SUPPLEMENTARY APPENDIX

CD36 defines primitive chronic myeloid leukemia cells less responsive to imatinib but vulnerable to antibody-based therapeutic targeting

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SUPPLEMENTARY INFORMATION TO

CD36 defines primitive chronic myeloid leukemia cells less responsive to imatinib but vulnerable to antibody based therapeutic targeting

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Supplementary Material and Methods

Cell growth and colony forming capacity upon leptin stimulation

The t(9;22)-positive cell lines KU812, BV173, LAMA84 and K562 (DSMZ, Germany) were cultured in RPMI medium supplemented with fetal bovine serum (Gibco, USA) at concentrations according to manufacturers instructions, or StemSpan (Stemcell Technologies, Canada) in 48-well or 96-well plates. For leptin stimulation assays, cultures were supplemented with 100ng/ml or 200ng/ml leptin (Peprotech, USA). Cell growth was evaluated with CountBright Absolute Counting Beads (Thermo Fisher Inc) and DAPI (4',6-Diamidine-2'-phenylindole dihydrochloride; Sigma-Aldrich, USA) as a marker for viability. Analyses were performed on a LSR Fortessa (BD Bioscience, USA). To investigate colony forming capacity, cells were seeded at 1000 $CD34^+$ cells per 1.1 ml of semisolid MethoCult H4434 medium (Stemcell Technologies) and replated after 2 and 4 weeks. Colonies were scored after 2, 4 and 6 weeks.

Fluorescent in situ hybridization (FISH) on primary CML cells sorted by FACS

Primary CML samples were sorted directly onto FISH slides, as previously described.[1] *BCR/ABL1* was detected using dual color, dual fusion *BCR/ABL1* probes (Abbott Molecular Inc, USA). All available cells were scored as *BCR/ABL1* positive, negative or inconclusive, the inconclusive group representing <1% of all cells scored. A mean of 280 cells per patient were scored.

REFERENCES

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Supplementary Table 1. CML patient characteristics

											BCR/ABL1 IS %			CD34+CD38low		
Patient #	Diagnosed	Gender	Age	Country	PB/BM	Treatment	Sokal	Hb (g/l)	Leu (10^9/l)	Trc (10^9/l)	dg	3mo	12mo	IL1RAP (%)	CD36 (%)	LEPR (MFI)
1	2014	F	46	SE	PB	Bosutinib	1,03	N/A	47,6	535	107,847	3,136	0,029	93	84	386
2	2014	F	32	SE	PB	Bosutinib	1,68	N/A	69	1286	16,920	0,009	N/A	60	72	
3	2015	F	65	FI	BM	Imatinib	1,01	N/A	117	526	N/A	N/A	N/A	88	60	218
4	2015	F	58	NO	PB	Imatinib	2	N/A	263,4	386	93,287	N/A	N/A	91	71	263
5	2015	M	54	NO	BM	Bosutinib	0,86	N/A	57,1	700	93,880	0,265	0,009	67	46	136
6	2015	F	55	NO	BM	Imatinib	0,78	N/A	71,3	298	92,555	23,141	0,363	97	59	180
7	2015	F	55	SE	BM/PB	Imatinib	1,09	N/A	127,1	762	22,803	3,663	N/A	90	76	299
8	2015	M	71	FI	PB	Imatinib	0,85	N/A	83,6	245	63,690	41,432	0,009	4	6	
9	2015	N/A	N/A	DK	BM	Imatinib	N/A	N/A	N/A	N/A	N/A	N/A	N/A	73	46	
10	2015	F	51	FI	BM	Imatinib	0,66	N/A	23,3	345	85,243	8,478	0,075	34	15	
11	2015	M	73	SE	BM	Imatinib	1,66	N/A	10,9	963	12,614	1,974	0,133	53	82	155
12	2015	M	52	NL	BM	Imatinib	0,65	N/A	143	274	104,142	28,653	32,845	3	2	
13	2015	M	53	NL	BM	Bosutinib	0,87	N/A	55,6	670	76,735	1,221	0,049	25	20	
14	2015	F	62	DK	BM	Bosutinib	0,78	N/A	55,3	474	78,990	N/A	N/A	30		
15	2015	F	77	FI	BM	Bosutinib	0,94	N/A	30,8	517	24,272	N/A	N/A	20		
16	2014	M	59	DK	BM	Dasatanib	2,78	97	306,9	514	49,720	3,571	0,010	97		
17	2014	M	69	NO	BM	Dasatanib	1,59	102	40,2	495	15,944	0,010	0,000	97		122
18	2014	M	45	NO	PB	Dasatanib	1,64	77	270	472	52,266	10,949	0,031	96		374
19	2014	M	19	NO	PB	Dasatanib	0,82	88	347,1	148	44,510	4,001	0,029	97		
20	2013	M	49	SE	BM	Dasatanib	0,69	156	76,6	269	23,195	2,592	0,007	57	20	
21	2009	F	51	SE	BM	Dasatanib	0,82	118	40	847	N/A	0,781	0,000	87		
22	2009	F	40	SE	BM	Dasatanib	0,67	137	11,4	706	N/A	0,008	0,000	82		
23	2010	M	71	SE	BM	Imatinib	0,93	123	37	275	N/A	4,375	0,279	89		
24	2010	M	55	SE	BM	Imatinib	0,75	141	65,1	462	N/A	1,366	11,239	92		
25	2010	F	59	SE	BM	Imatinib	0,8	111	97	401	N/A	89,587	22,889	88		
26	2011	F	87	SE	BM	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	80		
27	2012	M	71	SE	BM	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	90		
28	2013	M	73	SE	BM	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	90		
29	2013	F	67	SE	BM	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	70		
30	2013	M	42	SE	BM	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	80		
31	2016	N/A		SE	PB	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	91	76	197
32	2004	N/A	79	SE	BM	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	45		
33	2003	N/A	36	SE	BM	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	93		
34	2003	N/A	N/A	SE	BM	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	86	74	

Abbreviations: N/A, not available; F, female; M, male; SE, Sweden; FI, Finland; NO, Norway; DK, Denmark; NL, Netherlands; Hb, hemoglobin; Leu, leukocytes; Trc, thrombocytes dg, diagnosis; 3mo, 3 months; 12mo, 12 months; RNA-Seq, RNA sequencing.

Supplementary Table 2. Antibodies used in flow cytometry

Target	Fluorochrome	Company	Isotype
CD34	AF488	BioLegend	mouse IgG1
CD38	BV786	BD	mouse IgG1
CD123	AF488	BioLegend	mouse IgG1
CD45RA	PE-Cy7	BD	mouse IgG2b
CD90	PerCP-Cy5.5	BD	mouse IgG1
* IL1RAP	Biotin	RnD Systems	mouse IgG1
CD25	BV421	BD	mouse IgG1
CD26	FITC	EBioScience	mouse IgG1
NCAM1	PE	BD	mouse IgG1
CD36	PE	BD	mouse IgM
CD36	Biotin	Innovagen	polyclonal rabbit
CD36	-	Innovagen	polyclonal rabbit
LEPR	AF647	BD	mouse IgG2b
LEPR	PE	RnD Systems	mouse IgG2b
LEPR	Biotin	Innovagen	polyclonal rabbit
LEPR	-	Innovagen	polyclonal rabbit
CD93	PE	BioLegend	mouse IgG1
ITGB3	PE	BD	mouse IgG1
TFRC	PE	BD	mouse IgG2a
IL12RB1	PE	BD	mouse IgG1
TYRO3	PE	RnD Systems	mouse IgG1
TNFRSF18	PE	BioLegend	mouse IgG1
CD7	PE	BD	mouse IgG1
GP6	PE	BD	mouse IgG1
CD32	PE	RnD Systems	mouse IgG2a
HMM	-	Abcam	polyclonal rabbit
ECE1	-	RnD Systems	rat IgG1
Isotype	PE	BioLegend	mouse IgG1
Isotype	PE	BD	mouse IgM
Isotype	PE	BioLegend	mouse IgG2a
Isotype	PE	RnD	mouse IgG2b
Isotype	PE	Abcam	polyclonal rabbit
Isotype	BV786	BD	mouse IgG1
Isotype	AF647	BioLegend	mouse IgG2b
Isotype	Biotin	LSBio	polyklonal rabbit
Isotype	Biotin	BioLegend	mouse IgG2a
Isotype	-	Abcam	polyclonal rabbit
Isotype	-	Abcam	polyclonal rabbit
Isotype	-	RnD Systems	rat IgG1
** Lineage-cocktail	Pacific Blue	BioLegend	-
Anti-rabbit	BV421	BD	polyclonal goat
Anti-rat	APC	RnD Systems	polyclonal goat
Anti-rabbit	PE	RnD Systems	polyclonal goat
Streptavidin	BV421	BD	-
Viability Dye	7AAD	BD	-
Viability Dye	DAPI	BioLegend	-
Viability Dye	Draq7	Biostatus	-

*, antibody conjugated to biotin by Innovagen; **, CD3, CD14, CD16, CD19, CD20, CD56

Supplementary Figure Legends

Supplementary Figure S1

FACS gates used for sorting primary CML cells for *in vitro* cultures. The $CD34^{+}CD38^{low}$ gate was set to include 5% of $CD34^{+}$ cells. The IL1RAP gates were based on previously determined IL1RAP expression and set with approximately 10% margin. The CD36 positive and CD36 negative gates were based on previously determined CD36 expression for the particular CML sample leaving 20% of cells as $CD36^{intermediate}$ in order to obtain more pure cell populations. The figure shows CML #7 as a representative example. For CML #4 no IL1RAP-targeting antibody was used, instead CD36 gates were directly added to the $CD34^{+}CD38^{low}$ cell fraction, the $CD34^{+}CD38^{low}$ compartment of CML #4 were separately shown to be >90% $IL1RAP^{+}$.

Supplementary Figure S2

RNA-sequencing of sorted primary CML $CD34^{+}CD38^{low}$ cells from 10 newly diagnosed chronic phase CML patients and corresponding healthy controls (n=4). The heat map shows down regulated cell surface associated genes in primitive CML samples compared to healthy hematopoietic stem cell-enriced cells (up regulated genes are shown in Figure 1C)

Supplementary Figure S3

Low or absent expression of CD36 and LEPR in progenitor cells of healthy bone marrow. (**A**) Histograms showing CD36 and LEPR expression in $CD34^{+}CD38^{+}$ cells from five and four normal bone marrow (NBM) donors, respectively. (**B**). Detailed analysis of CD36 and LEPR expression in stem and progenitor populations from two normal bone marrow donors. Isotype based on all CD34 expressing cells. LMPP, lymphoid-primed

multipotent progenitors ($Lin^-CD34^+CD38^-CD45RA^+$); HSC, hematopoietic stem cells ($Lin^-CD34^+CD38^-CD90^+CD45RA^-$); MPP, multipotent progenitors ($Lin^-CD34^+CD38^-CD90^-CD45RA^-$); MEP, megakaryocyte-erythroid progenitors ($Lin^-CD34^+CD38^+CD123^-CD45RA^-$); GMP, granulocyte-macrophage progenitors ($Lin^-CD34^+CD38^+CD123^+CD45RA^+$); CMP, common myeloid progenitors ($Lin^-CD34^+CD38^+CD123^+CD45RA^-$).

Supplementary Figure S4

The individual LEPR flow cytometry datapoints and their corresponding isotype control plotted in pairs show LEPR overexpression in CML samples (n=10) but not NBM (n=4). Two asterisks (**) indicates $p<0.01$, ns; not significant.

Supplementary Figure S5

Leptin does not confer a growth advantage to CML cells. (**A**) The cell growth of KU812 and K562 cells is not affected by the addition of 100ng/ml leptin to in vitro cultures in RPMI or StemSpan media. (**B**) Cell growth of primary $CD34^+$ CML cells is not affected by the addition of 100ng/ml leptin to the cell cultures, evaluated in three different CML patient samples. (**C**) No proliferative advantage is seen on FACS sorted primitive ($CD34^+CD38^{low}$) CML patient cells when cultured with and without the addition of leptin (100 ng/ml or 200 ng/ml) for 7 days. (**D**) The colony forming capacity of primary $CD34^+$ CML is not altered by the presence of 100ng/ml leptin in the semisolid cultures. CFU-GEMM, colony forming unit - granulocyte, erythrocyte, monocyte, megakaryocyte; BFU-E, burst forming unit - erythroid; CFU-M, colony forming unit - macrophage; CFU-G, colony forming unit - granulocyte.

Supplementary Figure S6

Co-expression analysis of IL1RAP and CD36 in $CD34^{+}CD38^{low}$ cells of CML patients. (**A**) Example of co-expression profiles of CD36 and IL1RAP. (**B**) Summary of IL1RAP and CD36 co-expression data in $CD34^{+}CD38^{low}$ cells from 14 CML patients show a majority of cells with an $IL1RAP^{+}CD36^{+}$ phenotype (median 43%), similar amounts of $IL1RAP^{+}CD36^{-}$ cells (median 17%) and $IL1RAP^{-}CD36^{-}$ (median 15%), and very few $IL1RAP^{-}CD36^{+}$ cells (median 5%). Bar plot showing median values and interquartile range.

Supplementary Figure S7

Sensitivity of primitive CML cells to nilotinib. (**A**) $CD34^{+}CD38^{low}IL1RAP^{+}$ CML cells sorted based on CD36 expression do not differ in cell growth and survival following in vitro culture for 72 hours. Mean of three ($CD36^{+}$) or two ($CD36^{-}$) CML samples is shown and error bars depict standard deviation. (**B**) $CD34^{+}CD38^{low}IL1RAP^{+}$ CML cells FACS sorted according to CD36 expression and treated with nilotinib at a concentration of 5µM for 72 hours show no differences in cell survival. Mean of three CML samples is shown and error bars depict standard deviation.

Supplementary Figure S8

Cell cycle analysis of progenitor ($CD34^{+}CD38^{+}$) CML patient cells. The histogram to the left shows the cell cycle status of a representative CML sample with 67% of the cells in the G0/G1-phase and 33% in the S/G2/M-phase. The bar-plot to the right shows the cell cycle distribution (mean of three CML samples) with error bars depicting standard deviation.

Supplementary Figure S9

$CD36^+$ and $CD36^-$ cells within the $CD34^+CD38^{low}IL1RAP^+$ population do not differ in expression of cell surface markers CD25 and CD26. (**A**) Gates used to define $CD38^{low}IL1RAP^+CD36^+$ and $CD38^{low}IL1RAP^+CD36^-$ cells. (**B**) Histograms from a representative CML patient show similar expression patterns of CD25 and CD26 in the $CD36^+$ and $CD36^-$ cells, respectively (**C**) Summary of protein expression from three CML patients show no difference in mean expression of CD25 ($p=0.761$) or CD26 ($p=0.860$), respectively.

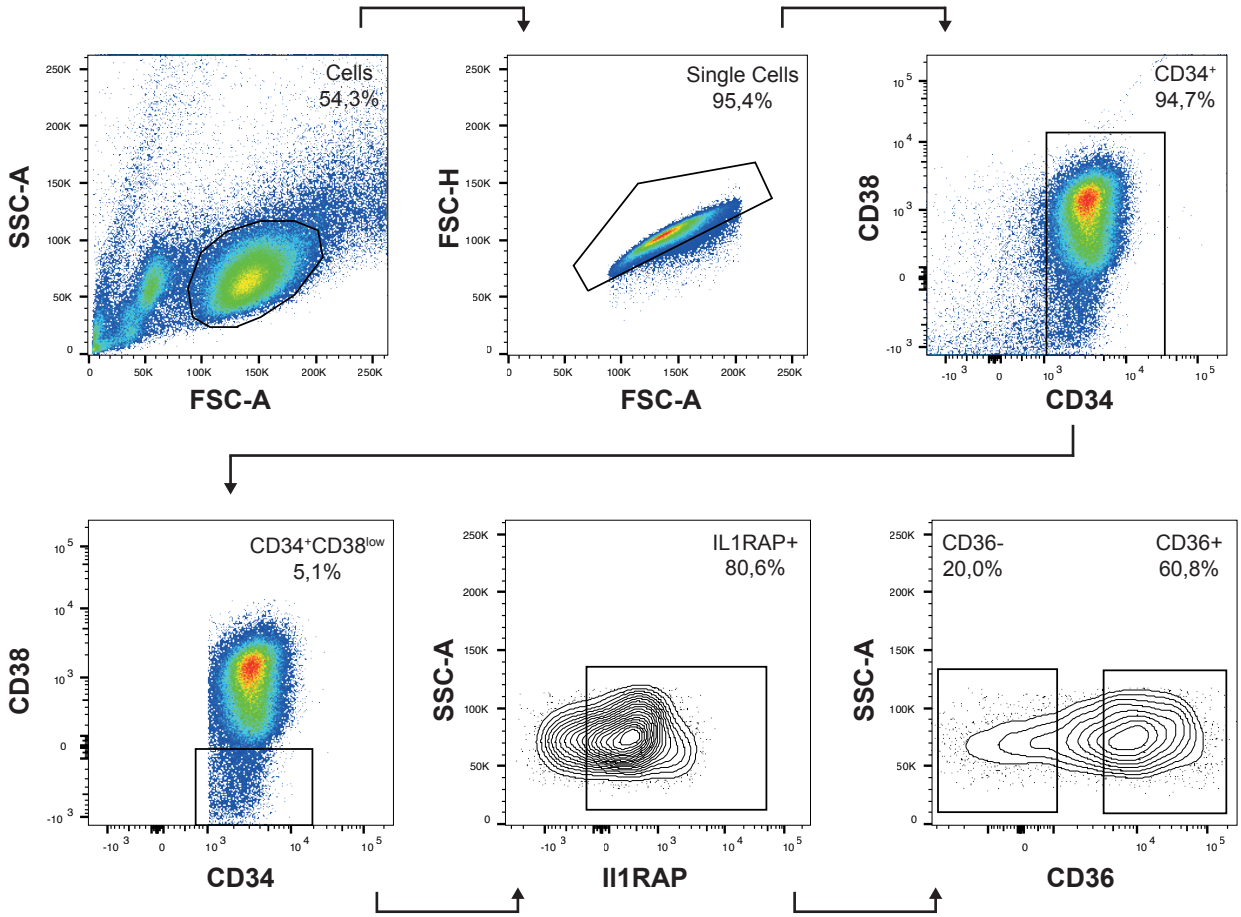
Supplementary Figure S10

CD36 expression on CML cells decrease following *in vitro* culture. (**A**) Ten thousand $CD34^+$ cells were sorted from two CML patients and kept in StemSpan medium for up to eight days. A reduction in CD36 expression was observed already after one day and further reduced during one week of culture. One representative CML patient sample is shown. (**B**) Two thousand five hundred $CD34^+CD38^{low}$ cells were sorted into StemSpan medium and cultured for 7 days. CD36 expression decreased spontaneously from 83% to 11% during the culture period. (**C**) Two thousand $CD34^+CD38^{low}CD36^+$ cells from one representative CML patient were sorted into StemSpan medium and cultured for three days. Prior to sorting 75% of viable $CD34^+CD38^{low}$ cells expressed CD36; the 60% with the highest expression were sorted to achieve 100% CD36 expressing cells at day 0 of the culturing period. After three days, 29% of viable cells still hade detectable CD36 expression.

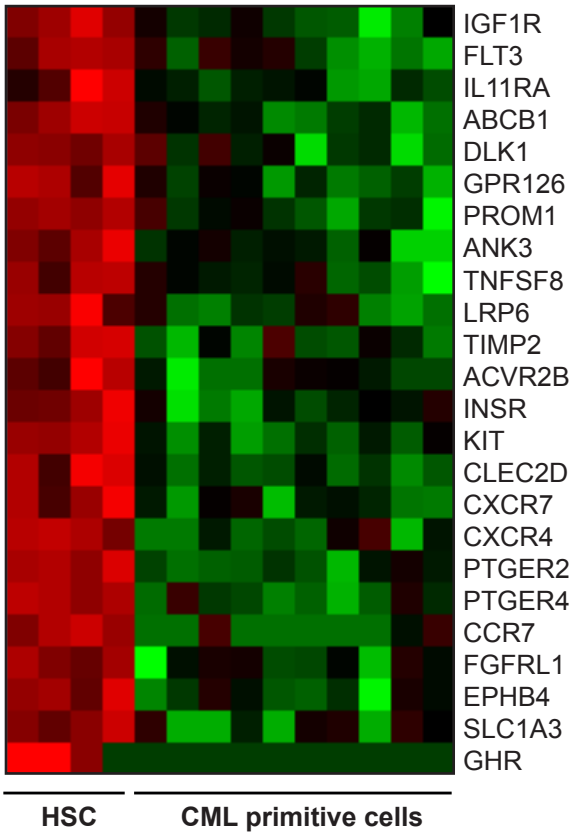
Supplementary Figure S11

Increasing antibody concentrations do not induce direct cell killing of KU812 cells in the absence of NK-cells when kept in culture with polyclonal rabbit anti-CD36 antibodies or corresponding isotype control antibodies

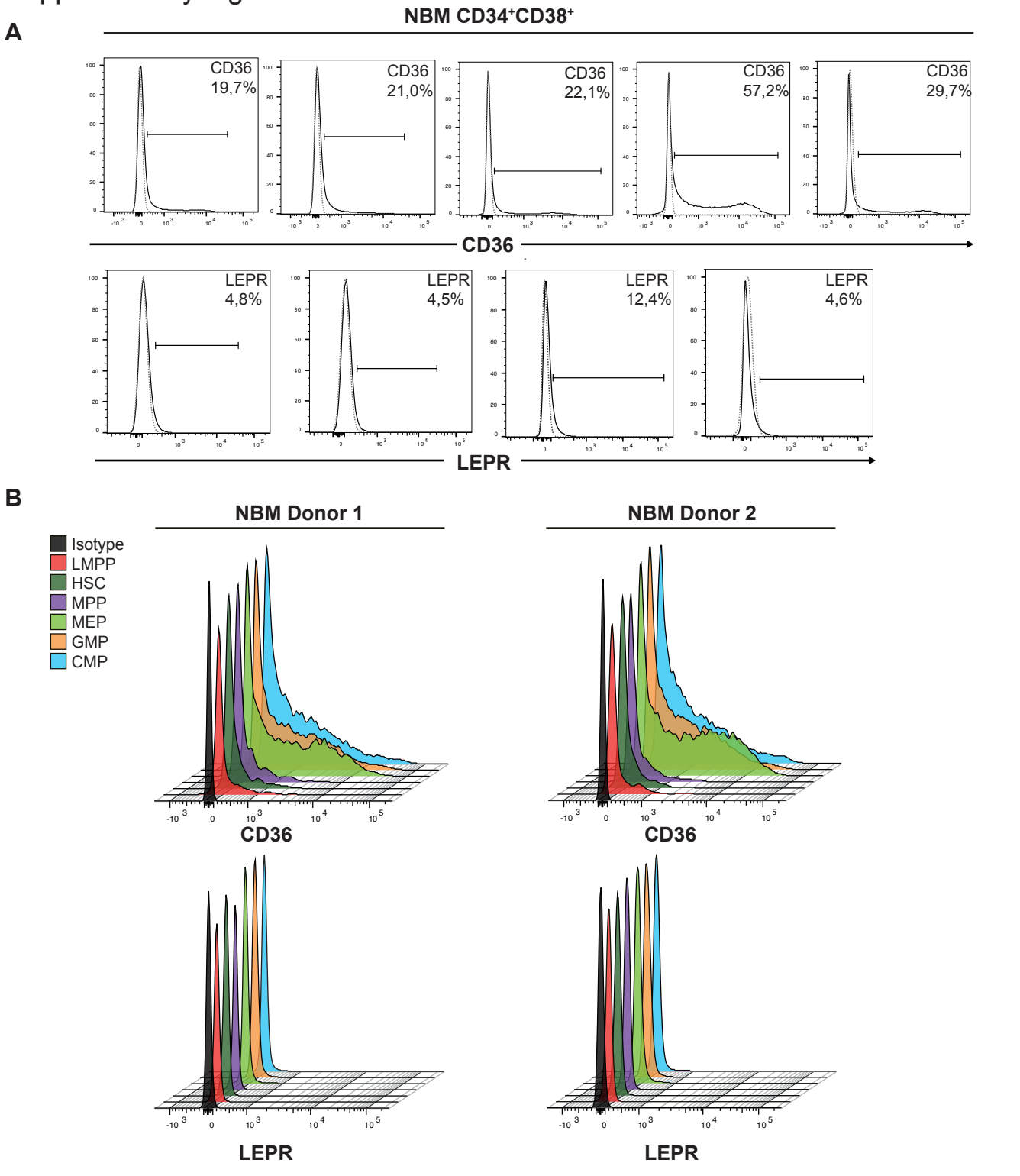
Supplementary Figure S1



Supplementary Figure S2



Supplementary Figure S3



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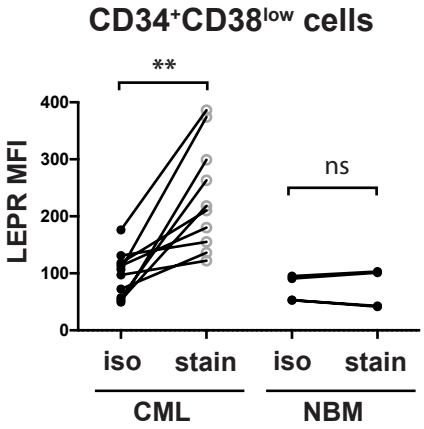

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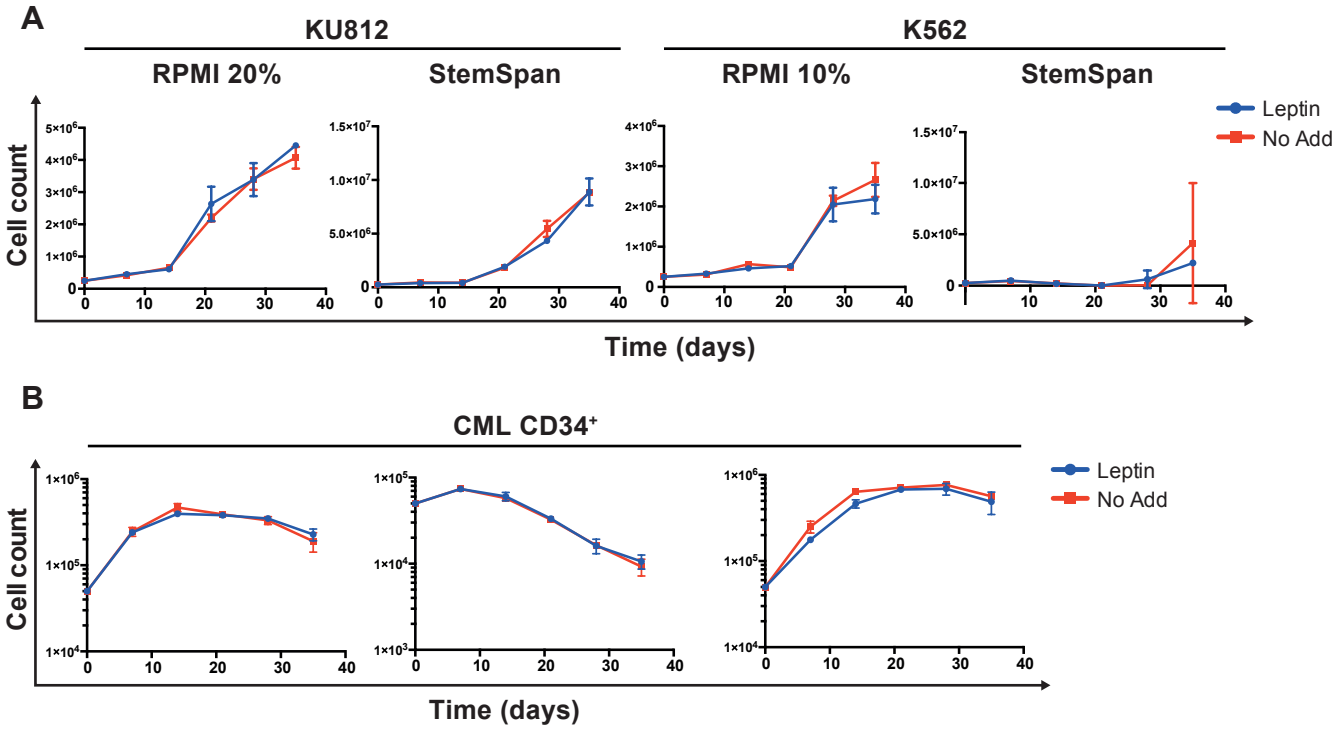

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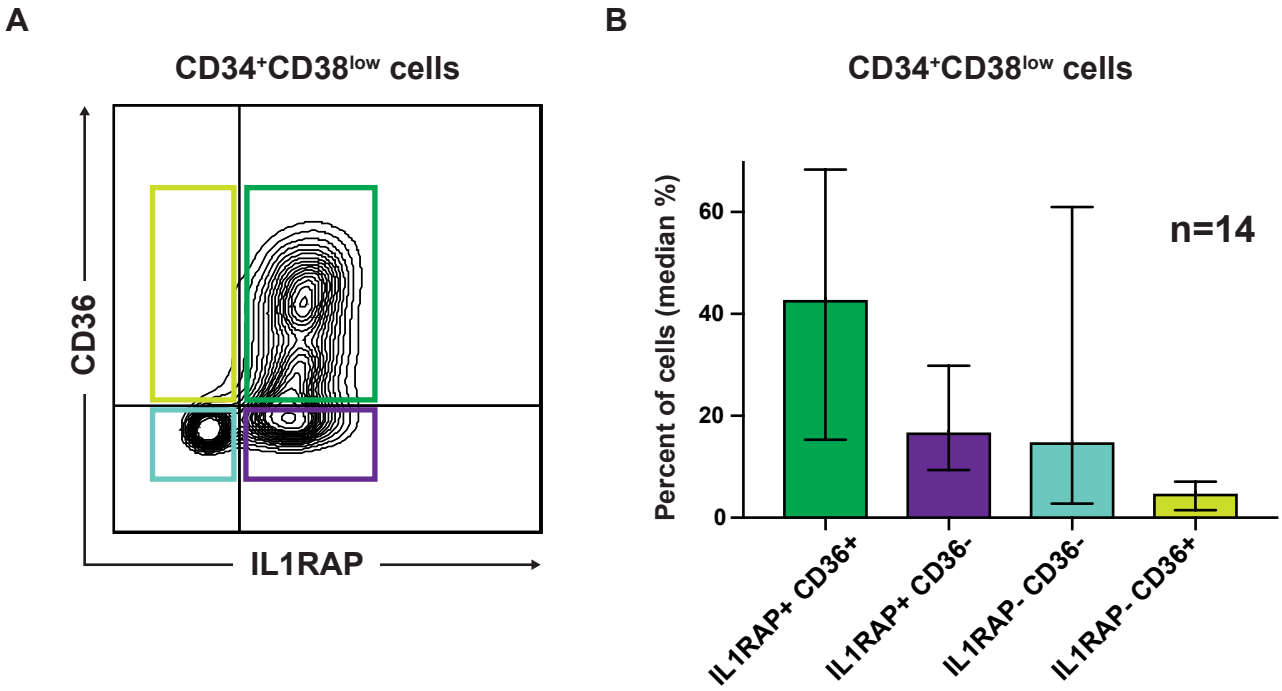
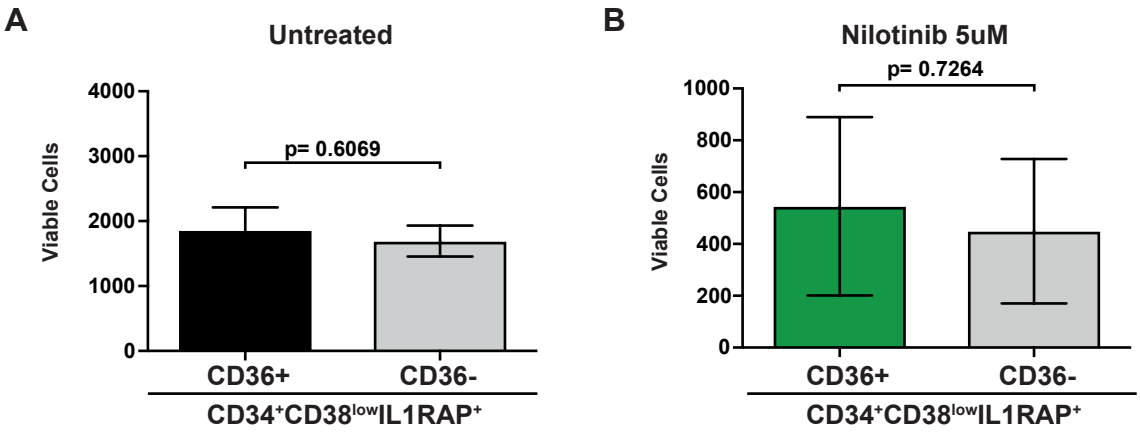
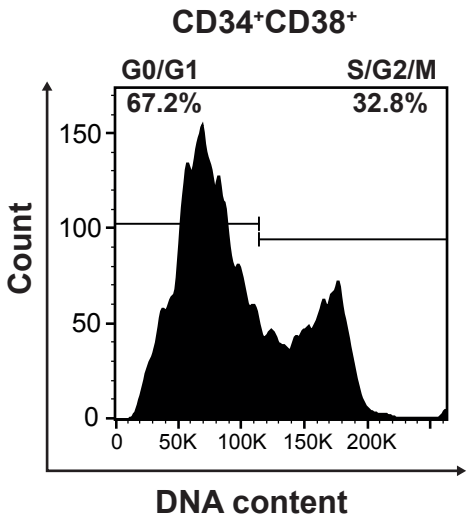
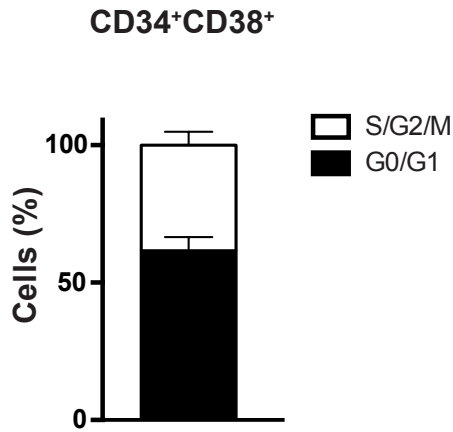


Supplementary Figure S7

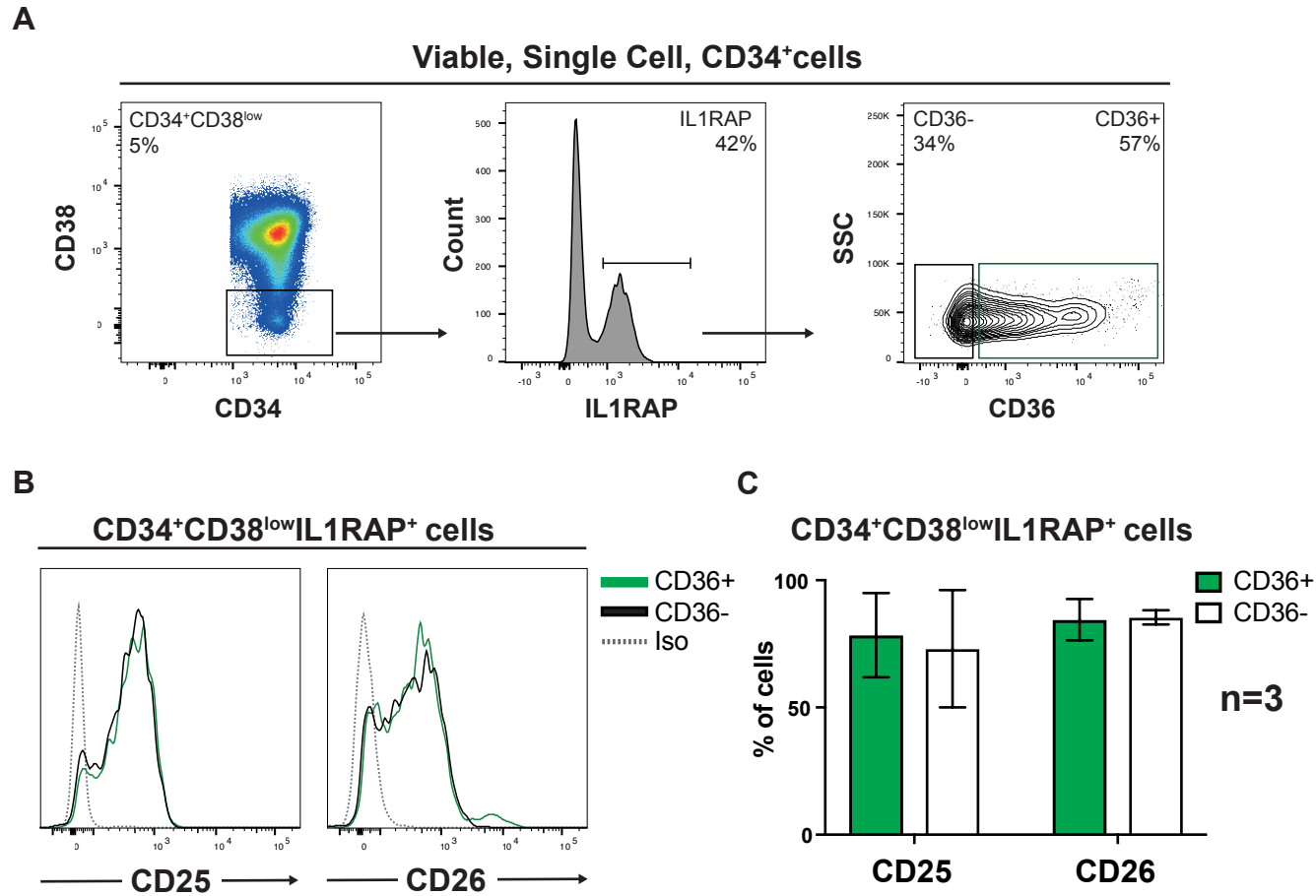


Supplementary Figure S8

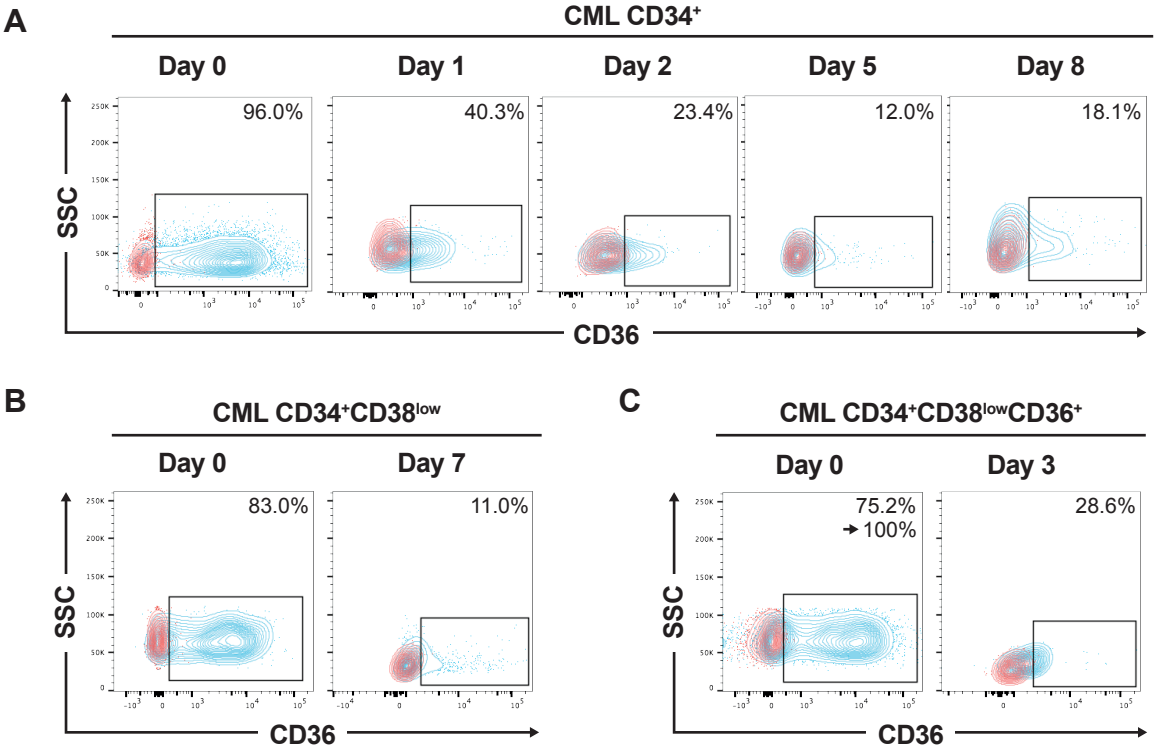




Supplementary Figure S9



Supplementary Figure S10



Supplementary Figure S11