Specific transgene expression in human and mouse CD4⁺ cells using lentiviral vectors with regulatory sequences from the CD4 gene

Gilles Marodon, Enguerran Mouly, Emma J. Blair, Charlotte Frisen, François M. Lemoine, and David Klatzmann

Achieving cell-specific expression of a therapeutic transgene by gene transfer vectors represents a major goal for gene therapy. To achieve specific expression of a transgene in CD4⁺ cells, we have generated lentiviral vectors expressing the enhanced green fluorescent protein (eGFP) reporter gene under the control of regulatory sequences derived from the CD4 gene—a minimal promoter and the proximal enhancer, with or without the silencer. Both lentiviral vectors could be produced at high titers (more than 10⁷ infectious particles per milliliter) and were used to transduce healthy murine hematopoietic stem cells (HSCs). On reconstitution of RAG-2–deficient mice with transduced HSCs, the specific vectors were efficiently expressed in T cells, minimally expressed in B cells, and not expressed in immature cells of the bone marrow. Addition of the CD4 gene-silencing element in the vector regulatory sequences led to further restriction of eGFP expression into CD4⁺ T cells in reconstituted mice and in ex vivo–transduced human T cells. Non–T CD4⁺ dendritic and macrophage cells derived from human CD34⁺ cells in vitro expressed the transgene of the specific vectors, albeit at lower levels than CD4⁺ T cells. Altogether, we have generated lentiviral vectors that allow specific targeting of transgene expression to CD4⁺ cells after differentiation of transduced mice HSCs and human mature T cells. Ultimately, these vectors may prove useful for in situ injections for in vivo gene therapy of HIV infection or genetic immunodeficiencies. (Blood. 2003;101: 3416-3423) © 2003 by The American Society of Hematology

Introduction

Achieving specific expression of a therapeutic transgene is a major hurdle of gene therapy. Given that CD4⁺ cells, whether T or non–T cells, are prominent players in pathologic conditions such as viral infection, autoimmunity, and cancer, specific expression of therapeutic genes in CD4⁺ cells has great potential clinical applications. CD4⁺-cell–mediated gene therapy could be achieved with ex vivo–transduced cells or hematopoietic stem cells (HSCs). Although transduced CD4⁺ cells, providing immediate but short-term immunotherapy, may be valuable for cancer treatment, many other clinical conditions are likely to require continuous generation of immune cells expressing therapeutic transgenes. In such cases, HSCs represent attractive targets for gene therapy, as illustrated by successful gene therapy of immunodeficient infants with severe combined immunodeficiency (SCID). When HSCs are transduced, it is often preferable and sometimes mandatory that transgene expression be limited to a certain subset of their progeny. Indeed, HSCs give rise to a diverse progeny in which differentiation and function could be impaired by some transgenes. For HIV gene therapy, transgene expression should be limited to CD4⁺ cells (for a review, see Buchschacher and Wong-Staal). We thus aimed at generating retroviral vectors driving CD4⁺-cell–limited transgene expression.

With retroviral vectors, CD4 specificity can be achieved in at least 2 ways—pseudotyping with CD4-specific envelopes and transcriptional control of transgene expression through CD4⁺-cell–specific regulatory sequences. Regarding the first strategy, early work has shown that a defective HIV-based vector comprising the native HIV envelope specifically infects CD4⁺ T cells. Further work using Moloney murine leukemia virus (MoMLV)–based vectors pseudotyped with a truncated HIV envelope also showed specificity of infection to CD4⁺ cells. However, targeting CD4⁺ cells through use of the HIV envelope is hampered by technical difficulties, such as low titers of the produced vectors. In addition, this method can only be applied to the generation of gene-modified CD4⁺ T cells through the transduction of mature T cells and not of HSCs.

Concerning the second strategy, we previously studied regulatory sequences of the CD4 gene in vitro and in vivo. These studies have shown that expression of a transgene through CD4 gene promoter and enhancer sequences was restricted to mature T cells. Addition of the CD4 gene-silencing element to these regulatory sequences limited transgene expression to CD4⁺ T cells in transgenic mice. We then designed a minimal transcriptional cassette (CD4pmE) that retained T-cell–specificity on cell lines in vitro and used it to successfully construct T-cell–specific MoMLV retroviral vectors. In other MoMLV-derived vectors in which transgene expression was driven by the viral long terminal repeat (LTR), the CD4 silencer conferred some specificity of expression to human CD4⁺ T cells in vitro. It is thus feasible to design T- and CD4⁺ T-cell expression-specific oncoretroviral vectors. However,
oncoretroviral vectors entail 2 major drawbacks when used to transduce HSCs: (1) expression requires integration into the host DNA that can only be achieved in dividing cells, and (2) expression is frequently silenced in vivo.13 Recent advances in lentiviral vector design (for a review, see Buchschacher and Wong-Staal14), together with the potential for transducing quiescent cells such as neurons15 or hematopoietic cells,16 make lentiviral vectors the vectors of choice for HSC-based gene therapy.17 In the work presented here, our aim was to develop lentiviral vectors achieving efficient HSC transduction and subsequent transgene expression limited to their CD4+ cell progeny. Specific vectors were tested in vivo in a mouse model of hematopoietic reconstitution and in vitro on human mature T cells and on CD34+ cells induced to differentiate into dendritic cells and macrophages.

Materials and methods

Mice

C57Bl/6-Ly5a (CD45.1) and C57Bl/6 RAG-2 knockout (KO) (CD5645.2) mice were bred at the animal facility of the Centre National de la Recherche Scientifique (CDTA, Orléans, France) and were maintained under pathogen-free conditions in our own animal facility. Mice between 8 and 20 weeks of age were used. RAG-2 KO mice were lethally irradiated at 9 Gy immediately before HSC transfer and were fed with neomycin-treated water up to 2 weeks after irradiation. Of note is that lethal irradiation of RAG-2 KO mice considerably increased the frequency of reconstituted animals, probably by providing space for donor-derived immature thymocytes previously occupied by immature precursors from RAG-2 KO mice.18

Lentiviral vector construction

The plasmid pSin.PGK.EGFP.WHV19 (kind gift of P. Salmon and D. Trono, University of Geneva, Switzerland) was digested with XhoI blunted/Clal, removing a fragment of 1383 base pair (bp); then a 1388-bp fragment of CD4pmE-eGFP (Ndel blunted/Clal) described previously20 was inserted, generating the pSin.CD4pmE.EGFP.WHV plasmid. In these vectors, a 400-bp deletion in the U3 region of the LTR completely abrogates the promoter activity of the viral LTR,20 thereby avoiding further transcription and subsequent transgene expression limited to their CD4+ cell progeny. Specific vectors were tested in vivo in a mouse model of hematopoietic reconstitution and in vitro on human mature T cells and on CD34+ cells induced to differentiate into dendritic cells and macrophages.

Lentiviral vector production and concentration

To produce lentiviral vectors, 4 × 10^8 293T cells were cotransfected using the calcium-phosphate method, as described,20 with 3.5 to 5 μg expression vector encoding the VSV-G envelope (pMAL.G), 6.5 to 10 μg packaging plasmid expressing gag, pol, tat, and rev but lacking the accessory genes nef, vpr, vpu, and vif (pCMVΔ8.91),20 and 10 μg gene transfer vector hPGK, LvCD4, or LvCD4-Sil. Cells were grown in Iscove modified Dulbecco medium (IMDM; Biological Industries, Kibbutz Beit Haemek, Israel) supplemented with 5% fetal calf serum (FCS), penicillin (100 μg/mL), streptomycin (100 U/mL), 2 mM l-glutamine (Life Technologies, Gaithersburg, MD). Lentiviral supernatants were collected at 18, 42, and 66 hours after cotransfection, spun down at 800 g, and passed through a 0.45-μm filter before aliquots were frozen at −80°C. To increase titers further, supernatants were concentrated by ultrafiltration using either the Ultraffare-15 or the Centricron Plus-80 filter devices according to the manufacturer’s instructions (Millipore, Bedford, MA). Briefly, 15 mL or 80 mL supernatant was centrifuged 1 hour at 2000g through Ultrafree-15 or CentricronPlus-80 apparatus at 20°C. Concentrated supernatants were separated into aliquots and kept at −80°C until further use.

Viral titers

CD4+ CEMx174 cells were grown in RPMI 1640 10% FCS and antibiotics (Life Technologies). Then 10^7 cells were transduced for 2 hours at 37°C, 5% CO_2 with 0.1 mL to 0.25 mL vector supernatants in the presence of 8 μg/mL polybrene (Sigma, St Louis, MO). Two more milliliter culture medium was added, and the cells were analyzed for enhanced green fluorescent protein (eGFP) expression 48 to 72 hours later. From the different dilutions assayed, only percentages of eGFP falling in the linear curve below 15% were taken into account for the calculation of viral titers. Percentages of eGFP+ cells were corrected by the number of cells infected, the dilution factor, and the volume used for infection to calculate the number of infectious particles per milliliter (ip/mL) supernatant. Titration experiments showed that no lentiviral vectors were present in the flow-through fraction after ultrafiltration (not shown).

Murine hematopoietic stem cell purification and transduction

Bone marrow from C57Bl/6-Ly5a was flushed from femurs and tibias using a 26-gauge needle in RPMI 1640 (Life Technologies). Lin− cells were obtained after incubation with the cocktail antibodies (CD4, CD8, B220, Mac-1, Gr-1, TER-119; kind gift of E. Schneider, CNRS URA1461, Paris, France) for 20 minutes at 4°C. Goat or sheep antirat immunoglobulin magnetic beads (Dynal, Oslo, Norway) were added to the cells at a ratio of 3 beads per total number of cells and were incubated under continuous agitation for 30 minutes at 4°C. The Lin− fraction was further enriched for Sca-1+ cells using the Sca-1 Multisort Kit according to the manufacturer’s instructions (Miltenyi Biotec, Paris, France). Typically, more than 80% of the Sca-1+ fraction expressed Sca-1 at a high density (data not shown). Purified Lin− Sca-1+ cells were cultured for 48 hours in RPMI 1640 (10% FCS, 50 μM β-mercaptoethanol, penicillin (100 μg/mL), streptomycin (100 U/mL), 2 mM l-glutamine [Life Technologies]) in the presence of 50 ng/mL murine stem cell factor (SCF), 20 ng/mL murine interleukin-3 (IL-3; R&D Systems, Abingdon, United Kingdom), and 100 U/mL human IL-6 (a kind gift from Dr L. Aarden, Amsterdam, The Netherlands) and were transduced in vitro in 24-well plates (Becton Dickinson Falcon; Mountain View, CA) coated with 50 μg/cm² fibronectin fragments (Retronectin; Takara Shuzo, Otsu, Japan) according to the manufacturer’s instructions. One hundred fifty microliters concentrated viral supernatants was added for 1 hour at 37°C, followed by spinoculation at 1000g for 2 hours. Cells were washed and resuspended in complete media for further overnight incubation. Cells were then washed and resuspended once in phosphate-buffered saline (PBS; Life Technologies). Between 1 × 10^3 and 2 × 10^3 Sca-1+ cells were injected intravenously into each mouse. For in vitro differentiation of HSCs, 3 × 10^4 total bone marrow cells of reconstituted RAG-2 KO mice were plated in semisedo colony-forming assay using Methocult media (Stem Cell Technologies, Meylon, France). Cells were allowed to grow for...
8 to 10 days before optical examination under ultraviolet (UV) light with a Diaphot microscope equipped with a mercury-filled UV lamp (Nikon SA, Champigny-sur-Marne, France).

Transduction of human lymphocytes

Peripheral blood mononuclear cells from healthy donors were collected by density-gradient centrifugation using Hystopaque-1077 (Sigma). Cells were either prestimulated for 48 hours with phytohemagglutinin (PHA-P; Sigma) at 5 μg/mL in RPMI 1640 10% FCS with penicillin (100 μg/mL), streptomycin (100 U/mL), 2 mM l-glutamine (Life Technologies), and 100 U/mL human IL-2 (Chiron, Emeryville, CA), or they were cultured only in the presence of 10 ng/mL human IL-7 (R&D Systems) without prestimulation. Cells were transduced with lentiviral vectors in the presence of 8 μg/mL protamine sulfate (Sigma) for 2 rounds of the following cycle of infection: 1 hour at 37°C, 5% CO₂, followed by 2-hour spinoculation at 2400g. Cells were analyzed for eGFP expression by flow cytometry from 48 hours to 16 days after infection.

Human hematopoietic stem cell purification and transduction

Samples that had undergone leukapheresis were obtained after informed consent of the patients and the approval by the institutional review board. CD34⁺ cells were purified from leukapheresis products collected from patients after stem cell mobilization with granulocyte–colony-stimulating factor (G-CSF) and cyclophosphamide using magnetic immunobeads as described elsewhere and were frozen at -80°C in FCS containing 10% dimethylsulfoxide (DMSO; Sigma) until further use. After thawing, CD34⁺ cells were cultured overnight at 10⁶ cells/mL on 12-well plates (Costar; Corning SA, Avon, France) in 2 mL IMDM containing 10% FCS, 1% L-glutamine, 1% antibiotics, SCF (300 ng/mL; a gift from Amgen, Corning SA, Avon, France), IL-3 (100 U/mL; Genzyme, Cambridge, MA), IL-6 (100 U/mL), insulin-like growth factor (IGF-1; 50 ng/mL; Valbiotech, Paris, France), and basic fibroblast growth factor (bFGF; 50 ng/mL; Peprotech, London, United Kingdom). CD34⁺ cells were then infected at 1.5 × 10⁵ cells/mL for 48 hours with different lentiviral supernatants supplemented with the same growth factor cocktail using an already published protocol that combined cell centrifugation in the presence of 8 μg/mL protamine sulfate followed by adhesion on wells precoated with Retronectin (Takara Shuzo). At day 2 after infection, cells were cultured in 24-well culture plates (Costar) at 2.5 × 10⁵ cells/mL under different conditions to induce differentiation into dendritic cells (DCs) or macrophages. For DC differentiation, cells were seeded in IMDM supplemented with 10% FCS, 300 ng/mL SCF, 50 ng/mL Flt3-L, 100 ng/mL granulocyte macrophage–colony-stimulating factor (GM-CSF; Schering Plough, Levallois-Perret, France), 50 ng/mL IL-4 (a gift from K. Thielemans, Université libre de Bruxelles, Belgium), 5 ng/mL tumor necrosis factor-α (TNF-α; Valbiotech). For macrophage differentiation, cells were cultured in IMDM containing 10% SVF and 10 U/mL macrophage–colony-stimulating factor (M-CSF; R&D Systems). In each culture condition, the medium was changed at day 5 after infection, and cultures ended at day 9 after infection. Cells were then counted, morphologically characterized, immunophenotyped, and assessed for eGFP expression.

Antibodies and flow cytometry

The following rat–antimouse unlabeled monoclonal antibodies (mAbs) were used for cell sorting: CD4 ( GK1.5), CD8 (8C6), B220 (6B2), Mac-1, Gr-1, and TER-119. For flow cytometry analysis, the following rat–antimouse or mouse antimouse mAbs were used, labeled with allophycocyanin (APC) or phycoerythrin (PE) (both from Pharmingen, San Diego, CA), tricolor (TC; Caltag, Burlingame, CA), quantum red (QR; Sigma), or phycoerythrin-Cy5 (PE-Cy5; Pharmingen). The following rat antimouse–or mouse antimouse mAbs were used, labeled with allophycocyanin (APC) or phycoerythrin (PE) (both from Pharmingen, San Diego, CA), tricolor (TC; Caltag, Burlingame, CA), quantum red (QR; Sigma), or phycoerythrin-Cy5 (PE-Cy5; Pharmingen). One to 2 million cells per well were pelleted in a 96-well plate (Corning SA) and stained with 20 μL 2.4G2 (F(ab)²/FcyRIIIb) antibody for blocking nonspecific binding. After that, 15 to 20 μL properly diluted antibody was added for a 20-minute incubation at room temperature under gentle agitation. Cells were fixed with 1% paraformaldehyde (PFA; Sigma) in PBS, and data were acquired with a FACScalibur (Becton Dickinson) and analyzed using CellQuest 3.3 (Becton Dickinson) or were acquired with an Epics Elite (Beckman Coulter; Reissy, France) and analyzed with FlowJo (TreeStar, San Carlos, CA).

Results

Generation and titration of CD4-cell–specific lentiviral vectors

Two lentiviral vectors with CD4-cell–specific promoters were designed (Figure 1). The LvCD4 and the LvCD4-Sil vectors were derived from LvPGK, a lentiviral vector expressing eGFP under the control of the ubiquitous phosphoglucomutase (PGK) promoter. The silencing element of the CD4 gene was added in the previously described 22 4pme transcriptional cassette to generate regulatory sequences specific for CD4-expressing cells. The minimal promoter of the CD4 gene with or without the silencer, the transgene eGFP, and the Wpre sequences were then inserted between the Clai and Asp718 sites of the LvPGK plasmid to generate the LvCD4 and LvCD4-Sil vectors expressing eGFP as a reporter gene. Infectious lentiviral vectors were titered by measuring the percentages of eGFP⁺ in infected CEMx174 T cells. As shown in Table 1, titers of CD4-cell–specific lentiviral vectors exceeded 10⁶ ip/mL. When lentiviral supernatants were concentrated by ultrafiltration, titers increased by 12- to 216-fold, in some cases exceeding 10⁸ ip/mL (Table 1).

In vivo reconstitution of RAG-2–deficient mice with HSCs transduced with eGFP-expressing cell-specific lentiviral vectors

To evaluate CD4 specificity on newly generated cells in vivo, Lin–Sca-1⁺ cells transduced with lentiviral vectors were injected into RAG-2–deficient mice. These mice are deprived of T and B cellsbecause of defective genetic rearrangements at the T-cell receptor (TCR) and B-cell receptor (BCR) loci,18 but they are fully able to support T- and B-cell differentiation from healthy mouse bone marrow.24 Furthermore, we chose RAG-2 KO mice as recipient mice to optimize the detection of eGFP⁺ cells in donor-derived T cells (earlier experiments in normal mice showed varying levels of engraftment that significantly impaired our ability to reliably evaluate the proportions of eGFP⁺ cells). Reporter gene

Figure 1. Schematic representation of the gene transfer vectors. Lentiviral vectors LvCD4 (A) and LvCD4-Sil (B). SD indicates splice donor; SA, splice acceptor; 5'- packaging signal: GA-RRE, truncated gag sequence with the rev-responsive element; cppT (central polypurine tract of HIV); C4pme, human CD4 minimal promoter/murine enhancer cassette (590 bp); Sil, human CD4 silencer (484 bp); Wpre, posttranscriptional cis-acting regulatory element of the woodchuck hepatitis virus (587 bp); LTR-SIN, self-inactivating 3' LTR (deleted of 400 bp in the U3 region). Enzymatic sites used for cloning the minimal promoter are indicated together with the eGFP and the Wpre (Clai/Asp718).
expression was quantified in secondary lymphoid organs, thymus, and bone marrow 2 months after reconstitution. At this time, the proportion and absolute numbers of T and B cells in the spleen, lymph nodes, thymus, and bone marrow were similar to control C57Bl/6 mice (not shown). This indicated that the transduction protocol used herein did not alter the stem cell competency of Lin−Sca-1+ cells. All mice analyzed were reconstituted with a sizable population of eGFP+ cells, ranging from 4% to 30% of CD45.1+ (donor-derived) cells in the spleen.

Specifcity of eGFP expression in spleen and lymph nodes of RAG-2−deficient mice reconstituted with transduced HSCs

Lymph nodes (LNs) and spleens of reconstituted animals were studied to assess the specificity of transgene expression conferred by the CD4 regulatory sequences. Results of a representative experiment showing eGFP expression in CD4+ and CD8+ T cells and in B220+ B cells are shown in Figure 2A. With the LvPGK vector, reporter gene expression was readily detectable in all subsets. With the LvCD4 vector, B cells expressed little eGFP whereas CD4+ and CD8+ T cells exhibited similar proportions of transduced cells. In contrast, with the LvCD4-Sil vector, eGFP expression was dramatically reduced in frequency and in intensity in CD8+ T cells compared with CD4+ T cells. In these experiments, 2 peaks of eGFP+ cells could sometimes be observed with either vector tested.

Although CD4+ T cells are the major cell subset expressing the CD4 molecule in mice, we also analyzed the specificity of our vectors in NK1.1+ natural killer (NK) cells and CD4+CD11c+ splenic DCs. To evaluate cell specificity independently of the variability of HSC transduction efficiencies, we present our results as an index relative to the frequency of eGFP+ CD4+ T cells (Figure 2B). However, it should be stressed that this index only partially reflects cell specificity because it does not confer the intensity of expression in positive cells. With the LvPGK vector, indexes were increased by an approximate factor of 2 in B cells, NK cells, and DCs, but they were comparable in CD8+ T cells. With the LvCD4 vector, the indexes were similar in CD8+ T cells, markedly reduced in B cells and DCs, and moderately reduced in NK cells. Compared with the LvCD4 vector, reporter gene expression was efficiently silenced with the LvCD4-Sil vector in CD8+ T cells, as evidenced by an index reduced by a factor of 4. When 2 peaks of eGFP+ cells could be observed in CD4+ T cells with the LvCD4-Sil vector, the strongest peak was efficiently silenced whereas the weakest peak appeared not to be, which called into question its specificity (not shown). These specificity index variations were correlated with variations in the intensity of eGFP expression in positive cells (Figure 2A). Altogether, CD4+ T cells appeared as the main lymphocyte subset expressing eGFP after in vivo maturation of Lin−Sca-1+ cells transduced with the LvCD4-Sil vector.

Specifcity of eGFP expression in the thymus of RAG-2−deficient mice reconstituted with transduced HSCs

We next sought to analyze at which stage of T-cell differentiation in the thymus the transgene would be expressed. Representations of immature CD4−CD8− double-negative (DN) and CD4+CD8+ double-positive (DP) cells and of mature CD4−CD8− and CD4+CD8− single-positive (SP) cells in eGFP+ and eGFP− thymocytes are illustrated from a representative experiment in Figure 3A for the LvCD4 vector and are summarized in Figure 3B. With the LvPGK vector, the proportions of DN, DP, CD4-SP, and CD8-SP were similar in eGFP+ and eGFP− cells, in agreement with the ubiquitous nature of this promoter. In contrast, compared with eGFP− cells, there was an increased proportion of SP cells and a decreased proportion of DP cells among the eGFP+ cells with the LvCD4 vector. Moreover, eGFP expression was clearly correlated with thymocyte maturation as indicated by the analysis of CD3 expression, which is absent or low in immature thymocytes and high in mature thymocytes. With LvCD4, almost all eGFP+ cells expressed high levels of CD3 (Figure 3A, inset); with the LvPGK vector, there was an even distribution of CD3-expressing cells among the eGFP+ and eGFP− cells (not shown). This shows that eGFP expression in DP cells occurs at a late stage of their

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**Table 1. Lentiviral vector titers and concentrations**

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<th>Vector</th>
<th>Concentration (range)</th>
<th>Fold enrichment (range)</th>
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<tr>
<td>LvPGK, n = 3</td>
<td>10⁶ ± 4.9 × 10⁵</td>
<td>57.6 (18-109)</td>
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<tr>
<td>LvCD4, n = 4</td>
<td>1.8 × 10⁹ ± 1.2 × 10⁹</td>
<td>44.6 (18-105)</td>
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<tr>
<td>LvCD4-Sil, n = 4</td>
<td>1.8 × 10⁹ ± 1.5 × 10⁹</td>
<td>70 (12-216)</td>
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Lentiviral vectors were produced by cotransfection of 293T cells with 5 μg VSV-G envelope plasmid, 10 μg packaging plasmid pCMV - R8.91, and 10 μg transfer vectors. The table shows the comparison in titers of concentrated versus nonconcentrated lentiviral vectors supernatants on CEM × 174 cells. Titers of lentiviral vectors (ip/mL) ± SD were calculated as described in “Materials and methods.” Enrichment in viral titers was calculated as the ratio between lentiviral titers before and concentration (range).
TER119/H11001

TER-119/H11001

fi

LvCD4-Silentiviral vectors. (C) Distribution of thymocytes transduced with the small cells in the bone marrow of the same animals.

the LvCD4 vector. Electronic gates were set around CD45.1 subsets was detected with the LvCD4 vector (Figure 4A). Despite (Figure 4A). In contrast, no significant expression of eGFP in these populations showed the same patterns of expression as those present in spleen and LNs (data not shown). To assess whether immature cells of the monocytic and erythroid lineages and of granulocytes expressed the transgene, reporter gene expression was studied in Mac-1−, TER119+ and Gr-1+ cells. Expression of the reporter gene with the LvPGK vector was readily detectable in 11 of 50 colonies tested for each of the LvCD4 and LvCD4-Sil vectors. In contrast, eGFP-expressing cells were readable detectable in the LvPGK vector in 11 of 50 BFU-E or CFU-GM colonies. Together these results indicated that the regulatory sequences of the CD4 gene were poorly expressed in immature cells of the bone marrow in vitro and in vivo.

Specificity of eGFP expression in ex vivo–transduced human lymphocytes

To assess whether silencing of the reporter gene would also be successful in human lymphocytes, PHA- or IL-7–activated human lymphocytes were transduced in vitro with the LvCD4 or LvCD4-Sil vectors. As shown in Figure 5, transduction with the LvCD4 vector resulted in similar proportions of eGFP+ cells in PHA-activated CD4+ and CD8+ subsets. In contrast, with the LvCD4-Sil vector, there was a 6-fold reduction in the proportion of eGFP+ CD8− cells compared with eGFP+ CD4+ cells. Furthermore, this decrease in the frequency of eGFP+ cells was accompanied by marked reductions in the intensity of eGFP expression in the remaining eGFP+ CD8− cells. Similar results were obtained with T cells from 3 different donors, yielding the following results in CD4+ and CD8+ T cells, respectively: 75.1% ± 12.2% versus 68.1% ± 8.1% for the LvCD4 vector and 63.3% ± 21.1% versus 18.2% ± 10.6% for the LvCD4-Sil vector. When IL-7–treated T cells were infected, a higher proportion of CD4+ cells than CD8+ T cells were transduced with the LvCD4 vector. With the LvCD4-Sil vector, CD4+ T-cell–specificity was evidenced by the concomitant reduction in the proportion and the intensity of eGFP staining. It should be noted that we did not observe the 2 peaks of fluorescence intensity (Figure 2A) in transduced human lymphocytes (Figure 5). The silencing element of the CD4 gene used herein allowed efficient repression of the transgene in PHA- and IL-7–activated human CD8+ T cells.

Figure 3. Thymic distribution of eGFP-expressing cells in reconstituted RAG-2 KO mice. (A) Typical distribution of donor-derived eGFP+ cells in the thymus of RAG-2 KO mice 2 months after engraftment with Lin− Sca-1+ cells transduced with the LvCD4 vector. Electronic gates were set around CD45.1 eGFP+ and CD45.1 eGFP− cells (left panel). Distribution of CD4/CD8-expressing thymocytes was then analyzed in eGFP+ and eGFP− cells. Histogram in the inset shows CD3 expression in total thymocytes (solid line), in total DP cells (dotted line), and in eGFP+ cells (bold line). (B) Summary of the results obtained as in panel A for nonspecific and T- or CD4-specific lentiviral vectors; areas represent the percentages of DN, DP, CD4 SP, and CD8 SP cells in eGFP+ or eGFP− cells with the LvPGK, LvCD4, and LvCD4-Sil lentiviral vectors. (C) Distribution of thymocytes transduced with the CD4-specific lentiviral vector LvCD4-Sil in eGFP+ or eGFP− cells analyzed as in panel A.

Figure 4. Bone marrow analysis of reconstituted RAG-2 KO mice. (A) eGFP expression on high-scatter large cells in the bone marrow of RAG-2 KO reconstituted animals with LvPGK (upper panels) and LvCD4 vectors (lower panels). Mac-1−, TER-119−, and Gr-1− cells were labeled with rat antirat antibodies followed by goat antimouse PE-labeled antibody. (B) CD45.1 and eGFP expression on low-scatter small cells in the bone marrow of the same animals.

Figure 5. eGFP expression in human CD4+ and CD8+ T cells transduced with the LvCD4 and LvCD4-Sil vectors in vitro. PHA- or IL-7–activated total human lymphocytes were transduced with the LvCD4 (upper panels) and the LvCD4-Sil (lower panels) vectors as described in “Materials and methods.” Cells were stained with monoclonal antibodies against human CD4 and CD8 and were analyzed by 3-color flow cytometry. Shown are the typical profiles of eGFP expression 4 days after transduction in gated CD4+ (left panels) and CD8+ (right panels) T cells. MFI indicates mean fluorescence intensity of eGFP+ cells. Dotted lines represent background of eGFP expression in nontransduced similar subsets of lymphocytes.

differentiation. Noteworthy, with the LvCD4-Sil vector, more than 75% of eGFP+ thymocytes were CD4+SP cells (Figure 3B), and there was a 5-fold reduction in the frequency of DP cells among eGFP+ cells compared with eGFP− cells (Figure 3B). As for LvCD4, eGFP expression in the thymus was only achieved in cells expressing high levels of CD3 (not shown). Approximately 7% of CD8-SP cells expressed the reporter gene in the thymus with the LvCD4-Sil vector, but it should be noted that levels were low (Figure 3C).

Specificity of eGFP expression in the bone marrow of RAG-2–deficient mice reconstituted with transduced HSCs

We assessed eGFP expression in the bone marrow of RAG-2 KO mice reconstituted with HSCs transduced with the lentiviral vectors. In mature T cells in the bone marrow, eGFP expression showed the same patterns of expression as those present in spleen and lymph nodes (data not shown). To assess whether immature cells of the monocytic and erythroid lineages and of granulocytes expressed the transgene, reporter gene expression was studied in Mac-1−, TER119+ and Gr-1+ cells. Expression of the reporter gene with the LvPGK vector was readily detectable in all these populations (Figure 4A). In contrast, no significant expression of eGFP in these subsets was detected with the LvCD4 vector (Figure 4A). Despite this lack of expression, there was a distinct population of eGFP+ cells within the CD45.1+ cells in the same mice (Figure 4B). Similar results were obtained with the LvCD4-Sil vector (not shown). It should be noted that eGFP+ cells with the LvPGK vector were found in CD45.1+ and CD45.1− cells within the small cells of the bone marrow (Figure 4B).

We also studied the specificity of expression in individual erythroid burst-forming unit (BFU-E) and granulocyte macrophage–colony-forming unit (CFU-GM) colonies derived in vitro from bone marrow of reconstituted RAG-2 KO mice. No eGFP expression could be detected in more than 50 colonies tested for each of the LvCD4 and LvCD4-Sil vectors. In contrast, eGFP-expressing cells were readily detectable with the LvPGK vector in 11 of 50 BFU-E or CFU-GM colonies. Together these results indicated that the regulatory sequences of the CD4 gene were poorly expressed in immature cells of the bone marrow in vitro and in vivo.
Table 2. Lentiviral transduction of non-T CD4+ cells

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Frequencies of eGFP-expressing cells (± SD) within dendritic cells (HLA-DR−CD4+) and macrophages (CD14+CD4+) on transduction with the indicated lentiviral vectors. Non-T-cell subsets were derived in vitro from transduced CD34+ hematopoietic precursors and analyzed for eGFP expression at day 12 of culture.

Specificity of eGFP expression in ex vivo non–T CD4+ cells derived from transduced human CD34+ hematopoietic precursors

We next sought to investigate whether expression of the reporter gene would be specific to non-T CD4+ cells. For this, we transduced G-CSF–mobilized CD34+ cells (more than 95% purity) with the different lentiviral vectors and induced them to differentiate in vitro into macrophages or dendritic cells. We focused the analysis of eGFP expression to cells that coexpressed CD4 and the phenotypic markers HLA-DR and CD14, phenotypes characteristic of dendritic cells and macrophages, respectively.25 At day 12 after infection, CD4+ cells represented more than 80% of HLA-DR+ or CD14+ cells (not shown). There were no significant differences in the frequencies of eGFP+ cells between the LvPGK and the specific vectors, although the frequency of eGFP+ cells tended to be lower with the LvCD4-Sil vector, irrespective of the cell type (Table 2). The intensity of eGFP expression with the specific vectors were reduced by a factor of at least 3 compared with the LvPGK vector and only represented minor shifts from negative controls, especially with the LvCD4-Sil vector (Figure 6). We conclude that the specific vectors were expressed in non–T CD4+ cells in these culture conditions at levels well below mature T cells (Figure 5). In marked contrast to CD8+CD4− T cells, non–T CD4− cells could also express the transgene (not shown), indicating a lack of specificity in these culture conditions.

Discussion

The present report establishes for the first time that CD4+–cell–specific expression of a transgene can be obtained by using regulatory sequences of the CD4 gene in the context of optimized lentiviral vectors. On reconstitution of RAG–2–deficient mice with transduced HSCs, the CD4+–cell–specific vector was efficiently expressed in T cells, not expressed in immature cells of the bone marrow, and minimally expressed in B cells. The addition of the CD4 silencing element in the LvCD4-Sil vector led to further restriction of expression to CD4+ T cells in the thymus, spleen, lymph nodes, and bone marrow of reconstituted animals. The LvCD4 vector was also expressed, but to a lower extent, in CD4+CD11c dendritic cells and NK1.1+ cells. For the latter cells, this could be attributed to high levels of Ets-1,26 a transcription factor that plays a role in CD4 promoter activation.6 Alternatively, it is possible that the NK1.1+ cells expressing the transgene are actually NKT cells, some of which express the CD4 molecule.27 Unfortunately, there were too few of these cells in reconstituted animals to reliably analyze eGFP expression in that subset. Noteworthy, eGFP expression from the LvCD4-Sil vector was reduced in CD4+ splenic dendritic cells and in NK1.1+ cells. This indicates that the effects of the positive and negative CD4 regulatory sequences are different in the context of lentiviral vectors or of the endogenous CD4 gene. As evidenced by the combined frequency and intensity of transgene expression, the specificity achieved herein was comparable to that obtained with erythroid or major histocompatibility complex class II+ cell–specific promoters.28,29 We also found that expression of eGFP in vivo was sometimes divided into 2 or more discrete peaks of fluorescence intensity, independent of the lentiviral vector specificity. When this was the case for the LvCD4-Sil vector, the peak of weaker intensity appeared less sensitive to silencing than the stronger one, questioning its specificity (not shown). The origin of this phenomenon is unclear, but it should be noted that a similar phenomenon has been seen with other lentiviral vectors carrying internal promoters and eGFP as a reporter gene.28,29 Our results, however, emphasize the good level of specificity that can be achieved with internal enhancers/promoters in lentiviral vectors. They are also the first demonstration that a silencer can be functional in the context of lentiviral vectors. Epigenetic silencing of CD4 has been proposed as a mechanism for the repression of cell surface CD4 expression in mature CD8+ T cells.30 Deletion of the silencer in mature CD8+ T cells using Cre recombinase was not accompanied by the reappearance of cell surface CD4 expression.30 Our results on human mature T cells show that eGFP expression was efficiently repressed on infection with the LvCD4-Sil vector carrying an exogenous silencer. This result indicates that the silencer–associated factors responsible for its function are operative in this subset. Therefore, the regulation of CD4 expression in mature CD8+ T cells may be tightly controlled by genetic and epigenetic mechanisms.

Detailed analysis of transgene expression during T-cell development in the thymus is important in view of the potential use of these vectors for the treatment of genetic immunodeficiencies that often result from blocks in T-cell development at distinct stages of their differentiation. With the LvCD4 vector, similar transgene expression was observed in CD4+ and CD8+ SP cells. The transgene was also expressed in the more mature fraction of the DP thymocytes—those expressing intermediate or high levels of CD3—a feature typical of cells that have undergone positive selection in the thymus.32 This expression pattern could not be predicted from the results observed in transgenic mice expressing the transgene from promoter/enhancer sequences of the CD4 gene.7 In these mice, the transgene was not expressed on DP thymocytes, and it was expressed only in the more mature fraction of the SP thymocytes. These expression discrepancies might have resulted from the differences in the promoter/enhancer elements used, a 600-bp engineered CD4 cassette for the LvCD4 vector compared with a natural 1.1-kbp sequence in transgenic mice. Transgene expression...
in DP cells in vivo with the LvCD4 vector is, however, in agreement with previous observations made on cell lines establishing that the minimal promoter included all elements necessary for transgene expression in DP cells. With the LvCD4-Sil vector, DP cells and a significant fraction of the CD8+ SP thymocytes still expressed the transgene. However, most of these cells expressed eGFP at low levels. The minor fraction of the CD8+ thymocytes expressing high eGFP levels, suggesting that they have escaped silencing, were not found at the periphery. We suggest that this persistent eGFP detection does not correspond to synthesis but does correspond to the long half-life of eGFP compared with the CD4 molecule. This could also explain the 2 peaks of fluorescence intensity observed in peripheral T cells. Use of another reporter gene in the LvCD4 and LvCD4-Sil vectors will help to resolve these issues.

We show that the LvCD4 and the LvCD4-Sil vectors were not expressed in the most immature thymocytes or in immature cells of the bone marrow. Similarly, expression was low to undetectable in murine splenic dendritic cells or monocytes. In contrast, our results on human cells show that non-T dendritic and macrophage cells consistently expressed the transgene with the specific vectors. Murine monocytes or splenic dendritic cells expressed no to low levels of the CD4 molecule, whereas human CD14+ and HLA-DR+ cells were uniformly CD4+. Differential levels of endogenous CD4 expression in mice and human cells seem sufficient to explain the results.

Of note is that high expression of eGFP was detected on mature CD4+ T cells with the LvCD4-Sil vector. In contrast, low levels of eGFP were detected on non–T CD4+ cells, and this correlated with lower levels of CD4 expression. This would suggest that the intensity of eGFP expression with the specific vectors parallels faithfully the endogenous CD4 transcriptional control. However, significant eGFP expression could be detected in CD4+ non–T cells derived from CD4+ cells, in contrast to the more physiological model of HSC differentiation in mice or to the lack of expression on human mature CD8+ T cells. Primate CD4+ cells and so-called lineage-expressing cells may express low levels of the CD4 molecule, and that may depend on the donor.

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