Peptide-Loaded Langerhans Cells, Despite Increased IL15 Secretion and T-Cell Activation In Vitro, Elicit Antitumor T-Cell Responses Comparable to Peptide-Loaded Monocyte-Derived Dendritic Cells In Vivo

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Abstract

Purpose: We compared the efficacy of human Langerhans cells (LC) as tumor immunogens in vivo with monocyte-derived dendritic cells (moDC) and investigated how interleukin 15 (IL15) supports optimal DC-stimulated antitumor immunity.

Experimental Design: American Joint Committee on Cancer stage III/IV melanoma patients participated in this first clinical trial comparing melanoma peptide-pulsed LC with moDC vaccines (NCT00700167, www.ClinicalTrials.gov). Correlative studies evaluated mechanisms mediating IL15 support of DC-stimulated antitumor immunity.

Results: Both DC vaccines were safe and immunogenic for melanoma antigens. LC-based vaccines stimulated significantly greater tyrosinase–HLA-A*0201 tetramer reactivity than the moDC-based vaccines. The two DC subtypes were otherwise statistically comparable, in contrast to extensive prior data in vitro showing LC superiority. LCs synthesize much more IL15 than moDCs and stimulate significantly more antigen-specific lymphocytes with a cytolytic IFN-γ profile even without exogenous IL15. When supplemented by low-dose IL15, instead of IL2, moDCs stimulate 5 to 6 logs more tumor antigen-specific effector memory T cells (TEMRA) over 3 to 4 weeks in vitro. IL2 and IL15 can be synergistic in moDC stimulation of cytolytic T cells. IL15 promotes T-cell expression of the antiapoptotic bcl-2 and inhibits candidate regulatory T-cell (Treg) expansion after DC stimulation, countering two effects of IL2 that do not foster tumor immunity.

Conclusions: MoDC-based vaccines will require exogenous IL15 to achieve clinical efficacy. Alternatively, LCs can couple the endogenous production of IL15 with potent T-cell stimulatory activity. Optimization of full-length tumor antigen expression for processing into multiple immunogenic peptides for presentation by both class I and II MHC therefore merits emphasis to support more effective antitumor immunity stimulated by LCs. Clin Cancer Res; 17(7); 1984–97. ©2011 AACR.

Introduction

Antigen-specific expansion of effector and memory CD8+ T cells is a central goal of immunotherapy against tumors. For stimulation of MHC-restricted, antigen-specific, CD8+, CTL in vitro (1), human Langerhans cells (LC) derived from CD34+ hematopoietic progenitor cells (HPC) have shown superiority over other known conventional or myeloid human dendritic cell (DC) subtypes, for example, monocyte-derived DCs (moDC) and dermal-interstitial...
Translational Relevance

Dendritic cell (DC) vaccination protocols most often use monocyte-derived DCs (moDC), although Langerhans-type DCs (LCs) have repeatedly proven superior to moDCs and other conventional DCs for stimulating immunity against viral and tumor antigens in vitro. We report a phase I comparison trial between melanoma peptide-pulsed LCs and moDCs, where LCs proved more potent than moDCs for one parameter but were otherwise comparable in eliciting immunity against melanoma antigens. We nevertheless established a critical role for IL15 in countering IL2-induced apoptosis and phenotypic Treg expansion during CTL generation by DC stimulation. MoDCs require exogenous IL15, whereas LCs do not. LCs providing endogenous IL15 therefore merit optimization as cancer immunogens in vivo, whereas continued use of moDCs will require exogenous IL15. Circumventing the inherent limitations of single peptides will require improved methods for loading full-length antigen for processing and presentation of multiple class I and II MHC-restricted epitopes.

DCs. This has held true for LCs either presenting peptide for recall responses against viral antigens or cross-presenting dying tumor cells to elicit tumor antigen–specific CTLs (1). Detailed comparisons between resident DC populations isolated from human skin have shown similar potency of LCs (2). Clinical trial data have also suggested greater efficacy of DC vaccines that contain LCs (3); but there have been no direct comparisons in vivo in humans between defined DC subtypes as vaccines. We therefore undertook a phase I clinical trial to determine safety and toxicity of melanoma peptide-pulsed LCs compared with the more commonly used moDCs. Laboratory studies then compared their immunologic efficacy based on T-cell tetramer reactivity and enzyme-linked immunosorbent spot (ELISpot) assay of IFN-γ secretion.

LCs achieve robust stimulation of CTLs without producing any IL-12p70 (1), yet they secrete more interleukin 15 (IL15) than any other conventional DC subtype (1, 4). IL15 is therefore of particular interest because of its role in lymphocyte homeostasis; the expansion of memory T cells, especially CD8+ CTLs; and its autocrine protection of DCs from apoptosis (2, 5–11). IL15 also has a contrasting role with IL2 in that it counters tolerance and supports autoimmunity (5), rendering IL15 an attractive cytokine to support targeted immune responses against self-differentiation antigens expressed by tumors like melanoma. Most IL15 data derive from mouse rather than human studies, although a recent non-human primate study provides important insight into the safety and cytotoxicity of the IL15 administered in vivo (12).

Monocyte precursors of moDCs have proven malleable in their differentiation, depending on cytokine exposure (13). Provision of IL15 during development leads to moDCs with Langerhans-like DC properties (14, 15). The moDCs and LCs used in the vaccine trial, however, were generated according to standard protocols without IL15 (1); and we wished to investigate the role of this cytokine on T-cell responses in the context of restimulation by the same DC subtype to which the T cells had been exposed in vivo during vaccination. Our findings establish an essential role for IL15 in the generation of cytolytic, IFN-γ-secreting T cells, even when stimulated by potent DCs. MoDCs depend on an exogenous source of IL15, yet LCs remain effective even with limiting or no exogenous IL15. These data have important implications for the design of DC-based immunotherapy trials, which going forward, must also include optimized approaches to provide full-length antigens for processing into multiple immunogenic peptides for presentation on both class I and II MHC.

Materials and Methods

Human cells, media, and cytokines

Human cell collection and use adhered to protocols approved by the Institutional Review and Privacy Board of Memorial Hospital, Memorial Sloan-Kettering Cancer Center (MSKCC). Healthy volunteers or patients provided peripheral blood mononuclear cells (PBMC) or granulocyte colony stimulating factor–elicited CD34+ HPCs for the isolation of T cells and the generation of moDCs and LCs (13). Provision of IL15 during development leads to moDCs with Langerhans-like DC properties (14, 15). The moDCs and LCs used in the vaccine trial, however, were generated according to standard protocols without IL15 (1); and we wished to investigate the role of this cytokine on T-cell responses in the context of restimulation by the same DC subtype to which the T cells had been exposed in vivo during vaccination. Our findings establish an essential role for IL15 in the generation of cytolytic, IFN-γ-secreting T cells, even when stimulated by potent DCs. MoDCs depend on an exogenous source of IL15, yet LCs remain effective even with limiting or no exogenous IL15. These data have important implications for the design of DC-based immunotherapy trials, which going forward, must also include optimized approaches to provide full-length antigens for processing into multiple immunogenic peptides for presentation on both class I and II MHC.

Phenotypic analyses by flow cytometry

Fluorescein isothiocyanate (FITC), phycoerythrin (PE), PE-cyanine-5 (PE-Cy5), PE-Cy7, peridinin chlorophyll protein-cyanine-5.5 (PerCP-Cy5.5), allophycocyanin (APC), APC-Alexa Fluor750, PE-Texas Red (ECD)-conjugated mouse anti-human mononclonal antibodies (mAb) included anti-CD3, anti-CD8, anti-CD25, anti-CD27, anti-CD28, and anti-CD69 (BD Biosciences); anti-CD4, anti-CD45RA, and anti-CD45RO (Beckman Coulter); anti-CD127, anti-human Foxp3, anti-CD8, and anti-CD62L (eBioscience); and anti-CCR7 (R&D Systems). Isotype controls included the appropriate fluorochrome-conjugated mouse IgG1 or rat IgG2a (Dako; eBioscience). Foxp3 detection required intracellular staining, using cell fixation and permeabilization buffers provided with the Foxp3 kit (eBioscience). HLA-A*0201–restricted tyrosinase, gp100, and flu matrix peptide (fluMP)-streptavidin-PE-labeled tetramers

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and negative tetramer controls (Beckman Coulter) detected antigen-specific T cells. Positive tetramer reactivity required a distinct population at least 1 log above the mean fluorescent intensity of the negative control.

Flow cytometry analyses used a Cytomics FC500 (Beckman Coulter) or a Cyan-ADP flow cytometer (DAKO). Gates were set for collection and analysis of 200,000 or more live events based on propidium iodide (PI) exclusion.

Immune responses to tumor antigenic peptide-pulsed dendritic cells in vivo

Clinical trial design. We conducted a phase I clinical trial to test safety and toxicity and to compare immune responses stimulated by tumor peptide-pulsed moDCs versus LCs in HLA-A*0201+ patients with American Joint Committee on Cancer (AJCC) stage III or IV melanoma (registered as no. NCT00700167 at www.ClinicalTrials.gov; Supplementary Fig. 1). There were no significant differences between assignment to either type DC vaccine with regard to demographics, Karnofsky performance score, or disease stage (Table 1). Phase Ia accrued cohorts of 3 patients to each of 3 vaccine doses (3 × 10⁶, 10 × 10⁶, or 30 × 10⁶), using either peptide-loaded (see below) moDCs or LCs for a priming dose, followed by 2 boosters of the same peptide-loaded DC subtype at approximately 4-week intervals. Patients received a total of 3 vaccines with no crossover between moDCs or LCs. moDCs or LCs were freshly made and used for the initial vaccine. Subsequent vaccines used thawed cells from the cryopreserved initial product (19). Table 2 lists release criteria for each vaccine, and Table 3 reports the viability and phenotype of all administered DC vaccines. Purity of the CD34+ -derived LC progeny was lower than that of the moDCs, reflecting the more variable differentiation potential of the starting populations of CD34+ HPCs compared with blood monocytes (1). The major contaminants of the LCs were immature myeloid cells, mostly eosinophils (1). Vaccines were dosed according to the absolute number of CD83+CD86brighthLA-DRbrighthCD14−moDCs or LCs by flow cytometry, so the DC numbers were equivalent.

Each moDC or LC vaccine, regardless of total dose, was administered as ten 0.1 mL deep intradermal injections divided equally between 2 sites in the proximal arms or thighs, excluding areas adjacent to prior lymph node resections. Surrounding erythema and induration were measured for delayed type hypersensitivity (DTH) reactions 48 hours after the second and third vaccines.

The dose that yielded a positive response by tetramer reactivity or ELISpot (see below), defined as greater than 2 SD above the prevaccine baseline response in at least 2 of the 3 initial phase Ia patients at each dose level, determined the optimal biologic dose. Hence, the 10 × 10⁶ dose level, which proved most feasible in terms of starting cell numbers, cytokine usage, costs, and labor, was also the optimal biologic dose for each DC subtype. Phase Ib of the trial then accrued 9 additional patients to each of the moDC or LC arm, resulting in a total of 12 patients who received either moDC or LC vaccines at the 10 × 10⁶ dose level in phases Ia and Ib combined.

Antigen-loading of moDCs or LCs. The clinical trial used 2 synthetic, HLA-A*0201–restricted, heteroclitic melanoma peptides: tyrosinase (TYR368–376) YMDGTMSQV and gp100 (gp100209–217) IMDQVPFSV (Research Genetics, Invitrogen). Synthetic influenza matrix peptide (fluMP58–66 GILGFVFTL; Research Genetics, Invitrogen) served as a positive control for HLA-A*0201–restricted responses. Keyhole limpet hemocyanin (KLH; high purity, endotoxin-free; Calbiochem) served as a positive control neoantigen for class II MHC-restricted responses.

### Table 1. Patient demographics and disease characteristics at protocol entry

<table>
<thead>
<tr>
<th>Variable</th>
<th>All patients, all dose levels</th>
<th>10 × 10⁶ dose level only</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LC (n = 18)</td>
<td>moDC (n = 18)</td>
</tr>
<tr>
<td>Gender, n (%), Male</td>
<td>10 (56)</td>
<td>11 (61)</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>8 (44)</td>
</tr>
<tr>
<td>Age, y</td>
<td>Mean 61.4</td>
<td>57.2</td>
</tr>
<tr>
<td></td>
<td>Range 43–81</td>
<td>31–83</td>
</tr>
<tr>
<td>Karnofsky performance score</td>
<td>Median 100</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Range 90–100</td>
<td>90–100</td>
</tr>
<tr>
<td>Disease stage (AJCC), n (%)</td>
<td>III, NED 15 (83)</td>
<td>16 (89)</td>
</tr>
<tr>
<td></td>
<td>IV, NED 2 (11)</td>
<td>1 (6)</td>
</tr>
<tr>
<td></td>
<td>IV, slow progressive disease</td>
<td>1 (6)</td>
</tr>
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</table>
Terminally maturing moDCs or LCs for the clinical trial were cultured at 1 x 10^6 cells/mL in 6-well plates, to which peptides (1 μmol/L) or KLH (10 μg/mL) were added overnight at 37°C, but in separate cultures to avoid MHC binding competition. Cells were washed and evaluated for release criteria (Table 2) before combining and resuspending to the appropriate concentration for vaccination.

Response assessments in vitro and correlative laboratory studies. Response assessments for the clinical trial used PBMC collected at prevaccine baseline and then approximately 3 weeks after each vaccine. We compared CD8+ T-cell reactivity with HLA-A*0201–peptide tetramers over time and ELISpot assay of IFN-γ secretion by total T cells over time as fold increase over baseline. These followed a single 6- to 7-day restimulation of the PBMC responders in vitro, using the same peptide-loaded autologous moDCs or LCs used for a patient’s vaccines, but without exogenous cytokines. Ten to 12 patients, out of total 12 in each DC subtype vaccine group at the optimal biologic dose of 1 x 10^6 cells/vaccine, were evaluable. Zero to 2 patients in each group became inevaluable due to inadequate cell yields or insufficient replicates (Table 4). For assessment of KLH-specific responses, PBMCs from moDC and LC vaccinees were harvested at baseline prevaccine and approximately 3 weeks after each of 3 vaccines and cultured with KLH (1 μg/mL). KLH-specific immunity was determined by the incorporation of ^3^HdTdR (1 μCi/well) by proliferating responder T cells in the last 8 hours of a 5-day culture.

All patients consented to the use of any residual cryopreserved cells for subsequent thaw and assessment in additional correlative laboratory studies. These used responder PBMCs, total T cells, or T-cell subsets selected by using immunomagnetic columns (Miltenyi Biotec) as indicated. Responder PBMCs or T cells were resuspended in 10% autologous serum (v/v) in complete RPMI-1640 and

### Table 2. Criteria for release of dendritic cell vaccines for patient administration

<table>
<thead>
<tr>
<th>Variable</th>
<th>Test</th>
<th>Result required for release</th>
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<tbody>
<tr>
<td>Bacteria and fungus</td>
<td>Culture in thioglycolate broth and soybean casein digest medium</td>
<td>No growth after 5 days of in-process culture; no growth confirmation of final product after administration</td>
</tr>
<tr>
<td>Endotoxin</td>
<td>Limulus amebocyte lysate (BioWhittaker; CBER/FDA biologic license number 709)</td>
<td>Negative on final product &lt;5 endotoxin units</td>
</tr>
<tr>
<td>Mycoplasma</td>
<td>PCR</td>
<td>Negative result, in process 48 hours before end of culture</td>
</tr>
<tr>
<td>Viability</td>
<td>PI staining of large FSC cells on flow cytometry</td>
<td>&lt;30% PI+ (or ≥70% viable)</td>
</tr>
<tr>
<td>Phenotype (flow cytometry)</td>
<td>Flow cytometry: gated population of large FSC, CD14−, class II MHC bright cells</td>
<td>≥50% CD83+, ≥ 50% CD86+</td>
</tr>
</tbody>
</table>

Abbreviation: FSC, forward scatter.

### Table 3. Viability and phenotype of dendritic cell vaccines administered to patients

<table>
<thead>
<tr>
<th></th>
<th>% viability of total (mean ± SEM)</th>
<th>% viable HLA-DR^{bright}CD14^{−} of total (mean ± SEM)</th>
<th>%CD83 of viable HLA-DR^{bright}CD14^{−} (mean ± SEM)</th>
<th>%CD86 of viable HLA-DR^{bright}CD14^{−} (mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood monocyte-derived DCs</td>
<td>89.6 ± 1.7</td>
<td>81.3 ± 2.1</td>
<td>89.6 ± 2.1</td>
<td>98.0 ± 0.5</td>
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<tr>
<td>CD34^{+}-derived LCs</td>
<td>93.1 ± 0.7</td>
<td>56.8 ± 1.9^a</td>
<td>65.6 ± 2.1^a</td>
<td>98.2 ± 0.4</td>
</tr>
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^aPurity of the CD34^{+}-derived LC progeny was lower than that of the moDCs, reflecting the more variable differentiation potential of the starting populations of CD34^{+} HPCs compared with blood monocytes (1). The major contaminants of the LCs were immature myeloid cells, mostly eosinophils (1). Regardless, vaccines were dosed according to the absolute number of CD83^{−}CD86^{+} HLA-DR^{bright}CD14^{−} moDCs or LCs.
plated at 2 × 10^6 cells/mL final volume per well of a 24-well plate (Costar) or at 1 × 10^5/100 μL final volume per well of a 96-well round bottomed plate (Costar). Mature autologous moDCs or LCs were resuspended at 10^6/mL in cytokine and serum-free RPMI-1640, then loaded with 10 μmol/L of a single 9-mer peptide for 1h at RT before restimulation. Target cells for ELISpot or CTL assays were similarly loaded with single peptides. Peptides were always separately loaded onto the respective DCs to avoid MHC binding competition. Peptide-loaded moDCs or LCs were added in graded doses to fixed numbers of responders. Where experiments required repeated stimulation,

<table>
<thead>
<tr>
<th>Phase la MoDCs</th>
<th>Total accrued (n)</th>
<th>Off study (n)</th>
<th>Reason for withdrawal from study</th>
<th>Completed all 3 vaccines (n)</th>
<th>Evaluable for response assessments (adequate cell yields)</th>
</tr>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Tetramer</td>
<td>ELISpot</td>
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<tr>
<td>gp100 tyr fluMP</td>
<td></td>
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<tr>
<td>gp100 tyr fluMP</td>
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<tr>
<td>Phase Ib MoDCs</td>
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<tr>
<td>Phase la + lb, all at 10 × 10^6 cells/vaccine</td>
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*Three evaluable patients were required at each dose level in phase la to select the optimal biologic dose (defined in Materials and Methods) to use in phase Ib. Hence, 1 additional patient was accrued to replace 1 who had inadequate cell yields for response assessments despite a normal complete blood count.

bResponse assessments for the trial overall were based on the evaluable patients at the 10 × 10^6 moDC or LC dose per vaccine in phase la, plus all evaluable patients in phase lb.
responder cells were harvested after 6- to 7-day initial stimulation and restimulated by the same DC subtype and exogenous cytokine combination each round.

The cultures described above were supplemented or not with either recombinant human IL2 (10 IU/mL; Chiron) and/or IL15 (10 ng/mL; R&D Systems; see also Supplementary Fig. 2). Although IL2 10 IU/mL is less than what DC-stimulated allogeneic T cells produce (20), we avoided supraphysiologic doses of IL2 in these autologous combinations that could activate natural killer (NK) cells among bulk PBMCs or T cells via their IL15Rs, which share common β- and γ-chains with the IL2R (21).

Inhibition of IL15 binding to IL15R-α on dendritic cells

LCs or moDCs were opsonized for 2 hours with a polyclonal goat anti-human IL15R-α (5μg/mL; R&D Systems) or its isotype control (5μg/mL; R&D Systems). These were then separately cocultured with responder PBMCs in functional assays at the indicated ratios. Anti-human IL15R-α or its isotype control was replenished at the same concentration in culture every 48 hours. When cells underwent a second round of stimulation after 1 week, responders were harvested, washed, and restimulated for 7 days by anti-human IL15R-α–opsonized gp100-loaded moDCs or LCs from the same population.

IFN-γ ELISpot assay

After antigen-specific opsonization, responder lymphocytes were tested for IFN-γ production by ELISpot assay (1-DIK ELISpot for human IFN-γ, Mabtech, DiaPharma Group; Vector ABC kit, Vector Laboratories; Automated ELISpot Reader System and KS 4.3 software, Carl Zeiss Vision) according to the manufacturer’s instructions. Targets for lymphocyte restimulation during overnight rechallenge in the ELISpot assays were either mature peptide-pulsed moDCs or LCs, whichever had been used for stimulations in vitro, plated in triplicates at 30:1 effector:target (E:T) ratio. Control wells contained effectors and unloaded targets. ELISpot reactivity against both moDC and LC targets without antigenic peptide was subtracted from reactivity against the peptide-loaded moDC and LC targets, because unloaded moDCs caused such high background.

Cytolytic T-cell assays

Cytolytic activity exerted by T lymphocytes responding to antigen-specific DC stimulation was assessed in standard 51Cr release assays against the same target cells used in ELISpot assays. A total of 5 × 104 51Cr-labeled target cells were added at the indicated T-cell: E/T ratios. Supernatants were collected after 4 to 6 hours from replicate microwells for calculating specific 51Cr release.

Assessment of early and late CD3+CD8+ T-cell apoptosis

Early and late apoptosis were evaluated by a FITC-conjugated Annexin V probe (BD Biosciences), together with PI (Sigma-Aldrich). Fluorochrome-conjugated mAbs costained the CD3+CD8+ T-cell subset of interest. Early apoptotic cells were Annexin V–PI+, whereas late apoptotic and necrotic cells were Annexin V+PI+.

Protein extraction and Western blotting

Positive immunomagnetic selection (Miltenyi Biotec) yielded CD3+CD8+ T cells with 97% to 99% purity. Protein extraction buffer (2 × 105 to 5 × 105) cells used RIPA lysis buffer (Pierce) with protease inhibitors (Roche Applied Science) for 20 minutes on ice. Twenty micrograms of cell lysate were applied to a 12% Bis-Tris gel (Invitrogen). Proteins were blotted onto a polyvinylidene difluoride membrane (Biorad), blocked with 5% nonfat dry milk in PBS/0.1% Tween20, and then probed with mouse anti-human Bcl-2 mAb (BD Biosciences). Mouse anti-human glyceraldehyde 3 phosphate dehydrogenase (GAPDH; Ambion) served as the loading control. Secondary antibody was horseradish peroxidase–conjugated goat anti-mouse IgG (PerkinElmer Life Sciences). Immunoreactive protein bands were identified by an ECL detection kit (Amersham).

Statistics

Responses to the moDC or LC vaccines in the clinical trial were tested by a stratified permutation (Wilcoxon) rank sum statistic. This compared moDCs with LCs where the outcomes were reactivity against gp100, tyrosinase, or control fluMP. The test was stratified by vaccine number over time.

For the correlative laboratory studies in vitro, replicate means from 3 or more independent experiments were averaged and SEM calculated as the measure of variability. Otherwise, SD was calculated for the replicate mean of 1 of 3 representative experiments. A stratified t test (stratified by the E/T or responder:stimulator ratio) was used for the functional assays. The t test was used for each comparison within a given time point or condition for all other analyses.

Online supplementary material

Supplementary Figure 1 shows the protocol schema. Supplementary Figure 2 is a dose-finding pilot study for IL2 and IL15 by using fluMP-pulsed moDC stimulators and fluMP–HLA-A*0201 tetramer reactive T-cell readouts. Supplementary Figure 3 illustrates the proliferation of total cells and the absolute number of CD3+CD8+ T cells reactive with tyrosinase, gp100, and fluMP–HLA-A*0201 tетramers over 3 to 4 rounds of weekly stimulation with and without IL2, IL15, or both in combination.

Results

Peptide-loaded moDCs and LCs are safe in patients with advanced melanoma and generate measurable tumor antigen-specific immune responses

Two groups of 12 patients each received 3 vaccines of either 10 × 106 peptide-pulsed moDCs or CD34+–derived LCs, which was the optimal biologic dose as explained in...
Materials and Methods. No patient experienced more than grade 2 toxicity according to Common Terminology Criteria for Adverse Events, version 3.0, possibly or probably related to these vaccines. All patients developed some degree of erythema (Fig. 1A), induration (Fig. 1B), and mild pruritus at the injection sites. Two patients experienced grade I vitiligo, possibly related to the vaccine. Grade 2 toxicities were because of combinations of grade 1 cutaneous toxicities. Prevaccine baseline and postvaccine responses were assessed simultaneously in vitro to avoid interassay variability. Proliferative responses to rechallenge with KLH (Fig. 1C) were greater in the moDC than LC vaccines (post 2, P = 0.002; post 3, P < 0.001; for comparisons between moDC vs LC vaccines). Figure 2A depicts the absolute tetrramer reactivity over time, including the prevaccine baseline. Figure 2B shows IFN-γ secretion measured by ELISpot as the fold increase over 2 SD above the prevaccine baseline mean. Background reactivity against targets without peptide was subtracted from each ELISpot condition, and resulting negative or zero values were normalized to 1 for calculation of fold increase.

Data comparing moDCs with LCs in vitro (1) led to the hypothesis that LCs would be superior immunogens. Only CD8+ T-cell reactivity with tyrosinase peptide–HLA-A*0201 tetramers reached statistical significance, however, slightly favoring LCs over moDCs (P = 0.04). There was a nonsignificant trend supporting moDCs for stimulating CD8+ T-cell reactivity with gp100 peptide–HLA-A*0201 tetramers (P = 0.11), but LCs and moDCs were equivalent in stimulating fluMP tetramer reactivity (P = 0.93). Limited cell numbers precluded isolation of CD8+ T cells for ELISpot assays. Total T-cell production of IFN-γ showed a trend without reaching statistical significance in favor of responses stimulated by tyrosinase peptide-pulsed moDCs (P = 0.08) and by fluMP-loaded LCs (P = 0.14), whereas the 2 subtypes showed no difference in stimulating gp100-specific responses (P = 0.30).

LCs maintain superior potency over moDCs in inducing IFN-γ–secreting lymphocytes at limiting doses of exogenous IL15

Pertinent to the generation of antigen-specific CTLs was our previous finding that LCs secreted significantly more...
IL15 than moDCs (1, 4). By titrating doses of exogenous IL15 down to zero, we evaluated the biologic dependence of LCs compared with moDC on exogenous IL15. T cells remaining after completion of the clinical trial response assessments underwent 3 weekly rounds of stimulation in vitro by the same gp100 peptide- or fluMP-pulsed DC subtype used for primary immunization in vivo. LCs stimulated significantly greater reactivity than the moDCs, especially at limiting doses of exogenous IL15 (Fig. 3A and B). Even in the absence of any exogenous IL15, LCs stimulated significantly greater T-cell responses than did comparable numbers of moDCs.

We then assessed the effect of blocking IL15R-α (Fig. 3C). After 2 weekly rounds of restimulation in vitro without addition of exogenous cytokine, ELISpot assays of IFN-γ secretion by responder PBMCs showed that LCs were again more potent APCs than moDCs. Blocking anti-IL15R-α significantly reduced the stimulatory capacity of...
IL15 and IL2 synergize in supporting development of antigen-specific CD3⁺CD8⁺ T cells with an IFN-γ–secreting cytolytic profile and effector memory phenotype

We used moDCs to distinguish the effects of IL15 from IL2, the receptors for which share common β- and γ-chains. MoDCs depend on exogenous IL15, unlike LCs, so endogenous IL15 would not confound the results. Pilot studies established that IL2 at 10 IU/mL was only minimally additive to IL15 at 10 ng/mL in supporting proliferation of antigen-specific T cells (Supplementary Figs. 2 and 3). Representative dot plots from the combined IL2 + IL15 condition in a single set of experiments illustrate the expansion of tetramer-reactive T cells in vitro against moDC-presented tyrosinase, gp100, and influenza matrix peptide antigens (Fig. 4A). These CD3⁺CD8⁺ tetramer-reactive T cells attained a cytotoxic IFN-γ–secreting profile (Fig. 4B). In contrast to cell proliferation (Supplementary Fig. 3), IL2 and IL15 synergized to support CTL development (P < 0.05 for IL2 or IL2 + IL15 vs. IL2 or no cytokine; and P < 0.005 by the third round of stimulation for the combination of IL2 + IL15 vs. IL15 alone).

Because limited cell numbers precluded isolation of CD8⁺ T cells for most ELISpot assays, we confirmed cytolytic activity in standard ⁵¹Cr release assays (Fig. 4C). Bulk responder T cells included CTLs capable of dose-dependent, MHC-restricted, tumor antigen–specific killing (Fig. 4C; P < 0.001). Although IL15 was more effective than IL2 alone in promoting robust expansion of tetramer-reactive CD3⁺CD8⁺ T cells, IFN-γ secretion and cytolytic function benefited from IL15 and IL2 synergy when stimulated by peptide-loaded moDCs.

After three 7-day rounds of stimulation in vitro in the presence of IL2 and IL15, we assessed activation and lymphoid homing markers on T cells from the previously vaccinated melanoma patients (Fig. 4D and E). CD3⁺CD8⁺ T-cell responders expressed neither of the lymphoid homing receptors, CCR7 or L-selectin (CD62L); nor did these T cells express CD28, all of which are usually found on central memory (TCM) or naive/precursor memory T cells (22). These tetramer-reactive T cells instead expressed the activation markers CD45RO, CD69, and variable amounts of CD27, as well as CD45RA (Fig. 4D). Data from 3 independent experiments gated on tyrosinase-reactive tetramers were pooled to illustrate the aggregate phenotype, as well as...
variability, characteristic of these CD45RA+ effector memory T cells (TEMRA: CD62L−, CCR7−, CD28−, CD127−, CD45RO−, CD69−, and CD45RA+; Fig. 5D; refs. 22–24). Figure 4E shows the dot plots from the 1 experiment of 3 with the highest number of tetramer-reactive cells among the gated CD3+CD8+ T cells.

IL15 prevents contraction of the human immune response by countering apoptosis and expansion of candidate Tregs

We then asked whether IL15 rescued T cells from IL2 activation–induced cell death or apoptosis. We again focused on moDCs in lieu of LCs, because we could better
control the amount of IL15 in the system in vitro. T cells from the gp100-pulsed moDC-vaccinated melanoma patients underwent a single 7-day restimulation with autologous gp100-pulsed moDCs in the presence of IL2 and/or IL15 or neither. CD3^+CD8^+ -gated responder T cells were analyzed cytofluorographically for early (AnnexinV^+PI^-) and late (AnnexinV^+PI^+) apoptosis (Fig. 5A). IL15 significantly reduced apoptosis, thus prolonging CD3^+CD8^+ T-cell survival after restimulation (P < 0.05). The control without cytokines was comparable to the condition with exogenous IL2 alone, because such low-dose IL2 did not enhance the higher amounts of endogenous IL2 already secreted by DC-stimulated T cells (20).

CD3^+CD8^+ responder T cells increased expression of the antiapoptotic protein, Bcl-2, after restimulation by gp100-pulsed moDCs with exogenous IL15 (Fig. 5B). IL15 also mitigated the reduction in Bcl-2 expression caused by IL2. Levels were nearly undetectable with IL2 alone.

Neither mouse nor human studies have addressed in detail whether IL15 could also attenuate regulatory T-cell (Treg) expansion by IL2. We therefore determined the percentage of Foxp3^+ Tregs among the gated CD3^+CD4^+CD25^bright^ T cells responding to gp100 peptide-pulsed (Fig. 5C) or fluMP-pulsed (Fig. 5D) human moDCs. IL15 reduced the proportion of candidate Tregs significantly below the proportions generated by moDCs alone or with only low-dose exogenous IL2 (n = 3 independent experiments, P < 0.01; Fig. 5C and D). These data show that IL15, while expanding total and tetramer-reactive T cells, reduced the proportion of candidate Tregs that would otherwise respond to IL2 produced in culture or added exogenously. In contrast to the mixed effect of IL15
and IL2 on Bcl-2 expression. IL15 suppression of these phenotypic Tregs overrode any effect of IL2.

Discussion

This clinical trial was the first head-to-head comparison between vaccines using peptide-pulsed LCs or moDCs in humans. Immune responses to tumor peptide and control antigens developed in vivo in patients with advanced melanoma in response to both DC subtypes, which proved safe and well tolerated in all patients. There was a trend toward greater erythema and induration 48 hours after peptide-pulsed moDC injections, possibly indicating less trafficking away from the local sites to draining nodes; but these reactions were not significantly different from those LC-based vaccines. The increased proliferative response to KLH rechallenge with moDCs may also account for their trend toward greater DTH responses. We set a high threshold to define a positive response, and LC-based vaccines stimulated significantly greater tyrosinase-HLA-A*0201 tetramer reactivity than the moDC-based vaccines. Beyond that, the results of this particular trial did not yet corroborate extensive data in vitro that had predicted LC superiority across the board (1, 2) or confirm the findings of some investigators suggesting that there is an advantage to including CD34+ HPC-derived LCs in DC vaccines (3).

We conducted additional experiments to establish mechanisms that could account for LC superiority over moDCs in vitro (1, 2), using T cells primed in vivo by DC vaccination and focusing on the activity of IL15 with a view toward future optimization of tumor antigen presentation. IL15 is a pleiotropic cytokine that affects homeostasis, activation, and homing of lymphocytes in both innate and adaptive immunity (5). LCs secrete more IL15 than the moDCs (1, 4), and when exogenous IL15 was either limiting or absent, only LCs could still stimulate a statistically significant enhancement of IFN-γ production by tumor and viral antigen-specific T cells. Anti–IL15R-α significantly inhibited both LC- and moDC-stimulated IFN-γ responses, yet blockade was incomplete for LCs. Limited cell numbers precluded isolation of CD8+ T-cell responders, so CD4+ T cells could have been less sensitive to IL15R-α blockade. Lacking a positive control to guide antibody dosing, complete blockade of LCs may also have required a higher mAb concentration. Other unmeasured factors could also have played roles downstream of the initial T-cell activation events, especially over two 7-day rounds of stimulation.

Somewhat paradoxically, we then concentrated on mature moDCs so that we could control the amount of IL15 in the system in vitro and not confound our assessments by what LCs could produce themselves. Exposure to IL15 during moDC development confers Langerhans-like properties on moDCs (14, 15), but does not provide IL15 during DC stimulation of tumor-specific T cells, which was the focus of our inquiry.

Our data showed that IL15 supported the long-term expansion by DCs of tumor antigen–specific T cells with effector memory phenotype (TEMRA) and cytolytic function. There was a modest additive effect on proliferation when IL2 combined with IL15. The 2 cytokines were synergistic in enhancing CTL activity stimulated by moDCs against melanoma antigens, however, with IL2 being an essential component of CD4+ T-cell help for the long-term expansion of CD8+ memory T cells (25–30). Low-dose exogenous IL2 avoided bystander activation of either IL15R (21) or NK cells in the responder populations, insofar as conventional DCs already stimulate T cells to secrete comparable or higher amounts of IL2 (20). Hence, IL2 did not add much to the DC-stimulated controls without exogenous cytokines.

More importantly, IL15 countered the apoptosis induced by IL2 activation, which otherwise impeded T-cell expansion by IL2 beyond the first or second week. IL15 achieved this by partially abrogating IL2’s reduction of the antiapoptotic protein, Bcl-2. Mouse data predicted these findings, but there have been no comparable studies with human cells. IL15 also trumped all of the IL2 effect on Treg expansion, which has not been previously reported. These data with human cells confirm that IL2 and IL15 have distinct roles in DC-stimulated T-cell apoptosis and survival.

Antigen-experienced T cells comprise both effector memory (TEM) and TCM, which home respectively to inflamed tissues and lymphoid organs (7, 22). Investigators have ascribed TCM development to IL15 and TEM generation to IL2 (6, 7), but IL15 supports TEM in HIV infection (8). Our data showed that conventional DCs with IL15 expanded both tumor and viral antigen-reactive human TEM cells lacking CCR7, CD62L, and CD127, yet coexpressing CD45RO, CD45RA, and CD69. This characterizes a CD45RA+ TEM subpopulation (TEMRA) (22), which has the largest amount of perforin and appears late in the immune response (31–33). Somewhat atypical was the persistent expression of CD27 on these cells, although a subset of CD27+ TEMRA cells with intermediate effector activity exists (34). Resting rather than activated populations like those in this study, however, have mostly established the phenotypes for TEM and TEMRA cells. Our data also showed that TEMRA cells developed under antigen-bearing DC-driven conditions with IL15, arguing against their development through homeostatic rather than antigen-driven pathways (31, 32).

Despite encouraging findings from the clinical trial establishing safety and efficacy of LC- and moDC-based vaccines, a clear winner did not emerge across all antigens tested, although LCs stimulated significantly greater tetramer reactivity at least against HLA-A*0201–restricted tyrosinase peptide. Among the outstanding challenges, foremost is the need to reconcile abundant data in vitro that LCs are in fact superior to moDCs for stimulation of virus and tumor antigen–specific CTLs, with the responses observed in vivo. The clinical trial data do not exclude the possibility that sufficient IL15 may have been present in vivo, as reported in a mouse model of influenza infection.
Given the trend toward greater DTH reactivity after moDC vaccination and the significant increase in moDC-stimulated KLH-specific proliferation on rechallenge, we also cannot exclude induction in vivo of IL15 in moDCs by KLH-specific CD4+ T cells.

Additional limitations when translating studies in vitro to a clinical trial included differences in peptide hydrophobicity that affected solubility and loading onto class I MHC. There was also no reliable way to confirm that sufficient peptide remained bound to MHC long enough to stimulate T cells in draining lymph nodes. These problems beg for approaches that achieve durable expression of full-length tumor antigens, which could in turn be processed into multiple epitopes for simultaneous presentation on both class I and II MHC. Furthermore, because LCs produce more IL15 (1, 2, 4) than the moDCs, and given that LCs can stimulate significantly higher numbers of virus- and tumor-specific T cells in the complete absence of exogenous IL15 or IL2, LCs merit further specific investigation as immunogens for the optimal induction of tumor immunity. Alternatively, continued use of moDCs as vaccines would benefit from supplementation with IL15 by transfection (36) or direct administration of the cytokine as a drug (NCT01021059, ClinicalTrials.gov). Available reagents and approaches have not yet detected IL15Rα by flow cytometry on human CD34+ HPC-derived LCs (1, 4), which is a prerequisite for binding and presenting IL15 in trans to responder lymphocytes. Alternative approaches are underway to resolve this conundrum and to ascertain distinctions between the mechanisms used by LCs versus moDCs for early T-cell activation events that depend on IL15.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References


Author Contributions

E. Romano, M. Rossi, G. Ratzer, J. Yuan, and J.W. Young designed, planned, and performed the experiments. J.W. Young, K.S. Panageas, P.B. Chapman, and A.N. Houghton designed the clinical trial. G. Ratzer, M. Rossi, M.A. de Cos, E. Romano, D.J. Chung, J. Yuan, and J.W. Young conducted the clinical trial. K.S. Panageas assisted with statistical design of the clinical trial, and G. Heller provided statistical design and analysis of the trial and lab correlative studies. J.W. Young conceived, planned, and reviewed the experiments. E. Romano and J.W. Young wrote the manuscript.

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