Supplemental Materials and Methods

Cell cultures, MTT and sphere formation assays

As reported in ref. 1, the HCT116 cell line (colorectal cells, CRCs) contains a high proportion of cancer stem cells (CSCs). To isolate this population, we cultured wild type cells in low attachment flasks, under selective sphere-forming conditions (Dulbecco's modified eagle's medium-F12 supplemented with ITS Liquid Media Complement, 5 mM HEPEs, 4 mg/ml BSA, 2 nM L-glutamine, 3 mg/ml glucose, 20 ng/ml EGF and 20 ng/ml bFGF) for two weeks.[2] Subsequently, spheres were collected and washed in PBS-EDTA medium, to obtain single-cell suspension, stained with antibodies anti-ALDH, anti-CD26, anti-CD44, anti-EpCAM, anti-CD133, anti-CD166 and analyzed using a BD Scientific Canto II Flow Cytometer (RRID:SCR_018056), to confirm their stem cell phenotype.[2-5] These colorectal (CR)-CSCs were maintained in low attachment flasks and cultivated with PluriSTEM-XF™ Human ES/iPS Cell Medium (Merk, Darmstadt, Germany), a complete xeno-free medium formulation for the feeder-free culture of human embryonic stem (ES) cells and induced pluripotent stem (iPS) cells.

Cell viability and proliferation were assessed using the CellTiter 96 AQueous One Solution Cell Proliferation Assay (#G3582; Promega, Madison, WI, USA), according to manufacturer's instructions.

Sphere-formation efficiency, and associated self-renewal ability, of CR-CSCs was assessed by using the Extreme Limiting Dilution Analysis (ELDA, RRID:SCR_018933).[6] 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% CO2, for 5-10 days, spheres containing ≥3 cells were counted under a Leica DM 2500 light microscope (Leica, Wetzlar, Germany).

D'Antonio L, *et al. J Immunother Cancer* 2023; 11:e006056. doi: 10.1136/jitc-2022-006056

CRISPR/Cas9-mediated Interleukin-30, WNT5A, RAB33A and STAT3 gene knockout

For CRISPR mediated *IL30* gene deletion in CRC and CR-CSC cells, we used two Trueguide Synthetic (s)gRNAs, designed and synthesized by Thermofisher (#CRISPR947384_SGM, IL27p28, Human and #CRISPR947272_SGM, IL27p28, Human; Thermo Fisher Scientific, Waltham, MA, USA). Cells were transfected with sgRNAs using CRISPRMAX™ Cas9 Transfection Reagent (#CMAX00001; Thermo Fisher Scientific), to derive two independent clonal cell populations (clone A and B). The Sanger sequencing, performed by Eurofins Scientific (Bruxelles, Belgium), confirmed the *IL30* gene deletion and Western blotting (WB) showed the absence of IL30 protein, in both clone A and clone B, therefore we used only clone A in subsequent experiments. To exclude off-target effects, we performed genetic rescue experiments transfecting knock-out cell clones with IL30 expressing vector, which restored the proliferation rates prior to genome editing.

For WNT5A and RAB33A gene deletion in IL30-CR-CSCs, we used the following Trueguide sgRNAs: #CRISPR816400_SGM, WNT5A, Human and #CRISPR847389_SGM, RAB33A, Human, respectively (Thermo Fisher Scientific).

For STAT3 gene deletion in CR-CSCs we used the Trueguide sgRNA #CRISPR917492_SGM, STAT3, Human (Thermo Fisher Scientific). Cells were transfected with sgRNAs using CRISPRMAX™ Cas9 Transfection Reagent (#CMAX00001; Thermo Fisher Scientific). The deletion of WNT5A, RAB33A and STAT3 genes was validated by Sanger-sequencing and WB.

Transfection with Interleukin-30 expressing vector

To generate the human *IL30* lentiviral expression vector, the *IL30* gene was amplified and cloned into 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, CRC cells and CR-CSCs cells were transfected with the

D'Antonio L, *et al. J Immunother Cancer* 2023; 11:e006056. doi: 10.1136/jitc-2022-006056

p-TWEEN-IL-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 and WB.

Real-time RT-PCR

RNA was extracted from human CRC and CR-CSC cells by using the RNeasy Mini Kit (#74104, Qiagen, Hilden, Germany), and reverse-transcribed with the RT2 First Strand Kit (#330401, Qiagen). For analyses of *IL30* mRNA levels, real-time RT-PCR was performed using the Human_IL27p28_1_SG QuantiTect Primer Assay (#QT00236250), the Quantifast SYBR Green PCR Kit (#204054) (both from Qiagen) and a MiniOpticon System (#CFB-3120, Bio-Rad, Hercules, CA, USA). The results, from experiments performed in triplicate, were pooled, and normalized to the median value of a set of housekeeping genes and changes in gene expression were calculated according to the ΔΔCt method, using the manufacturer's software.

Immunoelectron microscopy

CR-CSCs were grown in monolayer and fixed in 2% PFA, with 0.2% glutaraldehyde, in 0.1 M PBS, pH 7.4, for 3 h at room temperature. Then, the cells were embedded into 12% gelatin in 0.1 M PBS, pH 7.4, solidified on ice, infused in 2.3 M sucrose overnight at 4°C, mounted on aluminum pins and frozen in liquid nitrogen. For immunogold labeling of CR-CSCs, ultrathin cryosections (55 nm) were cut at -110°C, using an Ultracut EM FC6 (Leica Microsystems, Wetzlar, Germany), collected with 1% methyl cellulose in 1.15 M sucrose, blocked in 1% bovine serum albumin in PBS and then immunolabeled with primary antibody, a rabbit polyclonal anti-IL27A (Abcam Cat# ab118910, RRID:AB_10898806).

Bound antibodies were visualized using either goat anti-rabbit conjugated with 10-nm gold particles (#AC-10-01-05, Cytodiagnostics, Burlington, Ontario, Canada) or 10 nm PrA-CG (acquired from G. Posthuma, Utrecht, The Netherlands). All incubations were performed in presence of 1% BSA.

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 Immuno-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 antibodies, following standard procedures. The following primary antibodies were used: rabbit anti-human IL-27-A (IL27/p28, i.e. IL30) (Abcam Cat# ab118910, RRID:AB_10898806); mouse anti-human WNT3A (Abcam Cat# ab81614, RRID:AB_1658404), rabbit anti-human WNT5A (Thermo Fisher Scientific Cat# MA5-35281, RRID:AB_2849183), rabbit anti-human MMP2 (#TA350971S, OriGene

Technologies, Rockville, MD, USA), rabbit anti-human MMP13 (GeneTex Cat# GTX100665, RRID:AB_2037446), rabbit anti-human RAB33A (Thermo Fisher Scientific Cat# PA5-37028, RRID:AB_2553873) and mouse anti-human/mouse STAT3 (Cell Signaling Technology Cat# 9139, RRID:AB_331757).

The following secondary antibodies were used: goat anti-rabbit IgG (H + L)-HRP Conjugate (Sigma-Aldrich Cat# A0545, RRID:AB_257896) and rabbit anti-mouse IgG (whole molecule)-Peroxidase (Sigma-Aldrich Cat# A9044, RRID:AB_258431. Antibodies specific for 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 WB Substrate (#32106; Thermo Fisher Scientific, Waltham, MA, USA).

ELISA

Quantitation of ANGPT2, CXCL10, EPO, EGF, IGF1, IL6 and VEGFA, in the supernatant derived from CRC cells and CR-CSCs, was carried out using the following ELISA kits, according to manufacturer's protocols: human ANGPT2 ELISA kit (#ab99971, Abcam, Cambridge, UK), human CXCL10 ELISA kit (KAC2361, Life Technologies, CA, USA), human EPO ELISA kit (BMS2035-2, Life Technologies), human EGF ELISA kit (#ab100504, Abcam, Cambridge, UK), Human IGF1 ELISA kit (#ab211651, Abcam), human IL6 ELISA kit (#EH2IL6, Thermo Fisher Scientific, Waltham, MA, USA) and human VEGFA ELISA kit (#ab119566, Abcam).

Mouse studies

Since all the animals were genetically identical-and housed in the same room, under the same conditions, no randomization was performed. All the mice received a standard diet

(sterilized pellets) and had free access to water (also sterile). They were not fasted and were housed in pressurized individually ventilated (PIV) cages with cardboard houses and plastic tubes as environmental enrichment. All treatments were carried out during the light cycle. Tumors were measured with calipers as soon as they were palpable.

Immunopathology and morphometric analyses

For histology, tissue samples were fixed in 4%-formalin, embedded in paraffin, sectioned at 4-µm and stained with H&E. Single or double immunostainings, on formalin fixed and paraffin-embedded, or frozen, tissue sections, were-performed as described,[7] by using the antibodies listed in the supplementary table S2. Proliferation index, microvessel and cell counts were assessed by light microscopy, at ×400 in an 85,431.59 μm^2 field, on single immunostained sections, using QWin image analysis software (Leica QWin, RRID:SCR_018940), which ensures the following highly reproducible steps: 1) image acquisition; 2) conversion of RGB image (true colors) to binary image (black and white); 3) filtering to remove noise; 4) counting of immunostained cells or measurement of positively stained area. Six to eight high-power fields were analyzed for each section and three sections per sample were evaluated. The histopathological evaluation was performed excluding necrotic areas. Results were expressed as mean ± SD of positive cells (F4/80, Ly-6G) per field, or mean percentage ± SD of positive cells/number of total cells (Ki67) per field. Microvessels were identified as small tubes or circles marked by CD31 Abs and results were expressed as mean ± SD of positive vessels/field.

The morphometric analysis of CRC samples was confined to the neoplastic areas of the colon tissue sections, and was performed by light microscopy, at ×400, in an 85431.59 μm^2 field, on single immunostained sections, with Qwin image analysis software (Qiagen) as described above. IL30 expression, in both neoplastic cells and infiltrating leukocytes, was determined as described below.

Expression of IL30 by neoplastic cells was evaluated using the following score, based on 1) the widening of the staining expressed as the percentage of tumor stained, i.e.: <50%, between 50% and 70%, and >70%, and 2) the strength of the staining: defined as absent (–), slight (±), distinct (+) or strong (++).

Thus, IL30 expression by neoplastic cells was defined as:

- *positive,* when a) the widening was >70% and its strength ranged from slight (±) to strong (++), or b) the widening was between 50% and 70% and its strength ranged from distinct (+) to strong (++);
- *weakly positive,* when a) the widening was between 50% and 70% and its strength was slight (±), or b) the widening was equal to 50% and its strength ranged from slight (±) to strong (++);
- *negative* when the widening was <50% and its strength was slight (±) to absent (–).

Expression of IL30 by infiltrating leukocytes was evaluated using the following score, based on 1) the percentage of leukocyte expressing the cytokine, i.e. <50%, between 50% and 70%, and >70%, and 2) the strength of the cytokine staining, that was defined as absent (–), scarce (±), distinct (+) or strong (++).

Thus, IL30 expression by infiltrating leukocytes was defined as:

- *strong,* when a) the staining involved more than 70% of leukocytes and its strength ranged from scarce (±) to strong (++), or b) the percentage of positively stained leukocytes was between 50% and 70% and the strength of the staining ranged from distinct (+) to strong (++);
- *distinct,* when a) the staining involved >50% and ≤70% of leukocytes and its strength was scarce (±), or b) the staining involved 50% of leukocytes and its strength ranged from scarce (±) to strong (++);
- *scanty,* when the staining involved <50% of leukocytes and its strength ranged from scarce (±) to absent (–).

Therefore, CRC samples with positive and strong IL30 expression were classified as $IL30^{+/+}$, CRC samples with negative and scanty IL30 expression were classified as $IL30^{-/-}$, whereas the remaining CRC samples with different expression levels of IL30, ranging from weakly positive to negative in neoplastic epithelia, and from distinct to scanty in the immune cell infiltrate, were defined as $IL30^{+/-}$ CRCs. Immunostained sections were examined by two pathologists in a blind fashion, with very good agreement (κ value = 0.89 and 0.80 for evaluation of IL30 staining in CRC cells and infiltrating leukocytes, respectively).

CIBERSORTx

CIBERSORTx (RRID:SCR_016955), used to investigate IL30 gene expression in immune cells populations infiltrating CRC samples, is a computational framework, which accurately infers infiltrating cell subpopulations and their gene-expression from the mRNA-profiles of tissue samples, using specific gene signatures. LM22 was used as signature matrix, that contains 547 genes, which-distinguishes 22 human hematopoietic cell phenotypes.[8] Differences, in IL30 gene expression, between immune cell subpopulations, were assessed by Student's *t*-test or ANOVA followed by Tukey-HSD test.

REFERENCES

1. Yeung TM, Gandhi SC, Wilding JL, Muschel R, Bodmer WF. Cancer stem cells from colorectal cancer-derived cell lines. *Proc Natl Acad Sci U S A* 2010;107:3722-7.
2. Olejniczak A, Szaryńska M, Kmieć Z. In vitro characterization of spheres derived from colorectal cancer cell lines. In*t J Oncol* 2018;52:599-612.
3. Levin TG, Powell AE, Davies PS, *et al*. Characterization of the intestinal cancer stem cell marker CD166 in the human and mouse gastrointestinal tract. *Gastroenterology* 2010;139:2072-82.e5.
4. Munro MJ, Wickremesekera SK, Peng L, Tan ST, Itinteang T. Cancer stem cells in colorectal cancer: a review. *J Clin Pathol* 2018;71:110-6.
5. Pang R, Law WL, Chu AC, *et al*. A subpopulation of CD26+ cancer stem cells with metastatic capacity in human colorectal cancer. *Cell Stem Cell* 2010;6:603-15.
6. 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.
7. Sorrentino C, Musiani P, Pompa P, Cipollone G, Di Carlo E. Androgen deprivation boosts prostatic infiltration of cytotoxic and regulatory T lymphocytes and has no effect on disease-free survival in prostate cancer patients. *Clin Cancer Res* 2011;17:1571-81.
8. Newman AM, Liu CL, Green MR, *et al*. Robust enumeration of cell subsets from tissue expression profiles. *Nat Methods* 2015;12:453-7.