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Potent Maturation of Monocyte-Derived Dendritic Cells After CD40L Lentiviral Gene Delivery

*Richard C. Koya, *†Nori Kasahara, *Patricia M. B. Favaro, †Roy Lau, *Huy Q. Ta, †Jeffrey S. Weber, and *†Renata Stripecke

**Institute for Genetic Medicine and †Norris Comprehensive Cancer Center, Keck School of Medicine, University of Southern California, Los Angeles, California*

Summary: Dendritic cells (DCs) are being evaluated in immunization protocols to enhance immunity against infectious diseases and cancer. Interaction of T-helper cells expressing CD40 ligand (CD40L) with its cognate CD40 receptor on DCs leads to a mature DC phenotype, characterized by increased capacity of antigen presentation to cytotoxic T cells. The authors examined the ability of third-generation self-inactivating lentiviral vectors expressing CD40L to induce autonomous maturation of ex vivo expanded human monocyte-derived dendritic cells. Transduction with lentiviral vectors achieved a highly efficient gene transfer of CD40L to DCs, which correlated with phenotypic maturation as shown by the expression of immunologic relevant markers (CD83, CD80, MHCI) and secretion of IL-12, whereas DC phenotype was not affected by a control vector expressing only the green fluorescent protein marker. Addition of recombinant IFN-γ to DCs at the time of CD40L transduction further enhanced IL-12 production, and when co-cultured with allogeneic and autologous $CD8^+$ and $CD4^+$ T cells, a potent activation was observed. Autologous responses against an HLA-A2-restricted influenza peptide (Flu-M1) and a tumor-associated antigenic peptide (gp100 210M) were significantly enhanced when CD40L transduced DCs were used as antigen-presenting cells for in vitro stimulation of $CD8^+$ cytotoxic T lymphocytes. These results demonstrate that endogenous expression of CD40L by lentivirally transduced DCs induced their autonomous maturation to a phenotype comparable to that induced by optimal concentrations of soluble CD40L, providing a novel tool for genetic manipulation of DCs. **Key Words:** dendritic cells, lentiviral vectors, immunotherapy, gene therapy, CD40L, melanoma

Dendritic cells (DCs) coordinate the initiation of immune responses by naïve T cells and B cells.[1] The evaluation of DC-based immunotherapy in the treatment of patients with cancer and infectious diseases has been pursued by multiple centers worldwide. DC precursors derived from peripheral blood, bone marrow, or cord blood can be differentiated in vitro and used for immunization after pulsing with antigenic peptides, proteins, or their transgenes.[2,3] Since blood is the most accessible tissue for clinical studies, various protocols have been developed using peripheral blood mononuclear cells (PBMCs) for the in vitro production of DCs. Recombinant GM-CSF and IL-4 added to peripheral blood monocytes in culture promote the generation of immature monocytic DCs within 5 to 10 days.[4,5] These functionally immature DCs can capture and process antigens: they show high endocytic activity and high levels of intracellular major histocompatibility complex (MHC) class II molecules.[5]

After exposure to inflammatory stimuli (bacteria, virus, T cells, or cytokines), DCs mature into cells capable of potent antigen presentation: MHC–peptide complexes

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Address correspondence and requests for reprints to Renata Stripecke, Department of Medicine, University of California Los Angeles, 675 Charles E. Young Dr. South, MRL-1541, Los Angeles, CA 90095; e-mail: RStripecke@mednet.ucla.edu

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are transported to the cell surface,[6] high levels of MHC-I, co-stimulatory ligands (CD80, CD86), and the CD83 DC marker are expressed, and inflammatory cytokines (IL-12, IL-10) are secreted.[7,8]

Although in vivo priming of $CD8^+$ cytotoxic T lymphocytes (CTLs) generally requires the participation of $CD4^+$ T-helper lymphocytes, the nature of the help provided to CTLs was until recently unknown. CD40 and its ligand, CD40L, were shown to activate the antigen presenting cells (APCs) to allow CTL priming.[9] CD40L, also known as CD154, is a transmembrane protein expressed on activated T-helper cells; it promotes activation of DCs upon its engagement with the CD40 receptor.[10] In vitro studies have shown that triggering CD40 on DCs induces higher IL-12 secretion and a greater level of antigen presentation in comparison with other inflammatory mediators such as bacterial lipopolysaccharide (LPS) or TNF-α.[8] It has been demonstrated in vivo that DCs require maturation via CD40 to generate protective antitumor immunity.[11] DCs treated with soluble trimeric CD40L plus IFN-γ stimulated potent T-cell proliferation to CASTA, a soluble protein from *C. albicans,* induced T cells with augmented antigen-specific lysis, and increased the yield of antigen-specific IFN-γ-producing T cells.[12]

Currently, several clinical protocols for ex vivo production of DCs include a 24- to 48-hour treatment of DCs with soluble trimeric CD40L after they have been loaded with immunologically relevant antigenic molecules to boost DC activity in immunizations.[13] Unfortunately, production of the most effective soluble form of trimeric CD40L has been discontinued, and substitute molecules are not available commercially yet. This has driven investigators to find alternative ways to efficiently mature DCs. In particular, gene transfer of the CD40L sequence may offer persistent endogenous exposure to this maturation factor.

Gene delivery into monocyte-derived DCs with plasmids or retroviral vectors has been reported but has not been shown consistently effective.[14–18] Adenoviral vectors, which are capable of transducing nonreplicating cells, can efficiently transduce DCs,[2,19–21] but they are themselves highly immunogenic, which may hamper immune responses to weaker "self" tumor antigens,[22] or trigger the rejection of transduced cells co-expressing adenoviral antigenic determinants.[23] Furthermore, transduction of mouse DCs with *null* adenoviral vectors at a high multiplicity of infection (MOI > 100) induces some degree of activation by itself,[24] with unpredictable effects on the instruction of immune responses by these DCs in vivo. In contrast to the inconvenience of using these vector systems, lentiviral vectors offer an approach by which simple, efficient, stable, nontoxic, and nonhazardous gene delivery into monocyte-derived DCs may be obtained.

Therefore, we sought to genetically program DCs with lentiviral vectors that provide persistent expression of CD40L. Lentiviral vectors derived from the human immunodeficiency virus (HIV) are good candidates for the development of genetically modified dendritic cells: DCs are one of the natural target cells for HIV, and transduction with improved lentiviral vectors does not generate cytopathic or cytotoxic effects. After transduction, the transgene integrates stably into the genome to provide long-lasting expression. No additional antigenic products are co-expressed by the integrated virus other than the transgene of interest. Hematopoietic cells, including early hematopoietic progenitors and acute leukemia cells, are efficiently transduced by lentiviral vectors.[25,26] It has been demonstrated that lentiviral vectors transduce immature and mature DCs efficiently and stably.[27–29] Transduction of DCs with lentiviral vectors expressing the green fluorescent protein (GFP) did not alter their viability, immunophenotype, or ability to differentiate into mature DCs capable of stimulating autologous T-cell responses.[28] Importantly, lentivirus-transduced DCs to express an antigenic HLA-A2.1 restricted Flu peptide were fully functional and effectively activated autologous Flu-specific CTL responses.[27]

We have previously demonstrated the applicability of lentiviral vectors expressing GM-CSF and CD80 as an efficient gene transfer method to enhance antigen presentation by acute myeloid leukemia cells.[30] In this work, we evaluated the ability of third-generation self-inactivating (SIN) lentiviral vectors to deliver the CD40L gene into immature monocyte-derived human DCs. We show that autocrine production of CD40L by the DCs correlated with augmented $CD8^+$ T-cell stimulation.

MATERIALS AND METHODS

Cell Culture

The 293T and T2 cell lines were obtained from the American Type Tissue Collection (ATCC, Rockville, MD). Human DCs were obtained from adherent peripheral blood cell-derived monocytes cultured in the presence of recombinant human GM-CSF and IL-4, as previously described.[12] PBMCs were obtained from adult healthy volunteers or from HLA-A2.1 haplotype melanoma patients after surgery and/or during remission. The samples were obtained from melanoma patients undergoing immunotherapy treatment, and studies performed

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in accordance with protocols approved by the Los Angeles County/University of Southern California Institutional Review Board. Mononuclear cells were separated by density gradient centrifugation and were used fresh or cryopreserved in 10% dimethyl sulfoxide (DMSO, Sigma, St. Louis, MO) and 90% FBS. Frozen cells were thawed at 37°C and washed immediately. PBMCs were placed in 3 mL serum-free, clinical grade AIM V medium (Gibco-BRL, Gaithersburg, MD) per well in a sterile six-well plate at a concentration of 5×10^6 cell/well, and allowed to adhere to the plate at 37°C with 5% CO_2 for 1 hour. Nonadherent cells were then removed by gently washing the surface of the plate with PBS. Two milliliters fresh AIM V plus 80 ng/mL human IL-4 and 80 ng/mL GM-CSF (R&D Systems, Minneapolis, MN) were added per well. The plates were incubated for 7 days at 37°C in 5% CO_2; on the third day of incubation, the cultures were replenished with additional GM-CSF and IL-4. On the seventh day of culture, DCs were transduced with lentiviral vectors. As a positive control performed in parallel, soluble trimeric CD40L (a generous gift from Immunex, Seattle, WA) was added at 500 ng/mL on day 7 to untransduced DCs; after overnight incubation, the cells were washed twice and replenished with AIM V medium. Thereafter, all procedures were performed in parallel exactly as for transduced DCs.

Construction of Lentiviral Vector

The backbone vector pRRL-sin.hCMV-GFP-pre construct[31] (in this manuscript abbreviated to RRL-GFP) was kindly provided by Dr. Luigi Naldini (University of Milano, Italy). The GFP cDNA was excised by XbaI/SalI digestion and replaced by a 1.1 kb XbaI/SalI fragment from a derivative of the plasmid pBS-hCD40L-6A9 (ATCC) containing the CD40L open reading frame in the correct orientation, resulting in the vector construct pRRL-CD40L (Fig. 1A). The structural integrity of this construct was reconfirmed by restriction digestion and sequencing analysis.

Production of Lentivirus

The constructs required for the packaging of third-generation SIN lentiviral vectors consisted of the plasmid pMD.G (for the production of the VSV-G viral envelope), pMDLg/pRRE and RSV-REV (for expression of the structural proteins, enzymes, and regulatory proteins of HIV), which were provided by Dr. Luigi Naldini (University of Milano, Italy). Lentiviral vectors were produced in large scale by transient co-transfection of 293T cells with the transfer vector and packaging plasmid constructs as described.[26,30] The viral supernatants, collected 48 and 72 hours after transfection, were concentrated by ultracentrifugation and the viral aliquots were cryopreserved at −80°C until use. Viral titer was determined by assessing viral p24 antigen concentration by ELISA (Coulter Immunotech, Miami, FL), and hereafter expressed as μg of p24 equivalent units per milliliter. One μg/mL of p24 measured in the preparation corresponds to approximately 10^7 GFP transduction units/mL, as assessed by titration in 293T cells.

Lentiviral Mediated Gene Transfer

One milliliter of viral suspension containing 5 μg p24 equivalent/mL was seeded per well of a six-well plate containing 2 to 5×10^6 DCs. 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 overnight. The next morning, DCs were washed twice with AIM V medium and maintained in 1 mL AIM V medium. Twenty-four hours later, the supernatant was collected for ELISA and the cells were harvested for FACS analysis or other assays. Where indicated, IFN-γ (Immunex, Seattle, WA) was added at 167 IU/mL at the time of transduction and at the post-washing step.

FACS Analysis

Two days after lentiviral transduction or exposure to soluble CD40L, DCs were detached from the wells by incubation in PBS without Mg^{++} and Ca^{++} for 30 minutes at 37°C/5% CO_2, followed by resuspension. The cells were collected and washed once with PBS, incubated with PBS containing mouse IgG (50 μg/mL) (Sigma, St. Louis, MO) for 15 minutes on ice, stained with the corresponding monoclonal antibody for 20 minutes, washed, and resuspended in 100 μL of 1% paraformaldehyde for fixation. Monoclonal antibodies reactive against CD40, CD86, MHCI (HLA-ABC), and respective isotype antibodies were conjugated with FITC; monoclonal antibodies reactive against CD40L, CD83, and respective isotype antibodies were conjugated with phycoerythrin; antibodies reactive against MHCII (HLA-DR), CD14, and the respective isotype antibodies were conjugated with PerCP (Becton Dickinson/Pharmingen, La Jolla, CA). Flow cytometric analysis was assessed with a FACScalibur cytometer equipped with a 488-nm argon laser (Becton Dickinson, La Jolla, CA). To establish background for fluorescence and to set gates for data acquisition, staining with isotype antibodies was used.

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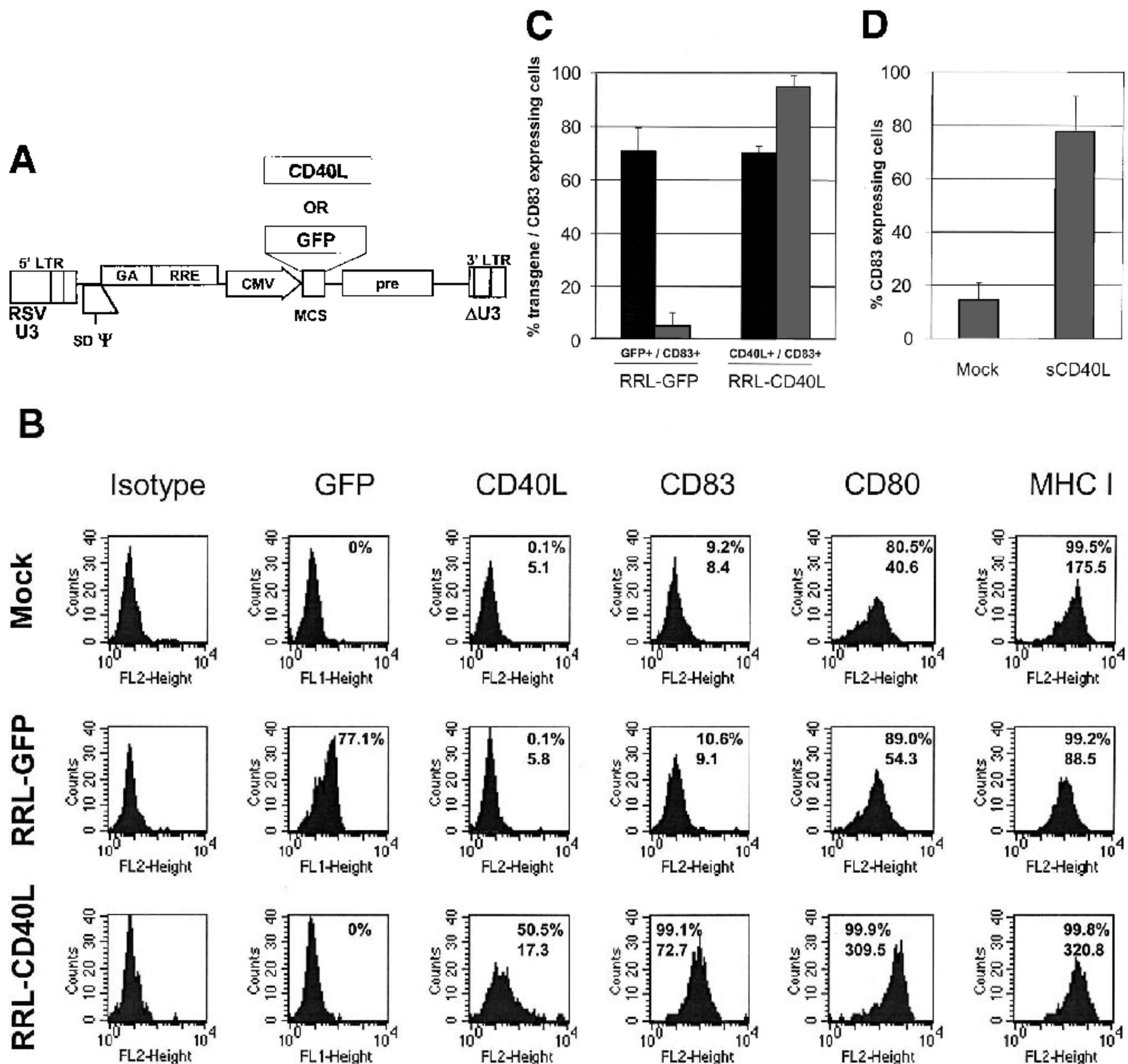

FIGURE 1. Transgene expression after lentiviral transduction of monocyte derived DCs. (**A**) Lentiviral vector construct for gene transfer of GFP and CD40L. The GFP and human CD40L coding sequence were inserted downstream of the internal CMV promoter. A deletion of the viral promoter has been engineered into the U3 region in the 3′ long terminal repeat (LTR), and results in a self-inactivating replacement of the RSV promoter in the U3 region of the 5′ LTR upon reverse transcription. RSV, Rous sarcoma virus promoter; pre, post-transcriptional regulatory element; Ψ, encapsidation signal; SD, splice donor element; SA, splice acceptor element; MCS, multiple cloning site. (**B**) Immunophenotypic analysis of nontransduced (Mock), RRL-GFP or RRL-CD40L-transduced dendritic cells. Cells were harvested 48 hours post-transduction and stained with the corresponding conjugated antibodies against the different markers, except for GFP expression, which was followed in the FL1 channel. Isotype nonspecific control antibodies were used to determine background levels. The annotated percentage of positive cells was calculated by subtracting the mean channel fluorescence values from the isotype control values. The geometric mean of expression for each marker is also noted. (**C**) CD40L transduction correlates with CD83 upregulation. (**D**) Control experiments using mock-treated DCs and DCs treated with soluble trimeric CD40L (sCD40L). Cells were harvested 48 hours post-transduction or sCD40L treatment and stained with conjugated antibodies against CD40L and/or CD83, whereas GFP expression was followed in the FL1 channel. Isotype nonspecific control antibodies were used to determine background levels. The percentage of positive cells was calculated by subtracting the mean channel fluorescence values from the isotype control values. The average and standard deviation values correspond to three independent transduction experiments.

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Cells present in the granulocyte/monocyte gate based on forward and side scatter characteristics were analyzed.

Analysis of IL-12

Production of IL-12 was analyzed by ELISA (R&D Systems) according to the manufacturer's instructions. The minimum detectable concentration of IL-12 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). For purification of $CD8^+$ T cells, we used a positive selection method (CD8 Microbeads, Miltenyi Biotec, Auburn, CA). The separation of the desired population of cells was performed by running the pre-bound cells through an LS magnetic separation column (Miltenyi Biotec). Where applicable, PBMCs were thawed and incubated with 0.1 mg/mL DNAse (StemCell Technologies) for 15 minutes at room temperature, before selection.

Allogeneic T-Cell Activation Analysis

Two days after lentiviral transduction or incubation with soluble CD40L, 1×10^3 DCs were co-incubated with 1×10^5 allogeneic purified $CD3^+$ T cells in 96-well U-bottom tissue culture microplates (Falcon) for 1 to 3 days (1:100 DC:T cell). Co-cultures from triplicate MLR wells were pooled and stained using the FastImmune kit (Becton Dickinson, San Jose, CA) according to manufacturer's specification. A mixture of anti-CD3 PercP/γ1 FITC/γ1 PE was used for the isotype staining, whereas for specific staining CD3 PerCP/CD4 FITC/CD69 PE or CD3 PerCP/CD8 FITC/CD69 PE mixtures were used. A gate (R1) set on the $CD3^+$ T-lymphocyte fraction was set for quantitative analysis of $CD4^+/CD69^+$ or $CD8^+/CD69^+$ subpopulations.

Generation of CTLs

Two days after transduction, 1×10^5 DCs were co-cultured 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) containing RPMI and 2% human serum. Ten ng/mL human interleukin-7 (IL-7, R&D Systems) and 10 U/mL recombinant human interleukin-2 (IL-2, R&D Systems) were added on day 1 and 3 of culture, respectively. On day 7, CTLs were collected for the cytotoxicity analysis.

Cytotoxicity Analysis

CTLs were assayed for specific lysis of peptide-pulsed Tap-deficient target cells (T2 cells). All peptides used were synthesized by the USC/Norris Cancer Center Microchemical Core Facility and were HLA-A2.1 restricted. The HPV E7 (86-93) peptide (TLGIVCPI) was used as a negative control peptide, whereas the Flu-M1 (58-66) peptide (GILGFVFTL) was used to detect $CD8^+$ T-cell immune responses against the influenza virus, and the gp100 210M (209-217) peptide (IMDQVPFSV) was used to detect $CD8^+$ T-cell responses against the gp100 melanoma-associated tumor antigen. T2 target cells were incubated with 10 μg peptide in 10 mL medium for 16 hours and then washed, and 1×10^6 cells were incubated for 2 hours in the presence of 100 μCi ^{51}Cr (1 μCi/μL) (Perkin-Elmer, Boston, MA). The labeled target cells were washed three times in RPMI 2% HS and plated in triplicate wells at a minimum of 3,000 targets per well. Effector cells were added to the target cells at different effector:target ratios in 96-well round bottom plates (Falcon). After incubation for 4 hours at 37°C/5% CO_2, 100 μL supernatant was collected from each well and the ^{51}Cr released was measured using a gamma counter. Specific lysis was calculated following the formula: (test lysis – spontaneous lysis): (total lysis – spontaneous lysis).

RESULTS

Third-Generation SIN Lentiviral Vectors Efficiently Transduce Human Monocyte-Derived DCs, Leading to Phenotype Maturation

Human immature monocyte-derived DCs were prepared from adherent PBMC-derived cultures using standard techniques, as previously described.[13] Generally, more than 80% of the initiating culture was $CD14^+$, and 7 days after culture in the presence of recombinant GM-CSF and IL-4, 80% to 90% of this population was observed to be immunophenotypically homogeneous, showing high expression of MHC and the co-stimulatory molecules CD80 and CD86 concomitant with complete disappearance of the monocytic marker CD14 (data not shown, but also see mock control, Fig. 1B).

Lentivirus vector-mediated transduction of this immature DC population was optimized under conditions of increasing p24 equivalent concentrations (data not

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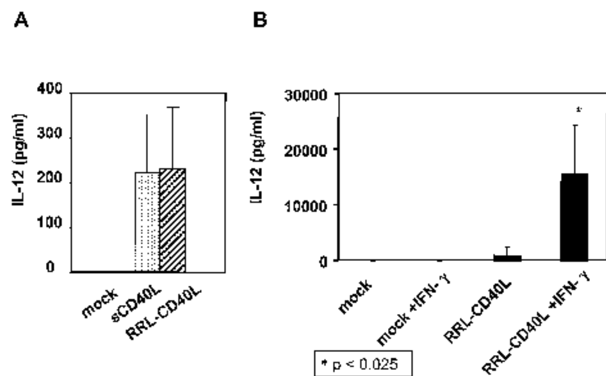

FIGURE 2. (**A**) IL-12 production after CD40L lentiviral transduction of DCs. DC controls (mock), those treated with soluble trimeric CD40L (sCD40L), or those transduced to express CD40L (RRL-CD40L) were analyzed 48 hours after treatment. Secretion of IL-12 by the different DC groups was analyzed by ELISA. The average and standard deviation values correspond to triplicates. (**B**) Presence of IFN-γ during transduction enhances the production of IL-12. DC supernatants were harvested 48 hours post-transduction, and secretion of IL-12 was analyzed by ELISA. Nondetectable IL-12 production was seen by untransduced cells incubated in the presence of IFN-γ. The average and standard deviation values correspond to four independent experiments. *P* value represents IL-12 production after RRL-CD40L transduction and IFN-γ treatment compared with RRL-CD40L transduction only.

shown). The efficiency of transduction was measured by the percentage of GFP- and CD40L-expressing cells, as analyzed by flow cytometry. The standard transduction protocol, which yielded the most consistent results, included the cultivation of DCs in the presence of 5 μg p24 equivalent/mL lentiviral vectors, which corresponds to an MOI of approximately 50. Under these conditions, the percentage of cells transduced with RRL-GFP and RRL-CD40L was comparable, typically in the range of 70% (see Figs. 1B, 1C).

The effects of lentiviral gene delivery on the DC immunophenotype were assessed by flow cytometry analysis. Expression of the DC maturation marker CD83 was observed at low percentages in the control GFP transduced DCs (<5%), whereas more than 90% of the population of DCs transduced with CD40 showed CD83 expression (see Figs. 1B, 1C). Upregulation of the costimulatory ligand CD80 and of MHC class I molecules was also consistently observed in RRL-CD40L transduced DCs, whereas transduction of DCs with a vector expressing GFP resulted in a pattern similar to the untransduced group (see Fig. 1B). Therefore, the immunophenotype analysis clearly indicated that expression of the CD40L transgene promoted DC maturation. As expected, upregulation of CD83 expression in DCs after exposure to CD40L was observed both after RRL-CD40L transduction and after treatment of DCs with soluble recombinant CD40L (see Figs. 1C, 1D).

CD40L Lentiviral Transduction Results in IL-12 Production, Which Is Enhanced by Co-treatment With Recombinant IFN-γ

The ability of mature DCs to induce T helper 1 (T_H1) responses has been correlated with the secretion of IL-12.[8] Therefore, DC maturation was assessed by measuring IL-12 secretion 48 hours after incubation with sCD40L or transduction with RRL-CD40L. Both soluble and transduced CD40L treatments resulted in increase of IL-12 in the supernatant compared with nontreated controls (Fig. 2A).

Activation of DCs by CD40L can be synergistically augmented by IFN-γ, to shift the immune response toward the T_H1 pathway.[32] Since enhancement of antitumor responses by CTL requires a polarization of T_H1 responses, we determined whether the presence of IFN-γ during DC transduction with RRL-CD40L could enhance the T_H1 stimulation pattern (i.e., by increasing IL-12 production). Whereas, as expected, treatment of untransduced DCs with IFN-γ alone did not stimulate production of detectable levels of IL-12, IL-12 production was significantly enhanced by IFN-γ treatment during RRL-CD40L transduction, resulting in a drastic increase of secreted cytokine (see Fig. 2B).

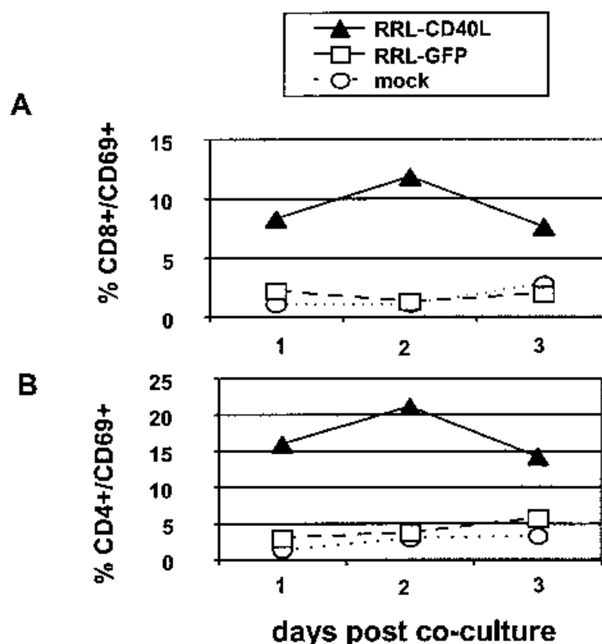

FIGURE 3. Kinetics of allogeneic T-cell activation. Allogeneic T cells co-cultured with DCs that were nontransduced (circles), transduced with RRL-GFP (squares), or transduced with RRL-CD40L (triangles) were analyzed by expression of the CD69 activation marker. (**A**) Activation profile of $CD8^+$ cells. (**B**) Activation profile of $CD4^+$ cells.

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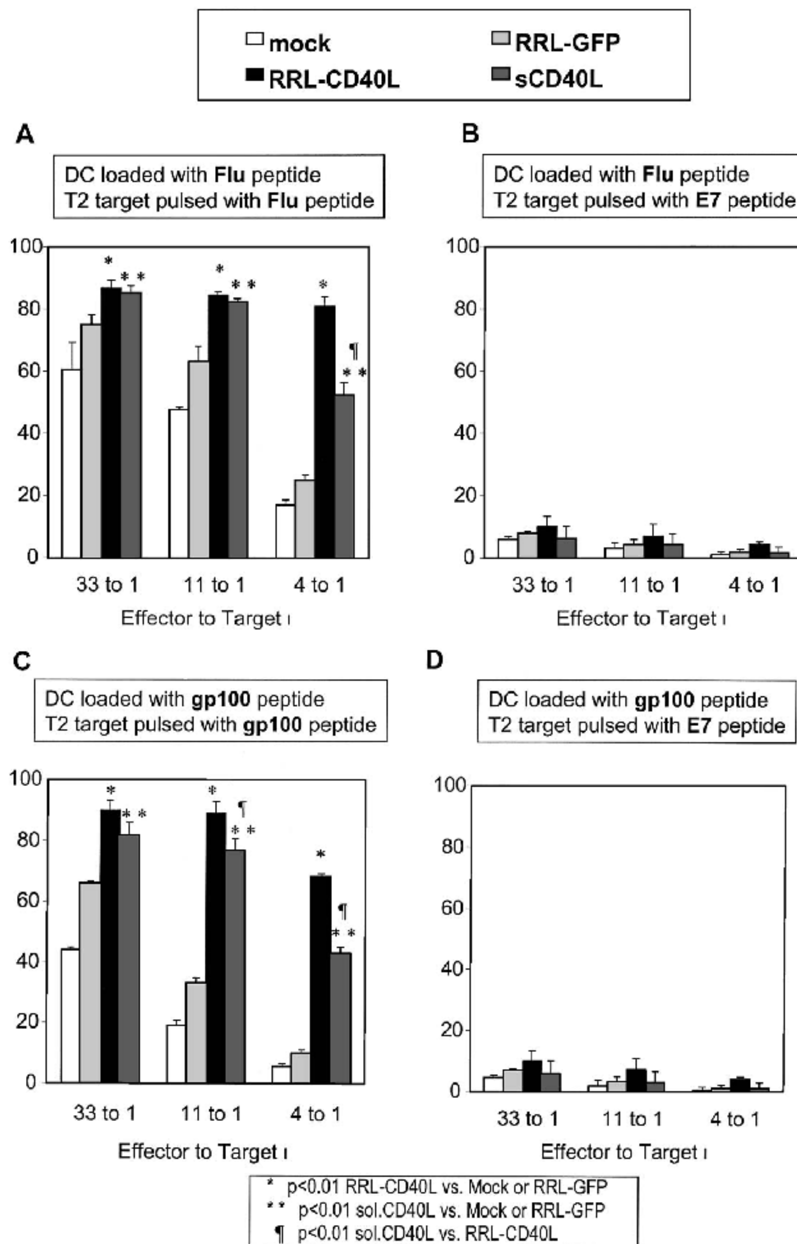

FIGURE 4. CTL assay against an influenza and melanoma-associated antigenic peptide. DCs that were not transduced (mock), transduced to express GFP or CD40L, or treated with soluble CD40L were pulsed with the Flu-M1 or gp100 target peptides and used for coculture with autologous purified $CD8^+$ cells in the presence of IL-2 and IL-7. The effector cells were harvested 7 days after coculture and allowed to react against T2 target cells labeled with ^{51}Cr and pulsed with two different peptides. (**A**) DCs: loaded with Flu antigen, T2: pulsed with Flu. (**B**) DCs: loaded with Flu, T2: pulsed with E7. (**C**) DCs: loaded with gp100, T2: pulsed with gp100. (**D**) DCs: loaded with gp100, T2: pulsed with E7.

Stimulation of Allogeneic $CD4^+$ and $CD8^+$ T Cells by RRL-CD40L-Transduced DCs

We first examined the functionality of phenotypically mature DCs after lentiviral gene transfer of CD40L using an allogenic MLR assay. Activation of T cells after coculture with CD40L-transduced DCs was assessed by cell surface expression of CD69, an early activation marker. Untransduced (mock) DCs and DCs transduced either with the control vector RRL-GFP or RRL-CD40L in the presence of IFN-γ were co-cultivated with allogeneic $CD3^+$ purified T cells for up to 3 days. As expected, coculture of T cells with mock- or RRL-GFP-transduced immature DCs produced only low levels of T-cell activation throughout the 3-day period (<5%) (Fig. 3). In contrast, RRL-CD40L-transduced DCs resulted in stimulation of $CD8^+$ and $CD4^+$ cells, the peak of activation being achieved 2 days after coculture (see Fig. 3). We then examined whether transduction of DCs with RRL-CD40L could support the activation of autologous T cells. Untransduced DCs (mock) and DCs transduced with RRL-GFP or RRL-CD40L were loaded with an

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HLA-A2.1 restricted Flu epitope (Flu M1) and used to stimulate autologous CD3$^+$ purified T cells. After 2 days, the cells were harvested and analyzed by flow cytometry for the expression of the activation marker CD69 by CD8$^+$ and CD4$^+$ cells. Compared with the percentage of autologous T cells showing baseline (1-2%) levels of CD69 expression, in the overall population, exposure to RRL-CD40L-transduced DCs resulted in approximately 2-fold stimulation of CD8$^+$ and CD4$^+$ cells (data not shown).

CTL Responses Against Flu-Specific and gp100-Specific Peptides Presented by RRL-CD40L-Transduced DCs

Next, the ability of RRL-CD40L-transduced DCs to stimulate CD8$^+$ CTLs in the absence of CD4$^+$ help was evaluated using Flu-M1 (Figs. 4A, 4B) and the melanoma-associated tumor antigen gp100 (see Figs. 4C, 4D). The matched autologous DC/T-cell samples used for CD8$^+$ restimulation in vitro were obtained from melanoma patients in remission, previously shown to have immune responses against Flu and gp100. Untransduced or transduced DCs were loaded with the Flu or gp100 peptides under standard conditions and used as stimulators of autologous CD8$^+$ purified T cells for 7 days. For comparison, soluble CD40L-treated DCs were used as a positive control in parallel. All CD8$^+$ T-cell treatment groups were then used in lysis assays with ^{51}Chromium-labeled T2 cells that had been previously loaded with the respective target peptide or an irrelevant control peptide (E7) at different effector:target ratios. For both Flu and gp100 target peptides, significantly higher stimulation of CTL activity was observed for the RRL-CD40L-transduced DC group in comparison to mock or negative control RRL-GFP-transduced DCs ($P < 0.05$; see Figs. 4A, 4C). In contrast, only overall low lysis was measured (<10%) when the irrelevant E7 peptide was used to pulse the T2 target cells, decreasing the possibility of non-HLA-mediated lysis (see Figs. 4B, 4D). Similar results were consistently obtained for CTL assays performed with different autologous matched samples, demonstrating a general phenomenon for the increased target-specific stimulation of CD8$^+$ cells by RRL-CD40L-transduced DCs. Of note, with either of the two target antigens, DC transduction with RRL-CD40L appeared to consistently provide higher stimulation than treatment with soluble CD40L at lower nonsaturating effector:target ratios (see Figs. 4A, 4C).

DISCUSSION

We were able to demonstrate the applicability of lentiviral vectors expressing CD40L to mature monocyte-derived DCs ex vivo. Compared with other gene delivery systems, lentiviral vectors offer significant advantages for genetic modification of DCs: lack of vector immunogenicity and cytotoxicity, ease of vector production, efficient gene transfer, and stable expression.

The results obtained here are consistent with previous observations that lentiviral vector transduction is a suitable methodology for efficient gene delivery into DCs.[27,28] Previous reports have explored first- or second-generation lentiviral vectors.[27,28] The lentiviral vectors evaluated here are third-generation vectors containing SIN mutations that eliminate the 5′ long terminal repeat (LTR) promoter after integration, thus drastically reducing the risks of virus rescue and/or production of replication competent lentivirus. In the SIN transfer vector construct, the 5′ LTR is chimeric, with the enhancer/promoter of RSV replacing the natural U3 region of the HIV LTR to rescue its transcriptional dependence on *tat*.[33] The 3′ LTR, which serves as the template to regenerate the U3 region in the 5′ LTR, has an almost complete deletion of its U3 region; therefore, upon reverse transcription, transcriptional inactivation of both LTRs in the integrated provirus occurs.[34] The RRL-SIN backbone was further designed to contain a constitutive hCMV internal promoter and a post-transcriptional regulatory element ("pre") from the woodchuck hepatitis virus in the 3′ untranslated region of the transgene, to increase RNA stability and therefore protein production.[31] We were able to demonstrate here that improved and safer third-generation SIN lentiviral vectors very efficiently delivered GFP and CD40L genes into DCs with an average transduction efficiency of 70% under the conservative MOI conditions used (=50). After transduction, DC activation was provided only by the immunomodulatory transgene (CD40L) but not by a vector expressing an immunologically inactive gene (GFP), indicating that lentiviral transduction by itself is unlikely to activate DCs. Further enhancement of the CD40L-induced maturation phenotype (i.e., IL-12 production) was achieved by co-incubation of DCs with recombinant IFN-γ during transduction. Interestingly, we consistently observed almost complete maturation of the entire population of DCs exposed to the CD40L vector, even when transduction levels were as low as 50%. This suggests that CD40L transduction also resulted in an indirect maturation effect on untransduced cells. Although the effects of CD40L on DC viability/apoptosis still remains an area of debate,[35,36] we have not observed significant effects from CD40L gene transfer upon DC cell viability as analyzed by proliferation and metabolic assays (data not shown).

In the in vitro assays here described, DC/CD40L were

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superior to nontransduced or GFP-transduced DCs for the stimulation of both allo- and auto-T cells. In particular, we were able to show that purified autologous $CD8^+$ cells directed against HLA-A2.1 restricted Flu or gp100 peptides were efficiently stimulated by CD40L-transduced DCs. The data presented demonstrated that RRL-CD40L lentiviral transduction is a good alternative to the use of soluble trimeric CD40L to stimulate DC maturation, as observed by immunophenotype, cytokine production, and T-cell activation. In conclusion, transduction of monocyte-derived DCs with third-generation SIN lentiviral vectors expressing CD40L represents a promising approach that warrants further preclinical and clinical evaluation.

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