Proposal

The University of Maryland, Gemstone Program

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We pledge on our honor that we have not given or received any unauthorized assistance on this assignment.

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Abstract

Allergies are a pervasive issue and require novel ways of alleviating symptoms. Existing research has established symptom management therapies and specific immunotherapy, but there has been an emerging emphasis on the potential of inhibiting molecules involved in the downstream signaling pathway of the allergic response. The research aims to identify and target the molecules involved in this pathway that result in the degranulation of mast cells which cause allergic symptoms. This study intends to block experimentally determined signaling molecules in the downstream FcεR1 signaling pathway in order to hinder mast cell degranulation. The results will be measured in mast cell lines with multiple cell degranulation assays. This project aims to find the most ideal small molecule in the signaling pathway in order to best reduce degranulation and thus reduce the allergic response.

Introduction

An allergy is a chronic condition due to an adverse, potentially life-threatening reaction to an otherwise harmless environmental substance within the immune system. Allergies impede both the dietary and social lives of those affected as well as the families and communities surrounding each person. According to the World Health Organization, the percentage of persons with sensitivity to allergens is 40% and climbing; it is the most pervasive disorder globally (World Health Organization, 2011). In the United States, allergies are the sixth leading cause of chronic illness (Centers for Disease Control and Prevention, 2017). While anyone at any age is at risk of developing allergies, they often manifest themselves at or before the age of five years old (American College of Allergy, Asthma, and Immunology [AAAAI], 2014). They are characterized by a range of symptoms from sneezing, runny nose, coughing and itching to life-threatening reactions such as anaphylactic shock (Centers for Disease Control and Prevention, 2017).

There are numerous types of allergies, from food allergies to seasonal ones, however all are caused by a reaction of the immune system to a specific allergen. In every allergic reaction, the immune cells known as mast cells are activated, triggering a cascade of molecules within the mast cell to bind, activate, or recruit each other in a complicated signaling pathway (Metcalfe, Peavy, & Gilfillan, 2009). If specific parts of that pathway were to be interrupted, the efficiency of the pathway and/or intensity of the product would be reduced. In this project, the question to be explored throughout the research is how small molecules within the downstream mast cell signaling pathway can be inhibited in order to reduce degranulation.

Literature Review

In this section, the basic functions of the immune system are outlined from first contact in order to juxtapose its mechanism with the sequence of the allergic response. Key components of the allergic pathway are presented, from the binding of IgE to the signaling pathway within the mast cell before the appearance of symptoms.

Overview of Immune Responses

In an adaptive immune response, the body utilizes many cell types to combat foreign substances known as antigens. When an antigen enters the body, it is detected and engulfed by antigen-presenting cells (APCs). Once the antigen is engulfed, it is broken up into its protein fragments which then are presented on surface proteins on the exterior of the APC (Warrington, Watson, Kim, & Antonetti, 2011). These surface proteins, known as the major histocompatibility complex (MHC), may activate immune T cells and aid the T cell into maturation and differentiation (Bonilla & Oettgen, 2010). If the T cell matures into a helper T cell, it can use the specificity of the original antigen to prompt B cells into producing antibodies. In different pathways, the B cell acts as an APC itself, and can use the MHC created using antigen fragments to make specific antibodies (Bonilla & Oettgen, 2010). This is the mechanism of antibody-mediated immunity, which is depicted in Figure 2.

Due to a DNA rearrangement event within the B cell, every antibody made by a specific B cell is unique to that antigen and to its B cell (Alberts, Johnson, & Lewis, 2002). This allows the B cells to make many antibodies to combat a specific antigen. With the encounter of an antigen, the B cell divides and differentiates into antibody-secreting effector cells. These effector cells are tasked with seeking out other copies of the same antigen (Alberts, Johnson, & Lewis, 2002). From there, the effector cell can secrete free-floating antibodies of the same structure as the antibodies presented on the surface of the B cell (Alberts, Johnson, & Lewis, 2002). Not all

cells produced by the B cells, however, become effector cells. Some of the cells function to retain the genetic memory for producing that specific antibody in order to ensure that a second exposure will trigger a more effective attack on the foreign substance (Warrington, Watson, Kim, & Antonetti, 2011).

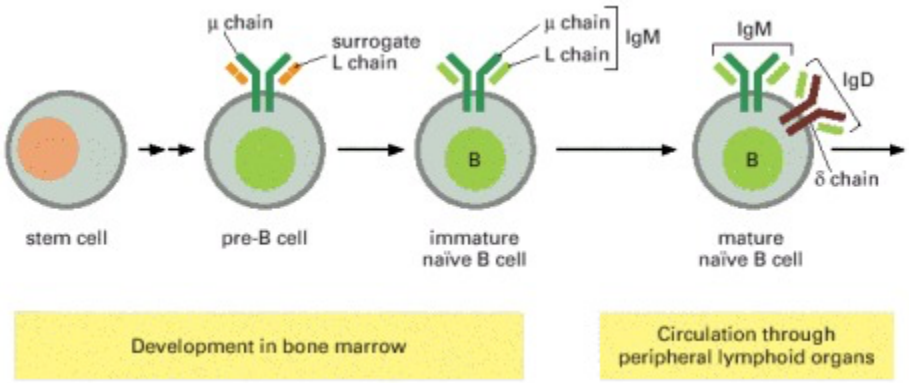


Figure 1. The main stages of development in B cells. Adapted from "B Cells and Antibodies" by B. Alberts, Johnson, A., Lewis, J., et al. (2002) *Molecular Biology of the Cell 4th Edition. Garland Science 2002*.

In a normal immune response, effector B cells produce the immunoglobulin G (IgG) antibodies. Using their specific Fab site, these antibodies are able to bind to antigens found in the bloodstream. Once bound to the antigen, the antibody can enact the antibody-mediated immune response, also known as the humoral immune response. Antibodies coat the antigen, which can block many of the pathogenic mechanisms of an antigen such as adhering to host tissues or binding to other mechanistic structures needed to carry out infection (Forthal, 2014). In another step of the process, opsonization occurs when the antibody coating of antigens promotes phagocytosis (Janeway et al., 2001). The presence of antibodies coating the antigens can also cause phagocytes of the immune system to recognize protein sequences within the Fc region of the IgG antibodies and bind to Fcγ receptors found on the surface of the phagocytes (Hiemstra & Daha, 1998). This binding increases phagocytosis by pulling the membrane of the phagocyte

around the antigen (Hiemstra & Daha, 1998). A final iteration of the humoral response occurs in the form of complement activation, where opsonization is aided by complement proteins such as C3 and C4 complement proteins to cause pathogen lysis (Noris & Remuzzi, 2013). In summary, the normal humoral immune response is largely dictated by the activity of immunoglobulin G and results in the neutralization of pathogenic substances to the body. This greatly differs from the allergic response, which is dictated by IgE rather than IgG.

Allergic Response Mechanism

In an allergic response, in addition to bringing the antigen back to the lymph nodes, the APC also present a co-stimulatory molecule on its surface (Bubnoff, Geiger, & Bieber, 2001). Once the naive T helper cell recognizes the antigen and the co-stimulatory molecule, it becomes a mature T Helper 2 cell (TH2). When interacting with the B cell, cytokines such as Il-4, Il-5, and Il-13 trigger the B Cell to start making Immunoglobulin E (IgE) instead of IgG (Bubnoff, Geiger, & Bieber, 2001). The IgE then binds to the FcεR1 receptor on the mast cell. If there is another exposure of this same antigen, the specific IgE antibody is already produced and bound to the mast cell (Warrington, Watson, Kim, & Antonetti, 2011). Once it recognizes the antigen, the Fab region of the IgE binds to the antigen. This causes the mast cell to degranulate and release proinflammatory mediators such as histamines, prostaglandins, and leukotrienes (Galli, Tsai, & Piliponsky, 2008). Depending on the proinflammatory mediator released, the body expresses different reactions, as shown in Figure 2.

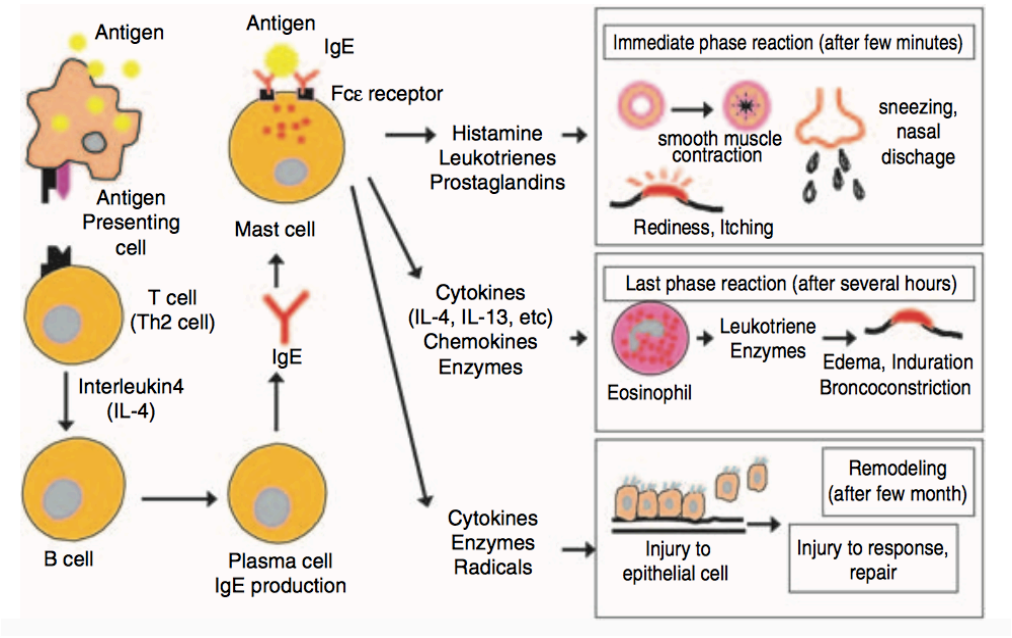


Figure 2. The mechanism of IgE mediated allergic reaction. Adapted from "Recent Advances in the Development of Anti-allergic Drugs," by H. Nagai et al., 2006, Allergology International, 55(1), 35-42.

Many proinflammatory mediators have overlapping effects, which can enforce symptoms such as anaphylaxis, coughing, sneezing, wheezing, hives, mucus secretion, and inflammation. In most cases, these symptoms are a result of acute inflammation in which the body is trying to heal itself and the symptoms will eventually fade (Galli, Tsai, & Piliponsky, 2008). Allergic diseases such as eczema, hay fever, sinusitis, and asthma, however, are the result of more long-term chronic inflammation (Galli, Tsai, & Piliponsky, 2008). Specific pathways and mechanisms of proinflammatory mediators are still areas that do not have complete scientific research and understanding.

Histamines are one of the major proinflammatory mediators released and they bind to H1 receptors which cause the smooth muscles of the bronchi to contract (Faustino-Rocha, Ferreira, Gama, Oliviera, & Ginja, 2017). This makes breathing difficult as the airways start to close. In addition, histamines cause blood vessel dilation and increased permeability of the blood vessel

walls. Anaphylactic shock, the rapid onset of severe allergic response, is a result of large amounts of histamine and can be life-threatening (Kim & Fischer, 2011). This allergic response can cause oral, skin, respiratory, cardiovascular, gastrointestinal, and neurologic symptoms, essentially attacking the entire body.

Prostaglandins are important in both early and late immune responses. They can also cause the smooth muscle to contract, causing coughing, wheezing, and shortness of breath (Satoh et al., 2006). Persistent bronchial hyperreactivity can lead to the diagnosis of asthma. Six hours after the exposure to the allergen, it is also involved in the pathogenesis of urticaria, allergic rhinitis, and allergic bronchial asthma (Satoh et al., 2006).

Leukotrienes are seen to have a role in many chronic inflammatory diseases (Liu & Yokomizo, 2015). For example, they are correlated with eczema, asthma, and hay fever. The molecules are also related to some more acute symptoms such as anaphylaxis, bronchoconstriction, and mucus secretion.

Immunoglobulin E and Immunoglobulin G

Human immunoglobulin (Ig) is generally a Y-shaped protein produced by plasma cells to aid the immune system in destroying foreign bacteria (Schroeder & Cavacini, 2010). Of the five antibody classes, IgG and IgE are the two isotypes directly involved in allergy mechanisms (Warrington et al., 2011). All immunoglobulin are composed of two heavy (H) and two light (L) chains, and a combination of variable (V) and constant (C) regions. The light chains are made up of two parts: one variable region and one constant region. The heavy chains are made up of four parts: one variable region and three constant regions (Schroeder & Cavacini, 2010). The variable and constant domains respectively bind to antigens and specify effector functions (Schroeder & Cavacini, 2010). Effector functions can include tasks such as binding to the Fc regions of mast cells. The variable regions of the heavy and light chain are paired together at the top of the

antibody to create an antigen binding site. The constant regions of the immunoglobulin determine the specific isotope of the immunoglobulin.

Immunoglobulin G (IgG) is the most abundant immunoglobulin within the blood and lymph nodes. IgG has four subclasses: IgG1, IgG2, IgG3, and IgG4. The subclasses have different numbers of disulfide bonds, have different roles within the body, and are named in the order of decreasing abundance (Vidarsson, Dekkers, & Rispens, 2014). One of IgG's roles is opsonization in which the antibody "coats" an antigen, so that the foreign substance can be easily identified and destroyed (Warrington et al., 2011). The IgG antibody binds to the antigen using its Fab region, while the Fc portion sticks out. The Fc portion can then be identified by receptors on a macrophage which engulfs the foreign substance. IgG also engages in agglutination in which the antibody can connect multiple antigen particles in order to create one big clump for easier and more efficient recognition and degradation (Warrington et al., 2011). Finally, IgG is the only immunoglobulin that can pass through the placenta. This is very important because mothers can pass their IgG into their offspring therefore passing certain immunities onto their offspring (Warrington et al., 2011).

Immunoglobulin E (IgE) is the antibody most directly related to allergies (Stone, Prussin, & Metcalfe, 2010). When the immune system produces an allergic mechanism, the B cells switch from producing IgG to producing IgE. The main purpose of IgE is to bind to the FcεR1 receptor on mast cells in order to induce an immune response to a foreign substance. It has a high affinity for mast cells and basophils, and when IgE is bound rather than free floating, its half-life increases from two days to about two weeks (Stone, Prussin, & Metcalfe, 2010). Mast cells have FcεR1 receptors on their surface for the Fc region of IgE antibodies to bind to. Once the IgE is bound, it can capture antigens, causing the mast cell to release its granules which contain proinflammatory mediators (Galli, Tsai, & Piliponsky, 2008).

FcεR1

FcεR1 is the receptor on mast cells and basophils to which all variants of human immunoglobulin bind. However, FcεRI has a high affinity for IgE (Metz, 2013). Structurally, FcεR1 is tetrameric and has 4 subunits, an α, β, and 2 disulfide-linked γ subunits. The β and γ chains are involved in signal transduction after binding of IgE, so they mediate the cells response to IgE (or other molecule) binding, and are responsible for the following signal cascades and histamine release. The FcεR1 receptor is located on the surface of several different types of cells. It is present in its complete form and expressed at a constitutive level only on basophils and mast cells (Metz, 2013), but it is also present in a reduced form $\alpha\gamma_2$, in which the β signaling subunit is not present. This reduced form of FcεR1 is expressed in cells like dendritic cells, eosinophils, and platelets. FcεR1 is also upregulated by binding of IgE to it on the surface of mast cells.

B Cell Activation

In the immune response, naive B cells undergo a process known as class switch recombination in which the class of antibody is changed from IgM to either IgG, IgE, or IgA by means of a chromosomal deletion along the locus for the antibody's heavy chain (Stavnezer, Guikema, & Schrader, 2009). There are two main components to class switching in T-dependent B cell activation. The first component is the bonding of the ligand on a T cell with the B-cell surface receptor CD40, and the second is the release of cytokines by that T cell (Maddaly et al., 2010). Once the B cell is activated, enzyme activation induced cytidine deaminase (AID) oversees the deletion along two splice sites known as switch (S) regions (He et al., 2015). The first S region is fixed just upstream of the coding sequences for IgM and IgD (Stavnezer, Guikema, & Schrader, 2009). The second S region to be cut is determined by cytokines produced by helper T cell as they bound to CD40. Cytokine interferon-γ (IFN-γ) causes a splice site just before the gene sequence for IgG to be cut, excising the sequence for IgM and IgD before

binding back the sites of excision (Kawano, Noma, Kou, Yoshizawa, & Yata, 1995). Cytokine interleukin 4 (IL-4) is responsible for the deletion resulting in the transcription of IgE antibodies, when the B cell then differentiates into a plasma cell where antibody production begins (Stavnezer & Schrader, 2015).

Pathways

The binding of IgE to FcεR1 results in a cascading web of signaling pathways between an intricate amount of adaptor proteins, kinases, and other molecules. These events lead in summary to two events specific to the allergic mechanism symptom manifestation: mast cell degranulation and cytokine production, as depicted in Figure 3.

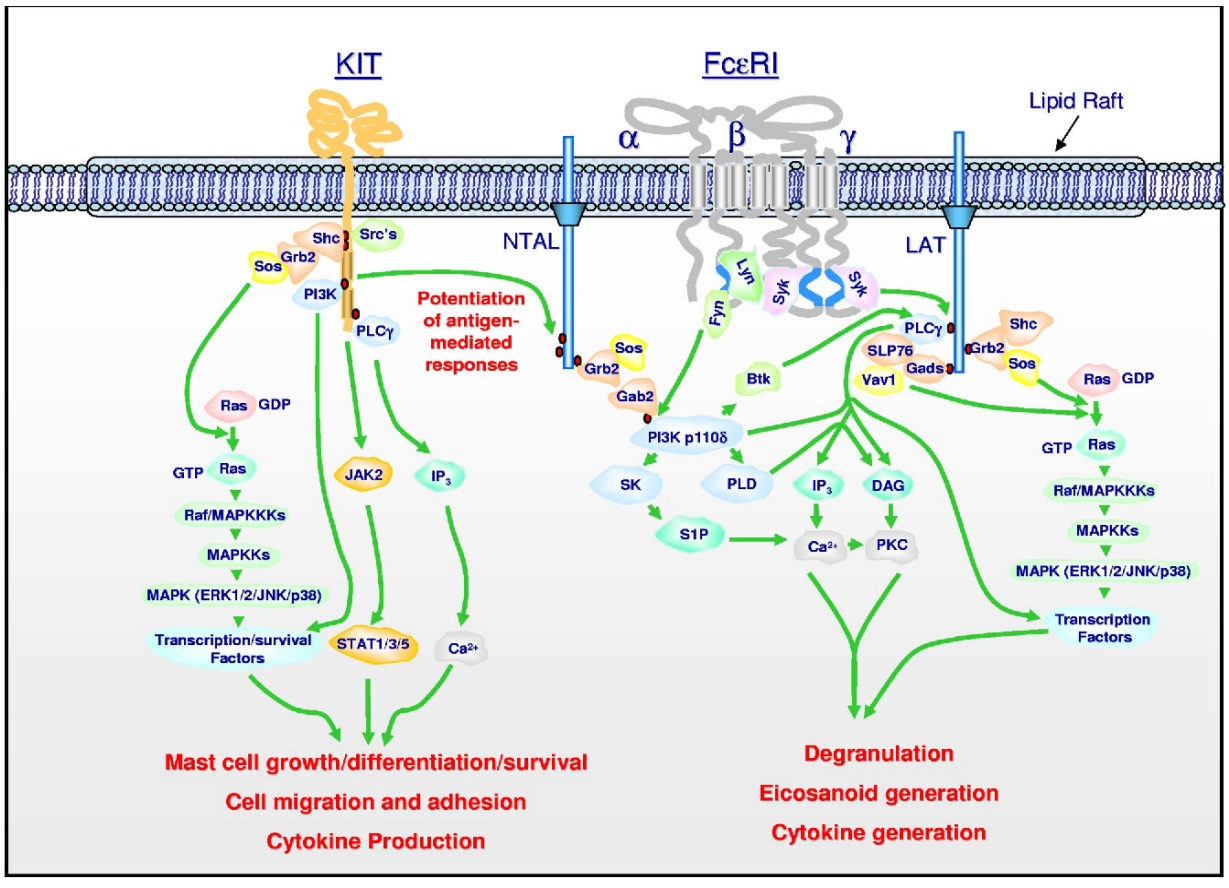


Figure 3: Overview of cell signaling pathways within mast cells. Adapted from "Integrated Signalling Pathway for Mast Cell Activation" by Gilfillan, A. M., & Tkaczyk, C. (2006), Nature Reviews Immunology, 6, 218-230. doi:10.1038/nri1782

Lipid Rafts, LYN, FYN, GAB2, PI3K

In the early part of the signaling pathway, IgE-antigen complexes bind to the high affinity FcεR1 receptor on mast cells and some basophils (Oettgen & Burton, 2015). Before this binding, FcεR1 receptors have a low affinity to form lipid rafts, or regions of the cell membrane where certain glycolipids cluster to form specific domains useful in signaling (Dykstra, Cherukuri, Sohn, Tzeng, & Pierce, 2003). After IgE-antigen binds to FcεR1, however, oligomeric antigens cross-link IgE bound to FcεR1 just long enough to assemble a "signalosome" made of adaptor proteins and other signaling proteins that promote raft clustering and bind to the actin cytoskeleton to stabilize the receptors in the lipid raft (Dykstra et al., 2003). Then, tyrosines in immunoreceptor tyrosine-based activation motif (ITAM) sequences, found in the cytosolic domains of the FεcR1 β and γ chains, become phosphorylated by Src kinase Lyn (Metcalfe, Peavy, & Gilfillan, 2009). Lyn kinase is preferentially found in these membrane microdomains and shifts the equilibrium from most inactive to mostly phosphorylated/active FcεR1 (Gilfillan & Tkaczyk, 2006).

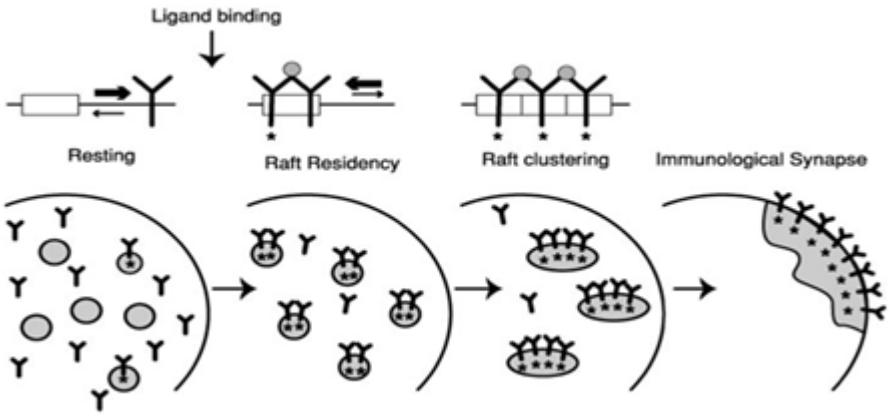


Figure 4. Process of creating lipid raft with IgE-antigen and FcεR1 oligomers Adapted from "Location is Everything: Lipid Rafts and Immune Cell Signaling," by M. Dykstra et. al, 2003, Annual Review of Immunology, 21, 457-481. 2003 by Laboratory of Immunogenetics.

One important thing to note was that Metcalfe et al. found Lyn kinase was not necessary to trigger degranulation and consequent histamine release, based on the differing genetic makeups of the rats the mast cells were isolated from. C57B1/6 rats expressed hyporesponsive degranulation phenotype (less histamine release) while 129/Sv rats expressed hyperresponsive degranulation phenotype (more histamine release), both reacting to the same Lyn kinase activity (Metcalfe, Peavy, & Gilfillan, 2009). In addition, residual degranulation was still observed in Lyn- mice, suggesting the importance of other components of the signaling pathway that contribute to downstream mast cell degranulation (Gilfillan & Tkaczyk, 2006).

Fyn kinase is also found in these lipid rafts and binds more tightly to the β subunit of FεcR1 when the receptor is activated (Parravicini et al., 2002). It is responsible for phosphorylating GRB2-associated binding protein 2 (Gab2), which is a process that is independent of Lyn and LAT, and is evidenced by the fact that Fyn-/- mice were shown to have a marked reduction in Gab2 phosphorylation (Parravicini et al., 2002). Gab2, like its analogs of Gab1 and Dos, the latter of which is found in drosophila, is a scaffold adaptor protein that signal molecules can bind to. Gab-/- mice were found to have both impaired mast cell degranulation and cytokine expression (Gu et al., 2001). Phosphorylation of Gab2 causes a conformational change to recruit molecules like protein tyrosine phosphatase 2 (SHP2) and phosphatidylinositol-3-OH kinase (Ptdlns-3K or PI3K).

Phosphatidylinositol-3-OH kinase, which can be abbreviated to Ptdlns-3K or PI3K, is a family of lipid kinases known to be involved in many cellular functions such as cell growth, development, proliferation, and survival (Hemmings & Restuccia, 2012). They are involved in pathways leading to endocytosis, vesicle trafficking, autophagy, signaling, cortical remodeling, secretion, and cytokinesis, to name a few, and deregulation of PI3K leads to tumorigenesis (Jean & Kiger, 2014). With so many functions, their scope will be simplified to the immediate

involvement within the pathway previously described as activated by Gab2. There are three distinctive classes of PI3K and eight total isoforms, all of which phosphorylate their 3' hydroxy group of the inositol ring (Koyasu, 2003). These classes are distinguished based on structural differences between additional domains, yet altogether all classes possess the foundation of three domains: C2, helical, and catalytic (Vanhaesebroeck, Guillermet-Guibert, Graupera, & Bilanges, 2010).

In the mast cell degranulation signaling pathway, PI3K is activated by the adaptor molecule Gab2. When recruited, the regulatory subunit of PI3K in conjunction with a tyrosyl phosphorylated SHP binds to sites on Gab2 (Gu et al., 2001). Once bound, class 1A PI3K containing p85 and p110 subunits convert the internally membrane bound phosphatidylinositol-(4,5)-bisphosphate (PIP_2) to phosphatidylinositol-(3,4,5)-triphosphate (PIP_3) (Kim, Rådinger, & Gilfillan, 2008). PIP_3 has docking sites that are made available to associating proteins at domains called pleckstrin-homology domains (Gilfillan & Tkaczyk, 2006). Some associating proteins called to PIP_3 include PLCγ, VAV, PLD, SK, and BTK.

PLD and PLCγ Pathways to DAG, PKC, Ca^{2+}

Phospholipase D (PLD) is a protein with an unknown effect on signaling for mast cell degranulation. This was determined after a dominant negative form of PLD1 actually increased mast cell degranulation in one study (Rivera, 2006). It has direct contact with kinases such as Protein Kinase C (PKC), ERK, and Tyrosine Kinase 2 (TYK). Activation of PLD has been linked to the production of phosphatidic acid (PA), diacylglycerol (DAG), and choline. PLD specifically plays a role in the first step, through the catalysis of the reaction from phosphatidylcholine to phosphatidic acid. PA itself also targets and regulates PKC.The conversion of phosphatidic acid to DAG is then usually catalyzed by the enzyme phosphatidate

phosphatase or phosphatidate phosphohydrolase (PAP) (Peng, 2005). DAG can also be phosphorylated through DAG kinase to reproduce PA (Becker, 2006).

There are two types of PLD: PLD1 and PLD2 (Rivera, 2006). PLD1 is mostly found in the granule membrane and intracellular vesicles, while PLD2 is mostly found in the plasma membrane (Rivera, 2006). Both forms of PLD are activated after FcεR1 activity, and therefore both play a role in produces downstream molecules, but it is thought that PLD1 has a larger effect in creation of phosphatidic acid. PLD1 specifically has an impact on the migration of secretory granules to the surface of mast cells. PLD2 then participates in the calcium dependent fusion of the plasma membrane and the vesicle (Rivera, 2006).

Research has found that many primary alcohols, such as ethanol and 1-butanol can deactivate PLD (Rivera, 2006). The alcohols will react with alcohols to generate phosphatidylbutanol, which inhibits PA formation through competitive inhibition with phosphatidylcholine (Peng 2005). This reaction has also been used as a good assay for PLD presence (Becker, 2006). In addition, silencing the RNA targeted at PLD has also been known as an effective silencer of the molecule and therefore pathway. It has been indicated the siRNA against PLD1 inhibits both PLD1 and PLD2, but siRNA against PLD2 only inhibits PLD2 (Peng 2005), suggesting more importance to PLD2, since the two silencers are similar enough to be comparable. This technique can be performed in culture, but is not practical for in patient use.

DAG is integral in PLD and PKC interaction. DAG production mainly comes from two pathways, one being the aforementioned PLD pathway, and another being through phospholipase C*y* (PLC*y*) (Peng, 2005). There are two forms of PLC*y* with PLC*y*-2 mainly involved in B-cells and the PLC*y*-1 form mainly expressed in mast cells (Kim, 2000). PLC*y* is the signaling enzyme that catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2), a phosphoinositide on the plasma membrane that is thus cleaved into the second messengers of membrane-bound

DAG as well as soluble inositol 1,4,5-trisphosphate (IP3) (Kim, 2000). These two cleavage products have important and independent roles in subsequent signaling that ultimately results in activation of calcium release (Gericke, 2013).

It has been suggested that PLC-produced DAG is more unsaturated and therefore may be a better activator of PKC. Production of DAG is what triggers PKC to bind and interact with PLD (Cazzoli, 2006). DAG competes with phorbol esters to bind to the C1 site on PKC (Becker, 2006). This could possibly become an area of interest for cascade inhibition. There are actually several different subgroups of PKC, some of which are dependent on DAG and calcium, some DAG alone, and some on neither (Singer, 1995). Of the calcium dependent groups, DAG generally binds to the C1 unit and calcium to the C2 unit, while in the calcium-independent groups, only DAG binds to the C1 unit (Huang 1989). Interaction of PLD1 by PKC seems to deactivate the molecule, and therefore trigger granule vesicle movement to plasma membrane, where PKC interaction with PLD2 somehow incites vesicle-membrane fusion and degranulation (Siddiqi, 2000). The PKC appears to interact with the PLD2 isoforms in a non-phosphorylating manner which is yet unknown (Singer, 1995). There is some dispute about which isoforms of PKC are actually active in this pathway, but some evidence suggests that PKCα is the form that interacts with PLD1 and PLD2 in FcεR1 pathway (Siddiqi, 2000). Other studies add that PKC-θ might also be activated in the FcεR1 pathway (Bell, 2015). In contrast, it is known that PKCs βI, βII, δ, and ε have some sort of role in the degranulation process (Rivera, 2006). Calcium(Ca^{2+}) also has a role in regulating PLD, but while it is known that there is a separate cascade of some sort, the exact mechanism is not yet understood (Siddiqi, 2000). What is known is that calcium release is triggered by the production of IP3 during the PLC pathway, which causes a transient release of the calcium in the endoplasmic reticulum, that in turns causes calcium to flow in from extracellular channels and spark the rest of the cascade (Rivera, 2006).

PKC is a group of lipid dependent kinases that phosphorylate protein structures and serve as effector molecules (Becker, 2006). In addition to calcium, PKC also has a high affinity for PLD, and is actually one of the regulatory molecules for PLD1 and PLD2. DAG generation recruits PKC to migrate to the plasma membrane, during the path in which it attaches to and activates PLD (Becker, 2006). It appears that the chain starts with DAG being produced by PLC, which then induces PLD to produce its own DAG in a much greater amount in order to activate PKC (Peng, 2005). The mechanism behind PKC regulation is very complicated though because there are a large variety of PKC enzymes that may be a part of the regulation. PKC inhibitors such as staturosphine and calphostin C have been seen to block PLD activation. Although, there are some studies that suggest that calphostin may act directly upon PLD.

SK to S1P Pathway

Sphingosine kinase (SK) is an enzyme found in the mast cell pathway that possesses isoforms SK-1 and SK-2. The role of SK is to catalyze the phosphorylation of sphingosine which forms S1P (Sun and Bonder, 2012). S1P stands for sphingosine-1-phosphate; it is a signaling sphingolipid that acts as an important regulator for many physiological processes and is the biological product of SK. S1P is known to have a role in the signaling processes of various diseases from cancers, to diabetes, osteoporosis, and allergies and asthma (Maceyka et al., 2011). In the allergic response, SK and S1P are involved in survival, differentiation, migration, and activation (Sun and Bonder, 2012).

There are two isoenzymes which form S1P, the SphK1 and the SphK2. These enzymes catalyst the phosphorylation of sphingosine to form S1P. SphK1 appears to regulate cell growth and survival, but when overexpressed SphK2 induces cell death (Olivera et al., 1999). Conversely, there is evidence that they have reductant functions that allows them to compensate for the lack of each other (Oskeritzian et al., 2007). This was shown by a study in which both

SphK1 and SphK2 were knocked out and resulted in loss of S1P and lethal effects but knockouts of one separately led to viable results. Both SphK1 and SphK2 were found to have increased activity when the FcεRI receptor was activated in mast cells (Mizugishi et al., 2005).

S1P has two receptors, $S1P_1$ and $S1P_2$ which are both expressed on the surface of mast cells. (Oskeritzian et al., 2007). $S1P_1$ regulates migration of mast cells towards antigens. $S1P_2$ is known to mediate mast cell activation and degranulation, which makes it a better target for this research. (Oskeritzian et al., 2007). The S1P that is secreted by the mast cell is able to bind to these receptors.

There are known inhibitors of SK and S1P receptor inhibitors, however only a few have gone onto clinical trials. One drug that targets S1P is FTY720 which is an immunosuppressive drug that aims to treat multiple sclerosis. It works by mimicking S1P and binding to the S1P receptors which caused down regulation of cell surface receptors which made the cells unable to respond to the real S1P (Olivera and Rivera 2005). This drug is one that is a first of its kind S1P receptor modulator. A downside to this treatment is that S1P is found in most cells and therefore unwanted effects have been reported. Research suggests that inhibiting the SK/S1P pathway may be an effective treatment for allergies, but there is a high possibility of adverse side effects. The side effects of the mentioned drug FTY720 include hypertension, dyspnea, elevated liver enzymes, and increased risk of infections (Sun and Bonder 2012). More research is required to form an effective treatment approach using S1P/SK inhibitors.

PKA and cAMP

Protein kinase A is an enzyme that plays an important role in a number of cellular processes. Its role is to phosphorylate other proteins to regulate their function. It is composed of two types of subunits. There are two catalytic subunits, which are responsible for adding phosphate groups to proteins. There are also two regulatory subunits, which are able to detect

levels of cyclic AMP (cAMP) and regulate the catalytic subunit based upon the detected level. When cAMP levels are low, the catalytic subunit is inactive, and when cAMP levels are high, the catalytic subunit is active. PKA is anchored to locations in the cell by A kinase anchor proteins (AKAPs), which also may be involved in PKA regulation (Landsverk et al, 2001). Research suggests that administering a treatment using an antagonist of cAMP or shRNA down-regulation of PKA reversed the EP_2-mediated inhibitory effect on MC degranulation (Serra-Pages, 2012). A molecule called PGE_2 can mediate the suppression of mast cell degranulation, which researchers found could be modulated to vary from activating to suppressing, depending on the relative ratio of EP_2 to EP_3 expression on these cells with suppression evident only in cells having increased EP_2 to EP_3 expression. (Serra-Pages, 2012).

Existing Research

Symptom Management

Currently, there is no cure for allergies. Relatively common and established treatment recommendations for allergies are focused on avoidance measures and pharmacotherapy-based symptom management (Lanser, Wright, Orgel, Vickery, & Fleischer, 2015). Although the recommendation of avoiding allergens will certainly lessen allergen exposure and thus the subsequent allergic response, the measure is impractical and inconvenient in daily practice (Marple et al., 2007).

Some treatments, such as decongestants and corticosteroids, simply focus on treating the symptoms of allergies. Decongestants relieve the nasal congestion associated with allergies by reducing swelling in nasal tissues and blood vessels (AAAAI, 2018). Corticosteroids also work to reduce inflammation in addition to ameliorating stuffiness, sneezing, and runny nose. There are also a number of over-the-counter treatments such as eye drops and nasal saline spray that

can also help to alleviate some of the symptoms. In emergency cases, Epinephrine can be administered to stop the serious allergic reaction of anaphylaxis in which the immune system overreacts to foreign substances and releases a flood of chemicals that can cause an individual to go into shock and can even lead to death if not treated properly (AAAAI, 2018).

Other treatments aim to stop or limit the allergy symptoms from occurring by interfering with a step in the molecular pathway of the allergic response. These include antihistamines, mast cell stabilizers, and antileukotriene agents (AAAAI, 2018). Antihistamines such as loratadine (Claritin) and cetirizine hydrochloride (Zyrtec) block histamine from attaching to its receptor after it has been released from the mast cells. Mast cell stabilizers can stop the histamine from ever being released from the mast cells in the first place. Antileukotriene agents work to limit the effects of leukotrienes, which are known to play an important role in some severe allergic symptoms. Some of these agents work by blocking the production of leukotrienes while others stop leukotrienes from ever binding to their receptors (AAAAI, 2018).

Although avoidance and symptom management treatments are able to reduce the effects of allergic responses, they are insufficient for many and are not measures working towards a curative or long-lasting therapy. Researchers have been seeking new and improved therapeutics such as through immunotherapy to better target the foundational mechanisms initiating allergic reactions which can even work in conjunction with the aforementioned treatments to increase the efficacy and endurance of treatment.

Allergy Immunotherapy

Allergy-specific immunotherapy (SIT) is a curative method of treatment for certain allergies as it is able to induce long-term allergen-specific tolerance through multiple mechanisms and routes of administration (Akdis, 2014). Desensitization with SIT involves a process similar to vaccinations in which increasing doses of extracts of the specific allergen are

administered followed by maintenance doses for several years. This process is hoped to trigger immunological change for sustained desensitization (Orengo et al., 2018). Immunotherapy, specifically allergy shots or desensitization, is one of the most effective treatments for those who experience chronic allergies. Currently, there are even oral tablets such as Grastek and Oralair that can be taken at home (AAAAI, 2018).

SIT focuses on inducing an increase in IgG production to outcompete IgE in binding to effector cell receptors (Orengo et al., 2018). Orengo et al. (2018) tested this method in mice and cat-allergic patients, finding that an increase in the blocking IgG/IgE ratio reduces the allergic response. Although SIT can provide lasting treatment, it has variable efficacy among patients. Some patients have no response to treatment, and others have adverse side effects. Side effects are most commonly associated with food allergies, and therefore SIT is only available for environmental aeroallergens (Orengo et al., 2018). To address some of the issues of this technology, more research is emerging on dosing schedules, routes of administration, and advanced vaccines (Akdis, 2014). While there are established methods of immunotherapy, improvements can be made for more effective actions that can be applied to all types of allergens.

Anti-Cytokine Drug Therapy

There is research underway in the vein of genetic exploration among the genes behind antibody production and class switch recombination. In a 2016 study, the effects of anti-IL-4 were investigated to combat asthma with variable changes among asthmatic symptom across asthma types (Bagnasco, Ferrando, Varricchi, Passalacqua, & Canonica, 2016). In 2001, clinical trials began testing anti-IL-4 drug pascolizumab against the effects of allergic asthma with mixed results that ultimately concluded inefficacy (Hart et al., 2002). Often, therapies with action to block IL-4 act in conjunction with inhibitors of IL-13 due to the redundancy that the two

cytokines possess in expressing many of the same pathways, functioning similarly (Kau & Korenblat, 2015). Several dual-blocking anti-IL-4 and anti-IL-13 drugs are in the process of testing yet are thus far ineffective to block allergic asthma entirely due to endotypes within the asthma subclass of allergies, meaning that the differences in function of various subtypes of asthma complicates the efficacy of the drugs (Kau & Korenblat, 2015). Overall there is lack of consistent efficacy in asthma studies and even less so within the scope of more complicated allergies, rendering anti-cytokine therapy unpromising.

Anti-IgE Drug Therapy

Anti-IgE drugs mediate the allergic response by binding to the Cε3 region within the Fc region of the IgE antibody (Holgate, 2014). One example of this type of drug is omalizumab, a recombinant humanized monoclonal antibody. It works by binding two of its molecules to the Fc region of the free-floating IgE so that is is unable to bind to FcεR1. This reduces the levels of free-floating IgE in the body as over time they are recycled. This is indicative of the drug's effectiveness against a long-term allergy such as allergic rhinitis (Easthope & Jarvis, 2001). Other allergies have been tested against the effects of omalizumab with varying degrees of success. In a review done in 2014, omalizumab was reported to have anecdotal or limited evidence against allergies such as Churg-Strauss Syndrome, bronchopulmonary allergic aspergillosis (fungal sensitivity), and eosinophilic otitis media (Holgate, 2014).

MEDI4212 is an anti-IgE monoclonal antibody that has the potential to eliminate IgE-expressing B cells and neutralize soluble IgE in asthma prone patients (Nyborg et al., 2015). This occurs through cell-mediated cytotoxicity, a type of immune reaction in which the target is coated with antibodies and killed by white blood cells. MEDI4212 variants also inhibited IgE-induced signaling and demonstrated enhanced cell killing. (Incorvaia & Mauro, 2015). MEDI4212's advantage over omalizumab is its ability to neutralize high amounts of soluble IgE

as well as remove the IgE expressing B cells. (Incorvaia & Mauro, 2015). The authors suggest that this drug is not likely to replace omalizumab, which has been brought to market, but could potentially be used as an additional treatment (Nyborg et al., 2015). More research needs to be done on this novel high-affinity anti-IgE monoclonal antibody, such as testing its abilities to reduce other allergies other than asthma.

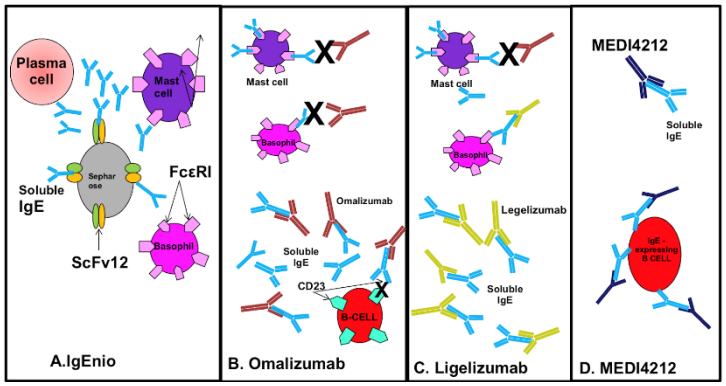


Figure 5. Incorvaia C., Riario-Sforzo G., Ridolo E. Adapted from "IgE Depletion in Severe Asthma: What We Have and What Could Be Added in the Near Future" Incorvaia C., Riario-Sforzo G., Ridolo E. (2017). Ebiomedicine. 16-17. 2017 The Authors.

Adenine in Cell Signaling

Adenine has a variety of biological functions other than being a component of DNA and RNA. A 2015 study shows that adenine possess anti-allergic effects by inhibiting FcεRI-mediated signaling events (Silwal et al., 2015). In this experiment, mice were induced for passive cutaneous anaphylaxis (PCA) which mimics the type I allergic response. Mast cells were collected from the bone marrow of mice. These cells were sensitized with DNP-specific IgEs and then stimulated with DNP-HSA antigen. Measurements were taken of degranulation, LTB_4 secretion (involved in inflammation) and cytokine secretion. This was then repeated but before stimulation of the antigen, the cells were treated either with adenine, adenosine, ADP, ATP or cytosine. Injecting adenine one hour before the antigen injection suppressed the PCA reaction -

almost entirely at 10 mg/kg. At this dose, adenine suppressed PCA better than ketotifen, an anti-allergy medicine. Adenine also prevented mast-cell degranulation in a dose dependent manner almost completely at 5mM. Interestingly, ATP-stimulated degranulation at 2mM but suppressed it by 40% at 5mM. adenosine and ADP-stimulated mast cell degranulation and cytosine had no effect. Adenine also blocked LTB_4 production as well as cytokine secretion by inhibiting FcεRI-mediated intracellular signaling (Silwal et al., 2015).

This study testing the effects of adenine is valuable because it leads us on the path to finding a medication that prevents the allergic response from occurring. Rather than antihistamines which merely alleviate symptoms caused by the degranulation of mast cells, administering adenine could terminate the allergic response earlier in its pathway.

After the current treatments for allergies were evaluated and the mast cell degranulation pathway that leads to allergies was illuminated, a method of minimizing allergic symptoms was chosen. This method involves manipulating parts of the pathway with small molecule inhibitors to measure the effects it has on the release of histamines and cytokines.

Methodology

The study aims to inhibit parts of the downstream FcεR1 in order to reduce or prevent mast cell degranulation. Existing allergy drugs mostly focus on post-degranulation events. These are more reactive approaches that deal with symptom management, while our research question aims to present a proactive approach. Due to the webbing complexity of the signaling pathways toward mast cell degranulation, specific small molecules within the pathway or a small chain of molecular reactions will be chosen based on their potency in minimizing the release of proinflammatory mediators. Inhibitors of these chosen molecules will be researched and taken into account when choosing the specific molecules. Then, a mast cell line will be purchased such as RBL-2H3 which are basophilic rat cells that are FceR1 receptor positive (ATCC® CRL-2256™). These cells are commonly used in degranulation assays because they can be activated to secrete histamine. Another mast cell line that may be used is MC/9 which are mouse liver mast cells that express FceR1 and will release histamine when exposed to antigens after incubating with anti-Fc-receptor antibodies (ATCC® CRL-8306™). Certain molecules within the pathways that lead to degranulation will be inhibited and degranulation will be measured with and without the presence of the inhibitors.

Small molecule inhibitors for the chosen signaling molecules within a pathway will be obtained. A mast cell degranulation assay will test for the efficacy of the cell to release histamines once the signaling molecule has been inhibited. Each of the chosen molecules will be inhibited, ensuring that only one molecule is inhibited in a given assay. This will pinpoint any reduction in degranulation to a specific molecule. Possible methods for such data collection and quantification may include ELISA (enzyme-linked immunosorbent assay), Western blots, or SDS-Page. An enzymatic isotopic assay can be performed to which can quantify amount of histamine directly from tissue, blood, or serum samples (Beaven, 1972). Following the protocol

for flow cytometry, the chemical and physical characteristics in mast cell samples can be analyzed. Other assays such as in-vitro kinase, and colorimetric assays, which measure the activity of β-hexosaminidase, and granule marker enzymes may be utilized. Indication of a reduction or prevention in degranulation by multiple specific molecules may lead to testing combinations of inhibitions that may enhance the effects.

Once promising targets are identified and if time permits, experimentation can be done with murine models in order to observe the effects of inhibiting the molecule(s) on other bodily functions. The chosen design is optimal because it is an intermediate step between mast cell degranulation and preventing the binding of IgE to FcεR1. The anticipated results would be an important contribution to the research field because it can elucidate the cell signaling pathway of allergic reactions and reveal a certain signaling molecule components that prevents mast cell degranulation and histamine release, effectively inhibiting the allergic response while minimizing adverse effects. While drugs targeting the binding of IgE to mast cells as well as drugs focused on antihistamine symptom relief are both well-researched and widely available, a goal of disrupting the signaling pathway is an innovative means by which the allergic response can be minimized.

Appendix

Glossary

Definitions are from Oxford Dictionary unless otherwise cited.

Adaptive immunity - immunity that has memory and occurs after exposure to an antigen either from a pathogen or a vaccination (Molnar, 2013).

Agglutination - (with reference to bacteria or red blood cells) clumping together.

Allergy-specific immunotherapy (SIT) - administration of allergen extracts to achieve clinical tolerance of allergens that cause symptoms in patients with allergic conditions (Frew, 2010).

Anaphylactic shock - An extreme, often life-threatening allergic reaction to an antigen to which the body has become hypersensitive.

Antibody - A blood protein produced in response to counter a specific antigen.

Antigen - A toxin or other foreign substance which induces an immune response in the body, such as the production of antibodies.

Antigen-presenting cell (APC) - Any cell that assists in the production of immune responses by presenting antigen; especially any of several types of cell with monocytic lineage that present antigen in association with class II MHC molecules, to helper T lymphocytes.

B cell - A lymphocyte not processed by the thymus gland, and responsible for producing antibodies. Also known as B lymphocyte.

Basophil - A basophilic white blood cell.

Corticosteroid - Any of a group of steroid hormones produced in the adrenal cortex or made synthetically. There are two kinds: glucocorticoids and mineralocorticoids. They have various metabolic functions and some are used to treat inflammation.

Cytidine deaminase (AID) - enzyme that in humans is encoded by the *CDA* gene (Kuhn,1993)

Cytokine - Any of a number of substances, such as interferon, interleukin, and growth factors, which are secreted by certain cells of the immune system and have an effect on other cells.

Decongestant - used to relieve nasal congestion.

Degranulation - (of a cell) lose or release granules of a substance, typically as part of an immune reaction.

ELISA (enzyme-linked immunosorbent assay) - A laboratory technique that uses antibodies linked to enzymes to detect and measure the amount of a substance in a solution, such as serum. The test is done using a solid surface to which the antibodies and other molecules stick. In the final step, an enzyme reaction takes place that causes a color change that can be read using a special machine. There are many different ways that an enzyme-linked immunosorbent assay can be done. Enzyme-linked immunosorbent assays may be used to help diagnose certain diseases. (NIC Dictionary).

FcεR1 - The high affinity Immunoglobulin E receptor which is a tetrameric membrane protein complex expressed on mast cells and basophils which belongs to the family of immunoreceptors involved in antigen recognition (Type I 2018).

Histamine - A compound which is released by cells in response to injury and in allergic and inflammatory reactions, causing contraction of smooth muscle and dilation of capillaries.

Humoral immune response - antibodies produced by B cells cause the destruction of extracellular microorganisms and prevent the spread of infections (Janeway, 2001).

Immunoglobulin (IgE, IgG, IgM, IgD, IgA) - Any of a class of proteins present in the serum and cells of the immune system, which function as antibodies.

Interferon-γ (IFN-γ) - an interferon that is produced by T cells, regulates the immune response, and in a form produced by recombinant DNA technology is used especially to control infections due to inability of white blood cells to destroy certain bacteria and fungi (Merriam-Webster).

Leukotriene - Any of a group of biologically active compounds, originally isolated from leukocytes. They are metabolites of arachidonic acid, containing three conjugated double bonds.

Major histocompatibility complex (MHC) - Major histocompatibility complex (MHC), group of genes that code for proteins found on the surfaces of cells that help the immune system recognize foreign substances. Also known as the human leukocyte antigen (HLA) system.

Mast cell - A cell filled with basophil granules, found in numbers in connective tissue and releasing histamine and other substances during inflammatory and allergic reactions.

Opsonization - Making (a foreign cell) more susceptible to phagocytosis.

Phagocytosis - The ingestion of bacteria or other material by phagocytes and amoeboid protozoans.

Pharmacotherapy - Medical treatment by means of drugs.

Proinflammatory - That promotes inflammation (Ex. When the lesions are traumatized, or rubbed firmly, the cutaneous mast cells may release *proinflammatory* mediators, causing edema, erythema, and even vesicle formation.) (Merriam-Webster)

Prostaglandin - Any of a group of compounds with varying hormone-like effects, notably the promotion of uterine contractions. They are cyclic fatty acids.

Sodium dodecyl sulfate - synthetic reagent used in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), which is a very common method for separating proteins by electrophoresis using a discontinuous polyacrylamide gel as a support medium and sodium dodecyl sulfate to denature the proteins (Caprette, 2018).

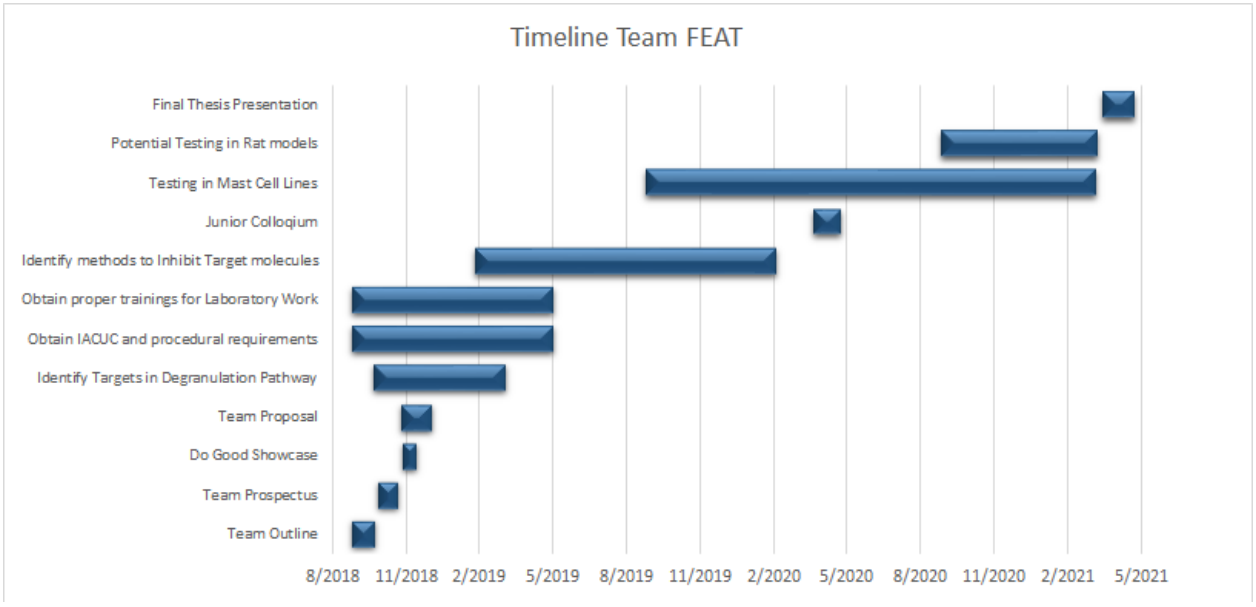
T cell - A lymphocyte of a type produced or processed by the thymus gland and actively participating in the immune response.

Tyrosine - A hydrophilic amino acid which is a constituent of most proteins and is important in the synthesis of some hormones.

Western blot - An adaptation of the Southern blot procedure, used to identify specific amino-acid sequences in proteins.

Timeline

In Fall 2018, the team will focus on selecting one methodology aim to pursue and focusing the research questions in order to complete the research proposal. Applications for the first round of funding opportunities and any necessary laboratory trainings will be fulfilled by the end of Winter 2018. By Fall 2019, data collection will commence. Preliminary research will be presented at the Gemstone Do Good Showcase. In Spring and Fall of 2020, the team will focus on finishing data collection and producing an initial draft of the thesis and begin to present research findings at conferences and Junior Colloquium. The final thesis will be submitted, presented, and defended at the Thesis Conference, and receive citations from the Gemstone program in Spring of 2021.



Anticipated Research Budget

The research budget may include use of lab space, lab machinery, lab materials, analytical programs, and potential animal models. Listed below are some of these anticipated expenses.

Anticipated Budget List

Items	Quantities	Estimated Prices
Western Blot • Blotting instrument ○ Including base • 2 cassettes to hold up to 2 midi blotting sandwiches • Blot roller • Midi nitrocellulose transfer pack	1	~$3,300.00
RBL-2H3 cell line	4	~$1464.00
MC/9 cell line	4	~$1464.00
Immunoglobulin E • 400 µg	4	~$1060.00
SDS - PAGE • PAGEprep resin slurry • Dimethyl Sulfoxide solution • Elution buffer • Sample buffer • Spin cups • Collection tubes	1	~$175.00
Histamine ELISA Kit • (20X) Wash Buffer • Antibody Diluent • Assay Buffer • Goat anti-Rabbit IgG coated microplate (12x 8 well strips) • Histamine Antibody • Histamine Standard Stock (250 ng/mL) • Histamine Tracer • Plate Sealer • SA-HRP Conjugate • Stop Solution	1	~$555.00

• TMB Substrate • Tracer Diluent		
In Vitro Kinase • Kinase Binding Assay	1	~$400.00
Gloves • Latex and allergy free • 100 count per box • Multiple sizes	4	~$100.00
Micropipettes • 0.1 to 2.5 µL • 2.0 to 20 µL • 20 to 200 µL • 100 to 1000 µL	1	$1080.00
Cell Culture Plates • 75 count per box • 24 wells per plate • Non-treated surface	1	~$301.00
Total		~$10,000.00

Mentor Feedback

Our mentor, Dr. Frauwirth read and gave feedback on our proposal. Most of the feedback was small remarks on how the team should reword certain phrases to make it clearer and more accurate to the readers. There were also some misunderstandings in the scientific background of our project which our mentor pointed out and helped us to clarify. He made suggestions for areas in the literature review that we could elaborate on and other parts that could be shorter. He has also urged us to research inhibitors and assays which would be added to our methodology.

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