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Combined effects of restriction factors and transduction adjuvants on lentiviral vector gene transfer efficacy

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Restriction factors include various cellular proteins that detect and impede viral infections. Among them, interferon-induced transmembrane (IFITM) and serine incorporator (SERINC) proteins interfere with the infectious cycle of HIV-1. Consequently, such restriction factors can also interfere with gene transfer efficacy when using recombinant lentiviral vectors derived from HIV-1, but these parameters remain incompletely understood. Here, using overexpressing human cell lines, we investigated the effects of IFITM and SERINC proteins on key parameters in the infectivity of lentiviral vectors, e.g. the nature of the vector envelope glycoprotein pseudotype and the use of transduction adjuvants. Vectors pseudotyped with glycoproteins from vesiculoviruses were mostly insensitive to the effects of restriction factors whereas those pseudotyped with glycoproteins of retroviral origin displayed contrasted responses. IFITM2 and IFITM3 very effectively restricted Syncytin1-pseudotyped vectors. Two transduction adjuvants, Vectofusin and cyclosporin H, counteracted these effects. Addition of either of these compounds led to reduced IFITM2 and IFITM3 protein levels. These results may rationalize the choice of pseudotypes as well as transduction conditions to use for gene transfer. Together, these parameters can strongly enhance the transduction efficacy, especially in target cells that naturally express IFITM proteins, including human hematopoietic cells that are of particular clinical interest.

Keywords Lentiviral vector, Gene therapy, Restriction factor, Viral transduction

Restriction factors (RFs) are involved in the response to numerous viral infections. They are part of the innate immune system and most of them are both activators and targets of the interferon response (reviewed in[1]). They are of various origins, are usually efficient against a wide variety of viruses and can interfere at multiple steps of the viral replicative cycles. Among this wide variety of factors, interferon induced trans-membrane (IFITM) and serine incorporator (SERINC) proteins constitute 2 unrelated families of genes encoding membrane-associated proteins, some of which possess antiviral activities. SERINC3 and SERINC5 are integral membrane proteins that can be incorporated in the membrane of enveloped viral particles and limit their infectivity. Both proteins have been described as active against HIV-1 derived particles, among others, in the absence of the HIV-1 encoded Nef protein that can counteract their activities[2–5]. SERINC3 and SERINC5 interfere with cell and virus membrane fusion steps via different mechanisms and also with innate immune responses through IFN-I and NF-κB signalling (reviewed in[6]). The IFITM family of proteins comprises 5 genes all situated in a locus on human chromosome 11. Among them, only IFITM1, 2 and 3 proteins are considered "immunity-related" and have been shown to be effective RFs. They are membrane-associated proteins, and can interfere with numerous viruses (including HIV-1), enveloped or not, via different mechanisms ([7], reviewed in[1,8,9]). Like SERINC proteins, they can interfere with the fusion step between the cellular and viral membranes early in the infection cycle of enveloped viruses and Nef has recently been reported to impair IFITM3 function[10]. However, unlike SERINC3 and SERINC5 that are incorporated on viral particles, IFITM1-3 block membrane fusion when expressed by the target cells.. They can also disturb other steps of viral cycles, including the production of viral particles and the incorporation of the viral glycoproteins during virus assembly ([11–13], reviewed in[1,8,9]). IFITM1 possesses a cellular sub-localisation different from IFITM2 and IFITM3 (cell membrane vs endosomes), and this difference has been reported to account for the different activities of the proteins: IFITM1 would block viruses whose membrane fuses with the cell surface membrane whereas IFITM2 and IFITM3 would block the entry of

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viruses that use the endosomal pathway ([14–16], reviewed in[1]). However a recent report indicate that coexpression of IFITM1 and IFITM3 partially relocalises IFITM1 to late endosomes and lysosomes where IFITM3 is present, suggesting that the respective roles of the 3 IFITM proteins may be more complex[17]. Altogether, the exact mechanisms of action are multiple, not all well understood and may sometimes appear different depending on the system used in the studies. In particular, RFs have initially been described with infectious replicative viruses, and their effects can vary when using non replicative pseudotyping systems.

In recent years, gene therapy projects have considerably expanded, with numerous potential applications investigated and new treatments marketed. Some of these promising products are based on HIV-1-derived lentiviral vectors which provide stable transduction of various cell types[18]. These vectors have been refined over the years to increase their safety and efficiency, removing all potentially harmful or unnecessary viral proteins from the HIV-1 element[18–20]. Among those suppressed factors are "accessory" proteins (namely Nef, Vif, Vpr and Vpu) that are not required for the infectious cycle of HIV-1 virus in some cell lines, and also not needed for single-cycle integration of non-replicative vectors. However, it was found that their role in vivo consists of (but is not necessarily limited to) counteracting the effects of several RFs (eg Nef, Vif and Vpu respectively can inhibit SERINC/IFITM3, APOBEG3G and BST2 proteins), permitting a productive HIV-1 infection[2,3,10], reviewed in[21]). The removal of these accessory proteins from lentiviral vectors (LVs) therefore potentially lowers/hampers their efficacy to transduce cells expressing RFs.

LVs are highly efficient tools to transduce most cell lines, but their transduction efficacy is often much lower in primary cells. For clinical applications, VSV-G is currently the choice glycoprotein for pseudotyping LVs because of its broad tropism, high titres and resistance allowing purification without infectivity loss. However, several cell types of major interest, including B cells and resting HSPCs and T cells, have been shown to express low levels of LDL receptors, the cellular proteins used to mediate entry via VSV-G, leading to a strongly reduced LV transduction rate[22]. Different glycoproteins whose receptor is better expressed in these cells can be used to replace VSV-G ([23–28], reviewed in [29]). These glycoproteins, especially those of retroviral origin, often requires the addition of compounds so called "transduction adjuvants" that enhance transduction efficiency. Cationic compounds such as polybrene, protamine sulfate or semen-derived enhancer of virus infection (SEVI) can be added to increase transduction efficiency by decreasing the natural repulsion between negatively-charged cells and viral membranes and also by promoting viral aggregation, but some of these factors can be toxic to sensitive cells[30–32]. Peptides such as those derived from fibronectin (e.g. Retronectin), have dual binding domains that bring cell membrane and viral particles closer, but this strategy requires coating the peptides on the culture dishes and is more cumbersome to use than soluble adjuvants[33]. Other compounds act as transduction enhancers by enhancing membrane fusion through their amphiphilic properties or by enhancing the adhesion or aggregation of particles towards the cells, for instance poloxamer derivatives (e.g. LentiBoost) or amphipathic peptide such as Vectofusin[34,35]. Using fibronectin fragments or Vectofusin is particularly useful to obtain high levels of transduction with LVs pseudotyped with retroviral envelopes such as GALV-TR or RD114[35,36]. More recently, it has been recognized that cells difficult to transduce with LVs such as CD34 + hematopoietic progenitor cells, express high levels of IFITM3 RFs that could be blocked by several compounds. Rapamycin, a mTor inhibitor, caraphenol A, a resveratrol trimer and cyclosporin H, a derivative of cyclosporin A without cyclophilin inhibitory activity have been used to decrease IFITM3 endosomal expression and improve HSPCs lentiviral transduction[37–40].

In this study, we developed a model system to systematically investigate the effect of the SERINC and IFITM RF expression in target cells on their transduction by LVs, depending on the nature of the envelope glycoprotein used and the transduction conditions used.

Results

1- Generation and characterisation of model cell lines

The model system is based on engineering the human HEK293T cell line to overexpress independently each of the RFs from IFITM and SERINC families, and measure the effects on lentiviral transduction efficiency. HEK293T cells are relevant models for this study as they are easy to grow and to transduce with low doses of LVs. They are also refractory to the interferon antiviral pathway activation during lentiviral transduction[41]. For all these reasons, the effect of each of the selected RFs can likely be tested independently of any other ones that could be otherwise induced by the transduction process. RF-overexpressing HEK293T stable cell lines were generated by lentiviral transduction using similar expression systems for each RF to obtain consistent study systems. The open reading frames (ORFs) of each of the 5 SERINC/IFITM RFs were expressed under the control of the short EF1alpha promoter with a common Kozak sequence upstream of the initiation codon and the stabilising WPRE sequence downstream of the ORF. The Hygromycin resistance gene was expressed from a separate promoter (PGK) within the LV. Following transduction with several doses of each RF-expressing LV, 293 T cells were selected with hygromycin and assessed for the number of integrated LV genomes. Populations of cells with an average vector copy number (VCN) of 1 were deemed comparable and were selected for the study. All cell lines had similar growth rates throughout the experiments and their viability was not affected, indicating that none of the overexpressed genes is susceptible to alter the results of the experiments through nonspecific mechanisms. Expression of the RF mRNAs of interest was specific in the different selected cell lines as expected (Fig. 1A). The protein expression level of IFITM proteins was detected specifically by Western blot in the IFITM-expressing cells but it was not possible in SERINC-expressing cells for which we had no antibody available for immunodetection Fig. 1B). The use of constructs with an identical structure (eg promoter, Kozak sequence and exact ORF sequence) and a VCN of 1 was chosen to compare the RF efficiency using conditions of expression as similar as possible. However qPCR data in Fig. 1A show variations in the mRNA fold increases for the 5 genes. These differences are in some part due to differences in the expression levels of the genes in the native HEK293T cells (indeed for similarly overexpressed mRNA levels, the fold increases will be higher if the

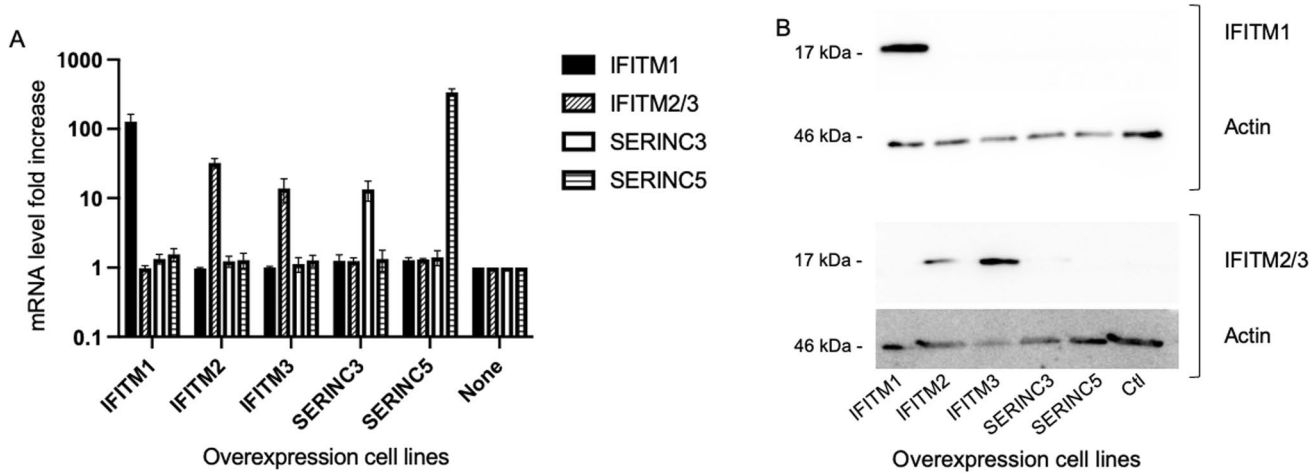

Fig. 1. RF levels in the overexpression HEK293T cell lines. (**A**). Quantification of RF mRNA expression in the overexpressing cell lines and control cells (Ctl) by qRT-PCR. Because of their very similar sequences (93,3% identity), IFITM2 and IFITM3 transcript levels were measured with the same primer pair. The results correspond to the means and standard deviations of two RNA extractions, each of them measured in duplicates. (**B**). Expression of IFITM proteins in the overexpressing cell lines detected by western blot. IFITM1 (Top) and IFITM2/3 (Bottom) were tested independently in all 6 cell lines and were detected only in the corresponding overexpressing cell lines (apparent MW for both 17 kDa, top panels). Total protein content in each well was checked by looking at actin content (MW 46 kDa, lower panels).

initial mRNA content is lower) but are also due to the intrinsic properties of the nucleic acid and amino acid sequences that can affect their stability. Indeed, using the same primers, mRNA levels are slightly higher in the IFITM2 overexpressing cells compared to IFITM3 overexpressing cells, but western blot in Fig. 1B show more IFITM3 protein than IFITM2 probably in relation with a higher stability of IFITM3 protein.

2- Variable sensitivity to RFs depending on the glycoprotein

We first tested the different cell lines for their sensitivity to transduction by LVs pseudotyped with the most commonly used glycoprotein in gene therapy, the vesicular stomatitis virus G glycoprotein (VSV-G), in the absence of any transduction adjuvant. As shown in Fig. 2, the 5 SERINC/IFITM RFs that were tested had only limited effect on the capacity of the cells to be transduced, with the maximal observed decrease in the transduction efficiency being around 25% in the cell line overexpressing IFITM3. Overexpression of IFITM2, a closely related protein, also led to a slight but significant decrease in the transduction sensitivity of the cells (approximately 10%). None of the other 3 RFs (IFITM1, SERINC3 and SERINC5) showed any effect in this assay. The observed effects were relatively small, but clearly statistically significant ($p < 0.0001$ for both in a paired t-test). We then tested the effects of the RFs on LVs pseudotyped with Cocal-G, a less well-studied glycoprotein, originating from the Cocal vesiculovirus, a close parent of VSV, and which is of notable clinical interest as it cannot be inactivated by normal human serum, unlike the VSV-G protein[42]. The results in Fig. 2 show that Cocal-G-pseudotyped LV proved very resistant to RF overexpression. Among the tested factors only IFITM3 could significantly reduce the transduction of the cells with Cocal-G LV, with a small effect limited to 13% ($p = 0.006$), clearly lower than that of VSV-G.

We also tested retroviral glycoproteins. First was Syncytin1 (Syn1), a human protein of retroviral origin involved in placenta formation, that can be used to pseudotype LVs[27,43,44] and requires the use of Vectofusin for high levels of transduction[27]. Unlike what was observed with the 2 vesiculovirus glycoproteins, 4 of the 5 RFs tested noticeably reduced the transduction efficiency with Syn1. Only the SERINC3-expressing cell line showed no modification of its transducability. The strongest decrease in transduction was observed when IFITM3 was overexpressed (approximately 75% decrease), followed by IFITM2 (55%), IFITM1 (18%) and SERINC5 (approx. 10%). All these effects were highly statistically significant. We then determined the sensitivity profile of another glycoprotein of clinical interest for its ability to transduce CD34 + cells, the baboon endogenous retrovirus (BaEV) glycoprotein used to transduce T and B cells[26,45]. As shown in Fig. 2, the transduction rates of all 6 cell lines are similar with LV pseudotyped with BaEV Env, indicating that this glycoprotein is not affected by any of the 5 RFs that we tested.

3- Modulation of the RF effects by transduction adjuvants

The previous series of experiments was performed in the absence of any transduction adjuvants, which is mostly relevant for standard cell lines that can easily be transduced. However, LV transduction is often much less efficient on primary cells, requiring the use of additional compounds to achieve the required levels of vector copies in such cells. We tested if the RF effects that we identified were modified by the use of such transduction adjuvants. Among those, we tested polybrene, protamine sulfate, cyclosporin H and Vectofusin that cover different chemical families and modes of actions[31,34,35,40]. The tested adjuvants were added individually at the same time

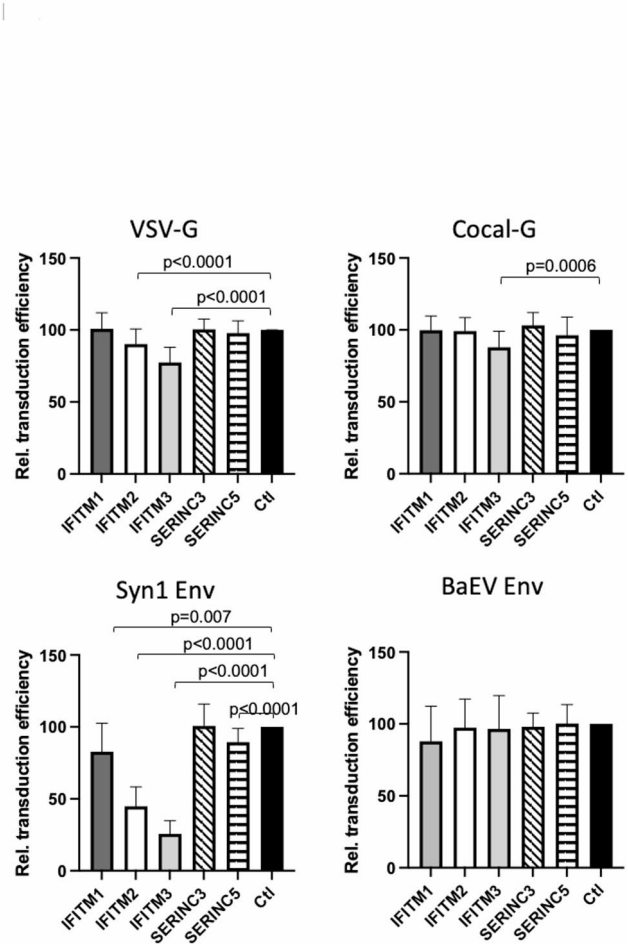

Fig. 2. Effect of RF expression on cell transduction. The 6 model cell lines overexpressing a RF (or control) were transduced with a GFP-expressing LV pseudotyped with 4 different viral glycoproteins (indicated above each graph) and the transduction efficiency measured by flow cytometry. For each experiment, the transduction level of each RF-expressing cell line was normalised to that of the control cell line. The graphs represent the means + /- SD of independent experiments (VSV-G n = 28, Cocal-G n = 16 , Syn1 n = 21, BaEV n = 5). Significant differences between the control cell line and the RF expressing ones are indicated (t-test).

as the LV and the transduction rates of each cell line were determined using flow cytometry as described above. For each condition (ie combination of glycoprotein, RF and nature of the adjuvant) we quantified the effect of the RF as the decrease in infectivity its expression induces (ie effect = [Titre Ctl cell line] / [Titre RF cell line]). The statistical significance of the differences in RF efficiency measured with or without each transduction adjuvants was tested in paired experiments. As shown in Fig. 3, the sensitivity of the vesiculovirus-derived glycoproteins to the tested RFs was not modified by adding polybrene or protamine sulfate. Similarly, the results obtained with BaEV Env pseudotyped LV showed little changes. It should be noted that this may be due to the fact that these 3 glycoproteins are the more resistant to the effects of the RFs, making variations harder to detect. On the contrary, Syn1-pseudotyped LV were strongly affected by some of the adjuvants, mostly cyclosporin H and Vectofusin. Both adjuvants significantly and strongly decreased the inhibitory effects of IFITM3, and of IFITM2 in a more limited way, while IFITM1-mediated restriction was not affected. The effects of cyclosporin H were expected based on previous studies showing that cyclosporin H can block IFITM3 action in human CD34 + cells and THP-1 cell line[40,46]. The effects of Vectofusin are novel and appeared to be slighly lower than those of cyclosporin H.

Effects of cyclosporin H and Vectofusin on IFITM2/3 protein levels

Cyclosporin H is a powerful compound to enhance lentiviral transduction of CD34 + cells[40,46]. The addition of cyclosporin H during the transduction of CD34 + cells with a VSV-G-pseudotyped LV decreases their IFITM3 content as measured by intracellular staining and results in higher vector copy number integration (Fig. 4A). Vectofusin is known to enhance the transduction of CD34 + hematopoietic progenitor cells with VSV-G-pseudotyped lentiviral vectors[35] but the effects are more modest than those of cyclosporin H (Fig. 4B) and therefore less likely to be detected by examining an effect on IFITM3 levels. To confirm the relationship between IFITM3 levels reduction capacity and transduction enhancement, the effects of cyclosporin H were compared to

Fig 3

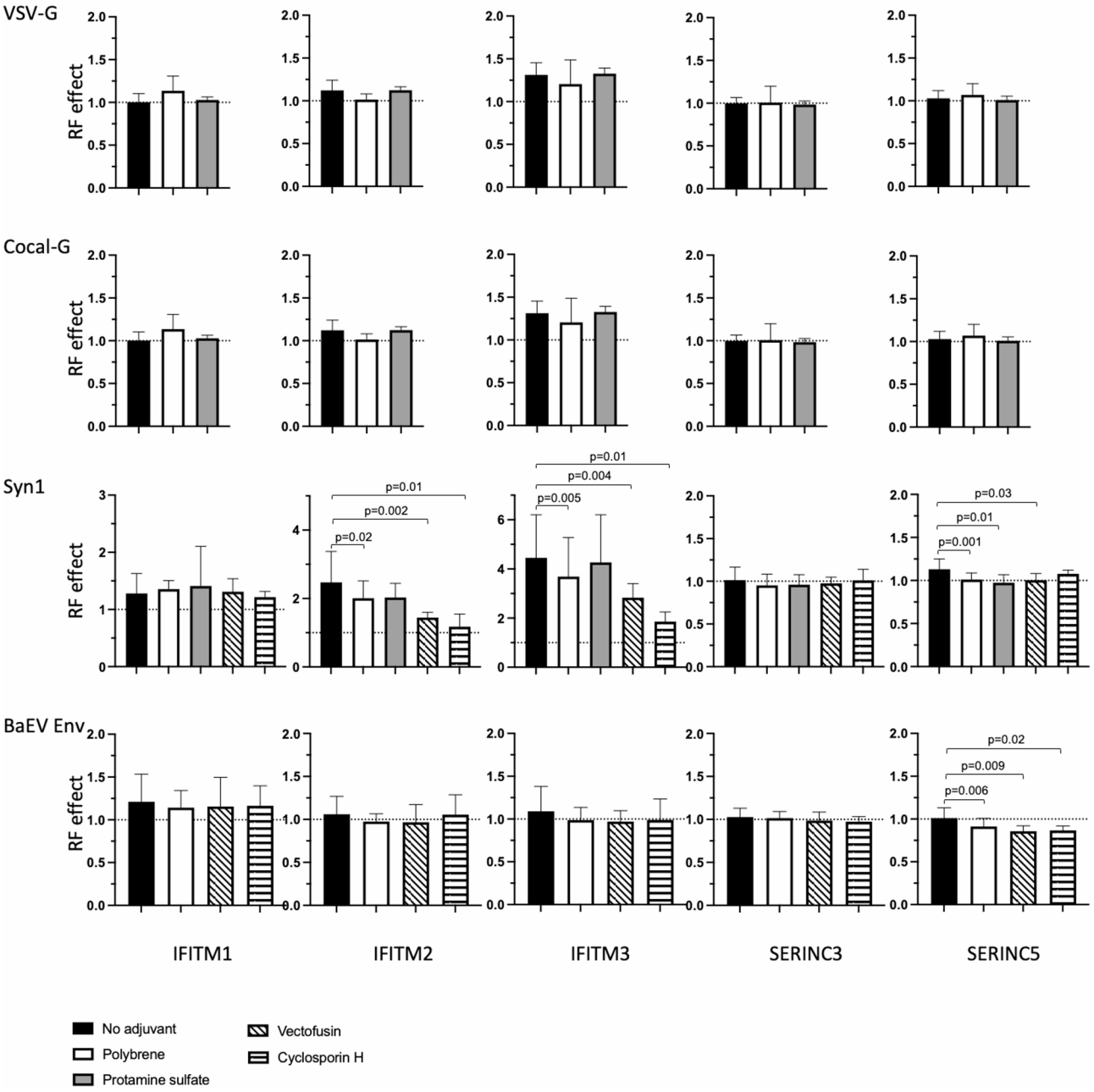

Fig. 3. Modulation of the effects of RFs on cell transduction in the presence of transduction adjuvants. The 6 model cell lines were transduced with eGFP-expressing LVs pseudotyped with different viral glycoproteins in the presence or absence of transduction adjuvants. The effect of each RF was measured for each condition as the fold decrease it induces in transduction (= [transduction efficiency Ctl] / [transduction efficiency RF]) and was plotted as mean + /- SD. Significant differences between the fold decreases measured with or without adjuvants in paired experiments are indicated (t-test). (graphs: $5 < n < 28$; stats: $4 < n < 15$).

that of Vectofusin in the overexpression model 293 T cell lines. Total IFITM2/3 content was quantified by flow cytometry after intracellular staining for IFITM2, IFITM3 in overexpressing cells and control after treatment or not with cyclosporin H or Vectofusin for 17 h (Fig. 5). Both compounds induced a decrease in IFITM2/3 expression level, and the differences in mean fluorescence intensity (MFI) were statistically significant (t-test in paired experiments). Interestingly, the decrease was stronger with cyclosporin H, consistent with the more pronounced inhibition of IFITM3 restriction seen in Fig. 3. Of note, both effects seem specific since the total

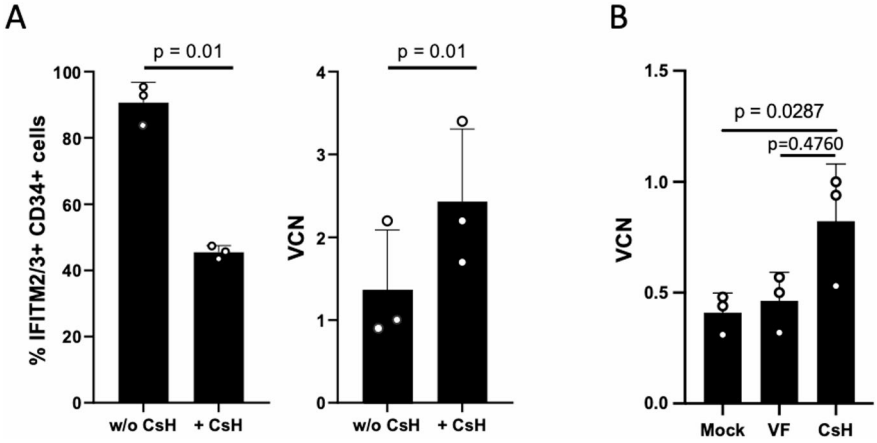

Fig. 4. Enhancement of CD34 + cell transduction using adjuvants. (**A**) The effect of cyclosporin H (CsH) addition on IFITM2/3 expression during the transduction of CD34 + cells with two rounds of VSV-G-pseudotyped LV was determined by flow cytometry 24 h after the start of the transduction (left). The corresponding transduction efficiencies were quantified by measuring the VCN using ddPCR 5 days adter transduction (right). Cells were isolated from 3 independent donors. (**B**)**.** Comparison between the effects of the addition of cyclosporin H and Vectofusin (VF) on CD34 + cell transduction efficiency with a single round of VSV-G pseudotyped LV. Transduction levels were determined by measuring the VCNs by ddPCR on cells isolated from 3 independent donors. Significant differences are indicated (t-test).

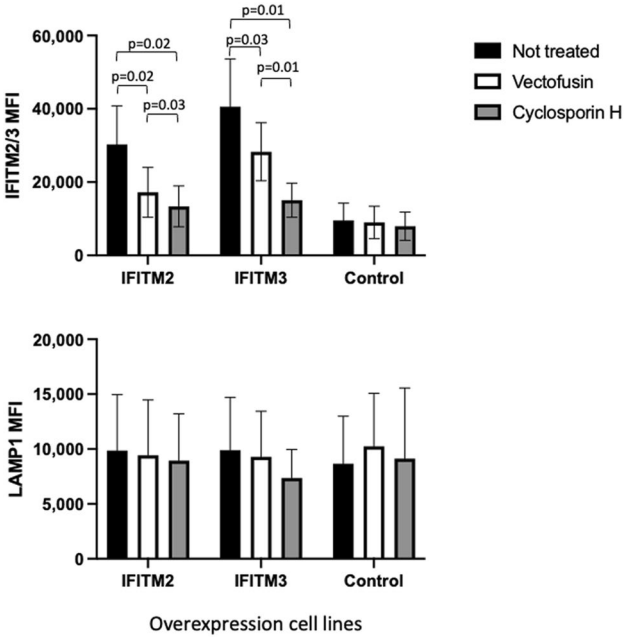

Fig. 5. Effects of Vectofusin and cyclosporin H treatment on the expression levels of IFITM2/3 and LAMP1 proteins in IFITM2, IFITM3 overexpressing cell lines and controls. Cells were treated for 17 h with Vectofusin or cyclosporin H and total amounts of IFITM2/3 and LAMP1 proteins were determined by flow cytometry after intracellular staining. Cells were gated based on SSC / FSC criteria and the the MFI of the whole population was measured for each protein. (n = 4 for IFITM2/3, n = 3 for LAMP1). The effects of Vectofusin and cyclosporin H on MFI of each cell population were tested (paired t-test) and statistically significant differences are indicated.

amount of LAMP1 protein, an endo-lysosomal protein that partially colocalises with IFITM3, was unaffected in the same conditions.

Discussion

In this study, we generated over-expressing cell lines to investigate the effect of IFITM and SERINC proteins to restrict LV transduction. These 2 families of proteins had already been shown in some settings to affect the fusion step between cell and virus membranes that occurs early in infection, where they play "symmetric roles" with SERINC proteins acting while incorporated on the virus membrane while IFITM are present in the membranes of the target cells. While no effect of SERINC proteins was identified in the system we tested (ie when RFs are expressed by the target cells), in agreement with previous findings (reviewed in[6]), we found that IFITM3 was the most potent RF in the model among those tested, and also demonstrated that IFITM2, the product of a recent IFITM3 gene duplication, had similar, albeit weaker, effects than IFITM3. Interestingly, the extent of the transduction inhibition depended on the nature of glycoprotein used to pseudotype the LVs with unexpected differences between retroviral envelope glycoproteins. We confirmed that cyclosporin H can decrease intracellular IFITM3 protein content and relieve the restriction effects on LVs, but describe for the first time that Vectofusin, which is known as a fusion enhancing and particle aggregating adjuvant, has similar properties.

Among the 5 RFs tested, we report that IFITM3 proteins restrict the transduction with VSV-G -pseudotyped LVs as already reported[40], but we also see an effect of IFITM2. IFITM2 arose from a recent gene duplication of IFITM3 and the 2 proteins have very similar sequences[47]. While many previous studies mostly concentrate on IFITM3 protein, the ancestral gene, IFITM2 effects are generally less studied but our data shows that IFITM2 retained some antiviral effects, although less potent than those of IFITM3. Among vesiculovirus glycoproteins, our assay shows that the Cocal-G protein is more resistant than its VSV counterpart to both IFITM2 and IFITM3 proteins. This adds up to other interesting properties of the Cocal-G variant for gene therapy, like its resistance to human serum inactivation[42]. Nowadays VSV-G protein is the gold standard for LV-based clinical applications, but our results may provide further justification to use Cocal-G-pseudotyped LVs in gene therapy especially since IFITM3 is known to be a major factor in the limitation to human CD34 + cell transduction with LVs[38–40,46].

We also tested the effect of the expression of IFITM and SERINC RFs on LVs pseudotyped with 2 retroviral envelope proteins that are good candidates to efficiently transduce B cells[26,27], unlike VSV-G whose receptor is not expressed in these cells[22]. Our results indicate that the sensitivity is independent of the viral family, with BaEV Env and Syncytin1-pseudotyped LVs showing very different behaviour. The BaEV Env is resistant to all 5 RFs, with no statistically significant effect detected, whereas Syn1-mediated transduction is impaired by all 3 IFITM proteins, with IFITM3 being the most efficient. This is all the more surprising that BaEV Env and Syn1 are using the same cellular proteins hASCT1 and hASCT2 as receptors for cell infection[44,48] and therefore were expected to use the same mechanisms of cell entry. It is however possible that the 2 proteins have different intrinsic properties that affect their sensitivity to IFITM proteins. Despite being phylogenetically a retroviral envelope protein, Syn1 is an endogenous envelope gene whose ancestor was coopted approximately 25 million years ago in the primate lineage for its highly fusogenic properties, the characteristics responsible for its present physiological role[49]. Syn1 conservation of infectious properties is not subjected to any selection pressure, and its hyperfusogenicity property is very likely associated with a lower stability. Retroviral Env interactions with a receptor (or pH acidification) lead to conformational changes in Env proteins enabling fusion of virus and cell membranes. The hyperfusogenicity of Syn1 associated with its decreased overall stability could result in different properties compared to BaEV Env even though they reportedly use the same receptors. Also, it was recently shown that, among other mechanisms, IFITM3 blocks viral infection/transduction by mimicking SNARE proteins and increasing heterotypic endosome fusion (with lysosomes), to the detriment of homotypic fusion, thus promoting viral particles degradation[13]. If for example BaEV induces cell and virus membrane fusion quicker than Syn1, BaEV-pseudotyped particles would be less sensitive to IFITM3 restriction, in agreement with our observations.

Finally, we tested the effects of several transduction adjuvants on IFITM-mediated restriction. No strong effect was detected for either vesiculovirus glycoproteins or the BaEV Env. These 3 proteins are the ones showing the less sensitivity to the tested RFs, and this means it would be much harder to detect any anti RF effect of the adjuvants because the initial variations are very low. However, we did not detect any enhancing effect of the adjuvants, indicating that they are at best option for LV transduction in the presence of RFs. The situation is different for Syn1-pseudotyped LV, that are much more sensitive to IFITM proteins restriction. Polybrene and Protamine sulfate show very limited, if any, effect on IFITM proteins restriction. However, Vectofusin and cyclosporin H are able to reduce the inhibiting effect of both IFITM2 and IFITM3, with cyclosporin H being more effective. The restriction effect observed with IFITM1 is unaffected by either of these 2 compounds, in agreement with IFITM1 and IFITM2/3 having distinct mechanisms of viral restriction. The effect detected with cyclosporin H is in agreement with studies that showed it can impair IFITM3 restriction in human CD34 + cells and show that the effect and extends to another cell type[40,46]. The mechanisms at play seem to be similar, with cyclosporin H treatment leading to a decrease in total amount of IFITM2/3, therefore counteracting their antiviral effect. Surprisingly, we observed that Vectofusin, a peptide thought to promote essentially entry by facilitating membrane fusion and viral particles adhesion and aggregation, also leads to a decrease in the protein levels of IFITM2/3. The lower IFITM2/3 inhibiting effect of Vectofusin compared to cyclosporin H correlates with a weaker effect on total IFITM proteins content, but we cannot exclude that cyclosporin H could also impact LV transduction efficiency through other independent mechanisms. Noteworthily, the IFITM2/3-inhibiting effect carried by Vectofusin is consistent with the modest effect on the transduction of CD34 + cells with VSV-G-pseudotyped lentiviral vectors. However, a previous study showed that Vectofusin strongly improves the transduction of human CD34 + cells with vectors pseudotyped with the retroviral envelope GALV-

TR presumably due to increased viral:cell membrane fusion, particle adhesion and aggregation, although no link to IFITM3 was made at the time[35,50]. Our data suggest that such enhancing effect of Vectofusin is linked to a greater sensitivity of some retroviral envelopes to restriction factors. The effects of Vectofusin and cyclosporin H seem rather specific since we did not detect any variation in the protein level of LAMP1, an endo-lysosomal protein that partially colocalises with IFITM2/3. The molecular mechanisms leading to IFITM2/3 depletion by these 2 very chemically distinct compounds are not yet characterised. Interestingly, a series of mTOR inhibitors have also been successfully used to decrease IFITM2/3 protein levels and enhance LV transduction in different cell models[38,51]. In particular, Caraphenol A addition enhances LV transduction of CD34 + cells while decreasing their IFITM2/3 content, similar to the effects of Vectofusin and cyclosporin H[39]. It would be very interesting to determine if these compounds use similar or independent mechanisms to alter IFITM2/3 content, and if additive effects could be reached. Further studies would be of great interest and could help improve both our understanding of cell intrinsic immunity and gene therapy protocols for cells resistant to LV transduction.

Methods

Procurement of human cells

The use of human cells was performed in accordance with international ethical guidelines and in accordance with french regulation applying to non-interventional research on human subjects. The use of human cell lines and the use of umbilical cord blood samples collected anonymously at the CHSF hospital in Evry, following uncomplicated birth and with the mother's informed consent, were approved by the french ministry of higher education and research under reference codecoh DC 2019–3578.

Plasmids

pMD2G (#12,259) and pRSV Rev (#12,253) were obtained from Addgene. pMD2 Cocal-G was a gift from HP Kiem, Fred Hutchinson Cancer Research Center). pMDLG-pRRE Kana was derived from pMDLG-pRRE (obtained from Addgene #12,251) by replacing the Ampicillin resistance cassette by a Kanamycin resistance cassette. BaEV-RD was obtained from Dr FL Cosset (CIRI, ENS Lyon France). pCAG Syn1 was generated by replacing eGFP with the human syncytin1 ORF (Genbank NM_014590.4) in the pCAG eGFP plasmid (Addgene #89,684). pCCL EF1short eGFP WPRE was generated from a custom generated pRRL EF1short eGFP WPRE (Vector Builder) by replacing the RSV promoter domain in the composite 5' LTR by the CMV promoter domain. LVs for RF expression were derived from a custom-generated pRRL EF1short eGFP WPRE PGK Hygro plasmid (Vector Builder) by replacing eGFP with the ORFs for the selected RF. SERINC3 ORF was obtained from Sino Biological (HG23919-U). IFITM1, IFITM2, IFITM3 and SERINC5 ORFs were custom-synthetised (Genecust) and are identical to Genbank NM_003641.5, NM_006435.3, NM_021034.3 and NM_001174072.3 respectively). The GLOBE-AS3 LV plasmid has been decribed elsewhere[52].

Cell culture, LV production and transduction

LentiX 293 T cells (TaKaRa) were grown in a humid atmosphere at 37 °C and 5% CO2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated foetal calf serum (Biowest), 100 mg/mL streptomycin, and 100 U/mL penicillin. For LV production, LentiX 293 T cells were seeded at 5×10^6 cells per 10 cm dish. The day after seeding, cells were transfected using 35 µL Fugene 6 (Promega) and 3 µg pMDLG-pRRE Kana (HIV-1 core proteins expression), 4.5 µg lentiviral transgene-expressing vector (pCCL EFlong eGFP (eGFP LV) or pRRL EFshort RF PGK Hygro, a dual expressing vector to concomitantly express a RF and hygromycin resistance (Hygro) , 1.1 µg pRev (HIV-1 Rev expression) and 1.5 µg of glycoprotein expressing plasmid (pMD2G for VSV-G expression, pCAG Syn1 for Syncytin1 expression, BaEV-TR for BaEV GP expression and pMD2 Cocal-G for Cocal-G protein expression). Cell medium was changed the day after transfection, and LV containing supernatants were collected 40–48 h later, filtered (0.45 µm) and stored at -80 °C before use.

Stable cell lines overexpressing RFs or controls were generated by transducing LentiX 293 T cells with varying doses of LVs encoding for individual RFs and hygromycin resistance. Cells were expanded 48 h post transduction and selected for 2 weeks using 300 u/mL hygromycin B (InvivoGen). After selection was completed, genomic DNA was extracted and VCNs of the transgene were measured in each population by ddPCR as described in[53]. Populations with one copy of transgene were used for overexpression experiments. Titration experiments were performed in 24-well plates seeded with 1×10^5 LentiX 293 T cells or derived populations the day before adding LVs (or 5×10^4 cells in 48-well plates). LV infectious titres (TU/mL) were determined by quantifying the proportion of eGFP expressing cells by flow cytometry 72 h post transduction, using conditions were 5–30% of the cells are eGFP-positive. In some cases, adjuvants were added for transduction following the manufacturer's instruction. Adjuvant concentrations were experimentally determined in the laboratory based on efficacy and are in the range used by others (eg[31,40]) or recommender by suppliers. Polybrene (Sigma), protamine sulfate (Sigma) and Vectofusin (Miltenyi) were used at 8 µg/mL, cyclosporin H (Sigma) was used at a concentration of 8 µM. No cell toxicity due to these compounds was detected. CD34 + cells were isolated from cord blood samples following uncomplicated births by magnetic bead cell sorting according to the manufacturer's instructions (Miltenyi). CD34 + cell experiments were performed as described in[46] using one or two rounds of LV transduction. Briefly, CD34 + cells (1E + 06 cells/mL) were cultured in X-VIVO15 serum-free medium without phenol red (Lonza), supplemented with penicillin (100 U/mL) and streptomycin (100 mg/mL) (Gibco), c-kit ligand (50 ng/mL), FLT3-ligand (100 ng/mL), interleukin-3 (20 ng/mL) and thrombopoietin (50 ng/mL) (Miltenyi Biotech). After 18 to 24 h of culture, the VSV-G pseudotyped, eGFP-expressing LV was added (5 × 10exp7 ig/mL) with or without adjuvant (Vectofusin 12 µg/mL or cyclosporin H 8 µM). The medium was changed after 6 h and cells were cultured in X-VIVO15 medium containing cytokines and 10% heat-inactivated fetal bovine serum (FBS) (Cytiva, Pasching, Austria) for several days before quantifying transduction by ddPCR.

qRT-PCR

Total RNA were extracted using RNA Plus kit (Macherey Nagel) and reverse transcribed (RT) with SuperScript First Strand kit (Invitrogen). qRT-PCR was done using Luna® Universal qPCR Master Mix (NEB) on a QS3 machine (Applied Biosystems). Efficacy of the PCR reactions was checked for each primer pair, and we also verified there was no amplification when no RT reaction was performed. Transcript levels were normalised to the TFIID gene and the no-RF control cell line using the ΔΔCt method. Primer sequences are provided in supplementary Table S1.

Western blotting

Cells were lysed in PBS—NP40 1%—DOC 0,1% supplemented with protease inhibitors (Complete EDTA-free, Roche) for 30 min on ice. Insoluble cell debris were removed by centrifugation (12 000 rpm 5 min at 4 °C). Samples were subjected to SDS-PAGE using Mini-PROTEAN TGX4-15% gels (BioRad) and proteins were transferred on PVDF membranes using a semi-dry system. Membranes were blotted with anti IFITM1 antibody (Proteintech 60,074–1-Ig, 1/5000) or anti IFITM2/3 antibody (Proteintech 66,081–1-Ig, 1/5000). These 2 antibodies have been chosen because they do not cross-recognise the other members of the IFITM family. After incubation with the primary antibodies, membranes were washed 3 times in PBS – Tween 0,1% and incubated with an HRP-conjugated anti-mouse IgG secondary antibody (Cytiva NA931). Membranes were washed and signal was revealed using HRP substrate (Amersham ECL Prime, Cytiva) and read using an Imager 680 (Life Technology). Exposition times were selected on the «integrated» images automatically provided by the Imager that combine the ECL signal with a normal light photo displaying the molecular weight marker («combined» jpeg files in the Supplementary data). Figure panels were generated from files containing only the ECL signal («native ECL» tif files in the Supplementary data), with contrast and brightness adjusted for better visibility («uncropped» » tif files in the Supplementary data). Anti actin antibody (Proteintech 66,009–1-Ig, 1/10,000) was used to check total protein content in each lane after membrane stripping (ReBlot Plus Strong, Millipore).

FACS staining

LentiX 293 T-derived cells were stained using FIX & PERM kit (Invitrogen) with a mouse anti IFITM2/3 antibody (Proteintech 66,081–1-Ig, 1/200) and a rabbit anti LAMP1 (CST #9091, 1/100) in some experiments. After washing, cells were incubated with secondary antibodies (Alexa 488 anti mouse antibody, 1/200, Life Technologies) or APC—anti mouse IgG1 (1/100, Biolegend) plus Alexa 488 anti rabbit antibody (1/50, Biolegend). Specificity of the staining was checked using no primary antibody and isotype controls.

Data availability

Data are available upon request and materials may be provided upon agreement with Inserm. The point of contact for any data or questions regarding the study is Marie Dewannieux, PhD, ART-TG, 30 Rue H. Desbruères, 91,100 Corbeil-Essones, France. marie.dewannieux@inserm.fr.

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Author contributions

MD, conceptualisation, supervision, funding, methodology, investigation, analysis, writing FF, investigation KH, investigation MM, investigation AG, conceptualisation, supervision, funding, writing, review and editing, re-

sources.

Declarations

Competing interests

The author A. Galy is inventor of a patent entitled " Stable pseudotyped lentiviral particles and uses thereof " published as US11993781B2. The other authors have no competing interest.

Additional information

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