MESSENGER RNA SILENCING
OF UBASH3A IN
TYPE 1 DIABETES

by
Nicholas R. Breehl

Master’s degree Biomedical Sciences (Hood College) 2021

MOCK GRANT PROPOSAL
Submitted in partial satisfaction of the requirements
for the degree of
MASTER OF SCIENCE
in
BIOMEDICAL SCIENCE
in the
GRADUATE SCHOOL
of
HOOD COLLEGE
April 2021

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DEDICATION

I would like to dedicate this work to my family and friends for the positive thoughts to continue towards this accomplishment. I would also like to thank Hood College for the magnificent leadership, mentorship and commitment to the students of the institution.
ACKNOWLEDGEMENTS

I would like to acknowledge the Hood faculty and staff for all of their dedicated work for the students of the institution.
Mock Grant Application
Modeled after Department of Health and Human Services
Public Health Services
(based on Form PHS 398)

1. TITLE OF PROJECT (Do not exceed 81 characters, including spaces and punctuation.)
   Micro RNA Towards UBASH3A for Type-1 Diabetes Treatment

2. RESPONSE TO SPECIFIC REQUEST FOR APPLICATIONS OR PROGRAM ANNOUNCEMENT OR SOLICITATION
   (If "Yes," state number and title)
   Number: Title:

3. PROGRAM DIRECTOR/PRINCIPAL INVESTIGATOR
   New Investigator ☐ No ☑ Yes
   
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   | 3e. DEPARTMENT, SERVICE, LABORATORY, OR EQUIVALENT | Department of Biology |
   | 3f. MAJOR SUBDIVISION | Biomedical Science Program |
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4. HUMAN SUBJECTS RESEARCH
   ☑ No ☑ Yes
   
   | 4a. Research Exempt | N/A |
   | 4b. Federal-Wide Assurance No. | N/A |
   | 4c. Clinical Trial | ☑ No ☑ Yes |
   | 4d. NIH-defined Phase III Clinical Trial | ☑ No ☑ Yes |

5. VERTEBRATE ANIMALS ☐ No ☑ Yes
   
   | 5a. Animal Welfare Assurance No. | N/A |

6. DATES OF PROPOSED PERIOD OF SUPPORT (month, day, year—MM/DD/YY)
   From 05/01/2021 Through 05/01/2022

7. COSTS REQUESTED FOR INITIAL BUDGET PERIOD
   
   | 7a. Direct Costs ($) | |
   | 7b. Total Costs ($) | |

8. COSTS REQUESTED FOR PROPOSED PERIOD OF SUPPORT
   
   | 8a. Direct Costs ($) | |
   | 8b. Total Costs ($) | |

9. APPLICANT ORGANIZATION
   Name Department of Biology
   Address Hood College
           401 Rosemont Ave
           Frederick, MD 21701

10. TYPE OF ORGANIZATION
    Public: ☑ Federal ☐ State ☑ Local
    Private: ☑ Private Nonprofit
              ☐ General ☐ Small Business
    For-profit: ☐ Woman-owned ☐ Socially and Economically Disadvantaged

11. ENTITY IDENTIFICATION NUMBER
    DUNS NO. N/A Cong. District N/A

12. ADMINISTRATIVE OFFICIAL TO BE NOTIFIED IF AWARD IS MADE
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14. APPLICANT ORGANIZATION CERTIFICATION AND ACCEPTANCE: I certify that the statements herein are true, complete and accurate to the best of my knowledge, and accept the obligation to comply with Public Health Services terms and conditions if a grant is awarded as a result of this application. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties.
    SIGNATURE OF OFFICIAL NAMED IN 13.
    (In ink. "Per" signature not acceptable.)
    N/A

DATE
This project is aimed to investigate micro-RNA as a potential therapeutic approach for silencing ubiquitin-associated and SH3-domain containing A (UBASH3A), a negative regulator of the NF-kB pathway in CD4+ T cell Receptor and CD-28 signaling. UBASH3A expression is upregulated in patients of Type-1 Diabetes (T1D) who harbor risk alleles RS11203203 and RS80054410 on chromosome 21 resulting in single nucleotide polymorphisms. Increases in UBASH3A decrease IL-2 and therefore T1Ds experience pathogenesis via lack of T regulatory (T reg/T-reg) cell development from the loss of the pleiotropic cytokine. It has previously been shown, using clustered regularly interspaced short palindromic repeats (CRISPR) CRISPR-associated protein 9 (Cas-9) edited CD4+ T cells, that the silencing of UBASH3A results in a decrease in the protein and an increase of IL-2 production in. This study will determine if the use of micro-RNA directed towards the UBASH3A messenger RNA can reduce UBASH3A protein and rescue IL-2 production in T cells. This work will pave the way for future studies that aim to treat patients with T1D.

This research will provide insight in the development of therapeutic microRNA approaches to circumvent genetically induced cell signal dysfunction contributing to Type 1 diabetes.
BIOGRAPHICAL SKETCH

Provide the following information for the key personnel and other significant contributors in the order listed on Form Page 2. Follow this format for each person. DO NOT EXCEED FOUR PAGES.

NAME
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eRA COMMONS USER NAME (credential, e.g., agency login)

EDUCATION/TRAINING (Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)

<table>
<thead>
<tr>
<th>INSTITUTION AND LOCATION</th>
<th>DEGREE (if applicable)</th>
<th>YEAR(s)</th>
<th>FIELD OF STUDY</th>
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<tr>
<td>Mount St. Mary’s University</td>
<td>B.S.</td>
<td>2011</td>
<td>Biology</td>
</tr>
<tr>
<td>Hood College, Frederick, MD</td>
<td>M.S.</td>
<td>2021 (anticipated)</td>
<td>Biomedical Science</td>
</tr>
</tbody>
</table>

A. Positions and Honors

Research Assistant, Uniformed Services University, Summer 2019 - Present

National Institutes of Health, Intramural Research Associate, Summer 2019

Regulatory Specialist, United States Pharmacopeia, March 2013 – June 2019

B. Selected peer-reviewed publications (in chronological order)

N/A

C. Research Support

N/A
FACILITIES: Specify the facilities to be used for the conduct of the proposed research. Indicate the project/performance sites and describe capacities, pertinent capabilities, relative proximity, and extent of availability to the project. If research involving Select Agent(s) will occur at any performance site(s), the biocontainment resources available at each site should be described. Under “Other,” identify support services such as machine shop, electronics shop, and specify the extent to which they will be available to the project. Use continuation pages if necessary.

Laboratory:
Hood College Laboratory

Clinical:
N/A

Animal:
N/A

Computer:
MacBook Pro equipped with FIJI, GraphPad Prism Software, and Microsoft Office Suite

Office:
N/A

Other:
N/A

MAJOR EQUIPMENT: List the most important equipment items already available for this project, noting the location and pertinent capabilities of each.

The following major equipment is available on site:

- Centrifuges - For collection of cell lysates
- MacBook Pro Computer
- Cloned T cells from Ge Laboratory
- PCR materials
- 
- 
- 
- 

The following consumables will be purchased with grant funding:

- Primary antibody - UBASH3A
- Western Blot materials
- ELISA kit
- T cell line
- miRNA clones and Luciferase Report Assay
- Cell Titer-Glo 2.0
- PCR primers
- 
-
SPECIFIC AIMS

Previous genome-wide association studies have revealed that a population of Type-1 diabetes (T1D) patients with high risk single nucleotide polymorphisms that are found on chromosome 21 encoding UBASH3A, a known negative regulator of CD4+ T cell activation. As a result, these patients have elevated concentration of protein and decreased secretion of IL-2, resulting in reduction in T reg cell populations. T reg cells contribute to autoimmunity, a part of the pathology of T1D.

The aim of this study is to investigate the potential of non-coding micro-RNA (miRNA) as a therapeutic approach to decrease the unwanted overexpression of the negative regulator UBASH3A. MiRNA has been previously explored for its therapeutic nature in many diseases to date. The study will make use of cultured Jurkat T cells, as well as clustered regularly interspaced short palindromic repeats and CRISPR-associated protein 9 (CRISPR-Cas9) edited cells T cells (a gift from the Ge laboratory) that overexpress UBASH3A and T cells that lack the protein from a double-knock-out, to observe the effects of administered miRNA.

After the administration miRNA is complete and evaluation of protein levels quantified with western blot analysis and FIJI software, we will stimulate the T cell cultures in 96-well plates coated in anti-CD-3 and anti-CD28 to perform a proliferation assay. Cytokine analysis for IL-2 production will be analyzed by ELISA Max Deluxe kit (BioLegend). It is hypothesized that an increase in IL-2 will be observed in cells that had relatively lower amounts of UBASH3A present. This research will be fundamental in
future studies that aim to test the rigor of miRNA in animal models before eventually progressing to human studies.
BACKGROUND AND SIGNIFICANCE

The immune system is a powerful and complex force that provides the means for survival against environmental threats as well as harmful mutations that arise within self, such as cancer and autoimmunity. A delicate balance exists within the immune system to discern between harmful and benign antigen or in other words, self vs non-self. In the case of cancer for example, mutations in self may arise that are harmful to the host, and in other cases, for example infection, where foreign exogenous protein (non-self) threaten the health of the host. For this reason, there are several mechanisms that will prevent the immune system from turning on in the host such as tolerance, cell-signaling, and co-stimulation. One important aspect of a functioning immune response lies within the activation of T-cells through the T-cell receptor (TCR) and co-stimulation of molecules such as CD-4, CD-8, and CD-28. Dysfunctional TCR signaling can potentially develop into autoimmunity from altered levels of cytokine interleukin-2 (IL-2) (Geng 2012). The alteration of specific signaling components and genetic factors, such as risk alleles, have been suggested to be involved in the pathogenesis of T1D (Kahaly and Hansen 2016).

Type-1 diabetes, also called insulin-dependent diabetes mellitus or juvenile-onset diabetes, is a T cell mediated autoimmune disease characterized by the destruction of self-antigen, in this case pancreatic beta-islet cells responsible for the production of insulin (Kahaly and Hansen 2016; Ehlers and Rigby 2015). Eventually, patients with T1D will progress to a stage where all insulin producing cells are eliminated and they become entirely reliant on therapeutic insulin. Many patients with T1D are deficient in their ability to produce IL-2 required for the differentiation of T regulatory cells responsible
for induction of tolerance (Seelig et al. 2018; Setoguchi et al. 2005). While the precise pathogenesis of T1D has been elusive (Bluestone JA 2010), mostly due to the many potential routes that can lead to the disease, researchers have made some advance towards the identification of genetic risk factors associated with alteration in specific proteins involved in TCR and CD28 signaling.

**Autoimmunity**

Autoimmunity can occur as a result of dysfunctional signaling cascades in T-cells (Ohashi 2002). To begin, we must first understand that T-cell signaling has many levels. One level in particular is that of central tolerance. Central tolerance is the ability to develop T-cells in the thymus that will not react to self-antigens. Positive and negative selection mechanisms are a means toward central tolerance. When a T-cell engages its receptor with an antigen presenting cell (APC) such as a dendritic cell or macrophage with a peptide loaded to the cell surface protein known as major histocompatibility complex-I (MHC-I) or MHC-II a signal will transduce. The strength of the signal will determine the fate of the T-cell where strong signals lead to apoptosis and weak signals lead to anergy (Kenneth Murphy 2017).

The concept of affinity and avidity come into play, where affinity is referring to the compatibility of the structures binding, while avidity is the specific strength of the bond that is formed. For example, in positive selection, weak signals are necessary for T-cell survival and maintenance. The positive selection process allows for T-cells to survive in the thymus and eventually patrol the periphery of the host for detection of foreign protein. Positive selection therefore teaches a T-cell the required interaction strength to
become active and mature. In negative selection at the thymic level, the developing T-cell will interact with MHC:Peptide complexes from APCs as well, but in this case, if the interaction is strong it will result in clonal deletion (Xing and Hogquist 2012) of that T-cell via apoptosis. These mechanisms exist to avoid development of a TCR repertoire that can react to self-antigen in the periphery and thereby avoid autoimmunity (Kenneth Murphy 2017; Ohashi 2002).

Central tolerance does a good job eliminating self-reactive T-cells before they leave the thymus. Unfortunately, not all self-antigens are present in the thymus and for this reason mechanisms exist to induce tolerance in the periphery, also known as peripheral tolerance. Normally, T-cells that interact with self-antigens in the periphery will activate for a short time before inducing apoptosis or if the T-cell does not undergo apoptosis it may become anergic or resistant to activation after TCR stimulation. In the absence of infection or signals that contribute to an inflammatory response, the anergic T-cell can be achieved (Kenneth Murphy 2017). Autoimmune diseases such as T1D may arise due to genetic factors, signal dysfunction or even co-infections that provide the necessary signals to awaken the T-cell. This project is focused on TCR signaling that can go awry as a result genetic mutations altering expression of proteins involved in TCR stimulation and CD-28 co-stimulation that ultimately reduce the pool of regulatory T cells required to avoid autoimmunity.

NORMAL T-CELL ACTIVATION SIGNAL CASCADE:

TF ACTIVATION: NFAT/AP-1/NFKB and CD28 Co-stimulation
Various signal pathways are required that will properly activate the T cell, allowing for the production of IL-2 (Figure 1). The three main transcription factors that signaling will converge on are Nuclear factor of activated T cells (NFAT), Nuclear factor kappa B (NF-kB), and Activator protein-1 (AP-1). Once achieved, the production of IL-2 is sustained with help from CD-28 co-stimulation.

![Figure 1. T cell activation signaling pathway.](image)

**T-CELL RECEPTOR ACTIVATION**

The T-cell receptor, that is made up of a complex of proteins including alpha:beta heterodimer, 4-CD-3 units (2-epsilon, 1-gamma and 1-delta) and a homodimer of zeta, all of which (excluding the alpha:beta) contain at least one Immunoreceptor Tyrosine-Activation Motif (ITAM). Upon appropriate TCR stimulation from an MHC:Peptide complex signal transduction occurs that allows the T-cell to produce interleukin-2, a pleiotropic cytokine essential for effector T-cell regulation and T regulatory cell
induction (Liao, Lin, and Leonard 2013). The activation of phospholipase-C gamma 1 (PLC-gamma) is essential before three transcription factors can be activated that together will contribute to IL-2 production (Hwang et al. 2020). A member of the Src-Family kinases known as LCK or Fyn is associated with CD-4 or CD-8 respectively, at the surface of the T-cell with the TCR complex. This close association allows the Src-Family kinase to phosphorylate the ITAMs, resulting in recruitment and activation of Zeta-chain-associated protein kinase 70, (ZAP-70). ZAP-70 phosphorylation allows for subsequent phosphorylations of phosphoinositide 3-kinase (PI3K), lymphocyte cytosolic protein 2 (SLP-76), and Linker for Activation of T cells (LAT). SLP-76 and LAT activation generates a complex formed with an adaptor protein, either Gads or Grb2, bringing them together. This complex can also contribute to the activation of PI3K. PI3K is an enzyme that catalyzes the production of Phosphatidylinositol (3,4,5)-trisphosphate (or PIP3) from Phosphatidylinositol (4,5)-bisphosphate (or PIP2). LAT:Gads/Grb2:SLP-76 and PIP3 will recruit PLC-gamma and Interleukin-2-inducible T-cell kinase (ITK). ITK activates PLC-gamma via phosphorylation. Now, PLC-gamma is free to act on PIP2 to create second messengers: Inositol trisphosphate (IP3) and diacylglycerol (DAG). It is from this point that the Nuclear factor kappa B (NF-kB), Nuclear factor of activated T cells (NFAT), and Activator protein-1 (AP-1) transcription factors required for IL-2 production can become activated through their unique signaling pathways (Kenneth Murphy 2017).
NFAT Signaling

After the successful activation of PLC-gamma, IP3 is generated and diffuses away to the endoplasmic reticulum where it engages with the Ca2+ receptors that will open and release calcium into the cytosol. This change in calcium concentration saturates Ca2+ binding to calmodulin that will target calcineurin, dephosphorylating it which permits nuclear translocation of NFAT (Kenneth Murphy 2017).

AP-1 Signaling

DAG, the product of PLC-gamma activation is required to bind to a guanine exchange factor (GEF), usually RAS guanyl nucleotide-releasing protein (RasGRP) or Son-of-Sevenless (SOS) that have been recruited via Grb2 adaptor protein. The GEF allows for RAS to exchange GDP for GTP and thereby becomes activated. A cascade of mitogen-activated protein kinases (MAPK) can begin phosphorylating one another in this order: RAF, MEK1 and ERK. These MAPKs are held at the membrane through a scaffold protein known as kinase suppressor of Ras (KSR). ERK can now enter the nucleus to phosphorylate ELK-1 transcription factor. ELK-1 along with serum response factor can bind to the serum response element in the promotor gene for FOS. Fos is a transcription factor that when combined with Jun will create a heterodimer known as the AP-1 transcription factor required for IL-2 production. The AP-1 transcription factor component Jun can be produced from DAG recruitment of protein kinase C-theta (PKC-theta). PKC-theta will phosphorylate another MAPK known as Jun kinase responsible for making Jun to complete the heterodimer with Fos for AP-1 transcription factor. (Kenneth Murphy 2017; Hendrik Gille 1995)
NF-κB Signaling

Nuclear Factor Kappa B (NF-κB), a homo- and hetero-dimer, is one of the three main transcription factors that becomes activated upon TCR and CD-28 stimulation, as a result of activated PLC-gamma. When functioning normally, NF-κB has the ability to signal in two ways, either through its canonical pathway or through its non-canonical pathway. In the canonical pathway, stimulation is received through various signals provided for example as antigen, microbials, stress agents, cytokines, growth factors, and mitogens. After stimulation of the TCR and subsequent PLC-gamma activation, PKC-theta is recruited to the membrane via DAG. PKC-theta kinase activity phosphorylates scaffold proteins called CARMA1. CARMA1 oligomerizes to form a complex with TRAF-6, a ubiquitin E3 ligase. A kinase called TAK1 phosphorylates another kinase known as inhibitory κB-kinase (IKK) which is responsible for the phosphorylation of inhibitory-κB (IκB). This phosphorylation serves as a signal for ubiquitination and degradation by the proteasome. The IκB, otherwise is responsible for keeping the NF-κB in the cytoplasm, therefore phosphorylation results in transient and immediate translocation of NF-κB to the nucleus. (Kenneth Murphy 2017; Oh and Ghosh 2013).

The second, non-canonical pathway is similar, however it responds to only particular stimuli from the TNFR superfamily ligands LTBR, BAFFR, CD40 and RANK. Upon stimulation NF-κB precursor protein, p100 is ubiquitinated. P100 is an inhibitor-κB-like protein that prevents NF-κB from activating, however, when NF-κB inducing-kinase (NIK) works with IKK, p100 becomes phosphorylated resulting in its ubiquitination and degradation thereby allowing NF-κB2 to move into the nucleus as a
complex with RelB, an NF-kB structurally related family member. When NF-kB moves into the nucleus it has the ability to mediate inflammation through gene activation that causes production of pro-inflammatory cytokines. (Liu et al. 2017)

**CD-28 Co-stimulation**

Another essential aspect of proper T-cell activation comes from co-stimulation of the CD-28(Yungping J. Chlang 2000) receptor and its engagement of B7.1 or B7.2 from the antigen presenting cell (APC), in most cases, the dendritic cell (DC) (Xing and Hogquist 2012). This interaction causes phosphorylation of CD-28 by LCK. Phosphorylation activates PI3K and thereby makes PIP3. PIP3 utilizes its PH domain and associates with 3-phosphoinositide-dependent protein kinase-1 (PDK1) and Akt, where PDK1 activates Akt by phosphorylation. Active Akt enhances T-cell survival and metabolism. Additionally, PIP3 brings associations with ITK, further activating PLC-gamma, that as we discussed above is an essential step towards activation of IL-2 transcription factors. Finally, CD28 co-stimulation via PIP3 recruits another GEF known as Vav(Yungping J. Chlang 2000) that activates Cdc42, a known transcription factor that activates actin polymerization which in turn aids in cellular survival (Kenneth Murphy 2017).

After review of the above signaling cascades that are initiated through MHC:peptide complexes that engage with and activate T-cell receptors as well as co-stimulatory molecules, one can appreciate the many opportunities in which autoimmunity may arise, and now we can explore how a negative regulatory protein known as
UBASH3A, that is increased in a population of individuals with T1Ds, might be contributing to the diseases pathogenesis.

**PRELIMINARY REPORT / PROGRESS REPORT**

As discussed in the Background section, T-cell mediated autoimmunity can occur as a result of aberrant signaling cascades. When dealing with IL-2 it appears that a balance needs to be found that can produce the correct amount of IL-2 for T regulatory cells while avoiding the expansion of T effector cells responsible for self-reactive T-cells (Berretta et al. 2011). When the TCR engages with APCs a series of signals are transduced intracellularly. UBASH3A, or Ubiquitin-associated and Src homology 3 domain containing A (also known as -2, TULA, and CLIP4) (Ge et al. 2017), comprised of a ubiquitin-associated domain (UBA), Src homology-3 (SH3) domain, and a COOH-terminal histidine phosphatase (Ge et al. 2017) is a novel protein involved in aberrant signaling.

Gene-wide-association studies have revealed that there are specific risk alleles that correlate with T1D (Barrett et al. 2009; Grant et al. 2009). These studies have found risk loci on chromosome 21 connected to the UBASH3A gene (Concannon et al. 2008). There is mounting evidence that UBASH3A is involved in many autoimmune diseases (Liu et al. 2015). The researchers have genotyped several thousand families with T1D using genome wide linkage scan data and family-based data to reveal strongly associated single nucleotide polymorphisms within the introns of the UBASH3A gene (Onengut-Gumuscu et al. 2015; Concannon et al. 2008; Grant et al. 2009). UBASH3A is reported to be expressed primarily in CD4+ T-cells (Ge and Concannon 2018) and single
nucleotide polymorphisms in UBASH3A are found to have roles in autoimmunity (Ge et al. 2017). For this reason, UBASH3A has become a potential target for the pathogenesis of diabetes.

A thorough study by Ge et al. revealed the normal function of UBASH3A in the T-cell to be a negative inhibitor of the NF-kB signaling pathway derived from stimulation of the TCR and CD-28 (Ge et al. 2017). The team showed that risk alleles rs11203203 and rs80054410 in the region encoding for UBASH3A in T1D patients, correlate with an increased expression of UBASH3A thereby inhibiting the contribution of the NF-kB pathway to produce IL-2. Their work began with a focus on determining where along the NF-kB pathway does UBASH3A lie. Their team generated a cell line deficient in UBASH3A using CRISPR/Cas9 to demonstrate increases in IL-2 production. Western blot analysis showed that UBASH3A null cells had more NF-kB p65 localizing in the nucleus after TCR and CD-28 stimulation. Next, they sought to determine the effects of the signaling components of the pathway to reveal deficient cells had increased phosphorylation levels of IKK complex while producing no changes in PKC-theta, TAK1 or TRAF6 activation. This analysis suggests UBASH3A is suppressing activation of IKK complex specifically (Figure 2).
Figure 2. Signal cascade involving UBASH3A negatively regulating IKK complex.

Of note, the group also confirmed that UBASH3A is able to successfully bind to ubiquitin chains on K48 and K63 of IkB (Ge et al. 2017). Lysine ubiquitination on 63 is the target of TRAF6 on TAK1 and K48 is the target for IKK on IkB, both ubiquitination processes that are essential for the translocation of NF-kB to the nucleus. The study continued their findings into CD4+ T-cells from healthy patients and T1D patients. T1D risk alleles result in higher levels of UBASH3A that result in less IL-2 production. This loss in IL-2 contributes to the inability of CD4+ T cells to differentiate into T regulatory cells required to induce tolerance to self-antigen (Seelig et al. 2018). These findings should be tested in a humanized animal model to determine if the knockout of UBASH3A in human T-cells results in an increase of T regulatory cells while also noting any changes of T effector cells. Chen et al. studied the role of UBASH3A in a Non-obese
Diabetic murine model by generating mutant null-UBASH3A to determine what deficiency in the protein might look like (Chen et al. 2020). The study revealed increased T-cell activation, confirming that UBASH3A has normal roles as a negative regulator. Unfortunately, these mutations still did not replicate the mutations seen in the human T-cells of T1D patients and therefore more work should be done to specifically replicate the polymorphisms found in the genome-wide association study.

In a separate study, UBASH3A was investigated for its role in TCR activation through its interaction with ZAP-70 and Syk (San Luis et al. 2011). Syk is a protein-tyrosine kinase (Agrawal, Carpino, and Tsygankov 2008). The results of this investigation revealed that UBASH3A may also have roles in phosphatase activity, responsible for the dephosphorylation of ZAP-70 and Syk, both of which are involved in the activation of the TCR through phosphorylation of their ITAMs. Although, the phosphorylation of these molecules appeared only to be slightly changed in UBASH3A deficient mice, suggesting that phosphatase activity may not be the primary role for UBASH3A. However, if the patient is harboring the risk alleles associated with increased amounts of UBASH3A, one might wonder if this increased phosphatase activity contributes to the lack of IL-2 production. Further studies will need to be conducted to glean this potential role in pathogenesis.

Other researchers have performed GST-pulldown assays to show the interactions of Cbl or Casitas B-lineage lymphoma, a negative regulator in T-cell activation, and UBASH3A (Kowanetz et al. 2004; Qiao et al. 2008). Cbl-b, a member of the Cbl family, is an E3 ligase that can ubiquitinate target proteins for proteasome or lysosome degradation, whereby a ubiquitin is attached by its carboxy-terminal glycine to lysine...
residues. Lysine 48 linkage usually results in proteasome degradation while K63 can result in lysosomal degradation. Research has shown that Cbl-b suppresses the NF-kB pathway and that mutations in Cbl-b may contribute to T1D (Qiao et al. 2008; Yokoi et al. 2002). One example of this interaction is through the SH3 domain of UBASH3A that binds with Cbl-b which can negatively regulate CD-28 co-stimulation(Ohashi 2002), reducing the activation of the T-cell further (Ge et al. 2019). For this reason, it has been suggested that UBASH3A may work synergistically with Cbl-b which has been identified as a potential contributor to the development of autoimmunity in general as well as in T1D (Ge et al. 2019).

Type-1 diabetes is diagnosed by the presence of autoantibodies in sera that recognize and destroy insulin producing pancreatic Beta-cells in the islets of Langerhans (Bluestone JA 2010). The loss of insulin results in the inability to regulate blood glucose levels which can lead to several pathologies and death. Treatment for T1D is to provide insulin on a daily basis, however, this does not mitigate the underlying conditions, the harmful autoantigens. A more personalized approach may be required for each individual, but genetic screening for risk alleles associated with the upregulation of UBASH3A expression would seem a prudent first step in determining if the T1D patient is carrying mutations for the UBASH3A gene. If risk alleles are present, a targeted approach to silence UBASH3A with RNA interference because UBASH3A is mostly expressed in CD4+ T cells only, but no studies currently exist for this approach.

To date, T1D remains a difficult autoimmune disease to cure (albeit relative to other autoimmune diseases, easy to treat if one can overlook the consumer cost of the treatment) however, studies are increasing our knowledge of the many ways in which the
disease may arise. It has become apparent that in cases such as these, a genetic screen is paving the way towards a more specific approach that will allow for a step towards precision medicine (Nyaga et al. 2018). In this case, the precision medicine would require more basic and fundamental research to determine the effects of specific inhibition of mutated genes contributing to the dysfunctional signaling cascade.

One route used to treat T1D may be to induce tolerance by providing the missing IL-2 cytokine to allow CD4+ T-cells to differentiate into T regulatory cells. The difficulty for this therapy arises in elucidation of the optimal balance of IL-2 administered due to the cytokines polytropic nature (Rosenzwajg et al. 2014). A study with NOD mice provided IL-2 and were able to show anergy (Godoy et al. 2019). Clinical trials have been conducted to perform this replacement therapy in a dose dependent manner that allow for a recombinant human IL-2 to be administered safely and effectively (Seelig et al. 2018). Replacement therapy was able to provide a balanced approach that allowed for T regulatory cells to increase while keeping T effector cells at bay. The largest risk for this study was noted as only adverse reactions to the administration site.

In this study, the aim is to continue the research strides that have been made in the field by investigating the therapeutic potential of micro-RNA, small non-coding RNA that can silence gene expression and reduce protein levels in the cell. The hope here is to apply the fundamental research from this study towards future micro-RNA therapeutic options for type-1 diabetic patients harboring the risk alleles that increase the UBASH3A protein. Micro-RNA has significant roles in gene regulation at the level of post-transcription, targeting the messenger RNA. This gene regulation can thereby degrade and inhibit protein. (Lam et al. 2015)
The fundamental purpose of this project is to investigate the therapeutic potential of microRNA to reduce the expression of UBASH3A in CD4+ T cells that have been genetically modified to have increased UBASH3A (mimicking the alterations seen in T1D patients harboring the risk alleles known to increase UBASH3A and allow for IL-2 cytokine production after T cell stimulation. This study will not currently utilize cells that have the single nucleotide polymorphisms, as it aims to continue the work done by the Ge laboratory.

**Aim 1: Determine if stimulated T cells have an effect on UBASH3A expression and utilize miRNA specific to UBASH3A messenger RNA to decrease total protein in stimulated CD4+ T cells and unstimulated CD4+ T cells.**

To begin the project, CD4+ T cells of the Jurkat cell line will be cultured, in addition to the gifted modified T cell clones from the Ge laboratory that provide examples of UBASH3A over and under expression. Clones have previously been validated in the Ge study to provide confirmation of expression levels of the protein. Cell groups will be starved of serum for 4-hours to synchronize the cell cycles and then stimulated for 24-hours in 96-well plates containing with 10ug/mL each of anti-CD3 (clone OKT3, BioLegend) and anti-CD28 (clone CD28.2, BioLegend) together. Cells will be prepared for protein and mRNA isolation through use of lysing buffer and centrifuged Supernatant will be removed for protein detection. For mRNA isolation, in preparation of RT-PCR we will purify the RNA from the culture and utilize DNase to degrade the DNA from the sample. Two primers (Forward:
CTTCGAGGCCTACCGTTGC, Reverse: CAGGGGAAATTCAGGTCTGGC) will be used specific to UBASH3A mRNA as well as reverse transcriptase, DNA polymerase and the 4-deoxyribonucleoside triphosphates needed for DNA synthesis. Quantification will rely on the relationship between the rate at which the PCR product is generated and original concentration of mRNA in the sample. For protein analysis, SDS-PAGE will separate the protein via gel electrophoresis. The proteins will move through the gel matrix with an electrical charge. Transfer after separation to a solid support membrane will occur Western blotting analysis will be performed to detect the amounts of UBASH3A protein in each group. Anti-UBASH3A antibody (ab197168) (Abcam) will be used to detect UBASH3A. Anti-GAPDH will be used as a control for the western blot analysis (ab8245). A reporter tagged secondary antibody specific to the Fc region of the primary antibody will be used for visualization.

Administration of miRNA (hsa-miR-425-3p; accession number; Product ID: HmiT057985 UBASH3A, Genecopeia – luciferase reporter clone and validation assay) will be applied as naked plasmids to T cells cultures targeting the Homo sapiens UBASH3A mRNA (NM_001243467.1) (Figure 3).

<table>
<thead>
<tr>
<th>Position 90-96 of UBASH3A 3 'UTR hsa-miR-425-5p</th>
<th>Predicted consequential pairing of target region (top) and miRNA (bottom)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Position 90-96</td>
<td>5 '... GAGCCACGGUGAUGU UGCAUA A ...</td>
</tr>
<tr>
<td>hsa-miR-425-5p</td>
<td>3 'AGUUGCCUCUAGC ACAGUA A</td>
</tr>
</tbody>
</table>

**Figure 3.** Sequence alignment of UBASH3A miRNA hsa-miR-425-3p; accession number to UBASH3A mRNA provided by Target-Scan-Human.

Administration of the miRNA will occur at minute-1. Timepoint 0-minute will therefore serve as a control, representing untreated T cells with no miRNA. UBASH3A overexpressing cell clones and knockout Jurkat cells targeting exon 2 of the UBASH3A
gene generated by CRISPR-Cas9 editing will be provided from the Ge laboratory as a gift and exposed to the micro-RNA. Cells will be prepared for mRNA and protein analysis as described above. A transfer after separation to a solid support membrane will occur and Western blotting analysis will be performed to detect the amounts of UBASH3A protein in each group at timepoints of 0 minutes, 30 minutes, 60 minutes and 90 minutes. Anti-UBASH3A antibody (ab197168) (Abcam) will be used to detect UBASH3A. Anti-GAPDH will be used as a control for the western blot analysis (ab8245). A reporter tagged secondary antibody specific to the Fc region of the primary antibody will be used for visualization. FIJI imaging software will be utilized for image quantification and GraphPad Prism for statistical t-test analysis. Expected results for the Jurkat T cells (unmodified) are to detect UBASH3A at time 0-mins and gradually fade to no detection over the time series. Expected results for the UBASH3A Knockout T cells will to see no detection of UBASH3A at any time point, including 0-mins. Expected results for the overexpressing UBASH3A T cells is to see a stronger band of UBASH3A detection at 0-minutes that gradually fades over the time series, but less than that of the unmodified Jurkat T cells. All experiments will be run in triplicate. Cell viability assay (Cell Titer-Glo® 2.0 Cell Viability Assay) will do used after all cell stimulations where light detection can occur as a result of ATP use in healthy cells.

**Aim 2: Determination of IL-2 production of stimulated and miRNA-treated-T-cells**

After T cells have been provided miRNA and UBASH3A protein expression determined, the cell groups will be starved of serum for 4 hours to synchronize the cell cycles and then stimulated for 24 hours in 96-well plates containing with 10µg/mL each of anti-CD3 (clone OKT3, BioLegend) and anti-CD28 (clone CD28.2, BioLegend)
together. Time point 0-minutes (no miRNA) will serve as a control for each group (Jurkat, UBASH3A KO and UBASH3A overexpression). Cells will be analyzed for IL-2 via ELISA Deluxe kit (BioLegend) from an appropriate time point after administration of miRNA shows reduction in UBASH3A protein expression (expecting to use 0mins, 30, 60, and 90-minutes time-points). Sandwich ELISA method will be used with anti-IL-2 coated to the wells. For detection, a biotinylated antibody for IL-2 will be added followed with streptavidin-horseradish peroxidase for binding. Color changes are produced by the addition of chromogenic enzyme substrate 3,3',5,5' tetramethylbenzidine (TMB). Quantification of ELISA will occur via color quantification with the creation of a standard curve for every plate to determine the value of the unknown. Statistical analysis (t-test) performed with GraphPad Prism to determine significance. Results expected are an increase in the cytokine IL-2 at all time points (30, 60, and 90-minutes) for the Jurkat cells and the cells overexpressing UBASH3A, when compared to time 0-minutes. Results for the KO UBASH3A, it is expected that there should be no change in the total IL-2 at 30, 60 and 90-minutes compared to 0-minutes. All experiments will be run in triplicate. Cell viability assay (Cell Titer-Glo® 2.0 Cell Viability Assay) will do used after all cell stimulations where light detection can occur as a result of ATP use in healthy cells.
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