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Langerhans-Type Dendritic CellsGenetically Modified to Express Full-Length Antigen Optimally Stimulate CTLs in a CD4-Dependent Manner

Jianda Yuan,*Paul Jean-Baptiste Latouche,‡Joanna Hodges,§Alan N. Houghton,††Michel Sadelain,*‡‡Isabelle Riviere,*‡‡† and James W. Young3*

Oncoretroviral vectors encoding either full-length Ag or a corresponding immunodominant peptide were expressed in Langerhans-type dendritic cells (LCs) differentiated from CD34+ progenitors. We used human CMV as a model Ag restricted by HLA-A*0201 to define parameters for eventual expression of cancer Ags by LCs for active immunization against tumors. Stimulation by CMVpp65495–503-pulsed LCs, CMVpp65495–503-transduced LCs, and full-length CMVpp65-transduced LCs respectively increased tetramer-reactive T cells with an effector memory phenotype by 10 ± 11, 34 ± 21, and 51 ± 24-fold (p < 0.05) from CMV-seropositive donors. CMV-specific CD8+ CTLs achieved respective frequencies of 231 ± 102, 583 ± 219, and 714 ± 281 spot-forming cells per 105 input cells (p < 0.01) in ELISPOT assays for IFN-γ secretion. LCs expressing full-length Ag stimulated greater lytic activity than either peptide-transduced or peptide-pulsed LCs (p < 0.05), all in the absence of exogenous cytokines. pp65-transduced LCs presenting class I and II MHC-restricted epitopes expanded IFN-γ-secreting CD4+ T cells, whereas pp65495–503-transduced LCs did not. CD4+ T cell numbers even declined after stimulation by pp65495–503 peptide-pulsed LCs. CD4+ T cell depletion confirmed their contribution to the more robust CTL responses. LCs, transduced with a retroviral vector encoding full-length Ag, stimulate potent Ag-specific T cells directed against multiple epitopes in a CD4+ TH cell-dependent manner. The Journal of Immunology, 2006, 176: 2357–2365.

Human CD34+ hemopoietic progenitor cells (HPCs) transduced by oncoretroviral constructs can differentiate into Langerhans-type dendritic cells (LCs) that express vector-encoded Ags (1). Gene-transduced LCs maintain the activated phenotype as well as the potent immunogenicity expected of mature dendritic cells (DCs) (1) and therefore offer enormous potential for manipulating human immune responses. DCs terminally differentiated from CD34+ HPCs over 2 wk in vitro also lose clonal or proliferative potential (2, 3). Hence, this loss mitigates concerns about malignant transformation due to insertional mutagenesis when administering gene-modified DCs in vivo.

Among the defined types of conventional human DCs, we have demonstrated that peptide-pulsed LCs are more potent than dermal-interstitial DCs or blood monocyte-derived DCs in stimulating CTL against a recall viral Ag in vitro (4). Monocyte precursors of monocyte-derived DCs also do not undergo further cell division and thus do not expand like CD34+ HPCs. We have recently developed a reproducible system for the oncoretroviral transduction of CD34+ HPCs-derived human LCs in vitro in the absence of FCS (1), as FCS would otherwise mitigate their clinical application as cell-based vaccines. Using an influenza viral recall Ag, we have additionally demonstrated that LCs, genetically modified to express influenza matrix peptide, are more potent stimulators of Ag-specific CD8+ T cell responses than are peptide-pulsed LCs.

Optimal manipulation of cellular immunity in vivo will most likely require an approach that encompasses multiple Ags restricted to class I and class II MHC. CD4+ T lymphocyte responses can play an important role, not only in generating more robust CTL responses, but also in maintaining those responses and generating immunologic memory (5–9). We have therefore evaluated their role in enhancing the immune response stimulated by LCs transduced to express class II as well as class I MHC-restricted Ags derived from the full-length immunogenic CMVpp65 (10).

The goal was to define parameters for the eventual expression of cancer Ags by LCs for active immunization against tumors, using human CMV as the model. Our intention was not to expand large numbers of T cells ex vivo for adoptive immunotherapy, where the role of CD4+ T cells in such expansion has been difficult to distinguish from the role of exogenous cytokines (11–14). Furthermore, even though most tumor Ags are either self or self-differentiation Ags, we considered recall responses against an immunogenic viral Ag useful for establishing the principal parameters of Ag presentation by gene-transduced LCs.
Most investigators have used monocyte-derived DCs for preclinical and clinical immunotherapy studies, but the blood monocyte precursors are no longer cycling and hence lack susceptibility to retroviral transduction. The breadth of the CTL response stimulated by CD34+ HPCs-derived LCs transduced with full-length Ag was therefore compared with that stimulated by LCs expressing only a single class I MHC-restricted epitope. Among the latter group of LCs, we compared those transduced with a vector encoding only the immunodominant HLA-A*0201-restricted CMVpp65\textsubscript{495–503} peptide with LCs pulsed by the same peptide, thereby assessing the contribution of more sustained Ag expression after gene transduction.

Overall, these experiments yield proof-of-principle data regarding the afferent or sensitization arm of cellular immunity against Ags encoded by oncoretroviral transduction. This approach should in turn provide a means of broadening the range of MHC alleles that could present immunogenic but undefined peptide Ags and should prove applicable to active immunotherapies against tumors and pathogens.

Materials and Methods

**Media**

X-VIVO 15 (BioWhittaker) without serum or plasma was used to culture CD34+ HPCs for generating LCs. Functional assays for stimulating and assessing T cell responses used complete RPMI 1640 (containing 10 mM HEPES (Invitrogen Life Technologies), 1% penicillin/streptomycin (Media Lab, Sloan-Kettering Institute, New York, NY), 50 \(\mu\)M 2-ME (Invitrogen Life Technologies), and 1% t-glutamine (Invitrogen Life Technologies)), supplemented with 10% heat-inactivated (56°C for 30 min) autologous or single donor human plasma or serum. PG13 viral packaging cells were cultured in DMEM (Invitrogen Life Technologies) with penicillin/streptomycin, t-glutamine, and 2-ME as described, but supplemented with 10% FCS (Gemini Bio-Products). Packaging cells were thoroughly washed free of FCS and recultured in X-VIVO 15 for collection of retroviral stocks.

**Cytokines**

Sterile recombinant, LPS-, pyrogen-, and mycoplasma-free human cytokines included GM-CSF (sargramostim, Leukine; Berlex); FLT-3 ligand, TNF-\(\alpha\), TGF-\(\beta\), thrombopoietin, c-kit ligand or stem cell factor, IL-1p, IL-6 (all R&D Systems); and PGE\(_2\) (Calbiochem). All cytokines were supplied carrier-free by the manufacturer but were reconstituted using HSA (1% final HSA, 25% HSA, NDCs 63546-251-05 pharmaceutical grade, manufactured by Swiss Red Cross, distributed by Alpine Biologies) in PBS. Cytokine doses are specified below or as recently published (4) for the generation and maturation of CD34+ HPCs-derived human LCs.

**Oncoretroviral vectors, packaging cell lines, and titration of retroviral supernatants**

The SFG retroviral vector (15) encoded the Ag of interest, expressed under the transcriptional control of the Moloney murine leukemia virus long-terminal repeat. Constructs were designed that carried the gene encoding the Ag of interest was placed under the transcriptional control of the Moloney murine leukemia virus long-terminal repeat. Bicistronic retroviral constructs were made that carried the gene encoding puromycin-N-acetyltransferase resistance (Puromycin\(^R\)). This gene was joined by an internal ribosomal entry site (IRES) to a gene that encoded eGFP, an immunodominant HLA-A*0201-restricted 9mer peptide (p495–503, NLVPMVATV) of CMV pp65, or the full-length structural CMV protein pp65 (p1–561). The splice donor (SD) site, splice acceptor (SA) site, and the extended packaging signal (\(p_p^p\)) are indicated.

**FIGURE I.** Schematic representation of the SFG-eGFP, SFG-CMVpp495, and SFG-CMVpp65 vectors. The SFG retroviral vector encoding Ags of interest was placed under the transcriptional control of the Moloney murine leukemia virus long-terminal repeat. Bicistronic retroviral constructs were made that carried the gene encoding puromycin-N-acetyltransferase resistance (Puromycin\(^R\)). This gene was joined by an internal ribosomal entry site (IRES) to a gene that encoded eGFP, an immunodominant HLA-A*0201-restricted 9mer peptide (p495–503, NLVPMVATV) of CMV pp65, or the full-length structural CMV protein pp65 (p1–561). The splice donor (SD) site, splice acceptor (SA) site, and the extended packaging signal (\(p_p^p\)) are indicated.

**Collection of human material for research**

All use of human specimens adhered to protocols reviewed and approved by the Institutional Review and Privacy Board of Memorial Hospital, Memorial Sloan-Kettering Cancer Center. All investigators were trained and certified in the protection of research rights in human research.

**Retroviral transduction and differentiation of CD34+ HPCs-derived DCs**

Bone marrow or G-CSF-mobilized peripheral blood stem cells were obtained as excess cells from donors already undergoing collection for other therapeutic protocols. CD34+ HPCs were isolated using positive immunomagnetic selection (CD34+ isolation and LS separation columns; Miltenyi Biotec) as previously reported (4). CD34+ HPCs were initially cultured at 2 \(\times\) 10\(^5\) cells/3 ml/well in a six-well tissue culture plate (Costar; Corning) in the presence of thrombopoietin (50 ng/ml; R&D Systems), c-kit ligand (20 ng/ml; R&D Systems), and FLT-3 ligand (50 ng/ml; R&D Systems) (1). Twenty-four hours from the initial start of culture, the cycling CD34+ HPCs were transferred to nontissue culture-treated plates coated with RetroNectin (Takara Bio) (1). The cells in X-VIVO 15 and in thrombopoietin, FLT-3 ligand, and c-kit ligand were then exposed to 1:1 volume ratio to FCS-free and packaging cell-free (0.45 \(\mu\)m filtration, Accrodisc; Pall Gelman Laboratory) retroviral stocks over 16 h in the presence of polybrene (8 \(\mu\)g/ml; Sigma-Aldrich). Target cells had already been plated overnight, and one replicate well was used at the time of infection to measure the exact viable target T cell number. At 72–96 h from the start of the transduction, the HeLa cells were analyzed. Enhanced GFP expression was assessed by flow cytometry. Puromycin resistance was determined by enumeration of viable colonies. The fraction of HeLa cells that yielded 10–50% eGFP-positive cells or viable puromycin-resistant colonies was used to calculate the titer using the percentage of eGFP-positive or the percentage of puromycin-resistant/viable cells multiplied by the HeLa cell number multiplied by dilution factor, resulting in the number of infectious particles per milliliter.

T lymphocytes and CD4\(^+\) T cell depletion

T cells were obtained from tissue culture plastic (BD Falcon brand tissue culture plates no. 35-3003; BD Labware) nonadherent PBMCs. T cells...
were further purified by nonadherence and elution from nylon wool columns (Polysciences). CD3+ purity was at least 95%. Where CD4+ T cell depletion was required, the bulk T cells were opsazonized with anti-CD4 magnetic beads (Dynal Biotech) at a final concentration of 4 × 10⁶ beads per 1 × 10⁶ T cells/ml. Bead-opsazonized cells were incubated for 30 min at 4°C, followed by magnetic depletion and washing of the remaining cells. Flow cytometry confirmed purity to be at least 95% CD³⁺ CD⁸⁻ T cells.

**Human CMV peptide (CMVpp65₄₉₅–₅₀₃) and HLA-A*0201-CMVpp65₄₉₅–₅₀₃ tetramer synthesis**

The CMV structural protein pp65 includes an HLA-A*0201-restricted, immunodominant, cytotoxic T lymphocyte (CTL) ligand, pp65₄₉₅–₅₀₃, which was commercially synthesized (ResGen; Invitrogen Life Technologies). The CMV peptide sequence and m.w. were confirmed upon receipt, resuspended in 50% (v/v) RPMI-DMSO (Sigma-Aldrich), and stored frozen at −20°C. Peptides were thawed once for dilution and use.

The HLA-A*0201-CMVpp65₄₉₅–₅₀₃ peptide tetramer was synthesized using the described synthetic peptide and conjugated with streptavidin-PE (Tetramer Core Facility, Sloan-Kettering Institute). Positive tetramer reactivity required a distinct population —1 log above the mean fluorescent intensity of the negative population.

**Induction of CMV-specific T cell responses**

Mature LCs, which had been transduced during pre-expansion in thrombopoietin, FLT-3 ligand, and e-kit ligand with retroviral vectors encoding either the CMVpp65₄₉₅–₅₀₃ peptide (p495-LCs) or the full-length CMVpp65 (p65-LCs), were washed free of cytokines and resuspended in complete RPMI 1640 –10% autologous heat-inactivated serum or plasma. For peptide loading in lieu of gene transduction, mature LCs (10⁶ cells/ml with 75 μg/ml human CMV pp65₄₉₅–₅₀₃ peptide for 1 h at room temperature (CMV-pulsed LCs). Each of the three types of CMV Ag-loaded LCs were then cocultured with 2 × 10⁶ purified autologous HLA-A*0201 T cells from human CMV-seropositive donors in 24-well tissue culture plates (Costar; Corning) at a ratio of 1:30 LCs to T cells. After an initial 6–7 days’ stimulation, the responder T cells were harvested, washed, and restimulated by the same type of LCs for a second round of stimulation over another 5–7 days. Although it was not critical to restimulate T cells using a potent viral Ag-like CMV, differences between experimental groups were more definitive after the second stimulation.

**Phenotypic analyses by flow cytometry**

Fluorescein (FITC), PE, CyChrome, and allophycocyanin-conjugated mouse anti-human Abs included anti-CD3, anti-CD4, anti-CD8, anti-CD45RA, anti-CD27, anti-CD62 ligand, anti-CCR7 (all from BD Biosciences), and anti-IFN-γ (Miltenyi Biotec). Isotype controls included IgG1-FITC, IgG1-PE, and IgG2a-FITC (DakoCytomation). Flow cytometry assessment used three- and four-color analyses on a FACSCalibur (BD Biosciences), and anti-IFN-γ Ab (clone 7-B6-1; Mabtech). After washing the wells five to six times with PBS/0.05% Tween 20, peroxidase-labeled streptavidin was added. The spots were developed with AEC substrate (3-amino-9-ethylcarbazole, Vector ABC kit; Vector Laboratories). Spot development was stopped after 4 min by washing with running distilled water. The plates were examined under a stereomicroscope, and spots were evaluated with an Automated ELISPOT Reader System with KS 4.3 software (Carl Zeiss).

**IFN-γ secretion assay**

Measurement of IFN-γ secretion by Ag-specific T cells was performed using a flow cytometry based assay (IFN-γ Secretion kit; Miltenyi Biotec). Targets for restimulation of T cells were either CMVpp65₄₉₅–₅₀₃ peptide-pulsed K562-HLA-A*0201 transfectants (a gift of Dr. Thomas Wölfel, University of Mainz, Mainz, Germany) (19) or the same mature transduced or peptide-pulsed LCs as had been used for initial sensitization. The tetramer-labeled T cells and the target cells for restimulation were cocultured overnight for 18 h at 37°C. Secreted IFN-γ was then detected on the T cell surface by opsonizing cells for 5 min at 4°C with an IFN-γ-specific, high affinity capture matrix, which is a bispecific Ab-Ab conjugate directed against both CD45 and IFN-γ. Cells were then transferred to warm medium at 37°C for 45 min to permit secretion of IFN-γ. Thereafter, the cells were washed and stained for 30 min at 4°C with Abs to any additional epitopes of interest, washed again, and immediately analyzed on a FACSComp (BD Biosciences) using CellQuest software.

**Chromium release CTL assay**

Cytotoxicity exerted by T lymphocytes responding to initial LCs sensitization but tested as effectors against radiolabeled targets was measured in a standard ⁵¹Cr release assay. Targets were K562-HLA-A*0201-transfected cells that were pulsed at 10 × 10⁶ cells/ml with 10 μM CMVpp65₄₉₅–₅₀₃ peptide for 1 h at room temperature or overnight at 2 × 10⁶ cells/ml with 1 μM CMVpp65₄₉₅–₅₀₃ peptide at 37°C. Target cells were labeled with 75 μCi of ⁵¹Cr (Na²⁹CrO₄; New England Nuclear) per 2 × 10⁶ cells/ml at 37°C for 1 h. A total of 2 × 10⁶ radiolabeled target cells was plated per round-bottom well of a 96-well microtiter plate (Costar; Corning). Graded doses of responder T cells, acting as effectors in this CTL assay, were added in triplicates to the microwells. T cell counts were based on total viable cells, not just T cell blasts or CD⁸⁺ T cells, so that total cytolytic activity generated per initial sensitization condition would be measured. A 30-μl supernatant was removed from each microwell after 4–6 h, transferred to a LumaPlate-96 (Yitrium Silicate Scintillator-coated white microplate, TopSeal-A; Packard BioScience) and counted in a gamma counter (Topcount NXT, Microplate Scintillation and Luminescence Counter; Packard BioScience). Cytolytic activity was calculated in the typical manner, using triplicate means to obtain the quotient of the experimental ⁵¹Cr release less the spontaneous ⁵¹Cr release, divided by the total ⁵¹Cr release less the spontaneous ⁵¹Cr release, and then multiplied by 100 to give a percentage. The percentage of lysis of K562 or empty K562 HLA-A*0201-transfected cells was subtracted from the lysis of CMVpp65₄₉₅–₅₀₃ peptide-pulsed targets to yield a final percentage of specific lysis.

**Statistics**

The test of equality of means between groups was based on the two-sample t statistic. A stratified t test was used for group comparisons in functional assays, in which the stratification variable was dose level.

**Results**

**Expression of the full-length CMVpp65 by retroviral transduction of human CD34⁺ HPCs-derived LCs enhances the expansion of HLA-A*0201-CMVpp65₄₉₅–₅₀₃ tetramer-reactive CD⁸⁺ T cells**

We first focused on class I MHC-restricted CD⁸⁺ T cell responses. We asked whether CD34⁺ HPCs-derived LCs expressing a transgene encoding CMV Ags, especially the full-length structural protein pp65, were more potent in promoting CD⁸⁺ T cell responses over LCs pulsed with a single immunodominant peptide derived from that same protein. The frequency of CD⁸⁺ T cells reacting with HLA-A*0201-CMVpp65₄₉₅–₅₀₃ peptide tetramers was determined by flow cytometry after two rounds of stimulation by each of the LCs populations (Fig. 2A). The transduced LCs stimulated a higher proportion of tetramer-reactive cells among the total CD⁸⁺ T cells than did the peptide-pulsed LCs. LCs transduced with the full-length pp65 also stimulated a higher proportion of tetramer-reactive CD⁸⁺ T cells than did LCs transduced only with...
the pp65\textsubscript{495-503} peptide. The expansion of HLA-A*0201 CMV tetramer-positive cells after stimulation with eGFP-LCs was negligible.

We confirmed these results in four different CMV-seropositive donors by calculating the overall fold increase in tetramer-reactive CD8\(^+\) T cells over baseline among the total responder population from each of the same four donors (p < 0.05 for each pairwise comparison as indicated) (Fig. 2B). Thus sustained expression of Ag by a retroviral vector enhanced the stimulation of tetramer-reactive CD8\(^+\) T cells. We hypothesized that expression of the full-length CMVpp65 also provided additional class II MHC-restricted helper epitopes that warranted further evaluation.

**FIGURE 2.** Gene modification of CD34\(^+\) HPCs-derived LCs to express CMV Ags, especially the full-length CMVpp65, supports a significantly greater expansion of HLA-A*0201/CMVp495–503 tetramer-reactive CD8\(^+\) T cells than does stimulation by CMVpp65\textsubscript{495-503} peptide-pulsed LCs. HLA-A*0201 T cells from a CMV-seropositive donor were stimulated by autologous CMVpp65-transduced LCs (pp65-LCs), CMVpp65\textsubscript{495-503} transduced LCs (p495-LCs), and CMV pp65\textsubscript{495-503} peptide-pulsed LCs (CMV-pep-LCs), or eGFP-transduced LCs (eGFP-LCs) at an LCs to T cell ratio of 1:30. A, One representative assessment among four donors is shown. The percentage listed in each dot plot (right upper quadrant) indicates the percentage of CD8\(^+\) T cells that were also tetramer-PE-reactive (right upper quadrant events divided by the sum of right upper quadrant plus right lower quadrant events \(\times 100\), after one and two rounds of stimulation each over 5–7 days. B, To represent the tetramer expansion across all four donors, we divided the total CD8\(^+\) tetramer-positive cells for each donor by the number of CD8\(^+\) tetramer-positive cells at the start of each round of stimulation. The overall fold increase in CD8\(^+\) tetramer-positive cells (far right) was 51.3 \(\pm\) 24.1, 33.8 \(\pm\) 20.9, 10.4 \(\pm\) 10.7, and 0.72 \(\pm\) 0.33-fold after stimulation with CMVpp65-transduced LCs (\(\square\)), CMVpp65\textsubscript{495-503} transduced LCs (\(\square\)), CMVpp65\textsubscript{495-503} peptide-pulsed LCs (CMV-pep-LCs) (\(\square\)), or eGFP-transduced LCs (eGFP-LCs) (\(\square\)) at an LCs to T cell ratio of 1:30. A, One representative assessment among four donors is shown. The percentage listed in each dot plot (right upper quadrant) indicates the percentage of CD8\(^+\) T cells that were also tetramer-PE-reactive (right upper quadrant events divided by the sum of right upper quadrant plus right lower quadrant events \(\times 100\), after one and two rounds of stimulation each over 5–7 days. B, 1st stim, 2nd stim, Overall expansion. Data are mean \(\pm\) SD (*, p = 0.045, **, p = 0.019).

We evaluated the expansion of CD4\(^+\) T cells after two rounds of stimulation by CMVpp65-transduced, CMVpp65\textsubscript{495-503} transduced, or CMVpp65\textsubscript{495-503}-pulsed LCs. Autologous T cells from CMV-seropositive donors were stimulated by each type of LCs at an LCs to T cell ratio of 1:30 without exogenous cytokines. The total number of CD4\(^+\) T cells recovered after two stimulations was greatest in response to the full-length CMVpp65-transduced LCs, was relatively static in response to the CMVpp65\textsubscript{495-503}-transduced LCs, and declined after stimulation by LCs pulsed only with the immunodominant HLA-A*0201-restricted 9mer CMV peptide (Fig. 3A).

We then investigated the relative contributions of CD4\(^+\) and CD8\(^+\) T cells within the population of IFN-\(\gamma\)-secreting T cells responding to each type of LCs stimulator, using a flow cytometry-based cytokine secretion assay (Fig. 3B). Target cells used to rechallenge lymphocytes were the same LCs used for initial sensitization (pp65-transduced LCs) (Fig. 3B, left). Rechallenge of responder T cells from the other conditions by the respective LCs used for their initial sensitization gave IFN-\(\gamma\) secretion profiles (data not shown) that were qualitatively similar to that in Fig. 3B (left), but with relative percentages of total IFN-\(\gamma\)-positive cells as shown in Fig. 3C (upper row). Controls included responder T cells that were not rechallenged (Fig. 3B, middle) and T cells stimulated by control LCs without Ag (Fig. 3B, right).

We restricted subsequent analyses to IFN-\(\gamma\)-positive cells only. This gating enriched for Ag-specific T cells, in contrast to the analyses of total responder T cells in Fig. 2. The same LCs used for primary sensitization (Fig. 3C, pp65, p495, CMV-peptide) were used as the targets for overnight rechallenge of each population of responding T cells in ELISPOT assays. The percentages of IFN-\(\gamma\)-secreting lymphocytes among the entire responder populations are indicated for each gate (Fig. 3C, inset, upper row). CD4\(^+\) T cell expansion was greater after stimulation by transduced LCs than by peptide-pulsed LCs (Fig. 3C, middle row). This was especially true upon stimulation by LCs expressing the full-length CMVpp65 (pp65-LCs), and hence class II MHC-restricted Ags derived from same. Given the fact that even the CMVpp65\textsubscript{495-503} peptide-transduced LCs (p495-LCs) could stimulate IFN-\(\gamma\)-secreting CD4\(^+\) T cells, we reasoned that the vector itself provided nonspecific helper epitopes presented by class II MHC. As for the trace number of IFN-\(\gamma\)-secreting CD4\(^+\) T cells responding to stimulation by the peptide-pulsed LCs (CMV-pep-LCs; range from 2.4 to 5% among IFN-\(\gamma\)-secreting cells and from 0.11 to 0.52% among total viable cells, n = 3 independent experiments), we inferred a low level of nonspecific bystander activation of CD4\(^+\) T cells within the bulk T cell responder population.

Although the peptide-pulsed LCs stimulated the fewest IFN-\(\gamma\)-secreting cells among the three LCs conditions tested (Fig. 3C, upper row), these cells included a larger proportion of HLA-A*0201/CMVpp65\textsubscript{495-503} tetramer-reactive CD8\(^+\) T cells than did the responder T cells stimulated by transduced LCs (Fig. 3C, lower row). LCs transduced to express only the peptide also stimulated proportionally more tetramer-reactive CD8\(^+\) T cells than did the LCs transduced to express the full-length CMVpp65. This pattern of CD8\(^+\) T cell responses within the IFN-\(\gamma\)-secreting responders (Fig. 3C, lower row) was the reverse of that seen for CD4\(^+\) T cell responses (Fig. 3C, middle row). Note also that each condition included an additional subpopulation of reactive IFN-\(\gamma\)-secreting CD8\(^+\) T cells that did not bind the IFN-\(\gamma\)-secreting CD4\(^+\) T cells comprise a larger proportion of the T cell response stimulated by gene-modified LCs, compared with peptide-pulsed LCs, especially when the transgene encodes the full-length CMVpp65.
HLA-A*0201-CMVpp65_{495-503} tetramer. This result was especially true for those T cells stimulated by transduced LCs and in particular by the LCs expressing the full-length CMVpp65.

**Human CD34+ HPCs-derived LCs stimulate greater functional T cell responses after retroviral gene transfer of CMV Ags, especially full-length pp65, than after CMV peptide pulsing**

We next turned to an evaluation of the functional activity of the responding CD8+ T cells. Autologous T lymphocytes from CMV-seropositive donors were sensitized by CMVpp65-transduced LCs (pp65-LCs), CMVpp65_{495-503} peptide-transduced LCs (p495-LCs), or CMVpp65_{495-503} peptide-pulsed LCs (CMV-pep-LCs) at a 1:30 ratio of LCs to T cells without exogenous cytokines.

After two rounds of stimulation, CD8+ T cells were re-exposed either to K562-HLA-A*0201 transfecant targets pulsed with the CMVpp65_{495-503} peptide or to the same LCs stimulators used for initial sensitization (respective LCs). The resulting IFN-γ secretion was measured in ELISPOT assays from 100,000 input CD8+ T cells plated in triplicate in flat-bottom 96-well plates (Fig. 4A). A representative well from each condition is shown after development of the enzyme substrate linked to the anti-IFN-γ Ab (Fig. 4B). When the target could present only class I HLA-restricted Ag, i.e., K562-HLA-A*0201 transfecants, the responder-effector T cells exhibited similar IFN-γ secretion regardless of which transduced LCs population had been used as the initial stimulator (Fig. 4A). Both transduced LCs populations, however, were significantly more immunogenic than the peptide-pulsed LCs (p < 0.01, two-sample t statistic) (Fig. 4A).

When the same LCs used for sensitization were tested again as targets (respective LCs), both transduced LCs stimulators still proved more immunogenic than the peptide-pulsed LCs (p < 0.01, two-sample t statistic) (Fig. 4A). Additionally, the full-length CMVpp65-transduced LCs were more immunogenic than the LCs carrying only the peptide transgene (p < 0.05, two-sample t statistic). There was also some background reactivity, probably due to the vector itself, given the CD8+ T cell IFN-γ response against the same eGFP-transduced LCs targets used for initial sensitization. The broader CD8+ T cell response against additional CMV Ags presented by the respective LCs but not by the K562-HLA-A*0201 targets, and the apparent contribution of CD4+ T cell help in boosting the Ag-specific CD8+ T cell response, was further confirmed in these ELISPOT assays, especially when using the transduced LCs.

Finally, we confirmed relative potency between the different LCs stimulators in a standard 51Cr release assay from targets killed by CTL (Fig. 4C). Effectors for this CTL assay were derived from the responder lymphocytes generated after two rounds of stimulation. Total cells, not just CD8+ T cells or blasts, were added to achieve the indicated E:T ratios. This procedure provided a readout of total cytolytic activity generated per initial sensitizing LCs condition. These data further confirmed the superiority of the LCs transduced with the full-length CMVpp65 (p < 0.01 compared with all other conditions). In turn, and as expected from prior studies using influenza Ags, the peptide-transduced LCs were superior to the peptide-pulsed LCs just as in the ELISPOT assay (p < 0.01).

**CD8+ T cell help during afferent sensitization by CMV Ag-bearing LCs supports a more robust CD8+ T cell response**

Although we had already characterized the proportional CD4+ T cell contribution to the responder or efferent side of the response to LCs sensitization against CMV (see also Fig. 3), the data presented in Fig. 4 indicated a role for CD4+ T cells during the afferent arm as well. We investigated this further by stimulating T cells with the
CMVpp65 generate more robust IFN-γ-secreting CD8+ T cell responses than do LCs transduced to express only the CMVpp65495–503 peptide, and both are more effective than CMVpp65495–503 peptide-pulsed LCs. HLA-A*0201 T cells from a CMV-seropositive donor were stimulated by autologous CMVpp65-transduced LCs (pp65-LCs), CMVpp65495–503 peptide-transduced LCs (p495-LCs), or CMVpp65495–503 peptide-pulsed LCs (CMV-pep-LCs) at an LCs to T cell ratio of 1:30 without addition of exogenous cytokines. The negative control for sensitization was eGFP-transduced LCs at an LCs to T cell ratio of 1:30 without addition of exogenous cytokines. The negative control for sensitization was eGFP-transduced LCs.

FIGURE 4. LCs transduced by a retroviral vector encoding full-length CMVpp65 generate more robust IFN-γ-secreting CD8+ T cell responses than do LCs transduced to express only the CMVpp665495–503 peptide, and both are more effective than CMVpp65495–503 peptide-pulsed LCs. HLA-A*0201 T cells from a CMV-seropositive donor were stimulated by autologous CMVpp65-transduced LCs (pp65-LCs), CMVpp65495–503 peptide-transduced LCs (p495-LCs), or CMVpp65495–503 peptide-pulsed LCs (CMV-pep-LCs) at an LCs to T cell ratio of 1:30 without addition of exogenous cytokines. The negative control for sensitization was eGFP-transduced LCs. A. After two rounds of stimulation, ELISPOT assays measuring IFN-γ secretion by positively selected CD8+ T cells were performed, using as target cells either HLA-A*0201-transfected K562 cells pulsed with CMVpp65495–503 peptide (middle group) or the same transduced or peptide-pulsed LCs as had been used for primary and secondary stimulation (far right group; respective LCs). Negative controls for this readout assay used empty or irrelevant peptide-pulsed (e.g., influenza virus matrix peptide) K562-HLA-A*0201 transfectants (far left group). One hundred thousand CD8+ T cells were plated per well in triplicates in a 96-well plate. Shown are the averaged triplicate means of IFN-γ spot-forming cells (SFC) per 10^5 input CD8+ T cells from each of three independent experiments, ± SEM. Brackets indicate significant differences between sensitizing LCs when responder-effector T cells were challenged in the ELISPOT assay by the target cells indicated. **p < 0.01 and *p < 0.05 by two-sample t statistic. All CMV-sensitizing LCs were significantly more potent than the negative control eGFP-transduced LCs (p < 0.01; two-sample t statistic; not bracketed). B. Example wells from each stimulator condition in one of three ELISPOT assays using the K562-HLA-A*0201 transfectants (far left group). One hundred thousand CD8+ T cells were plated per well in triplicates in a 96-well plate. C. Actual killing activity was also measured in a standard [3H]Cr release assay, using radiolabeled HLA-A*0201-transfected K562 target cells that had been pulsed with the CMVpp665495–503 peptide. Responder T cells were added as total viable T cells (not just as morphologic T cell blasts) to 2 × 10^7 radiolabeled targets in triplicate for each of the E:T ratios indicated. This experiment assessed total cytolytic activity generated by each type of sensitizing LCs. Data point symbol represents the averaged triplicate (means ± SEM) from three independent experiments. Differences between any two of the conditions were considered significant at p < 0.01 (using stratified t test with E:T dose as the stratification variable).

FIGURE 5. CD4+ T cell help supports a more robust CD8+ T cell response than the response achieved by purified CD8+ T cells, in proportion to the amount of class I MHC-restricted recall viral Ag available for presentation by the sensitizing LCs. HLA-A*0201/CMVpp665495–503 peptide-restricted tetramer-positive cells were generated by two 7-day stimulations by CMVpp65-transduced LCs (pp65-LCs), CMVpp65495–503-transduced LCs (p495-LCs), or CMVpp65495–503-pulsed LCs (CMV-pep-LCs) at an LCs to T cell ratio of 1:30 without addition of exogenous cytokines. Each round of stimulation was conducted with or without prior depletion of CD4+ T cells, as indicated. A. Responder T cells were analyzed by flow cytometry for coexpression of CD8 and HLA-A*0201-CMVpp665495–503 tetramer reactivity. Values indicated (upper right quadrant) in each dot plot are the percentage of CD8+ T cells that were also tetramer-reactive (events in right upper quadrant divided by the sum of events in right upper quadrant plus right lower quadrant). B. Data are the mean ± SD of the percentage of HLA-A*0201/CMVpp665495–503 tetramer-reactive CD8+ T cells (as analyzed in A) from three independent experiments after two rounds of stimulation. Values for p (n.s., not significant) are indicated above bracket for each type of LCs stimulator.
LCs-presenting CMV Ags, especially those carrying the transgene encoding full-length CMVpp65, but also support a more robust CD8⁺, tetramer-reactive T cell response. The sensitizing class II MHC-restricted Ags are again presumed to be those encoded by the full-length CMVpp65 as well as some nonspecific Ags associated with the retroviral vector itself.

Regardless of the Ag-loading method, human CD34⁺ HPCs-derived LCs bearing CMV Ags stimulate T cell responders with an effector memory phenotype

After two stimulations by the three types of CMV Ag-bearing LCs, the autologous T cell responders from seropositive donors were analyzed for markers of activation or lymphoid homing (Fig. 6). Analysis was limited to CD8⁺ T cells (Fig. 6A) and to both CD4⁺ and CD8⁺ T cells (Fig. 6B). None of the CD8⁺ T cells that were also HLA-A*0201-CMVpp65₄₉₅–₅₀₃ peptide tetramer-reactive, nor any of the IFN-γ-secreting lymphocytes, expressed the lymphoid homing receptors, L-selectin (CD62 ligand), CD27, or CCR7, or the naive T cell epitope, CD45RA. This phenotype is characteristic of the effector memory phenotype (20). Data shown are T cells responding to LCs transduced with the vector encoding the full-length CMVpp65, but T cell responders to the peptide-transduced or peptide-pulsed LCs displayed the same effector memory phenotype (data not shown).

Discussion

With respect to stimulating robust CD8⁺ CTL responses, these experiments have demonstrated a clear advantage of retroviral transduction over peptide pulsing for Ag-loading and sustained presentation by CD34⁺ HPCs-derived LCs in vitro. Comparisons between gene-modified LCs further demonstrate an advantage for those transduced by vectors encoding full-length Ag as opposed to vectors encoding only a corresponding immunodominant peptide. LCs transduced with full-length Ag provide additional class I MHC-restricted epitopes that stimulate a broader CTL response.

Our data demonstrate that gene-modified LCs expressing full-length Ag also present class II MHC-restricted epitopes that provide T cell help. We confirmed this in two ways, with respect to both the afferent or sensitization arm, as well as the effector arm of the lymphocyte responses. CD4⁺ T cell depletion had the greatest effect on stimulation by LCs transduced to express full-length Ag, the least effect on stimulation by peptide-pulsed LCs, and an intermediate effect on LCs transduced with the peptide construct.

Regarding the effector arm, LCs bearing the transgene encoding full-length CMVpp65 stimulated robust CD4⁺ responses, which we detected in IFN-γ secretion assays upon re-exposure to targets also expressing the full-length Ag. We additionally noted that LCs transduced with the vector expressing only the immunodominant peptide provided some nonspecific help, most likely derived from other components of the vector construct. Even the peptide-pulsed LCs stimulated a background CD4⁺ T cell response (~5%), presumably due to a bystander effect from cytokines secreted during the initial stimulatory culture. Importantly, the CD4⁺ T cell responses that we detected were not confounded by the introduction of exogenous cytokines that are often used to expand T cells for adoptive immunotherapy (11–14, 21).

We also identified a tetramer nonreactive subpopulation among the IFN-γ secreting CD8⁺ T cells, especially after stimulation by transduced LCs. This finding suggested that either 1) transduced LCs provided stronger Ag-signaling compared with peptide-pulsed LCs, resulting in down-modulation of TCR (22–24) and inaccessibility to tetramer binding, or 2) responses developed against additional class I MHC-presented epitopes derived from CMV or from the vector itself, which the available tetramer could not detect. The fact that the proportion of tetramer nonreactive CD8⁺ T cells was roughly similar after stimulation and rechallenge by each of the transduced LCs would favor the first explanation. That the peptide-pulsed LCs, which could not present additional class I MHC-restricted epitopes, also generated a small population of tetramer nonreactive CD8⁺ T cells would further support the first explanation. In contrast, our data do not exclude broader responses against additional class I MHC-restricted epitopes presented by the transduced LCs, which were not detected by the HLA-A*0201/CMVpp495–503 tetramers.

We have focused on the LCs for these studies because we have previously demonstrated that peptide-pulsed LCs are much more potent than other DC types, including the commonly used blood monocyte-derived DCs, in stimulating Ag-specific CTLs (4). LCs also develop ex vivo from cycling CD34⁺ HPCs and are thus amenable to retroviral transduction that can only target dividing cells (4). The alternative differentiation pathway from cycling CD34⁺ HPCs yields dermal-interstitial DCs (4, 25–27); and although these could be modified by retroviral transduction, they remained less potent stimulators for these CD8⁺ CTL responses than did their LCs counterparts (data not shown and Ref. 4).

FIGURE 6. HLA-A*0201-CMVpp65₄₉₅–₅₀₃-reactive CD8⁺ tetramers, as well as all IFN-γ-secreting T cells in the responder population, have an effector memory phenotype. Responder T cells, without depletion of CD4⁺ T cells, were stimulated twice by LCs that had been transduced to express the full-length CMVpp65 or the CMVpp65₄₉₅–₅₀₃ peptide or had been pulsed by the CMVpp65₄₉₅–₅₀₃ Peptide alone. Flow cytometry analysis is shown for the T cells stimulated by LCs bearing the vector-encoded full-length CMVpp65. Tetramer-reactive CD8⁺ T cells and all IFN-γ-secreting T cells stimulated by either CMVpp65₄₉₅–₅₀₃-transduced or CMVpp65₄₉₅–₅₀₃-pulsed LCs displayed similar phenotypes (data not shown). A, Dot plots show the gated CD3⁺ CD8⁺ T cells analyzed for tetramer reactivity and absence of the CD62L, CD45RA, CD27, and CCR7 epitopes, consistent with an effector memory phenotype. B, Dot plots show similar absence of the same epitopes on IFN-γ-secreting cells among all responder cells in the lymphocyte gate (FSC vs SSC as indicated), again consistent with an effector memory phenotype. These data are representative of three independent experiments.
Monocyte-derived DCs differentiate from nondividing precursors and would therefore be susceptible to genetic modification only by constructs that do not require cycling targets, e.g., adenoviral or adenoviral-associated lentiviral or fowlpox vectors (12, 14, 21, 28–32). Immunogenicity of the constructs themselves, however, has often limited the efficacy of these approaches. In some cases infection has also interfered with normal DCs immunogenicity (32), which we have not observed with retroviral vectors (1, 33). Lentiviral vectors have been used to genetically modify monocyte-derived DCs (34, 35). Although these and the other aforementioned vectors have the potential advantage of targeting non-cycling cells like monocyte precursors, durable expression can prove more challenging than with retroviral constructs (1). Other methods like uptake of inactivated CMVs or inactivated CMV-infected cell lysates (11) have not been addressed by these studies. All conventional DCs can take up exogenous Ag for cross-presentation on class I MHC (4). LCs, however, cross-present Ag more effectively than do monocyte-derived DCs, which by inference must sequester and degrade a greater proportion of phagocytosed Ag than they process and present (4).

Methods of loading complete Ag onto DCs are critical to simultaneous stimulation of CD4+ T cells, which are important to the maintenance of CD8+ CTL responsiveness and memory (5–9). In this respect it will be important to compare the DCs-based approach defined in this study with that using artificial APCs (16). An important unknown is the relative efficacy of artificial APCs vs DCs, the latter of which typically do not require additional cytokine adjuvants to elicit T cell responses, perhaps even in seronegative individuals and possibly by extension to tumor-bearing patients. Artificial APCs can be generated in single defined lots, however, which are then available for immediate and potentially broad use, bypassing the need for autologous cells. There may nevertheless be issues about the immunogenicity of mouse fibroblast-derived artificial APCs used in humans, which are irrelevant to the use of autologous DCs.

Finally, recent data indicate that vaccine approaches will need to target multiple viral Ags to restore sufficiently broad immunity (36). DCs may prove advantageous in this respect as well, owing to their excess capacity for MHC-bound Ags (37) and their pan-ophyly of surface costimulators and secreted cytokine and chemokine molecules (38, 39). The extent to which retroviral vectors can sustain protein expression through multiple divisions of CD34+ progenitors en route to fully differentiated DCs, however, compared with lentiviral-transduced proteins in nondividing cells, is an important unknown that merits further assessment.

We have used CMVs as the model Ag for these studies and tested the capacity of gene-modified LCs to stimulate adaptive T cell immune responses in vitro from CMV-seropositive individuals. The rationale was to define parameters for the eventual expression of cancer Ags by LCs for active immunization against tumors. The goal was not to apply this approach to the cytokine-supported expansion ex vivo of DCs-stimulated, Ag-specific T cells for adoptive immunotherapy. CMV responses from seropositive donors provided a system with a known immunodominant Ag, its corresponding immunodominant peptide, and restriction to a commonly expressed HLA allele. These results have thus established an important proof-of-principle, which we are now pursuing in the context of antitumor immunity. Any approach that allows DCs to process and present antigenic epitopes from the complete Ag, tailored to their own MHC molecules, obviates the need for prior knowledge of immunodominant Ags and their restricting MHC alleles. The additional advantage of retroviral transduction, however, is that Ag presentation is sustained for at least 28 days, and normal immunogenicity of transduced DCs is maintained (1, 33). The sustained presentation of both class I and II MHC-restricted antigenic epitopes should also prove very effective in overcoming the apparent tolerance toward tumor Ags, most of which are self or altered-self Ags, as well as in maintaining immunologic memory.

Disclosures

The authors have no financial conflicts of interest.

References


