Cholangiocarcinoma stem-like subset shapes tumor-initiating niche by educating associated macrophages

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Supplementary materials and methods

Mice

The Humanitas Animal Care and Research Advisory Committee approved housing and experimental animal procedures. NOD/SCID/IL2rγ null (NSG) mice (Jackson Laboratory) were used in all experiments. The mice were used at 6 weeks of age, in accordance with the guidelines and with the approval of the local Experimental Animal Committee [16].

Sphere Assay

To calculate sphere-forming efficiency, CCA cells were single-cell sorted into 96-well plates coated with an Ultra-Low Attachment Surface (Corning) using a FACS Aria (BD Biosciences). The cells were grown in anchoring-independent conditions with selective serum-free DMEM/F12 medium supplemented with 1X B27 supplement minus vitamin A (Life Technologies), human recombinant EGF (hrEGF) (R&D System) (20 ng/mL), and bFGF (R&D System) (20 ng/mL). After 10 days, the spheres formed were counted, and the sphere-forming efficiency (SFE) was calculated by dividing the number of spheres formed by the original number of single cells seeded and expressed as a percentage. An Olympus IX81 confocal microscope in a closed humidified chamber at 37°C in a CO_2-enriched atmosphere with a 20x objective was used to obtain pictures of sphere morphology [11, 15].

Additionally, large-scale sphere cultures were established plating 1.8 x 10^3 cells/cm^2 into poly (2-hydroxyethyl methacrylate) (poly-HEMA [Sigma Aldrich]) coated dishes. To prevent cell aggregation, 1% methylcellulose (R&D System) was added to the culture medium.

Monocyte Isolation and Macrophage Differentiation

Human monocytes were obtained from healthy blood donor buffy coats by gradient centrifugation using a Ficoll gradient (GE Healthcare) and further purified from peripheral blood mononuclear cells (PBMC) by magnetic-activated cell sorting (MACS) using CD14 microbeads (Miltenyi Biotech). After MACS purification, two fractions were obtained: the CD14+ fraction and the eluate (composed of all PBMC CD14- cells). The purity of CD14+ cells was >90%.

Macrophages and tumor-conditioned macrophages were obtained by culturing 1 x 10^6/mL monocytes for 6 days in RPMI 1640 with 10% FBS supplemented with 100 ng/mL of recombinant human M-CSF (Peprotech) or in presence of 30% CCA sphere- or monolayer-CM. M1 and M2 polarized macrophages were obtained by culturing 1 x 10^6/mL monocytes for 6 days in RPMI 1640 with 10% FBS supplemented with 100 ng/mL of recombinant M-CSF. M1 cells were polarized by stimulating M-CSF macrophages for 24 h with LPS (100 ng/mL) (Peprotech) and IFN-γ (20 ng/mL) (Peprotech). M2 cells were polarized by stimulating human M-CSF macrophages for 24 h with IL4 20 ng/mL (Peprotech).

Cell Cultures

HUCCT1, CCLP1 and SG231 cells, from intrahepatic bile ducts, were a kind gift from Dr. A.J. Demetris (University of Pittsburgh, Pittsburgh,PA) and were cultured as described (21, 22). The primary CCA4 cell line (mucinous intrahepatic CCA, female 50years old) was a kind gift from Dr. D. Alvaro (University La Sapienza, Rome, Italy) [25]. Human immortalized non-malignant cholangiocyte cell line H69 from Dr. G. J. Gores, Mayo Clinic, Rochester, MN and primary human intrahepatic cholangiocyte cell line HiBEC from ScienCell. Experiments were performed using cells between passages 2 and 8. All cell lines were incubated at 37°C in a humidified chamber supplemented with 5% CO_2 [22].

Preparation of Conditioned Medium (CM)

To collect tumor-CM, 1.8 x 10^3 CCA cells/cm^2 were grown as spheres for 7 days, while 5.5 x 10^3 CCA cells/cm^2 were grown as monolayer for 5 days. Then, the medium was discarded, the plates were rinsed two times with saline solution, and the cells were incubated with fresh medium for 24 h. CM was harvested and clarified by centrifugation, and the supernatants were filtered at 0.20 µm and used fresh or stored at -80°C [35].

ELISA

The concentration of 37 molecules (cytokines, chemokines, interleukins and other factors described in the Results section) in the sphere- or monolayer-CM was measured using commercially available ELISA kits according to the manufacturer's instructions (R&D Systems). RPMI 1640 with 10% FBS, DMEM with 10% FBS, or serum-free DMEM/F12 was used as a negative control. A seven-point standard curve was used to calculate the concentration of the molecules. Elisa expression values were log2 transformed and log Fold-change values were created by subtracting control from treated log2 intensities, for each molecule and each cell line analyzed. When both treated and control Elisa expressions were null, then "NA" was used. Molecules with "NA" in all conditions and cell lines were excluded from further analysis. A matrix with four columns (cell lines) and 23 rows (proteins with at least one log Fold-change available) was loaded in TMeV (http://www.tm4.org/) [27] and hierarchical clustering with "Euclidean distance" as similarity metrics and "average" as linkage method was applied to cluster proteins and cells according to their expression profiles upon treatment.

Lymphocyte Reaction Assay

Human peripheral blood lymphocytes (PBL) were obtained from healthy blood donor buffy coats by gradient centrifugation using a Ficoll gradient (GE Healthcare) and further purified from peripheral blood mononuclear cells (PBMC) by magnetic-activated cell sorting (MACS) using CD4+ T cell isolation kit (Miltenyi Biotech). CD4+ were collected and labeled with CFSE 2 µM (eBioscience) for 10 min at 37°C. Cells were washed extensively and 2 x 10^5 cells/well were cultured in round-bottomed 96-well plates in RPMI-1640 with 10% FBS with 30% of sphere- and monolayer-CM for 5 days. As a negative control we used CD4+ non stimulated. At day 5, cells were collected and stained against CD4 using the described conjugated antibody.

Flow Cytometry

A total of 1 x 10^5 purified monocytes, *in vitro*-differentiated macrophages, CD4+ or patient-derived PBMCs were washed and resuspended in FACS buffer (phosphate buffered saline [PBS] plus 2% FBS). The anti-human antibodies used included anti-CD14-PerCP/Cy5.5, anti-CD45-PB, anti-CD4-PE and anti-CD3-APC (Biolegend); anti-CD206-PE, anti-CD56-APC, anti-CD68-PE, and anti-HLA-DR-PerCP/Cy5.5 (BD Biosciences); and anti-CD115-PE (R&D System). Dead cells were excluded using the LIVE/DEAD Fixable Aqua or Violet Dead Cell Stain Kit (Life Technologies). The cells were stained for 20 min at 4°C and detected using an LSRFortessa (BD Biosciences). A fluorescence minus one (FMO) sample, containing all antibodies except the one of interest, was used as a negative control. Data analysis was performed with the FlowJo software (FlowJo, LLC).

Tube Formation Assay

The tube formation assay was carried out as described [40], with slightly modifications. In brief, 96-well plates were coated with 70 µl of pre-thawed growth factor-reduced Matrigel (BD

Biosciences). The plate was then kept at 37 °C for 1 h to allow the matrix solution to gel. 1.5 x 10^4 HUVEC cells were resuspended in the respective conditioned media, added to each well and incubated at 37°C for 24 h. Images were acquired under an inverted microscope. Pro-angiogenic activity was quantified by measuring the number of tube structures formed between discrete endothelial cells in each well. Each experiment was performed in triplicate.

Gene Expression

The total RNA was extracted with the RNeasy kit (Qiagen) according to the manufacturer's instructions. The RNA concentration and quality were measured using an optical Nanodrop ND1000 spectrophotometer (Thermo Scientific).

Total RNA (500 ng) from the CCA spheres or monolayer was reverse transcribed into cDNA using an RT² First Strand Kit (SabBioscience, Qiagen) according to the manufacturer's instructions. RT2 Profiler™ human cancer stem cell (PAHS-1776Z), human embryonic stem cell (PAHS-081Y) and human liver cancer cell (PAHS-133Z) 384-well PCR arrays (SabBioscience, Qiagen) were performed for quantitative PCR using the ABI ViiA™ 7 System (Applied Biosystems) with the following cycling conditions: 10 min at 95°C, 40 cycles of 15 s at 95°C followed by 1 min at 60°C and a final infinite 4°C hold. Data were centered and normalized, and hierarchical clustering of genes/samples using centered correlation metrics with complete linkage was performed. The grouping of the genes from top to bottom is given for each heatmap.

Total RNA (500 ng) from tumor-conditioned macrophages was transcribed with a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). PCR was performed on the cDNA with gene-specific primer pairs (see table below). The qPCR human primers for CD163

were purchased from Qiagen. Changes in the mRNA expression level of target genes were detected using FAST SYBR-Green PCR Master Mix and the 7900HT Fast Real Time PCR System (Applied Biosystems). The cycling conditions consisted of 20 s at 95°C, 40 cycles of 1 s at 95°C followed by 20 s at 60°C, and a final infinite 4°C hold. The mRNA levels of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were used for normalization. The fold difference ($2^{-\Delta\Delta Ct}$) was calculated using the ΔCt of monolayer-conditioned macrophages as a control. All reactions were performed in triplicate.

List of Primers

Gene	Primer Forward	Primer Reverse
CD80	GGGAAAGTGTACGCCCTGTA	GCTACTTCTGTGCCCACCAT
CXCL9	TTTTCCTCTTGGGCATCATC	TCAATTTTCTCGCAGGAAGG
CXCL10	AGCCAATTTTGTCCACGTGT	TGATGGCCTTCGATTCTGGA
ALOX15	CTTGCTCTGACCACACCAGA	GCTGGGGCCAAACTATATGA
CCL18	GTGGAATCTGCCAGGAGGTA	CCCAGCTCACTCTGACCACT
CCL17	CACCCCAGACTCCTGACTGT	CCCTCACTGTGGCTCTTCTT
CCL5	TATTCCTCGGACACCACACC	ACACACTTGGCGGTTCTTTC
OA	TCCGACGAAACCTTCCTCAA	TCCCCGAAGCTCCACTTCTA
OPN	TTGCAGTGATTTGCTTTTGC	GTCATGGCTTTCGTTGGACT
AD10	CTGGCCAACCTATTTGTGGAA	GACCTTGACTTGGACTGCACTG
AD17	GAAGGCCAGGAGGCGATTA	CGGGCACTCACTGCTATTACC
FN	ATCAACCTTGCTCCTGACAG	GTCTCAGTAGCATCTGTCAC
MMP2	TTGACGGTAAGGACGGACTC	ACTTGCAGTACTCCCCATCG
EDA	CGGGATCCAACATTGATCGCCCTAAAGG	TCCCCCGGGTGTGGACTGGGTTCCAATC
GAPDH	GATCATCAGCAATGCCTCCT	TGTGGTCATGAGTCCTCCCA
CD13	CAGTGACACGACGATTCTCC	CCTGTTTCCTCGTTGTCCTT
LGR5	CTTCCAACCTCAGCGTCTTC	TTTCCCGCAAGACGTAACTC
VEGFR1	ATTTGTGATTTTGGCCTTGC	CAGGCTCATGAACTTGAAAGC
VEGFR2	GTGACCAACATGGAGTCGTG	CCAGAGATTCCATGCCACTT
VEGFR3	CATCCAGCTGTTGCCCAGG	GAGCCACTCGACGCTGATGAA
IL4R	GCGTCTCCGACTACATGAGC	GGTTGCTCCAGGTCAGCAGC
IL13Ra1	AGGATGACAAACTCTGGAG	CTCAAGGTCACAGTGAAGG
IL13Ra2	ATACCTTTGGGACTTATTCC	TGAACATTTGGCCATGACTG
CD44	TTATCAGGAGACCAAGACAC	ATCAGCCATTCTGGAATTTG
ITGA5	AAGCTTGGATTCTTCAAACG	TCCTTTTCAGTAGAATGAGGG
ITGB3	CTCCGGCCAGATGATTC	TCCTTCATGGAGTAAGACAG
SDC4	CAGGGTCTGGGAGCCAAGT	GCACAGTGCTGGACATTGACA
SDC1	TACTAATTTGCCCCCTGAAG	GATATCTTGCAAAGCACCTG

Cell Migration and Invasion Assay

CD14+ cells (1.5 x 10^6/well) were seeded into the upper chamber of 6-well transwell supports with a membrane with an 8 µm pore size (Corning) in serum-free RPMI 1640 (migration assay). Tumor-conditioned macrophages (9 x 10^4/well) were placed into the upper chamber of a 24-well BioCoat™ Matrigel® Invasion Chamber with a membrane with a 0.4 µm pore size (BD Biosciences) in RPMI 1640 supplemented with 10% FBS (invasion assay). The cells were allowed to migrate or invade toward the lower compartment of the system, which contained either 30% tumor-CM or RPMI 1640 alone. After 6 h (migration assay) or 18 h (invasion assay) of incubation at 37°C, the cells that had not penetrated the filter were removed with cotton swabs, and cells that had migrated to the lower surface of the filter were fixed with methanol, stained with Diff Quick solutions, and photographed with an Olympus BX51 microscope with a 20x objective. The values for migration/invasion were expressed as the average number of migrated/invaded cells per microscopic field (20x) over five fields. Each experiment was performed in triplicate [36].

Adhesion Assay

Tumor-conditioned macrophages (15 x 10^4/dish) were seeded onto 60 mm dishes coated with FN (Millipore) in RPMI 1640 supplemented with 10% FBS and allowed to adhere to the surface. After 10 min, non-adherent cells were discarded, and cells that had adhered to the surface were fixed with methanol, stained with Diff Quick solutions, and photographed with an Olympus BX51 microscope with a 10x objective. The values for adhesion were expressed as the average number of adherent cells per microscopic field (10x) over five fields. Each experiment was performed in triplicate [36].

Drug Treatment and MTT Assay

The cell viability was measured with the 3-(4,5-dimethylthiazolo-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich) assay. CCA cell lines were cultured as spheres or monolayers in 96-well plates and then exposed to the following drugs or relative vehicles: cisplatin (0-112 µM) (Sigma-Aldrich) for 24 h, oxaliplatin (0-500 µM) (Sigma-Aldrich) for 24 h, 5-Fluoruracil [5-FU (0-40 mM)] (Sigma-Aldrich) for 48 h and gemcitabine (0-1000 µM) (Axxora) for 72 h. The optimal drug concentrations were determined by calculating the IC50 in CCA-monolayer cultures. Five replicates were performed for each condition. At the end of the treatments, 20 µL of 5 mg/mL MTT solution was added to each well, and the plates were incubated for 3 h at 37°C. Next, 100 µL of 100% DMSO per well was added to solubilize the precipitate, and the absorbance of each well was measured with a VersaMax microplate reader (Molecular Devices Corporation) at a wavelength of 570 nm. The percent viability was calculated as follows: (sample OD570-blank control OD570)/(control OD570- blank control OD570) x100 .

Immunohistochemistry

Frozen tissues form SPH and MON derived tumors were cut at 8 µm put on SuperFrost slides and stored at -80°C. For Sirius red staining, tissues were fixed in 4% neutral buffer formalin for 5 min. Tissues were placed in 0.1% of Sirius red solution for 1 hour and then washed under tap water for 20 min. Haematoxylin were used for counterstaining and then mounted with Eukitt (Sigma-Aldrich). For immunohistochemistry tissues were fixed with 4% paraformaldehyde for 15 minutes, endogenous peroxidase was blocked with H2O2 0.03% for 5 min, then unspecific binding sites were blocked with Rodent Block M (Biocare Medical) for F4/80 and αSMA while PBS+2%BSA was used for CD31 for 30 minutes. Rat anti mouse F4/80 (MCA497g, clone Cl:A3-1, AbD Serotec) were diluted 1:400, goat anti mouse CD31 (AF3628, R&D System) were

diluted 1:1000 and mouse anti mouse αSMA (A 2547, clone 1A4, Sigma-Aldrich) were diluted 1:200 and then incubated for 1 hour at room temperature. As secondary antibody Rat on mouse HRP polymer (RT517 L, Biocare Medical) were used for F4/80, goat on rodent (GHP516 L, Biocare Medical) were used for CD31, mouse on mouse HRP (MM620H, Biocare Medical) were used for αSMA. Reactions were developed with 3,3'-Diaminobenzidine -DAB- (Biocare Medical) and counterstained with haematoxylin. Slides were then dehydrated through an ascending scale of alcohols and xylene, and mounted with Eukitt.

Human CCA specimens were fixed in 10% formalin before being processed in paraffin. Hematoxylin and eosin-stained sections from each tissue block were evaluated to obtain the diagnosis and the pathological T stage. A representative block for each case was selected for immunohistochemical analysis with CD68 and CD163 markers. The primary antibodies included anti-CD163 (Rabbit Monoclonal clone EPR4521 dilution 1:250, Epitomics), anti-CD68 (Mouse Monoclonal clone PGM-1 dilution 1:60), anti-CD44 (555476, 1:50 dilution; BD Pharmingen), anti-EPCAM (clone VU-1D9; 1:1000 dilution; Merck Millipore). The chromogen diaminobenzidine (DAB) was used for CD163, CD44 and EPCAM whereas FAST RED was used for CD68. The tissue sections were counterstained with Mayer's hematoxylin. The negative control was performed by substituting the primary antibody with non-immune serum at the same concentration. The control sections were treated in parallel with the samples.

The ratio between CD163 intensity values evaluated in tumor front and intralesion were calculated and log transformed. One sided Student t-test was then applied to log ratios in order to compare G1 to G2-G3 and such test yielded a *p-value* of 0.006, with average G2-G3 values bigger than average G1 values. Fisher Exact test was applied as well, to test any association between grade and CD163 log ratios and this gave a *p-value* of 0.05, with 1.1 used as cut-off.

In Vivo Study

In vivo experiments were performed in accordance with the guidelines and approval of the local Experimental Animal Committee. The following materials were used: 1,000 sphere- or monolayer-derived cells to measure the tumorigenic potential of CCA cells and 1,000 monolayer-derived cells and 300 macrophages for TAMs + CCA monolayer co-injection experiments. The cells were dissociated into single-cell suspensions and resuspended in 100 µL of DMEM and reduced Matrigel growth factor (BD Bioscience) (1:1), and the mixture was s.c. injected into the right flank of 6-week-old NSG mice. Tumor growth was monitored, and the diameter of the growing tumors was measured in millimeters every week using a caliper. The animals were sacrificed when the xenografts reached 2.0 cm in diameter [15, 16, 26]. The limiting dilution analysis was performed by sorting 1,000/100/10 alive cells from dissociated CCA monolayer and spheres. Web-based Extreme Limiting Dilution Analysis (ELDA) statistical software (http://bioinf.wehi.edu.au/software/limdil/index.html) was used [28].

TAM Isolation from Human CCA Resections

Human TAMs were isolated from fresh CCA samples as previously described with slight modifications [16]. Briefly, the tissues were minced into small (1 to 2 mm) pieces and digested with PBS containing 2 mg/ml collagenase D (Roche) at 37°C for 2 h. The resulting suspension was sequentially filtered through sterile 100- and 70-µm nylon cell strainers. The cells were then centrifuged at 1,400 RPM for 30 min with Ficoll (GE Healthcare). CD14+ macrophages were further isolated by MACS using CD14 Microbeads (Miltenyi Biotec) according to the manufacturer's instructions.

Statistical analysis, CCA patient data base

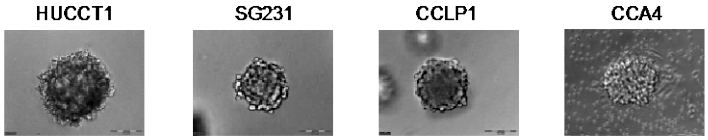
Graphpad Prism v5 was used for data analysis. The error bars represent 1+/- SEM. The *p value* was calculated with Student's *t* test. The statistical significance and *p-value* are shown when relevant.

The GSE26566 series matrix containing expression values from Illumina humanRef-8 v2.0 expression beadchip Array [transcript (gene) version] of 104 CCA samples was downloaded from GEO [1]. Differences in gene expression of specific genes of tumor tissue (T) versus surrounding liver (SL) and CCA epithelial compartment (EPI) versus stromal component (S) were evaluated.

Pearson correlation between gene pairs was calculated using R and the "cortest" function, yielding correlation coefficients and *p-values*.

Supplementary Fig. 1

A



B

Half Maximal Inhibitory Concentration (IC50) %					
		Drugs			
		Oxaliplatin (μM)	5-Fluorouracil (mM)	Cisplatin (μM)	Gemcitabine (μM)
HUCCT1	MON	1318.3 ± 48	44.7 ± 16	37.2 ± 7	10.5 ± 2
	SPH	1698.2 ± 37	158.5 ± 19	1412.5 ± 72	251.2 ± 22
	p value	*0.0000*	*0.0000*	*0.0000*	*0.0000*
SG231	MON	44.7 ± 10	61.7 ± 14	79.4 ± 11	18.6 ± 4
	SPH	245.5 ± 17	416.9 ± 31	1174.9 ± 105	95499.3 ± 230
	p value	*0.0000*	*0.0000*	*0.0000*	*0.0000*
CCLP1	MON	91.2 ± 21	30.9 ± 12	13.3 ± 5	0.01 ± 0.005
	SPH	1659.6 ± 143	380.2 ± 67	309.0 ± 23	251188.6 ± 209
	p value	*0.0000*	*0.0001*	*0.0000*	*0.0000*
CCA4	MON	43.7 ±	0.7 ±	26.3 ±	147.9 ±
	SPH	1584.8 ±	229.1 ±	31.6 ±	181970.1 ±
	p value	*0.0000*	*0.0000*	*0.1676*	*0.0000*

The data are mean ± SEM (n=5, *p value* versus MON by Student t test)

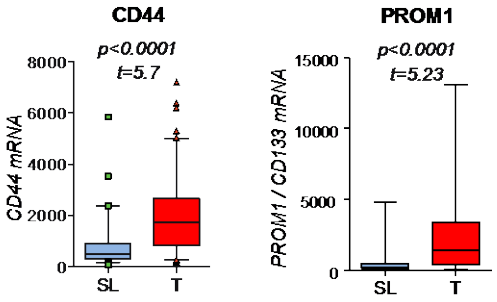
Supplementary Fig. 1 CCA-derived spheres: morphology and drug-response

A) Representative images of CCA-derived spheres.

B) IC50s of CCA monolayers and spheres assayed for sensitivity to oxaliplatin, 5-fluorouracil, cisplatin and gemcitabine (n=5). Data are mean ± SEM (*p value* versus MON by Student t test).

Supplementary Fig. 2

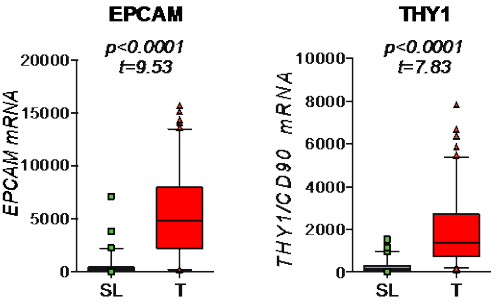
Up-regulated genes in CCA-spheres			
Cell Lines	**Liver Cancer**	**CSC**	**ESC**
HUCCT1	61% (51/84)	69% (58/84)	27% (23/84)
SG231	42% (35/84)	48% (40/84)	37% (31/84)
CCLP1	75% (63/84)	55% (46/84)	24% (20/84)
CCA4	77% (65/84)	64% (54/84)	19% (16/84)



B

Cell Line	Marker	MON MFI ± SEM	SPH MFI ± SEM	*p value*
HUCCT1	CD13	624.7 ± 58	3359.8 ± 97	*0.0000*
	CD44	1070.9 ± 75	3852.9 ± 103	*0.0002*
SG231	CD13	60.6 ± 14	102.5 ± 17	*0.0459*
	EPCAM	334.5 ± 52	930.6 ± 34	*0.0005*
CCLP1	CD13	35.3 ± 6	51.2 ± 9	*p>0.05*
	THY1	2.5 ± 0.4	29.2 ± 8	*0.0287*
CCA4	CD13	7 ± 2	49.9 ± 7	*0.0095*
	THY1	66.6 ± 14	196.9 ± 26	*0.0047*

The data are mean ± SEM (n=3, *p value* versus MON by Student t test)



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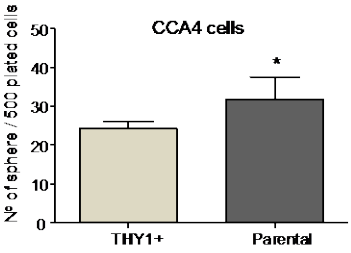
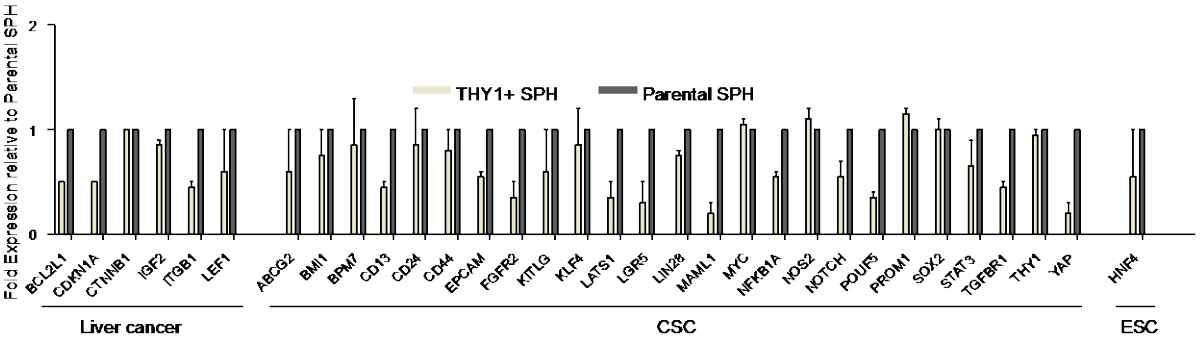


N° of injected cells	1000	100	10	Tumor Initiating Fraction (TIF) (95% CI)	*p value*
	N° of tumors formed/ total n° of transplants				
THY+ SPH	8/8	6/8	3/4	1/47.8 (1/108-1/21.3)	*0.2028*
Parental SPH	4/4	3/4	3/4	1/35.7 (1/110-1/11.8)	

The data are mean ± SEM (*p value* versus Parental SPH by Student t test)



Supplementary Fig. 2 CCA-derived spheres: molecular features and comparison with THY enriched spheres

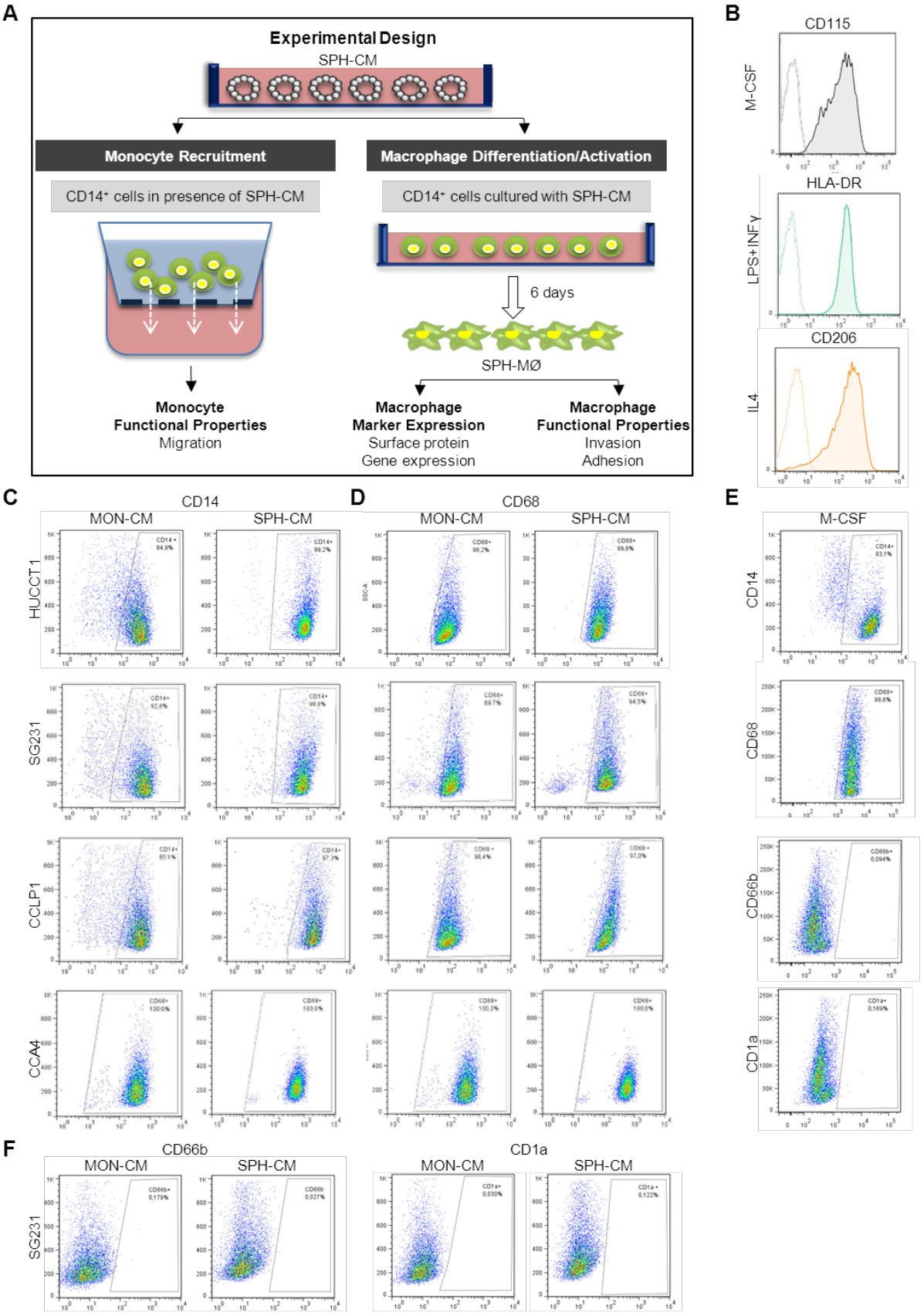
A) Up-regulated sphere-specific genes using pathway-focused arrays. Significant up-regulated genes compared to monolayers expressed as a percentage of the total number.

B) Mean Fluorescence Intensity (MFI) of CSC surface markers in spheres (SPH) and monolayers (MON) by FACS.

C) Validation of the key significant CSC surface markers (CD44, PROM1, THY and EPCAM) in tumor lesions (T) and surrounding liver (SL) using transcriptome data from 104 CCA patients. Pearson correlation between gene pairs was calculated using R and the "cortest" function, yielding correlation coefficients and *p-value* versus SL.

D) Upper left: capacity of spheroid formation by THY+ sorted and Parental CCA4 cells. Upper right: limiting-dilution cell transplantation assay. Tumor initiating fraction (TIF) of THY+ and Parental CCA4 derived spheres. A web-based calculation was performed by the ELDA program (see Methods section). *p value* versus Parental-tumor initiating frequency (TIF) by Student t test. Lower: comparison of gene expression of CCA4 THY+ SPH and Parental SPH. All mRNA levels are presented as fold changes normalized to 1 (mean expression of Parental-SPH). Data are mean ± SEM (*p value* versus Parental by Student t test, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$).

Supplementary Fig. 3



Supplementary Fig. 3. Phenotypic analysis of monocytes cultured with SPH- and MON-CM for 6 days.

A) Flow chart of the experimental approach. The chemotactic effect of the stem-like component on monocytes was evaluated by testing the migratory capacity of CD14+ cells in presence of SPH-CM. In addition, the effect on macrophage differentiation was evaluated by surface protein analysis (FACS) and gene expression profiling (qRT-PCR), as well as by the analysis of functional properties (invasion and adhesion).

B) CD115, HLA-DR and CD206 staining of M0 classically differentiated macrophages in the presence of M-CSF and *in vitro*-activated M1 (LPS+INFγ) and M2 (IL4) macrophages.

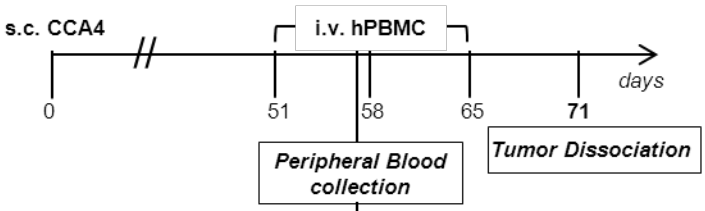
C) CD14 and D) CD68 profile expression determined by FACS. The dot plots are representative of three independent experiments using macrophages from three different healthy donors.

E) CD14, CD68, CD66b, and CD1a expressing monocytes differentiated in the presence of M-CSF as a classical control for M0 macrophages. The dot plots are representative of three independent experiments using macrophages from three different healthy donors.

F) CD66b and CD1a staining determined by FACS was used to exclude differentiation toward granulocytes and dendritic cells. The dot plots are representative of three independent experiments using macrophages from three different healthy donors. Data are mean ± SEM (*p value* versus Parental by Student t test, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$).

Supplementary Fig. 4.

A



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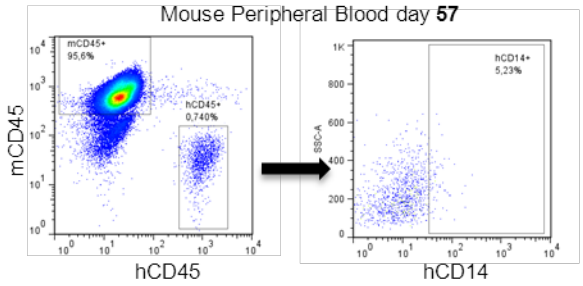


C

s.c. injected CCA4 cells	% hCD45+ (Mean ± SEM)	*p value*	% hCD45+ hCD14+ (Mean ± SEM)	*p value*
MON	0.626 ± 0.161		4.985 ± 0.346	
		0.452		*0.078*
SPH	0.747 ± 0.182		6.520 ± 0.707	

The data are mean ± SEM (n=3, *p value* versus MON by Student t test)

Supplementary Fig. 4. Phenotype of CCA-infiltrated macrophages in humanized mice

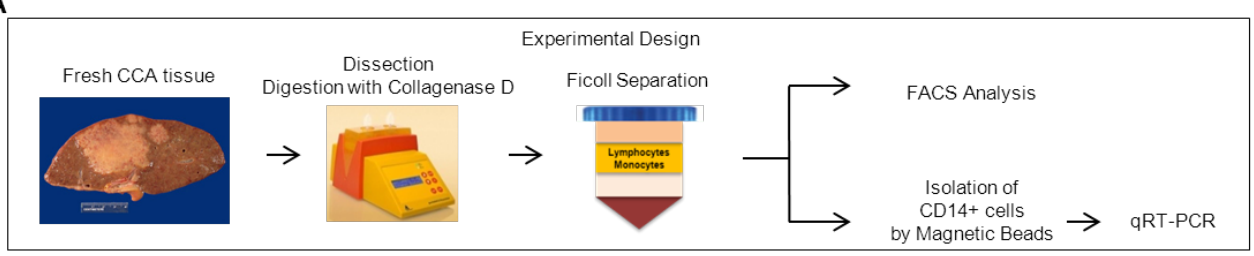
A) Upper: schematic illustration of experimental protocol for human CCA4-SPH derived cells and human PBMC co-injection. CCA4-SPH engrafted NSG mice were injected with three doses of human PBMC. At day 71, tumors were removed and associated-mononuclear component analyzed.

B) Presence of human CD45+ and human CD45+ CD14+ cells was assessed in mouse peripheral blood 6 days after first human PBMC engraftment in NSG mice (day 57) and expressed as percentage. The dot plots are representative of three independent experiments, using three different mice.

C) Presence of human CD45+ and human CD45+CD14+ cells determined by FACS. Data are mean ± SEM (n=3, *p value* versus MON-injected mice by Student t test).

Supplementary Fig. 5.

A



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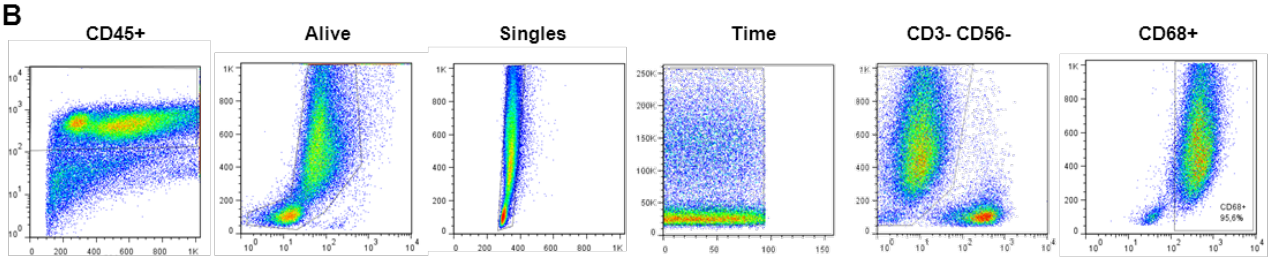


C

Clinical and pathological parameters of CCA patients used for molecular characterization (FACS and RT-PCR)

Patient	Grade	TNM classification			Tumor size (cm)	Perineural invasion	Angio-invasion
#CCA23	G2	T2b	NX	MX	8.5+1.5	no	yes
#CCA24	G3	T4	N0	MX	9.5	no	no
#CCA25	G2	T1	N0	MX	4.6	no	no
#CCA26	G3	T3a	N0	MX	9.5+3.7	no	no

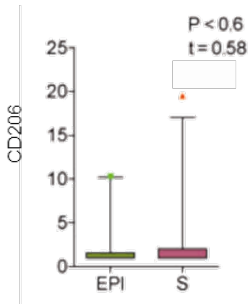
D

MFI of CD115, HLA-DR and CD206 in CCA-infiltrated MØs by FACS, included both T and PT regions of three CCA patients (#23, #24, #25 patients).

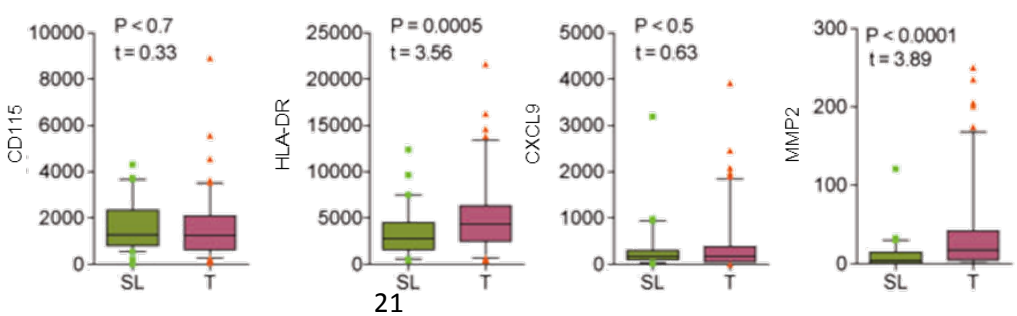
Marker	Condition	MFI ± SEM	*p value*
CD115	T	172.8 ± 20	*0.0031*
	PT	40.0 ± 17	
CD206	T	100.8 ± 31	*0.0372*
	PT	9.9 ± 04	
HLA-DR	T	353.6 ± 24	*0.0348*
	PT	288.6 ± 19	
CD68	T	194.7 ± 28	*0.0264*
	PT	85.7 ± 14	

The data are mean ± SEM (n=3, *p value* versus PT by Student t test)

E



F



Supplementary Fig. 5. Phenotype of CCA-infiltrated macrophages in human samples

A) Experimental design to isolate mononuclear subset from freshly resected CCA tissue.

B) FACS setting to identify macrophage components associated with CCA specimens.

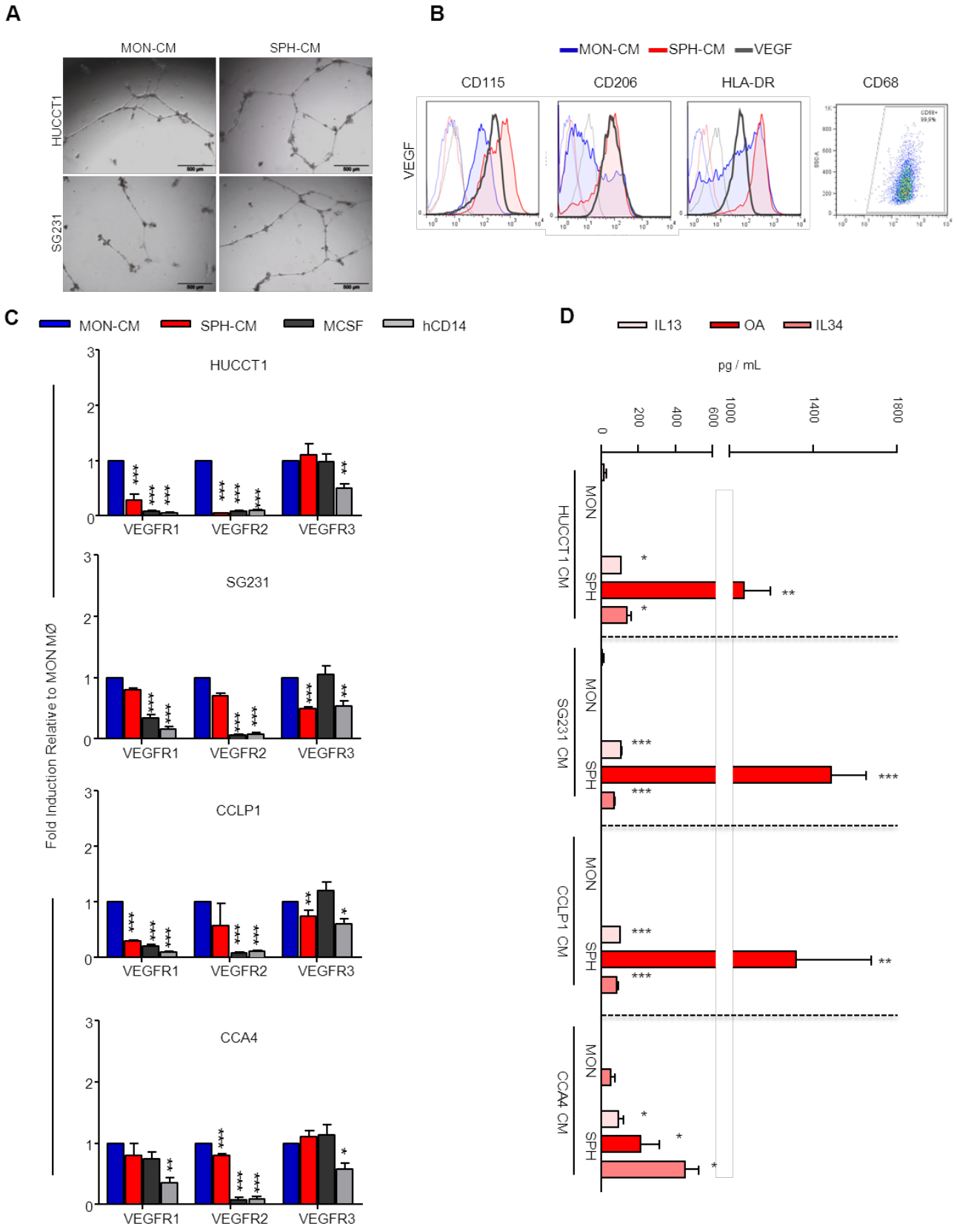
C) Clinical and pathological parameters of CCA patients used for molecular characterization (FACS and RT-PCR).

D) Mean Fluorescence Intensity (MFI) of CD115, HLA-DR and CD206 in CCA-infiltrated MØs by FACS, included both tumor (T) and peritumor (PT) regions of three CCA patients (#23, #24, #25 patient). Data are mean ± SEM (n=3, *p value* versus MON-injected mice by Student t test).

E) CD206 gene expression was evaluated in a set of CCA patients (n=23) where paired intratumoral epitheIial (EPI) and stromal compartments (S) were obtained by laser micro-dissection. Pearson correlation between gene pairs was calculated using R and the "cortest" function, yielding correlation coefficients and *p value* versus EPI.

F) Validation of the key significant markers (CD115, HLA-DR, CXCL9, MMP2) in tumor lesions (T) and surrounding liver (SL) using transcriptome data from 104 CCA patients. Pearson correlation between gene pairs was calculated using R and the "cortest" function, yielding correlation coefficients and *p value* versus SL.

Supplementary Fig. 6.



Supplementary Fig. 6. Biological implication of CCA-SPH released factors

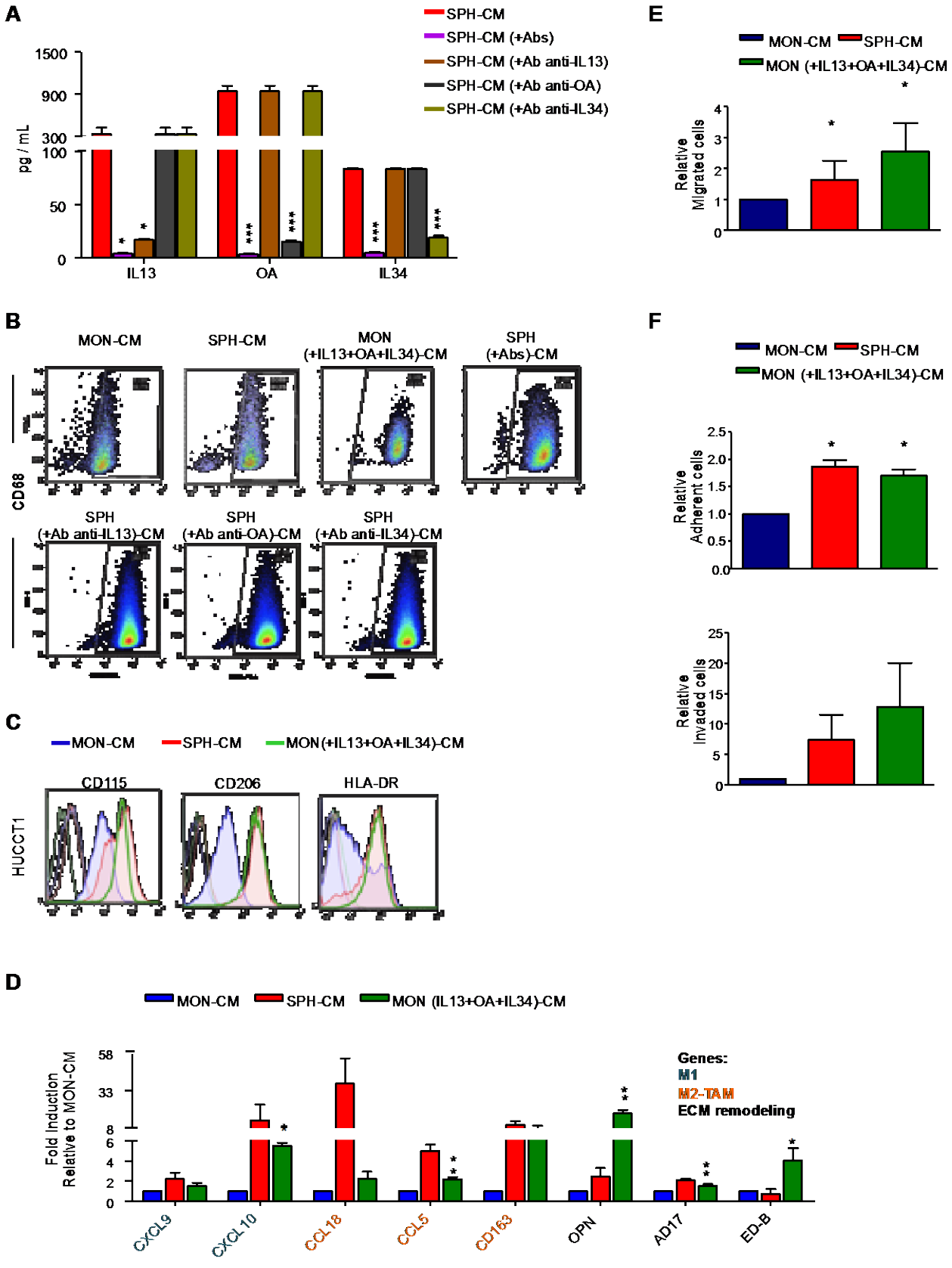
A) Representative pictures of tubules formed by HUVEC growth in MON and SPH-CM of both HUCCT1 and SG231 cells. Scale bar: 500 µm.

B) Effect of VEGF on macrophage differentiation**.** FACS histograms and percentage of positive cells for the CD115, CD206, HLA-DR and CD68 after addition of VEGF in fresh medium (in grey), as well as the addition of SPH-CM (in red) and MON-CM (in blue). The histograms and dot plots represent three independent experiments using macrophages from three different healthy donors.

C) Relative expression of transcript-encoding receptors for VEGF (VEGFR1, VEGFR2, VEGFR3) in macrophages differentiated in presence of SPH-CM (SPH MØ) as well as MON-CM (MON MØ). Freshly isolated human CD14+ cells used as control. GAPDH was used as an internal control. All mRNA levels are presented as fold changes normalized to 1 (mean expression of MON MØ). Data are mean ± SEM (*p value* versus MON MØ by Student t test, * p≤0.05, ** p≤0.01, *** p≤0.001). C) IL13, OA and IL34 levels in SPH and MON conditioned medium (n=3). Data are mean ± SEM (*p value* versus MON MØ by Student t test, * p≤0.05, ** p≤0.01, *** p≤0.001).

D) ELISA test of IL13, OA and IL34 concentration of SPH-CM and MON-CM in all tested CCA cells.

Supplementary Fig. 7.



Supplementary Fig. 7. Macrophage differentiation induced by the combination of IL13, OA and IL34

A) ELISA test of IL13, OA and IL34 concentration to evaluate the inhibitory effect of single antibody or combination (antibodies anti IL13 (800ng/mL, 10.000X), OA (2700ng/mL, 3000X), IL34 (800ng/mL, 10.000X)) added to SG231 SPH-CM (in violet, brown, dark grey and dark green, respectively). The effect of SPH-CM (in red) was also shown.

B) Representative CD68 dot plots of macrophages derived by culture of CD14+ cells in the presence of IL13 (80pg/mL), OA (0,9ng/mL) and IL34 (80pg/mL), added to the SG231 MON-CM. Additionally, the inhibitory impact of human antibodies anti IL13 (800ng/mL, 10.000X), OA (2700ng/mL, 3000X), IL34 (800ng/mL, 10.000X) added alone or in combination to SG231 SPH-CM were shown. The effects of both SPH- and MON-CM are also shown. The dot plots represent three independent experiments using macrophages from three different healthy donors.

C) FACS profile of CD115, CD206A and HLA-DR positive cells after addition of a combination of IL13 (80pg/mL), OA (0,9ng/mL) and IL34 (80pg/mL) to HUCCT1 MON-CM (in green). The effects of both SPH- (in red) and MON-(in blue) CM are also shown. The histograms represent three independent experiments using macrophages from three different healthy donors.

D) Relative expression of transcript-encoding markers for M1 and M2 features as well for genes involved in ECM remodeling, adhesion, invasion in macrophages differentiated in presence of: HUCCT1 SPH-CM (in red), MON-CM (in blue), MON (+IL13 (80pg/mL)+OA (0,9ng/mL)+IL34 (80pg/mL))-CM (in green). GAPDH was used as an internal control. All mRNA levels are presented as fold changes normalized to 1 (mean expression of macrophages differentiated with MON-CM). Data are mean ± SEM (n=3, *p value* versus MON MØ by Student t test, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$).

E) CD14+ cells migrated towards the combination of IL13 (80pg/mL), OA (0,9ng/mL) and IL34 (80pg/mL) added to HUCCT1 MON-CM (in green) (6 h). The effects of both SPH- (in red) and MON-(in blue) CM are also shown. The CD14+ cells that migrated were counted and normalized to the number of CD14+ cells that migrated in the presence of MON-CM. Data are mean ± SEM (n=3, *p value* versus MON-CM by Student t test, * p≤0.05).

F) Invasion and adhesion assay of macrophages differentiated in presence of a combination of IL13 (80pg/mL), OA (0,9ng/mL) and IL34 (80pg/mL) to HUCCT1 MON-CM (in green). The effects of both SPH- (in red) and MON-(in blue) CM are also shown. The cells were counted and normalized to the number of MON-derived macrophages (MON M□). Data are mean ± SEM (n=3, *p value* versus MON MØ by Student t test, * p≤0.05).

Supplementary Table 1

Expression of CD115, CD206 and HLA-DR by MØ cultured in presence of CCA SPH- and MON-CM as well as in presence of M-CSF, IL4, LPS/INFγ and VEGF

Marker	Cell Line-CM	Conditions	MFI ± SEM	*p value*
CD115	HUCCT1	SPH	532.7 ± 13	*0.0001*
		MON	159.3 ± 20	
	SG231	SPH	575.7 ± 23	*0.0003*
		MON	132.9 ± 30	
	CCLP1	SPH	1390.9 ± 27	*0.0000*
		MON	175.9 ± 32	
	CCA4	SPH	1275.3 ± 53	*0.0000*
		MON	240.9 ± 34	
		M-CSF	929.1 ± 41	
		VEGF	252.4 ± 14	
CD206	HUCCT1	SPH	372.4 ± 28	*0.0040*
		MON	178.2 ± 31	
	SG231	SPH	244.9 ± 26	*0.0019*
		MON	58.9 ± 17	
	CCLP1	SPH	172.5 ± 21	*0.2992*
		MON	152.8 ± 18	
	CCA4	SPH	341.3 ± 33	*0.0031*
		MON	124.9 ± 27	
		IL4	299.2 ± 26	
		LPS/INFγ	49,2 ± 19	
		VEGF	93.9 ± 12	
HLA-DR	HUCCT1	SPH	77.9 ± 05	*0.0038*
		MON	40.0 ± 06	
	SG231	SPH	112.1 ± 23	*0.0180*
		MON	12.7 ± 05	
	CCLP1	SPH	113.4 ± 10	*0.0032*
		MON	52.0 ± 07	
	CCA4	SPH	221.2 ± 26	*0.0131*
		MON	97.2 ± 31	
		IL4	14,8 ± 19	
		LPS/INFγ	170,8 ± 32	
		VEGF	94.5 ± 7	

The data are mean ± SEM (n=3, *p value* versus MON-CM by Student t test)

Supplementary Table 2

Clinical and pathological parameters of CCA patients used for CD163 immunohistochemical staining

Patient	Grade	TNM classification			Tumor size (cm)	Perineural invasion	Angio-invasion	CD163		CA19.9 (IU/mL)
								Intralesion	Tumor Front	
#CCA9	G1	T1	N0		3.8	no	no	75	75	42
#CCA14		T1	N0		2.8	no	no	35	35	2
#CCA21		T1	N0		3.5	no	no	35	75	15
#CCA27		T1	N0		2.5	no	no	30	40	nd
#CCA28		T1	N0		3.3	no	no	25	60	nd
#CCA29		T1	N0		3.6	no	no	45	35	nd
#CCA1	G2	T2b	NX	M1	33(multilobed)	nd	no	35	75	nd
#CCA2		nd	nd	nd	5	nd	no	7.5	5	5
#CCA3		T2b	N0	MX	6.3	yes	yes	7.5	35	nd
#CCA7		T1	N0	nd	3.1	yes	no	5	150	nd
#CCA8		T2	N0	nd	11	nd	yes	5	130	40
#CCA10		T2	N0	M1	5.7	nd	no	35	150	50
#CCA11		T2	N0	nd	7.5	nd	yes	5	125	28
#CCA13		T3	NX	nd	7	nd	no	5	40	nd
#CCA15		T1	N0	nd	11 (multilobed)	nd	no	5	160	6
#CCA17		T2b	NX	M1	33 (multilobed)	nd	no	35	70	nd
#CCA18		Nd	nd	nd	5	nd	no	7.5	5	5
#CCA19		T2a	N0	MX	6.3	yes	yes	15	25	12
#CCA22		T1	N1	MX	4	no	no	37	175	75
#CCA4	G3	T1	N1	nd	3.5	nd	no	7.5	140	23
#CCA5		nd	nd	nd	6.5	yes	yes	75	135	58
#CCA6		T3	N0	MX	6.9	nd	no	100	150	57
#CCA12		nd	nd	nd	6.3	nd	yes	35	165	95
#CCA16		T2b	N0	nd	14 (multinodular)	nd	yes	5	90	55
#CCA20		T1	N0	nd	2.8	no	no	35	145	16

Supplementary Table 3

List of all 37 soluble mediators tested in sphere (SPH) and monolayer (MON) conditioned medium (CM) in all CCA cell lines

Mediators	Production	HUCCT1-CM		SG231-CM		CCLP1-CM		CCA4-CM	
		MON	SPH	MON	SPH	MON	SPH	MON	SPH
VEGF	MON and SPH	1213±334	1165±44	1150±623	567±489	513±395	20±28	293±11	150±8
TNFa	MON or/and SPH	126±178	7±10	nd	nd	nd	4±5	nd	31±3
MCSF		216±239	nd	nd	nd	nd	nd	nd	nd
CCL21		35±49	50±71	53±75	48±68	43±60	47±67	43±60	151±23
TGFb		146±207	nd	26±36	119±169	nd	nd	nd	27±6
GM-CSF		1058± 913	214±157	14±20	nd	nd	nd	nd	nd
CCL22		nd	nd	33±6	3±4	nd	nd	nd	nd
CCL20		286±405	278±393	98±139	44±62	nd	nd	nd	nd
CXCL8		968±288	1008±355	328±234	89±98	102±145	nd	102±145	nd
CCL2		217±246	137±193	nd	nd	63±55	nd	63±55	nd
IL1b		nd	35±50	nd	nd	nd	nd	nd	nd
IL6		484±209	460±202	nd	nd	nd	nd	nd	nd
CXCL11		nd	nd	nd	nd	nd	nd	18±7	nd
IL17		nd	nd	nd	nd	nd	nd	nd	830±33
CXCL1		562±795	551±779	10±14	8±11	9±13	9±13	nd	nd
CCL5		1±1	26±36	nd	nd	nd	nd	nd	nd
CXCL9		nd	6±9	1±2	2±3	nd	nd	nd	40±13
CXCL12		nd	13±19	nd	13±18	nd	nd	nd	nd
IL2	No	nd	nd	nd	nd	nd	nd	nd	nd
IL10		nd	nd	nd	nd	nd	nd	nd	nd
IL12 p40		nd	nd	nd	nd	nd	nd	nd	nd
IFNg		nd	nd	nd	nd	nd	nd	nd	nd
CCL18		nd	nd	nd	nd	nd	nd	nd	nd
CCL17		nd	nd	nd	nd	nd	nd	nd	nd
CCL1		nd	nd	nd	nd	nd	nd	nd	nd
CCL7		nd	nd	nd	nd	nd	nd	nd	nd
CXCL10		nd	nd	nd	nd	nd	nd	nd	nd
IL7		nd	nd	nd	nd	nd	nd	nd	nd
CCL3		nd	nd	nd	nd	nd	nd	nd	nd
CCL8		nd	nd	nd	nd	nd	nd	nd	nd
CCL4		nd	nd	nd	nd	nd	nd	nd	nd
GDNF		nd	nd	nd	nd	nd	nd	nd	nd
IL12p70		nd	nd	nd	nd	nd	nd	nd	nd
IL13	SPH	15±10	107±1	nd	110±2	nd	103±7	nd	97±46
OA		nd	1069±223	nd	1483±295	nd	1318±620	nd	216±166
IL34		nd	142±34	nd	73±4	nd	87±13	53±37	453±126

Supplementary Table 4. Clinical and pathological parameters of 12 CCA patients used for IL13, OA, and IL34 serum quantification.

Clinical Variables		No. (*n*=12)	%
Age (years)	<70	3	25.0
	>70	9	75.0
Gender	Male	7	58.3
	Female	5	41.7
TNM	I-II	0	00.0
	III-IV	9	75.0
	nd	3	25.0
Tumor stage	IIIA-B	2	16.7
	IVA-B	9	75.0
	nd	1	08.3
Relapse	yes	0	00.0
	no	12	100.0
Distant Metastasis	with	4	33.3
	without	3	25.0
	nd	5	41.7
Invasion	with	9	75.0
	without	0	00.0
	nd	3	25.0
Tumor resection	yes	4	33.3
	no	8	66.7
Chemotherapy	yes	7	58.3
	no	5	41.7
CA19.9 (IU/mL)	40-100	1	08.3
	100-1000	6	50.0
	> 1000	4	33.3
	nd	1	08.3
CA125 (UI/mL)	< 37	5	41.7
	> 37	6	50.0
	nd	1	08.3
1-year survival		4/10	40.0

Supplementary Table 5. Correlation of SPH stem-like genes and SPH MØ in clinical CCA (n=104 patient tumors) using a microarray database (Andersen et al., 2012)

Correlation of SPH stem-like and SPH MØ genes in clinical CCA (n=104 patient tumors)		
SPH genes	**CCL5**	
	R	*p value*
LEF1	0.2709	*0.0054*
MAML1	0.2553	*0.0089*
SPH genes	**CCL18**	
	R	*p value*
CTNNB1	0.2563	*0.0086*
SPH genes	**CD115**	
	R	*p value*
LEF1	0.2942	*0.0024*
THY1	0.2517	*0.0100*
SPH genes	**CD163**	
	R	*p value*
ABCG2	0.2583	*0.0081*
CTNNB1	0.2941	*0.0024*
HNF4A	0.3300	*0.0006*
STAT3	0.3017	*0.0018*
SPH genes	**CD206**	
	R	*p value*
BMI1	0.3368	*0.0005*
KLF4	0.3241	*0.0008*
MYC	0.3056	*0.0016*
NOS2	0.2479	*0.0112*
SPH genes	**CD68**	
	R	*p value*
CD44	0.2392	*0.0145*
LGR5	0.2315	*0.0181*
SPH genes	**HLA-DRA**	
	R	*p value*
BMI1	0.3944	*0.0000*
CTNNB1	0.4342	*0.0000*
LEF1	0.5412	*0.0000*
THY1	0.3587	*0.0002*
SPH genes	**MMP2**	
	R	*p value*
CD44	0.3561	*0.0002*
CTNNB1	0.5208	*0.0000*
KLF4	0.2790	*0.0041*
LEF1	0.7343	*0.0000*
THY1	0.8105	*0.0000*
YAP1	0.2786	*0.0042*
SPH genes	**OPN**	
	R	*p value*
BCL2L1	0.3511	*0.0002*
EPCAM	0.2446	*0.0123*
FGFR2	0.3939	*0.0000*
ITGB1	0.5934	*0.0000*

Pearson correlation between gene pairs was calculated using R and the "cortest" function, yielding correlation coefficients and p-values.

Supplementary Table 6. Expression of CD115, CD206 and HLA-DR by MØ cultured in presence of CCA SPH- and MON-CM as well as in presence of MON-CM added with 3 sphere-specific released molecules and SPH-CM added with antibodies for the 3 sphere-specific released molecules

Marker	Cell Line-CM	Conditions	MFI ± SEM	
CD115	HUCCT1	SPH	532.7 ± 13	
		MON	159.3 ± 20	***
		MON+IL13+OA+IL34	463.3 ± 22	**
		MON+IL13	47.9 ± 13	***
		MON+IL34	113.2 ± 21	*
		MON+OA	76.4 ± 16	
	SG231	SPH	575.7 ± 23	
		MON	132.9 ± 30	***
		MON+IL13+OA+IL34	547.2 ± 35	***
		SPH+Abs	130.7 ± 27	***
		SPH+Ab anti-IL13	241.0 ± 32	***
		SPH+Ab anti-OA	160.1 ± 29	***
		SPH+Ab anti IL34	172.0 ± 33	
CD206	HUCCT1	SPH	372.4 ± 28	
		MON	178.2 ± 31	**
		MON+IL13+OA+IL34	358.3 ± 33	**
		MON+IL13	1333.9 ± 130	**
		MON+IL34	1825.2 ± 104	**
		MON+OA	1071.0 ± 86	
	SG231	SPH	244.9 ± 26	
		MON	58.9 ± 17	**
		MON+IL13+OA+IL34	264.7 ± 31	**
		SPH+Abs	25.3 ± 12	**
		SPH+Ab anti-IL13	94.0 ± 22	***
		SPH+Ab anti-OA	45.6 ± 13	**
		SPH+Ab anti-IL34	90.2 ± 19	
HLA-DR	HUCCT1	SPH	77.9 ± 5	
		MON	40.0 ± 6	*
		MON+IL13+OA+IL34	86.4 ± 17	ns
		MON+IL13	90.4 ± 21	ns
		MON+IL34	56.4 ± 14	**
		MON+OA	149.8 ± 28	
	SG231	SPH	112.7 ± 23	
		MON	12.7 ± 5	**
		MON+IL13+OA+IL34	200.6 ± 24	*
		SPH+Abs	14.7 ± 7	*
		SPH+Ab anti-IL13	40.2 ± 15	**
		SPH+Ab anti-OA	14.8 ± 7	*
		SPH+Ab anti-IL34	55.7 ± 18	

The data are mean ± SEM (n=3, *p value* versus MON- or SPH-CM by Student t test, * p≤0.05, ** p≤0.01, *** p≤0.001, ns=not significant)