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

Jay M. Lee¹,², Mi-Heon Lee¹,², Edward Garon³, Jonathan W. Goldman³, Ramin Salehi-Rad⁴, Felicita E. Baratelli⁴, Dörthe Schaeu⁵, Gerald Wang¹,⁴, Fran Rosen¹,⁴, Jane Yanagawa¹,², Tonya C. Walser¹,⁴, Ying Lin¹,⁴, Stacy J. Park¹,⁴, Sharon Adams⁶, Francesco M. Marincola⁶, Paul C. Tumeh¹,⁷, Fereidoun Abtin⁵, Robert Suh⁵, Karen L. Reckamp⁸, Gina Lee¹,⁴,⁹, William D. Wallace¹⁰, Sarah Lee¹¹, Gang Zeng¹¹, David A. Elashoff¹,¹², Sherven Sharma¹,⁴,¹³, and Steven M. Dubinett¹,⁴,⁸,⁹,¹⁴

Abstract

Purpose: A phase I study was conducted to determine safety, clinical efficacy, and antitumor 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 × 10⁶, 5 × 10⁶, 1 × 10⁷, or 3 × 10⁷ DCs/injection) by CT- or bronchoscopy-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 mm²). 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...
**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 (Ad-CCL21-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).
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A

Assessed for eligibility, check baseline tumor burden

Leukapheresis, blood collection, PBMC preparation, cryopreservation, HLA typing

Intratumoral Ad-CCl2-DC

Baseline evaluation

Day –14

Day 0

Day 7

Day 14

Day 28

Day 56

Tumor biopsy

Check tumor burden

Collect blood for immune and safety monitoring

B

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C

LGC Gated

D

% Positive cells

E

Average %

± SD (n = 16)

LGC

CD68/HLA-DR

CCR7/CD83

CD80

CD14

CD19

CD3

LGC

CD68/HLA-DR

CCR7/CD83

CD80

CD14

CD19

CD3

Average %

± SD

(n = 16)
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 Pharamingen), 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 × 10⁶ 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-ctgtctgacccacccaac, reverse-ggttctgaaatcactc), NY-ESO-1 (size 205 bp, forward-cgctgctgttctact, reverse-gagacagacgtgatgag), MAGE-1 (size 105 bp, forward-ctcgaggagtccattcc, reverse- ggagacagacgtgatgag), Her2/neu (size 127 bp, forward-tgtgactgcctgtccctaca, reverse-ggtgacttcaactagagtag), p53 (size 105 bp, forward-tctgtgagggcaaggttt, reverse-ggtgacttcaactagagtag), MAGE-3 (size 227 bp, forward-tggaagagagatgcgag, reverse-gtggtctacactagagta), survivin (size 184 bp, forward-agacagccccattaggaacacaca, reverse-gacratcgctcacctctg), and SXX-2 (size 166 bp, forward-agcctggtctcataacttac, reverse-gggtggtcgactcattactg). The primer sequences for PD-L1 gene (size 142 bp) were forward-TGTGACCCAGCAGACTGAGAA and reverse-AGTCCCTTCATATTGGAGGATGT. 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 40 cycles of 15 seconds at 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 C₀ values that were obtained from a β-actin standard curve–derived equation (18). TAA that expressed more than 100 copies per 10⁶ β-actin copies were considered overexpressed and selected for the ELISPOT assay. PD-L1 expression was shown as gene copy number per 10⁶ 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-vivo 10 medium (Lonza Inc.) with 10% AB serum (Gemini) overnight. Rested PBMCs (2 × 10⁵ cells/well) were cocultured with patients' HLA-matched peptides derived from TAA 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 × 10⁵ PBMC was calculated after subtracting background using C.T.L. Immunospot system (Cellular 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)
with absolute number of more than 20 spots per $2.5 \times 10^5$ PBMCs. Subjects that had a high response with TAA nonspecific and vaccine-independent IFN response 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 $-80^\circ$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: ExtR 5' - TAC GGG ACT TTC CTA CTT GGC AGT -3', and IntF 5' - GTT TCT GTG GGG -3'.

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% DC culture, transduction of DC with a clinical grade adenovirus (Fig. 1B). Following 6 days of culture, the cells showed high expression of DC surface marker phenotype, 97.3% ± 5.8% CD86+/HLA-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 mm2 (Fig. 4B). SLC04, SLC06, SLC12, SLC15, SLC23, SLC26, 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).
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

### Table 1. Summary of adverse events

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*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
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

<table>
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<th>Patient ID</th>
<th>TAA Selected for IFNγ ELISPOT assay (HLA/peptide sequence)</th>
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<td>SLC01</td>
<td>NY-ESO-1α (A2/SLLMWITQC); NY-ESO-1β (DR4/WITQCFLPVFLAQPPSGQRR); CEA (DR4/YACFVSNLATGRRNNS); HER2-a (A2/KIFGSLALF); HER2-b (DR4/KIILEAYMVAGVSPYVY)</td>
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<tr>
<td>SLC07</td>
<td>CEA (DR4/YACFVSNLATGRRNNS); MAGE-1 (A1/EADPTGHSS); MAGE-3 (DP4/KKLTQHVFQENLYE); NY-ESO-1α (A31/ASGGPGGAPR); NY-ESO-1β (DR4/PGVVLKKEFTVSG)</td>
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<tr>
<td>SLC12</td>
<td>CEA-a (A24/QYSWFVNGTF); CEA-b (A24/TYACFVSNL); NY-ESO-1 (DP4/WITQCFLPVFLAQPPSGQRR); HER2 (DR1/KIILEAYMVAGVSPYV)</td>
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<tr>
<td>SLC23</td>
<td>MAGE-1 (A1/EADPTGHSS); MAGE-3 (DR1/KKLTQHVFQENLYE); SSX-2 (DR1/KEILDEAYMVAGVSPYV)</td>
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<tr>
<td>SLC28</td>
<td>CEA (DR4/YACFVSNLATGRRNNS); HER2-a (A2/KIFGSLALF); HER2-b (DR4/KIILEAYMVAGVSPYV); p53α (A2/PPGTRV); p53β (A2/LLGRNSFEV)</td>
</tr>
<tr>
<td>SLC30</td>
<td>p53-1 (A2/PPGTRV); p53-2 (A2/LLGRNSFEV); HER2-a (A2/KIFGSLALF); HER2-b (DR7/KIILEAYMVAGVSPYV); MAGE-1 (A2/CLGSLYDGL); SSX-2 (A2/KASEKIFV)</td>
</tr>
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</table>
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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 immuno-therapy 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 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 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.
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

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.
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 chemotactic 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 TAAAs 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.

Authors’ Contributions


Study supervision: J.M. Lee, G. Lee, S.M. Dubinett

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