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TITLE: Development of the Anti-inflammatory Mast Cell

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CONTRACTING ORGANIZATION: SRI International, Menlo Park, CA

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14. ABSTRACT

The purpose of the project is to develop a real time pre-exposure prophylactic for severe food allergies and prevent life threatening anaphylaxis. We have developed the methodology needed to generate knockout Mast cell lines that are not capable of releasing histamine or prostaglandins. We are currently working towards generating a knockin mast cell to counter the allergic response.

15. SUBJECT TERMS
None listed.

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRDC
Unclassified	Unclassified	Unclassified	Unclassified	12	**19b. TELEPHONE NUMBER** *(include area code)*

TABLE OF CONTENTS

1. INTRODUCTION:

We have generated a stable LUVAΔHDC cell line, LUVAΔCOX2 cell line and a LUVAΔHDCΔCOX double knockout cell lines. We have discovered that over time the LUVA Mast cells lose their expression of the FcεR1 expression. We are currently knocking in the FcεR1 into our knockout cell lines to stabilize the expression of this receptor. This was unexpected but we are working through this hurtle. Once our knockout cell lines are expressing the FcεR1 stably, we can then test the effect of an allergen on the release of histamine as well as prostaglandins, For the knockin genes, we will be using a lentiviral mediated delivery approach.

2. KEYWORDS:

Mast Cell, AIM cell, Nucleofection, Cas9, CRISPR, gRNA, knockout (KO), histidine decarboxylase (HDC), cyclooxygenase (COX), lentivirus

3. ACCOMPLISHMENTS:

What were the major goals of the project?

The major goals are to

1. Generate a Mast cell line that is unable to produce histamine by knocking out histidine decarboxylase (HDC) and also prostaglandins synthesis by generating a cyclooxygenase KO.
2. Generate knock-in mast cell cell lines that stably express FcεR1 and also express histamine N-methyltransferase (HNMT) to counter the histamine response.

Generation of a Mast cell line unable to produce histamine by knocking out histidine decarboxylase (HDC). Transfection of the LUVA cell pool was done by specific guide RNAs complexed together with spCas9 to form a ribonucleoprotein (RNP). The RNP are then delivered to the cells via optimized electroporating setting. To confirm successful editing, PCR and Sanger sequencing was performed to choose the pool with the highest editing efficiency. For HDC, two homozygous KO clones were produced (LUVA HDC-KO Clone H7 and LUVA HDC-KO clone B6). Clone H7 had a 100% KO score and clone B6 had a 99% KO score. The analysis of Clone H7 is shown (Figure 1 - 2).

We have also successfully generated the LUVA COX2 KO cell line using the same approach as the HDC as shown in figures 1-3. In addition, we have generated a LUVA HDC and COX2 double knockout cell line. All cell lines were confirmed with Sanger sequencing as described for the LUVA HDC knock out cell line (Figures 1-2).

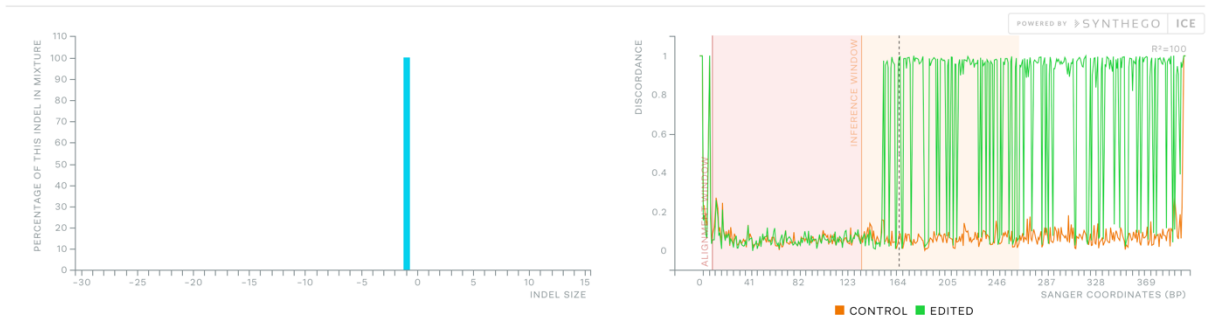


Figure 1. **LUVA HDC-KO Clone H7 Intel distributions**. The indel plot displays the inferred distribution of indels in the entire edited population of genomes. The plot shows one indel at -1 of 100%. The right plot shows the discordance plot detailing the level of alignment per base between the control and edited sample in the inference window (region around the cut site). It displays the average amount of signal that disagrees with the reference sequence. The green and orange lines are close together before the cut site and a jump in discordance near the cut site continuing to remain far apart after the cut site = high level of discordance

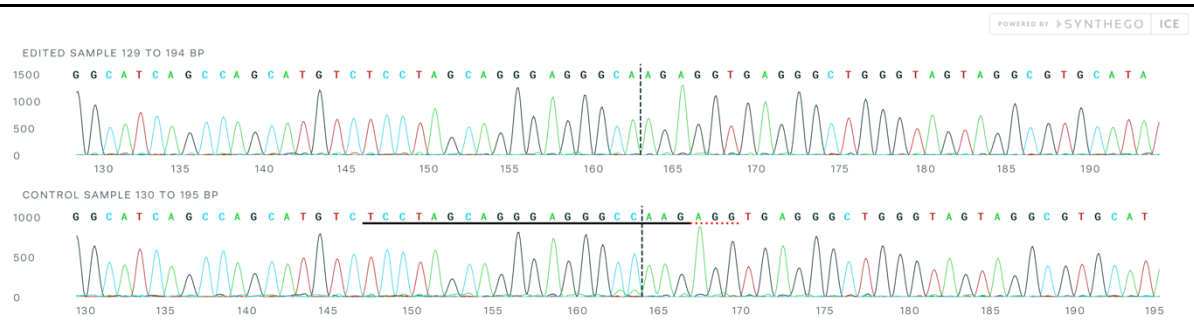


Figure 2. LUVA HDC-KO Clone H7 Sanger sequence. The edited and wild-type (control) sequences in the region around the guide sequence is shown. The horizontal black underlined region represents the guide sequence. The horizontal red underline is the PAM site (PAM sequence AGG). The vertical black dotted line represents the actual cut site. Cutting and error-prone repair results in mixed sequencing bases after the cut.

We have discovered that the LUVA Mast cell line loses expression of the FcεR1 on its surface. We have been able to confirm the knockout cell lines do not express histamine using a mitogen stimulation of PMA/Ionomycin, we have not been able to confirm this using an allergen. We are now knocking in the FcεR1 into all our KO cell lines to generate a stable expression of FcεR1 so that we can test the functional assay using allergens. The design of the FcεR1 knock in plasmid is shown in figure 4. Once we have the stable expression of the FcεR1 in our cell lines we will move forward to test the allergen assay for histamine and prostaglandin D2 release.

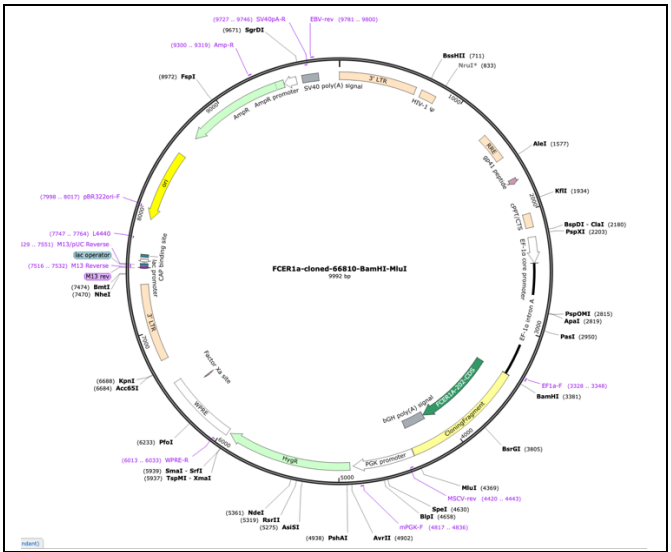


Figure 3. Knock in plasmid design expressing FcεR1.

Currently we are designing a knock in plasmid to express histamine N-methyltransferase (HMNT) in our AIM cells. This will include a peptide sequence from TNFα that has been shown to be required to guide TNFα to Mast cell granules. Both knock in approaches are currently underway.

What was accomplished under these goals?

1. The major activities for this reporting period was to overcome the difficulties of transforming the Mast cell and generate the AIM cell.
2. Our specific objectives was to generate a Mast cell line that is unable to produce Mast cell mediators of the allergic response: Histamine and prostaglandins.
3. We have been able to successfully generate 2 single knock out Mast cell clones, a histidine decarboxylase (HDC) knock out and also a cyclooxygenase (COX) KO. We have also succeeded in generating a HDC and COX double knock out Mast cell clone. We have begun to generate in vitro functional data but have realized that the Mast cell line has lost the expression of FcεR1. We are therefore unable to test the AIM cells in a true allergic assay since the FcεR1 is required to test IgE activation of the AIM cell in the presence of an allergen. We are now working on adding back genes to the double knockout cell line to generate a Mast cell that stably expresses FcεR1 and expresses histamine N-methyltransferase, an enzyme that can break down histamine.
4. No other achievements to note.

What opportunities for training and professional development has the project provided?

Nothing to report

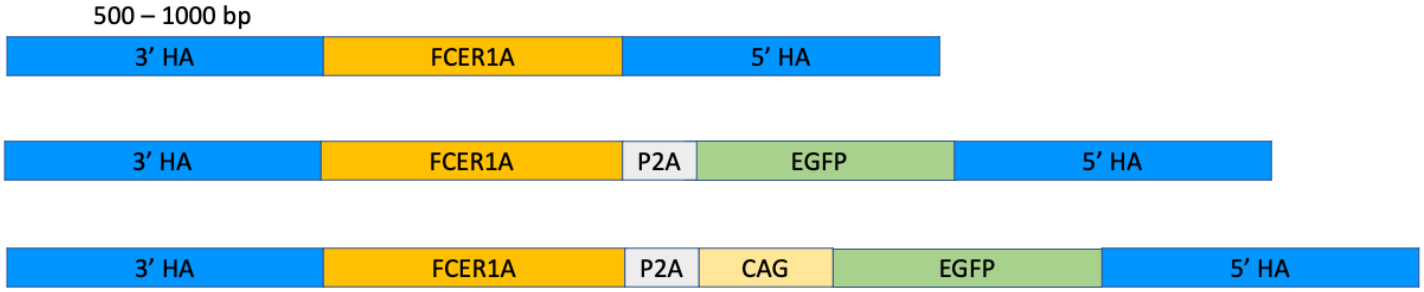
How were the results disseminated to communities of interest?

Nothing to report

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

In the next reporting period, we plan to accomplish knocking in genes necessary to complete the project and enable us to test the AIM cell function when exposed to allergens. The following is our general approach for these knockin studies.

Options for DNA donor structure. Shown only FCER1A, but structure would be similar for both genes, using different selection markers (if allowed). Homology arms (HAs) to match the different safe harbors for each gene.



Proposed outline:

1. Sequence target loci (2-4 weeks)
2. Test RNP transfection efficiency (2 weeks)
    a. Test references in the literature using Lonza Nucleofector
    b. Go/No-Go step: if 3 rounds of transfection testing prove to be difficult, outsource project
3. *Optional:* Test serotype preference (2 weeks)
    a. If serotype preference described in literature, can be skipped
    b. If no reference found, then purchasing a test kit with several serotypes is necessary
    c. Try ~5 MOIs
    d. Optional Go/No-Go step: if AAV transduction efficiency is low in all tested serotypes, outsource project
4. Design homology arms (HA) and gene fragments (1 week) – can be parallel to step 2
    a. 750pb and 1kb HA
    b. Design with AAV size constraints in mind just in case
5. Order fragments from Twist in AAV backbone (1-3 weeks)– can be parallel to step 2
    a. Keep AAV option open in case want to try it later
    b. Total of 4 plasmids
    c. Order maxipreps enough to start with experiments
6. *Optional:* Order AAV synthesis (~2 weeks)
    a. *Order a CMV*-GFP control of same serotype if not enough left from test kit
7. *Optional:* RNP+AAV electroporation (two singles and one double)
8. RNP+ plasmid electroporation (two singles and one double)
9. FACS to select cells with KI or antibiotic selection (1-2 weeks) OR skip to clonal selection
10. Clonal selection (1-4 weeks, depends how fast cells grow)
11. PCR + Sanger sequencing of isolated clones (1-2 weeks)
12. Cell ready for functional validation

CDS information from Emsembl

ENST00000693622.1 FCER1A-203

UniProtID

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>sp|P12319|FCERA_HUMAN High affinity immunoglobulin epsilon receptor subunit
alpha OS=Homo sapiens OX=9606 GN=FCER1A PE=1 SV=1
MAPAMESPTLLCVALLFFAPDGVLAVPQKPKVSLNPPWNRIFKGENVTLTCNGNNFFEVS
STKWFHNGSLSEETNSSLNIVNAKFEDSGEYKCQHQQVNESEPVYLEVFSDWLLLQASAE
VVMEGQPLFLRCHGWRNWDVYKVIYYKDGEALKYWYENHNISITNATVEDSGTYYCTGKV
WQLDYESEPLNITVIKAPREKYWLQFFIPLLVVILFAVDTGLFISTQQQVTFLLKIKRTR
KGFRLLNPHPKPNPKNN
```

Nucleotide Sequence (774 nt):

ATGGCTCCTGCCATGGAATCCCCTACTCTACTGTGTGTAGCCTTACTGTTCTTCGCTCCAGATGGCGTGT
TAGCAGTCCCTCAGAAACCTAAGGTCTCCTTGAACCCTCCATGGAATAGAATATTTAAAGGAGAGAATGT
GACTCTTACATGTAATGGGAACAATTTCTTTGAAGTCAGTTCCACCAAATGGTTCCACAATGGCAGCCTT
TCAGAAGAGACAAATTCAAGTTTGAATATTGTGAATGCCAAATTTGAAGACAGTGGAGAATACAAATGTC
AGCACCAACAAGTTAATGAGAGTGAACCTGTGTACCTGGAAGTCTTCAGTGACTGGCTGCTCCTTCAGGC
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CAAATGCCACAGTTGAAGACAGTGGAACCTACTACTGTACGGGCAAAGTGTGGCAGCTGGACTATGAGTC
TGAGCCCCTCAACATTACTGTAATAAAAGCTCCGCGTGAGAAGTACTGGCTACAATTTTTTATCCCATTG
TTGGTGGTGATTCTGTTTGCTGTGGACACAGGATTATTTATCTCAACTCAGCAGCAGGTCACATTTCTCT
TGAAGATTAAGAGAACCAGGAAAGGCTTCAGACTTCTGAACCCACATCCTAAGCCAAACCCCAAAAACAA
CTGA

Translation (257 aa):

MAPAMESPTLLCVALLFFAPDGVLAVPQKPKVSLNPPWNRIFKGENVTLTCNGNNFFEVSSTKWFHNGSL
SEETNSSLNIVNAKFEDSGEYKCQHQQVNESEPVYLEVFSDWLLLQASAEVVMEGQPLFLRCHGWRNWDV
YKVIYYKDGEALKYWYENHNISITNATVEDSGTYYCTGKVWQLDYESEPLNITVIKAPREKYWLQFFIPL
LVVILFAVDTGLFISTQQQVTFLLKIKRTRKGFRLLNPHPKPNPKNN

HNMT ENSG00000150540

https://www-ncbi-nlm-nih-gov.sri.idm.oclc.org/CCDS/CcdsBrowse.cgi?REQUEST=CCDS&DATA=CCDS2181

Nucleotide Sequence (879 nt):

ATGGCATCTTCCATGAGGAGCTTGTTTTCTGACCACGGGAAATATGTTGAATCTTTCCGGAGGTTTCTCA
ACCATTCCACGGAACACCAGTGCATGCAGGAATTCATGGACAAGAAGCTGCCAGGCATAATAGGAAGGAT
TGGAGACACAAAATCAGAAATTAAGATTCTAAGCATAGGCGGAGGTGCAGGTGAAATTGATCTTCAAATT
CTCTCCAAAGTTCAGGCTCAATACCCAGGAGTTTGTATCAACAATGAAGTTGTTGAGCCAAGTGCTGAAC
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GGAGACATCATCTGAATACCAAAGTAGAATGTTGGAGAAAAAGGAGCTTCAAAAGTGGGACTTTATTCAT
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TCTCAGAGCAGAGCTTGGGAAAGATCTACAAGAGCCTGAATTTAGTGCTAAGAAAGAGGGGAAGGTTCTT
TTTAATAATACTCTGAGTTTCATAGTGATTGAGGCATAA

Translation (292 aa):

MASSMRSLFSDHGKYVESFRRFLNHSTEHQCMQEFMDKKLPGIIGRIGDTKSEIKILSIGGGAGEIDLQI
LSKVQAQYPGVCINNEVVEPSAEQIAKYKELVAKTSNLENVKFAWHKETSSEYQSRMLEKKELQKWDFIH
MIQMLYYVKDIPATLKFFHSLLGTNAKMLIIVVSGSSGWDKLWKKYGSRFPQDDLCQYITSDDLTQMLDN
LGLKYECYDLLSTMDISDCFIDGNENGDLLWDFLTETCNFNATAPPDLRAELGKDLQEPEFSAKKEGKVL
FNNTLSFIVIEA

4. **IMPACT:**

We have developed a LUVAΔHDC, a LUVAΔCOX2, and a LUVAΔHDCΔCOX2 cell lines and once we confirm that it is unable to generate histamine or prostaglandins in response to allergens, this will be a first step towards development of a therapy to counter a systemic allergic response in real time to prevent anaphylactic shock. This will be an enormous step forward in treating severe allergic responses. We are currently submitting invention disclosures for the knockout Mast cell clones.

What was the impact on the development of the principal discipline(s) of the project?

Nothing to report

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:

We have surprisingly found that the LUVA cells lose expression of FcεR1 and we have therefore been unable to test our knockout cell lines in an allergen stimulation assay using allergen and IgE. We are therefore going to knock in FcεR1 to generated stable FcεR1 expression to test in our in vitro assays. There are no significant changes to the methods or anticipated overall results.

Changes in approach and reasons for change

No changes in approach will be made, we will be adding a knockin of the FceR1 receptor for stable expression of this normal Mast cell receptor

Actual or anticipated problems or delays and actions or plans to resolve them

We have asked for a one year no cost extension and it was approved. This year we will complete the original aims.

Changes that had a significant impact on expenditures

Nothing to report on expenditures

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

N/A

Significant changes in use or care of human subjects

N/A

Significant changes in use or care of vertebrate animals

N/A

Significant changes in use of biohazards and/or select agents

N/A

6. PRODUCTS:

Publications, conference papers, and presentations

Nothing to report

Journal publications.

Nothing to report

Books or other non-periodical, one-time publications.

Nothing to report

Other publications, conference papers and presentations.

Nothing to report

Website(s) or other Internet site(s)

Nothing to report

Technologies or techniques

Nothing to report

Inventions, patent applications, and/or licenses

Nothing to report

Other Products

Nothing to report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

Name:	Mary Premnko-Lanier
Project Role:	Researcher PI
Contribution to Project:	Design knock out/in plasmids and will help with transfections, Sanger sequencing of all knock in cell lines. Design all in vitro assays and direct all experiments done by scientists and research assistants
Funding Support:	

Faculty Scholarship Grant Premenko-Lanier (PI) 06/01/18 – 6/01/23
Title: Influenza M2e protein conjugated to tetanus toxin as a universal influenza vaccine booster
Funding Agency: Samuel Merritt University
Point of contact: Veronica Paniagua, 510-879-0784
Level of Effort: 20%
Goals: The major goals of this project are to generate a safe conjugate booster vaccine to target stable influenza antigens to be used as a supplement or booster complement to the current annual influenza vaccine.

Faculty Scholarship Grant Premenko-Lanier (PI) 04/01/23 – 3/31/26
Title: Understanding vaccine hesitancy in populations that experience health disparities
Funding Agency: Samuel Merritt University
Point of contact: Veronica Paniagua, 510-879-0784
Level of Effort: 20%
Goals: The major goals of this project are to develop surveys and outreach programs to increase vaccine and disease awareness in patients that experience health disparities. The ultimate goal is to determine mechanisms to intervene and increase vaccine use in such populations

CDMRP PR202214 Premenko-Lanier (PI) 03/01/21 – 02/28/24
Title: Development of the Anti-Inflammatory Mast Cell.
Funding Agency: CDMRP
Point of Contact: Sherry M. Apperson
Proposed Level of Effort: 25%
Goals: The goal is to develop a Mast cell (anti-inflammatory mast cell, AIM)that has the pro-allergy mediators such as histamine and eicosanoids knocked down and anti-histamine enzymes knocked in. This AIM cell would counter severe food allergies in real time.

SRI International Premenko-Lanier (Director) Annual
Title: Virology and Immunology Commercial projects: SARS-CoV-2, LCMV, Herpes Simplex Virus, *in vitro* and *in vivo*
Funding agency: SRI International
Goals: to provide services to commercial clients for the testing and validation of various anti-pathogen and immune based therapies to virus infections. I lead a core group of virologists and immunologists that perform multiple tasks including basic virology, basic immunology, high through put analysis with or without GLP standards.

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

Nothing to report

What other organizations were involved as partners?

Nothing to report

8. SPECIAL REPORTING REQUIREMENTS

Nothing to report

9. APPENDICES: Nothing to report