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TITLE: A Milk Protein-Hitchhiking Strategy for the Oral Delivery of Amphiphilic Vaccines

PRINCIPAL INVESTIGATOR: Jiahe Li

CONTRACTING ORGANIZATION: Northeastern University

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14. ABSTRACT

We are grateful for the one-year no-cost extension. Following the last report, we have made extensive efforts in two major directions: (1) identification of a more potent adjuvant system targeting the STING pathway to augment the *in vivo* performance. (2) Repurpose the platform developed during the Discovery Award as a powerful platform to fight against the COVID-19. At the end of this grant period, we have published two papers resulting from the support of this grant.

15. SUBJECT TERMS

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TABLE OF CONTENTS

Page

1. Introduction

Service members and civilian populations are potentially exposed to many emerging infectious diseases that are life-threatening. These include, but are not limited to, Zika virus (ZIKV), dengue, HIV, norovirus, hepatitis, and Middle East Respiratory Syndrome. However, many of these diseases remain among the leading causes of illness and death in the battlefield, and account for substantial spending on the related consequences of infection even after soldiers come back from the service. Many infectious diseases, however, can be prevented through vaccination. Developing a convenient and cost-effective vaccine delivery technology that can be deployed to service members and civilians remains highly desirable. Oral vaccination is a very powerful and effective approach for vaccination, because it can not only induce a broad immunity through the blood and lymphatics, but also help patrol a very thin lining in the respiratory, digestive, and reproductive systems for potential virus invasion. The latter is particularly important as many pathogens invade the body via sexual or respiratory transmission. However, the stomach presents a major barrier to successful delivery of protein-based vaccines to the intestines, in which immune cells would otherwise receive alarm signals. The stomach is highly acidic and also contains proteins that recognize vaccines as food sources to chop up. To address this challenge, we draw inspiration from two seemingly irrelevant disciplines − dairy science and bioconjugation chemistry − to develop a “plug and play” type of approach for oral delivery of vaccines. Our strategy is to repurpose a milk protein, α-lactalbumin, as a vehicle, which is known to be resistant to degradation by stomach. Since this protein naturally associates with many bioactive lipids such as Vitamin D, we propose to explore a range of lipid molecules derived from a common supplement (e.g. Vitamin D) and vegetable oil (e.g. oleic acid). As a proof of principle study, we link lipid molecules to a short strand of amino acids (an antigenic peptide) that instructs the host immune system to mount potent humoral and cellular immune responses against the pathogen of interest. We envision that co-administering these two components with α-lactalbumin will help them from being cleared by the stomach, and therefore a stronger signal will be sent to immune cells lying underneath the intestines.

2. Keywords

Oral vaccine, subunit vaccine, α-lactalbumin, immunization

3. Accomplishments (based on the SOW)

Major Task 1 (Aim 1): Chemical conjugation of lipids to peptide antigens and adjuvants

This task was completed and documented in the last annual report.

Major Task 2 (Aim 2): Complexation of lipid conjugates with α-lactalbumin

This task was completed and documented in the last annual report.

Major Task 3 (Aim 3): Evaluate the therapeutic efficacy in mice

Explore alternative immune adjuvants to enhance the oral vaccination

In contrast to existing delivery strategies such as nanoformulations or synthetic depots to overcome the challenges in encapsulation and intracellular delivery of STING agonist (e.g., cGAMP), we have repurposed the natural receptor STING as a highly modular and simple platform to efficiently bind and deliver cGAMP *in vitro* and *in vivo*. Specifically, we took advantage of previous biochemical studies, in which the recombinant C-terminal domain of STING protein (STINGΔTM, 139-379aa for human and 138-378aa for mouse) is known to bind cGAMP with high affinity and stability(17,18). Additionally, in our previous work, we serendipitously uncovered that the recombinant STINGΔTM could form complexes with cGAMP, and activate the downstream STING signaling following delivery of the complexes by commercial transfection reagents in HEK293T that do not express endogenous STING. On the contrary, recombinant STINGΔTM proteins with catalytically inactive mutations, including S366A and deletion of the last 9 amino acids (i.e., ΔC9), failed to activate the STING pathway in HEK293T. Building on this serendipitous discovery, to bypass the need for transfection reagents, here we developed a cell-penetrating (CP)-STINGΔTM to deliver cGAMP into different cell types via genetic fusion of a cell-penetrating protein (**Fig. 1A** and **B**). Notably, in contrast to cell-penetrating peptides such as trans-activating transcriptional activator (TAT), we have chosen the Omomyc mini-protein as our cell-penetrating moiety for three reasons: (1) Omomyc (91 amino acids) is derived from a dominant-negative form of the human *MYC* oncogene and has recently shown specific targeting and potent tumor cell penetration capabilities in human cancer cell lines and xenograft mouse models; (2) The natural dimer conformation of Omomyc coincides with STINGΔTM, which also exists as a dimer in the absence of cGAMP; (3) Omomyc may not cause an immunogenicity issue owing to its human origin.

Since the C terminal amino acids of STING directly interact with downstream effector proteins, including TBK1 and IRF3, we genetically fused the cell-penetrating protein Omomyc to the N terminus of STINGΔTM to prevent any steric hindrance posed by Omomyc (**Fig. 1C**). In addition, we generated two essential CP-STINGΔTM mutants to help dissect the mechanisms underlying enhanced delivery of cGAMP: one lacks the effector function to engage with the downstream STING signaling pathway and the other fails to bind cGAMP (**Table 1**). After recombinant protein expression in *E. coli*, we purified 6x Histidine (His) tagged proteins via the metal affinity purification and size exclusion chromatography.

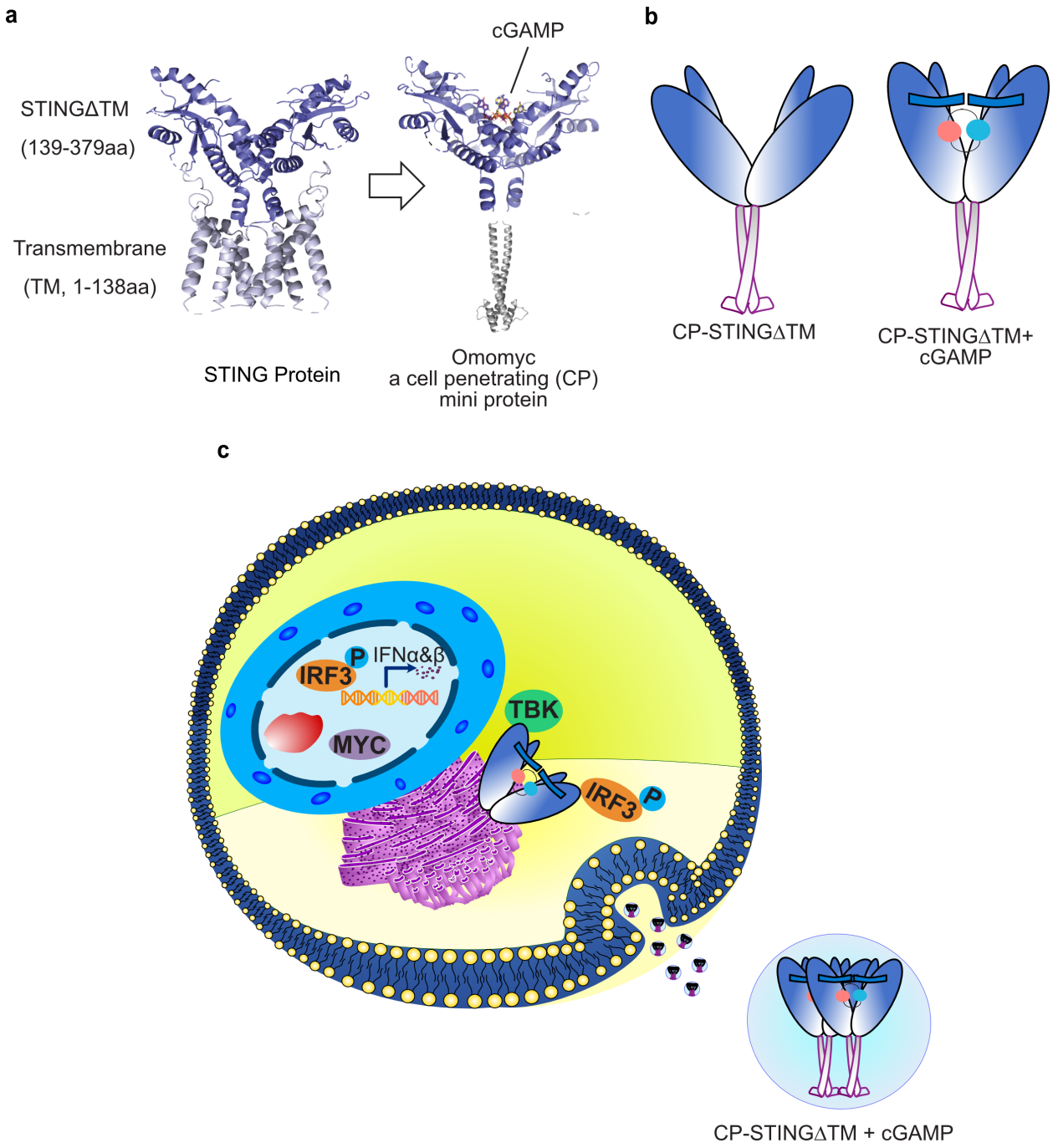

Figure 1. Schematic of using recombinant cell-penetrating (CP)-STINGΔTM as a biologically functional platform for cGAMP delivery. (a) To bypass the need for synthetic vehicles, we designed and engineered a CP-STINGΔTM by replacing the transmembrane (TM) of the full-length STING with Omomyc, a cell-penetrating mini protein. (b) A cartoon model illustrating how CP-STINGΔTM binds cGAMP. (c) By fusing with the cell-penetrating domain, the CP-STINGΔTM is capable of penetrating cells, delivering cGAMP, and engaging with downstream proteins such as TBK1 and IRF3, which result in the production of type I IFNs.

STING variants*	Description
STINGΔTM	STING lacking the N terminal transmembrane domain
STINGΔTMΔC9	9-amino acid deletion at the C terminus that abolishes type 1 IFN induction
STINGΔTM(R238A/Y240A)	Deficient for cGAMP binding
CP-STINGΔTM	Inclusion of cell-penetrating domain -- Omomyc to bypass transfection reagent
CP-STINGΔTMΔC9	
CP-STINGΔTM(R238A/Y240A)	
CP-STINGΔTM-dsred	

Table 1: STING variants used in this study. * Amino acid positions represent the human STING (1-379aa), which are conserved in the mouse STING (1-378aa)**.**

The chaperon role of CP-STINGΔTM/cGAMP in mice bearing STING-positive melanoma

We first tested CP-STINGΔTM/cGAMP in a syngeneic mouse melanoma model bearing Yummer1.7 cells ($STING_{positive}$), which carry *Braf* mutation and *Pten* loss that mimic mutations in melanoma (52). Before the treatment study, we confirmed that CP-STINGΔTM was detectable throughout tumor slices even at 96 hr after a single i.t. administration, while STINGΔTM lacking Omomyc did not (**Fig.2A**). Next, we initiated the treatment study in a separate cohort when tumors reached ~170 mm^3. Over the course of treatment, no significant weight loss was detected among different treatment groups in comparison to the vehicle control group. Of note, both CP-STINGΔTM and CP-STINGΔTMΔC9 markedly reduced tumor progression compared to CP-STINGΔTM(R237A/Y239A) and STINGΔTM through co-delivery of cGAMP (**Fig.2B**). The findings agreed with our *in vitro* studies: (1) The cGAMP-binding deficient mutant CP-STINGΔTM(R237A/Y239A) cannot effectively deliver cGAMP into target cells. (2) STINGΔTM alone cannot efficiently penetrate cells due to the absence of Omomyc. (3) because Yummer 1.7 and hematopoietic cells in tumors express endogenous STING, CP-STINGΔTM plays a chaperon role in enhancing the intracellular delivery of cGAMP such that there was no detectable difference between wildtype CP-STINGΔTM and the IFN defective mutant CP-STINGΔTMΔC9.

The functional role of CP-STINGΔTM/cGAMP in mice carrying STING-negative NSCLC

We next extended our approach to a syngeneic NSCLC model with STING silencing (**Fig.2D**). The Lewis lung cancer cell line LLC1 was chosen because it is poorly immunogenic and is resistant to anti-PD-1 (53). Moreover, in the literature cGAMP alone failed to cure established LLC1 tumors (54), which prompted us to test whether CP-STINGΔTM/cGAMP can improve the therapeutic efficacy. To mimic the loss of STING in a subset of NSLCL, we depleted endogenous STING in LLC1 via CRISPR, and verified the STING loss by immunoblotting (data not shown). We first profiled the uptake of CP-STINGΔTM/cGAMP versus STINGΔTM/cGAMP in tumors via intracellular anti-FLAG staining by flow cytometry. It was found that CP-STINGΔTM/cGAMP were internalized by ~50% tumor cells, while MDSC, macrophages and DCs exhibited partial uptake (**Fig.2C**). Importantly, while CP-STINGΔTM/cGAMP did not penetrate every single tumor cell, our ongoing survival study, that pools two biological repeats (a total of N=10 mice per treatment), has been very promising—only cGAMP/CP-STINGΔTM cured 40% mice with established tumors (~170 mm^3), while cGAMP/CP-STINGΔTMΔC9 (IFN defective), cGAMP/CP-STINGΔTM(R237A/Y239A) (cGAMP-binding deficient) or cGAMP/STINGΔTM (no cell penetrating capability) did not (**Fig.2D**).

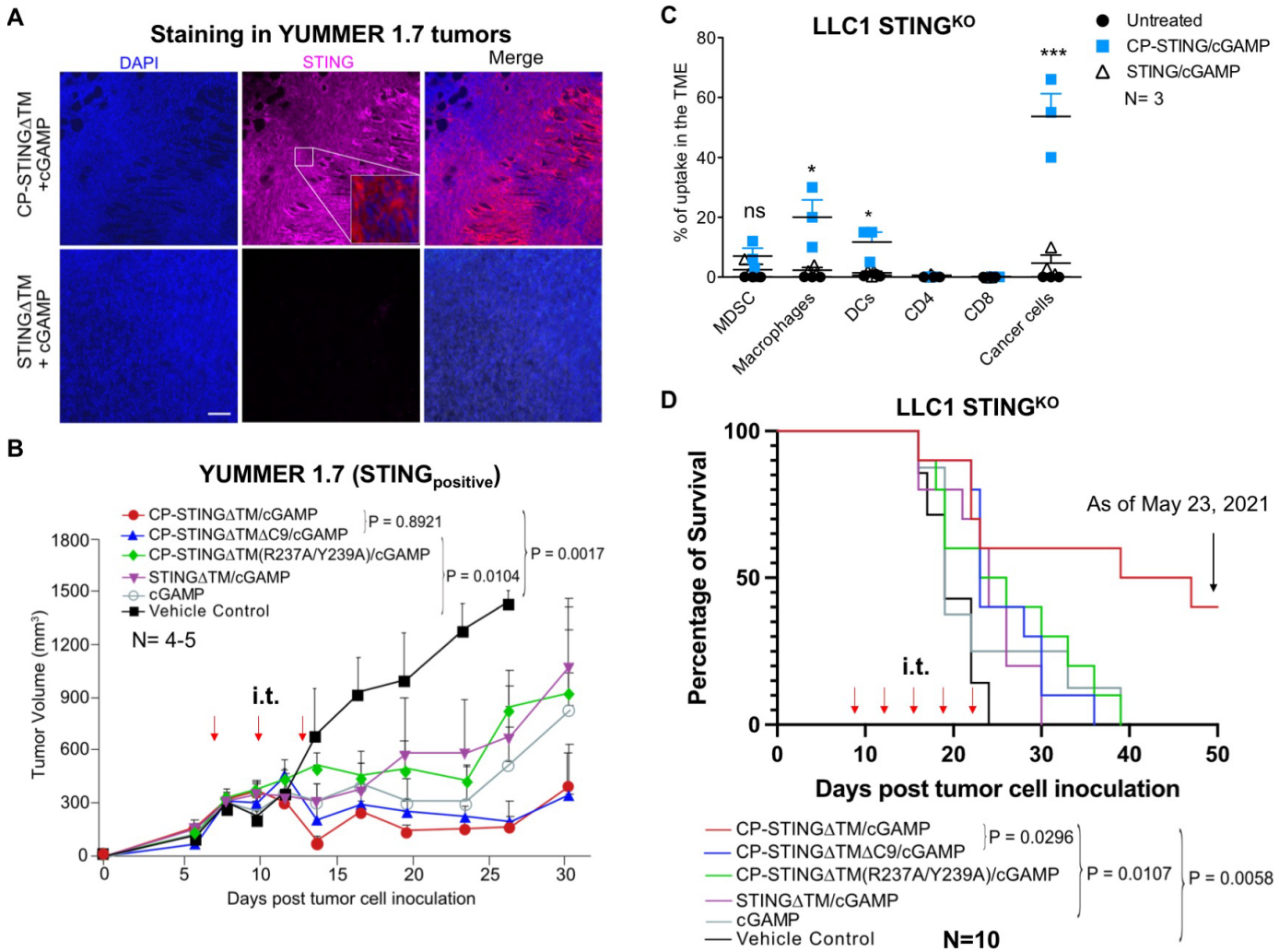

Figure 2. ***In vivo*** **studies. (A)** CP-STINGΔTM+cGAMP but not STINGΔTM+cGAMP remained in the TME at 96 hr after a single local administration. Scale bar = 100 μm. **(B)** Tumor size measurement in YUMMER 1.7-bearing mice. **(C)** Uptake of CP-STINGΔTM+cGAMP versus STINGΔTM+cGAMP by tumor-infiltrating lymphocytes and cancer cells ($CD45^-$) 24 hr after injection in LLC1 $STING^{KO}$. Data were analyzed by flow cytometry. *P<0.05, ***P<0.001. Values = mean ± SEM, N=4. **(D)** Survial rates in mice bearing LLC1 $STING^{KO}$ as of May 23, 2021. Treatment was initiated when tumors cells reached ~170 mm^3. Arrows indicate the timepoints of *in situ* injections.

4. Impact

At the end of the grant period, we have published four relevant papers, which are in part funded by the Discovery award. Notably, my first PhD student, Dr. Xin Sun, is the first author of three papers, and successfully defended her PhD in May 2021.

5. Changes/Problems

As discussed above, we identified the lack of in vivo immune responses from oral vaccination of our lactalbumin-based complexes despite encouraging data in vitro. Later on, we changed from in vivo to in vitro using the gut model to understand whether it was caused by poor bioavailability of the complexes. In parallel, in addition to the CpG adjuvant, we explored the STING pathway as a more potent immune adjuvant for oral vaccination. As a proof-of-concept, we showed that co-delivery of the recombinant STING protein with STING agonists such as cGAMP can enhance the efficacy of STING agonist delivery by ~100-fold. Encouraged by the preliminary data, our direction has slightly changed to focusing on the STING pathway to augment the efficacy of vaccination in mice.

6. Products

Journal articles that acknowledge this Discovery Award:

Sun X, Yang S, Al-Dossary AA, Broitman S, Ni Y, Guan M, Yang M, **Li J***. Nanobody-Functionalized Cellulose for Capturing SARS-CoV-2, *Applied and Environmental Microbiology*, 2022 Jan 5. https://journals.asm.org/doi/10.1128/aem.02303-21

Sun X, Ni Y, He Y, Yang M, Tani T, Kitajima S, Barbie DA, **Li J*.** Engineering the Immune Adaptor Protein STING as a Functional Carrier, *Advanced Therapeutics*, 2021. https://onlinelibrary.wiley.com/doi/10.1002/adtp.202170017 Selected as the Cover Picture in the Special Issue:Next Generation Immunotherapies

Sun X, Hay I, Doran P, Basireddy S, Scott M, Wu Y, Al-Dossary AA, **Li J***. Chapter Thirteen – Delivery strategies for STING agonists. Systemic Drug Delivery Strategies, Volume 2 of Delivery Strategies and Engineering Technologies in Cancer Immunotherapy. 2021, Pages 333-357. https://doi.org/10.1016/B978-0-323-85781-9.00013-0

He Y, Hong C, Yan Z.E, Li Y, Zhu G, Yang M, Li Y, Sun X, Irvine DJ, **Li J***, Hammond PT*. Self-Assembled cGAMP-STING ΔTM Signaling Complex as a Bioinspired Platform for cGAMP Delivery, *Science Advances*, 2020. https://advances.sciencemag.org/content/6/24/eaba7589

Provisional patent applications filed by Northeastern University:

Nothing to report

7. Participants & Other Collaborating Organizations

Name: Jiahe Li
Project Role: PI
Nearest Person-Month Worked: 1 month (summer) (Academic salary covered by NEU)

Name: Xin Sun
Project Role: Graduate student
Nearest Person-Month Worked: 12 months
Contribution to Project: 1st authors in three publications

Name: Shaobo Yang
Project Role: Graduate Student
Nearest Person-Month Worked: 4 months (tuition is waived in the college of engineering at NEU)
Contribution to Project: co-author in one publication

Name: Mengdi Yang
Project Role: Graduate Student
Nearest Person-Month Worked: 4 months (tuition is waived in the college of engineering at NEU)
Contribution to Project: co-author in three publications

8. Special Reporting Requirements

9. **Appendices** Copies of papers published and in press are included in the Appendix