussed for subclinical *Neospora caninum* infections of calves [28]. The raise in total monocyte numbers was apparently not because of the selective increase of a certain monocyte subset (Fig. 3F–H).

The changed composition of peripheral blood leukocytes was paralleled by their altered gene expression. The significantly upregulated expression of *TNF, CXCL8,* and *IL12* in blood leukocytes is in contrast to previous findings where no differences in the expression of *TNF, IL6, IL1B, CXCL8,* and *IL10* were observed comparing non-stimulated blood monocytes of animals with and without endometritis [15]. The differences may rely on the techniques used. Galvao, et al. [15] assessed gene expression in separated blood monocytes whereas we decided to use the PAXgene system to analyze gene expression in whole blood leukocytes. The latter may avoid the described separation-induced alterations of gene expression, phenotype, and function of blood cells [29–31], which has been validated also for bovine blood [32]. The upregulation of abovementioned genes could both indicate an activation of MNCs and/or neutrophilic granulocytes in peripheral blood of SCE cows. In addition to circulating bacterial products, the most likely stimulating factors are circulating plasma ingredients originating from affected uterine tissue or metabolic factors and/or hormones. For instance, elevated concentrations of BHB during early lactation were found in cows with clinical and SCE compared with healthy cows and high serum BHB concentrations in the first 2 weeks PP [14,33]. However, in the present study we found no significant differences between BHB serum levels of SCE versus uterine healthy cows. The same appeared to be the case for serum Ca^{2+} levels which have been described as lower in SCE animals compared with healthy animals between 20 to 31 days PP [10]. The time frame (45–55 days PP) during which we took samples obviously masked such potential differences between animal groups, and makes it unlikely that these metabolic substances induced blood leukocyte gene expression differences. In addition, we found no significant correlation between age, parity, milk yield or age, and either of the studied parameters (data not shown).

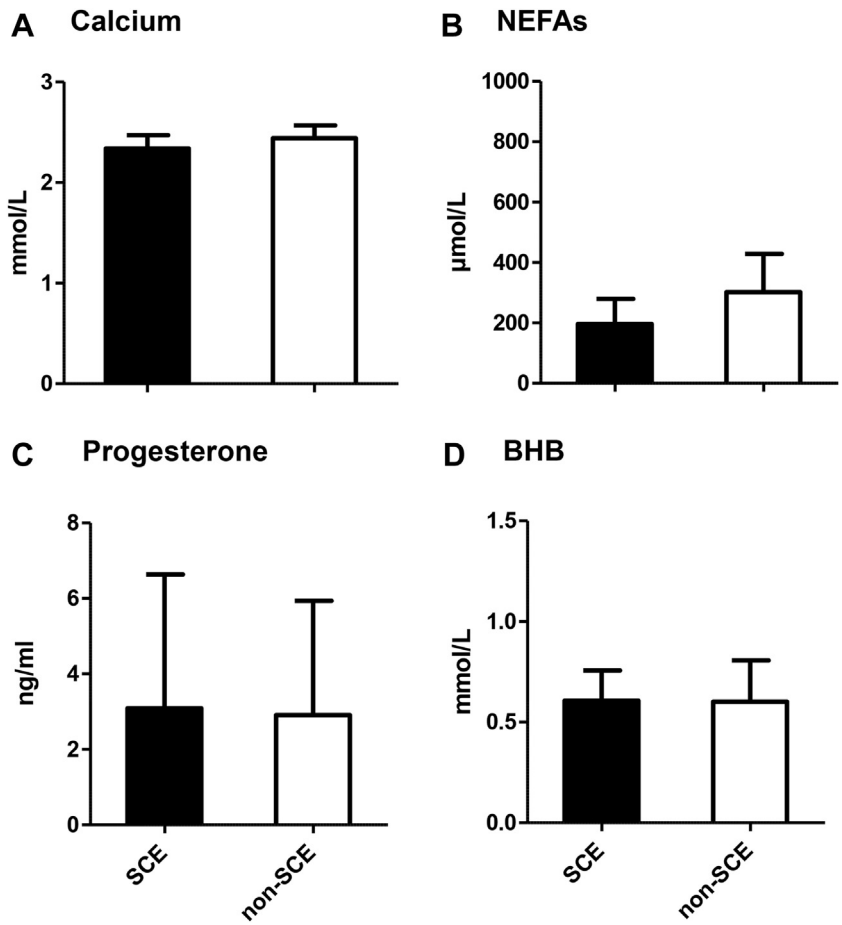

Fig. 7. Serum concentrations of calcium (A), nonesterified fatty acids (B), progesterone (C), and β-hydroxybutyrate (D) in SCE cows (n = 8) and non-SCE animals (n = 14) at Days 45 to 55 PP. Mean values ± SD; * Denotes P less than 0.05. BHB, β-hydroxybutyrate; NEFA, nonesterified fatty acids; PP, postpartum; SCE, subclinical endometritis; SD, standard deviation.

The lack of differences in BHB, NEFAs, calcium, and progesterone concentrations between diseased and healthy animals suggests that other, yet not identified plasma factors might have altered gene expression in blood leukocytes. The assessment of gene expression with the PAXgene system inhibited the identification of the cellular subpopulation responsible for the higher expression of *TNF, CXCL8,* or *IL12.* In the first attempt to address this question, we exchanged blood plasma from non-SCE cows with plasma from SCE cows followed by PAXgene analysis, which did not demonstrate an altered gene expression in normal blood leukocytes. Also, the incubation of separated total blood leukocytes or PMN with plasma of SCE cows did not show the characteristic and significant TNF expression increase in either cellular population (Fig. 5A, B). This lack of effect might have been because of a masking effect of the cell separation [34–36].

Although total leukocytes or PMN from healthy cows showed no altered expression of the analyzed genes after incubation with blood plasma from SCE cows (data not shown), the observed upregulation of *IL12, TNF,* and *CXCL8* after PAXgene analysis might have been because of an activation of distinct myeloid cell subsets. We, therefore, analyzed the response of monocyte subpopulations toward plasma from SCE and non-SCE animals, and observed a significant upregulation for *IL12, IL1B,* and *CXCL8* in intermediate monocytes stimulated with plasma from SCE cows. The significantly higher *IL1B* expression of intermediate monocytes after exposure to SCE plasma is also reflected by the higher *IL1B* expression in blood leukocytes of SCE cows (Figs. 4 and 6). This result proves at least, that circulating factors in blood plasma of SCE animals can alter the baseline expression of selected cellular subsets in blood, although we could not identify the cellular population of SCE cows which expresses higher level of *TNF* (compared with healthy animals). Whether the enhanced level of two neutrophil-attracting chemokines (*CXCL8* and *CXCL1*; Fig. 6) in intermediate monocytes are maintained after their migration into tissues, and whether this accounts for the attraction of neutrophils into uterine tissue warrants further attention.

4.1. Conclusions

Subclinical endometritis of dairy cows is reflected by an altered cellular composition and gene expression pattern in

the peripheral blood. It remains open whether the observed changes are characteristic for this uterine disease. The data emphasize the role of certain monocyte subsets for bovine SCE, and may point the way for analysis of potential candidate genes associated with SCE.

Acknowledgments

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