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TUMOUR-ASSOCIATED MACROPHAGES INFLUENCE CANINE MAMMARY CANCER STEM-LIKE CELLS ENHANCING THEIR PRO-ANGIOGENIC PROPERTIES

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Cancer stem-like cells as cells with ability to self-renewal and potential to differentiate into various types of cells are known to be responsible for tumour initiation, recurrence and drug resistance. Hence a comprehensive research is concentrated on discovering cancer stem-like cells biology and interdependence between them and other cells. The aim of our study was to evaluate the impact of macrophages on cancer stem-like cells in canine mammary carcinomas. As recent studies indicated presence of macrophages in cancer environment stimulates cancer cells into more motile and invasive cells by acquisition of macrophage phenotypes. From two canine mammary tumour cell lines, CMT-U27 and P114 cancer stem-like cells were stained with Sca1, CD44 and EpCAM monoclonal antibodies and isolated. Those cells were next co-cultured with macrophages for 5 days and used for further experiments. Canine Gene Expression Microarray revealed 29 different expressed transcripts in cancer stem-like cells co-cultured with macrophages compared to those in mono-culture. Up-regulation of C-C motif chemokine 2 was considered as the most interesting for further investigation. Additionally, those cells showed overexpression of genes involved in non-canonical Wnt pathway. The results of 3D tubule formation in endothelial cells induced by cancer stem-like cells co-cultured with macrophages compared to cancer stem-like cells from mono-cultures and with addition of Recombinant Canine CCL2/MCP-1 revealed the same stimulating effect. Based on those results we can conclude that macrophages have an impact on cancer stem-like cells increasing secretion of pro-angiogenic factors.

Key words: *cancer stem-like cells, tumour-associated macrophages, monocyte chemoattractant protein-1, metastasis, macrophage, canine mammary tumour*

INTRODUCTION

Solid tumours consist of heterogeneous populations of cells. The small subpopulation of cancer stem-like cells (CSLCs) is thought to be an underlying mechanism of tumour initiation and dissemination. Since the first discovery of CSLCs in non-hematologic tumours by Al-Hajj *et al.* in 2003, research has been focused on their identification in broad spectrum of solid tumours as well as on investigation of their unique characteristics (1). However, recent studies of tumour microenvironment showed that investigators should not concentrate only on individual subpopulation of cells but explore the molecular interactions occurring inside the solid tumour mass (2). Diverse type of cells e.g. fibroblast, tumour-associated macrophages (TAMs), neuroendocrine cells or the blood and lymphatic vascular network create complex structure of tumour microenvironment. Currently, special attention is paid to immune cells which seem to play a crucial role in tumour progression.

TAMs represent the majority of immune cells infiltrating solid tumours and particularly mammary tumours (3). Significant infiltration of tumour by TAMs is correlated with drug resistance and poor prognosis in women with breast cancer (4-6). Different TAMs subsets show diverse pro-tumoural effect depending on their location. Situated in the area of basement membrane breakdown TAMs enhance epithelial-mesenchymal transition (EMT) and local invasion of tumour cells, whereas TAMs located in perinecrotic site are responsible for angiogenesis switch enabling tumour growth and spread (7). Noy and Pollard showed that macrophages are also involved in creating pre-metastatic site and by secreting various cytokines and chemokines they act as a chemoattractant for tumour cells encouraging them to extravasate (3).

Chemokines are a family of small (7–15 kDa) regulatory proteins (8) and currently great attention is paid on their role in cancer progression and metastasis (9-11). Particularly, CCL2 (monocyte chemoattractant protein-1) is known to be involved in cross talk between cancer cells and tumour microenvironment, enhancing infiltration of TAMs (12, 13). In breast cancer, increased production of CCL2 by cancer cells has been shown to correlate with lymph node metastasis and shorter overall survival (14, 15) while the inhibition of CCL2/CCR2 axis in MDA-MB-231-bearing mice significantly inhibited metastasis (13). However, nothing is known so far about the influence of CCL2 and TAMs on canine CSLCs.

The aim of this study was to investigate the influence of TAMs and CCL2 on CSLCs biology. The study was conducted using the canine mammary cancer model. Molecular and etiological

similarities between breast cancer and canine mammary tumour indicated that dog could be a good model for human studies. Therefore, we presume that presented results can provide mutual benefits both for human and veterinary medicine (16, 17).

MATERIALS AND METHODS

Cell culture

In this study two canine mammary cancer cell lines were used. An anaplastic carcinoma cell line (P114) was kindly donated by Dr. Gerard Rutteman (Utrecht University, The Netherlands) and a simple carcinoma cell line (CMT-U27) was established by Prof. Eva Hellmen (Swedish University of Agricultural Sciences, Sweden). Information about these cell lines has been widely published before (18-20). Cells were cultured in RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated foetal bovine serum, penicillin-streptomycin (50 IU/mL), and fungizone (2.5 mg/mL; Sigma Aldrich, USA) at 37°C in a humidified 5% CO_2 incubator. The human umbilical venous endothelial cell line (HUVEC) was purchased from Life Technologies (USA). HUVECs were cultured in 200PRF medium (Gibco, USA) with Low Serum Growth Supplement (LSGS) (Gibco) in a standard culture conditions. For the 3D tubule formation assays, early passages were used (P2-P4).

Canine macrophages were obtained from anti-coagulated whole blood from blood bank and subjected to mononuclear cell separation using Accuspin System-Histopaque 1077 (Sigma Aldrich), according to the manufacturer's protocol. To selectively extract monocytes, the received pellet of cells was suspended in Monocyte Attachment Medium (Promo Cell) and plated in a number of 15 millions per T-75 flask for 1.5 hours incubation at 37°C in a humidified 5% CO_2 incubator. Cells, that attached to the flask were then suspended in M2 Macrophage generation Medium DXF (Promo Cell) and incubated for 6 days without medium change in order to obtain M2 phenotype of macrophages present in the cancer stem-like cells niche in the tumour mass (21). After that time fresh amount of M2 Macrophage generation Medium DXF constituting 50% of whole amount of medium was added. After 10 days of incubation macrophages were obtained and used within 3 weeks for further experiments.

Cell staining and flow cytometry sorting

To distinguish the CSLCs subpopulation in the P114 and CMT-U27 cell lines a panel of three antibodies was used. In brief, we used the rat anti-mouse Ly-6A/E (Sca-1) FITC-conjugated (BD Bioscience USA) (22), the rat anti-mouse CD326 (EpCAM) PE-conjugated (eBioscience USA) and the mouse anti-canine CD44 APC-conjugated (EXBIO Czech Republic) antibodies. Staining was performed using 20 µl, 4.5 µl and 4.5 µl of antibodies, respectively per 2×10^6 cells for one hour in 4°C. Next, cells were analysed and sorted using FACSAria II (BD Bioscience) into two tubes in order to obtain canine mammary CSLCs (Sca-1^{pos}/EpCAM^{pos}/CD44^{pos}) and tumour cells (Sca-1^{neg}/EpCAM^{neg}/CD44^{neg}). FACS sorting isolated a 97 – 99% pure population on the post-sort, as assessed using BD FACS Diva 5.0 software. Unstained CMT-U27 and P114 cells were used as a negative control.

Co-culture

Sca-1^{neg}/EpCAM^{neg}/CD44^{neg} and Sca-1^{pos}/EpCAM^{pos}/CD44^{pos} cells were seeded on 24-well plates in triplets 300 cells in each well. In a similar manner they were co-cultured with macrophages using transwell system. Neoplastic cells were cultured in the bottom chamber and macrophages were seeded into transwell with 0.4-µm polyester, porous membrane which, allowed cells not to contact with each other at the ratio 1:5. The co-cultures were maintained for 5 days.

For evaluation of vascular endothelial growth factor (VEGF) and integrin subunit beta 3 (IGTB3) expression Sca-1^{pos}/EpCAM^{pos}/CD44^{pos} cells were seeded on 24-well plates in three repetitions in number of 700 cells on each well. In a similar manner they were co-cultured with Recombinant Canine CCL2/MCP-1 (R&D Systems) added 0.3 µl per well. Co-cultures were maintained for 24 hours.

Tissue samples

For this study 40 tumour samples were collected from female dogs during a standard mastectomy in the Department of Small Animal Diseases with Clinic, Faculty of Veterinary Medicine, Warsaw University of Life Sciences and two private veterinary clinics in Warsaw. All bitches underwent standard clinical examination before the procedure, including: patient history, complete physical examination, documentation of tumour characteristics, haematological examination, serum biochemistry profile and thoracic radiographs in three projections (right and left lateral and dorsoventral). Owners of the animals gave written or oral permission to use tissues of their dogs for scientific studies. Samples were obtained within study approved by the III Local Ethical Committee (approval no. 8/2012, 17.01.2012) of the Warsaw University of Life Sciences. The remaining 18 samples were kindly donated by Prof. Robert Klopfleisch from Freie Universitaet Berlin (Germany). Samples obtained during mastectomy were fixed in 10% neutral buffered formalin and routinely embedded in paraffin. The 3 micrometre (µm) thick sections from each representative paraffin block were set on slides and stained with haematoxylin and eosin (H & E) for further histological evaluation. Part of each tumour sample was also immersed in RNA Later (Invitrogen), frozen and stored at –80°C. All tumours were diagnosed according to the WHO criteria for canine mammary neoplasms at Department of Pathomorphology, Faculty of Veterinary Medicine, Warsaw University of Life Sciences and validated in blind manner by two independent pathologists (23). Depending of the grade of malignancy we obtained four groups consisted of either nine or ten tumours: benign, grade 1, grade 2, and grade 3. To the analysis we also included nine tumour samples from female dogs with metastatic cancer and ten samples of the normal mammary gland. Histological characteristics of samples are presented in *Table 1*.

RNA isolation, validation, amplification, reverse transcription, labelling and hybridization

Total-RNA (t-RNA) was isolated from the sorted cancer cells (mono-cultured or co-cultured with macrophages) using miRNeasy Mini Kit (QIAGEN) according to the manufacturers protocol. The quantity of t-RNA was measured using a NanoDrop instrument (NanoDrop Technologies, USA), and final RNA quality and integrity were evaluated using BioAnalyzer (Agilent, USA).

The Quick Amp Labeling Kit (Agilent, USA) was used to amplify and label target RNA to generate complementary RNA (cRNA) for oligo microarrays used in gene expression profiling and other downstream analyses. The gene expression for cancer stem like cells from CMT-U27 and P114, grown as co-culture with macrophages, was compared against the gene expression of the same CSLCs grown as a mono-culture (gene expression in CMT-U27 CSLCs grown as a co-culture with macrophages was compared to gene expression in CMT-U27 CSLCs grown as a mono-culture; gene expression in P114 CSLCs grown as a co-culture with macrophages was compared to gene expression in

Table 1. Histological classification of tumour samples.
Empty space - the factor does not concern the sample (for control samples), x - the factor does not concern the sample (for benign tumours).

Control/tumour type	Histological type	Grade o malignancy	Metastasis	No	Σ
Control				10	10
Benign	benign mixed tumor	x	no	4	9
	simple adenoma	x	no	2	
	complex adenoma	x	no	2	
	atypical papilloma	x	no	1	
Malignant	simple carcinoma	I	no	6	10
	complex carcinoma	I	no	3	
	fibrosarcoma	I	no	1	
	simple carcinoma	II	no	7	10
	complex carcinoma	II	no	2	
	mucinous carcinoma	II	no	1	
	simple carcinoma	III	no	5	10
	complex carcinoma	III	no	5	
	simple carcinoma	III	yes	3	9
	complex carcinoma	III	yes	4	
	squamous cell carcinoma	III	yes	1	
	lipid-rich carcinoma	III	yes	1	

P114 CSLCs grown as a mono-culture). Each sample was examined in a dye-swap to eliminate the effect of label factor. Thus, each biological condition was labelled once by Cy3 and once by Cy5. Taking the average of two labelled arrays, the dye effect on any particular gene was cancelled. The hybridization was performed with canine-specific AMADID Release GE 4 × 44 K microarrays (Agilent, USA) using Gene Expression Hybridization Kit (Agilent, USA) according to the manufacturer's protocol.

Signal detection, quantification and analysis

Acquisition and analysis of the hybridization intensities were performed using DNA microarray scanner (Agilent, USA). Then, the results were extracted using Agilent's Feature Extraction Software with normalization and robust statistical analyses. Results were analysed for statistical purposes using Feature Extraction and Gene Spring software (Agilent, USA). The unpaired t-test with Benjamin-Hochberg FDR < 5% (false discovery rate) correction was applied (with p value cut-off < 0.05). For further analysis we hierarchically clustered the genes and chose only those with values within upper and lower cut-off (100.00 and 20.00, respectively) in each of the slide. We analysed only genes that were regulated in all the examined samples within the group (that is: in both CMT-U27 and P114 CSLCs grown as a co-culture with macrophages and in both CMT-U27 and P114 CSLCs grown as a mono-culture) whose expression changed at least 1.5 IN each of examined slide. In this experimental model we examined each of the sample in duplicate (dye-swap), whereas significant genes were chosen from two biological repetitions (CMT-U27 and P114 cell line). The area of the analyses covered in this publication has been deposited in NCBI's Gene Expression Omnibus and is accessible *via* GEO Series accession number GSE73230.

RNA isolation and reverse transcription from tumour samples

Total-RNA was isolated from tumour pieces and normal mammary gland tissue (ϕ 1 cm) using miRNeasy Mini Kit (QIAGEN) according to the manufacturer's protocol. Before extraction each sample was washed with RNase Away Reagent (Ambion) and disrupted in Tissue Lyser LT (QIAGEN, Germany) at 50 Hz for 30 min. The quantity of obtained t-RNA was measured using a NanoDrop instrument (NanoDrop Technologies, USA), and final RNA quality and integrity were evaluated using BioAnalyzer (Agilent, USA). Synthesis of cDNA was performed using Transcriptor First Strand cDNA Synthesis Kit (Roche) and run in Eppendorf Master Cycler Personal thermal cycler (Eppendorf, Germany).

Real-time RT-PCR

Sequences of key genes were obtained from NCBI database. Primers were designed using Primer3 software (free online access) and were verified using Oligo Calculator (free online access) and Primer-Blast (NCBI database). Primer sequences are listed in *Table 2*. RPS19 gene was used for a normalisation of target gene expression as an internal control (24, 25). RT-PCR was performed using a fluorogenic Lightcycle Fast Strand DNA SYBR Green kit (Roche) according to the manufacturers protocol on Strategene Mx3005P qPCR System (Agilent Technologies). Data was analysed using the comparative Ct method (25). The experiment was conducted in triplets.

Tubule formation assay (angiogenesis in vitro assay)

In this study 3D tubule formation by human endothelial cells (HUVECs) was stimulated using canine mammary CSLCs grown as mono-culture (pre-treated or not with CCL2) and co-culture. Lower chamber of 96-transwell system plate was previously coated with 14 µl Growth-Factor Reduced Matrigel Matrix (BD Biosciences) per well and allowed to solidify in 37°C for 30 minutes. Next, HUVECs were plated 1.4×10^4 per well according to the manufacturers protocol. Into the upper chamber 4000 CSLCs isolated from each cancer cell line were seeded per well according to the following pattern: CSLCs grown as a co-culture with macrophages; CSLCs grown as a mono-culture; CSLCs grown as a mono-culture with addition of 0.3 µl Recombinant Canine CCL2/MCP-1 (R&D Systems) into each well. As a positive inducer control we used LSGS-supplemented Medium 200PRF, and a negative control, LSGS-supplemented medium with 30 µM Suramin (Sigma-Aldrich). After incubating at 37°C for 6 hours, each well was visualised using phase contrast microscope to evaluate HUVECs reorganisation into 3D vessel tubes. The quantification of tube formation in each environment was calculated using ImageJ software.

Table 3. Up/down-regulated genes in canine mammary cancer stem-like cells grown as co-culture with macrophages.
The list of up- (↑) and down- (↓) regulated genes in canine mammary cancer stem-like cells grown as co-culture with macrophages. The unpaired t-test with Benjamin- Hochberg FDR < 5% (false discovery rate) correction (with P value cut-off < 0.05) (Gene Spring, Agilent, USA) and further PANTHER analysis were conducted.

No	Fold change	P value	Gene symbol	Gene name
1	↑3.52	0.004	GRAP	PREDICTED: Canis lupus familiaris GRB2-related adaptor protein
2	↑2.51	0.007	CCL2	Canis lupus familiaris chemokine (C-C motif) ligand 2
3	↑2.34	0.04	IFI44L	interferon-induced protein 44-like
4	↑2.26	0.01	RASD1	RAS, dexamethasone-induced 1
5	↑2.08	0.01	RARRES3	retinoic acid receptor responder (tazarotene induced) 3
6	↑2.07	0.04	LTB	Canis lupus familiaris lymphotoxin beta
7	↑1.84	0.009	TNFRSF14	PREDICTED: Canis familiaris similar to TNF receptor superfamily member 14 precursor
8	↑1.83	0.01	KIT	Canis lupus familiaris v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog
9	↑1.75	0.004	NFKBIA	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha
10	↑1.70	0.03	IKZF4	IKAROS family zinc finger 4 (Eos)
11	↑1.60	0.04	LOC477558	Q6IPV7_HUMAN (Q6IPV7) Glutathione S-transferase theta 2
12	↑1.59	0.02	EP400	E1A-binding protein p400
13	↑1.59	0.02	MCOLN1	mucolipin 1
14	↑1.54	0.02	AMPD1	adenosine monophosphate deaminase 1
15	↑1.53	0.02	CRYBA4	Canis lupus familiaris crystallin, beta A4
16	↑1.52	0.04	RPL24	ribosomal protein L24
17	↑1.51	0.04	ASB11	ankyrin repeat and SOCS box containing 11
18	↓1.50	0.04	ENKUR	enkurin, TRPC channel interacting protein
19	↓1.50	0.04	GNAO1	guanine nucleotide binding protein , alpha activating activity polypeptide O
20	↓1.54	0.03	CRYBB2	crystallin, beta B3
21	↓1.54	0.04	GULP1	GULP, engulfment adaptor PTB domain containing 1
22	↓1.54	0.04	LIPG	lipase, endothelial
23	↓1.60	0.04	LOC610944	PREDICTED: Canis familiaris hypothetical protein
24	↓1.66	0.008	MME	membrane metallo-endopeptidase
25	↓1.69	0.01	OPRD1	opioid receptor, delta 1
26	↓1.76	0.03	PLEKHB1	pleckstrin homology domain containing, family B (evectins) member 1
27	↓1.85	0.04	SESN1	sestrin 1
28	↓1.94	0.03	SNN	stannin
29	↓2.20	0.02	cOR52Z4	PREDICTED: Canis lupus familiaris cOR52Z4 olfactory receptor family 52 subfamily Z-like

Invasion assay

The BD BioCoat Matrigel™ 96-multiwell tumour invasion system (BD Biosciences, USA) pre-coated with BD Matrigel matrix was used according to the manufacturer's protocol. The insert plates were prepared by rehydrating the BD Matrigel Matrix layer with phosphate buffered saline (PBS) for two hours at 37°C. The rehydration solution was then carefully removed

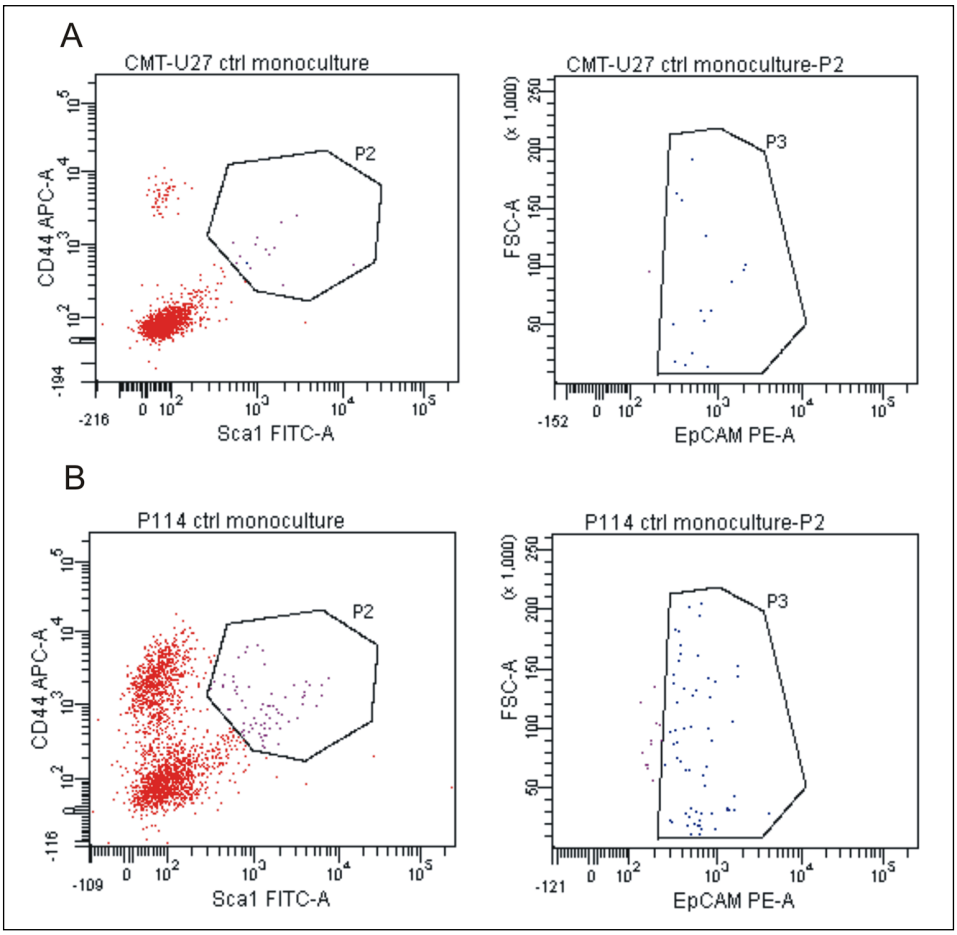

Fig. 1. Percentage of isolated canine mammary CSLCs. Representative plots showing canine mammary cancer stem-like cells (Sca-1^{pos}/EpCAM^{pos}/CD44^{pos}) isolated from CMT-U27 (A) and P114 (B) cell lines. Left plot presents Sca1 vs. CD44 staining and gating of the double positive population of cells. This population is presented on the right plot (FSC vs. EpCAM) showing that majority of the Sca-1^{pos}/CD44^{pos} cells is also EpCAM^{pos}.

Table 2. Primers used for real-time qPCR.
Primers sequences used in this study and their annealing optimal temperature and time. The mRNA sequences of key genes were obtained from NCBI database. Primers were designed using PRIMER3 software (free on-line access) and checked using Oligo Calculator (free on-line access) and Primer-Blast (NCBI database). RPS19 genes were used as non-regulated reference genes for normalization of target gene expression.

Gene symbol	**Forward primer**	**Reverse primer**	**Optimum annealing temp.**(°C)	**Optimum annealing time** (sec)
RPS19	CCTTCCTCAAAAAGTCTGGG	GTTCTCATCGTAGGGAGCAAG	61	10
Wnt5a	TGCCACTTGTATCAGGACCA	GCTGCCTATCTGCATGACC	61	10
Wnt2	GCATCCTTTCCCTTCCTTTC	TCAGCTGGAGTTGTGTTTGC	60	8
Wif1	TCTGTGTCACTCCTGGGTTC	ACCTCCATTTCGACAGGGTT	58	10
Rac1	GGGAGACGGAGCTGTAGGTA	ACATCTGTTTGCGGATAGGAT	56	10
Dvl2	TCACCATCCCTAACGCCTTT	GCACTGCTCAGAAAACGTGA	58	10
Fzd10	ACTTCCTTCATCCTGTCCGG	TAACAGGCGATCACACAGGT	62	10
CCL2	CTCCAGTCACCTGCTGCTAT	CACAGCTTCTTTGGGACACT	60	4
CCR2	AGAATGCGATGTGGACAGCA	CGACCAGTGGATGGCTTCTT	61	12
VEGF	TGCAGATTATGCGGATCAAA	TTTCTTGCGCTTTCGTTTTT	56	7
ITGB3	CGGCGTCGGAGTGTCCAA	TTCCTTCAGGTTACAGCGGG	61	6

and CSLCs and CSLCs previously co-cultured with macrophages (ratio 6:1) and sorted out using FACS AriaII cell sorter (Becton Dickinson) were plated into upper chamber at 500 cells per insert. Cells were seeded in serum-free RPMI-1640 medium whereas bottom chamber contained medium with chemoattractant (20% FBS). Assay plates were incubated for 22 hours at standard culture conditions. Afterwards, medium from apical chamber was removed and the whole insert system transferred to the second 96-well plate coated with 2.5 µg/ml Calcein AM in Hanks' Balanced Salt solution (HBSS). Plates were incubated for 1 hour at standard culturing conditions. The fluorescence of invaded cells was measured at excitation wavelength 485 nm and emission wavelength 530 nm using a fluorescence plate reader with bottom reading capabilities, Infinite 200 PRO Tecan (Tecan, Switzerland). The experiment was repeated three times.

Statistical analysis

The analysis for statistical purposes was conducted using Prism version 5.00 software (GraphPad Software, USA). The one-way ANOVA, ANOVA + Tukey HSD (Honestly Significant Difference) post-hoc test and *t*-test were applied. The differences were considered as significant when $P < 0.05$ or highly significant when $P < 0.01$ or $P < 0.001$.

RESULTS

Canine mammary neoplastic cell lines comprise of cancer stem-like cells subpopulation of cells

CSLCs (Sca-1^{pos}/EpCAM^{pos}/CD44^{pos}) constituted from 1.16 to 3.75 % of CMT-U27 and P114 cell lines respectively (*Fig. 1*). The self-renewing potential of Sca-1^{pos} CSLCs expressing higher level of EpCAM and CD44 expression as well as the proliferation trend has been previously presented by our group (22).

Gene expression in canine mammary cancer stem-like cells co-cultured with tumour-associated macrophages

The microarray experiment revealed differences in gene expression between canine mammary CSLCs as a co-culture with TAMS and CSLCs grown in mono-culture. The un-paired t-test Benjamin-Hochberg with P-value cut off < 0.05 showed 29 significantly deregulated genes (fold change > 1.5) (*Table 3*) amongst which 17 were up-regulated and 12 were down-regulated. The analysis using PANTHER classification system showed that identified up-regulated genes were mostly involved in inflammation mediated by chemokine and cytokine and apoptosis signalling pathway whereas down-regulated were associated with encephalin release and heterotrimeric G-protein signalling

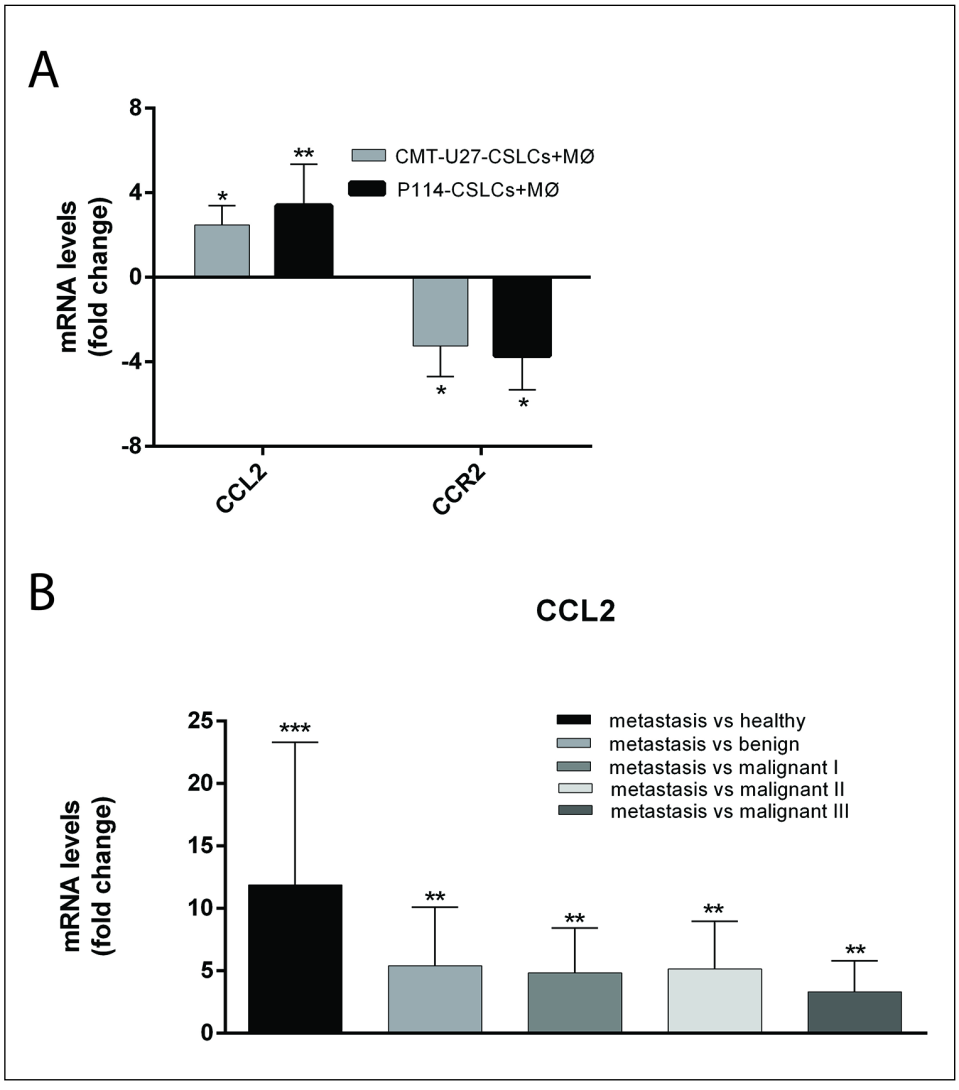

Fig. 2. CCL2 and CCR2 gene expression in canine mammary cancer stem-like cells co-culture with macrophages; CCL2 gene expression in metastatic canine mammary tumours. (A) Graph showing the expression of CCL2 and CCR2 genes in canine mammary cancer stem-like cells co-cultured with macrophages (CMT-U27-CSLCs+Mϕ, P114-CSLCs+Mϕ). The data was calculated relative to the control, mono-culture of CSLCs. (B) Graph showing the expression of CCL2 gene in metastatic canine mammary tumour samples compared to healthy tissue, benign and malignant tumours of grade 1, 2 and 3. Data is presented as a fold change. All bars represent means ± S.D.

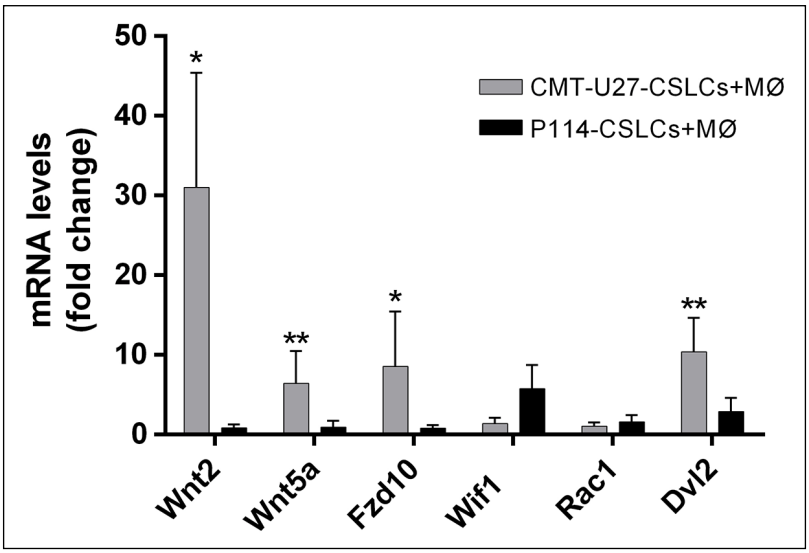

Fig. 3. The expression of selected Wnt pathway genes in cancer stem-like cells co-cultured with macrophages. Graph showing Dvl2, Rac1, Wnt2, Wif1, Fdz10 and Wnt5a genes expression in canine mammary cancer stem-like cells co-culture with macrophages (CMT-U27-CSLCs+Mϕ, P114-CSLCs+Mϕ). The data was calculated relative to the control (expression of these genes in CSLCs grown as mono-culture) and is presented as a fold change. All bars represent means ± S.D.

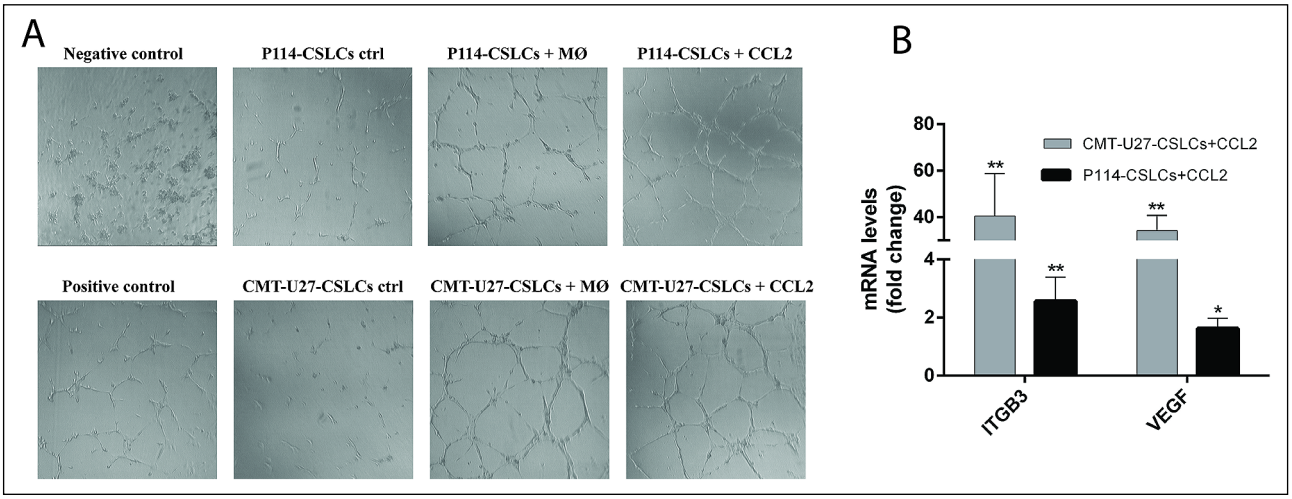

Fig. 4. Vessel formation (3D) by HUVEC; ITGB3 and VEGF gene expression in canine mammary cancer stem-like cells treated with CCL2. Representative pictures showing 3D vessel formation by HUVECs due to stimulation by co-culture with canine mammary cancer stem-like cells (P114-CSLCs, CMT-U27-CSLCs), canine mammary cancer stem-like cells co-cultured with macrophages (P114-CSLCs+Mϕ, CMT-U27-CSLCs+Mϕ) and canine mammary cancer stem-like cells treated with CCL2 (P114-CSLCs+CCL2, CMT-U27-CSLCs+CCL2) (A). Graph showing ITGB3 and VEGF gene expression in canine mammary cancer stem-like cells treated with CCL2 (P114-CSLCs+CCL2, CMT-U27-CSLCs+CCL2). The data was calculated relative to untreated control CSLCs and is presented as a fold change. All bars represent means ± S.D (B).

pathway-Gq alpha and Go alpha mediated pathway. Biological processes analysis showed that deregulated genes were mainly involved in cellular, metabolic and apoptotic processes. Due to known involvement of CCL2 in the interactions between cancer cells and TAMs this gene was chosen for the purposes of microarray experiment validation. CCL2 was up-regulated (fold change = 2.5) in CSLCs due to co-culture with TAMs. We observed 2.34 ($P < 0.05$) increase of expression in CMT-U27 and 3.54 increase of expression in P114 ($P < 0.001$). Interestingly, the expression of CCR2 (C-C chemokine receptor type 2), the receptor for CCL2 was 3.03 ($P < 0.05$) fold lower in CMT-U27 and 3.85 ($P < 0.05$) in P114 CSLCs co-cultured with TAMs compare to mono-culture (*Fig. 2A*).

Increased expression of CCL2 is associated with higher grade of malignancy and metastasis

Forty-eight tumour samples collected from female dogs with CMT were used to evaluate CCL2 expression in various stages of cancer. We studied benign (n = 9), malignant: grade 1 (n = 10), grade 2 (n = 10), grade 3 (n = 10) and metastatic tumours (n = 9). Control group consisted of 10 normal mammary tissues. In all groups we showed increased expression of CCL2 with statistically significant overexpression in metastatic tumours. Expression of CCL2 in metastasis tumour samples was higher 11.64 fold ($P < 0.001$), 5.59 fold ($P < 0.01$), 4.7 fold ($P < 0.01$), 5.0 fold ($P < 0.01$) and 3.23 fold ($P < 0.01$) compared to healthy tissue, benign and malignant tumours of grade 1, 2 and 3, respectively (*Fig. 2B*).

Macrophages induced higher expression of genes involved in non-canonical Wnt signalling in cancer stem-like cells

Comparison of the expression of genes involved in non-canonical Wnt pathway in CSLCs grown as mono-culture and co-culture with macrophages revealed significant overexpression of Wnt2 ($P < 0.01$), Wnt5a ($P < 0.01$), Fzd10 ($P < 0.05$) and Dvl2 ($P < 0.01$) due to co-culture with TAMs in CMT-U27 cell line. Despite expressions of Wif1, Rac1 and Dvl2

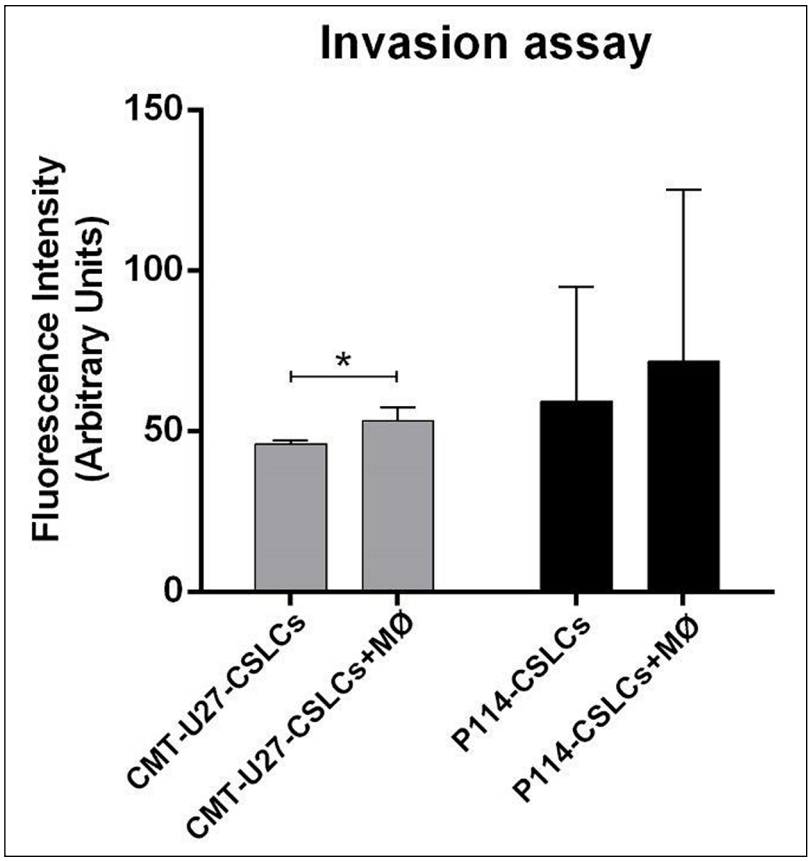

Fig. 5. Changes in invasion capacity of cancer stem-like cells upon co-culture with macrophages. The graph showing fluorescence intensity related to invasion of CSLCs grown as mono-culture (control) and as a co-culture with macrophages (CSLCs+MQ). All bars represent means ± S.D.

were higher in P114 CSLCs co-cultured with macrophages these differences were not significant (*Fig. 3*).

Co-culture of cancer stem-like cells with tumour-associated macrophages and CCL2 enhance angiogenesis in vitro

The angiogenesis in vitro assay revealed that CSLCs grown in co-culture with TAMs stimulated HUVECs to form 3D vessels compared to the positive control. Similar results were obtained by HUVECs induced by CSLCs treated with CCL2. The average total number of nets comprised of 36.9 and 40.5 for CMT-U27 and 44.1 and 54 for P114 respectively. In contrary, stimulation by CSLCs grown in mono-culture showed no impact on tubule formation as observed in the negative control (*Fig. 4A*).

Cancer stem like cells co-cultured with CCL2 show increased expression of VEGF and ITGB3

RT-PCR analysis confirmed higher expression of genes associated with angiogenesis in CMT-U27 and P114 CSLCs treated with CCL2 compared to control. We observed 1.64 fold ($P < 0.05$) increased expression of VEGF in P114 and 34.06 fold ($P < 0.01$) increased expression of VEGF in CMT-U27. A similar trend was observed in ITGB3 (down-stream signalling gene in VEGF-dependent pathway) expression with 40.13 fold ($P < 0.01$) increased expression in CMT-U27 CSLCs and 2.56 fold ($P < 0.01$) in P114 CSLCs treated with CCL2 compared to control (*Fig. 4B*).

Macrophages promote invasiveness of cancer stem-like cells

The invasion assay showed higher invasive capacity of CSLCs co-cultured for 5 days with macrophages compared to control CSLCs (grown as mono-culture). In CMT-U27 cell line, the mean fluorescence intensity related to invasiveness of CSLCs was 45 whereas to the CSLCs co-cultured with macrophages was 53.3 ($P < 0.05$). Macrophages did not influence invasiveness of CSLCs isolated from P114 cell line (*Fig. 5*).

DISCUSSION

Tumour-associated macrophages (TAMs) are the most abundant population of immune cells in tumour microenvironment (3). High infiltration of these cells has been associated with progression and metastasis in cancer (26). Research performed by Krol *et al.* (2012) on molecular interplay between canine mammary cancer cells and TAMs showed extensive genes deregulation in cancer cells upon co-culture with TAMs. The majority of those genes were involved in macrophage activation as well as cell motility and led to the acquisition of invasive phenotype by cancer cells (27). In this article, investigating the same CMT cell lines we presented the influence of TAMs on subpopulation of cancer stem-like cells and discuss their role in tumour progression.

Previously, higher expression of a potent macrophages attracting factor CCL2 have been indicated in cancer cells co-cultured with TAMs (27). Consistently, our microarray analysis revealed overexpression of this gene in CSLCs grown with macrophages. Whether CCL2 secreted by CSLCs affects TAMs only still remains under discussion. However, the increased expression of CCL2 receptor (CCR2) has been reported in circulating monocytes, macrophages and TAMs (13, 28). Interestingly, we found that CSLCs co-cultured with macrophages showed lower expression of CCR2 compared to mono-culture. This cross talk however needs further investigation in CSLCs isolated from tumour samples as already

established immortal population cells may not reflect adequate tumour complexity (29). Based on our results we suggest that TAMs induce expression of chemo-attractants in CSLCs enhancing TAMs infiltration into the tumour mass.

The positive correlation between increased number of TAMs and breast cancer progression has been shown before by many authors (30, 31). Higher density of TAMs within tumour mass significantly reduces both relapse-free and overall survival (32). In this study, we showed that macrophages might promote invasion capacity in CSLCs. Moreover, our investigation of CCL2 expression in canine mammary cancer tissues confirmed that its level was correlated with higher grade of tumour malignancy and metastasis. These results are in accordance with our previous studies showing that in metastatic tumours the number of macrophages were significantly higher than in other tumours (33, 34). Increased macrophage infiltration and CCL2 expression in metastatic tumours may also be related to more efficient blood vessel formation in these tumours. The CCL2 not only stimulates angiogenesis but also helps cancer cells to intravasate (35, 36). In the current study, the angiogenesis *in vitro* assay was performed to demonstrate that TAMs enhance pro-angiogenic properties of CSLCs. The results showed similar 3D vessel formation by HUVECs stimulated by CSLCs grown in co-culture with macrophages to those stimulated by CCL2. As reported by Stamatovic *et al.* formation of new blood vessels can be regulated by CCL2 through β_3-integrin (37). Notably, the expression of β_3-integrin as well as VEGF, up-stream in the signaling pathway, the widely known angiogenesis inducer and apoptosis inhibitor, were significantly higher in CSLCs treated with CCL2 (38). These results suggested that CCL2 might be the key factor in angiogenesis induced by TAMs infiltration.

The Wnt signalling pathway is known to be critical in embryonic development as well as in adult tissue self-renewal. Disturbances mostly in advanced age have been shown to cause pathological stages including cancer (39). Although our microarray analysis did not revealed changes in Wnt pathway gene expression, previous studies in canine cancer cells demonstrated that TAMs enhance non-canonical Wnt signalling activity in them (27). We observed similar effect on Wnt pathway in CSLCs co-cultured with TAMs. The expression of Wnt2, Wnt5a, Fzd10 and Dvl2 was significantly higher in CMT-U27 cell line upon co-culture. Significant differences in level of expression of selected genes between these cell lines might be caused by their distinct histopathological origin (22, 27). Recently, research has been focused at Wnt pathway association with angiogenesis. Above 19 secreted Wnt glycoproteins promoting role of Wnt1, Wnt3a, Wnt5a and Wnt2 in endothelial proliferation has been demonstrated (40-43). Remarkably, Wnt2 and Wnt5a overexpression was also reported in TAMs population during progression of human colorectal cancer (44). Our study revealed up-regulation of the non-canonical Wnt pathway in CSLCs grown in co-culture with TAMs. Stimulation of new tubules formation in angiogenesis assay might therefore also be triggered by overexpression of the indicated genes.

In conclusion, results presented in this study support the hypothesis of TAMs multidirectional role in tumour progression. Our data indicate that TAMs enhance CSLCs to overexpress the macrophage attracting factor CCL2 recruiting macrophages into the tumour mass. Then, TAMs stimulate CSLCs to promote angiogenesis.

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