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Inhibition of HIV replication: A powerful antiviral strategy by IFN-β gene delivery in CD4+ cells

Fabienne Brule [a], *Emmanuel Khatissian* [a], *Alexandre Benani* [d], *Audrey Bodeux* [a], *Luc Montagnier* [c], *Jacques Piette* [a,*], *Evelyne Lauret* [b], *Emmanuel Ravet* [a,**]

[a] *Laboratory of Virology & Immunology, University of Liège, B-4000 Liège, Belgium*
[b] *INSERM, U790, 39 rue C. Desmoulins, F-94805 Villejuif, France*
[c] *World Foundation AIDS Research and Prevention, F-75732 Paris, France*
[d] *CNRS UMR5241, Université Paul Sabatier, F-31000 Toulouse, France*

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ABSTRACT

In this study, we demonstrated the efficiency and feasibility of a gene therapy protocol against HIV infection using the antiviral effects of IFN-β expression. Lentiviral vectors containing the human or the simian IFN-β sequences under the influence of the murine moderate H2-kb promoter were constructed. To examine the capacity of IFN-β to inhibit the replication of HIV in human CD4+ cells, a transduction protocol permitting to efficiently transduce CD4+ cells or PBMC (85 ± 12% of CD4+-transduced cells) with a moderate expression of IFN-β was developed. Results indicate that enforced expression of IFN-β has no negative effects in terms of apoptosis and proliferation. In human CD4+ cells, it drastically inhibits (up to 99.9%) replication after challenging with different strains of HIV-1. The expression of exogenous IFN-β leads to an amplification of the CD4+ cells (11-fold) and to a drastic decrease of the p24 protein. Micro-array analyses indicated that antiviral effect of IFN-β could be due to a major regulation of the inflammatory response. These results are encouraging for the development of a clinical study of gene therapy against AIDS using IFN-β.

1. Introduction

Despite intensive research efforts and an abundant number of new treatments, infection with HIV-1 is still a worldwide health problem. Current medical protocols consist in an anti-retroviral drug therapy named highly active anti-retroviral therapy (HAART). This therapy limits both morbidity and mortality by a reduction of the viral load, and an increase of the number of CD4+ cells, thereby restoring immunity [1]. However, this strategy presents several drawbacks because of its duration and the difficulty to obtain a good compliance [2]. Moreover, the toxicity of HAART is clearly established, particularly with cases of lipodystrophy syndrome and hepatotoxicity [3,4]. The number of failures increases with the impossibility to purge completely the viral reservoir [5] and with the apparition of numerous HIV resistant variants due to drug pressure [6,7].

Alternative protocols are required to limit the virus replication, either alone or in combination with those currently practised. Gene therapy protocols offer a good

* *Corresponding author at*: Unit of Virology & Immunology, GIGA-Research, GIGA B34, University of Liège, 4000 Liege, Belgium. Tel.: +32 4 366 24 42; fax: +32 4 366 24 33.
** *Corresponding author*. Present address: UMR5018 Neurobiologie, plasticité tissulaire et métabolisme énergétique, CHU Rangueuil, 31000 Toulouse, France. Tel.: +33 5 61 32 34 94.
E-mail addresses: jpiette@ulg.ac.be (J. Piette), ravetemm@toulouse.inserm.fr (E. Ravet).
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opportunity to control the disease evolution. It is well known that virus-derived vectors are able to transduce $CD4^+$ cells [8]. Lentivirus-derived vectors are defined by their capacity to transduce quiescent cells such as haematopoietic stem cells and $CD4^+$ cells [9,10]. Such vectors allow the transgene integration in the genome of transduced cells, leading to a persistent long-term transgene expression. A major problem in gene therapy is the potential deleterious consequences of multiple integrations of the provirus in the transduced cells. Indeed, three cases of T cell leukaemia were identified almost three years after a gene therapy treatment for X-linked severe combined immune deficiency using a MoMuLV-derived vector in $CD34^+$ cells. In two of them, an insertion of the vector close to the LMO2-proto-oncogene promoter was identified, leading to an aberrant expression of LMO2. These results underline the necessity for a good control of the effects of vector integration in host cells [11,12]. Although one paper has reported that a lentiviral vector is able to integrate in the known tumour suppressor gene BRCA1 [13], a recent study showed that an HIV-1-derived lentiviral vector injected in a mouse model never induced detectable oncogenic effects by opposition to an EIAV-derived vector [14]. However it will be more secure to use cell population exempt of progenitors such as $CD34^+$ haematopoietic cells.

Previous HIV-1 gene therapy studies have used transdominant proteins [15,16], RNA decoys, ribozyme, antisense RNA [17–21] and small interfering RNA (siRNA) [22-24]. While a large part of these studies have used viral targets, few of them have exploited cellular mechanisms such as cellular co-factors or innate immunity. Such strategies present several advantages such as (i) the use of native proteins to limit the possibility of immune response and (ii) the possibility to inhibit all strains of HIV-1.

A well-described mechanism restricting HIV-1 replication is the pleiotropic antiviral effect of interferon (IFN)-β which is known to affect various stages of HIV life cycle from the uptake of the viral particles to the release of the newly formed virions [25–31]. Several studies demonstrated that a moderate and continuous IFN-β production in human PBMC or $CD4^+$ cells transduced by a MoMuLV-derived vector can reduce *in vitro* HIV-1 replication [25,32]. In addition, IFN-β contributed to the restoration of normal immune cell functions by enhancing IFN-γ and IL-12 production and by returning IL-4, IL-6, IL-10 and TNF-α production to normal levels. Moreover, the cytotoxic response of $CD8^+$ cells against HIV-infected cells was also improved [32,33]. *In vivo* studies showed that IFN-β-transduced human $CD4^+$ cells transferred into a hu-PBL-SCID mouse model supporting a constitutive HIV replication displayed a reduction of the virus replication and an enhanced $CD4^+$ cell survival [34]. Conversely, similar experiments performed in the macaque model have only shown a transient presence of a small number of cells producing IFN-β and no particular effect on SIVmac251 viremia. These disappointing results may be in relation with the low transduction efficiency of the MoMuLV-derived vector and the small number of transduced cells infused into the animals [35]. To overcome this problem, lentiviral vectors represent a suitable alternative.

In this study, we developed lentiviral vectors carrying the human or simian sequences, named LT-huIFN and LT-siIFN, respectively. Culture conditions allowing high levels of gene transfer in human $CD4^+$ cells were developed. The effects of constant expression of IFN-β on apoptosis and cell proliferation were determined. The inhibition of replication of different HIV-1 strains was analyzed in $CD4^+$ isolated cells or in total PBMC cells when IFN-β expression was restored. In these conditions, we also followed the $CD4^+$ cells survival after HIV-1 infection. In order to understand the mechanisms of IFN-mediated inhibition, micro-arrays and real-time PCR analyses were carried out.

2. Materials and methods

2.1. DNA constructs

TRIP-ΔU3-H2kb-huIFNβ (LT-huIFN) and TRIP-ΔU3-CMV-EGFP [36] plasmids were obtained from Virogenix (Le Plessis Robinson, France), and puc0,6-siIFNβ [37] from E. Lauret (Villejuif, France). pHCMV-G [38] and pCMVΔ8.91 [39] plasmids were kindly donated by Dr. Dubart-Kupperschmitt (Paris, France).

2.2. Cell lines

The CEMx174 human lymphoid cells, highly permissive for replication of various HIV/SIV strains [40] were cultured in RPMI complete medium (RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum (Cambrex), 2 mM glutamine, 100 U/ml of penicillin, and 100 μg/ml of streptomycin). 293FT cells (Invitrogen) grown in DMEM medium supplemented with 10% fetal calf serum (Hyclone), 2 mM glutamine, 100 U/ml of penicillin, and 100 μg/ml of streptomycin, 0.1 mM MEM Non-Essential Amino Acids (Invitrogen) and 500 μg/ml of G418 (Invitrogen).

2.3. Vector production

Vector particles were produced by transient calcium phosphate co-transfection of 293 FT cells with the vector plasmid carrying the human or simian IFN-β or EGFP, pCMVΔ8.91 and pHCMV-G as previously described [36]. Vector concentrations were determined by measuring p24 (HIV-1 capsid protein). The viral titres were evaluated on the CEMx174 cells and expressed in transduction unit/ml (TU/ml). The batches of the different vectors used were prepared together to ensure similar transduction efficiencies [41].

2.4. Virus strains and production

HIV-1 (strains X4 LAI and ELI) and HIV-2 (strain A ROD) were obtained from the NIBSC (National Institute for Biological Standards and Control, http://www.nibsc.ac.uk), SIVmac (strains 251 and 239) were obtained from the Pasteur Institute (Paris, France). HIV and SIV strains were amplified on CEMx174. Cell supernatants were centrifuged to eliminate the debris and viral stocks were titrated by determination of the SIV or HIV gag antigenemia by ELISA p27 (Coulter) or ELISA p24 (Innogenetics) respectively and stored at −80 °C.

2.5. Isolation of primary cells

Buffy coats were obtained from the blood transfusion centre (CHU, Liège, Belgium). $CD4^+$ cells were purified from PBMC (Peripheral Blood Mononuclear Cells) using the CD4 Dynabeads (Dynal) according to the manufacturer's instructions.

2.6. Flow cytometry and ELISA

Surface staining was performed with CD4-PE MAb (Monoclonal Antibody, clone L200, Becton Dickinson). Intracellular staining were performed with specifics monoclonal antibodies directed against the viral p24 protein (KC57-FITC MAb, Coulter) and human/simian IFN-β (US Biologicals), using a cell permeabilization kit according to the manufacturer's recommendations (Oxford Biotechnology). Phenotype analyses were realized by Flow cytometry (FACSvantage, Becton Dickinson). Apoptosis was determined using the Annexin-V FITC kit (Becton Dickinson) following the manufacturer's instructions. Extracellular hu- and siIFN-β detections were assessed by ELISA (Biosource).

2.7. Transduction and infection protocols

CEMx174 cells were plated at 5×10^5 cells/ml. Lentiviral particles were added (500 ng/ml of viral p24). After 12 h of transduction cells were washed and cultured in the same conditions for 72 h. EGFP expression in the cells transduced with the LT-EGFP vector was determined by flow cytometry analysis.

Human PBMC or $CD4^+$ cells were plated at 10^6 cells/ml in X-Vivo15 medium (Cambrex) complemented with 10% huSAB (human Serum AB, Cambrex), 50 IU/ml rHu-IL-2 (Gentaur), CD3-CD28 Dynabeads (three beads:one cell-ratio, Dynal) and protamin sulfate (4 μg/ml, Sigma) in plates coated with retronectin (25 μg/ml, Takara). Vector particles were added at the concentration of 1250 ng of viral p24/ml, twice at a 24-hour interval for a total of 48 h. Cells were then washed and cultured in conditions of T-cell expansion. EGFP expression in the $CD4^+$ cell population was analysed by flow cytometry after 72 h of culture. LT-mock- and LThuIFN-transduced cells were washed twice, and beads were removed.

Transduced cells were challenged with several common strains of HIV at a dose of 10 ng of p24/ml for 12 h. Infected cells were then washed three times and cultured for 10 days at 1.5×10^6 cells/ml.

2.8. Affymetrix GeneChip® Microarray analyses

Micro-array analyses were performed on duplicate on two independent samples transduced with two different vectors production. Total mRNAs were extracted from 4×10^6 LT-huIFN and LT-Mock transduced cells at J5 post transduction using the RNeasy kit following manufacturer's instructions (Qiagen). The integrity of the RNA was confirmed with the Agilent Bioanalyser using the RNA 6000 Nano kit (Agilent). We used the GeneChip® Expression 3′ Amplification One-Cycle Target Labeling kit (Affymetrix, Santa Clara, CA) (http://www.affymetrix.com) to label the RNA following the manufacturer protocol. The cRNA was hybridized to Affymetrix Human U133_2 arrays according to the manufacturer protocol. Briefly, double-stranded cDNA was synthesized routinely from five micrograms of total RNA primed with a poly-(dT)-T7 oligonucleotide. The cDNA was used in an *in vitro* transcription reaction (IVT) in the presence of T7 RNA polymerase and biotin-labelled modified nucleotides during 16 h at 37 °C. Biotinylated cRNA was purified and then fragmented (35–200 nucleotides), together with hybridization controls and hybridized to the micro-arrays for 16 h at 45 °C. Using the Fluidics Station (Affymetrix), the hybridized biotin-labeled cRNA was revealed by successive reactions with streptavidin R-phycoerythrin conjugate, biotinylated antistreptavidine antibody and streptavidin R-phycoerythrin conjugate. The arrays were finally scanned in an Affymetrix/Hewlett-Packard GeneChip Scanner 3000.

2.9. Real-time PCR analyses

Total mRNAs were extracted using the procedure described above. Total RNAs (500 ng) were reverse transcribed using random primers and the High Capacity cDNA archive kit (Applied) according to the manufacturer's instructions. To quantify IL-16, IL-8 and IL-1RAP mRNA expression, real-time PCR were performed using the ABI7000 (Applied Biosystem) and the SYBR green mastermix kit (Applied). Primers used were as follows:

- IL-16: S: TCGGCCCACAGACCAAGT;
  AS: CATCCGAGCCTGCCTCTTAA.
- IL-8: S: CTGGCCGTGGCTCTCTTG;
  AS: TTAGCACTCCTTGGCAAAACTG.
- IL-1RAP: S: TTGCTGCGCCCTCTCA;
  AS: TAGGGCCTGCTGATGTTCTAGTT.

After amplification, a melting curve was plotted to check the specific Tm of each PCR product. Their sizes were determined on a 2% agarose gel stained with SYBR green. The transcript level of the housekeeping gene β-actine was evaluated to normalize data.

2.10. Statistics

Statistical analysis was performed using the paired Student *t* test. The statistical analysis of the micro-arrays data was analyzed using GeneChip® Operating Software (GCOS) and GeneChip® DNA Analysis Software (GDAS) (Affymetrix) provided by the affymetrix micro-array plateform and expressed as *p* value.

3. Results

3.1. Construction of LT-siIFNβ, LT-Mock and LT-EGFP vectors

The BamHI-EcoRI fragment of the pUC0.6-siIFN-β was subcloned in pBluescript KS-II (Stratagene) generating the plasmid pBKS-II-siIFN. The LT-siIFNβ plasmid was obtained after the replacement of the BamHI-XhoI fragment containing the human IFN-β cDNA of LT-huIFNβ with the BamHI-XhoI fragment of pBKS-II-siIFN. LT-EGFP was obtained by cloning the BamHI-XhoI fragment of the TRIP-ΔU3-EF1α-EGFP plasmid

in LT-siIFN-β. LT-Mock was obtained by substituting the SacI-KpnI fragment of LT-EGFP containing the EGFP with the 102-bp SacI-KpnI fragment of the pBluescript KS-II multicloning site (Fig. 1A).

3.2. Production of IFN-β by transduced cells

Firstly, the time course of lentiviral transduction in CEM × 174 cells transduced with the control vector LT-EGFP was monitored. FACS analysis performed 72 h after the transduction procedure showed a very high proportion (95 ± 3%; $n = 4$) of EGFP-expressing CEM × 174 cells (Fig. 1B). Since several studies have shown that lentiviral vectors produced concommitantly present similar transduction efficiencies, we decided to confirm this observation. Therefore, intra-cytoplasmic IFN-β expression was analyzed by flow cytometry (Fig. 1C). The levels of transduction obtained with LT-huIFN and LT-siIFN, 89 ± 5% ($n = 3$) and 91 ± 4% ($n = 4$) were comparable to those obtained with LT-EGFP. ELISA analysis of LT-huIFN-transduced cells supernatants carried out at the same time point indicated the presence of IFN-β at a level of 560 ± 77 international unit/ml (IU/ml) ($n = 3$). IFN-β expression was never detected in cells transduced with control vectors.

3.3. Inhibition of HIV and SIV replication in IFN-β-transduced CEMx174 cells

IFN-β is known to inhibit the replication of several viruses, and particularly HIV-1 [32]. To evaluate the ability of IFN-β to control HIV/SIV replication in our system, CEM × 174 cells were transduced with 500 ng of p24/mL of the LT-Mock or LT-hu/siIFN vectors. Then, the transduced cells were challenged with different strains of HIV and SIV at a concentration of 10 ng of p24 or p27/mL, respectively. Viral replication in the culture supernatants was measured at different time points by an ELISA titration of the p24 or p27 proteins, for HIV and SIV strains, respectively. Table 1 shows that IFN-β expression in CEM × 174 cells drastically reduced HIV and SIV replication. The amounts of HIV-1_{ELI} and HIV-2_{ROD} released in the culture supernatant was reduced by a factor close to 99.9 ± 0.1% and 99.8 ± 0.1%, respectively, when CEM × 174 cells were transduced with LT-huIFN, compared to cells transduced with LT-Mock vector. Similar results were observed when IFN-transduced cells were infected with pathogenic SIV strains, with an inhibition of 99.3 ± 0.3% for SIVmac-239 and of 98.8 ± 1.3% for SIVmac-251. The level of inhibition was identical when LT-siIFN was used (Table 1). These results demonstrated that huIFN-β and siIFN-β expression conferred similar antiviral protection against several HIV and SIV pathogenic strains.

3.4. huIFN-β production has no detectable effects on cell growth and apoptosis

A simplified protocol was then developped to transduce primary human T lymphocytes (see Section 2). This protocol allowed us to obtained a transduction efficiency of 85 ± 12% ($n = 10$) of $CD4^+$ cells. This optimised protocol was also able to

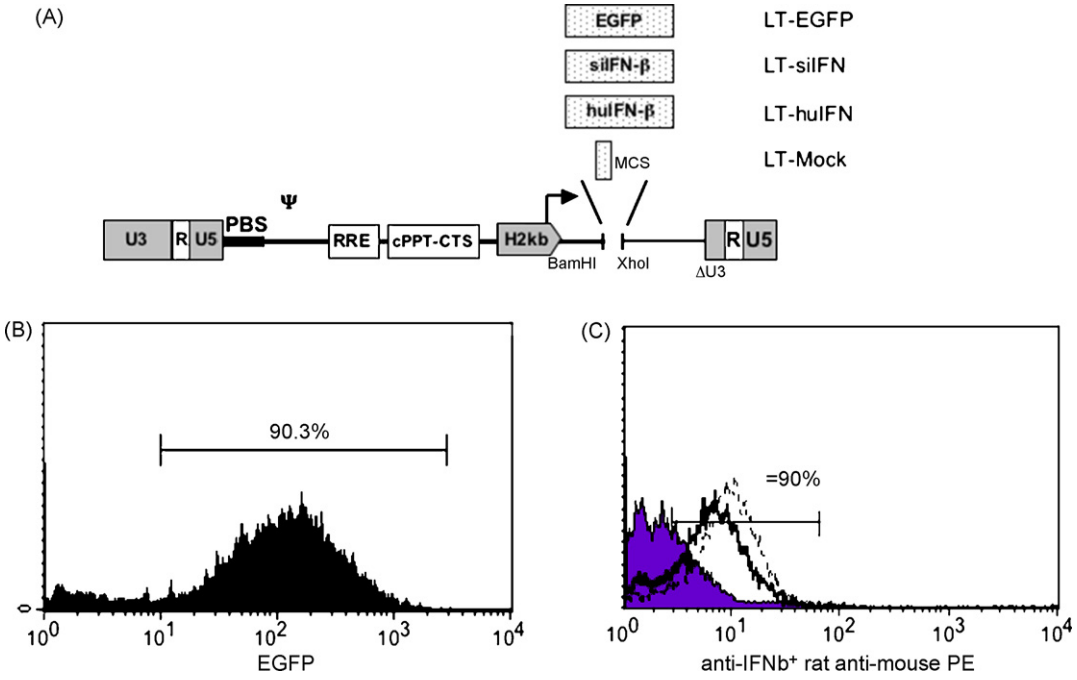

Fig. 1 – Constructs description and enforced expression of siIFN-β and huIFN-β in CEMx174 cells. (A) Structure of the lentiviral vector (LT) encoding EGFP, siIFN-β, huIFN-β and a MCS under the control of the H2-kb promoter. Batches of the four used particle vectors were produced simultaneously to acquire same transduction efficiency. (B) FACS analysis of EGFP expression of CEMx174 cells with LT-EGFP, percentage of GFP positive cells is indicated in the dot-plot. (C) FACS analyses of IFN-β expression in transduced cells. Cells were permeabilized, labelled with a mAB specific of huIFN-β (clone 8.PL.1) and analysed by FACS for the presence of IFN-β. Full area, dotted line, and full line represent IFN-β expression in LT-Mock, LT-siIFN and LT-huIFN transduced cells, respectively.

Table 1 – Inhibition of different strains of HIV/SIV in LT-IFN CEMx174 transduced cells

	HIV-1_{LAI} ($n = 3$)	HIV-1_{ELI} ($n = 3$)	HIV-2_{ROD} ($n = 5$)	SIVmac239 ($n = 6$)	SIVmac251 ($n = 6$)
LT-huIFN	97.0 ± 7 (0.092[a])	99.96 ± 0.05 (<0.05)	99.8 ± 0.1 (<0.05)	99.4 ± 0.3 (<0.05)	98.8 ± 1.3 (<0.05)
LT-siIFN	97.4 ± 4 (0.090[a])	99.98 ± 0.02 (<0.05)	99.2 ± 0.5 (0.089[a])	99.7 ± 0.2 (<0.05)	98.2 ± 1.2 (<0.05)

Inhibition level is expressed in average of percentage ± S.D. (p). Student's t-test was performed to determined whether the inhibition rate observed is significant.

[a] Values of p superior to 0.05 could be explained by the low number of experiments ($n = 3$).

reach transduction efficiencies of PBMC rather similar to those shown above (76 ± 3%; $n = 4$) (Fig. 2A and B). To evaluate the effects of huIFN-β expression during T cell expansion, $CD4^+$ cells purified from PBMC of healthy donors were transduced with the different LT vectors using the transduction protocol described above. Production of huIFN-β was monitored in the supernatants after 8 days of culture. LT-Mock-transduced cells did not produce detectable levels of huIFN-β by opposition to LT-huIFN-transduced cells that produced 14 ± 5 IU/10^5 cells per 3 days ($n = 4$). In these experiments, huIFN-β expression did not alter the proliferation rate as compared with cells transduced with the Mock vector (Fig. 2C). Furthermore, such a level of huIFN-β expression has no effect on cell apoptosis: 51 ± 12% and 50 ± 11% of LT-Mock- and LT-huIFN-transduced cells were AnnexinV positive (Fig. 2D and E, $n = 4$), respectively. So these experiments have demonstrated that the expression of IFN-β was not associated with adverse effect on $CD4^+$ cells.

3.5. *Inhibition of HIV replication on LT-IFN transduced primary cells*

We next examined the antiviral resistance of huIFN-β-expressing lymphocytes. In a first series of experiments, transduced $CD4^+$ cells challenged with several common HIV strains were cultured for 10 days. Microscopic analysis of transduced cells challenged with HIV-2_{ROD} showed that the number of syncitia was drastically diminished in LT-huIFN-transduced cells (Fig. 3A and B). The measurement of viremia in culture supernatants by p24 ELISA at different times post-challenge during 10 days revealed that, in LT-mock-transduced cells, HIV replication increased rapidly and became maximal after 6–8 days of culture. Conversely, LT-huIFN-transduced cells displayed a high anti-HIV resistance, with an almost undetectable viremia as illustrated by the 99.8 ± 0.1%, 99 ± 1% and 99.2 ± 0.5% reduction of p24 release

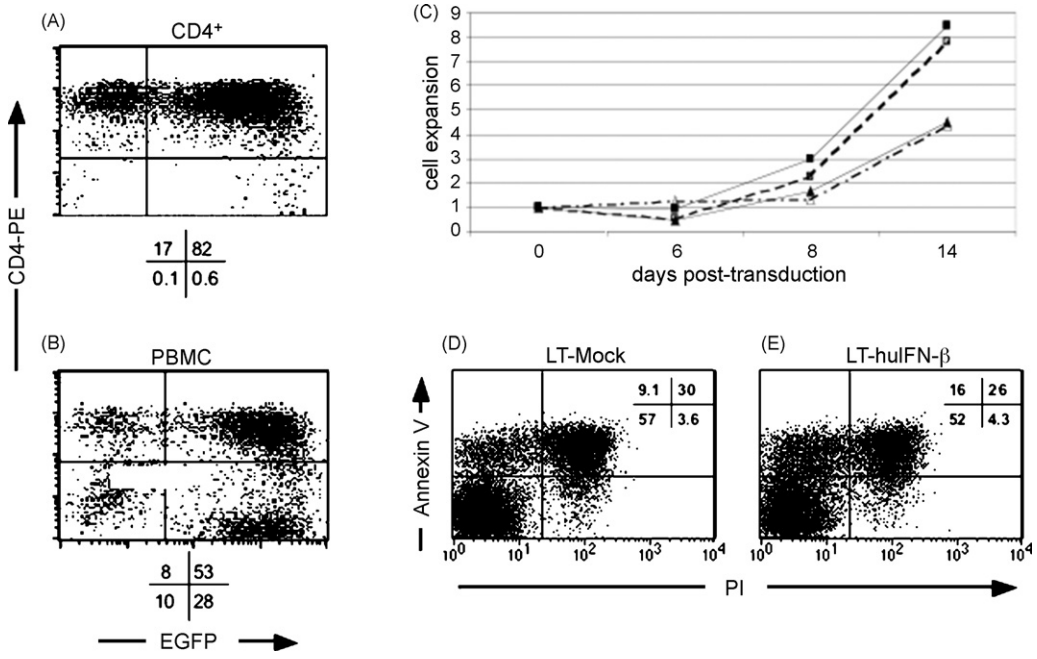

Fig. 2 – Enforced huIFN-β-expression does not affect proliferation and apoptosis in $CD4^+$ cells. $CD4^+$ cells purified from PBMC of healthy donors were transduced with 1250 ng p24/ml of the vectors, this dose corresponding to 0.7 TU/ml or 2.13 ± 1.07 of MOI. Cells were numerated and replated at 1×10^6 cells/ml at days 6, 8 and 10 of culture. (A and B) Transduction protocol of human PBMC or purified $CD4^+$ cells allows efficient transgene expression in $CD4^+$ cells. Percentages of cells in every quadrant are indicated beneath the dot-plots. (C) Proliferation of $CD4^+$ cells is expressed as the rate of cells in the culture as compared with day 0. Two representative experiments are showed. Dotted lines represent the LT-Mock transduced cells whereas full lines represent LT-huIFN ones. Square points and triangular points are for each experiment. (D and E) Apoptosis is analyzed after staining with Annexin V and propidium iodide (PI) at day 10 of culture. FACS analysis of one representative experiment is shown here. LT-Mock and LT-huIFN transduced cells are respectively represented. The percentages of cells in every quadrant are indicated beneath the dot-plots.

in the supernatants of CD4+ cells infected with HIV-1_{LAI}, HIV-1_{ELI} and HIV-2_{ROD}, respectively ($p < 0.005$) (Fig. 3C; Table 2). Therefore, the viremia observed after HIV-1_{LAI} infection was <5 ng of P24/ml in LT-huIFN-transduced cells and ranging between 1340 and 11200 ng of P24/ml in LT-Mock-transduced cells.

Interestingly, when IFN-β-transduced PBMC were challenged with the same strains of virus, the same range of

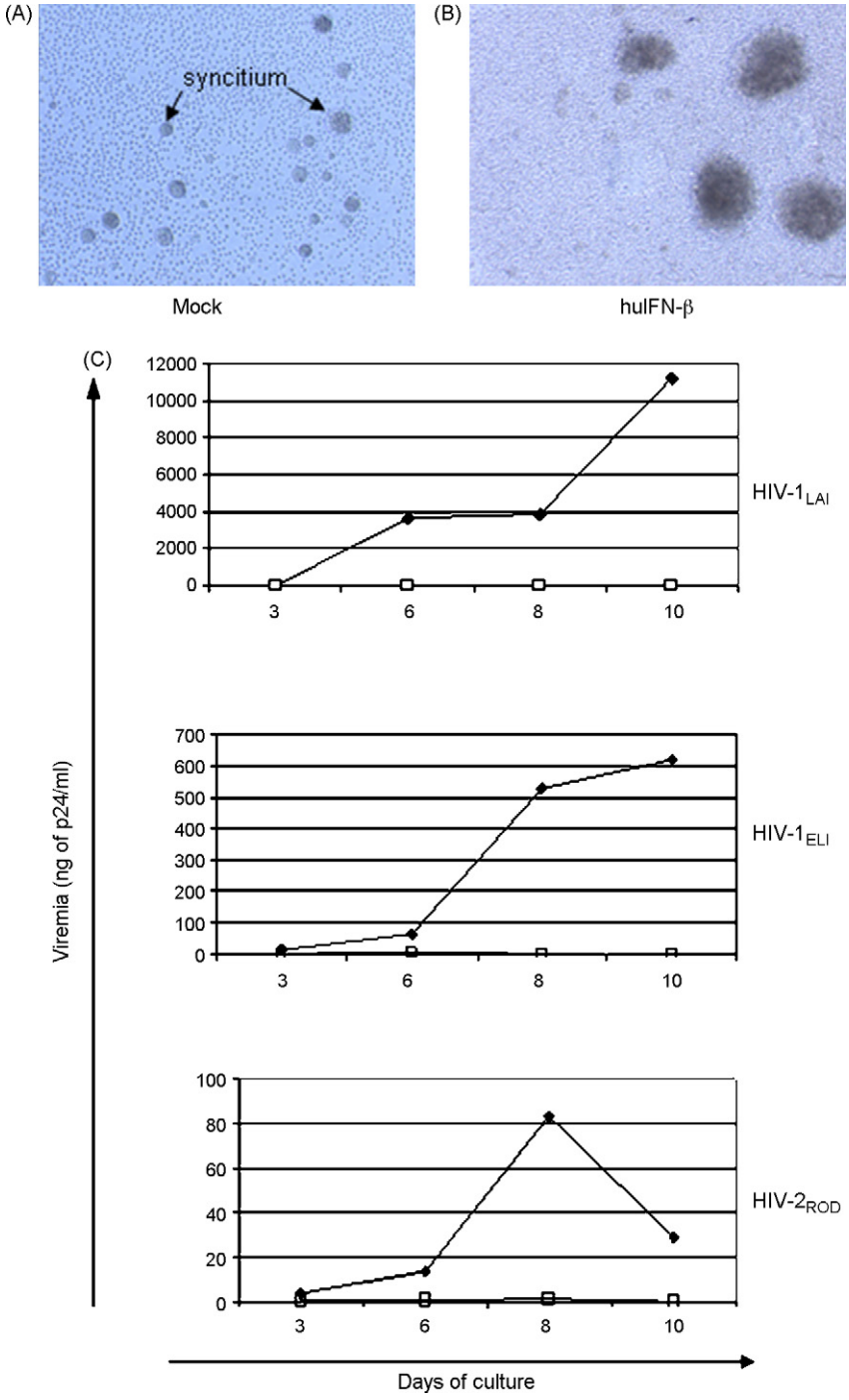

Fig. 3 – Effective inhibition of different strains of HIV in LT-huIFN transduced cells. One representative experiment is shown. CD4+ cells were transduced in parallel with the vectors, washed twice and cultured for 3 days. Transduced cells were infected overnight with 10 ng P24/ml of different strains of HIV (LAI, ELI and ROD), washed twice and cultured at 1.5×10^6 cells/ml. Cells counts were normalized after each passage and culture supernatants were harvested for viral load analysis. (A and B) Microscopy analysis of CD4+ cells infected with HIV-2_{ROD} was carried out 5 days post-infection. LT-Mock transduced cells showed specific formation of numerous syncitia as opposed to LT-huIFN transduced ones. (C) Evolution of viremia during post-infection culture was represented in ng of P24 for different days of culture. Used virus strains are listed next to each specific graph. LT-Mock transduced cells and LT-huIFN transduced cells are respectively represented by open symbols and full symbols.

Table 2 – Exogenous IFN-β inhibits HIV replication in LT-IFN transduced cells

		HIV-1 LAI	HIV-1 ELI	HIV-2 ROD
$CD4^+$ cells	% of inhibition (p)	99.9 ± 0.1 (<0.005; $n = 9$)	99 ± 1 (<0.005; $n = 7$)	99.3 ± 0.6 (<0.005; $n = 3$)
PBMC		99.3 ± 1.1 (<0.005; $n = 6$)	99.1 ± 1.1 (<0.005; $n = 4$)	94.9 ± 8.8 (<0.005; $n = 4$)

Transduced cells were challenged with different strains of HIV 72 h after the end of transduction. Viral load was determined at different times by ELISA p24 on culture supernatants. Inhibition level is expressed in average of percentage ± S.D. (p, Student's t-test was performed to establish whether the inhibition rate observed is significant).

inhibition was obtained. Percentages of inhibition were $99.2 \pm 1.1\%$, $99 \pm 1\%$ and $95 \pm 9\%$ in cells infected with HIV-1_{LAI} ($n = 6$), HIV-1_{ELI} ($n = 4$) and HIV-2_{ROD} ($n = 3$), respectively ($p < 0.005$).

These results clearly indicated that enforced expression of huIFN-β drastically inhibits the replication of different HIV strains.

3.6. *Enforced expression of huIFN-β preserves HIV-infected $CD4^+$ cells by inhibiting apoptosis*

HIV-1 infection led to the expression of HIV protein such as gp120, nef or vpu [42] and to apoptosis of infected $CD4^+$ cells. To follow the $CD4^+$ cell population and the proportion of HIV-infected cells, transduced cells were subsequently challenged with HIV-1_{LAI} as previously described. Infected cells were then counted throughout the culture, and expression of CD4 and intra-cellular p24 were assayed on day 10.

Enforced expression of huIFN-β drastically altered the formation of the p24 subunit of Gag (recognized by the KC57 antibody, Fig. 4A,B,E). In fact, the proportion of $KC57^+$ cells was decreased from $31 \pm 20\%$ in control cells to $0.4 \pm 0.3\%$ in LT-huIFN-β-transduced cells ($n = 3$, $p < 0.05$). Infected cells were nearly undetectable in huIFN-β-transduced cells.

Analysis of cell proliferation revealed that 10 days after the onset of infection, cell expansion was 1.2 ± 0.3-fold and 3.5 ± 1.3-fold for LT-Mock- and LT-huIFN-transduced cells, respectively ($n = 3$, $p < 0.05$). Furthermore, mock-transduced population contained $15 \pm 5\%$ of $CD4^+$ cells, compared to $51 \pm 14\%$ in huIFN-β expressing cells, indicating that enforced expression of huIFN-β preserves $CD4^+$ cells after HIV infection (Fig. 4A,B). Therefore, the absolute number of $CD4^+$ cells was greatly enhanced (11 ± 6-fold) in hu-IFNβ–expressing cells, in contrast with population transduced with the control vector (Fig. 4C).

To further gain insight into the mechanisms responsible for the preservation of $CD4^+$ cells in huIFN-β-expressing cells, cell apoptosis was analysed. Our data revealed that the proportion of Annexin V^+ cells was clearly diminished in LT-huIFN-transduced cells ($26.7 \pm 4.6\%$ versus $82.1 \pm 17.2\%$ in LT-huIFN- and LT-Mock-transduced cells, respectively ($n = 3$, $p < 0.05$) (Fig. 4D).

These results indicated that enforced expression of IFN-β in cells challenged with HIV protect $CD4^+$ cells against cell apoptosis mediated by infection.

3.7. *Effect of interferon beta expression on gene regulation in $CD4^+$ cells*

To identify the molecular signatures induced by IFN-β, we compared the transcriptional gene expression profiles for two different healthy donors LT-huIFN and LT-Mock transduced

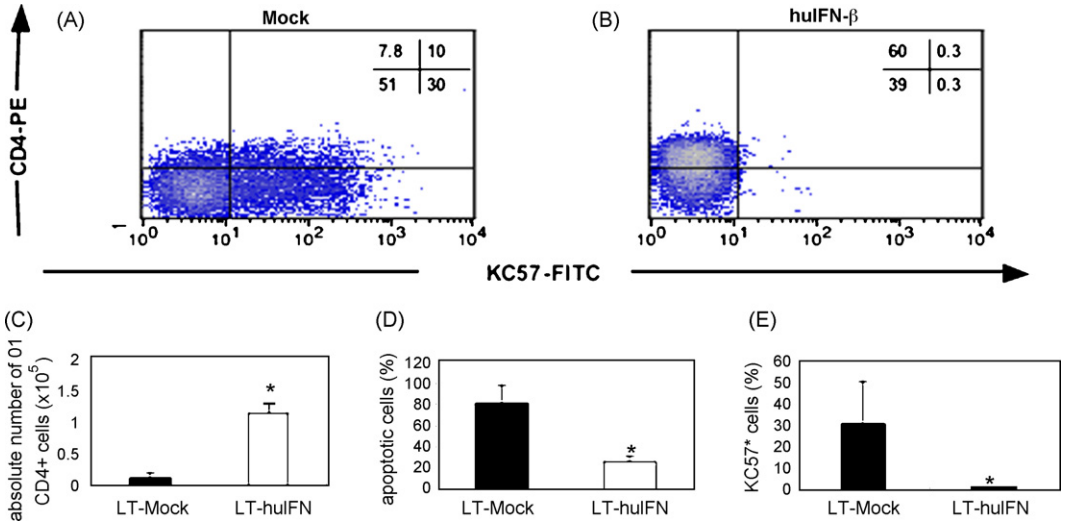

Fig. 4 – Expression of huIFN-β inhibits infection by HIV and preserves $CD4^+$ cells. Transduced $CD4^+$ cells were infected by 10 ng/ml of HIV-1_{LAI}. Presence of intracellular HIV and expression of CD4 were tested 5 days post-infection. Cells were harvested, counted, permeabilized and labelled with KC57-FITC and CD4-PE. (A and B) FACS analysis of one representative experiment is presented. (C) Absolute number of $CD4^+$ cells generated in the culture. Shown are average ± S.D. (D) Absolute number of death cells. In order to study the apoptosis, infected cells were Trypan Blue treated and numerated at day 6 post-infection. Shown are average ± S.D. (E) Percentage of $KC57^+$ cells at 5 days post-infection. Shown are the means ± S.D.

cells. Transduced cells were cultured for 5 day-post-transduction, total mRNA were isolated and gene expression analyses were carried out using Affymetrix GeneChip® Microarray. Genes were selected based on a more than twofold modification compared to controls (a twofold variation is an acceptable level of difference in expression as indicated by several studies [43–45]). Among 22,000 genes on the array, IFN-β enforced expression up-regulated 37 genes, while it reduced the expression of 39 genes (Tables 3 and 4, $n = 2$).

IFN-β expression was accompanied by the regulation of genes implicated in the inhibition of apoptosis: NOL3, PBEF1, BNIP3 were down-regulated while FAIM-3 was up-regulated.

Our data also indicated that huIFN-β expression led to the up-regulation of 6 genes involved in the inflammatory

Table 3 – Genes up-regulated in LT-huIFN transduced CD4⁺ cells

Gene symbol	Fold	Gene name
Inflamatory response		
IL8	8.46	Interleukin 8
IL1RN	7.11	Interleukin 1 receptor antagonist
IL1RAP	6.73	Interleukin 1 receptor accessory protein
CCL3	4.08	Chemokine (C-C motif) ligand 3
CEBPB	3.94	CCAAT/enhancer binding protein (C/EBP), beta
IL12RB2	3.14	Interleukin 12 receptor, beta 2
Immune response		
IFI27	17.51	Interferon, alpha-inducible protein 27
LAMP3/CD208	5.39	Lysosomal-associated membrane protein 3
DPP4	2.79	Dipeptidylpeptidase 4 (CD26)
TNFRSF11A (RANK)	2.6	Tumor necrosis factor receptor superfamily, member 11a, NFKB activator
IL18RAP	2.3	Interleukin 18 receptor accessory protein
Transcription factor		
HLF	6.63	Hepatic leukemia factor
FOSL2	4.23	FOS-like antigen 2
NFE2L1	2.51	Nuclear factor (erythroid-derived 2)-like 1
Growth factor		
VEGF	3.56	Vascular endothelial growth factor
Signalisation		
SNFT	5.1	Jun dimerization protein p21SNFT
Metabolism		
CTS	14.72	Cystathionine-beta-synthase
ADM	12.55	Adrenomedullin
CTH	10.06	Cystathionase (cystathionine gamma-lyase)
PHGDH	8	Phosphoglycerate dehydrogenase
EIF4EBP1	4.44	Eukaryotic translation initiation factor4E binding protein 1
GALNT3	4	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 3
ASNS	3.94	Asparagine synthetase
MARS	3.32	Methionine-tRNA synthetase
PSPH	3.32	Phosphoserine phosphatase
WARS	3.32	Tryptophanyl-tRNA synthetase
RPS6KA2	6.5	Ribosomal protein S6 kinase, 90 kDa, polypeptide 2
THBS4	5.46	Thrombospondin 4
GARS	2.99	Glycyl-tRNA synthetase
AARS	2.64	Alanyl-tRNA synthetase
PYCR1	2.6	Pyrroline-5-carboxylate reductase 1
YARS	2.46	Tyrosyl-tRNA synthetase
SARS	2.38	Seryl-tRNA synthetase
Cell cycle		
CCNA1	8.75	Cyclin A1
Apoptosis		
NOL3	3.2	Nucleolar protein 3 (apoptosis repressor with CARD domain)
PBEF1	3.14	Pre-B-cell colony enhancing factor 1
BNIP3	2.6	BCL2/adenovirus E1B 19 kDa interacting protein 3

Transduced cells were cultured for 5 days post-transduction. IFN-β-regulated genes identified by array analysis were divided into different functional categories.

Table 4 – Genes down-regulated in LT-huIFN transduced CD4⁺ cells

Gene symbol	Fold	Gene name
Immune response		
KLRB1	9.8	Killer cell lectin-like receptor subfamily B, member 1
IL16	8.3	Interleukin 16 (lymphocyte chemoattractant factor)
CD24	6	CD24 antigen
IGKC	5.5	Immunoglobulin kappa constant
CXCL9	5.2	Chemokine (C-X-C motif) ligand 9
TOX	4	Thymus high mobility group box protein TOX
CCR4	3.6	Chemokine (C-C motif) receptor 4
LIME1	3.6	Lck interacting transmembrane adaptor 1
TNFSF4	3.2	Tumor necrosis factor (ligand) superfamily, member 4
LGMN	3.2	Legumain
KLRK1	2.7	Killer cell lectin-like receptor subfamily K, member 1
Transcription factor		
MXD4	4.3	MAX dimerization protein 4
TP73L	2.9	Tumor protein p73-like
Extracellular matrix		
COL6A1	12.1	Collagen, type VI, alpha 1 Extracellular matrix
COL6A2	9.2	Collagen, type VI, alpha 2
CD44	2.3	CD44 antigen (homing function and Indian blood group system)
PECAM1	2.9	Platelet/endothelial cell adhesion molecule (CD31 antigen)
Signalisation		
CHN1	6.3	Chimerin (chimaerin) 1
KIT	5.7	v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog
GNG4	5.7	Guanine nucleotide binding protein (G protein), gamma 4
DACT1	4.3	Dapper, antagonist of beta-catenin, homolog 1 (Xenopus laevis)
PDE7B	3	Phosphodiesterase 7B
PTPLA	2.5	Protein tyrosine phosphatase-like member a
Metabolism		
FXYD7	12.6	FXYD domain containing ion transport regulator 7
HS3ST1	11.6	Heparan sulfate (glucosamine) 3-O-sulfotransferase 1
APOD	7	Apolipoprotein D
ABAT	4.2	4-Aminobutyrate aminotransferase
CHST2	3.7	Carbohydrate(*N*-acetylglucosamine-6-O)sulfotransferase 2
UCP2	3.2	Uncoupling protein 2 (mitochondrial, proton carrier)
AK1	3.2	Adenylate kinase 1
PPGB	3	Protective protein for beta-galactosidase
MRPS14	2.8	Mitochondrial ribosomal protein S14
NUDT3	2.7	Nudix (nucleoside diphosphate linked moiety X)-type motif 3
Divers		
APOB48R	4.5	Apolipoprotein B48 receptor non mais ApoB oui
GPRASP1	4	G protein-coupled receptor associated sorting protein 1
PDLIM2	3	PDZ and LIM domain 2
THADA	2.7	Thyroid adenoma associated
PMCH	2.7	Pro-melanin-concentrating hormone
Apoptosis		
FAIM3	4.4	Fas apoptotic inhibitory molecule 3

response and five genes involved in the immune response. Among these, we can mention the up-regulation of five genes classified as positive regulators of inflammation such as IL-8, IL-1RAP, CCL3 (MIP-1α), CEBPB, IL-12RB2 and the up-regulation of IL-1RN which is an inhibitor of inflammation. In addition, there is an up-regulation of 5 other genes implicated in immune response, particularly important seems to be the up-regulation of DPP4/CD26 which is known to be necessary to MIP1-β processing and to be inhibited by Tat [46]. On the other hand, we also noticed a down-regulation of 11 genes involved in the immune response. We observed the down-regulation of genes implicated in the attraction and activation of LT and Natural Killer (NK) such as KLRB1 and IL-16 (Table 4). Moreover IFN-β-expression led to the down-regulation of four genes coding for proteins of the extra-cellular matrix.

The third category of genes largely represented is implicated in protein metabolism. We detected that IFN-β expression up-regulated 17 genes of this category. Conversely, 10 genes in this class were down-regulated by IFN-β.

Quantitative RT-PCR analyses were carried out to confirm these micro-arrays results. Level of expression in LT-Mock transduced cells constitute the basal mRNA input. These data confirm that IFN-β expression led to a down-regulation of IL-16 mRNA (0.47 ± 0.13; $n = 6$, $p < 0.05$) compared with the basal level in LT-Mock transduced cells. Contrarily there is an up-regulation of IL-8 and IL-1RAP by 3 ± 1.3-fold ($n = 6$,

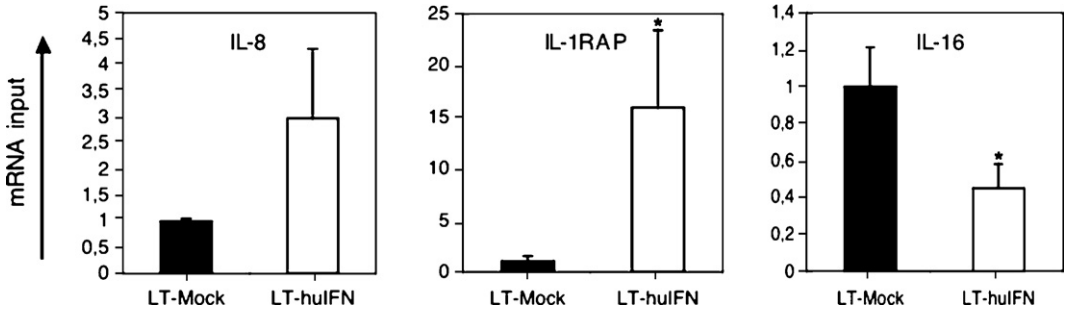

Fig. 5 – Real-time PCR analyses of. transduced CD4⁺ cells LT-Mock and LT-huIFN transduced cells were cultured for 5 days post-transduction. Il-16, IL-8 and IL-1RAP expressions were assessed by real time PCR.

$p = 0.08$) and 15.9 ± 7.5-fold ($n = 6$, $p < 0.05$), respectively (Fig. 5).

These results strongly suggested that the powerful IFN-β antiviral effect stemmed from the regulation of the expression of keys genes implicated on immune/inflammatory response.

4. Discussion

In this study, we evaluated the protection against several strains of HIV by enforced-expression of the interferon β gene using a lentiviral vector. We developed a very efficient protocol of lymphocyte transduction that led up to 93% transduced cells ($n = 10$). Using this protocol, we demonstrated that moderate expression of IFN-β had no effect on cell growth and survival, and was able to inhibit quasi completely the replication of several HIV strains in CD4⁺ human cells.

Our gene therapy protocol displays several important advantages compared to several others reported in the literature [22]. Firstly, to limit the ex-vivo manipulation of patient cells, we decreased the multiplicity of infection and the transduction time and still provided a very high transduction levels for PBMC and CD4⁺ isolated cells. The possibility to efficiently transduce PBMC could be very useful (i) for treating patients with to low CD4⁺ cell counts, and (ii) by limiting the number of steps in a clinical procedure. Moreover, we never detected CD34⁺ cells, known as being rich in haematopoietic progenitors [47], not only in transduced CD4⁺ cells, but also in the PBMC transduced cells (data not shown). These data indicate that at the end of the transduction, haematopoietic progenitors were probably absent of the transduced cells, avoiding the possibility to inject transduced haematopoietic stem cells in patients. Secondly, our protocol does not display any cytotoxic side effects. Indeed, despite very good results, it was established that siRNA (in general or against IFN) could have cytotoxic effects and could induce changes in the level expression of untargeted protein [48,49]. It is also known that expression or administration of IFN-β could have toxic effects on treated cells or patients [37,50]. In our conditions, IFN production in LT-huIFN human transduced cells was 14 ± 5 UI per 10^5 cells ($n = 4$), and no toxicity at this level of expression was observed on the proliferation rate and cell survival. Therefore, we have developed a very efficient transduction protocol without adverse side effect.

Importantly, such huIFN-β production was sufficient to fully inhibit the replication of different strains of HIV (>99%) in CD4⁺ cells. Similar results have been obtained in PBMC.

Furthermore, huIFN-β expression diminished the number of p24-expressing cells, indicative of a protection of IFN-β-expressing cells and/or a decrease in p24 formation in already-infected cells. We observed that IFN-β expression greatly enhanced the absolute number of CD4⁺ cells. In addition, we observed a drastic diminution of HIV-mediated apoptosis in IFN-β-transduced cells. These results argued for a survival of infected CD4⁺ cells and/or a protection against HIV infection mediated by IFN-β expression. The number of experiments and HIV strains tested has to be improved in the future, in order to enhance statistical value and confirmed these pre-clinical data.

Micro-arrays analyses showed that the antiviral effects observed in our experiments could be due to the activation of the inflammatory response mediated by cytokines such IL-1, IL-12 and IL-8 and by MIP-1α, CD26 and CEBPB. It is well described that HIV infection down-regulated the expression or activation of many of these factors such as IL-12 and CD26 [51,52]. IL-8 and MIP-1α are able to inhibit HIV replication [53,54] and could participate actively to the IFN-β-antiviral effects. Interestingly, many of these inflammatory factors could be finely regulated by NF-κB which was recently described as a potent vaccine adjuvant [55]. This is also consistent with the up-regulation of RANK, an activator of the NF-κB alternative pathway [56].

An enhanced expression of anti-apoptotic agents, NOL-3 and PBEF1 was also observed, whereas BNIP3, a pro-apoptotic factor inhibited during viral infection, was up-regulated [57]. Real-time PCR results showed IL-16 down-regulation and IL-1RAP/IL8 up-regulation. These data strongly confirm the micro-arrays analyses.

Micro-arrays and real-time PCR analyses were performed 5 days post-transduction, this time corresponding to 36 hours after infection by HIV strains in inhibition experiments. This time point has been chosen in order to permit an optimal expression of IFN-β and his partners. It should be very interesting to perform micro-arrays analyses at different time points to obtain a well understanding of the kinetics of gene expression after IFN transduction. The gene expression profile of LT-IFN transduced cells before and after HIV-infection also consist an important way to explore.

All these results suggest that the IFN-β expression profoundly modulates the gene expression profile in $CD4^+$ cells, with particular drastic changes in genomic expression of immune/inflammatory response actors. In order to confirm the gene expression results, proteomics analyses have to be realized. This could confirm the implication of immuno/inflammatory agents in IFN mediated HIV inhibition.

Numerous projects actually developed the use of siRNA or antisense RNA against HIV proteins [22]. These strategies were found efficient but currently limited by the number of HIV strains efficiently inhibited. Additionally, it seems that HIV-1 could bypass the inhibition through a siRNA strategy [58,59]. All these data confirmed that our protocol represent a powerful therapeutic tool to inhibit several HIV strains in primary human $CD4^+$ cells and PBMC.

Our therapeutic model could likely be used in strategies against several diseases in which IFN-β is known to be an important mediator. Indeed, several studies showed that IFN-β is a good candidate for gene therapy against different diseases such as cancers [60–63] or deregulation of the CNS [64,65].

We demonstrated that the effect of hu- and siIFN-β was similar to inhibit HIV and SIV replication indicating that the antiviral effect in our model was effective for at least two species. So it would be feasible to test the efficiency and innocuity of the LT-huIFN vector in a macaque model of experimental infection by SIVmac, which is the most appropriate animal model of AIDS [66].

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