HUMAN GENE THERAPY 15:733–748 (August 2004)
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Making Dendritic Cells from the Inside Out: Lentiviral Vector-Mediated Gene Delivery of Granulocyte-Macrophage Colony-Stimulating Factor and Interleukin 4 into CD14$^+$ Monocytes Generates Dendritic Cells *In Vitro*

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ABSTRACT

We have evaluated a one-hit lentiviral transduction approach to genetically modifying monocytes in order to promote autocrine and paracrine production of factors required for their differentiation into immature dendritic cells (DCs). High-titer third-generation self-inactivating lentiviral vectors expressing granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin 4 (IL-4) efficiently achieved simultaneous and persistent codelivery of the transgenes into purified human CD14$^+$ monocytes. Coexpression of GM-CSF and IL-4 in CD14$^+$ cells was sufficient to induce their differentiation into a DC-like phenotype, as evidenced by their morphology, immature immunophenotypic profile (CD14$^-$, CD1a$^+$, CD80$^+$, CD86$^+$, MHC-I$^+$, MHC-II$^+$), and their ability to further develop into a mature phenotype (CD83$^+$) on further treatment with soluble CD40 ligand. Mixed lymphocyte reactions showed that the T cell-stimulating activity of lentivirus-modified DCs was superior to that of DCs grown by conventional methods. Lentivirus-modified DCs displayed efficient antigen-specific, MHC class I-restricted stimulation of autologous CD8$^+$ T cells, as shown by IFN-γ production and CTL assays. DCs coexpressing GM-CSF and IL-4 could be kept metabolically active and viable in culture for 14 days in the absence of exogenously added growth factors, unlike conventionally produced DCs. Coexpression of FLT3 ligand did not improve the viability, expansion, or immunologic performance of lentivirus-modified DCs. This article demonstrates the proof-of-concept to genetically convert monocytes to DC-type antigen-presenting cells with lentiviral vectors.

OVERVIEW SUMMARY

This work demonstrates the proof-of-concept that the one-hit lentiviral cotransduction approach efficiently modifies monocytes to promote the production of factors required for their autonomous differentiation into immature dendritic cells (DCs). We show that cells with typical morphology and immunophenotype of DCs were successfully produced after gene cotransfer of granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin 4 (IL-4) with high-titer self-inactivating lentiviral vectors into purified human peripheral blood monocytes. We demonstrate that the genetically programmed DCs have full antigen presentation capability. In addition, lentivirus-programmed DCs were more viable *in vitro* than conventionally generated DCs, persisting for 2–3 weeks in culture in the absence of exogenous growth factors. Important from the clinical perspective, we showed that 7–10 days of *ex vivo* culture required by the conventional methodology could be substituted by 1 day of transduction. Therefore, this advance in the genetic manipulation of DC precursors will certainly offer a novel and simpler strategy to engineer DC vaccines for a broad range of diseases.

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INTRODUCTION

The induction of an adaptive, antigen-specific immune response requires the activation of T lymphocytes by antigen-presenting cells (APCs). Dendritic cells (DCs) are the most potent "professional" APCs in the immune system, with multiple characteristics contributing to this capability, including efficient molecular machinery for antigen uptake, processing, and presentation of both exogenous and endogenous molecules. Mature DCs express high levels of MHC class I and II molecules as well as costimulatory molecules such as CD40, CD80, and CD86 (Sallusto and Lanzavecchia, 1994; Schuler and Steinman, 1997; Banchereau and Steinman, 1998) that are prerequisites for their potent T cell-stimulating capacity. DCs reside in peripheral tissues in an immature state for optimal antigen uptake and, in response to inflammatory signals, they mature and migrate to secondary lymphoid organs (such as the spleen and lymph nodes) to activate antigen-specific T cells. The majority of DCs, defined as myeloid-derived DCs, originate in the bone marrow from common myeloid cell precursors, and are widely distributed in both lymphoid and nonlymphoid tissues. Myeloid DC precursors derived from peripheral blood, bone marrow, or cord blood can be manipulated to differentiate *in vitro* and used as adjuvants for immunization after loading them with pathogen- or cancer-specific antigenic peptides, protein, cDNA, RNA, or cell extracts (for a review, see Ribas *et al*., 2003).

$CD14^+$ monocytes are a naturally abundant cell population in the peripheral blood, which is an accessible source for production of DCs. Plastic-adherent peripheral blood monocytes can differentiate into "immature DCs" if a mixture of cytokines is added to the culture (Romani *et al*., 1994; Sallusto and Lanzavecchia, 1994). Hematopoietic growth factors commonly used to generate DCs *in vitro* include granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin 4 (IL-4), and FLT3 ligand (FLT3L). GM-CSF regulates cellular proliferation, differentiation, and upregulation of MHC and costimulatory molecules in myeloid cells through binding to its receptor and is required to generate cells with morphologic characteristics of DCs from mouse bone marrow cultures (Inaba *et al*., 1992). IL-4 interacts with its cognate receptor IL-4R, modulating the functions of diverse cell types. IL-4 engagement on monocytes downregulates CD14, which is a lipopolysaccharide receptor, and blocks the development of macrophages (Heidenreich, 1999). In combination with GM-CSF, IL-4 is required to promote differentiation of mature DCs from human peripheral monocytes (Sallusto and Lanzavecchia, 1994). Furthermore, for mouse DC cultures originated from bone marrow precursors, IL-4 has a minimal effect on DC maturation when GM-CSF is used at high doses (200 U/ml), but the inclusion of IL-4 appears to compensate for low doses of GM-CSF (2 U/ml) to promote development into mature DCs (Lutz *et al*., 2000). FLT3L interacts with the FLT3 receptor to stimulate a variety of hematopoietic lineages, including primitive hematopoietic cells, dendritic cells, and B cells. Culture of $CD34^+$ hematopoietic progenitors in a mixture of growth factors with addition of FLT3L leads to higher DC production (Siena *et al*., 1995), presumably by sustaining the long-term expansion of primitive $CD34^+DR^-$ DC precursors (Curti *et al*., 2001). There is an established antitumor activity of FLT3L, suggesting that this protein plays a major role in activating the immune system via its ability to stimulate the production of dendritic cells (Maraskovsky *et al*., 1996). Monocytes express the FLT3 receptor (Visser *et al*., 1996) and it was shown that when FLT3L is added to monocyte-derived DC cultures there is an increase in the DC population compared with cultures with GM-CSF and IL-4 alone (Hubert *et al*., 1998).

A major limitation to the production of dendritic cell-based vaccines is the need for 1 to 2 weeks of *ex vivo* culture in order to generate patient-customized vaccines, with the high cost of specialized facilities and personnel. Furthermore, the yield of DCs derived in the presence of GM-CSF and IL-4 cannot be expanded beyond the number of initial monocytes, because they do not proliferate on differentiation (Cavanagh *et al*., 1998). Therefore, the viability of monocytes during such extended culture periods remains a critical issue. The successful immunization potential of DCs is further complicated by the fact that the myeloid-related DC subset turns over rapidly, in about 10 days *in vivo*, with a $t_{1/2}$ of only 2 days in lymph nodes, as determined by continuous bromodeoxyuridine (BrdU)-labeling experiments (Ruedl *et al*., 2000). Thus, it has been shown in both mice and humans that the vast majority of *ex vivo*-derived DCs (95–99%) administered subcutaneously fail to migrate from vaccination sites to regional lymph nodes or to the spleen, as determined by monitoring the activity of ^{111}In-labeled DCs 48 hr postinjection (Eggert *et al*., 1999; Morse *et al*., 1999). The viability of *ex vivo*-produced DCs is particularly critical after they are administered *in vivo*, because once removed from culture they lack the supraphysiological levels of growth factors to maintain their phenotype and function. Therefore, methods to facilitate the generation, and prolong the life span, of *ex vivo*-derived myeloid DCs are warranted. We proposed to devise a simplified method to genetically engineer monocytes for autocrine and paracrine production of growth factors required for their eventual differentiation and maintenance *in vivo*.

We have previously shown that second-generation lentiviral vectors, pseudotyped with the vesicular stomatitis virus envelope glycoprotein (VSV-G), efficiently transduce human hematopoietic progenitor and leukemia cells (Case *et al*., 1999; Stripecke *et al*., 2000). In addition to their efficient infectivity of human hematopoietic cells, later generations of lentiviral vectors do not generate cytopathic or cytotoxic effects per se, and only the transgenes integrate stably into the genome to provide long-lasting expression. To demonstrate improved safety and performance, we demonstrated the efficient transduction of acute myeloid leukemia cells by third-generation self-inactivating lentiviral vectors (Koya *et al*., 2002), and more recently showed the applicability of these vectors to provide simultaneous delivery of four genes (GM-CSF, CD80, IL-4, and CD40 ligand [CD40L]) into acute myelogenous leukemia (AML) cells in order to trigger their differentiation into AML-APCs (Stripecke *et al*., 2003). It has been demonstrated that lentiviral vectors transduce immature and mature DCs efficiently and stably (Gruber *et al*., 2000; Schroers *et al*., 2000; Dyall *et al*., 2001; Koya *et al*., 2003). Transduction of DCs with lentiviral vectors does not alter DC viability or immunophenotype, or their ability to differentiate into mature DCs capable of stimulating autologous T cell responses (Gruber *et al*., 2000). We evaluated the ability of lentiviral vectors to deliver the CD40L gene into immature monocyte-derived human DCs and showed that autocrine production of CD40L by the DCs correlated with a potent maturation phenotype and augmented $CD8^+$ T cell stimulation (Koya *et al*., 2003).

We here demonstrate the "proof-of-concept" of promoting the differentiation of monocytes into immature DCs through direct lentiviral vector-mediated constitutive coexpression of GM-CSF, IL-4, and FLT3L, which was evaluated *ex vivo* by analysis of phenotype, function, and cell viability.

MATERIALS AND METHODS

Cell culture

293T and T2 cell lines were obtained from the American Type Tissue Collection (ATCC, Manassas, VA).

CD14$^+$ selection and culture

Peripheral blood mononuclear cells were obtained from HLA-A2.1-positive melanoma patients undergoing immunotherapy treatment and studies were performed in accordance with protocols approved by the Los Angeles County/University of Southern California (Los Angeles, CA) Institutional Review Board. Mononuclear cells were separated by density gradient centrifugation, and cryopreserved in 10% dimethyl sulfoxide (DMSO; Sigma, St. Louis, MO) and 90% human AB serum (Omega Scientific, Tarzana, CA). Immediately before use, frozen cells were thawed at 37°C and washed with phosphate-buffered saline (PBS). CD14$^+$ cells were obtained by positive immunomagnetic cell selection (Miltenyi Biotec, Auburn, CA). Up to $3–4 \times 10^6$ cells were placed in the wells of a sterile 6-well plate containing 2 ml of serum-free, clinical-grade X-VIVO 15 medium (Cambrex Bio Science Walkersville, Walkersville, MD) plus human IL-4 (80 ng/ml) and GM-CSF (80 ng/ml) (R&D Systems, Minneapolis, MN). The plates were incubated at 37°C with 5% CO_2 for 8 hr before transduction. When applicable, soluble trimeric CD40L (R&D Systems) was added at 500 ng/ml on day 7 post-transduction.

Construction of LV-GMCSF, LV-IL4, and LV-FLT3L vectors

The backbone vector used for insertion of the immunomodulatory genes consisted of a derivative of the pRRL-sin.hCMV-GFP-pre (LV-GFP) construct (Zufferey *et al*., 1998), from which the green fluorescent protein (GFP) cDNA was deleted by *Xba*I–*Sal*I digestion and replaced with a polylinker containing multiple cloning site (MCS) CTAGAACTAGTGGATCCCCCGGGCTGCAGGAATTCGATATCAAGCTTATCGATACCG, resulting in the vector pRRL-sin.hCMV-MCS-pre. The *Eco*RI site present in the backbone of the original pRRL-sin.hCMV-GFP-pre construct was eliminated by a point mutation introduced by polymerase chain reaction (PCR), resulting in the backbone vector pRRL-sin.hCMV-MCS-pre ΔEco (Koya *et al*., 2002). For construction of LV-GMCSF, a DNA fragment obtained by *Eco*RI digestion of pHR-GM-CSF was introduced into the *Eco*RI site present in the multiple cloning site of the vector (Koya *et al*., 2002). For construction of LV-IL4 a 470-bp *Xba*I–*Bam*HI fragment from the plasmid pNGVL3-hIL4 (National Gene Vector Laboratories Vector Production Facility, Indiana University, Indianapolis, IN) was inserted into the backbone vector pRRL-sin.hCMV-MCS-pre ΔEco digested with *Xba*I and *Bam*HI (Stripecke *et al*., 2003). For construction of LV-FLT3L, the plasmid pNGVL3-hFLex, which carries the extracellular domain (secreted form) of the human FLT3L gene, was obtained (National Gene Vector Laboratories, Vector Production Facility) and digested with *Hin*dII. The DNA was blunt-ended and digested with *Bam*HI, and the 600-bp *Hin*dII–*Bam*HI fragment encoding a soluble form of FLT3L was ligated with the backbone vector pRRL-sin.hCMV-MCS-pre ΔEco digested with *Xba*I, blunt-ended, and further digested with *Bam*HI. The structural integrity of all constructs was reconfirmed by restriction digestion and sequencing analysis.

Production of lentivirus

Constructs required for the packaging of third-generation self-inactivating lentiviral vectors consisted of the plasmid pMD.G (for production of the VSV-G viral envelope) and pMDLg/pRRE and RSV-REV (for expression of the structural proteins, enzymes, and regulatory proteins of human immunodeficiency virus [HIV]) (Dull *et al*., 1998), and were kindly provided by L. Naldini (San Raffaele Telethon Institute for Gene Therapy, Milan, Italy). Lentiviral vectors were produced in large scale by transient cotransfection of 293T cells with the transfer vector and packaging plasmid constructs as described (Stripecke *et al*., 2003). Viral supernatants, collected 48 and 72 hr posttransfection, were concentrated by ultracentrifugation and viral aliquots were cryopreserved at –80°C until use. Viral titer was determined by assessing viral p24 antigen concentration by enzyme-linked immunosorbent assay (ELISA) (Coulter Immunotech, Miami, FL), and hereafter expressed as micrograms of p24 equivalent units per milliliter. One microgram of p24 per milliliter measured in the preparation corresponds to approximately 10^7 GFP transduction units/ml, as assessed by titration in 293T cells.

Lentivirus-mediated gene transfer

Five micrograms of p24 equivalent per milliliter was added per well of a six-well plate containing purified monocytes. Protamine sulfate was added at a final concentration of 5 μg/ml and the transduction plates were incubated at 37°C in 5% CO_2 for 12–16 hr. The monocytes were extensively washed twice with X-VIVO 15 medium, and maintained in the presence or absence of human IL-4 (80 ng/ml) and GM-CSF (80 ng/ml). At various time points, supernatant was collected for ELISA and cells were harvested for fluorescence-activated cell-sorting (FACS) analysis or other assays.

FACS analysis

Transduced and control cells were detached from the wells by incubation in PBS without Mg^{2+} and Ca^{2+} for 30 min at 37°C in 5% CO_2, followed by resuspension. Cells were collected and washed once with PBS, incubated with PBS containing mouse IgG (50 μg/ml; Sigma) for 15 min on ice, stained with the corresponding monoclonal antibody for 20 min, washed, and resuspended in 100 μl of 1% paraformaldehyde for fixation. Monoclonal antibodies reactive against CD86, and respective isotype antibodies, were conjugated with fluorescein isothiocyanate (FITC); monoclonal antibodies reactive against CD83, CD1a, CD80, and MHC-II (HLA-DR), and respective isotype antibodies, were conjugated with phycoerythrin; antibodies reactive against MHC-I (HLA-ABC) and CD14, and respective isotype antibodies, were conjugated with peridinin

chlorophyll protein (PerCP; BD Biosciences Pharmingen, San Diego, CA). Flow cytometric analysis was performed with a FACSCalibur cytometer equipped with a 488-nm argon laser (BD Biosciences Immunocytometry Systems, San Jose, CA). To establish background for fluorescence and to set gates for data acquisition, staining with isotype antibodies was performed. Cells present in the granulocyte/monocyte gate based on forward and side scatter characteristics were analyzed.

Analysis of secreted GM-CSF, IL-4, and FLT3L

Production of soluble cytokines was analyzed by ELISA (R&D Systems) according to the manufacturer's instructions. The minimum detectable with this assay is typically less than 3 pg/ml.

T cell enrichment

Mononuclear cell suspensions prepared from peripheral blood were incubated with a cocktail of monoclonal antibodies and a magnetic colloid to enrich total human T cells by negative selection, using reagents and instructions provided by the manufacturer (StemCell Technologies, Vancouver, BC, Canada). Separation of the desired population of cells was performed by passing the prebound cells through an LS magnetic separation column (Miltenyi Biotec). When applicable, peripheral blood mononuclear cells (PBMCs) were thawed and incubated with DNase (0.1 mg/ml; StemCell Technologies) for 15 min at room temperature, before selection.

Mixed lymphocyte reactions

Irradiated (3200 cGy) nontransduced or transduced monocytes were used as stimulators. Stimulators (1×10^5 cells) were cocultured at various ratios with allogeneic T cells in 96-well round-bottom plates in 200 μl of medium and incubated for 6 days at 37°C in a 5% CO_2 incubator. All mixed lymphocyte reaction (MLR) cultures were carried out in RPMI 1640 supplemented with 5% heat-inactivated human AB serum. All microcultures were performed in triplicate. Cells were pulsed with 1 μCi of [^{3}H]thymidine (PerkinElmer Life and Analytical Sciences, Boston, MA) for the last 18 hr of the culture period, and then harvested onto glass fiber filters. [^{3}H]Thymidine incorporation was measured by liquid scintillation spectrophotometry. The stimulation index (SI) was calculated for each experiment as follows: SI = cpm (T cells + stimulator cells)/cpm (T cells).

Enzyme-linked immunospot

Mononuclear cells (5×10^5 cells per well) were cocultured with day 7 mock-transduced or transduced autologous monocytes (5×10^4 cells per well) treated overnight with sCD40L in the presence of a mixture of immunogenic HLA-A2.1-restricted peptides (Flu-M1 [GILGFVFTL, 10 μg/ml], gp100 210M [IMDQVPFSV, 10 μg/ml], and CMV [$pp65_{495–503}$, NLVPMVATV, 25 μM]), CMV-B7-restricted peptides ($pp65_{265–275}$ [RPHERNGFTVL] and $pp65_{417–426}$ [TPRVTGGGAM], 25 μM), and CMV-B35-restricted peptide ($pp65_{188–195}$ [FPTKDVAL], 25 μM). The mixture of cells was seeded in triplicate wells of Immobilon polyvinylidene difluoride (PVDF) 96-well plates (Millipore, Bedford, MA) coated with anti-interferon γ (IFN-γ) capture antibodies (Mabtech, Stockholm, Sweden) for 24 hr. Detection of spots reflecting IFN-γ production was performed with a biotinylated anti-IFN-γ antibody (Mabtech) followed by reaction with avidin–peroxidase complex (Vector Laboratories, Burlingame, CA) and subsequent bromochloroindolyl phosphate (BCIP)–nitroblue tetrazolium (NBT) substrate color development. Spots were enumerated with the Zeiss (Thornwood, NY) enzyme-linked immunospot (ELISpot) system, which incorporates a Zeiss microscope, a camera, and computer imaging software. As a quality positive control for the ELISpot, PBMCs were incubated with pokeweed mitogen (Sigma).

Generation of cytotoxic T lymphocytes

Seven days after transduction, 1×10^5 control or transduced monocytes were mixed with HLA-A2 antigenic peptides (10 μg/ml each) and incubated overnight with sCD40L (500 ng/ml) and IFN-γ (167 IU/ml). All peptides used were synthesized by the Norris Cancer Center Microchemical Core Facility (University of Southern California) and were HLA-A2.1 restricted. Human papillomavirus (HPV) E7(86–93) peptide (TLGIVCPI) was used as a negative control peptide, whereas Flu-M1(58–66) peptide (GILGFVFTL) was used to detect $CD8^+$ T cell immune responses against influenza virus and gp100 210M(209–217) peptide (IMDQVPFSV) was used to detect $CD8^+$ T cell responses against gp100 melanoma-associated tumor antigen. The next day, the cells were cocultured with 2×10^6 enriched autologous $CD8^+$ T cells (obtained from melanoma patients with the HLA-A2.1 haplotype) in a 24-well plate (Falcon; BD Biosciences Discovery Labware, Bedford, MA) containing RPMI and 2% human serum. Human interleukin 7 (IL-7, 10 ng/ml; R&D Systems) and recombinant human interleukin 2 (IL-2, 10 U/ml; R&D Systems) were added on days 1 and 3 of culture, respectively. On day 7, cytotoxic T lymphocytes (CTLs) were assayed for specific lysis of peptide-pulsed TAP-deficient target cells (T2 cells). T2 target cells were incubated with 10 μg of peptide in 10 ml of medium for 16 hr and washed, and 1×10^6 cells were incubated for 2 hr in the presence of 100 μCi of ^{51}Cr (1 μCi/μl; PerkinElmer Life and Analytical Sciences). The labeled target cells were washed three times in RPMI–2% human serum and plated in triplicate wells at a minimum of 3000 targets per well. Effector cells were added to the target cells at various effector-to-target ratios in 96-well round-bottom plates (Falcon). After incubation for 4 hr at 37°C and 5% CO_2, 100 μl of supernatant was collected from each well and the ^{51}Cr released was measured with a γ counter. Specific lysis was calculated according to the following formula: (test lysis − spontaneous lysis)/(total lysis − spontaneous lysis).

Analysis of viability

Transduced and control monocytes were plated in triplicate at a density of 1×10^4 cells per well of a 96-well plate and cell viability was assessed by MTS assay (Promega, Madison, MI), according to the manufacturer's instructions. On days 1, 7, 14, 21, and 28 posttransduction, cells in the 96-well plate were incubated with the reaction substrate and absorbance was measured 3 hr later at 490 nm. The viability index was calculated as VI = Abs_{490} (transduced and/or cytokine-treated monocytes)/Abs_{490} (mock controls cultured in the absence of cytokines). Viable cells were determined by trypan blue exclusion, and absolute cell counts were performed with the aid of a microscope.

Statistical analysis

Data were analyzed by analysis of variance (ANOVA) followed by Scheffé post hoc analysis.

RESULTS

Efficient and persistent transduction of CD14$^+$ monocytes with lentiviral vectors

Each third-generation self-inactivating (SIN) vector used in this study contains HIV *cis*-acting sequences and an expression cassette for the transgene GFP, GM-CSF, IL-4, or FLT3L (Fig. 1A). The transgenes are the only portion transferred to target cells and, on integration, the vector does not carry wild-type copies of the HIV long terminal repeat (LTR) to the host DNA. The 5′ LTR is chimeric, with the enhancer/promoter of Rous sarcoma virus (RSV) replacing the U3 region of the HIV LTR to rescue its transcriptional dependence on *tat*. The 3′ LTR has an almost complete deletion of the U3 region, which includes the TATA box. As the latter also serves as the template to regenerate the U3 region in the 5′ LTR, on reverse transcription, transcriptional inactivation of both LTRs in the integrated provirus occurs. The RRL-sin backbone used in our studies also contains the human cytomegalovirus (hCMV) internal promoter and a posttranscriptional regulatory element ("pre" in Fig. 1A) from the woodchuck hepatitis virus in the 3′ untranslated region of the transgenes, to increase RNA stability and therefore protein production (Zufferey *et al*., 1999). Batches of SIN third-generation lentiviral vectors were produced by transient cotransfection of 293T cells. The virus preparations were concentrated by ultracentrifugation, and the viral titer was quantified by measuring the concentration of *gag* p24. We determined by transduction experiments with the GFP reporter

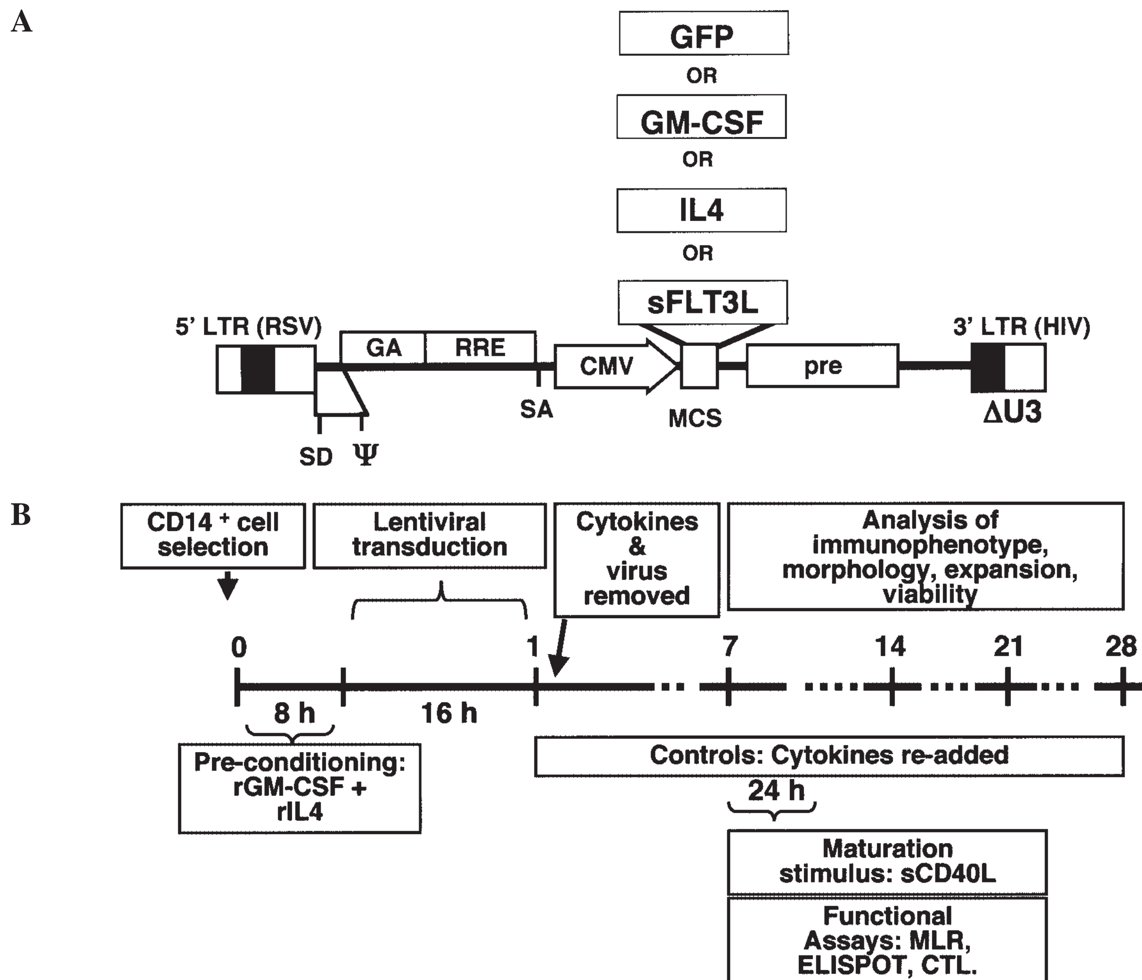


FIG. 1. (**A**) Structure of HIV-1-derived lentiviral vectors. LTR, long terminal repeat; SD, splice donor; SA, splice acceptor; Ψ, encapsidation signal; GA, *gag*-truncated open reading frame; RRE, Rev-responsive element. All transgenes (GFP, GM-CSF, IL-4, and FLT3L) were inserted downstream of the internal cytomegalovirus (CMV) promoter. A deletion of the viral promoter has been engineered into the U3 region of the 3′ LTR (ΔU3), and results in a self-inactivating (SIN) replacement of the Rous sarcoma virus (RSV) promoter in the U3 region of the chimeric 5′ LTR on reverse transcription. The woodchuck posttranscriptional regulatory element ("pre") was inserted to increase RNA stability. (**B**) Schematic temporal representation of the selection, preconditioning, and lentiviral transduction of CD14$^+$ cells, followed by a listing of the various analysis methods applied.

gene that 5 μg of p24 equivalent of lentiviral vector produced consistently high levels of gene delivery with undetectable cellular toxicity after transduction of hematopoietic cells (Koya *et al*., 2002; Stripecke *et al*., 2003).

As a source of human monocytes, we employed cryopreserved PBMCs obtained by leukapheresis. Enriched $CD14^+$ cells (>90% pure; data not shown) were maintained in culture in serum-free X-VIVO 15 medium in the presence of recombinant human GM-CSF and recombinant human IL-4 for 8 hr before transduction (Fig. 1B). We included this preconditioning step after performing pilot experiments showing that (1) GM-CSF improves lentiviral transduction; and (2) IL-4 prevents the outgrowth of contaminating macrophages in the culture and favors DC growth until autonomous constitutive coexpression of transduced LV-GMCSF and LV-IL4 is achieved. Monocytes were typically transduced with 5 μg of p24 equivalent of lentiviral vector per milliliter and, 16 hr afterward, the cells were washed and cultured for an additional 24 hr. Under these conditions, we consistently obtained an average of 70% transduced cells, as shown by flow cytometry analysis of the reporter gene GFP. The persistence of transgene expression after transduction of monocytes was assessed by GFP expression 7, 14, and 21 days after transduction. Kinetic analysis of GFP expression demonstrated consistently high percentages (55–83%) of GFP^+ cells throughout the 21-day period (Fig. 2A). The inclusion or lack of recombinant cytokines in the medium did not alter the patterns of GFP expression throughout the culture (Fig. 2A).

Culture supernatants from monocytes transduced with LV-GMCSF, LV-IL4, or LV-FLT3L were analyzed 2 days after transduction by ELISA. GM-CSF, IL-4, and FLT3L production was typically in the range of 500–2500 pg/ml for 10^6 cells/ml per 24 hr, whereas nontransduced or LV-GFP-transduced cells did not show detectable levels of any of the cytokines. Accumulated levels of GM-CSF, IL-4, and FLT3L produced by monocytes that were transduced singly or in combinations, and maintained in the absence of exogenously added factors, were measured on days 7, 14, and 21 after transduction. After harvesting the supernatants weekly, the cells were washed and seeded in fresh medium. Mock- and LV-GFP-transduced cells did not show detectable levels of the cytokines (Fig. 2B), whereas GM-CSF, IL-4, and soluble FLT3L were readily measured in the respective transduction groups. Interestingly, there was an increase in cytokine production over time, particularly for GM-CSF and FLT3L, indicating positive feedback for the production of these cytokines (Fig. 2B). Cotransduction of $CD14^+$ cells with different combinations of vectors (LV-GM-CSF plus LV-IL4, LV-GMCSF plus LV-FLT3L, and LV-GM-CSF plus LV-IL4 plus LV-FLT3L) demonstrated that the cytokines were variably coexpressed at slightly reduced (GM-CSF) or similar (IL-4 and FLT3L) levels than when transduction was performed with a single vector (Fig. 2B). These results represent duplicate experiments.

Monocytes transduced to coexpress GM-CSF, IL-4, and FLT3L acquire a typical DC phenotype

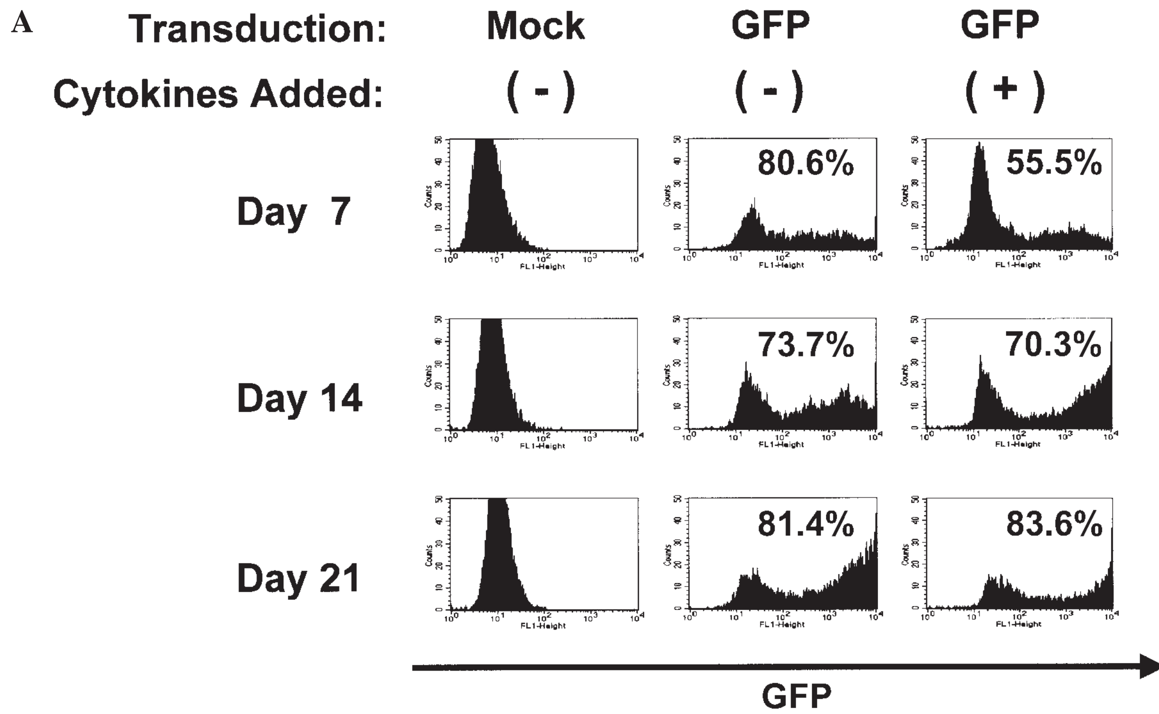
Microscopic examination of cells that were spread onto microscope slides by Cytospin centrifugation (Shandon Cytocentrifuge; Thermo Electron, Waltham, MA) and stained with Giemsa revealed the presence of dendritic processes and cell vacuoles, and a reticulate appearance, typical of dendritic cells. This was noticeable for the control group maintained for 14 days in the presence of recombinant cytokines and transduced with LV-GFP, and in the test groups maintained in the absence

TABLE 1. FLOW CYTOMETRY ANALYSIS OF RELEVANT DENDRITIC CELL MARKERS AND OF GREEN FLUORESCENT PROTEIN[a]

Transduction	Recombinant cytokines	Day 7							
		CD1a	*CD14*	*CD80*	*CD83*	*CD86*	*HLA-DR*	*HLA-ABC*	*GFP*
Mock	–	0	8.7	4.2	1.2	70.2	95.9	86.9	0
	+	18.5	1.2	21.5	34.5	93.5	92.1	90.4	0
LV-GFP	–	0.4	18.1	6.1	0.4	NA	98.1	94.4	79.3
	+	38.2	0.1	33.5	2.9	NA	92.2	64.9	65.5
LV-GMCSF + LV-IL4	–	40.5	1.1	60.5	8.6	76.9	93.6	87.6	0
LV-GMCSF + LV-IL4 + LV-FLT3L	–	37	1.2	57.5	10.2	85.9	92.4	92.5	0

Transduction	Recombinant cytokines	Day 14							
		CD1a	*CD14*	*CD80*	*CD83*	*CD86*	*HLA-DR*	*HLA-ABC*	*GFP*
Mock	–	0	4.5	5	0	49.6	95	77.5	0
	+	2.8	1	20.7	27.4	94.5	93.3	79.6	0
LV-GFP	–	0	9.7	1	0	NA	99.2	82.5	71.2
	+	25.5	1.5	43.8	14.8	NA	98.9	84.8	63.0
LV-GMCSF + LV-IL4	–	16.6	1.5	30.5	6.7	96.3	98.6	80.3	0
LV-GMCSF + LV-IL4 + LV-FLT3L	–	14.8	0.5	33.6	8	98.1	97.5	90.8	0

[a]Monocytes were not transduced (Mock) or transduced to express GFP, GM-CSF plus IL-4, or GM-CSF plus IL-4 plus FLT3L and maintained in culture for 7 or 14 days in the absence (–) or presence (+) of exogenously added recombinant GM-CSF and IL-4. Numbers represent the percentage of marker-positive cells calculated by subtracting the frequency of cells stained with specific immunoconjugated antibodies from cells stained with isotype control antibodies.



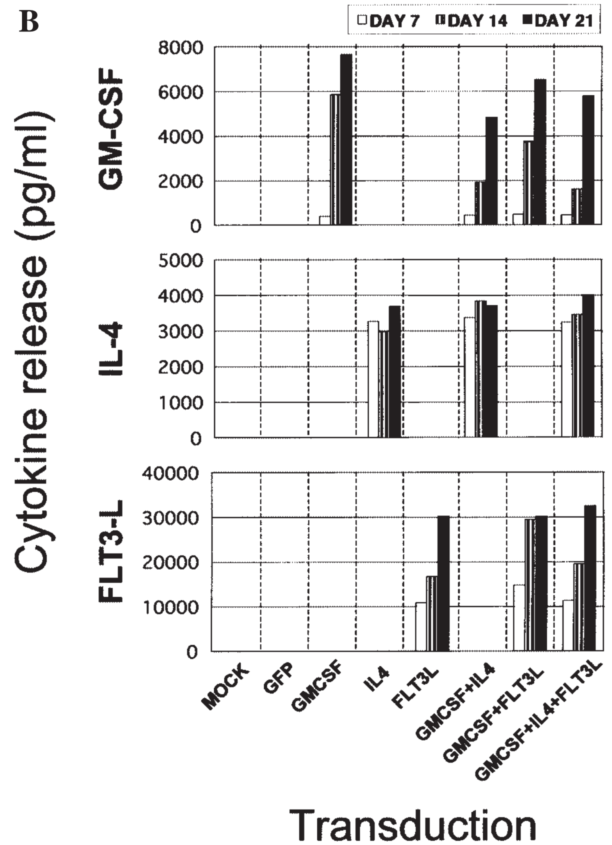
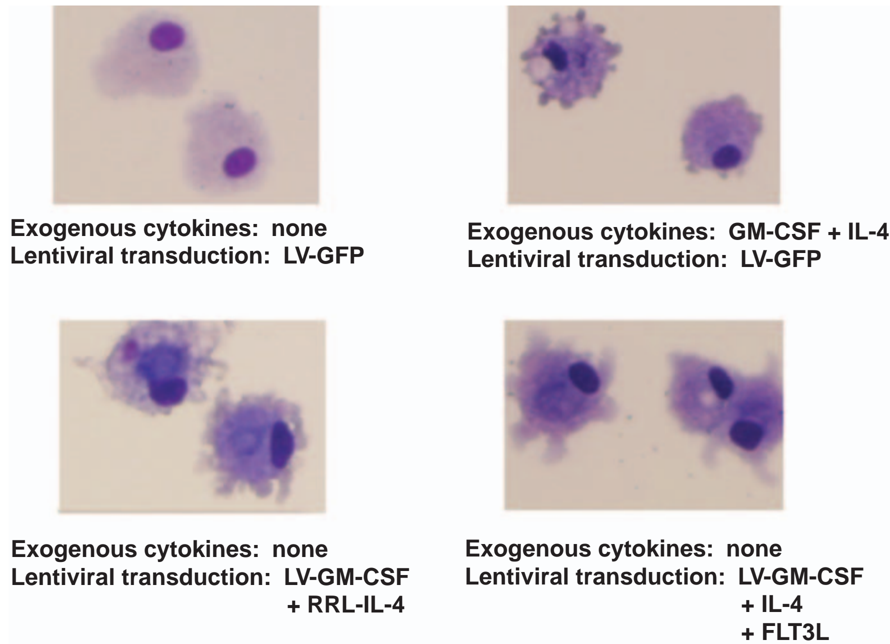


FIG. 2. (**A**) Flow cytometry analysis of GFP after transduction of $CD14^+$ cells shows efficient and persistent transgene expression. Purified $CD14^+$ cells were transduced with the LV-GFP lentiviral vector and subsequently maintained in the absence (−) or presence (+) of recombinant GM-CSF and IL-4. Flow cytometry analysis of the GFP-expressing cells was performed 7, 14, and 21 days after transduction. (**B**) Persistent expression of GM-CSF, IL-4, and FLT3L after transduction of $CD14^+$ cells. Purified $CD14^+$ cells nontransduced (Mock) or transduced with LV-GFP, LV-GMCSF, LV-IL4, and LV-FLT3L, singly or in combinations, were maintained in culture for 3 weeks. Every week, the cell supernatant was harvested for analysis of the secreted cytokines by ELISA, the cells were washed, and medium was replenished.



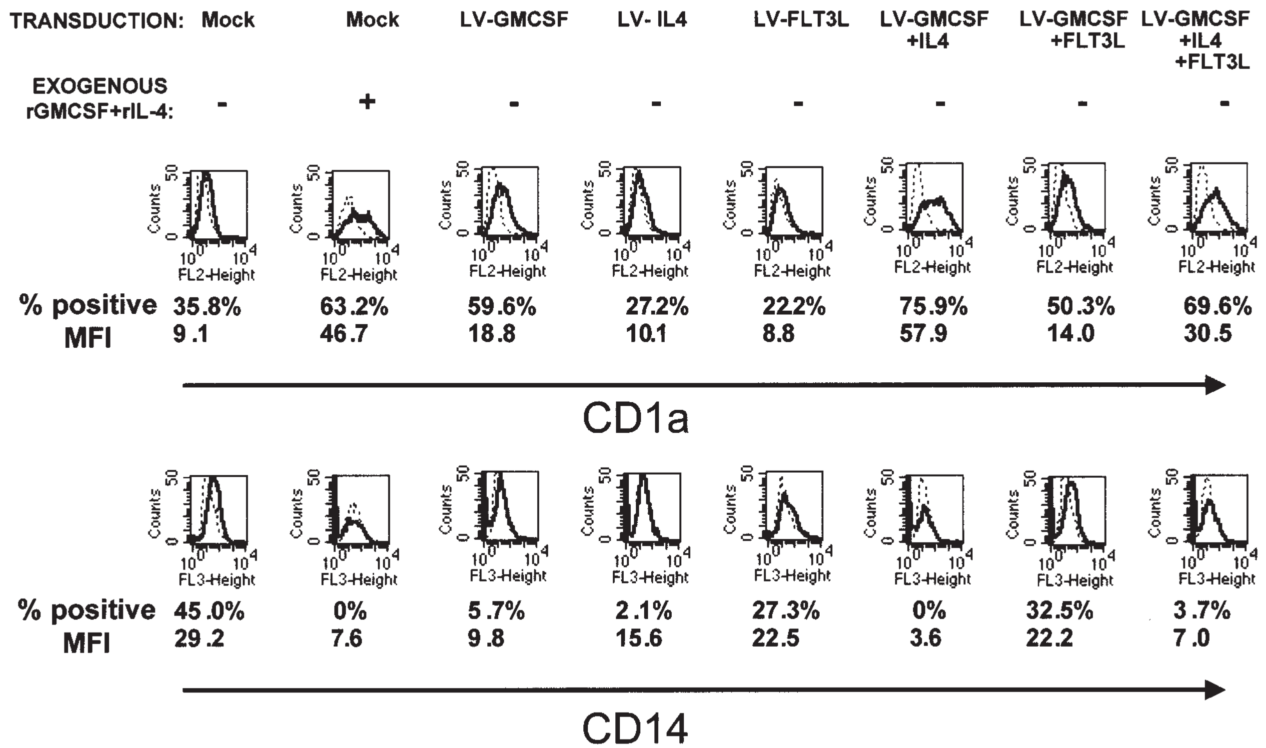


FIG. 3. (**A**) $CD14^{+}$ cells transduced to express GM-CSF, IL-4, and FLT3L develop the morphology of dendritic cells. The results depicted are micrographs of 14-day cultured cells that were spread on microscope slides by Cytospin centrifugation followed by Giemsa/Wright staining. Controls were transduced with LV-GFP and maintained in the absence or presence of exogenously added GM-CSF and IL-4, whereas no exogenous cytokine treatment was included for cells transduced with LV-GMCSF, LV-IL4, and LV-FLT3L. (**B**) $CD14^{+}$ cells transduced to express GM-CSF, IL-4, and FLT3L acquire CD1a expression and lose CD14 expression. Shown is the expression of CD1a and CD14 of 7-day cultured cells that were not transduced (Mock) or transduced to express the immunomodulatory molecules GM-CSF, IL-4, and FLT3L, singly or in combinations. The positive control group was the only group maintained in the presence of exogenous GM-CSF and IL-4 (Mock +). The percentage of $CD1a^{+}$ and $CD14^{+}$ cells was calculated by subtracting the frequency of cells stained with the specific immunoconjugated antibodies (boldface lines) from the cells stained with isotype control antibodies (broken lines). MFI, Mean fluorescence intensity. These data are representative of triplicate experiments.

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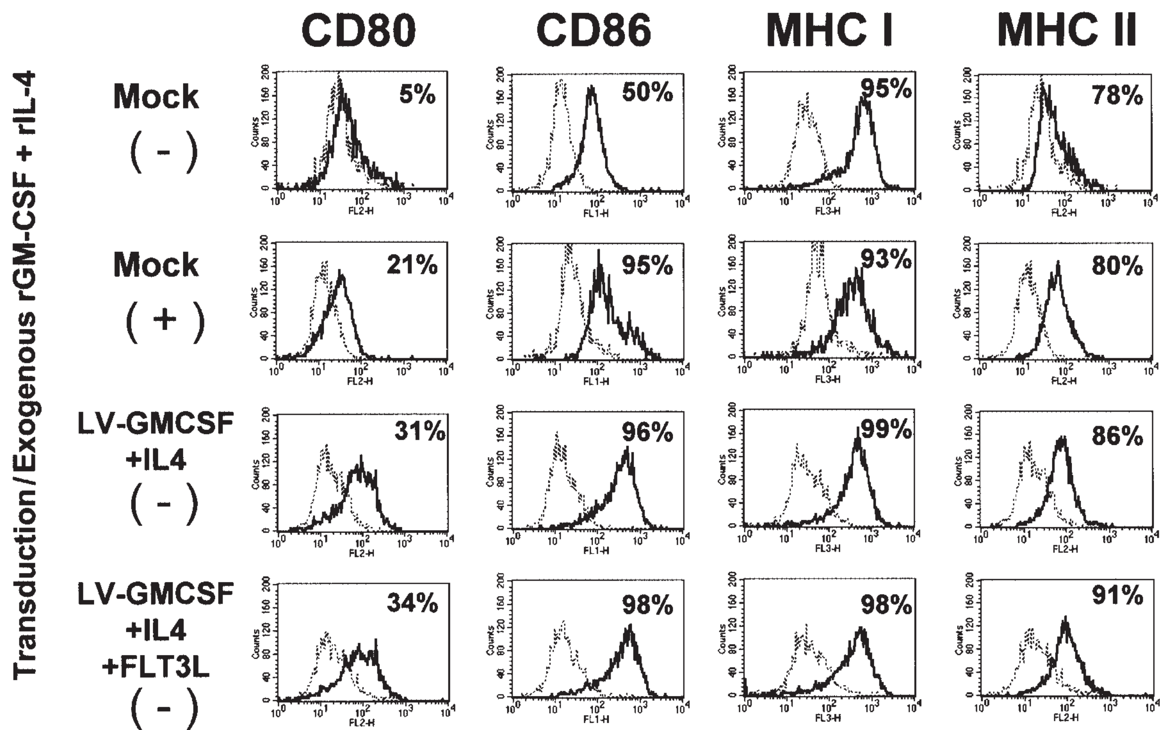

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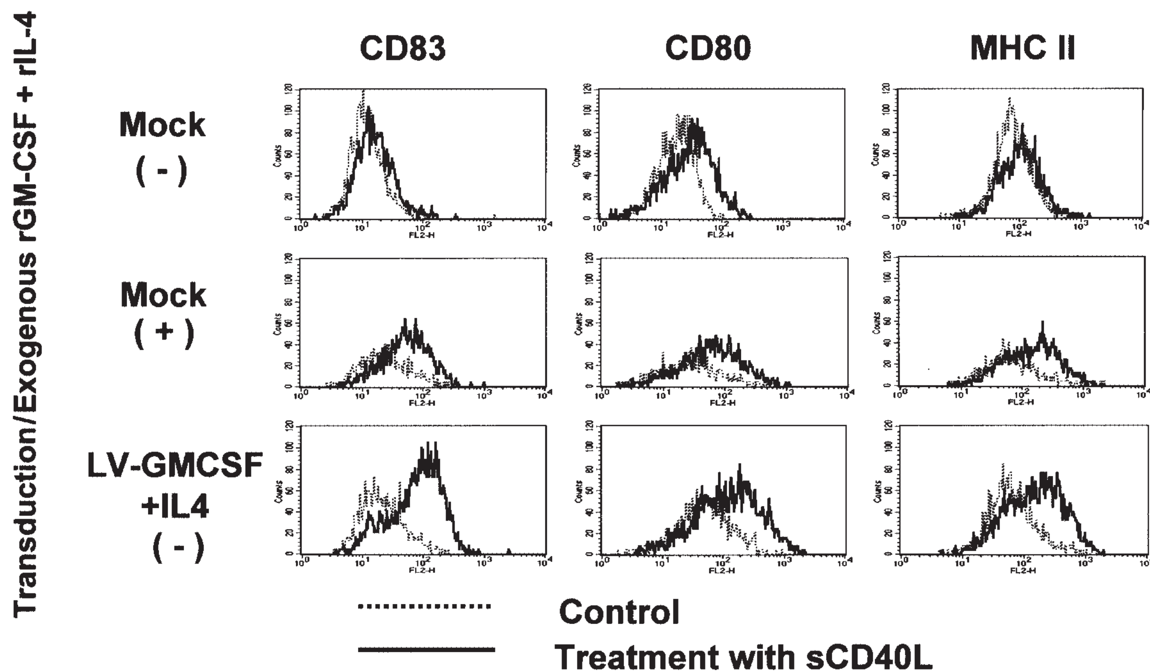

FIG. 3. (*continued*) (**C**) CD14^{+} cells transduced to express GM-CSF, IL-4, and FLT3L differentiate into a typical DC immunophenotype. Nontransduced (Mock) cells corresponding to untreated monocytes (−) or DCs grown in the presence of exogenous GM-CSF and IL-4 (+) were compared with monocytes that were transduced to coexpress GM-CSF, IL-4, and FLT3L and maintained in culture for 14 days. The percentage of CD80^{+}, CD86^{+}, MHC-I^{+}, and MHC-II^{+} cells was calculated by subtracting the frequency of cells stained with the specific immunoconjugated antibodies (boldface lines) from the cells stained with isotype control antibodies (broken lines). These data are representative of duplicate experiments. (**D**) Genetically programmed DCs transduced to express GM-CSF and IL-4 can be matured in the presence of soluble CD40L. Nontransduced (Mock) cells corresponding to untreated monocytes (−), DCs grown in the presence of exogenous GM-CSF and IL-4 (+), and DCs transduced to coexpress GM-CSF and IL-4 were maintained in culture for 7 days and, when applicable, were treated with exogenously added soluble CD40L for 1 day. The histograms show expression of CD83, CD80, and MHC-II by control cultures (broken lines) or by cultures that received sCD40L treatment (boldface lines). These data are representative of duplicate experiments.

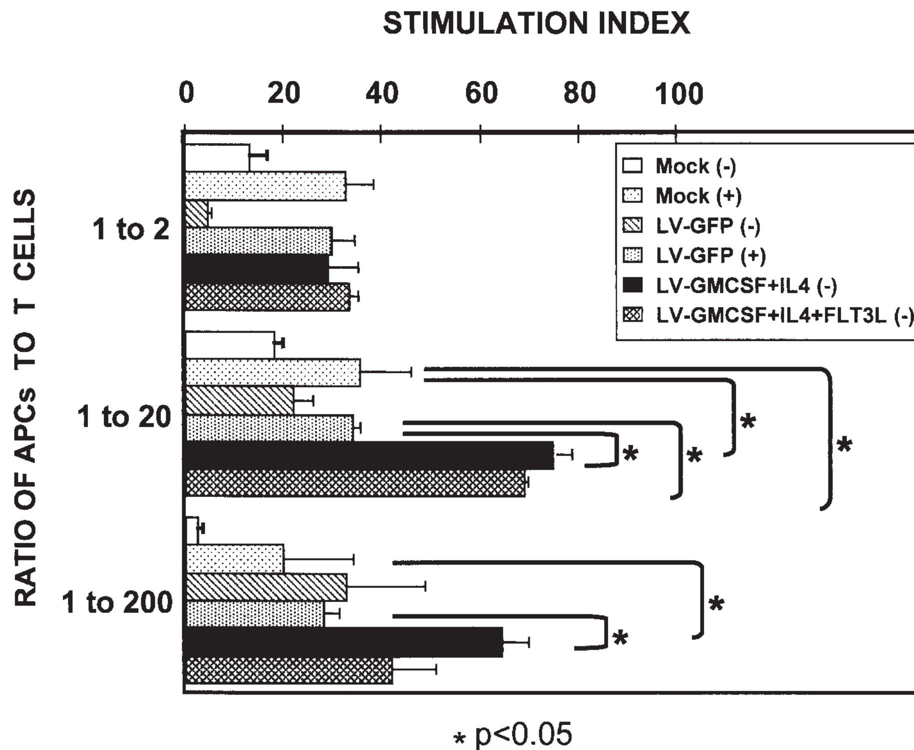

FIG. 4. Genetically programmed DCs efficiently stimulate allogeneic mixed lymphocyte reactions (MLRs). MLR was performed with nontransduced cells (Mock), cells transduced with reporter control LV-GFP, or cells transduced with experimental combination LV-GMCSF plus LV-IL4 or LV-GMCSF plus LV-IL4 plus LV-FLT3L, maintained in culture for 7 days, irradiated (3200 CGy), and incubated with enriched allogeneic T cells. The stimulation index was calculated on the basis [^{3}H]thymidine incorporation. *Significant difference ($p < 0.05$). These data are representative of triplicate experiments.

of recombinant growth factors, but transduced with LV-GMCSF plus LV-IL4 plus LV-FLT3L (Fig. 3A). The negative control group transduced with LV-GFP and kept in culture in the absence of GM-CSF and IL-4 displayed the typical morphology of monocytes (Fig. 3A).

Flow cytometry analysis of CD1a (upregulated during differentiation of immature DCs) and CD14 (lost on DC differentiation) was performed 7 days after transduction, which was done singly or in combinations. Expression of CD1a was remarkably higher for cells maintained continuously in the presence of exogenously added cytokines and cells transduced with LV-GMCSF, and was particularly high for cells cotransduced with LV-GMCSF plus LV-IL4 (Fig. 3B). CD1a expression persisted for cells analyzed 14 days after transduction (Table 1). In contrast, CD14 expression decreased markedly in cells maintained in the presence of exogenously added cytokines and in cells transduced with LV-GMCSF and LV-IL4. Interestingly, cells transduced with LV-FLT3L showed consistently lower levels of CD1a and higher levels of CD14 expression (Fig. 3B and Table 1).

Additional relevant DC immunophenotypic markers were analyzed on days 7 (Fig. 3C and Table 1) and 14 (Table 1) after transduction. The levels of expression of costimulatory molecules CD80 and CD86 and of MHC class I and II were higher for the groups cotransduced with LV-GMCSF plus LV-IL4 plus LV-FLT3L (triple combination) than for the nontransduced groups maintained in the presence or absence of exogenous cytokines (Fig. 3C and Table 1). Of note, transduction with LV-GFP by itself did not induce any immunophenotypic changes compared with mock transduction, either in the presence or absence of recombinant factors (Table 1). CD83 expression, a marker for mature DCs, was observed in a small subset (approximately 10%) of cells transduced LV-GMCSF plus LV-IL-4 plus LV-FLT3L (Table 1).

We subsequently examined the effects of exogenously added soluble CD40L on day 7 after initiation of the cultures. As expected, soluble CD40L triggered upregulation of CD83, CD80, and MHC-II for both nontransduced DCs and DCs transduced with LV-GMCSF plus LV-IL4 (Fig. 3D).

The immunophenotypic analysis confirmed that DC were generated after lentiviral transduction of CD14$^+$ cells to express GM-CSF and IL-4. Cotransduction with LV-GMCSF plus LV-IL4 plus LV-FLT3L typically produced lower amounts of CD1a$^+$ cells, but this combination did not affect the expression of other relevant DC markers.

Genetically programmed DCs efficiently stimulate immune responses

We performed functional assays to evaluate the immunostimulatory capacity of lentivirally modified dendritic cells harvested 7 days after transduction. First, we compared the allo-

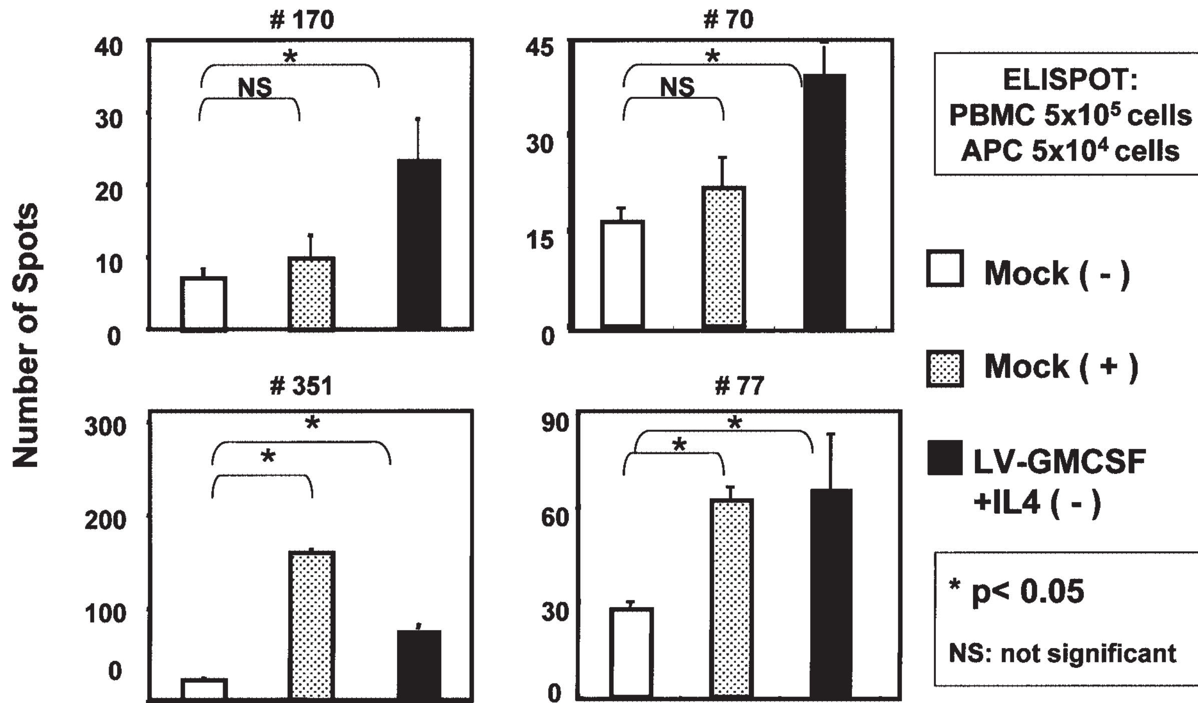

FIG. 5. Genetically programmed DCs efficiently present antigens to $CD8^+$ T cells. Four different patient samples were used. IFN-γ ELISpot was performed with APCs that were nontransduced (Mock) and maintained in the absence ($-$) or presence ($+$) of exogenously added GM-CSF and IL-4, or cotransduced with LV-GMCSF plus LV-IL4, and maintained in culture for 7 days. The APCs were mixed with antigenic epitopes from gp100, influenza virus, or CMV and used to activate autologous PBMCs for production of IFN-γ. The number of spots corresponding to the number of progenitor T cells activated *in vitro* was determined from triplicate wells. *Significant difference ($p < 0.05$).

geneic T cell-stimulatory activity of cells that were not transduced or were transduced with LV-GFP, and maintained in the presence or absence of exogenously added cytokines, with that of DCs differentiated under autonomous expression of GM-CSF, IL-4, and FLT3L (Fig. 4). Irradiated APCs were cocultured with allogeneic $CD3^+$ purified T cells in a mixed lymphocyte reaction (MLR) at various stimulator:T cell ratios. Alloreactivity, measured as a stimulation index after [^{3}H]thymidine incorporation, was observed in all cultures (Fig. 4). APCs maintained in the absence of exogenously added GM-CSF and IL-4 [mock ($-$)] were weak stimulators. At nonsaturating ratios (APC:T cell ratio, 1:20 and 1:200), the genetically programmed DCs coexpressing GM-CSF and IL-4 were significantly more stimulatory ($p < 0.05$) than DCs grown in the presence of exogenously added factors (Fig. 4). Notably, DCs coexpressing GM-CSF, IL-4, and FLT3L were outperformed by DCs expressing GM-CSF and IL-4 only.

The HLA-restricted capacity for antigen presentation of genetically modified DCs was evaluated by IFN-γ secretion in ELISpot assays. APCs (5×10^4 cells) harvested on day 7 posttransduction were loaded with a mixture of peptide epitopes present in CMV, Flu, and gp100 and cocultured with PBMCs (5×10^5 cells) in triplicate wells of ELISpot plates for 48 hr. IFN-γ spots detected in the wells indicated the presence of responding epitope-specific $CD8^+$ T cells. Four different patient samples were analyzed independently (Fig. 5). For all cases, APCs obtained after transduction with LV-GMCSF plus LV-IL4 stimulated significantly higher numbers of IFN-γ-producing cells than nontreated mock-transduced APCs ($p < 0.05$; Fig. 5). In two of four cases, the stimulation of IFN-γ-producing cells was significantly higher for genetically derived DCs than for DCs obtained by cytokine treatment and in one of four cases the reverse was observed (Fig. 5). In agreement with the MLR data, addition of FLT3L to the combination of GM-CSF plus IL-4 was not beneficial for antigen presentation as assessed by ELISpot (data not shown). Thus, genetically programmed DCs coexpressing GM-CSF and IL-4 were consistently the most efficient combination for antigen presentation to autologous T cells.

Class I-restricted cytotoxic activity elicited by genetically modified DCs was investigated by chromium release assays. APCs pulsed with HLA-A2.1-restricted peptides were used to stimulate purified autologous $CD8^+$ T cells and cytotoxicity was evaluated against homologous peptides, using T2 cells as targets (Fig. 6). APCs, generated after 7-day culture in the presence of exogenous factors or after LV-GMCSF plus LV-IL4 transduction, were matured with soluble CD40L and incubated with HLA-A2.1-restricted peptides to generate immune responses against influenza (Flu), a melanoma antigen (gp100), or an irrelevant control peptide (E7). The CTL assays demonstrated the capacity of genetically programmed DCs to stimulate $CD8^+$ lytic cells at significantly higher rates ($p < 0.05$) than mock ($-$) controls, albeit at significantly reduced efficiency compared with conventional DCs (Fig. 6). These results

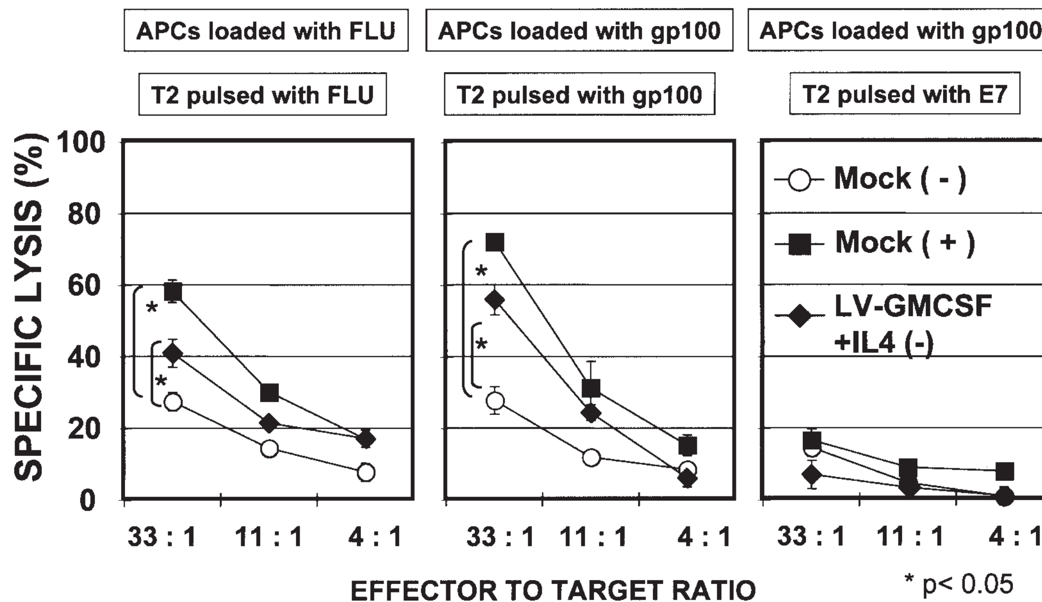

FIG. 6. Cytotoxicity against influenza- and melanoma-associated antigenic peptides. Monocytes that were not transduced (mock) and maintained in the absence (−) or presence (+) of exogenously added GM-CSF and IL-4, or monocytes transduced to coexpress GM-CSF and IL-4, were maintained in culture for 7 days. The APCs were matured in the presence of sCD40L and loaded with Flu-M1 or gp100 target peptides and used for coculture with autologous purified $CD8^+$ cells in the presence of IL-2 and IL-7. The effector cells were harvested 7 days after coculture and allowed to react at various effector:target ratios against T2 target cells labeled with ^{51}Cr and pulsed with three different peptides. Lysis was measured on the basis of ^{51}Cr released into the medium supernatant. *Left*: APCs loaded with Flu antigen, T2 cells pulsed with Flu. *Middle*: APCs loaded with gp100, T2 cells pulsed with gp100. *Right*: APCs loaded with gp100, T2 cells pulsed with E7, an irrelevant peptide control. *Significant difference ($p < 0.05$). These data are representative of triplicate experiments performed with PBMCs from three different patients.

were reproducible in triplicate experiments performed with different matched patient samples.

Autonomous expression of GM-CSF and IL-4 promotes a transient, but consistent, increase in cell viability in vitro

The effect of the autonomous coexpression of GM-CSF, IL-4, and FLT3L by genetically modified DCs on cell viability was assessed *in vitro* for 28 days after transduction. Control $CD14^+$ cells that were nontransduced or transduced to express GFP were maintained throughout the culture period in the presence of exogenously added GM-CSF and IL-4. These cells were compared with DCs obtained after transduction with lentivirally encoded GM-CSF, IL-4, and FLT3L, and maintained in the presence or absence of exogenously added cytokines. The first analysis, to monitor the rate of cell death in culture, was performed weekly by determining the total number of viable cells. As expected, mock or GFP-transduced cultures maintained in the presence of exogenously added cytokines showed a pronounced decrease in cell numbers during the first 2 weeks of culture (Fig. 7A). In contrast, cells transduced to coexpress GM-CSF and IL-4, or GM-CSF, IL-4, and FLT3L, showed significantly higher numbers of viable cells for the same period ($p < 0.01$), but eventually decreased to low numbers by the third week of culture (Fig. 7A). Addition of exogenous recombinant cytokines to the lentivirally transduced cells expressing GM-CSF and IL-4 did not promote higher cell viability, indicating that cytokine levels produced by lentiviral transduction were not limiting. Importantly, coexpression of FLT3L was unnecessary, as it did not promote the generation of higher numbers of viable cells at any time point (Fig 7A).

The absolute cell count was performed in parallel with the MTS assay, which measures cellular metabolic activity. Metabolic rates among the various experimental groups were statistically undistinguishable during the first week of culture (Fig. 7B). In the following 3 weeks, however, the metabolic rates of cells autonomously coexpressing GM-CSF and IL-4 were significantly higher ($p < 0.05$) than for the mock and GFP-transduced groups maintained in the presence of exogenous cytokines. Also, addition of exogenous cytokines to cells autonomously expressing GM-CSF and IL-4 did not further increase the viability of the cells (Fig. 7B). These results represent three independent experiments.

DISCUSSION

The modulation of immune responses by genetically engineering immune cells is an expanding field with broad poten-

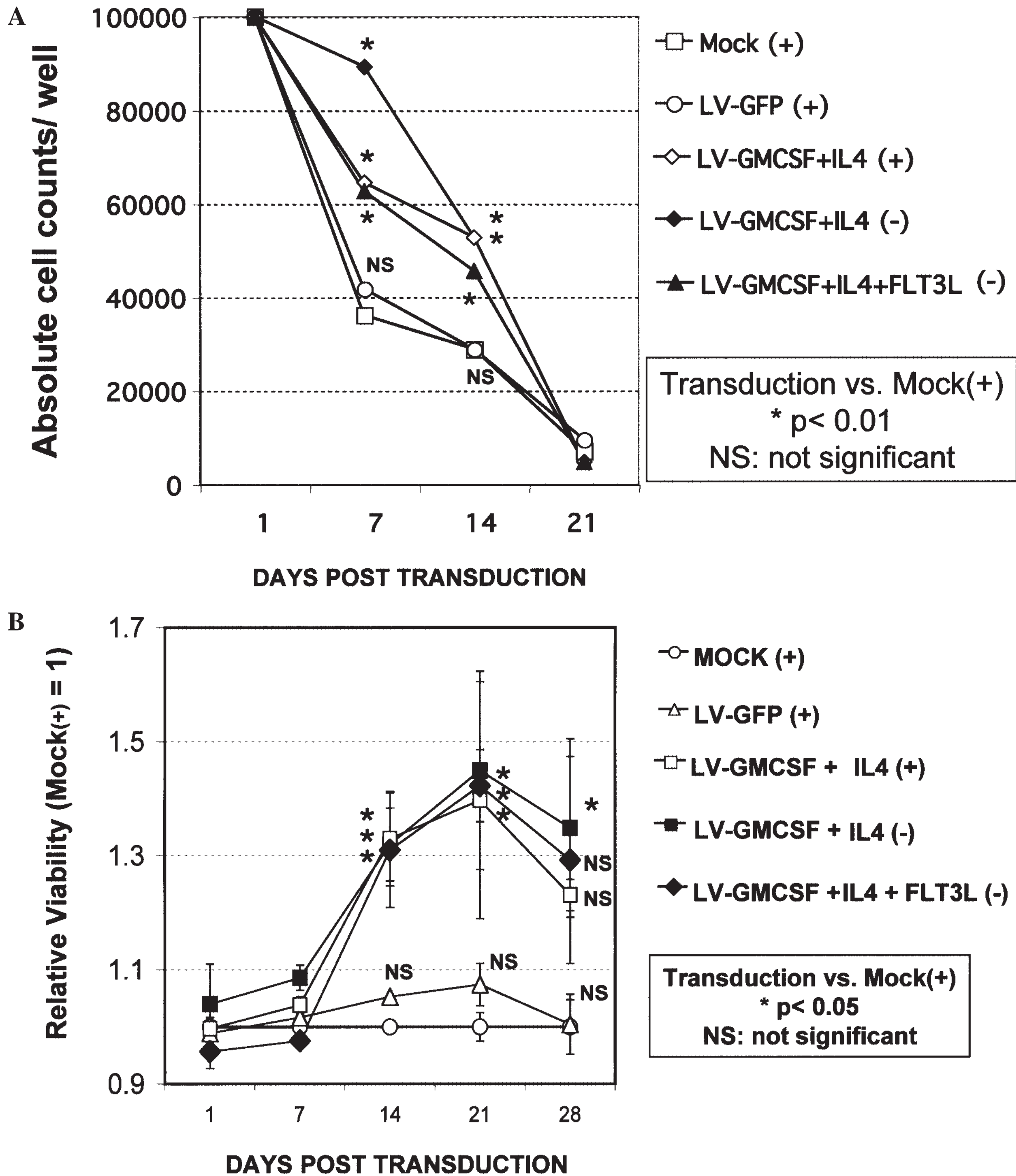

FIG. 7. Viability and metabolic activity of genetically programmed DCs. Control groups maintained in the presence of exogenously added GM-CSF and IL-4 (+) and corresponding to nontransduced (Mock), transduced with LV-GFP, or transduced with LV-GMCSF plus LV-IL4 were compared with cells maintained in the absence of exogenous cytokines (−) and cotransduced with LV-GMCSF plus LV-IL4 or with LV-GMCSF plus LV-IL4 plus LV-FLT3L. (**A**) Absolute cells counts: On day 1 after culture, 1×10^5 cells were seeded in triplicate wells and viable cells were counted at weekly intervals. (**B**) Viability measured by MTS assay: On day 1 after culture, 1×10^4 cells were seeded in triplicate wells and viability was monitored by absorbance measurements of the metabolized assay substrate performed at weekly intervals. Values obtained for the different experimental groups were divided by the values obtained with the reference Mock (+) group. *Significant difference relative to the Mock (+) group. These data are representative of triplicate experiments.

tial in achieving potent and specific immunity against cancer and infectious diseases. *Ex vivo*-derived DCs represent a potent adjuvant for use in vaccination protocols, because they are specific, nontoxic, and abrogate the need for systemic cytokine infusions. DCs can be generated from monocyte precursors in peripheral blood or stem cell progenitors after 1 to 2 weeks of culture *ex vivo* in the presence of GM-CSF and IL-4. These standard methods are costly, as they require clinical-grade laboratory practices and continuous monitoring during the culture period. Furthermore, *ex vivo*-grown DCs maintained in supraphysiological concentrations of GM-CSF and IL-4 lack these growth factors once administered *in vivo*, which leads to a small subset of cells that ultimately reaches the secondary lymphoid organs for antigen presentation. Our approach was to engineer DCs to maintain their growth factor milieu during their differentiation through autocrine and paracrine production of these factors. Here we report a gene delivery strategy, consisting of a single hit of transduction of monocytes with lentiviral vectors to induce autocrine and paracrine expression of GM-CSF, IL-4, and FLT3L. Gene delivery into monocyte-derived DCs by the experimental lentiviral vectors offered an approach by which simple, efficient, persistent, and nontoxic gene expression was achieved. We obtained a consistently high transgene transduction rate (usually 70%) into human CD14$^+$ cells purified from cryopreserved PBMCs, using third-generation SIN lentiviral vectors at high concentrations (5 μg of p24 equivalent/ml). Preconditioning the monocytes with recombinant GM-CSF and IL-4 for 8 hr reduced macrophage contamination (through IL-4) and improved transduction efficiency (through GM-CSF). As previously demonstrated for acute myeloid leukemia cells (Stripecke *et al*., 2003), simultaneous codelivery of multiple genes into monocytes was successfully achieved under the transduction conditions employed.

We demonstrated that coexpression of GM-CSF and IL-4, which are the growth factors most commonly used to generate immature DCs, was sufficient and necessary to promote differentiation of the monocytes into cells with typical immature DC morphology and immunophenotype (CD14$^-$, CD1a$^+$, CD80$^+$, CD86$^+$, MHC-I$^+$, and MHC-II$^+$) which could be stimulated to further mature into CD83$^+$ DCs by exogenously added CD40L. Coexpression of FLT3L in combination with GM-CSF and IL-4 was unnecessary to induce the development of DCs.

Genetically derived DCs significantly outperformed DCs grown in the presence of recombinant cytokines in *in vitro* allogeneic T cell stimulation, and were more consistent in MHC class I-restricted antigen presentation analyzed by ELISpot assay.

Importantly, autonomous expression of GM-CSF and IL-4 promoted significantly higher viability of the cells for the initial 2-week period *in vitro*. One concern regarding the persistent autocrine and paracrine production of growth factors by DCs is the disruption of the normal regulation of DC differentiation and proliferation, which could lead to ongoing proliferation with potential malignant transformation. However, our attempts to obtain long-term culture of cells from monocytes expressing GM-CSF, IL-4, and FLT3L have so far failed on extended *in vitro* culture (>4 weeks). These results confirm our previous observation with primary AML cells transduced to express GM-CSF. Thus, although we observed higher cell proliferation and viability of the leukemic blasts during the first week after transduction, we were not able to maintain the cells in culture for more than 1 to 2 weeks, except in rare cases (Koya *et al*., 2002). These results are in full agreement with studies from Stocking and Ostertag evaluating retroviral vector-mediated gene delivery of GM-CSF or IL-3 into hematopoietic cells; on conversion of factor-dependent myeloid cells to factor independence, autocrine stimulation was not associated with tumorigenicity (Stocking *et al*., 1989). This may be explained by the fact that growth-stimulatory signals often trigger a negative feedback loop, as was shown for the stimulatory effect of GM-CSF, which was also accompanied by proapoptotic signals in AML (Faderl *et al*., 2003). Thus, concurrent with its stimulation of AML cell growth, GM-CSF upregulated suppressor of cytokine signaling (SOCS) proteins, procaspases, and caspases (Faderl *et al*., 2003). Additional clinical evidence that GM-CSF per se does not support leukemogenesis is the fact that GM-CSF is often used as an adjunct to induction chemotherapy of myelodysplastic syndrome (MDS) and AML to sensitize the blasts to the cytotoxic effects of chemotherapy regimens. GM-CSF is also used in AML patients to reduce neutropenia and therefore the risk of infections (Rossi *et al*., 2002). In addition, the administration of IL-4 between courses of chemotherapy has also been shown to reduce or abrogate regrowth of leukemia cells, with the proposed mechanism being through suppression of telomerase activity (Preisler *et al*., 2000). Although preclinical evidence will be necessary to demonstrate that the autonomous expression of GM-CSF and IL-4 after lentiviral vector integration does not lead to malignancy *in vivo*, it is noteworthy that murine bone marrow DCs genetically modified to express GM-CSF showed increased capacity to induce primary immune reactions in syngeneic mouse tumor model systems, which correlated with a better migratory capacity of DCs to lymph nodes, whereas no malignancy was observed (Curiel-Lewandrowski *et al*., 1999; Klein *et al*., 2000).

Genetic immunization with tumor antigen-transduced DCs generates antigen-specific cellular responses (Ribas *et al*., 2003). This method takes advantage of the ability of endogenous expression of tumor antigen genes to continuously provide antigenic epitopes for both MHC class I- and II-restricted expression. However, in the therapeutic setting both for mouse models and human clinical settings, despite detectable antitumor immune responses, immunizations with tumor antigen-transduced DCs were not potent enough to significantly promote rejection of the tumors. One possible reason for this problem is that conventionally generated DCs, once removed from culture, lack the supraphysiological levels of exogenously added growth factors required to function adequately *in vivo*. A potential approach to circumvent this is described here, by genetically programming DCs to maintain their own growth factor milieu during differentiation/maturation by autocrine and paracrine production of these factors. Of note, it was shown in murine tumor models that GM-CSF gene transfer into monocyte-derived DCs augmented their antitumor effect, and this was correlated with their increased life span *in vivo* (Curiel-Lewandrowski *et al*., 1999; Klein *et al*., 2000). In particular, expression of GM-CSF by DCs led to enhanced cytotoxic T lymphocyte activity, potentially mediated by increased numbers of DCs in draining lymph nodes (Klein *et al*., 2000). Therefore, we anticipate that genetically programmed DCs will outperform conventionally generated DCs in viability, in biodis-

tribution into lymphoid areas, and in immunopotency to significantly enhance immunotherapeutic efficacy against cancer.

In summary, we have demonstrated the feasibility of genetically programming monocytes to autonomously differentiate into DCs with lentiviral transduction for coexpression of GM-CSF and IL-4, which maintained the phenotype and viability for at least 2 weeks. Addition of FLT3L coexpression did not improve the viability, expansion, or immunologic performance of DCs. This approach involved 1 day of cell manipulation, compared with 1 to 2 weeks when using conventional recombinant cytokine cocktail methods. We are currently evaluating the capacity of the genetically modified monocytes to attain autonomous differentiation into DCs *in vivo* for simultaneous genetically programmed antigen presentation.

AKNOWLEDGMENTS

This work was supported by a Howard Temin Award from the National Cancer Institute (K01 CA878864; R.S.), by a Career Development Award from Stop Cancer (R.S.), and by research grants from the Wright Foundation (R.S.) and from the Margaret E. Early Research Trust (R.S.). The authors thank Luigi Naldini for providing the third-generation lentiviral vector system, Mark Hechinger for assistance with flow cytometry analysis, and Huy Q. Ta for technical assistance.

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Received for publication March 29, 2004; accepted after revision June 16, 2004.

Published online: July 12, 2004.