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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 anti-tumor immune responses in patients with advanced non-small cell lung carcinoma (NSCLC) following intratumoral (IT) 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 dendritic cells/injection) by CT- or bronchoscopic-guided IT injections (days 0 and 7). Immune responses were assessed by tumor antigen-specific peripheral blood lymphocyte induction of IFN-γ in ELISPOT assays. Tumor biopsies were evaluated for $CD8^+$ T cells by immunohistochemistry (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 1) induction of systemic tumor antigen-specific immune responses, 2) enhanced tumor $CD8^+$ T cell infiltration, and 3) 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.

Keywords

CCL21; dendritic cell immunotherapy; adenoviral immunotherapy; lung cancer

Introduction

Lung cancer is the leading cause of cancer death in the United States, and immunotherapy with checkpoint inhibitors is transforming therapeutic approaches [1, 2]. While approximately 20% of patients respond to antibody-mediated therapies that block PD-1 or PD-L1, patients without tumor-infiltrating $CD8^+$ T cells and PD-L1 expression appear to be less likely to respond [3, 4]. Thus, it has been suggested that those tumors with a non-immunogenic 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 DC and $CD8^+$ T-cell densities constitutes a powerful and independent prognostic factor for

overall survival [7]. Importantly, these DC 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].

Based on 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 dendritic cell (DC)-based intratumoral (IT) 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, co-localizing 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+ dendritic cell 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]. Based on 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.

Methods

Study design

A phase I, dose escalation, multi-cohort trial was conducted to enroll patients with advanced stage of lung cancer at UCLA Medical Center and the West Los Angeles Veterans Administration (VA) Medical Center (Figure 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 (PD) 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 (Figure 1A). Clinical evaluation of tumor shrinkage and disease progression following Ad-CCL21-DC vaccination was assessed using the revised Response Evaluation Criteria in

Solid Tumors (RECIST; version 1.1). Patient characteristics including smoking history, medical co-morbidities, lung cancer stage, and previous therapies are described in 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 (Frederick, MD) under FDA good manufacturing practice (GMP) standards [11]. Peripheral blood mononuclear cells (PBMC) from patients were obtained by leukocyte-enriched buffy coat (leukapheresis, LK) from patients with UCLA Institute Review Board (IRB) approval. Informed consent was obtained from each donor. Human monocyte-derived dendritic cells were prepared as described [11]. These cells were cultured for 6 days in complete RPMI with 5% autologous serum, 800 U/ml GM-CSF (Bayer, Bothell, WA), and 400 U/ml IL-4 (Schering-Plough, Kenilworth, NJ) [11]. On day 6 of culture, monocyte-derived DC were harvested and cell viability was determined by Trypan Blue (Mediatech Inc., Herdon, VA) exclusion while the viral vectors thawed on ice. Cells were equilibrated to room temperature (RT) and transduced with AdCCL21 with 1167 viral particles (VP)/cell, equivalent to 100:1 multiplicity of infection (MOI) [11]. The manufactured Ad-CCL21-DC were resuspended in 1 ml of RPMI containing 5% autologous serum for vaccine injection (days 0 and 7, Figure 1A).

Phenotypic analysis of DC by flow cytometry

DC were characterized on day 6 of culture before transduction by flow cytometry using the following panel of monoclonal antibodies: HLA-DR-FITC, CD86-PE, CCR7-PE, CD14-FITC, CD80-PE, CD3-FITC, CD19-PE (BD Biosciences Pharmingen, San Diego, CA), CD83-FITC (Coulter Immunology, Hialeah, FL) [11].

DNA isolation and HLA typing

An aliquot from patients' leuko pak was used for HLA (Human Leukocyte Antigen) 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 (National Cancer Institute) for HLA typing.

Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Lung tumor tissues from patients were removed by the core needle biopsies on day 0 and day 7 after the vaccination and were frozen in RNAlater® solution (Life technologies, Grand Island, NY) until used for qRT-PCR. Total RNA was isolated from the frozen tissues using RNeasy Protect Kit (Life technologies, Grand Island, NY) and transcribed into cDNA using ThermoScript™ RT-PCR Systems (Life technologies, Grand Island, NY) according to the manufacturer's instruction. Tumor associated antigen (TAA) expression profiles and PD-L1 expression were measured by qRT-PCR using the patients' cDNA, SYBRGreenER qPCR SuperMix Kit (Life technologies, Grand Island, NY), and TAA primers in a iCycler® (Bio-Rad, Hercules, CA). 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 15min 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 Ct values that was 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, National Institutes of Health. Based on the HLA types of the patients, peptides that match predicted TAA commonly seen in NSCLC were designed and synthesized (Genscript Corporation, New Brunswick, NJ). The primers used for TAA profiling and gene frequencies in the population corresponding to the HLA alleles of our representative peptides are described in Table S2 and S3 (supplemental data). Specific tumor antigen peptides from our available panel of HLA class I- and HLA class II-restricted peptide epitopes were selected (Table S4).

Immune monitoring by IFN-γ ELISPOT assay

Pre- and post-vaccinated PBMC were collected and frozen until used. Frozen PBMC were rested in X-vivo10 medium (Lonza Inc, Allendale, NJ) with 10% AB serum (Gemini, West Sacramento, CA) overnight. Rested PBMC (2×10^5 cells/well) were co-cultured with patients' HLA matched peptides derived from tumor associated antigens for 24 hours at 37°C in the presence of IL-2 (100 IU/ml) in pre-coated 96 well ELISPOT plate with anti-human IFN-γ monoclonal antibody (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. PBMC cultured with medium alone or anti-CD3 at a dilution of 1:1000 (Mabtech, Cincinnati, OH) were used as negative and positive controls, respectively. The IFN-γ spots were revealed following the manufacturer's instructions (Mabtech, Cincinnati, OH). Briefly, cells were removed after 24 hours and the plates were washed five times with PBS. Biotinylated secondary antibody in PBS plus 0.5% fetal bovine serum (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 1:1000 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, Cincinnati, OH) 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, Cleveland, OH) in the UCLA Immunology Core Facility.

Immune response was defined as TAA specific and vaccine dependent IFN-γ production that showed more than a 2-fold increase in spot number compared to background (no peptide) with absolute number of more than 20 spots per 2.5×10^5 PBMC. Subjects that had a high response with TAA non-specific and vaccine independent IFN-γ 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 PBMC before and after vaccination was determined by a commercially available antibody ELISA (Diagnostic Automation Inc. Calabasas, CA) 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 pre-coated with Adenovirus antigen and incubated for one hour at room temperature. After a 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, Valencia, CA) and used for PCR. The primers were designed to specifically detect CCL21 DNA driven by 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, Grand Island, NY) and the external PCR product after 1:1000 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.

Immunohistochemistry

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 micro-sectioned 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, 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 Phosphate Buffered Saline 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 tumor-associated antigens

Recombinant proteins of NY-ESO-1, P53, CEA, survivin, and MAGE-A3 were purchased from Abcam (Cambridge, MA) and were used to coat 96-well maxSorb ELISA plates (Nunc, Denmark) at 50–150 ng/well/100 μl in PBS overnight at 4 degrees. 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 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 PBL to test the presence of autoantibodies. The following antibodies were evaluated: Anti-nuclear 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's t-test. P value of <0.05 was considered statistically significant.

Results

Generation of Ad-CCL21-DC vaccine

The PBMC from 16 subjects were evaluated for the generation of Ad-CCL21-DC vaccine (Figure 1B). Following leukapheresis, cryopreservation and thawing of mononuclear cells (MNC) resulted in 94.9 ± 3.0% cell viability (Figure 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 (Figure 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 (Figure 1C, 1D and 1E). Additionally, DC maturation surface marker expression was low, 0.4 ± 0.5% $CCR7^+/CD83^+$ consistent with an immature DC phenotype (Figure 1C, 1D and 1E). Of note, there was one additional patient (SLC10) that received only one vaccination but was excluded from the second vaccination due to non-compliance. 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 (4/16) of patients at day 56 had stable disease (SD) following Ad-CCL21-DC vaccination. Median survival was 3.9 months.

All AE reported to the Food and Drug Administration (FDA) are listed in Table 1. Four possibly vaccine related AE occurred in 3 of 17 patients (includes SLC10 who received only one vaccination) with no clear association to dose or schedule. These included 1) SLC15, who experienced flu-like symptoms and blood-tinged sputum after each injection, 2) SLC18, who experienced nausea after receiving the first vaccination, and 3) SLC12, who experienced fatigue after day 14 follow-up (Table 1).

Peripheral blood immune monitoring

Because the DC 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 (CTLs) 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 TAA were chosen based on the known expression of these antigens in the literature in NSCLC patients. These TAA included CEA (60% expression) [28, 29], NY-ESO-1 (40%) [30], MAGE-1 (21%) [31], MAGE-3 (46%) [31, 32], P53 (37%) [32], HER2/neu (50%) [33], and SSX-2 (17%) [34, 35]. In our study, all subjects expressed at least one or more of the selected TAA on day 0 and 7 tumor biopsies (Figure 2A). Among the TAA selected for IFN-γ ELISPOT assay in this study, CEA (88% of patients), HER2/neu (94%), and p53 (94%) were the most frequently expressed (Figure 2A). The TAA HLA/peptide sequences of both HLA class I and class II categories for the responder subjects are shown in Figure 2B.

The IFN-γ ELISPOT assay revealed 6 of 16 total immune responders (Figure 3A and 3B). Of these, 3 responders (Figure 3B) had a high response with non-specific and vaccine-independent IFN-γ 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 RT-PCR for PD-L1 mRNA expression did not correlate with IFN-γ response (Figure 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; Figure 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 (Figure 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 (Figure 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 (Figure 5A). PD-L1 expression was seen in membranous and cytoplasmic locations (Figure 5A and 5B).

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 (Figure 4B). SLC04, SLC06, SLC12, SLC15, SLC23, SLC28, and SLC30 demonstrated induction of $CD8^+$ T cells following vaccination (Figure 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 (Figure 4B).

Humoral response against tumor-associated antigens

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 (Figure 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 anti-adenoviral 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 (Table S5 in supplemental data).

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 DC 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 IFN-γ release by ELISPOT. Humoral responses against tumor-associated antigens 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 co-stimulatory 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 anti-tumor immune response [36, 38]. One of the critical checkpoint pathways responsible for mediating tumor-induced immune suppression is the programmed death-1/programmed death ligand-1 (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 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]. While lung cancers express tumor antigens, they often fail to function well as antigen presenting cells (APCs) [46]. In fact, the tumor's lack of co-stimulatory 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.

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 antigen presenting cells, 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's 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. DC are uniquely potent APCs involved in the initiation of immune responses. Serving as immune system sentinels, DC 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 chemo-attractive properties of CCL21 encourages 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 (WES) will be employed to assess the mutational load and define neo-epitopes 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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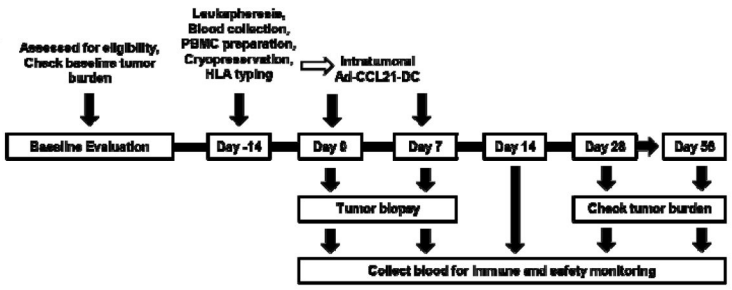
Translational Relevance

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 interactions. In pre-clinical model systems, we have previously demonstrated that intratumoral administration of dendritic cells (DC) 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.

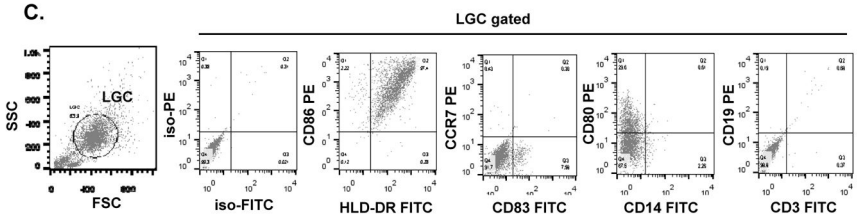
A.



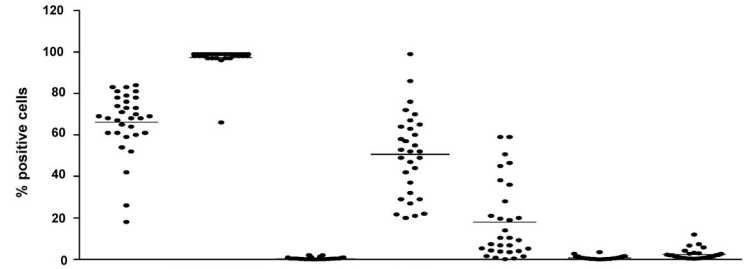
B.

	MNC Yield from LK (BC) x10^9	MNC Yield from LK (BC)%	MNC* Thawed (AC) x10^9	MNC Thawed (AC) Viability %	DC Yield (BT) x10^6	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.

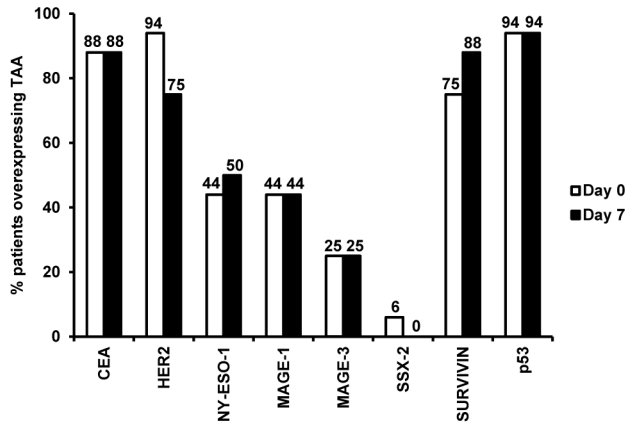
	LGC	CD86$^+$/HLAR$^+$	CCR7$^+$/CD3$^+$	CD80$^+$	CD14$^+$	CD19$^+$	CD3$^+$
Average % ±SD (N=16)	66.1 ±15.1	97.3 ± 5.8	0.4 ± 0.5	50.6 ± 20.1	17.9 ± 18.5	0.7 ± 0.8	2.3 ± 2.5

Figure 1. Protocol summary and generation of adenoviral CCL-21 transduced DC from lung cancer patients

(A) Patients were assessed for eligibility by checking baseline tumor burden and underwent leukapheresis if eligible. PBMC 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 one-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) DC were

generated following 6 days of culture with GM-CSF and IL-4 from cryopreserved mononuclear cells (MNC) obtained by leukapheresis, as described in Materials and Methods. The summary of the yield of MNC and DC, recovery of DC 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 IL-4, 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/MNC Thawed) x100, LGC; large granular cells

A.



B.

Patient ID	TAA selected for IFN-γ 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. Tumor associated antigen (TAA) expression profiles in NSCLC patients and HLA restricted synthetic peptides of TAA used for IFN-γ ELISPOT assay

(A) Lung tumor biopsy for 16 patients was performed on day 0 and day 7 after the vaccine administration and tumor associated antigen expression profiles were determined by qRT-PCR using the tumor antigen panel as described in Material and Methods. Over expression 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 tumor associated antigen derived synthetic peptides were selected and added to the 96 well plate of INF-γ ELISPOT assay. Six of 16 patients showed immune responses to TAA specific INF-γ production.

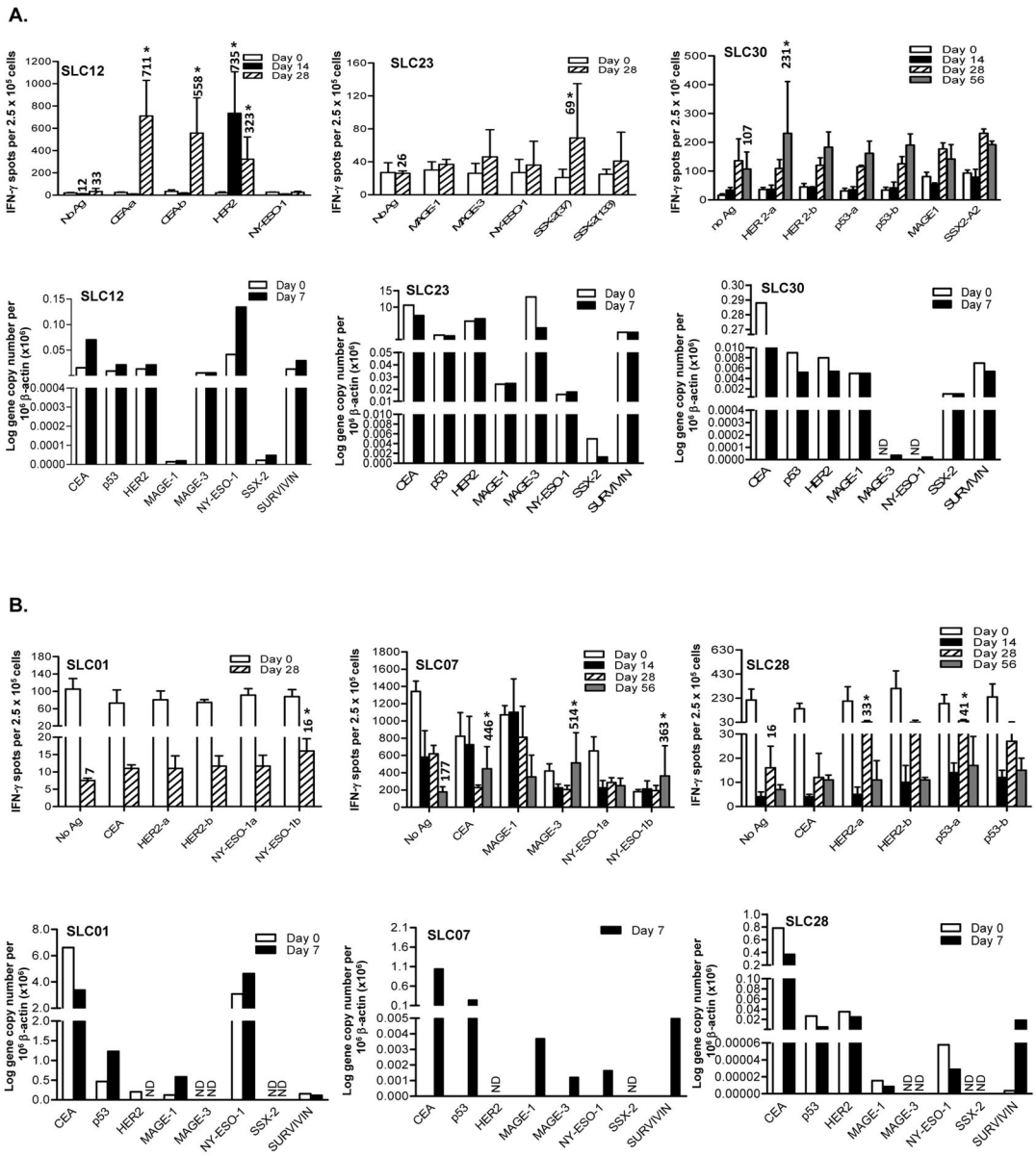
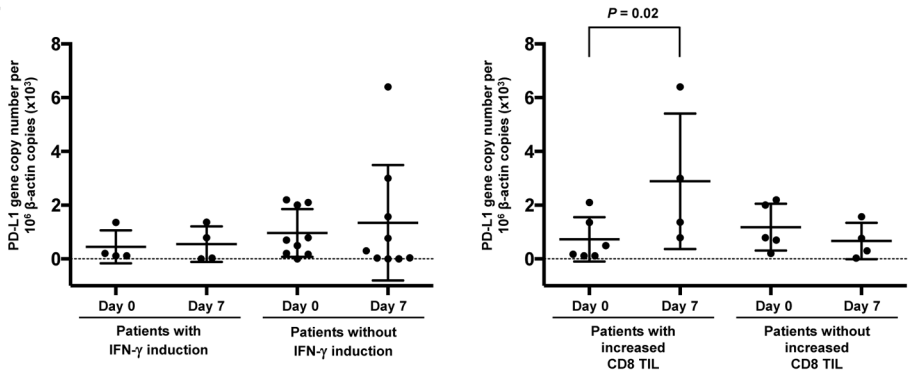


Figure 3. Immunologic responses to vaccination

PBMC were collected pre- and post-vaccination and were co-cultured with patient's HLA matched peptides and derived from tumor associated antigens for 24 hours to monitor immune responses by IFN-γ ELISPOT assay as described in Materials and Methods. (A and B) Six of 16 patients showed vaccine dependent response to IFN-γ production. (B) Three of 6 responders (SLC01, SLC07, and SLC28) had a high response with TAA non-specific and vaccine independent IFN-γ production at baseline that declined after vaccination yet met criteria for TAA-specific and vaccine dependent immune responses. Profiles of tumor associated antigen for each patient are shown in the lower panel.

A.



B.

Cohort	Patient ID	IFN-γ ELISPOT Response†	CD8 TIL/mm²	
			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 Day 14, 28, or 56; I – IFN-γ induction; NI – no IFN-γ induction; NA – no viable tumors

Figure 4. Association between PD-L1 expression and IFN-γ ELISPOT assay response or $CD8^+$ T cells infiltration into tumor

(A) Left; PD-L1 gene copy numbers were compared between patients with (n=4) and without (n=9) IFN-γ 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 IFN-γ 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 are shown.

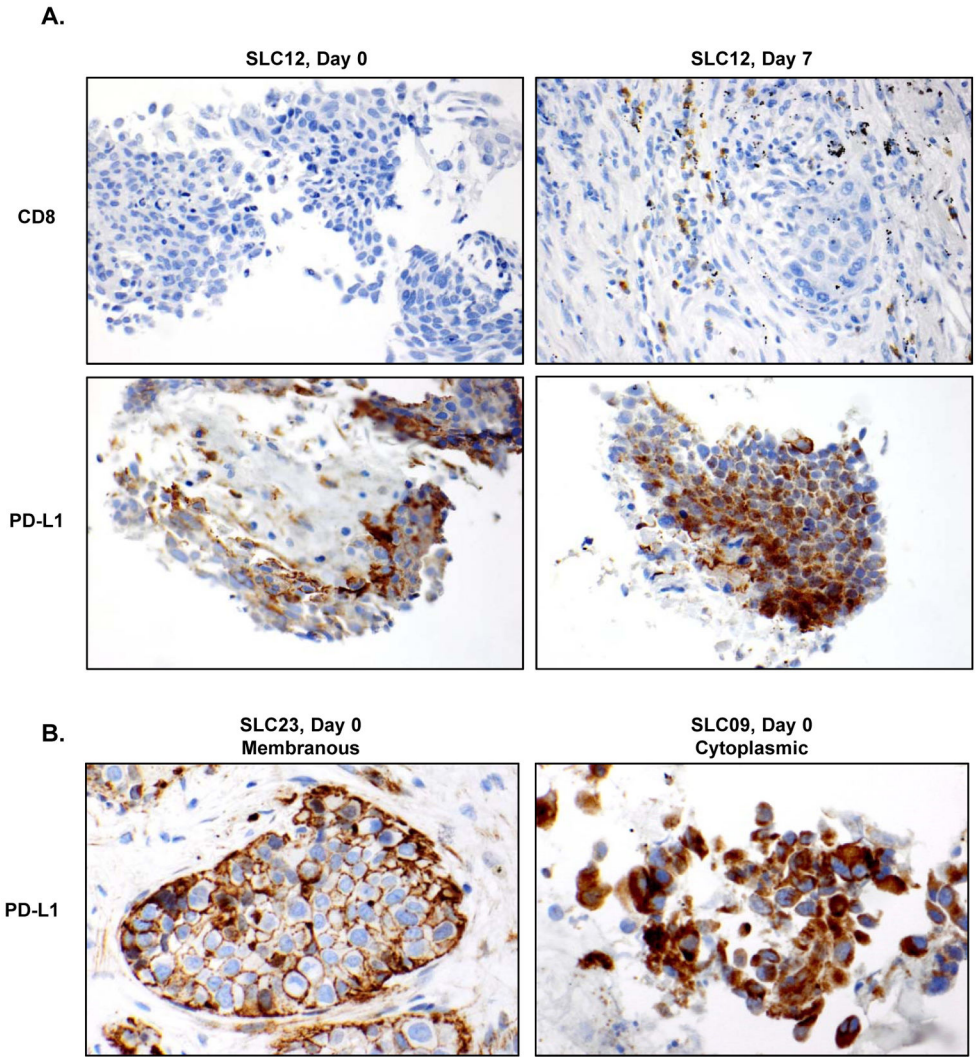


Figure 5. Tumor immunohistochemical staining with CD8 and PD-L1

(A) Paraffin-embedded tumor tissues were stained with anti-CD8 and anti-PD-L1 on day 0 and day 7 and representative immunohistochemical staining images from SLC12 are shown. (B) Representative immunohistochemical staining images of membranous and cytoplasmic expression of PD-L1 on day 0 from patients SLC23 and SLC 09, respectively, are shown.

Table 1

Summary of adverse events

Adverse Event (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†		1			4
Vomiting			1			1
General						
Cachexia	1					1
Edema	1	1	1			3
Fever	1					1
Fatigue	2†	3	3	1		9
Flu-like symptoms	1†					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						

Adverse Event (n = 17 patients)	Grade 1	Grade 2	Grade 3	Grade 4	Grade 5	Total
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[†]					
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

Adverse Event (n = 17 patients)	Grade 1	Grade 2	Grade 3	Grade 4	Grade 5	Total
Wheezing	1					1
Catheter placement for leukapheresis			2			2
Progressive disease			1	6		7

† 4 adverse events were possibly related to the vaccine