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TITLE: Developing Novel Immunotherapeutics for Acute Myeloid Leukemia

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CONTRACTING ORGANIZATION: University of Southern California

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14. ABSTRACT
Acute myeloid leukemia (AML)is the most common type of acute leukemia in adults and the second most common pediatric leukemia. The goal of this funded project is to develop novel form of therapeutic agents for selective killing of AML cells through activating and redirecting cellular immunity. This will be achieved by exploiting a multidisciplinary approach to design, generate, and characterize the proposed therapeutic exosomes using cellular and animal models of AML. During the first year of the performance period, we rationally designed and analyzed multiple therapeutic fusion constructs and performed initial characterization of the physicochemical properties and biological activity for the resulting genetically engineered exosomes. While we continue the evaluation of the designed exosomes, we are designing and generating additional types of engineered exosomes for further analysis and characterization.

15. SUBJECT TERMS
Nothing listed

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1. **INTRODUCTION:**

Acute myeloid leukemia (AML) is the most common type of acute leukemia in adults and the second most common pediatric leukemia. Currently, chemotherapy is the standard of care for AML. But the 5-year survival rate of AML is below 30%, which demands for rapid development of new therapeutic approaches with improved effectiveness. Exosomes are naturally occurring nanosized vesicles secreted by various types of cells. Emerging studies revealed pivotal roles for exosomes in mediating intercellular communication. In comparison to conventional synthetic and viral particles, these native extracellular vesicles are found to possess some unique and important properties for therapeutic development, such as protein-enriched membrane structure for enhanced cellular uptake, tolerance by host immune cells, and great biocompatibility. The study is aimed to harness endogenously derived exosomes for eliciting immune responses against AML cells. By combining knowledge and technologies in exosome biology, antibody engineering, and nanotechnology, engineered exosomes will be rationally designed, synthesized, and evaluated using cellular and animal models of AML. By modulating cellular immunity through different strategies, the designed exosomes are expected to provide new and more effective approaches for fighting AML. Mechanistic analysis of the modes of action for these therapeutic exosomes will provide insights into the improvement of the efficacy and safety profiles of the immunotherapeutic exosomes for clinical applications.

2. **KEYWORDS:**

Acute myeloid leukemia; immunotherapy; exosome; protein engineering; nanomedicine

3. **ACCOMPLISHMENTS:**

- **What were the major goals of the project?**
  - Major Task 1: Design of SMART exosomes derived from different fusion partners
  - Major Task 2: Design of SMART exosomes specific for T cells and AML cells
  - Major Task 3: Application for regulatory approval for the animal use
  - Major Task 4: In vitro and in vivo evaluation of the generated SMART-Exos
  - Major Task 5: Design of SMART-Exos expressing immunomodulatory proteins
  - Major Task 6: Design of SMART-Exos carrying immunomodulators
  - Major Task 7: In vitro and in vivo assessment of the SMART-Exos with immunomodulatory agents
  - Major Task 8: Characterize in vitro SMART-Exos-mediated immune attach of AML cells
  - Major Task 9: Investigate in vivo elicited anti-cancer immune responses by the SMART-Exos

- **What was accomplished under these goals?**
  - **Major Task 1. Design of SMART exosomes derived from different fusion partners.** To generate engineered exosomes targeting specific cell types, we designed different fusion protein constructs for displaying monoclonal antibodies on exosomes surface. To this end, we attempted to exploit several different transmembrane proteins in full-length or truncated form, which include both type I and type II proteins, such as platelet-derived growth factor receptor (PDGFR) and lysosome-associated membrane protein 2. Based on sequence analysis of these membrane proteins, fusion constructs were designed by connecting monoclonal antibodies to the N- or C-terminus of the fusion partners via linkers varying in sequence and/or length. These designed fusion constructs were further installed with epitope tag at their termini for downstream analysis. By performing molecular cloning using the designed and synthesized gene fragments together with appropriate primers, fusion constructs were generated by overlap extension polymerase chain reaction (PCR). The amplified fusion genes were then subcloned into mammalian expression vectors. Through extensive screening of transformants and subsequent sequencing, fusion constructs with sequences fully

matched to our initial designs were successfully generated. During this reporting period, we generated more than 6 fusion proteins featured with different fusion partners or displaying strategies and confirmed DNA sequences.

- **Major Task 2: Design of SMART exosomes specific for T cells and AML cells.** While we were designing fusion constructs for displaying monoclonal antibodies on exosome surface, we were also designing and generating different antibody-based constructs for anchoring on exosome surface as targeting moiety. To this end, different antibodies clones that can specifically recognize T-cell CD3 and AML cell-associated C-type lectin-like molecule-1 (CLL-1) were explored. By utilizing different fusion strategies and linkers, monoclonal antibodies in varied formats that are compatible for expression of exosome surface were generated and analyzed for binding affinity and specificity toward their cognate antigens. The selected lead antibody clones were then genetically fused with membrane fusion partners for exosome display. By varying the antibody orientation, linker sequence and length, and fusion partners, different antibody-membrane protein fusions were designed. Epitope tags were added at the termini of the fusion constructs. Using the same approach for molecule cloning, the designed fusion genes were generated by overlap PCR, followed by ligation into target expression vectors. By screening all the transformants and sequencing candidate plasmids, positive hits with confirmed DNA sequences were identified. For this reporting period, we designed and generated more than 8 antibody fusion protein constructs specific for CD3 and/or CLL-1 molecule.
- **Major Task 3: Application for regulatory approval for the animal use.** In addition to designing and generating fusion protein constructs, we were preparing required animal protocol for the planned animal research. The prepared IACUC animal use protocol for studies proposed in this project has been approved by our institutional IACUC committee. The IACUC-approved animal use protocol was then submitted to USAMRMC ACURO along with our prepared ACURO Animal Use Appendix and other relevant documentation. After providing all the required institutional approval and documents, we have received ACURO approval for the use of mice to conduct the planned research.
- **Major Task 4: In vitro and in vivo evaluation of the generated SMART-Exos.** The designed fusion constructs were then used to express engineered exosomes in mammalian cells. The produced exosomes encoding the designed fusion proteins were then collected and examined for expression by performed immunoblots using different antibodies. The fusion constructs showing the expression of fusion proteins were then assessed for stability, size

distribution, as well as binding affinity and specificity. For stably expressed exosomes with displayed antibodies, their binding affinity for CD3 and/or CLL-1 were evaluated by ELISA or flow cytometry using recombinant antigens and cell lines expressing CD3 or CLL-1 molecule. Currently, we are continuing to evaluate the expression levels, stability, size distribution, binding affinity and specificity for all the designed SMART-Exos constructs. Once these evaluations are completed, the developed exosomes will be further analyzed for cellular efficacy and specificity. Selected lead exosomes will then be studied in animal models of AML for effectiveness and toxicity.

- **Major Task 5: Design of SMART-Exos expressing immunomodulatory proteins.** We performed initial design of fusion protein constructs for display proteins targeting certain immune checkpoint pathways. The fusion proteins were designed using different strategies for simultaneously expressing both targeting moieties and effector proteins. The designed fusion constructs were generated by molecular cloning through overlap extension PCR, followed by subcloning into expression vector. Several of designed fusion proteins have been confirmed by DNA sequencing. We are currently in the process of producing the designed engineered exosomes. Meanwhile, we are designing new exosomes with expressed immunomodulatory proteins by utilizing above identified lead exosomes and other fusion partners and linkers. Our aim is to generate a panel of engineered exosomes with different immunomodulatory proteins for functional analysis and selection.
- **Major Task 6: Design of SMART-Exos carrying immunomodulators.** We performed pilot studies to load immunomodulators into the generated SMART-Exos. The resulting exosomes are being analyzed for loading capacity, efficiency, and stability. Once we confirm the lead exosomes with adequate binding affinity and specificity, they will be loaded with the selected immunomodulators using different strategies, followed by comparative analysis. We expect to develop a panel of SMART-Exos with loaded immunomodulators for subsequent analysis of biological and pharmacological activities.
- **Major Task 7: In vitro and in vivo assessment of the SMART-Exos with immunomodulatory agents.** This part of work will be performed once we complete the design and generation of various types of SMART-Exos with immunomodulatory agents. It is expected that this task will be completed during the second and third year of the performance period.
- **Major Task 8: Characterize in vitro SMART-Exos-mediated immune attach of AML cells.** This part of work will be carried

out once we identify lead SMART-Exos with adequate potency and specificity. It is anticipated that this part of work will be finished during the second and third year of the reporting period.

- **Major Task 9: Investigate in vivo elicited anti-cancer immune responses by the SMART-Exos.** This part of work will be performed once we identify lead SMART-Exos without and with immunomodulatory agents. It is expected that this task will be completed during the second and the third year of the performance period.

- **What opportunities for training and professional development has the project provided?**
  - This Career Development Award offers me an excellent opportunity to focus on my independent research program for making important progress toward the goals of this project. The findings from the study will not only help publish a few important research publications to demonstrate my productivity and contribution to this field of research but also provide critical results for competing for other federal grants. Moreover, I have benefited significantly from the guidance of my Career Guide in designing and implementing the research program, collaborating with other researchers, accessing to various research resources, and preparing myself for further professional development. Additionally, the project provides opportunities to train graduate students and postdoctoral researchers who would have chances to work on this project, which will develop their skills in research design, experimental performance, critical thinking, and trouble shooting. By participating in this project, they will also benefit from presentation and publication of the research findings, guidance from advisor, and interactions with other researchers, which will promote their professional development.
- **How were the results disseminated to communities of interest?**
  - Part of the results was presented in research seminars given to undergraduate and graduate students interested in biomedical research.
- **What do you plan to do during the next reporting period to accomplish the goals?**
  - We are performing the in vitro evaluation of the designed and generated SMART-Exos. Once these experiments are finished, we plan to evaluate their biological activities using cellular models of AML, followed by studies of their plasma stability. We are also designing and generating different forms of SMART-Exos with immunomodulatory agents. In vitro characterization will then be performed for the SMART-Exos with expressed or loaded

immunomodulatory agents. Once the in vitro cytotoxicity and specificity are determined, we will start analyzing the immune responses from the cellular samples treated by the generated exosomes.

4. **IMPACT:**

- **What was the impact on the development of the principal discipline(s) of the project?**
  - The findings from this project will provide knowledge on how the designed fusion proteins and loaded therapeutics will affect the stability and biological function of native exosomes as well as how the membrane structure would affect the folding and function of the display proteins and antibodies. These results will provide fundamental guidance for future design of therapeutic exosomes with improved functions and properties.
- **What was the impact on other disciplines?**
  - Nothing to report.
- **What was the impact on technology transfer?**
  - Nothing to report.
- **What was the impact on society beyond science and technology?**
  - Nothing to report.

5. **CHANGES/PROBLEMS:**

- **Changes in approach and reasons for change**
  - Nothing to report.
- **Actual or anticipated problems or delays and actions or plans to resolve them**
  - Due to the COVID-19 pandemic, the research lab was ordered to close in the middle of March. While we could continue designing fusion constructs, no benchwork could be performed to validate our designs until the lab was slowly reopened in the middle of June. Currently, the lab personnel can only work on limited schedule (5 hours per day and 5 days per week). We anticipate significant delay in upcoming months. In compliance with institutional guidelines on lab research under COVID-19 pandemic, we will maximize the hours for benchwork, increase work efficiency, and improve management of research design, experimental work, and data analysis to minimize the delay.
- **Changes that had a significant impact on expenditures**
  - Nothing to report.
- **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**
  - Nothing to report.
- **Significant changes in use or care of human subjects**
  - Nothing to report.
- **Significant changes in use or care of vertebrate animals**
  - Nothing to report.
- **Significant changes in use of biohazards and/or select agents**
  - Nothing to report.

6. **PRODUCTS:**

- **Publications, conference papers, and presentations**
  - **Publications**
    - Nothing to report.
  - **Conference papers**
    - Nothing to report.
  - **Presentations**
    - Devising Artificial Biomolecules at the Interface of Chemistry and Biology, the Minority Opportunities in Research (MORE) Programs at California State University, Los Angeles, CA, October 2019
- **Website(s) or other Internet site(s)**
  - Nothing to report.
- **Technologies or techniques**
  - We designed and generated multiple fusion proteins for genetic display for exosome surfaces. The resulting engineered exosomes possess new or enhanced functions and properties in comparison to native exosome nanoparticles.
- **Inventions, patent applications, and/or licenses**
  - Nothing to report.
- **Other Products**
  - Nothing to report.

7. **PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS:**

- **What individuals have worked on the project?**

Name:	Yong Zhang
Project Role:	PI
Research Identifier (e.g. ORCID ID):	0000-0002-3132-8557
Nearest person month worked:	No change
Contribution to Project:	No change
Funding Support:	
Name:	Heinz-Josef Lenz
Project Role:	Career Guide
Research Identifier (e.g. ORCID ID):	
Nearest person month worked:	No change
Contribution to Project:	No change
Funding Support:	
Name:	Alan L. Epstein
Project Role:	Co-I
Research Identifier (e.g. ORCID ID):	
Nearest person month worked:	No change
Contribution to Project:	No change
Funding Support:	
Name:	Qinqin Cheng
Project Role:	Postdoctoral researcher
Research Identifier (e.g. ORCID ID):	
Nearest person month worked:	No change
Contribution to Project:	No change
Funding Support:	
Name:	Zhefu Dai
Project Role:	Graduate student
Research Identifier (e.g. ORCID ID):	
Nearest person month worked:	No change

Contribution to Project:	No change
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?**
  - **The following grants awarded to the PI were completed,**
    - "Mapping Post-Translational Modification-Dependent Interactome in Cancer Cells" funded by The V Foundation for Cancer Research, 02/19 – 01/20
    - "Targeting Metastatic Colorectal Cancer with Novel Immuno-Nanoparticles" funded by AAPS Foundation, 08/17 – 07/19
    - "Development of Novel Immunotherapeutics for Breast Cancer" funded by USC Ming Hsieh Institute for Engineering Medicine for Cancer, 07/18 – 08/19
    - "Novel Immunotherapeutics for Colorectal Cancer" funded by PhRMA Foundation, 09/18 – 08/19
  - **The following grants were awarded to the PI in the reporting period,**
    - "Targeting Hepatocellular Carcinoma with Inhibitory Antibodies" funded by USC Undergraduate Research Associates Program Grant, 07/19 – 06/20
    - "Targeting Renal Cell Carcinoma with Novel Immunotherapeutics" funded by AACR-Bayer Innovation and Discovery Grant, 07/19 – 06/20
    - "Immunotherapeutic Exosomes for Triple Negative Breast Cancer" funded by California Breast Cancer Research Program, 08/19 – 01/21
    - "Novel Immunotherapeutics for Cigarette-Smoking Associated Acute Myeloid Leukemia" funded by Tobacco Related-Disease Research Program, 09/19 – 08/20
    - "Developing a Novel Therapy for Hispanic Americans with Colorectal Cancer" funded by USC Norris Comprehensive Cancer Center, 04/20 – 03/21
- **What other organizations were involved as partners?**
  - Nothing to report.

8. **SPECIAL REPORTING REQUIREMENTS:**

- **COLLABORATIVE AWARDS:** Not required.
- **QUAD CHARTS:** Not required.

9. **APPENDICES:** Nothing to report.