Mast Cell Responses to Food Toxins

by

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ABSTRACT

Mast cells, components of the immune system, promote allergic symptoms such as itching, sneezing, and increased intestinal motility. Although mast cells have a detrimental role in allergies, they might have unrecognized physiological functions. Indeed, mast cells have been reported to protect against lethal envenomation. I hypothesized that mast cells have a protective role in the defense against toxins. Because toxin-induced diarrheal diseases are one of the top five causes of mortality in children worldwide (induced by cholera toxin, for example), I tested the role of mast cells in sensing relevant dietary toxins. My goals were to a) establish an in vitro model of mast cell activation using foodborne toxins and b) determine the mast cell transcriptional programs induced by these toxins. To establish the in vitro model, I generated mast cells from murine bone marrow precursors and cultured them in mast cell-specific media for 5 weeks. Mature mast cells were then stimulated with toxins from phylogenetically distinct origins. I found that, surprisingly, no toxin was able to induce significant cell death, even after 24h of culturing, suggesting that mast cells are resistant to the toxic effects of these compounds. To assess mast cell activation, I quantified the levels of TNF-α 6h after toxin exposure. None of the toxins were able to induce TNF-α production by mast cells, suggesting that toxins might not induce inflammation in mast cells. However, I found that mast cells induced expression of activation-related transcripts like *Il1b, Tpsab1, Alox5, Egr1, Tnfa and Hdc* in response to cholera toxin, when compared with controls. Mast cells stimulated with retrorsine induced the expression of *Tph1, Alox5, Il1b and Hdc.* Deoxynivalenol induced *Ltc4, Il6, Tpsab1, Tnfa, Hdc, and Alox5* while okadaic acid

induced *Il6, Tnfa, Tph1, Alox5, Egr1, Il1b and Hdc* expression. Aconitine only induced *Il6, Hdc, and Tpsab1*. Lastly, Ochratoxin A induced expression of *Il1b, Il6, Tpsab1, Egr1 and Hdc*. Altogether, these results suggest that mast cells directly sense and respond to food toxins, which was unknown. How exactly mast cells contribute to toxin defenses will be crucial to investigate as they impact both toxin-induced and inflammatory diseases.

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CHAPTER 1

INTRODUCTION

Mast cells are crucial components of the immune system and primarily are known for their role in promoting allergic symptoms (Krystel-Whittemore et al., 2016). However, recent studies have suggested that mast cells may have unrecognized physiological functions beyond their involvement in allergies (Palm et al., 2012). One such function is their ability to protect against lethal envenomation (Galli et al., 2020). This finding led to the hypothesis that mast cells may also play a protective role in defending against ingested food toxins. This will be explored by stimulating bone marrow-derived mast cells with food toxins and examining the cytokine release and inflammatory-associated gene expression.

Mast cells, vital components of the immune system, play a crucial role in detecting and responding to harmful substances, including toxins. As leukocytes, they are found in connective and mucosal tissues throughout the body, serving as evolutionarily conserved innate immune cells and acting as key effector cells in allergic reactions (Urb & Sheppard, 2012). Activation and degranulation of mast cells have profound effects on both physiological and pathological conditions. Mast cells are recognized for their ability to induce vasodilation, maintain vascular homeostasis, modulate innate and adaptive immune responses, promote angiogenesis, and participate in venom detoxification (Krystel-Whittemore et al., 2016). During allergic inflammation, mast cell degranulation can be triggered by allergens through IgE antibody crosslinking in exposed tissues; specifically, with FcεR1, the high affinity IgE receptor (Daëron et al., 1995). The

granules of mast cells contain various mediators, including cytokines, amines (e.g., histamine, serotonin), lipid mediators (class of bioactive lipids that are produced locally through specific biosynthetic pathways in response to extracellular stimuli such as toxins), and proteases (Lundequist & Pejler, 2011).

Prior to initial encounter with allergens or pathogens, allergy-related cells belonging to the innate immune system, such as eosinophils, mast cells, and basophil, are already present. However, activation of the adaptive allergic response, including the production of IgE antibodies and cytokine proteins, requires exposure to an allergen or pathogen (Akahoshi et al., 2011). On the other hand, innate immunity alone is responsible for safeguarding the host against toxins and other non-living stressors. Mast cell's ability to quickly respond underlines many allergic inflammations likely evolved as a defensive strategy against venoms and noxious substances (Palm et al., 2012).

Research has shown that mycotoxins such as deoxynivalenol activate cytokine production through T helper cells known as CD4+ and CD8+; these cells play a significant role in the adaptive immune system. Toxins such as cholera toxin have been shown to induce symptoms similar to food intolerance such as intestinal inflammation and increased gut motility Additionally, it has been suggested that mycotoxins are implicated in human chronic intestinal inflammatory disease. Although more research on the mechanisms is needed, a compromised intestinal barrier has been linked to inflammatory disease. (Gao et al., 2020). Furthermore, research supports the notion that the degradation of reptile or anthropoid venoms by mast cell-derived proteases can enhance resistance to these venoms (GALLI et al., 2017). This is exemplified by the Th2

immune response associated with IgE antibody production induced by honeybee venom, which can increase resistance to lethal doses of honeybee venom (GALLI et al., 2017).

Mast cell immune responses have demonstrated the ability to enhance the host's defense against certain venoms. However, there is still debate on whether or not the development of IgE antibodies venoms enhances resistance to lethal doses or induces the detrimental anaphylaxis reaction, Some studies suggest that the development of IgE antibodies to insect venoms increases the risk of anaphylactic reactions upon subsequent exposure, similar to allergic disorders. (Mukai et al., 2016).

Understanding the direct response of mast cells to food toxins is crucial for expanding our knowledge of their involvement in the detoxification process and overall immune response. This study aims to establish an in vitro model of mast cell activation using food toxins and investigate the relevant transcriptional programs induced by their activation. The food toxins used in this study include various gastrointestinal toxins such as cholera toxin, deoxynivalenol, retrorsine, okadaic acid, deoxynivalenol, aconitine, and Ochratoxin A. These toxins were chosen because they were chemically and phylogenetically distinct and known to cause illness when ingested and primarily impact effect the digestive tract, allowing for their classification as food toxins.

Vibrio cholera is a gram-negative oxidase-positive rod that is typically found in contaminated food or water and is highly prevalent in developing countries (Fanous & King, 2023). Worldwide, cholera is estimated to cause upwards of four million cases per year (Fanous & King, 2023). Cholera toxin ingestion, produced by the bacterium *Vibrio cholerae*, is responsible for causing the severe gastrointestinal infection known as

cholera. Vibrio cholerae produces intestinal secretion of water through its enterotoxin: cholera toxin (Rocha et al., 2003). Additional reports suggest that cholera toxin may stimulate intestinal inflammatory response such as intestinal secretion, primarily through the release of histamine, prostaglandins, Il1β and TNF-α during *in vivo* studies (Rocha et al., 2003), possibly through faecal leukocyte secretion, indicating an inflammatory response in the gut (Saha et al., 2011).

Retrorsine is a natural hepatotoxic pyrrolizidine alkaloid (PA) and is the main PA in S. formosus leaves (Lehmann et al., 2023). The hepatotoxic effects of PA include the inhibition of hepatocyte cell division (Zhou et al., 2006). Exposure to retrorsine is associated with liver damage and can lead to symptoms such as jaundice, abdominal pain, and hepatic failure (Lehmann et al., 2023). Although the specific interactions between retrorsine and mast cells are currently unknown, retrorsine in combination with gut dysbiosis causes intestinal inflammation and compromises the integrity of the gut barrier (Xiao et al., 2022). One study found that retrorsine did not successfully suppress the proliferation of mouse liver cells compared to rat liver cells, supporting the claim that mice are resistant to the effects of retrorsine (Zhou et al., 2006). Variations in response to retrorsine between mice and rat models have indicated that the specific metabolic and detoxification pathways lead to retrorsine resistance in mice (Zhou et al., 2006). Additional studies have shown that retrorsine is a mechanism based inactivator of P450 3A4, an enzyme essential for the metabolism of medicine and endogenous compounds (Lehmann et al., 2023).

Deoxynivalenol, also known as vomitoxin, is a mycotoxin produced by certain species of Fusarium fungi commonly found in cereals such as wheat, barley, and maize (Williams, n.d.). Ingesting food contaminated with deoxynivalenol can lead to symptoms like nausea, vomiting, abdominal pain, and diarrhea (Sobrova, n.d.). In a study determining the impact of epithelial stress or damage on allergic sensitization, it was found that deoxynivalenol induced impairment of the intestinal barrier contributes to the development of whey-induced food (Bol-Schoenmakers et al., 2016). It was also shown that deoxynivalenol also resulted in the rapid induction of IL-33, which is a known activator of the immune system, including mast cell activation (Bol-Schoenmakers et al., 2016). Together, this suggests that deoxynivalenol compromises the intestinal barrier which induces IL-33 production and therefore immune response; further suggesting the potential for mast cell activation in response to deoxynivalenol stimulation.

Okadaic acid is a marine toxin produced by certain species of dinoflagellates, commonly found in shellfish and other seafood. Consumption of contaminated seafood can lead to symptoms like nausea, vomiting, and diarrhea (Valdiglesias et al., 2013). Okadaic acid has been shown to target serine/ threonine phosphates, which play a significant role in in the maintenance of essential cellular processes (Valdiglesias et al., 2013). Additionally, okadaic acid has been shown to inhibit IL-1 while upregulating IL-8, which is strongly associated with inflammation (Valdiglesias et al., 2013). Although the effects of okadaic acid on the immune system is poorly studied, it has been suggested that okadaic acid induces immunostimulation and inflammatory responses in response to okadaic-acid induced cytokine production (Valdiglesias et al., 2013). Additionally,

treatment of mast cells with okadaic acid has been shown to increase IL-6 production in a dose dependent mechanism through p38 MAPK pathway (Boudreau et al., 2004).

Aconitine is a highly toxic alkaloid found in several species of the Aconitum plant, commonly known as monkshood or wolfsbane. Ingestion or even skin contact with aconitine-containing plants can cause severe toxicity, with symptoms including gastrointestinal distress, cardiac arrhythmias, and neurological effects (Chan, 2009). Although there is limited information on mast cell interaction with aconitine, it has been shown that aconitine toxicity can be limited through hydrolysis. Mast cells have been shown to induce water secretion in response to activation, possibly as a way to reduce toxicity (Albert-Bayo et al., 2019). It has also been shown that aconitine can induce natural killer cell mediated immunity (Wang et al., 2023), which have been known to interact with mast cells during pathogenesis.

Ochratoxin A is a mycotoxin produced by several species of Aspergillus and Penicillium fungi. It can contaminate a wide range of food commodities, including cereals, coffee, and dried fruits. Chronic exposure to Ochratoxin A has been linked to kidney damage, immunosuppression, and increased risk of certain cancers (Ochratoxin A and Human Health Risk: A Review of the Evidence - PMC, n.d.). Although there is limited research on interactions between Ochratoxin A and the immune system, one study showed that within intestinal porcine cell line, Ochratoxin A induces the expression of Il6 but not Tnfa (Yoon & Lee, 2022).

Toxin	**Chemistry**	**Origin**	**Clinical effect**
Cholera Toxin (CT)	3 polypeptide chains: alpha, beta and gamma Comprised of 2 subunits:	*Vibrio cholera* gram-negative oxidase positive	Diarrhea, abdominal discomfort, and vomiting

	A subunit (CTxA) contains 2 domains; B subunit (CTxB) consists of 5 B subunit monomers in pentameric ring structure	rod	
Retrorsine (RT)	Bicyclic backbone containing a pyrrolizidine ring system and tertiary amine functional group	Natural hepatotoxic pyrrolizidine alkaloid (PA); main PA in S. Formosus leaves	Liver damage leading to symptoms such as jaundice, abdominal pain and hepatic failure
Deoxynivalenol (DN)	Tricyclic 12,13-epoxytrichothec-9-ene ring system, with a macrocyclic ring. Includes an epoxide group, a ketone group, and multiple hydroxyl groups	Mycotoxin produced by Fusarium fungi	Nausea, vomiting, abdominal pain, and diarrhea
Okadaic Acid (OA)	Polyketide-derived compound with multiple rings and functional groups; key features include oxygen-containing rings, ester groups and multiple hydroxyl groups.	Marine toxin produced by dinoflagellates	Nausea, vomiting, diarrhea and abdominal pain; chronic exposure can cause immunotoxicity, neurotoxicity and tumor promotion
Aconitine (AC)	Polycyclic diterpenoid framework with diterpenoid backbone, ester group, multiple hydroxyl groups, amino groups, 6-membered C ring and 5-membered D ring	Toxic alkaloid found in aconitum plant	Gastrointestinal distress, cardiac arrhythmias and neurological effects
Ochratoxin A (OT)	Fused dihydroisocoumarin ring system (cyclic structure)	Mycotoxin produced by aspergillus and	Kidney damage, immunosuppression, and increased risk of

	linked to a phenylalanine derivative by an amide bond; chlorine atom (Cl) is attached to the phenylalanine ring.	penicillium fungi	cancer

To determine the effects of these toxins on a genetic level, several inflammatory-associated genes will be studied. Tryptase Alpha/Beta 1, or Tpsab1, encodes for tryptase alpha/beta 1, a protein associated with gastrointestinal smooth muscle activity. During inflammation, tryptase induces the expression of Il1b, which may be important for the recruitment of inflammatory cells to the site of activation (Payne & Kam, 2004). Tnfa is typically released during mast cell activation in order to induce the production of the cytokine TNF-α (Saggini et al., 2011). Interleukin-6 (IL-6) is a pro-inflammatory cytokine involved in various immune reactions and host defense mechanisms. Mast cells are known to increase Il6 expression and therefore IL-6 production in response to activation (Conti et al., 2002). IL-1β (Interleukin 1 Beta) is another inflammatory cytokine released by mast cells in response to activation and has been shown to increase vascular permeability (Tanaka et al., 2014). IL-1β production is induced by the expression of Il1b gene (Tanaka et al., 2014). Early growth response factor-1 (Egr1) is a transcription factor involved in cellular responses to various stimuli, including inflammation and shown to induce IL-13 production which is an important cytokine during innate immune response (Li et al., 2008). Hdc encodes for Hdc (histidine decarboxylase) which is the enzyme responsible for catalyzing histidine into histamine (Fitzsimons et al., 2001). Histamine is produced by mast cells in response to allergens

(Thangam et al., 2018) and has been shown to induce several protective mechanisms such as mucus production and therefore pathogen immobilization (Urb & Sheppard, 2012). The objective of this study is to establish an in vitro mast cell activation model using foodborne toxins and identify the mast cell transcriptional programs induced by the toxin stimulation. The hypothesis of this study is that mast cells are able to directly sense and respond to toxins as a protective mechanism.

Gene	**Function/ Pathway**
Ltc4s	Key role in the process of inflammation as the rate limiting enzyme in the conversion of arachidonic acid to cysteinyl-leukotrienes
Il6	Primarily produced at sites of acute and chronic inflammation, where it is secreted into the serum and induces a transcriptional inflammatory response
Tnfa	An inflammatory cytokine produced by macrophages, monocytes, and mast cells during acute inflammation
Tph1	Tryptophan hydroxylase is an enzyme that converts tryptophan to 5-hydroxytryptophan, which is then decarboxylated to produce serotonin
Tpsab1	Beta tryptases appear to be the main isoenzymes expressed in mast cells, which can signal neurons
Alox5	Enzyme essential for synthesis of leukotriene D4, a proinflammatory mediator in mast cell granules
Ccl11	Chemokine, primarily associated with eosinophils, believed to be involved in inflammatory responses
Egr1	Transcription factor, most associated with differentiation, Regulates the expression of proteins such as IL- beta and CXCL2 that are involved in inflammatory processes and development of tissue damage after ischemia
Hdc	Catalyzing the decarboxylation of histidine to form histamine
Il1b	Important mediator of the inflammatory response, and is involved in a variety of cellular activities, including cell proliferation, differentiation, and apoptosis

CHAPTER 2

MATERIALS AND METHODS

Animals

BALB/c mice were bred in our animal facility and used at 4-8 weeks of age. BALB/c mice were chosen because I would anticipate that C57BL/6 mice would exhibit a weaker immune response to the toxins. This is because it is relatively easier to stimulate a Th2 response in BALB/c mice when compared to C57BL/6 mice.

BALB/c mice were maintained on a 12-hour light/ dark cycle with food and water provided ad libitum. Animal experiments were approved and conducted according to the IACUC guidelines of Arizona State University (Protocol #21-1864R). Mice were euthanized through carbon dioxide asphyxiation before bone marrow collection.

Preparation of bone marrow-derived mast cells (BMMCs)

Bone marrow cells were collected from the femurs and tibias of BALB/c mice. They were then cultured for 4-8 weeks in DMEM Medium supplemented with 10% FBS, 1% Pen/Strep, 1% L-Glutamine, 20mM HEPES, 1% pyruvate, 1% nonessential amino acids and 50µM BME. ACK lysis was used to remove red blood cells from the bone marrow. Additionally, mast cell growth factors SCF and IL-3 were supplemented to the media. Bone marrow cell culture was replenished every 3 or 4 days and maintained at 37°C and 5% CO2.

Flow cytometry was utilized to assess the maturity and purity of mast cells by quantifying the expression levels of c-kit and FcεR1, respectively. This was done by reconstituting the cells in FACs Buffer (PBS solution with 2% FBS). The resuspended

cells were treated with 0.5 mg/mL FC Block (BD Pharmingen), 0.2 mg/mL CD117 and 0.2 mg/mL FcR1 antibodies (both antibodies from Invitrogen) to assess the expression of the maturity. The cells were treated with PE-Cy7, the fluorochrome for c-kit, and APC, the fluorochrome for FcεR1. Analysis was conducted using the Attune Flow Cytometer and results analyzed using the FlowJo software. Cells were considered suitable for further experimentation if their expression levels exceeded 90%, indicating a high degree of maturity and purity. Cells meeting this criterion were selected for subsequent analysis and experimental procedures.

Viability

Flow cytometry was utilized to assess the maturity and purity of mast cells by quantifying the expression levels of zombie yellow, a marker of cell death. Mast cells were plated at 100,000 cells in 200 µL and treated with zombie yellow according to the Biolegend protocol.

Stimulation

Mast cells were plated at 4,000,000 cells/2 mL the night before toxin stimulation. After toxin application, bone marrow mast cells (BMMCs) were incubated at 37°C for 6 hours. Ionomycin, a known mast cell activator, was used as a positive control at a concentration of 3 µM. Negative control included media and vehicles of the toxins. The supernatants and cell pellets were separated, and cell pellets were given 350µL RNA Stat 60 and frozen at -80° C in preparation of RNA extraction for qPCR analysis. Supernatants were stored at -20° C in preparation of ELISA assay. RNA Stat 60 (Trizol): ABP Biosciences Cat. No. FP312

Cytokine assay

Supernatants of the stimulated cells was analyzed for TNF-α using an ELISA (enzyme linked immunosorbent assay) for each respective cytokine. The concentration of TNF-α in the supernatants was determined using commercially available ELISA kits according to the manufacturer's instructions (Invitrogen, Thermo Fisher Scientific). The absorbance of each well was measured using a microplate reader at the appropriate wavelength of 450nm, and the concentration of TNF-α and IL-6 in the samples was determined by comparing the absorbance values to a standard curve.

RNA Extraction

Cells treated with RNA Stat 60 were used for RNA extraction using the Qiagen® RNeasy Micro Kit (Cat. No. / ID: 217084). To generate cDNA, 1.5μg/μL total RNA was reverse transcribed using 5x1st strand buffer, 10 mM dNTPs RNAse-free, 100 mM DTT, and RT Smart MMLV

qPCR analysis

RNA from stimulated cells were converted to cDNA using commercially available cDNA kit. Primers were synthesized by Sigma-Aldrich. SYBR Green was used to detect the targeted sequences. Expression levels of *Rpl13a*, housekeeping gene, was also measured for comparison. There were no technical replicates per run.

CHAPTER 3

RESULTS

Mast cells are resistant to lethal doses of food toxins.

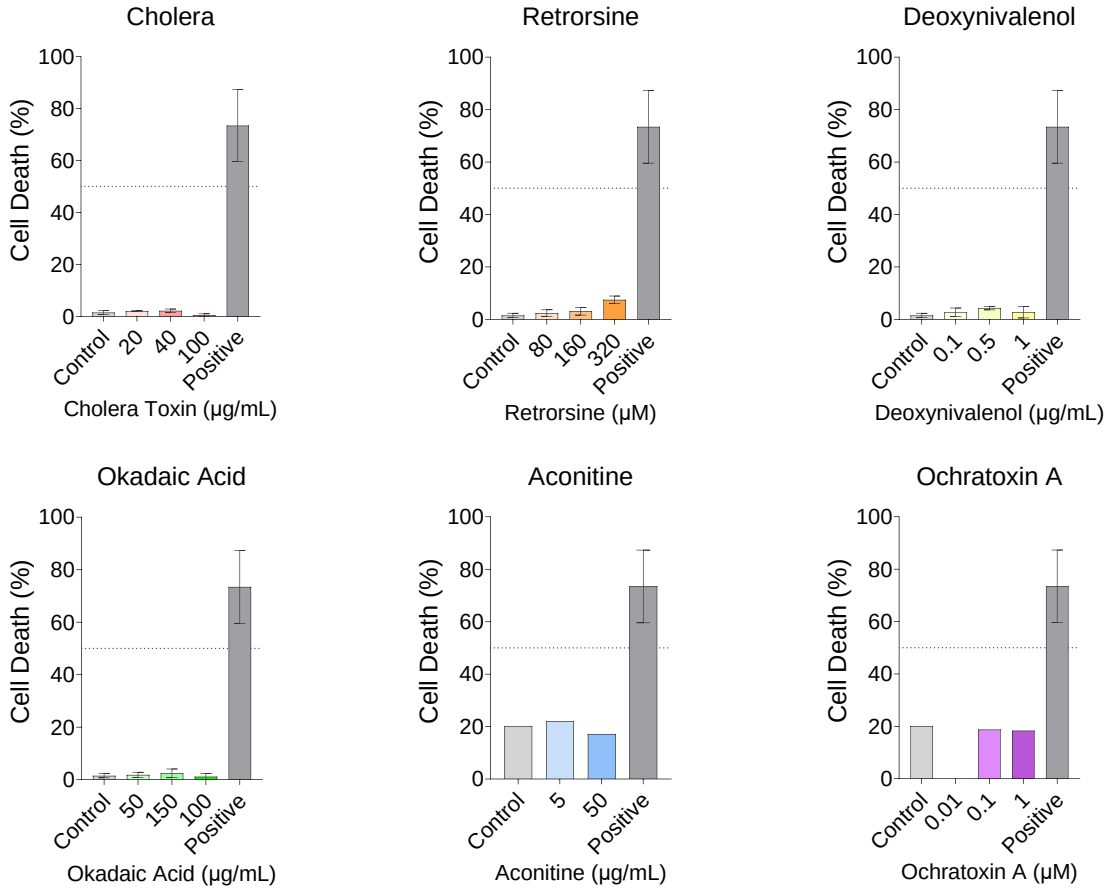

Figure 1 The graph shows the percentage of mast cell death after a 24-hour toxin stimulation. This was the combination of 2 independent experiments and an SEM was utilized to analyze the data (2 technical replicates, insufficient for STDEV). Positive control for cell death was induced by boiling mast cells at 100F for 5 minutes.

When determining the role of mast cells during toxification, I first had to ensure mast cells were able to survive potentially lethal doses of food toxins, therefore ensuring their ability to then sense and respond. To address this, I incubated mature mast cells for 24 hours in media at 3 doses for each of the 6 toxins. As a positive control for cell death,

mast cells were boiled for 5 minutes at 100°F while for negative controls mast cells were given media. The cells were then collected and prepared for flow cytometry analysis using the marker for cell death, Zombie Yellow. All of the toxins induced cell death comparable to the negative control (Figure 1). Results showed that mast cells were resistant to the lethal effects of all the toxins at all doses.

Mast cells do not release TNF-α upon toxin stimulation.

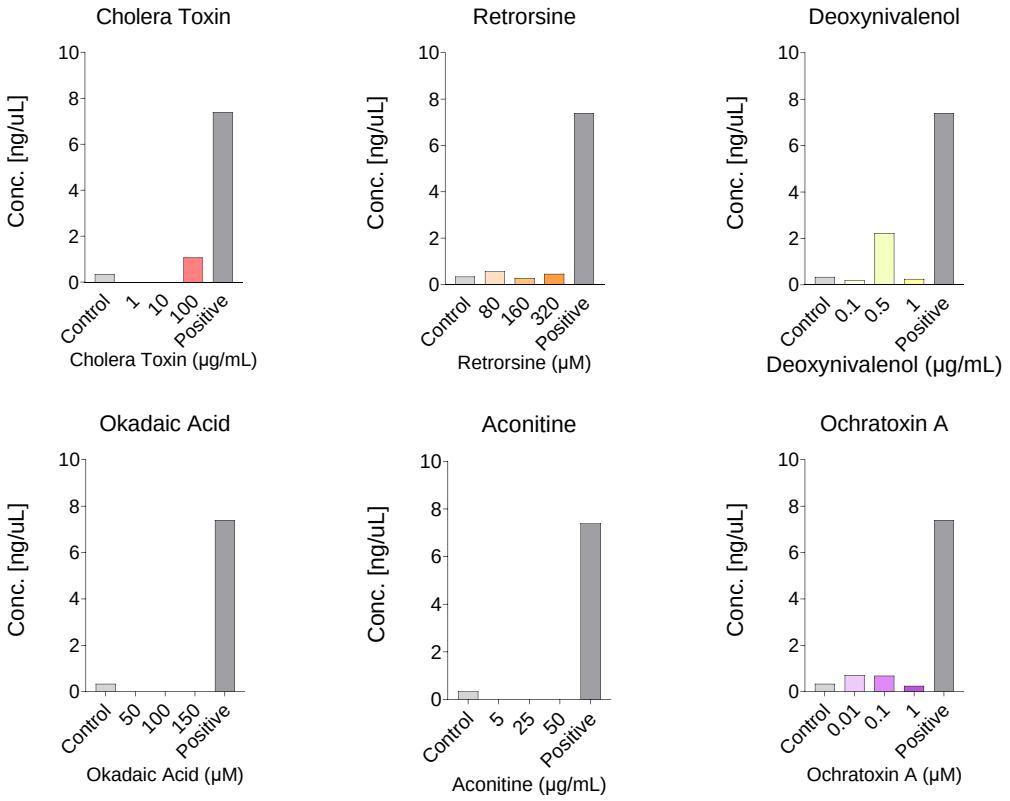

Figure 2: The graphs show the concentration of TNF-α produced (µg/mL) by mast cells as a result of a 6-hour toxin, media, and ionomycin stimulation (positive control). Toxins at 3 varying concentrations were tested. There were no replicates.

Once activated, mast cells will typically release the cytokine TNF-α in order to enhance the immune response. Therefore, I wanted to determine if mast cells release

TNF-α in response to direct stimulation with food toxins through an ELISA assay of the supernatant of the stimulated cells. Although cholera toxin and deoxynivalenol showed minimal levels of TNF-α release, none of the toxins were able to induce significant production compared to the controls (Fig. 2). This is indicative that mast cells do not produce TNF-α in response to food toxins.

Cholera toxin stimulation induces the expression of *Tnfa, Tpsab1, Alox5, Egr1, Hdc,* and *Il1b*

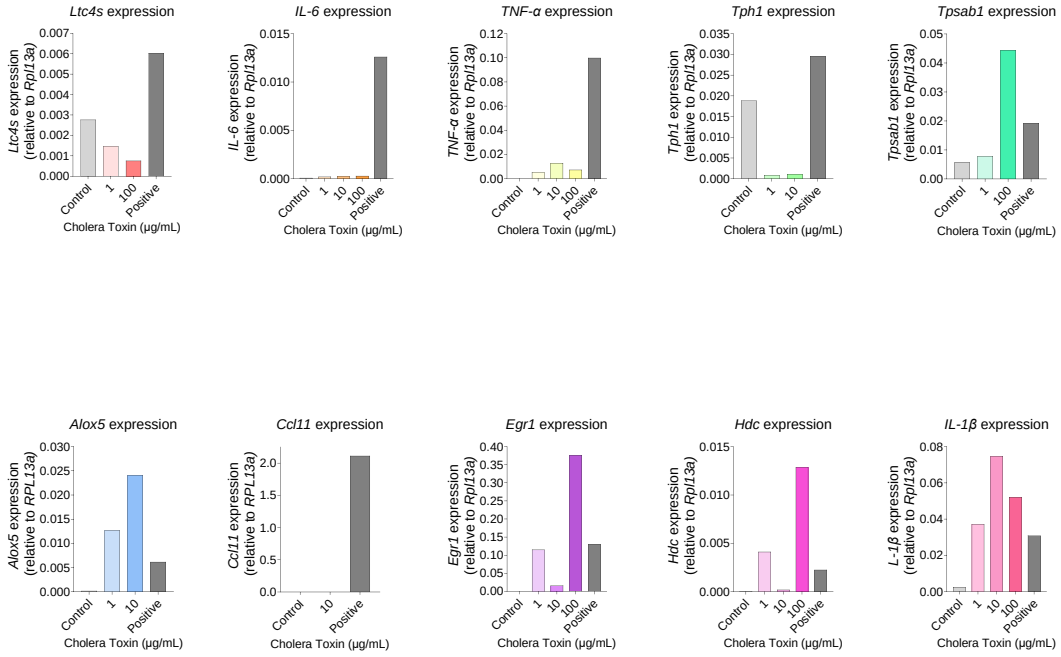

Figure 3 The graphs depict the relative expression of inflammatory genes (relative to the housekeeping gene Rpl13a gene expression). Mast cells were treated for 6 hours with either 1µg/mL, 10µg/mL, and 100µg/mL of cholera toxin. Ionomycin stimulation was used as a positive control while cells stimulated with media was used as a negative control.

To investigate the transcriptional effects of cholera toxin on mature mast cells, I stimulated $4x10^6$ MCs at doses of 1 µg/mL, 10 µg/mL, and 100 µg/mL. I then investigated the relative expression of several inflammatory associated genes typically associated with mast cell activation. *Ltc4s, Il6, Tph1,* and *Ccl11* expression in stimulated

mast cells at all concentrations was below the negative control, showing no upregulation of the expression of these genes in response to cholera toxin. *Tnfa* expression for all the doses was higher than the negative control but significantly lower than the positive control; additionally, 10 µg/mL showed the highest expression compared to the other doses. Although previous research linked *Tnfa* production to cholera toxin stimulation, this was established *in vivo* and therefor implies that there could be a systemic response that mast cells are not the sole contributor of. *Tpsab1* and *Alox5* gene expression showed signs of a dose-dependent increase in expression, however further testing at additional doses is necessary. Interestingly, there was a similar pattern of expression for genes *Egr1* and *Hdc* in which the middle dose, 10 µg/mL, showed the lowest expression but 100µg/mL showed significantly higher expression compared to the positive. The expression of *Il1b* was comparable to the positive for 1µg/mL, but 10 µg/mL showed the highest expression with 100 µg/mL being expressed more than the positive but less than 10 µg/mL. Altogether, these results indicate that mast cells induce expression of *Tpsab1, Alox5, Egr1, Hdc,* and *Il1b* upon direct stimulation with cholera toxin (Fig 3).

Stimulation of mast cells with retrorsine leads to an upregulation in the expression of *Tph1, Alox5, Hdc,* and *Il1b*

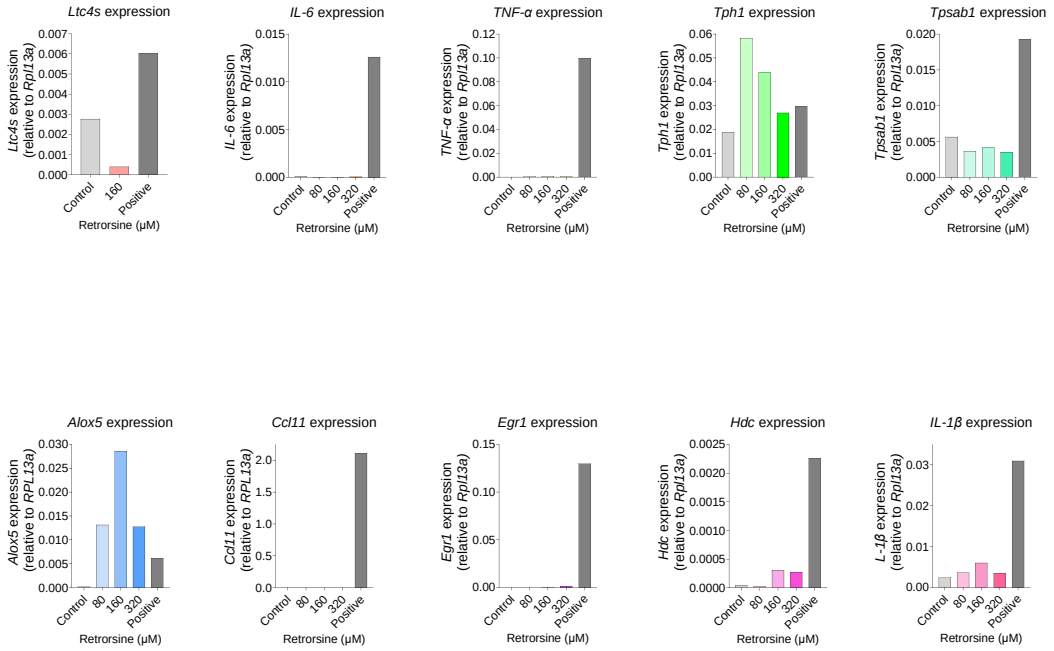

Figure 4 The graphs depict the relative expression of inflammatory genes (relative to the housekeeping gene Rpl13a gene expression). Mast cells were treated for 6 hours with either 80μM, 160μM, and 320μM of retrorsine. Positive control was the use of ionomycin stimulation while media was used as a negative control. There were no replicates.

Next, I investigated the effects of retrorsine on mast cells on a transcriptional level. The concentrations of the toxin included 80 μM, 160 μM, and 320 μM. Expression of inflammatory genes were investigated in order to determine sensing and response to the toxin retrorsine. *Ltc4s, Il6, Tnfa, Tpsab1, Ccl11* and *Egr1* showed either no expression or no expression above the negative control. For both genes *Alox5* and *Il1b*, the middle dose of 160 μM showed the highest expression while the lowest and highest dose (80 μM and 320 μM respectively). However, the expression of *Alox5* at all doses was shown to be significantly above the positive control while *Il1b* expression was above the negative but still below the positive control. *Tph1* expression indicated an inverse dose-dependent response to the concentrations with 80 μM having the highest level of

expression and 320 µM the lowest level. *Hdc* expression showed the same level of expression at 160 µM and 320 µM but no expression at the 80 µM (Fig 4).

Stimulation of mast cells with deoxynivalenol results in an increase in the expression of *Ltc4s, 1l1b, Il6, Tnfa, Tpsab1, Alox5, Hdc,* and *Egr1*

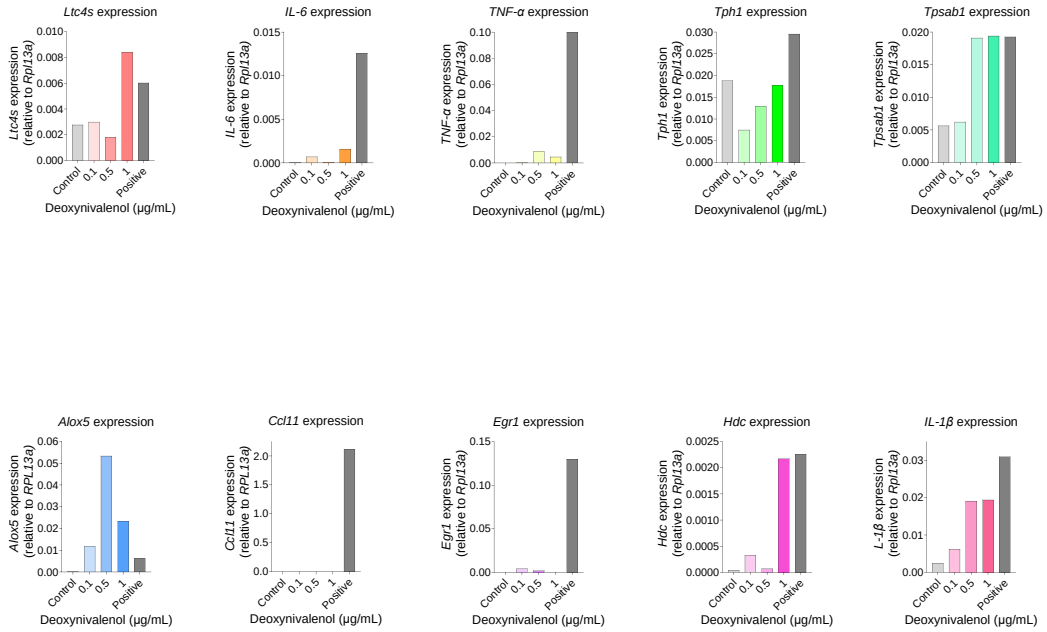

Figure 5 The graphs depict the relative expression of inflammatory genes (relative to the housekeeping gene Rpl13a gene expression). Mast cells were treated for 6 hours with either 0.1µg/mL, 0.5µg/mL, and 1.0µg/mL of deoxynivalenol toxin. Positive control was the use of ionomycin stimulation while media was used as a negative control. There were no replicates.

To determine the transcriptional effects of deoxynivalenol on mast cells, the relative expression of several inflammatory cytokines was measured. *Tph1*, *Ccl11*, and *Egr1* expression was not induced upon toxin stimulation. The highest dose, 1µg/mL, induced *Ltc4s* expression above the positive control, but the lower doses induced expression at or below the negative controls. *Il6* expression was minimally induced at 0.1µg/mL and 1µg/mL compared to the positive control. The middle dose, 0.5µg/mL, induced the highest expression in *Tnfa* and *Alox5*; however, all doses of deoxynivalenol

induced *Alox5* expression higher than the positive. *Il1b* and *Tpsab1* both saw similar levels of expression between the 0.5 µg/mL and 1 µg/mL doses. *Il1b* expression was above the negative control for all the doses but was not expressed more than the positive control. On the other hand, *Tpsab1* expression for 0.5 µg/mL and 1 µg/mL was equivalent to the positive controls while 0.1µg/mL was equivalent to the negative control. Another gene, *Hdc*, also induced expression at 1 µg/mL that was equivalent to the positive controls; however, 0.5 µg/mL induced expression equal to the negative control while 0.1 µg/mL was slightly upregulated (Figure 5).

Okadaic Acid stimulation induces *Il6, Tnfa, Tph1, Alox5, Egr1, Hdc* and *Il1b* expression.

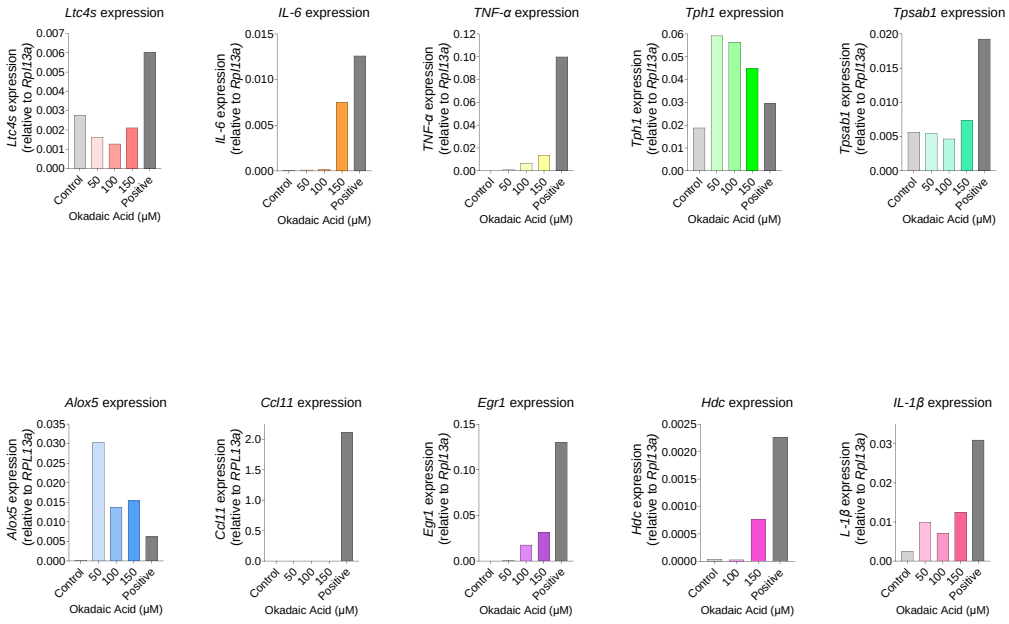

Figure 6 The graphs depict the relative expression of inflammatory genes (relative to the housekeeping gene Rpl13a gene expression). Mast cells were treated for 6 hours with either 50µM, 100µM, and 150µM of okadaic acid. Positive control was the use of ionomycin stimulation while media was used as a negative control. There were no replicates.

Next, the transcriptional effects of okadaic acid on mast cells were analyzed. *Ltc4s* and *Ccl11* did not induce expression at any of the doses, showing that mast cells do

not upregulate these genes in response to the toxin. Only the highest dose, 150 µM, induced expression of *Il6* and *Hdc* when mast cells are stimulated with okadaic acid. We could see a dose-dependent increase in the expression of *Tnfa* and *Egr1*, indicating a direct relationship between *Tnfa* and *Egr1* gene expression and okadaic acid concentration. Interestingly, *Tph1* expression indicated an inverse dose dependent effect in response to the toxin; additionally, all doses of okadaic acid induced expression at levels higher than the positive control. *Alox5* expression also indicated an inverse dose dependent effect in response to the toxin with all doses also above that of the positive control. Okadaic acid concentration of 150 µM was shown to be the only concentration that could induce *Tpsab1* expression, however the expression was minimal compared to the positive control. *Il1b* expression was at the highest at toxin concentration of 150 µM yet did not follow a dose dependent pattern with 100 µM inducing the lowest expression (Fig 6).

Stimulation of mast cells with the Aconitine results in the induction of *Il6, Tpsab1, Egr1,* and *Hdc* expression

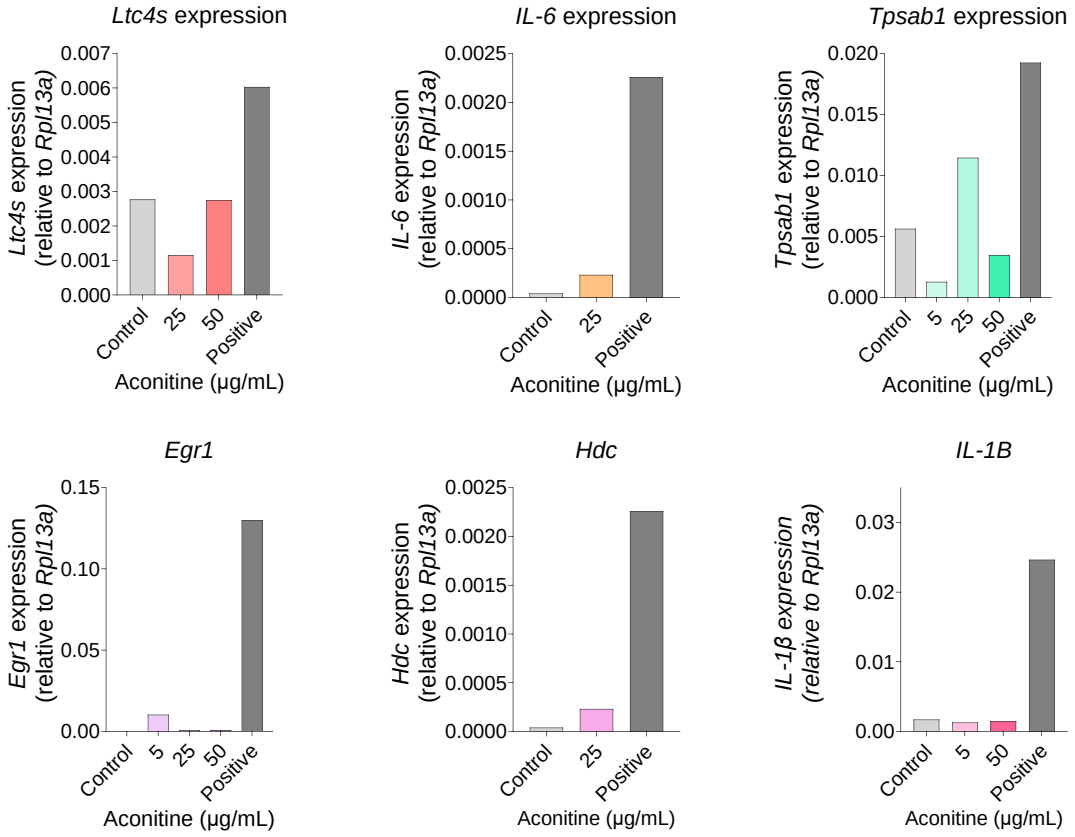

Figure 7 The graphs depict the relative expression of inflammatory genes (relative to the housekeeping gene Rpl13a gene expression). Mast cells were treated for 6 hours with either 5µg/mL, 25µg/mL, and 50µg/mL of aconitine. Positive control was the use of ionomycin stimulation while media was used as a negative control. There were no replicates.

To determine the effects of aconitine on mast cells, the levels of expression of several inflammatory genes were measured. *Ltc4s* and *Il1b* expression was not induced at any of the tested aconitine toxin concentrations. Interestingly, *Tpsab1* expression was only induced at a concentration of 25 µg/mL, but not at 5 µg/mL or 50 µg/mL. *Il6* and *Hdc* expression was minimal compared to the positive control for the toxin concentration

of 25 µg/mL. There was no *Egr1* expression for either the 25 µg/mL or 50 µg/mL concentrations of aconitine, but minimal expression at 5 µg/mL (Fig 7).

Ochratoxin A stimulation induces *Il6, Tpsab1, Egr1, Hdc and Il1b* expression

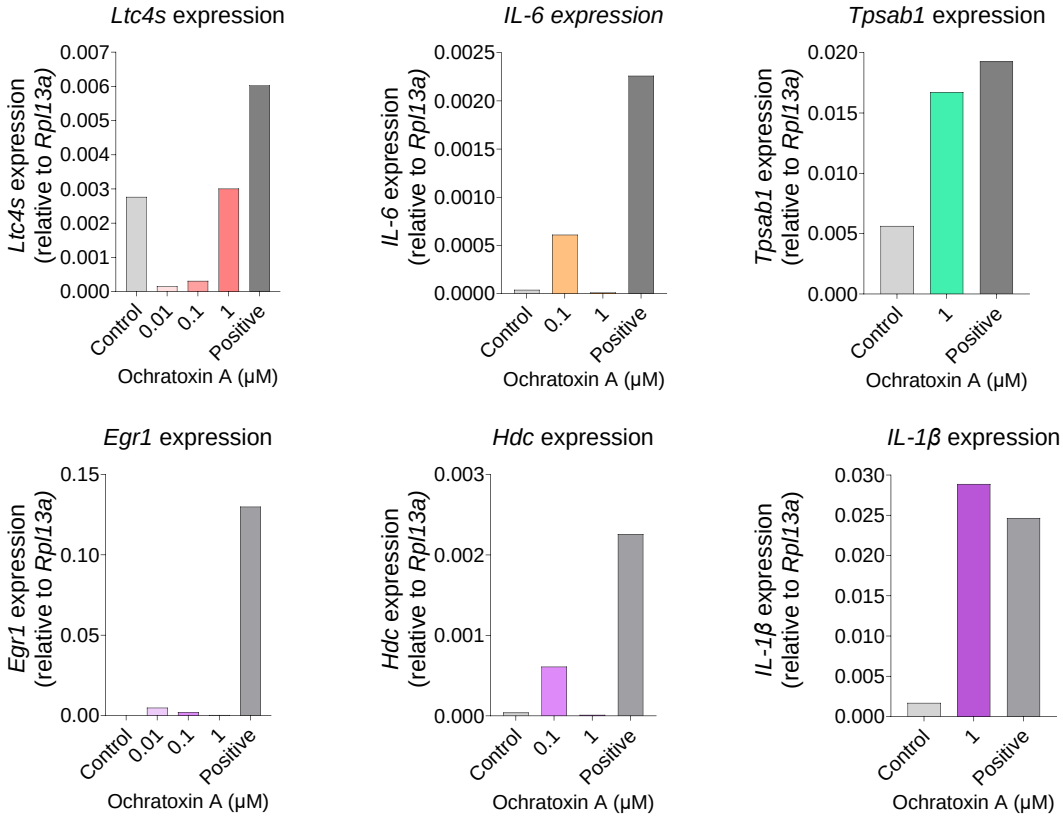

Figure 8 The graphs depict the relative expression of inflammatory genes (relative to the housekeeping gene Rpl13a gene expression). Mast cells were treated for 6 hours with either 0.01µM, 0.1µM, and 1µM of Ochratoxin A. Positive control was the use of ionomycin stimulation while media was used as a negative control. There were no replicates.

Ochratoxin A stimulation transcriptional effects on mast cells was investigated through the analysis of various inflammatory gene expressions. *Ltc4s* expression was below that of the negative control, showing that ochratoxin did not induce the expression of this gene. Ochratoxin A at a concentration of 0.1 µM induced the highest gene expression of the doses for genes *Il6* and *Hdc*; intriguingly, the highest concentration of 1

µM did not induce any expression for either of the genes as well. *Egr1* expression was minimally upregulated in an inverse dose dependent manner in mast cells stimulated with Ochratoxin A. Both *Tpsab1* and *Il1b* expression was induced at a toxin concentration of 1 µM, but *Il1b* expression was higher than that of the positive control (Fig 8).

Fold change of genes at different doses of toxins

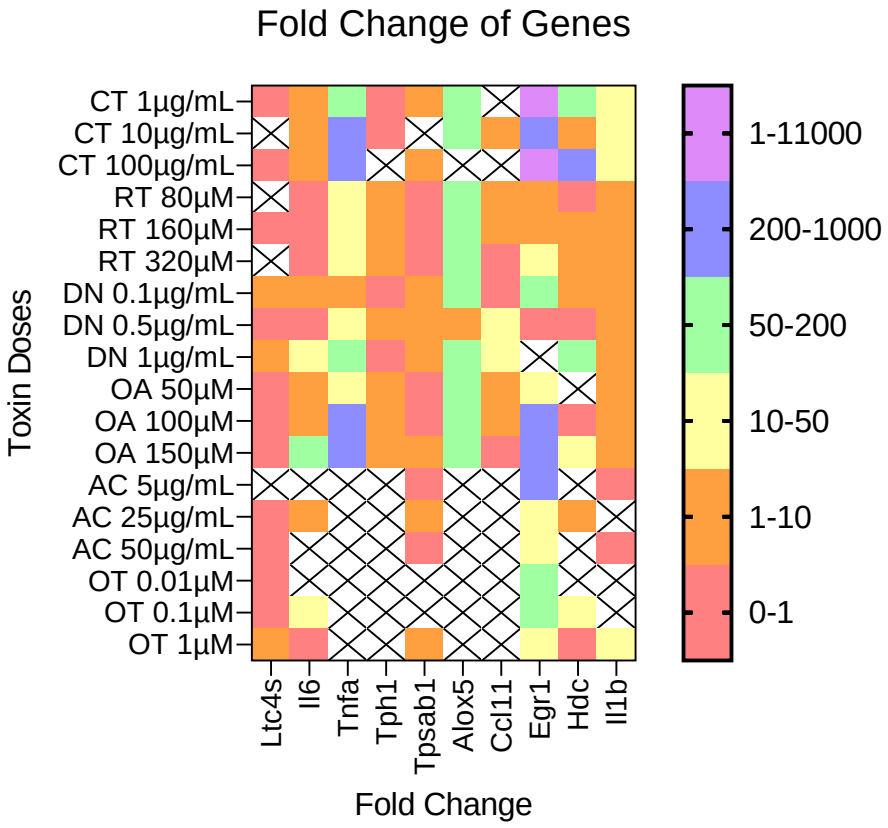

Figure 9. Fold change of genes at different doses of toxins. The graph compiles the data from the previously shown experiments. Fold change was calculated using the relative expression of the gene at a toxin dose/ relative expression of the gene at the negative control. Blocks with an "x" through it were not calculated due to insufficient data.

The graph above shows a heat map of the fold change of the different genes at different toxin doses. As we can see, a majority of the toxins induced a 1-10-fold change. This shows that the toxins induced activation in mast cells 1-10-fold change above the normal levels.

CHAPTER 4

DISCUSSION

The objective of this study was to investigate the role of mast cells in sensing dietary toxins and understand their potential contribution to toxin defenses. To achieve this, I established an in vitro model using mast cells generated from murine bone marrow precursors and cultured them in mast cell-specific media for 5 weeks. Surprisingly, none of the tested toxins induced significant cell death in mast cells, even after 24 hours of exposure. This suggests that mast cells are resistant to the toxic effects of these compounds, which allows them to be able to sense and respond to these toxins even at otherwise lethal concentrations. These findings imply that mast cells might have unrecognized physiological functions beyond their involvement in allergic symptoms. Furthermore, we examined the activation of mast cells in response to various dietary toxins. This was done by measuring the concentration of the inflammatory cytokine TNF-α in the supernatant of mast cells stimulated with toxins for 6 hours. While the toxins did not induce significant TNF-α production, a marker of inflammation, we observed the upregulation of specific activation-related transcripts in mast cells exposed to different toxins.

For instance, cholera toxin induced the expression of *Il1b, Tpsab1, Alox5, Egr1, Tnfa,* and *Hdc*. Additionally, *Ltc4s, Il6, Tph1,* and *Ccl11* expression was not induced. The activation of the above-mentioned genes indicates that mast cells are able to directly sense and upregulate these genes in response to the toxin. Interestingly, all of the induced genes saw an expression level above the positive control for at least one of the tested

doses, with the exception of *Tnfa;* however, *Tnfa* expression level confirm the results of the ELISA, showing that mast cells do not significantly increase the production of TNF-α in response to cholera toxin. Additionally, *Il6* expression was not induced. Both TNF-α and IL-6 are cytokines mast cells are known to release during activation, therefore the lack of expression for both suggests that mast cells do not release these cytokines in response to cholera toxin. Expression above the positive control indicates a higher level of activation. This shows that cholera toxin induces the expression of several inflammatory genes that are typically associated with mast cell's protective role in the immune system. Another study confirmed mast cell's ability to respond to cholera toxin (20 µg/mL) by showing that mast cells induce the expression *Il4* upon toxin stimulation (Feng et al., 2008). In the study, mast cell derived IL-4 drove naïve CD4 T cells to become antigen specific Th2 cells. Another study found that mast cells induce histamine production in a dose- dependent manner in response to cholera toxin (concentration ranges from 0.1 µg/mL to 1000 µg/mL) (Saito, n.d.). Additionally, another study focused on the effects of cholera toxin on allergic sensitization to whey protein in mice. The study showed that mast cell protease-1 levels, a measure for mucosal mast cell degranulation, was elevated after oral allergen stimulation in cells treated with both cholera toxin and oral allergen; showing that cholera toxin increases mast cell presence in conjunction with oral allergens (Bol-Schoenmakers et al., 2016). Interestingly, resident peritoneal macrophages induced the expression of *Il1b* in response to cholera toxin (Orimo et al., 2019). Despite the different cell types, this indicates that peritoneal immune cells induce *Il1b* expression in response to cholera toxin, supporting my results seen in Figure 3.

Overall, it can be concluded that mast cells directly sense and respond to cholera toxin through the upregulation of several inflammatory- associated genes.

Retrorsine stimulation led to the upregulation of *Tph1, Alox5, Il1b,* and *Hdc*; however, there was upregulation of *Ltcs4, Il6, Tnfa, Tpsab1, Ccl1* and *Egr1*. Interestingly, retrorsine stimulated the expression of *Tph1* and *Alox5* at levels above the positive control, showing an activation level above the ionomycin control. *Hdc* and *Il1b* showed minimal expression compared to the negative control. Interestingly, none of the genes induced a dose- dependent expression, however further testing at additional doses would be required to confirm this. Together, the results demonstrate that mast cells respond to retrorsine by inducing the expression of several inflammatory genes, but significantly induce *Tph1* and *Alox5* expression. Similar to what we saw with cholera toxin, the lack of expression of *Il6* and *Tnfa* also indicates that mast cells do not respond retrorsine by releasing cytokines TNF-α and IL-6. Although there is no published information about how mast cells respond to retrorsine (that I could find), one study suggested that retrorsine in conjunction with gut dysbiosis (imbalance in the gut microbial community) leads to intestinal inflammation and compromises the integrity of the gut barrier (Xiao et al., 2022). This study could confirm the transcriptional analysis described above due to the upregulation of several inflammatory genes, suggesting mast cells may play a role in the inflammatory response to retrorsine. Overall, the data suggest that mast cells do not induce TNF-α or IL-6 cytokine release but rather express certain inflammatory genes in response to retrorsine stimulation.

Deoxynivalenol induced *Ltc4, Il6, Tpsab1, Tnfa, Hdc, Il1b* and *Alox5* expression in mast cells stimulated with the toxin for 6 hours. At the highest dose of 1µg/mL, deoxynivalenol stimulation resulted in *Ltc4s* expression higher the positive control, indicating mast cells exhibit heightened activation with deoxynivalenol stimulation compared to ionomycin activation. Both *Tnfa* and *Il6* showed very low expression levels, signifying that mast cells induce cytokine production upon deoxynivalenol stimulation; however, since the expression levels were extremely low, further testing would be required. The TNF-α ELISA and *Tnfa* expression together suggest that mast cells do not release TNF-α in response to deoxynivalenol. *Alox5* expression was not induced in a dose-dependent manner but was highly expressed at levels above the positive control. There was no *Ccl1* or *Egr1* expression in response to the toxin. Interestingly, *Tpsab1* and *Hdc* induced expression in mast cells at levels comparable to positive control, indicating a high level of activation. The expression *Il1b* also indicated mast cell activation, however, the levels were below the positive. One paper has shown that deoxynivalenol can facilitate allergic sensitization to food proteins (Bol-Schoenmakers et al., 2016); however, this paper did not identify mast cell protease-1 (MCPT-1) has the effector of this sensitization. Additionally, another paper suggested that phosphorylation of proteins involved in lymphocyte activation and development was significantly altered, suggesting that mast cells were impacted by deoxynivalenol exposure (Pan). Overall, deoxynivalenol induced expression of several inflammatory genes in mast cells, indicating that mast cells can sense and respond to deoxynivalenol.

Mast cells stimulated with okadaic acid induced *Il6, Tnfa, Tph1, Alox5, Egr1, Il1b,* and *Hdc* expression. In contrast to the other toxins, the expression of inflammatory genes seems to be dose dependent which 150 µM showing the highest expression for *Il6, Tnfa, Egr1,* and *Hdc.* In a study focusing on *Pseudomonas aeruginosa* infection, an infection characterized by airway inflammation and significant cytokine production, okadaic acid was shown to potentiate *P. aeruginosa* induced IL-6 production (Boudreau et al., 2004). This study and the *Il6* expression levels suggest okadaic acid facilitates the production of the cytokine IL-6, however primarily at high concentrations. *Il1b* showed relatively consistent expression levels despite the different toxin concentrations, indicating that there might be a concentration-based threshold of activation below 50 µM. It is also interesting to note that both *Tph1* and *Alox5* expression was higher than the positive control, indicating a significant upregulation of the enzymatic functions of these genes. Although as mentioned there was a possible dose- dependent increase in expression of *Tnfa* in response to okadaic acid, the ELISA results showed no TNF-α in the supernatant. This could indicate that mast cells are only beginning to produce and release TNF-α, which could be determined through a stimulation with additional timepoints. At the highest dose of the toxin, *Hdc* expression exhibited the highest level of expression, indicating an increase in the catalysis of histamine. A study on the involvement of okadaic acid on histamine release indicated that treatment with the toxin resulted in the enhancement of IgE-mediated histamine release in rat peritoneal mast cells (during suboptimal challenge) (Kitani et al., 2009). Despite the difference in cell type, this study supports the results indicating that *Hdc* expression is induced during mast cell

activation through toxin stimulation, likely through a similar enhancing effect. Okadaic acid was shown to induce expression of several inflammatory genes in mast cells through direct stimulation.

Mast cells induced *Il6, Tpsab1, Egr1,* and *Hdc* expression in response to aconitine toxin. Interestingly, none of the genes tested induced expression above the positive control or in a dose- dependent manner nor did aconitine induce TNF-α cytokine release. *Il6* and *Hdc* both showed similar expression levels for stimulation with 25 µg/mL concentration, however further testing at additional doses would be required to determine whether or not there is a dose dependent relationship. *Tpsab1* only induced expression also only at a concentration of 25 µg/mL but suggest that mast cells can respond to aconitine stimulation through the expression of *Tph1*. *Egr1* only induced expression at 5 µg/mL but not at the other doses, also showing the ability of mast cells to respond to aconitine through the expression of *Egr1*. However, if mast cells are able to respond to aconitine through the upregulation of these genes, we would also expect expression at higher concentrations of toxins to also induce at least similar expression levels. Aconitine is a known toxin plant that has been used as an anti-inflammatory, anti- cancer and anti-viral treatment in traditional medicine (Gao, n.d.). *Aconitum soongoricum Stapf.,* Despite minimal information about the relationship between dose and toxicity, hydrolysis is known to reduce the toxicity of aconitine. Mediators released by mast cells in the intestinal mucosa has been shown to affect the epithelial integrity and viability as well as promote ion and water section in response to activation (Albert-Bayo et al., 2019). However, this could also be a protective mechanism that mast cells induce in order to

minimize the toxic effects of aconitine interactions. In conclusion, the results imply that mast cells directly sense and respond to aconitine by inducing the expression of inflammatory genes.

Additionally, Ochratoxin A induced the expression of *Il1b, Il6, Tpsab1, Egr1*, and *Hdc*. Expression of *Il6, Egr1* and *Hdc* were minimal in response to Ochratoxin A and further testing would be required. Lastly, mast cells did not release TNF-α or induce the expression of *Tnfa,* indicating mast cells do not respond to Ochratoxin A by producing and releasing TNF-α. Although more data at additional doses is needed, it is important to note that *Tpsab1* and *Il1b* both induced high levels of expression in mast cells, with levels either almost equal to or greater than the ionomycin control. On a study researching the effects of Ochratoxin A on IPEC-J2, intestinal porcine epithelial cell line, ochratoxin was shown to activate the expression of *Il6* but not *Tnfa* (Yoon & Lee, 2022). Although there are several different variations, such as cell origin, this study can offer a certain level of support for the observed expression of *Il6* and *Tnfa* when cells are stimulated with ochratoxin. In summary, while further testing and additional data are needed, the findings from this study, along with the evidence from the research conducted on the intestinal porcine epithelial cell line, collectively provide a degree of support for the conclusion that mast cells can sense and respond to Ochratoxin A through the activation of various inflammatory-associated genes.

When referring to Figure 9, we can see that there is substantial fold change in the gene expression in mast cells during toxin stimulation. Interestingly, *Egr1* is the only gene with a fold change greater than 1,000 during cholera toxin stimulation; indicating

that this was the gene that was the most change compared to the negative control. Additionally, *Ltc4s, Tnfa, Ccl11* and *Tpsab1* did not induce fold change greater than 10 in any of the toxin doses.

These results provide evidence that mast cells directly sense and respond to food toxins, shedding light on a previously unknown aspect of mast cell functionality. When looking at the genes commonly expressed among all toxin stimulations, *Hdc* is the only gene that was induced across all toxin stimulations. *Hdc* is the gene associated with catalyzing histidine into histamine (Fitzsimons et al., 2001), indicating that mast cells will induce histamine production in response to toxin stimulation, regardless of toxin origin (ex. Fungal vs bacterial). It has been established that mast cells produce histamine in order to induce inflammation during allergic reactions (Thangam et al., 2018). Previous research has shown that histamine plays a role in activating endothelial cells, leading to vasodilation, and increased vascular permeability. Histamine also activates smooth muscle cells, resulting in bronchoconstriction and various mechanisms that aid in expulsion, such as coughing, sneezing, vomiting, and diarrhea (Urb & Sheppard, 2012). Histamine has also been shown to enhance mucus production of epithelial cells, therefore aiding in pathogen immobilization and cytoprotection, as well as recruit additional inflammatory cells such as eosinophils and natural killer cells (Urb & Sheppard, 2012). My data shows mast cells induce *Hdc* expression, suggesting that food toxins might also trigger these protective mechanisms via histamine.

Genes *Il6, Tpsab1, Alox5, Egr1* and *Il1b* were all induced by four out of the six tested toxins. *Il6* encodes for the cytokine IL-6 which has been shown to contribute to

host defense through hematopoiesis and immune reactions (Tanaka et al., 2014). IL-6 functions as a warning signal in the event of tissue damage through damage-associated molecular patterns (DAMPs), which are released from damaged or dying cells, resulting in inflammation (Tanaka et al., 2014). My data indicates that the increase in *Il6* expression, specifically in mast cells treated with deoxynivalenol, okadaic acid, Ochratoxin A and aconitine, might also trigger DAMPs in order to induce further inflammation via the production of IL-6. It would be interesting to further explore the mechanism of IL-6 during toxification and determining if and what the specific protective mechanism is. Tryptase Alpha/Beta 1, protein associated with gastrointestinal smooth muscle activity, is encoded by the gene *Tpsab1*. During inflammation, tryptase induces the expression of *Il1b*, which may be important for the recruitment of inflammatory cells to the site of activation (Payne & Kam, 2004). However, only cholera toxin and Ochratoxin A both induced *Tpsab1* and *Il1b* expression. This indicates that there may be a different role of *Tpsab1* expression, such as functioning as an amplification signal similar to histamine (Payne & Kam, 2004), but further testing is required. The cytokine Il1β is a pro-inflammatory cytokine, encoded by *Il1b* (Lopez-Castejon & Brough, 2011). IL-1 is known to increase pro-inflammatory actions, thereby increasing vascular permeability at the site of section, thus additionally increasing leukocyte migration to the site (Solimando et al., 2022). Mast cell derived IL-1β is typically associated with skin inflammation and arthritis (Solimando et al., 2022). Together, this information indicates that mast cell derived IL-1β is primarily involved in inflammation. Although further research is required, inflammation can be a protective mechanism and the expression of

Il1b could be a part of this mechanism in response to toxification. *Egr1*, the gene coding for early growth response factor-1, was shown to be expressed in four out of the six toxins in my data. One study found that Egr1 is required for SCF-induced IL-13 expression, describing the likely role of Egr1 during IL-13-mediated allergic inflammation (Li et al., 2008). Additionally, Egr1 deficient mice showed decreased levels of SCF- induced cytokine production, IL-13, indicating Egr1 is necessary for cytokine production within mast cells (Li et al., 2008). IL-13 has been shown to be an important cytokine in novel innate immune response, as it is released from damaged or inflamed gut epithelium (Mannon & Reinisch, 2012). Therefore, *Egr1* expression in response to food toxins can be an innate immune response within gut epithelium through IL-33 expression. Mucosal mast cells have been determined to contain both Alox5 and the activating protein FLAP, allowing for the synthesis of leukotrienes (Widmayer, 2022). Leukotrienes have been shown to increase neutrophil migration, degranulation as well as capillary permeability and smooth muscle contractions (Rask-Madsen, 2001). They are also the major mediators of inflammation (Sun et al., 2019).Therefore, it can be suggested that expression of *Alox5* induces Alox5 and therefore leukotriene production, during toxin stimulation in order to induce the potentially protective mechanisms of leukotrienes. Although my data did not show *Ltc4s* expression, this could be because Alox 5, the enzyme coded by *Alox5*, induces the synthesis of other leukotrienes, such as leukotriene B4 which is linked to chronic inflammatory bowel disease (Dreyling et al., 1987).

The direct sensing of food toxins by mast cells has significant implications. By uncovering this ability, our findings open new avenues for understanding the interactions

between mast cells and toxins in the context of toxin-induced and inflammatory diseases. Given that toxin-induced diarrheal diseases are among the leading causes of mortality in children worldwide, elucidating the role of mast cells in toxin defenses becomes crucial in building our understanding of these diseases and possible therapeutic solutions. The resistance of mast cells to the toxic effects of tested compounds suggests a potential protective mechanism that may limit the detrimental impact of toxins.

Although my results suggest mast cells directly sense and respond to food toxins, further investigation is warranted to elucidate the exact mechanisms by which mast cells contribute to toxin defenses and their impact on both toxin-induced and inflammatory diseases. This could be done using mast cell knockout mice and stimulating with the toxins to determine the exact role of mast cells in detoxification. Additionally, due to the small sample size, replicates of this research should be done to confirm results found. Future studies should explore the signaling pathways and molecular mechanisms involved in mast cell toxin sensing and response. Understanding these mechanisms may unveil potential therapeutic targets for toxin-related conditions and provide insights into the development of interventions that could mitigate the adverse effects of dietary toxins. Investigating the role of thrombotic, thrombolytic or complement proteases, if any, would also be interesting to further determine the full immune response to toxins. Because histamine has a variety of functions, further examining the specific receptors impacted by toxin-induced histamine release would give further insight into the role of histamine and mast cells during toxification. Another potential investigation could include examining the effects of mast cells stimulated with the supernatant of epithelial cells treated with

varying toxins, giving insight into how mast cells might respond to stimulated epithelial cells. Finally, future studies should investigate if mast cells can sense and respond to other toxins such as aflatoxins and ciguatoxins.

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