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Isoprenylation Inhibition Significantly Reduces IgE Mediated Mast Cell Function and Allergic Disease

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Abbreviations

3-hydroxymethyl-3-methylglutaryl coenzyme A reductase - HMGCR

Alternaria- ALT

Antigen-induced clustering of IgE-FcεRI complexes- crosslinking or IgE XL

Area under the curve- AUC

Bone marrow derived mast cells- BMMC

Dendritic cells- DCs

DNP-coupled human serum albumin- DNP-HSA

Dual farnesyl and geranylgeranyl transferase inhibitor- FGTIs

Extra cellular acidification rate- ECAR

Fluvastatin- FLU

FT inhibitors- FTIs

GGT-1 inhibitors- GGTIs

goblet cells- GBC

Half maximal inhibitory concentration- IC₅₀

Hematoxylin and eosin stain- H&E

Immunoglobulin- Ig

immunoreceptor tyrosine-based activation motif- ITAM

Low density lipids- LDL

Mouse mast cell proteases- mMCPT

Ovalbumin- OVA

Passive systemic anaphylaxis- PSA

Periodic acid-Schiff stain- PAS

Propidium iodide -PI exclusion

Small interfering RNA- siRNA

T follicular helper cells- Tfh

Abstract

Allergic disease is the 6th leading cause of chronic illness in the US and accounts for billions of dollars in healthcare annually. Mast cells are tissue resident innate immune cells linked to allergic disease and activated by IgE and other ligands. Upon activation, they release histamine, cytokines, chemokines, proteases, and lipid mediators evoking allergic symptoms. New ways of targeting mast cells could greatly benefit allergic disease therapy. Previous findings supported repurposing statin drugs, such as Fluvastatin, as a therapeutic treatment of allergic disease reduced allergic symptoms *in vitro* and *in vivo*. We found that Fluvastatin suppressed IgE-mediated mast cell activation by inhibiting isoprenylation of proteins. However, statin suppression is dependent on genetic background. To avert genetic background dependency, we tested the hypothesis that a dual farnesyl and geranylgeranyl transferase inhibitor (FGTI 2734) would suppress allergic disease without having variable efficacy. We show that FGTI 2734 reduced IgE-mediated degranulation and cytokine production similar to Fluvastatin in bone marrow derived mast cells from multiple genetic backgrounds. FGTI-2734 also significantly reduced cytokine production from human derived skin mast cells stimulated through the IgE receptor. We show that FGTI 2734 suppressed a mast cell-dependent model of IgE-mediated anaphylaxis *in vivo* in multiple genetic backgrounds. Among the >400 proteins targeted by prenylation, Ras family proteins have been shown to play a substantial role in IgE-mediated mast cell signaling. We found that loss of K-Ras, but not N-Ras, can mimic the effects of statins on IgE-mediated mast cell activation, suggesting a differential role of K-Ras and N-Ras in mast cell function. We show that in an airway model of allergic disease, FGTI-2734 treatment can significantly reduce airway inflammation and hyperresponsiveness. These data indicated

that dual farnesyl and geranylgeranyl transferase inhibitors may be an effective therapeutic treatment for mast cell-associated diseases.

Introduction

Brief Introduction to the immune system

The immune system is a complex network of cells throughout the body that are essential for the protection and clearance of outside harms such as bacteria, parasites, and toxins. This defense can be broken down into two branches of cells and soluble mediators that have distinct and critical roles, the adaptive and innate immune systems (Fig 1).

The adaptive immune system consists of a pathogen-specific response employing B and T cells. After the first pathogen exposure these cell types can “remember” and respond quickly to subsequent infection by the pathogen. Two subsets of T cells are cytotoxic CD8⁺ T cells and helper CD4⁺ T cells. Cytotoxic T cells are important in clearing intracellular pathogens and in cancer cell clearance. They respond to cell-targeted antigen specific activation by releasing granzyme and perforin to kill the infected cell (Raskov et al. 2021). Helper T cells have 3 main subsets Th1, Th2, and Th17. Th1 cells are characterized by being T-bet transcript positive and help in clearance of bacterial infections, through the secretion of IFN- γ , a signature cytokine that activates macrophages and dendritic cells (DCs) (Annunziato et al. 2014). Th17 cells are associated with IL-17 expression and secretion and are important to clearance of bacterial and fungal infections (Tesmer et al. 2008). Th2 cells are characterized by being GATA3 transcript positive and are critical for clearance of parasitic infections.

Despite their critical benefits to the host, adaptive immune cells have pathological potential. Most relevant to the current studies is Th2-driven allergic disease largely caused by these cells producing IL-4, IL-5, and IL-13 (Walker and McKenzie 2018). Cytokine release from Th2 cells is associated in allergic disease with cell proliferation, tissue eosinophilia, goblet cell

hyperplasia, polarization to an M2 macrophage phenotype that remodels tissues, and smooth muscle contraction in the lungs and gut (Zhu and Paul 2008; Fallon et al. 2002).

T follicular helper cells (Tfh) secrete IL-21 and are associated with B cell activation in lymph node germinal centers (Crotty 2019). Tfh also express CD40L that activates antigen-specific B cells via CD40 (Wishnie et al. 2021). In allergic disease Tfh2 and Tfh13 induce B cell class switching to IgE (Zhu and Paul 2008; Fallon et al. 2002). B cells produce immunoglobulin (Ig) specific to an antigen peptide. These Igs help in opsonization and eliminate pathogens in an antigen-specific manner. Activated B cells produce immunoglobulin M (IgM) and can be promoted by secondary signals such as IL-4 to undergo Ig class switching. IgM is the first antibody produced and is the primary immunoglobulin response (Schroeder and Cavacini 2010). IgG subclasses are important for secondary response, neutralizing toxins and clearing of viruses (Schroeder and Cavacini 2010; Vidarsson, Dekkers, and Rispens 2014). IgA is important in responding and clearing mucosal infections (Schroeder and Cavacini 2010; Pabst and Slack 2020; Breedveld and van Egmond 2019). IgE is important in parasitic infections and allergic disease (Schroeder and Cavacini 2010; Gould and Sutton 2008).

The adaptive immune response is slow to develop taking about 7 days to activate upon the first antigen exposure of a new pathogen. During this time the innate immune system plays a critical role, responding within the first few hours to days of an infection. The innate immune system consists of tissue resident cells such as: dendritic cells, macrophages, and mast cells; and circulating cells such as neutrophils, eosinophils, and basophils that once a pathogen is present are trafficked from the blood into the tissue. Dendritic cells are classically identified recognized by CD11c⁺ in murine cells and are important in antigen presentation by MHCII to

naïve CD4 T cells and induction of CD4 T cell subset differentiation (Zanna et al. 2021). Dendritic cells have also been shown to cross present to CD8 T cells and activate them using MHC I. Macrophages are characterized by CD11b and F4/80 dual positivity, and are important to phagocytosis and pathogen clearance (Ross, Devitt, and Johnson 2021). Neutrophils are characterized by CD11b and Ly6G markers, and are the first cell type to be recruited to an infection, they are critical to the release of reactive oxygen species and hydrolytic enzymes, and clearance of bacterial and fungal infections (Németh, Sperandio, and Mócsai 2020). Basophils are identified by the cell surface markers CCR3 and CD123 as well as CD63 and CD203 when activated and are important to hypersensitivity and anaphylactic reaction (Chirumbolo 2012; Kim et al. 2016). Mast cells and eosinophils are important to parasitic infection clearance and are the hallmark cell type in allergic response. Eosinophils are characterized by the presence of SiglecF and CCR3 cell markers and are maturation, activated, and survival by IL-5. These cell types release a large quantity of granule proteins and chemical mediators that are important in clearing parasitic infection but in allergic disease can cause tissue damage and remodeling (Abdala-Valencia et al. 2018). Mast cells express c-Kit, ST2 (the IL-33 receptor), and the high affinity IgE receptor, FcεRI. IgE-mediated mast cell function is critical for killing parasites and degrading toxins or venoms (Galli et al. 2016).

All these cell types play a key role in the immune system and its protection from infection (Fig 2).

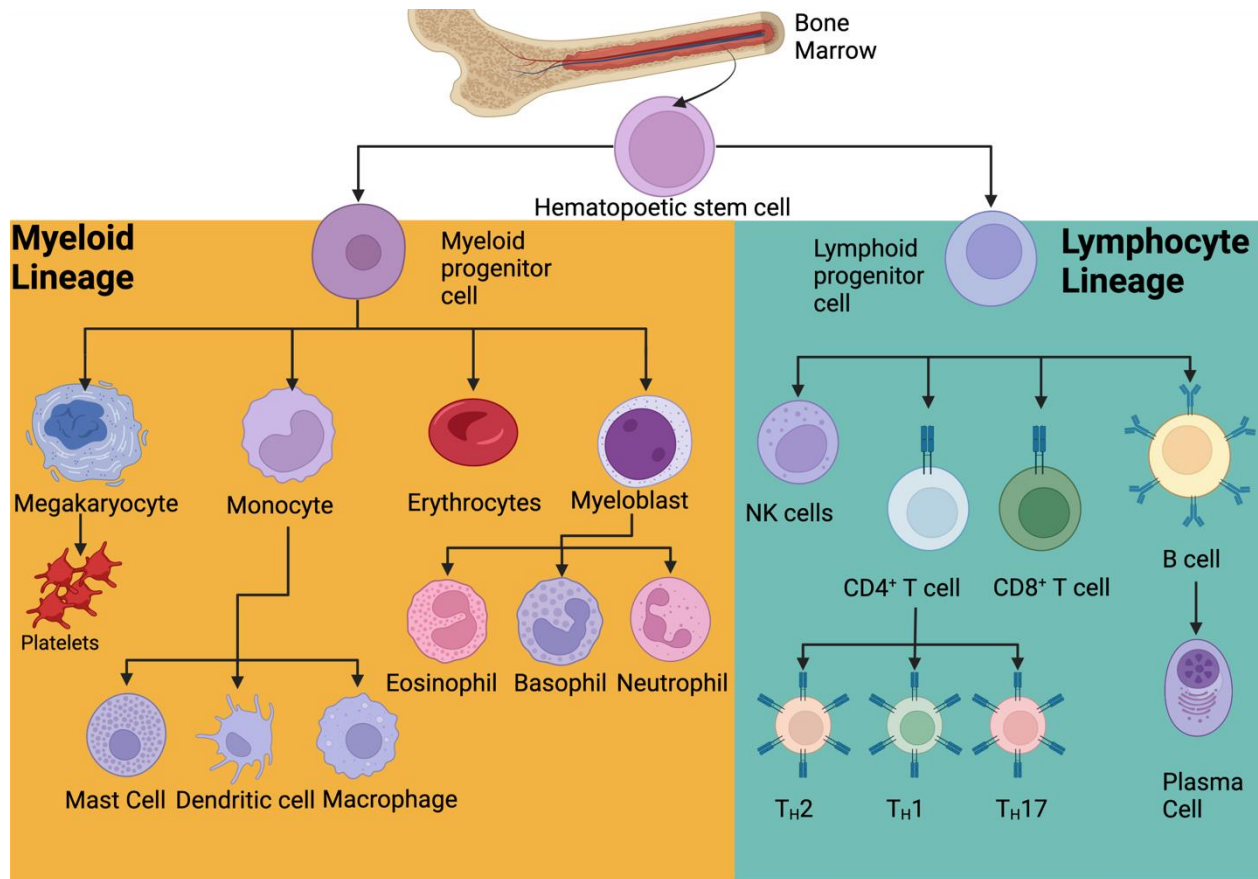


Figure 1: Cell lineage of the Immune cells. The immune system is made up of a complex system of cells derived from the bone marrow. These cells make up the innate (myeloid derived cells in yellow) and adaptive cells (lymphocyte derived cells in blue).

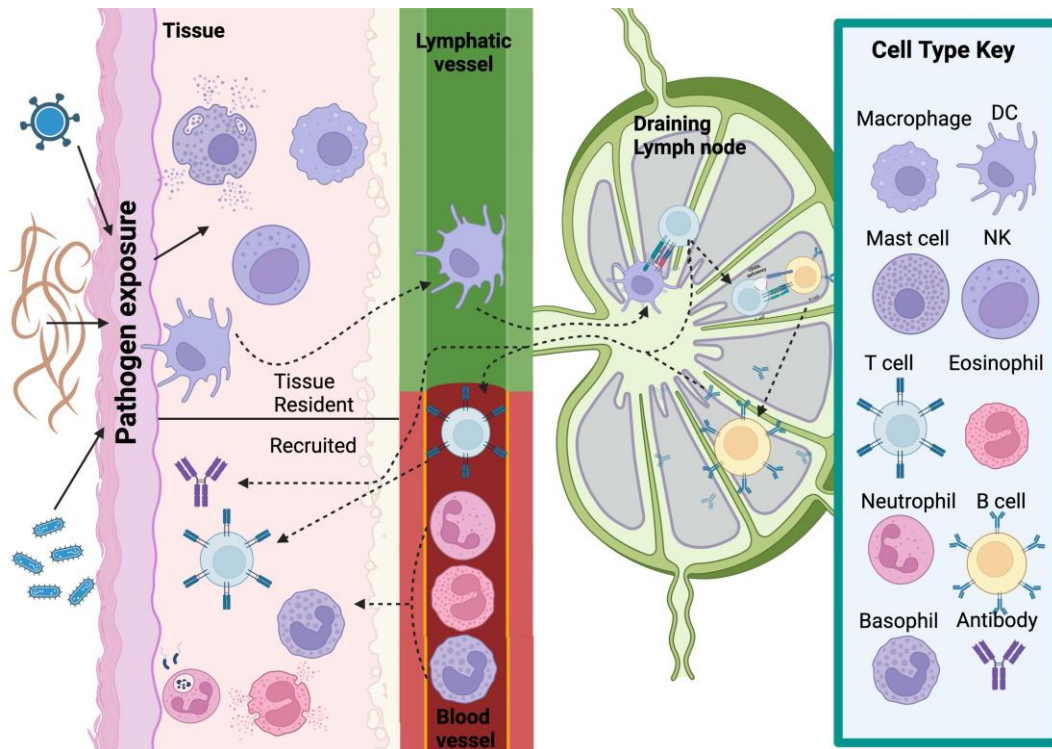


Figure 2: Pathogen exposure causes activation of the innate and adaptive immune system. Initial pathogen exposure such as bacteria, viruses, and parasites causes immediate activation of tissue resident cells. Once activated these cells help clear the pathogens but also release chemokines and cytokines to recruit circulating immune cells to the site of infection. Resident dendritic cells uptake pathogen antigen to present on MHC II+ and traffic to the drain lymph node to activate corresponding T cells. Antigen specific T cells will then traffic out of the draining lymph node back to the site of infection. T cells then help to activate corresponding B cells to release immunoglobulins.

Brief Introduction allergic disease and asthma

Allergic asthma is estimated to affect >25 million individuals in the United States and have an estimated cost in the United States of \$82 billion in 2013 (To et al. 2012; Nurmagambetov, Kuwahara, and Garbe 2018). It is characterized by Th2-type lung inflammation, excessive mucus production, and airway hyperresponsiveness, remodeling, and obstruction. Allergic asthma can be induced by common allergens such as house dust mite or fungal allergens (Salo et al. 2006; Li et al. 2014). These allergens induce a Th2-type inflammation and elicit IgE production (Kobayashi et al. 2009; Li et al. 2014).

Current treatments rely heavily on avoidance of allergen and reducing allergic response with drugs such as antihistamines and corticosteroids (Damask et al. 2021). Patients with non-severe asthma can rely on short acting β_2 agonists and low dose inhaled corticosteroids as effective controllers and relievers of symptoms with little to no side effects (Sobieraj and Baker 2018). For severe asthmatics the options are limited to oral corticosteroids, which have negative side effects, and are not effective at maintaining control of asthma in all patients. A more recent option is monoclonal antibody therapies which also have negative side effects and are not feasible for most people due to high cost and frequent doctor visits for infusions (Currie, Douglas, and Heaney 2009). For these reasons, it is imperative to continue to study new possible therapies and therapeutic targets.

There is evidence for mast cell involvement in asthma, including pulmonary mast cell hyperplasia along with high levels of leukotrienes, tryptase, chymase, and histamine in the sputum and bronchial alveolar lavage fluid of asthmatics (Broide et al. 1991; Brightling et al. 2002; Wang et al. 2015) (Fig 3). In addition, one of the most promising therapy Omalizumab

which inhibits IgE is effective in more than 50% of asthma cases(Godse et al. 2015). Thus, targeting IgE-mediated mast cell activation has therapeutic benefits and will increase our understanding of allergic asthma (Martin et al. 1993; Bousquet et al. 2004).

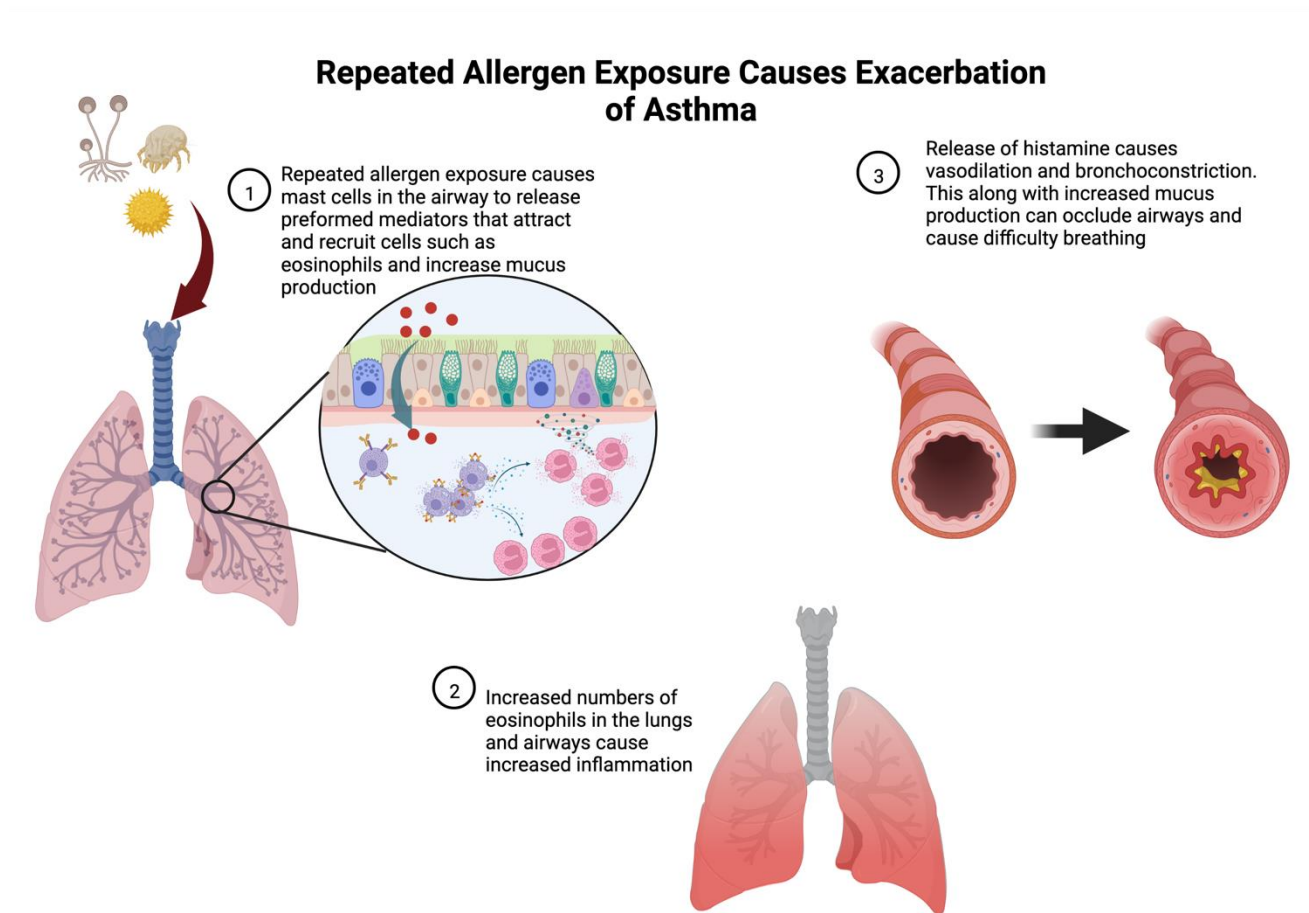


Figure 3: Continued allergen exposure leads to exacerbation of asthma. Repeated allergen exposure in the lungs activates mast cells by $\text{Fc}\epsilon\text{RI}$ crosslinking with antigen-specific IgE. These mast cells degranulate and release preformed mediators, which recruit cells such as eosinophils and cause goblet cells to produce and release mucus. The increase in cells cause increased inflammation, tissue damage, and lung remodeling. Release of preformed mediators such as histamine cause vasodilation and bronchoconstriction.

Mast cells and their roles in allergic disease

Mast cells were named by Paul Ehrlich in 1878, although they were first noted 15 years before by von Recklinghausen (Blank, Falcone, and Nilsson 2013). Ehrlich noted these cells contained tightly packed granular vesicles that he inferred were linked to cellular feeding/ingestion and named them “mastzellen” or “feeding cells” (Beaven 2009). These cellular granules were later shown to contain preformed mediators such as histamine, proteases, and lysosomal enzymes that play a key role in anaphylaxis and allergic disease symptomology (Moon, Befus, and Kulka 2014). Mast cells also release arachidonic acid metabolites that cause inflammation. Once released, prostaglandins and leukotrienes cause redness, swelling, pain, and increase contractile and inflammatory responses through specific interactions with G protein coupled receptors (Ricciotti and FitzGerald 2011; Singh et al. 2013). Finally, mast cells also produce key cytokines and chemokines. For example, they secrete IL-5, important in eosinophil activation, IL-13 that promotes mucus production (Tukler Henriksson et al. 2015), TNF- α and IL-6 that have critical roles in vasodilation and leukocyte recruitment (Popko et al. 2010). Chemokines such as MCP-1 and MIP-1 α are also produced and are important in the trafficking of macrophages and monocytes which perpetuate the inflammatory process (Desireddi et al. 2008).

There are two subtypes of mouse mast cells, connective tissue mast cells and mucosal mast cells. Their analogous pairs in humans are termed MC_{TC} (connective tissue) and MC_T (mucosal), indicating their production of tryptase and chymase enzymes. Connective tissue and MC_{TC} mast cells are mostly found in the submucosa of the gut, the peritoneum, and skin, while the mucosal and MC_T mast cells are predominately in the intestinal and lung mucosa

(Kurashima and Kiyono 2014). While these subtypes are activated by many of the same stimuli, their preformed mediators differ. Connective tissue mast cells produces high levels of mast cell proteases (mMCP)-4,5,6,&7 but not mMCP-1 and -2 (Xing et al. 2011). Mucosal mast cells express mMCP-1 and -2 but not mMCP-4, 5, & 6 (Li et al. 2018; Kataoka et al. 2005). Of note, bone marrow derived mast cells (BMMC), a common in vitro-derived population from which much of our data are derived, tend to have a mucosal phenotype (Akula et al. 2020).

Mast cells can be activated by many stimuli including cytokines released as danger signals (IL-33) and damage-associated molecular patterns (DAMPs) such as ATP, ADP, and AMP (Krystel-Whittemore, Dileepan, and Wood 2015). In allergic disease, mast cells have been best characterized in antigen-specific recognition by IgE bound to its high affinity receptor, Fc ϵ RI (Fig. 4). Fc ϵ RI is composed α , β , and γ chains (Kinet 1999). The β and γ chains both contain an immunoreceptor tyrosine-based activation motif (ITAM) in their cytoplasmic domain. Antigen-induced clustering of IgE-Fc ϵ RI complexes (referred to as crosslinking or XL) activates pre-associated Lyn kinase, which then recruits and phosphorylates Syk kinase (Kovářová et al. 2001). This sets off a cascade of signaling leading to rapid release of granule mediators in seconds to minutes, an intermediate generation and release of prostaglandins, leukotrienes, and eicosanoids through cPLA2-mediated arachidonic acid metabolism within an hour, and delayed production and release of cytokines and chemokines within several hours. The signaling cascade is depicted in Figure 4 but in brief, Syk activation leads to LAT phosphorylation, which acts as a docking protein for PLC γ . PLC γ promotes a calcium influx that prompts degranulation (Lee and Oliver 1995). Syk also phosphorylates PI3K, which leads to AKT phosphorylation, promoting both degranulation and cytokine production (Takayama et al.

2013). Phosphorylation of LAT allows for binding of Grb2 and SOS which then activates the classical Ras/Raf-1/MEK/ERK signaling cascade leading to cytokine transcription (Turner and Cantrell 1997). Ras activation has also been linked to PI3K activation and important roles in cytoskeleton remodeling, a key component of degranulation (Dráber, Sulimenko, and Dráberová 2012). ERK phosphorylates cPLA2 and hence leads to the arachidonic acid cleavage and eicosanoids production (Taketomi and Murakami 2022; Lin et al. 1993). Collectively this protein-driven cascade controls FcεRI signaling and mast cell function in allergic disease.

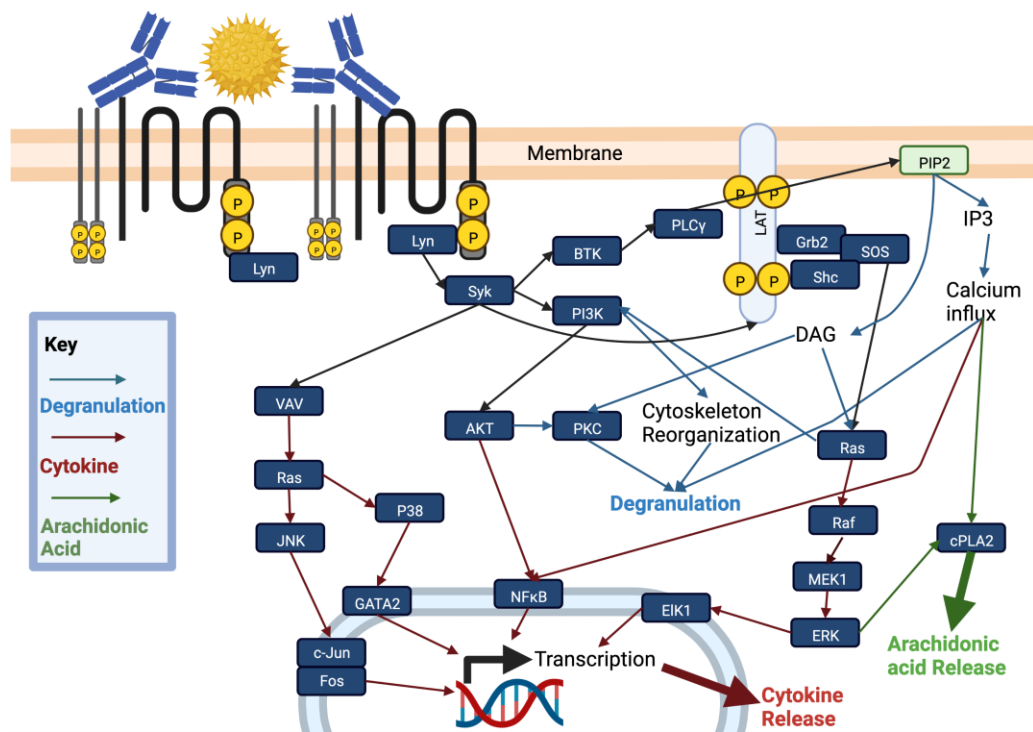


Figure 4: FcεRI signaling during antigen specific IgE crosslinking in the presence of allergen. Once crosslinked, FcεRI β and γ ITAM subunits become phosphorylated leading to a cascade of signaling that causes Degranulation, arachidonic acid release, and cytokine production.

Statins as a potential therapy

Statins are one of the most prescribed drugs in America and the leading therapy for hypercholesterolemia to reduce cardiovascular morbidity and mortality (Sirtori 2014). The first statin to be described, Mevastatin, was derived from *Penicillium Citrinium* extract in the 1970s by Akira Endo (Endo, Kuroda, and Tsujita 1976; Endo 2010). Since then, three other statins (pravastatin, simvastatin, and lovastatin) have been derived from the fermentation of fungi and five have been synthesized chemically (atorvastatin, venvastatin, pitavastatin, fluvastatin, and rosuvastatin) (Hoffman et al. 1986; Lee et al. 1991; Tulbah 2020). Statins work by blocking the rate limiting step of cholesterol synthesis, mediated by 3-hydroxymethyl-3-methylglutaryl coenzyme A reductase (HMGCR) (Ramkumar, Raghunath, and Raghunath 2016). By lowering cholesterol synthesis, this class of drugs effectively reduces the circulating levels of low-density lipoprotein cholesterol (Fig. 5). Reduced circulating cholesterol was reason for the drugs' effectiveness in reducing cardiovascular morbidity and mortality (Greenwood, Steinman, and Zamvil 2006). Over the last decade however, many studies have found that statin effects may actually be attributed to their ability to reduce inflammation (Montecucco et al. 2009; Diamantis et al. 2017). One meta-analysis study found that statin use in patients with sepsis and other infections had a positive correlation to a protective outcome (Janda et al. 2010). More important for our work in allergic disease, 3 large (10,000+ patient) studies found that increasing both dose and duration of statin use reduced asthma-related emergency department and hospitalizations visits (Wang et al. 2018; Tse et al. 2013; Huang et al. 2011). Finally, one study using a mouse model of allergic airway inflammation and airway hyperreactivity showed that simvastatin reduced the inflammatory phenotype seen in the model (Zeki et al. 2009).

These studies reveal a relevant role for the use of statins in reducing inflammation and allergic disease.

While the anti-inflammatory effects could be from lowering circulating LDL, which itself has proinflammatory effects, others have found statin effects to be from their ability to modulate the immune response (Quist-Paulsen 2010; Kim et al. 2019). *Link et al.* found that 20mg/day of rosuvastatin therapy could reduce Th-1 related TNF- α and IFN- γ levels (Link et al. 2006). Other studies have found that statin treatments can reduce dendritic cell, T cell, mast cell, and macrophage activation both in vivo and in vitro, independent of circulating LDL reduction (Kolawole et al. 2016; Paez et al. 2020; Yilmaz et al. 2004; Forero-Peña and Gutierrez 2013; Healy et al. 2020). We previously showed that fluvastatin can reduce mast cell activation. Fluvastatin reduced IgE-mediated degranulation and cytokine production both in vivo and in vitro (Kolawole et al. 2016). This study also found that statin effects were not phenocopied by inhibiting cholesterol synthesis alone. We also noted that some mouse strains were resistant to fluvastatin effects by upregulating HMGCR expression when treated with fluvastatin. These data were mirrored by variable fluvastatin responses among human derived skin mast cells from 8 different donors. A reduction in statin efficacy has been shown by others to be linked to a gene variant that is regulated by cholesterol concentrations, with lower cholesterol levels leading to increased HMGCR expression (Reiner 2014; Ness, Chambers, and Lopez 1998; Chasman et al. 2004). This variability in statin's ability to reduce allergic inflammation depending on genetic background reduces its possible therapeutic utility. For this reason, further study into the mechanism in which statins reduce inflammation and allergic disease

needs to be further explored. We postulated that studying how statins reduce IgE-mediated mast cell activation could lead to novel therapeutic options.

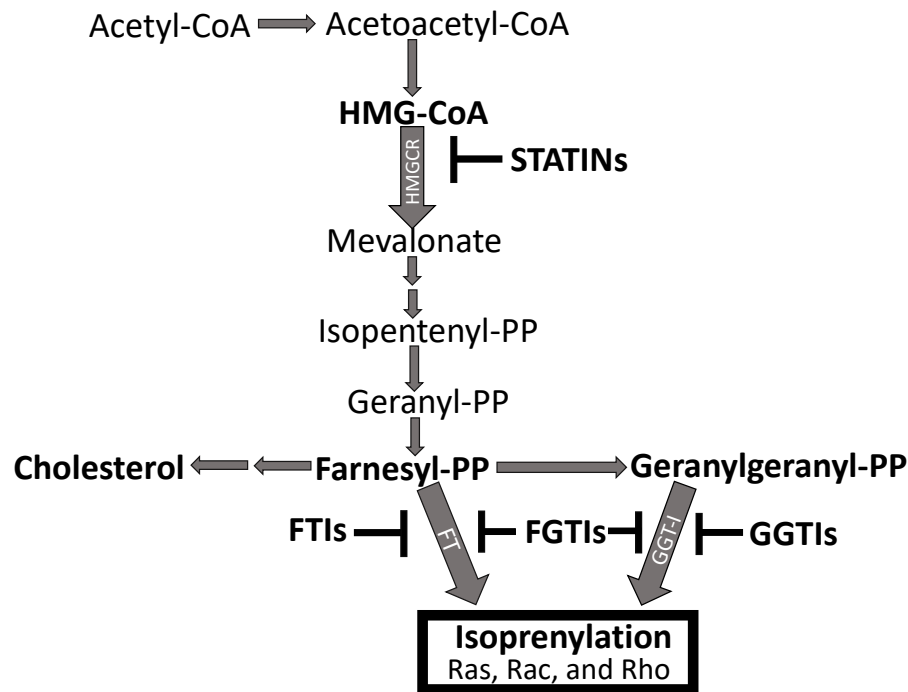


Figure 5: Cholesterol synthesis pathway. Statins reduce cholesterol synthesis by inhibiting the rate limiting step HMGCR. Along with cholesterol synthesis statins also inhibit isoprenylation, a key protein lipid modification that allows >400 proteins to localize to cell membranes for various signaling pathways. Isoprenylation inhibitors FTIs, GGTIs, and FGTIs inhibit the lipid modification of small GTPases such as Ras and Rho family proteins.

Isoprenylation

One important intermediate of the cholesterol biosynthesis pathway that could explain the effects of statins on the immune system is the isoprenylation of proteins (Fig. 5) (Jeong et al. 2018). Isoprenylation is the addition of a 15-carbon chain by enzyme farnesyl transferase (FT) or 20-carbon chain by enzyme geranylgeranyl transferase (GGT-1) to the C terminus of a protein with a CAAX motif (Casey and Seabra 1996). The CAAX motif is a sequence where C is a cysteine, A is any aliphatic amino acid, and X is any amino acid – although FT prefers methionine and GGT-1 prefers leucine at this last position. A third transferase protein, geranylgeranyl transferase type-2 (GGT-2), adds two 20-carbon chain to proteins ending in a CCAX motif, typically members of the Rab protein family (Kalinin et al. 2001). Isoprenylation is crucial for over 400 proteins' ability to associate with the phospholipid bilayer of membranes (Berndt, Hamilton, and Sebti 2011; Casey and Seabra 1996; Wang and Casey 2016).

There are data to support a role for isoprenylation in allergic disease. When treating plasmacytoid dendritic cells with statins, it was found that they produced significantly less type 1 IFN when stimulated with TSLP, an important step in a Th2 allergic response. Statin treatment reduced the phosphorylation of STAT6 and NF κ B and could be mimicked by use of geranylgeranyl inhibitors and Rho inhibitors, suggesting a role for geranylgeranylation of Rho proteins in TSLP DC activation (Inagaki-Katashiba et al. 2019). The effects of isoprenylation blockade have also been linked to airway remodeling in allergic disease. Takeda et al. found that clinically relevant doses of simvastatin reduced remodeling of smooth airway muscle, a phenotype they linked to blocking isoprenylation of RhoA proteins (Takeda et al. 2006). In IgE-mediated mast cell function, it was found that fluvastatin reduced activation by reducing

isoprenylation and could be phenocopied by a geranylgeranyl transferase inhibitor (Kolawole et al. 2016). These studies, along with others in T cells, show an important role for isoprenylation, a side reaction of the cholesterol synthesis pathway inhibited by statins, in inflammation and allergic disease (Srikanth et al. 2016).

The potential anti-inflammatory and anti-proliferative effects of blocking isoprenylation have spurred drug development. These include specific inhibitors in development, in pre-clinical trials, in clinical trials, and a few isoprenylation inhibitors already FDA-approved (Karasic et al. 2019; Van Cutsem et al. 2004). There are four classes of isoprenylation inhibitors: GGT-2 inhibitors, GGT-1 inhibitors (GGTIs), FT inhibitors (FTIs), and dual farnesyl and geranylgeranyl transferase inhibitors (FGTIs) which inhibit both GGT-1 and FT. These specific inhibitors work as peptide mimics of the CAAX box recognized by farnesyl and/or geranylgeranyl transferases. These classes of inhibitors have been highly studied and developed for possible cancer therapies, with the newest iterations of these drugs being highly selective for their targets (Storck et al. 2019; Berndt, Hamilton, and Sebt 2011). These inhibitors offer a new possibility in isoprenylation inhibition and related therapeutic options. In this study we will be focusing on the inhibition of farnesyl transferase and geranylgeranyl transferase by GGTIs, FTIs, and FGTIs.

Ras, Rac, Rho family proteins in mast cell activation

Understanding how isoprenylation of specific proteins affects IgE-mediated mast cell activation will elucidate fundamental aspects of mast cell biology. Over 400 unique proteins are either farnesylated or geranylgeranylated; notably Rab, Ras, and Rho family proteins (Storck et al. 2019). In particular, Ras family proteins have been linked to mast cell signaling, while Rho

family proteins have been linked to mast cell cytoskeleton remodeling (Baier et al. 2014; Sheshachalam, Baier, and Eitzen 2017). The previous research for Ras's involvement in IgE signaling was demonstrated in both mouse and human mast cells (Turner and Cantrell 1997; Mor et al. 2010). One Ras isoform, K-Ras, has been shown to play a substantial role in mast cell survival and activation in c-KIT signaling (Khalaf et al. 2007). Transcript data from mouse mast cells show that mast cells can express K-Ras, N-Ras, and H-Ras (Dwyer, Barrett, and Austen 2016). Previous studies have shown that K-Ras and N-Ras, while preferentially farnesylated, can also be geranylgeranylated under conditions lacking farnesylation. In contrast, H-Ras has only been shown to be geranylgeranylated (Berndt, Hamilton, and Sebti 2011). Once Ras is activated, it signals through the classical Ras/Raf-1/MEK/ERK signaling cascade (Fang et al. 2012; Yamasaki and Saito 2008) (Fig. 4). Ras has also been linked to regulating PI3K activation in FcεRI signaling, where Ras activation leads to PI3K phosphorylation and subsequent cytoskeleton reorganization and mast cell degranulation (Dráber, Sulimenko, and Dráberová 2012). Rho family proteins, which are generally isoprenylated by GGT-1, have a substantial role in IgE-mediated mast cell activation. Rho proteins include the Rac subfamily. Rac2 is a key player in cytoskeleton remodeling crucial to mast cell degranulation and histamine H4 Receptor-mediated chemotaxis (Baier et al. 2014; Kuramasu et al. 2018; Yang et al. 2000). Another Rho family protein of note is cdc42 which also promotes degranulation and chemotaxis (Hong-Geller et al. 2001).

Because Ras and Rho family proteins have been shown to play a role in FcεRI signaling, it stands to reason that targeting isoprenylation would reduce FcεRI signaling and mast cell

function in allergic disease. In this study, we will be testing isoprenylation inhibitors for their ability to reduce IgE-mediated mast cell function in vitro and in vivo.

Methods

Animals

Mouse strains C57BL/6J, 129/SvJ, and BALB/cJ breeding pairs were purchased from The Jackson Laboratory (Bar Harbor, ME). Breeding colonies were maintained in a specific pathogen-free facility. Experiments used age- and sex-matched mice housed in the same facility. Bone marrow was extracted from mice at a minimum of 8 weeks old and *in vivo* models were conducted on mice older than 12 weeks under protocol approval from the Virginia Commonwealth University Institutional Animal Care and Use Committee.

Mouse mast cells

Mouse bone marrow-derived mast cells (BMMCs) were developed by culturing bone marrow from femurs in complete RPMI (cRPMI) 1640 medium (Invitrogen Life Technologies, Carlsbad, CA) containing the following purchased from Corning (Corning, NY): 10% FBS, 2mM L-glutamine, 100 U/ml penicillin, 100 ug/ml streptomycin, 1mM sodium pyruvate, and 1 mM HEPES. Cultures were supplemented with supernatant from IL-3-producing WEHI-3B cells and recombinant SCF, to contain IL-3 and SCF at a final concentration of 1 and 10ng/ml, respectively. BMMC were used after at least 21 days of culture and consisted of >95% mast cells based on cell surface staining for c-Kit and FcεRI.

Peritoneal mast cells were obtained from peritoneal cavity lavage with PBS containing 1 mM EDTA. Cells were cultured in the same media used for BMMCs for no longer than 2 weeks. Cultures contained for >95% of c-Kit- and FcεRI-positive cells.

Human skin MC cultures

All protocols involving human tissues were approved by the Institutional Review Board at Virginia Commonwealth University. Cells were cultured as previously described (Kambe et al. 2001). Human skin mast cells were cultured with vehicle or drugs as described.

Cytokines and reagents

Recombinant mouse IL-3 and SCF were purchased from Shenandoah Biotechnology (Warwick, PA) for in vitro studies. Purified mouse IgE anti-DNP for in vitro activation (clone C38-2, κ isotype) was purchased from BD Biosciences (San Diego, CA). Mouse anti-DNP IgE for in vivo use was produced from a hybridoma line (H1 ϵ 26) and purified by HPLC. Propidium iodide, fluvastatin, and DNP-coupled human serum albumin (DNP-HSA) were from Sigma-Aldrich (St. Louis, MO). Antibodies against N-Ras (sc-31), HDJ2 (sc-59554), *pggt1b* (sc-376854), FT α (sc-23906) and Rap1a (sc-1482) were obtained from Santa-Cruz (Dallas, TX). Antibodies against CD107a (cat 121614), CD63 (cat 143904), TNF α (cat 506327), IL-6 (cat 504508), c-Kit (cat 105827), Fc ϵ RI (cat 134316), and p-p38 (cat 690203) obtained from BioLegend. p-SRC (cat 560094), p-ERK (cat 612592), p-AKT (cat 562599), and p-JNK (cat 562480) were obtained from BD Biosciences (Franklin Lakes, NJ). Anti-K-Ras (H00003845) was obtained from Abnova. Anti-B-actin (84575) was obtained from Cell Signaling. Histamine was obtained from Sigma-Aldrich (St. Louis, MO). OVA-HSA was obtained from Sigma-Aldrich (St. Louis, MO). *Alternaria alternata* was obtained from Greer Labs (Lenoir, NC). siRNAs for N-Ras (sc-36005), K-Ras (sc-43876), and scramble control were obtained from Santa Cruz (Dallas, TX). siRNAs for *pggt1b* (GGT; SIO1376228, SIO1376221, SIO1376207, and SIO1376207) and *fnta* (FT; SIO1004360, SIO1004353,

SI01004339, SI01004346) were obtained from Qiagen (Redwood City, CA). IC Fixation buffer and permeabilization buffer were purchased from Thermofisher. 10% formaldehyde was obtained from Macron Fine Chemicals. GolgiStop was obtained from BD Biosciences (Franklin Lakes, NJ). Cell Tak was purchased from Millipore Sigma (St. Louis, MO). Zombie aqua was purchased from BioLegend (San Diego, CA).

FTI2153, GGTI-2417 and FGTI-2734

Farnesyl transferase inhibitor (FTI-2153) and geranylgeranyl transferase inhibitor (GGTI-2417), were synthesized as described previously (Sun et al. 1999; Peng et al. 2006) by the Chemical Biology core facility at the Moffitt Cancer Center. The dual farnesyl and geranylgeranyl transferase inhibitor, FGTI-2734, was either purchased from MedChem Express (Monmouth Junction, NJ) or synthesized by the chemical biology core facility at the Moffitt Cancer Center as described previously (Fletcher et al. 2010).

Secreted Cytokine measurements

BMMCs were sensitized overnight with DNP-specific mouse IgE (0.5 $\mu\text{g/ml}$), then washed and resuspended 1×10^6 cells/ml in cRPMI with IL-3 and SCF (10 ng/ml). Cells were treated with the indicated inhibitors for 24hrs and stimulated with DNP-HSA (50 ng/ml) for 16hrs unless otherwise indicated. Supernatants were measured for IL-6, TNF α , and MCP-1 content using ELISA kits from Biolegend (San Diego, CA). IL-13 and MIP-1 α using ELISA kits from Peprotech (Rocky Hill, NJ). ELISAs were developed using BD OptEIA reagents from BD Biosciences (Franklin Lakes, NJ).

Human mast cells were cultured in AIM-V serum-free medium with 100 ng/ml of recombinant human SCF (100 ng/ml) at 1×10^6 cells/ml and treated with either FGTI-2734 (2.5 μ M) or DMSO for 24hrs. Cells were then activated with anti-Fc ϵ RI (22E7 mAb; 1 μ g/ml) for 16hrs. TNF α , MCP1, and GM-CSF levels were measured by ELISAs from Peprotech (Rocky Hill, NJ). ELISAs were developed using BD OptEIA reagents from BD Biosciences (Franklin Lakes, NJ).

Degranulation

BMMCs were sensitized overnight with DNP-specific mouse IgE (0.5 μ g/ml), then washed and resuspended 1×10^6 cells/ml in cRPMI with IL-3 and SCF (10 ng/ml). Cells were treated with the indicated inhibitors for 24hrs unless otherwise indicated and stimulated with DNP-HSA (50 ng/ml) for 15 minutes, then stained for CD107a and CD63 on ice for 30 minutes. Flow cytometry where gated by single cell exclusion using FSC-H and FSC-A and then isotype controls were used to set gates for CD107a and CD63 positive cells. MFI and percent positive were then measured by flow cytometry using a BD FACSCelesta.

Human mast cells were cultured in AIM-V serum-free medium with 100 ng/ml of recombinant human SCF (100 ng/ml) at 1×10^6 cells/ml and treated with either FGTI-2734 (5 μ M) or DMSO for 24hrs. Cells were then activated with anti-Fc ϵ RI (22E7 mAb; 1 μ g/ml) for 30 minutes. Activation was stopped by adding three volumes of ice-cold PBS. The cells were centrifuged at 1000 rpm for 10 min at 4°C. Supernatants were transferred into a separate tube. Cell pellets were resuspended in PBS, sonicated in a Branson sonifier (model 350; power 5, 50% pulse cycle 4 pulses) and microfuged. Beta-hexosaminidase was assayed by measuring release

of p-nitrophenol from the substrate p-nitrophenyl N-acetyl-D-glucosaminide as described (Zhao et al. 2005). Absorbance values were read at 405 nm.

Propidium iodide staining

To determine cell viability, BMMCs were sensitized overnight with DNP-specific mouse IgE (0.5 $\mu\text{g/ml}$), then washed and resuspended 1×10^6 cells/ml in cRPMI with IL-3 and SCF (10 ng/ml). Cells were treated with the indicated inhibitors for 36hrs, then stained with propidium iodide (10 $\mu\text{g/ml}$) and fluorescence was measured using a BD FACSCelesta. Gating strategy was as follows: Cells were gated by single cell exclusion using FSC-H and FSC-A and then non-stained cells were used for isotype controls for gating PI positive populations.

siRNA gene targeting

BMMCs were transfected with 2 μM siRNAs specific for the target or scramble control sequences. Transfection experiments were done using 1) Amaxa Nucleofector from Lonza (Allendale, NJ) using program T-5 in Dulbecco's modified Eagle's medium with 20% FBS and 50 mM HEPES (pH 7.5) or 2) Neon transfection system by ThermoFisher, where cells were electroporated using 1450 V 10 ms and 1 pulse setting in buffer T at 1×10^6 cells per transfection. Cells were used 48 hours after being transfected, with successful inhibition verified by Western blotting for proteins.

Western Blot

Western blot analysis was performed as described previously (Fernando et al. 2013). Blots were visualized and quantified using a LiCor Odyssey CLx infrared imaging system (Lincoln, NE). In

brief: protein concentration was measured using ThermoFisher kit Pierce BCA Protein Assay Kit (Ref 23227), 40ug per lane was loaded and 4-20% or 15% (only used for HDJ-2 blots) SDS page gel (BioRad) was used for gel electrophoresis. Proteins were then transferred for 45 minutes at 25 V onto nitrocellulose paper. Blots then blocked for 30 minutes with 50% Casein buffer in TPBS. Membranes were then incubated with appropriate antibody concentrations as recommended by manufacturers overnight in 4 degrees C (except β -actin which was incubated for 1hr at room temp). Membranes were washed and then incubated with appropriate secondary antibody for 1hr.

Intracellular cytokine staining

BMMCs were sensitized overnight with DNP-specific mouse IgE (0.5 μ g/ml), then washed and resuspended 1×10^6 cells/ml in cRPMI with IL-3 and SCF (10ng/ml). Cells were treated with the indicated inhibitors for 24 hours then stimulated with DNP-HSA (50 ng/ml) for 90 minutes at 37°C in a CO₂ incubator. GolgiStop (BD science) was added for an additional 4hrs. Cells were then fixed with IC Fixation buffer (ThermoFisher) for 20 minutes, washed twice and permeabilized with PermBuffer (ThermoFisher), and then stained overnight at 4°C with anti-cytokine antibodies and measured by flow cytometry the next day. Gating strategy was as follows: cells were gated by single cell exclusion using FSC-H and FSC-A and then isotype controls were used to set gates for IL-6 and TNF- α positive cells.

Seahorse metabolic flux analysis

BMMCs were sensitized overnight with DNP-specific mouse IgE (0.5 µg/ml), then washed and resuspended 1×10^6 cells/ml in cRPMI with IL-3 and SCF (10 ng/ml). Cells were treated with the indicated inhibitors for 24 hours then resuspended in RPMI media without SCF/IL-3 (Agilent, Santa Clara, CA). The media contained glucose (10mM), pyruvate (1mM), L-glutamine (2 mM), and 1% FBS and adhered to wells with Cell Tak (3.45 µg/ml) at 200,000 cells/well for 45 minutes. The protocol included 3 readings every 3 minutes for baseline, 5 readings every 3 minutes after SCF/IL-3 (10 ng/ml) injection, and 5 readings every 3 minutes after DNP-HSA (50 ng/ml) injection.

RT-qPCR

RNA was harvested from BMMCs stimulated with DNP-HSA for 4 hours using TRIzol reagent (Life Technologies, Grand Island, NY). Nucleic acid purity was measured using a Nanodrop 1000 UV-Vis Spectrophotometer (Thermo Scientific, Waltham MA). To measure mRNA expression, cDNA was synthesized using the qScript cDNA Synthesis Kit (Quanta Biosciences, Beverly Hills, CA) following the manufacturer's protocol. qPCR analysis was performed with Bio Rad CFX96 Touch™ Real-Time PCR Detection System and SYBR® Green detection using a relative Livak Method. Reactions consisted of a heat-activation step at 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds, 55°C for 30 seconds and 60°C for 1 minute. Fluorescence data were collected during the extension step of the reaction. Primers used included: IL-6 (forward: 5'TCCAGTTGCCTTCTTGGGAC3', reverse: TCCAGTTGCCTTCTTGGGAC3'), β-actin (forward: 5'GATGACGATATCGCTGCGC3', Reverse: 5'CTCGTCACCCACATAGGAGTC3') and IL-13 (forward: ATGGCGTCTGGGTGACTGCAGTCC', Reverse: GAAGGGGCGTGGCGAAACAGTTGC)

Phosphorylation measured by flow cytometry

BMMCs were sensitized overnight with DNP-specific mouse IgE (0.5 $\mu\text{g/ml}$), then washed and resuspended 1×10^6 cells/ml in cRPMI with IL-3 and SCF (10 ng/ml). Cells were treated with the indicated inhibitors for 24hrs. Cells were stimulated with DNP-HSA (50 ng/ml) for indicated times (0, 1, 5, 15, or 30 minutes) then collected and fixed using 4% formaldehyde (Macron Fine Chemicals) for 20 minutes. Cells were permeabilized (PermBuffer ThermoFisher) and stained for phosphorylated proteins overnight at 4 C. Mean fluorescence intensity was measured by flow cytometry. Gating strategy was as follows: cells were gated by single cell exclusion using FSC-H and FSC-A and then isotype IgG controls were used to set gates for p-SRC, p-ATK, p-P38, and p-JNK positive cells.

Cell membrane fractions

BMMCs were treated with inhibitors for 24hrs (40 million cells per sample) and lysed with hypotonic lysis buffer (20 mM Tris 8.0, 1mM MgCl_2 and protease inhibitors) on ice for 20 minutes. Cells further lysed with Dounce homogenizer until 80-90% of cells are lysed (checked via trypan blue). NaCl (0.15 M) was added, the solution was mixed gently, then spun at 400g for 10 minutes to remove nuclei and unlysed cells. Lysates were then ultracentrifuged at 32.7K for 1hr (100,000xg). Supernatant cytosolic proteins and pellet containing membrane-associated proteins were harvested. Protein fractions were then measured by western blot, compared by using equal amounts of protein.

Passive systemic anaphylaxis

Mice were weighed and then administered 1mg/kg doses of FGTI-2734 or equivalent dilution of DMSO in 200μL of PBS via intraperitoneal (i.p.) injections, followed by 200μL PBS containing 50μg mouse IgE anti-DNP. The following day, mice were again administered 200μL PBS containing 1mg/kg of FGTI-2734 or DMSO equivalent 2 hours before DNP-HSA (50μg) was administered via i.p. injection. In some experiments, 2mg histamine was injected in place of DNP-HSA. The core body temperature of each mouse was measured using a rectal microprobe (Physitemp Instruments, Clifton, NJ). Mice were euthanized and blood was collected by cardiac puncture to analyze plasma.

Alternaria-induced pulmonary inflammation

Mice were sensitized with 30μl PBS containing OVA (100μg/mouse) +/- *Alternaria alternata* extract (10μg/mouse) via intranasal (i.n.) route on days 0 and 7. Mice were treated with FGTI-2734 (1 mg/kg) or equivalent dilutions of vehicle (40% w/v 2-hydroxypropyl-β-cyclodextrin (Sigma) in sterile water) in 30 μl via i.n. route on days 12-16. Challenge by OVA (50μg/mouse) +/- *Alternaria* (5μg/mouse) in 30 μl i.n. on days 14-16. Mice were euthanized on day 17. Blood was collected by cardiac puncture and bronchiolar lavage was collected by flushing the lungs twice with 800 μl of PBS with 0.5 mM EDTA.

The left lung was excised, fixed, and stained with H&E and PAS for histology. Histology staining for H&E and PAS was done by StageBio (Mt. Jackson, VA) and samples were scored blinded. PAS scores: 0= no goblet cells (GBC), 1=<25%, 2= 25-50%, 3=51-75%, and 4= > 75% of

epithelial lining are GBC; H&E scores: 0= no cell infiltration in alveoli/bronchi space, 1=<25%, 2= 25-50%, 3=51-75%, and 4= > 75% cell infiltration in alveoli/bronchi space.

The right lung was extracted and homogenized by incubating at 37C for 30 minutes in DNase (10mg/ml, Worthington) and Liberase TM (5mg/ml, Roche) to digest the lungs. Single cell suspension was used to analyze cell types via flow cytometry. Cell types from lung and BALF were analyzed using flow cytometry with gating on single cells (doublet exclusion), live cells (Zombie aqua negative). Eosinophils were detected as double positive for CCR3 and SiglecF; Neutrophils as double positive for CD11b and Ly6G.

Single cell suspensions of the mediastinal lymph node (from individual mice) were resuspended in cRPMI and activated with 300 µg/ml OVA for 3 days. Supernatants were then analyzed for IL-13 and IL-5 levels by ELISA.

***Alternaria*-induced Airway Hyperresponsiveness**

Mice were sensitized with 30µl PBS containing OVA (100ug/mouse) +/- *Alternaria alternata* extract (10ug/mouse) via intranasal (i.n.) route on days 0 and 10. Mice were treated with FGTI-2734 (5 mg/kg) or equivalent dilutions of vehicle (40% w/v 2-hydroxypropyl-β-cyclodextrin (Sigma) in sterile water) in 30 µl via i.n. route on days 18-23. Challenge by OVA (50ug/mouse) +/- *Alternaria* (5ug/mouse) in 30 µl i.n. on days 20-23. Airway hyperresponsiveness was measured by FlexiVent with increasing doses of methacholine (5, 10, 25, 50, 100 mg/ml).

Statistics

p values were calculated with GraphPad Prism software v9 by paired or unpaired two-tailed Student's t-test when comparing 2 samples. For a comparison of 3 or more samples, ANOVA was performed followed by Tukey's post hoc test. P values of <0.05 were considered statistically significant. Data are expressed as mean \pm standard error of mean (SEM) with statistical significance: * $p < .05$, ** $p < .01$, *** $p < .001$, **** $p < .0001$. Biological replicates for in vitro experiments were BMMC populations derived from separate mice. Technical replicates are samples from the same cultures or mice analyzed in replicates.

Results

Dual isoprenylation inhibitor FGTI-2734 suppresses IgE-mediated mast cell function.

Previously, we showed that fluvastatin significantly reduced IgE-mediated mast cell function by inhibiting protein isoprenylation (Kolawole et al. 2016). Statins such as fluvastatin target HMGCR, which affects many cellular functions dependent on cholesterol biosynthesis pathway intermediates downstream of HMGCR (Fig. 5). Targeting HMGCR with statins to inhibit isoprenylation was also challenging because it required high statin concentrations (Kolawole et al. 2016). With the recent development of FGTI-2734, isoprenylation is now a more clinically relevant target that could circumvent these statin challenges (Kazi et al. 2019). Here, we tested three highly selective isoprenylation inhibitors: a GGT-1 inhibitor (GGTI-2417) (Falsetti et al. 2007), an a FT inhibitor (FTI-2153) (Crespo et al. 2002), and a dual FT/GGT-1 inhibitor (FGTI-2734) (Kazi et al. 2019) to determine their effects on mast cell function. C57BL/6J bone marrow-derived mast cells (BMMCs) were sensitized with IgE and treated with fluvastatin, FTI-2153, GGTI-2717, FGTI-2734 (all at 5 μ M final concentration) or vehicle control for 24 hours before antigen-induced IgE crosslinkage (IgE XL). Only fluvastatin and the dual inhibitor FGTI-2734 significantly reduced expression of the degranulation markers CD107a and CD63 and inhibited IL-6 and IL-13 production (Fig 6). Even at doses of 20 μ M, neither GGTI-2417 nor FTI-2153 significantly reduced IgE-mediated cytokine production (Fig. 7).

To demonstrate that FTI-2153, GGTI-2417 and FGT-2734 were effective at inhibiting their targets in intact mast cells, we determined their effects on the exclusively farnesylated FT

substrate, HDJ2, and the exclusively geranylgeranylated GGT-1 substrate, Rap1a. To this end, BMMCs were treated with FTI-2153, GGTI-2417, or FGTI-2734 for 24hrs and lysates were subjected to western blotting as described in Methods. Figure 8 shows that FTI-2153 was highly selective for inhibiting HDJ2 farnesylation (as documented by the well-established upward shift of the non-farnesylated HDJ2 (Berndt, Hamilton, and Sebti 2011; Peng et al. 2006; Sun et al. 1999)), but not Rap1a geranylgeranylation. Conversely, GGTI-2417 selectively inhibited Rap1a geranylgeranylation (as demonstrated by the appearance of the non-geranylgeranylated Rap1a (Berndt, Hamilton, and Sebti 2011; Peng et al. 2006; Sun et al. 1999)), but not HDJ2 farnesylation. The dual inhibitor FGTI-2734 suppressed both HDJ2 farnesylation and Rap1a geranylgeranylation. These isoprenylation inhibition data, coupled with the data from Figures 6 and 7, confirm inhibitor specificity and suggest that both the FT and GGT-1 arms of the isoprenylation pathway must be inhibited to mimic statin effects on FcεRI function.

Dose dependence for FGTI-2734 was measured by inhibition of IgE-induced IL-6 secretion. Concentrations above 0.5 μM showed significant effects, with a maximum 50% reduction in IL-6 secretion by C57BL/6 BMMCs (Fig 9). We found similar effects on IL-13, TNF-α, MCP, and MIP-1α (Fig 9) and no significant loss of cell viability at these concentrations (Fig. 10). Importantly, BMMCs from two other genetic backgrounds, BALB/cJ and fluvastatin-resistant 129/SvJ, yielded similar results (Fig. 11). The IC₅₀ for FGTI-2734 suppression of IL-6 production was approximately 0.6 μM and not significantly different between strains (Fig. 11). FGTI-2734 time-dependency was measured by reduction in FcεRI-induced degranulation because, unlike cytokine secretion, this response is very rapid. BMMCs were treated with FGTI-2734 for

increasing amounts of time before antigen activation. The maximum inhibitory effect was seen at 6 hours of drug treatment with a $T_{1/2}$ of ~3 hours (Fig. 11).

To test mast cells beyond culture-derived BMMCs, we first examined mouse peritoneal mast cells. As with BMMC, FGTI-2734 reduced Fc ϵ RI-mediated IL-6, IL-13, TNF- α , MCP-1, and MIP1 α release (Fig. 12). Finally, we assessed FGTI-2734 effects on human skin-derived mast cells. Like mouse mast cells, human mast cells from 5 donors showed a significant reduction in Fc ϵ RI-induced TNF- α , MCP-1, and GM-CSF secretion (Fig. 13). In contrast, FGTI-2734 had modest effects on Fc ϵ RI-mediated degranulation, with 3 of 5 donor populations showing no statistical significance (Fig. 13). Overall, these data showed that FGTI-2734 reduced IgE-mediated mast cell function with its most consistent effects on cytokine production. These data indicate the importance of isoprenylation in Fc ϵ RI signaling.

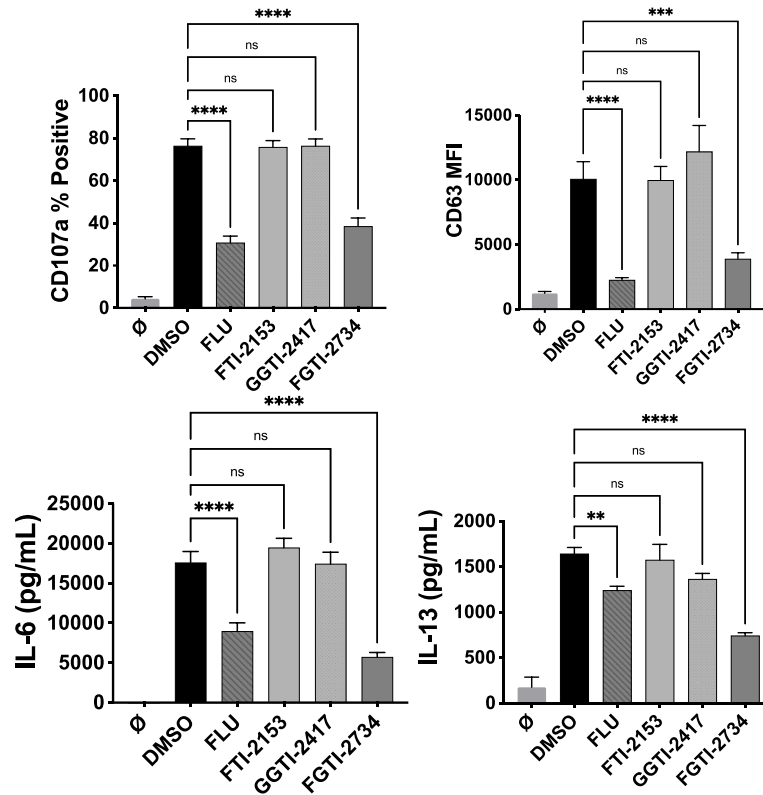


Figure 6: Blocking both isoprenylation pathways is required to phenocopy statin effects on IgE-mediated mast cell activation. C57BL/6J BMMCs were sensitized with IgE and treated with fluvastatin (FLU; 5 μ M), FTI-2717 (5 μ M), GTI-2418 (5 μ M), FGTI-2734 (5 μ M), or vehicle control for 24 hours, then activated by IgE XL. To measure degranulation, IgE XL was carried out for 15 minutes and CD107a and CD63 were measured by flow cytometry. To quantify cytokine secretion, BMMCs were activated for 16 hours, and cytokines were measured in culture supernatant by ELISA. Data shown are from 3 independent experiments with at least 3 biological replicates and at least 2 technical replicates. ANOVA was used for statistics.

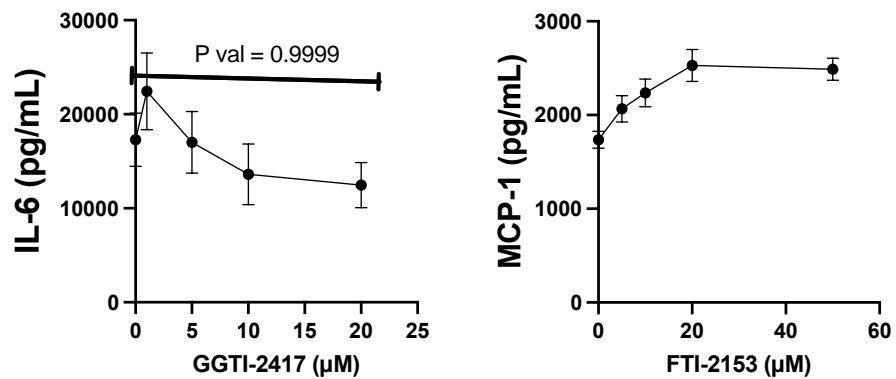


Figure 7: GGT1- and FT-specific inhibitors GGTI-27417 and FTI-2153 do not reduce cytokine production, even at higher concentrations. BMMCs were treated with increasing amounts of GGTI-2718 or FTI-2717 for 24 hours and activated by IgE for 16 hours. Cytokines were measured by ELISA. Data shown are from a minimum of 3 BMMC populations analyzed in duplicate.

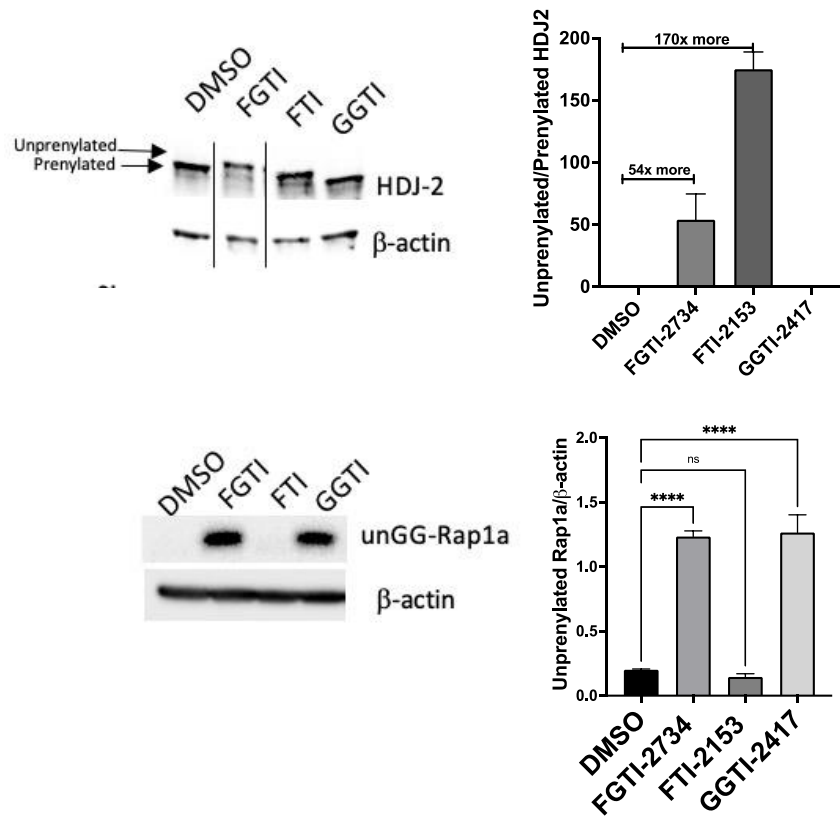


Figure 8: Isoprenylation inhibitors inhibit their intended targets in mast cells.

BMMCs were treated with the indicated inhibitors for 24 hours and protein concentrations were measured using Western Blot, normalized to DMSO control. Data shown on the right are mean and SEM from 3 experiments.

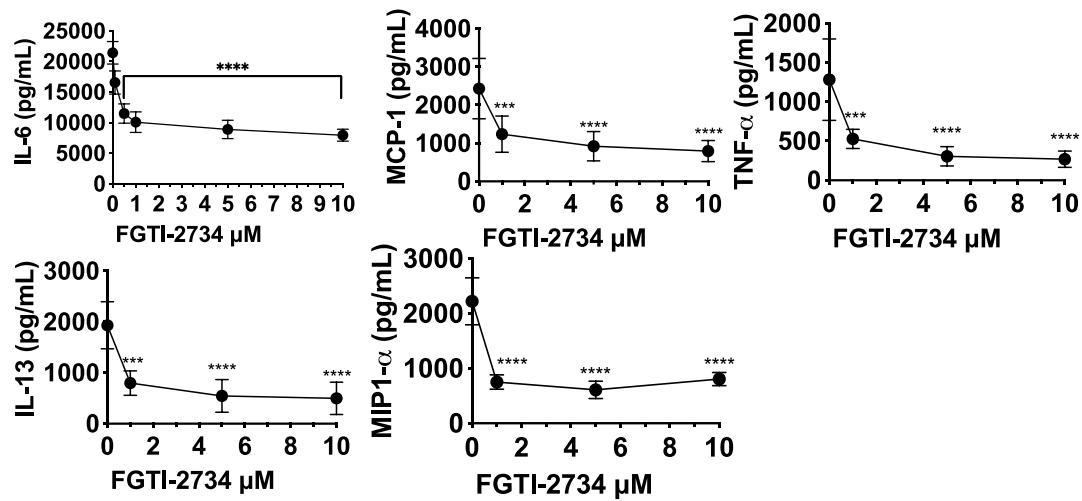


Figure 9: FGTI-2734 inhibits IgE mediated cytokine production in a dose dependent manner. BMMCs were treated with increasing doses of FGTI-2734 for 24 hours prior to IgE XL for 16 hours. Cytokines were measured by ELISA. Data shown are from 3 independent experiments with at least 3 biological replicates and at least 2 technical replicates. ANOVA was used for statistics.

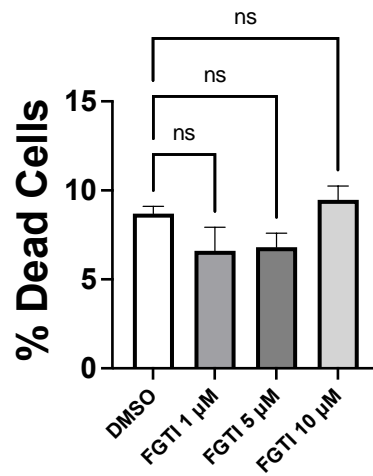


Figure 10: FGTI-2734 does not induce cell death. BMMCs were treated with increasing concentrations of FGTI-2734 for 48 hours and cell death was measured using PI exclusion by Flow cytometry. Data are from 4 BMMC populations analyzed in triplicate.

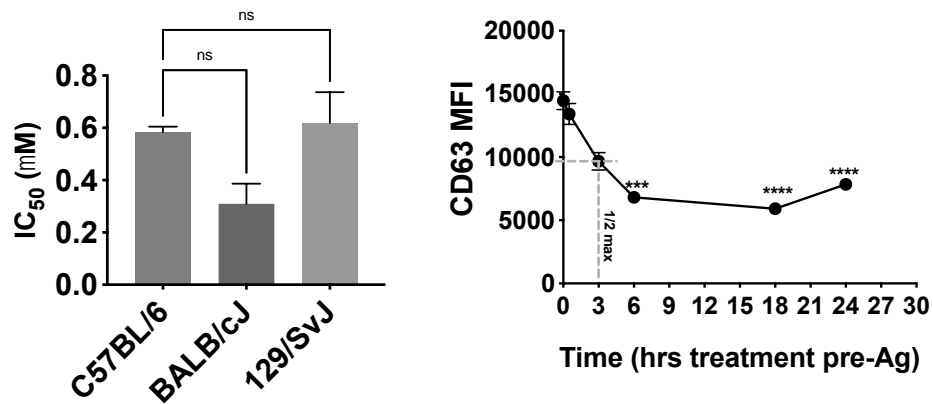


Figure 11: FGTI effects are not dependent on genetic background and reduce IgE-mediated degranulation in a time-dependent manner. BMMCs derived from C57BL/6J, BALB/cJ, or 129/SvJ were treated as in the dose response experiments shown in Figure 9 and IC_{50} values for suppressing IL-6 production were calculated. On the right side, BMMCs were treated with FGTI-2734 (5 μ M) for the indicated times before IgE XL for 15 minutes. CD63 expression was measured by flow cytometry and $t_{1/2}$ for inhibition was calculated. Data shown are from 3 independent experiments with at least 3 biological replicates and at least 2 technical replicates. ANOVA was used for statistics.

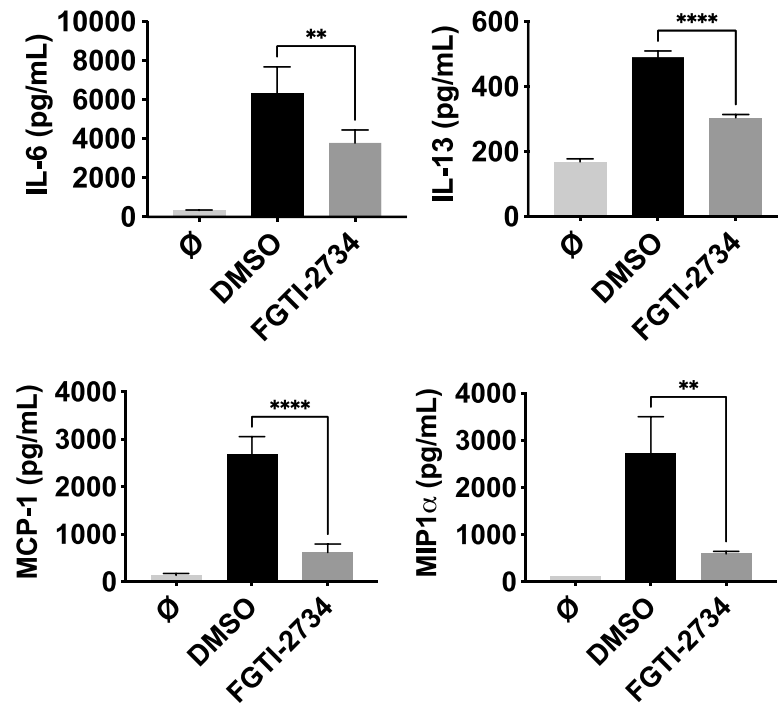


Figure 12: FGTI-2734 reduces IgE-mediated cytokine secretion by peritoneal mast cells. Peritoneal mast cells from 10 mice were treated with FGTI-2734 (5 μ M) for 24 hours and activated by IgE XL for 16 hours. Cytokines in culture supernatant were measured by ELISA. Data shown are from 3 independent experiments with at least 3 biological replicates and at least 2 technical replicates. ANOVA was used for statistics. ϕ = unstimulated control cells in DMSO.

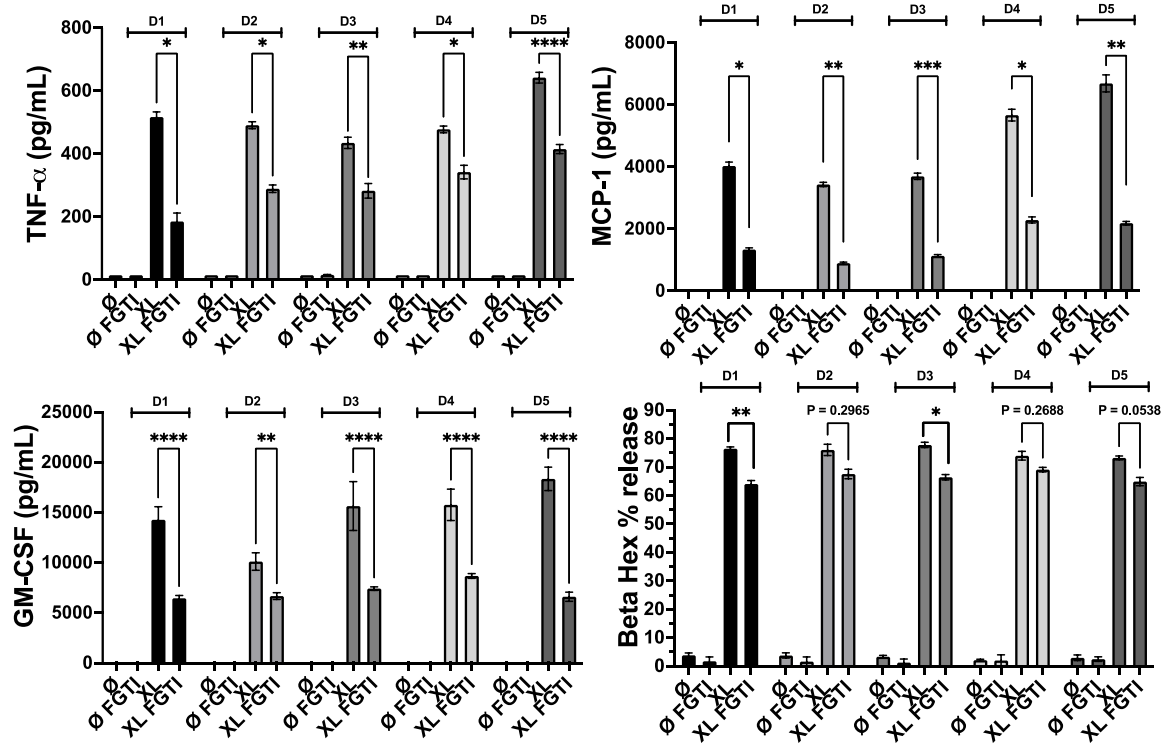


Figure 13: FGTI-2734 treatment reduces IgE induced cytokine production in skin-derived human mast cells. Human skin-derived mast cells from 5 donors were treated with either FGTI-2734 (5 μ M) or vehicle control for 24 hours and cells were then activated by anti-Fc ϵ RI for 16 hours to measure secreted cytokines were measured by ELISA or 30 minutes to measure beta-hexosaminidase release by colorimetric assay. Each donor cell population is numbered separately. ANOVA was used for statistics.

siRNA targeting isoprenylation enzymes phenocopies FGTI-2734 effects.

To confirm FGTI-2734 effects are not due to unexpected off-target activities, FT and GGT-1 expression was suppressed with siRNA. FT and GGT-1 are heterodimer enzymes that share an alpha structural subunit but have distinct catalytic beta subunits, FT beta and GGT-1 beta. The individual beta subunits were targeted with siRNA, which yielded a ~67% loss of GGT-1 beta and a ~35% loss of FT beta (Fig. 14A). Even with this partial suppression, BMMCs showed a significant decrease in IgE-mediated degranulation and cytokine secretion compared to control transfection (Fig. 14 B&C). These data argue that FGTI-2734 effects can be attributed to loss of isoprenylation.

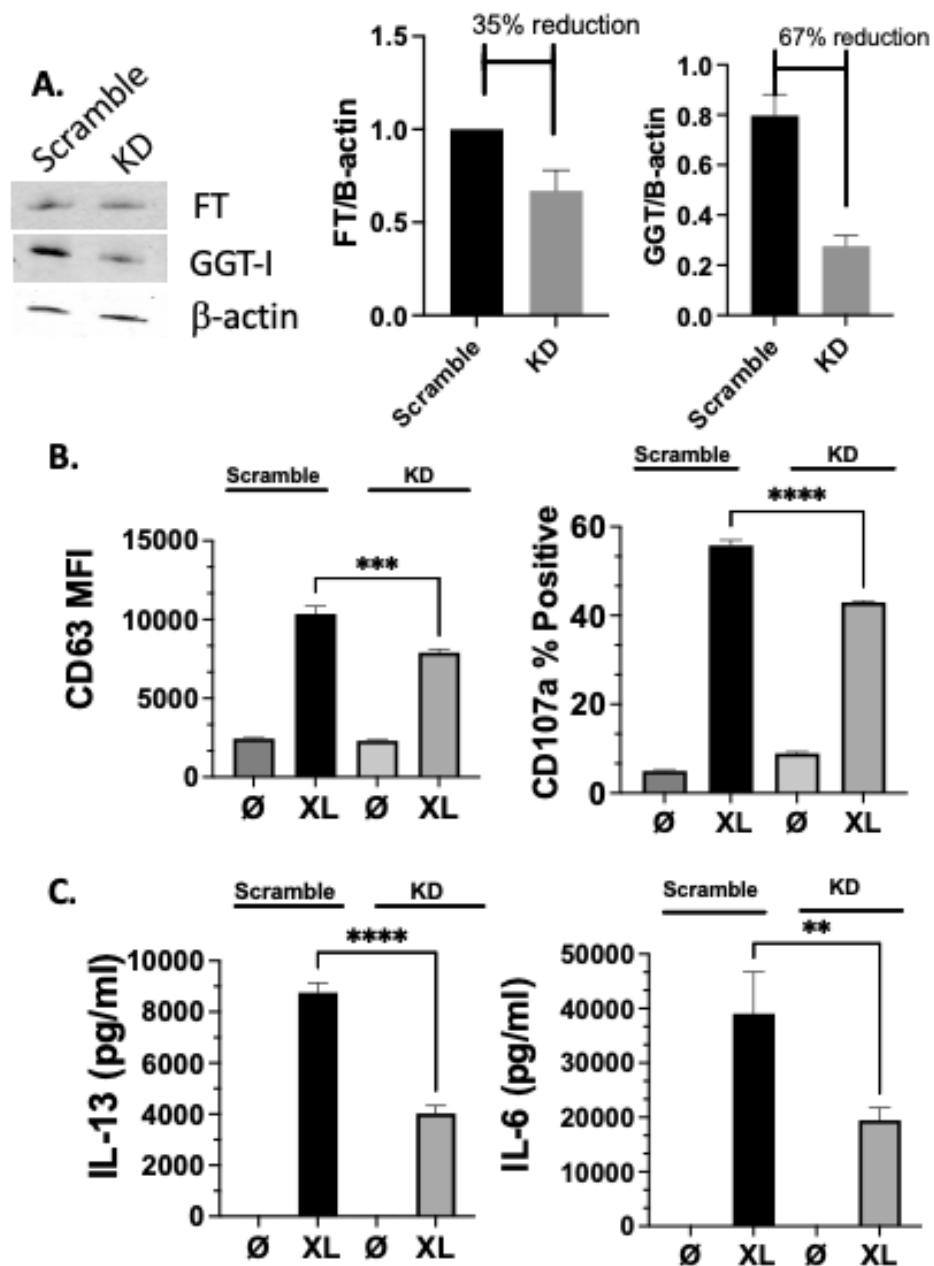


Figure 14: siRNA targeting GGT-1 and FT reduce IgE-mediated degranulation and cytokine production. C57BL/6J BMMCs were transfected with siRNAs targeting GGT-1 and FT or with scrambled control siRNAs. **A)** Cell lysates were assessed by Western blot for GGT-1 and

FT expression, which was normalized to actin loading control for quantification. **B)** Transfected cells were activated by IgE XL for 15 minutes and degranulation markers CD63 and CD107a were measured by flow cytometry. **C)** Transfected cells were activated by IgE XL for 16 hours, and cytokines were measured by ELISA. Data shown are from 2 experiments using 6 samples.

FGTI-2734 treatment inhibits multiple FcεRI signaling events.

Because FcεRI signaling relies on glycolysis for ATP generation (Mendoza, Fudge, and Brown 2021), we tested FGTI-274 effects on glucose metabolism. Using Seahorse metabolic flux analysis, we found that FGTI-2734 had no effect on IgE-mediated extracellular acidification rate, a measure of glycolysis (Fig. 15 A). FGTI-2734 also had no significant effect on FcεRI surface levels or expression of c-Kit, which can amplify FcεRI signaling (Gilfillan and Tkaczyk 2006) (Fig. 15 B).

To identify how FGTI-2734 is reducing cytokine secretion, we next measured its effects on cytokine synthesis. Using intracellular staining, we found that FGTI-2734 reduced both staining intensity and the fraction of cells producing TNF- α and IL-6 (Fig. 16). Coupled with ELISA data, this indicated that the drug suppressed both cytokine synthesis and secretion. We then measured cytokine mRNA by qPCR and found that FGTI-2734 significantly reduced IL-6 and IL-13 transcripts (Fig. 17), suggesting a decrease in cytokine transcription or perhaps mRNA stability. We also found the FGTI-2734 treatment reduced FcεRI-mediated Ca²⁺ influx, corroborating FGTI-2734's ability to reduce degranulation (Fig. 18).

These data suggest that FGTI-2734 acts downstream of FcεRI signaling with a net effect of decreasing cytokine synthesis and Ca²⁺ influx. Therefore, we measured FcεRI phosphorylation events that precede cytokine transcription. We found that FGTI-2734 did not significantly reduce phosphorylation of Src kinases, the earliest events in FcεRI activation. In contrast, downstream phosphorylation of AKT and the MAP kinases ERK, p38, and JNK were significantly reduced (Fig. 19 A, C, & D). Interestingly, FGTI-2734 treatment, while not reducing SRC (such as Lyn and Fyn) kinase phosphorylation, did reduce Syk phosphorylation (Fig. 19 B). These data

indicate that FGTI-2734 disrupts FcεRI signaling between the apical Src kinases and the downstream PI3K/AKT and MAPK pathways, suggesting that the isoprenylated proteins inhibited by FGTI-2734 may mediate PI3K/AKT and MAPK pathway activation and separately regulate a previously unknown isoprenylated protein regulates Syk phosphorylation.

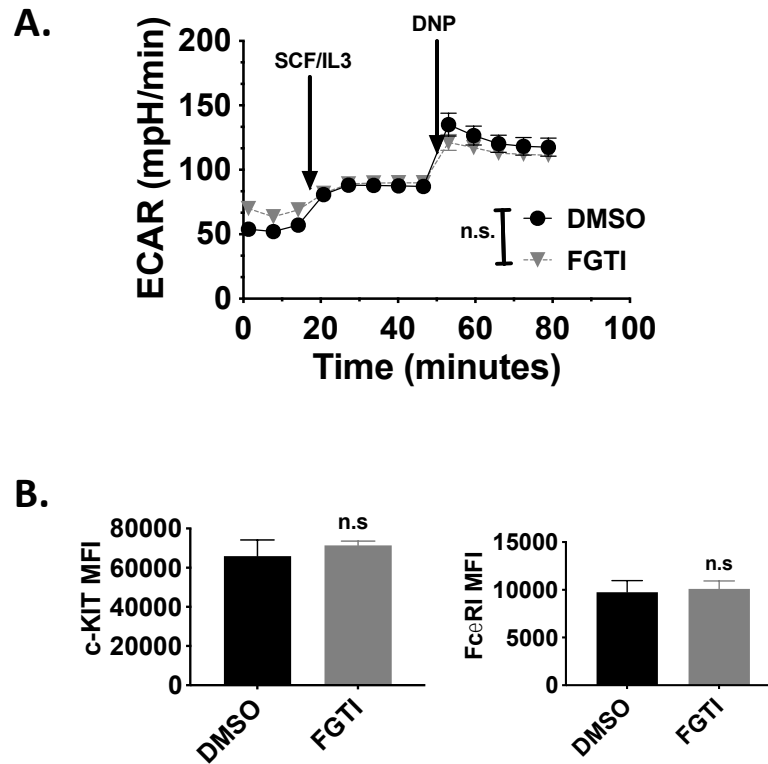


Figure 15: FGTI does not decrease glycolysis, nor does it decrease FcεRI or c-KIT receptor expression. A) BMMCs were treated for 24 hours with FGTI-2734 (5μM) then starved of growth factors (SCF/IL-3) and ECAR was measured using Agilent Seahorse. **B)** BMMCs were treated with vehicle control (DMSO) or FGTI-2734 (5μM) for 24 hours and FcεRI and c-KIT expression was measured by flow cytometry.

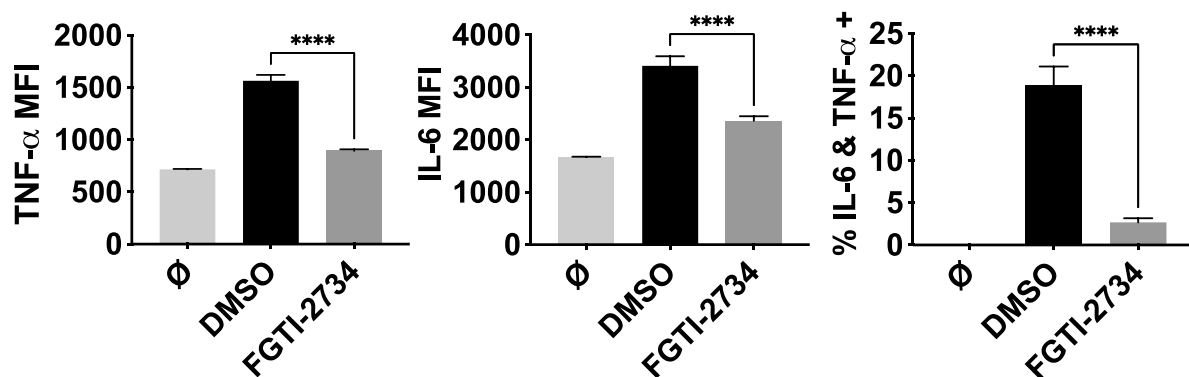


Figure 16: FGTI-2734 treatment decreases intracellular cytokine staining. BMMCs were treated with vehicle or FGTI-2734 (5 μ M) for 24 hours then activated with IgE XL for 5.5 hours and cytokine production was assessed by flow cytometry. Data shown are from 3 experiments using 3 samples. ϕ = unstimulated control cells in DMSO.

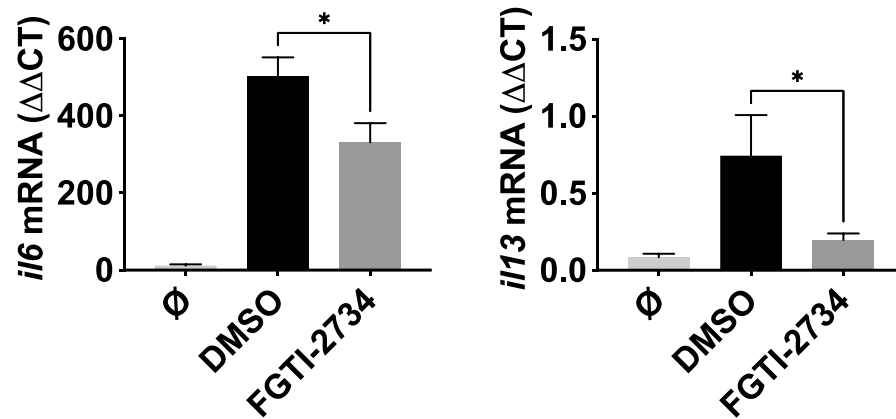


Figure 17: FGTI-2734 reduces cytokine mRNA. BMMCs were treated with FGTI-2734 (5 μ M) for 24 hours and activated with IgE XL for 4 hours. RT-qPCR was used to measure cytokine mRNA levels. Data shown are from 3 experiments using 3 samples.

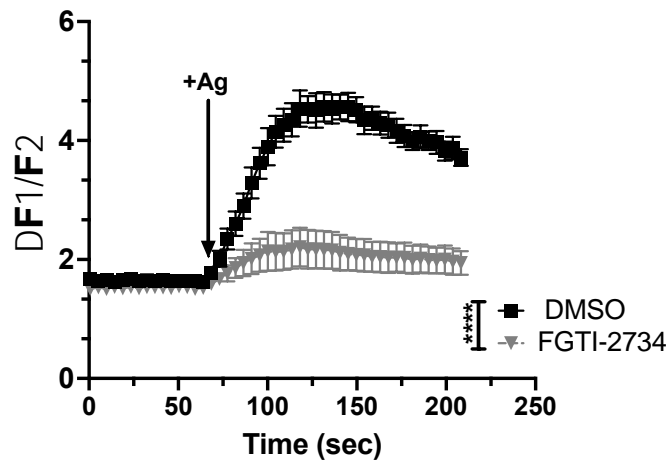


Figure 18: FGTI decreases IgE-mediated Ca^{+} influx. BMMCs were treated with DMSO or FGTI-2734 (5 μ M) for 24hrs before cells were activated with DNP-HSA while measuring Fura-2 fluorescence intensity. Data are from 3 independent experiments. Statistics measured by AUC and Student's t-test.

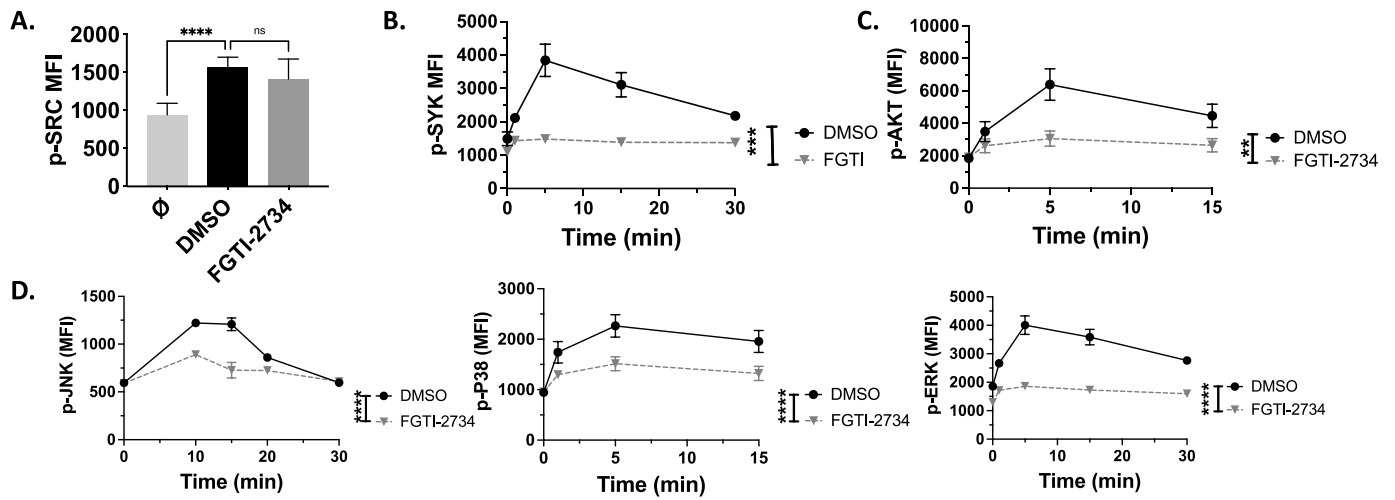


Figure 19: FGTI suppresses FcεRI signaling cascades. BMMCs were treated with FGTI-2734 (5μM) or vehicle for 24 hours and placed in media without growth factors for 2 hours before IgE XL for 5 minutes. Cells were fixed and permeabilized prior to flow cytometry analysis of phospho-protein staining for **(A)** p-SRC, **(B)** p-SYK, **(C)** p-AKT, **(D)** p-MAPKs. Data shown are from 3 experiments using 3 samples.

FGTI-2734 effects may be due to loss of K-Ras localization.

Over 400 proteins are known to be isoprenylated, including Ras, Rac, and Rho family proteins

(Storck et al. 2019). In particular, Ras proteins are important for FcεRI signaling through MAP

kinases. Studies of K-Ras and N-Ras function in cancer have shown that while these proteins are

exclusively farnesylated under normal physiological conditions, they become

geranylgeranylated if FT is blocked (Berndt, Hamilton, and Sebti 2011). To determine if FGTI

inhibits K-Ras isoprenylation, which in turn reduces its membrane anchoring, we treated

BMMCs with DMSO or FGTI-2734 for 24hrs, then isolated membrane and cytoplasmic proteins.

We found that FGTI-2734 decreased K-Ras membrane localization and concomitantly increased

K-Ras in the cytosol. The net effect was a ~50% decrease in membrane-associated K-Ras (Fig

20). To determine if reduced access to K-Ras is sufficient to mimic FGTI-2734 effects, we

inhibited K-Ras expression with siRNA. Several attempts with siRNA pools yielded ~30% K-Ras

loss (Fig 21 A). Interestingly, even this modest reduction decreased FcεRI-mediated

degranulation and IL-6 and IL-13 secretion, much like FGTI-2734 (Fig 21 B&C). In contrast, a 50%

reduction of N-Ras using siRNAs had no effect on degranulation or cytokine secretion (Fig 22).

These data indicate that inhibiting K-Ras isoprenylation and the subsequent reduction of its

membrane localization phenocopies FGTI-2734 and could partly explain the mechanism by

which FGTI-2734 suppresses mast cell function.

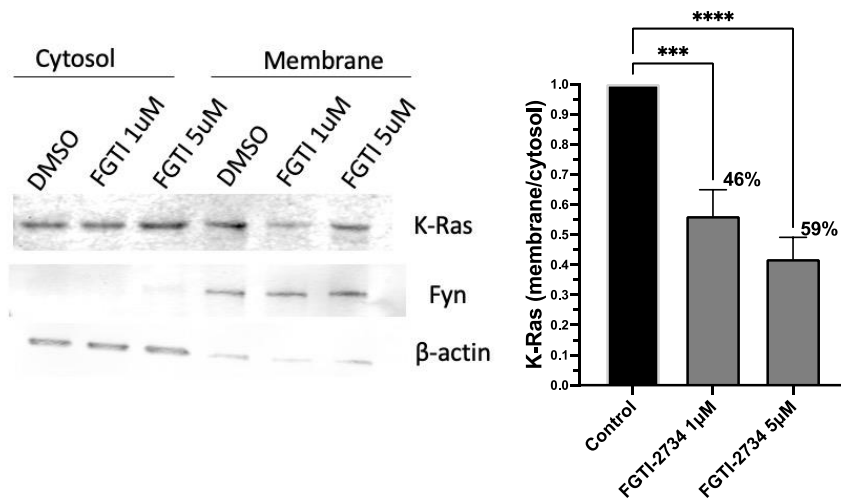


Figure 20: FGTI decreases K-Ras isoprenylation and localization to the cell membrane. BMMCs were treated with either FGTI-2734 or vehicle control for 24 hours and lysed. Membrane and cytosolic fractions were assessed for K-Ras by Western blot. Fyn, a membrane-associated protein that is not isoprenylated, and actin, a cytosolic protein, were used to determine the effectiveness of cell fractionation. Data are from 2 independent experiments, with p values calculated by ANOVA.

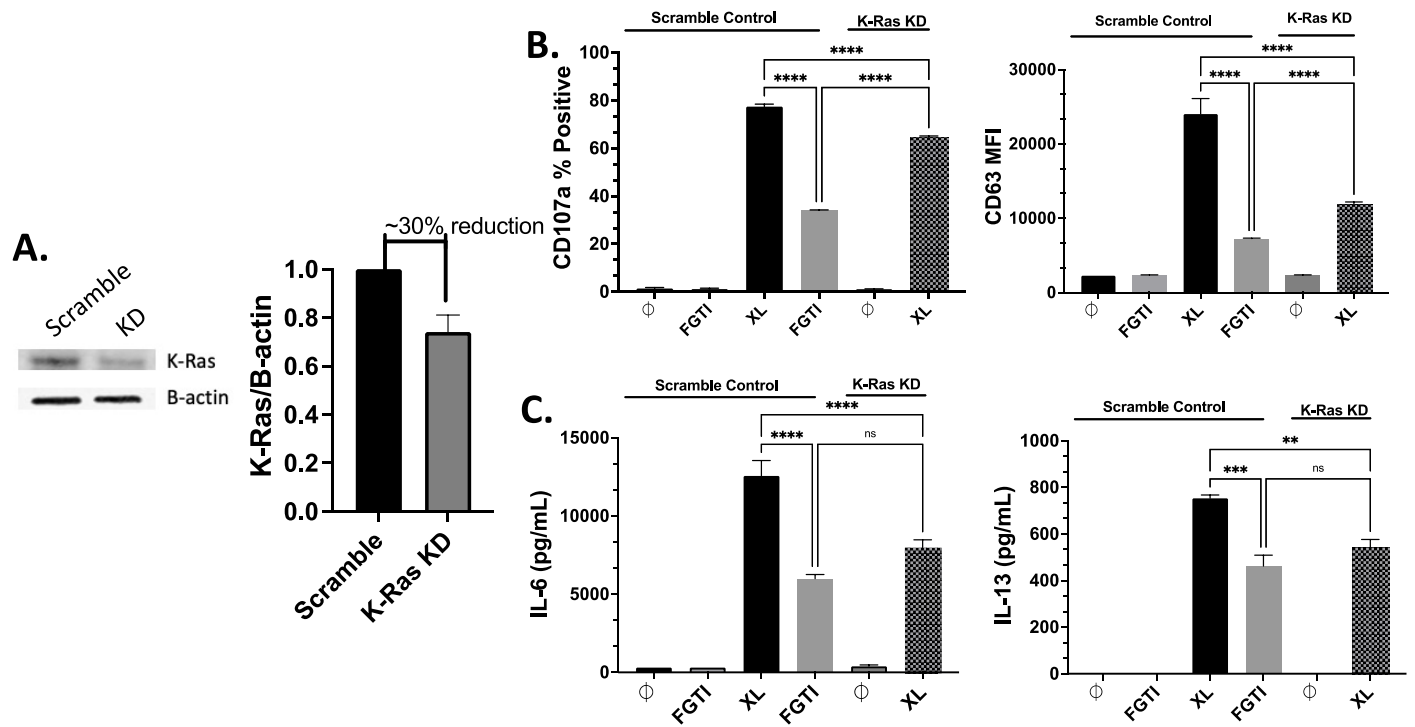


Figure 21: K-Ras knockdown reduces IgE-mediated activation, mimicking the

effects of FGTI-2734. (A) BMMCs were transfected as in Fig. 14 with siRNA targeting K-Ras.

Lysates were assessed for K-Ras expression by Western blot. **(B)** Cells from (A) were activated by

IgE XL for 15 minutes and degranulation markers CD107a and CD63 were measured by flow

cytometry. **(C)** Cells from (A) were activated by IgE XL for 16 hours and cytokines were measured

by ELISA. Data are representative of at least 2 independent experiments. P values were

calculated by ANOVA.

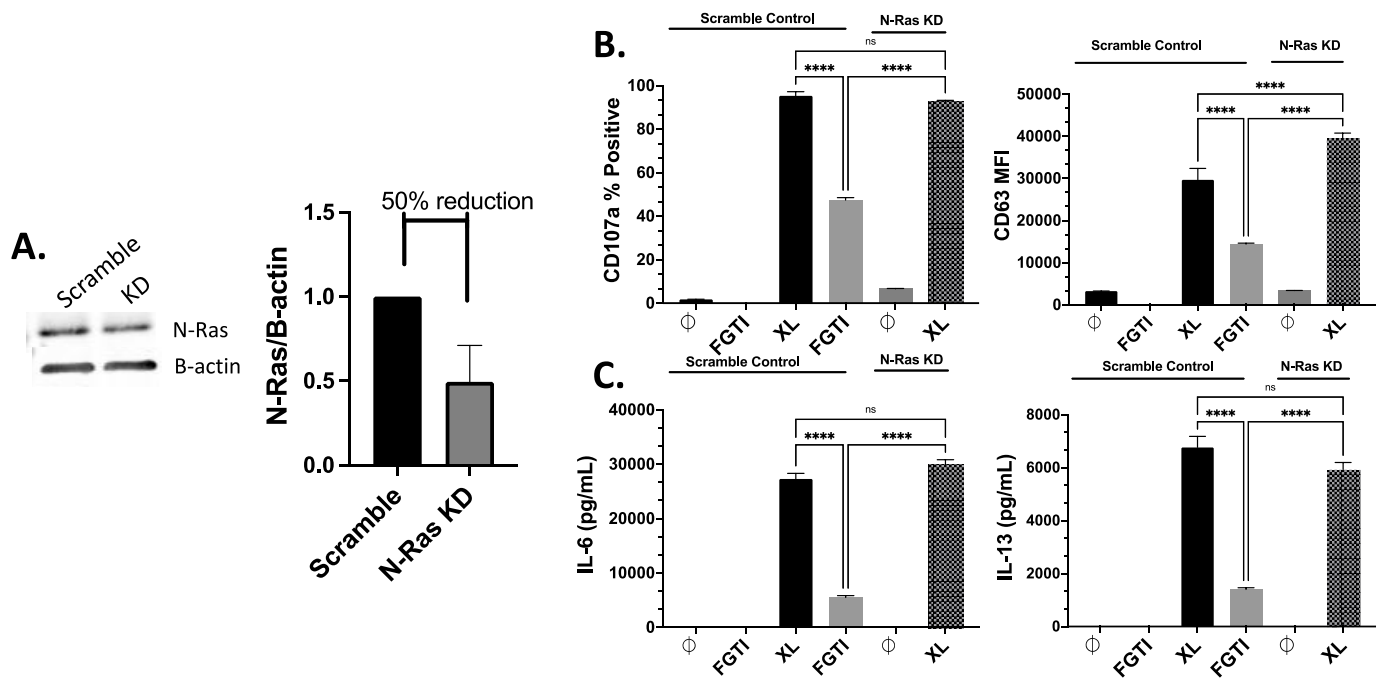


Figure 22: N-Ras does not reduce IgE-mediated activation or phenocopy FGTL.

BMMCs were transfected as in Fig. 14 with siRNA targeting N-Ras. Cell lysates were assessed for N-Ras expression by Western blot. IgE XL-induced degranulation and cytokine production were assessed as described for K-Ras targeting (Fig. 21). Data are representative of at least 2 independent experiments. P values were calculated by ANOVA.

FGTI-2734 inhibits IgE-mediated systemic anaphylaxis.

To determine if FGTI-2734 can suppress mast cell function *in vivo*, we employed a mast cell- and histamine-dependent model of passive systemic anaphylaxis (PSA) (Kanjrawi et al. 2013). C75BL/6J mice were injected with IgE and treated with vehicle or FGTI-2734 (1 mg/kg) 20 hours before and again 2 hours before antigen injection. Core body temperature was used to assess anaphylaxis and peripheral blood plasma was used to measure mast cell protease (mMCPT-1) and IL-6. As shown in Figure 23A, FGTI-2734 significantly diminished hypothermia over the course of 2 hours post-antigen, reducing the area under the curve by >50% (Fig 23 A). We also found that FGTI-2734 significantly reduced plasma levels of the mast cell protease mMCPT-1 as well as IL-6 (Fig 23 B). Consistent with our *in vitro* data, FGTI-2734 also suppressed PSA in fluvastatin-resistant 129/SvJ mice (Fig. 24). To determine if FGTI-2734 effects were due to inhibiting mast cell function rather than acting directly on the vasculature, mice were injected with histamine. Unlike IgE-antigen challenge, FGTI-2734 had no effect on histamine-induced hypothermia, indicating the drug does not antagonize histamine receptor signaling (Fig. 25). These data support the conclusion that FGTI-2734 can significantly reduce FcεRI-mediated mast cell function *in vivo*.

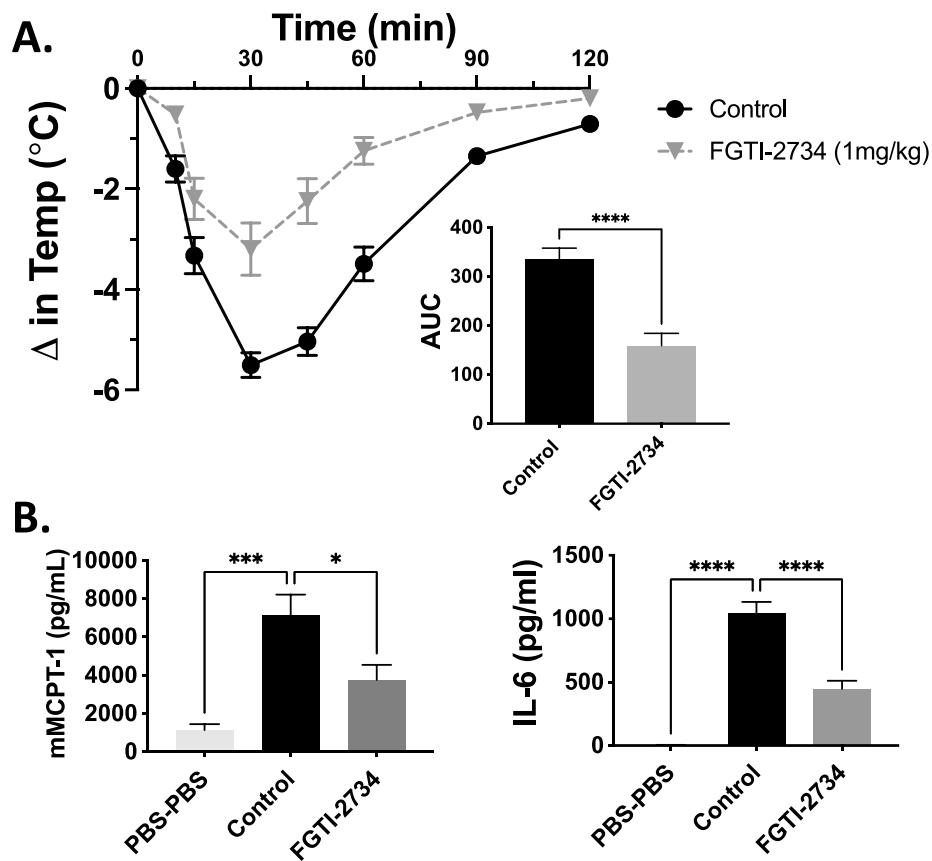


Figure 23: FGTI reduces mast cell-dependent anaphylaxis on the C57BL/6J mouse background. Female C57BL/6J mice were treated with vehicle or FGTI-2734 (1mg/kg, i.p.) and subjected to PSA as described in Materials and Methods. **(A)** Change in core body temperature. AUC values were compared using Student's t-test. **(B)** Plasma levels of mMCPT-1 ten minutes after antigen injection or IL-6 two hours after antigen injection were measured by ELISA. Data shown are from 3 experiments using N=13 mice/point. P values were calculated by ANOVA.

*p<.05; ***p<.001; ****p<.0001.

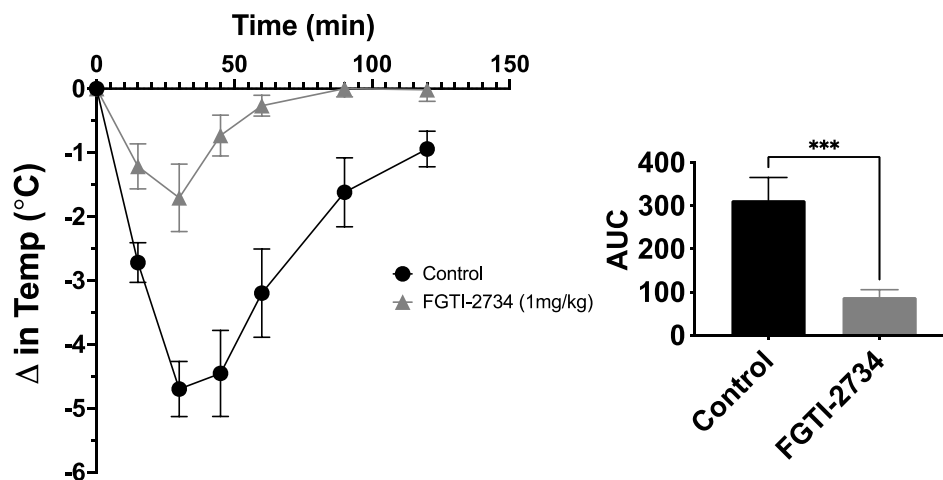


Figure 24: FGTI-2734 reduces anaphylaxis in statin-resistant 129/SvJ mice. Female

129/SvJ mice were treated with vehicle (N=7) or FGTI-2734 (1mg/kg, i.p.) (N=8) and subjected to PSA as described in Materials and Methods. Change in core body temperature measured.

***p<.001. AUC values were compared using Student's t-test.

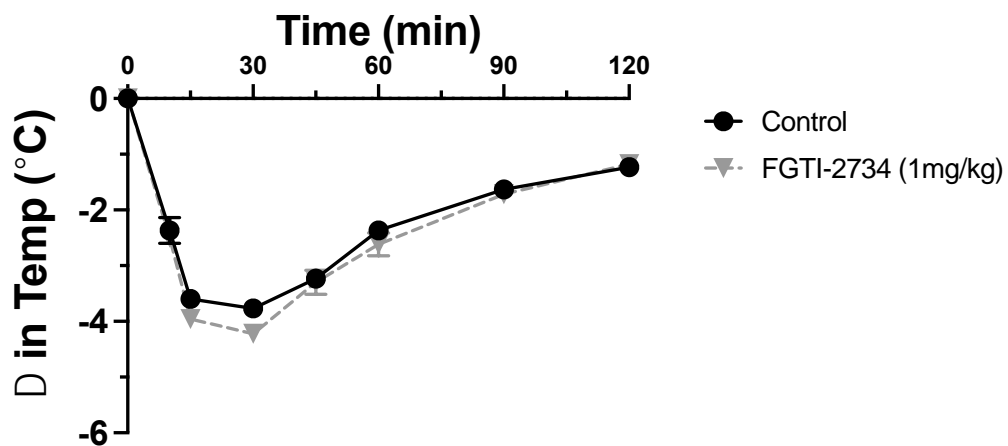


Figure 25: FGTI treatment does not inhibit histamine-induced anaphylaxis. Female C57BL/6J mice were treated with vehicle or FGTI-2734 (1mg/kg, i.p.) 20 hours and again 2 hours before histamine injection (2mg, i.p.), and core body temperature was measured. Data shown are from 3 experiments, N=13/treatment group.

FGTI-2734 reduces inflammation in *Alternaria*-induced airway inflammation.

PSA is mast cell-dependent but circumvents many aspects of the atopic response such as antigen presentation, Th2 differentiation, and IgE production. To test FGTI-2734 effects in a more clinically relevant allergy model, we employed *Alternaria* (ALT)-induced airway inflammation (Kobayashi et al. 2009). This model elicits pulmonary eosinophilia and other indicators of the Th2 response. The experimental design is depicted in Figure 26A. In brief, mice were sensitized via i.n. route with OVA (100 µg/mouse, used to track antigen-specific IgE production) +/- ALT (10 µg/mouse) on days 0 and 7 and then challenged with i.n. OVA (50 µg/mouse) +/- ALT (5µg/mouse) on days 14-16. Vehicle or FGTI-2734 (1mg/kg) was administered via i.n. route on days 12-16 and samples were harvested on day 17.

We found that FGTI-2734 significantly diminished ALT-induced BALF infiltrate. In fact, total cellular infiltrate and eosinophil number were restored almost to basal levels (Fig 26B). In both BALF and lung tissue digest, the percent eosinophils was reduced by at least half (Fig 26 B&C). This model of allergic disease does not induce significant neutrophil infiltration (Fig 27), and FGTI did not affect the modest increase in neutrophils noted. Blinded scoring of H&E- and PAS-stained lung sections showed that FGTI-2734 significantly reduced inflammation and mucus production (Fig. 28). The drug also significantly reduced plasma levels of mast cell mMCPT-1 protease (Fig. 29). Consistent with FGTI-2734 treatment beginning after antigen sensitization, the drug did not affect the 5-fold increase in OVA-specific IgE levels (Fig. 29). When single cell suspensions of the draining mediastinal lymph nodes were re-stimulated with OVA ex vivo, we found that FGTI-2734 significantly reduced IL-5 and IL-13 secretion (Fig 30).

Finally, we tested FGTI-2734 effects on airway hyperresponsiveness. We found that the model depicted in Figure 26A elicited little airway hyperresponsiveness and that FGTI-2734 at 1mg/kg had no effect (data not shown). Therefore, we extended the model to elicit increased airway resistance. The experimental design is depicted in Figure 31A. Briefly, mice were sensitized via i.n. route with OVA (100 µg/mouse) +/- ALT (10 µg/mouse) on days 0 and 10 and then challenged with i.n. OVA (50 µg/mouse) +/- ALT (5µg/mouse) on days 20-23. Vehicle or FGTI-2734 (5mg/kg) was administered via i.n. route on days 18-23, and airway hyperresponsiveness was measured by FlexiVent on day 24. We found that FGTI-2734 treatment significantly reduced both resistance (Rrs) and elastance (Ers) as judged by AUC (Fig 31 B). These data show that FGTI-2734 can reduce airway inflammation and bronchoconstriction in a relevant model of allergic asthma.

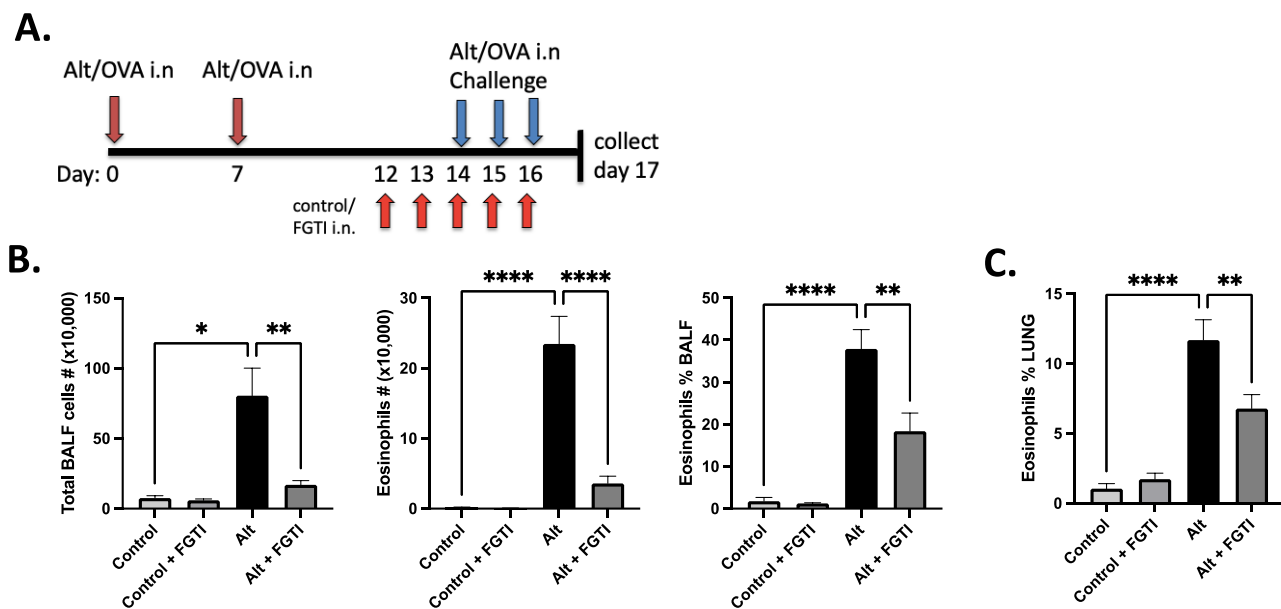


Figure 26: FGTI reduces Eosinophil infiltration in model of allergic disease. A)

Schematic of ALT model. FGTI-2734 was used at 1 mg/kg. **B)** Total BALF cells and BALF eosinophil number and percentage of CD45+ cells were measured by flow cytometry analysis as described in Materials and Methods. **C)** Lung tissue eosinophils were measured by flow cytometry. Data shown are from 4 experiments using N=12 mice, analyzed by ANOVA.

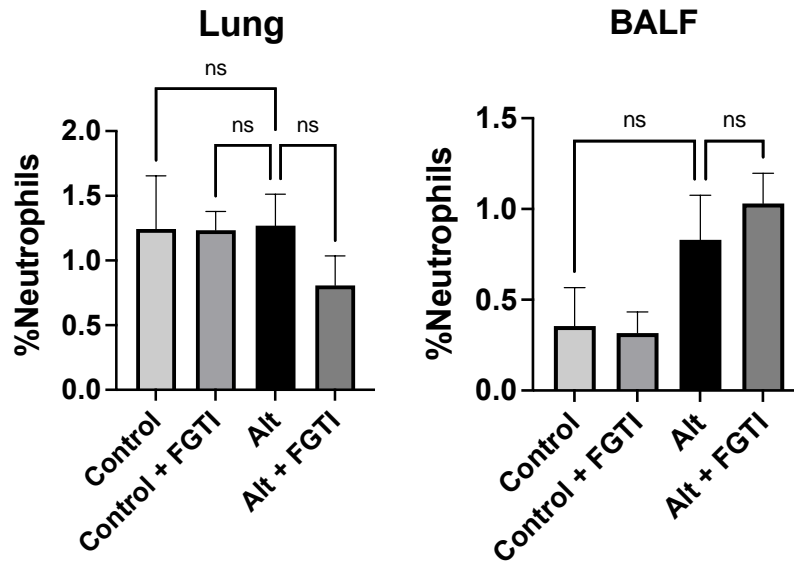
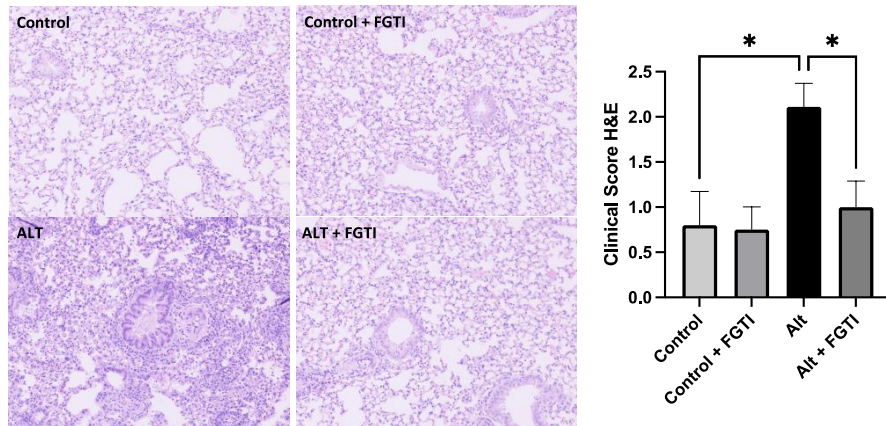


Figure 27: FGTI does not affect neutrophil infiltration. Lung tissue neutrophils were measured by flow cytometry analysis as described in Materials and Methods. BALF neutrophils were measured by flow cytometry. Data shown are from 4 experiments using N=12 mice analyzed by ANOVA.

A.



B.

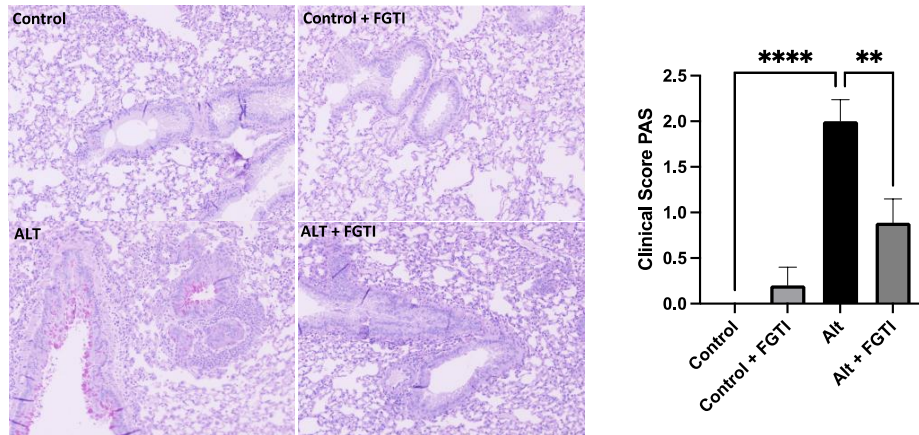


Figure 28: FGIT reduces lung pathology. Scoring of lung inflammation in **(A)** H&E-stained sections and **(B)** mucus production in PAS-stained sections was calculated as described in Materials and Methods. Bar charts show summary data from 4 experiments using 12 mice/group, with samples analyzed by ANOVA.

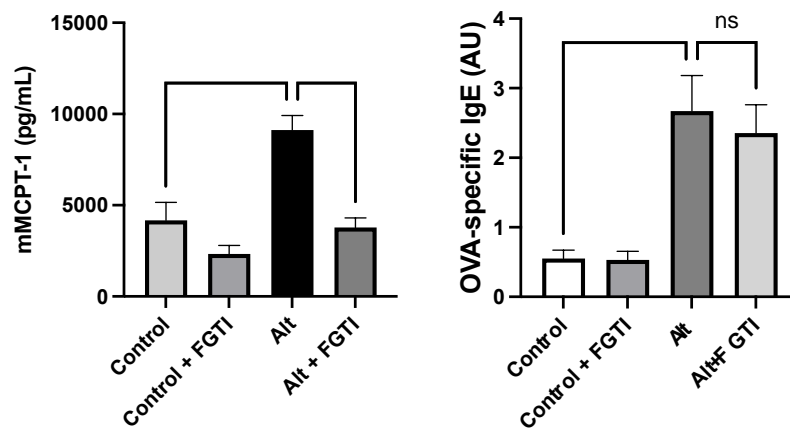


Figure 29: FGIT reduces mast cell protease but not circulating antigen-specific

IgE. Plasma mMCPT-1 and OVA-specific IgE concentration on day 17 were measured by ELISA.

Data are from 4 experiments using 12 mice/group, with samples analyzed by ANOVA.

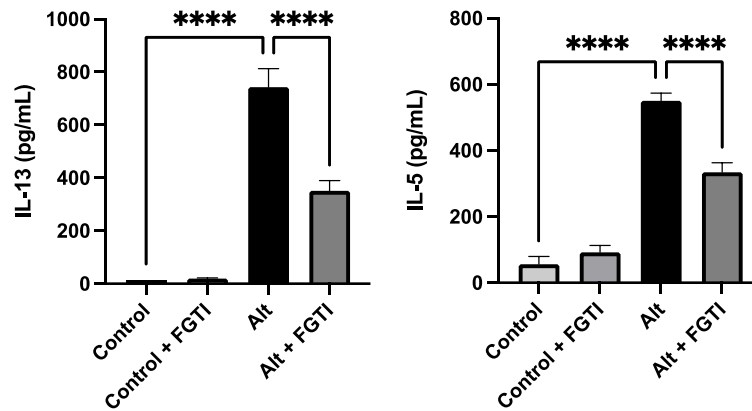
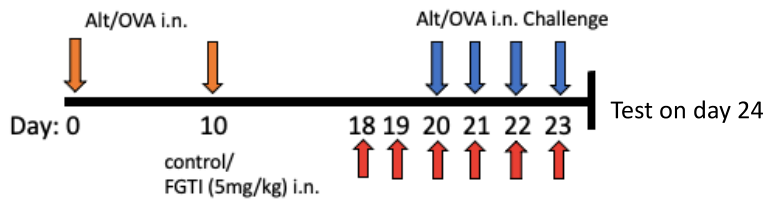


Figure 30: FGTI reduces ex vivo lymph node simulation. Mediastinal lymph node cells were collected on day 17. Cells were restimulated with OVA (300 ng/ml) for 3 days, and cytokines in culture supernatants were measured by ELISA. Data are from 4 experiments using 12 mice/group, with samples analyzed by ANOVA.

A.



B.

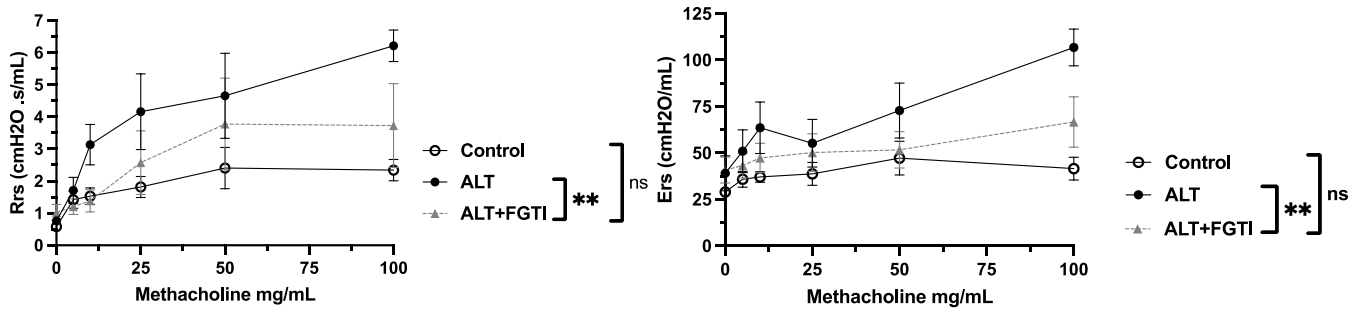


Figure 31: FGTI reduces airway resistance and elastance. A) Schematic of ALT-

induced airway hyperresponsiveness model. FGTI-2734 was used at 5 mg/kg. **B)** FlexiVent was

used to measure lung resistance (Rrs) and elastance (Ers) elicited by increasing doses of

methacholine. AUC and ANOVA were used to measure significance. N=8/group.

Discussion

Asthma and allergic disease are abundant in developed and some developing nations (To et al. 2012; Nurmagambetov, Kuwahara, and Garbe 2018). While current interventions are effective for many, novel approaches are needed to address severe disease, reduce cost, and improve access. Several large studies found an association between statin use and reduced asthma severity, prompting us to ask how these frequently-prescribed and relatively safe drugs might be repurposed (Wang et al. 2018; Tse et al. 2013; Huang et al. 2011). Mast cells play a key role in allergic disease, with their most studied contributions being through FcεRI signaling. Because cholesterol and isoprenylated proteins play critical roles in IgE-mediated mast cell function, FcεRI is a logical target for statin-based inhibition. Our initial finding that statins could suppress FcεRI-induced mast cell activation was complicated by varying levels of statin resistance among mouse strains and human donors (Kolawole et al. 2016). Therefore, we sought a consistent means of attacking the same signaling cascades while circumventing drug resistance.

In our previous study, we showed evidence that rescuing isoprenylation reversed statin effects (Kolawole et al. 2016). In the current work, we found that inhibiting isoprenylation mimics statin effects, but that both FT and GGT-1 need to be inhibited. Our previous work identified an independent role for geranylgeranylation; however, this conclusion was based on GGT-1 inhibitor effects at concentrations that could have blocked both FT and GGT-1 (Kolawole et al. 2016; Goossens et al. 2005). The recent development of FGTI-2734 and the more selective FT and GGT-1 inhibitors, FTI-2153 and GGTI-2417, respectively, allowed us to better parse isoprenylation and its role in mast cell function (Kazi et al. 2019). We also expanded our work to a relevant asthma model and sought to identify isoprenylation substrates critical for FcεRI

function. The data support the idea that targeting isoprenylation and specifically K-Ras may be productive avenues for clinical translation.

Our current findings support the conclusion that FT and GGT-1 provide cooperative support for FcεRI signaling. While both enzymes must be antagonized to suppress mast cell function, doing so appears to avoid the limits of statin resistance. Our previous studies found statin resistance in the 129/SvJ mouse strain, partial resistance in other strains, and considerable variation among human donors. In response to statin treatment, 129/SvJ mast cells upregulated HMGCR expression, a known mechanism of statin resistance (Chasman et al. 2004). This inconsistent drug response reduced our optimism that statins might be repurposed for allergy therapy. Therefore, it was encouraging that FGTI-2734 showed no variability on different mouse backgrounds, including 129/SvJ.

Isoprenylation is the enzymatically-catalyzed addition of a 15-carbon or 20-carbon lipid to the C-terminus of proteins, allowing membrane localization that is pivotal for cell signaling. Isoprenylation occurs on a cysteine residue located 4 amino acids from the C-terminus. The last amino acid in the CAAX box motif determines if the protein is preferentially lipid-modified by FT or GGT-1. It is estimated that FT and GGT-1 modify at least 400 proteins (Storck et al. 2019), many of which are small GTPases such as RAS that play a role in cell signaling, vesicle trafficking, and protein scaffolding for signal transduction. Importantly, isoprenylation inhibitors such as FGTI-2734 are in pre-clinical or clinical studies targeting cancers with defective Ras pathways (Karasic et al. 2019). These studies have greatly expanded our knowledge of Ras function.

All 3 Ras isoforms, H-Ras, N-Ras and K-Ras are farnesylated. However, N- and K-Ras can alternatively become geranylgeranylated when FT is inhibited with a FTI (Berndt, Hamilton, and

Sebti 2011). Mast cells can express all 3 Ras isoforms, but their individual roles in FcεRI signaling are unclear (Dwyer, Barrett, and Austen 2016). Our findings suggest that H-Ras is dispensable, because it is only farnesylated and FT inhibition had no effect on FcεRI-induced cytokine production or degranulation.

The need to block both FT and GGT-1 to suppress mast cell function suggests a role for N-Ras or K-Ras. Our findings show that a 50% decrease in N-Ras had no effect on cytokine production or degranulation, while a 30% loss of K-Ras largely mimicked FGTI-2734 treatment. Other studies have reported that a small loss of Ras function significantly decreases lymphoid cell activation by antigen receptor signaling (Das et al. 2009). It is interesting that modest K-Ras depletion had less effect on degranulation than cytokine secretion. One interpretation is that cytokine induction is heavily dependent on K-Ras function, while degranulation proceeds with a lower threshold of K-Ras-mediated signals. (Baier et al. 2014; Hong-Geller et al. 2001). We found that FGTI-2734 treatment correlated to a >50% loss of K-Ras membrane localization. Therefore, a 30% K-Ras loss via siRNA may equate to less effective inhibition of degranulation. However, loss of isoprenylation on proteins other than K-Ras may explain the more potent effects of FGTI-2734 compared to the siRNA approach. Other possible candidate proteins could be Rac2 and Cdc42 have important roles in granule formation and trafficking, thus K-Ras may simply be less critical (Baier et al. 2014; Hong-Geller et al. 2001). This could be best assessed using K-Ras KO cells, avoiding the issues of partial depletion with siRNA. If this approach were paired with forced K-Ras membrane localization by transfecting a myristoylated-K-Ras mutant prior to FGTI-2734 treatment, we could determine if K-Ras targeting is necessary and sufficient for FGTI-2734 effects.

These data may also have relevance to differences we observed between mouse BMMC and human skin mast cells. The latter group was mostly resistant to FGTI-2734 when measuring IgE-induced degranulation, whereas cytokine secretion was significantly reduced in mouse and human cells. One explanation is that human skin mast cells may require minimal K-Ras signaling or use a protein distinct from mouse BMMC to elicit degranulation. Also, it is important to note that human skin mast cells are a connective tissue phenotype mast cells, while BMNCs are more closely related to mucosal mast cells (Akula et al. 2020). While mucosal and connective tissue mast cells share many key similarities, their protein and transcription signatures differ (Kurashima and Kiyono 2014). Further study of isoprenylated proteins is needed to better understand the role of FT and GGT-1 in mast cell function, especially in human mast cells comparing mast cells from different anatomical locations.

The reduction of Syk phosphorylation could also explain the phenotype seen with FGTI-2734 treatment. Because Syk phosphorylation occurs very early in the FcεRI cascade – likely the second step after Lyn activation - decreased Syk activity could be a major contributor to FGTI-2734 effects. Syk KO mast cells have significantly reduced mast cell function (Wex et al. 2011; Costello et al. 1996). Diminished Syk phosphorylation could also explain the reduced Ca²⁺ influx seen in Fig. 18. Because Syk does not possess a CAAX box, it is unlikely the FGTI-2734 acts directly on this protein. A review of the literature did not suggest an isoprenylated GTPase protein that would likely affect Syk phosphorylation, including by acting on proteins like SHP-1 and -2 that dephosphorylate Syk and FcεRI β (Alsadeq et al. 2014). Future research into isoprenylated protein(s) that can modulate Syk could improve our understanding of FcεRI

signaling in allergic disease and provide new therapeutic targets. It is also important to consider off-target effects of FGTI-2734 that unexpectedly inhibit Syk.

The importance of isoprenylation and the efficacy of FGTI-2734 was clear in two mouse models relevant to allergic disease. PSA is a mast cell-dependent model in which FcεRI crosslinkage elicits systemic cytokine production as well as histamine-mediated vasodilation and hypothermia. This relatively simple model circumvents antigen presentation, Th2 differentiation, and IgE production, allowing mast cell function to be more clearly assessed. Because FGTI-2734 decreased IgE-mediated hypothermia and plasma cytokines - but had no effect on direct histamine challenge – the drug appears to suppress mast cell function rather than vascular histamine responsiveness. This was effective even on the statin-resistant 129/SvJ background, supporting the potential advantage of targeting isoprenylation directly.

Alternaria-induced pulmonary inflammation is considerably more complex and more clinically relevant than PSA (Salo et al. 2006). It maintains the complexity of antigen processing and lymphocyte differentiation and manifests as airway eosinophil recruitment and pulmonary hyperresponsiveness (Kobayashi et al. 2009). By waiting to treat with FGTI-2734 on day 12, we sought to target established allergic inflammation rather than prevent it. This model also employed the drug through intranasal administration, which could have significant advantages for clinical use. Drug efficacy at the 1mg/kg dose was striking. Eosinophil numbers, circulating mMCPT-1, and pulmonary inflammation were reduced to nearly control levels. Mucus production and *ex vivo* lymphocyte cytokine secretion was halved, and airway hyperresponsiveness was greatly diminished. These data suggest that isoprenylation is foundational to inflammation in this fungal challenge model and warrant further study of the

proteins involved. Because the drug had no effect on antigen-specific IgE production at the time points tested, our data suggest FGTI-2734 could be effective in established disease.

There are important limits to our interpretations. In the airway inflammation model, FGTI-2734 is likely acting directly on lymphocytes and eosinophils, so we cannot conclude that mast cell inhibition explains all or even most of the effects seen. Inducible cell-specific deletion of the critical isoprenylation enzymes and perhaps K-Ras is needed to draw such conclusions. Of the >400 known isoprenylation substrates, K-Ras is likely one among several that are critical to FcεRI-mediated mast cell function. Importantly, and unlike statins, FGTI-2734 is in pre-clinical models and has no safety profile. Thus, it remains well removed from allergic disease therapy for now.

In sum, our study supports an important role for isoprenylation, and specifically the substrate K-Ras, in mast cell function. It appears that FT and GGT-1 must both be suppressed for efficacy and that this approach might circumvent statin resistance. We urge further development of dual FT/GGT-1 inhibitors and their study in allergic disease.

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