Published in final edited form as:
Mol Ther. 2015 February ; 23(2): 352–362. doi:10.1038/mt.2014.193.

Cyclosporin A and Rapamycin relieve distinct lentiviral restriction blocks in hematopoietic stem and progenitor cells

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Abstract

Improving hematopoietic stem and progenitor cell (HSPC) permissiveness to HIV-derived lentiviral vectors (LVs) remains a challenge for the field of gene therapy as high vector doses and prolonged *ex-vivo* culture are still required to achieve clinically relevant transduction levels. We report here that Cyclosporin A (CsA) and Rapamycin (Rapa) significantly improve LV gene transfer in human and murine HSPC. Both compounds increased LV but not gammaretroviral transduction and acted independently of calcineurin and autophagy. Improved gene transfer was achieved across all $CD34^+$ subpopulations, including in long-term SCID repopulating cells. Effects of CsA were specific of HSPC and opposite to its known impact on HIV replication. Mutating the Cyclophilin A binding pocket of the viral capsid (CA) further improved transduction in combination with CsA. Tracking of the LV genome fate revealed that CsA relieves a CA-dependent early block and increases integration, while Rapa acts early in LV infection independently of the viral CA. In agreement, only Rapa was able to improve transduction by an integrase-defective LV harboring wild-type CA. Overall, our findings pave the way for more efficient and sustainable LV gene therapy in human HSPCs and shed light on the multiple innate barriers specifically hampering LV transduction in these cells.

Introduction

Hematopoietic stem cell (HSC) gene therapy has tremendous potential to treat human disease. Therapeutic benefits have already been achieved using γ-retroviral (γRV) [1-3] as well as lentiviral vectors (LV) as gene delivery vehicles [4-6]. Nevertheless, suboptimal target cell permissivity still imposes the use of multiple hits of high vector doses and

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For details see supplementary materials.

The authors declare no conflict of interest.

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prolonged *ex-vivo* culture to reach the high transduction levels observed in some of the recent LV-based clinical trials [5, 6]. This remains a drawback for the field as it implies large-scale vector productions and may lead to impaired preservation of HSC in culture. Therefore efforts to improve LV transduction efficiency are still needed, as even relatively small improvements in cell permissivity to transduction could greatly impact on sustainability of vector production and the number of patients that could be treated with each batch of vector.

Antiviral factors referred to as restriction factors (RFs) targeting specific steps of the retroviral life cycle [7] could be responsible, at least in part, for poor permissiveness of HSC to gene transfer with HIV-derived LV. Some of these RFs are inducible by specific danger signals such as type I IFN. In this regard, innate immune signaling and in particular IFN-mediated responses have been implicated in impaired LV transduction *in vivo* [8, 9] and immunosuppressive treatment has been shown to improve LV transduction in hepatocytes [8]. Among immunosuppressive compounds, Cyclosporin A (CsA), and the structurally related macrolides FK506 and rapamycin (Rapa), are currently approved for use in prevention of allograft rejection. CsA forms a complex with the host peptidyl-prolyl isomerase (PPIase) cyclophilin A (CypA), inhibiting the Ca^{2+}-dependent phosphatase calcineurin and consequent activation of pro-inflammatory cytokines such as IL-2 [10]. Rapa and FK-506 exert their immunosuppressive functions through binding and inhibition of another host PPIase, FKBP12 [11]. Besides its immunomodulatory functions, CsA has been widely used in the field of HIV-1 research to investigate the role of CypA in modulating HIV-1 replication, as it disrupts the CypA-capsid (CA) interaction [12]. The beneficial effects of this interaction on HIV-1 replication in human cells has been demonstrated through multiple approaches over the years, although the exact mechanisms remain unknown [13]. In the context of HSC, only a handful of studies have investigated the impact that engineering the viral CA or modulating its interactions with host factors could have on lentiviral transduction efficiency [14-16]. Rapa has been shown to improve engraftment and SCID repopulating capacity of cord blood (CB)-derived $CD34^+$ cells [17] and to increase the number of long-term HSCs *in vivo* when combined with an activator of the Wnt–β-catenin [18]. We investigated here the impact CsA alone or in combination with Rapa could have on transduction efficiencies in human and murine HSPC and provide evidence that both compounds significantly increase LV-mediated gene transfer in long-term SCID repopulating HSCs through distinct mechanisms.

Results

Cord-blood (CB)-derived $CD34^+$ cells were stimulated for one day with early-acting cytokines (interleukin-6 [IL-6], stem cell factor [SCF], thrombopoietin [TPO], and Flt3 ligand [Flt3L]) [15] and transduced with a self-inactivating (SIN) lentiviral vector expressing GFP under the control of the PGK promoter (SINLV-GFP) at increasing MOI, in presence or absence of increasing concentrations of CsA. While the two lowest concentrations of CsA did not improve LV transduction, both 10 μM and 50 μM CsA led to a marked increase in the percentage of GFP^+ cells (Fig. S1a), but increased toxicity was observed with 50 μM CsA (Fig. S1b). To further investigate the concentration range at which CsA improves transduction, we titered the compound in the 1 to 10 μM range.

Improved transduction was observed only at the two highest doses without significant differences in cell growth between them (Fig. S1c,d). The 10 μM concentration was then chosen for further studies. At this concentration, CsA treatment consistently led to a 3-fold increase in the percentage of GFP^+ cells (Fig. 1a, CsA vs. DMSO, $p<0.0001$) and increased the integrated vector copies by 5 to 6-fold on average in CB and BM-derived HSPC (Fig. 1b, CsA vs. DMSO, $p=0.0003$ for CB and $p=0.0237$ for BM-derived HSPC, left and right panels respectively). This effect was specific for HSPC, as transduction in presence of 10 μM CsA led to a 2.5-fold decrease in GFP^+ cells in $CD4^+$ T cells activated with PHA and IL-2 for three days prior to transduction as well as in monocyte-derived macrophages (MDM) (Fig. 1c, CsA vs DMSO, $p<0.0001$ and Fig. 1d, CsA vs DMSO, $p=0.0023$, respectively). Activation of $CD4^+$ T cells with antibodies against CD3 and CD28 in presence of IL-7 and IL-15 did not alter the negative impact CsA has on LV transduction in these cells (Fig. S2).

To test calcineurin involvement, we transduced CB and BM-derived $CD34^+$ cells, $CD4^+$ T cells and MDM in presence of FK-506, a compound which blocks calcineurin similarly to CsA but independently of CypA [10]. FK-506 had no impact on LV transduction efficiency in HSPCs and actually tended to increase it in activated $CD4^+$ T cells and MDM (Fig. S2 and S3). CypA mRNA and protein levels could not explain this discrepancy in the CsA impact, as $CD34^+$ HSPC expressed similar levels of CypA as $CD4^+$ T cells and MDM (Fig. 1e and 1f, respectively). Comparable levels were also detected in the myelogenous leukemia cell line K562, in which CsA had little or no effect on transduction (Fig. S4).

We then compared the impact of CsA on LV transduction to that of Rapamycin alone or in combination with CsA in both CB and BM-derived HSPC. We chose the 10 μg/ml concentration for our experiments as lower doses of Rapa failed to improve transduction as efficiently (Fig. S5). Rapamycin led to a statistically significant 2-fold increase in the percentage of GFP^+ cells in both CB and BM-derived HSPC (Fig. 2a, left panel, Rapa vs. DMSO, $p<0.0001$; Fig. 2b, left panel, Rapa vs. DMSO, $p<0.001$). Observed increases in transduction efficiencies in terms of percentages of GFP^+ cells were mirrored by a comparable, if not even greater, increase in terms of VCN/cell measured after two weeks of culture (Figs. 2a, b, right panels). As opposed to CsA, the effect of Rapa on LV transduction efficiencies was not specific for the HSPC compartment as a trend to increase was observed in $CD4^+$ T cells, whether activated with PHA/IL-2 or through CD3/CD28 stimulation, while it significantly decreased transduction by 3-fold in MDM (Fig. S2 and S6). Combining the two compounds together during LV transduction led to a further increase in terms of transduction efficiency in both CB and BM-derived HSPC, in which a 4.5-fold increase was observed on average (Fig. 2a, b, left panels, DMSO vs. CsA+Rapa, $p<0.0001$) at the expense of acute cell loss and survival of a fraction of treated cells (Fig. S7). Both CsA and Rapa, as well as their combination, yielded equally improved transduction levels in all HSPC subpopulations ranging from the more committed $CD34^-$ progenitors to the most primitive $CD34^+CD133^+CD90^+$ cells, reaching on average 3 to 4-fold more GFP^+ cells as compared to controls (Fig. 2c, $p<0.05$ for Rapa, $p<0.001$ for CsA, $p<0.0001$ for CsA+Rapa). No significant alterations in the relative percentages of the different subpopulations were observed among the treatments (Fig. S8). Similarly to human HSPC, both CsA and Rapa, as well as their combination, significantly improved transduction also in murine Lin^- HSPC

(Fig. S9a), no major differences in the transduction levels between the different conditions were observed in murine $CD4^+$ T cells (Fig. S9b).

We also tested the capacity of CsA and Rapa to improve transduction efficiency of the same SINLV vector used in previous experiments but produced in a large-scale setting and with a downstream processing that has been applied in the context of recent clinical trials conducted at our Institute [5, 6]. Both CsA and Rapa yielded increased transduction efficiencies at different MOI also in this context, reaching a 2 to 3-fold increase in the percentages of GFP^+ cells as compared to controls at an MOI of 10 (Fig. 3a, $p<0.001$ for Rapa, $p<0.0001$ for CsA and CsA+Rapa). This increase was accompanied by a 5- and 10-fold increase in VCN/cell at an MOI of 10 for CsA and CsA+Rapa-treated cells, respectively (Fig. 3b).

We addressed whether CsA and Rapa are able to improve LV transduction in clonogenic progenitors without hampering their colony-forming capacity. No significant differences were observed between the different treatments in the absolute numbers of total, colony-forming unit-granulocyte/macrophage (CFU-GM) (myeloid) and burst-forming unit erythroid (BFU-E) (erythroid) colonies deriving from equal CB $CD34^+$ cellular inputs, although a trend towards lower CFU counts was seen for CsA+Rapa (Fig. 4a). Similarly, the percentages of myeloid ($CD33^+$) and erythroid ($CD235a^+$) cells harvested from pooled colonies did not vary among the different treatments after two weeks of culture (Fig. 4b). Transduction efficiencies were significantly increased within both myeloid and erythroid cells, reaching around 90% of GFP^+ cells in all treatment groups as compared to ~60% reached in controls (Fig. 4c, $p<0.001$ for Rapa; $p<0.0001$ for CsA and CsA+Rapa). Increased transduction efficiency was confirmed by measurement of VCN/cell on pooled colonies isolated after 14 days of culture (Fig. 4d). Results were confirmed also in BM-derived $CD34^+$ cells in which no differences in colony counts were observed, except for the CsA+Rapa combination (Fig. 4e). None of the treatments affected the percentages of myeloid ($CD33^+$) and erythroid ($CD235a^+$) progenitors (Fig. 4f) and increased transduction was maintained in all clonogenic progenitors in terms of GFP^+ cells, reaching ~80% in all treatment groups as compared to 40% observed in the control (Fig. 4g). Improved transduction efficiency was reflected as a 3-fold increase in average in VCN/cell for all treatment groups as compared to the control (Fig. 4h).

In order to investigate the impact of CsA and Rapa on HSC, we transplanted the treated $CD34^+$ cells into NSG mice and assessed the extent of GFP marking in human hematopoietic cells in the long-term repopulated mice. FACS analysis of the peripheral blood of mice at 11 weeks after transplant showed similar engraftment levels of human $CD45^+$ cells (~40%) in all treatment groups (Fig. 5a) and no significant differences were observed in the percentages of human B ($hCD19^+$), T ($hCD3^+$) and myeloid cells ($hCD33^+$) within the human $CD45^+$ population when compared to controls (Fig. 5b). Mice transplanted with CsA-, Rapa- as well as CsA+Rapa-treated human HSPC showed comparable high levels of transduction (around 80% of $GFP^+/hCD45^+$ cells) in all the different human hematopoietic lineages, which were significantly higher than those observed in the control group (Fig. 5c, $p=0.0001$).

The engraftment levels of human $CD45^+$ cells in the BM of mice 20 weeks post-transplantation were comparable among the DMSO-, CsA-, and CsA+Rapa-treatment groups, while the Rapa-treatment group showed a statistically significant increase in the levels of engraftment with respect to controls (Fig. 5d, $p<0.047$). No significant differences were observed in the composition of the different lineages within the $hCD45^+$ fraction in the BM among the treatment groups (Fig. S10a). Moreover, the VCN per human genome measured at the end of the experiment in the BM cells of mice transplanted with CsA, Rapa and CsA+Rapa-treated HSPC was 3 to 4-fold higher with respect to the VCN found in BM cells of the control mice (Fig. 5e, $p<0.002$) and increased transduction was maintained also among the different lineages in the BM (Fig. S10b). Finally, no significant difference in the percentages of phenotypically identified cell subsets enriched for HSCs ($CD34^+CD38^{low}$), progenitors ($CD34^+CD38^+$) and differentiated cells ($CD34^-CD38^+$) were detected among all treatment groups (Fig. 5f).

The levels of total human $CD45^+$ cells and of the different hematopoietic lineages (B, T and myeloid cells) were comparable among the different groups in the spleens of transplanted mice (Fig. 5g, Fig. S10c). Moreover, a significant increase in the overall percentage of GFP^+ cells in the CsA, Rapa and CsA+Rapa treatment groups with respect to DMSO treatment was also detected and was maintained among the different lineages (Fig. 5h, CsA 88.7±2, Rapa 80.8±1 or CsA+Rapa 87.0±0.6 vs. DMSO 49.0±5, $p<0.0001$, Fig. S10d). The transduction efficiencies observed *in vivo* in terms of percentages of GFP^+ cells and VCN were comparable to those observed in liquid culture seeded in parallel with the transplanted cells (Fig. S11).

Since both CsA and Rapa are reported to induce autophagy [19], which is emerging as a mechanism of escape to innate immune responses in the case of some viral pathogens [20], we transduced the $CD34^+$ HSPC in presence or absence of the inhibitor of autophagy 3-methyladenine (3-MA) [19], to investigate whether this could be the common denominator behind the beneficial effect that both compounds have on LV transduction. Although 3-MA alone had little or no effect, its combination with both CsA and Rapa led to a further improvement of transduction, both in terms of percentages of GFP^+ cells as well as VCN/cell (Fig. S12a, b), indicating that blocking autophagy does not hamper their beneficial effect on LV transduction.

A recent report suggests that quiescent HSPC are poorly permissive to VSV-g-pseudotyped LV transduction because of low levels of expression of its receptor, the low-density lipid receptor (LDL-R) [21]. Increased permissivity to transduction upon cytokine-stimulation was shown to correlate with increased LDL-R expression [21]. We examined whether CsA and Rapa could further increase LDL-R levels in cytokine-stimulated human HSPC. No major differences in LDL-R surface expression were observed between the different groups at 6h post-exposure (Fig. S13). We then tested whether Csa/Rapa could benefit also VSV-g pseudotyped γ-retroviral vectors in cytokine-stimulated HSPC. However, neither compound was able to significantly increase the percentage of GFP^+ cells in either CB or BM-derived HSPC (Fig. S14), suggesting that LV-specific pathways and/or viral components are involved in the beneficial effects observed for SINLV. In order to address whether the LV CA-CypA interaction could be involved in CsA-mediated improvement of transduction in

HSPC, we generated SINLVs harboring individual CA mutants with specific phenotypes described in Table 1. All CA mutants showed impaired transduction efficiencies in $CD4^+$ T cells, in which CypA is an LV promoting factor, despite similar infectivity as the wild-type (WT) vector in 293T cells (Table S1) (Fig. 6a, white bars, mutants vs. WT, $p<0.0001$ for all except A92E which did not reach statistical significance). Impact of Rapa did not vary among the different CA mutants, while CsA inhibited WT LV transduction by around 2-fold (Fig. 6a, DMSO vs. CsA, $p<0.01$) but did not affect the V86M, A105T, N74D and A88T mutants. The P90A and A92E mutants tended to benefit from CsA in $CD4^+$ T cells, although not reaching statistical significance (Fig. 6a). In $CD34^+$ cells all CA mutants, except A92E and A88T, performed less well than the WT vector in control conditions (Fig. 6b, white bars, mutants vs. WT, $p<0.0001$ for all except A92E and A88T). However, CsA improved transduction efficiency of all vectors (Fig. 6b, DMSO vs. CsA, $p<0.0001$ for all). Interestingly, higher transduction efficiency was observed for A88T as compared to the WT vector in presence of CsA (Fig. 6b, A88T vs. WT with CsA, $p<0.05$). Such additive effects were not observed with Rapa despite increased efficiency for all CA mutants.

To further dissect the mechanisms through which CsA and Rapa increase LV transduction in HSPC, we tracked the LV genome fate by measuring viral DNA replication intermediates at an early time post-transduction in Rapa, CsA and control treated CB-derived $CD34^+$ cells transduced with LV harboring either WT or the A88T viral CA. Rapa led to a 2 to 3-fold increase in late-RT products independently of the CA (Fig. 7a, Rapa vs. DMSO, $p<0.05$), followed by a consequent increase in both 2-long-terminal-repeat (2LTR) circles (Fig. 7b, Rapa vs. DMSO, $p<0.01$ for WT, $p<0.05$ for A88T), surrogate of nuclear entry of HIV-1 DNA [22], as well as in integrated vector copies (Fig. 7c, Rapa vs. DMSO). Interestingly, CsA did not alter late-RT nor 2LTR circle formation for the WT LV (Figs. 7a,b) while a 2 to 3-fold increase in both products was observed for the A88T LV (Figs. 7a, b, CsA vs. DMSO, $p<0.01$ and $p<0.05$, respectively). CsA lead to a significant increase in the amount of integrated proviral copies for both vectors (Fig. 7c, CsA vs. DMSO, $p<0.05$) and the A88T CA mutant LV yielded a higher VCN/cell as compared to WT vector in presence of CsA (Fig. 7c and Fig. S15, CsA WT vs. CsA A88T, $p<0.01$), although not reaching statistical significance at the MOI of 10.

To further test the hypothesis that CsA would act after nuclear entry in the context of WT CA, we measured the capacity of CsA and Rapa to increase transduction of integrase-defective LV (IDLV) in CB-derived $CD34^+$ cells. Similarly to integrating vectors, Rapa lead to an increase in IDLV transduction, measured both by FACS (Fig. 7d, left panel, DMSO vs. Rapa, $p<0.05$) as well as retrotranscribed viral DNA copies measure 3 days post-transduction (Fig. 7d, right panel, DMSO vs. Rapa, $p<0.01$). CsA instead was not able to improve IDLV transduction (Fig.7d), further suggesting that it acts during the LV integration step in human HSPC.

Discussion

We report here that LV gene transfer efficiency can be significantly increased in murine and human HSPC, including in the clinically relevant BM-derived human $CD34^+$ cells, by a short exposure to CsA or Rapa during the transduction period. Both compounds increased

LV transduction in all $CD34^+$ subpopulations suggesting that they overcome restriction blocks common to all HSPC. This observation is of importance as the true stem cells endowed with self-renewal and repopulating capacity are a minority comprised within the most primitive $CD34^+CD133^+CD90^+$ HSPC fraction [23]. Along these lines, improved LV transduction efficiencies were maintained also in clonogenic progenitors without affecting their colony-forming capacity and no differences in transduction efficiencies were measured among all human peripheral blood subsets monitored in transplanted NSG mice, indicating overall improved gene transfer in the multipotent progenitors/HSC *in vivo.* Importantly, increased transduction efficiencies were maintained long-term *in vivo* and no effects on HSPC engraftment or SCID repopulating capacities were observed with the exception of the Rapa-treatment group in which we saw, in line with previous reports [17, 18], a statistically significant increase in HSPC engraftment. This could be of further benefit in clinical settings in which engraftment kinetics are crucial for successful gene therapy outcome [24]. Improved gene transfer efficiency was obtained also with a SINLV that had been processed with the same downstream protocols as vectors used in current clinical trials [5, 6]. This observation is of value bearing in mind that clinical-grade vector preparations, although largely devoid of contaminant byproducts such as plasmid DNA and cell debris, are often less infectious than the standard laboratory-grade stocks [25].

A recent report suggests that one of the components of poor HSPC permissiveness to VSV-g-pseudotyped LV transduction resides in low levels of expression of the low-density lipid receptor (LDL-R) when cells are maintained quiescent [21]. Nevertheless, neither CsA nor Rapa altered LDL-R expression levels in cytokine-stimulated human HSPC at 6 hours post-exposure, when most of the viral entry has occurred. Furthermore, VSV-g pseudotyped SINRV did not benefit from Rapa/CsA-exposure in $CD34^+$ cells suggesting that Rapa/CsA relieve other envelope/receptor-independent restrictions in HSPC and that the LDL-R levels are not rate-limiting in this cell compartment when activated by growth-promoting cytokines.

Rapamycin, CsA as well as proteasomal inhibition, previously shown to increase LV transduction efficiency in human HSPC [15], induce autophagy in a variety of cell types [19, 26]. However, autophagy does not seem to be involved in either case as the autophagy inhibitor 3-MA together with either Rapa or CsA actually further improved gene transfer efficiency rather than impaired it. As autophagy-inducing agents have been shown to inhibit HIV replication in primary human MDM [27], blocking its induction may therefor further improve transduction efficiency in presence of these compounds. No increase with 3-MA alone was observed suggesting that basal levels of autophagy are not sufficient *per se* to impact on LV transduction in HSPC.

The effects of Rapa were independent of the viral CA as all tested mutants still benefitted from it. Furthermore, we observed that Rapa leads to an increase in all LV replication intermediates, suggesting it acts early in the viral life cycle. Our observations are in accordance with the recent study by Wang et al., reporting that Rapamycin increases LV transduction of murine and human HSPC in an autophagy and CA-independent manner by enhancing post-binding endocytic events, leading to increased levels of reverse-transcription and genomic integration [28].

Interestingly, the effects of CsA were HSPC-specific and opposite to what occurs in differentiated hematopoietic cells. These observations were confirmed also in the murine context, suggesting an evolutionary conserved mechanism of action, although CsA failed to impair LV transduction in murine $CD4^+$ T cells as reported also previously [29]. In the context of human HSPC, we observed that CsA was able to improve LV transduction only at high concentrations required to disrupt CypA-CA interaction [30] but not in its nanomolar immunosuppressive range [31]. This concentration-dependent impact of CsA on LV transduction could explain, at least in part, why in previous studies it did not improve LV transduction in HSPC, as concentrations below 8 μM were used [14, 16]. CsA-mediated increase in LV transduction is usually observed in the context of cross-species infection due to disruption of the interaction of the lentiviral core with the host restriction factor TRIM5α and only specific HIV-1 CA mutants have been reported to benefit from CsA in human cells [22], a trend we observed for the P90A and A92E CA mutant LV in CsA-exposed $CD4^+$ T cells. A recent report suggests that variable levels of TRIM5α expression in human HSPCs correlated with donor-dependent variability in permissivity to LV transduction [32]. However, such mechanisms unlikely explain our observations as most of the $CD4^+$ T cells and MDM, in which CsA had the opposite effect, were donor-matched with the CB-derived HSPC. Furthermore, all CA mutants tested still benefited from CsA suggesting CA-independent relief of restriction, most likely after nuclear import as only integrated proviral copies were increased in the context of WT LV upon CsA exposure. In agreement, CsA, as opposed to the early-acting Rapa, was not able to increase transduction efficiency of an integrase-defective LV.

A previous study has shown that MG-132-mediated proteasomal inhibition increases LV transduction efficiency in human and murine HSPC independently of the CypA-CA interaction [15] and high concentrations of CsA have been shown to increase transduction of primary murine bone-marrow derived cells in a CA-independent manner [29]. Interestingly however, we observed that mutating the CypA binding pocket of the LV CA did alter the impact of CsA on the viral replication intermediates in human HSPC suggesting that also the CA-CypA interaction may be involved in LV restriction in HSPC. The A88T mutant has been reported to no longer bind CypA and to escape to the antiviral action of the recently identified IFN-induced host factor Mx2 [33]. Interestingly, the antiretroviral activity of Mx2 has been reported to involve CypA [33] but may not depend solely on the CA-CypA interaction as CsA failed to rescue HIV-1 infection in Mx2-overexpressing $CD4^+$ MT-4 cells [34]. However, although the A88T CA mutant did perform significantly better than the WT vector in human HSPC in presence of CsA, no benefit was observed in control conditions. Furthermore, we observed an increase also in late-RT products in presence of CsA for the A88T CA mutant while Mx2 has been suggested to act during nuclear import and/or viral integration [33, 35]. Also direct involvement of CypA in CsA-mediated relief of restriction in HSPC remains unclear, as another CypA-independent mutant, P90A, failed to transduce HSPC to a greater extent than the WT vector. Of note, CsA inhibits only the CypA-CA interaction while P90A, in addition to CypA, no longer interacts with Nup358, a key factor during HIV-1 nuclear entry [36]. Lack of Nup358 interaction could hamper detection of benefits related to escape from CypA-mediated restriction in HSPC for P90A. Based on our observations, it will be of interested to further investigate involvement of Mx2

and CypA in poor HSPC permissiveness to LV transduction. Also other yet to be discovered factors binding the viral CA may contribute and testing of a broader panel of CA mutants, including recently identified Mx2-resistants [34], may help to further dissect the host and viral determinants involved in LV restriction in HSPC. Host factors directly impairing the HIV-1 integration process, such as TRIM28 [37] or the cyclin-dependent kinase inhibitor CDKN1A (p21) [38] could instead be involved in the CA-independent impact of CsA on LV integration in HSPC.

It has recently been reported that altering interactions of the HIV-1 core with host factors, including disruption of the CypA-CA complex by CsA, during the early phases of transduction can lead to IFN production in MDM [39]. However, we did not detect IFNα secretion in transduced HSPC cultures, whether treated or not with CsA/Rapa (Fig. S16). This suggests that overt triggering of IFN secretion does not occur in this cell compartment, favoring safe implementation of CsA/Rapa in clinical HSPC transduction protocols for future applications. Nevertheless, only the use of single compounds can be envisaged as visible toxicity of the combination of CsA and Rapa was observed. In this regard, less toxic non-immunosuppressive variants of CsA that retain the capacity to disrupt the LV CA-CypA interaction have been described [29, 39, 40] and could provide a means to improve transduction efficiency in human HSPC while reducing toxicity. Careful investigation of the impact of CsA on LV integration profiles in human HSPC will also be required as CsA has been reported to redirect integration of LV into regions of higher gene density in human cell lines [36], potentially impacting transgene expression levels and long-term safety.

Overall, our findings provide an important step forward in the development of more efficient and sustainable LV gene therapy protocols as they should enable scaling down of required vector doses as well as the shortening of HSPC *ex-vivo* transduction protocols that currently employ two transduction hits to reach high levels of gene marking. In the future, the protocols described in this work can be tailored for each specific disease application to achieve the most appropriate range of transduction efficiency and VCN/cell for clinical implementation. Furthermore, we shed light on the multiple restriction bottlenecks hampering lentiviral gene transfer in human HSPC and bring novel perspectives for the study of defense mechanisms safe-guarding them from lentiviral invasion.

Materials and Methods

Vectors, cells and transduction

Vectors were prepared and used as previously described [41]. Cyclosporin A (CsA), FK506, Rapamycin (Rapa) and 3-MA (Sigma-Aldrich) were added during transduction. Human $CD34^+$ hematopoietic stem and progenitor cells (HSPC), $CD4^+$ T cells and $CD14^+$ monocytes were isolated through magnetic bead selection according to manufacturer's instructions (Miltenyi Biotec) 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. 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). All cells were transduced at the indicated multiplicity of infection (MOI) as calculated by titration of vector stocks on 293T cells.

Colony-forming cell (CFC) assay and mice

CFC assays and transplantation of NOD-SCID-IL2Rg$^{-/-}$ (NSG) mice with human CB-derived CD34^{+} cells were performed as previously described [42]. All animal procedures were performed according to protocols approved by the Animal Care and Use Committee of the Ospedale San Raffaele (IACUC 661) and according to the Italian law.

RNA, DNA and Proteins

For replication intermediate analysis, cells were lysed and processed 24 hours post-transduction in Monini lysis buffer as previously described followed by semi-quantitative Taqman assays [22, 43]. VCN, copies of IDLV and gene expression were assessed as previously described [15, 44]. Western Blot was performed as previously described [45].

Statistical analysis

Data are expressed as mean ± standard error of the mean (SEM). Unpaired Student's t test or ANOVA for multiple comparisons were used. Percentages were converted into Log ODDs for statistical analysis. Significance was considered at $p<0.05$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by grants from the Italian Ministry of Health and the Italian Telethon Foundation to A.K.R. (Bando Giovani Ricercatori GR-2009-1471693, TGT14-D04); from Italian Telethon Foundation (Telethon D1) to E.M. and from Italian Telethon Foundation and the European Research Council (TARGETINGGENETHERAPY) to L.N. C.P. conducted this study as partial fulfillment of her Ph.D. in Molecular Medicine, Program in Cellular and Molecular Biology, International Ph.D. School, Vita-Salute San Raffaele University, Milan, Italy. We wish to thank Bernhard Gentner for helpful discussions and critical reading of the manuscript, Erika Zonari, Tiziana Plati and Giulia Escobar for technical advice, Pierangela Gallina for assistance with the mouse experiments, Molmed SpA and Alessandro Aiuti for the purified SINLV-GFP and Andrea Annoni for help with isolation of primary murine cells. C.P designed and performed experiments, analyzed and interpreted data and wrote the manuscript; D.C. designed, performed and analyzed the *in vivo* experiments and wrote the manuscript, F.P and S.B. performed experiments, L.N. provided intellectual input, interpreted data and revised the manuscript, E.M supervised all *in vivo* experiments, provided intellectual input, interpreted data and revised the manuscript. A.K.R conceived, designed and supervised the project and wrote the manuscript.

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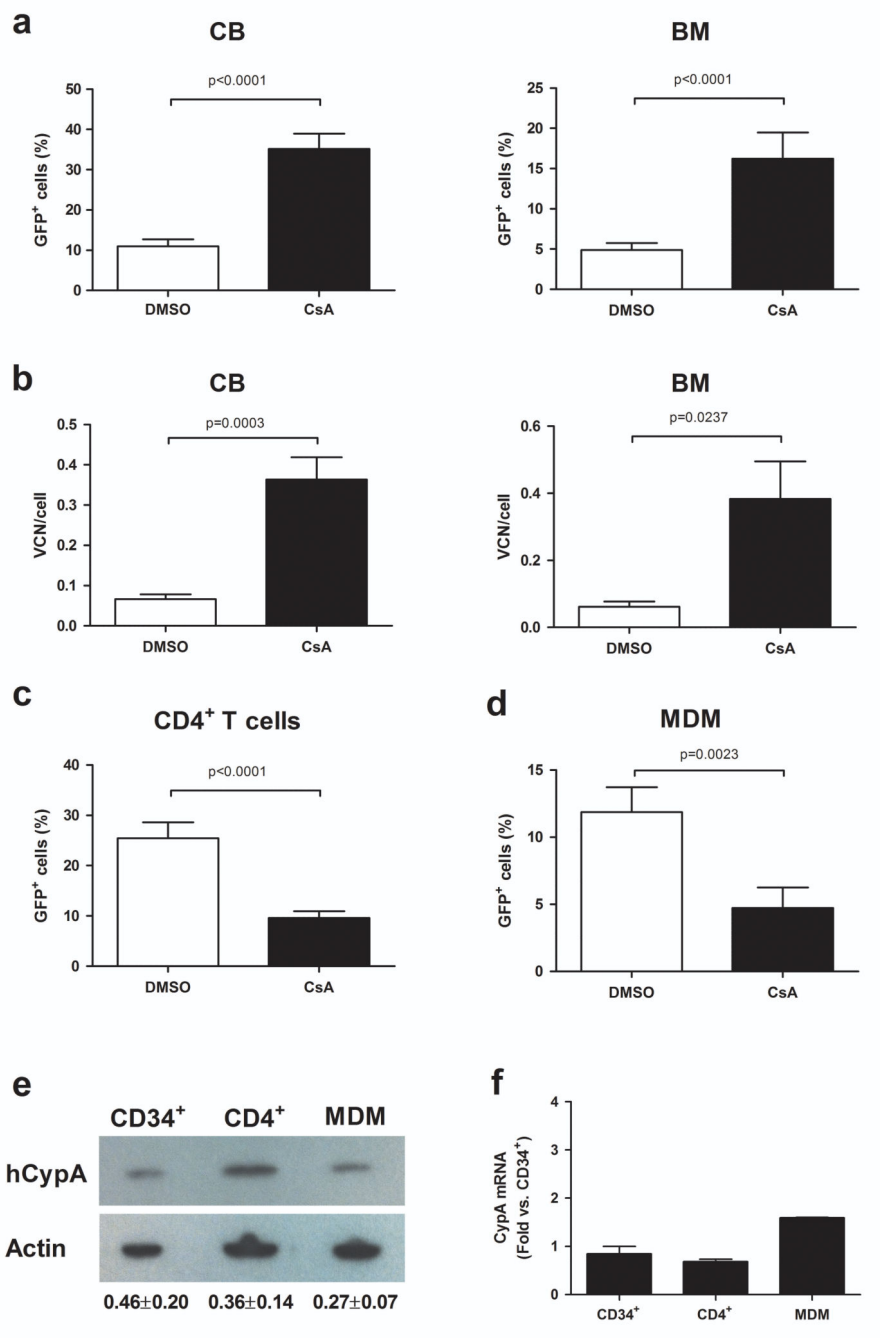

Figure 1. CsA increases LV transduction efficiency specifically in human HSPC

CB and bone marrow (BM)-derived CD34$^+$ cells (**a,b**), CD4$^+$T (**c**) cells and monocyte-derived macrophages (MDM) (**d**) were transduced with SINLV-GFP at an MOI of 1, in presence or absence of 10 μM CsA. The percentage of GFP$^+$ cells was evaluated by FACS 5 days after transduction. Vector copy numbers (VCN) were assessed by qPCR 14 days post-transduction in CB (**b**, left panel) and BM-derived CD34$^+$ cells (**b**, right panel). Data are the mean ± SEM of at least six independent experiments. p values are for paired Student's t-test. (**e**) CypA protein levels in CB-derived CD34$^+$, CD4$^+$ T cells and MDM were analyzed by

Western blot, actin was used as normalizer. One representative out of 2 gels is shown. Numbers below are the mean ± SD of the signal quantified by ImageJ software and normalized to actin. (**f**) CypA mRNA was detected from total RNA by qPCR. Data are represented as fold differences vs. one of the two CB-CD34^{+} donors.

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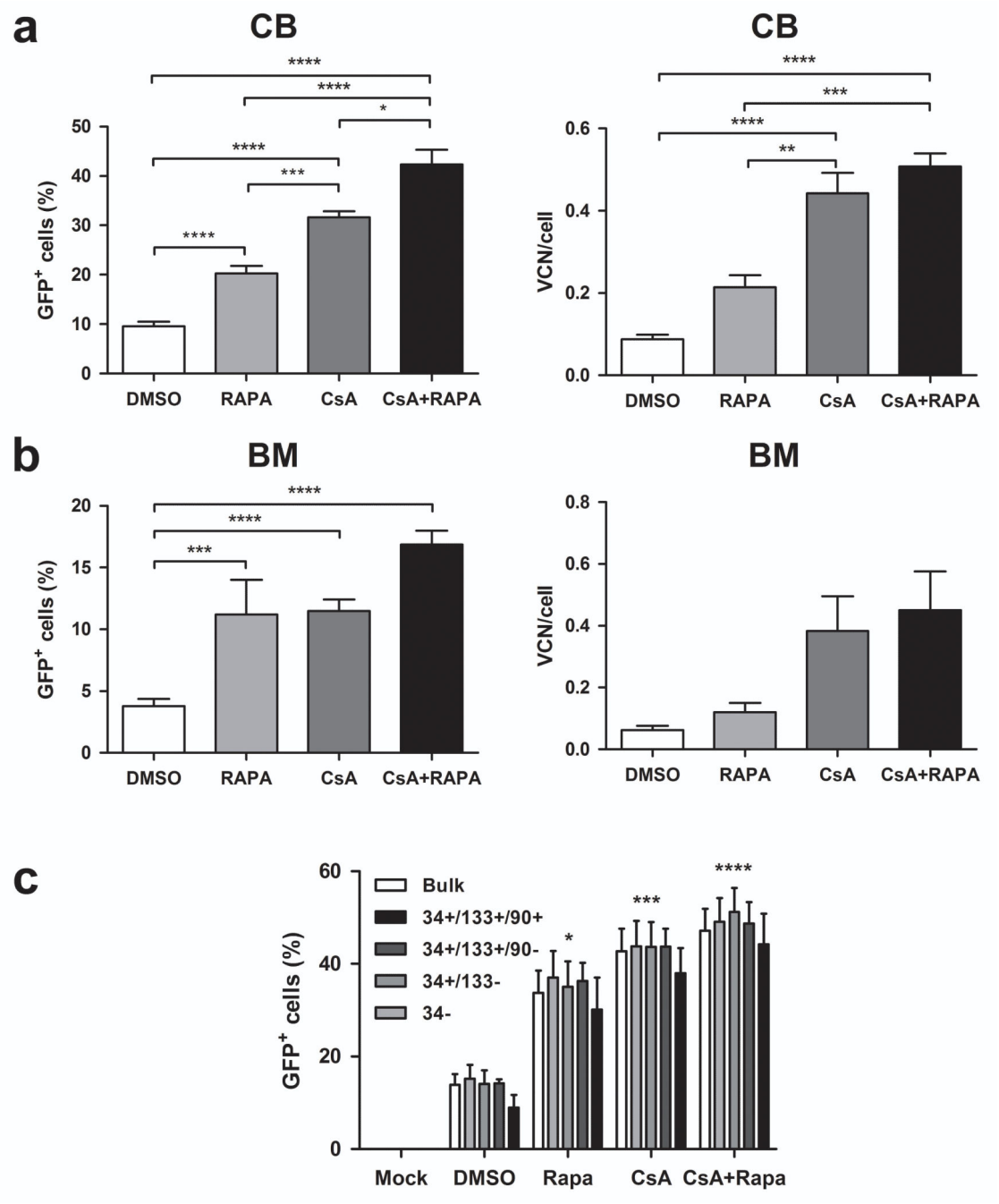

Figure 2. Impact of CsA, Rapa and their combination on HSPC transduction

CB (**a,c**) and BM-derived (**b**) $CD34^+$ cells were transduced with SINLV-GFP at an MOI of 1, in presence or absence of 10 μM CsA or 10 μg/ml Rapamycin or their combination. (**a, b**, left panels) The percentage of GFP^+ cells was evaluated by FACS 5 days post-transduction and (**a, b**, right panels) VCN were assessed 14 days post-transduction in CB and BM, respectively. Data represent the mean ± SEM of at least three independent experiments. p value are for One way ANOVA with Bonferonni's multiple comparison. (**c**) The percentage of GFP^+ cells within the different subpopulation was evaluated by FACS three days after

transduction. Data represent the mean ± SEM of five independent experiments, p values are equal for all subpopulations within the same treatment group, * for $p<0.05$; *** for $p<0.001$ and **** for $p<0.0001$ in two-way ANOVA with Bonferroni's multiple comparison vs. DMSO.

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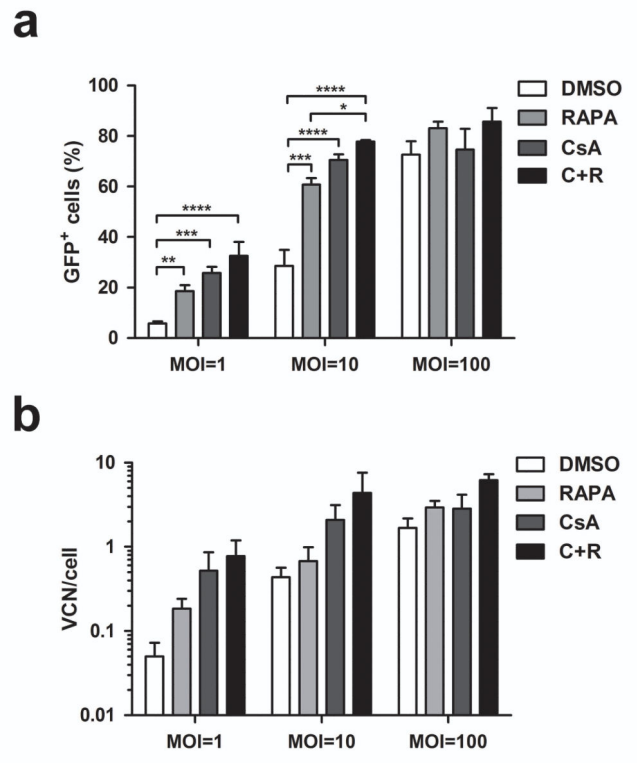

Figure 3. Impact of CsA and Rapa on transduction of human HSPC with a highly purified vector

CB-derived $CD34^+$ cells were transduced with a clinical grade SINLV-GFP at an MOI of 10, in presence or absence of 10 μM CsA or 10 μg/ml Rapamycin or their combination. (**a**) The percentage of GFP^+ cells was evaluated by FACS 5 days post-transduction. (**b**) VCN were assessed 14 days post-transduction. Data represent the mean ± SEM of four independent experiments. p values are for One way ANOVA with Bonferroni's multiple comparison. * for $p<0.05$, ** for $p<0.01$, ***for $p<0.001$, **** for $p<0.0001$.

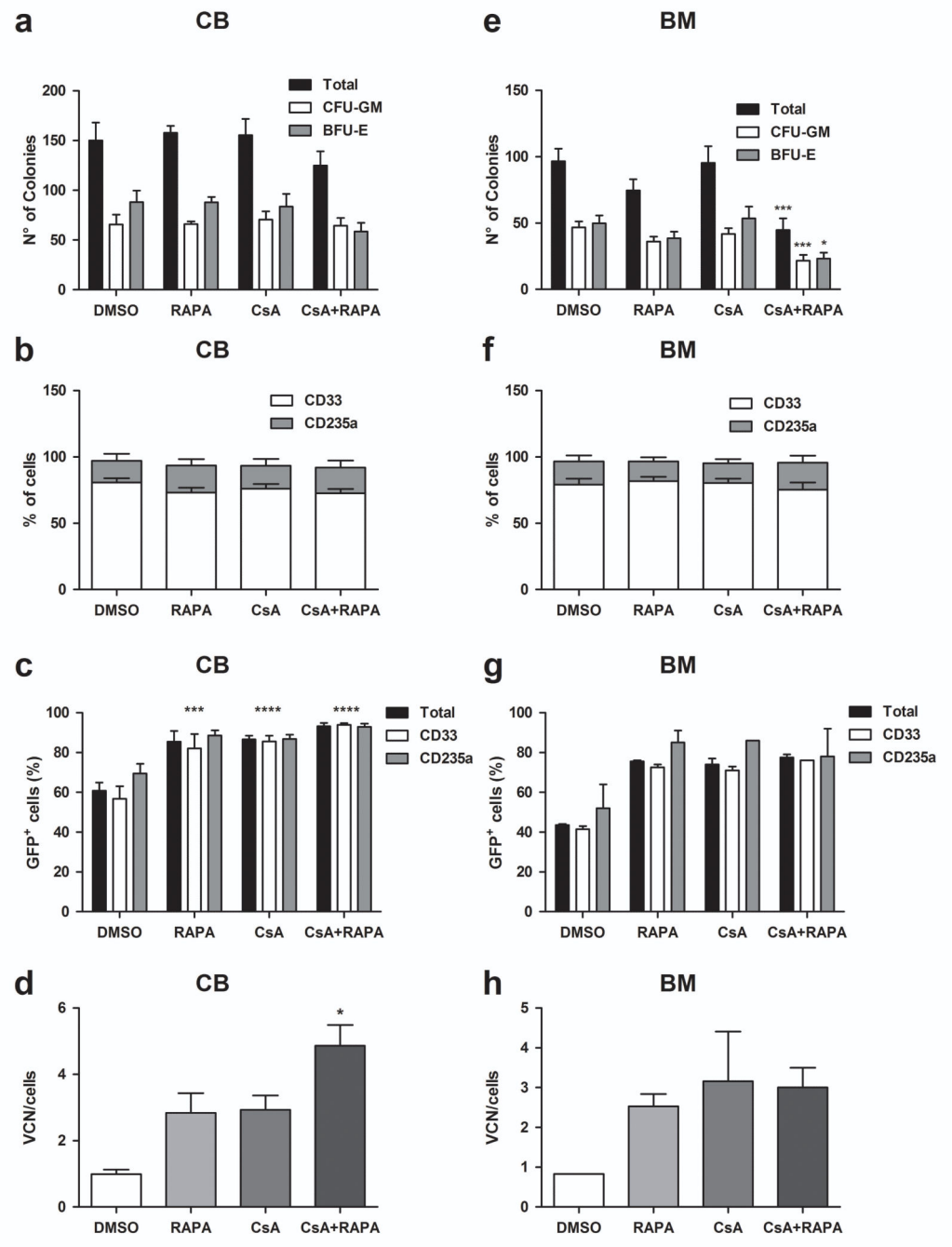

Figure 4. Impact of CsA and Rapa on colony-forming capacity of human HSPC

CB (**a-d**) and BM-derived (**e-h**) $CD34^+$ cells were transduced with SINLV-GFP at an MOI of 10, in presence or absence of 10 μM CsA or 10 μg/ml Rapamycin or their combination, followed by plating of equal numbers of vital cells the day after in semi-solid methylcellulose. (**a** and **e**). CFU count was performed 14 days after plating. (**b,c**) and (**f,g**). The percentage of myeloid (CD33) and erythroid (CD235) progenitors was measured by FACS after 15 days of culture together with the percentage of GFP^+ cells within the different progenitors. (**d** and **h**) VCN/cell in bulk colonies was measured by q-PCR. Data are

the mean ± SEM of four independent experiments in triplicate each for CB and of two in triplicate each for BM-derived HSPC, p values are for One-way (**d**) or Two-way (**e**) ANOVA with Bonferonni's multiple comparison vs. DMSO, * for $p<0.05$, ***for $p<0.001$, **** for $p<0.0001$

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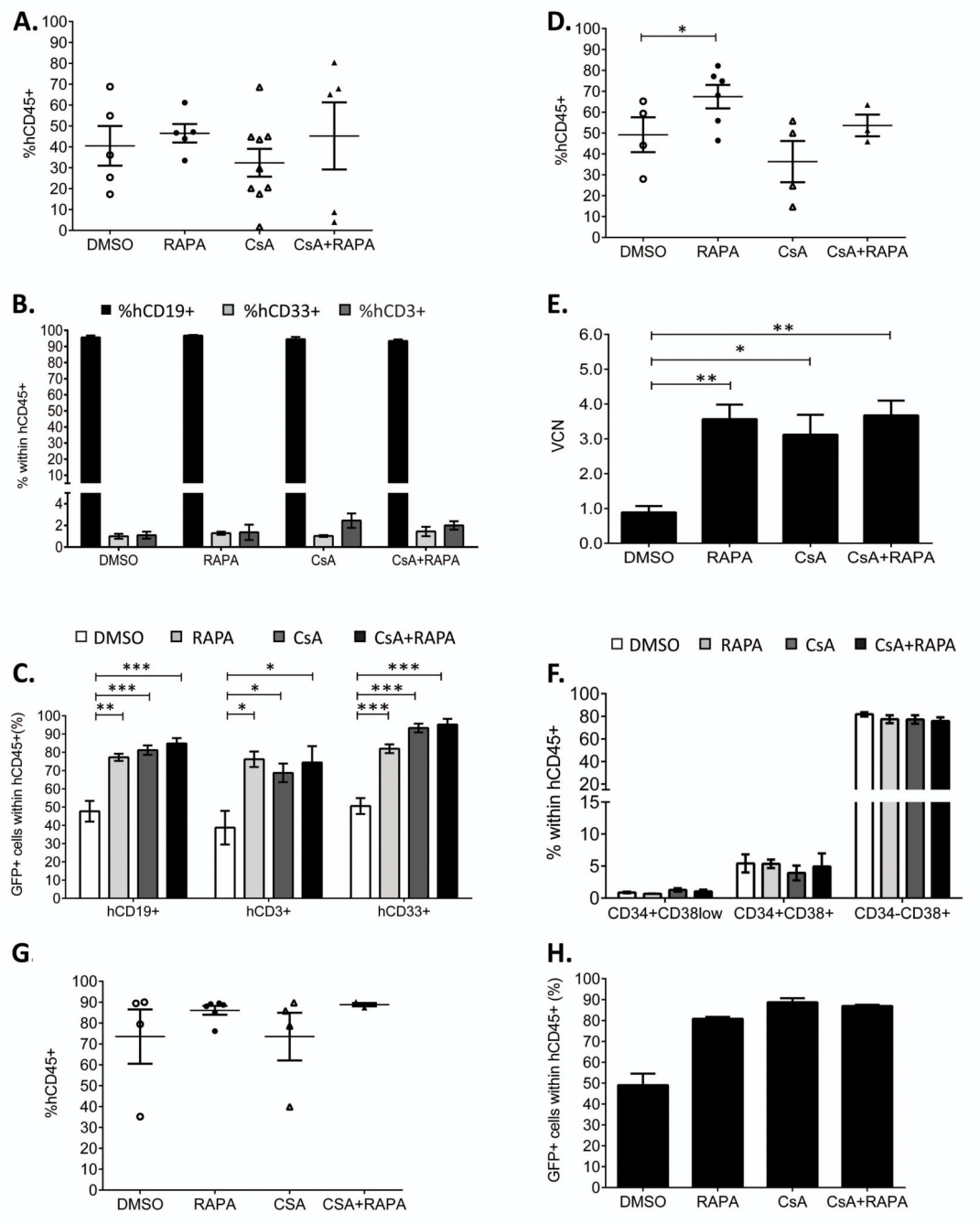

Figure 5. Impact of CsA and Rapa on transduction efficiency and engraftment capacity of HPSC *in vivo*

(**a-c**) Peripheral blood analyses in NSG mice at 11 weeks post-transplant: **(a)** engraftment levels evaluated as percent of human $CD45^+$ cells over the total of blood mononuclear cells (y-axis) in mice from different treatment groups (indicated in x-axis), **(b)** percentages of human B, T and myeloid cell linages ($hCD19^+$, $hCD3^+$ and $hCD33^+$ respectively) within human $CD45^+$ cells and, **(c)** frequency of GFP^+ cells (y-axis) within the indicated hematopoietic lineages (x-axis). (**d,f**). Analyses of the BM of NSG mice at 20 weeks post-

transplant: **(d)** engraftment levels of human $CD45^+$ cells, **(e)** VCN and **(f)** percentages of the indicated hematopoietic populations within human $CD45^+$ cells. **(g,h)** Analyses of the Spleen of NSG mice 20 weeks post-transplant: **(g)** Engraftment levels of human $CD45^+$ cells and **(h)** frequency of GFP^+ cells within $hCD45^+$ cells. All values are expressed as mean ± SEM. p values are for One way ANOVA with Bonferroni's multiple comparison.

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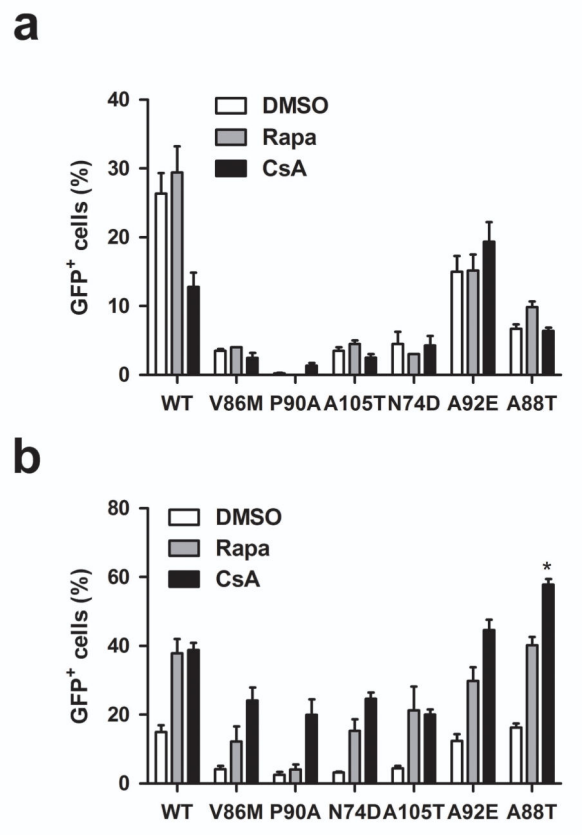

Figure 6. Impact of CA mutations on CsA/Rapa-mediated effects on LV transduction

$CD4^+$T (**a**) and CB-derived $CD34^+$ cells (**b**) were transduced with SINLVs harboring single CA point mutations at an MOI of 1 in presence or absence of 10 μM CsA or 10μg/ml Rapa. The percentage of GFP^+ cells was evaluated by FACS 5 days post-transduction. Data represent the mean ± SEM of at least three independent experiments. p values are for One-Way ANOVA with Bonferroni's multiple comparison vs. WT.

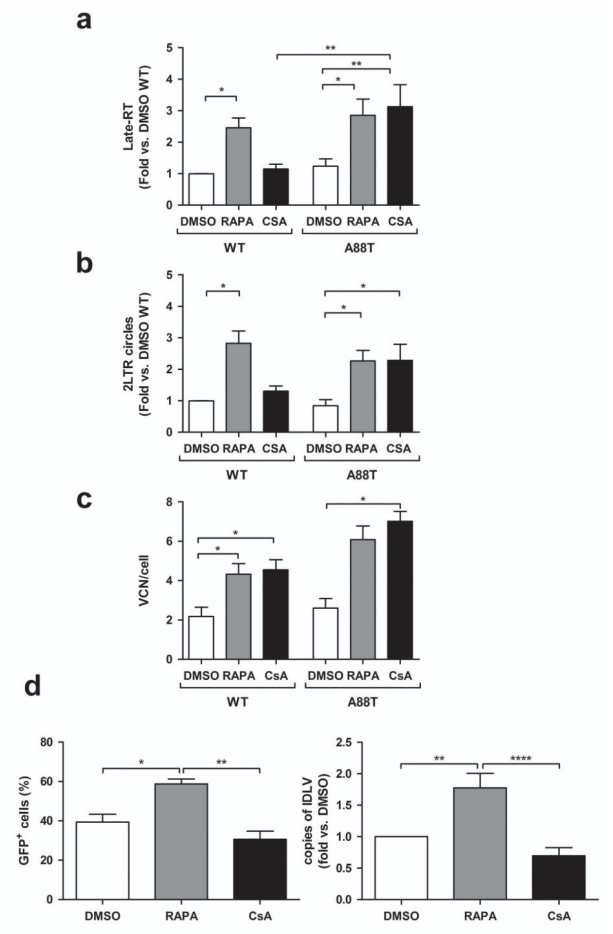

Figure 7. CsA and Rapa act on distinct steps on the LV life cycle

CB-derived CD34$^+$ cells were transduced with WT or A88T SINLV-GFP at an MOI of 10 (**a-c**) or an IDLV harboring WT CA at an MOI of 50 (**d**), in presence or absence of 10 μM CsA or 10 μg/ml Rapamycin. The LV genome fate was tracked by measuring late-RT product (**a**) and 2LTR circles (**b**) 24h after transduction and integrated proviral copies two weeks post-transduction (**c**). Data represent the mean ± SEM of four independent experiments expressed as fold vs DMSO WT (**a**, **b**) or as absolute copies/cell (**c**). (**d**) IDLV transduction efficiency was measured 3 days post-transduction by FACS (left panel) and by measuring the retrotranscribed LV DNA products by PCR (right panel). Data are the mean ± SEM of three independent experiments. p values are for One-way with Bonferonni's multiple comparison.* for $p<0.05$, ** for $p<0.01$, **** for $p<0.0001$.

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Table 1

Phenotypes reported for the CA mutants tested in this study

CA Mutants	Phenotypes	References
P90A	CypA and Nup358-independent	[39]
N74D	TNPO3 and Nup153-independent, CPSF6-358 escape mutant, affects CypA interaction	[46, 47]
V86M	Alters CypA-CA interaction relieving hT5α restricted mutants	[48]
A105T	Relieves CypA-mediated restriction of CsA-dependent CA mutants	[49]
A92E	CypA-independent, CsA-dependent in some cell lines, still interacts with CypA	[22, 50]
A88T	MxB escape mutant, no longer binds CypA	[33]