

CCR5 antagonism by maraviroc inhibits Hodgkin lymphoma microenvironment interactions and xenograft growth

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Supplementary methods

Drugs

The following drugs used in the study were surplus samples obtained from the clinical pharmacy of CRO Aviano: doxorubicin (Pfizer), brentuximab vedotin (Takeda), cisplatin (Teva), gemcitabine (Actavis), and vinorelbine (Pierre Fabre).

Preparation of primary MSCs from cHL-involved lymph nodes.

Three separate frozen lymph node preparations obtained from a patient affected by a nodular sclerosis variant of HL of non-T, non-B phenotype (CD15+, CD30+, CD40+, CLA-, CD20-, CD79a-, CD3-, ALK-) were employed as sources of MSCs from HD-involved lymph nodes (HL-MSCs)¹. Single cell suspension obtained by mechanical dissociation of lymph node fragments was cultured in Mesenchymal-Stem-Cell Growth Medium Bulletkit (Lonza) to avoid differentiation. After non-adherent cells had been removed, adherent cells (MSCs)² were cultured in Mesenchymal-Stem-Cell Growth Medium Bulletkit.

Fluorescence-assisted transmigration invasion and motility assay

Cells (MSCs: 50,000 cells/insert; purified CD14+ monocytes: 200,000/insert) were tagged with the lipophilic CellTracker™ CM-Dil Dye (Thermo Fisher Scientific) as described.³ Cells were then seeded in 150 µl serum-free medium in the upper side of fibronectin-coated (20 µg/mL) Boyden chambers inserted into the wells of 24-well cell culture plates. Below the Boyden chambers, the wells contained 700 µL of RPMI-1640 plus 10% FCS (control medium) or conditioned medium from cHL cell lines (cHL CM) or from tumor-educated MSCs (E-MSC CM) for testing as a chemoattractant. In some

experiments, the CCR5 antagonist maraviroc (0.1 to 100 μ M final concentration) was added to cells, or a mouse anti-human CCL5 antibody (5 μ g/mL; clone 21418, R&D Systems) was added to the chemoattractant 1 h prior to migration. Transmigrated cells were revealed using a computer-interfaced GeniusPlus microplate reader (Tecan).

Proliferation, clonogenic growth and senescence assays

In some experiments, MSCs were plated in culture medium or in cHL conditioned medium (500 cells/well) containing neutralizing anti-human FGF2 (AF233, R&D Systems; 1 μ g/mL), anti-human TGF β 1 (TB21, GeneTex; 2 μ g/mL), or anti-TNF α (AF-210, R&D Systems; 0.5 μ g/mL) antibodies or no antibody. Half the volume of culture medium was replaced every 2 days. After 9 days, growth was evaluated using the MTT assay. In other experiments, MSCs (100 cells/well, 24-well plates) or monocytes (2.0×10^4 cells/well, 96-well plates) were cultured for 9 days in RPMI-1640 with 10% cHL conditioned medium (final concentration of FCS, 1%) or in RPMI-1640 containing 1% FCS (medium control) and, then fixed with methanol and stained with crystal violet.

cHL cells (2.0×10^5 cells/mL) were cultured for 72 h with increasing concentrations of maraviroc; then viable cells were counted using the trypan blue dye exclusion method. Clonogenic growth was assessed as previously described.⁴ Briefly, L-1236 (10^3 /mL), HDLM-2 (5×10^2 /mL), and L-540 (2.5×10^2 /mL) cells were suspended in 1 mL of RPMI-1640 medium containing 15% FCS and 0.8% methylcellulose (Methocel, Fluka) in the presence of E-MSC conditioned medium or E-monocyte conditioned medium, with or without maraviroc (50 μ M) or a neutralizing anti-CCL5 antibody (5 μ g/mL; clone 21418, R&D Systems) and seeded in 100 μ L aliquots (8 replicates) in 96-well flat-

bottomed microplates. After 14 days of incubation, plates were observed under phase-contrast microscopy, and aggregates with 40 cells were scored as colonies.

To study senescence, MSCs (1000/well) were cultured in serum-free medium for 15 days, with and without cHL conditioned medium (10%, v/v). β -galactosidase activity was evaluated with the Senescence Cells Histochemical staining kit (Sigma-Aldrich).

Flow cytometry

Surface expression of CD206 and PD-L1 was evaluated using PE-Cy5 Mouse Anti-Human CD206 and FITC Mouse Anti-Human PD-L1/CD274 (clone MIH1) antibodies (Becton Dickinson). Intracellular IDO expression was examined using a rabbit anti-IDO mAb (clone D5j4ETM; Cell Signaling Technology) followed by phycoerythrin (PE)-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories). Annexin-V (Becton-Dickinson Pharmingen) binding, staining with propidium iodide (PI) and 7-aminoactinomycin D (7-AAD; Becton-Dickinson) , and detection of activated caspase 3/7 with CaspaTag Caspase 3/7 (Chemicon International, Milan, Italy) were evaluated as previously described.⁵ CCR5 expression was analyzed using the anti-CCR5 mAb (clone 45531; R&D Systems). KG-1 cells (human acute myeloid leukemia) were used as a negative control for CCR5 expression. Cell cycle progression after a 24 h treatment with maraviroc (100 μ M) was evaluated by propidium iodide staining. These analyses were done on a FACScan flow cytometer (Becton Dickinson), using CellQuest software. Cell cycle was analyzed using Mod-Fit LT software (Verity Software House, Topsham (ME), USA).

ELISAs

Cytokines/chemokines were quantified using commercially available ELISA kits for CCL3, CCL4 and CCL5, M-CSF, IL-10 and CCL17/TARC (all from Immunological Sciences) and TGF- β 1 (Invitrogen).

Allogeneic lymphocytes treated with E-monocyte conditioned medium

Peripheral blood mononuclear cells (PBMC) were isolated from healthy donor blood by density gradient centrifugation on Ficoll-PaqueTM PLUS (GE Healthcare), and subsequently incubated with Pharm Lyse Lysing Buffer (BD Biosciences) for 5 min at 4 °C to minimize the contamination with red blood cells. Monocyte depletion was carried out by plastic adherence (2 h).

Lymphocytes (1×10^5 cells/well in 96-well dishes) were prestimulated for 1 h with 2 μ g/mL phytohemagglutinin (Sigma). Then, they were cultured with increasing concentrations of E-monocyte conditioned medium. After 72 h, cell growth was measured using the Cell Proliferation ELISA, Brd-U (colorimetric) kit (Sigma).

Synergy

cHL cells (2×10^5 /mL) were cultured for 72 h with one or more drugs in combination, and viable cells were counted by trypan blue dye exclusion. Synergy was determined by calculating the combination index (CI)⁶ using CalcuSyn software (Biosoft, Ferguson, MO, USA). The combined drug effect was calculated using the diagonal constant ratio combination. A CI value ranging from 0.9 to 1.1 indicates an additive effect between two drugs. CI values <0.9 indicate synergy, the lower the value the stronger the synergy. On the contrary, CI values >1.1 indicate antagonism.

Evaluation of heterospheroid formation by fluorescence microscopy and tumor cell purification

To monitor heterospheroid formation, cells were stained before they were placed in cocultivation in non-adherent conditions. For this purpose, MSCs were stained with the fluorescent dye Dil (red),

CD14+ monocytes with DiD (blue) and HDLM-2 cells with CellTrace CFSE Cell Proliferation Kit (green; all dyes from Thermo Fisher Scientific). After 24 h, images were acquired using a confocal microscope (Leica DM IRE2). To isolate single tumor cells, heterospheroids were trypsinized and cHL cells (CD30+) were recovered using CD30 microbeads (Miltenyi Biotec). Then viable cells were counted, analyzed for colony growth and cell cycle progression.

Tumor xenograft: immunofluorescence and survival analysis

Mice were killed (L-540 xenografts after 12 days of treatment; L-428 xenografts after 38 days of treatment) and tumors were excised and frozen in the Killik embedding medium (Bio Optica, Milano, Italy). Frozen tumor sections were stained with rat anti-mouse CD68 antibody (MCA1957GA, Bio-Rad), rabbit anti-human CD30 antibody (EPR4102, ABCAM) and TO-PRO-3 (Invitrogen). Specific Alexa Fluor-conjugated secondary antibodies were applied. Fluorescence intensity (CD68+) was quantified using Volocity software (PerkinElmer).

To study survival of mice bearing L-540 xenografts, in adherence of rules of the CRO Aviano committee on animal experimentation (OBPA) and of the Italian Ministry of Health, we used a predefined cutoff volume of 800 mm³ as a surrogate for mortality.

Statistical analysis

Statistical analysis was performed using GraphPad Prism v6 software. Student's *t* test was used to compare two groups, and one-way analysis of variance (ANOVA) was used for three or more groups; consecutive multiple comparisons were performed using Dunnett's or Tukey's test. Spearman's test was used to correlate CCL5 and CD68 expression. Survival was analyzed with Kaplan-Meier plots and log-rank test. Hazard ratios and 95% confidence intervals were calculated. **P*<0.05 indicated

statistical significance.

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- 6 **Chou TC, Talalay P. Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. Adv.Enzyme Regul. 1984;22:27-55.**

Supplementary Table S1. Clinical characteristics of 65 cHL patients, with a median age of 31 years (range, 6-83 years).

Characteristic		Patients, n (%)
Sex	Male	39 (65)
	Female	21 (35)
cHL subtype	Nodular sclerosis	51 (7)
	Mixed cellularity	5 (8)
	Nodular lymphocyte predominant	8 (12)
	Not otherwise specified	1 (2)
Epstein-Barr virus	Positive	19 (29)
	Negative	46 (71)
Stage	I/II	23 (61)
	III/IV	15 (39)
Bulk	No	31 (82)
	Yes	7 (18)
B symptoms	No	22 (58%)
	Yes	16 (42%)
Treatment	Chemotherapy	16 (41)
	Combined modality	21 (54)
	Radiotherapy	2 (5)

Supplementary Table S2. Combination index (CI) values for L-1236 and HDLM-2 cell lines treated with maraviroc (MVC) (first column) and with doxorubicin (Doxo) or brentuximab vedotin (BV) for 72 h.

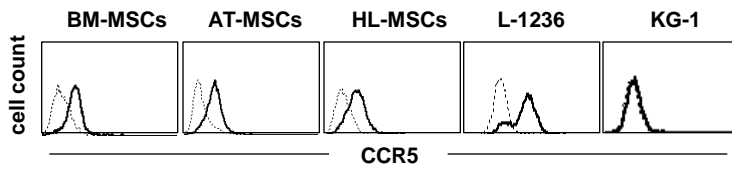
	MVC (μ M)	Doxo (ng/mL)	CI	BV (μ g/mL)	CI
L-1236	2.5	6.3	0.453	3.8	0.560
	5.0	12.5	0.714	7.5	0.421
	10.0	*25.0	0.623	*15.0	0.363
HDLM-2	2.5	3.1	0.408	75	0.509
	5.0	6.3	0.473	150	0.338
	10.0	*12.5	0.675	*300	0.441
L-1236	0.25	6.3	0.807	3.8	0.951
	0.50	12.5	1.130	7.5	0.852
	1.00	*25.0	1.125	*15.0	1.232
HDLM-2	0.25	3.1	1.105	75	1.332
	0.50	6.3	1.146	150	0.726
	1.00	*12.5	1.211	*300	0.948

Cell viability was determined by trypan blue dye exclusion. Combination indexes were calculated using CalcuSyn software.

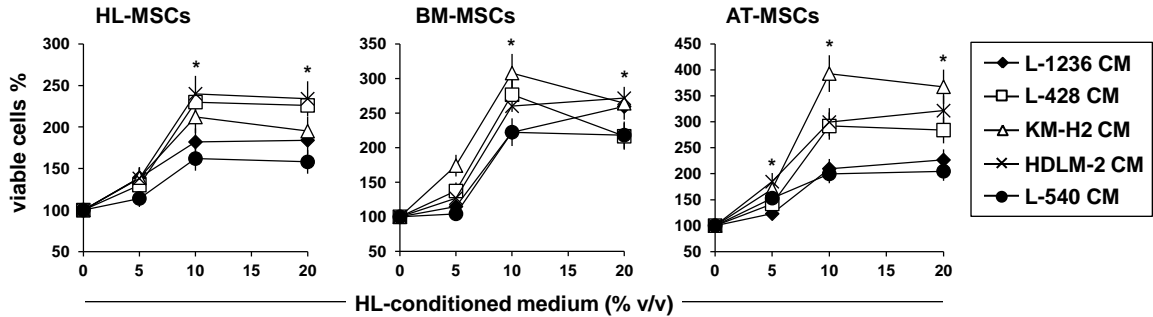
* indicates the IC₅₀ for each drug.

Supplementary Figure S1

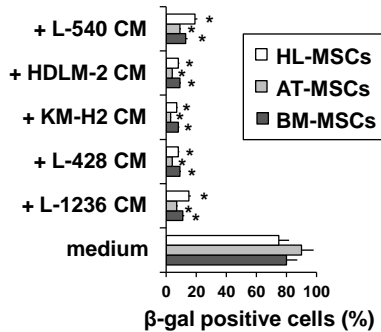
S1A



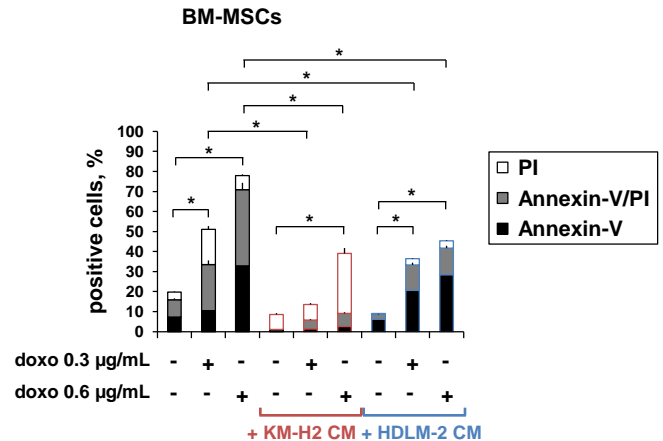
S1B



S1C

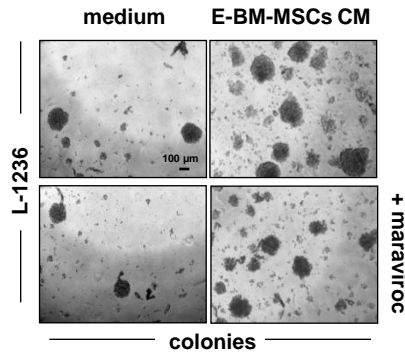


S1D

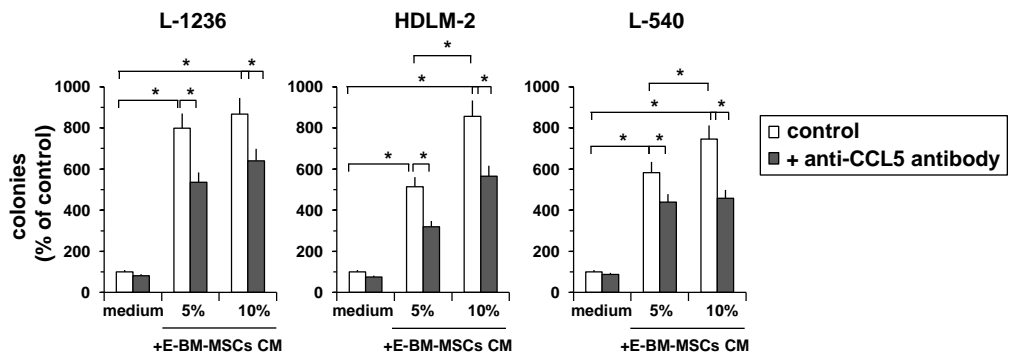


Supplementary Figure S1. CCR5 expression by MSCs and effects of cHL cell conditioned medium on MSC growth, senescence and doxorubicin cytotoxicity. (A) CCR5 expression was evaluated using the anti-CCR5 mAb (clone 45531; R&D Systems) in BM-MSCs, AT-MSCs, HL-MSCs, L-1236 cells and KG-1 cells (negative control). (B) BM-MSC, AT-MSC and HL-MSC (500/well) were cultured in 96-well plates in RPMI-1640 medium in increasing concentrations (percentage, v/v) of cHL conditioned medium (CM) that was replaced twice. After 6 days, growth was evaluated using the MTT assay. Results are means and SD of three replicate wells from three independent experiments. (C) Senescence-associated beta-galactosidase (β -gal) activity. Briefly, 1,000 MSCs were cultured in 24-well plates in RPMI-1640 medium in the absence or presence of 10% (v/v) cHL CM. After 15 days, β -gal-positive cells were counted. Three different experiments were evaluated. (D) BM-MSCs were treated with doxorubicin (doxo) (0.3 and 0.6 μ g/mL) in the presence or absence of 20% KM-H2 or HDLM-2 CM. After 6 days, cells were harvested, double stained with annexin-V-FITC and propidium iodide (PI) and analyzed by flow cytometry. Bar charts show the percentages of stained cells. Values are means and SD of three experiments.

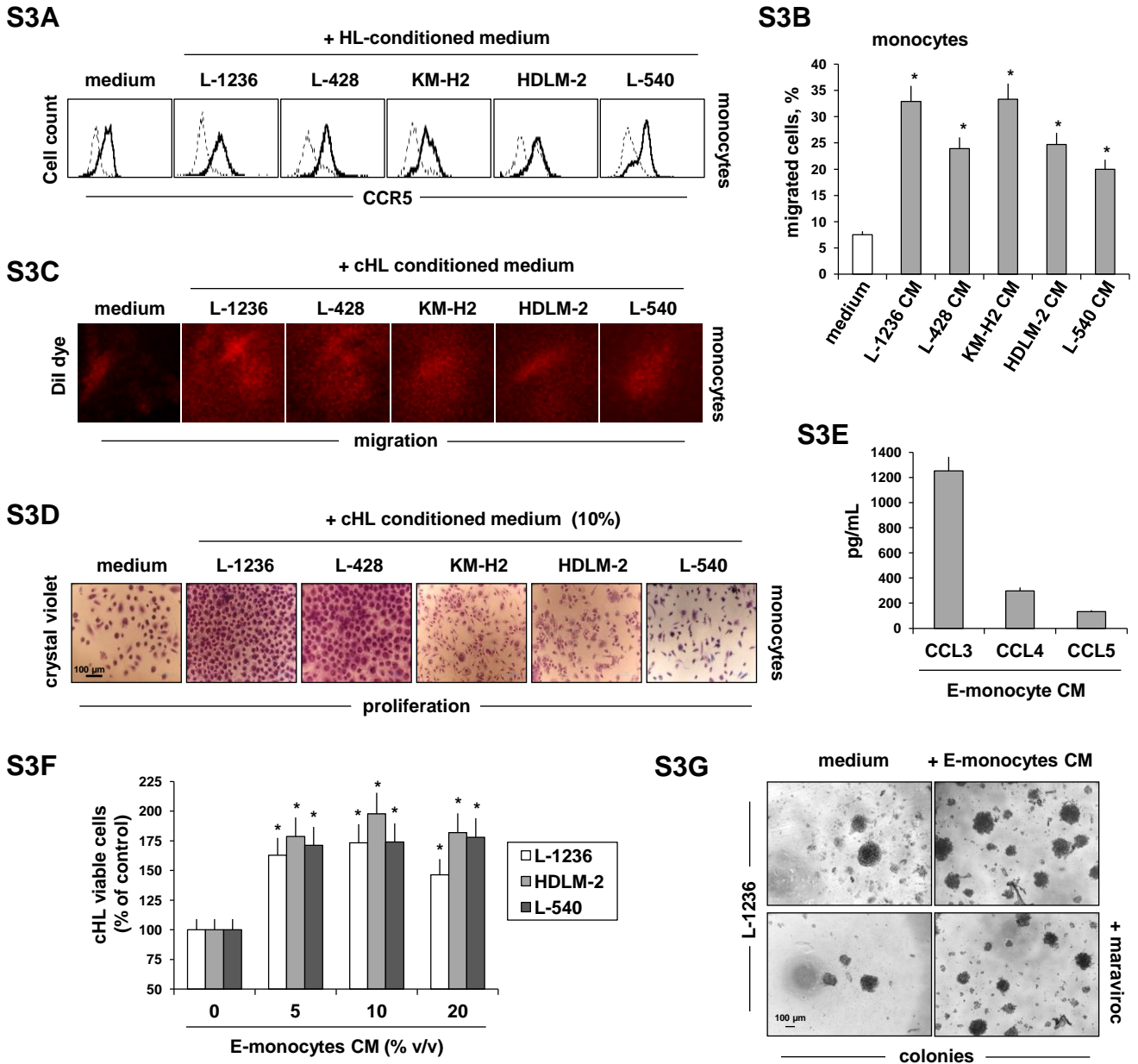
S2A



S2B

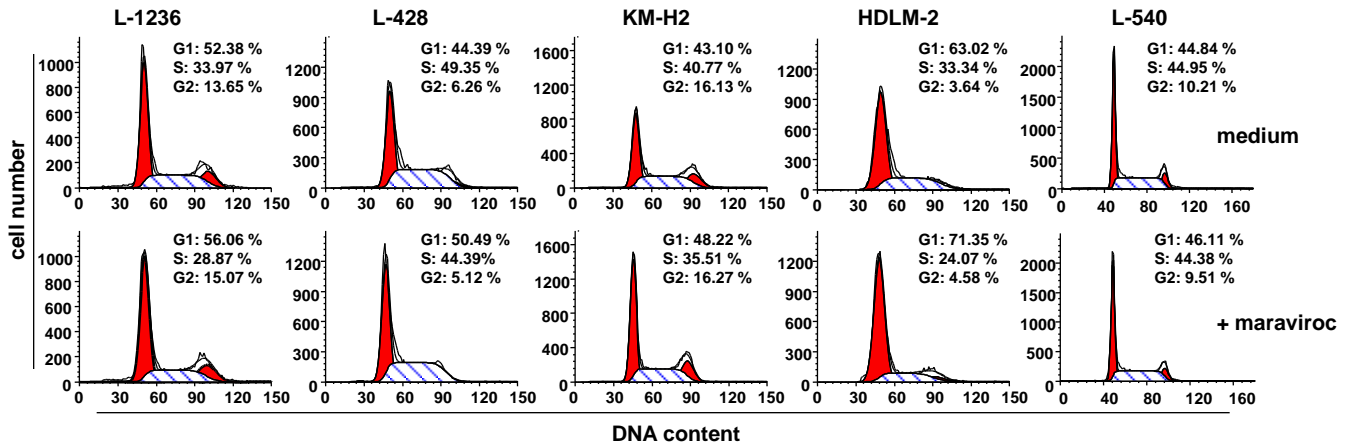


Supplementary Figure S2. Maraviroc and an anti-CCL5 antibody reduce the clonogenic growth of cHL cells enhanced by MSC CM. (A) Clonogenic growth. L-1236 cells ($10^3/\text{mL}$) were cultured in methylcellulose-containing medium in the presence or absence of E-BM-MSC conditioned medium (CM), with or without maraviroc ($50 \mu\text{M}$). After 14 days, colonies were photographed using an inverted microscope (Eclipse TS/100, Nikon). **(B)** L-1236 ($10^3/\text{mL}$), HDLM-2 ($5 \times 10^2/\text{mL}$), and L-540 ($2.5 \times 10^2/\text{mL}$) cells were cultured in methylcellulose medium in the presence or absence of E-BM-MSC CM, with or without a neutralizing anti-CCL5 antibody ($5 \mu\text{g}/\text{mL}$). Bar charts show the percentages of colonies normalized to control samples. Three different experiments were evaluated.

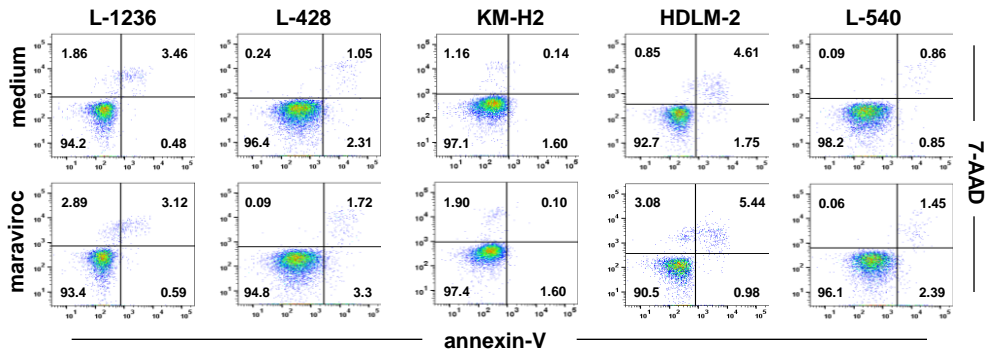


Supplementary Figure S3. Effects of cHL CM on monocyte migration, proliferation, CCR5-ligand secretion and of E-monocytes CM on cHL growth. (A) Purified CD14⁺ monocytes were cultured for 6 days with 20% CM from L-1236, L-428-, KM-H2, HDLM-2, and L-540 cells, inducing monocytes into becoming E-monocytes. CCR5 expression was evaluated by flow cytometry. **(B)** 1 h migration of human CD14⁺ monocytes across fibronectin-coated Boyden chambers towards cHL CM. CM from different experiments were evaluated. **(C)** Representative photomicrographs of Boyden chamber membranes showing transmigrated CD14⁺ monocytes stained with red fluorescent dye Dil. **(D)** Monocytes (2.0×10^4 /well) were cultured in 96-well flat-bottomed plates and exposed to increasing concentrations (%) of cHL CM that was replaced twice. After 6 days, monocytes were fixed with methanol and stained with crystal violet. **(E)** Monocytes were cultured with 20% L-1236 CM for 6 days. The medium was changed and, after another 3 days, E-monocyte CM was recovered. CCL3, CCL4 and CCL5 release was evaluated by ELISA. **(F)** HDLM-2, L-1236, and L-540 cells (2.5×10^3 /mL) were treated for 9 days with E-monocyte CM, replaced twice. Viable cells were evaluated by CellTiter 96 AQueous One Solution Cell Proliferation Assay (MTS) (Promega). Values are means and SD of three experiments. **(G)** Clonogenic growth in methylcellulose-containing medium. L-1236 cells were cultured in the presence of E-monocyte CM (5%) and treated or not with maraviroc (50 μ M). Colonies were counted and L-1236 cells were photographed using an inverted microscope.

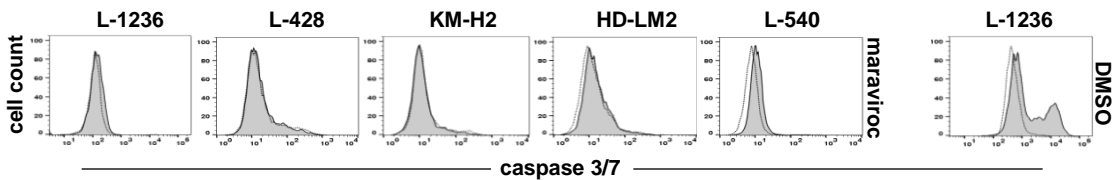
S4A



S4B

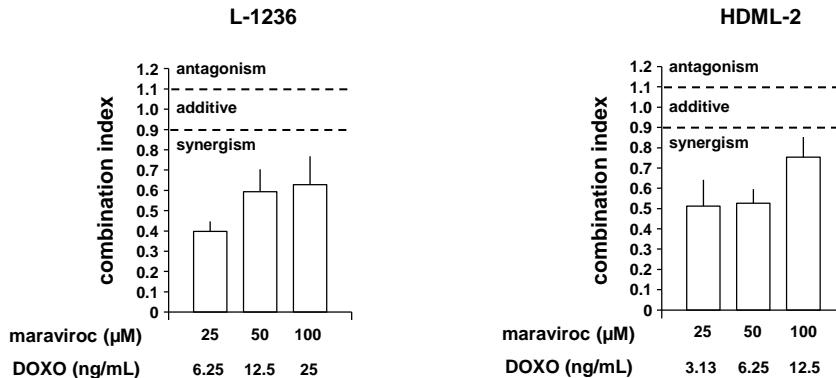


S4C

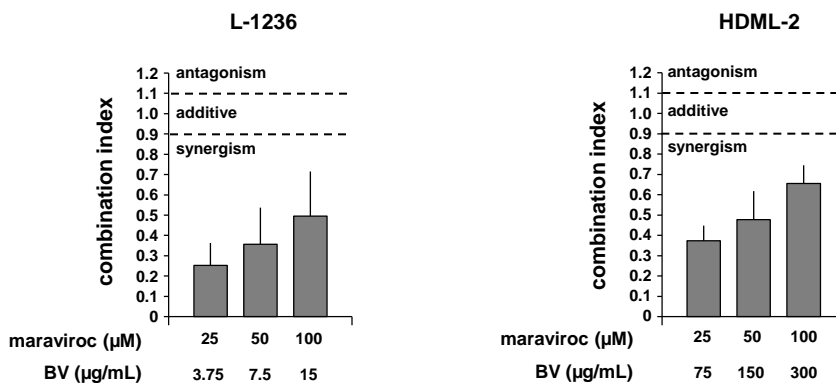


Supplementary Figure S4. Maraviroc slightly increases the percentage of cHL cells in G1 phase but does not induce apoptosis. (A) Representative cytofluorimetric histograms of the cell cycle progression. cHL cells (2.0×10^5) were treated for 24 h with maraviroc (100 μ M), and then stained with propidium iodide (PI) and analyzed by flow cytometry. **(B, C)** cHL cells were treated for 72 h with maraviroc (100 μ M) and **(B)** then doubly stained with annexin-V-FITC and 7-AAD or **(C)** resuspended in complete medium supplemented with CaspaTag Caspase 3/7 (Chemicon) for 1 h at 37°C to evaluate caspase 3/7 activation by flow cytometry. L-1236 cells treated with DMSO were used as positive control.

S5A

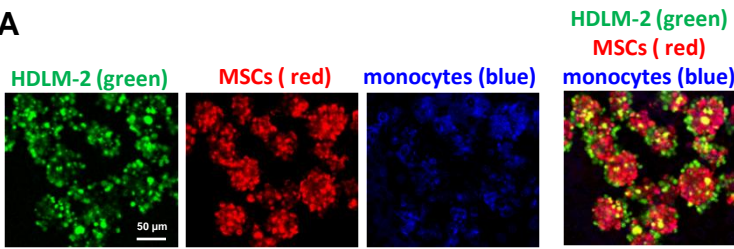


S5B



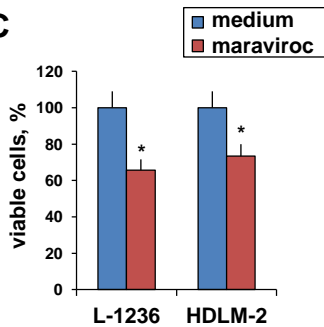
Supplementary Figure S5. Drug combination curves of maraviroc with doxorubicin or brentuximab vedotin. L-1236 and HDLM-2 cells were cultured with increasing concentrations of maraviroc and **(A)** doxorubicin (DOXO) or **(B)** brentuximab vedotin (BV), alone or in combination. Viable cells were evaluated by trypan blue dye exclusion after 72 h. Synergy was determined using CompuSyn software (CI, combination index). Values of bar charts are mean CI values and SD of three experiments each run in triplicate.

S6A

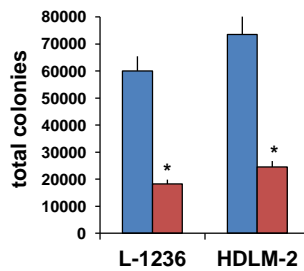


3D heterospheroids

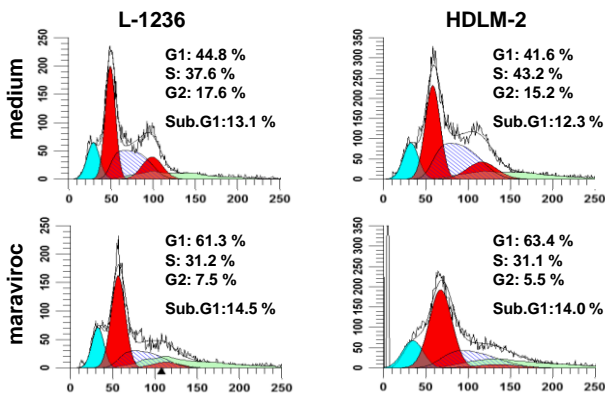
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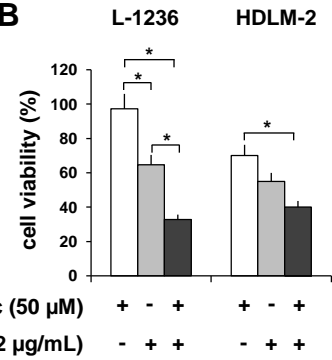
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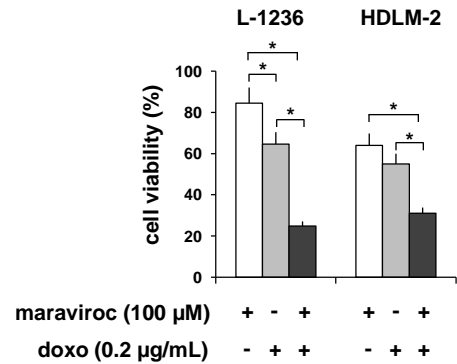
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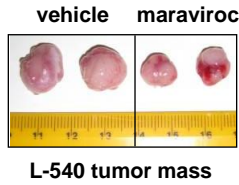
MVC (μM)	Doxo (μg/mL)	CI L-1236	CI HDLM-2
50	0.1	0.669	0.607
50	0.2	0.321	0.453
50	0.4	0.335	0.567



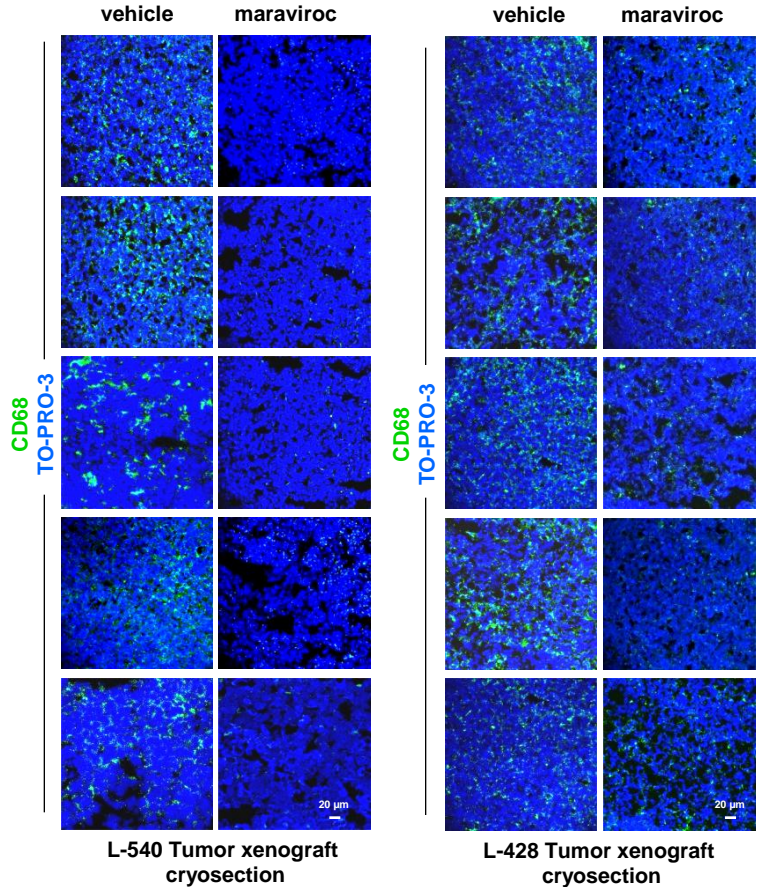
MVC (μM)	Doxo (μg/mL)	CI L-1236	CI HDLM-2
100	0.1	0.588	0.629
100	0.2	0.418	0.271
100	0.4	0.46	0.293

Supplementary Figure S6. Effects of maraviroc on heterospheroids. (A) 3D heterospheroids were generated by culturing HDLM-2 cells (stained green with CFSE), MSCs (red with fluorescent dye DiI), and monocytes (blue with DiD) under non-adherent conditions on poly-HEMA-coated wells. Photomicrographs were acquired using a confocal microscope (Leica DM IRE2). (B) Maraviroc-doxorubicin drug combination was assessed in heterospheroids generated with L-1236 cells, HL-MSCs and monocytes (2.0×10^3 cells/well; 96-well plates) in RPMI-1640 medium plus 1% FCS with increasing concentrations of doxorubicin (doxo) and either 50 μM (upper panel) or 100 μM (lower panel) maraviroc, alone or in combination. After 6 days, cell viability was evaluated using PrestoBlue Cell Viability kit. Values are means and SD of three experiments each run in triplicate. Synergy was determined using CompuSyn software (CI, combination index). CI values <0.9 indicate synergy. (C) Viable L-1236 or HDLM-2 cells isolated from heterospheroids (also containing HL-MSCs and monocytes) after a 48 h treatment with maraviroc (100 μM). Values are percentages normalized to untreated samples. (D) Total number of colonies formed in methylcellulose-containing medium from cHL cells recovered from heterospheroids. (E) Representative flow cytometric plots showing cell cycle progression of L-1236 or HDLM-2 cells purified from heterospheroids treated or not for 48 h with maraviroc (100 μM).

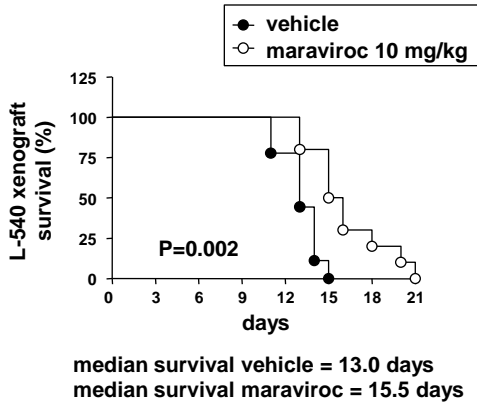
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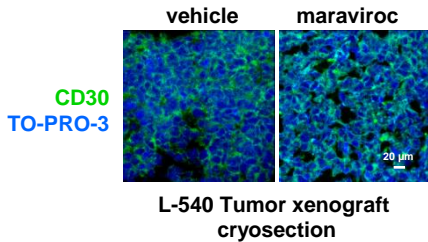
S7D



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S7C



Supplementary Figure S7. Maraviroc slows tumor xenograft growth, enhances mice survival and reduces TAM infiltration. (A) Representative photographs of tumors excised from mice bearing L-540 xenografts treated or not with 10 mg/kg maraviroc. (B) Kaplan-Meier survival analysis of mouse xenograft models of L-540 cHL cells treated with 10 mg/kg maraviroc. Survival was estimated using a predefined cutoff of 800 mm³ as a surrogate (log-rank test). (C) Immunofluorescence staining of cHL cells (CD30+) in L-540 tumor xenograft cryosections from vehicle-treated and maraviroc-treated mice. Nuclei were stained with TO-PRO-3 dye. Images were acquired using a confocal microscope (Leica DM IRE2). (D) Immunofluorescence staining of intratumoral TAMs (CD68+ cells) in tumor cryosections from vehicle-treated and maraviroc-treated L-540 and L-428 xenograft mice. Nuclei were stained with TO-PRO-3 dye.