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Review

CCL21-DC tumor antigen vaccine augments anti-PD-1 therapy in lung cancer

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Abstract: Targeting inhibitory immune checkpoint molecules has highlighted the need to find approaches enabling the induction and activation of an immune response against cancer. Therapeutic vaccination, which can induce a specific immune response against tumor antigens, is an important approach to consider. Although this approach has shown low clinical efficacy when combined with other treatment modalities, therapeutic cancer vaccines will have a better outcome when combined with immune checkpoint blockade therapy with potential for cancer free survival. In this review, we will discuss the results of our two recent publications in preclinical lung cancer models. Our studies reveal that anti-PD-1 administered in combination with CCL21-DC tumor antigen therapeutic vaccines eradicate lung cancer. The results of these studies highlight the importance of combination therapy of immune checkpoint blockade and therapeutic cancer vaccines for lung cancer patients.

Keywords: lung cancer; PD-1; immune checkpoint; CCL21; dendritic cells (DC); T cells; immune therapy; tumor peptide antigens (Ag); tumor lysate Ag; tumor microenvironment (TME)

1. Introduction

Lung cancer remains the leading cause of cancer death worldwide with more than 1.9 million deaths in 2017 [1]. Even after advances in radiation therapy, chemotherapy, molecular targeted therapies and lung cancer surgical resection, the survival is low. Novel immune approaches could complement existing therapies for improving cancer free survival. CCL21 promotes T cell immune activity by attracting dendritic cells (DC) and T cells to form ectopic lymph node architectural structures that correlate with beneficial outcomes in cancer patients [2,3]. We have previously shown in a clinical trial that intratumoral administration of CCL21 gene modified dendritic cells (CCL21-DC) in advanced stage lung cancer induces systemic CD8T cell responses, increases tolerability, and induces tumor shrinkage [4]. Although CCL21 induces immune responses, an immune tolerant tumor microenvironment (TME) through PD-L1/PD-1 interaction evades immune responses. PD-Ll and PD-1 inhibitors have been approved for non-small cell lung cancer (NSCLC) [5]. Although PD-1 and PD-L1 inhibitors are effective for NSCLC, only a minimum number of patients respond [6]. Combination immunotherapy can be achieved with therapeutic vaccines and immune checkpoint blockade to increase systemic activated anti-tumor T-cell responses. PD-L1/PD-1 ligand receptor interaction confers T cell immune tolerance [7,8]. CCL21 initiates T cell activation through CCR7 and CXCR3 receptors [9]. We have demonstrated that therapeutic vaccine administered *s.c.* will enable systemic T cell anti-tumor responses [10,11]. These studies provide an understanding of the molecular and cellular mechanisms of PD-1 blockade and therapeutic vaccination responses in pre-clinical lung cancer models. We conducted these pre-clinical studies based on the hypothesis that therapeutic vaccine dependent induction of specific systemic immunity will benefit patients. These are the first reports that anti- PD-1 and CCL21-DC tumor Ag vaccine eradicate lung cancer and has potential to augment therapy in patients who do not respond to immune checkpoint blockade therapy [11,12].

2. Therapeutic cancer vaccine

2.1. CCL21 transduction and K-Ras peptide or tumor lysate pulsing of DC

DC were genetically modified with a replication-deficient adenovirus vector expressing murine CCL21 or control virus without CCL21 insert at a MOI of 100: 1 [10]. The transduced DC were pulsed with MHC Class I [LVVVGADGV] and MHC Class II [MTEYKLVVVGADGVG] peptides or tumor lysates as previously described [10,11].

2.2. Tumorigenesis

The method of tumorigenesis has been described by us earlier [10,11]. Briefly tumorigenesis was conducted to test if CCL21-DC peptide or tumor lysate vaccine combined with anti-PD-1 would augment therapy over monotherapy. To determine the induction of immunological memory, mice that eradicated tumors in response to therapy were challenged by *s.c.* injection on the left flank with 1×10^6 parental tumor cells (K-RasG12Dp53null tumor cells). The vaccine was administered by subcutaneous injection on the left flank of the tumor on day 12 and then once a week for three weeks. These tumor cell lines form reproducible tumors after subcutaneous injection and were isolated from the lung of (K-RASG12Dp53 null or KrasG12 D) transgenic mice. We used 6-8- week-old pathogen-

free 129-E mice (Charles River Lab) were used in the experiments, and tumor volume was determined using the following formula: Tumor Volume = $0.4ab^2$, where a = large diameter and b = small diameter. The tumor burden was monitored by measuring by dissecting diameter with calipers. The mice with 150 mm^3 tumors, were injected with CCL21-DC lysate Ag pulsed vaccine (5×10^6) once per week for 3 weeks by *s.c.* administration on the left contra-lateral flank of the tumor. Mice were also injected *i.p.* with anti-PD-1 (200 μg/dose) or isotype IgG2b Ab (200 μg/dose) every 48 h for 3 weeks. Mice were re-challenged one month following tumor eradication with 1×10^6 tumor cells. The depleting anti-CD4 (L3T4) and anti-CD8 (YTS169.4) were from BioXCell. The monitoring anti-CD4 monoclonal antibody (GK1.5) was from eBioscience. The monitoring anti-CD8 monoclonal antibody (5H10) was from Invitrogen. Tumors were harvested to conduct the experiments described below.

3. Results

3.1. Combined therapy augments antitumor activity

We tested the antitumor efficacy of (1) diluent, (2) anti-PD-1, (3) CCL21-DC tumor lysate vaccine, and (4) CCL21-DC tumor lysate vaccine plus anti-PD-1 in the K-RasG12Dp53null tumor model. The treatments induced the following fold reductions in tumor burden compared to diluent control: (1) anti-PD-1 induced a 2-fold reduction, (2) CCL21 DC tumor lysate vaccine induced a 2-fold reduction, and (3) Anti-PD-1 plus CCL21-DC tumor lysate vaccine induced a 17-fold reduction. The anti-PD-1, CCL21-DC tumor lysate vaccine, and anti-PD-1 plus CCL21-DC tumor lysate vaccine treatments resulted in 3-, 3-, and 19-fold weight changes of tumors at the end of therapy in comparison to control, respectively. In comparison to monotherapy, combined therapy led to 80% tumor eradication and rejected secondary tumor challenge demonstrating long-term immunological memory. Individual depletion of CD4T cells or CD8T cells abrogated the antitumor activity of combined therapy. Compared to diluent control, combined therapy caused a 19-fold tumor weight reduction, whereas that of monotherapy was 3-fold. DC lysate and anti-PD-1 plus control vector (CV)-DC lysate caused 10% tumor eradication. This result indicates that CCL21 secretion by DCs pulsed with tumor Ags enhanced tumor eradication by 8-fold in comparison to DCs or anti-PD-1 plus CV-DC tumor lysate treatment groups [11]. Similar results were obtained of combined therapy in the LKR13 G12D model [10]. Control peptide did not have anti-tumor effect. In comparison to monotherapy that reduced tumor burden without causing tumor eradication, treatment groups receiving three vaccinations (once/week ×3) with anti-PD-1 (every 48 h × 3 weeks) led to 75% tumor eradication. Sequential therapy led to better outcome with 100% tumor eradication [10].

3.2. Granzyme B expressing CTL and tumor cytolysis were enhanced following combined therapy

T cells from the TME one week following the end of combined therapy had increased cytolytic activity (8-fold) against parental K-RasG12Dp53null tumor cells *in-vitro* in comparison to diluent control, whereas monotherapy had a 2-fold increase.

Flow cytometry of Tregs and NK cells was not conducted because there were no changes in the activity of these cells. Purified Tregs from the TME did not alter the proliferation of anti-CD3/anti-CD28 stimulated T cells. Purified NK cells from the TME did not alter the cytolysis of tumor cells *in-vitro*. Flow cytometry analyses were conducted to evaluate $CD8^+$T cells expressing granzyme B

following therapy. Flow cytometry analyses revealed increased frequency (4-fold) of activated CD8 T cells expressing granzyme B in the anti-PD-1 plus CCL21-DC tumor lysate vaccine treatment group in comparison to monotherapy [11]. Similar results were obtained in the LKR-G12D model. We conducted flow cytometry analyses to evaluate T cell activation in the TME. The data revealed increased frequency (3-fold) of activated CD8 T cells expressing perforin in the CCL21-DC peptide vaccine plus anti-PD-1 treatment group in comparison to monotherapy. T cells from the TME of the combined therapy had increased cytolytic activity against parental LKR-13 tumor cells in comparison to other groups [10,11]. Vaccine administered prior to combined therapy had the highest cytolytic activity against LKR-13 tumor cells compared to the other groups. RNA sequencing data confirmed the flow analysis and details are described [10].

3.3. H&E staining revealed enhanced anti-tumor activity

Immunohistochemistry (IHC) staining was conducted on tumor sections one week following the 2nd week of therapy. IHC of the tumor sections demonstrated increased CD3T cells, caspase-3 apoptotic tumor cells, perforin and granzyme B expression in the therapeutic peptide or tumor lysate vaccine, CCL21-DC peptide or tumor lysate vaccine plus anti-PD-1 treatment groups compared to diluent control. Immunofluorescence (IF) staining of tumor sections showed enhanced activated T cells (CD4 and CD8T cells), but reduced tumor cells in combined and sequential therapy in comparison to Anti-PD-1 and diluent control [10,11].

3.4. Enhanced immune infiltrates and reduced tumor following CCL21-DC tumor Ag vaccine plus anti-PD-1 therapy

In comparison to controls and monotherapy, H&E staining of tumor sections revealed enhanced immune infiltrates with reduced tumor burden in the CCL21-DC peptide or tumor lysate vaccine plus anti-PD-1 treatment groups compared to diluent control [10,11].

3.5. RNA sequencing

RNA sequencing analysis revealed enhanced: CD4 T cells (3-fold), CD8 T cells (10-fold), B cells (13-fold), IFN γ (11-fold), TNF α (2-fold), perforin (3-fold), granzyme B (2-fold), INOS (2-fold), Arginase (2-fold), CXCR3 (7-fold), CXCL9 (5-fold), CXCL10 (6-fold) and DC were increased according to the following markers (IL3ra, Itgam, Anpep, Sell, CD68) in the CCL21-DC peptide vaccine plus anti-PD-1 treatment in comparison to control [10,11]. There was increase expression of VISTA in the TME following combined therapy [12].

4. Discussion and conclusions

This approach has not been evaluated in a clinical trial but has promising potential. Our data reveals that anti-PD-1 enhances activity of T cell infiltrates induced by therapeutic vaccine to eradicate tumors with potential to improve the clinical outcome of lung cancer patients. Similar studies have been performed by other investigators with consistent findings [13–15]. The results of our studies demonstrate activation of T-cells to effectively target and eradicate an aggressive K-Ras mutant lung

cancer. Our results demonstrate that the models respond to individual therapeutic vaccination or immune checkpoint blockade therapy, although the therapies administered individually are sub-optimum. These models have low baseline activated T cell infiltrates. The K-Ras model is representative of a significant mutational phenotype responsible for 15–25% of NSCLC [16] and provides understanding of therapy against driver mutation induced tumorigenesis. The K-Ras model is an adenocarcinoma common in both smokers and non-smokers [17–22]. Tobacco mutagens cause a high mutation frequency in the somatic genomes of smoking-associated tumors [23,24]. Our data demonstrates that tumor Ag pulsed CCL21-DC vaccine results in tumor growth inhibition, but the vaccine therapy alone is not optimum [10,11]. PD1 antibody therapy in cancer have shown efficacy in clinical trials [25–27]. The K-Ras model does not have the high mutation frequency seen in human lung cancer [22,28–30]. PD-1 immune checkpoint blockade therapy is more effective in tumors harboring a high mutation burden [31,32]. T cells respond broadly to mutation changes that render proteins immunogenic and the high mutation load provides additional targets for T cells that may increase the efficacy of response. We found that combined therapy rescued TIL activity causing increased tumor cytolysis [10,11]. In these models, tumor eradication was T cell dependent because depletion of T cells abrogated the effect [10,11]. We also noticed that therapeutic vaccine plus anti-PD-1 induced the highest expression of perforin and granzyme B expression in the TME compared to monotherapy by IHC and RNA sequencing [10]. Further, our RNA sequencing data demonstrated increased markers of (IL3ra, Itgam, Anpep, Sell, CD68). There was also increased expression of VISTA that is expressed on MDSC [10]. Currently up to 50–60% of patients being treated with PD-1 checkpoint inhibitors have low T cell infiltration and have reduced anti-tumor response [33,34]. Our data demonstrates that CCL21-DC tumor Ag vaccine overcome this limit and can be utilized to develop effective technologies for long-term cancer free survival. Other targets that can be exploited for cancer immunotherapy include FLT3L, TLR9, TLR3, TLR7/8, CTLA-4, IL-12, CD137, IL-15, CXCL10, A29/A2b, IL-23, LHG3, CD73 and GITR in combination with immune checkpoint blockade.

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Ethical disclosure

All animal work was conducted in accord with the Veterans Affairs Institutional Animal care and Use Committee guidelines: id D16-00002. The Animal Care and Use Committee review board approved all the studies involving animals.

Conflict of interest

All the authors declare that there are not biomedical financial interests or potential conflicts of interest in writing this manuscript.

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