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Resistin-Like Molecule Alpha Lentivirus and Utilizing Community Engaged Research To Tackle COVID-19 Health Disparities

A Thesis submitted in partial satisfaction
of the requirements for the degree of

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in

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by

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ABSTRACT OF THE THESIS

Resistin-Like Molecule Alpha Lentivirus and Utilizing Community Engaged Research to Tackle COVID-19 Health Disparities

by

Sumaya Troy Alaama

Master of Science, Graduate Program in Biomedical Sciences
University of California, Riverside, September 2023
Dr. Meera G. Nair, Chairperson

Macrophage-derived proteins play critical roles throughout all stages of the immune response to a variety of pathogens. Depending on the immunological challenge and the subsequent cytokines produced in response, macrophages will become classically (IFNγ) or alternatively activated (IL-4), with an entire spectrum of macrophage phenotypes existing between these two classifications. Upon activation, macrophages will produce a diverse array of proteins and cytokines to guide the immune response depending on the stimulus. Resistin-like molecule alpha (RELMα) is an example of such a protein that is secreted by alternatively activated M2-macrophages. Our laboratory has previously identified a variety of roles for RELMα *in vivo* and *in vitro*. However, knowledge gaps remain with regards to potential cell-extrinsic versus cell-intrinsic differences of immune regulating proteins on cell behavior and function. These include whether there is a difference in how a cell behaves when it expresses the protein or whether it encounters it extracellularly, or whether the function differs dependent on the cell-type, given that RELMα is expressed by immune and non-immune cells. To begin addressing these questions, new tools are needed that allow overexpression in cell lines and primary cells.

In the first part of this thesis, I sought to generate a RELMα over-expressing lentivirus to be later utilized in the efficient transduction of both cell lines and primary cells.

Community-based research has the potential to improve the possibility of reducing certain health disparities. The COVID-19 pandemic brought health disparities to the forefront, many of which existed before and were exacerbated by it. It then becomes the responsibility of researchers to engage within a community-based framework to use these opportunities and address the factors driving these disparities. Research conducted at the UC Riverside School of Medicine on COVID-19 in Riverside County has identified such disparities in the population, where Hispanic individuals were more likely to be severely infected with COVID-19. When opportunity arises in which community-engaged research should be performed, we offer the methodology and framework that was used in planning for our community-engaged research symposium that fostered a rich environment of academic and community collaboration. In the second part of my thesis, I will go into the community-engaged research I performed that allowed for the successful execution of a community-focused COVID-19 symposium.

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CHAPTER ONE – Utilizing Community Engaged Research to Tackle COVID-19 Health Disparities

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Introduction

SARS-CoV-2 (severe acute respiratory syndrome coronavirus 2) led to the COVID-19 global pandemic, resulting in the infection of much of the worldwide population. In the United States alone, there have been more than a million deaths attributed to COVID-19 [11]. Publicly available state and national datasets tracking COVID-19 infections have revealed significant disparities, highlighting that individuals of color not only face a higher likelihood of contracting COVID-19 but also experience more severe infections, leading to hospitalization and an increased risk of death [12]. One study demonstrated that among the counties in the United States identified as COVID-19 hotspots, 96.2% of them exhibited disparities in COVID-19 cases affecting one or more underrepresented racial/ethnic groups. Among these groups, Hispanic/Latino (Hispanic) individuals constituted the largest population (3.5 million people) residing in hotspot counties, where a disproportionate number of cases were observed within this group [13]. Riverside County is prominently recognized as one of the most heavily impacted regions in California during the COVID-19 pandemic, surpassing other counties in the state. The area has experienced a significant surge in demand for enhanced access to primary care, primarily driven by the disproportionately high number of COVID-19 cases within the Hispanic population [14]. These factors collectively contribute to the severity of the disease within this geographic area. Differences in disease outcome have brought to light the long-standing health and social inequities that contribute to the increased risk for infection, severe illness, and death among at-risk populations. As investigations on

COVID-19 continue, an expansion for research into the drivers behind these health and social inequities has been crucial in addressing differences in disease outcome.

Research conducted in our laboratory, in collaboration with other labs in the UCR School of Medicine, has shown that Hispanic/Latino populations were overrepresented in the ICU population in Riverside during the height of the COVID-19 pandemic [1]. These findings were ascertained with demographic data I collected on study participants including age, gender, medical history, treatments during hospital stay, laboratory test results, and ethnicity. After this data was collected, I generated study demographics by group (Healthy Control, Moderate Infection, Severe Infection, and Recovered) and compared each experimental group and the healthy control for the specific characteristic using an un-paired student's t-test). This revealed that the severe infection group is significantly skewed towards Hispanic individuals (n=23, 69.7%, p=0.012) compared to other groups (control and moderate infection) and the demographic data for Riverside County (51.6% Hispanic) [1, 2]. These participants also report higher instances of comorbidities, particularly diabetes, hypertension, and obesity which highlights the likely healthcare disparities in preventative healthcare access in the severe group. According to the RUHS, 84% of their payor population uses Medi-Cal insurance [3]. Statewide Medi-Cal enrollment data shows that 59% of those patients enrolled are Hispanic/Latino youth, which is more than double the enrollment of Asian/Pacific Islander and White ethnicities. Furthermore, the Riverside area is home to a high number of uninsured individuals [4]. It has been reported that Medi-Cal patients with comorbidities requiring specialist visits

have difficulties in accessing specialist care (i.e., endocrinologists for those with diabetes or cardiologists for those with hypertension). According to the U.S. Bureau of Labor Statistics 2021 data, the average Hispanic/Latino median household income was $55,321 compared to $74,912 for non-Hispanic white households [5]. In 2020, the U.S. Census Bureau reported that 17% of Hispanic individuals were living at poverty level [6]. As a result, few insurance options are available in these cases, and a large percentage of underserved populations utilize Medi-Cal, where they experience difficulties accessing routine preventative care and specialist visits, potentially leading to the worse outcomes seen in the severe infection cohort. Furthermore, limited healthcare access, fear of financial burden, or avoidance of the healthcare system due to documented status can result in patients waiting longer to seek care and therefore receiving treatment only after the disease has advanced to a more severe stage, also leading to increased mortality risk [7].

Characteristic	Healthy Control (n=23)	Moderate Infection (n=33)	P-value	Severe Infection (n=33)	P-value	Recovered (n=23)	P-value
Sex							
Male	12	13	0.27	23	0.39	10	0.76
Female	9	20		10		11	
Age, years							
Mean (SD)	27.60 (7.27)	52.88 (16.68)	**0.0003**	56.09 (13.86)	**<0.001**	31.05 (12.17)	0.27
Median (IQR)	26.00 (19.00-52.00)	48.00 (22.00-84.00)		52.00 (23.00-80.00)		27.00 (19.00-67.00)	
Ethnicity							
Hispanic	7	8	0.55	23	**0.012**	9	0.75
Non-Hispanic	14	24		10		12	
Co-morbidity							
Yes	8	27	**0.002**	30	**<<0.001**	8	1
No	13	6		3		13	
Vaccinated							
Yes	18	10	**0.001**	9	**<<0.001**	21	0.48
No	1	12		23		0	

Table 1. Study Demographics. Statistical tests for each experimental group compared to "Healthy Control" are as follows: 1) for "Age" parameter, One-way ANOVA with multiple comparisons was performed using the mean, standard deviation (SD), and "n" of each group; 2) for all other parameters, Fisher's exact test was used to determine significance.

It has been shown that community-based participatory research is a paradigm to reduce health disparities [8]. Therefore, further investigation into the underlying causes of the inequities we identified in our research study required community input. To address this, I helped organize a community-engaged symposium called the 'COVID-19 Community Chat,' which was open to the public event to present our study's findings on the impact of COVID-19 on immune and lung health, with the additional goal of addressing the health disparities in COVID-19 outcomes among individuals in Riverside County identified in our study. Our goal was to foster a collaborative and engaging environment that provided a platform for the community to express their experiences with the pandemic and share their thoughts on the research. The valuable feedback obtained from this interaction will guide future research directions.

COVID-19 Community Chat

Community Engaged Research Event Planning

In academic community-engaged research, it is essential to share the significant findings with the recruited participants, their family members, and the broader community in which the research is conducted. To accomplish this, we adopted a less formal approach by hosting a “community chat” event style. The objective of our 'COVID-19 Community Chat' event was to offer the community a platform to explore and discuss the multifaceted impacts of COVID-19 in Riverside, encompassing perspectives from medical, scientific, and public policy. Throughout the event planning process, the Center for Health Disparities Research at the UC Riverside School of

Medicine (HDR@UCR) collaborated to ensure that all event advertising and study recruitment materials were culturally competent and made accessible in Spanish language as well as English. Presentations were designed in such a way that when the study findings were disseminated, they were accessible and members of the community outside of academia could understand them. The objective was to empower participants to actively engage in discussions and address any questions they had during the presentation. One method for achieving this was consciously to avoid using jargon associated with academia. For example, we opted to name the event the 'COVID-19 Community Chat' rather than the 'COVID-19 Symposium'. In addition, we made sure to schedule the event so that it was outside the work week, so community members did not have to call off work to attend. Since the event was several hours as well, we provided free breakfast and lunch for all attendees as well as on-site parking. This was done to optimize accessibility and encourage all members of the community to attend.

Fostering Academic and Community Collaboration

Our research brought to light the disparities in COVID-19 disease outcomes that exist in Hispanic populations in Riverside County, and our symposium provided a platform that allowed for the community to provide their feedback and share their experiences on how COVID-19 has impacted them. We achieved this by hosting roundtable sessions, with tables focused on specific topics including the public policy perspective, physicians treating COVID-19 and the associated health outcomes, the ongoing scientific research on COVID-19 being conducted at UCR, and the community

perspective on the impacts of the pandemic in Riverside, led by professionals within the field of the corresponding topic who would guide the roundtable discussions. After providing ample time for discourse and addressing community questions, we identified and collected the main discussion points from each table for further research. In the final phase of our event, community members had the opportunity to share the key points from their respective roundtable discussions with the rest of the attendees, highlighting the most significant insights, and future directions focused on addressing the points brought up by the community. With this approach, collaboration and meaningful engagement between research institutions and the community allows research to be conducted in a manner that considers the community's needs. By engaging in open discussions and actively involving community members, future research can be directed in a way that addresses their concerns and priorities. This collaborative approach ensures that the research aligns with the community's needs and enables progress to be made in a mutually beneficial direction.

Discussion

Community feedback during the symposium brought forth a diverse range of perspectives of how COVID-19 has impacted individuals of Riverside County, with some of these perspectives having been echoed in several studies on COVID-19. For example, community members expressed their concerns over the effects of “Long COVID.” One community member stressed how ever since getting COVID, they are unable to work the same as before due to the fatigue they experience, despite being fully recovered from

their active infection. Several reports of similar post-COVID-19 symptoms have raised concerns over the consequences of Long COVID, which is characterized by symptoms four weeks after infection such as fatigue that interferes with daily life, difficulty breathing, cough, dizziness, headache, diarrhea, or joint or muscle pain. One survey investigating the relationship between Long COVID and employment status revealed those with self-reported long COVID symptoms are more likely to be unemployed [9]. Members of the community expressed their concerns with the possibility of losing their health insurance from being unable to work due to symptoms of Long COVID, leaving them unable to access healthcare. The inability to access healthcare can then further exacerbate symptoms after COVID-19 infection due to them not being treated, addressed, or investigated. Related to access to healthcare, is how intrinsically tied insurance is to employment in the United States. Small business was disproportionately affected during the pandemic, with small business owners explaining how they were concerned over their business failing leading to a loss in health insurance, leaving them and their families without healthcare [10]. Other studies have reported that small businesses have been disproportionately affected compared to large businesses, especially minority businesses [11].

Other perspectives came from students at local schools, who expressed one positive outcome of the pandemic. This outcome was the expansion of online and hybrid learning. Many individuals relayed how online learning is overall more accessible. Studies have reported positive perceptions towards hybrid learning [12], and although schools are

moving back towards in person learning, students expressed how “keeping online, distance, and hybrid options alive will benefit students from other backgrounds,” such as those who are those who are chronically ill and therefore disabled. Students of the community also voiced other more negative perspectives of how the pandemic affected them. Students who were in on-campus housing during the lockdown reported feeling “siloed” in the early stages of lockdown. Unsure of how to access information regarding the virus, specifically from more easily accessible resources such as those readily available on campus, left students feeling isolated. A study reported that one result of the isolation and quarantine programs were increased symptoms of PTSD and depression [13]. It was evident from testimonies of students at the event that isolation took a toll on their mental health, especially those with limited access to mental health resources.

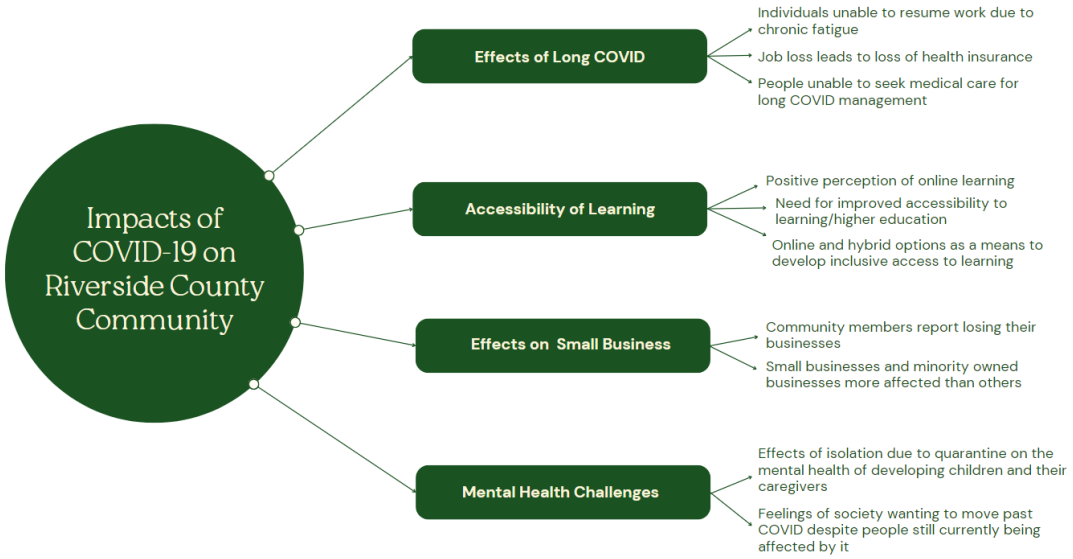

Figure 1. Impacts of COVID-19 on Riverside County Community. Feedback and perspectives recorded from ‘COVID-19 Community Chat’ community roundtable detailing how COVID-19 has impacted individuals of Riverside County.

In conclusion, the COVID-19 pandemic has highlighted significant health and social inequities, particularly impacting underserved populations. The disproportionate burden of COVID-19 on communities of color, as exemplified by the experiences of Hispanic/Latino populations in Riverside County, underscores the urgent need for research that addresses these disparities. Health disparities are preventable differences in the burden of disease, injury, and violence, and the factors driving these disparities are often intricate and multi-faceted. Therefore, the approach in which further research is conducted should also be multi-faceted. Community-engaged research offers one such approach. Unlike traditional research approaches, community-engaged allows the community to be more involved in a meaningful way by providing space and opportunities for partnership, cooperation, and negotiation regarding local health issues. It also enables academic researchers to come up with meaningful and effective interventions and programs that are designed with the community in mind. For community-engaged research to be successful, community engagement must be a core focus. Studies on community engagement have identified main themes that emanate across all strategies, including how engagement is an ongoing an iterative process, how community partner roles must be well-defined and clearly communicated, and mutual trust and transparency are central [14]. Community-engaged research has emerged as a powerful approach to involve communities in shaping research agendas, sharing findings, and fostering collaboration between academia and the community. The "COVID-19 Community Chat" symposium served as a platform for meaningful engagement, allowing community members to share their experiences, provide feedback, and contribute to the

direction of future research. By hosting roundtable discussions that covered various perspectives, the symposium fostered collaboration and ensured that the voices and needs of the community were heard and incorporated into research endeavors. This community-engaged approach is vital in understanding and addressing the drivers behind health disparities, ultimately paving the way for more equitable and effective interventions to combat COVID-19 and improve overall community health.

CHAPTER TWO - Generation of Resistin-like molecule alpha Lentivirus

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Introduction

Resistin-like molecule alpha, or RELMα, is a small-cysteine rich secreted protein with other names including hypoxia-induced mitogenic factor (HIMF) and found in inflammatory zone 1 (FIZZ1). RELMα belongs to the FIZZ/RELM protein family. Like it's other "relatives" (resistin, RELMβ, and RELMγ), it is composed of three domains: an N-terminal sequence containing a secretory signal peptide, a variable middle portion, and a highly conserved C-terminal signature sequence containing 10 cysteine residues [15]. Under homeostasis, resistin-like molecule alpha is expressed in the lung, heart, and white adipose tissue [16, 17]. It was first discovered in the bronchoalveolar lavage fluid of mice with experimentally induced allergic pulmonary inflammation [18] and later shown to be expressed by alternatively activated (IL-4) macrophages [19] and type II alveolar epithelial cells [20]. It is induced in a many different immune contexts. It has been shown to be strongly induced in a type 2 immune response such as infection with parasitic worms [21] as well as known Th2 immune contexts like hypoxia [18]. The function of RELMα is dependent on the immune context. In certain contexts, RELMα acts as immune regulator, such as infection with *N. brasiliensis,* where RELMα will downregulate inflammation and worm killing to minimize host tissue damage. There are other contexts in which RELMα has been shown to be protective, such as the skin. The skin microbiome induces RELMα expression, where it kills bacteria through membrane disruption, and is dependent on and upregulated by dietary vitamin A [22].

While RELMα upregulation is observed across different immunological contexts, the mechanisms involved in the transcription of it remain to be fully elucidated. Signal transducers and activators of the STAT6 and C/EBP pathways coordinate RELMα induction during a Th2 inflammatory response [23] and other experiments have demonstrated that RELMα promoter gene constructs respond to Th2 cytokines (IL-4 and IL-13). The involvement of STAT6 in RELMα production has also been shown in other immune contexts, such as the mouse model of acute pulmonary inflammation. A great number of signaling pathways are activated by RELMα, but the receptor for it remains unclear. Previous research has defined binding partners for RELMα, such as Bruton's tyrosine kinase [24], and work done in one study suggests that RELMα may act as a ligand of G-protein coupled receptors [25], although the particular GPCRs remain undetermined.

To create a better model for studying this protein, we aimed to generate RELMα expressing lentivirus to utilize for lentiviral transduction. Lentiviruses are a subclass of retroviruses that are capable of infecting dividing and nondividing cells through mitosis-independent transport of the viral DNA into the nucleus [26]. Use of these lentiviral vectors is attractive because of their natural ability to enter cells and deliver their genetic material for the goal of their own propagation. The basic genes required for their function include the *gag, pol, and env* genes; *gag* encodes the structural proteins, *pol* encodes for the reverse transcriptase and enzymes required for the integration into the host cell genome, and *env* encodes for the viral envelope glycoprotein [27]. Today, the different

portions of the lentiviral genome that are utilized in commercially available lentiviral systems vary between generations. Third-generation systems, like the one utilized in this protocol, split the necessary viral genes across different plasmids. The *gag* and *pol* genes are encoded on a plasmid separate from the plasmid containing the *rev* or *env* genes. For our experiments, we utilized the pLenti6.3/V5™-TOPO™ TA Cloning™ Kit (Invitrogen, Carlsbad, CA) which is composed of a third-generation lentiviral system. This third-generation system that employs the destination vector in which the gene of interest is cloned, and a mixture of three packaging plasmids (pLP1, pLP2, and pLP/VSVG), which supply the helper functions as well as the structural and replication proteins *in trans* required to produce lentivirus, rendering the virus replication incompetent and therefore safer. This lentiviral system also contains an internal CMV promoter for robust expression of the gene of interest. In brief the workflow is as follows: generate PCR products containing the coding sequence of your gene of interest, clone gene of interest into lentiviral destination vector, use destination vector and packaging plasmids to generate lentivirus, and transduce target cells.

Materials and Methods

Lentiviral Transduction Workflow

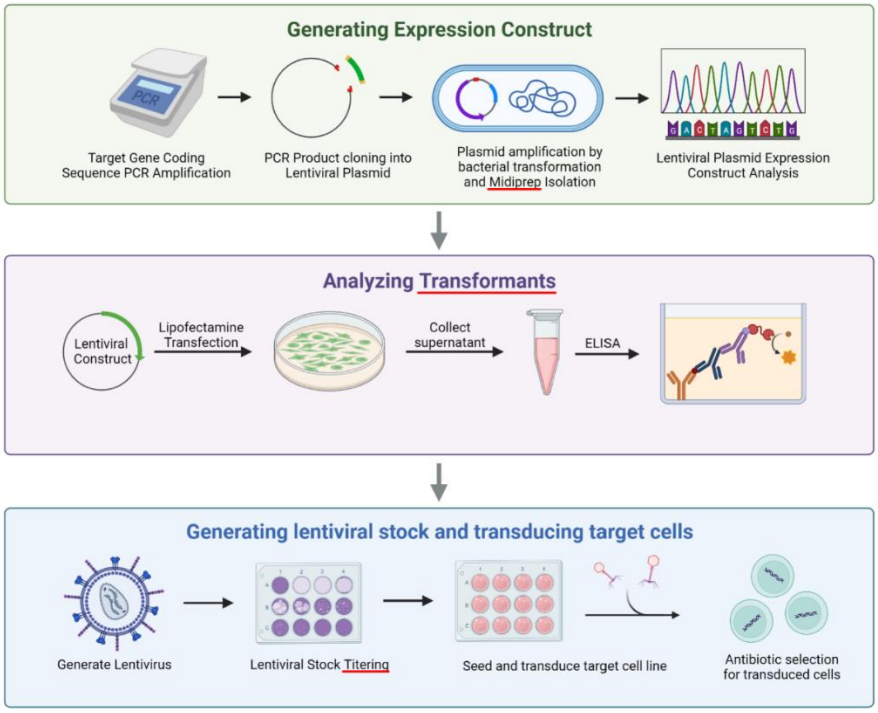

Figure 2. Lentiviral Transduction Workflow. Generating Expression Construct. RELMα coding sequence amplified from plasmid by Polymerase Chain Reaction and subsequently cloned into lentiviral vector. *E. coli* then transformed with lentiviral vectors followed by colony selection and plasmid isolation. Lentiviral vectors analyzed with PCR and Sanger Sequencing. **Analyzing Transformants.** HEK293T cells transfected with lentiviral vector followed by incubation period and subsequent ELISA on supernatant to confirm target gene expression. **Generating lentiviral stock and transducing target cells.** HEK293T cells seeded then co-transfected with lentiviral vector and packaging plasmids to generate lentivirus. Following lentivirus generation, HEK293T cells were seeded and infected with lentivirus in 10-fold serial dilutions ranging from 10^-2 to 10^-6 to titer stock. After titering lentiviral stock the target cells seeded, infected with lentivirus, and antibiotics added to culture to select for stably transduced cells.

RELMα coding sequence amplification

Primers were designed in SnapGene software (http://www.snapgene.com/) using a sequence map of a pCMV6-sport6-mRetlna plasmid made in house containing the coding

sequence for RELMα. PCR amplifications were performed in a 25 µL volume containing 2mM MgCl2, 0.2 mM dNTPs, 10 µM of each primer (forward: 5′-GCCAACTTTGAATAGGATGAAGCC-3′; reverse: 5′-TCATTCTTAGGACAGTTGGCA-3′), 1 U Platinum™ *Taq* DNA Polymerase High Fidelity (Life Technologies, Carlsbad), CA, USA), and 180 ng of pCMV6-sport6-mRetlna plasmid as template. The expected PCR product is 358 bp. Conditions of PCR consisted of initial denaturation at 94 °C for 30, followed by 30 cycles of 94 °C for 15 s, 55 °C for 30 s, and extension of 68 °C for 30 s. After PCR, products were run on % agarose 1× TAE gel (40 mM Tris–HCl, 20 mM acetic acid, 1 mM EDTA, pH 8.0), containing GelRed® Nucleic Acid Gel Stain (Biotium, Fremont, CA, USA) for DNA staining and visualization. The gel was run in 1X TAE buffer and photographed using a gel documentation system (Gel Doc EQ, Biorad, Hercules, CA, USA). GeneRuler™ 1 Kb (ThermoFisher scientific™, Lithuania, EU) molecular-weight DNA size markers were included for calculation of amount and size of DNA fragment. Selected DNA bands were cut out of agarose gels with surgical blades. The excised gel was as small as possible to avoid diluting the recovered DNA. Recovered DNA was purified from agarose gel with the PureLink Quick Gel Extraction Kit (Invitrogen, Carlsbad, CA) and concentration of PCR product was quantified using a DS-11 Series Spectrophotometer (Denovix, Wilmington, Delaware).

RELMα lentiviral expression construct

Purified PCR product from RELMα coding sequence amplification was cloned into the lentiviral destination vector according to the manufacturer's instructions. In brief, 1 µl of PCR product and 1 µl were mixed gently in a tube utilizing a 1:1, 1:3, or 3:1 ratio of vector:insert along with salt solution and sterile water to a final volume of 5 µl and allowed to incubate for 5 minutes at room temperature. Cloning reaction was then added to one vial of One Shot® Stbl3™ chemically competent cells (Invitrogen, Carlsbad, CA) and incubated on ice for 30 minutes. After incubation, cells were heat-shocked at 42°C in the water bath, and then placed on ice again for 2 minutes. 225 µl S.O.C. media that was pre-warmed to room temperature was then added to cells prior to being placed in a shaking incubator at 37°C for 1 hour at 225 rpm. Transformed cells were spread on pre-warmed LB agar plates containing ampicillin (50-100 µg/mL) to select for resistant colonies and cultured overnight at 31°C. For positive control, 10 pg of pUC19 DNA was added to a separate vial of competent cells to verify transformation efficiency. Fifteen ampicillin resistant colonies were inoculated in LB Broth containing ampicillin and cultured overnight at 31°C. Following inoculation, plasmid DNA was isolated from bacteria utilizing the PureLink™ HQ Mini Plasmid Purification Kit (Invitrogen, Carlsbad, CA). To probe for successfully ligated clones, PCR amplifications were performed with RELMα primers as described above utilizing a GoTaq® DNA Polymerase (Promega, Madison, WI) and the pCMV6-sport6-mRetlna plasmid as a positive control. Colonies with positive bands upon PCR were sent for Sanger Sequencing. Sequencing results were checked for RELMα coding sequence and run on

BLAST to verify coding sequence is within frame [28]. Plasmid containing RELMα lentiviral expression construct complete with coding sequence in frame containing no nucleotide mismatches was selected and transformed into *E. coli* as described above. Following transformation, culture, and inoculation plasmid DNA was isolated from bacteria utilizing the PureLink™ HQ Midi Plasmid Purification Kit (Invitrogen, Carlsbad, CA), then PCR and Sanger Sequencing were performed again.

RELMα lentiviral plasmid generation

Before generating the lentivirus, RELMα protein expression in the cell culture supernatant of HEK29T cells transfected with RELMα lentiviral expression construct using Lipofectamine™ 2000 Reagent was confirmed by enzyme-linked immunosorbent assay (ELISA). On day 1, HEK293T cells were seeded on 24-well plate to be 80% confluent at the time of transfection. The following day, cells were transfected with RELMα lentiviral expression construct, a pCMV-GFP expressing plasmid as a positive control, or an empty destination vector with no insert for a negative control. Cells transfected with positive control were confirmed with imaging on BZ-X00 (Keyence, Osaka, Japan). Cells were incubated for 1-3 days at 37°C before supernatant was collected for ELISA. For ELISA, Greiner 96-well plates were coated with primary antibodies to RELMα (0.5μg/ml; Peprotech) in γ-coat overnight at 4°C. After blocking the plates with 5% newborn calf serum in PBS for 2 h at 37°C, supernatant was added at various dilutions and incubated at room temperature for 2 h. Detection of RELMα was done with biotinylated antibodies (0.5 μg/ml; Peprotech) for 1 h, followed by incubation

with streptavidin-peroxidase (Jackson Immunobiology) for 1 h in the dark. The peroxidase substrate 3,3′,5,5′-tetramethylbenzidine (TMB) (BD) was added, followed by the addition of 2 N H_2SO_4 as a substrate stop, and the optical density (OD) was captured at 450 nm. Samples were compared to a serial fold dilution of recombinant protein.

RELMα lentivirus generation

To then generate lentivirus, HEK293T cells were seeded on a 10cm tissue culture plate to be at 90% confluency at the time of transfection. The following day, cells were co-transfected with RELMα lentiviral expression construct and ViraPower™ Packaging Mix that contains an optimized mixture of the three packaging plasmids, pLP1, pLP2, and pLP/VSVG. Transfection with a *lacZ* lentiviral plasmid in addition to was used as a positive control to generate *lacZ* lentivirus. Empty destination vector with no insert was used for a negative control. Cell culture medium was replaced the following day with antibiotic free medium. Cells were incubated for 24 h at 37°C, and supernatant containing virus was harvested. Supernatant was centrifuged at 2,000 × g for 15 minutes at 4°C to pellet debris and filtered through PVDF filter before storing in 1 mL cryovials at –80°C. Lentiviral stock was then tittered in HEK293T cells. HEK293T cells were seeded on a 6-well plate such that they would be 45% confluent at the time of transduction. On the day of transduction, 10-fold serial dilutions of lentiviral stock were prepared in culture medium to a final volume of 1 mL. Culture medium was removed from the wells and replaced with medium containing lentivirus and Polybrene® at a final concentration of 6 μg/mL. Cells were incubated overnight at 37°C and culture medium was removed the

following day and replaced with complete culture medium containing Blasticidin (10 ug/mL) to select for stably transduced cells. Cells were incubated for 4 days with culture medium renewal every 2-3 days, until no live cells were observed in the mock well under microscopy. After selection, cell culture medium was removed, and cells were washed twice with PBS before staining with 1 mL crystal violet solution. Cells were incubated for 10 minutes at RT before crystal violet stain was removed for lentiviral stock tittering.

Results

RELMα coding sequence amplification

PCR primers (forward: 5′-GCCAACTTTGAATAGGATGAAGCC-3′; reverse: 5′-TCATTCTTAGGACAGTTGGCA-3′), on pCMV6-sport6-mRetlna plasmid successfully yields products at two different annealing temperatures, 53°C and 56°C (Fig 2). Product size between 300-400bp compared to DNA ladder, in alignment with expected RELMα coding sequence size plus primers.

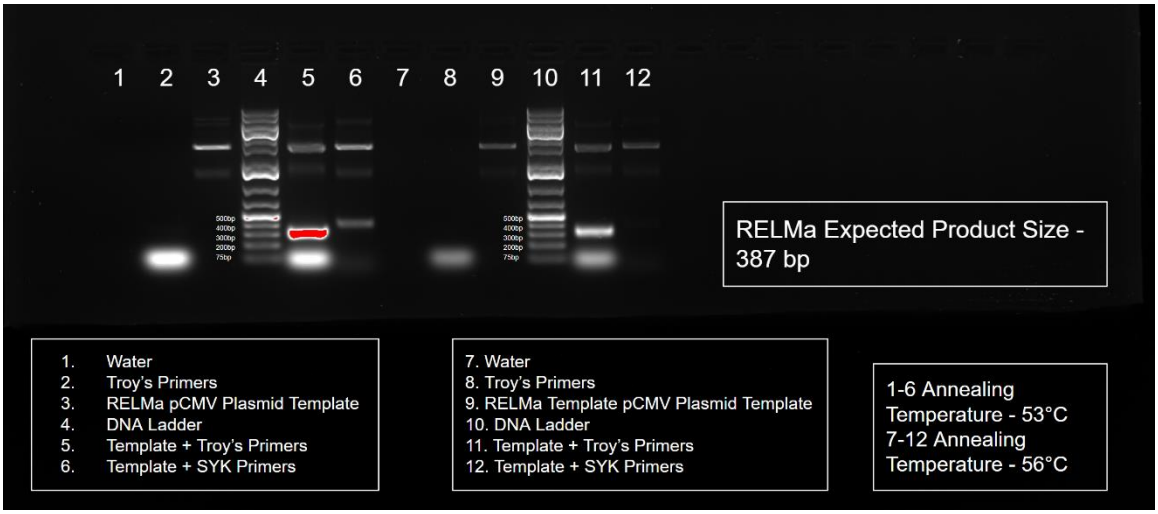

Figure 3. PCR Amplification of RELMα Coding Sequence. Agarose gel electrophoresis (1% agarose) of PCR amplified products using specific PCR primer sets. Lanes 4 and 10 contain 1 kb plus DNA size marker.

Plasmid samples 7, 8, and 9 are candidates for successfully cloned inserts.

After cloning, bacterial transformation, and plasmid isolation, miniprep colony PCR using previously described RELMα primers yielded positive bands in all lanes, with brightest bands present in lanes 12, 13, and 14 (corresponding to samples 7, 8, and 9) (Fig 3). Sample lanes were compared to lane 5 containing positive control, and lanes with similar level of brightness in band product were selected as candidates for Sanger sequencing.

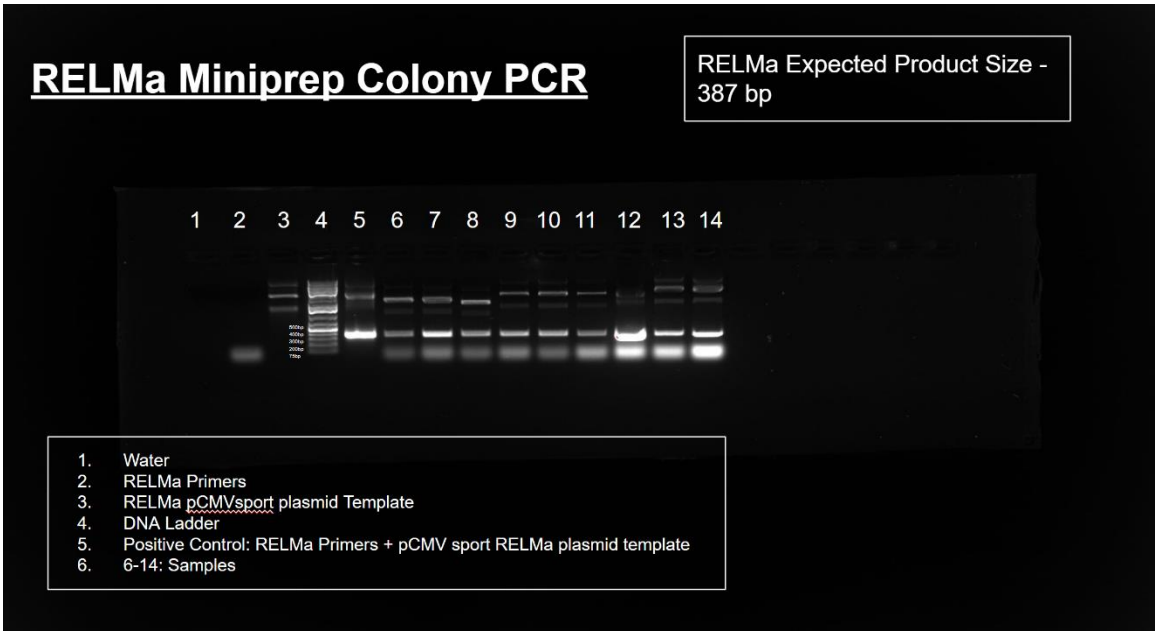

Figure 4. Bacterial Colony Miniprep PCR of Isolated Lentiviral Plasmids Agarose gel electrophoresis (1% agarose) of PCR products amplified from plasmids isolated from transformed bacterial colonies using specific PCR primer sets. Lane 4 contains 1 kb plus DNA size marker.

Sample 7 contains plasmid with successfully cloned insert that is in open reading frame and contains no nucleotide mismatches.

Alignment of Sanger sequencing results of plasmid from sample 7 to RELMα coding sequence and nucleotide BLAST of sequencing results reveals successful cloning of coding sequence into lentiviral destination plasmid (Fig 4A and 4B).

Figure 5. Verifying successful cloning of RELMα coding sequence into lentiviral plasmid with sequencing and alignments. Samples with positive band in miniprep colony PCR sent for Sanger Sequencing. (A) Sample 7 nucleotide sequence from sequencing aligned with template nucleotide sequence with primers and start codon identified. (B) Nucleotide BLAST of sample 7 sequencing data with 100% match to RELMα coding sequence. (C) Chromatogram data of sample 7.

Cells transfected with RELMα Lentiviral Expression Construct secrete RELMα protein into the supernatant.

To confirm expression of recombinant protein prior to generating lentivirus, transfection of HEK293T cells with RELMα Lentiviral Expression Construct and subsequent ELISA on cell culture supernatant after 3 days of incubation yields 2 ng/mL of RELMα protein in supernatant (Fig 5C). Calculation of target protein concentration done with comparison to standard curve (Fig 5B).

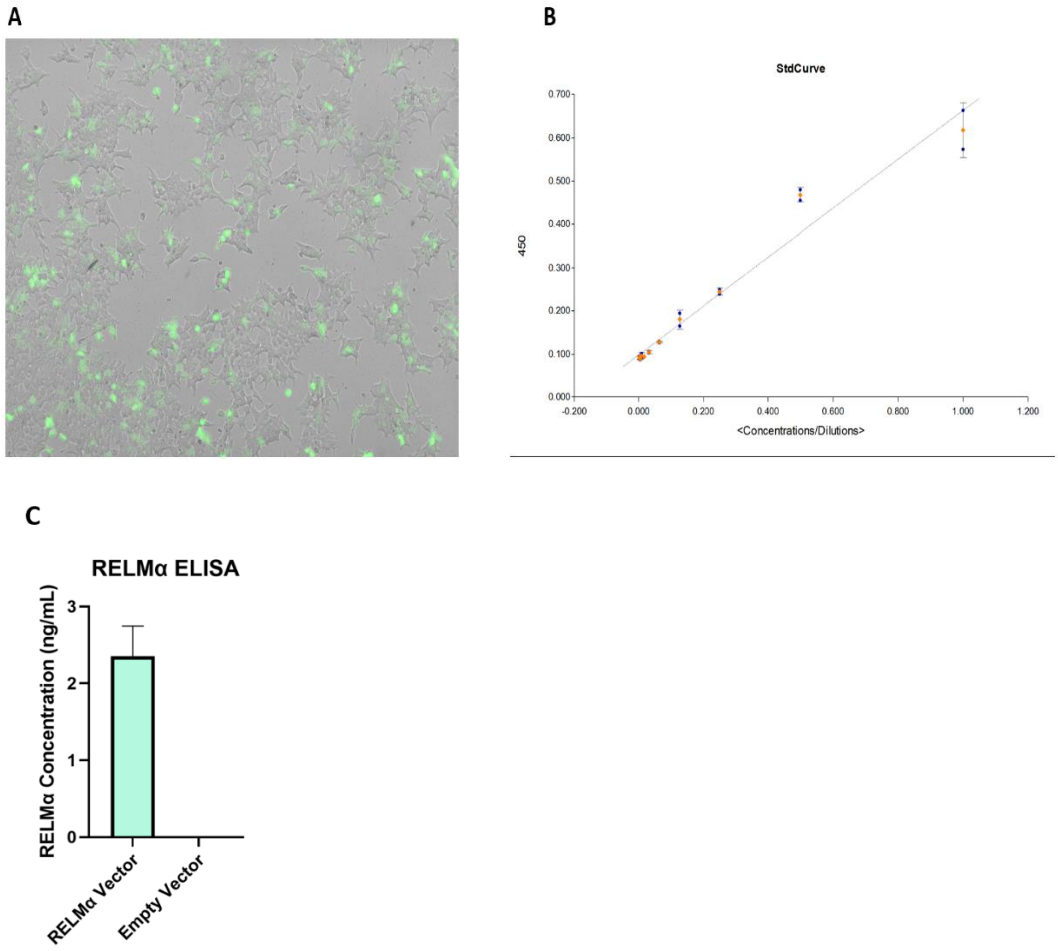

Figure 6. Cells transfected with RELMα Lentiviral Expression Construct secrete RELMα protein into the supernatant. HEK293T cells were seeded in 24-well plates to be 80% confluent at time of transfection. On the day of transfection, media was removed and replaced with cell culture medium without antibiotics, and RELMα Lentiviral Expression Construct was co-transfected with GFP-expressing plasmid using Lipofectamine 2000 reagent. Cells were incubated for 3 days at 37° C before supernatant was collected for ELISA. (A) HEK293T cells co-transfected with RELMα Lentiviral Expression Construct and GFP plasmid imaged at 24 hours post transfection by fluorescent microscope. (B) Standard curve of RELMα ELISA generated from ten 1:2 dilutions of 1ng/mL stop standard. (C) Concentration of RELMα on supernatant from HEK293T transfected with either RELMα Lentiviral Expression Construct or lentiviral plasmid without insert for negative control.

Cells transduced with RELMα Lentivirus have successful incorporation of lentiviral genes into host cell genome.

HEK293T cells transduced with RELMα Lentivirus and subjected to Blasticidin in the media for 4 days after transduction survive antibiotic selection indicating successful integration of lentiviral genes into host cell genome compared to mock cells.

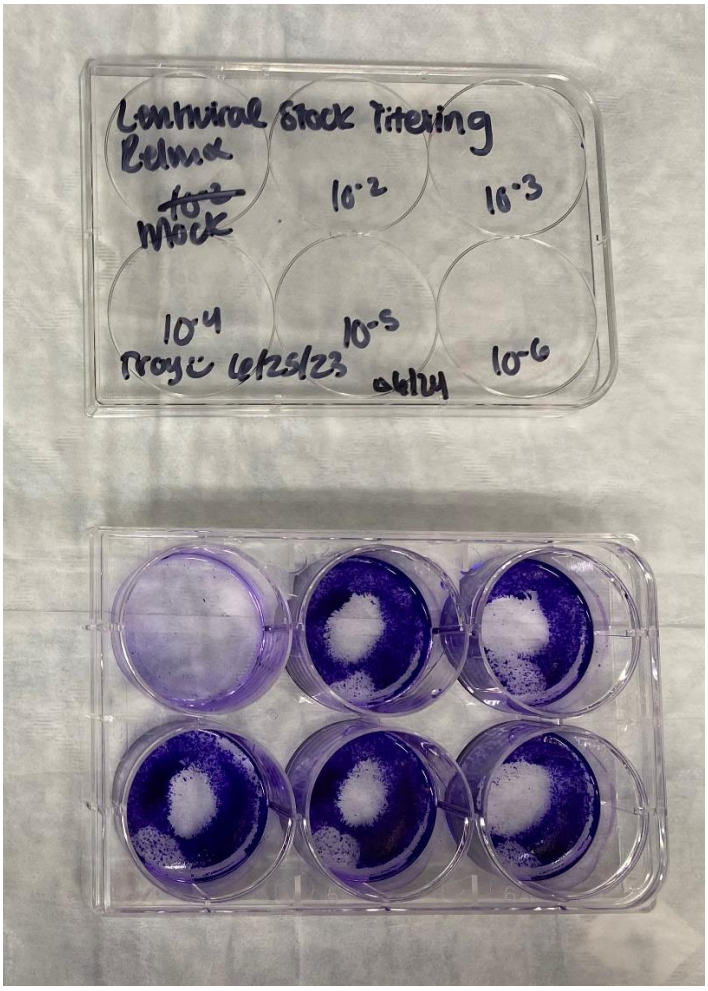

Figure 6. Cells transduced with RELMα Lentivirus have successful incorporation of lentiviral genes into host cell genome.
HEK293T cells were seeded on a 6-well plate to be 45% confluent at the time of transduction. On the day of transduction, 10-fold serial dilutions of lentiviral stock were prepared in culture medium to a final volume of 1 mL. Culture medium was removed from the wells and replaced with medium containing lentivirus and Polybrene® at a final concentration of 6 μg/mL. Cells were incubated overnight at 37°C and culture medium was removed the following day and replaced with complete culture medium containing Blasticidin (10 ug/mL) to select for stably transduced cells. Cells were incubated for 4 days with culture medium renewal every 2-3 days, until no live cells were observed in the mock well under microscopy. After selection, cell culture medium was removed, and cells were washed twice with PBS before staining with 1 mL crystal violet solution for stock tittering.

Conclusions and Future Directions

In conclusion, RELMalpha is secreted from cells that are transfected with the RELMα lentiviral plasmid and cells transduced with the RELMα lentivirus survived the antibiotic selection indicating successful integration of genes from the RELMα lentiviral plasmid into the target cell genome. For future directions, we would like to transduce different cell lines and primary cells to further investigate changes in phenotype. The first cell line we would like to transduce is the mouse lung epithelial, or MLE-12, cell line that is used as an *in vitro* model for cells of the lung epithelium. After transducing these cells, the next step would be to investigate for any differences in phenotype via cell viability or chemokine secretion assays in the stable RELMα expressing cells compared to MLE-12 cells that are not transduced but are treated with RELMα in the supernatant. After investigating these phenotype changes, we can then utilize them in various experiments such as potentially investigating if there is a difference in cell proliferation on lung scaffolds when comparing non-transduced cells treated with RELMα in the supernatant versus seeding the MLE-RELMα expressing cells alone. We would also like to transduce bone marrow derived macrophages and do similar assays for changes in phenotype and do gene expression changes by NanoString.

CHAPTER THREE – Conclusion

Summary

Generation of Resistin-like molecule alpha overexpressing lentivirus

To investigate cell-intrinsic versus cell-extrinsic differences of RELMα expression and secretion, a RELMα lentivirus was generated to be used for downstream lentiviral transduction. In summary: the coding sequence for the protein of interest was amplified, cloned into a destination vector, and transfected into cells to confirm recombinant protein secretion. This plasmid was then co-transfected with viral packaging plasmids to produce the RELMα lentivirus, which will be used to transduce a target cell line.

COVID-19 Pandemic and Academic Led Community Engaged Research

Research on the impact of the COVID-19 pandemic on underserved populations, particularly communities of color, with a focus on Hispanic/Latino populations in Riverside County, California has emphasized the need for research to address health disparities and understand the drivers behind them. We describe our community-engaged research strategies, including those for planning and executing an event called the "COVID-19 Community Chat" that engaged the community and gathered feedback on future research directions. Roundtable discussions were held on various topics, allowing community members to share their experiences and contribute to future research directions. This work highlights the importance of involving the community in shaping research agendas, sharing findings, and fostering collaboration with academia. We

advocate for the use of community-engaged strategies to address health disparities and improve interventions for vulnerable populations.

Future Directions

RELMα stable expressing cell lines

Using the RELMα lentivirus generated in this thesis, primary cells and immortalized cell lines can be transduced and assayed for changes in morphology and function that differ from their non-transduced counterparts. The first cell line we would like to transduce is the mouse lung epithelial, or MLE-12, cell line that is used as an *in vitro* model for cells of the lung epithelium. After transducing these cells, the next step would be to investigate for any differences in phenotype via cell viability or chemokine secretion assays in the stable RELMα expressing cells compared to MLE-12 cells that are not transduced and not transduced but are treated with RELMα in the supernatant. One designated experiment for this cell line is to utilize them in influenza infection. Previous work done in the laboratory has shown that RELMα upregulates IAV infection of lung epithelial cells. Infection of these MLE- RELMα stable expressing cells with influenza should reflect this phenotype, but there might be some discernable differences in infection that come to light when comparing the cell intrinsic RELMα-expressing MLE cells to the not transduced MLE-12 cells treated with RELMα extrinsically. We would also like to transduce bone-marrow macrophages, and possibly bone-marrow monocytes.

COVID-19 Community Chat

For any future research that addresses the complex questions that arise from the complexity that is real life such those that deal with health disparities, we have demonstrated the importance behind utilizing community-engaged research. We will

continue to use these strategies moving forward with research that uncovers health disparities and makes discoveries to improve treatments in diseases that affect our community.

Conclusion

The overarching theme of this thesis is that complex problems often require intricate solutions. To address the complex question regarding cell-extrinsic versus cell-intrinsic effects of RELMα, I created a RELMα overexpressing lentivirus. First, we amplified the RELMα coding sequence using PCR prior to cloning it into the lentiviral destination vector. Then we transformed bacteria with our cloning product, isolated resistant colonies, and confirmed successful ligation with PCR and sequencing. Secretion of RELMα protein into the supernatant was confirmed by ELISA on supernatant of HEK293T transfected with destination plasmid. After further plasmid amplification and verification, the RELMα lentiviral destination vector was co-transfected with the other viral packaging plasmids into HEK293T cells to generate lentivirus. HEK293T cells were then used to titer the lentiviral stocks and confirm successful integration of lentiviral genes into target cells using antibiotic selection. To address the complex questions regarding the health disparities identified in our COVID-19 research, we describe our approach to community-engaged research in the form of an event focused on presenting the main findings from the study but also focusing on making the event as inclusive as possible to further engage the community and foster a collaborative environment for moving the research forward.

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