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TITLE: CRISPR Screen to Identify Neutrophil Regulators of Interferon-Gamma; Signaling in Acute Lung Injury

PRINCIPAL INVESTIGATOR: Robert S. Hagan, MD/PhD

CONTRACTING ORGANIZATION: University of North Carolina, Chapel Hill, NC

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Robert S. Hagan, MD/PhD

E-Mail: robhagan@med.unc.edu

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UNIVERSITY OF NORTH CAROLINA AT CHAPEL HILL
OFFICE OF SPONSORED RESEARCH
104 AIRPORT DR STE 2200
CHAPEL HILL NC 27599-5023

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13. SUPPLEMENTARY NOTES

14. ABSTRACT Acute lung injury (ALI) can be caused by infections, trauma, blast injuries, and inhalation of toxic material such as burn pit fumes. Neutrophils constitute the initial response to many injuries and infections and secrete a number of mediators such as interferon gamma (IFNγ) that contribute to both pathogen clearance and host tissue damage. Neutrophils are an attractive target for host-directed therapies intended to limit organ damage. However, neutrophil signaling is notoriously difficult to study due to their short lifespan, the lack of immortalized cell lines that recapitulate neutrophil biology, and an absence of tools that allow neutrophil-specific gene modification *in vivo* and *in vitro*. This project uses CRISPR-Cas9 technology in mice to perform large-scale *in vivo* screening and identify regulators of neutrophil IFNγ in the setting of *Streptococcus pneumoniae*-induced ALI. The overall goal is to identify targetable pathways that may be used to treat ALI and to create a platform for efficient genome-wide studies of neutrophil function.

In the initial reporting period, we have been successful in establishing the relevant Cas9-GFP mouse line and optimized antibody panels for fluorescence-activated cell sorting (FACS). We have also optimized murine hematopoietic stem cell (HSPC) purification. We have established, amplified, purified, and tested a lentiviral library for expression of sgRNAs; based on published data from collaborators we have altered our choice of library to assist adequate recovery of guide RNAs. In February 2019 work on this project was largely paused by the COVID-19 pandemic when UNC halted non-essential and non-COVID research projects and reduced animal colonies.

15. SUBJECT TERMS
None listed.

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1. INTRODUCTION:

Acute respiratory distress syndrome (ARDS) is a serious problem caused by burns, infections, and blast and inhalational injuries. Neutrophils are major contributors to ARDS in humans and mice, but it is impossible to apply genome-wide approaches to neutrophils due to their short lifespan outside the host. This proposal uses *in vivo* CRISPR screening in mice to solve a major problem in neutrophil and inflammation research: the inability to rapidly and efficiently introduce genetic changes to neutrophils. This method is generally applicable and can be used in other injury models or other illnesses. The proposed aims focus on identifying regulators of neutrophil expression of the cytokine interferon gamma (IFNγ).

2. KEYWORDS:

Pneumonia, ALI, ARDS, neutrophil, cytokine, CRISPR, mouse, immunology, interferon

3. ACCOMPLISHMENTS:

What were the major goals of the project?

Specific Aim 1: Identification of regulators of neutrophil IFNγ by CRISPR screening
Major Task 1: Generation of mice transplanted with lentiviral library-transduced HSPCs
Subtask 1: Obtain IACUC and ACURO approval (1-3 months) completed
Subtask 2: Lentiviral library production, purification, titering, and testing (1-3 months) completed

Subtask 3: Breeding, genotyping of Cas9-GFP donor and CD45.1 recipient mice (3-18 months)
completed and ongoing

Subtask 4: Harvest and purification of Cas9-GFP HSPCs (3-18 months)
completed and ongoing

Subtask 5: Lentiviral transduction of HSPC, resconstitution of recipient mice (4-6 months)
50% complete, paused due to pandemic

Major Task 2: CRISPR screen for regulators of IFN gamma expression Awaiting subtask 5

Specific Aim 2: Secondary screen of identified regulators
Major Task 1: Design and synthesis of custom lentiviral sgRNA library Awaiting Aim 1
Major Task 2: Secondary screen with custom focused library Awaiting Aim 1

What was accomplished under these goals?

Major activities

We established and genotyped sufficient colonies of donor (Cas9-GFP) and recipient (CD45.1) mice and characterized Cas9-GFP expression in HSPCs and neutrophils from bone marrow, blood, and spleen. We optimized HSPC enrichment using magnetic bead purification and FACS sorting (Figure 1) . We see that Cas9-GFP is robustly expressed in >99% of Lin-/c-Kit+/Sca-1+ HSPCs (Figure 2) and expression is maintained in immature neutrophils, though it declines somewhat in mature neutrophils (data not shown); this suggests that Cas9-GFP should be broadly and sufficiently expressed for genome editing during the differentiation of all neutrophils from HSPCs. We also validated and optimized antibody panels for high purity FACS purification of sgRNA+/Cas9-GFP+/IFNγ+ neutrophils from the mouse lung (data not shown).

We obtained DNA for a lentiviral library encoding 10,000 element targeting ~1000 genes (see section 5 regarding library selection). The library was amplified and used for production of lentiviral particles that were ultraconcentrated and then titered by 4 methods (flow cytometry for mCherry as shown in Fig 3), plaque/cytopathic assay, p24 ELISA, and RT-qPCR). Several rounds of library amplification and production were required to generate sufficient virus to allow adequate coverage in transplanted HSPCs. In conjunction with the High Throughput Sequencing Facility, we designed and obtained nested sets of primers allowing efficient amplification and identification of sgRNAs from pools of sorted lung neutrophils during the screen.

Specific objectives – The objectives of this part of the project were to establish the machinery for *in vivo* CRISPR mutagenesis followed by performance of a screen for regulators of IFNγ expression in neutrophils during bacterial pneumonia.

Significant results – Awaiting completion of *in vivo* screen and validations

Other Achievements – Nothing to report

Stated goals not met – As discussed in section 5, the COVID-19 pandemic necessitated a pause in the project prior to starting the screening experiments (Major Task 1, subtask 5 and Major Task 2). The University was placed on lockdown, non-essential activities and core facilities were halted, and project personnel including the PI were redirected to clinical care of COVID-19 ICU patients. Subtask 5 uses precious, hard-to-replace reagents (highly purified sgRNA library), takes place over 4-6 weeks (immune reconstitution of irradiated mice), and requires 2 sorting rounds in the FACS core; for this reason we have not started the experiment until we are sufficiently confident that it can be completed without interruption to personnel or facilities. Because of this, we intend to apply for a no-cost extension to

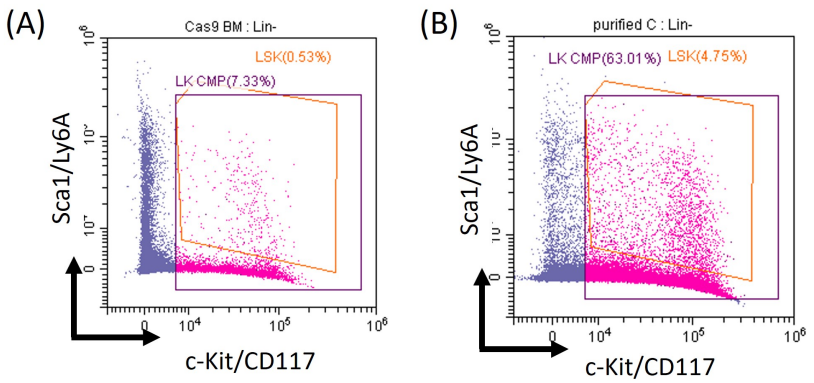

Fig 1: Purification of mouse Cas9-GFP-expressing hematopoietic stem cells from bone marrow. **(A)** Pre-purification bone marrow Lineage-negative (CD3/CD4/CD8/CD19/Ter119/NK1.1/Gr1 negative) cells expressing stem cell markers Sca1 and c-Kit. Gates depict HSPCs (LSK gate) and common myeloid progenitors (LK CMP gate). **(B)** Enrichment of HSPCs and CMPs pre-sorting by CD117 affinity purification. Gates depict roughly 9 fold enrichment pre-sort.

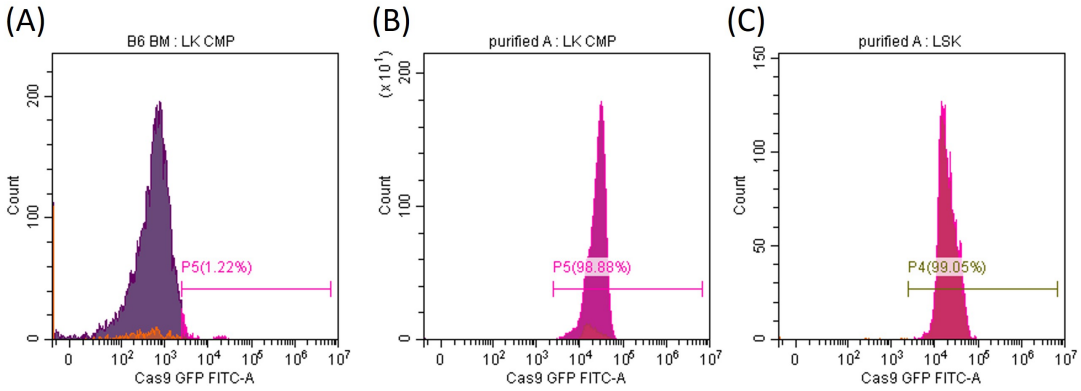

Fig 2: Cas9 expression after enrichment and sorting of hematopoietic stem cells. Cas9-GFP expression in purified HSPCs (LSK gate) from **(A)** control C57Bl/6 mice, **(B)** common myeloid progenitors from Cas9-GFP mice, and **(C)** Lin-negative Sca1+/c-Kit+ from Cas9-GFP mice.

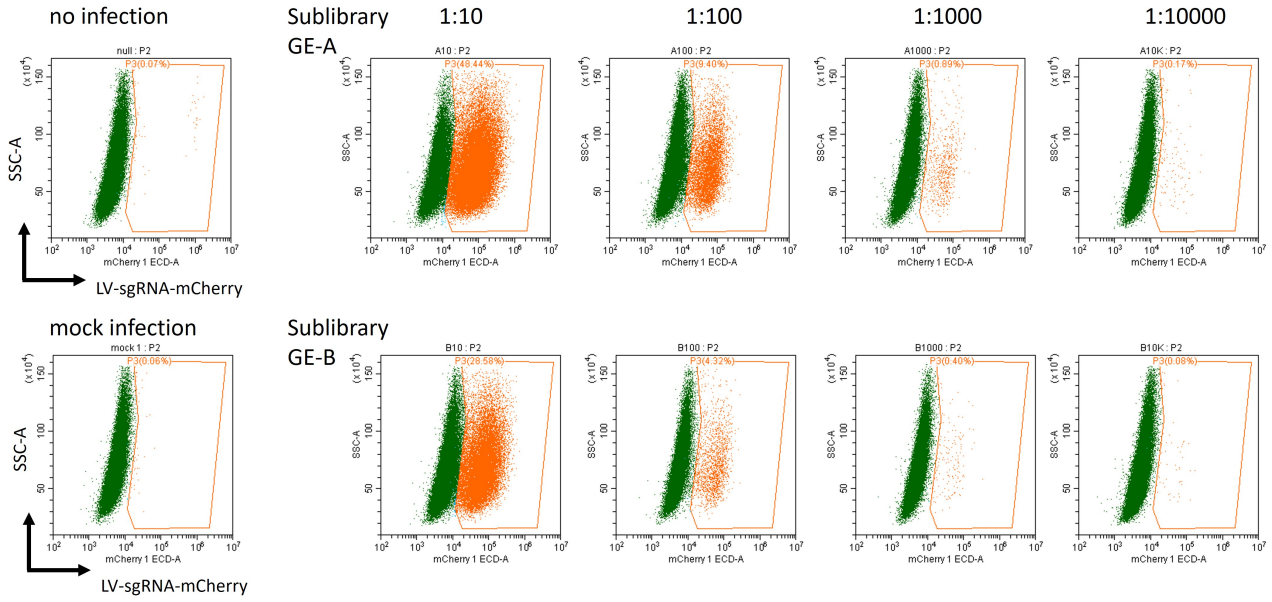

Fig 3: Lentiviral sgRNA expression from two sgRNA sublibraries in bone marrow cells. Lentiviral-mediated sgRNA expression is indicated by mCherry 24 hours after infection of CD117-purified bone marrow cells. mCherry-positive gate depicts sgRNA-expressing cells across a 1000-fold titration of lentivirus

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?

Because of the interruption due to the COVID-19 pandemic, we intend to apply for a no-cost extension. Donor and recipient mice are bred and ready for Specific Aim 1, Subtask 5. We are performing now the immediate steps of lentiviral transduction, irradiation, and reconstitution of mice. Primer sets for Next Generation Sequencing of amplified sgRNA sequences from FACS-isolated cell fractions have arrived and we await confirmation from the FACS core and High Throughput Sequencing Facility core that they will be able to remain open if there is a change in University status due to the pandemic.

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:

As the project was beginning, the groups that first described *in vivo* CRISPR screening published three papers characterizing more fully the extent of sgRNA recovery from transplanted HSPCs in mice (Doench, J, Nature Review Genetics, Feb 2018; Giladi, A, et al, Nature Cell Biology, July 2018; LaFleur, MW et al, Nature Communications, Apr 2019). Based on their calculations, it became clear that adequate sgRNA recovery would not be achieved with the 90,000 sgRNA Yusa library and this would not yield conclusive results. We elected to use an alternative library from Michael Bassik's lab (Addgene 1000000123) with 10,000 sgRNAs targeting ~1000 genes.

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

As of February 2020 the project was largely on track with timely acquisition and optimization of reagents and mice. The COVID-19 pandemic essentially paused the project for four major reasons. First, the PI was mobilized to care for COVID patients in the UNC MICU. Second, the university limited non-essential and non-COVID-related research activities. We asked for permission to continue because this is an ARDS-related project but it was deemed non-essential and non-COVID. Third, the university asked for reduction in animal colonies due to limited veterinary staff. Fourth, the experiments in Specific Aim 1, Subtask 5 take a minimum of 6 weeks per round and require participation of the FACS and High Throughput Sequencing Facility cores; because it could not be guaranteed that these would be open and that would be available without interruption, the experiment had to be paused.

Changes that had a significant impact on expenditures

As described above, multiple budgeted components including the FACS core, High Throughput Sequencing Facility core, and bioinformatics support have not been used yet due to the COVID-19 slowdown.

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

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**

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

What individuals have worked on the project?

Name:	Robert S. Hagan, MD/PhD
Project Role:	PI
Researcher Identifier:	ORCID ID 0000-0002-1504-0086
Nearest person month worked:	4 months
Contribution to project:	Dr. Hagan has overseen the project and performed bench and animal work including mouse HSPC purification, flow cytometry, lentiviral titering, and sequencing primer design.
Funding Support:	NHBLI
Name:	Jose L. Torres-Castillo
Project Role:	Research Technician
Research Identifier:	ORCID ID: 0000-0001-8456-8717
Nearest person month worked:	5 months
Contribution to project:	Mr. Torres-Castillo has performed mouse breeding, genotyping, and colony management, bone marrow and lung harvesting, ordering, reagent optimization, and lab management.
Funding Support:	NHBLI
Name:	Claire M. Doerschuk, MD
Project Role:	Collaborator
Research Identifier:	ORCID ID: 0000-0003-2638-3321
Nearest person month worked:	1 month
Contribution to project:	Dr. Doerschuk has contributed to experimental design and execution
Funding Support:	NHBLI

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

Dr. Hagan submitted and received a fundable score on an invited NHLBI R03 (1 R03 HL155249-01) to support a project examining the function of the innate immune kinase TBK1 (TANK-binding kinase 1) in lung neutrophils. This project represents an offshoot from Dr. Hagan's NHBLI K08 project (1 K08 HL143271-01A1) studying TBK1 in macrophages.

Dr. Doerschuk was awarded an R01 (1R01HL145396-01) to support a project studying macrophage trafficking and function in the lung microenvironment during pneumonia.

What other organizations were involved as partners?

Nothing to report

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS:

QUAD CHARTS:

9. APPENDICES: