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TITLE: Development of Base Editing for Gene Therapy of ELANE-Mutated Severe Congenital Neutropeniaa

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13. SUPPLEMENTARY NOTES

14. ABSTRACT

We propose to develop a novel approach to gene therapy for severe congenital neutropenia (SCN), a life-threatening bone marrow failure syndrome. Patients present early in life with recurrent infections and sepsis; without treatment, most die by age 2 years. Current treatment with lifelong filgrastim injections has dramatically improved survival, but at the cost of progression to myelodysplasia and acute myeloid leukemia. Allogeneic hematopoietic stem cell transplant is the only currently curative treatment, but is limited by donor availability, transplant-related morbidity and mortality, and risk for graft-versus-host disease. Thus there is an unmet need for safe and effective therapy for SCN.

Approximately 40% of cases derive from autosomal dominant monoallelic mutations in *ELANE*, the gene encoding neutrophil elastase. All known pathogenic mutations preserve expression but alter the structure of the protein product resulting in an unfolded protein response or abnormal intracellular trafficking leading to excess apoptosis.

Our specific aims are unchanged from the original proposal:

Aim 1: We will define optimal sgRNAs and ABE8e constructs to maximize ELANE inactivation and minimize off-target editing in transformed cell lines. Aim 1a. We will define the optimal promoter target sites for ABE8e inactivation of ELANE expression in highly permissive cell lines such as HEK293T for target optimization and NE-expressing myeloid cell lines for ELANE expression. Aim 1b. We will maximize base editing efficiency and specificity by assessment of both on- and off-target editing in highly permissive cells.

Aim 2: We will optimize therapeutic base editing conditions in human CD34+ cells and establish the consequences of editing on ELANE expression. Aim 2a. We will optimize base editing efficiency and specificity in CD34+ HSPCs, starting from and further adapting conditions from Aim 1. 2b. We will determine the effects of edits on ELANE expression in culture-derived neutrophils produced in vitro by differentiation of CD34+ HSPCs.

Aim 3: We will determine the functional competence of elastase-deficient neutrophils. Aim 3a. We will assess known protease-dependent functions of NE-deficient neutrophils. Aim 3b. We will assess likely protease-independent functions of NE-deficient neutrophils.

In the current grant period, we have established neutrophil elastase-expressing myeloid cell lines NB4 and THP1, established protocols for myeloid differentiation of these cells, optimized methods for transfection of adenine base editor (ABE) mRNA into these cells, adapted a HEK293T reporter cell line carrying a mCherry coding sequence that can be activated by adenine base editing, and developed flow cytometric assays for measurement of planned outcome data including neutrophil elastase expression, phagocyte oxidase activity, and pathogen phagocytosis.

15. SUBJECT TERMS

Neutropenia, severe congenital neutropenia, ELANE, gene editing, gene therapy

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1. INTRODUCTION:

We propose to develop a novel approach to gene therapy for severe congenital neutropenia (SCN), a life-threatening bone marrow failure syndrome. Patients present early in life with recurrent infections and sepsis; without treatment, most die by age 2 years. Allogeneic hematopoietic stem cell transplant is the only currently curative treatment. Thus there is an unmet need for safe and effective therapy for SCN. Approximately 40% of cases derive from autosomal dominant monoallelic mutations in *ELANE*, the gene encoding neutrophil elastase. All known pathogenic mutations preserve expression but alter the structure of the protein product resulting in an unfolded protein response or abnormal intracellular trafficking leading to excess apoptosis. The recent development of base editing tools has allowed the precise conversion of one base pair to another at a target genomic locus without requiring DSBs, homology directed repair or donor DNA templates. Adenine base editors (ABEs) thus provide an attractive, novel modality of genomic DNA modification. Our goal is to develop a novel universal therapeutic approach for *ELANE*-associated SCN by utilizing a recently described ABE, termed ABE8e, for mutagenesis in the *ELANE* promoter to silence gene expression and thus prevent cell death due to misfolded elastase protein.

2. KEYWORDS

Neutropenia, severe congenital neutropenia, ELANE, gene editing, gene therapy

3. ACCOMPLISHMENTS:

What were the major goals of the project?

Our **specific aims** are: **Aim 1**: **We will define optimal sgRNAs and ABE8e constructs to maximize *ELANE* inactivation and minimize off-target editing in transformed cell lines.** Aim 1a. We will define the optimal promoter target sites for ABE8e inactivation of ELANE expression in highly permissive cell lines such as HEK293T for target optimization and NE-expressing myeloid cell lines for *ELANE* expression. Aim 1b. We will maximize base editing efficiency and specificity by assessment of both on- and off-target editing in highly permissive cells. **Aim 2: We will optimize therapeutic base editing conditions in human CD34+ cells and establish the consequences of editing on *ELANE* expression**. Aim 2a. We will optimize base editing efficiency and specificity in CD34+ HSPCs, starting from and further adapting conditions from Aim 1. 2b. We will determine the effects of edits on *ELANE* expression in culture-derived neutrophils produced *in vitro* by differentiation of CD34+ HSPCs. **Aim 3: We will determine the functional competence of elastase-deficient neutrophils**. Aim 3a. We will assess known protease-dependent functions of NE-deficient neutrophils. Aim 3b. We will assess likely protease-independent functions of NE-deficient neutrophils.

What was accomplished under these goals?

1. Major activities: At this stage, we are focused on setting up necessary cell transfection systems and assays for neutrophil function.
a) We have established neutrophil elastase-expressing myeloid cell lines NB4 and THP1 and established protocols for myeloid differentiation of these cells using ATRA and phorbol ester, respectively.
b) We are optimizing methods for transfection of adenine base editor (ABE) mRNA into these cells, as our supply of ABE protein (our original plan) has been extremely limited. However, Over the past year, we have focused on improving the expression and purification of ABE8e adenine base editors. Previously, we have been able to produce ABE8e protein, but the quantity of purified protein per liter of cells has been modest (~1 to 2 nanomoles/liter). Consequently, we reoptimized the protein expression construct through the incorporation of a new linker sequence between the tadA8e adenine deaminase domain and the Cas9 nickase domain to reduce proteolysis. We also shifted from a 6xhistidine tag to a 12xhistidine tag, which improves the capture of the protein on the Ni-NTA resin. In addition, we optimized the protein induction conditions, shifting to a 15 °C induction temperature, which reduces the toxicity of ABE8e expression within the bacteria. All these changes lead to dramatically improved yields of purified ABE8e protein (~8 nmoles/liter). We have purified 50 nmoles of the ABE8e protein, and it is ready to use for the assessment of base changes within the ELANE promoter that will downregulate transcription of the toxic allele.
c) We have adapted for plasmid transfection evaluation a HEK293T reporter cell line carrying a stop codon inside a mCherry coding sequence that can be corrected upon on target activity by using two separate plasmids expressing ABE and sgRNA targeting the stop codon sequence.
d) We have established an in vitro assay for the visualization of neutrophil extracellular traps (NETs) by confocal microscopy in mature neutrophils stimulated by phorbol ester and are developing flow cytometric measurements for more specific characterization of NETs by staining for DNA, histone H3, myeloperoxidase and neutrophil elastase. A quantitative method for the evaluation of bacterial killing by the neutrophils is also being developed. Together with our well-established assays of oxidative burst and phagocytosis, these techniques will be key components for the evaluation of consequences of the base editing therapeutics on neutrophil function.

2. Specific objectives: see major goals, above. We plan to move these studies into CD34 cells and incorporate functional studies of edited cells in the next grant period.

3,4. Significant results and other achievements: still too early in the study

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

Dr. Josias Soares de Brito has become increasingly independent in the planning and conduct of experiments and has presented at work-in-progress seminars for the research group and department. His professional development has been sufficient to be put up for promotion to a faculty position as Instructor in the Department of Pediatrics.

Nothing to report

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

We will use the methods developed and optimized in the present grant period to start base editing of the ELANE promoter in cell lines and then CD34 cells. We investigate the functional effects of ELANE knockdown using the assays developed in this grant period, plus additional assays (e.g. NETosis, microbial killing) to be developed in the next grant period.

4. **IMPACT:**

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:

The primary problem has been the lack of availability of sufficient quantities of ABE protein to carry out the proposed experiments. As a result, we had to develop methods to transfect with mRNA and plasmids, which are more difficult because of the robust innate immune systems of myeloid cells. We can now use those systems as alternatives if necessary, but recent progress from Dr. Wolfe's laboratory (see section 3, above) promise to make the sufficient ABE protein available in the coming grant year.

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

As above.

Changes that had a significant impact on expenditures

None

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

Significant changes in use or care of human subjects

Not applicable

Significant changes in use or care of vertebrate animals

Not applicable

Significant changes in use of biohazards and/or select agents

Not applicable

6. PRODUCTS:

- **Publications, conference papers, and presentations**

Journal publications.

None

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

None

Other publications, conference papers and presentations.

None

- **Website(s) or other Internet site(s)**

None

- **Technologies or techniques**

None

- **Inventions, patent applications, and/or licenses**

None

- **Other Products**

None

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	Peter E. Newburger
Project Role:	PI
Researcher Identifier (e.g. ORCID ID):	0000-0002-8615-673X
Nearest person month worked:	2.4
Contribution to Project:	Dr. Newburger supervised Dr. Soares de Brito, provided overall direction of the project, and was responsible for fiscal management and communication with the DoD.
Funding Support:	NIH
Name:	Scot Wolfe
Project Role:	co-investigator
Researcher Identifier (e.g. ORCID ID):	0000-0002-2881-5573
Nearest person month worked:	0.6
Contribution to Project:	Dr. Wolfe was responsibility for the production and application of base editors and for advice on their application and interpretation of results.
Funding Support:	NIH
Name:	Josias Soares de Brito
Project Role:	Post-doctoral associate
Researcher Identifier (e.g. ORCID ID):	0000-0003-0690-0467
Nearest person month worked:	12
Contribution to Project:	Planned and performed experiments, developed new procedures and assays.
Funding Support:	none

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

Change in support for Scot Wolfe:
New grant does not change effort on the current DoD grant.
1 R61 NS123756-01A1

Title:	***Generation of a large animal model of Sialidosis to enable future translation of novel therapeutics***
Time Commitment:	0.12 calendar months
Supporting Agency:	NIH/NINDS
Grants Officer:	Mark Langer mark.langer@nih.gov
Performance Period:	01/01/2023 – 12/31/2023
Level of Funding:	Total Award Amount (including indirect costs)
Brief Description:	Describe genome editing to make a large animal model to advance development of a gene therapy to treat Sialidosis.
Specific Aims:	The specific aim on this project is to generate three human relevant NEU1 mutations in sheep to model sialidosis utilizing CRISPR/Cas9 and PRIME genome editing.
Roles:	Co-I
Overlap:	None

What other organizations were involved as partners?

Nothing to report

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS:

QUAD CHARTS:

9. APPENDICES: