ANTI-GLUTEN SYNTHETIC NOTCH RECEPTOR T CELLS FOR THE TREATMENT OF CELIAC DISEASE

by

Benjamin Andrew Clark

B.S. (Salisbury University) 2013

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

Accepted:

Rebecca Erwin-Cohen, Ph.D. Committee Member	Ann Boyd, Ph.D. Director, Biomedical Science Program
Ann Boyd, Ph.D. Committee Member	
Craig Laufer, Ph.D. Project Adviser	April Boulton, Ph.D. Dean of the Graduate School

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DEDICATION

This is dedicated to sufferers of autoimmune diseases. For which there is an enormous unmet need for treatments and relief. And to my parents who were immensely patient through this whole process.

ACKNOWLEDGEMENTS

Thank you to the reading committee Drs. Rebecca Erwin-Cohen, Craig Laufer, and Ann Boyd. I would also like to thank Drs. Rebecca Erwin-Cohen, Kevin Tosh, and all my friends for giving me a real push to overcome setbacks and complete this challenge.

Mock Grant Application

Modeled after Department of Health and Human Services
Public Health Services
(based on Form PHS 398)

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Type	Activity	Version – HCBMS.011712
Review Group		Formerly
Council/Board (Month, Year)		Date Received

1. TITLE OF PROJECT *(Do not exceed 81 characters, including spaces and punctuation.)*
Customized Synthetic Notch Receptor T Cells for the Treatment of Celiac Disease

2. RESPONSE TO SPECIFIC REQUEST FOR APPLICATIONS OR PROGRAM ANNOUNCEMENT OR SOLICITATION ☒ NO ☐ YES
(If "Yes," state number and title)
Number: Title:

3. PROGRAM DIRECTOR/PRINCIPAL INVESTIGATOR — **New Investigator** ☐ **No** ☒ **Yes**

3a. NAME (Last, first, middle)
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3b. DEGREE(S)
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3h. eRA Commons User Name
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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)*		7. COSTS REQUESTED FOR INITIAL BUDGET PERIOD		8. COSTS REQUESTED FOR PROPOSED PERIOD OF SUPPORT	
From	Through	7a. Direct Costs ($)	7b. Total Costs ($)	8a. Direct Costs ($)	8b. Total Costs ($)
1/1/2021	1/1/2022				

9. APPLICANT ORGANIZATION
Name: Benjamin Clark
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401 Rosemont Ave
Frederick, MD 21701

10. TYPE OF ORGANIZATION
Public: → ☐ Federal ☐ State ☐ Local
Private: → ☒ Private Nonprofit
For-profit: → ☐ General ☐ Small Business
☐ 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
4/16/2021

Face Page

PROJECT SUMMARY (See instructions):

Celiac disease is a complex autoimmune disorder affecting approximately 0.5-1.7% of the general population. Currently, there are no treatments for Celiac disease aside from a lifelong adherence to a strict gluten-free diet. There is a largely unmet need for alternative treatment options for patients due to the difficulty in maintaining such a diet. Recent advancements in understanding the underlying Celiac disease pathology have identified IL-15 as a key player driving disease morbidity. Synthetic Notch receptors (synNotch) are chimeric Notch receptors in which the extracellular sensing domain and intracellular response domain can be changed to sense and respond to a custom signal. The customizability of these receptors allows for the engineering of cells that can sense a chosen target and induce a chosen response. Here, I propose engineering a synNotch receptor capable of sensing immunogenic gluten peptides and linking its function to the production of the peptide, BNZ-2. BNZ-2 is a γc cytokine receptor antagonist that selectively inhibits IL-15 and IL-21 signaling without impacting IL-2 signaling. I propose engineering T cells to express this synNotch BNZ-2 receptor to disrupt improper IL-15 upregulation driving Celiac disease morbidity.

RELEVANCE (See instructions):

IL-15 is a key driver of disease morbidity in Celiac disease. This study will evaluate the feasibility of using SynNotch receptors linked to BNZ-2 production to disrupt the effect of IL-15 in Celiac disease.

PROJECT/PERFORMANCE SITE(S) (if additional space is needed, use Project/Performance Site Format Page)

Project/Performance Site Primary Location			
Organizational Name: Department of Biology, Hood College			
DUNS:			
Street 1: 401 Rosemont Ave		Street 2:	
City: Frederick	County: Frederick		State: MD
Province:	Country: United States		Zip/Postal Code: 21702
Project/Performance Site Congressional Districts: 6th Congressional District			

Additional Project/Performance Site Location			
Organizational Name:			
DUNS:			
Street 1:		Street 2:	
City:	County:		State:
Province:	Country:		Zip/Postal Code:
Project/Performance Site Congressional Districts:			

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	POSITION TITLE
Benjamin Andrew Clark	B.S.
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.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
Salisbury University	B.S.	2013	Biology
Hood College, Frederick, MD	M.S.	2021 (anticipated)	Biomedical Science

A. Positions and Honors

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

C. Research Support

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:

AstraZeneca Facility: Fully available. Full BSL-2 capabilities. Full access to everything needed to complete this proposal.

Clinical:

N/A

Animal:

N/A

Computer:

AstraZeneca Facility: Fully equipped with all necessary software for data analysis.

Office:

AstraZeneca Facility: For general use.

Other:

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:

- BD FACS Symphony – for flow cytometry
- BD FACS Aria II – for cell sorting
- Centrifuges – for cell isolations and transductions
- Biosafety Cabinets – for cell culture
- Computers with all necessary software for analysis
- Gel electrophoresis equipment – for western blot
- Incubators
- Cell counters – for measuring T cell expansion

The following consumables will be purchased with grant funding:

- Leukopaks – for T cell isolation
- Plasmids – for synNotch receptors and response elements
- pPackH1 HIV Lentivector Packaging kits – for lentivirus production
- Dynabeads Human T-Activator CD3/CD28 – for T cell stimulation
- Polybrene – for transductions
- Antibodies – for flow cytometry and western blot
- Fixation/Permeabilization Kit – for intracellular staining
- Cytokines – for T cell culture and stimulation

SPECIFIC AIMS

Specific Aim 1: Design a Synthetic Notch (synNotch) Receptor Capable of Sensing Immunogenic Gluten Peptides and Link its Activity to BNZ-2 Expression.

Using the approach described by Roybal *et al.*, a synNotch receptor capable of sensing immunogenic gluten peptides linked to BNZ-2 production will be created, cloned into a modified pHR_PGK (Addgene #79125) backbone, and packaged into a VSV-G-pseudotype lentivirus using a pPACKH1 HIV Lentivector Packaging Kit (System Biosciences, #LV500A-1). Response elements containing BNZ-2 and mCherry will be produced using a similar pHR_PGK (Addgene #79123) backbone.

Specific Aim 2: **Transduce primary T cells with synNotch receptor and assess *in vitro* characteristics.**

The SynNotch receptor vector and response elements will be transduced into primary T cells isolated from a healthy donor (Hemacare Leukopak). A) Transduced T cells will be assessed for viability, expansion, and potential toxicity. B) transduction efficiency, and phenotype will be assessed via flow cytometry. Modified T cells will be sorted based on anti-myc staining and blue fluorescent protein (BFP) expression, then C) co-cultured with HEK-293 cells stably expressing gluten peptides containing the target epitope (Steinsbø *et al.* 2014). Cellular response will be measured by assessment of mCherry production. D) Following co-culture, T cells stimulated with IL-15 and IL-21 will be treated with synNotch BNZ-2 T cell supernatant. Flow cytometry and western blot will be carried out post treatment to analyze the effect of BNZ-2 secreted by synNotch BNZ-2 T cells on IL-15 and IL-21 STAT signaling in IL-15 and IL-21 stimulated T cells (Ciszewski *et al.* 2020).

BACKGROUND AND SIGNIFICANCE

Introduction

Celiac disease (CD) is an autoimmune disorder that primarily affects the small intestine in genetically pre-disposed individuals triggered by the ingestion of gluten, a protein found in wheat, rye, and barley (Kupfer and Jabri 2012). It results from an improper T-cell mediated immune response to ingested gluten that results in significant immune-mediated damage to the small intestine (Kupfer and Jabri 2012). CD is among the most common autoimmune disorders, with projections ranging from 0.5-1.7% of the general global population being affected (Caio *et al.* 2019; Singh *et al.* 2018; Tye-Din *et al.* 2018.). Incidence of the disease has increased 4- to 5-fold over the past three to four decades (Rubin and Crowe 2020). The factors driving the increase in the number of cases are not fully elucidated. However, an important factor of the increasing number of diagnoses is the fact that there has been a significant increase in awareness of the disease and improvement in the methods used for diagnosing CD (Kelly *et al.* 2015, Rubio-Tapia *et al.* 2009). Given the increased awareness and improved diagnostic methods, many factors regarding the disease's prevalence are poorly understood. For instance, the availability and abundance of the cereal crops containing high levels of gluten has dramatically increased over recent years and these cereals are heavily consumed in western diets (Rubio-Tapia *et al.* 2009). Along with the increased usage and abundance, the genetics of these gluten-containing cereals have been heavily altered because of industrial farming, food processing, and modification of gluten peptides (Rubio-Tapia *et al.* 2009). How any of these practices impact genetically susceptible people, while interesting, is difficult to test experimentally (Rubio-Tapia *et al.* 2009).

CD is a systemic disease. The disease can affect almost every part of the body. Aside from the digestive system, the disease most commonly impacts the dermatologic, hematologic, neurologic, musculoskeletal, endocrine, and reproductive systems (Rubin and Crowe 2020). Primary symptoms revolve around gastrointestinal distress, including diarrhea, vomiting, abdominal pain, abdominal distension, weight loss, failure to gain weight, and malabsorption (Kelly *et al.* 2015). Untreated CD leads to increased severity of the symptoms as the immune-mediated destruction of the small intestinal epithelium continues, causing villus atrophy (Caio *et al.* 2019; Kupfer and Jabri 2012). Nutrient deficiencies are common due to disrupted absorption of nutrients in the intestines and are often monitored in CD (Kelly *et al.* 2015). Major deficiencies include iron, vitamin D, vitamin B12, vitamin B6, folate, and zinc (Kelly *et al.* 2015). Less common symptoms include headaches, depression, paresthesia, joint symptoms, rashes, and fatigue (Rubin and Crowe 2020). Secondary symptoms also increase in severity if CD is left untreated (Rubin and Crowe 2020). CD is associated with other autoimmune disorders, particularly type 1 diabetes mellitus, autoimmune thyroid disease (Hashimoto's thyroiditis and Graves' disease) (Caio *et al.* 2019; Kupfer and Jabri 2012).

CD is clinically characterized by the presence of specific serologic markers and histological changes in the small intestinal mucosa (Kelly *et al.* 2015). Presence of IgA antibodies against tissue transglutaminase (tTG), endomysium (EmA), and IgG against deamidated gliadin peptide (DGP) in patient serum are highly indicative of CD (Caio *et al.* 2019; Kelly *et al.* 2015; Kupfer and Jabri 2012; Sandström *et al.* 2013; Tye-Din *et al.* 2018). In addition to serology, endoscopic biopsies are performed, and findings most commonly include blunting or atrophy of intestinal villi, crypt hyperplasia, and an increase

in the number of intra-epithelial lymphocytes (IELs) (Caio *et al.* 2019; Kelly *et al.* 2015; Kupfer and Jabri 2012; Tye-Din *et al.* 2018.). The combined results of serology and diagnostic biopsy are currently the standard for diagnosing CD as neither the serology nor biopsy alone is enough of a basis for diagnosis (Kelly *et al.* 2015).

The only treatment for CD is lifelong adherence to a gluten free diet (GFD) (Tye-Din *et al.* 2018; Caio *et al.* 2019). Although the symptoms can be severe, most patients following a strict GFD will have near normal lifespans. Adherence to a GFD relieves symptoms and allows the intestines to heal, but if gluten is re-incorporated into a patient's diet, even accidentally, symptoms will resume (Caio *et al.* 2019; Kupfer and Jabri 2012; Malamut and Cellier 2018; Tye-Din *et al.* 2018). Accidental gluten exposure is extremely common and hard to prevent, leading to lessened quality of life for CD patients (Itzlinger *et al.* 2018; Kelly *et al.* 2015). Sharing food preparation areas with gluten containing foods or utilizing the same equipment for gluten containing and gluten free foods is enough to trigger a response in CD patients (Itzlinger *et al.* 2018; Kelly *et al.* 2015; Yoosuf and Makharia 2019). A full CD immune response can be triggered with as little as a 50 mg of gluten or $1/100^{th}$ of a slice of bread (Itzlinger *et al.* 2018; Kelly *et al.* 2015; Yoosuf and Makharia 2019). Additionally, gluten free foods are almost always more expensive and often less available than gluten containing foods, leading to further quality of life issues (Itzlinger *et al.* 2018; Kelly *et al.* 2015). Patients with undiagnosed CD or not adhering to the GFD will have more severe symptoms and increased mortality (Rubio-Tapia *et al.* 2009; Itzlinger *et al.* 2018). Undiagnosed CD being associated with a 4-fold increased risk of death compared to patients without CD (Rubio-Tapia *et al.* 2009).

A small subset of patients of with CD will have the disease progress into refractory celiac disease (RCD) (Malamut and Cellier 2018, Malamut *et al.* 2019). RCD is diagnosed when the patient's condition does not improve with GFD. The incidence of RCD is not known but suspected to be around 1.5% of diagnosed CD cases (Malamut and Cellier 2018). In RCD, the immune response to gluten does not cease when gluten is removed from the patient's diet (Malamut and Cellier 2018; Malamut *et al.* 2019). There are two types of RCD, type I and type II with type I appearing to be more common and less severe (Malamut and Cellier 2018). RCD type I presents similarly to active CD but with increased intestinal damage and increased normal IELs. RCD type II has a more specific diagnosis due to significant alteration of IEL phenotype in the intestines (Malamut and Cellier 2018). Three different combined techniques identify abnormal IELs phenotypes associated with RCD type II: >25% of CD103+ or CD45+ IELs lacking CD3 T-cell receptor complexes, >50% of IELs expressing intracellular CD3ε but no CD8, or presence of clonal rearrangement of the gamma chain T-cell receptor (Malamut and Cellier 2018).

Of the two types, RCD type II is associated with poor prognosis with a 5-year survival rate of 44-58% due to more severe malnutrition and increased chance for malignancy (Malamut and Cellier 2018). Type I RCD carries a higher risk of mortality than uncomplicated CD, but overall follows the same trends in terms of symptoms as active uncomplicated CD (Malamut and Cellier 2018; Malamut *et al.* 2019; Singh 2018). The presence of CD or RCD increases the risk of developing enteropathy-associated T-cell lymphoma (EATL) and EATL can occur in any CD patient (Malamut and Cellier 2018). RCD type II confers the highest risk at 33-52% of patients, followed by type I at ~14% and uncomplicated CD where the risk is much less (Malamut and Cellier 2018). Expression of

CD103 and identical TCR gamma chain clonality indicate that the origin of the malignancy lies with the RCD type II IELs (Malamut and Cellier 2018). EATL is an extremely aggressive malignancy with a 20-25% 5-year survival rate with increasing likelihood of survival in patients with less severe, uncomplicated CD (Malamut and Cellier 2018). The potential complications of CD coupled with increased prevalence highlight the need for new treatments to supplement or outright cure CD.

Risk Factors for CD

Genetics play a strong role in CD pathogenesis. Class II human leukocyte antigen (HLA) receptors, such as HLA-DQ, are composed of αβ heterodimers encoded by their respectively named genes (*i.e.*, HLA-DQA1 and HLA-DQB1). Located on antigen presenting cells (APCs), the αβ heterodimer is a cell surface receptor that binds to and presents peptides 8 to 20 amino acids long. These peptides are typically taken up by pinocytosis, macrocytosis, or endocytosis of extracellular debris and presented to T cells. In the case of CD, gluten proteins taken up by APCs are presented as peptides by the HLA molecule to T cells which recognize and become activated by the peptide:HLA receptor complex (Kupfer and Jabri 2012).

Nearly all confirmed CD patients test positively for HLA-DQ2, HLA-DQ8, or both (Caio *et al.* 2019; Kelly *et al.* 2015; Kupfer and Jabri 2012; Megiorni *et al.* 2009; Tye-Din *et al.* 2018). Specific alleles of HLA-DQB1 and HLA-DQA1 confer differing levels of risk for developing CD. CD is heavily influenced by the gene dose effect, a phenomenon where more copies of a gene can significantly influence the amount of gene product. In the case of CD, this gene dose effect has been associated with higher risk of developing

disease, earlier onset of disease, and more severe symptoms (Caio *et al.* 2019; Kupfer and Jabri 2012; Megiorni *et al.* 2009; Tye-Din *et al.* 2018; Vader *et al.* 2003).

HLA-DQB1*02 and HLA-DQA1*05, which form HLA-DQ2.5, are the primary alleles associated with CD (Kupfer and Jabri 2012; Megiorni *et al.* 2009). Through the gene dose effect, homozygotes for DQB1*02 have a 5-fold increased risk of developing the disease over heterozygotes only carrying one allele (Megiorni *et al.* 2009; Tye-Din *et al.* 2018). Homozygosity of DQB1*02 accounts for 25% of CD cases (Megiorni *et al.* 2009). HLA-DQB1*02/DQA1*05 is the most common HLA configuration, accounting for up to 50% of CD cases (Megiorni *et al.* 2009). HLA-DQ2.2 (DQB1*02/DQA1*02), which is highly homologous to HLA-DQ2.5, does not confer as much risk of developing CD, due to decreased stability of bound peptides (Bodd *et al.* 2012; Kupfer and Jabri 2012). HLA-DQ8 is encoded by DQA1*03:01 and DQB1*03:02 and has a prevalence of 5-10%. HLA-DQ8 along with HLA-DQ2 carries the highest risk of CD (Megiorni *et al.* 2009). As with DQ2, DQ8 follows the same gene dose principle with homozygosity conferring a higher risk (Megiorni *et al.* 2009). Only 0.9% of CD patients carry none of the aforementioned genes (Megiorni *et al.* 2009).

Non-HLA genes also contribute to CD risk, though not nearly as much. More than 70 non-HLA genes have collectively been implicated in in CD risk (Tye-Din *et al.* 2018; Sharma *et al.* 2016). These genes encode proteins heavily involved in the immune system and affect T and B cell activation, chemokine receptor activity, cell migration, cytokine binding, stress pathways, and innate immunity (Kupfer and Jabri 2012; Sharma *et al.* 2016; Tye-Din *et al.* 2018). The fact that the genes affecting development of CD are nearly all

immune based, highlights the importance of immune dysregulation in disease manifestation.

HLA-DQ compatibility, however, is not the only factor involved in developing CD. HLA-DQ2 and HLA-DQ8 are both common among the general population with 25-35% carrying them, but only 3% of these individuals will develop the disease (Caio *et al.* 2019; Singh 2018). There are environmental factors that play a role in disease development. There is a high prevalence of CD observed among populations living in areas with high consumption of wheat products. While, intake of gluten is necessary for CD to develop, it does not explain why not all genetically predisposed individuals consuming gluten develop CD and why the disease can develop later in life despite many decades of gluten intake (Kupfer and Jabri 2012; Tye-Din *et al.* 2018).

Several studies potentially indicate that viral gastrointestinal infections, including rotavirus, reovirus, and adenovirus, can play a role in CD development (Bouziat *et al.* 2017; Kupfer and Jabri 2012; Tye-Din *et al.* 2018; Zanoni *et al.* 2006). How infections influence CD development remains unclear. Current research indicates that infection could increase gastrointestinal permeability or elevate tTG expression, leading to more immunogenic gluten peptides. Furthermore, molecular mimicry is possible if foreign antigens share structural similarities to self-antigens and therefore activate autoreactive T or B cells.

The microbiome has been implicated in a range of autoimmune and inflammatory diseases including CD. Clinical studies have described differences between healthy individuals and individuals with active or treated CD (Caio *et al.* 2019; Kupfer and Jabri

2012; Tye-Din *et al.* 2018). The presence of certain rod-shaped bacteria (*Clostridium spp*, *Prevotella spp*, and *Actinomyces spp*) in duodenal biopsies was highly associated with CD (Kupfer and Jabri 2012; Tye-Din *et al.* 2018). Genetically at-risk individuals were shown to harbor different microbial composition than those with a lower risk (Tye-Din *et al.* 2018). Whether or not the altered microbiome is a cause of CD or caused by CD remains poorly understood.

Disease Mechanism, Innate Immune Response, Cellular Immune Response

Gluten is the primary antigen driving CD morbidity (Caio *et al.* 2019; Kelly *et al.* 2015; Kupfer and Jabri 2012; Tye-Din *et al.* 2018). Gluten is a general term used to describe the disease activating peptides found in wheat. The primary component of wheat gluten responsible for disease is a family of closely related proline and glutamine-rich proteins called gliadins (Kim *et al.* 2004; Shan *et al.* 2002). Similar proteins found in barley (hordein), and rye (secalin) are closely related to gliadin and are collectively termed prolamins (Kim *et al.* 2004; Tye-Din *et al.* 2018;). Due to the presence of large amounts of proline and glutamine residues, these proteins are largely resistant to gastric, pancreatic, and intestinal proteases (Kim *et al.* 2004; Shan *et al.* 2002). Gluten T cell epitopes largely cluster to these regions of high proline and glutamine (Kim *et al.* 2004; Tye-Din *et al.* 2018). Typically, responses against food proteins are stopped via oral tolerance. Why this tolerance breaks down for gluten in CD is unknown (Tye-Din *et al.* 2018).

In CD, loss of intestinal barrier integrity has been noted and is critical in pathogenesis due to gluten trafficking to the lamina propria and interacting with immune cells. Gliadin has been shown to cause an increase in intercellular tight junction

permeability of intestinal epithelial cells linked to the release of zonulin, a protein that facilitates tight junction disassembly (Caio *et al.* 2019; Lammers *et al.* 2008). Gliadin peptides binding to chemokine receptor CXCR3 causing an MyD88-dependent release of zonulin, leading to tight junction disassembly (Lammers *et al.* 2008). Additionally, transferrin receptor CD71 has been shown to be overexpressed on the luminal side of the intestinal epithelium in active CD (Caio *et al.* 2019; Matysiak-Budnik *et al.* 2008). Normally, CD71 is localized to the to the basolateral membrane of epithelial cells (Matysiak-Budnik *et al.* 2008). Due to CD71 being overexpressed on the luminal side, CD71 is able to facilitate apical to basal retrotranscytosis of IgA-gliadin complexes to the lamina propria, feeding into intestinal inflammation further (Caio *et al.* 2019; Matysiak-Budnik *et al.* 2008).

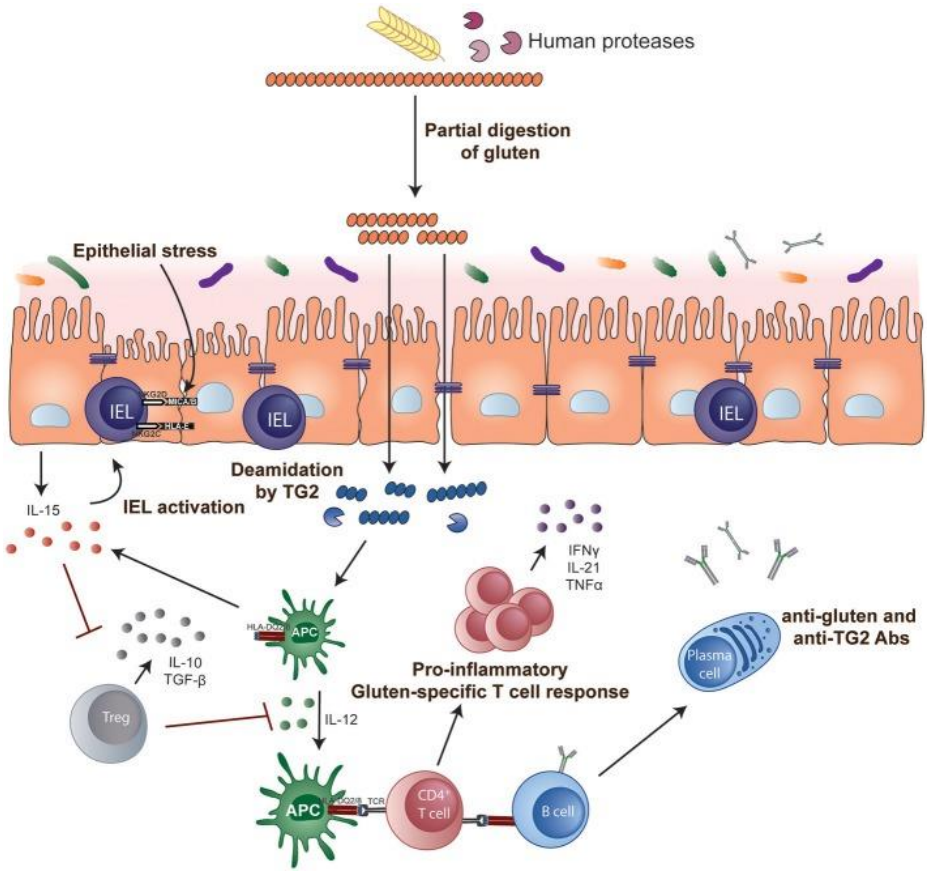

Figure 1. Summary of key steps in the pathogenesis of Celiac disease.
Gluten peptides resist gastrointestinal degradation. Peptides are trafficked into the lamina propria from the lumen through disassembled tight junctions. tTG catalyzes the deamidation of gluten peptides, allowing them to bind more efficiently to HLA-DQ2 or -DQ8 molecules on APCs. Activated gluten-specific CD4+ T cells begin secreting pro-inflammatory cytokines such as IFN-γ and IL-21. These cytokines promote the activation of IELs and B cells. Activated IELs take on an NK-like phenotype that allow them to mediate destruction of enterocytes expressing stress signals. IL-15 renders effecter T cells resistant to the suppressive effects of regulatory T cells and endows mucosal dendritic cells with inflammatory properties promoting pro-inflammatory responses. Figure adapted from Tye-Din *et al.* 2018.

Once trafficked, gliadin peptides trigger the upregulation of cytokines notably interleukin 15 (IL-15) (Caio *et al.* 2019). These cytokines activate dendritic cells, upregulate stress markers on epithelial cells, and license IELs to kill intestinal epithelial cells (Abadie and Jabri 2014; Caio *et al.* 2019). IL-15 is overexpressed not only by epithelial cells but by innate immune cells as well and will often stay elevated after removal of gluten (Abadie and Jabri 2014). The mechanisms responsible for maintaining IL-15 upregulation on this scale have not been fully elucidated (Abadie and Jabri 2014).

To initiate the improper immune response, HLA-DQ2 or -DQ8 bind gliadin peptides that have been deamidated by tTG in the small intestinal epithelium. The deamidation process converts selected glutamine residues to negatively charged glutamic acid residues which facilitate stable binding to HLA-DQ2 or -DQ8 expressed on the surface of local APCs (Kim *et al.* 2004; Kupfer and Jabri 2012; Tye-Din *et al.* 2018). Deamidated gliadin peptides are presented to gluten specific, HLA-DQ2/-DQ8-antigen restricted CD4+ T helper cells, initiating a response. The gluten specific CD4+ T cells exhibit a Th1 phenotype characterized by secretion of large amounts of pro-inflammatory cytokines including IFNγ, tumor necrosis factor α (TNFα), and IL-21 (Caio *et al.* 2019; Tye-Din *et*

al. 2018). Furthermore, tTG specific B cell activation and subsequent production of anti-tTG antibodies is thought to be mediated by gluten specific T cells. This is potentially due to tTG-specific B cells picking up tTG-gluten complexes and presenting them to the gluten specific T cells (Malamut *et al.* 2019). However, the role of tTG-specific B cells and anti-tTG antibodies in disease pathology is still unclear, despite being a hallmark of CD (Caio *et al.* 2019; Kupfer and Jabri 2012; Tye-Din *et al.* 2018).

In all traditional cases of CD, gluten specific CD4+ T cells are required to trigger CD. However, tissue damage requires the activation of cytotoxic IELs through IL-15. IL-15 produced by intestinal epithelial cells and inflammatory dendritic cells in the lamina propria drives massive expansion and increase in cytotoxic activity of CD8+TCRαβ+ IELs (Malamut *et al.* 2019). The coordination of gluten specific CD4+ T cells and IL-15 is not fully understood but is necessary to drive the activation and expansion CD8+TCRαβ+ as demonstrated in mouse models (Korneychuk *et al.* 2014; Malamut *et al.* 2019). The current hypothesis is that IL-2 and IL-21 from gluten specific CD4+ T cells in conjunction with IL-15 is enough to drive expansion, enhance granzyme B and NK marker expression without the need for any specific antigen (Malamut *et al.* 2019). In fact, a specific cytotoxic IEL against gluten has not been identified (Setty *et al.* 2015).

IL-15 induced upregulation of NK receptors NKG2D and CD94/NKG2C, as well as costimulatory activity for the NKG2D cytolytic pathway allow IELs to kill in a T cell receptor (TCR)-independent manner. These IELs have been directly shown to kill targets expressing major histocompatibility complex class I polypeptide-related sequence A (MICA), MICB, and HLA-E (Abadie and Jabri 2014; Hüe *et al.* 2004). Due to gluten-related inflammation of the epithelium, MICA, MICB, and HLA-E are highly expressed

on epithelial cells resulting in IEL mediated cytotoxicity (Caio *et al.* 2019; Korneychuk *et al.* 2014; Malamut *et al.* 2019; Tye-Din *et al.* 2018). Interestingly, only epithelial cells expressing IL-15 and ligands for activating NK receptors will be destroyed (Abadie and Jabri 2014).

While IL-15 is the major driver of tissue destruction, IL-21 is also a key player in the improper immune response. Gluten specific CD4+ T cells have been shown to produce high amounts of IL-21 (Sarra *et al.* 2013). This increased production of IL-21 leads to inhibition of T reg suppressive functions which further drives inflammation in CD (Sarra *et al.* 2013). IL-21 is positively regulated by IL-15 production. Sarra *et al.* demonstrated that intestinal CD4+ T cells stimulated with IL-15 produce significantly more IL-21 and IFN-γ than intestinal CD4+ T cells in the absence of IL-15.

Selective blocking peptide BNZ-2

BNZ-2 is a peptide designed to selectively block IL-15 and IL-21 while preserving IL-2 function (Ciszewski *et al.* 2020). IL-15 and IL-21 are both part of the γ-chain (γc) cytokine family along with IL-2, IL-4, IL-7, and IL-9. They all display a similar 4-helical bundle structure and a common receptor signaling subunit (Nata *et al.* 2015; Ciszewski *et al.* 2020). The D-helices of these cytokines primarily interact with the γc (Nata *et al.* 2015). Advancement in structural information regarding the binding of cytokines to receptors showed that there is a mildly conserved region across all γc cytokine D-helices (Nata *et al.* 2015). Complex inflammatory autoimmune diseases, like CD, often involve multiple cytokines driving immune responses. Therefore, Nata *et al.* hypothesized that the common structures shared by γc cytokines can be targeted and selectively inhibited. To do this, Nata *et al.* combined natural sequences of the γc cytokines and synthesized peptides

to target the interface of the cytokine and γc interaction. Figure 2 shows the sequence alignment of BNZ-2 with IL-15, IL-21, and IL-2.

Peptide	Sequence																				Key
IL-21	P	K	E	F	L	E	R	F	K	S	L	L	Q	K	M	I	H	Q	H	L	S	Conserved
IL-15	I	K	E	F	L	Q	S	F	V	H	I	V	Q	M	F	I	N	T	S	--	--	IL-15/IL-21 Conserved
IL-2	I	V	E	F	L	N	R	W	I	T	F	C	Q	S	I	I	S	T	L	T	--	IL-15 Specific
BNZ-2	P	K	E	F	L	E	R	F	V	H	L	V	Q	M	F	I	H	Q	S	L	S	IL-21 Specific

Figure 2. Alignment of BNZ-2 with IL-21, IL-15, and IL-2.
The amino acid sequences of the D-helices for IL-21, IL-15, and IL-2 were aligned with BNZ-2. Shared residues are color coded: orange highlighted residues are common to all, green highlighted residues are common to IL-15, IL-21 and BNZ-2, yellow highlighted residues are common to IL-21 and BNZ-2, pink highlighted residues are common to IL-15 and BNZ-2. BNZ-2 and IL-2 share no residues outside those common to all γc cytokines. Figure adapted from Ciszewski *et al.* 2020.

Both IL-15 and IL-21 interact with two different, non-redundant signaling pathways, pSTAT5 and pSTAT3 respectively, they both enhance pSTAT1, indicating the combination of the two play a key role in IEL mediated tissue destruction (Ciszewski *et al.* 2020; Sarra *et al.* 2013). Furthermore, it was observed that there was minimal crossover between the two cytokines when independently administered to T cells. IL-15 was shown to induce pSTAT5 and pERK in a dose-dependent manner (Ciszewski *et al.* 2020). IL-15 induction of pAKT and to a lesser degree, pSTAT3, leads to increased IL-21 production and IFN-γ (Sarra *et al.* 2013).

IL-15 induced pSTAT5 and pERK starting at a low concentration of 5 pM but required a higher concentration of 140 pM to induce pSTAT1 and pAKT (Ciszewski *et al.* 2020). IL-15 was inefficient at inducing pSTAT3, requiring a much higher concentration of around 700 pM, but still influences the pathway (Ciszewski *et al.* 2020). IL-21, however, induced pSTAT1 and pSTAT3 at the same low concentration that IL-15 induced pSTAT5 (Ciszewski *et al.* 2020). IL-21 failed to induce pSTAT5, pERK, and pAKT even

when using high concentrations (Ciszewski *et al.* 2020). To observe the additive effect of IL-15 and IL-21 Ciszewski *et al.* then tested the effect on phosphorylation of STAT molecules by adding both in tandem at submaximal concentrations. The results showed a clear additive effect on induction of pSTAT1 when IL-15 and IL-21 were combined compared with IL-21 alone (Ciszewski *et al.* 2020). pSTAT3 remained solely driven by IL-21 and pSTAT5, pERK, and pAKT remained solely driven by IL-15 (Ciszewski *et al.* 2020). In the presence of BNZ-2, these effects were reversed.

Having shown that IL-15 and IL-21 act in tandem to induce changes in the gene transcriptional profile, Ciszewski *et al.* examined the cyotkines' effect on granzyme B production and proliferation, and if BNZ-2 would reduce the effects. They found that IL-15 and IL-21 drive an increase in granzyme B production and proliferation, and addition of BNZ-2 resulted in a reversal of the effect (Ciszewski *et al.* 2020). Furthermore, it was determined in organ cultures that BNZ-2 reduced interferon gamma production in response to gluten through its ability to block IL-15 and IL-21 signaling (Ciszewski *et al.* 2020).

SynNotch Receptor

Synthetic Notch receptors or SynNotch receptors are an emerging tool in cellular therapy that allows for customization of cell behaviors that are induced by user determined signals (Roybal *et al.* 2016). Notch receptors contain three elements: an extracellular recognition domain, a core regulatory domain, and intracellular transcriptional domain (Figure 3). In SynNotch receptors, the extracellular and intracellular domains are synthetic, allowing the researcher to select what the receptor recognizes (Roybal *et al.* 2016). When the SynNotch receptor encounters the targeted ligand, the receptor undergoes transmembrane cleavage, releasing the intracellular transcriptional domain which enters

the nucleus to drive transcription of target genes (Morsut et al. 2016; Roybal *et al.* 2016). SynNotch circuits can be used to create a specific, customized cellular response to a particular signal or signals. Therefore, synNotch receptors can be used in a wide variety of applications due to the programmability of synNotch modified cells, including autoimmune diseases like CD.

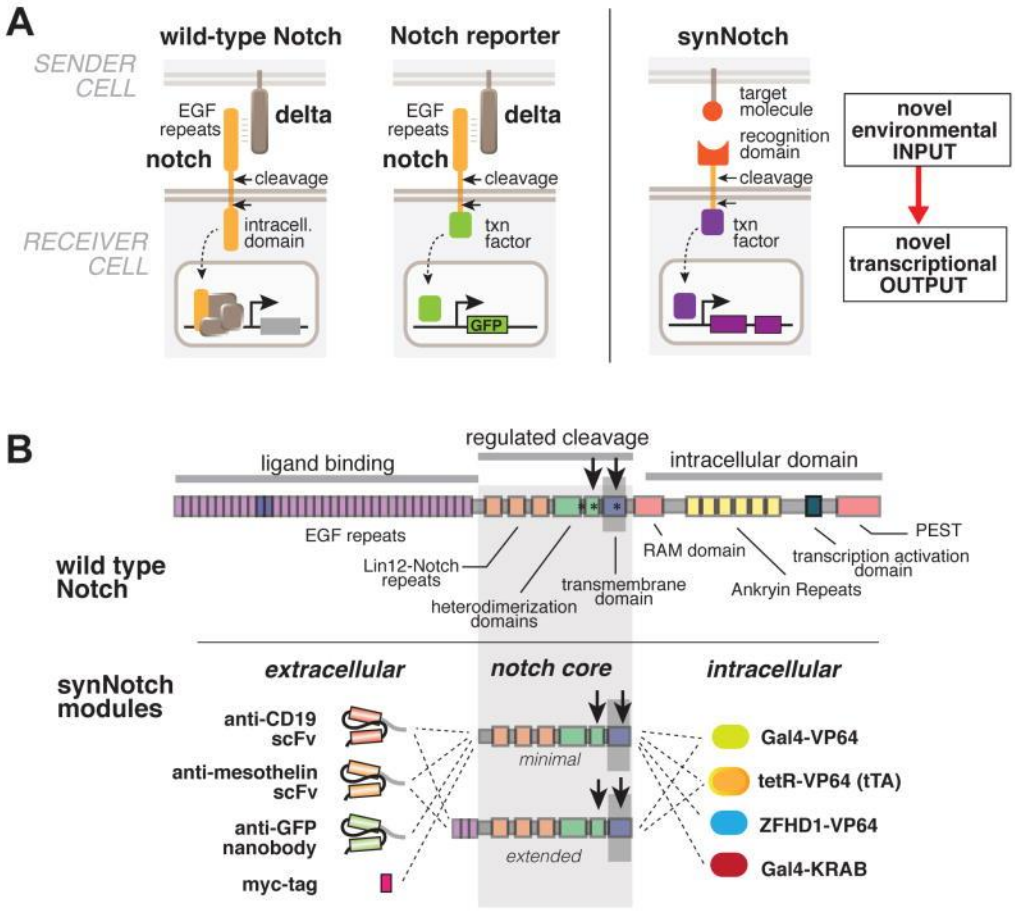

Figure 3. Modular Configuration of SynNotch Receptors

(A) Design of SynNotch Receptors. Left: Wild-type notch extracellular domain binds to its ligand, delta, expressed on another cell. An intracellular transcriptional regulatory domain is released by ligand binding induced cleavage. Middle: Notch receptor with only intracellular transcriptional regulatory domain changed to an orthogonal transcription factor. Right: synNotch receptors have both their extracellular and intracellular domains replaced, allowing for custom inputs and outputs. (B) The modularity of synNotch receptors. The extracellular and intracellular domains can be swapped with various recognition and effector domains, respectively. Figure adapted from Morsut *et al.* 2016.

Current Celiac Disease Treatment Research

As stated before, the only treatment for CD is a lifelong adherence to a GFD. However, several studies in adults with CD indicate that failure to achieve mucosal healing is common even when demonstrating proper adherence to the diet over many years, highlighting the need for new treatments (Tye-Din *et al.* 2018). Currently, there are several things being tested in the laboratory and clinic to treat CD. So far, no treatment has passed a phase 3 clinical trial (Tye-Din *et al.* 2018).

Different approaches are being taken to address CD. The first approach is to attempt to reduce the impact of gluten on the system or prevent it from triggering an immune response. Genetically modified wheat is one such approach (Tye-Din *et al.* 2018). The genes known to code for the toxic components for gluten are known, and attempts are being made to modify the genes to produce something harmless to CD patients. No such combination of wheat plants with altered gliadin producing genes safe for CD patients and commercial viability has been found due to the plant being adversely affected (Tye-Din *et al.* 2018). Recent developments, however, could potentially lead to finding the appropriate combination because wheat DNA sequencing data has become more comprehensive and widely available (Tye-Din *et al.* 2018).

Another approach in CD treatment utilizes enzymes to break down gluten either in the body or prior to entering the body. For gluten to be rendered incapable of producing an immune response, the peptides must be degraded into 9 amino acids or less (Tye-Din *et al.* 2018). Therefore, any enzymes that are to be explored must thoroughly degrade gluten peptides into fragments that are less than 9 amino acids in length. Due to the proline and

glutamine-rich nature of gluten peptides (Kim *et al.* 2004), most of the candidate enzymes are proline or glutamine specific (Tye-Din *et al.* 2018). A few of these enzymes are currently in clinical trials, notably latiglutenase a 1:1 mix of EP-B2 (endoprotease B, isoform 2) and SC-PEP (*Sphingomonas capsulate*-proline specific endoprotease), and AN-PEP (*Aspergillus niger*-prolyl endopeptidase). While the enzymes are effective at degrading gluten, their efficacy as an oral treatment for patients has not been fully determined (Tye-Din *et al.* 2018).

Larazotide acetate is an intestinal tight junction regulator that may enhance the function of the intestinal barrier, is currently in the first ever phase 3 clinical trial for CD (Tye-Din *et al.* 2018). In a Phase 2 trial larazotide acetate was shown to reduce symptoms in CeD patients on a GFD better than a GFD alone, but only at the lowest dose of 0.5 mg (Leffler *et al.* 2015; Yoosuf and Makharia 2019). More studies will be required to fully determine whether these treatments will be beneficial to CD patients.

A more comprehensive and potentially curative approach is being taken with Nexvax 2. Nexvax 2 is a therapeutic vaccine composed three gluten peptides encompassing immunodominant HLA-DQ2.5-restricted T cell epitopes (Daveson *et al.* 2017; Yoosuf and Makharia 2019). The objective of this treatment is to re-establish robust immune tolerance to ingested gluten. A phase 1 trial concluded that the recall immune response was indeed modified in patients, after initially causing CD symptoms in patients when compared to placebo. The symptoms were no different when compared to the placebo group after later administration of Nexvax 2 (Daveson *et al.* 2017; Yoosuf and Makharia 2019). Re-establishing immune tolerance would be a significant breakthrough in treating CD.

AMG-714 is a monoclonal antibody against IL-15. Recently it was utilized in a phase 2a clinical trial where it was utilized to treat RCD type II (Cellier *et al*. 2019; Yoosuf and Makharia 2019). Researchers found that the effect was not different from the placebo group. AMG-714 did not prevent mucosal damage caused by gluten challenge. However, it did have an effect on the density of IELs in the highest dose group. IELs in each dose group increased, but the highest dose group increased the least overall. Therefore, potential applications for AMG-714 could still exist (Cellier *et al.* 2019; Yoosuf and Makharia 2019).

The goal of this proposal is to generate a synNotch receptor modified T-cell targeting tTG-deamidated gluten peptides. The synNotch circuit would then drive the expression and secretion of BNZ-2 which would then block IL-15 and IL-21 signaling in the gut. The first step is to incorporate an anti-gluten single chain variable fragment (scFv) (extracellular recognition domain) and Gal4 DNA binding domain fused to viral transcriptional activator domain (intracellular transcriptional domain), VP64 into a synNotch receptor (Morsut *et al.* 2016; Roybal *et al.* 2016). Next, the receptor and BNZ-2 cassette will be transduced into primary T-cells and tested to determine functionality.

RESEARCH DESIGN AND METHODS

In this grant proposal, primary T cells from healthy donors will be modified to produce a synNotch receptor targeting the most common toxic epitope of gluten. The synNotch receptor function will be linked to production of BNZ-2, a peptide that selectively blocks IL-21 and IL-15 but no other gamma-chain cytokines such as IL-2. The synNotch receptor circuit will be packaged into a lentiviral vector which will be transduced into T cells using the centrifugal inoculation technique. Modified T cells will first be assessed *in vitro* for phenotype, transduction efficiency, viability, expansion, and functionality.

Primary Human T-cell Isolation and Culture

Leukopaks from 4-6 anonymous healthy donors, 50% male, 50% female, can be acquired from HemaCare (# PB001F-1). PBMCs will be isolated from leukopaks using ficoll density gradient centrifugation. T cells will be isolated from PBMCs via negative selection using StemCell EasySep Human T Cell Isolation Kits (StemCell Technologies, #17951). Isolated T cells will be cryopreserved using CryoStor CS10 media (StemCell Technologies, #07952). After thawing, T cells will be cultured in X-VIVO 15 (Lonza, #BE02-060Q), 5% human AB serum (GeminiBio, #100-512), and 50 IU/mL IL-2 (Peprotech, #200-02).

Cell Lines

HEK-293 cells will be transduced to stably express the toxic gluten epitope PLQPEQPFP fused to platelet-derived growth factor transmembrane domain and green fluorescent protein (GFP) reporter. Transduced HEK-293 cells will be sorted based on GFP expression using fluorescence activated cell sorting (FACS) and cultured in DMEM

supplemented with 10% fetal bovine serum (FBS). HEK-293 cells will be acquired from ATCC (ATCC® CRL-1573™).

Lentivirus Production and T Cell Transduction

VSV-G-pseudotype lentiviruses will be produced via transduction of Lenti-X 293 cells (Takara, #632180) using a pPACKH1 HIV Lentivector Packaging Kit (System Biosciences, #LV500A-1). All lentiviruses produced will be titered prior to transduction in AD-293 cells (Agilent #240085) using flow cytometry to detect anti-myc Alexa Fluor 647 or BFP to determine viral titer. T cells will be thawed and cultured for 24 hours before being stimulated with Dynabeads Human T-Activator CD3/CD28 (Life Technologies, #111.32D) at a bead-to-cell ratio of 3:1. Lentiviruses will be added at a multiplicity of infection (MOI) of 5 along with 1ug/mL Polybrene to T cells 24 hours post stimulation and centrifuged at 1000g for 2 hours at 37C. On day 4 post-transduction, beads will be removed, and cells will be expanded until day 7. On day 7, T cells will be sorted on a FACS Aria II (BD Biosciences) based on anti-myc Alexa Fluor 647 antibody (BioLegend #626810) staining and BFP expression. Sorted T cells will then be cryopreserved or used in assays.

Specific Aim 1: Design a Synthetic Notch (synNotch) Receptor Capable of Sensing Immunogenic Gluten Peptides and Link its Activity to BNZ-2 Expression

Using the approach described by Roybal *et al.*, the synNotch receptor construct will contain an extracellular domain targeting gluten (Steinsbø *et al.* 2014), the mouse Notch1 minimal regulatory region, Gal4 DNA binding domain fused to viral transcriptional activator domain VP64 (intracellular transcriptional domain). The receptor will also contain an n-terminal CD8a signal peptide (MALPVTALLLPLALLLHAARP) for

membrane targeting and a myc-tag (EQKLISEEDL) for determination of surface expression with anti-myc Alexa Fluor 647 antibodies. The synNotch extracellular scFv will contain the genes IGHV3-23 and IGLV4-69 in V_H-V_L format that recognize the epitope PLQPEQPFP (Steinsbø *et al.* 2014). The scFv portion will be cloned into pHR_PGK_antiCD19_synNotch_Gal4VP64 (Figure 4) (Addgene #79125) in place of the CD19 scFv (Morsut *et al.* 2016).

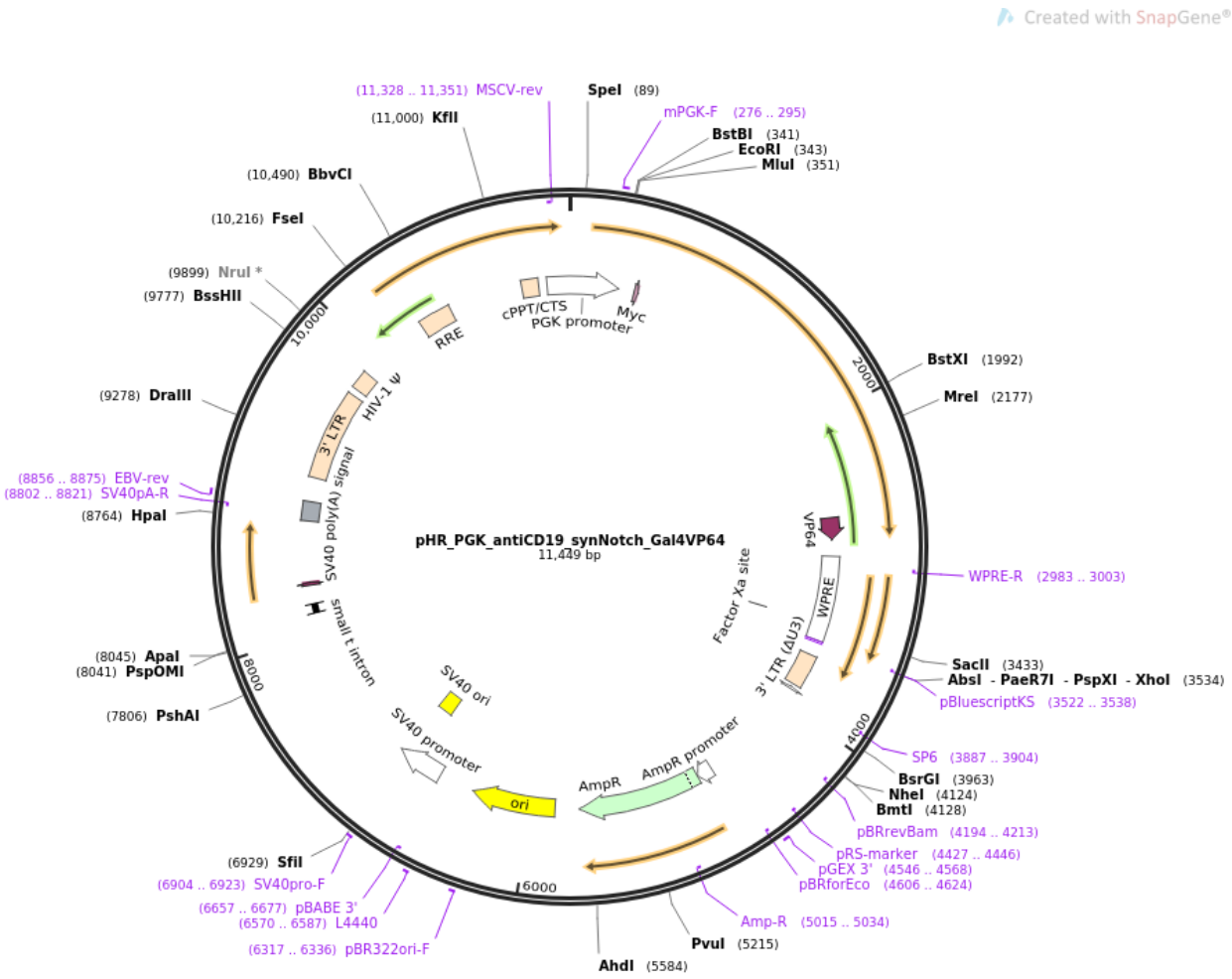

Figure 4. SynNotch receptor vector map.
pHR_PGK_antiCD19_synNotch_Gal4VP64 (Addgene #79125), from Wendell Lim's laboratory (Morsut *et al.* 2016; Roybal *et al.* 2016). The ORF containing the anti-CD19 scFv will be replaced with the scFv specific for gluten epitope PLQPEQPFP (Steinsbø *et al.* 2014). Figure obtained from the Addgene website.

The synNotch response elements will be cloned into a second vector, pHR_Gal4UAS_IRES_mC_pGK_tBFP (Figure 5) (Addgene #79123), which is an empty backbone lentiviral vector designed by Wendell Lim (Morsut *et al.* 2016) for inducible expression of genes by synNotch receptors. 5 copies of the GAL4 DNA binding domain target sequence (GGAGCACTGTCCTCCGAACG) will be cloned 5' to a minimal CMV promoter, mRNA codon optimized BNZ-2 (Ciszewski *et al.* 2020), and IRES mCherry reporter (Roybal *et al.* 2016). The vector also contains a PGK promoter that drives constitutive expression of BFP (Roybal *et al.* 2016). SynNotch and response element cassettes can be ordered as strings from Thermo Fisher and cloned into the vector backbone using Gibson assembly, a type of cloning that does not rely on restriction enzyme digestion.

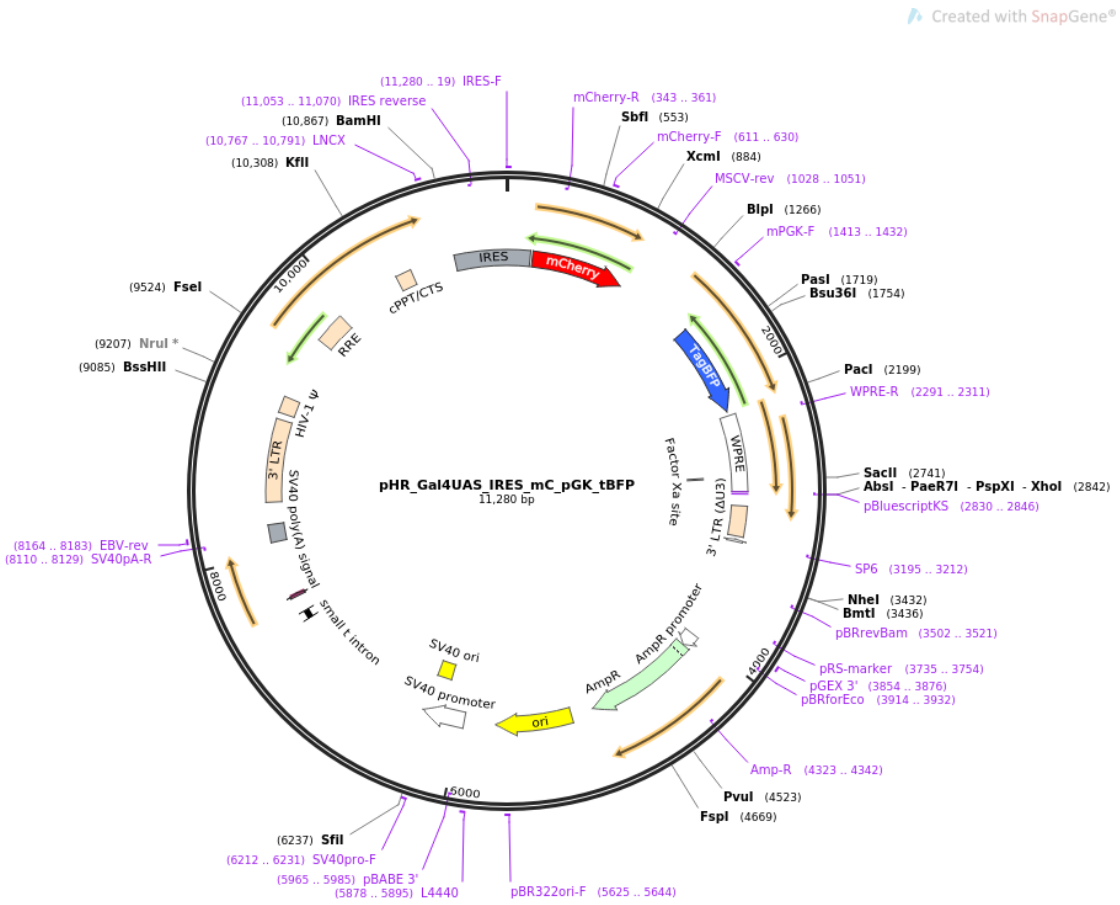

Figure 5. SynNotch response element vector map. pHR_Gal4UAS_IRES_mC_pGK_tBFP (Addgene #79123), from Wendell Lim's laboratory (Morsut *et al.* 2016; Roybal *et al.* 2016). The response elements will be cloned into this vector backbone. Figure obtained from the Addgene website.

Expected Result:

Lentiviruses produced are expected to yield a titer of $>10^7$-10^8 TU/mL measured by droplet digital PCR (ddPCR). The swapping of anti-CD19 scFv with the anti-gluten scFv should not impact the ability of the vector to be packed into a lentivirus nor should the addition of BNZ-2 to the response vector. A major highlight of the synNotch system described by Morsut *et al.* (2016) and Roybal *et al.* (2016), is the ability to freely exchange the extracellular and intracellular components of the receptor (Figure 3).

Specific Aim 2: Transduce Primary T Cells with synNotch Receptor Circuit and Assess *in vitro* Characteristics

Aim 2a: Transduced T cells will undergo several assays to determine their *in vitro* characteristics. First, cells will be assessed for viability, expansion, and transduction efficiency. Viability and expansion will be measured via acridine orange/propidium iodide (AO/PI) staining to both count cells and determine if the synNotch circuit is toxic to the cells. Cell counts and viability will be taken at multiple time points throughout the entirety of the experiment, day 0, day 1, day 4, day 7, day 14. AO/PI staining can be carried out using the Nexcelom Cellometer following the manufacturer's protocol.

Expected Result:

Stimulated T cells double approximately every 24 hours. Lentiviral transductions do not significantly interfere with T cell expansion and viability. Therefore, there should be robust growth throughout the duration of *in vitro* testing. Viability should remain high, >85%, and average cell diameter should remain between 10-13 μm. If the construct is toxic, disruptions will be observed in the expansion rate, viability, and size of the cells. If there are any disruptions, monitoring cell counts and viability via AO/PI staining (Figure 6) will be able to detect unwanted changes from a high level prior to further testing. Stimulation should begin to wane around 7-10 days according to the manufacturer's insert (Life Technologies, #111.32D), but cells can be re-stimulated if necessary.

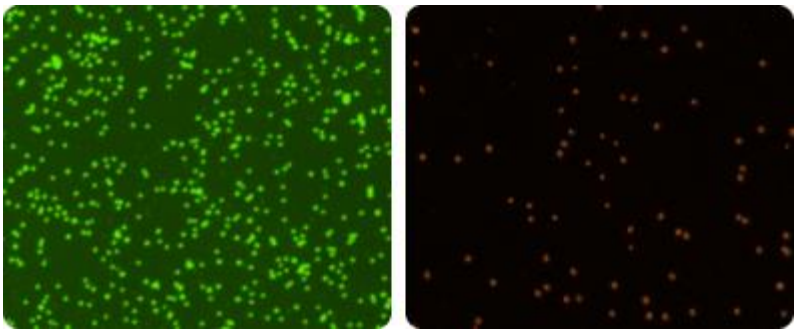

Figure 6. AO/PI staining of peripheral blood mononuclear cells.
Figure adapted from Nexcelom's website. Live nucleated cells stained with acridine orange (AO) appear green (left) and dead nucleated cells stained with propidium iodide (PI) appear red (right). Figure obtained from the Nexcelom website.

Aim 2b: To measure transduction efficiency, FACS will be employed. Cells will be stained with anti-myc Alexa Fluor 647 (BioLegend #626810) and sorted for anti-myc positivity and BFP expression on day 7 to assess stable integration of the synNotch circuit. Sorting will be carried out using a BD FACS Aria II. Sorted T cells will then be cryopreserved or used in assays.

Expected Result:

Lentiviral transductions carry a certain amount of variability. However, by day 7 a stably transduced population of T cells should be apparent via anti-myc alexa fluor 647 staining and BFP expression. Sorting these cells using the BD FACS Aria II, will yield a pure population of synNotch BNZ-2 T cells for use in downstream assays. I expect that prior to sorting, I will see between 5-70% (Figure 7) of the T cells expressing the synNotch receptor and response elements. Only cells that are double positive for anti-myc and BFP will be used for subsequent testing (Figure 7). The variability would be due to donor differences and the lentiviruses.

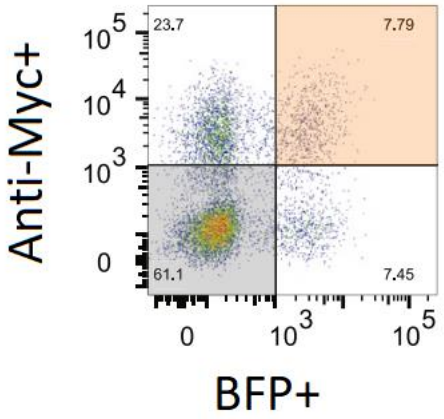

Figure 7. 2-parameter dot plot of T cells transduced with the synNotch receptor and response elements. T cells that are double positive for the synNotch receptor (anti-myc AF647) and response elements (BFP) are highlighted in the orange box. Cells highlighted in grey are untransduced. Figure adapted from Roybal *et al.* 2016.

Aim 2c: Next, the synNotch circuit will be tested to determine if the circuit is functional. Stably transduced HEK-293 cells expressing the synNotch target will be cultured 1:1 together with synNotch T cells for 24 hours. After which, the cultures will be analyzed for mCherry expression to determine if the cells respond to the signal.

Expected Result:

After 24 hours of co-culture, I should see mCherry expression via flow cytometry in T cells containing both the synNotch receptor and response elements. mCherry expression is directly tied to GAL-VP64 transcriptional activation. If the synNotch circuit does not work, no mCherry expression will be observed (Figure 7). Roybal *et al.* (2016) demonstrated that there is no basal secretion of what the authors describe as "a la carte" cytokine profile expression in the absence of synNotch antigen sensing. Therefore, given these results I should expect to see something similar where there is no mCherry expression

unless the gluten peptides trigger the synNotch circuit. Figure 8, adapted from Roybal *et al.* (2016), shows no basal secretion of BNZ-2 in the absence of antigen sensing.

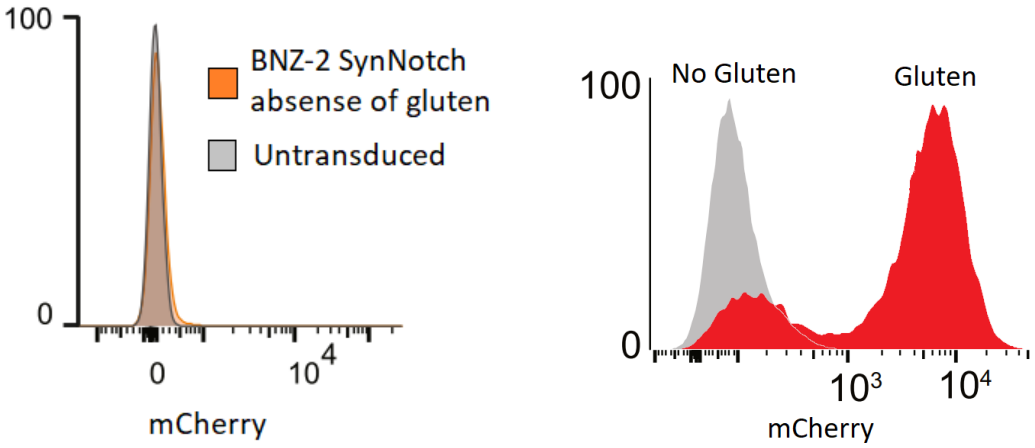

Figure 8. Basal expression of mCherry reporter in the absence of antigen sensing. Overlayed histograms of mCherry expression in both synNotch T cells (orange) and untransduced T cells (grey) in the absence of target (left). In the presence of gluten, synNotch BNZ-2 T cells should produce mCherry (right). Figure adapted from Roybal, *et al.* 2016.

Aim 2d: Finally, the effect of BNZ-2 produced by synNotch T cells *in vitro* will be analyzed by adapting the methods described by Ciszewski *et al.* (2020). Unaltered T cells stimulated with 140 pM IL-15 and 14 pM IL-21 will be treated with supernatant from synNotch BNZ-2 T cell and HEK-293 co-cultures. These T cells will be assessed by analyzing phosphorylation of signaling molecules, granzyme b production, and proliferation via flow cytometry and western blot.

It was demonstrated by Ciszewski *et al.* (2020) that the effects of upregulation of IL-15 and IL-21 could be reversed using BNZ-2. Therefore, analysis of the STAT molecules involved will be carried out in a similar manner as described by Ciszewski *et al*.

(2020) in which T cells will be sorted for CD45+ (BD Biosciences), CD103+ (BD Biosciences), CD3+ (BioLegend), CD8+ (BD Biosciences), TCRαβ+ (BioLegend) and grown with Dynabeads Human T-Activator CD3/CD28 (Life Technologies, #111.32D) and IL-2 (Peprotech, #200-02) for 10-12 days before bead removal and starvation. Cells will be deprived of IL-2 post bead removal for 48 hours before addition of IL-15 and IL-21. Starved T cells will be treated with synNotch T cell co-culture supernatant containing BNZ-2 or control supernatant without BNZ-2. Western blot and flow cytometry will be performed after 20 minutes and 2 hours of cytokine stimulation, respectively (Figure 9).

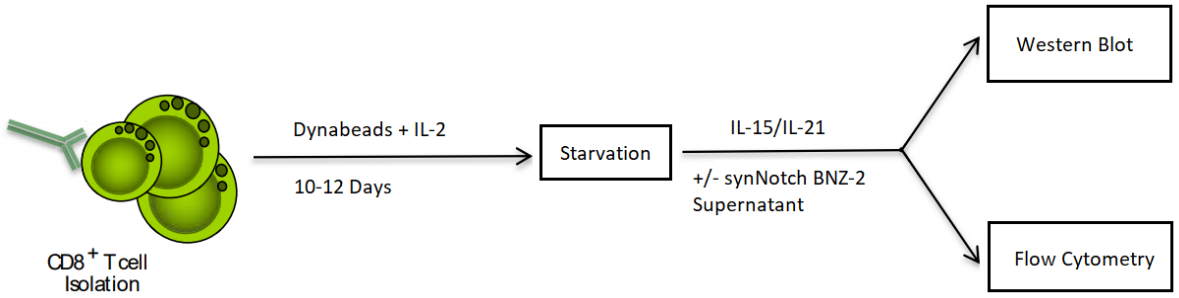

Figure 9. *In vitro* workflow schematic.

T cells will be isolated as described above. CD45+CD103+CD3+CD8+TCRαβ+ will be cultured with Dynabeads and IL-2 for 10-12 days before being deprived of IL-2 for 48. Cells will then be stimulated with IL-15 and IL-21 in the presence or absence of synNotch BNZ-2 supernatant. Western blotting will be performed on samples 20 minutes post-stimulation and flow cytometry analysis of samples 2 hours post-stimulation. Figure adapted from Ciszewski *et al.* 2020.

Western blotting will be performed to detect pSTAT1(Cell Signaling Technology #9167), pSTAT3 (Cell Signaling Technology #9145), pSTAT5 (Cell Signaling Technology #9359), pERK (Cell Signaling Technology #4370), pAKT (Cell Signaling Technology #4060), and β-actin (Cell Signaling Technology #3700) (loading control). Extracted proteins will be separated by sodium dodecyl sulfate-polyacrylamide gel

electrophoresis using 4% to 12% gradient polyacrylamide gels (Invitrogen #NP0322), transferred to a polyvinylidene fluoride membrane (Invitrogen #88518), blocked overnight with 5% nonfat dry milk in Tris-buffered saline containing 0.1% Tween-20 buffer, and immunoblotted with antibodies against the above targets. Bands will be detected with a Li-Cor C-digit blot scanner (Li-Cor).

Starved T cells treated with synNotch BNZ-2 T cell supernatant will be fixed and stained with anti-STAT3 (pY705) (Cell Signaling Technology), anti-STAT5 (pY694) (eBioscience), anti-granzyme B (eBioscience), or anti-Ki-67 (BD Biosciences) antibodies and acquired on a BD FACS Symphony flow cytometer to assess the effects of BNZ-2 produced by synNotch BNZ-2 T cells.

Expected Result:

Starved, IL-15 and IL-21 stimulated cells treated with BNZ-2 should show decreased phosphorylation of STAT proteins, ERK, and AKT when compared to cells treated with control supernatant (Figure 10).

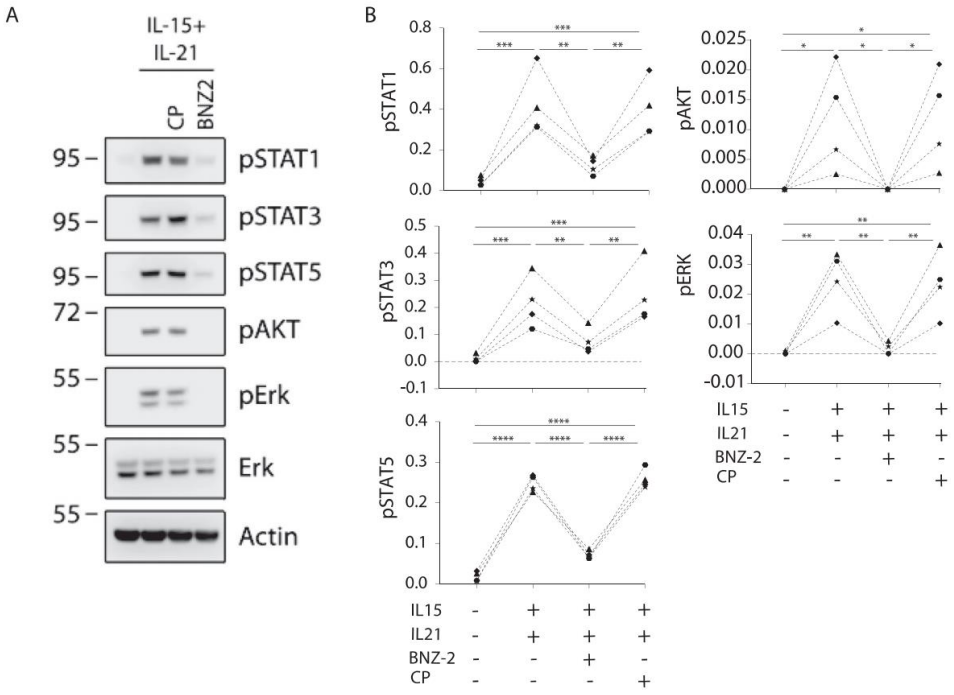

Figure 10. SynNotch BNZ-2 T cell supernatant blocks phosphorylation of STAT, AKT, and ERK molecules in T cells stimulated with 140 pM IL-15 and 14 pM IL-21. (A) Western blot showing phosphorylation of STAT, AKT, and ERK molecules in the presence and absence of synNotch BNZ-2 T cell supernatant. ERK and Actin were used as equal loading controls. (B) Quantification of western blots performed in (A). Figure adapted from Ciszewski *et al.* 2020.

I expect similar results with flow cytometric analysis of the treated T cells. Cells stained for anti-STAT3 (pY705) and anti-STAT5 (pY694) should show a reduced signal when compared to control supernatant (Figure 11).

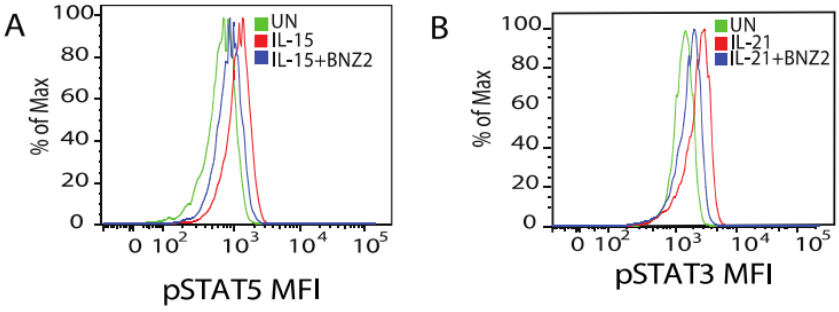

Figure 11. BNZ-2 blocks STAT5 and STAT3 signaling in T cells. Mean fluorescence intensity (MFI) histograms (left) of (A) pSTAT5 and (B) pSTAT3 in response to IL-15 (140 pM) and (B) IL21 (14 pM). Stimulated T cells are shown in red (control supernatant), cells treated with BNZ-2 supernatant are in blue and unstimulated cells are in green. Figure adapted from Ciszewski *et al.* 2020.

Similar results are expected with granzyme b and Ki-67 staining. Granzyme b production is significantly increased in the absence of BNZ-2. Therefore, the amount of granzyme b produced by IL-15 and IL-21 stimulated T cells should be reduced in the presence of BNZ-2. Ciszewki *et al.* (2019) demonstrated the ability of BNZ-2 to essentially reverse the effects of IL-15 and IL-21 in T cells (Figure 12).

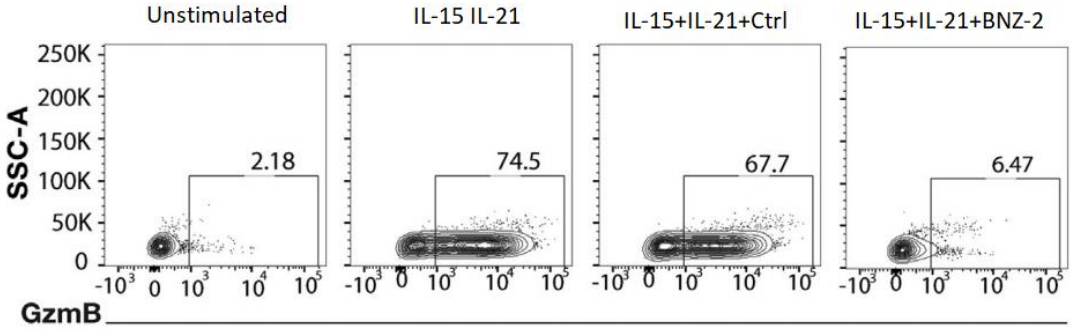

Figure 12. BNZ-2 produced by synNotch BNZ-2 T cells reverses the production of granzyme b in IL-15 and IL-21 stimulated T cells. Representative dot plots depicting the reduction of granzyme b production in the presence (right plot) and absence (middle plots) of synNotch BNZ-2 T cell supernatant compared to unstimulated T cells (left plot). Figure adapted from Ciszewski *et al.* 2020.

In CD, the synergistic effects of IL-15 and IL-21 result in increased IEL proliferation. IL-15 and IL-21 stimulated T cells in the presence of synNotch BNZ-2 T cell supernatant should show less Ki-67 positivity when compared to control supernatant (Figure 13). I fully expect to demonstrate that BNZ-2 produced by synNotch BNZ-2 T

cells functions in the same manner as synthesized BNZ-2 and that it is produced in sufficient quantity to have a significant impact on activated IL-15 and IL-21 activated T cells.

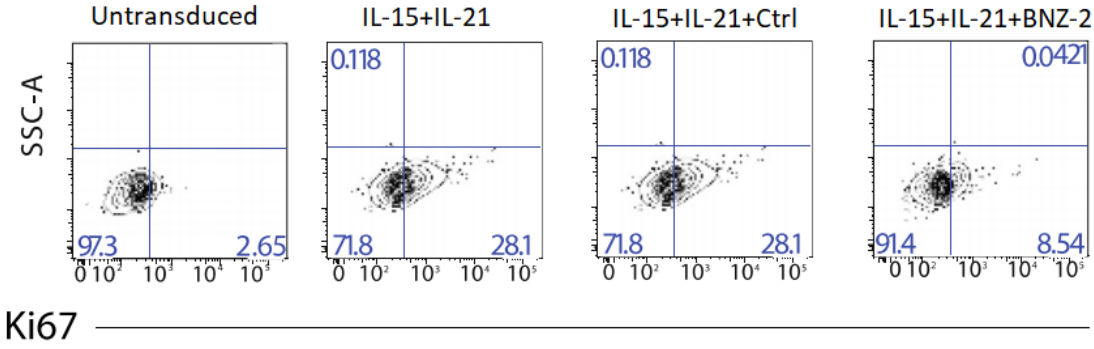

Figure 13. Reduced proliferation in IL-15 and IL-21 stimulated T cells. Dot plots depicting the frequency of Ki-67 in the presence (right plot) or absence (middle plot) of synNotch BNZ-2 T cell supernatant compared to unstimulated T cells (left plot). Figure adapted from Ciszewski *et al.* 2020.

Conclusions and Future Directions

Celiac disease is a complex autoimmune disease that currently has only one treatment option, a lifelong adherence to a strict GFD. With 1-3% of the general population thought to be affected by Celiac disease, more treatment options are going to be required to reduce disease morbidity and improve quality of life for patients. In this grant proposal, I propose the use of cellular therapy involving a synthetic Notch receptor (Roybal *et al.* 2016) targeted at one of the most common immunotoxic gluten peptides (Steinsbø *et al.* 2014) tied to the production of BNZ-2 (Ciszewski *et al.* 2020) to disrupt the improper immune response that is the hallmark of CD. The customizable synNotch receptor (Morsut *et al. 2016*; Roybal *et al.* 2016) circuit offers a hopeful approach in creating a treatment that can either augment or reduce strict adherence to a GFD.

Ciszewski *et al.* (2020) demonstrated that the synergistic effects of IL-15 and IL-21 dysregulation in CD are critical in driving tissue destruction by IELs. BNZ-2 is a novel peptide designed to selectively inhibit both IL-15 and IL-21 but not interfere with other γc cytokines such as IL-2 (Ciszewski *et al.* 2020). The ability to target both IL-15 and IL-21 specifically is a potentially significant advantage over blockading one of them, since AMG-714 which blocks only IL-15, failed to improve active CD symptoms in the clinic (Cellier *et al.* 2019). Currently, BNZ-2 is beginning a phase 1 trial where they are delivering the peptide subcutaneously. BNZ-3 (Bioniz 2021) is another product being developed that works in the same capacity as BNZ-2 except that it is being developed for oral delivery.

The key advantage of utilizing synNotch receptor cellular therapy for this purpose is the ability to create an adaptive response to target exposure (Roybal *et al.* 2016). Subcutaneous or orally administered treatments will often require the patient to utilize the

treatment consistently to maintain a certain level of effectiveness. I aim to demonstrate that by using a cellular therapy approach is the potential to have a longer lasting adaptive response to gluten exposure that can quell the improper immune response but become inactive in the absence of gluten (Figure 8). IL-15 and IL-21 have a proper function in a normal immune response to pathogens, and potential issues could arise if a patient is consistently blocking IL-15 and IL-21 signaling with consistent oral or subcutaneous delivery.

A potential problem with this system could arise since the synNotch receptor, like wildtype Notch receptors, require the target to be associated with a different cell. Unbound gluten peptides could potentially outcompete gluten peptides that are bound by tTG or MHC and render the system ineffective. Additionally, if the peptides are bound to tTG or MHC, the immunotoxic epitope the scFv in the synNotch receptor binds to may not be accessible. However, there has been some work on creating monoclonal antibodies against gluten/MHC complexes, which could be used to improve results if this synNotch receptor is ineffective in vivo. Additionally, Roybal *et al.* (2016) demonstrated that one could further augment the response element to produce more than one gene product. In the context of this grant, it could be beneficial to include immunosuppressive cytokines in addition to the BNZ-2 in the response element. The downside to adding more to the response element is that the size of the vector needed to deliver such a payload may exceed what is possible to package into a lentivirus.

Furthermore, synNotch BNZ-2 T cells may not be able to produce a clinically relevant amount of the BNZ-2 peptide in vivo. Future experiments could be designed to determine how much of the peptide would need to be produced to protect against gluten

challenge. The amount of peptide produced could potentially be modified with a stronger promoter, for example. In the case, of too much peptide being produced, a kill switch for these cells could be incorporated into the system in order to eliminate the cells from the patient's system and reverse the effect.

This proof-of-concept approach for Celiac disease could be applied for other diseases where there are known targets driving the disease. Due to the modularity of the synNotch system, the only challenge would be to design the proper extracellular domains, intracellular domains, and response elements (Morsut *et al.* 2016; Roybal *et al.* 2016). Furthermore, this system is not limited to T cells. Theoretically, any cell type can be used for this purpose. In the context of celiac disease Tregs, B cells or dendritic cells, for instance, could be used.

There exists even more future potential with this system in that this therapy could be turned into a gene therapy rather than cellular therapy. If for instance, the synNotch receptor and response elements could be delivered directly to patient intestinal stem cells, these receptors would then be omnipresent, and could act in a lifelong preventive manner or curative manner depending on the application. Overall, the dynamic ability of the synNotch receptor to have custom inputs and outputs coupled with advances in traditional treatments such as BNZ-2, provide a powerful platform for research.

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