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PD-L1 Blockade Immunotherapy with B-Cell Epitope Vaccines Elicit Antitumor Effects in a Syngeneic Tumor Model of Colon Carcinoma

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ABSTRACT

The programmed Death 1 receptor (PD-1) and its PD-L1/2 ligands induce a signal transduction pathway that inhibits tumor-infiltrating cytotoxic T lymphocyte activity and promotes tumor growth and metastasis. PD-L1 expression seems to be one of the main immune escape mechanisms in many cancers. Many studies have shown the effectiveness of PD-1 or PD-L1 blocking antibodies with specific monoclonal antibodies such as Pembrolizumab or Atezulizumab that showed clinical effectiveness across a wide range of subtypes of cancer. Nonetheless, it has been shown many times that immunotherapy with PD-1 and/or PD-L1 inhibitors alone is only effective in one sixth of patients. Previous studies have shown the desired efficacy of different immunotherapeutic approaches can be achieved when using the combinations of checkpoint inhibitors. In particular, synthetic peptide vaccines which are targeting B-cell epitopes have the benefit of developing a particular immune response that can potentially trigger B- and T-cell memory responses thereby reducing immune evasion. In addition, when these molecules formulated with ISA720 adjuvant, they elicited robust immune responses and inhibited tumor growth in mouse syngeneic models. Here, we asked whether PD-L1 vaccination could confer tumor control in mouse tumor models and we show the production of polyclonal antibodies, which block PD-L1 signaling, by our novel B-cell epitope vaccine which trigger similar anticancer effects to the standard monoclonal antibody Atezulizumab. In addition, we show that in a set of preclinical studies, the PD-L1 vaccine has a strong tumor inhibition effect in a syngeneic mouse model of colon cancer. The PD-L1 vaccine as well as the combination vaccines were safe and no evidence of tissue damage or toxicity was found. We further showed that the anti-PD-L1 sera is able to block the PD-1: PD-L1 interactions, and also is capable of apoptosis induction in the tumor cells in vitro. Overall these data suggest that this vaccine could be a promising strategy for cancer therapy.

KEYWORDS

Immuno-oncology; Peptide cancer vaccines; B-cell epitopes; PD-L1 blockade

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INTRODUCTION

As one of the immune checkpoint inhibitors, PD-1 and its ligand PD-L1 mediate a signaling pathway critical for maintenance of peripheral tolerance [1,2]. PD-1 is highly expressed on the surface of activated immune cells, including T cells, B cells, natural killer cells, macrophages, and dendritic cells. PD-L1 is also expressed on some immune cells [3-7]. In addition, the expression of PD-L1 on the surface of many tissue cells, including those of the heart, lung, thymus, spleen, and kidney, is upregulated after interferon γ (INF-γ) stimulation [8,9]. The PD-1/PD-L1 pathway plays an important role in T cell antigen receptor signaling inhibition and hence blocking cytokine production, tumor immune evasion, T cell proliferation and differentiation inhibition, and apoptosis induction of effector T cells [10]. PD-L1 is overexpressed in a variety of cancer types and tumor-associated antigen-presenting cells. Such overexpression in the tumor microenvironment is associated with a poor prognosis in many cancer patients [11-14]. When PD-1 on activated T cells interacts with PD-L1 on tumor cells, tumor cells are protected from cytotoxic lysis, whereas T cells are functionally impaired and lapse into exhaustion or apoptosis. Monoclonal antibodies (mAbs) targeting PD-L1 or PD-1 have been approved for clinical treatment of several cancer types, particularly advanced solid tumors [15-18]. The receptor tyrosine kinases (ErbBs) family of the epidermal growth factor plays an essential role in controlling cell proliferation, development, differentiation and migration. Loss of regulation of the ErbB receptors underlies many human diseases, most notably several human cancers including colorectal, lung and mammary carcinomas, and is linked to more aggressive forms of cancer, Increased metastasis risk, increased invasion of tumors, and decreased overall survival [9,19]. In the last 20 years, our understanding of the function and complex control of these receptors has fueled the production of selective therapeutic agents for human malignancies. HER-2 is thus a primary therapeutic target in multiple cancers [20-25]. Trastuzumab was the first humanized monoclonal antibody targeting HER-2 combined with chemotherapy to be approved for clinical application in patients with metastatic breast cancer. The introduction of pertuzumab, a humanized recombinant mAb that prevents the dimerization of HER-2 with other members of the HER family, improved cancer patients survival rate [26,27]. Despite the benefits of Trastuzumab, in average 25% of patients with metastatic HER-2 positive breast malignancies experience primary resistance and most responding patients ultimately develop resistance within one year of treatment [6,7,28-32]. Despite the role in survival improvements, they demonstrate significant toxicities and acquired resistance in many patients. Hence, peptide-based and small molecule inhibitors have received attention in recent years in drug development strategies [33-36]. However, compared to monoclonal antibodies, small molecule immune checkpoint inhibitors need substantial improvements. Although a few of them have been shown in some patent applications, so far no further details have been released. These peptide vaccines provide an attractive immunotherapeutic alternative in cancer treatment, with substantial benefits in terms of protection, cost-effectiveness and ease of administration [37-39]. Over the years, we have introduced a new immunotherapy concept that focuses on humoral responses based on B-cell epitope vaccines. In particular, the Ohio State University laboratory has successfully developed two new B-cell epitope specific vaccines based on the extracellular domain of the HER-2 structure, which are the binding sites of pertuzumab and trastuzumab [40-44]. Using the crystal structures of these two complexes, we have designed the specific trastuzumab-binding epitope (597-626) as well as the pertuzumab-binding epitope (266-

296). Two HER-2s combo vaccines produced high affinity antibodies that recognizes the native HER-2 receptor and has shown antitumor characteristics both in vivo and in vitro in a number of preclinical studies [41-43]. The two mentioned vaccines displayed similar properties to those of trastuzumab and pertuzumab, which led to their application in a phase-I clinical trial. The findings of this first-in human study as well as the phase-I dose escalation study were recently reported [43]. The study vaccine was nontoxic and safe, well tolerated, showed anti-tumor activity and preliminary evidence that peptide vaccination could prevent therapeutic resistance and would be an ideal alternative to monoclonal antibody therapies. However, therapeutic mAbs have their limits for clinical application because of the requirement of frequent administration and high cost [45,46]. In this report, we have tried to address these problems by taking an active immunization strategy. We present the characterization and development of a new B-cell peptide epitope targeting the human PD-L1, which causes the production of polyclonal antibodies in the body that is capable of blocking PD-1/PD-L1 interaction, which mimics the effects of Atezolizumab, and assessed its immunogenicity and anti-tumor efficacy in mice. Using sophisticated methods of peptide mapping and predictive antigenicity algorithms based on 3D structure of PD-L1, we show the preclinical development of the PD-L1 vaccine to design a chimeric B-cell vaccine based on the extracellular domain of PD-L1, which we linked to a promiscuous T helper cell measles virus fusion protein (MVF). We first evaluated the immunogenicity of each peptide epitope in rabbits and Balb/c mice, which elicited antibodies that recognized the immunogenic synthetic peptides as well as the recombinant human PD-L1 protein. The syngeneic colorectal CT-26/HER2 mouse tumor model was used to evaluate the efficacy of vaccination with our four MVF-PD-L1 peptides. The PD-L1(130-147) epitope showed significant inhibition of tumor growth. Nonetheless, therapeutic blockage of the signaling axis between PD-1/PD-L1 and monoclonal antibodies (mAbs) in some cancer patients has shown remarkable clinical success, however, vast majority of patients receiving monotherapies, stay resistant to this treatment and will not respond or relapse [47-50]. Clinical trials assess combinations of checkpoint-blocking antibodies, which include conventional therapies (such as radiation and chemotherapy), targeted therapies, other immunological modulators, tumor vaccines, and viral oncolytic. Growing research has indicated that the combinations of checkpoint blocking antibodies are more powerful than single inhibitors, but also cause greater toxicity to the immune system. Therefore, the therapeutic potential of immunotherapy has been tempered by several clinical failures and awaits novel approaches [49,51-56].

MATERIALS AND METHODS

Identification and Synthesis of Peptide Epitopes for hPD-L1

B-cell epitopes of PD-L1 were designed and selected using Peptide Companion TM algorithm by six correlates of antigenicity, as follows: (a) The profiles of chain flexibility and mobility of individual sequences were calculated according to Karplus and Schultz; (b) Hydropathic profiles were generated over a seven residue span setting and then smoothed with a three residue span using the scale of Kyte and Doolittle; (c) Hydrophilicity profiles over a six residue window were generated using the program of Hopp and Woods; (d) Analysis of the exposure of an amino acid residue to water was carried out by the solvent exposure algorithm of Rose et al.; (e) Protrusion indices were calculated by the method of Thornton et al. (f) Welling et al. method was used to determine the probability that a five-residue sequence is antigenic; Sequences were given a score of 1 to 6 based on their respective index values and were ranked: the highest ranking sequences had the highest individual score for the analyses examined, and successive

candidates had the next highest score, etc. The best scoring epitopes were further ranked by correlation with their secondary structural attributes; e.g., an amphiphilic α-helical sequence or a β-turn loop region are preferred over a random coil fragment. Computer algorithms by 'Chou and Fasman' and Novotny et al. were used for secondary structure prediction (α-helix, β- strand/sheet, β-turn/loop, random coil). Electrostatic ion pairs and helix dipole interaction in helical segments were also considered (e.g., hydrophobic/hydrophilic balance). Using algorithms of antigenicity, we used peptide epitope mapping to identify four epitopes of PD-L1. We have modelled all the four epitopes using PyMOL 3-D modeling software (DeLano WL 2002, The PyMOL User's Manual). Four novel peptide sequences were then synthesized using a 9600 Milligen/Biosearch solid-phase peptide synthesizer (Millipore, Bedford, MA, USA) with Fmoc/BOP chemistry and PyBOP/HOBT coupling reagents (PB Biosystems, Louisville, KY, USA) on CLEAR amide resin (Peptides International, Louisville, KY, USA). All synthesized peptides were designed and built as chimeric constructs using a promiscuous T helper epitope which is derived from the measles virus fusion protein (MVF, amino acids 288- 302) using a four-residue linker (GPSL). Peptides were cleaved from the resin using cleavage reagent R (TFA)/thioanisole/EDT/anisole (90/5/3/2), and crude peptides were purified by semi preparative (C-4 Vydac columns) reversed phase high performance liquid chromatography (RP-HPLC; Waters, Bedford, MA, USA). RP HPLC fractions showing the same retention time were pooled together and lyophilized. All peptides showed purity in excess of 95%. Samples were then characterized by MALDI mass spectroscopy and analyzed on an analytical RP-HPLC system (Waters, Bedford, MA, USA). All peptides showed the correct molecular mass.

Immunization with hPD-L1 Peptide Epitopes

For each peptide, vaccine antibodies were raised in New Zealand white rabbits, purchased from Charles River Laboratories (Wilmington, MA, USA). Rabbits were immunized with 1mg of MVF chimeric peptide emulsified in Montanide ISA 720 (Seppic, Paris, France) and boosted 3 times at three week intervals. For mouse experiments, 6 weeks - 8 weeks old mice were immunized with 0.1 mg of peptide emulsified in ISA 720 (1:1 ratio). Mice were boosted with the respective doses at 3 weeks interval. Blood was then collected and sera were tested for antibody titers. Sera were collected weekly and peptide vaccine antibodies were purified by affinity chromatography using a protein A/G column and the concentration was measured using Spectrophotometer. All experiments were performed in accordance with the approved protocols of the Ohio State University Institutional Animals Care and Use Committee.

Mouse Isotyping Assay

Antibody isotypes (i.e. IgA, IgM, IgG1, IgG2a, IgG2b, and IgG3) were determined using the Mouse Typer Isotyping Kit (BIO-RAD, Hercules, CA). Briefly, wells of a 96-well assay plate were coated with 200ng peptide antigen in ddH2O, and incubated at 4°C overnight. The plate was then washed with washing buffer (0.05% Tween-20 and 1% horse sera in PBS), and was blocked with 1% BSA in PBS at room temperature for 1 hour. 100 µl of diluted mice sera was added to each well for 2 hours and after washing the wells, 100 µl ready to use rabbit anti-mouse subclasses antibodies were added to each well respectively and incubated at room temperature for another 2 hours. The wells were washed again, 100 µl of 1/3000 dilution of goat anti-rabbit conjugated to HRP antibody (BIO-RAD, Hercules, CA) was added to each well and incubated for 1 hour at room temperature in dark. The plate was then washed and 50µl prepared substrate solution was added to each well. The reaction was stopped with a 25 µl 5% SDS stopping buffer.

Absorbance at 415 nm was determined using an ELISA plate reader. Dilutions of each sera sample were determined by the ELISA titers shown in absorbance of 0.4 or higher after subtracting the background.

Invivo Studies of Four Peptide Vaccine hPD-L1 Epitopes: CT-26 and CT26/HER2 Tumor Model in Mice

6 weeks - 8 weeks old Balb/c mice (Charles River, Wilmington, MA) were used as standard animal models. Mouse CT26 colon carcinoma cell line was purchased from American Type Culture Collection Cells and were regularly cultured in RPMI1640 medium containing 10% FBS. The CT26/HER-2 mouse model expressing HER2 has been previously established and used. Vaccines were dissolved in sterile water and emulsified in Montanide ISA 720 (1:1). Female Balb/c mice (Charles River Laboratories) at the age of 6 weeks - 8 weeks were immunized three times at 3 weeks intervals with 100 µg of each peptide vaccine, and 10 days after the third immunization, the mice were challenged with CT26 and CT26/HER-2 tumor cells, which were implanted subcutaneously (s.c.) on the right flank (1 × 105 per mouse). 200 µg of rat α-mouse PD-L1 mAb 10F.9G2 (Bio X Cell, West Lebanon, NH) were administered intraperitoneal (i.p.), twice weekly following tumor inoculation in the control group. Mice were monitored and scored for the formation of palpable tumors on a daily basis and sacrificed if tumors became necrotic or exceeded the predetermined size of 2,000 mm^3. Tumor volumes were measured in cubic millimeters with calipers and calculated with the following formula: A × B2 × 0.5, where A is the largest diameter, and B is the widest point perpendicular to length. During immunization, blood was drawn weekly and used in ELISA to monitor antibody titers. The mice were euthanized at the end of treatment and tumors as well as some internal organs were extracted and saved for further study.

PD-1/PD-L1 blockade bioassay

The assay was performed following the manufacturer's protocol for the PD-1/PD-L1 blockade bioassay (Promega). In brief, 4 × 105 aAPC/CHO-K1 or PD-L1 aAPC/CHO-K1 cells were seeded into 96-well plates in RPMI-1640 with 10% FBS. After overnight culturing, the medium was aspirated, and serially diluted purified PD-L1 or PD-1 antibodies, as well as Nivolumab and Atezolizumab antibodies (as reference antibodies) were added. Next, PD-1 effector cells were co-cultured and the plate was kept for 5 hours at 37°C in a humidified 5% CO_2 incubator. Following mixing with Bio-Glo Reagent, the luminescence was measured with a luminescence plate reader, SpectraMax M3 plate reader (Molecular Devices).

Antibody-dependent cell-mediated cytotoxicity (ADCC) reporter bioassay

ADCC Reporter Bioassay (Promega) was used per the manufacturer's instruction to assess ADCC activity. Briefly, the target WIL2-S and MC38 cells were seeded in each well of a 96-well assay plate one day prior. The MC38 cells were treated with IFN-γ (25 ng/ml for 16 hours at 37°C) to ensure induction of PD-L1 expression. The next day, either serially diluted reference antibody (Atezolizumab) or CD20 control antibody, and serially diluted purified PD-L1 130 polyclonal antibodies were added to the assay plate. Next, Jurkat cells (immortalized T-lymphocyte cells engineered to stably express FcγRIIIa receptors, as effector cells) were co-cultured with antibody treated target cells at an effector to target cell ratio of 4:1 for 6 hours at 37°C in a humidified 5% CO_2 incubator. Once bound to the antibody, engineered Jurkat T-lymphocyte cells activate gene transcription through the nuclear factor of activated T-cells (NFAT) pathway, inducing the expression of firefly luciferase. Lastly, luciferase activity was quantified using luciferase assay reagent by a SpectraMax M3 plate reader (Molecular Devices).

Apoptosis Assay (Caspase 9 Assay)

The Caspase-Glo 9 assay kit (Promega, Madison, WI) was used for caspase detection in treated cells in vitro. The reagent provides a pro-luminescent caspase-9 substrate, in combination with luciferase and a cell-lysing agent. Cells were plated in 96-well plates at 1 × 104 cells per well and cultured in complete media overnight. Then, the cells were treated with anti-PD-L1 antibodies as well as the reference antibody Atezolizumab and β-Lapachone (10 μM, Sigma Aldrich) for 24 hours. Caspase activity was measured by adding 50 μl of Caspase-Glo® 9 reagent directly to the assay well which results in cell lysis, followed by caspase cleavage of the substrate, and the generation of luminescence. The luminescence was detected using a microplate reader (Molecular Devices). The amount displayed on the readout is proportional to the amount of caspase activity in the sample.

Cytokines Quantification in Sera

Blood collected in serum tubes was allowed to clot and tubes were centrifuged at 1700 g for 10 minutes at room temperature. A portion of the serum was used for ELISA and other routine biochemical analysis while the rest was stored at -20°C until it was used to analyze cytokines. Prior to analysis, samples were thawed, vortexed, and centrifuged at 1000 g for 10 minutes to separate particles. Cytokine profiles were determined using a commercially available fluorescence-based system, MILLIPLEX MAP Mouse High Sensitivity Cytokine/Chemokine Magnetic Bead Panel (MHSTCMAG-70K Millipore/sigma, Burlington, MA) with an automated analyzer (Luminex 200, Luminex Corporation, Austin, TX). The concentrations of granulocyte-macrophage colony stimulating factor (GM-CSF), keratinocyte chemoattractant KC-like (KC/CXCL1), IFN-γ, interleukin-1α (IL-1α), IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-10, IL-12 (p70), IL-13, IL-17A, LIX, MCP-1, MIP-2, TNF-α were analyzed. Cytokine/chemokine's concentration were measured in duplicate, following the protocol provided by the manufacturer. Briefly, the plate was washed with the wash buffer for 10 minutes at room temperature prior to use. Standards, controls and samples were added to the appropriate wells and were incubated overnight at 2°C - 8°C on a shaking platform to allow the beads and cytokines to couple. After a series of washing, the detection antibodies were added to each well for 1 hour at room temperature. After incubation and adding the Streptavidin-Phycoerythrin followed by washes, the plate was run on a Luminex machine. The median fluorescence intensity (MFI) was analyzed using a 5-parameter logistic and spline curve-fitting method for calculating the concentrations of analytes in samples and Controls using standard curves.

Statistical Analysis

Tumor sizes were observed daily and measured by calipers. Tumor volumes were calculated by formula: Volume = (Length * Width * Width)/2. Data statistical analysis was performed using GraphPad Prism 8.1.2 (GraphPad Software, Inc.). One-way analysis of variance (one-way ANOVA) followed by the Tukey's multiple comparisons test were used to compare data in multiple groups or data between groups in multiple groups. P-value or adjusted p-value less than 0.05 was accepted as statistically significant different. * indicates $p<0.05$, ** indicates $p <0.01$.

DISCUSSION

In this study, we found that immunization of mice with an inhibitory PD-L1 B-cell epitope vaccine, that elicit a polyclonal antibody response that mimics the effects of Atezolizumab, delayed tumor growth and increased survival time. The antibodies induced by the MVF-PD-L1 vaccine blocked PD-L1 binding to PD-1 in vitro (PD-1/PD-L1 blockade bioassay) and conferred tumor growth control in a syngeneic colon carcinoma mouse model. Our analyses revealed that the blocking ability of antibodies was significantly associated with antibody titer. We also

found that, compared with control groups, the MVF-PD-L1 vaccine increased $CD8^+$ T cell infiltration. As many immune cell types express PD-L1, one of the main safety concern regarding the PD-L1 vaccine is off-target toxic effects. Our results indicate that, in a period of over 50 days of immunization, these effects were not evident in MVF-PD-L1 immunized mice. A longer term of safety monitoring is warranted for future vaccine development. According to the data, the major effect of MVF-PD-L1 therapy is shown in the preventive model. Compared with mAbs or chemotherapy, the vaccine needs time to trigger the immune response and is more dependent on the status of the immune system. In the present CT26 mouse therapy model, tumor progression is so quick that the immune system does not have enough time to control solid tumor growth. The increased volume of tumors does not only weaken the immune response but also promotes the formation of a tumor-suppressive microenvironment. This highlights the role and importance of an active immunotherapy approach. Moreover, complications arising from monotherapy with monoclonal antibodies and problems associated with that such as adverse/side effects that patients face, led us to the design of a wide range of peptide-based B-cell epitope vaccines with the focus on the design of chimeric B and T cell vaccines. Our previous studies have successfully shown the design of such vaccines (e.g., HER2, VEGF, etc.) which elicit robust high affinity antibodies 21-23, 25, 26, 42, 44. Furthermore, we have successfully implemented those vaccines into a phase I clinical trial 44. Upregulation of PD-L1 and consequently its ligation to PD-1 (on activated T cells) is the mechanism of action that cancer tissues limit the host immune response. Successful cancer vaccines require the selection of appropriate antigens, which in turn is able to induce robust immune responses, and strategies to overcome immune evasion and suppression. The PD-L1 vaccine described here showed high immunogenicity and antigenicity to human PD-L1 and induced tumor inhibition in mouse models of colon cancer. As discussed previously, using novel design methodology including identification of B-cell epitopes and chimeric peptides incorporating “promiscuous” T cell epitope, we were able to identify four potential PD-L1 peptide B-cell epitopes that represent novel agents aimed to elicit a polyclonal antibody response that could be useful for clinical translation and highlight the paradigm of peptide vaccine for future drug development. The recognition of the recombinant PD-L1 protein by all four epitopes further validates them as potential vaccine candidates. In order to determine the validity of the chosen epitopes, initial preliminary studies were conducted in vivo. Syngeneic Balb/c mice were immunized with all the four different PD-L1 constructs (PD-L1 36-53, PD-L1 50-67, PD-L1 95-112, PD-L1 130-147) and challenged with murine colon carcinoma CT26 and CT26/HER2 tumor cells to provide an initial assessment of the validity of the epitopes in inhibiting tumor growth. All vaccinated mice showed a high immunogenicity profiling by developing high titers of antibodies. Upon challenge with CT26 carcinoma cell lines, only mice vaccinated with MVF-PD-L1 (130-147) showed significant inhibition of tumor growth, leading us to conclude that this epitope would be a prime candidate for a vaccine. Here, mice engrafted with CT26 tumor and treated with PBS served as negative control. Results show that PD-L1 vaccine treatment had a significant effect in tumor growth reduction at day 18 post challenge as compared to negative control. In recent years, there has been a rise in the need to develop small molecules or peptide vaccines targeting PD-1/PD-L1 to build more efficient and less toxic therapeutics with better pharmacokinetic profiles. In accordance to that, results of antibody dependent cell-mediated cytotoxicity also showed no evidence of toxicity or autoimmunity. Atezolizumab (Tecentriq, Genentech/Roche), is an IgG1 mAb against PD-L1, which has been approved to treat

metastatic non-small cell lung cancer and locally advanced or metastatic urothelial cancer in first- and second-line setting. Atezolizumab, which exclusively binds to the ligand PD-L1 on the surface of tumor cells, results in the blockade of the PD-L1 binding to its inhibitory receptor PD-1. In addition, FDA has approved two other fully humanized IgG1 monoclonal antibodies (Avelumab and Durvalumab targeting PD-L1). Similar to Atezolizumab, our anti-MVF-PD-L1 130 antibodies did not induce the ADCC. However, mAb Atezolizumab as well as all anti-peptide PD-L1 antibodies were able to initiate apoptosis in PD-L1 expressing cells in vitro. This suggests that such vaccines may have clinical advantages for the treatment of cancer. We believe this is the first B-cell vaccine that targets the PD-L1 ligand in PD-1/PD-L1 complex. The vaccine targeting PD-L1 can be used for cancer patients after surgery for persistent tumor control. Compared with mAbs, vaccines targeting PD-L1 can reduce the economic burden, especially for people in less developed countries. This highlights the fact that moving toward new immunotherapeutic approaches such as small peptide vaccines are inevitable. We concluded that the PD-L1 vaccine induced a specific immune response, which leads to a delayed tumor growth in vivo. The heterogeneity and diversity of tumors indicate that tumor cells have different morphological and phenotypic profiles. The complex nature of tumor microenvironment is why single therapy has restricted efficacy. Thus, combination therapy offers a superior way to combat cancer. In conclusion, this novel PD-L1 (130-147) peptide incorporating a promiscuous T cell epitope derived from measles virus protein MVF epitope represents a vaccine agent useful for clinical translation. Overall our cytokine profiling analysis also indicates that tumor growth inhibition is very effective without the negative effects of cytokines overexpression responsible for CRS.

RESULTS

The Design and Purification of Four Novel Human PD-L1 (hPD-L1) Peptide Epitopes

The tertiary structure of PD-L1 is composed of an extracellular domain, a transmembrane domain, and an intracellular region. Because the extracellular domain is responsible for binding of PD-1, as well as being accessible for anti-peptides antibodies, we have chosen this domain as an immunogenic region. We identified four B-cell epitope sequences of human PD-L1 for further investigation: amino acid 36-53, 50-67, 95-112 and 130-147 (Table 1). Then the four peptide sequences were synthesized as chimeric constructs with a “promiscuous” T helper epitope derived from the measles virus fusion protein (MVF, amino acids 288-302).

Peptides	Amino Acid sequences of synthesized Peptides
PD-L1 (36-53)	LIVYWEMEDKNIIQFVHG
MVF-PD-L1 (36-53)	KLLSLIKGVIVHRLEGVE-GPSL- LIVYWEMEDKNIIQFVHG
PD-L1 (50-67)	FVHGEEDLKVQHSSYRQR
MVF-PD-L1 (50-67)	KLLSLIKGVIVHRLEGVE-GPSL- FVHGEEDLKVQHSSYRQR
PD-L1 (95-112)	YRCMISYGGADYKRITVK
MVF-PD-L1 (95-112)	KLLSLIKGVIVHRLEGVE-GPSL- YRCMISYGGADYKRITVK
PD-L1 (130-147)	VTSEHELTCQAEGYPKAE
MVF-PD-L1 (130-147)	KLLSLIKGVIVHRLEGVE-GPSL- VTSEHELTCQAEGYPKAE

Table 1: Amino acid sequences of peptide vaccines.

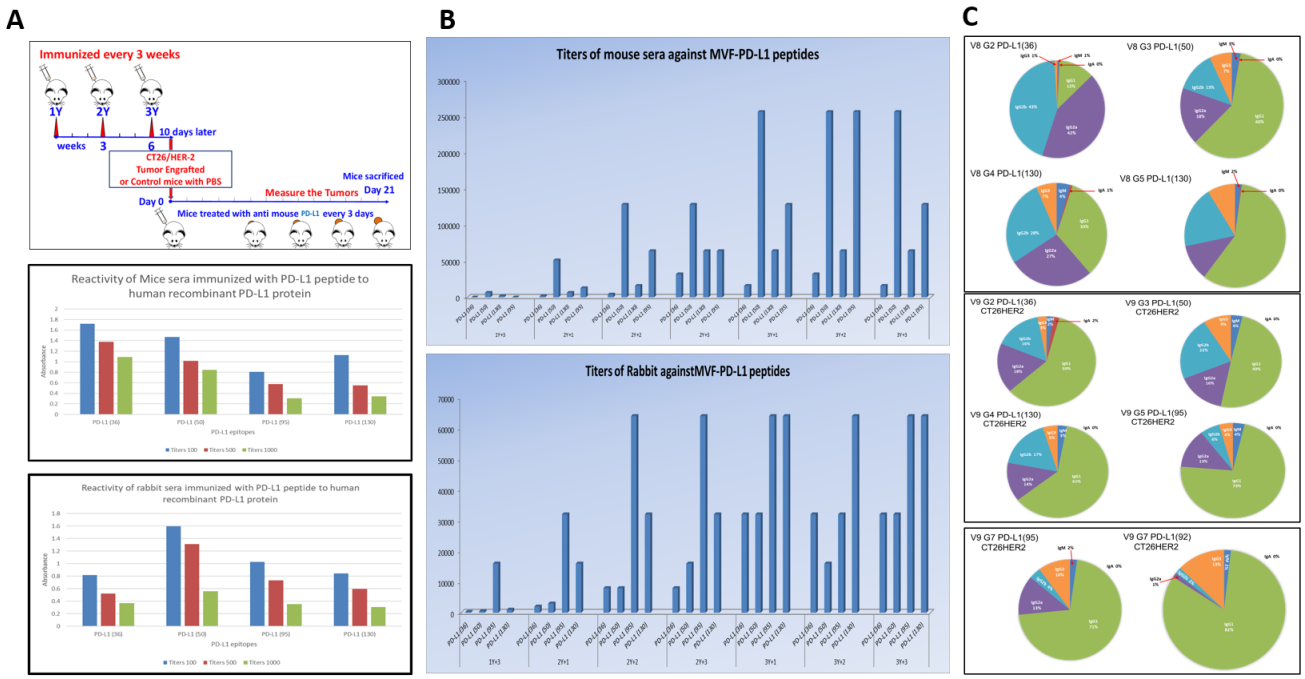

Figure 1: Immunogenicity and preliminary assessment of human PD-L1 peptide epitopes in Balb/c mice. (A) Mice were immunized as shown. Bleeds were collected weekly after the primary immunization and ELISA were used to detect antibody titers in sera (Shown in B), both in mice as well as New Zealand White rabbits. Rabbits were immunized with 1 mg of peptide dissolved in ddH2O emulsified (1:1) in Montanide ISA720. Rabbits were boosted with the same doses at 3-week intervals. Blood was collected via the central auricular artery in rabbits and sera were tested by ELISA on 200ng/well peptide or 1ug/well of recombinant PD-1 protein. On the right panel (C), isotypes in Balb/c mice after immunization with MVF-PD-L1 peptides: ISA720 are shown.

Invivo Immunization with PD-L1 Epitopes Results in Robust Antibody Response

Immunogenicity of all four peptides were evaluated in outbred rabbits. Upon immunization, high-tittered antibodies (>30,000) were observed. In addition, antibodies were capable of recognizing the human recombinant PD-L1 protein. (Figure 1A & Figure 1B). Sequencing database shows a 69% overall homology between mouse and human PD-L1 sequences.

Therefore, in order to evaluate the binding capacity of our synthesized human epitopes to the mouse PD-L1, we produced rabbit raised antibodies for all of our four epitopes. The purified antibodies showed reactivity with human recombinant protein (Figure 1A & Figure 1B). The isotypes of antibodies elicited in the mice were determined to be predominantly of the IgG1 class (Data shown in Figure 1C).

Efficacy of the PD-L1 Vaccine Epitopes in Inhibiting Tumor Growth in a Syngeneic Balb/c Model Challenged with Colon Carcinoma Cell Line

In order to verify the efficacy of the PD-L1 epitope vaccine *in vivo*, Balb/c mice (10 mice/group) were immunized with MVF-PD-L1 (36-53), MVF-PD-L1 (50-67), MVF-PD-L1 (95-112) and MVF-PD-L1 (130-147), where vaccines were emulsified with Montanide 720 (1:1 ratio). Mouse syngeneic tumor models, especially CT26, is a widely used tool to demonstrate activity of novel anti-cancer immunotherapies. Mice were immunized 3 times at 3 weeks intervals. Ten days after the third immunization (3Y), the mice were inoculated subcutaneously (s.c.) with either CT26 or CT26/HER2 tumor cells (1X105 cells per mouse) and tumor formation was monitored on a daily basis afterwards. Mice treated with PBS served as negative control, and twice weekly treated mice with 200 µg/dose injections of anti-mouse PD-L1 monoclonal antibody (mAb clone 10F.9G2)

served as positive control. Tumor growth was monitored daily and measured by calipers. As shown, bleeds were collected one, two and 3 weeks after each vaccination and are shown as 1Y+3, 2Y+1, 2Y+2, 2Y+3, 3Y+1, 3Y+2, 3Y+3, accordingly where Y indicates the number of immunization. All PD-L1 epitopes elicited high titers of PD-L1 anti-peptide antibodies. Mice vaccinated with all MVF-PD-L1s showed significant tumor inhibition, and anti-mouse PD-L1 monoclonal antibody and PD-L1 vaccine treatment had similar and significant reduction in tumor growth at day 18 post challenge as compared to negative control treatment with PBS. However, most notably MVF-PD-L1 (130-147) showed the strongest tumor growth inhibition at day 18, indicating its potential therapeutic application as a useful inhibitory vaccine. Tumor growth pattern for each individual mouse is shown in Figure 2.

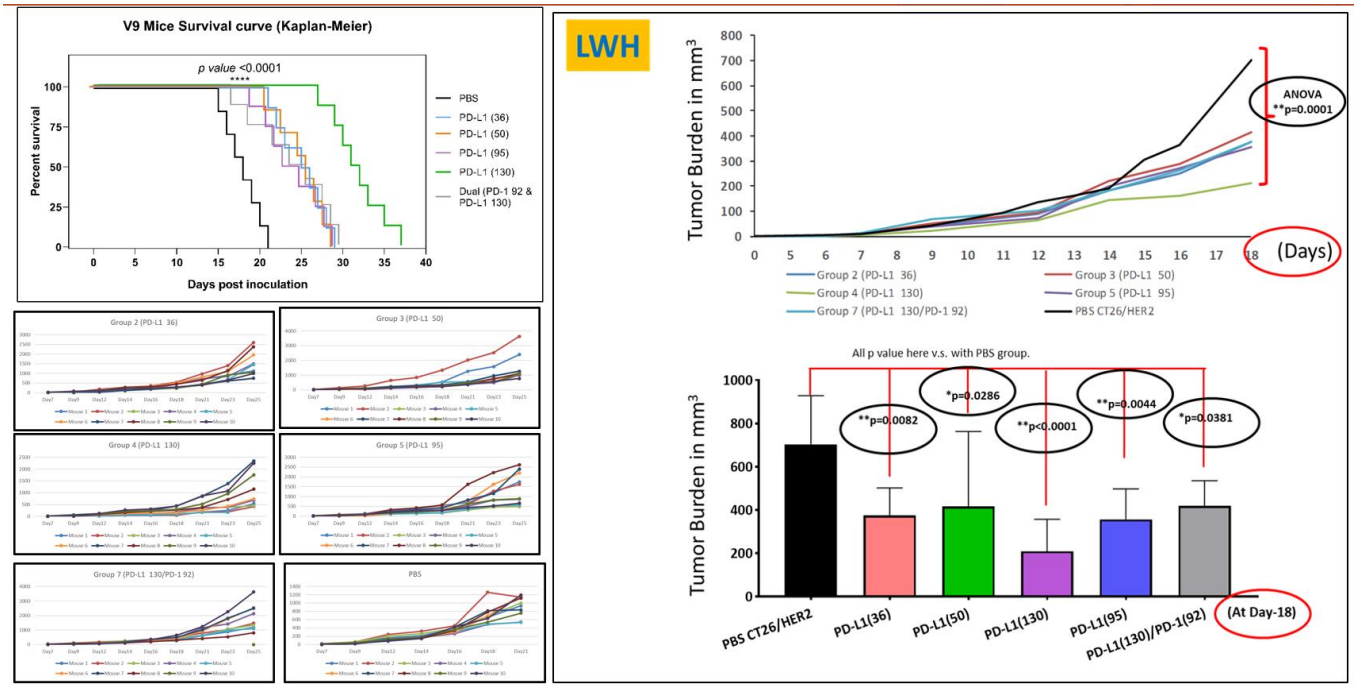

Figure 2: Efficacy of MVF-PD-L1 vaccine candidates in Balb/c mice syngeneic model challenged with CT26WT or CT26/HER2 colon carcinoma cell line. Mice were immunized with MVF-PD-L1 vaccines emulsified in Montanide ISA720, 3 times at 3-week intervals. 10 days after the third immunization the mice were engrafted with either CT26WT or CT26/HER2 tumor cells. Tumor growth was monitored daily and measured by calipers. Individual plots of tumor growth and tumor burden by days in Balb/c mice as well as plots of tumor volume LWH at day 18 for each of the treatment groups are shown. * indicate $p<0.05$, ** indicate $p<0.01$.

Tumor sizes at various times are also shown and one-way analysis of variance (one-way ANOVA) followed by the Tukey's multiple comparisons test were used to compare data in multiple groups or data between groups. Significant higher percentage of tumor growth inhibition as well as the highest survival rate and lowest levels of lesions was observed in PD-L1 (130-147) vaccinated group as shown in figure 2 compared to negative control analyzed by Kaplan-Meier method. This shows immunization with PD-L1 (130-147) significantly increases life expectancy in mice by over 200%. We also analyzed the different isotypes of antibodies being generated by vaccination. We also examined and observed that combined immunization of PD-L1 vaccines with PD-1 (92-110) does not provide any syngeneic effects, perhaps due to peptide overload and competing effects of antibodies to bind to the ligands (data shown in Figure 2).

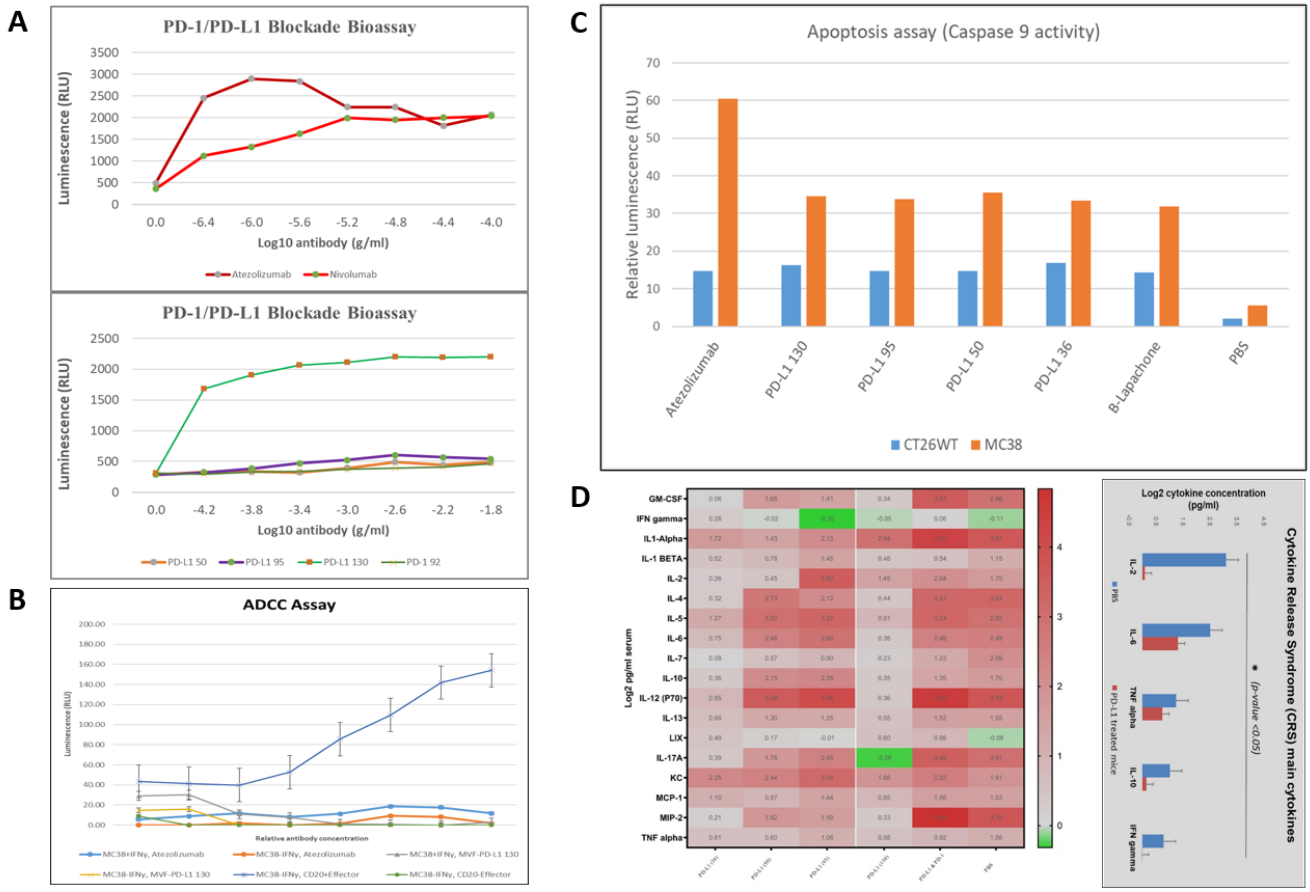

Figure 3: In vitro assays of purified polyclonal PD-L1 antibodies. (A) PD-1/PD-L1 blockade bioassay; Purified anti-peptides polyclonal antibodies bind to PD-L1 at the same site as PD-1. The bioassay shows an increase in luminescence signal, demonstrating the ability of anti-PD-L1 antibody from immunized rabbits to block PD1/PD-L1 interaction, similar to commercial anti-human PD-L1 antibody Atezolizumab, thus demonstrating that the antibody is biologically functional. (RLU: relative light units). (B) Atezolizumab, as well as anti-MVF-PD-L1 130 antibodies do not initiate the antibody-dependent cellular cytotoxicity (ADCC). CD20 antibody as positive control, once added with effector cells, is able to mediate ADCC. (C) Anti-PD-L1 vaccine antibodies are capable of evoking cancer cell apoptosis. Apoptosis was evaluated by measuring caspase 9 activity after treatment with 100 µg/mL peptide vaccine antibodies. Cells were plated in 96-well plates and treated with inhibitors for 24 h prior to the addition of the caspase reporter reagent. Caspase activity of a normal rabbit IgG was used as a negative control. Results showed that the purified rabbit PD-L1 antibodies increased caspase activity, indicating induction of apoptosis. Results shown are averages of different experiments. (D) Heatmap visualization applied to all PD-1 treated Mice cytokine data. Cytokine profiling was performed using an 18-plex marker panel on Luminex platform. GM-CSF, IFN-gamma, IL-1 alpha, IL-1 beta, IL-2, IL-4, IL-5, IL-6, IL-7, IL-10, IL-12(p70), IL-13, Il-17A, LIX, KC, MCP-1, MIP-2 and TNF-alpha were assessed for concentration in sera by quantitative multiplexing. Values are log2-transformed normalized cytokine concentration. This visualization illustrates some differences across different treatments and which cytokines are secreted in response to treatment. Each column corresponds to a single treatment group, while rows correspond to individual cytokines. Color pattern is relative with respect to cytokine concentration within each treatment, with red indicating higher concentrations (expression) and green indicating cytokine downregulation.

Anti-MVF-PD-L1 130 Antibody is Capable of Blocking PD-1/PD-L1 Interaction

The currently available commercial bioluminescent cell-based PD-1/PD-L1 blockade bioassays give a great opportunity for a fast and reliable screening of therapeutic antibodies which are designed and developed to interfere with PD-1-signaling interaction. In order to study if the anti-MVF-PD-L1 130 antibodies are functional, and if rabbits vaccinated with B-cell epitope PD-L1 (130-147) peptide secrete functional anti-PD-L1 antibodies, first we raised the antibodies in white New Zealand rabbits. The animals were immunized 3 times at 3 weeks interval each with 1mg of the peptide. After the last immunization, bleeds were collected and antibodies were purified using

column A/G. Next, PD1/PD-L1 blockade assay was used to test the functionality of anti-PDL1 (130-147) antibody. It was observed that purified polyclonal anti-PD-L1 antibody from the rabbits could block PD1/PD-L1 interaction similar to commercial anti-human PD-L1 antibody (Figure 3A). These results suggest that functional anti-PD-L1 is produced in rabbits immunized with the B-cell epitope peptides.

ADCC Activity

Antibody-dependent cell-mediated cytotoxicity (ADCC) is the critical mechanism of action of anti-cancer mAbs, through which antibodies recruit FcγR-bearing effector cells to target "diseased" cells for destruction by components of the cell-mediated immune system, such as natural killer cells. However, It is known that most of PD-1/PD-L1 antibodies in clinical development do not mediate antibody-dependent cell mediated cytotoxicity, like atezolizumab (MPDL3280A), which is an FcγR-binding deficient IgG1 isotype humanized monoclonal antibody engineered to eliminate ADCC activity. While ADCC induction could potentially enhance tumor death by inducing apoptosis, it can also result in depletion of PD-L1-expressing T cells, thereby blunting the immune response. In order to eliminate ADCC and CDC at clinically relevant doses, the Fc domain of atezolizumab is engineered to reduce its interaction with the FcyR. To evaluate ADCC activity of our purified polyclonal antibodies, we used MC38 cells treated for 16h with 25ng/ml IFN-y, and WIL2-S target cells together with Jurkat effector cells. The simultaneous binding of mAbs with both target and effector cells leads to the induction of measurable nuclear factor of activated T-cells (NFAT) luciferase fluorescence. A linearity test for ADCC response was performed at various concentration of antibody as shown in figure 3B to assess the performance of the assay. Antibodies elicited by the peptide vaccine had similar effects to the mAb Atezolizumab and were not capable of mediating antibody-dependent cellular cytotoxicity (Figure 3B).

Apoptosis determination by caspase activity assay

The potential for disease-specific targeting and low toxicity profiles have made monoclonal antibodies attractive therapeutic drug candidates. Antibody-mediated target cell killing is frequently associated with immune effector mechanisms such as ADCC, but they can also work through apoptotic processes. Depending on their mechanism of action, monoclonal antibodies can induce targeted cell-specific killing alone or can enhance target cell susceptibility to chemo- or radio-therapeutics by affecting the modulation of anti-apoptotic pathways. To further examine this, we evaluated whether anti-PD-L1 peptide vaccine antibodies were capable of inducing apoptosis of cancer cells via a caspase activation assay. MC38 (as PD-L1 expressing cells) and CT26WT cancer cells were seeded in 96-well plates and incubated overnight at 37 °C. The following day, Low serum growth media containing peptide vaccine antibodies as well as reference antibody Atezolizumab and β-Lapachone as apoptosis inducer were added to the wells. The plates were then incubated for an additional 24 hours at 37 °C. The caspase-Glo reagent was then added, and caspase 9 release was determined using an illuminometer as a measure of apoptotic induction. Caspase-9 is a member of cysteine aspartic acid specific protease (caspase) family, which plays a key initiator role in the intrinsic apoptotic pathway of mammalian cells. We found that the PD-L1 antibody caused a significant increase in the amount of caspase activity in treated cells as compared to negative controls (PBS) and comparable to mAb Atezolizumab. The increased caspase release was more than 3 fold (Figure 3C), clearly indicative of increased apoptosis.

Determination of Cytokine Profiles

It is well documented that treatment with checkpoint inhibitors can result in cytokine release syndrome (CRS)

resulting in life-threatening toxicity causing potential damage in some tissues and organs. The "cytokine storm" results in excessive or uncontrolled release of both pro-inflammatory cytokines (such as TNF-alpha, IL-1, and IL-6) as well as anti-inflammatory ones (such as IL-10 and IL-1R). Thus, we were interested in determining whether PD-L1 vaccination had deleterious cytokine responses versus monoclonal antibody treatment. This could lead to a better understanding of the causes mitigating any potential CRS and how best to design pre-clinical studies that can better predict the risks of novel immunotherapies in humans. We analyzed cytokine concentrations of immunized mice using a commercially available fluorescence-based system, MILLIPLEX MAP Mouse High Sensitivity Cytokine/Chemokine Magnetic Bead Panel (MHSTCMAG-70K Millipore/sigma, Burlington, MA) with an automated analyzer (Luminex 200, Luminex Corporation, Austin, TX). We observed higher levels of cytokines (e.g. MIP-2, INF-gamma, IL-1beta, IL-2, IL-4, IL-5, IL-6 and IL-10) in PBS group compared to the rest of the PD-L1 treated ones. Higher levels of IL-6 and IL-4 expression show B-cell activation, as these cytokines are highly expressed in naïve B-cells compare to T-cells [56,57]. Our cytokine quantification/profiling data shows IL-2, IL-6 and IL-13 are expressed at a higher level after immunization. In our study, we found that IL-1, IL-2 and IL-17 levels were significantly lower in the PD-L1 immunized mice. Furthermore, our analysis shows a mosaic pattern of cytokines expression in PD-L1 immunized mice, some cytokines are upregulated (TNF-alpha, IL-13, IL-1 beta, IL-6, MCP-1 and KC) where others are downregulated (IL-17A, LIX, IFN-gamma, GM-CSF), as opposed to the PBS group where all of cytokines are upregulated and hence maybe responsible for a 'cytokine storm'. As discussed earlier our treatment has a great effect on tumor growth inhibition without the negative effects of potential CRS. Several recent studies have shown repeatedly IL-2, IL-6, IL-10 and TNF-alpha are the key cytokines in CRS and hence their overexpression might have adverse consequences [58-61]. In a recent study, Stebbings et al. reported a life-threatening cytokine storm following CD28-specific mAb treatment in a Phase I clinical trial [62]. In line with these reports, our data shows these cytokines are expressed significantly lower (p-value <0.05) in our PD-L1 treated mice compare to negative control (Figure 3D).

PD-L1 Vaccines are Safe, No Adverse Tissue Damage or Toxicity found in Vaccinated Mice

To examine the safety of the PD-L1 vaccines in vivo, the mouse body weight and appearance were monitored and examined on a daily basis and were similar among experimental groups. These data suggest that PD-L1 vaccination is a safe and tolerable approach for cancer therapy with no signs of toxicity or autoimmunity. Moreover, the treated and untreated animals showed no difference in the morphology or behavior of the animals and if so they were required to be removed from the study by the protocol. However, no differences were observed between all groups.

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