Supplementary Materials

Supplementary Materials and Methods

Vectors, cells and transduction

Third generation self-inactivating LV and integrase-defective LV (IDLV) expressing GFP under the control of an internal PGK promoter (SINLV-GFP) stocks were prepared, concentrated and titered as previously described [1, 2]. Bald vector, an entry-incompetent LV was produced omitting the Env-encoding plasmid during vector production. For SINLV capsid mutants, vectors were produced as previously described [1], except that the wild-type pMDLg/pRRE construct was replaced with a packaging plasmid harboring a specific point-mutation in the p24 coding region as follows: pMDLg/pRRE-N74D; pMDLg/pRRE-P90A; pMDLg/pRRE-A105T; pMDLg/pRRE-V86M; pMDLg/pRRE-A92E; pMDLg/pRRE-A88T. All modified packaging plasmids were purchased from GenScript Inc, Piscataway, NJ. The SIN-retroviral vector (SIN-RV) was produced as previously described [3]. Titers and infectivities of the different WT and CA mutant LV stocks used in this study are reported in **Table S1**. Cytokine-stimulated HSPC were then transduced at a concentration of 1×10^6 cells/ml with SINLV-GFP or IDLV for 20 hours at the indicated multiplicity of infection (MOI) as calculated by titration on 293T cells. MDM were transduced 7 days after differentiation. $CD4^+$ T cells were transduced at a concentration of 10^6 cells/ml, after 3 days of stimulation. Cyclosporin A (CsA), FK506, Rapamycin (Rapa) and 3MA (all from Sigma-Aldrich, St. Louis, MO) were added during transduction at the indicated concentration and washed out with the vector 16-20 hours later.

Cells

The human embryonic kidney 293T cells (HEK293T) and the human K562 myelogenous leukemia cell lines were maintained in Iscove's modified Dulbecco’s medium (IMDM; Sigma-Aldrich,

St. Louis, MO) supplemented with 10% fetal bovine serum (FBS; Life Technologies, Carlsbad, CA), penicillin (100 IU/ml), streptomycin (100 μg/ml) and 2% glutamine (complete IMDM). Human $CD34^+$ hematopoietic stem and progenitor cells (HSPC), $CD4^+$ T cells and $CD14^+$ monocytes were isolated through positive magnetic bead selection according to manufacturer's instructions (Miltenyi Biotec, Bergisch Gladbach, Germany) from umbilical cord blood (CB) collected upon informed consent from healthy volunteers according to the Institutional Ethical Committee approved protocol (TIGET01). Otherwise, CB and bone marrow (BM)-derived $CD34^+$ were directly purchased from Lonza (Lonza, Basel, Switzerland). Experiments report results obtained both from single donors as well as from pools of up to 4 donors. Human CB and BM-derived HSPC were stimulated and cultured as previously described [4]. $CD4^+$ T cells were activated in complete Roswell Park Memorial Institute medium (RPMI; Lonza, Basel, Switzerland), supplemented with phytohaemagglutanin (PHA) (2μg/ml, Roche, Basel, Switzerland) and IL-2 (300IU/mL, Novartis, Basel, Switzerland) for three days and maintained in complete RPMI, supplemented with IL-2 (300 UI/ml) only afterwards. Otherwise, negatively selected $CD4^+$ T cells were cultured in presence of beads coated with anti-CD3 and anti-CD28 antibodies (Life Technologies, Carlsbad, CA) and IL-7 and IL-15 (PeproTech, Rocky Hill, NJ) for two days and maintained in complete IMDM, supplemented with IL-7 and IL-15 afterwards as previously described [5]. Monocyte-derived macrophages (MDM) were differentiated from isolated $CD14^+$ monocytes in complete DMEM supplemented with 5% human serum AB (Lonza, Basel, Switzerland) for seven days. Murine Lin^- and $CD4^+$ cells were isolated from bone marrow and spleen of euthanized C57BL/6 mice respectively, using magnetic bead selection according to manufacturer's instructions (Miltenyi Biotec, Bergisch Gladbach, Germany) and cultured as previously described [6, 7]. All cells were maintained in a 5% CO_2 humidified atmosphere at 37°C.

Transplantation of human HSPC in NSG mice

NOD-SCID-IL2Rg-/- (NSG) mice were purchased from Jackson laboratory. Human CB-derived $CD34^+$ cells were pre-stimulated and transduced as described above with SINLV-GFP at an MOI of 10 in presence of DMSO/CsA/Rapa or their combination as indicated. After transduction $1.3x10^5$ cells were infused into the tail vein of sublethally irradiated 10-week-old NSG mice (radiation dose: 200 cGy for mice weighting 18-25g and of 220 cGy for mice above 25g of weight). All animal procedures were performed according to protocols approved by the Animal Care and Use Committee of the Ospedale San Raffaele (IACUC 661) and communicated to the Ministry of Health and local authorities according to the Italian law.

Colony-forming cell assay

Colony-forming cell (CFC) assays were performed by plating $8x10^2$ human CB-derived $CD34^+$ cells transduced with SINLV-GFP at an MOI of 10 in presence of the different compounds in a methylcellulose-based medium (Methocult; Stem Cell Technologies, Vancouver, Canada). Fifteen days later colonies were scored by light microscopy for colony numbers and morphology. CFU-E and BFU-E were scored as erythroid colonies, while CFU-G, CFU-M and CFU-GM and CFU-GEMM as myeloid colonies.

Flow cytometry

All cytometric analyses were performed using the FACS Canto III instrument and LSRFortessa instruments (BD Biosciences, San Jose, CA) and analyzed with the FACS Express software (De Novo Software, Glendale, CA). GFP expression in transduced cells was measured 5-7 days post-transduction. To stain for erythroid and myeloid colony-forming cells (CFC), differentiated cells were harvested from Methocult plates, pooled, washed and resuspended in PBS containing 2% FBS for immunostaining. Percentages and transduction efficiencies in the different $CD34^+$ subpopulations were assessed two days post-transduction according to previously described markers [8, 9]. LDL-R suface staining was performed at 6 hours post-exposure to CsA/Rapa. To exclude dead cells from the analysis,

cells were washed and resuspended in PBS containing 10 ng/ml 7-aminoactinomycin D (7-AAD, Sigma-Aldrich, St. Louis, MO). To analyze peripheral blood, spleen and bone marrow from transplanted NSG mice, samples were prepared and stained as previously described [7] (for all antibodies see **Table S2**).

Gene expression, replication intermediates and vector copy numbers (VCN)

For gene expression total RNA was extracted using the RNeasy Plus micro Kit according to manufacturer's instructions (Qiagen, Venlo, Netherlands) and retrotranscribed using the SuperScript Vilo kit (Life Technologies, Carlsbad, CA). For replication intermediate analysis, cells were lysed and processed 24 hours post-transduction in Monini lysis buffer as previously described [10] followed by semi-quantitative Taqman assays. For VCN, total DNA was extracted using a Maxwell 16 instrument (Promega, Madison, WI) or Blood & Cell Culture DNA micro kit (Qiagen, Venlo, Netherlands) according to manufacturer's instructions and VCN were assessed as previously described [11].

Primers and probes

For gene expression, the following Taqman probes from Applied Biosystems (Life Technologies, Carlsbad, CA) were used: PPIA (Hs99999904_m1), HPRT1 (Hs01003267_m1). For replication intermediates, the following primers: for late-RT products: LATE RT (5NC2rev) 5'-GAGTCCTGCGTCGAGAGAG -3' ; LATE RT fw DU3 sense 5'-TCACTCCCAACGAAGACAAGATC-3' . For 2LTR circles, primers specifically detecting the 2-LTR circle junction were used as previously described [12]. Results were normalized using the human TERT gene (hTelo fw 5'-GGCACACGTGGCTTTTCG -3' ; hTelo rev 5'-GGTGAACCTCGTAAGTTTATGCAA-3'; hTelo probe VIC-5'-TCAGGACGTCGAGTGGACACGGTG-3'-TAMRA).

Western blot

Whole cell extracts were prepared as previously described [13]. Samples were subjected to SDS-PAGE, transferred to PVDF membrane by electroblotting, and blotted with a mouse polyclonal antibody (Ab) raised against CypA (Santa-Cruz Biotechnology, Dallas, Texas). A mouse polyclonal anti-βactin Ab (Sigma-Aldrich, St. Louis, MO) was used as a normalizer.

ELISA

Supernatants from transduced cells were harvested 24 hours post-transduction and the IFNα concentration was assessed using the VeriKineTM Human IFN Alpha ELISA Kit (PBL Assay Science, Piscataway, NJ) according to manufacturer's instructions.

Table S1. Titers and infectivity of WT and CA mutant vector stocks used in this work.

Vector	Titer (TU/ml)	p24 (µg/ml)	Infectivity (TU/ng of p24)
WT	**1.25E+10**	**194**	**6.45E+04**
	1.21E+10	**141**	**8.60E+04**
	1.47E+10	**200**	**7.32E+04**
	1.12E+10	**60**	**1.86E+05**
V86M	**5.10E+09**	**150**	**3.40E+04**
	5.97E+09	**94**	**6.35E+04**
P90A	**4.19E+09**	**141**	**2.98E+04**
	5.93E+09	**102**	**5.81E+04**
A105T	**9.20E+09**	**185**	**4.98E+04**
	4.99E+09	**42**	**1.19E+05**
N74D	**8.69E+09**	**106**	**8.23E+04**
	6.36E+09	**61**	**1.04E+05**
A92E	**8.79E+09**	**102**	**8.64E+04**
	1.71E+10	**146**	**1.17E+05**
	5.38E+09	**103**	**5.22E+04**
A88T	**1.72E+09**	**103**	**1.67E+04**
	2.62E+09	**152**	**1.73E+04**
	2.79E+09	**94**	**2.97E+04**

Table S2. List of anti-human antibodies used for flow cytometry.

Antibody	Fluorochrome	Dilution	Clone	Company	Code
hCD235a	APC	1:25	GA-R2	BD Biosciences	551336
hCD33	BV421	1:25	WM53	BD Biosciences	562854
Anti human FCR Blocking		1:50		Miltenyi Biotec	120-000-442
hCD45	APCh7	1:25	2D1	BD Biosciences	641417
hCD19	PE	1:25	SJ25C1	BD Biosciences	345789
hCD33	PeCy7	1:25	P67.6	BD Biosciences	333952
hCD3	APC	1:25	UCHT1	BD Biosciences	555335
hCD13	BV	1:25	WM15	BD Biosciences	562596
hCD34	PeCy7	1:25	8G12	BD Biosciences	348811
hCD38	V450	1:25	HB7	BD Biosciences	646851
hCD90	APC	1:25	5E10	BD Biosciences	559869
Hcd133/2	PE	1:25	293C3	Miltenyi Biotec	130-090-853
Anti-human LDL R Ab		1:200	472413	R&D Systems	MAB2148
Anti-mouse IgG	AlexaFluor 488	1:500		Invitrogen	A-21202
Mouse IgG1 isotype control	FITC	1:200		BD Biosciences	51-35404X

Supplementary Figure Legends

Figure S1. Optimal conditions for CsA effect in human HSPC. Cord blood (CB)-derived $CD34^+$cells were transduced with SINLV-GFP at increasing multiplicities of infection (MOI) in presence or absence of increasing concentrations of CsA. (**a,c**) The percentage of GFP^+ cells was measured by FACS 5 days post-transduction. (**b,d**) Cell growth was assessed by cell count over time. Data are the mean ± SEM of two independent experiments.

Figure S2. Impact of Rapa, CsA and FK-506 in TCR-activated $CD4^+$ T cells. $CD4^+$ T cells were negatively selected from cord blood and activated with anti-CD3/CD28 beads in presence of IL-7 and IL-15 for two days prior to transduction with SINLV-GFP at a MOI of 1 in presence or absence of 10 µg/ml Rapa, 10 µmol/l CsA or 20 µmol/l FK-506. The percentage of GFP^+ cells was measured by FACS 5 days post-transduction. Data are the mean ± SEM of three independent transductions. p value are for One way ANOVA with Bonferroni's multiple comparison, * for $p<0.05$; ** for $p<0.01$ and **** for $p<0.0001$.

Figure S3. The effect of CsA does not depend on calcineurin. (**a,b**) CB and BM-derived $CD34^+$ cells, (**c**) PHA/IL-2 activate $CD4^+$T cells and (**d**) MDM were transduced with SINLV-GFP at an MOI of 1, in presence or absence of 10 µmol/l CsA or 20 µmol/l FK506. The percentage of GFP^+ cells was evaluated by FACS 5 days after transduction (**a,b**, left panels and **c,d**) and VCN were assessed 14 days post-transduction (**a,b**, right panels). Data are the mean ± SEM of at least three independent experiments. p value are for One way ANOVA with Bonferroni's multiple comparison.

Figure S4. Impact of CsA on LV transduction in K562. (**a**) CypA protein levels in K562 and CB-derived $CD34^+$ cells were detected by WB, actin was used as normalizer. One representative out of two gels is shown. (**b**) K562 cells were transduced with SINLV-GFP at an MOI of 1, in presence or absence

of 10 µmol/l CsA. The percentage of GFP^+ cells was evaluated by FACS 5 days after transduction. Data represent the mean ± SEM of six independent experiments.

Figure S5. Titration of Rapa in $CD34^+$ cells. CB-derived $CD34^+$ cells were transduced with SINLV-GFP at a MOI of 1 in presence or absence of increasing concentrations of Rapa. The percentage of GFP^+ cells was measured by FACS 5 days post-transduction. Data are the mean ± SEM of two independent experiments. p value are for One way ANOVA with Bonferroni's multiple comparison.

Figure S6. Impact of Rapa on LV transduction in $CD4^+$ T cells and MDM. (**a**) PHA/IL-2 activated $CD4^+$T cells and (**b**) MDM were transduced with SINLV-GFP at an MOI of 1, in presence or absence of 10µg/ml Rapamycin. The percentage of GFP^+ cells was evaluated by FACS 5 days post-transduction. Data represent the mean ± SEM of five independent experiments. p values are for Student's t-test.

Figure S7. The combination of CsA and Rapa leads to overt toxicity in HSPC. CB-derived $CD34^+$ cells were transduced with SINLV-GFP at an MOI of 1, in presence or absence of 10 µmol/l CsA or 10 µg/ml Rapamycin or their combination. (**a**) Cell count over time was performed and data are the mean ± SEM of four independent experiments. p values are for Two-way ANOVA with Bonferonni's multiple comparison vs DMSO. (**b**) Annexin staining for apoptotic cells was performed two days after transduction by FACS. Data represent the mean ± SEM of three independent experiments. p value are for Two way ANOVA versus untransduced (UT).

Figure S8. Impact of CsA and Rapa on HSPC subpopulation composition. CB-derived $CD34^+$ cells were transduced with SINLV-GFP at an MOI of 1, in presence or absence of 10 µmol/l CsA or 10 µg/ml Rapamycin or their combination. The percentages of the different subpopulation was assessed, using the gating strategy shown in (**a**), by FACS 3 days after transduction (**b**). Data represent the mean ± SEM of five independent experiments.

Figure S9. Impact of CsA and Rapa on murine HSPC and CD4$^+$ T cells. (**a**) Murine Lin$^-$ HSPC and (**b**) activated CD4$^+$ T cells were transduced with SINLV-GFP at an MOI of 1, in presence or absence of 10 µmol/l CsA or 10 µg/ml Rapamycin or their combination. The percentage of GFP$^+$ cells was evaluated by FACS 5 days post-transduction. Data are the mean ± SEM of three independent experiments for HSPC and three independent transductions for CD4$^+$ T cells. p value are for One way ANOVA with Bonferroni's multiple comparison.

Figure S10. Impact of CsA and Rapa on hematopoietic cell subsets in BM and Spleen at 20 weeks post-transplant. (**a,c**) Percentages of the indicated hematopoietic populations within human CD45$^+$ cells in BM (**a**) and spleen (**c**). (**b,d**) Frequency of GFP expression in the indicated hematopoietic subpopulations in BM (**b**) and spleen (**d**). All values are expressed as mean ± SEM.

Figure S11. *In vitro* transduction efficiency of HSPC transplanted in NSG-mice. (**a**) The percentage of GFP$^+$ cells was evaluated by FACS 5 days post-transduction. (**b**) VCN were assessed 14 days post-transduction. Data are representative of two independent experiments.

Figure S12. Inhibition of autophagy enhances both CsA and Rapa-mediated improvement of transduction. (**a**) CB and (**b**) BM-derived CD34$^+$ cells were transduced with SINLV-GFP at an MOI of 1, in presence or absence of 10 µmol/l CsA, 10 µg/ml Rapamycin or 5 mmol/l 3MA or their combinations. Left panels, The percentage of GFP$^+$ cells was evaluated by FACS 5 days post-transduction. Right panels, VCN were assessed 14 days post-transduction. Data represent the mean ± SEM of three independent experiments.

Figure S13. Impact of Rapa and CsA on LDL-R surface expression in HSPC. CB-derived CD34$^+$ cells were exposed to10 µmol/l CsA or 10 µg/ml Rapamycin for 6h and LDL-R surface expression was measured by FACS. Data are representative of two independent experiments.

Figure S14. Effects of CsA and Rapa are lentivirus specific. (**a**) CB and (**b**) BM-derived CD34$^+$ cells were transduced with SINRV-GFP at an MOI of 10, in presence or absence of 10 µmol/l CsA or

10 µg/ml Rapamycin or their combination. The percentage of GFP^+ cells was evaluated by FACS 5 days post-transduction. Data represent the mean ± SEM of four independent experiments.

Figure S15. A88T yields more integrated proviral copies that WT LV in presence of CsA. CB-derived $CD34^+$ cells were transduced with SINLV-GFP harboring either the WT or the A88T capsid at a MOI of 1, in presence or absence of 10 µmol/l CsA. VCN/cell were determined by Taqman assay 14 days post-transduction. Data represent the mean ± SEM of four independent experiments. p value are for One way ANOVA with Bonferroni's multiple comparison.

Figure S16. IFNα is not detected upon exposure of HSPC to CsA/Rapa. Supernatants from CB-derived $CD34^+$ cells were harvested 24 hours post-transduction with at an MOI of 10, in presence or absence of 10 µmol/l CsA or 10 µg/ml Rapamycin. Medium supplemented with recombinant human IFNα was used as positive control and the medium in which the cells were cultured (SS+PS+hcyt) as the negative one. Data are the mean ± SEM of three independent experiments. Nd for not detected (detection limit 12.5pg/ml).

Supplementary References

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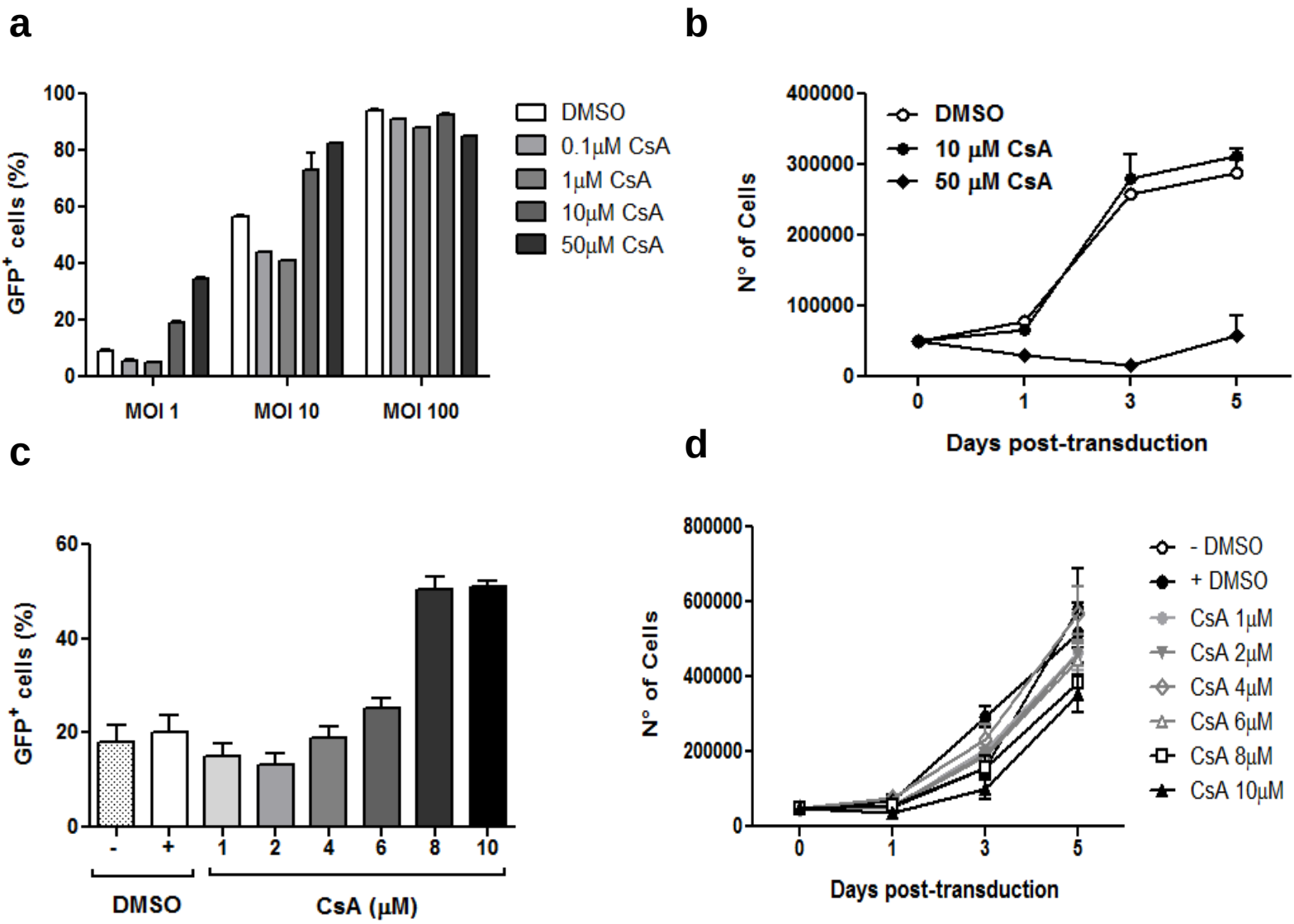

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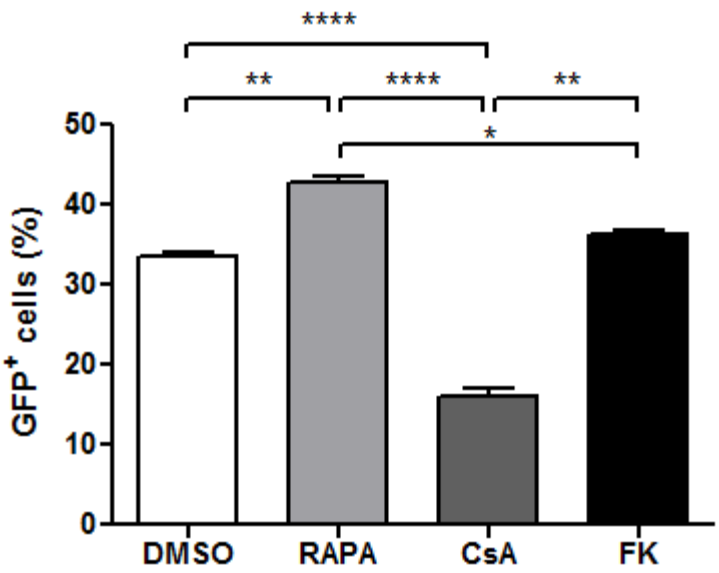

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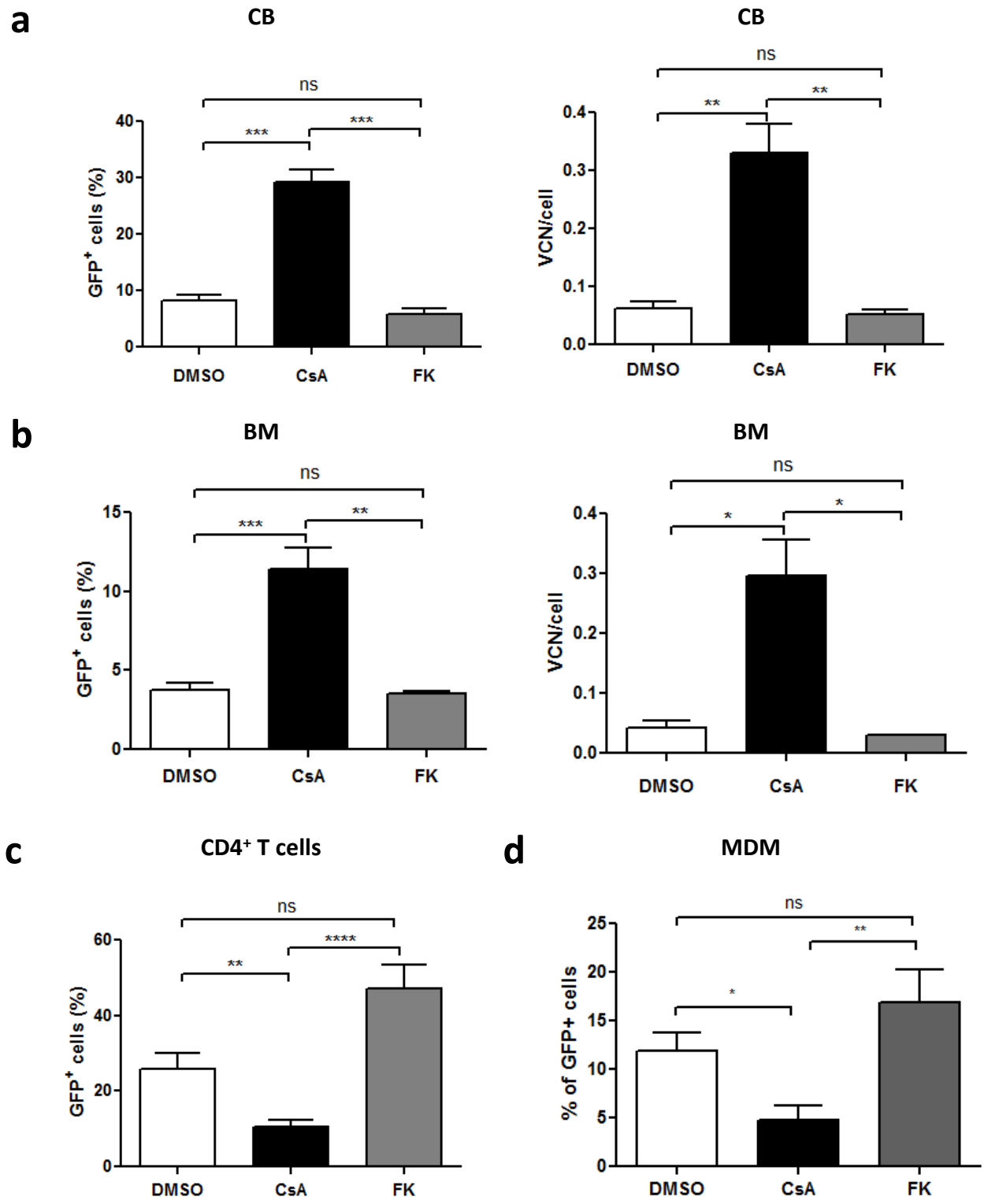

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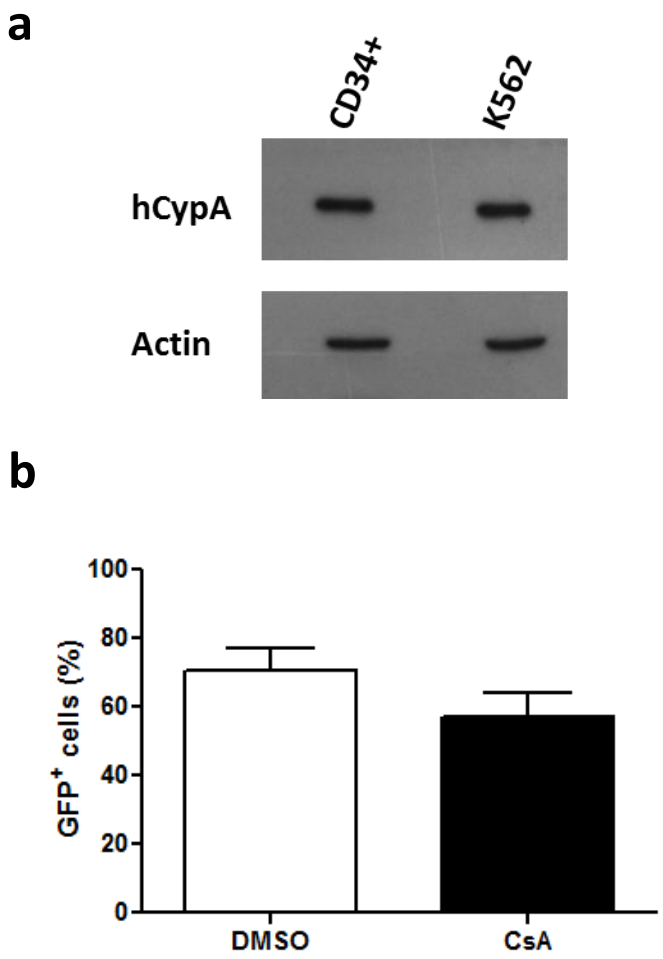

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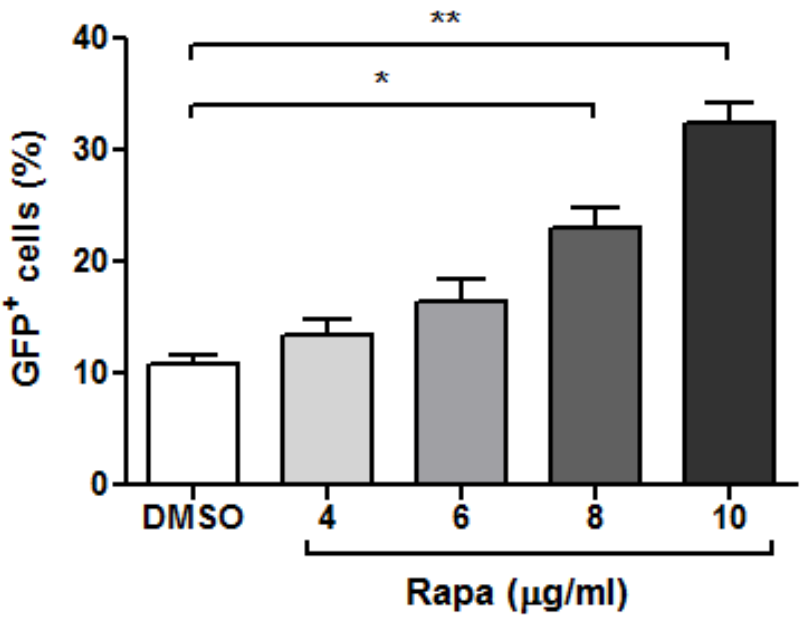

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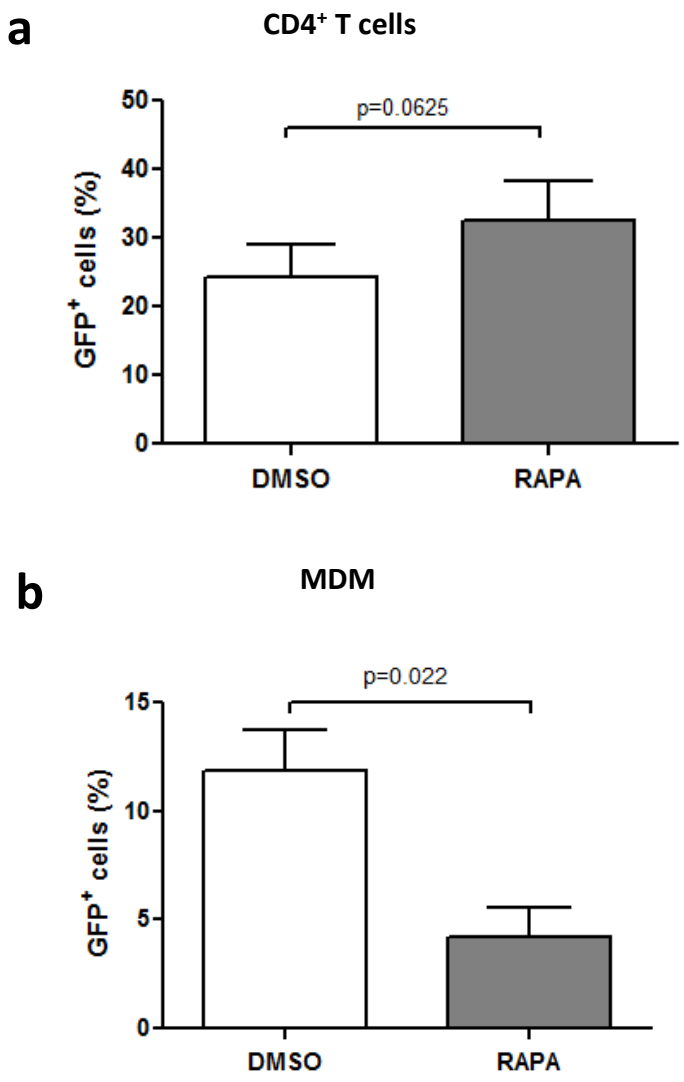

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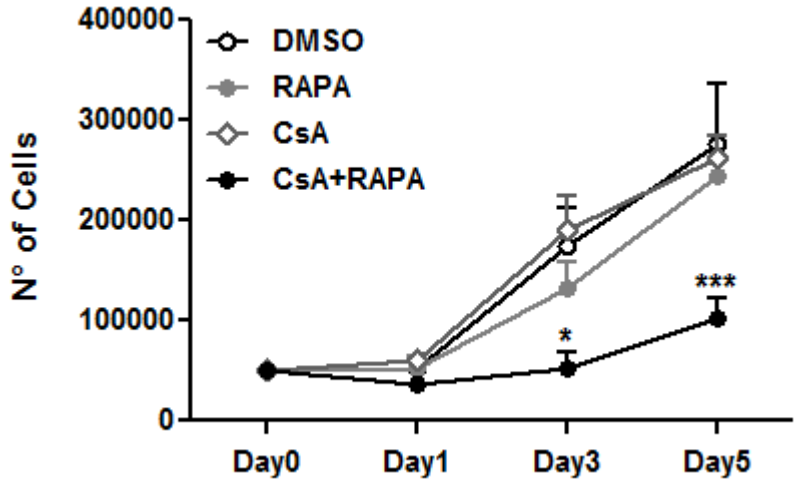
a



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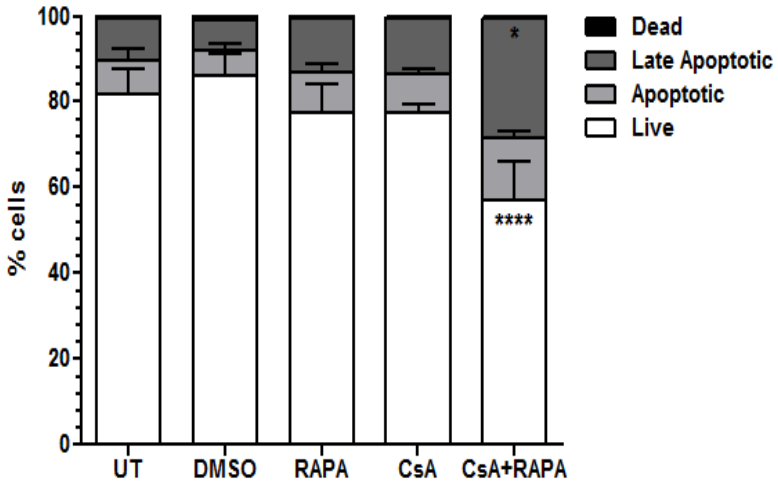

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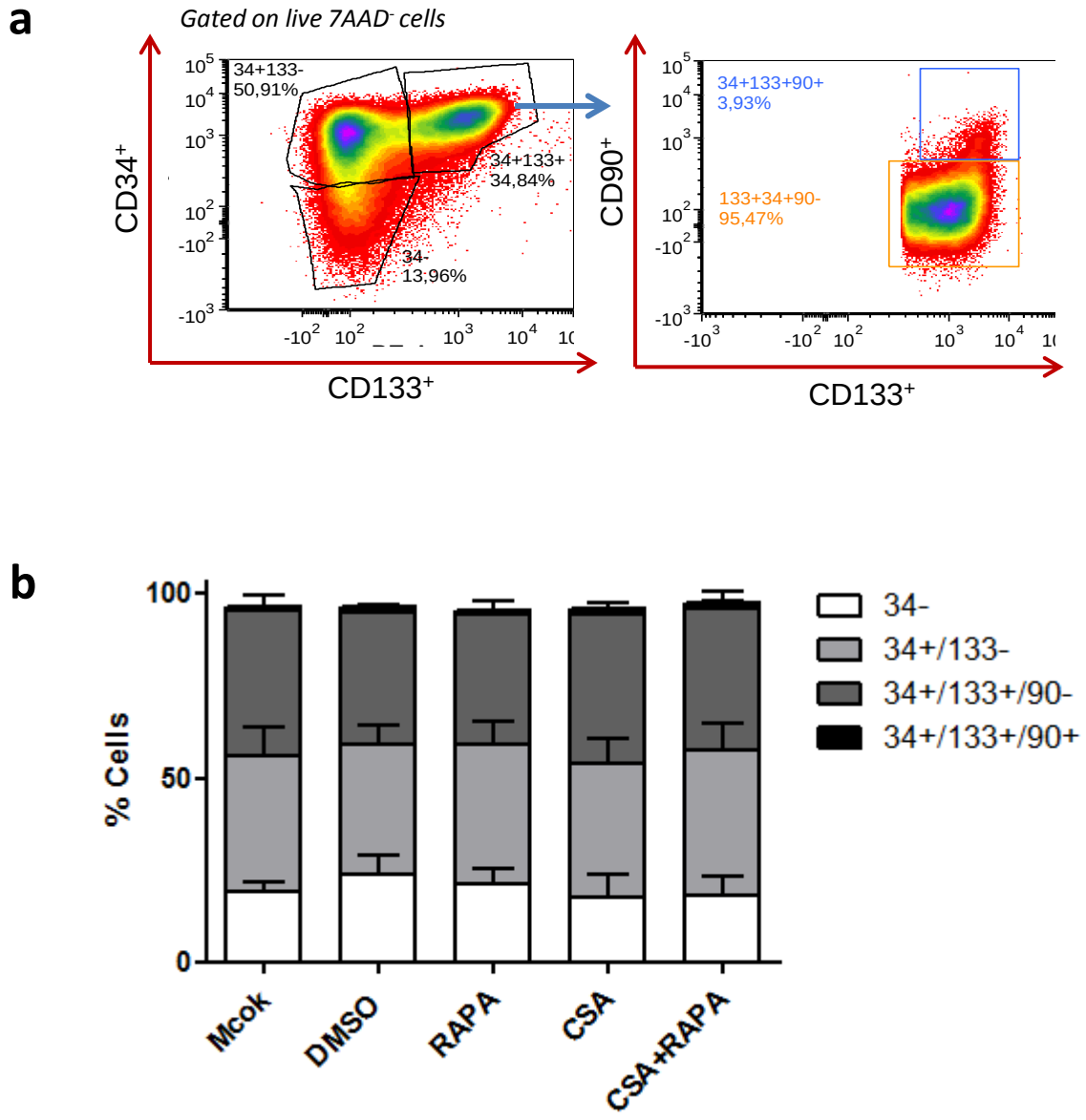
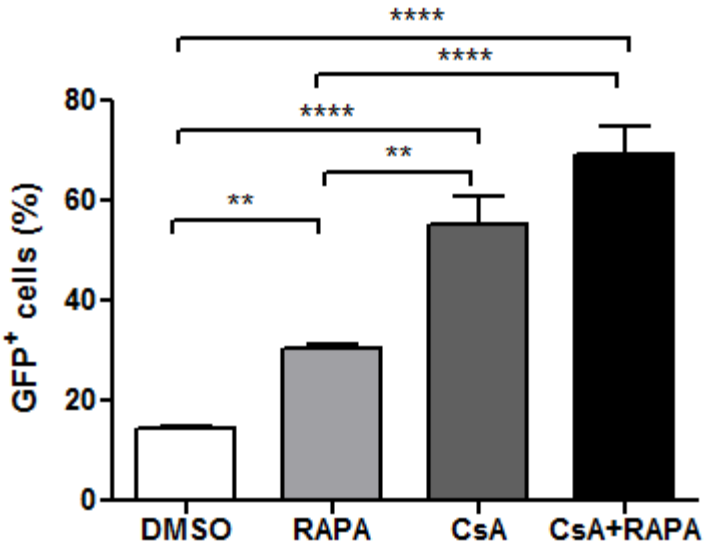


Figure S8. Impact of CsA and Rapa on HSPC subpopulation composition. CB-derived CD34⁺ cells were transduced with SINLV-GFP at an MOI of 1, in presence or absence of 10 µmol/l CsA or 10 µg/ml Rapamycin or their combination. The percentages of the different subpopulation was assessed, using the gating strategy shown in (**a**), by FACS 3 days after transduction (**b**). Data represent the mean ± SEM of five independent experiments.

a



b

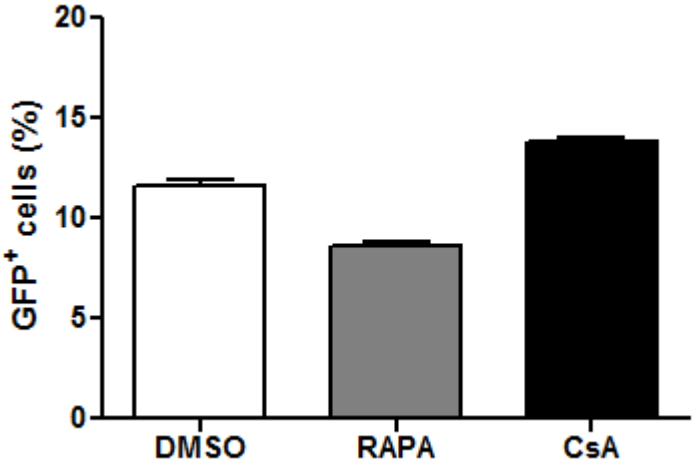


Figure S9. Impact of CsA and Rapa on murine HSPC and CD4$^+$ T cells. (**a**) Murine Lin$^-$ HSPC and (**b**) activated CD4$^+$ T cells were transduced with SINLV-GFP at an MOI of 1, in presence or absence of 10 µmol/l CsA or 10 µg/ml Rapamycin or their combination. The percentage of GFP$^+$ cells was evaluated by FACS 5 days post-transduction. Data are the mean ± SEM of three independent experiments for HSPC and three independent transductions for CD4$^+$ T cells. p value are for One way ANOVA with Bonferroni's multiple comparison.

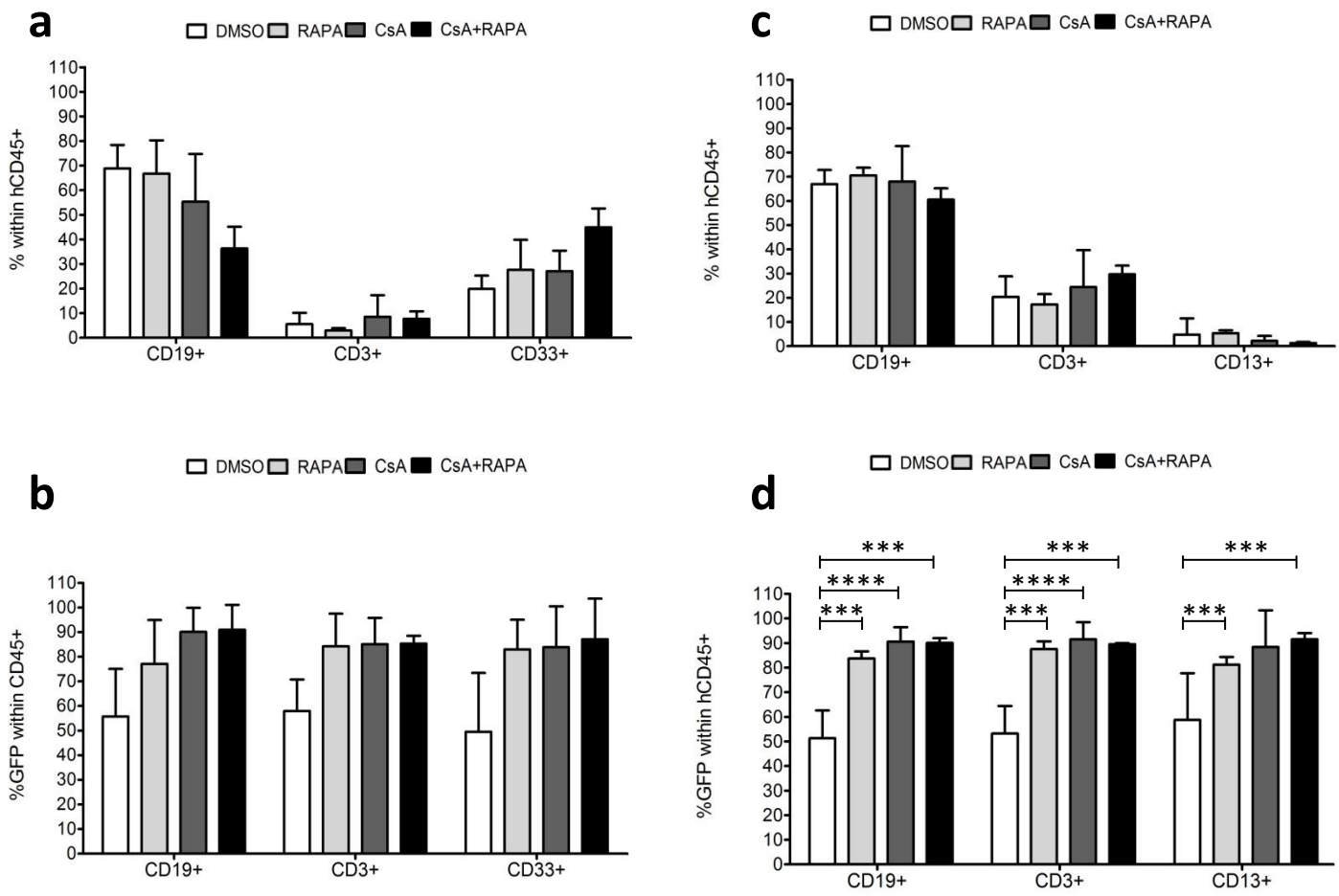


Figure S10. Impact of CsA and Rapa on hematopoietic cell subsets in BM and Spleen at 20 weeks post-transplant. (**a,c**) Percentages of the indicated hematopoietic populations within human $CD45^+$ cells in BM (**a**) and spleen (**c**). (**b,d**) Frequency of GFP expression in the indicated hematopoietic subpopulations in BM (**b**) and spleen (**d**). All values are expressed as mean ± SEM.

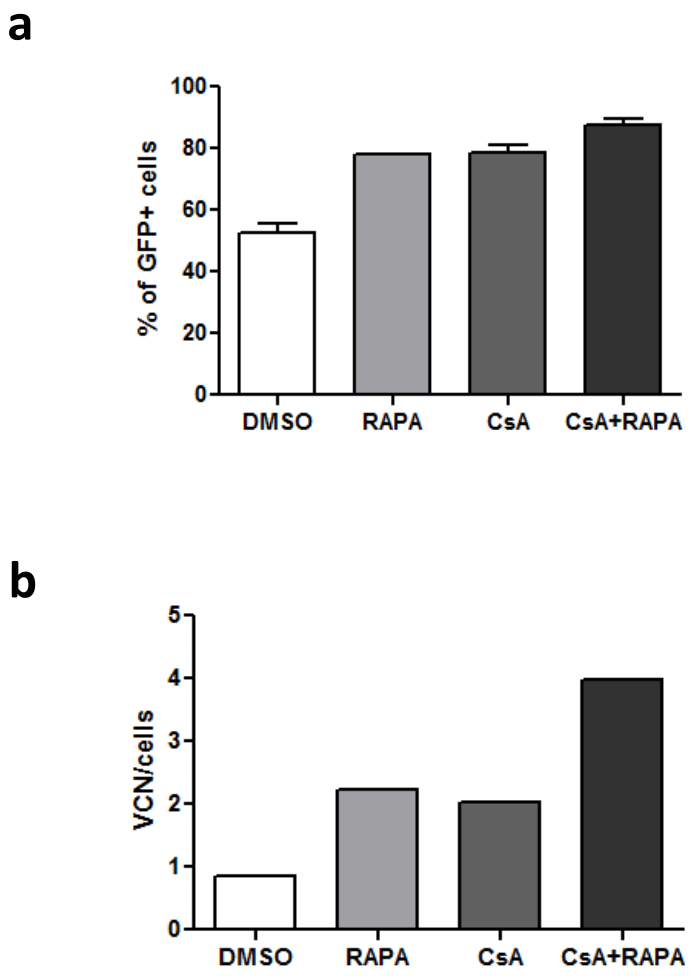


Figure S11. ***In vitro*** **transduction efficiency of HSPC transplanted in NSG-mice.** (**a**) The percentage of GFP^+ cells was evaluated by FACS 5 days post-transduction. (**b**) VCN were assessed 14 days post-transduction. Data are representative of two independent experiments.

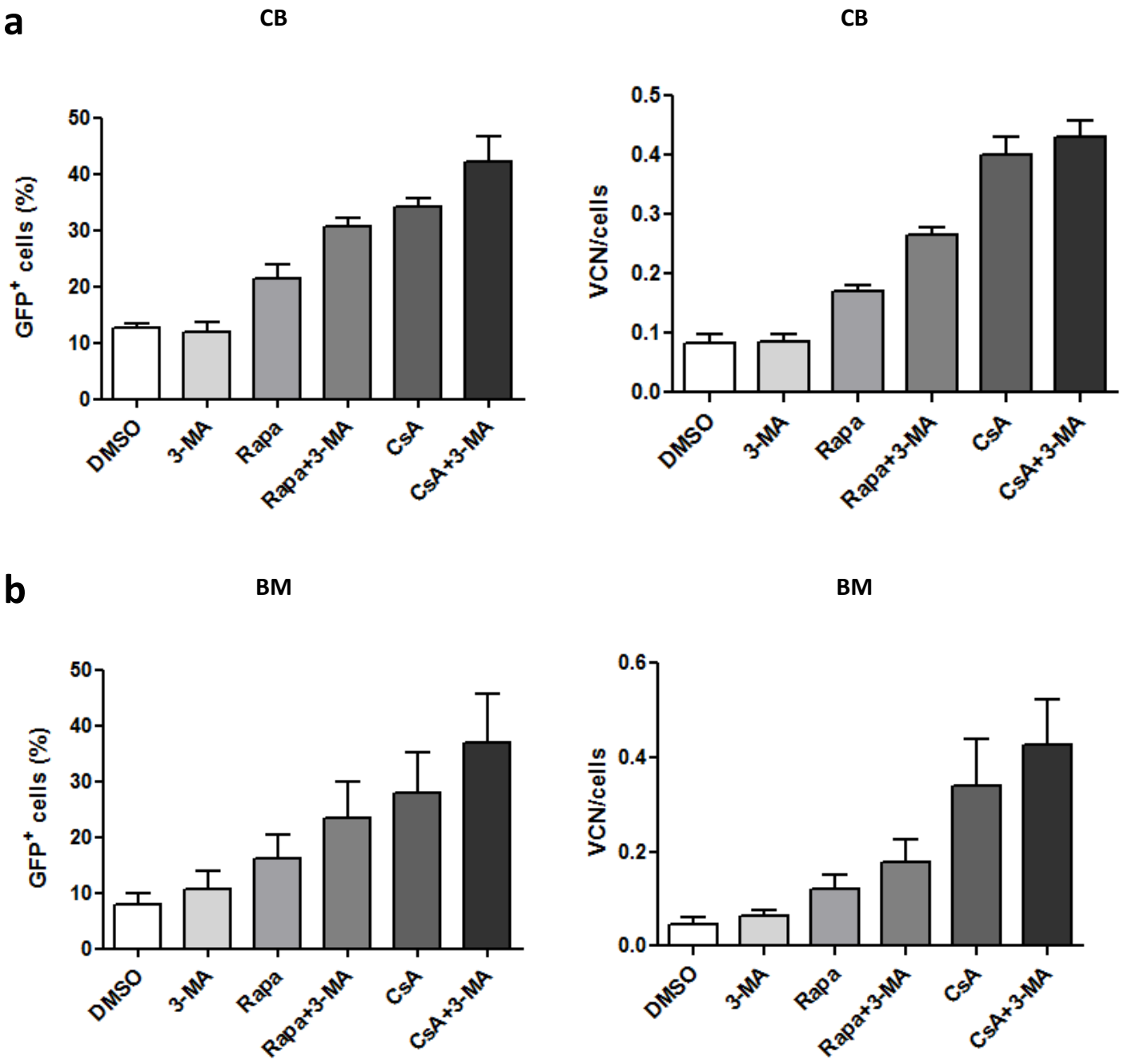


Figure S12. Inhibition of autophagy enhances both CsA and Rapa-mediated improvement of transduction. (**a**) CB and (**b**) BM-derived CD34$^+$ cells were transduced with SINLV-GFP at an MOI of 1, in presence or absence of 10 µmol/l CsA, 10 µg/ml Rapamycin or 5 mmol/l 3MA or their combinations. Left panels, The percentage of GFP$^+$ cells was evaluated by FACS 5 days post-transduction. Right panels, VCN were assessed 14 days post-transduction. Data represent the mean ± SEM of three independent experiments.

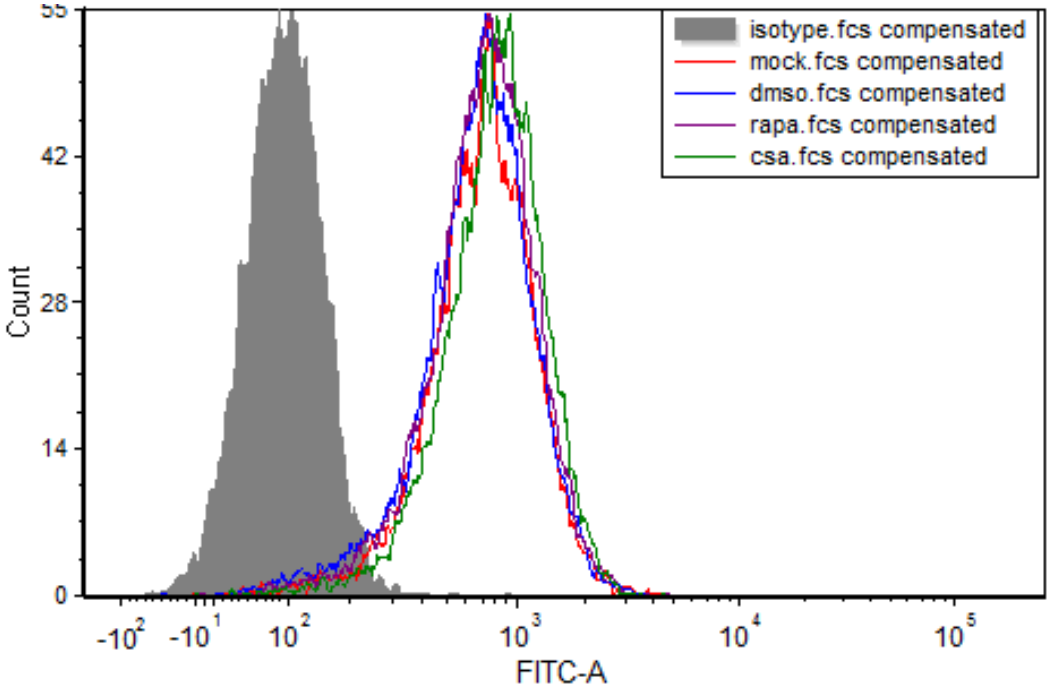


Figure S13. Impact of Rapa and CsA on LDL-R surface expression in HSPC. CB-derived $CD34^+$ cells were exposed to10 µmol/l CsA or 10 µg/ml Rapamycin for 6h and LDL-R surface expression was measured by FACS. Data are representative of two independent experiments.

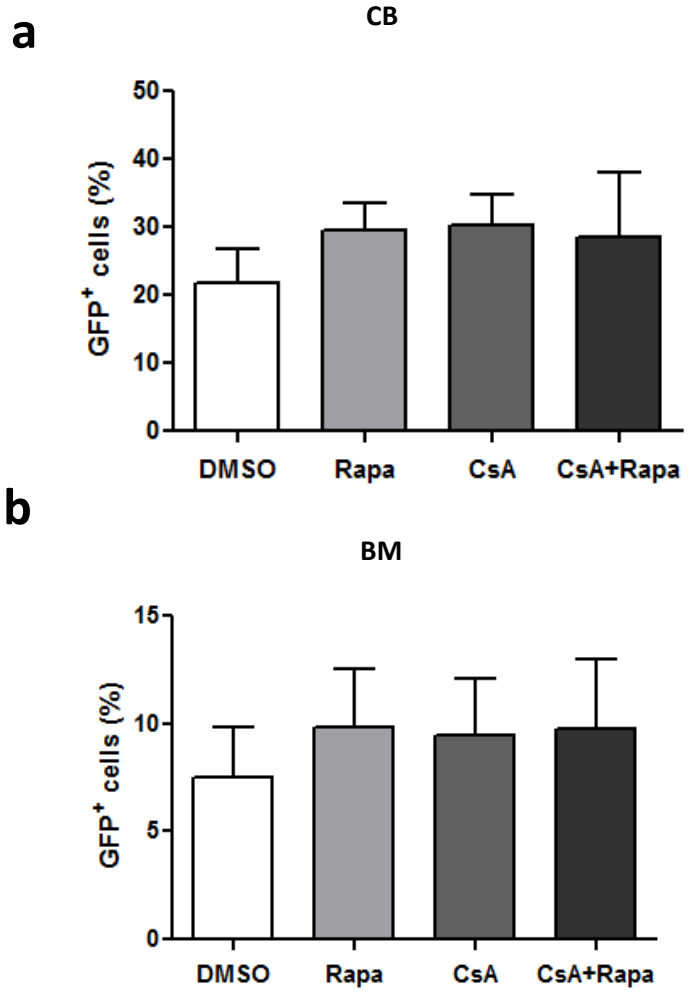


Figure S14. Effects of CsA and Rapa are lentivirus specific. (**a**) CB and (**b**) BM-derived $CD34^+$ cells were transduced with SINRV-GFP at an MOI of 10, in presence or absence of 10 µmol/l CsA or 10 µg/ml Rapamycin or their combination. The percentage of GFP^+ cells was evaluated by FACS 5 days post-transduction. Data represent the mean ± SEM of four independent experiments.

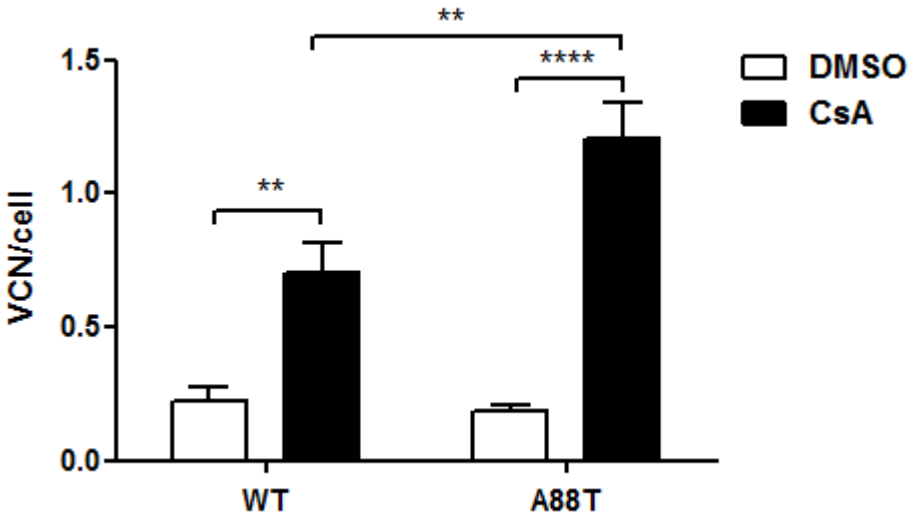


Figure S15. A88T yields more integrated proviral copies that WT LV in presence of CsA. CB-derived $CD34^+$ cells were transduced with SINLV-GFP harboring either the WT or the A88T capsid at a MOI of 1, in presence or absence of 10 µmol/l CsA. VCN/cell were determined by Taqman assay 14 days post-transduction. Data represent the mean ± SEM of four independent experiments. p value are for One way ANOVA with Bonferroni's multiple comparison.

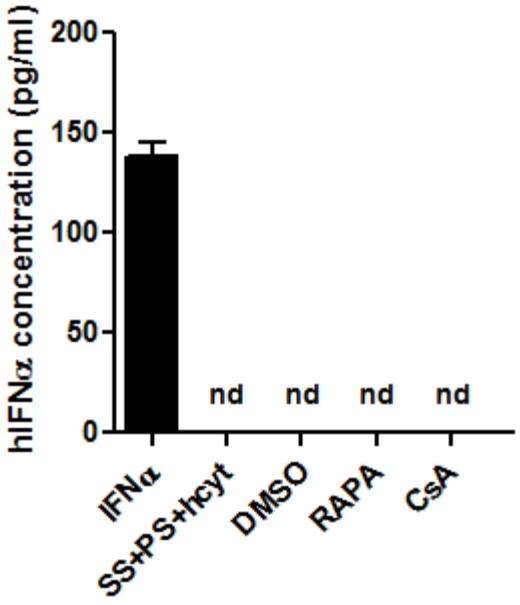


Figure S16. IFNα is not detected upon exposure of HSPC to CsA/Rapa. Supernatants from CB-derived CD34$^+$ cells were harvested 24 hours post-transduction with at an MOI of 10, in presence or absence of 10 µmol/l CsA or 10 µg/ml Rapamycin. Medium supplemented with recombinant human IFNα was used as positive control and the medium in which the cells were cultured (SS+PS+hcyt) as the negative one. Data are the mean ± SEM of three independent experiments. Nd for not detected (detection limit 12.5pg/ml).