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14. ABSTRACT

The object of this research is to develop a therapeutic strategy against lung cancer by utilizing novel vault bioparticles to deliver CCL21, an immunotherapeutic chemokine. Our hypothesis is that delivering CCL21 to the tumor, will make the entire repertoire of tumor antigens available in situ and thus increase the likelihood of an immune response and reduce the potential of phenotypic modulation. We have developed a bioparticle to deliver CCL21 that is based on the human vault particle. Vaults needed for initial animal studies (CCL21-vaults and empty control vaults) have been purified. We have secured ACURO approval for our animal studies and initial anti-tumor efficacy studies have been initiated in three animal models of lung cancer with the CCL21-vault as monotherapy. One model showed significant efficacy.

15. SUBJECT TERMS
Vault nanoparticles, CCL21 chemokine, Major Vault Protein (MVP), Non-small-cell lung carcinoma (NSCLC), Programmed cell death protein 1 (PD1).

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Introduction:

The object of this research is to develop a therapeutic strategy against lung cancer by utilizing novel vault bioparticles to deliver CCL21, an immunotherapeutic chemokine. Our hypothesis is that delivering CCL21 to the tumor, will make the entire repertoire of tumor antigens available in situ and thus increase the likelihood of an immune response and reduce the potential of phenotypic modulation. We have developed a bioparticle to deliver CCL21 that is based on the human vault particle. Vaults are nano-scale particles consisting of an outer protein shell comprised of 78 copies of a single protein called the major vault protein (MVP). We have taken advantage of a high-affinity binding domain (termed INT) to package the human CCL21 chemokine into human vault shells. Using a mouse lung tumor syngeneic model, we showed that intratumoral (IT) injection of CCL21-vaults could promote the recruitment of T lymphocytes and antigen presenting cells into the tumor environment leading to a robust antitumor response. The Specific Aims of this proposal will confirm this approach and gather pre-clinical data in three animal models of lung cancer with the CCL21-vault as monotherapy and in combination with a checkpoint inhibitor. We will also carry out preliminary toxicology studies in mice. This proposal will develop a therapeutic strategy against lung cancer and has the potential to be an effective therapy for other solid tumors.

Keywords:

PD-1 – Programmed cell death protein 1, also known as PD1 and CD279 (cluster of differentiation 279), is a cell surface receptor that plays an important role in down-regulating the immune system and promoting self tolerance by suppressing T cell inflammatory activity.

PD-L1 - Programmed death-ligand 1 (PD-L1) also known as cluster of differentiation 274 (CD274) or B7 homolog 1 (B7-H1) is a protein that in humans is encoded by the CD274 gene. PDL1 is the ligand for PD1. Both PD1 and PDL1 are immune regulatory checkpoint markers.

CD8+ T cells - A cytotoxic T cell that is a T lymphocyte that kills cancer cells, cells that are infected (particularly with viruses), or cells that are damaged in other ways. Also called cytolytic T cells or CTL.

CD4+ T cells – A type of T helper cell (Th cell) that play an important role in the immune system, particularly in the adaptive immune system. These cells express the surface protein CD4 and are referred to as CD4+ T cells.

TIL – Tumor infiltrating leukocytes are white blood cells that have left the bloodstream and migrated into a tumor. They are mononuclear immune cells, a mix of different types of cells (i.e., T cells, B cells, NK cells, macrophages) in variable proportions, T cells being the most abundant cells.

Treg – Regulatory T cells, formerly known as suppressor T cells, are a subpopulation of T cells which modulate the immune system, maintain tolerance to self-antigens, and prevent autoimmune disease.

MDSC - Myeloid derived suppressor cells are a heterogenous group of immune cells from the myeloid lineage.

NSCLC - Non-small-cell lung carcinoma (NSCLC) is any type of epithelial lung cancer other than small cell lung carcinoma (SCLC). NSCLC accounts for about 85% of all lung cancers.

MVP – Major Vault Protein (MVP) is the structural protein of the vault bioparticle. There are 78 copies of MVP in a vault shell.

INT – The Major Vault Protein Interaction Domain (INT) – is a protein domain derived from the vault protein VPARP that binds with nanomolar affinity to the inside of the vault particle.

CCL21 - A multifunctional chemokine that mediates migration of lymphocytes and antigen-stimulated DCs into T cell zones of secondary lymphoid organs.

CCL21-vault – A vault particle packaged with the CCL21 chemokine fused to the INT packaging domain (CCL21-INT).

Overall Project Summary:

The object of this proposal is to develop a therapeutic strategy against lung cancer by utilizing novel vault bioparticles to deliver an immunotherapeutic chemokine. CCL21 is a multifunctional chemokine that mediates leukocyte migration and activation. 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 to recruit and activate T cells. Our hypothesis is that delivering CCL21 to the tumor, will will make the entire repertoire of antigens available in situ and thus increase the likelihood of an immune response and reduce the potential of phenotypic modulation. We have developed a bioparticle to deliver CCL21 that is based on the human vault particle. Endogenous vault particles are found in great abundance in the cytoplasm of all nucleated human cells. Vaults are nano-scale particles consisting of an outer protein shell comprised of 78 copies of a single protein called the major vault protein (MVP). In human cells this protein shell protects two other vault proteins and a small RNA, however, by expressing MVP alone in insect cells (which lack endogenous vaults), empty vault shells are produced that are morphologically indistinguishable from endogenous human vaults, but devoid of normal internal contents. We have taken advantage of a high-affinity binding domain (termed INT) to package the human CCL21 chemokine into human vault shells. Using a mouse lung tumor syngeneic model, we showed that intratumoral (IT) injection of CCL21-vaults could promote the recruitment of T lymphocytes and antigen presenting cells into the tumor environment leading to a robust antitumor response. Recent preliminary data suggests that subcutaneous delivery of the CCL21-vault as a vaccine may be more effective than the IT route.

The Specific Aims of this proposal are directed at confirming this approach and gathering pre-clinical data in three animal models of lung cancer with the CCL21-vault as monotherapy and in combination with a checkpoint inhibitor. We will also carry out preliminary toxicology studies in mice. This proposal will develop a therapeutic strategy against lung cancer and has the potential to be an effective therapy for other solid tumors. If successful, this work will have a clear path to human testing since human vaults have been produced at commercial scale and GMP purity through an NCI SBIR.

Key Research Accomplishments:

What were the major goals of the project?

Specific Aim 1: Evaluate the efficacy of human CCL21-human vault therapy in three murine models of lung cancer (months 1-12)

Major Tasks: 1. Prepare Vaults for Aim 1 Animal Testing

Milestone(s) Adequate vaults purified and characterized for carrying out Tasks 2 and 3. (100% complete)

2. Quantify efficacy of human CCL21-vaults in 3 murine models of lung cancer & optimize dose and route of CCL21-vault delivery

Milestone: ACURO Approval (100% complete)

Milestone(s): Efficacy determined in 3 mouse models, optimal dose and route determined. (50% complete)

3. Evaluate the determinants of anti-tumor response

Milestone: Determinants of anti-tumor response analyzed. (10% complete)

Specific Aim 2: Assess the efficacy of combination therapy with anti-PD-1 and human CCL21-vaults in murine models of lung cancer (months 10-24)

Major Tasks: 1. Prepare Vaults for Aim 2 Animal Testing

Milestone: Adequate vaults purified and characterized for carrying out Tasks 5 and 6. (50% complete)

2. Assess the efficacy of combination therapy with anti-PD-1 and human CCL21-vaults in murine models of lung cancer.

Milestone: Efficacy determined in 3 mouse models, optimal dose and route determined. (0% complete)

3. Evaluate the determinants of anti-tumor response

Milestone: Determinants of anti-tumor response analyzed. (0% complete)

Specific Aim 3: Assess safety of CCL21-vaults following systemic administration in mice (months 12-24)

Major Tasks: 1. Prepare Vaults for Aim 3 Animal Testing

Milestone: Adequate vaults purified and characterized for carrying out Task 8. (0% complete)

2. Assess safety of CCL21-vaults following systemic administration in mice.

Milestone: Assessment of CCL21-vault safety. Determination of LD50 for single dose and repeated dose systemic, MTD and no observable effect level of the treatment. (0% Complete)

What was accomplished under these goals?

Although this research began in July 2018, we were unable to initiate the animal work until securing our ACURO approval at the end of November. Because of this animal experiments were delayed 3 months. Never-the-less we are currently in full swing making solid progress that is summarized here.

Specific Aim 1, Major Task 1: Over the first 3-6 months we prepared vaults for the aim 1 animal testing experiments. The vaults for these studies are produced at GLP purity. We have utilized baculovirus protein expression system that we had previously developed to produce the human CCL21-human vault and the control, empty human vault. Vaults were purified from infected cell pellets. Briefly, the cell pellets are lysed followed by centrifugation at 20,000xg to

remove nuclei, mitochondria and other large particulate matter. The vaults are then pelleted from the supernatant fraction at 100,000xg. Vaults are further purified by salt precipitation and affinity chromatography on an FPLC. The preparation yields are ~10 mg/L of infected cells. The quality and quantity of vaults produced were evaluated by BCA protein assay, SDS-PAGE, Western blotting, CCL21-INT packaging, and negative-stain electron microscopy. In addition, we utilized a quantitative ELISA for measuring CCL21-INT fusion protein packaging so that copy numbers could be accurately measured. Purified INT standards and CCL21-INT vaults were serially diluted and bound to an ELISA plate. These plates were probed with a polyclonal rabbit anti-INT antibody followed by peroxidase conjugated secondary antibody prior to colorimetric quantitation. CCL21-INT copy number was calculated from the ELISA quantitation by comparison to a mass-spectrometry verified CCL21-INT vault standard.

We have used the baculovirus expression system (which uses sf9 insect cells) for many years in the research setting. While a good research tool, the baculovirus approach is complex and costly for industrial scale applications and therefore partnering with a company with on-going baculovirus expression has been necessary for development of vaults using this expression system. The system also has stability problem which is another reason it has found limited utility in the commercial space. We formed a partnership with a vaccine company, Protein Sciences Corp. (PSC), one of the only commercially and FDA approved baculoviral manufacturers. With PSC we developed a GLP purification of sf9-expressed vaults funded by phase I and II SBIR grants from the National Cancer Institute. Unfortunately as we were finishing our GLP process and product stability studies, PSC was purchased by Sanofi and we were informed that Sanofi was not interested in producing our GMP vaults.

Fortunately for us we had been working in parallel on expression of vaults in yeast (*Pichia pastoris*). Like insects, yeast also lack native vaults and, more importantly, yeast are a much more robust commercial manufacturing system. In addition to demonstrating that the yeast—expressed human vaults were structurally-indistinguishable from sf9-expressed vaults, we also showed that our commercial-scale GLP purification procedures were also transferrable to yeast expressed vaults. Because of the above reasons, we are now in the process of switching our vault manufacturing to yeast and, this change will be discussed below under the **Changes/Problems** section of this report.

Specific Aim 1, Major Task 2: As discussed above, due to a delay in securing our ACURO approval, we initiated these experiments in January 2019. To date we have evaluated the efficacy of human CCL21-vaults in 3 novel murine models of lung cancer with increased mutational loads.

Traditional conditional genetically-engineered murine models (GEMMs) of NSCLC bear driver mutations of the disease, recent studies reveal these GEMMs possess low mutational burden, and have shown limited utility in preclinical studies of immunotherapy. We have established novel GEMMs that better recapitulate the mutational landscape of human NSCLC by bearing common driver mutations and varying mutational loads. Previously propagated cell lines with known driver mutations ($kras^{G12D}$ (LKR-13); $kras^{G12D}/p53^{-/}$ (KP); and $kras^{G12D}/p53^{-/-}$, $lkb1^{-/-}$(KPL)) were exposed to MNU treatment three times to increase the mutational load. These cell-lines are labeled as LKR-13-3M, KP-3M, and KPL-3M.

We evaluated the efficacy of the CC21-vault in these three-cell line. CCL21-vault monotherapy did not show efficacy in the KP-3M and KPL-3M mouse models. However, treatment of

LKR13-3M with human CCL21-vaults as a single arm trial revealed significant anti-tumor responses (Fig 1).

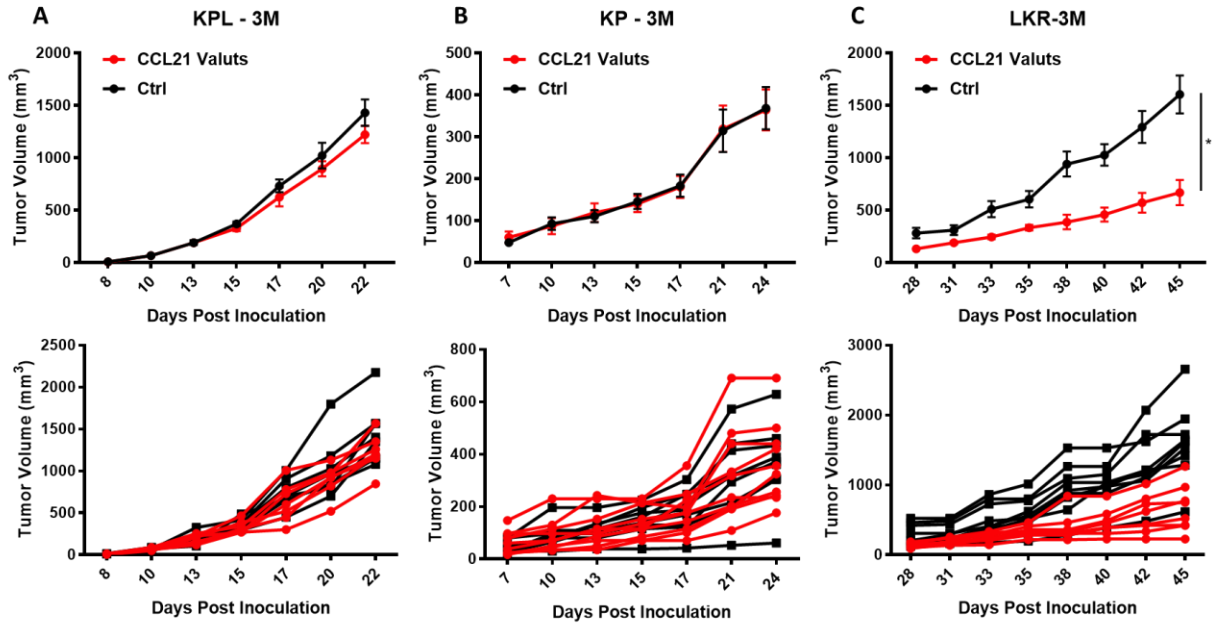

Figure 1. A) Post-tumor inoculation ($1x10^5$ KPL-3M cells delivered SC), FVB mice bearing <50mm^3 tumors (~d5-7) were treated with i) vehicle, ii) Human CCL21-vault (50µg/dose IT every three days for 3 doses), and tumor growth was measured with caliper. **B)** Post-tumor inoculation ($1x10^6$ KP-3M cells delivered SC), FVB mice bearing <50mm^3 tumors (~d5-7) were treated with i) vehicle, ii) Human CCL21-vault (50µg/dose IT every three days for 3 doses), and tumor growth was measured with caliper. **C)** Post-tumor inoculation ($2x10^6$ LKR-13-3M cells delivered SC), 129/E mice bearing <50mm^3 tumors (~d5-7) were treated with i) vehicle, ii) Human CCL21-vault (50µg/dose IT one times per week for 4 doses), and tumor growth was measured with caliper. *P* values were determined by non-paired *t*-test. *, $P<0.05$; **, $P< 0.005$; ***, $P<0.0005$; ****, $P< 0.0001$.

Conclusions:

We have made solid progress on the Aims of year one and we are on track for successfully completing all of our aims by the end of year two. The animal experiments were delayed approximately 3 months by a delay in securing ACURO approval, however we are now fully engaged in this part of the work.

What opportunities for training and professional development has the project provided?
Nothing to Report.

How were the results disseminated to communities of interest?
Nothing to Report.

What do you plan to do during the next reporting period to accomplish the goals?

During the next reporting period we plan to accelerate the testing of CCL21-vaults in animal models & optimize the dose and route of CCL21-vault delivery (Specific Aim 1). We will also evaluate the determinants of anti-tumor response (Specific Aim 1). We will confirm efficacy of the CCL21-vault in the LKR-3M1 and 3LL tumor models (this later model was previously shown to be suppressed by CCL21-vaults) and move on to Specific Aim 2 where we will assess the efficacy of combination therapy with anti-PD-1 and human CCL21-vaults in these two

murine models of lung cancer. Finally we will assess safety of CCL21-vaults following systemic administration in mice (Aim 3).

For the experiments described in Aims 2 and 3, we plan to prepare the vaults using the yeast protein expression system described above.

Impact: Nothing to Report.

Changes/Problems: Although there are no significant changes in the project or its direction. We have decided to change our vault manufacturing system from the baculovirus system to yeast. The reason for this change is described here.

We have used the baculovirus expression system (which uses sf9 insect cells) for many years in the research setting. While a good research tool, the baculovirus approach is complex and costly for industrial scale applications and therefore required that we partner with a company with on-going baculovirus expression for development of vaults using this expression system. The system also has stability problem which is another reason it has found limited utility in the commercial space. We formed a partnership with a vaccine company, Protein Sciences Corp. (PSC), one of the only commercially and FDA approved baculoviral manufacturers. With PSC we developed a GLP purification of sf9-expressed vaults funded by phase I and II SBIR grants from the National Cancer Institute. Unfortunately as we were finishing our GLP process and product stability studies, PSC was purchased by Sanofi and we were informed that Sanofi was not interested in producing our GMP vaults.

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Products: Nothing to Report.

Participants & Other Collaborating Organizations
What individuals have worked on the project?

Name:	*Leonard H. Rome*
Project Role:	*PI*
Researcher Identifier (e.g. ORCID ID):	*0000-0002-1236-2063*
Nearest person month worked:	*1.2*
Contribution to Project:	*Dr. Rome is overseeing the entire project and participating in assessment of vault purity.*
Funding Support:	

Name:	*Steven Dubinett*
Project Role:	*Co-Investigator*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*0.3*
Contribution to Project:	*Dr. Dubinett is in charge of evaluating specific immune responses in the tumor models (Aims 1 and 2). He is directly supervising Dr. Bin Liu.*
Funding Support:	

Name:	*Joye Yang*
Project Role:	*Staff Research Associate*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*4.9*
Contribution to Project:	*Ms. Yang is responsible for purifying vault particles for use in the animal experiments.*
Funding Support:	

Name:	*Ramin Salehi-rad*
Project Role:	*Co-Investigator*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*1.2*
Contribution to Project:	*Dr. Salehi-rad is in charge of all of the animal experiments.*
Funding Support:	

Name:	*Bin Liu*
Project Role:	*Co-Investigator*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*2.4*
Contribution to Project:	*Dr. Liu is in charge of determining the determinants of response to the CCL21-vaults. In addition she is assisting Dr. Salehi-Rad with the animal experiments.*
Funding Support:	

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

There has been one change in key personnel since the grant was funded. Stacy Park, Ph.D., Co-Investigator has taken another position and has been replaced by Dr. Bin Liu. Dr. Liu is an Adjunct Professor in the Department of Medicine with expertise in immunology, molecular and cell biology. She will oversee the flow cytometry analysis in order to determine the determinants of response to the CCL21-vaults. In addition she has been assisting Dr. Salehi-Rad with the animal experiments.

There has also been a change in the “Other Personnel” category. Dr. Rome’s technician, Hedi Roseboro, retired from UCLA and was replaced by Ms. Joye Yang, Staff Research Associate. Ms. Yang was trained to take over Ms. Roseboro’s responsibilities in the lab and she has been providing assistance with all aspects of the project that require production and purification of CCL21-vaults and empty vaults. She is currently leading the conversion of vault production from insect cells to yeast and she is in charge of day-to-day particle production and purification for the experiments described in Aims 1-3.

What other organizations were involved as partners?
Nothing to Report