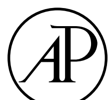
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# The use of lentiviral vectors in gene therapy of leukemia: combinatorial gene delivery of immunomodulators into leukemia cells by state-of-the-art vectors☆

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**Abstract**

Our goal is to develop cell vaccines against leukemia cells, genetically modified to express molecules with potent immune-stimulatory capacities. Pre-clinical evaluation of this approach in murine models has demonstrated efficient anti-leukemic responses with the expression of immunomodulators, in particular GM-CSF and CD80, in irradiated cell vaccines. We have previously shown efficient insertion of GM-CSF and CD80 genes into primary human leukemia cells with the use of second and third generation self-inactivating (SIN) lentiviral vectors (Blood 96 (2000), 1317; Leukemia 16 (2002), 1645). The advantages of lentiviral vectors for development of autologous leukemia cell vaccines include: (1) efficient and consistent gene delivery; (2) high levels of transgene expression; (3) persistent expression of the transduced gene; (4) no viral proteins, as only the transduced gene is expressed; (5) no undesirable cytotoxic effects, and; (6) simplicity of use [leukemia cells are exposed to vector(s) only once]. In this work, we evaluated the insertion of the central polypurine tract and the central termination sequence into a SIN lentiviral vector encoding for GM-CSF and CD80, which significantly enhanced the transduction efficiency of primary leukemia cells and provided higher levels of GM-CSF and CD80 co-expression. We also demonstrate a methodology to deliver simultaneously a combination of immunomodulatory molecules (GM-CSF, CD80, IL-4, and CD40L) to activate different pathways of immune stimulation. Therefore, lentiviral vectors offer a simple, versatile, and reliable approach for engineering leukemic cells for use as cell vaccines.
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## Introduction

Long-term, disease free survival occurs only in 25–35% of adults with acute myeloid leukemia (AML) [1] and 30–40% of those with acute lymphocytic leukemia (ALL) [2]. Relapse remains the single most important clinical problem in AML; thus, immune therapeutic strategies designed to eradicate residual disease hold promise and are an attractive addition to therapy.

The demonstration of immune responses against a few leukemia-associated antigens in AML patients supports the concept that normal immune mechanisms can effectively target leukemia cells. These antigens include HLA-A2.1 restricted peptides present in Wilms tumor gene encoded transcription factor WT1 [3,4] and in the serine protease, proteinase-3, [4,5]. The role of the immune response against AML cells has also been shown in the model of allogeneic transplantation, with demonstration of graft versus leukemia effects [6]. Major improvements in long-term survival for leukemia patients could be achieved if a host immune response to several leukemia antigens could be enhanced to

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eradicate minimal residual disease after use of induction and consolidation chemotherapy. Peptide vaccination may become a feasible approach for AML immunotherapy, although specific epitopes can only be employed in patients expressing these antigens within the context of the proper MHC I molecule. Therefore, the clinical usefulness of these leukemia-associated antigens as therapeutic targets remains to be determined. In contrast, leukemia cell vaccines would be expected to result in the presentation of multiple leukemia-associated antigens without requiring knowledge of the identity of these antigens. Acute leukemia cells are, however, inefficient antigen presenting cells (APCs), and the poor reactivity of autologous anti-leukemia T cell mediated immunity is associated with the inability of leukemia cells to provide sufficient co-stimulation to autologous T cells. This provides the rationale to modify leukemic cells into efficient APCs by genetic manipulation [7].

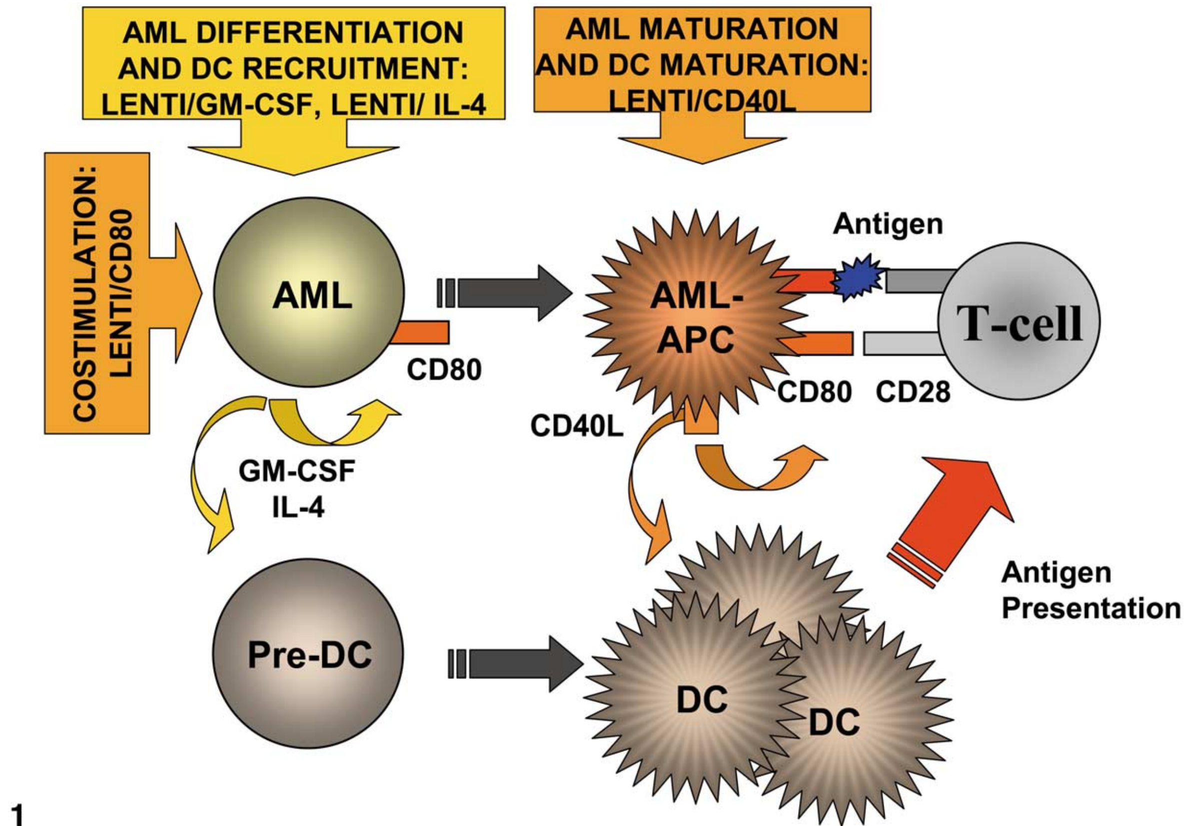
In previous reports, we have demonstrated that gene delivery of CD80 and/or GM-CSF (granulocyte macrophage colony-stimulating factor) into primary leukemia cells enhanced their capacity for allogeneic and autologous T cell stimulation in vitro. Functional experiments with human ALL cells and autologous T cells demonstrated that CD80 gene delivery into the leukemia cells was required to provide T cell stimulation through CD28 [8]. We subsequently demonstrated that transduction of GM-CSF was associated with a maturation of AML cells toward a dendritic cell (DC) phenotype, with improved stimulation of allogeneic and autologous T cells in vitro [9]. These results are in agreement with data obtained from animal models, demonstrating the ability of irradiated murine leukemia cell vaccines genetically modified to express CD80 and/or GM-CSF to render anti-leukemia immunity in syngeneic mice [10–12].

We now want to determine the effect of delivery of other genes and combinations of genes in the immune-stimulatory capacity of AML cells. Therefore, we sought to develop a combinatorial gene transfer approach to promote autogenous effects to activate different pathways of leukemia cell maturation in a more efficient antigen presenting cell phenotype: gene delivery of the CD80 co-stimulatory ligand to facilitate T cell priming through its direct interaction with CD28; expression of GM-CSF and IL-4 to promote the differentiation of AML cells into DC-like cells; and, finally, expression of CD40L to lead to the maturation and effective antigen presentation by AML-APC cells (Fig. 1). Concomitantly, production of GM-CSF/IL-4 and CD40L in the vaccination site could also potentially promote exogenous effects, e.g., recruitment of DCs, to amplify the immune response (Fig. 1).

Primary leukemia cells are very fragile and non-proliferating in culture, which hampers their potential to be transduced with a range of genetic vectors, with the exception of lentiviral vectors, which are not cytotoxic and do not require active cell replication for infection [13,14]. We have therefore standardized a protocol to genetically modify primary leukemia cells using high titer batches of human immunodeficiency virus (HIV-1)-derived lentiviral vectors [9] (see below, Materials and Methods). Hematopoietic cells (macrophages, DCs, T cells) are natural targets for HIV. Importantly, however, transduction with designed lentiviral vectors does not generate cytotoxic or cytopathic effects. Lentiviral transduction leads to stable integration of the transgene into the genome to provide long-lasting expression. Due to the simple structure of the lentiviral transfer vector, no additional antigenic products are co-expressed by the integrated virus other than the transgene of interest. The lentiviral vector production system consists in co-transfection of 293T packaging cells with 3–4 plasmids encoding for the genomic RNA and for different genes present in wt HIV-1: *env, gag, pol, tat,* and *rev* and the viral envelope which may be a pseudotype (Fig. 2A) [15]. For production of lentiviral vectors, the vesicular stomatitis G protein (VSV-G) envelope gene (which replaces the natural HIV-I envelope gene) and the genes encoding for *gag, pol, tat,* and *rev* are expressed in trans from different plasmids used in the transfection of 293T cells.

In our first endeavor to deliver CD80 and GM-CSF genes into human leukemia cells, we used a second-generation lentiviral vector packaging system, in which the four accessory genes of HIV (*vif, vpr, vpu,* and *nef*) were removed from the viral assembly process [16]. The transfer vector for the second generation vectors contains the HIV 5′ and 3′ long terminal repeats (LTRs), which contain elements required for poly-adenylation, reverse transcription, and transcription of the genomic RNA, the latter being dependent on the transactivator *tat*. The expression of the transgene is driven by an internal constitutive human cytomegalovirus (hCMV) promoter (Fig. 2A and B). The second-generation lentiviral vector pHR-GM/CD contained a bi-cistronic open-reading-frame interspaced by an internal ribosome entry site (IRES) for co-expression of GM-CSF and CD80 (Fig. 2B). This vector design provided consistent delivery of CD80 into primary AML and ALL cells, but GM-CSF production was variable and low [8].

Later, we evaluated a third-generation self-inactivating (SIN) lentiviral vector co-expressing GM-CSF and CD80 (Fig. 2C) [9]. In the SIN transfer vector construct the 5′ LTR is chimeric, with the enhancer/promoter of RSV replacing the natural U3 region of the HIV LTR to rescue its transcriptional dependence on *tat* [17]. The 3′ LTR, which serves as the template to regenerate the U3 region in the 5′ LTR, has an almost complete deletion of its U3 region. Therefore, upon reverse transcription, transcriptional inactivation of both LTRs in the integrated provirus occurs [18], thus drastically reducing the risks of virus rescue and/or production of replication competent lentivirus. The third-generation RRL-SIN backbone contained the hCMV internal promoter and a post-transcriptional regulatory element (Wpre) from the woodchuck hepatitis virus in the 3′ untranslated region of the transgene to increase RNA stability and therefore protein production [19]. We demonstrated that RRL-SIN lentiviral vectors transduced AML cells and did

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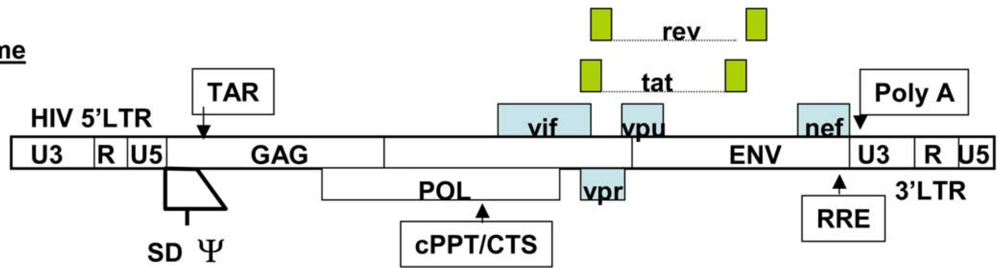


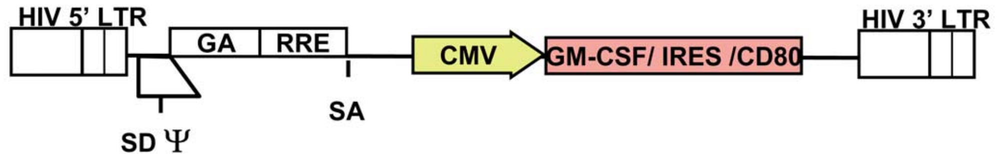


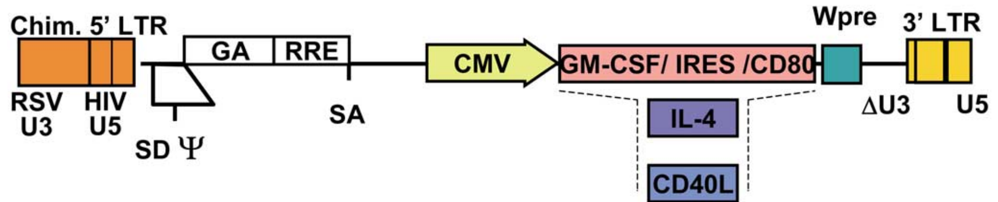


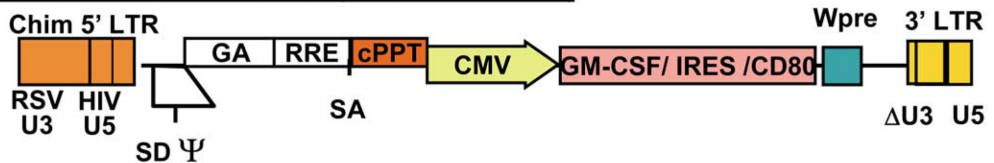


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so significantly more efficiently, providing higher expression of both GM-CSF and CD80 [9].

More recently, it was observed that an element within *pol,* which is required in *cis* to promote high integration of lentiviral vectors, had inadvertently been removed from the lentiviral vector backbone. This element, the so-called central polypurine tract and termination sequences (cPPT/CTS), consisted of a 118-bp element, which when re-introduced in lentiviral vectors, produced higher efficiency of transduction of several types of human primary cells [20].

In this paper we show the effects of the cPPT/CTS element insertion into SIN lentiviral vectors used for GM-CSF and CD80 gene transfer into leukemia cells. We also demonstrate the usefulness of the lentiviral vector system to promote simultaneous gene transfer of GM-CSF, CD80, IL-4, and CD40L by one single hit of transduction of primary leukemia cells.

## Materials and methods

### *Cell culture*

The 293T cell line was obtained from ATCC (Rockville, MD), 293T cells were cultured in D10 medium consisting of Dulbecco's modified Eagle's medium with L-glutamine (DMEM, BioWittaker, Walkerville, MD), 10% fetal bovine serum (FBS), and penicillin/streptomycin (50 U/ml). The Nalm-6 human leukemia cell line was kindly provided by Dr. Dario Campana (St. Jude Children's Research Hospital, Memphis, TN) and was cultured in RPMI 1640 medium with L-glutamine (BioWittaker, Walkersville, MD), 10% FBS, and penicillin/streptomycin (50 U/ml). Cells were incubated at 37°C and 5% $CO_2$. Experiments were performed with cells in exponential growth phase and all transductions were performed in AIM-V serum free medium (Gibco/BRL, Grand Island, NY).

### *Primary cells*

Primary cells were obtained from peripheral blood samples and bone marrow aspirates from adult AML patients at diagnosis. The samples were obtained and studies performed in accordance with protocols approved by the Institutional Review Board of the University of Southern California, after informed consent. Mononuclear cells were separated by density gradient centrifugation, cryopreserved in 10% dimethyl sulfoxide, and 90% FBS and stored in the Tissue Procurement Cell Bank of the University of Southern California. These cells were thawed at 37°C for 1 h using a thawing medium containing AIM-V, 30% FBS, 20 U/ml heparin, and 0.2 U/ml DNAse (Roche, Indianapolis, IN). After thawing, primary cells were washed twice with AIM-V medium. Primary AML cells were cultured in AIM-V medium containing 10 ng/ml human interleukin-3 (IL-3) and 50 ng/ml human stem cell factor (R&D Systems) overnight, prior to transduction.

### *Construction of lentiviral vectors*

The GFP cDNA of pRRL–GFP was excised by *Xba*I/*Sal*I digestion and replaced by a polylinker containing multiple cloning sites (MCS; CTAGAACTAGTGGATCCCCCGG-GCTGCAGGAATTCGATATCAAGCTTATCGATACCG), resulting in the vector pRRL–MCS–pre. The *Eco*RI site present between the Wpre element and the 3′ LTR of the original pRRL–GFP construct was eliminated by a point mutation introduced by PCR resulting into the backbone vector pRRL–sin.hCMV–MCS–preΔEco. DNA fragments obtained by a partial digestion of the bi-cistronic pHR–GM/CD plasmids and encoding GM-CSF/IRES/CD80 [8] was introduced into the *Eco*RI site present in the MCS of the vector pRRL–MCS–preΔEco to generate RRL–GM/CD [9]. For construction of pRRL–IL-4, the human IL-4-open-reading-frame was excised from the plasmid pNGVL3–hIL4 (National Gene Vector Laboratory, University of Michigan) with *Xba*I/*Bam*HI digestion and inserted in frame into the pRRL–MCS–preΔEco vector digested with *Xba*I/*Bam*HI. For construction of pRRL–CD40L, the GFP cDNA of pRRL–GFP was excised by *Xba*I/*Sal*I digestion and replaced by a 1.1-kb *Xba*I/*Sal*I fragment from a derivative of the plasmid pBS–hCD40L-6A9 (ATCC) containing the CD40L open-reading-frame in the correct orientation, resulting in the vector construct pRRL–CD40L. The RRL–

Fig. 1. Model system for acute leukemia cell vaccines modified into antigen presenting cells by gene transfer methods. Acute myeloid leukemia (AML) cells can be genetically manipulated with lentiviral vectors to express immunomodulators (e.g., CD80, GM-CSF, IL-4, and CD40L) to direct their autocrine differentiation into AML-APC. Secretion of factors (GM-CSF, IL-4) by genetically modified leukemia cells that trigger the differentiation of normal antigen presenting cell precursors, e.g., dendritic cells (DC), will assist in the cross presentation of leukemia antigens to T cells. Through competent antigen presentation, the AML-APC and DCs will provide stimulation of T cells, leading to the generation of anti-leukemia immune responses.

Fig. 2. Structure of the genome of HIV-1 and of HIV-1 derived lentiviral vectors co-expressing immunomodulators. (A) Schematic representation of the HIV-1 provirus, showing the coding region for viral (gag, pol, env) and accessory proteins (tat, rev, vif, vpu, vpr) and regulatory elements (long terminal repeat, LTR; splice donor, SD; splice acceptor, SA; encapsidation signal, $\psi$; polyadenylation site, poly A; transactivating region, TAR; rev responsive element; RRE; central polypurine tract and central termination sequences, cPPT). (B) Structure of the pHR–GM/CD vector used for production of second-generation lentiviral vector encoding for GM-CSF and CD80, which were inserted downstream of the internal cytomegalovirus (CMV) promoter and interspaced by an internal ribosome entry site (IRES). (C) For construction of the third-generation lentiviral vector RRL–GM/CD, RRL–IL-4, and RRL–CD40L, a deletion of the viral promoter has been engineered into the U3 region in the 3′ LTR (ΔU3) and results in a self-inactivating replacement of the Rous sarcoma virus (RSV) promoter in the U3 region of the chimeric 5′ LTR upon reverse transcription. The woodchuck post-transcriptional regulatory element (Wpre) was inserted to increase RNA stability; (D) the vector RRL-cPPT-GM/CD was obtained by insertion of the central polypurine tract and central termination sequences (cPPT) into the RRL–GM/CD.

cPPT–GFP construct was created by insertion of a PCR product containing the 118-bp cPPT element flanked by *Hpa*II. The PCR product was obtained using the pMDLg/p vector as template and the oligonucleotide primers 5′-TCGCGACCGGTTAACTTTTAAAAGAAAAGGGGGG-3′ and 5′-AAGCTTCCGGAAAATTTTGAATTTTTGTAAT-TTG-3′. After PCR, the DNA product was subsequently digested with *Hpa*II and the fragment corresponding to the cPPT/CTS element was inserted into the *Cla*I site of pRRL–GFP. For construction of pRRL–cPPT–GM/CD, a fragment containing the GFP gene of was excised from the RRL–cPPT–GFP with *Xba*I and *Sac*II and replaced with the corresponding fragment containing GM-CSF/IRES/CD80 obtained after *Xba*I and *Sac*II digestion of pRRL–GM/CD. The structural integrity of all constructs was reconfirmed by restriction digestion and sequencing analysis.

*Production of lentivirus*

The constructs required for the packaging of third-generation self-inactivating lentiviral vectors were previously described [17,19]. The construct pMD.G was used for the production of the VSV-G viral envelope in combination with the packaging constructs pMDLg/pRRE and pRSV–REV, whereas the pRRL constructions correspond to the different transfer vectors. Lentivirus vectors were produced by transient co-transfection of 293T as previously described [9]. In brief, eight T175 tissue culture flasks coated with poly-L-lysine and containing $18 \times 10^6$ 293T cells per flask were used for each transfection. For each flask, the plasmids pRRL-(60 $\mu$g), pMDLGg/p (39 $\mu$g), pRSV–REV (15 $\mu$g), and pMD.G (21 $\mu$g) were dissolved in water in a total volume of 3.0 ml. A total of 300 $\mu$l of 2.5M $CaCl_2$ was added to the DNA mixture, which was then filtered through a 0.2-$\mu$m filter. A total of 2.8 ml of the DNA/$CaCl_2$ mix was added dropwise, under constant vortexing to 2.8 ml of 2× HBS (pH 7.12) buffer. The DNA/$CaPO_4$ suspension was added to each flask and incubated in a 5% $CO_2$ incubator at 37°C overnight. The next morning, the medium was discarded, the cells were washed, and 30 ml D10 medium containing 20 mM Hepes and 10 mM sodium butyrate was added. The butyrate shock was performed for 8 to 12 h. After that, the cells were washed once and 30 ml fresh D10 with 20 mM Hepes was added onto the 293T cells, which were further incubated in a 5% $CO_2$ incubator at 37°C for 12 h overnight. The medium supernatants were then collected for the first round of ultracentrifugation, the medium was replenished, and supernantants were re-collected 24 h later. The supernatants were spun at 1000*g* for 15 min and filtered through a 0.4-$\mu$m filter to remove cellular debris and finally ultracentrifuged for 2 h and 20 min at 15°C and 50,000*g*. The viral pellets were suspended in 1 ml DMEM, pooled, and vortexed at low speed for 2–3 h at room temperature to make sure the virus particles were thoroughly resuspended. The virus suspensions were spun at 700*g* for 10 min to remove any macroscopic debris formed during concentration. The cleared concentrated supernatants were cryopreserved at −80°C.

*Titer determination*

Viral titer was determined by assessing viral p24 antigen concentration by ELISA (Coulter Immunetech, Miami, FL) and hereafter expressed as $\mu$g of p24 equivalent units per milliliter. One microgram per milliliter of p24 measured in the preparation corresponds to approximately $10^7$ GFP transduction units/ml, as assessed by titration in 293T cells. For each production round, we typically obtained 10 ml of concentrated virus in a concentration of 5–40 $\mu$g/ml p24 equivalent.

*Lentiviral mediated gene transfer*

Leukemia cells were washed twice with AIM-V and for each transduction point $1–2 \times 10^6$ cells were mixed with AIM-V and lentiviral vectors at various concentrations of p24 equivalent per milliliter. For primary leukemia cells the concentration of 5 $\mu$g/ml p24 equivalent was consistently effective to promote high levels of gene transfer (50–100%). One milliliter of the viral suspension was added to the cells and seeded on a well of a 6-well plate, protamine sulfate was added at the final concentration of 6 $\mu$g/ml, and the transduction plates were incubated at 37°C, 5% $CO_2$, overnight. Leukemia cells were washed twice with AIM-V medium and seeded at a density of $10^6$ cells/ml in AIM-V. Twenty-four hours later, the supernatant was collected for ELISA and the cells were harvested for FACS analysis.

*FACS analysis*

For analysis of GFP expression, cells were washed once with phosphate-buffered saline (PBS) and resuspended in 100 $\mu$l of 1% paraformaldehyde for fixation. For analysis of surface membrane antigens, cells were washed once with phosphate-buffered saline, incubated with PBS containing mouse IgG (50 $\mu$g/ml) for 15 min on ice, stained with the corresponding monoclonal antibody for 20 min, washed, and resuspended in 100 $\mu$l of 1% paraformaldehyde for fixation. The monoclonal antibodies to CD80 and CD40L and respective isotype monoclonal antibodies were conjugated with FITC and the monoclonal antibodies against CD33 and respective isotype monoclonal antibodies were conjugated with phycoerythrin (PharMingen, La Jolla, CA). Flow cytometric analysis was accomplished with a FACScalibur cytometer equipped with a 488-nm argon laser. To establish background for fluorescence and to set gates for data acquisition, staining with isotype antibodies was used. Care was taken to analyze cells present in the CD33+ blast gate based on forward and side scatter characteristics.

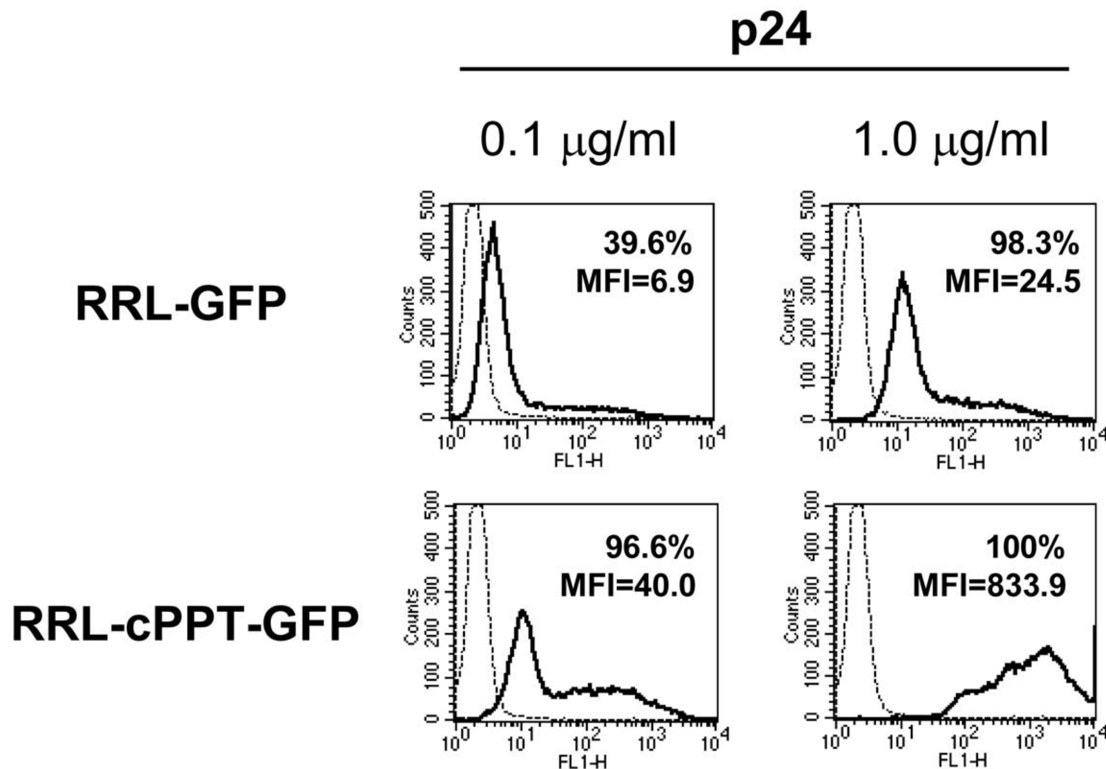


Fig. 3. Nalm-6 leukemia cells were transduced with the RRL–GFP or the RRL–cPPT–GFP vectors at 0.1 and 1.0 $\mu$g of p24 equivalents per milliliter. Forty-eight hours after transduction, GFP expression was followed by flow cytometry. The percentage of GFP+ cells was calculated by subtracting the frequency of the transduced cells (boldface lines) from the background, mock controls (broken lines). MFI, mean fluorescence intensity.

*Analysis of GM-CSF and IL-4*

Secretion of GM-CSF and of IL-4 after leukemia transduction was analyzed by ELISA (Pierce/Endogen, Rockford, IL) according to the manufacturer's instructions. The minimum detectable concentration of GM-CSF following this assay is typically less than 3 pg/ml.

## Results

In this work, we advanced to study a new design of self-inactivating lentiviral vectors containing the cPPT/CTS element and tested the feasibility of simultaneous multiple gene deliveries into primary leukemia cells.

We first compared the transduction of the leukemia cell line Nalm-6 with lentiviral vectors expressing the GFP reporter gene. Transduction was performed in parallel with 0.1 and 1.0 $\mu$g/ml p24 equivalent of the vectors RRL–GFP or the new construct RRL–cPPT–GFP. Transduction with the RRL–cPPT–GFP vector was superior at both viral concentrations, but particularly noticeable at the lower 0.1 $\mu$g/ml p24 equivalent concentration. Under this condition, only 40% of the Nalm-6 cells expressed the GFP gene after transduction, compared to >96% GFP$^+$ cells in the RRL–cPPT–GFP transduced group, demonstrating the higher efficiency of the latter to infect leukemia cells. Parenthetically, transduction with 1.0 $\mu$g/ml p24 equivalent concentration, which produced nearly 100% transduction for both vectors, the mean fluorescence intensity measured for the RRL–cPPT–GFP transduced cells was more than 30 times superior than for the cells transduced with the RRL–GFP. This result indicated that, as expected, the cPPT element conferred a higher integration rate of the transgene, leading to higher expression levels (Fig. 3). These results are representative of three independent experiments performed with different viral batches. Similar results were obtained when expression of CD80 and GM-CSF was followed after transduction of Nalm-6 with varying p24 equivalent/ml concentrations of RRL–GM/CD or RRL–cPPT–GM/CD, confirming the higher expression of the transgenes for the latter design (data not shown).

Therefore, we evaluated the function of the cPPT/CTS element in the RRL–cPPT–GM/CD vector used in transduction of primary leukemia cells. Four different primary AML samples were transduced with 5 $\mu$g/ml p24 equivalent of RRL–GM/CD or RRL–cPPT–GM/CD followed by analyses of GM-CSF secretion and CD80 membrane expression by ELISA and FACS, respectively. Expression levels of GM-CSF and CD80 2 days post-transduction were consistently and significantly superior for the RRL–cPPT–

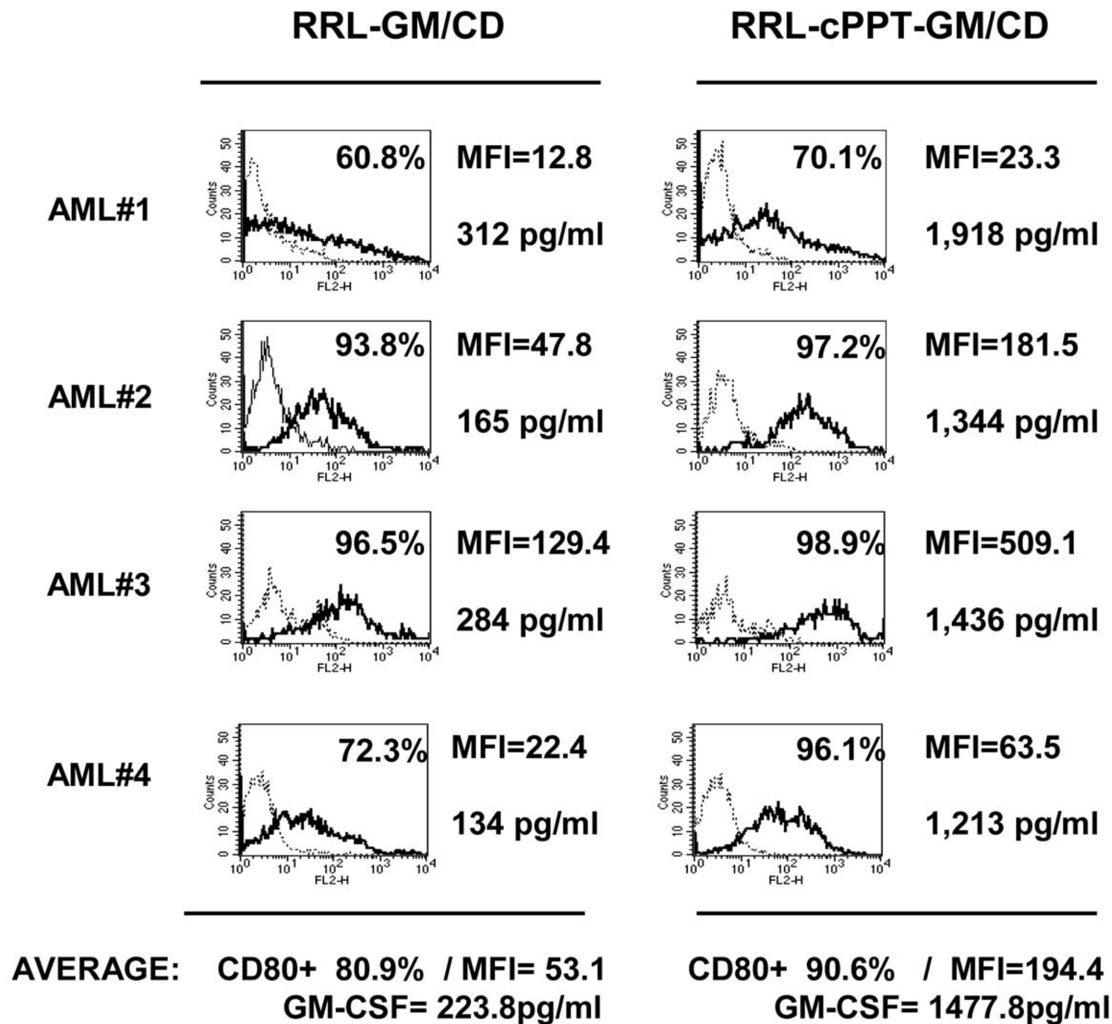


Fig. 4. Comparative transduction of cryopreserved primary AML cells samples with 5.0 $\mu$g of p24 equivalents per milliliter of RRL–GM/CD and RRL–cPPT–GM/CD vectors. The percentage of CD80+ cells was calculated by subtracting the frequency of the transduced cells (boldface lines) from the background, mock controls (broken lines). MFI, mean fluorescence intensity, corresponds to the relative fluorescence values of cells in the CD80+ gate. GM-CSF expression was measured as picograms per $10^6$ cells in 1 milliliter per 24 h.

GM/CD (average GM-CSF = 1,477.8 pg/ml; $CD80^+$ = 90.6%; MFI = 194.4) compared with the RRL–GM/CD vector group (average GM-CSF = 223.8 pg/ml; $CD80^+$ = 80.9%; MFI = 53.1) (Fig. 4). Expression of the GM-CSF and CD80 transgenes 5 days after transduction stayed constant, producing similar levels of CD80 and GM-CSF production (data not shown).

Eventually, we evaluated the feasibility of simultaneous co-transduction of primary leukemia cells with three lentiviral vectors for delivery of four different genes: CD80, GM-CSF, IL-4, and CD40L. Five micrograms per milliliter p24 equivalent of each vector (RRL–cPPT–GM/CD, RRL–IL-4, and RRL–CD40L) was used in transduction of two primary AML cell samples (Tables 1 and 2 and Fig. 5). Expression of CD80 was detectable at high percentages (40–98%) in all transduction groups: RRL–cPPT–GM/CD;

Table 1
Expression of transgenes after lentiviral vector co-transduction of primary AML cells of subtype 5

| AML/M5 | GM-CSF (pg/ml) | IL-4 (pg/ml) | CD80 (%) | CD80 (MFI) | CD40L (%) | CD40L (MFI) |
|---|---|---|---|---|---|---|
| Mock | 58 | — | 3.3 | 2.2 | 3.3 | 2.1 |
| RRL–cPPT–GM/CD | 8177 | — | 99.3 | 10.7 | 3.8 | 1.9 |
| RRL–cPPT–GM/CD + RRL–IL-4 | 9190 | 3405 | 98.8 | 14.6 | 3.6 | 1.8 |
| RRL–cPPT–GM/CD + RRL–IL-4 + RRL–CD40L | 13,365 | 5195 | 99.3 | 32.6 | 44.9 | 12.0 |

Table 2
Expression of transgenes after lentiviral vector co-transduction of primary AML cells of subtype 2

| AML/M2 | GM/CSF (pg/ml) | IL-4 (pg/ml) | CD80 (%) | CD80 (MFI) | CD40L (%) | CD40L (MFI) |
|---|---|---|---|---|---|---|
| Mock | — | — | 4.9 | 1.9 | 3.2 | 1.9 |
| RRL–cPP T–G M/CD | 5495 | — | 41.1 | 124.8 | 4.2 | 2.4 |
| RRL–cPP T–G M/CD + RRL–IL-4 | 6375 | 2740 | 50.3 | 106.6 | 3.0 | 2.5 |
| RRL–cPP T–G M/CD + RRL–IL-4 + RRL–CD40L | 5234 | 2195 | 71.6 | 92.0 | 40.9 | 12.0 |

RRL–cPPT–GM/CD plus RRL–IL-4, and RRL–cPPT–GM/CD plus RRL–IL-4 plus RRL–CD40L (Tables 1 and 2 and Fig. 5). Of note, only minor variations in the percentage of $CD80^+$ cells and in the levels of CD80 expression were observed, indicating that under these conditions, transduction with multiple vectors was additive. Expression of GM-CSF was also detected in all groups ranging from approximately 5200–13,400 pg/ml, with minor variations upon co-transductions. The RRL–IL-4 and RRL–CD40L produced a range of approximately 3400–5200 pg/ml IL-4 (for $10^6$ cell/ml in 24 h) and approximately 40% $CD40L^+$ cells, respectively (Tables 1 and 2 and Fig. 5).

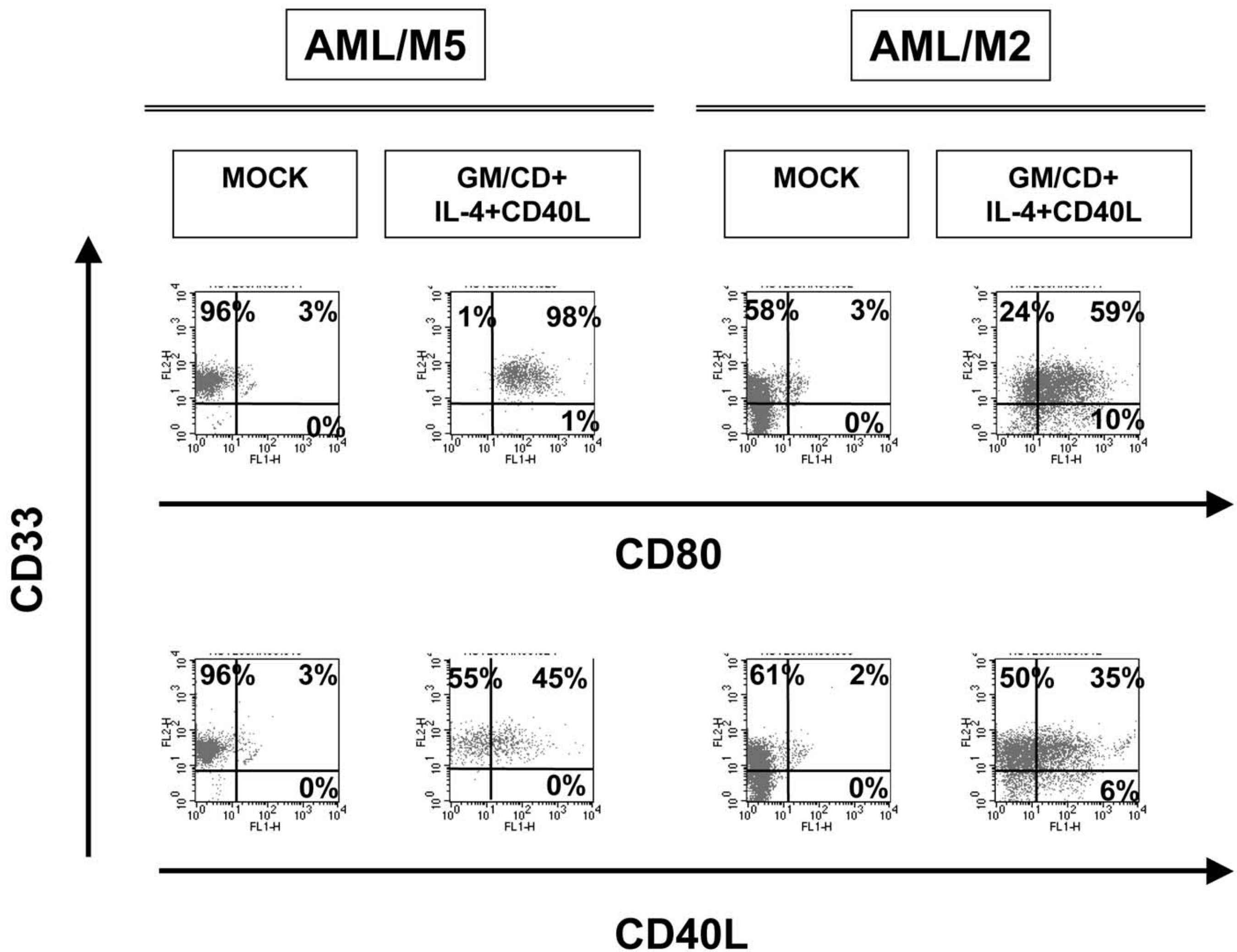


Fig. 5. Co-transduction of cryopreserved primary AML cells samples with three vectors simultaneously: RRL–cPPT–GM/CD, RRL–IL-4, and RRL–CD40L. The right and left groups of panels represent two distinct samples, non-transduced (mock) or transduced with the different vectors, and correspond to dot-plot analysis of the double staining for the CD33 myeloid lineage marker (phosphatidylethanolamine-conjugated anti-CD33) and CD80 transgene expression (fluorescein isothiocyanate-conjugated anti-CD80, top) or CD40L transgene expression (fluorescein isothiocyanate-conjugated anti-CD40L, bottom). Isotype nonspecific control antibodies were used to set up the background levels. The numbers correspond to the relative percentage of each cell population per quadrant.

## Discussion

We have recently evaluated third-generation self-inactivating lentiviral vectors, which have the potential advantage of improved efficiency and safety for clinical application [9]. The SIN vectors, once integrated in the genome of the target cell, lack crucial regions of the 5′ and 3′ LTRs, required for virus rescue. Therefore, in practical terms, SIN vectors are exceedingly unlikely to generate replication competent lentiviruses, which remains the major biosafety concern for human testing and use. We here demonstrated that SIN vectors containing the cPPT/CTS elements were more efficient than previous generation vectors in producing high levels of CD80 and GM-CSF in Nalm-6 leukemia and in primary AML cells. These results were expected, because the cPPT, a sequence found in the *pol* segment of HIV, is required in cis in the transfer vector to allow efficient reverse transcription of the genomic RNA in target cells. Therefore, "re-insertion" of this element into self-inactivating vectors provided higher transduction rates in primary AML cells, which we now consider the "state-of-the-art" lentiviral vector design for future studies of leukemia cell vaccine improvement.

We also developed and tested lentivirus expressing IL-4 and CD40L to demonstrate the feasibility of transducing primary AML cells with several genes simultaneously. Co-transduction of primary leukemia cells with RRL–cPPT–GM/CD, RRL–IL–4, and RRL–CD40L demonstrated that the four different transgenes could be simultaneously inserted and expressed, without substantially affecting the expression of the single genes. Therefore, co-transduction of primary leukemia cells with lentiviral vectors expressing different immunomodulators to elicit complementary pathways of antigen presentation by leukemia cells (Fig. 1) will next be characterized. The combination of CD80 and GM-CSF, which has already been demonstrated in preliminary studies to trigger the immunophenotypic differentiation of leukemia to APCs and to elicit potent immune stimulatory activities, will serve as the standard for comparisons with the multiple gene transduction strategy demonstrated in this work. Furthermore, the ease of the multiple delivery of genes with lentiviral vectors into primary hematopoietic and leukemia cells will be an advantageous method for evaluating the influence of different genes in multi-factorial pathways, e.g., signal transduction pathways, cell cycle control, and apoptosis.

For the advancement of the cell vaccine approach for leukemia, in vitro and in vivo validation of the best combination of immunomodulatory transgenes to promote potent AML-APC phenotype and function will be necessary as the field progresses to future clinical applications. We predict that, for a phase I study with 10 patients, using one sub-cutaneous administration of $10^7$ irradiated transduced leukemia cells, one would need a viral batch containing approximately $10^{10}$ infective particles. This amount of virus is feasible and can be achieved by up scaling our current experimental production system by 10 times. Federal approval of the lentiviral vector gene delivery system for clinical use has not occurred, however, and will be required to move the lentivirally modified AML-APC vaccine field forward.

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