Peptide-Lipid Conjugates for Enhancing *mRNA-LNP Delivery* and *Colon Cancer Vaccines*

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NEXT-GENERATION DELIVERY INNOVATIONS

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Patent Application Title:
PEPTIDE-LIPID CONJUGATES FOR ENHANCED NUCLEIC ACID DELIVERY AND METHODS OF MAKING AND USING SAME. Application no. US App. No. 63/745,773

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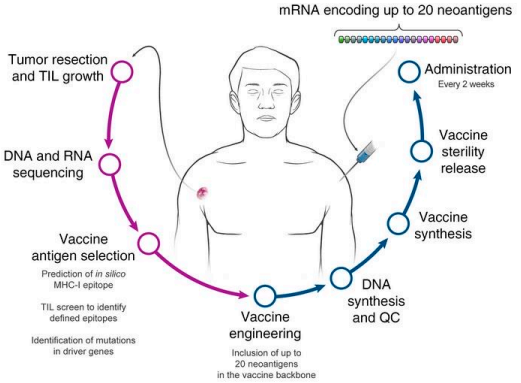
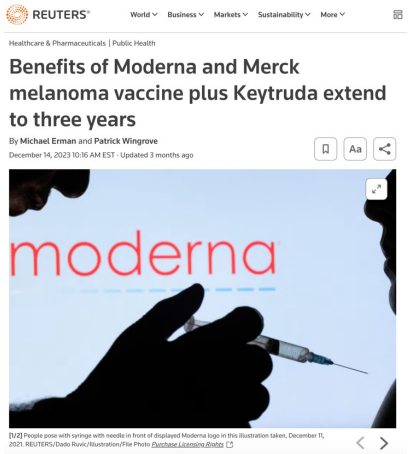
Trends in mRNA-LNPs *beyond COVID-19*
→ Personalized mRNA Cancer Vaccines



Cafri et al., J Clin Invest. 2020

mRNA-Lipid Nanoparticles (mRNA-LNPs)



The Delivery Platform:
LNPs are not surface functionalized to target dendritic cells (DCs) preferentially.

Opportunity for Improvement:
Can we develop a simple yet effective method for delivering mRNA-LNPs to DCs more effectively?

Inspiration: Chemotherapy-Induced *Immunogenic Cell Death (ICD)* = generation of "*Cancer Cells Vaccine*"

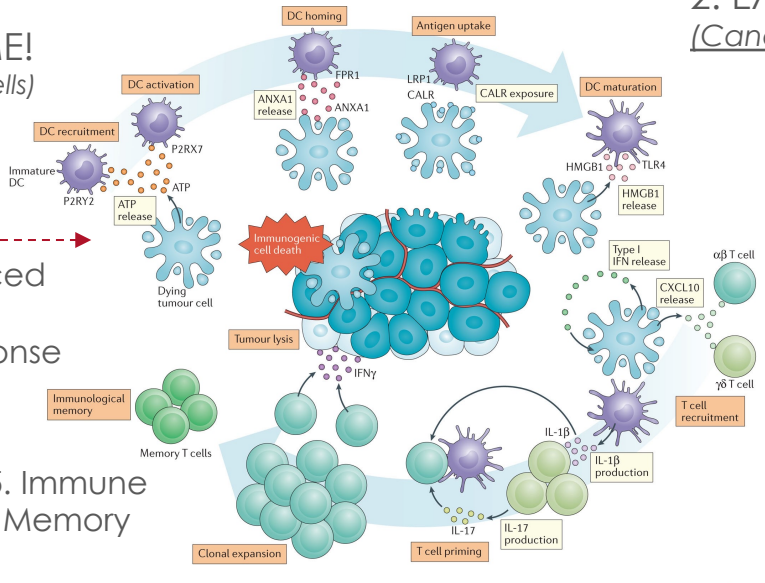
1. FIND ME!
(Dendritic Cells)

2. EAT ME!
(Cancer Antigen Uptake by DCs)

3. DANGER!
(Immune Adjuvant)

ICD Inducer:
- Doxorubicin
- Mitoxantrone
- Oxaliplatin

Chemotherapy-induced Immunogenic Immunotherapy response



5. Immune Memory

4. Cytotoxic T Lymphocyte (CTL) Response

Galluzzi et al., Nat Rev Immunol 17, 97–111 (2017).

Inspiration: Calreticulin (CRT) Exposure: The "Eat Me" Signal Driving Phagocytic Uptake of Dying Cancer Cells by Dendritic Cells (DCs)

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Article | Published: 24 December 2006

Calreticulin exposure dictates the immunogenicity of cancer cell death

Michel Obeid, Antoine Tesniere, François Ghiringhelli, Gian Maria Fimia, Lionel Apetoh, Jean-Luc Perfettini, Maria Castedo, Grégoire Mignot, Theoharis Panaretakis, Noelia Casares, Didier Métivier, Nathanael Larochette, Peter van Endert, Fabiola Ciccosanti, Mauro Piacentini, Laurence Zitvogel & Guido Kroemer

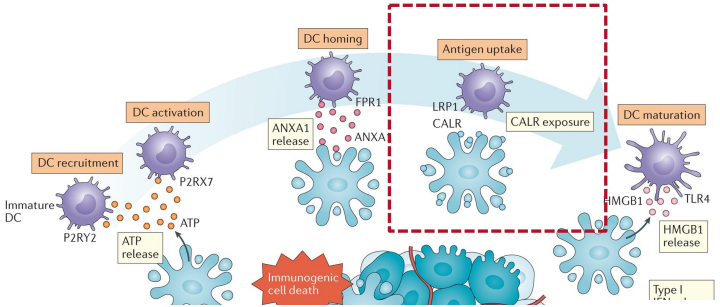
Nature Medicine **13**, 54–61 (2007) | Cite this article

32k Accesses | **29** Altmetric | Metrics

Obeid et al., Nat Medicine, 13, 54–61 (2007).

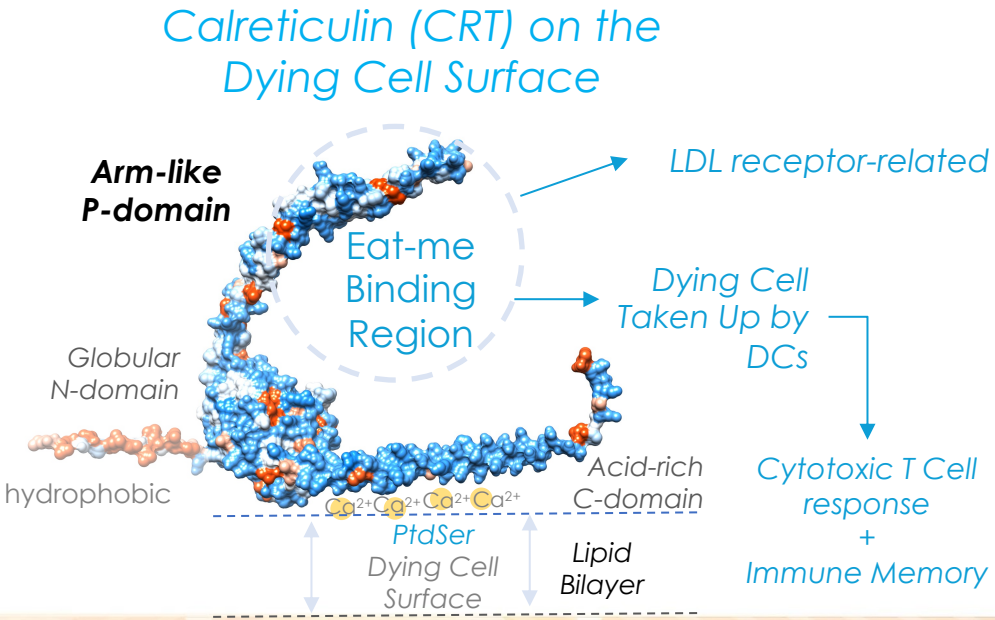
2. CRT = the EAT ME Signal!

Cancer Antigen Uptake/Delivery by DCs



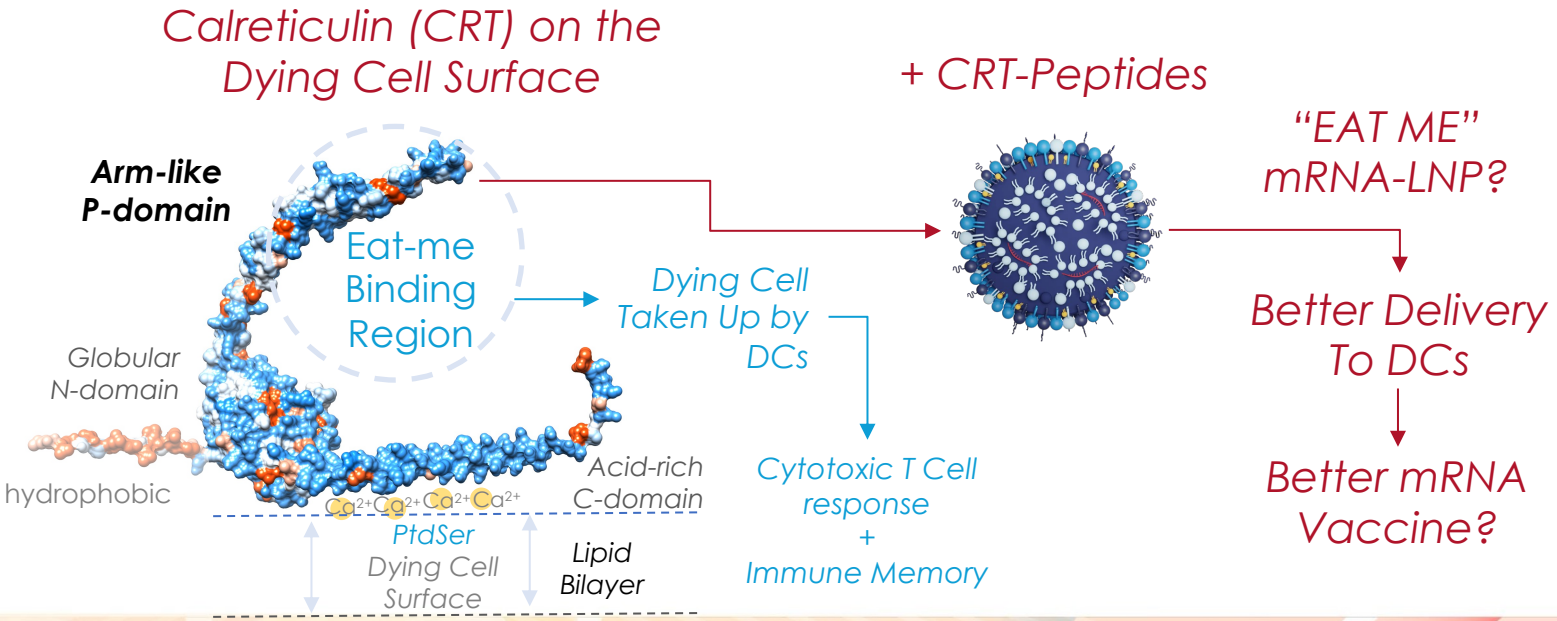
Galluzzi et al., Nat Rev Immunol 17, 97–111 (2017).

How does Calreticulin sit on the dying cell surface following ICD to serve as the *Eat Me* signal?



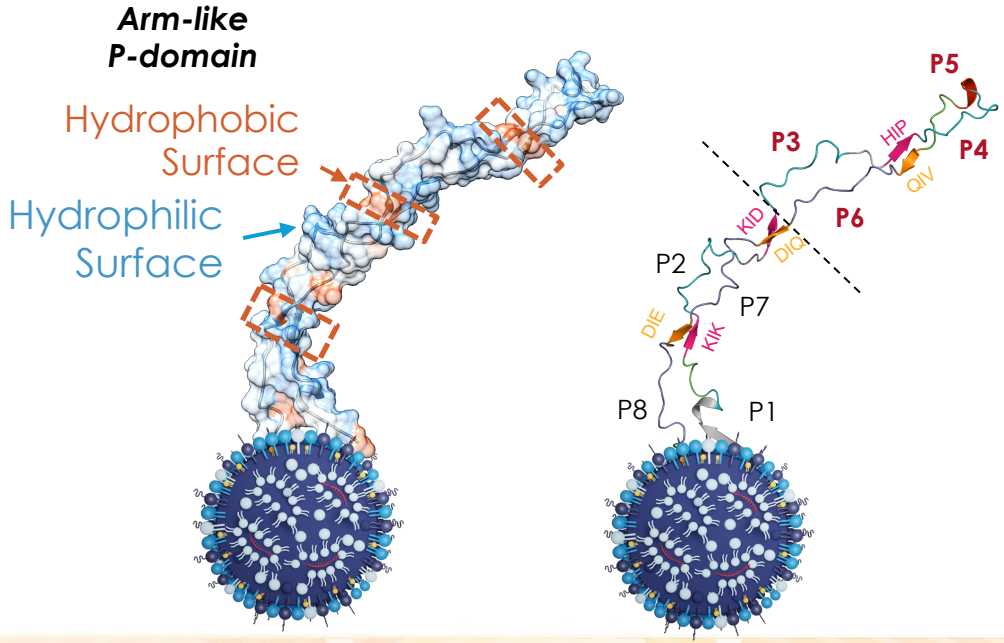
Hypothesis 1: CRT peptides are responsible for the Eat Me signal

Hypothesis 2: CRT peptides-functionalized LNPs
= better mRNA delivery to DC → better vaccine?



LNP photo credit: Inside Therapeutics

Approach: Mapping Hydrophilic P-Domain Peptides of Calreticulin to Enable Surface Presentation on LNPs



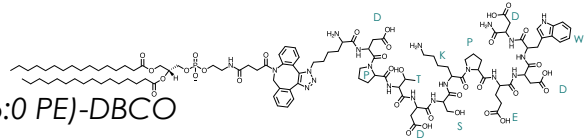
Hydrophilic P-domain Peptides Fragments: P1-8

Peptide Name	CRT Residual#	Amino Acid Sequence	pI (pH)*	Net Charge (pH 7)*	GRAVY†
P1	199-206	DWDFLPPK	3.88	-1.0	-1.05
P2	210-220	DPDASKPEDWD	3.36	-4.0	-2.23
P3	227-237	DPTDSKPEDWD	3.36	-4.0	-2.54
P4	244-254	DPDAKKPEDWD	3.77	-3.0	-2.51
P5	264-254	PPEWEGDMEED	2.79	-6.0	-2.15
P6	278-268	RPKWEGKYEPN	9.93	1.0	-2.60
P7	292-282	PHIWTGKYDPN	7.66	0.1	-1.46
P8	306-296	YISPDPSYEPN	3.01	-2.0	-1.36

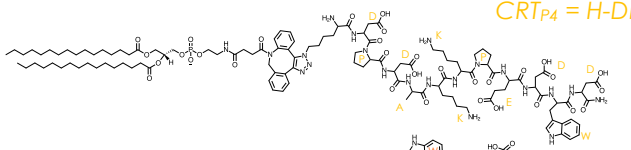
LNP photo credit: Inside Therapeutics

Method: Conjugation of CRT-Derived Peptides to Lipids for LNP Surface Display via Click Chemistry

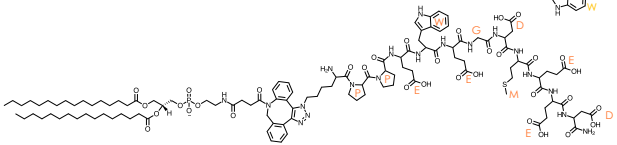
Lipid =
DPPE (16:0 PE)-DBCO



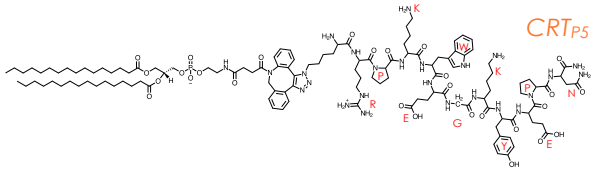
CRT_{P3} = DPTDSKPEDWD-NH_2



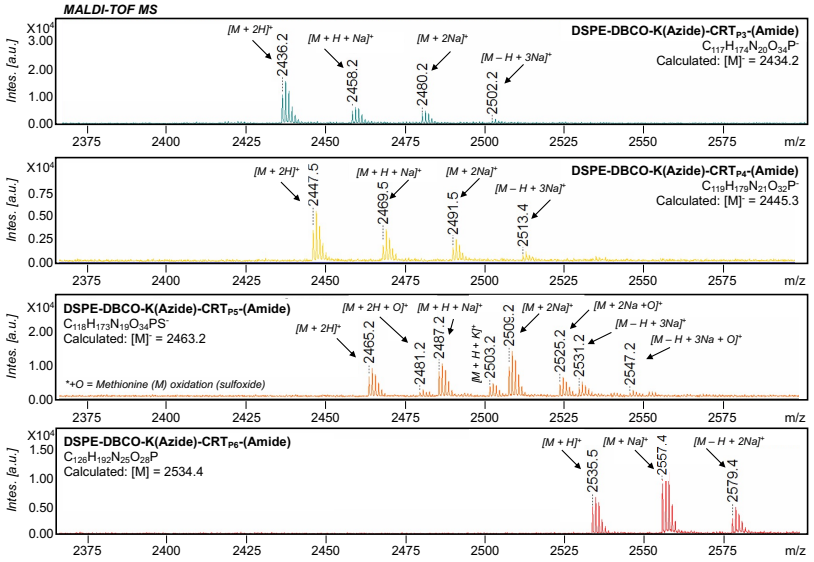
CRT_{P4} = H-DPDAKKPEDWD-NH_2



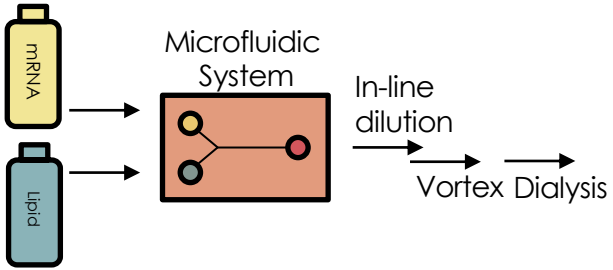
CRT_{P5} = PPEWEGDMEED-NH_2

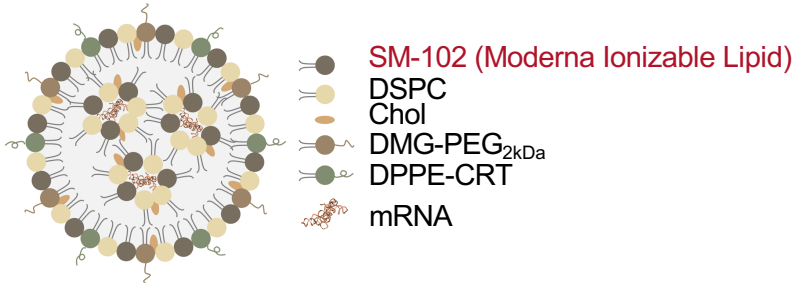


CRT_{P6} = RPKWEGKYEPN-NH_2

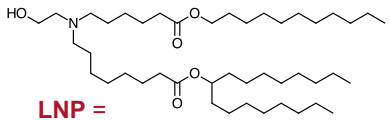


Method: Formulating CRT-Peptide Functionalized mRNA-LNPs with Ionizable Lipids (e.g., SM-102) via Microfluidic



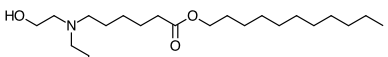


Formulation	LNP	CRT$_{P3/P4/P5/P6}$-LNPs
Ionizable lipid:	50%	50%
DSPC:	**10%**	5%
Chol:	38.5%	38.5%
DMG-PEG$_{2kDa}$:	1.5%	1.5%
DSPE-CRT$_{P3/P4/P5/P6}$:	**0%**	**5%**

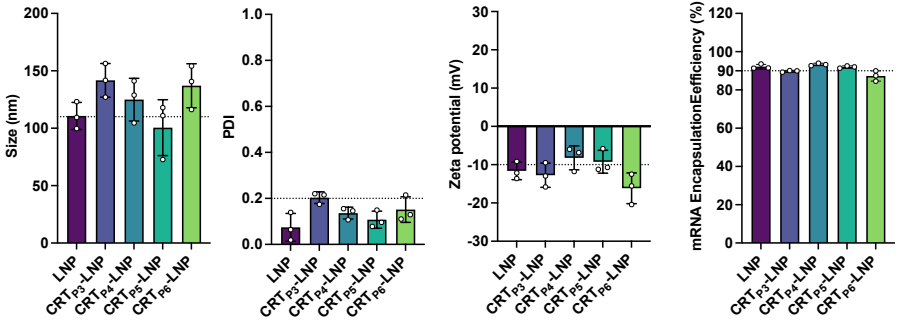


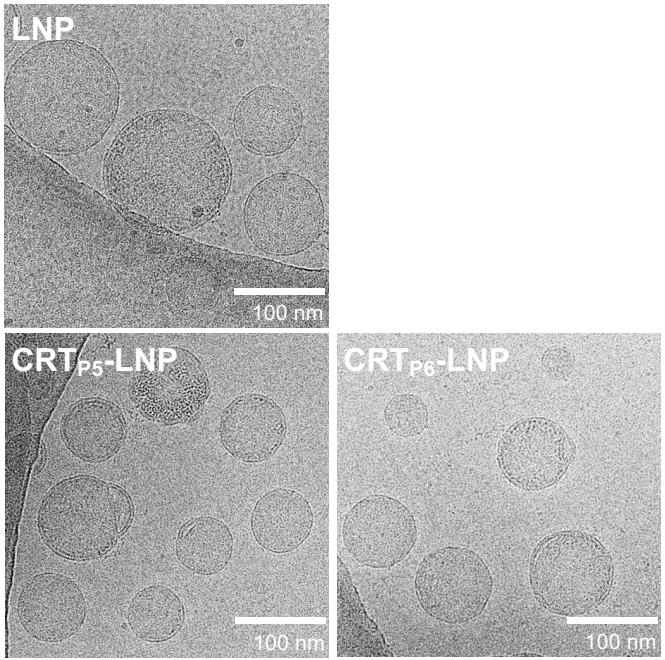
LNP =
Ionizable Lipid = **SM-102** (Moderna)

Results: $CRT_{P3/4/5/6}$ Peptides are LNP Compatible with mRNA-LNPs and Preserve Nanoparticle Integrity

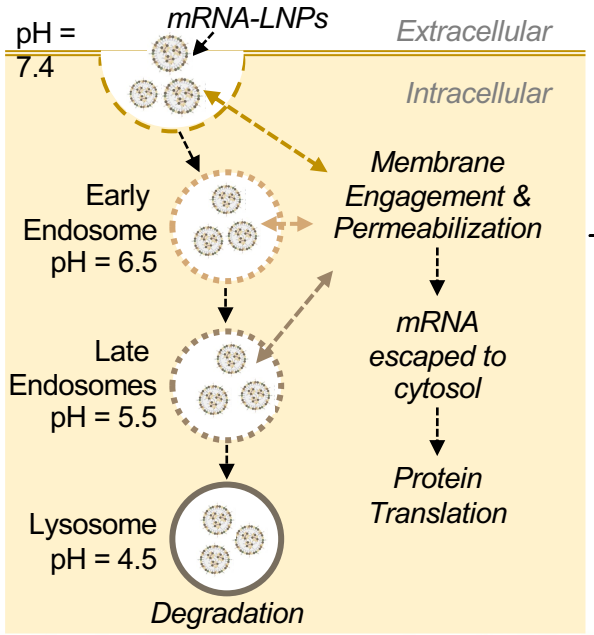


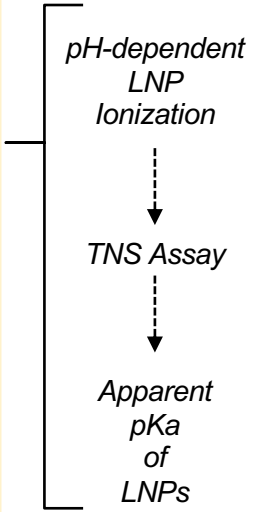
LNP =
Ionizable Lipid = **SM-102**
(Moderna)

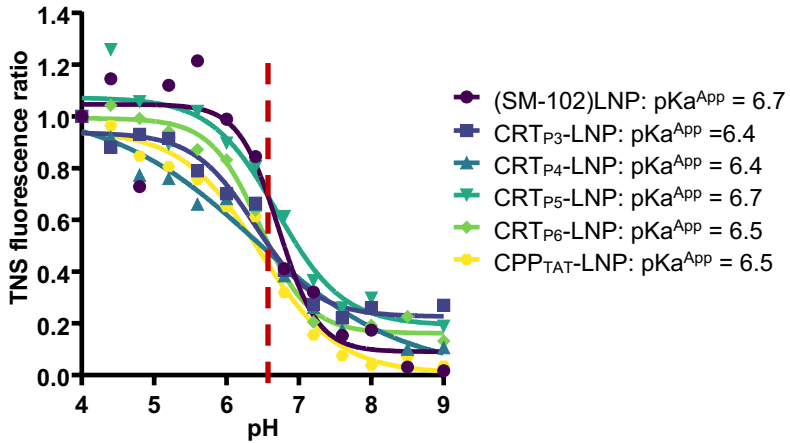




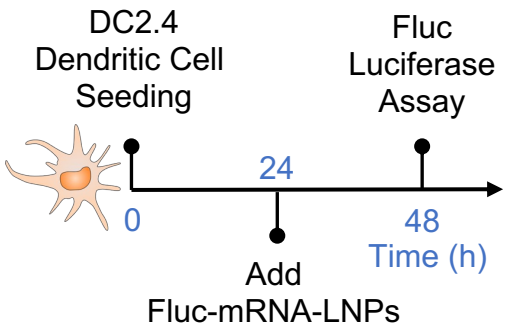
Results: Apparent pKa (pKa^{App}) of mRNA-LNPs Remains Unchanged Upon Incorporation of $CRT_{P3/P4/P5/P6}$ Peptides



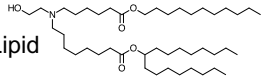




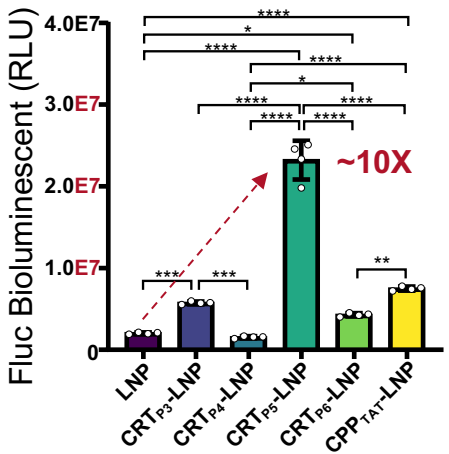
Results: CRT_{P5} Significantly Increased mRNA Delivery to Dendritic Cells without Affecting Cell Viability

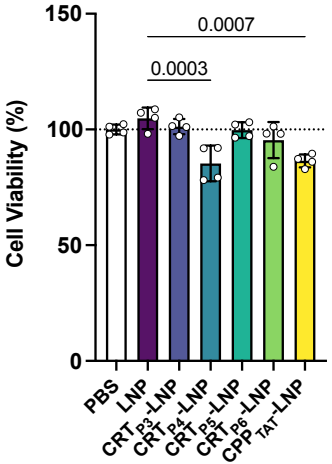


LNP
Ionizable Lipid
= **SM-102**
(Moderna)

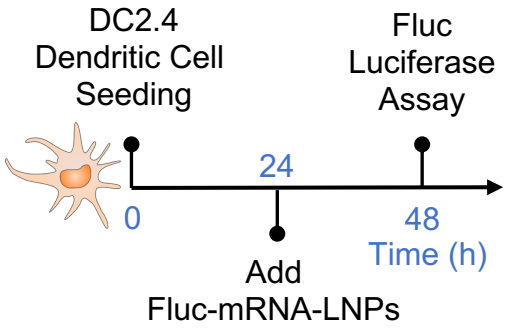


Positive Peptide Control:
*^The **CPP_{TAT}** peptide (GRKKRRQRRRPQ)*
*= **C**ell **P**enetrating **P**eptide (**CPP**) from the transactivator of transcription (**TAT**) of human HIV*

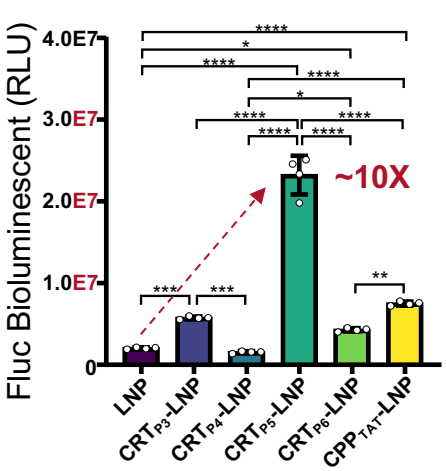




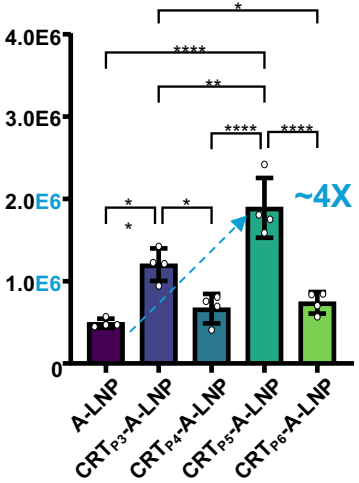
Results: CRT_{P3} and CRT_{P5} Promote Efficient mRNA Delivery to Dendritic Cells via LNPs of Different Ionizable Lipids



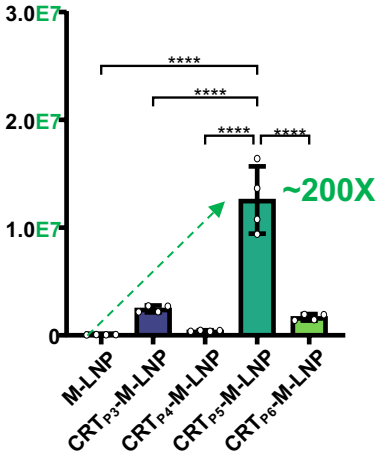
LNP
Ionizable Lipid
= **SM-102**
(Moderna)



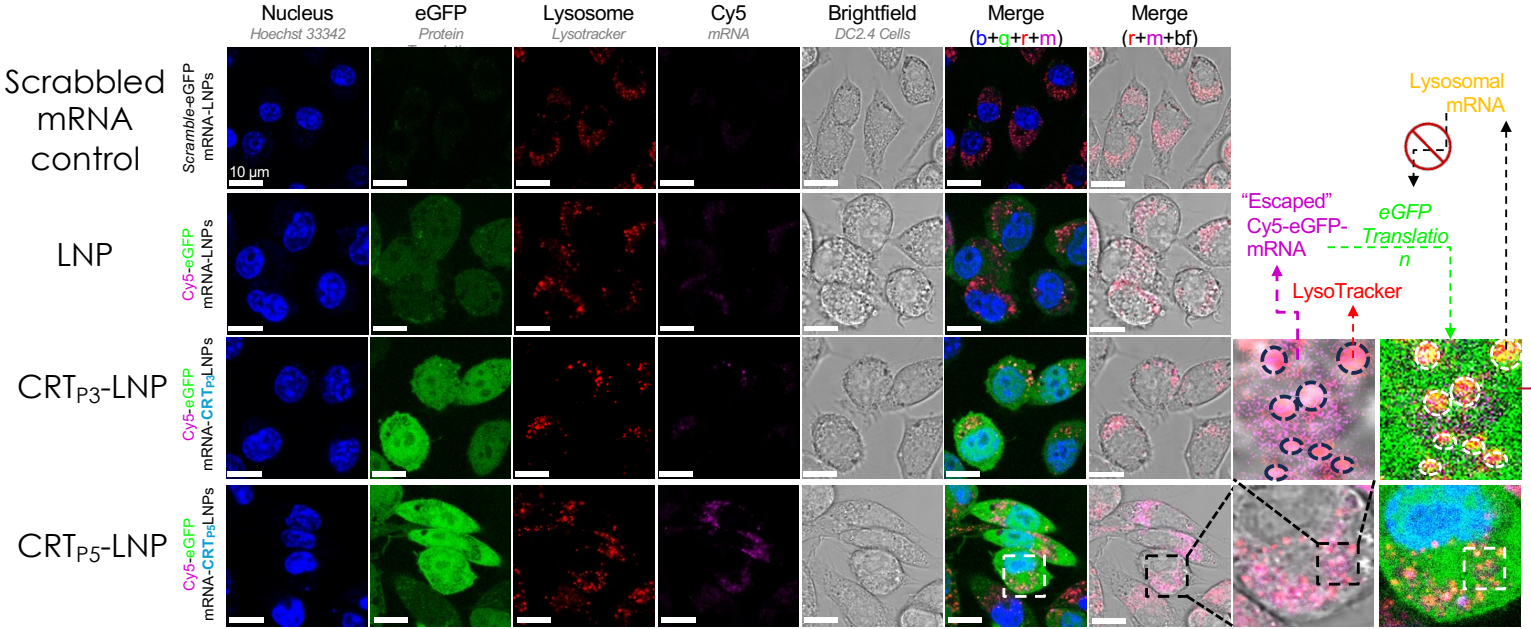
A-LNP
Ionizable Lipid
= **ALC-0315**
(Pfizer/BioNTech)



M-LNP
Ionizable Lipid
= **MC3**
(Alnylam)

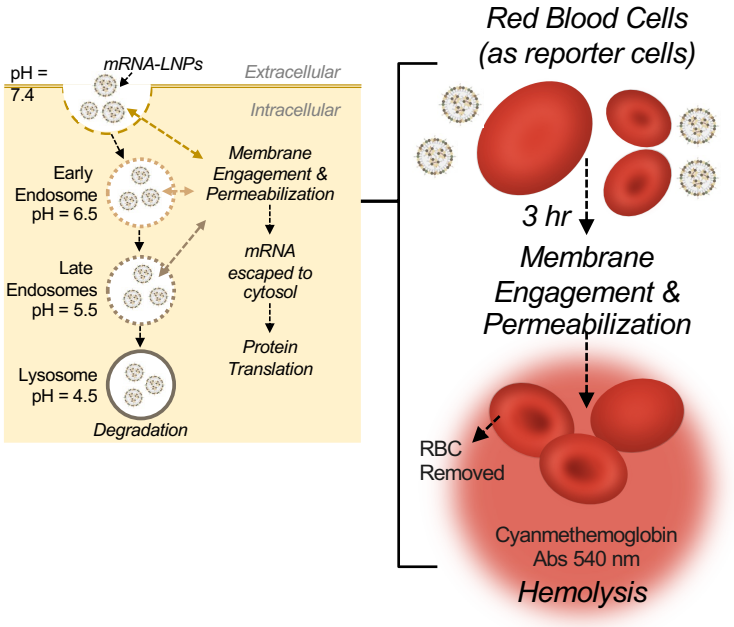


CRT_{P5}-mRNA-LNPs to DC2.4: Confocal microscopy studies using Cy5-labeled eGFP-mRNA for evaluating mRNA-LNPs delivery



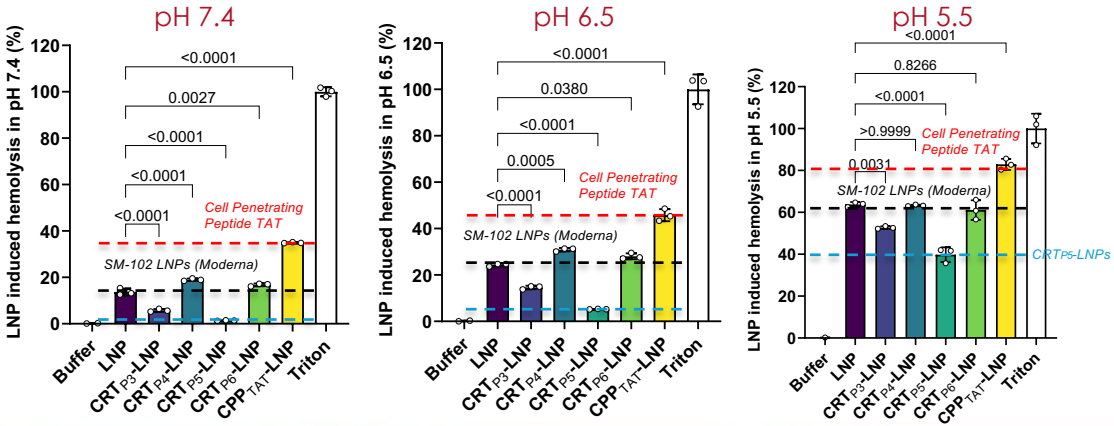
Question:
Is it hemolytic or fusogenic?

Results: $CRT_{P3/P5}$ Peptides Show Lower Hemolytic Activity Than LNPs and Cell-Penetrating Peptide functionalized LNPs



RBC Hemolysis Evaluation

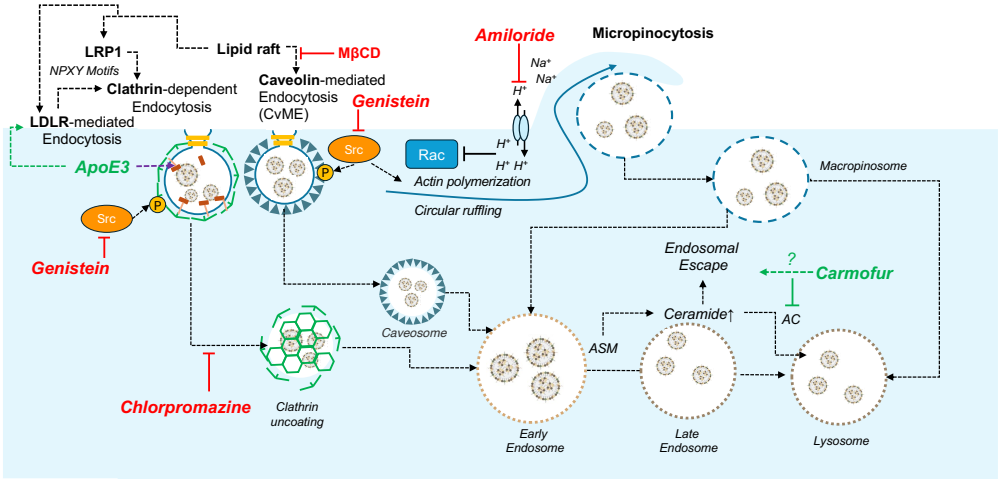
- *Negative Control: PBS Buffer*
- *Positive Control: Triton*
- *Peptide Negative Control = LNP Only*
- *Peptides Positive Control = Cell Penetrating Peptide TAT^ + LNP*
- *Experimental Groups: $CRT_{P3/P4/P5/P6}$ Peptides + LNP*



^The ***CPP$_{TAT}$*** *peptide (GRKKRRQRRRPQ) = **C**ell **P**enetrating **P**eptide (**CPP**) from the transactivator of transcription (**TAT**) of human HIV*

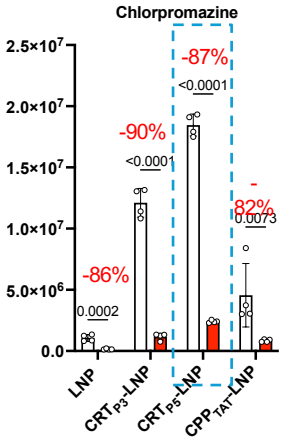
Question: How did CRT_{P5}-mRNA-LNP enter DC2.4 cells?

Approach: Probing Cellular Uptake and Trafficking of CRT_{P5}-mRNA-LNPs Using Pathway Inhibitors and Enhancers

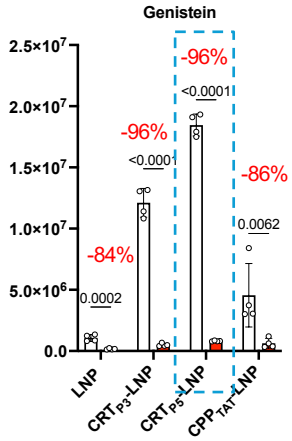


ASM: Acid SphingoMyelinase, responsible for converting sphingomyelin in the endosomal membrane into ceramide.
AC: Acid Ceramidase, primary function is to catalyze the hydrolysis of ceramide, breaking it down into sphingosine and a free fatty acid.

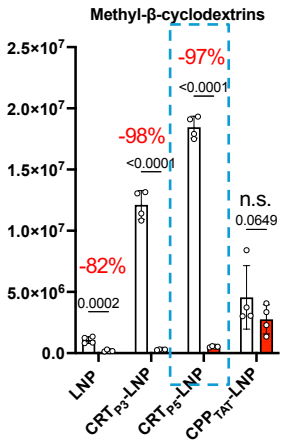
Results: CRT_{P5}-mRNA-LNPs Entry to DC2.4:
Endocytosis-dependent | LRP1/LDLR/Ceramide Facilitated



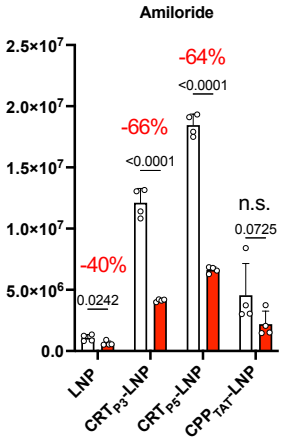
Clathrin-dependent Endocytosis Inhibition



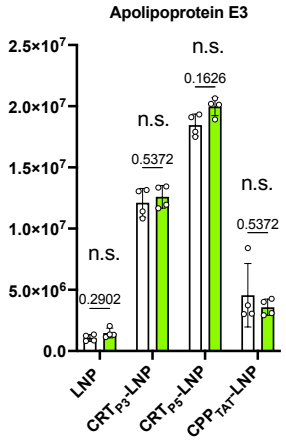
Src kinase Dependency Inhibition



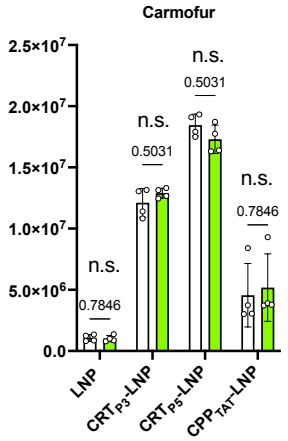
Lipid Raft Dependency Inhibition



Macropinocytosis Inhibition



LDLR Utility Enhancement



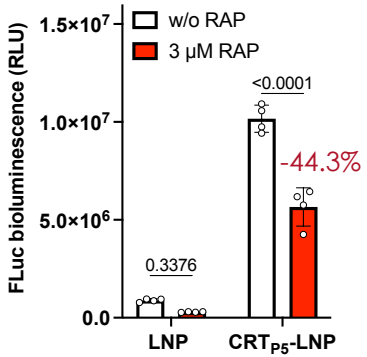
Ceramide Utility Enhancement

No Enhancement
= likely already utilizing the mechanism

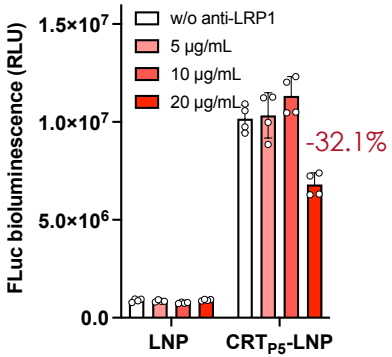
Dose: 2 mM amiloride, 20 μM chlorpromazine, 0.2 mM genistein, 5 mM Methyl-β-cyclodextrin (MβCD), 0.1 μg/mL Apolipoprotein E3 (ApoE3) or 10 μM Carmofur

CRT_{P5}-mRNA-LNPs to DC2.4 is both Receptor-dependent & Peptide-sequence-dependent

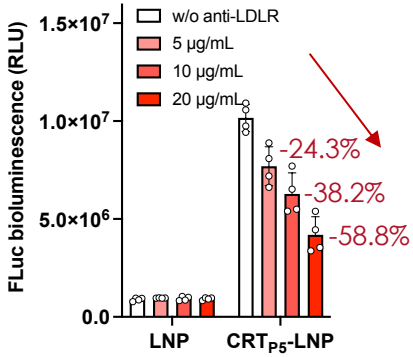
Inhibition by
Receptor Associated Protein (RAP)
(LRP1 binding competing ligand)

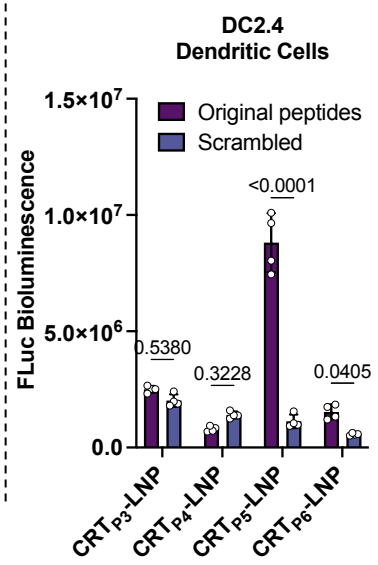


Inhibition by
Anti-LRP1



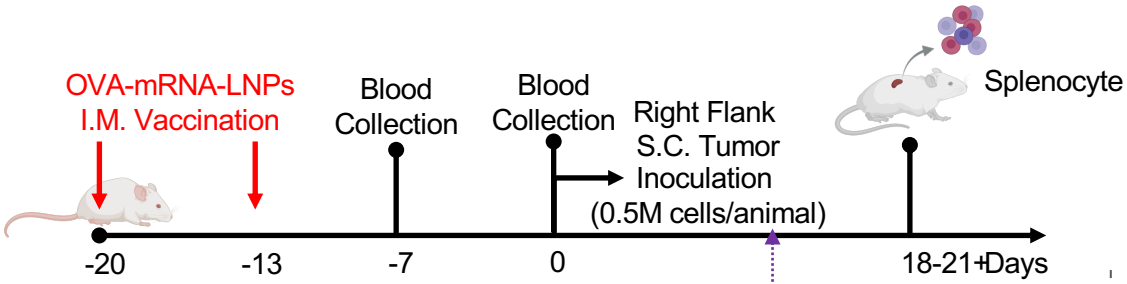
Inhibition by
Anti-LDLR





The CRT_{P5} function relies on both receptor targeting (LRP1/LDLR) and, in a sequence-dependent way.

Proof of Concept – mRNA-LNPs Cancer "Prevention" Vaccine in the CT26WT$^{OVA+/-}$ Colon Cancer Model @ Balb/c Mice



N = 16 (8 Male + 8 Female per group)

Study Groups:

- *Untreated (UT/Saline)*
- *Vehicle Control (VC – Empty LNPs)*
- *OVA-mRNA-LNP (SM-102, no peptides)*
- *CRT_{P5}-OVA-mRNA-LNPs*

Model Antigen:

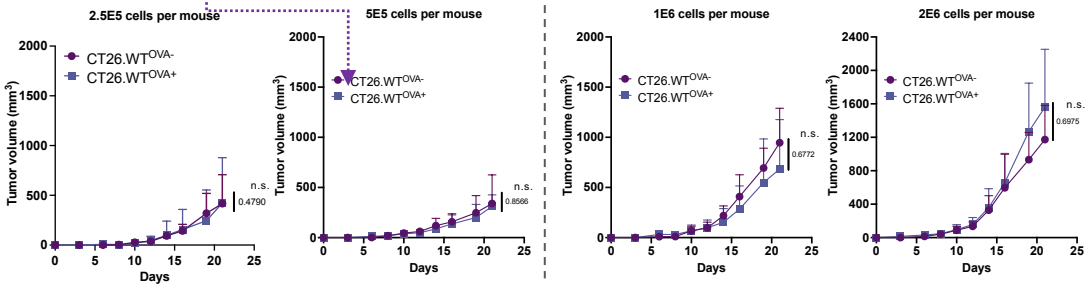
- Ovalbumin (OVA)

Colon Cancer Tumor Model:

- CT26WT^{OVA-} @ Balb/c mice
- CT26WT^{OVA+} @ Balb/c mice

mRNA Vaccine Doses:

- 0.1μg/20g BW
- 1.0μg/20g BW

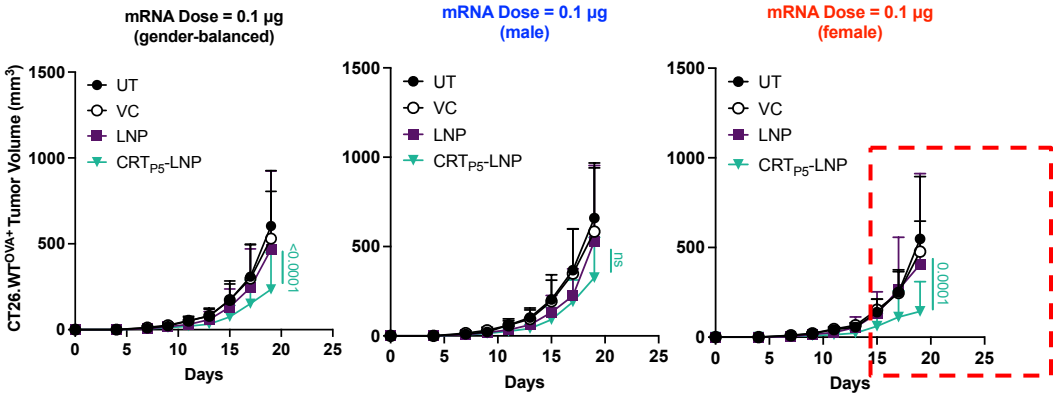


CT26WT^{OVA-} vs CT26WT^{OVA+}: Max at 0.5M cells/mouse to have the same growth curve

mRNA Dose = 0.1 µg

US Patent App. No. 63/745,773

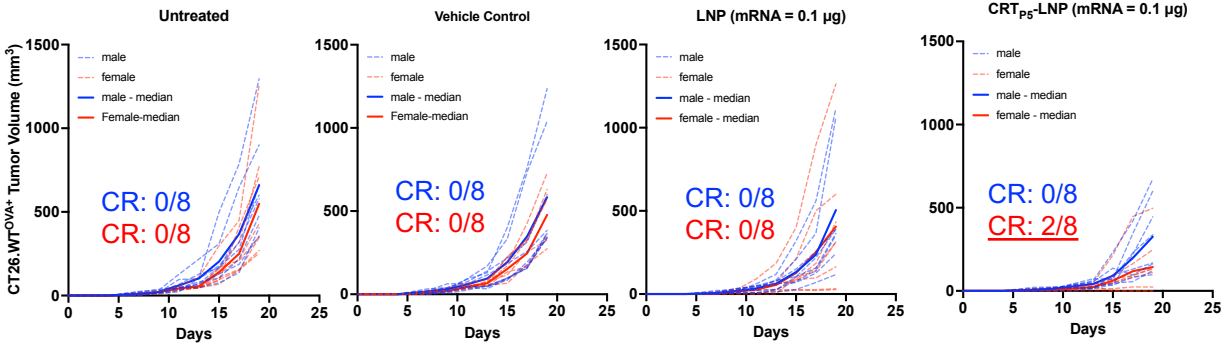
Untreated (UT) = PBS only
Vehicle Control (VC) = Empty LNPs



Tumor-burden in "Female" mice was significantly smaller in the CRT$_{P5}$-LNP groups

Complete response (CR)
= the disappearance of all tumors

Male = Blue
Female = Red

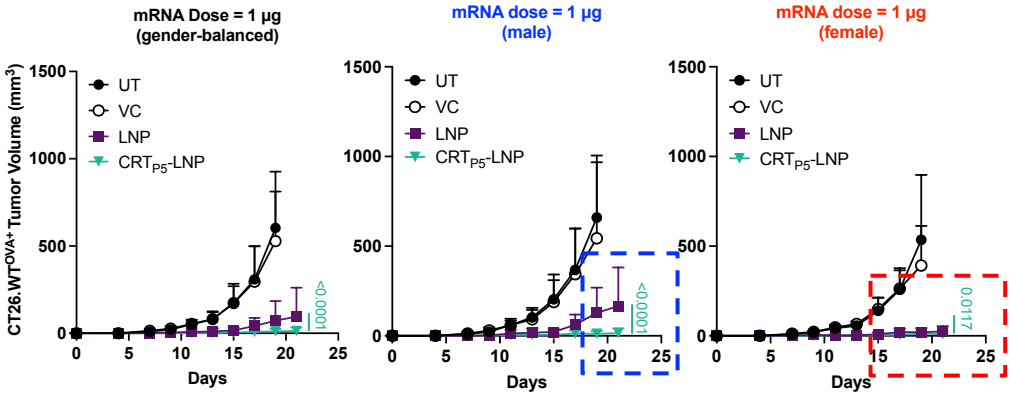


Tumor-free "Female" mice ONLY in the CRT$_{P5}$-LNP groups

mRNA Dose = 1.0 µg

US Patent App. No. 63/745,773

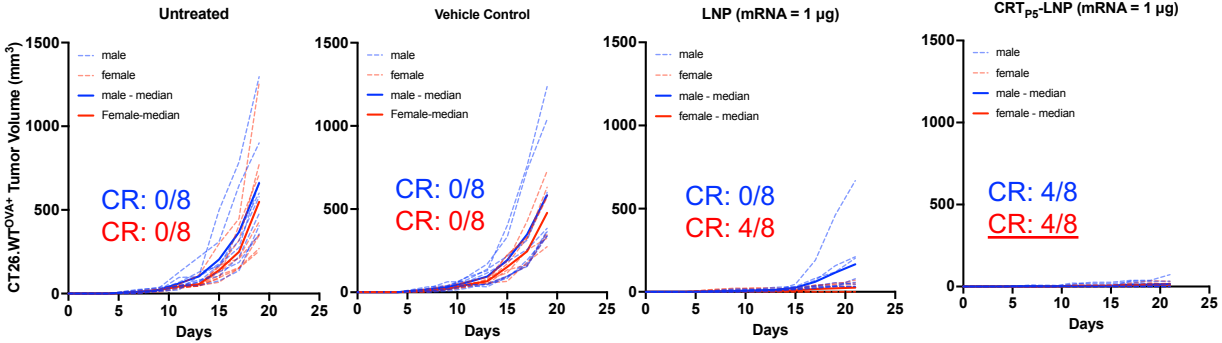
Untreated (UT) = PBS only
Vehicle Control (VC) = Empty LNPs



Tumor burden in both "Male" & "Female" mice was significantly smaller in the CRT$_{P5}$-LNP groups than in the standard LNP group

Complete response (CR)
= the disappearance of all tumors

Male = Blue
Female = Red

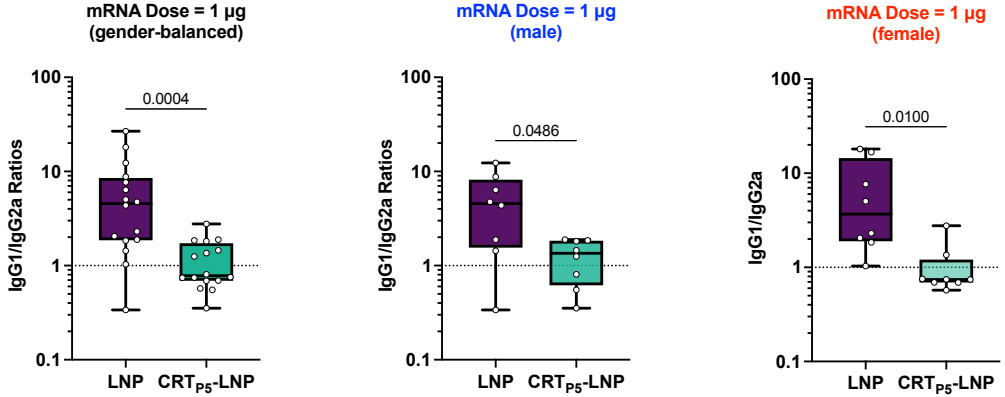


Tumor-free "Male" mice ONLY in the CRT$_{P5}$-LNP groups

IgG1(Th2)/IgG2a (Th1) Ratios

2 weeks after the booster & Immediately before the tumor challenge

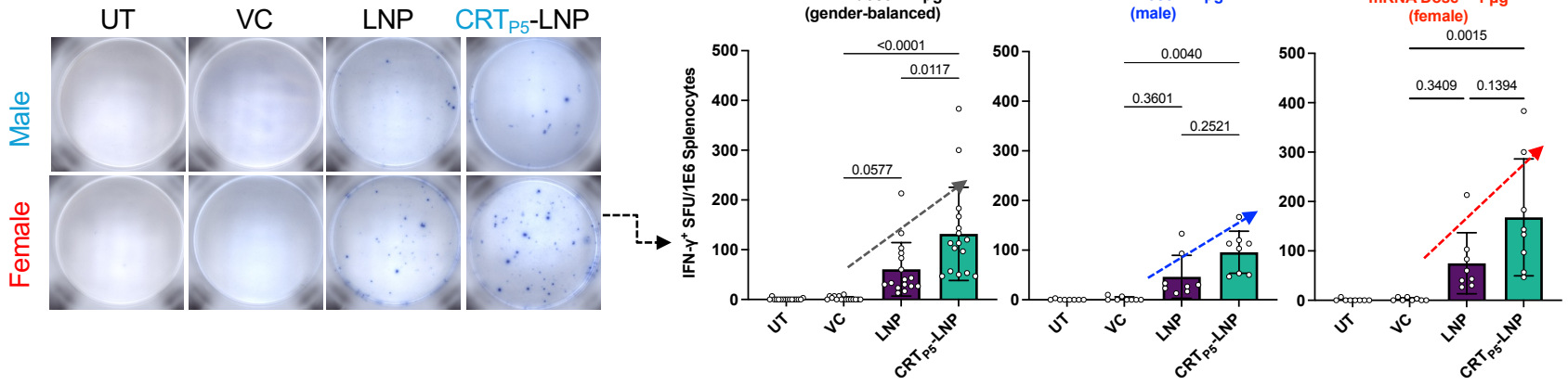
Th2 response (IgG1): Enhances antibody production
Th1 response (IgG2a): Enhances cytotoxic T lymphocyte (CTL) response



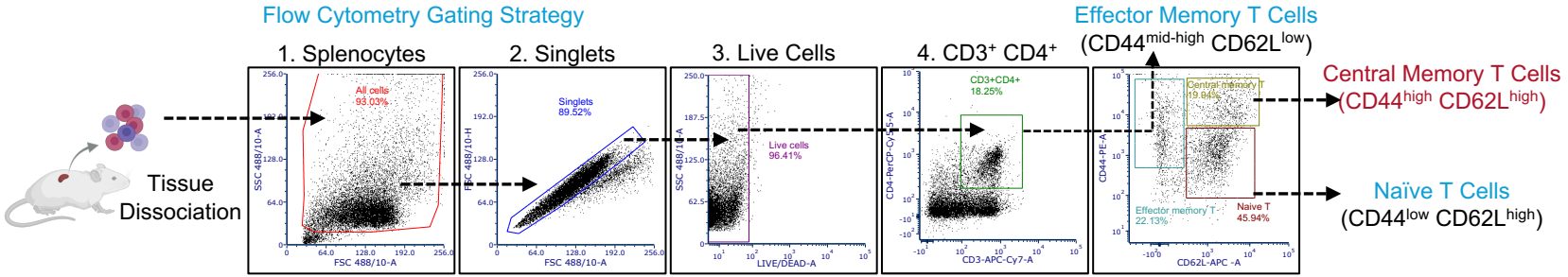
Standard LNP is very good at generating antibody body responses (good for infectious diseases).
But CRT$_{P5}$-LNPs generate more balanced Th1 (IgG2a)/Th2 (IgG1) response than standard LNPs

CRT_{P5}-LNPs Induced Higher Antigen-specific Immune Cell Responses Than Standard LNPs

IFN-γ ELISOPT

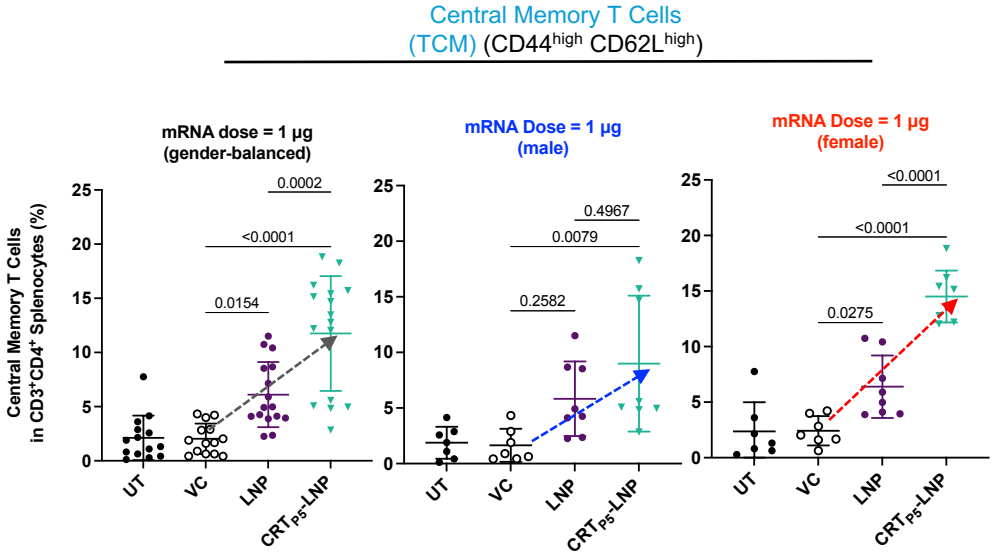


Central/Effector Memory T-Cell Ratios in Spleens

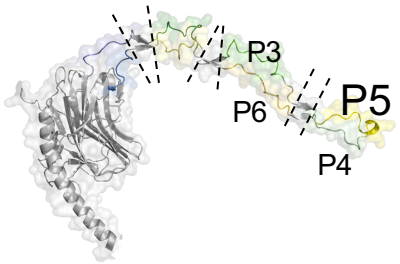


Feature	Central Memory T Cells (TCM)	Effector Memory T Cells (TEM)
Location	Lymphoid Tissue	Non-Lymphoid Tissue
Phenotype	CD44^{high} CD62L^{low} CCR7^{+}	CD44^{high} CD62L^{low} CCR7^{-}
Function	**Prolonged surveillance, quick proliferation upon antigen encounter**	Immediate effector functions (e.g., cytokine secretion, cytotoxicity)
Longevity	**Long-lived**	Short-lived
Recall Response	Slower activation Higher proliferative potential	Rapid response Lower proliferative potential

CRT_{P5}-LNP generates higher Central Memory Cells than standard LNPs



Conclusion & Future Works



1.$CRT_{P3/4/5/6}$ peptides are hydrophilic and compatible with LNP formulations

2.CRT_{P5}-mRNA-LNPs enhance uptake, endosomal escape, and antigen expression in dendritic cells for LNPs formulated with SM-102, ALC-0315, and D-Lin-MC3 ionizable lipids.

3. CRT_{P5}-mRNA-LNPs uptake in DC2.4 dendritic cells is predominantly driven by endocytosis over macropinocytosis.

4. CRT_{P5}-mRNA-LNPs delivery utilizes LRP1 and LDLR receptors.

5. CRT_{P5}-mRNA-LNPs cancer vaccine promotes more balanced Th1/Th2 responses and more antigen-specific cellular responses than standard LNPs, which leads to better outcomes, especially in male mice.

Future Works:
→ Evaluate the intracellular trafficking and kinetics of CRT-mRNA-LNPs
→ Evaluate different conjugation chemistries and peptide display density
→ Evaluate biological sex as a variable at the tumor microenvironment and in DCs influencing CRT_{P5}-mRNA-LNP vaccine delivery and immunogenicity.

Thank you! Questions?

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