Clinical Cancer Research

Phase I Trial of Intratumoral Injection of *CCL21* Gene–Modified Dendritic Cells in Lung Cancer Elicits Tumor-Specific Immune Responses and CD8$^+$ T-cell Infiltration

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Abstract

Purpose: A phase I study was conducted to determine safety, clinical efficacy, and antitumor immune responses in patients with advanced non–small cell lung carcinoma (NSCLC) following intratumoral administration of autologous dendritic cells (DC) transduced with an adenoviral (Ad) vector expressing the *CCL21* gene (Ad-CCL21-DC). We evaluated safety and tumor antigen–specific immune responses following *in situ* vaccination (ClinicalTrials.gov: NCT01574222).

Experimental Design: Sixteen stage IIIB/IV NSCLC subjects received two vaccinations (1×10^6, 5×10^6, 1×10^7, or 3×10^7 DCs/injection) by CT- or bronchoscopic-guided intratumoral injections (days 0 and 7). Immune responses were assessed by tumor antigen–specific peripheral blood lymphocyte induction of IFNγ in ELISPOT assays. Tumor biopsies were evaluated for CD8$^+$ T cells by IHC and for PD-L1 expression by IHC and real-time PCR (RT-PCR).

Results: Twenty-five percent (4/16) of patients had stable disease at day 56. Median survival was 3.9 months. ELISPOT assays revealed 6 of 16 patients had systemic responses against tumor-associated antigens (TAA). Tumor CD8$^+$ T-cell infiltration was induced in 54% of subjects (7/13; 3.4-fold average increase in the number of CD8$^+$ T cells per mm^2). Patients with increased CD8$^+$ T cells following vaccination showed significantly increased PD-L1 mRNA expression.

Conclusions: Intratumoral vaccination with Ad-CCL21-DC resulted in (i) induction of systemic tumor antigen–specific immune responses; (ii) enhanced tumor CD8$^+$ T-cell infiltration; and (iii) increased tumor PD-L1 expression. Future studies will evaluate the role of combination therapies with PD-1/PD-L1 checkpoint inhibition combined with DC-CCL21 *in situ* vaccination. *Clin Cancer Res; 23(16); 4556–68. ©2017 AACR.*

Introduction

Lung cancer is the leading cause of cancer-related death in the United States, and immunotherapy with checkpoint inhibitors is transforming therapeutic approaches (1, 2). Although approximately 20% of patients respond to antibody-mediated therapies that block programmed death-1 (PD-1) or programmed death ligand-1 (PD-L1), patients without tumor-infiltrating CD8$^+$ T cells and PD-L1 expression appear to be less likely to respond

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

Clinical Trial registration: ClinicalTrials.gov NCT01574222

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Translational Relevance

Intratumoral infiltration by activated immune effector cells is associated with a significantly better prognosis; however, tumor-associated immunosuppression is frequently evident in lung cancer. C-C motif chemokine ligand 21 (CCL21) is required for normal lymphoid tissue organization that is ultimately essential for effective T cell–dendritic cell (DC) interactions. In preclinical model systems, we have previously demonstrated that intratumoral administration of DCs overexpressing CCL21 (Ad-CCL21-DC) led to both local and systemic antitumor responses. We evaluated the safety and efficacy of intratumoral vaccination with Ad-CCL21-DC in patients with advanced NSCLC. We observed induction of systemic tumor antigen–specific immune responses, $CD8^+$ T-cell infiltration at the tumor sites, and increased tumor PD-L1 expression. Intratumoral administration of Ad-CCL21-DC was safe and feasible. These findings suggest that antitumor responses in patients receiving PD-1/PD-L1 checkpoint blockade may be further improved when combined with *in situ* vaccination of Ad-CCL21-DC.

(3, 4). Thus, it has been suggested that those tumors with a nonimmunogenic microenvironment may be best treated in combination with vaccines that evoke T cell–mediated immune responses (1, 5). Thus far, however, vaccines for NSCLC have yielded disappointing results (6).

Studies of the immune contexture in human NSCLC indicate that the combination of mature dendritic cell (DC) and $CD8^+$ T-cell densities constitutes a powerful and independent prognostic factor for overall survival (7). Importantly, these DCs were associated with tertiary lymphoid structures (TLS) exhibiting the structural features of secondary lymphoid organs (8). These lymphoid aggregates, hypothesized to be the result of chronic immune stimulation and lymphoid neogenesis, may contribute to the generation of primary or secondary antitumor immune responses (9).

On the basis of these findings, one potential approach is to enhance T-cell responses by *in situ* vaccination that takes advantage of the full repertoire of available tumor antigens by providing effective antigen uptake and presentation at the tumor site (10). We have found that DC-based intratumoral vaccination augments antigen presentation, resulting in effective T-cell responses (10–13). The creation of chemokine gradients that favor lymphocyte and DC entry into the tumor also facilitates *in situ* vaccination (10–15). Chemokines are a group of homologous yet functionally divergent proteins that directly mediate leukocyte migration and activation. CCL21, expressed in high endothelial venules and T-cell zones of spleen and lymph nodes, strongly attracts effector T cells and DCs by interacting with CCR7 and CXCR3 receptors (16, 17). CCL21 recruits lymphocytes and antigen-stimulated DCs into T-cell zones of secondary lymphoid organs, colocalizing these early immune response constituents and culminating in cognate T-cell activation (17). In our preclinical murine models, CCL21 treatment resulted in an increase in CD4, CD8, and $CD11c^+DEC205^+$ DC infiltrates into the tumor, creating a lymphoid-like microenvironment (12).

We hypothesized that DCs and CCL21 were important immune mediators to evaluate for immunotherapy (10). On the basis of these findings, we conducted a phase I trial of intratumoral injection of autologous DC overexpressing CCL21 (AdCCL21-DC). Here, we report tumor antigen–specific systemic immune reactivity and safety in advanced NSCLC.

Materials and Methods

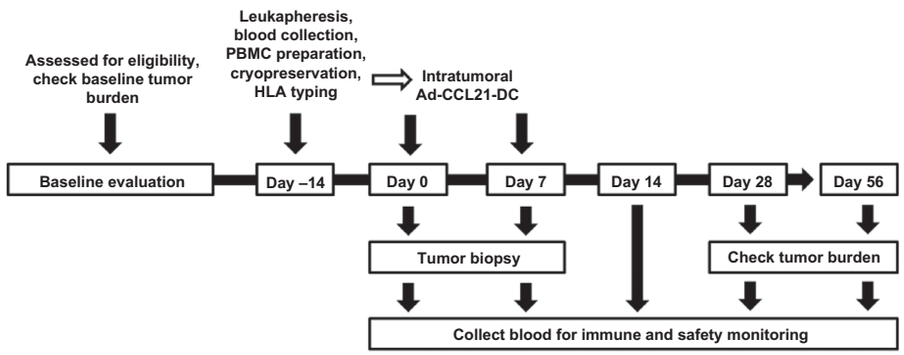
Study design

A phase I, dose escalation, multicohort trial was conducted to enroll patients with advanced stage of lung cancer at UCLA Medical Center (Los Angeles, CA) and the West Los Angeles Veterans Administration (VA) Medical Center (Los Angeles, CA; Fig. 1A). Patients enrolled into a given cohort received the same Ad-CCL21-DC dose (1×10^6, 5×10^6, 1×10^7, or 3×10^7 cells/injection) by CT-guided or bronchoscopic intratumoral injection on both days 0 and 7. The starting dose was 1×10^6 cells/injection in the first cohort (A) and was increased to 5×10^6, 1×10^7, or 3×10^7 cells/injection in subsequent cohorts (B, C, and D, respectively). Dose escalation proceeded only if all 3 patients enrolled in the lower dose cohort experienced no dose-limiting toxicity (DLT) over a 28-day period or only 1 of 6 patients in a cohort had a DLT. All subjects were monitored for clinical and biologic responses for a total of 56 days. All enrolled patients were followed by a participating study physician and underwent a history and physical examination every 3 months until progressive disease or withdrawal from the study. Eligible patients were assigned to a cohort and received intratumoral vaccine injections in conjunction with tumor sampling and patient monitoring (Fig. 1A). Clinical evaluation of tumor shrinkage and disease progression following Ad-CCL21-DC vaccination was assessed using the revised RECIST (version 1.1). Patient characteristics, including smoking history, medical comorbidities, lung cancer stage, and previous therapies, are described in Supplementary Table S1.

Vaccine generation

We previously reported our methodology for vaccine generation (11). The CCL21 adenoviral construct (AdCCL21), lot# L0604006, was manufactured for clinical use by the Biopharmaceutical Development Program at SAIC-Frederick under FDA good manufacturing practice standards (11). Peripheral blood mononuclear cells (PBMC) from patients were obtained by leukocyte-enriched buffy coat (leukapheresis) from patients with UCLA Institute Review Board approval. Informed consent was obtained from each donor. Human monocyte-derived DCs were prepared as described previously (11). These cells were cultured for 6 days in complete RPMI with 5% autologous serum, 800 U/mL GM-CSF (Bayer), and 400 U/mL IL4 (Schering-Plough; ref. 11). On day 6 of culture, monocyte-derived DCs were harvested, and cell viability was determined by Trypan blue (Mediatech Inc.) exclusion, while the viral vectors were thawed on ice. Cells were equilibrated to room temperature and transduced with AdCCL21 with 1167 viral particles (VP)/cell, equivalent to 100:1 multiplicity of infection (11). The manufactured Ad-CCL21-DCs were resuspended in 1 mL of RPMI containing 5% autologous serum for vaccine injection (days 0 and 7, Fig. 1A).

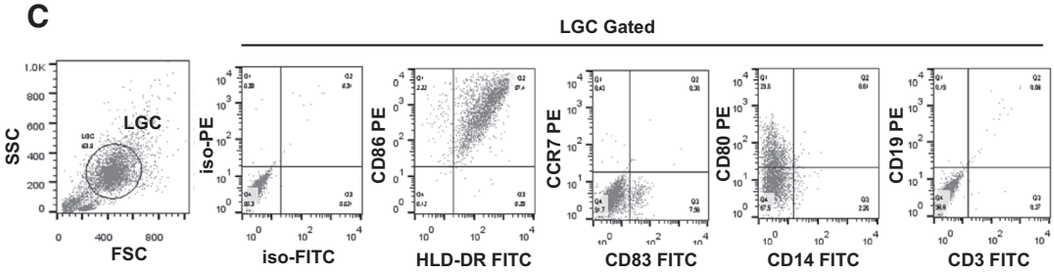
A



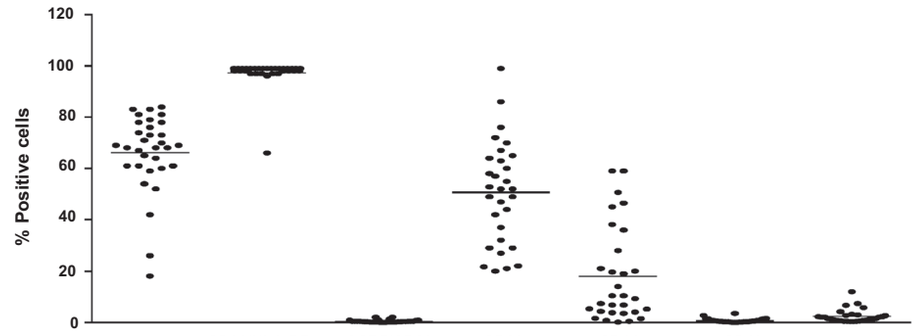
B

	MNC Yield from LK (BC) $\times 10^9$	MNC Yield from LK (BC)%	MNC* Thawed (AC) $\times 10^9$	MNC Thawed (AC) Viability %	DC Yield (BT) $\times 10^6$	DC Yield** (BT) %	DC Viability (BT) %	DC Recovery (AT) %	DC Viability (AT) %
Average ± SD (*n* = 16)	6.3 ± 3.6	9.5 ± 8.6	1.3 ± 1.0	94.9 ± 3.0	76.5 ± 70.4	6.4 ± 4.4	95.6 ± 3.3	63.3 ± 19.7	89.2 ± 7.5

C



D



E

	LGC	CD86$^+$/HLAR$^+$	CCR7$^+$/CD3$^+$	CD80$^+$	CD14$^+$	CD19$^+$	CD3$^+$
Average % ±SD (*n* = 16)	66.1 ± 15.1	97.3 ± 5.8	0.4 ± 0.5	50.6 ± 20.1	17.9 ± 18.5	0.7 ± 0.8	2.3 ± 2.5

Phenotypic analysis of DCs by flow cytometry

DCs were characterized on day 6 of culture before transduction by flow cytometry using the following panel of mAbs: HLA-DR-FITC, CD86-PE, CCR7-PE, CD14-FITC, CD80-PE, CD3-FITC, CD19-PE (BD Biosciences Pharmingen), and CD83-FITC (Coulter Immunology; ref. 11).

DNA isolation and HLA typing

An aliquot from patients' leuko pak was used for HLA typing. DNA was isolated from 1 to 5×10^6 cells using a QIAamp DNA Blood Mini Prep Kit (Qiagen) and stored at −20°C until shipped to NCI (Rockville, MD) for HLA typing.

Quantitative reverse transcription polymerase chain reaction (qPCR)

Lung tumor tissues from patients were removed by the core needle biopsies on day 0 and day 7 after vaccination and were frozen in RNAlater solution (Life Technologies) until used for qPCR. Total RNA was isolated from the frozen tissues using RNeasy Protect Kit (Life Technologies) and transcribed into cDNA using ThermoScript RT-PCR Systems (Life Technologies) according to the manufacturer's instructions. Tumor-associated antigen (TAA) expression profiles and PD-L1 expression were measured by qPCR using the patients' cDNA, SYBR GreenER qPCR SuperMix Kit (Life Technologies), and TAA primers in a iCycler (Bio-Rad). TAA primers included CEA (size 239 bp, forward-ctatgcagagccacccaaac, reverse- cgttctggattccacactca), NY-ESO-1 (size 205 bp, forward- cgcctgcttgagttctacct, reverse- ggagacaggagctgatggag), MAGE-1 (size 105 bp, forward- tctgtgaggaggcaaggttt, reverse- atgaagacccacaggcagat), MAGE-3 (size 227 bp, forward-tgaaggagaagatctgccag, reverse- ggtgacttcaactagagtag), p53 (size 127 bp, forward- acaacgttctgtccccttg, reverse- ggagcagcctctggcattct), Her2/neu (size 186 bp, forward- tgtgactgcctgtccctaca, reverse- gtaactgccctcacctctcg), survivin (size 184 bp, forward-gacgaccccatagaggaaca, reverse- gcactttcttcgcagtttcc), and SSX-2 (size 166 bp, forward- acggttggtgctcaaatacc, reverse-gggtggccttgaaacctagt). The primer sequences for PD-L1 gene (size 142 bp) were forward- TGTGACCAGCACACTGAGAA and reverse- AGTCCTTTCATTTGGAGGATGT. Amplification for TAA expression was carried out for 40 cycles of 15 seconds at 95°C and 60 seconds at 60°C. Amplification for PD-L1 expression was carried out for 95°C for 15 minutes and 40 cycles of 15 seconds at 94°C, 60 seconds at 60°C, and 30 seconds at 72°C. All samples were run in triplicate. TAA gene and PD-L1 expression levels were expressed as a gene copy number using C_t values that were obtained from a β-actin standard curve–derived equation (18). TAA that expressed more than 100 copies per 10^6 β-actin copies were considered overexpressed and selected for the ELISPOT assay. PD-L1 expression was shown as gene copy number per 10^6 β-actin copies.

HLA typing and TAA synthetic peptides

Molecular typing of patients' HLA was conducted at the Department of Transfusion Medicine, NIH (Bethesda, MD). On the basis of the HLA types of the patients, peptides that match predicted TAA commonly seen in NSCLC were designed and synthesized (Genscript Corporation). The primers used for TAA profiling and gene frequencies in the population corresponding to the HLA alleles of our representative peptides are described in Supplementary Tables S2 and S3 (Supplementary Data). Specific tumor antigen peptides from our available panel of HLA class I- and HLA class II–restricted peptide epitopes were selected (Supplementary Table S4).

Immune monitoring by IFNγ ELISPOT assay

Pre- and postvaccinated PBMCs were collected and frozen until used. Frozen PBMCs were rested in X-vivo10 medium (Lonza Inc.) with 10% AB serum (Gemini) overnight. Rested PBMCs (2×10^5 cells/well) were cocultured with patients' HLA-matched peptides derived from TAAs for 24 hours at 37°C in the presence of IL2 (100 IU/mL) in precoated 96-well ELISPOT plate with anti-human IFNγ mAb (15 mg/mL) overnight at 4°C. TAA-derived synthetic peptides for HLA class I and class II were added 1 μg/mL and 10 μg/mL, respectively. PBMCs cultured with medium alone or anti-CD3 at a dilution of 1:1,000 (Mabtech) were used as negative and positive controls, respectively. The IFNγ spots were revealed following the manufacturer's instructions (Mabtech). Briefly, cells were removed after 24 hours, and the plates were washed five times with PBS. Biotinylated secondary antibody in PBS plus 0.5% FBS (Life Technologies, Inc.) was added, and the plates were incubated at room temperature for 2 hours, followed by five PBS washes. Streptavidin-alkaline phosphatase at a dilution of 1:1,000 was added and incubated for 1 hour at room temperature in the dark, followed by five PBS washes. Plates were developed with nitroblue tetrazolium/5-bromo-4-chloro-3-indolylphosphate substrate (Mabtech) for 15 to 45 minutes until distinct spots emerged and then rinsed extensively with deionized water and allowed to dry. The number of specific T cells shown as spot-forming cells per 2.5×10^5 PBMC was calculated after subtracting background using C.T.L. Immunospot system (Celluar Technology Ltd.) in the UCLA Immunology Core Facility.

Immune response was defined as TAA-specific and vaccine-dependent IFNγ production that showed more than a 2-fold increase in spot number compared with background (no peptide)

Figure 1.

Protocol summary and generation of adenoviral CCL21 transduced DCs from lung cancer patients. **A,** Patients were assessed for eligibility by checking baseline tumor burden and underwent leukapheresis if eligible. PBMCs were isolated from the leuko pak and cryopreserved until use and an aliquot was used for HLA typing. Patients received intratumoral injection of vaccine twice with a 1-week interval in between. Needle biopsy was performed just prior to injection on the same day (protocol day 0 and day 7) to allow collection of samples from the tumor. Peripheral blood samples were drawn for safety monitoring (at screening and on days 0, 7, 14, and 28) and immune monitoring studies (days 0, 7, 28, and 56). **B,** DCs were generated following 6 days of culture with GM-CSF and IL4 from cryopreserved MNCs obtained by leukapheresis, as described in Materials and Methods. The summary of the yield of MNCs and DCs, recovery of DCs before adenoviral transduction (BT) and after adenoviral transduction (AT) with AdCCL21, and viability by Trypan blue staining is reported. **C,** After a 6-day culture of MNC in the presence of GM-CSF and IL4, cells were analyzed for surface markers such as CD86, HLD-DR, CCR7, CD83, CD80, CD14, CD19, and CD3 by flow cytometry, as described in Materials and Methods. The results were expressed as a representative phenotype in 1 of 16 patients. **D** and **E**, Summaries of the phenotypic results from 16 patients are shown. Note that there are two data points from 2 preparations of the DC culture per one patient. LK, leuko pak; BC, before cryopreservation; AC, after cryopreservation; BT, before transduction; AT, after transduction; *, cell number used for DC culture; **, (DC yield/MNCs thawed) × 100; LGC, large granular cells.

with absolute number of more than 20 spots per 2.5×10^5 PBMCs. Subjects that had a high response with TAA nonspecific and vaccine-independent IFNγ production at baseline that declined after vaccination but met criteria for TAA-specific and vaccine-dependent immune responses were included as immune responders in the analysis.

Detection of adenovirus IgM and IgG antibodies from patient's plasma

The presence of adenovirus-specific IgG and IgM antibodies in patient plasma collected from PBMCs before and after vaccination was determined by a commercially available antibody ELISA (Diagnostic Automation Inc.) and the manufacturer's instructions. Plasma samples from patients were thawed from −80°C, serially diluted. Diluted patient plasma and the ready-to-use standards were added to a 96-well ELISA plate precoated with adenovirus antigen and incubated for 1 hour at room temperature. After washing, the ready-to-use anti-human IgG or IgM peroxidase conjugate was added and incubated for 30 minutes. After a further washing step, the color development was performed by adding the substrate (TMB) solution and incubating for 20 minutes, and terminated by the addition of a stop solution. The plate was measured at the wavelength of 450 nm.

Nested PCR for detection of free AdCCL21 from patients' plasma

To detect free adCCL21, DNA was extracted from patient's plasma using a kit (Qiagen) and used for PCR. The primers were designed to specifically detect CCL21 DNA driven by cytomegalovirus (CMV) from the Ad vector, but not genomic DNA or RNA. Two pairs of primers were used for the nested PCR as follows: pair #1 (external), primers from the mid-human CMV IE promoter (between AP1 and CAAT box sites) to the 3′ UT region of the hCCL21 cDNA cloned into the Ad vector for the outer PCR; pair #2 (internal), from the 3′ end of the CMV promoter (3′ CAAT box, overlapping the TATA box) to the translation termination of the CCL21 cDNA (bases) for the inner PCR. External and internal primer sequences used are as follows: ExtF 5′- TAC GGG ACT TTC CTA CTT GGC AGT -3′/ ExtR 5′- AGA TTC TCC AGG GCT CCA G -3′ and IntF 5′- CGT GTA CGG TGG GAG GTC TA -3′/IntR 5′- GTT TCT GTG GGG ATG GTG TC -3′, respectively. First, external PCR using external primers was performed using a kit (Invitrogen) and the external PCR product after 1:1,000 dilution was further utilized for internal PCR. The resulting internal PCR was analyzed on an agarose gel. PCR conditions were as follows: external PCR, an initial step at 95°C for 2 minutes, followed by 30 cycles of 95°C for 30 seconds, 55.5°C for 30 seconds, and 60°C for 60 seconds, and internal PCR, an initial step at 95°C for 2 minutes, followed by 30 cycles of 95°C for 30 seconds, 57.7°C for 30 seconds, and 60°C for 60 seconds.

IHC

Lung tumor tissues from patients were removed by the core needle biopsies on day 0 and day 7 after the vaccination, were embedded in paraffin, and microsectioned onto slides. Slides were then placed in xylene to remove paraffin, followed by ethanol. Following a wash in tap water, the slides were incubated in 3% hydrogen peroxide/methanol solution for 10 minutes. For CD8 immunostaining, the slides were washed in distilled water and then incubated for 25 minutes in EDTA Solution pH8 (Invitrogen, #005501) at 95°C using a vegetable steamer. The slides were brought to room temperature and rinsed in PBS containing 0.05% Tween-20 (PBST). The slides were then incubated at room temperature for 90 minutes with anti-CD8 antibody (Dako, M7103) at a dilution of 1:50. The slides were rinsed with PBST and then were incubated with the polyclonal rabbit anti-mouse immunoglobulins (Dako, Z0259) at a dilution of 1:200 at room temperature for 30 minutes. After a rinse with PBST, the slides were incubated with DAB (3,3′-diaminobenzidine) for visualization. Subsequently, the slides were washed in tap water, dehydrated in ethanol, and mounted with media. For PD-L1 staining, slides were baked for 1 hour at 65°C. Slides were then deparaffinized in xylene and rehydrated through graded ethanol to deionized water. Antigen retrieval was performed in a pressure cooker for 5 minutes with Tris-EDTA pH9 buffer and then cooled for 15 minutes at room temperature. IHC was performed on a Leica Bond III autostainer programmed for primary antibody, PDL1 Clone EP314 (Epitomics), at a dilution of 1:300 in Bond Antibody Diluent for 60 minutes, polymer for 15 minutes, peroxidase block for 5 minutes, DAB for 10 minutes, and hematoxylin for 5 minutes followed by 0.5% cupric sulfate for 10 minutes with bond washes between steps. The Bond Refine Polymer Detection Kit (DS9800) was utilized for all steps after primary antibody exposure.

ELISA screening for antibody responses against TAAs

Recombinant proteins of NY-ESO-1, P53, CEA, survivin, and MAGE-A3 were purchased from Abcam and were used to coat 96-well maxSorb ELISA plates (Nunc) at 50 to 150 ng/well/100 μL in PBS overnight at 4°C. BSA was used as a negative control protein for coating. Plasma samples from 8 available patients: SLC17, 18, 23, 25, 28, 29, 30, and 31 at various time points were tested under three different dilutions of 1/100, 1/200, and 1/400 using a previously published protocol for measuring class-switched IgG Ab (19, 20). A reaction was designated positive when specific optical density (OD) at 450 nm (OD against a target minus OD against BSA) was at least 0.1 and at least 2-fold above the specific OD against the same target on day 0. A patient was designated to have strong Ab responses when all three dilutions of the serum had positive reactions, otherwise as weak Ab responses when only 1 or 2 of the dilutions were positive.

Autoimmune antibody serologic testing

Serum samples were obtained from the patient's peripheral blood leukocytes (PBL) to test the presence of autoantibodies. The following antibodies were evaluated: antinuclear antibodies (ANA), rheumatoid factor (RF), anti-double stranded DNA antibodies (Anti-dsDNA), anti-ribonucleoprotein antibodies (anti-RNP), anti-Ro (ssA), anti-La (ssB), and anti-thyroglobulin antibody (anti-TG).

Clinical adverse events

The NCI Common Terminology Criteria for Adverse Events, version 3.0 (CTCAE) was utilized for adverse event (AE) reporting. A grading (severity) scale was used for each AE term.

Statistical analysis

Differences in the PD-L1 gene copy numbers between groups were analyzed by Student *t* test. P value of <0.05 was considered statistically significant.

Results

Generation of Ad-CCL21-DC vaccine

The PBMCs from 16 subjects were evaluated for the generation of Ad-CCL21-DC vaccine (Fig. 1B). Following leukapheresis, cryopreservation and thawing of mononuclear cells (MNC) resulted in 94.9% ± 3.0% cell viability (Fig. 1B). On day 6 of DC culture, transduction of DC with a clinical grade adenovirus expressing CCL21 (AdCCL21) revealed 89.2 ± 7.5% cell viability (Fig. 1B). Following 6 days of culture, the cells showed high expression of DC surface marker phenotype, 97.3% ± 5.8% $CD86^+$/$HLA\text{-}DR^+$, by flow cytometry (Fig. 1C–E). In addition, DC maturation surface marker expression was low, 0.4% ± 0.5% $CCR7^+$/$CD83^+$ consistent with an immature DC phenotype (Fig. 1C–E). Of note, there was one additional patient (SLC10) that received only one vaccination but was excluded from the second vaccination due to noncompliance. As such, there were no specimens for data analysis after day 0 vaccination.

Clinical outcomes in response to Ad-CCL21-DC vaccination

A total of 16 patients received both (day 0 and day 7) vaccinations. Twenty-five percent of patients (4/16) at day 56 had stable disease (SD) following Ad-CCL21-DC vaccination. Median survival was 3.9 months.

All AEs reported to the FDA are listed in Table 1. Four possibly vaccine-related AEs occurred in 3 of 17 patients (includes SLC10 who received only one vaccination) with no clear association to dose or schedule. These included (i) SLC15, who experienced flu-like symptoms and blood-tinged sputum after each injection; (ii) SLC18, who experienced nausea after receiving the first vaccination; and (iii) SLC12, who experienced fatigue after day 14 follow-up (Table 1).

Peripheral blood immune monitoring

Because the DCs were immature at the time of injection, we anticipated that their capacity for antigen uptake and subsequent maturation would facilitate *in situ* vaccination. Therefore, we hypothesized that intratumoral administration of Ad-CCL21-DC would induce $CD8^+$ cytotoxic T lymphocytes (CTL) against multiple TAA. Monitoring systemic T-cell responses against defined peptide epitopes of TAA, pertinent to the expression profile of the patient's tumor and HLA types, provided a measurement of TAA-specific T cells within patients' peripheral blood and may provide a marker for evaluating vaccine immunologic efficacy. Several studies have identified a correlation between the ability to induce a specific T-cell response assessed by ELISPOT assay with clinical outcomes (21–27). Specific TAAs were chosen based on the known expression of these antigens in the literature in NSCLC patients. These TAAs included CEA (60% expression; refs. 28, 29), NY-ESO-1 (40%; ref. 30), MAGE-1 (21%; ref. 31), MAGE-3 (46%; refs. 31, 32), P53 (37%; ref. 32), HER2/neu (50%; ref. 33), and SSX-2 (17%; refs. 34, 35). In our study, all subjects expressed at least one or more of the selected TAA on day 0 and 7 tumor biopsies (Fig. 2A). Among the TAA selected for IFNγ ELISPOT assay in this study, CEA (88% of patients), HER2/neu (94%), and p53 (94%) were the most frequently expressed (Fig. 2A). The TAA HLA/peptide sequences of both HLA class I and class II categories for the responder subjects are shown in Fig. 2B.

The IFNγ ELISPOT assay revealed 6 of 16 total immune responders (Fig. 3A and B). Of these, 3 responders (Fig. 3B) had a high response with nonspecific and vaccine-independent IFNγ production at baseline that declined after vaccination yet met criteria for TAA-specific and vaccine-dependent immune responses on subsequent monitoring.

Expression of PD-L1 and T-cell infiltrates in the primary lung cancer

PD-L1 expression was evaluated in the primary lung cancer before (day 0) and after (day 7) vaccination. Quantitative PCR for PD-L1 mRNA expression did not correlate with IFNγ response (Fig. 4A, left). PD-L1 mRNA expression increased significantly with increased $CD8^+$ T-cell infiltration (day 0, 740 ± 781 vs. day 7, 2910 ± 2213, $P = 0.02$; Fig. 4A, right). These results suggest that vaccine-mediated $CD8^+$ T-cell infiltration is associated with induction of PD-L1 mRNA expression. For example, patient SLC12 (Fig. 4B) had an increase in $CD8^+$ T-cell infiltration on day 7 after AdCCL21-DC vaccination in the setting of high baseline PD-L1 expression on day 0 and resultant increase in PD-L1 expression with vaccination on day 7 (Fig. 5A). These findings also suggest that CCL21 chemokine gene-modified DC immunotherapy can induce vaccine-mediated $CD8^+$ T-cell infiltration with parallel induction of PD-L1 protein expression (Fig. 5A). PD-L1 expression was seen in membranous and cytoplasmic locations (Fig. 5A and B).

Following vaccination (day 0 vs. 7), tumor $CD8^+$ T-cell infiltration was induced in 54% (7/13) of subjects (1.3–7.7 range and 3.4 average fold increase) as measured by the number of $CD8^+$ T cells per mm^2 (Fig. 4B). SLC04, SLC06, SLC12, SLC15, SLC23, SLC28, and SLC30 demonstrated induction of $CD8^+$ T cells following vaccination (Fig. 4B). Three subjects (SLC01, SLC18, and SLC25) were excluded in the $CD8^+$ T-cell analysis due to the absence of viable tumor seen histologically at one or both biopsy timepoints (Fig. 4B).

Humoral response against TAAs

Overall, 5 targets (NY-ESO-1, P53, CEA, survivin, and MAGE-A3) were tested utilizing plasma samples from 8 available patients: SLC17, 18, 23, 25, 28, 29, 30, 31 (Supplementary Fig. S1). Among these 8 patients, strong vaccine-induced Ab was present in SLC23 against MAGE-A3. Weak Ab responses were detected in SLC23 against NY-ESO-1, SLC30 against survivin, SLC31 against survivin, and SLC25 against P53 and NY-ESO-1.

Two of the 4 patients with Ab responses also had vaccine induced cellular immune responses, although not all matched the targets of the cellular immune responses (Ab responses against SSX2 and Her2 were not evaluated).

Safety monitoring

The safety of Ad-CCL21-DC vaccination when administered as an intratumoral injection into a tumor site of patients with advanced NSCLC was assessed. Nested PCR to detect free adenovirus (AdCCL21) in the peripheral blood revealed no evidence of free virus following vaccination on days 0 (day of vaccination), 14, and 28 (data not shown). The titer of antiadenoviral antibody (Anti-Ad Ab) for serotype 5 adenovirus was determined by ELISA in subject serum samples, and it revealed no significant change in the titers of IgG and IgM antibodies on days 0 and 28 (data not shown).

Autoimmune serologies of the peripheral blood revealed no significant change comparing antibody titers before and after AdCCL21-DC vaccination (Supplementary Table S5 in Supplementary Data).

Table 1. Summary of adverse events

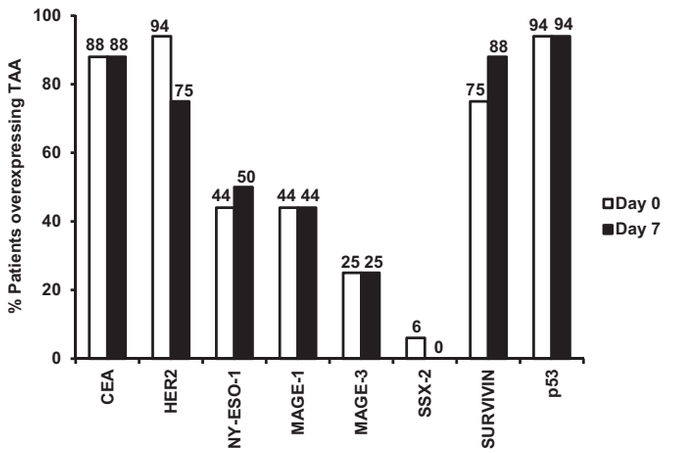
AE ($n = 17$ patients)	Grade 1	Grade 2	Grade 3	Grade 4	Grade 5	Total
Blood and lymphatics						
Anemia		2				2
Leukocytosis	1					1
Cardiac						
Chest pain	1	1	1			3
Sinus tachycardia	1		1	1		3
Superior vena cava and brachiocephalic vein stenosis			1			1
Gastrointestinal						
Abdominal pain	2					2
Dysphagia	1					1
Constipation	2					2
Gastroesophageal reflux disease	1					1
Hematemesis	1					1
Nausea	3[a]		1			4
Vomiting			1			1
General						
Cachexia	1					1
Edema	1	1	1			3
Fever	1					1
Fatigue	2[a]	3	3	1		9
Flu-like symptoms	1[a]					1
General discomfort/pain	1		1			2
Night sweats		1				1
Pallid	1					1
Swelling (head)	1					1
Infections and infestations						
Sinusitis	1					1
Metabolism and nutrition						
Alkalosis	1					1
Alcohol intoxication		1				1
Anorexia	1	1				2
Hypercalcemia	1					1
Hyponatremia	1					1
Musculoskeletal						
Fracture (cervical spine)				1		1
Pain/Muscle weakness	1	7	4	1		13
Nervous system						2
Headache	1	1				
Stroke					1	1
Psychiatric						
Anxiety		3				3
Confusion	1					1
Delirium	1					1
Depression		1				1
Renal and urinary						
Nocturia		1				1
Pyuria	1					1
Urinary tract infection	1					1
Respiratory						
Blood-tinged sputum after injections	1[a]					
Chronic obstructive pulmonary disease exacerbation			1			1
Dyspnea	2	4	5	1		12
Hypoxia	1		1			2
Pleural effusion	3	1	1			5
Possible pneumonia			1			1
Productive cough	1	3	1			6
Pulmonary hypertension	1					1
Rhonchi	1					1
Sore throat	1					1
Wheezing	1					1
Catheter placement for leukapheresis			2			2
Progressive disease			1	6		7

[a]Four adverse events were possibly related to the vaccine.

Discussion

Here, we report the first-in-man administration of CCL21 as well as the first trial of DC intratumoral *in situ* vaccination in human NSCLC. We found that *CCL21* gene–modified DCs could be administered safely intratumorally to patients with advanced NSCLC. In addition, in response to therapy, several of the treated

A



B

Patient ID	TAA Selected for IFNγ ELISPOT assay (HLA/peptide sequence)
SLC01	NY-ESO-1a (A2/SLLMWITQC); NY-ESO-1b (DR4/WITQCFLPVFLAQPPSGQRR); CEA (DR4/YACFVSNLATGRNNS); HER2-a (A2/KIFGSLAFL); HER2-b (DR4/KEILDEAYVMAGVGSPYVS)
SLC07	CEA (DR4/YACFVSNLATGRNNS); MAGE-1 (A1/EADPTGHSY); MAGE-3 (DP4/KKLLTQHFVQENYLEY); NY-ESO-1a (A31/ASGPGGGAPR); NY-ESO-1b (DR4/PGVLLKEFTVSG)
SLC12	CEA-a (A24/QYSWFVNGTF); CEA-b (A24/TYACFVSNL); NY-ESO-1 (DP4/WITQCFLPVFLAQPPSGQRR); HER2 (DR1/KEILDEAYVMAGVGSPYVS)
SLC23	MAGE-1 (A1/EADPTGHSY); MAGE-3 (DR13/AELVHFLLLKYRAR); SSX-2(37) (DR13/KMKASEKIFYVYMKRKYEAMT); SSX-2(133) (DR13/QNDGKELCPPGKPTTSEKIHERSGPKRGEH); NY-ESO-1 (DP4/WITQCFLPVFLAQPPSGQRR)
SLC28	CEA (DR4/YACFVSNLATGRNNS); HER2-a (A2/KIFGSLAFL); HER2-b (DR4/KEILDEAYVMAGVGSPYVS); p53a (A2/PPGTRVRAM); p53b (A2/LLGRNSFEV)
SLC30	p53-1 (A2/PPGTRVRAM); p53-2 (A2/LLGRNSFEV); HER2-a (A2/KIFGSLAFL); HER2-b (DR7/KEILDEAYVMAGVGSPYVS); MAGE-1 (A2/CLGLSYDGLL); SSX-2(A2/KASEKIFYV)

Figure 2.

TAA expression profiles in NSCLC patients and HLA-restricted synthetic peptides of TAA used for IFNγ ELISPOT assay. **A,** Lung tumor biopsy for 16 patients was performed on day 0 and day 7 after the vaccine administration, and TAA expression profiles were determined by qPCR using the tumor antigen panel as described in Materials and Methods. Overexpression of TAA was defined as an expression of more than 100 gene copies per 10^6 β-actin gene copies. Percentage of patients that overexpress each TAA were shown. **B,** Patients' HLA-matched TAA-derived synthetic peptides were selected and added to the 96-well plate of IFNγ ELISPOT assay. Six of 16 patients showed immune responses to TAA-specific IFNγ production.

tumors revealed enhanced $CD8^+$ lymphocyte infiltration, and immune monitoring showed specific systemic immune responses against autologous tumor antigens as evidenced by PBL IFNγ release by ELISPOT. Humoral responses against TAAs were also found in 4 of 8 patients evaluated.

The T-cell response, which is initiated through antigen recognition by the TCR, is regulated by the balance between costimulatory and inhibitory signals, including immune checkpoints (36). These immune checkpoints are important for the maintenance of self-tolerance and prevention of autoimmunity (36). The expression of immune checkpoint proteins can be dysregulated by tumors resulting in immune resistance, particularly against tumor-specific T cells (36, 37). Blockade of immune checkpoints can amplify the antitumor immune response (36, 38). One of the critical checkpoint pathways responsible for mediating tumor-induced immunosuppression is the PD-1/PD-L1 pathway (39).

Recent studies reveal responses in approximately 20% of NSCLC patients treated with inhibitors of the PD-1/PD-L1 checkpoint. This includes robust and durable responses in previously treated patients with progressive locally advanced or metastatic NSCLC (40–43). However, a large percentage of patients do not

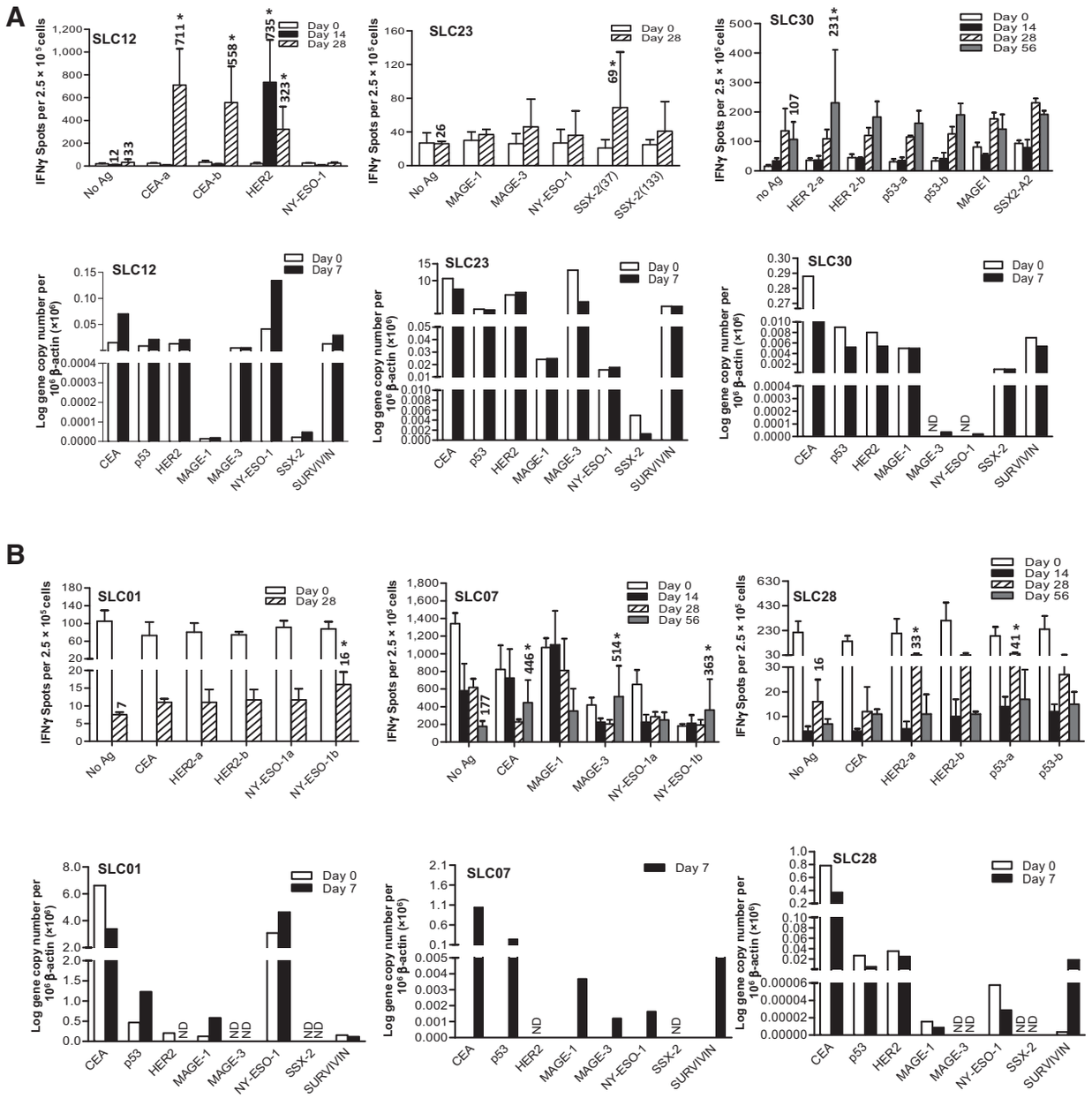
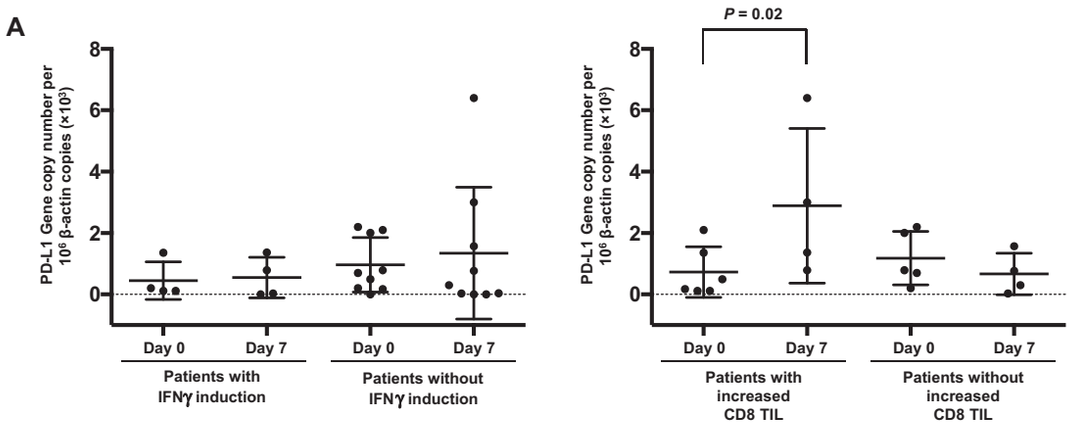


Figure 3.

Immunologic responses to vaccination. PBMCs were collected pre- and postvaccination and were cocultured with patient's HLA-matched peptides and derived from TAAs for 24 hours to monitor immune responses by IFNγ ELISPOT assay as described in Materials and Methods. **A** and **B**, Six of 16 patients showed vaccine-dependent response to IFNγ production. **B,** Three of 6 responders (SLC01, SLC07, and SLC28) had a high response with TAA nonspecific and vaccine-independent IFNγ production at baseline that declined after vaccination yet met criteria for TAA-specific and vaccine-dependent immune responses. Profiles of TAA for each patient are shown in the bottom panel.

respond to checkpoint inhibitors delivered as single agents. Studies in melanoma and NSCLC patient-derived tumor specimens reveal that responses to PD-1/PD-L1 blockade require baseline PD-L1 expression and an existing T-cell response at baseline (3, 44, 45). Although lung cancers express tumor antigens, they often fail to function well as antigen-presenting cells (APC; ref. 46). In fact, the tumor's lack of costimulatory molecules, in combination with its production of inhibitory factors, promotes a state of suppressed cell-mediated immunity (47–51). Therefore, our efforts to build on recent gains in NSCLC immunotherapy are focused on methods to restore tumor T-cell infiltration, tumor antigen presentation, and T-cell responsiveness to extend the effectiveness of checkpoint inhibitors to additional NSCLC patients.

A



B

Cohort	Patient ID	IFNγ ELISPOT response†	CD8 TIL/mm^2	
			Day 0	Day 7
A	SLC01	I	NA	111.2
	SLC04	NI	62.7	241.6
	SLC05	NI	41.4	30.2
	SLC06	NI	19.0	147.1
B	SLC07	I	180.2	72.5
	SLC09	NI	136.6	47.2
	SLC12	I	6.9	17.5
C	SLC15	NI	8.0	28.0
	SLC17	NI	110.1	115.5
	SLC18	NI	NA	NA
	SLC23	I	23.0	63.5
	SLC25	NI	18.7	NA
D	SLC28	I	43.3	91.2
	SLC29	NI	113.9	70.6
	SLC30	I	88.0	111.3
	SLC31	NI	48.1	45.7

†ELISPOT response on days 14, 28, or 56; I –IFNγ induction; NI – no IFNγ induction; NA – no viable tumors

Figure 4.

Association between PD-L1 expression and IFNγ ELISPOT assay response or CD8$^+$ T-cell infiltration into tumor. **A,** Left, PD-L1 gene copy numbers were compared between patients with ($n = 4$) and without ($n = 9$) IFNγ induction on days 0 and 7 after vaccine administration; right, PD-L1 gene copy numbers were compared between patients with ($n = 4$) and without ($n = 9$) tumoral infiltration of CD8$^+$ T cells on day 0 and day 7 after vaccine administration. **B,** A summary of IFNγ ELISPOT assay response on day 14, 28, or 56 after vaccine administration, and tumoral infiltration of CD8$^+$ T cells on day 0 and day 7 after vaccine administration. Results are shown from a total of 16 patients.

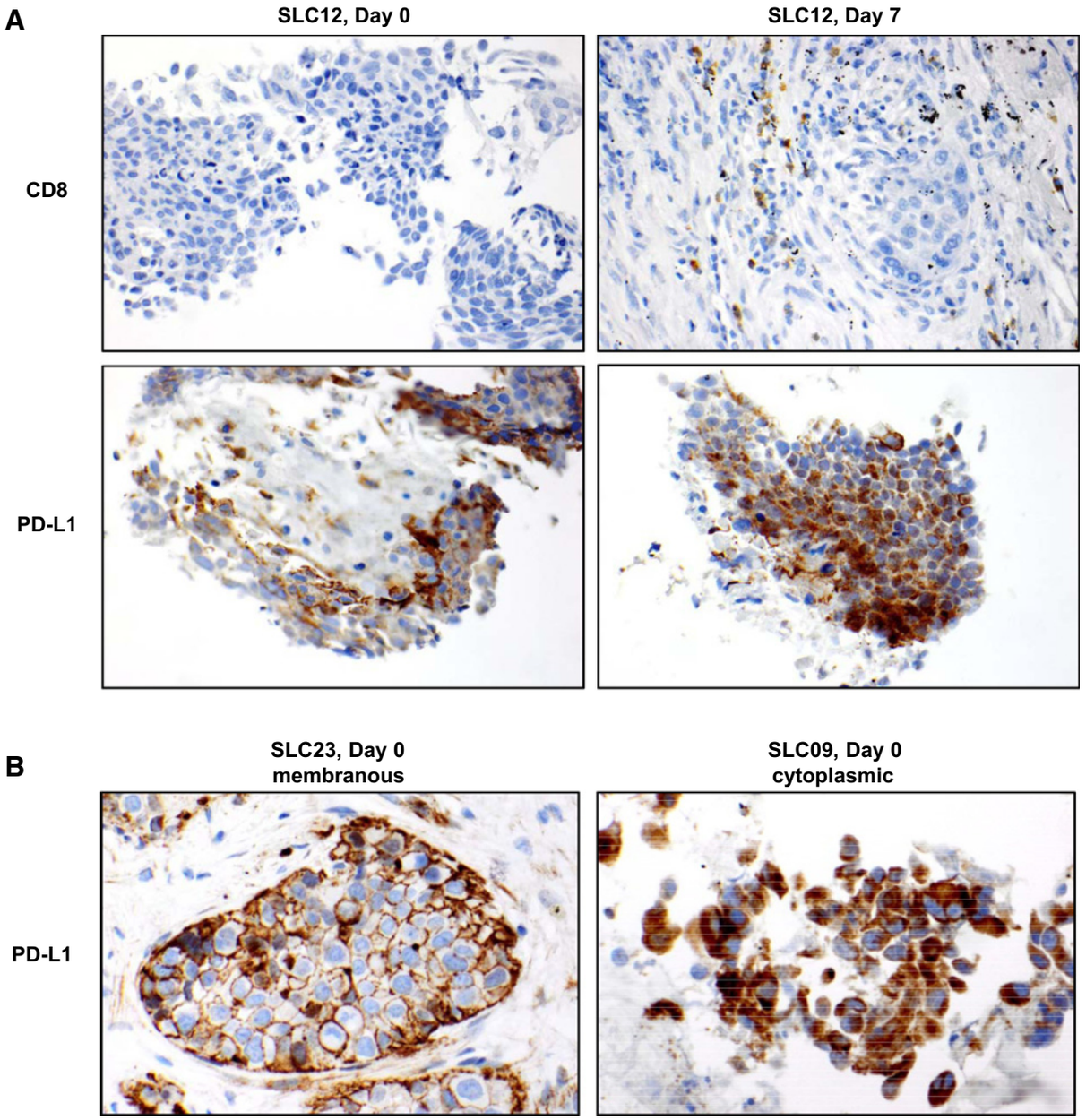


Figure 5.

Tumor IHC staining with CD8 and PD-L1. **A,** Paraffin-embedded tumor tissues were stained with anti-CD8 and anti-PD-L1 on day 0 and day 7, and representative IHC staining images from SLC12 are shown. **B,** Representative IHC staining images of membranous and cytoplasmic expression of PD-L1 on day 0 from patients SLC23 and SLC09, respectively, are shown.

The current study focuses on restoration of tumor antigen presentation and antitumor effector activities in lung cancer patients by utilizing intratumoral DC-based genetic immunotherapy in an attempt to generate specific systemic responses. The concept of *in situ* vaccination suggests that effective cancer vaccines can be generated *in vivo* without the need to first identify and isolate the TAA (52). Thus, *in situ* vaccination has the potential to exploit the TAA at the tumor site to induce a systemic response. DCs, the most potent APCs, have the capacity to modulate immune tolerance and immunity and could play a central role for *in situ* vaccination (5).

CCL21 (also previously referred to as Exodus 2, 6Ckine, or secondary lymphoid tissue chemokine) has been identified as a lymphoid chemokine that is predominantly and constitutively expressed by high endothelial venules in lymph nodes and Peyer patches, lymphatic vessels, and stromal cells in spleen and

appendix (53).This chemokine, along with CCL19, is required for normal lymphoid tissue organization that is ultimately essential for effective T cell–DC interactions. DCs are uniquely potent APCs involved in the initiation of immune responses. Serving as immune system sentinels, DCs are responsible for antigen acquisition in the periphery and subsequent transport to T-cell areas in lymphoid organs where they prime specific immune responses. Thus, chemokines that attract both DC and lymphocyte effectors into the tumor can serve as potent agents in immunotherapy. In addition, by emulating the lymph node environment, the chemoattractive properties of CCL21 encourage the localization of those immune effectors previously found to engender a favorable prognosis in NSCLC (7–9).

Tumor mutational load may be an important predictor of response to immune-based therapies. One limitation to our study is that only selected TAAs were assessed for immune recognition. This may have underestimated the true extent of autologous antigen recognition following vaccination. In future studies, whole-exome sequencing will be employed to assess the mutational load and define neoepitopes that are recognized *in situ*. An additional limitation to this study is the limited number and dosing of injected DCs. Now that we have determined that DC *in situ* vaccination is safe and feasible in this patient population, increased dosing and DC numbers can be assessed.

In addition to the induction of TAA-specific CTL and helper T cells in the peripheral blood, our vaccination strategy was effective in eliciting tumor $CD8^+$ T-cell infiltration (54% of all subjects), and there was a parallel increase in PD-L1 expression. These findings suggest that the vaccination itself increased PD-L1 expression as a result of antigen recognition and $CD8^+$ T-cell infiltration at the tumor site. Therefore, vaccination may be an effective approach to increasing efficacy to PD-1/PD-L1 checkpoint inhibition therapies in low PD-L1 baseline-expressing tumors and those that show a paucity of $CD8^+$ T-cell infiltration. Our findings provide a strong rationale for initial *in situ* vaccination immunotherapy to induce a baseline immune response that facilitates antigen uptake, presentation, and effector activation in patients receiving checkpoint inhibitor therapy for NSCLC.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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