SUPPLEMENTAL MATERIALS AND METHODS

Cell cultures and MTT assay

For BCSCs, the culture medium consisted of serum-free DMEM:F12 (1:1), enriched with GlutaMAX-I supplement (#A1286001; Thermo Fisher Scientific, Waltham, MA, USA), 50 ng/mL heparin (Sigma-Aldrich), 20 ng/mL EGF and 10 ng/mL βFGF (#3718-FB; R&D Systems, Minneapolis, MN, USA).

E0771 cells were cultured in RPMI 1640 medium, supplemented with 10% FBS and 10 mM HEPES, meanwhile Py230 cells were cultured in F-12K Medium (#21127022; Thermo Fisher Scientific, Waltham, MA, USA), enriched with 5% FBS and 0.1% Corning™ MITO+ Serum Extender (#355006; Thermo Fisher Scientific, Waltham).

Cell proliferation was assessed using the CellTiter 96® AQueous One Solution Cell Proliferation Assay (#G3582; Promega, Madison, WI, USA), according to manufacturer's instructions. Briefly, cells were seeded on a 96-well plate, at a density of 3×10^3 cells per well, for mBCSC cell line, and 1×10^3 cells per well, for BCSC-608 cell line, using the following experimental conditions:

- mBCSCs (CTRL), mBCSCs treated with rIL30 (50 ng/ml) (#7430-ML, R&D Systems, Minneapolis, MN, USA), mBCSCs transfected with IL30 gene expressing vector (IL30mBCSC) or empty vector (EVmBCSCs), IL30mBCSCs treated with (0.5 µg/ml) anti-IL30 antibodies (Abs) (R&D Systems Cat# AF1834, RRID:AB_355012);
- mBCSCs (CTRL), mBCSCs treated with rIL30 (50 ng/ml), mBCSCs treated with rIL30 (50 ng/ml) and with (40 µg/ml) anti-CXCL10 Abs (Thermo Fisher Scientific Cat# MA5-23774, RRID:AB_2609319), mBCSCs treated with the supernatant (sup) of IL30mBCSCs, mBCSCs treated with IL30mBCSC sup and with (40 µg/ml) anti-CXCL10 Abs;
- mBCSC and mBC cells untreated or treated with 30-150 ng/ml of rCXCL10 (#250-16, PeproTech, London, UK);

- hBCSCs untreated or treated with 0.5 µg/ml anti-IL30 Abs (R&D Systems Cat# AF1834, RRID:AB_355012);
- IL30mBCSCs, EVmBCSCs and mBCSCs;
- hBCSCs untreated or treated with 5-100 ng/ml of rhCXCL10 (#300-12, PeproTech, London, UK);
- hBCSCs untreated or treated with 5-100 ng/ml of rhIL23 (#200-23, PeproTech, London, UK).
- IL30-overexpressing BCSC-608 cells treated with 0.5-1.0 µg/ml anti-CXCL10 Abs (R&D Systems Cat# MAB266, RRID:AB_2261309), or anti-IL23 Abs (LSBio Cat# LS-B1366-50, RRID:AB_1015843), or with both anti-CXCL10 and anti-IL23 Abs;
- IL30-overexpressing BCSC-105 cells treated with 0.5-1.0 µg/ml anti-CXCL10 Abs (R&D Systems Cat# MAB266, RRID:AB_2261309)

Then, the plates were incubated for 48 hours and read at 490 nm, using the SpectraMax 190 microplate reader (RRID:SCR_018932; Molecular Devices San Jose, CA, USA). Proliferation was measured using untreated control cells as reference and the results were given as mean ± SD of three independent experiments carried out in triplicate.

Transfection with *IL27p28* (*IL30*) expressing vector

To generate the mouse IL30 lentiviral expression vector, a 728 bp fragment, carrying the gene for IL30, was amplified from the cDNA of Py230, using the Platinum Taq polymerase High Fidelity (#10966018; Thermo Fisher Scientific, Waltham, MA, USA) with the following primers: IL30 forward (5′-GCTCTAGAATGGGCCAGGTGACAG-3′) and IL-30 reverse (5′-CCGCTCGAGTTAGGAATCCCAGGCT-3′).

The amplified fragment was cloned into the XbaI-XhoI unique sites of a Tween lentiviral vector, under the control of a CMV promoter. This vector constitutively expresses GFP (Green Fluorescent Protein), under the control of a PGK promoter. Then, Py230 cells were

transfected with the p-TWEEN-mIL-30 or the empty vector p-TWEEN, using Calcium Phosphate Transfection Kit (#K278001; Thermo Fisher Scientific, Waltham, MA, USA).

The infected cells were sorted using a FACSAria II Cell Sorter (RRID:SCR_018934; BD Biosciences, Franklin Lakes, NJ, USA) using GFP as marker. Expression of IL30 was confirmed by real-time RT-PCR, Western Blotting (WB) and ELISA assay.

PCR array and real-time RT-PCR

RNA was extracted by using the RNeasy Mini Kit (#74104; Qiagen, Hilden, Germany), and reverse-transcribed with the RT^2 First Strand Kit (#330401; Qiagen, Hilden, Germany). PCR array analyses were run on a Qiagen Rotor Gene Q (Qiagen Rotor-Gene Q, RRID:SCR_018976), using the RT^2 Profiler Human Cancer Inflammation & Immunity Crosstalk PCR Array (#PAHS-181Z, Qiagen, Hilden, Germany) or the RT^2 Profiler Mouse Cancer Inflammation & Immunity Crosstalk PCR Array (#PAMM-181Z, Qiagen, Hilden, Germany) and RT^2 SYBR Green ROX FAST Master mix (#330501, Qiagen, Hilden, Germany). The results from each plate were normalized to the median value of a set of housekeeping genes. Changes in the gene expression were calculated using the ΔΔCt method. Results from experiments performed in triplicate were pooled and analyzed with the manufacturer's software. A significant threshold of a 2-fold change in gene expression corresponded to a $p<0.001$.

For analyses of murine stemness-related genes and human *CXCR3* isoforms, real-time RT-PCR was performed using the Quantifast SYBR Green PCR Kit (#204054, Qiagen, Hilden, Germany) and a MiniOpticon System (#CFB-3120, Bio-Rad, Hercules, CA, USA).

Primers for *Bmi1, Klf4, Notch1, Snai1, Snai2, Sox2, Twist1, Zeb1* and *CXCR3* isoforms were designed and synthesized by Sigma-Aldrich Corporation (St. Louis, MO, USA): *Bmi1* forward 5'-GGAAGAGGTGAATGATAAAAGG-3' and *Bmi1* reverse 5'-CATGACGTCAATCTGGAAAG-3'; *Klf4* forward 5'-CCCCTCTCTCCATTATCAAG-3' and

Klf4 reverse 5'-CTCTTGGTATAGGTTTTGCC-3'; *Notch1* forward 5'-GCATATGTATGCCAGGTTATG-3' and *Notch1* reverse 5'-CACTCATCCACATCATACTG-3'; *Snai1* forward 5'-AGTTGACTACCGACCTTG-3' and *Snai1* reverse 5'-AAGGTGAACTCCACACAC-3'; *Snai2* forward 5'-GACACATTAGAACTCACACTG-3' and *Snai2* reverse 5'-GACATTCTGGAGAAGGTTTTG-3'; *Sox2* forward 5'-ATGAGAGATCTTGGGACTTC-3' and *Sox2* reverse 5'-TCTATACATGGTCCGATTCC-3'; *Twist1* forward 5'-GAGACTTAGATGTCATTGTTTC-3' and *Twist1* reverse 5'-GAATTTGGTCTCTGCTCTTC-3'; *Zeb1* forward 5'-ATATGAGCACACAGGTAAGAG-3' and *Zeb1* reverse 5'-TTCATGTGTTGAGAGTAGGAG-3'; *CXCR3A* forward 5′-ACCCAGCAGCCAGAGCACC-3′ and *CXCR3A* reverse 5′-TCATAGGAAGAGCTGAAGTTCTCCA-3′; *CXCR3B* forward 5′-TGCCAGGCCTTTACACAGC-3′ and *CXCR3B* reverse 5′-TCGGCGTCATTTAGCACTTG-3′; *CXCR3-alt* forward 5′-CCAATACAACTTCCCACAGGGGT-3′ and *CXCR3-alt* reverse 5′-GTCTCAGACCAGGATGAATCCCG-3.

Primers for *Lag3* (#QT00113197), *Twist2* (#QT00101598), *Zeb2* (#QT00148995) and the housekeeping gene *hypoxanthine phosphoribosyltransferase 1* (*Hprt*) (#QT00166768) were purchased from Qiagen, (Hilden, Germany).

Melting curve analysis was done to assess the specificity of PCR products and the efficiency of reaction for each target was evaluated by amplifying serial dilutions of cDNA. Relative quantification of mRNA was done according to the comparative threshold cycle method with Hprt as calibrator, using the Bio-Rad CFX Manager software.

For RT-PCR of human *CXCR3* isoforms, RNA extraction, reverse transcription and amplification were performed as described for real-time RT-PCR. The PCR products were separated on a 2% agarose gel, stained with ethidium bromide, and DNA bands were visualized with a Transilluminator 2000 (#170-8110; Bio-Rad, Hercules, CA, USA).

All samples were processed in triplicate, and wells without added cDNA served as negative controls.

ELISA

Quantitation of the IL30 protein in the supernatant derived from murine and human BCSCs was assessed with the Mouse IL27p28/IL30 Quantikine ELISA Kit (#M2728; R&D Systems, Minneapolis, MN, USA) or the Human IL27 ELISA Kit, specific for p28 subunit of IL27 (#CSB-E08464h; Cusabio, Wuhan, Hubei, China), respectively, according to manufacturer's instructions.

CXCL10 and IL23 levels, in the supernatant collected from mBCSCs, were assessed using the IP10 (CXCL10) Mouse ELISA Kit (Thermo Fisher Scientific Cat# BMS6018, RRID: AB_2575626) or the IL23 Mouse ELISA Kit (Thermo Fisher Scientific Cat# BMS6017, RRID:AB_2575624), according to manufacturer's instructions. CXCL10, IL23, CXCL1 and CSF2 levels in the supernatant from hBCSCs were assessed with Human CXCL10/IP10 Quantikine ELISA (#DIP100; R&D Systems, Minneapolis, MN, USA), Human IL23 Quantikine ELISA (#D2300B; R&D Systems, Minneapolis, MN, USA), Human GM-CSF (CSF2) ELISA kit (#KHC2011; Thermo Fisher Scientific, Waltham, MA, United States) and Human GRO alpha (CXCL1) ELISA Kit (Thermo Fisher Scientific Cat# BMS2122, RRID:AB_2575448), according to manufacturer's protocols.

Western blotting

For total protein extraction, cells were collected by centrifugation and lysed with ice cold RIPA Lysis buffer (Thermo Fisher Scientific, Waltham, MA, USA), supplemented with Protease and Phosphatase Inhibitors Cocktails (Thermo Fisher Scientific, Waltham, MA, USA). Total proteins were then quantified using the Bradford assay. For cytosolic and plasma membrane protein extraction, Minute Plasma Membrane Protein Isolation and Cell

Fractionation Kit (#SM-005; Invent Biotechnologies, Plymouth, MN, USA) was used, according to manufacturer's protocol. Cytosolic and plasma membrane fractions were then quantified using Pierce BCA Protein Assay Kit (#23225; Thermo Fisher Scientific, Waltham, MA, USA). Subsequently, whole cell lysates, cytosolic and plasma membrane fractions were loaded on Mini-PROTEAN TGX Gels 4-20% (#4561094; Bio-Rad, Hercules, CA, USA) and proteins were transferred from the gels on Immun-Blot PVDF Membranes (#1620177; Bio-Rad, Hercules, CA, USA) in transfer buffer (glycine, tris [pH 8.4] and methanol) using Mini Trans-Blot Cell apparatus (Bio-Rad). Membranes containing the transferred proteins were then blocked with 5% milk (Sigma-Aldrich, St. Louis, MO, USA) in TBST and, subsequently, probed with primary and horseradish peroxidase conjugated secondary Abs, following standard procedures. The following primary and secondary Abs were used: rabbit anti-human IL-27-A Ab (Abcam Cat# ab118910, RRID:AB_10898806) and goat anti-mouse IL-27p28/IL30 Ab (R&D Systems Cat# MAB7430, RRID:AB_11129241); goat anti-rabbit IgG (H + L)-HRP Conjugate (Sigma-Aldrich Cat# A0545, RRID:AB_257896) and rabbit anti-goat IgG (whole molecule)-Peroxidase (Sigma-Aldrich Cat# A5420, RRID:AB_258242). β-Actin (Sigma-Aldrich Cat# A2228, RRID:AB_476697) and Sodium Potassium ATPase (Thermo Fisher Scientific Cat# MA5-32184, RRID:AB_2809472) were used as loading controls for total/cytosolic proteins and plasma membrane proteins, respectively. Membranes were then washed with TBST, and developed with Pierce ECL Western Blotting Substrate (#32106; Thermo Fisher Scientific, Waltham, MA, USA).

Sphere formation assay

The sphere-formation efficiency, and the associated self-renewal ability, of murine and human BCSC cells was assessed by using the Extreme Limiting Dilution Analysis (ELDA, RRID:SCR_018933).[1]

The experimental conditions for mBCSCs were as follows:

- mBCSCs (CTRL), mBCSCs treated with rIL30 (50 ng/ml), EVmBCSCs, IL30mBCSCs and IL30mBCSCs treated with 0.5 μg/ml anti-IL30 Abs (R&D Systems Cat# AF1834, RRID:AB_355012).

The experimental conditions for hBCSCs were as follows:

- BCSC-608 and BCSC-105 untreated or treated with (0.5 μg/ml) anti-IL30 Abs (Abcam Cat# ab118910, RRID:AB_10898806);
- hBCSCs untreated or treated with (50 ng/ml) of rCXCL10 (#300-12, PeproTech, London, UK); or treated with 10 ng/ml (BC-SLC-105) or 50 ng/ml (BC-SLC-608) of rIL23 (#200-23, PeproTech, London, UK).

Cells were seeded at concentrations of 1 cell, 2 cells, 4 cells or 8 cells per well, on 96-well plates. After incubation at 37°C, in a humidified incubator with 5% CO_2, for 5-10 days (mBCSCs treated with rIL30, and hBCSCs treated with anti-IL30 Abs) or 8 days (hBCSCs treated with rCXCL10 or rIL23), spheres containing ≥3 cells were counted under a Leica light microscope.

Mouse studies

Since limiting dilution analyses revealed a 100% of tumor take with a number of orthotopically implanted mBCSCs up to $1X10^{3,2}$ to determine the most appropriate conditions for tumor growth, we first inoculated orthotopically $1X10^3$ cells, which gave rise to tumors, 5 to 6 months later. We then implanted $1X10^4$ mBCSCs, which developed tumors with a shorter latency time (Figure 5A), but had comparable microenvironmental contexture, as revealed by immunopathological analyses (Supplemental Figures S1A, B).

Histopathology, immunohistochemistry and confocal microscopy

Mouse and human tissue samples were fixed in 4% formalin and embedded in paraffin. For histology, paraffin-embedded samples were sectioned at 3 µm and stained with hematoxylin and eosin (H&E). Immunohistochemistry and immunofluorescence were performed as reported,[3 4] by using the Abs listed in Supplemental Table S2.

Single, double (NKp46/RORγt and CD11b/Gr-1, in murine tumor samples), (CD133/IL30, CD133/IL23, and CD133/CXCL10, in human BC samples) and triple (CD133/IL30/IL23, and CD133/IL30/CXCL10, in human BC samples) immunostainings were performed by using the DoubleStain IHC Kit M&R on human tissue (#ab210059; Abcam, Cambridge, UK) and the TripleStain IHC Kit R&R&M on human tissue (#ab183288; Abcam, Cambridge, UK), according to manufacturer's instructions.

For single immunofluorescent staining for IL30, hBCSCs were fixed with 4% paraformaldehyde (10 min), incubated with anti-IL30 Ab, overnight at 4 °C, and subsequently with Alexafluor 488 Ab (Thermo Fisher Scientific Cat# A32731, RRID:AB_2633280), for 2h at room temperature.

For double immunofluorescent staining IL30/ATPase, $5x10^4$ of hBCSCs were seeded on chambered cell culture slides (Nunc™ Lab-Tek™ II CC2™ Chamber Slide System, #154852; Thermo Fisher Scientific, Waltham, MA, USA) and incubated at 37°C for 48h. After coverglass removal, cells were fixed with 4% paraformaldehyde and stained with anti-ATPase Na+/K+ Transporting Subunit Alpha 1 (ATP1A1) Ab, for 2h at room temperature, followed by incubation with Alexafluor 594 Ab (Thermo Fisher Scientific Cat# A11037, RRID:AB_2534095). Subsequently, slides were incubated with anti-IL30 Ab, overnight at 4°C, followed by incubation with Alexafluor 488 Ab (Thermo Fisher Scientific Cat# A32731, RRID:AB_2633280).

For double immunofluorescent stainings IL30/CXCL10 and IL30/IL23, slides were incubated overnight, at 4 °C, with the primary Ab against IL30, followed by incubation with Alexafluor 488 Ab (Thermo Fisher Scientific Cat# A32731, RRID:AB_2633280). Then,

slides were incubated with the secondary Ab (rabbit anti-CXCL10 or -IL23) followed by incubation with Alexafluor 594 Ab (Thermo Fisher Scientific Cat# A11037, RRID:AB_2534095), for 2h at room temperature.

Lastly, nuclei were counterstained with diamidino-2-phenylindole (DAPI) (Thermo Fisher Scientific, Waltham, MA, USA) for 2 minutes and slides were analyzed under an LSM 510 Meta confocal microscope (Zeiss, Oberkochen, Germany; RRID:SCR_018062).

All staining experiments were performed in triplicate and representative results of one experiment are shown.

REFERENCES

1. Hu Y, Smyth GK. ELDA: extreme limiting dilution analysis for comparing depleted and enriched populations in stem cell and other assays. *J Immunol Methods* 2009;347:70-8.
2. Bao L, Cardiff RD, Steinbach P, *et al*. Multipotent luminal mammary cancer stem cells model tumor heterogeneity. *Breast Cancer Res* 2015;17:137.
3. Airoldi I, Tupone MG, Esposito S, *et al*. Interleukin-27 re-educates intratumoral myeloid cells and down-regulates stemness genes in non-small cell lung cancer. *Oncotarget* 2015;6:3694-708.
4. Di Meo S, Airoldi I, Sorrentino C, *et al*. Interleukin-30 expression in prostate cancer and its draining lymph nodes correlates with advanced grade and stage. *Clin Cancer Res* 2014;20:585-94.