SUPPLEMENTARY APPENDIX

CD40 signaling instructs chronic lymphocytic leukemia cells to attract monocytes via the CCR2 axis

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Monocyte isolation and *in vitro* differentiation

Monocytes for differentiation experiments were obtained by isolation from HDs after obtaining written informed consent. To this end, PBMCs were isolated using ficoll gradient purification according to manufacturer's instructions, after which monocytes were separated from peripheral blood lymphocytes using percoll gradient purification (GE healthcare, Milwaukee, USA). Next, monocytes were incubated to adhere at 37°C in 5% CO_2 for 40 min at a concentration of $0.75*10^6$ cells/mL in 6-well plates (3 mL) in IMDM supplemented with 1% Fetal Bovine Serum and washed to remove non-adherent cells. The monocytes were then differentiated using $1.5*10^6$ CLL cells/mL in $IMDM^{+/+}$ or 200 ng/mL NAMPT in $IMDM^{+/+}$ for 72 h. Alternatively, monocytes were differentiated using IMDM-/- supplemented with 25% serum from different CLL patients or 25% pooled serum from HDs (Human Serum Type AB, Sigma) for 72 h. Control monocytes were differentiated to either M1 using 10 ng/mL IFN-Y or M2 using 10 ng/mL IL-4 (both R&D systems, Minneapolis, MN, USA) in $IMDM^{+/+}$. Next, the differentiated macrophages were removed from the plates using 80 mM Lidocaine (Sigma) in PBS/10 mM EDTA (Merck, Darmstadt, Germany). They were then stained for the indicated markers using CD80-FITC (eBioscience), CD163-PE (Beckton Dickinson Biosciences [BD], San Jose, CA), CD206-APC (BD), or relevant isotype controls, after which fluorescence signals were measured on a FACS Canto II (BD). Analysis was then performed using FlowJo software (TreeStar, San Carlos, CA, USA). For cell viability, cells were analyzed by flow cytometry using a Dioc6-PI staining as described before[23].

LN material and Immunofluorescence

Four-micron sections from paraffin-embedded whole LN extirpations (N = 9) were obtained from the AMC Pathology department. All material was derived from either untreated patients or patients at least 3 months after chemotherapy. No patients received kinase inhibitor therapy. Localization of CLL was proven by standard CD5/CD19/CD20/CD3 immunohistochemistry as performed by standard diagnostic pathology work-up. For immunofluorescence, sections were de-waxed by immersion in xylene and hydrated by serial immersion in ethanol and PBS. Antigen retrieval was performed by heating sections for 20 min in sodium citrate buffer (10 mM sodium citrate, 0.05% Tween20, pH 6.0). Sections were washed with PBS (2 x 10 min) and blocking buffer (TBS containing 10% BSA and 0.3% Triton X-100) was added for 1 h. For triple stainings, sections were incubated with primary antibody, anti-CD20 (eBioscience, 14-0202-80), anti-CD68-biotin (eBioscience, 13-0687-80) and anti-CD3 (Santa Cruz, sc-59008), in blocking buffer overnight at 4°C. Subsequently, the slides were washed with PBS (2 x 10 min) and incubated with Alexa Fluor 488 labeled goat anti-mouse and Alexa Fluor 647 labeled goat anti-rat

antibodies (Invitrogen) for 1 hour, after which the slides were washed with PBS. Subsequently, slides were incubated with streptavidin conjugated to Alexa Fluor 594 (Invitrogen) for 1 hour. Next the slides were stained for 10 minutes with DAPI (0.1 μg/mL in PBS). For the macrophage differentiation stainings, sections were incubated with primary antibody, pan-macrophage marker CD68 (clone PG-M1, DAKO, Carpinteria, CA, USA) in combination with either M1 marker CD80 (ab134120, Abcam, Cambridge, MA, USA) or M2 marker CD206 (ab64693, Abcam) in SignalStain (Cell Signaling) or blocking buffer, respectively, overnight at 4°C. Subsequently, the slides were washed with PBS (2 x 10 min) and incubated with goat anti-rabbit Alexa594 or goat anti-mouse Alexa488 (both Life Technologies, Carlsbad, CA, USA) for 1 h, after which the slides were stained for 10 min with DAPI (0.1 μg/mL in PBS). Sections were mounted with Fluoromount-G (eBioscience) and immunofluorescent imaging was performed using a Leica DMRA fluorescence microscope equipped with a cooled camera. Images were acquired using Image Pro Plus and composed in Adobe Photoshop CS3. For signal quantification, a Cell Profiler (http://cellprofiler.org) pipeline was created to measure the red intensity (CD80/CD206) of green cell objects (CD68), using "IdentifyPrimaryObjects" for identification with automatic thresholding and "shape" as distinction method. Averages of these per-cell intensities were subsequently calculated in R (https://www.r-project.org/).

rhCD40L stimulation and intracellular CCL2 measurements

CLL cells were stimulated for 48 hours with CD40L expressing NIH3T3 fibroblasts, 100 ng/mL recombinant multimeric CD40L (Adipogen AG, Liestal, Switzerland), or PBMCs that were activated with anti-CD3/CD28 (Sanquin, Amsterdam, the Netherlands) for 72 hours prior to co-culture. Cells were treated with 10 μg/ml brefeldin A (Sigma) and monensin (BD, San Jose, CA, USA) for 6 hours, followed by surface staining with antibodies directed against: CD3 (ThermoFisher, Waltham, MA, USA), CD5 (BD), CD19 (BD), CD20 (BD), CD95 (BD). Subsequently, CLL cells were fixed and permeabilized using BD cytofix/cytoperm and stained intracellularly with anti-CCL2 (ThermoFisher). Fluorescence signals were then measured on a LSR Fortessa and analyzed using FlowJo.

Statistical analysis

Two-way analysis of variance (ANOVA) with Bonferroni post-hoc tests were performed to test for significant differences between multiple groups using Graphpad Prism software (Graphpad, La Jolla, CA, USA). A (paired) t-test was used to test for significant differences between two groups. *P* values <.05 (*),

<.01 (**) , <.001 (***) and <.0001 (****) were considered statistically significant. Error bars represent standard error of the mean (*SEM*).

(A) Ex vivo samples

ID#	Sample#	Figure	Age (years)	WBC count x10^9/L	RAI stage	CD3 (%)	IGHV status	Chromosomal aberrations	Last therapy
01	12-09835	1B	74	11	2				
02	96-12335	S1	58	n.d.	n.d.				
03	98-03391		68	7	1				
04	05-02147		63	84	1				
05	08-09306		68	31	1				
06	09-01534		83	n.d.	n.d.				
07	09-08848		52	5	1				
08	10-14157		66	6	1				
09	11-13183		53	20	1				

(B) In vitro samples

01	49	1E	54	21	2	1	M	12+	n.d.
02	70		65	16	0	4	P	none	n.d.
03	74		63	40	1	4	M	n.d.	CAB
04	135		63	15	n.d.	8	M	13q-	none
05	163		41	182	n.d.	10	U	12+,13q-	F + R
06	224		44	102	2	4	U	n.d.	none
07	302		59	155	n.d.	3	M	13q-	F + C + R
08	455		65	117	1	2	M	n.d.	CAB
09	912		77	40	0	8	U	12+	none
10	927		58	7	n.d.	11	U	13q-	F
11	1049		74	40	0	7	M	n.d.	none
12	1072		27	160	n.d.	2	U	11q-,13q-	none
13	1119		79	12	2	10	n.d.	n.d.	none
14	1157		56	18	0	9	M	13q-	none
15	1167		58	170	2	1	M	13q-	F + CAB
16	1171		73	23	0	5	M	n.d.	none
17	1174		59	124	1	4	n.d.	12+,17p-	CHOP
18	1233		79	100	0	6	U	12+	none
19	1235		68	134	1	3	n.d.	11q-	F + R
20	1251		75	120	n.d.	3	n.d.	n.d.	none
21	1317		70	422	n.d.	1	U	17p-	F + C + R
22	1331		76	112	0	4	M	n.d.	none
23	1407	1F,2,3	78	216	n.d.	1	n.d.	n.d.	none
24	1408		63	161	0	3	n.d.	n.d.	none
25	1465		65	124	0	3	M	13q-	none
26	1177		75	29	0	6	M	none	none
27	1200		77	28	0	5	n.d.	n.d.	none
28	1266		74	17	0	7	n.d.	n.d.	none
29	1314		77	28	0	5	n.d.	n.d.	none
30	1322		87	39	0	8	n.d.	11q-	none
31	1353		86	28	n.d.	5	M	none	CAB
32	1357		76	38	0	8	M	n.d.	none
33	1365		73	34	1	11	n.d.	n.d.	CAB
34	1605		68	21	0	5	n.d.	n.d.	n.d.

Figure S1: Additional Immunofluorescence stainings for macrophage differentiation markers

(A) 8 additional CLL LN, 1 control Systemic lupus erythematosus (SLE), and 1 HD reactive LN slides were stained as in Figure 1B. Yellow scale bars correspond to 20 μm. Note the $CD206^+$ high endothelial venule structures in some sections.

(B) Example gating strategy used for the quantification of flow cytometry data presented in Figure 1E.

(C) Monocytes were differentiated and stained as in Figure 1E, this time using 72 h conditioned medium from CLL cells.

Figure S2: CLL cells stimulated by autologous T cells induce monocyte migration and T cells only do not induce migration

(A) After 72 h stimulation of T cells using α-CD3/α-CD28 antibodies (see methods) expression levels of CD40L on $CD3^+$ cells were determined using flow cytometry. The geometrical mean (Geomean) of N=4 stimulated T cell samples compared to unstimulated samples is shown.

(B) Autologous T cells from CLL patient samples were separated from CLL cells by positive CD4/CD8 magnetic selection to a purity of around 80% CD3+ cells. Co-cultures using both fractions were performed as indicated, all in presence of α-CD3/α-CD28 antibodies. Freshly isolated HD monocytes were then seeded in the upper chambers of a trans-well migration plate to migrate towards these conditioned media and migration was quantified as in Figure 2A.

(C) For each of the 3 experiments presented in Figure 2A, the average migration was calculated for each condition and compared to the migration induced by T cells only. PBMCs used as a T cells source were not enriched for T cells (left panel). The migration effect induced by CLL cells after T cell stimulation presented in Figure S2A was compared to the migration effect of T cells only. In this experiment, T cells were enriched to around 80% (right panel).

Figure S3: The CCR2 inhibitor INCB3284 has no cytotoxic effect on CLL cells after extended culture

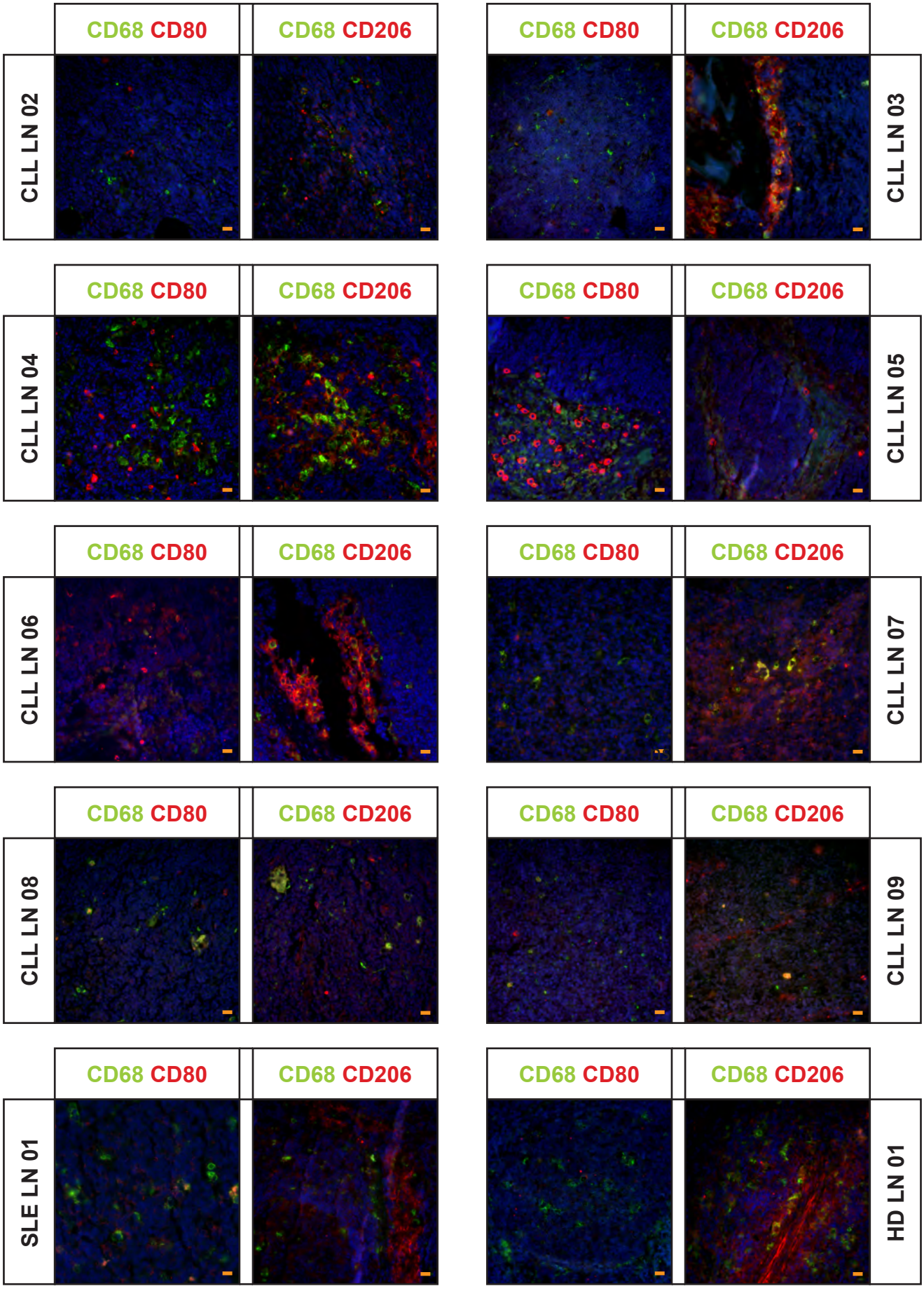
(A) CLL cells were cultured in the presence or absence of 1 μg/mL CCR2 inhibitor INCB3284 and viability was measured after 72h using a Dioc6-PI staining. ns, not significant using a paired t-test.

(B) CLL cells (N=3) were treated with 100 ng/mL recombinant multimeric CD40L for 48 h or left untreated and CCL2 expression was determined by intracellular flow cytometry staining.

Table S1: Characteristics of CLL patients that provided samples for this study

(A) Sample ID, age, white blood cell (WBC) count, and RAI stage are given for the CLL patients used for the LN stainings in Figure 1B and S1. Sample ID numbers match with the numbers used in the Figures. If multiple lab results were available for one patient, the lab results that matched closest in time with the LN extirpation were taken. (B) For the PBMC samples used in *in vitro* experiments, percentage CD3 cells, IGHV mutation status, chromosomal aberrations and last therapy are provided in addition. Each patient was treatment-free for at least 3 months. The Figures for which samples were used are also indicated. A minus sign indicates chromosomal deletion, a plus sign a duplication, M: mutated, U: unmutated, P: polyclonal. F: Fludarabine, C: Cyclophosphamide, R: Rituximab. CAB: Chlorambucil. CHOP: cyclophosphamide + doxorubicin + vincristine + prednisolone. n.d.: not determined/unknown.

A


CD68 CD80
CD68 CD206
CLL LN 02
CLL LN 03
CLL LN 04
CLL LN 05
CLL LN 06
CLL LN 07
CLL LN 08
CLL LN 09
SLE LN 01
HD LN 01

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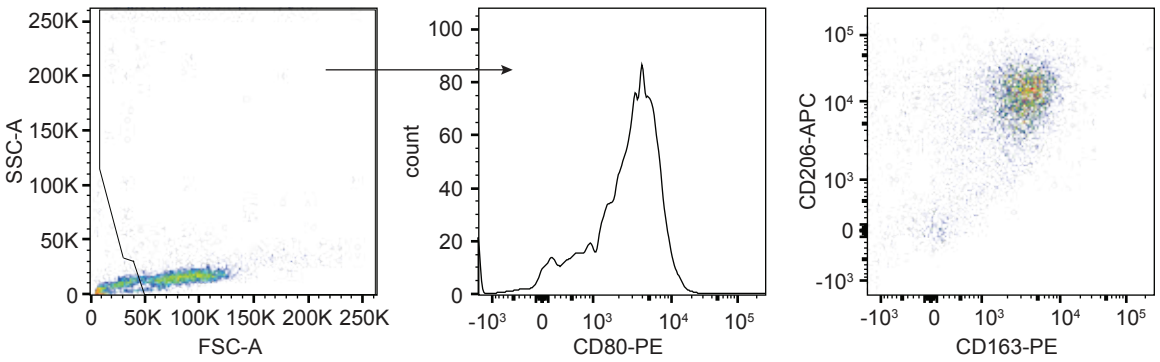
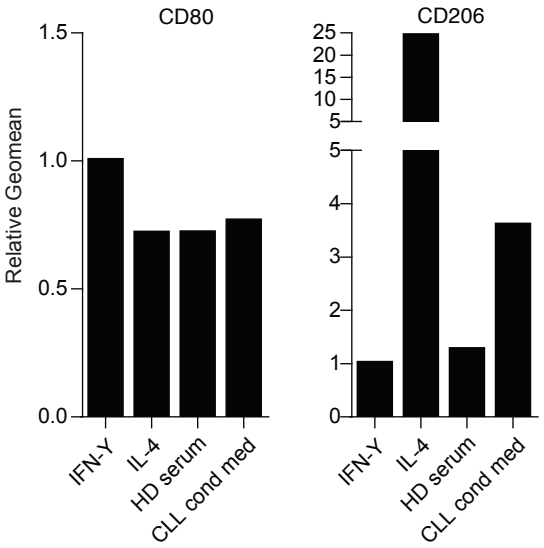
B



C



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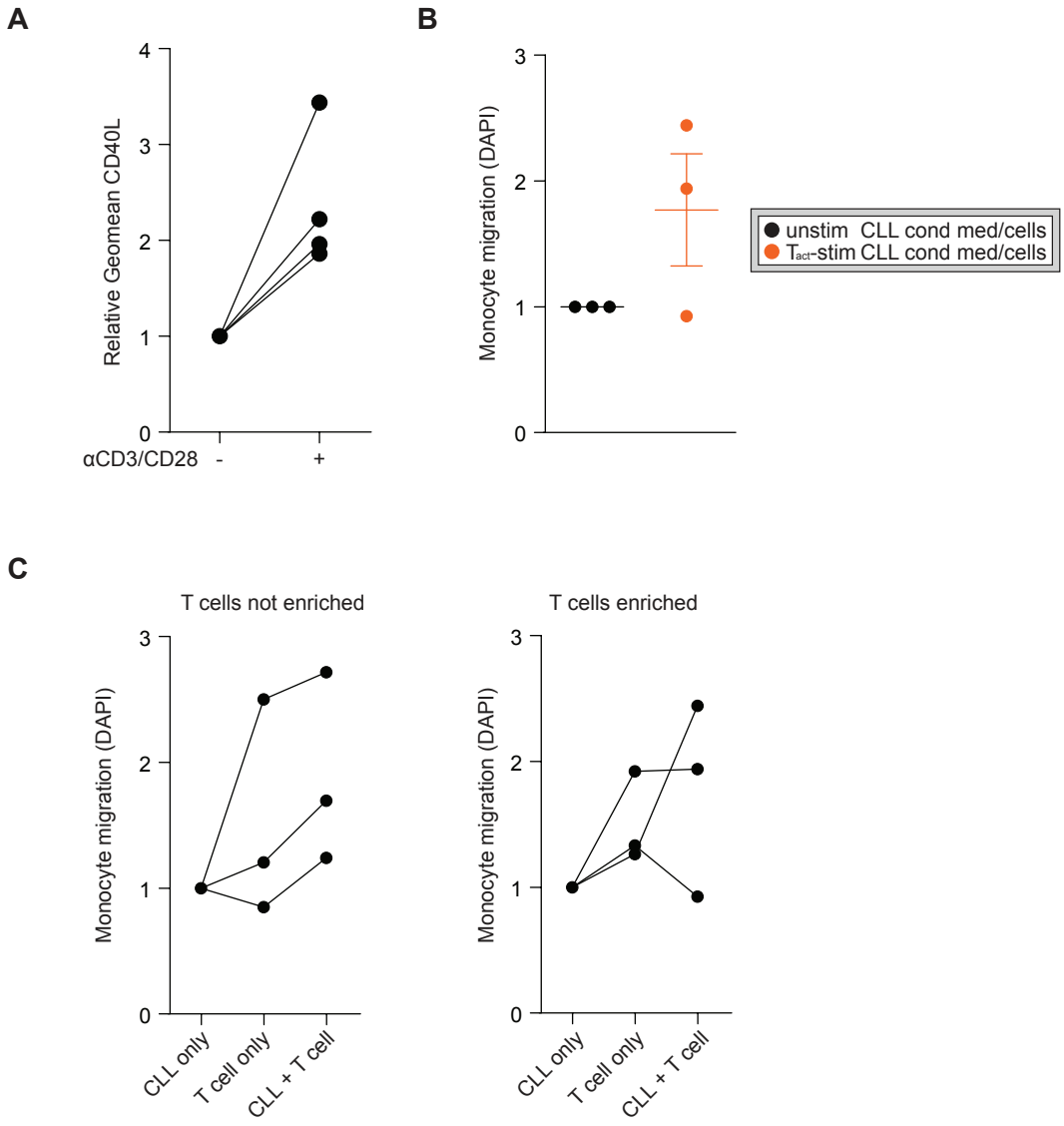

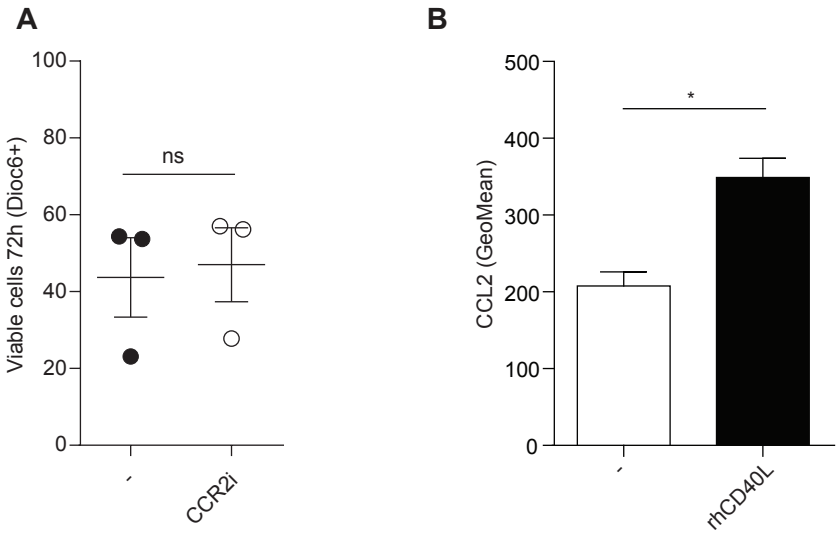
A
Viable cells 72h (Dioc6+)
100
80
60
40
20
0
ns
-
CCR2i
B
CCL2 (GeoMean)
500
400
300
200
100
0
*
-
rhCD40L