

ORIGINAL ARTICLE

A tissue-specific, activation-inducible, lentiviral vector regulated by human CD40L proximal promoter sequences

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The application of new protocols for gene therapy against monogenic diseases requires the development of safer therapeutic vectors, particularly in the case of diseases in which expression of the mutated gene is subject to fine regulation, as it is with CD40L (CD154). *CD40L*, the gene mutated in the X-linked hyper-immunoglobulin M syndrome (HIGM1), is tightly regulated to allow surface expression of its product only on T cells stimulated by antigen encounter. Previous studies in an HIGM1 animal model showed that transduction of progenitor cells corrected the syndrome but caused a thymic lymphoproliferative disease because of the unregulated expression of the transgene by constitutive vectors. To develop a tissue-specific, activation-inducible, lentiviral vector (LV) for gene therapy to counter HIGM1, we have constructed two self-inactivating LVs, pCD40L-eGFP and pCD40L-CD40L, regulated by a 1.3 kb fragment of the human CD40L proximal promoter. The expression of pCD40L-eGFP LV is restricted to cells in which mRNA transcripts of the endogenous *CD40L* gene can be detected. Moreover, the expression of the reporter gene in primary T lymphocytes depends on the activation state of the cells. Remarkably, primary HIGM1 lymphocytes transduced with pCD40L-CD40L LV expressed CD40L only after T-cell stimulation. Therefore, the CD40L-promoter-driven vectors are able to achieve a near-physiological expression pattern that follows very closely that of the endogenous CD40L gene. *Gene Therapy* (2011) **18**, 364–371; doi:10.1038/gt.2010.144; published online 25 November 2010

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INTRODUCTION

Gene therapy to counter hematopoietic diseases has met with great success in recent years. Patients suffering from X-linked severe combined immunodeficiency, adenosin-deaminase deficiency, X-linked chronic granulomatous disease and Wiskott–Aldrich syndrome have benefited from the reconstitution of their immune systems by gene therapy (reviewed in the study by Thrasher¹ and Aiuti *et al.*²). Nevertheless, some patients have experienced undesired side effects in the form of lymphoproliferation because of integration of the transgene in the vicinity of transcriptionally active areas.^{3,4} This severe setback has pointed to the need for improvement in the biosafety of gene therapy and the vectors used.

Several strategies have been devised to achieve this objective. Some authors, for instance, have inserted insulators based on β -globin gene sequences into the vector's backbone to protect promoters from the influence of regulatory elements found in the proximity of the genomic areas where the vectors are inserted.^{5,6} Another strategy for improving biosafety has been to encourage tissue-specific expression of the transgene at physiological levels. In fact, the transcriptionally targeted expression of therapeutic genes in the hematopoietic lineage has been achieved by including tissue-specific promoters such as CD19,⁷ CD4⁸ or WASP^{9,10} in the vector. This strategy ultimately aims to overcome the risk of ectopic expression of the transgene if transduced hematopoietic precursors differentiate into non-hematopoietic tissues. Although this

issue remains controversial, clear-cut data do exist to indicate that hematopoietic stem cells may differentiate into or fuse with several non-hematopoietic cell types, such as hepatocytes, endothelial, epithelial, neuron or muscle cells.^{11–13} Therefore, the expression of transduced hematopoietic genes in non-hematopoietic tissues may result in severe adverse effects. For instance, we have recently shown that the transduction of non-hematopoietic cells with unregulated lentiviral vectors (LV) expressing the protein encoded by the defective gene in the Wiskott–Aldrich syndrome (*WAS*) disrupts the cytoarchitecture and causes significant cytoskeletal abnormalities.¹⁴ We also showed that the transduction of non-hematopoietic cells with a *WAS* complementary (c)DNA in the context of a transcriptionally regulated vector overcomes cytoskeletal defects by preventing the ectopic expression of the protein.¹⁴

The hurdles faced by gene therapy are even greater in diseases in which the expression of the defective gene is finely regulated. This is true for X-linked hyper-immunoglobulin (Ig) M syndrome (HIGM1), a primary immunodeficiency caused by mutations in the *CD40L* gene.^{15,16} CD40L (CD154) is transiently expressed on the surface of activated T cells after antigen engagement of the T-cell receptor complex.^{17,18} The molecule has a critical role in the mechanisms of the Ig isotype switch by providing appropriate signals to B cells after interaction with its ligand, the CD40 molecule expressed on B cells.¹⁹ Patients with hyper-IgM also have defects in T-cell functions linked

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to abnormalities in the maturation of antigen-presenting cells and in the antigen priming of lymphocytes.²⁰

As with other immunodeficiencies, hyper-IgM patients show a high mortality rate, commonly due to opportunistic infections.¹⁶ As the overall outcome of patients who undergo bone marrow transplantation is unsatisfactory,¹⁶ we need to explore new therapeutic approaches to cure the disease, and gene therapy appears to be a viable alternative. In fact, attempts to correct the disease by transduction of hematopoietic precursors with unregulated vectors expressing CD40L led to the correction of humoral and cell immune responses.²¹ Nevertheless, the majority of reconstituted mice developed a T-lymphoproliferative disorder as a consequence of the unregulated expression of the therapeutic gene in thymic cells.²¹ Further complexity was added to CD40-CD40L interactions by the fact that CD40L transgenic mice generated by a construct controlled by a ubiquitous promoter showed severe lymphoid abnormalities.²² This evidence has hindered further attempts to counter HIGM1 with gene therapy because of the potential risk of the procedure in humans in the absence of a tightly regulated therapeutic approach.

To explore new gene therapy alternatives in the fight against HIGM1 and other diseases that require stringent gene regulation mechanisms, we have constructed two LVs in which transgene expression is controlled by a 1.3 kb fragment of the human CD40L gene promoter. Our results demonstrate that both vectors express the transgene following a pattern that mimics that of the endogenous *CD40L* gene. Furthermore, both vectors obtained transgene expression depending on the activation state of the transduced cells. To our knowledge, this is the first LV capable of achieving tissue-specific, activation-dependent expression. This may be a key development in the treatment of diseases in which fine regulation of the transgene is essential.

RESULTS

A self-inactivating (SIN) LV controlled by a 1.3 kb fragment of the human CD40L promoter follows the expression pattern of the endogenous *CD40L* gene

CD40L is subject to tight regulatory mechanisms that are of critical functional importance,¹⁹ and in fact the adverse effects described in animal models of hyper-IgM gene therapy have been linked to the constitutive expression of the constructs.^{21,22} Because of this precedent, we reasoned that a critical requirement for hyper-IgM gene therapy must be the ready availability of tissue-specific, activation-inducible, stringent vectors. We hypothesized that these requirements might be fulfilled by constructing a vector that regulates the expression of the transgene by a fragment of the human CD40L promoter. Figure 1 shows a schematic map of the two SIN LVs controlled by a 1.3 kb fragment of the human CD40L promoter.²³ The resulting plasmid, pCD40L-eGFP, expresses the reporter *eGFP* gene, whereas pCD40L-CD40L is a SIN LV in which the expression of the human *CD40L* gene is controlled by its endogenous promoter.

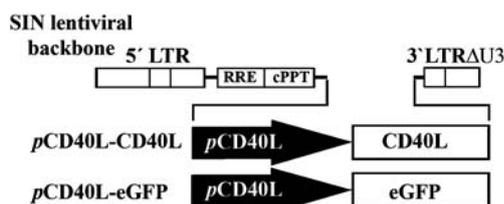


Figure 1 Maps of SIN LV under the control of a CD40L promoter fragment. A 1.3 kb human CD40L proximal promoter sequence was subcloned into a SIN lentiviral backbone to control the expression of either the human CD40L cDNA or the *eGFP* reporter gene.

First, we addressed the ability of the 1.3 kb fragment of the human CD40L promoter to drive a tissue-specific expression of the transgene when incorporated into a SIN LV backbone. Three hematopoietic cell lines (Jurkat, K562 and U937) and two non-hematopoietic lines (293T and Ecv-304) were transduced with the pCD40L-eGFP LV and expression of the reporter transgene was assessed by flow cytometry. The results of a representative experiment are shown in Figure 2a, wherein eGFP expression was observed in Jurkat cells but not in 293T, in spite of an efficient transduction of this cell line, yielding 1.64 vector integrations per cell. A quantitative analysis of pCD40L-eGFP expression levels related to the endogenous expression levels of CD40L mRNA in each cell line is shown in Figure 2b. A correlation between the expression of eGFP (regulated by the pCD40L-eGFP vector) and CD40L (regulated by the endogenous CD40L promoter) can be seen. Thus, the pCD40L-eGFP vector was expressed in cell lines that showed

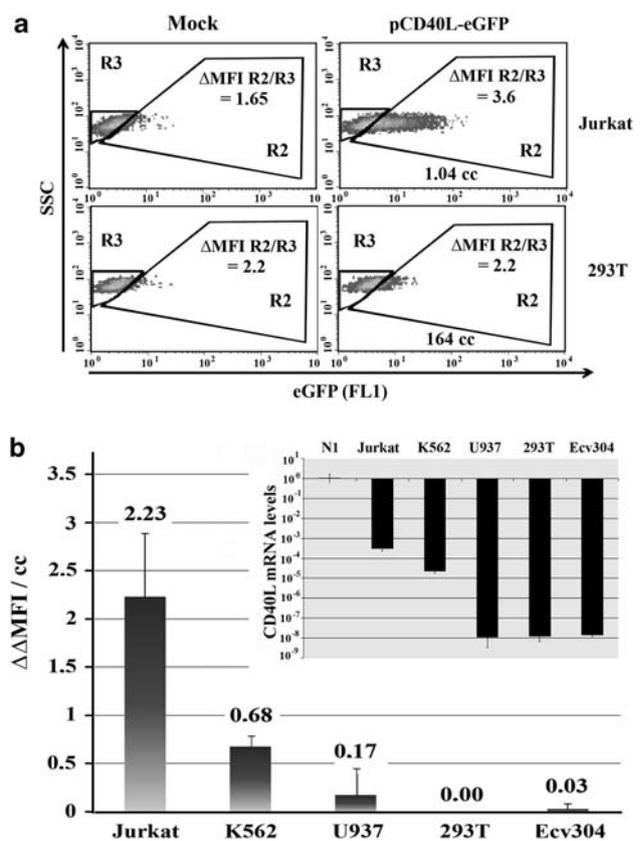


Figure 2 Expression of the pCD40L-eGFP lentiviral vector is restricted to CD40L-expressing cells. A panel of cell lines was transduced with the pCD40L-eGFP lentiviral vector at high multiplicity of infections. Expression of the *eGFP* reporter gene was assessed by flow cytometry and the vector copy number per cell (cc) was estimated by real-time PCR. (a) An example of the results obtained for all transduced cells is represented by Jurkat (hematopoietic) and 293T (non-hematopoietic) cell lines. Lentiviral vector expression levels were calculated in terms of the increase in mean fluorescence intensity (MFI) of the expressing cells (R2) over the non-expressing cells (R3) (MFI R2/MFI R3) compared with the vector copy number per cell obtained for each transduced cell line. (b) Summary of the pCD40L-eGFP expression levels for the different cell lines analyzed. The expression of endogenous CD40L-mRNA is shown for direct comparison of endogenous CD40L and LV-transgene expression. CD40L expression was detected by quantitative PCR and the values obtained were normalized against the expression levels of N1 cells as follows: Jurkat 10^{-3} ; K562 $10^{-4.5}$; U937, 293T and Ecv304 10^{-8} (background levels) and represented in the inset graph. cc: vector copy number per cells.

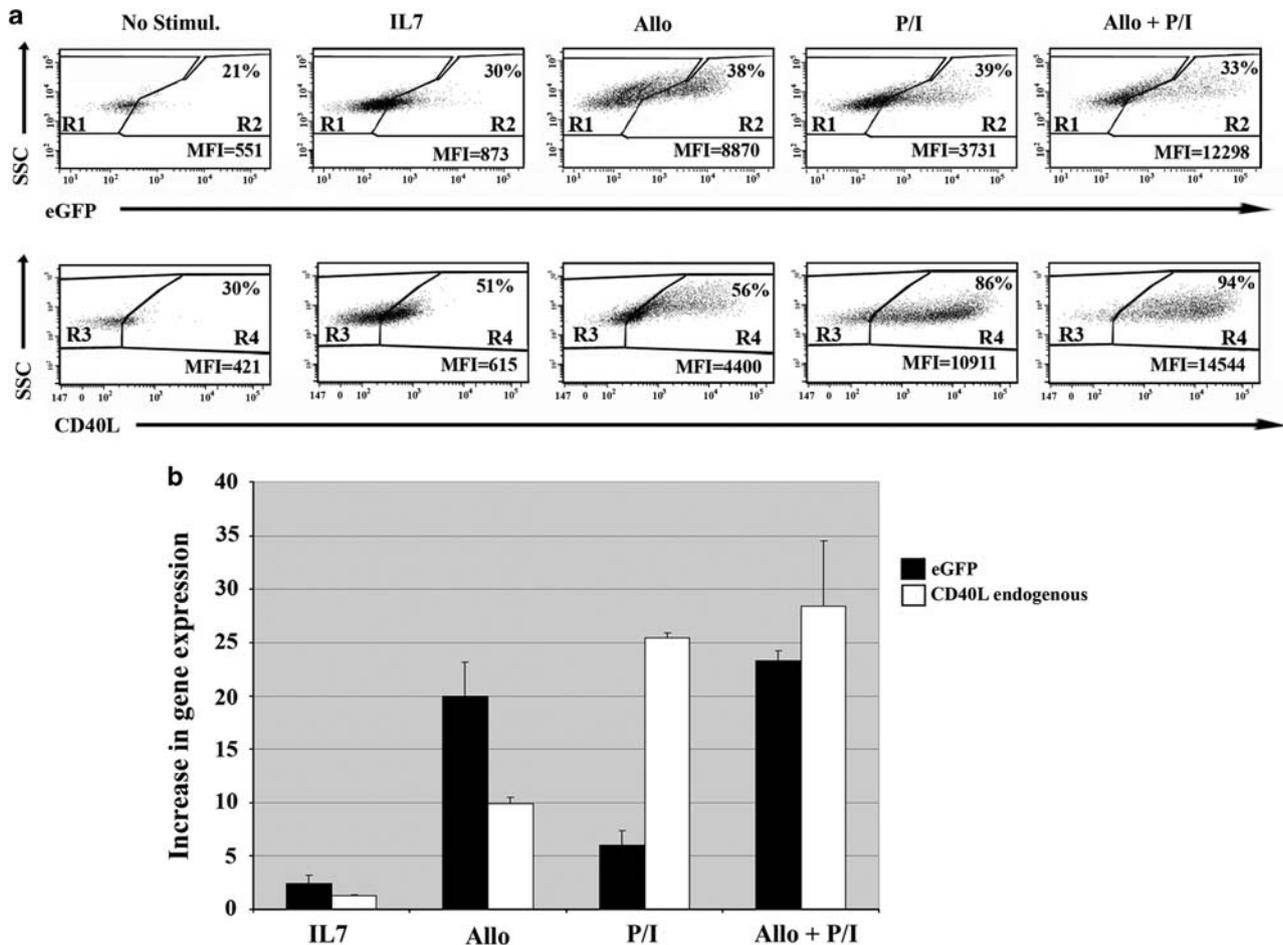


Figure 3 Expression of the pCD40L-eGFP lentiviral vector in primary human lymphocytes is activation dependent. Fresh peripheral lymphocytes from a healthy individual were transduced with the pCD40L-eGFP vector (multiplicity of infection=1) and the following day exposed to different conditions, as described in the Materials and methods section. (a) Results of a representative experiment showing eGFP expression levels (top panels) and endogenous CD40L (bottom panels) of transduced cells under different activation conditions: unstimulated cells (No Stimul.); IL-7 (IL7); allostimulation (Allo); PMA+ionomycin (P/I); and a combination of allostimulation and P/I (Allo+P/I). Mean fluorescence intensity (MFI) values and percentage of positive cells are indicated within the relevant gated regions. (b) Graph showing the relative expression levels of eGFP (solid bars) and endogenous CD40L (open bars) in pCD40L-eGFP-transduced lymphocytes under the different activation conditions. The relative expression values of eGFP and endogenous CD40L were obtained by dividing the MFI value obtained for each group of cells by the MFI value of unstimulated cells.

CD40L mRNA by quantitative PCR. The expression levels of endogenous CD40L in Jurkat cells compared with those of N1 cells were 10^{-3} and $10^{-4.5}$ for K562, as detected by a quantitative PCR (Figure 2b, inset). No expression of the construct was to be found in CD40L-negative non-hematopoietic cells 293T and Ecv-304, even after phorbol 12-myristate 13-acetate (PMA) stimulation (data not shown). Although the hematopoietic cell line U937 showed no detectable amounts of endogenous CD40L, with or without stimulation with PMA, a minimal expression of the transgene was obtained (Figure 2b).

The expression of LVs controlled by the CD40L promoter is activation dependent

The characteristics of the CD40L promoter fragment that governs the pCD40L-eGFP vector were further investigated in fresh peripheral lymphocytes. It was of interest to determine whether pCD40L was able to drive the expression of the transgene following the same expression pattern as the endogenous CD40L gene on different types of stimulation. We found that neither unstimulated nor interleukin (IL) 7-treated primary cells expressed significant levels of CD40L or the eGFP reporter gene (Figure 3a, left-hand panels). Interestingly, a highly

fluorescent population was detected after allostimulation of eGFP-transduced cells (Figure 3a, upper-row panels; Allo), which mimicked the increase in surface CD40L observed in the same population (Figure 3a, lower-row panels; Allo). Although stimulation of transduced lymphocytes with PMA+ionomycin yielded a similar percentage of eGFP-positive cells as that obtained by allostimulation (Figure 3a, upper row; P/I), the level of transgene expression, as detected by the increase in fluorescence intensity, was significantly lower (Figure 3b, solid bars). The highest endogenous expression of CD40L was achieved, however, when cells were treated with PMA+ionomycin but not by allostimulation (Figure 3a, lower-row panels and Figure 3b). Therefore, it would appear that the pCD40L-eGFP vector functions as the endogenous CD40L promoter in the absence of stimuli, in the presence of IL7 and on allostimulation, whereas response to stronger signals such as PMA+ionomycin diminishes.

Characterization of an activation-dependent therapeutic vector for HIGM1

The ability of the pCD40L promoter contained in the pCD40L-eGFP vector to drive a tissue-specific, inducible expression of the reporter

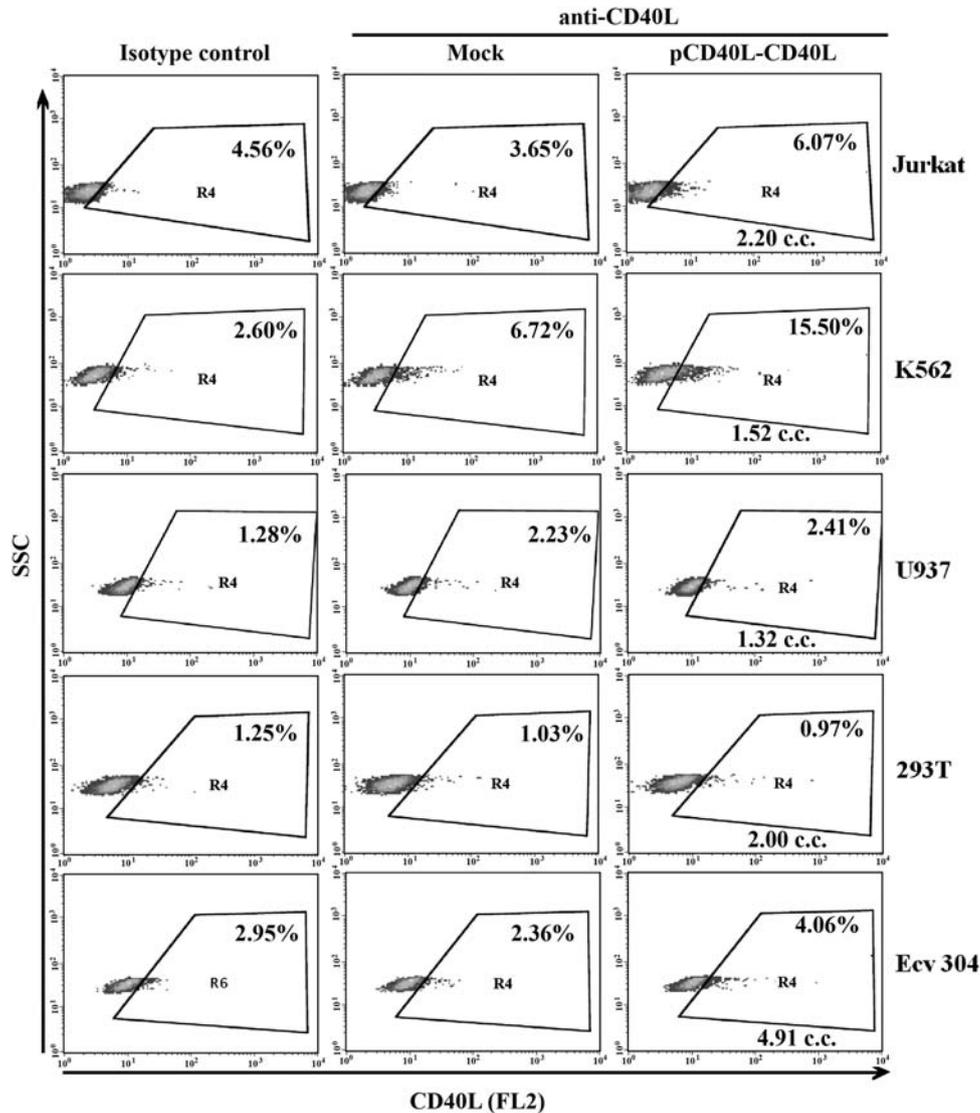


Figure 4 pCD40L-CD40L LV is weakly expressed or undetectable in unstimulated hematopoietic and non-hematopoietic cell lines. The pCD40L-CD40L lentiviral vector was used to transduce three hematopoietic (Jurkat, K562 and U937) and two non-hematopoietic (293T and Ecv304) cell lines. Mock-transduced cells (central panels) and pCD40L-CD40L-transduced cells (right-hand panels) were stained with the 24–31 anti-CD40L monoclonal antibody and analyzed by flow cytometry. Cells stained with an isotype-matched irrelevant monoclonal antibody are shown in the left-hand panels. cc: vector copy number per cell.

gene prompted us to develop a potentially therapeutic vector for HIGM1. The resulting SIN LV controls the expression of CD40L by a 1.3 kb fragment of the human promoter. The CD40L-negative Jurkat T cells were transduced with the pCD40L-CD40L LV in parallel to K562, U937, 293T and Ecv304 cells. We found no significant expression of the transgene in any of the cell lines tested, despite the efficient integration of the vector as determined by real-time PCR, with the exception of a small percentage of Jurkat and K562 cells (Figure 4, right-hand panels).

As untreated Jurkat cells transduced with the pCD40L-CD40L vector showed a minimal expression of CD40L, we investigated whether PMA+ionomycin stimulation of transduced cells led to the surface expression of the CD40L transgene. Stimulation of CD40L-negative Jurkat variant cells led to only a small net increase of 4.45% in CD40L surface expression (Figure 5, top panels). Interestingly, stimulation of pCD40L-CD40L-transduced Jurkat cells resulted in a higher

increase (8.8%) in the percentage of cells expressing CD40L after stimulation, (Figure 5, bottom panels) as a result of CD40L transgene expression. We determined by quantitative PCR that the stimulation of transduced Jurkat cells with PMA resulted in a twofold increase in endogenous CD40L mRNA levels (data not shown).

To confirm these results and to address the physiological expression pattern of the construct in primary cells, we generated primary long-term allospecific CD4⁺ T-cell lines from two HIGM1 patients harboring different mutations in the *CD40L* gene. The cells were transduced with pCD40L-CD40L LV and the expression of CD40L was determined under different conditions. Untransduced cells from a normal individual expressed CD40L on activation (Figure 6, top panels), whereas a cell line derived from an HIGM1 patient, PH3, failed to express surface CD40L after treatment with PMA+ionomycin (Figure 6, central-row panels). Interestingly, we were able to rescue activation-dependent CD40L expression in PH3 cells transduced with

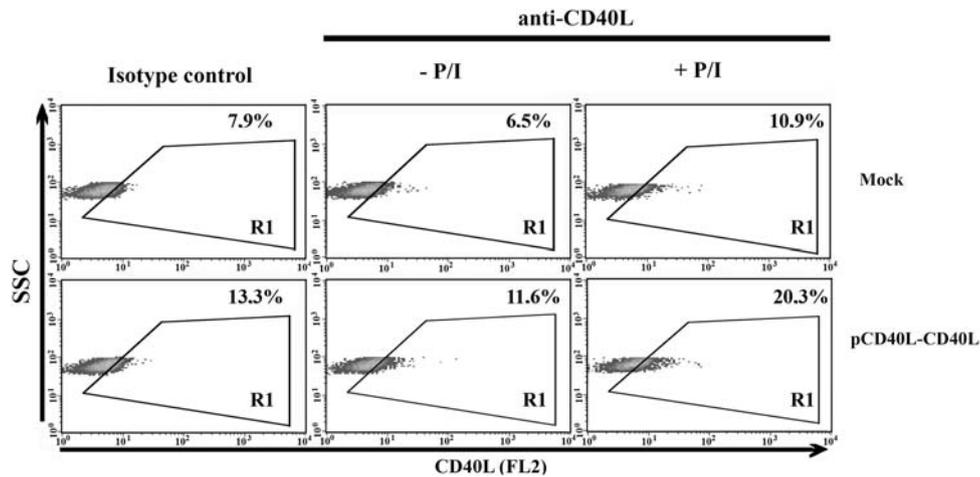


Figure 5 Jurkat cells transduced with pCD40L-CD40L LV express the CD40L molecule at their surface after stimulation. The CD40L-negative variant Jurkat cells were transduced with the pCD40L-CD40L vector and expression of the molecule was analyzed 7 days after transduction. Unstimulated cells (central panels) and cells stimulated overnight with PMA+ionomycin (right-hand panels) were stained with the anti-CD40L monoclonal antibody and analyzed by flow cytometry. Cells stained with an isotype-matched irrelevant antibody are shown in the left-hand panels. The percentage of cells contained within the R1 region is indicated in each panel. A significant increase in CD40L expression is detected in pCD40L-CD40L-transduced Jurkat cells after stimulation.

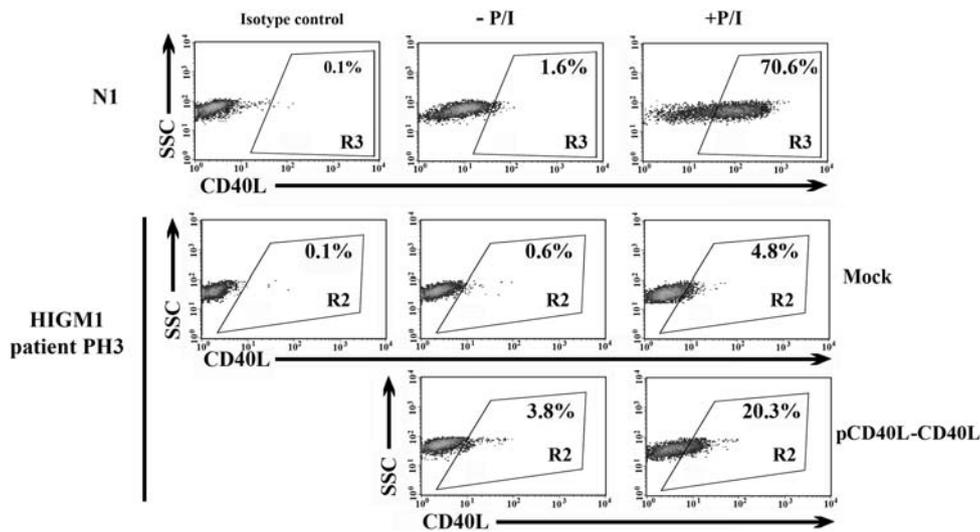


Figure 6 Primary T cells from a patient with HIGM1 transduced with the pCD40L-CD40L vector express the transgene only after stimulation. Long-term allospesific cells obtained from HIGM1 patient PH3 (harboring a Q174R mutation in the *CD40L* gene) were mock-transduced (middle-row panels) or transduced with the pCD40L-CD40L lentiviral vector (bottom-row panels). Surface expression of CD40L was studied 14 days after allostimulation (-P/I) and after 18h of PMA+ionomycin treatment (+P/I). The surface expression of CD40L on allospesific cells from a normal individual (N1) is shown in the top-row panels. Results from staining cells with an isotype-matched irrelevant antibody are shown in the left-hand panels. The percentage of cells contained within the gated regions is indicated in each of the panels.

pCD40L-CD40L LV (Figure 6, bottom panels). Similar results were obtained with transduced cells obtained from HIGM1 patient PH2, who has an IVS3+5g/a mutation (data not shown).

To further characterize the expression pattern of CD40L on PH3 cells transduced with the pCD40L-CD40L therapeutic vector, transduced cells that had been allostimulated 14 days earlier, and thus were at near-resting state, were cocultured with Raji cells. The surface expression of CD40L was assessed at days 1, 3 and 5 after allostimulation. N1 cells revealed a vigorous expression of surface CD40L, which peaked at day 3 and fell significantly by day 5 (Figure 7, top panels). Untransduced cells from patient PH3 showed a leaky expression of CD40L, which reached a maximum on day 3 and subsequently decreased by day 5 (Figure 7, middle-row panels). Remarkably, PH3

cells transduced with the pCD40L-CD40L vector showed a similar expression pattern to that of normal cells. Thus, the surface expression of CD40L was not only higher on transduced cells compared with their untransduced counterparts (Figure 7, bottom panels) but also the maximum level of expression reached on day 3 after allospesific stimulation fell rapidly by day 5 to the levels obtained in unstimulated cells. Similar results were obtained on stimulation of cells with anti-CD3/CD28-coated immunobeads (data not shown).

DISCUSSION

The need to develop safer approaches for gene therapy became evident after the severe adverse effects observed in two human clinical trials of a primary immunodeficiency.^{3,4} Other results obtained in animal

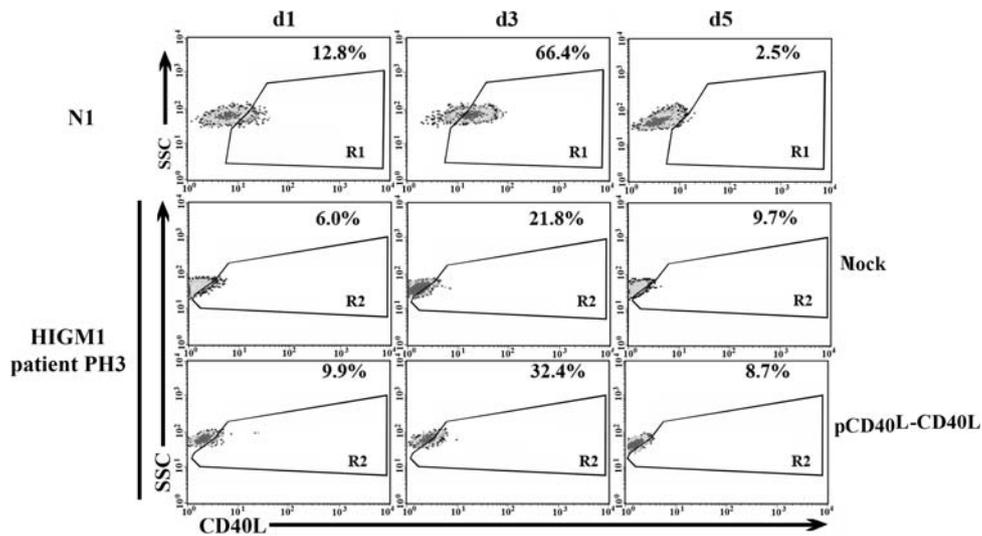


Figure 7 The activation-dependent CD40L expression of HIGM1 cells transduced with the pCD40L-CD40L vector reverts in resting cells. Resting long-term allospecific cells (14 days post stimulation) from a normal individual (N1; top panels) and from HIGM1 patient PH3 untransduced (middle panels) or transduced with pCD40L-CD40L (bottom panels) were cocultured with Raji cells and the expression of CD40L was assessed 1 day (left-hand panels), 3 days (central panels) and 5 days (right-hand panels) from the day of stimulation. Gate regions R1 (N1) and R2 (PH3) were drawn over the results obtained with cells stained with an isotype-matched control.

models are consistent with the dangers associated with the unregulated expression of therapeutic transgenes, as has been demonstrated in mouse models of hyper-IgM syndrome.^{21,22}

We have designed an LV that regulates the transcription of the transgene by a 1.3 kb fragment of the human CD40L promoter, which includes all transcriptionally active sequences present in this DNA fragment. In particular, we have included the four NF-AT binding motifs that appear to be critically important for optimum mRNA production.^{23,24} This fragment also contains potential sites for binding Egr, Stat, AKNA, nuclear factor- κ B and a CD28 response element.²⁴ Studies previous to ours had also demonstrated that the 1.3 kb proximal promoter was transcriptionally active in primary CD4⁺ T cells, as demonstrated by the luciferase assay.²⁵

We therefore assumed that this 1.3 kb proximal promoter sequence would retain the ability to drive gene transcription in a lentiviral setting, and this led us to construct the pCD40L-eGFP LV. Our results show that this vector achieves efficient expression of the reporter gene, with two important features: first, that the eGFP expression regulated by the pCD40L promoter fragment is restricted to cells in which constitutive transcripts of the endogenous CD40L mRNA are detected, thereby fulfilling the tissue-specificity requirement; second, that the expression of the reporter gene in transduced primary lymphocytes depends on cell stimulation, the other significant hurdle in the path toward safer gene therapy protocols.

The response of the promoter element of the pCD40L-eGFP vector is, however, slightly different from that of the endogenous CD40L promoter. In particular, we found that the increase in gene expression when transduced cells were treated with PMA+ionomycin was significantly lower with the vector than it was with endogenous CD40L (Figure 3b). When interpreting these results, we have to take several facts into consideration. For example, the presence of a T-cell-specific transcriptional enhancer rich in GATA and NFAT proteins has recently been discovered. This enhancer functions as a CD4-specific hypersensitivity site located upstream of the promoter sequences included in our promoter fragment²⁶ and is therefore not present in our construct. A second T-cell-specific nuclear factor- κ B/Rel enhancer

has been located in a 1284 bp region 3' flanking the CD40L gene,²⁵ a region that was not included in our vector either. This enhancer element is particularly sensitive to PMA+ionomycin stimulation.²⁵ Finally, it has also been found that the CD40L 3' region is important for the regulation of CD40L expression, as it controls mRNA stability during T-cell activation.²⁷ It remains to be determined whether the presence of any of the above-mentioned elements would have had a significant impact on the behavior of the promoter fragment or on the stability of the transgene mRNA.

As the 1.3 CD40L promoter fragment fulfilled our two biosafety requirements (tissue specificity and activation-dependent expression), we went on to study the ability of this promoter to regulate the expression of a potential therapeutic vector for HIGM1. The pCD40L-CD40L vector efficiently transduced a number of hematopoietic and non-hematopoietic cells, as determined by assessing the number of vector integrations by real-time PCR. Once more, the expression of the CD40L transgene remained restricted to stimulated cells in which endogenous CD40L mRNA transcripts were detected, such as our CD40L⁻ Jurkat variant cells. These results were further confirmed in an *in vitro* model of HIGM1, that is, long-term allospecific T-cell lines from patients with HIGM1. The patients' cells were efficiently transduced by the pCD40L-CD40L vector, which achieved significant expression of the therapeutic gene. Most importantly, transgene expression was strictly dependent on cell activation, as transduced resting cells from two HIGM1 patients did not express any detectable surface CD40L, a molecule that was readily detected on cells from both patients after stimulation. It is noteworthy that the expression pattern of transduced cells stimulated with alloantigen or anti-CD3/CD28-coated immunobeads followed that of their normal counterparts. One critical feature of the pCD40L-CD40L vector is that not only does its triggering of the expression of CD40L depend strictly on stimulation but this expression also diminishes as the cells return to their resting state.

As mentioned in the Introduction section, attempts to develop gene therapy protocols for HIGM1 using constitutive vectors have in the past caused lymphoproliferative disease in thymic cells.^{21,22} These adverse effects are, however, related to the unregulated expression of

the therapeutic protein and are not due to insertional mutagenesis. An elegant study in CD40L-deficient mice showed that a trans-splicing repair strategy was able to correct the mutant gene and allowed expression of the protein under the control of endogenous regulatory mechanisms.²⁸ Under these conditions, none of the treated mice developed lymphoproliferation, whereas they all showed an improvement in the hallmark symptoms of the immunodeficiency. Unfortunately, the implementation of gene therapy in humans based on trans-splicing approaches is not feasible because of their technical complexity and low efficiency.

Gene therapy for HIGM1 is therefore a sound therapeutic alternative, provided that the therapeutic transgene is delivered efficiently in a tightly regulated setting. Our pCD40L-CD40L vector has proved that it can fulfill the requirements for tissue specificity and activation-dependent expression. Furthermore, we have found that the expression of the therapeutic protein in transduced cells is abolished as the cells progress toward a resting state, a fact that is of critical importance in the avoidance of adverse thymic effects,²¹ as it is known that overexpression of CD40L disrupts normal thymic organization.²⁹ To our knowledge, the vectors described in this work are the first tissue-specific, activation-inducible LVs and we believe that they represent a significant step forward in the application of gene therapy to diseases with stringent regulatory requirements, such as HIGM1. Nevertheless, it remains to be determined whether the pCD40L-CD40L vector is able to rescue the functional defects of HIGM1 cells and, in particular, whether CD40L-deficient mice reconstituted with this vector suffer from the lymphoproliferative syndrome observed after reconstitution with constitutive vectors. We are currently conducting experiments to ascertain the true therapeutic potential of this vector.

MATERIALS AND METHODS

Cell lines and culture media

Primary allospecific T-cell lines from healthy individuals and from two patients with HIGM1 were generated and maintained, as described elsewhere,³⁰ in Panserin medium (PAN Biotech, Aidenbach, Germany) supplemented with 5% human serum, glutamax, penicillin/streptomycin and 50 U ml⁻¹ of recombinant human IL-2 (kindly supplied by the National Institute of Health's AIDS reagent program, Rockville, MD, USA). Patient PH2 has a confirmed IVS3+5 g/a mutation, whereas patient PH3 has a Q174R mutation. The following cell lines, Jurkat (T-cell acute leukemia), K562 (chronic myelogenous leukemia), U937 (promyelocytic leukemia) and Ecv 304 (bladder carcinoma), were cultured in RPMI 1640 (Bio-Whittaker, Verviers, Belgium) supplemented with 10% fetal calf serum (FCS), glutamine and antibiotics as described above, and 293T (human embryonic kidney) was cultured in Dulbecco's modified Eagle's medium (Gibco-Invitrogen, Carlsbad, CA, USA). The Jurkat cell line used throughout this study is a serendipitous variant that is CD40L negative at surface level but expresses CD40L mRNA, as detected by reverse transcriptase-PCR.

Construction of plasmids and lentiviral vectors

The human immunodeficiency virus packaging (pCMVDR8.91), which encodes *gag*, *pol*, *tat* and *rev* genes, and the vesicular stomatitis virus-G envelope (pMD.G) plasmids were kindly provided by Dr D Trono (University of Geneva, Switzerland). The full-length *CD40L* cDNA was amplified by reverse transcriptase-PCR from stimulated T lymphocytes from a healthy individual using high-fidelity Pfu DNA polymerase (Promega, Madison, WI, USA) with the primers forward-CD40L (5'-CATTTCACCTTTAACACAGC-3') and reverse-CD40L (5'-AGCTCCACCACAGCCTGC-3'), which yield an 837 bp cDNA. PCR conditions were 1×(95 °C, 2 min); 35×(94 °C, 30 s/50 °C, 1 min/72 °C, 3 min); 1×(72 °C 10 min). The cDNA obtained was subcloned into the plasmid pCRII-Blunt-TOPO (Invitrogen) and sequenced to confirm DNA amplification.

To construct the pCD40L-CD40L LV, we first generated the S-CD40L plasmid as an intermediate construct by excising the eGFP and WPRE elements present in the pHR' SIN cppt-SEW lentiviral plasmid³¹ by BmaHI-XhoI digestion and then subcloning the CD40L cDNA. The resulting plasmid was further digested with BamHI-ClaI to eliminate the SFFV constitutive promoter, which was replaced by a fragment of the human CD40L promoter, spanning 1.3 kb upstream of the initiation codon³² (GENEART, Regensburg, Germany).

The CD40L-eGFP plasmid was constructed by replacing the BamHI-XhoI fragment of the pCD40L-CD40L vector containing CD40L cDNA with the BamHI-XhoI fragment of the pHR' SIN cppt-SE containing eGFP cDNA.

Vector production

LVs were produced by co-transfecting exponentially growing 293T cells with vector plasmid, either pCD40L-CD40L or pCD40L-GFP, the packaging plasmid pCMVDR8.91 and the envelope plasmid pMD.G, as described elsewhere (27 µg total DNA; plasmid proportions of 3:2:1, respectively).⁹ Briefly, 80% confluent cells were resuspended in 1.5 ml of OPTI-MEM media (Gibco) and mixed at room temperature for 20 min with 60 µl of Lipofectamine 2000 (Invitrogen) and then diluted in 1.5 ml of OPTI-MEM. The plasmid-Lipofectamine mixture was added to prewashed cells and incubated for 6–8 h at 37 °C and 10% CO₂. The producer cells were then washed and cultured for a further 48 h in 8 ml of OPTI-MEM medium. Viral supernatants were collected and filtered through a 0.45 µm filter (Nalgene, Rochester, NY, USA), aliquoted and immediately frozen at –80 °C.

Cell transduction and stimulation

Before the lentiviral transduction of cell lines, 10⁵ cells per well were seeded in 24-well plates in 300 µl of medium and the viral supernatants were then added in serial dilution for 8 h. The medium was replaced and the cells were cultured for a further 5 days before being collected and analyzed for CD40L or eGFP expression by flow cytometry in a FACScan flow cytometer (Becton-Dickinson, San Jose, CA, USA). Fresh peripheral lymphocytes from a normal individual were isolated by Ficoll-Hypaque gradient centrifugation and maintained overnight in RPMI complete medium in the presence of 10 ng ml⁻¹ of rIL-7. The cells were pooled in one well of a six-well plate and transduced overnight in the presence of rIL-7. The next day they were washed to remove rIL-7 and divided into the following groups: unstimulated cells kept in complete medium (which includes IL-2); cells kept in complete medium plus 10 ng ml⁻¹ of IL-7; and cells allostimulated as indicated above. On day 6, aliquots of the unstimulated cells were cultured overnight with 5 ng ml⁻¹ of PMA (Sigma, St Louis, MO, USA) and 0.5 µg ml⁻¹ of ionomycin. Another aliquot of allostimulated cells was treated in a similar way with PMA+ionomycin. On day 7, after transduction the expression of the *eGFP* reporter gene or CD40L was determined by flow cytometry as indicated below.

DNA extraction and calculation of vector copy numbers by quantitative PCR

For genomic DNA extraction, cells were resuspended in Tris HCl 20 mM (pH 8), EDTA 5 mM (pH 8), NaCl 400 mM, 1% sodium dodecyl sulfate and proteinase K (100 µg ml⁻¹) and then incubated overnight at 55 °C with continuous shaking. Proteinase K was inactivated at 95 °C and RNase (5 µg µl⁻¹) was then added for 30 min at 60 °C, followed by phenol:chloroform:isoamyl-alcohol extraction. DNA was finally precipitated with absolute ethanol.

Real-time PCRs were performed in a thermocycler ABI Prism 7000 PCR Detection System (Applied Biosystem, Foster City, CA, USA) using iQ SYBR Green Supermix (BioRad, Hercules, CA, USA). The vector copy number was determined by amplification of a 127 bp fragment from cDNA encompassing exons 2 and 3 from the *CD40L* gene. The primers were F-Exon2-CD40L (5'-G ATACAGAGATGCAACACAGGA-3') and R-Exon3-CD40L (5'-GCTGTTTTCT TCTTCGCTCC-3'). The parameters for the PCR were 1×(95 °C, 2 min) and 40×(95 °C, 20 s/55 °C, 30 s/72 °C, 30 s).

The vector copy number per cell in eGFP vectors was determined by amplification of the *eGFP* gene using primers F-GFP (5'-GCCCGACAACCACT ACCT-3') and R-GFP (5'-CGTCCATGCCGAGAGTGA-3'). The parameters for the PCR were 1×(95 °C, 2 min); 40×(95 °C, 15 s/63 °C, 30 s/72 °C, 30 s); 1×(72 °C, 2 min). The number of vector copies per cell was calculated by

interpolating the results in a standard curve constructed with 10-fold-increasing quantities of plasmid DNA (S-CD40L or pCD40L-GFP plasmids). Quantitative analysis of CD40L mRNA was carried out by retrotranscription of total RNA of the cell lines, followed by amplification using primers F-exon -CD40L (5'-CCAGGTTACCAAGTGTGCTCA-3') and R-exon -CD40L (5'-GCGG CACATGTCATAAGTGAGGC-3'). Amplification conditions were 1×(50 °C, 2 min); 1×(95 °C, 10 min); 40×(95 °C, 15 s; 60 °C, 1 min); and 1×(95 °C, 15 s).

Immunostaining and flow cytometry

Transduced cells were collected 7 days after transduction, unless otherwise indicated, washed, blocked and stained with the anti-CD40L monoclonal antibody 24-31 (Calbiochem, Darmstadt, Germany), followed by a phycoerythrin-labeled goat antimouse IgG antibody (Caltag Laboratories, Burlingame, CA, USA). For double fluorescence experiments, an allophycocyanine-labeled second antibody was used (BD-Pharmingen, San Diego, CA, USA). Cells transduced with the pCD40L-eGFP vector were washed and analyzed on a BD FACSCanto II flow cytometer (Becton Dickinson, San Jose, CA, USA).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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