12-LIPOXYGENASE PROMOTES MACROPHAGE INFILTRATION AND PANCREATIC ISLET DYSFUNCTION IN THE VERTEBRATE MODELS OF DIABETES PATHOGENESIS

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DEDICATION

To my parents, I am eternally indebted to you for all your sacrifices, support and encouragement as I pursue my dreams.

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As famously said by John F. Kennedy. "*We must find time to stop and thank the people who make a difference in our lives*". I would like to take this opportunity to thank everyone for being there for me on this journey. This dissertation would not have been possible without the guidance and the help of several individuals who contributed and extended their valuable assistance in the preparation and completion of this study.

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Abhishek Anant Kulkarni

12-LIPOXYGENASE PROMOTES MACROPHAGE INFILTRATION AND PANCREATIC ISLET DYSFUNCTION IN THE VERTEBRATE MODELS OF DIABETES PATHOGENESIS

Diabetes is a morbid metabolic disorder that affects almost 500 million people worldwide. Although multiple factors contribute to diabetes pathogenesis, pancreatic islet inflammation and dysfunction are shared characteristics of its major forms. 12-lipoxygenase (12-LOX), an enzyme involved in lipid metabolism, has been implicated in islet inflammation. 12-LOX generates reactive oxygen species (ROS) that activate inflammation and serve as major contributors to islet dysfunction. Importantly, since ROS are transient moieties, they are challenging to study *in vivo*. Hence, establishing better animal models of ROS-mediated stress is critical to facilitate the discovery and preclinical testing of novel diabetes therapeutics. Here, I have adapted a zebrafish model of conditional β-cell injury, which is regulated by the administration of the prodrug metronidazole (MTZ), to study responses to ROS *in vivo*. I demonstrate that with MTZ treatment, ROS are generated within β-cells and subsequently exhibit recruitment of macrophages into the islet and induction of β-cell death. I utilized this model to uncover roles for macrophages and 12-LOX during islet injury. Excessive macrophage infiltration exacerbates islet inflammation and dysfunction. Interestingly, on the depletion of macrophages in zebrafish, I observed that β-cells recovered normal function upon cessation of prodrug treatment. This suggests that infiltrating macrophages promote maladaptive inflammation and premature removal of damaged β-cells. Thus, limiting the macrophage infiltration may be a therapeutic approach to restoring β-cell function. Based on the established roles of 12-

LOX in other contexts, I hypothesized that its inhibition would prevent the localized infiltration of proinflammatory macrophages. To test this, I used both zebrafish and mouse models and observed a significant reduction in macrophage migration upon loss of 12-LOX activity. Furthermore, I found that expression of CXCR3, a crucial receptor regulating migration, was significantly reduced in 12-LOX loss-of-function macrophages. These data suggest a role for 12-LOX in macrophages, which is conserved across species. Collectively, my study reveals novel roles for 12-LOX in macrophage function and provides testable therapeutic targets for the resolution of inflammation-induced damage in the pancreatic islets.

Maureen Harrington, Ph.D., Chair

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1.1 Pancreatic islet structure and function:

Metabolism is a process of conversion of the complex metabolites obtained from the food, like carbohydrates, proteins, and fats, to simpler molecules like sugars, amino acids, and fatty acids, respectively (Beauvoit et al., 2018). Different organs are involved in this intense metabolic process that ultimately leads to energy production, which is necessary to run all the cellular processes. One such organ is the pancreas, which has a variety of crucial roles, including regulation of macronutrient digestion and hence, energy homeostasis by releasing various digestive enzymes and pancreatic hormones. The pancreas is located behind the stomach within the left upper abdominal cavity and has three parts: the head, body, and tail. Anatomically, the pancreas can be divided into two compartments: exocrine and endocrine (Röder et al., 2016).

The exocrine compartment encompasses about 95-98% of the total pancreatic mass. It is composed of cells that have a primary role in facilitating the digestion of food. The two major cell types of the exocrine pancreas are the acinar cells and the ductal cells. The acinar cells produce pancreatic fluid that contains a variety of enzymes, including trypsin and chymotrypsin, for the breakdown of proteins, amylase for carbohydrates, and lipase for fats. This pancreatic fluid gets ultimately released via the pancreatic ductal system into the duodenum of the small intestine where the digestion of the food occurs. The ductal cells produce a bicarbonate-rich secretion that helps in maintaining the pH of the duodenum (Motta et al., 1997; Pandiri, 2014). The endocrine compartment accounts only for about 2-5% of the total pancreatic mass. The primary function of this compartment is regulating glucose homeostasis. The endocrine cells are clustered together to form small island-like

structures within the pancreas called the islets of Langerhans. These highly vascularized islets contain five different cell types that release specific hormones (Figure 1). The more abundant cell types are the α-cells and β-cells. In humans, the α-cells constitute about 15-20% of total islet cells and produce the hormone called glucagon, while the β-cells constitute about 65-80% of total islet cells and produce the hormone called insulin. These β-cells also co-secrete amylin with insulin. The less abundant cell types of the islets include the γ- cells and δ-cells. Both these cell types constitute about 3-10% of total islet cells and produce the hormones pancreatic polypeptides and somatostatin, respectively. Finally, the least abundant islet cell type is the ε-cells that constitute approximately 1% of the total islet cells and produce the hormone ghrelin. Each of these hormones has a different function (Figure 1). Glucagon functions in upregulating blood glucose levels while insulin downregulates blood glucose levels. Furthermore, glucagon inhibits the secretion of insulin. Somatostatin inhibits the secretion of insulin as well as glucagon. Pancreatic polypeptide inhibits exocrine pancreatic secretions while ghrelin stimulates appetite and promotes fat storage. In summary, these hormones help in maintaining blood glucose levels in response to nutrient stimulus (Batterham et al., 2003; Brereton et al., 2015; Da Silva Xavier, 2018; Göke, 2008; Hauge-Evans et al., 2009; Hellman and Grapengiesser, 2014; Katsuura et al., 2002; Steiner et al., 2010; Wierup et al., 2002).

Inadequate synthesis or secretion of pancreatic hormones can lead to various metabolic syndromes. The focus of this thesis is, in part, the function of the β-cells, the cell-type that releases insulin to reduce blood glucose levels. Dysfunction or death of β-cells leads to a metabolic disorder called as diabetes that is characterized by chronic hyperglycemia (Kharroubi and Darwish, 2015).

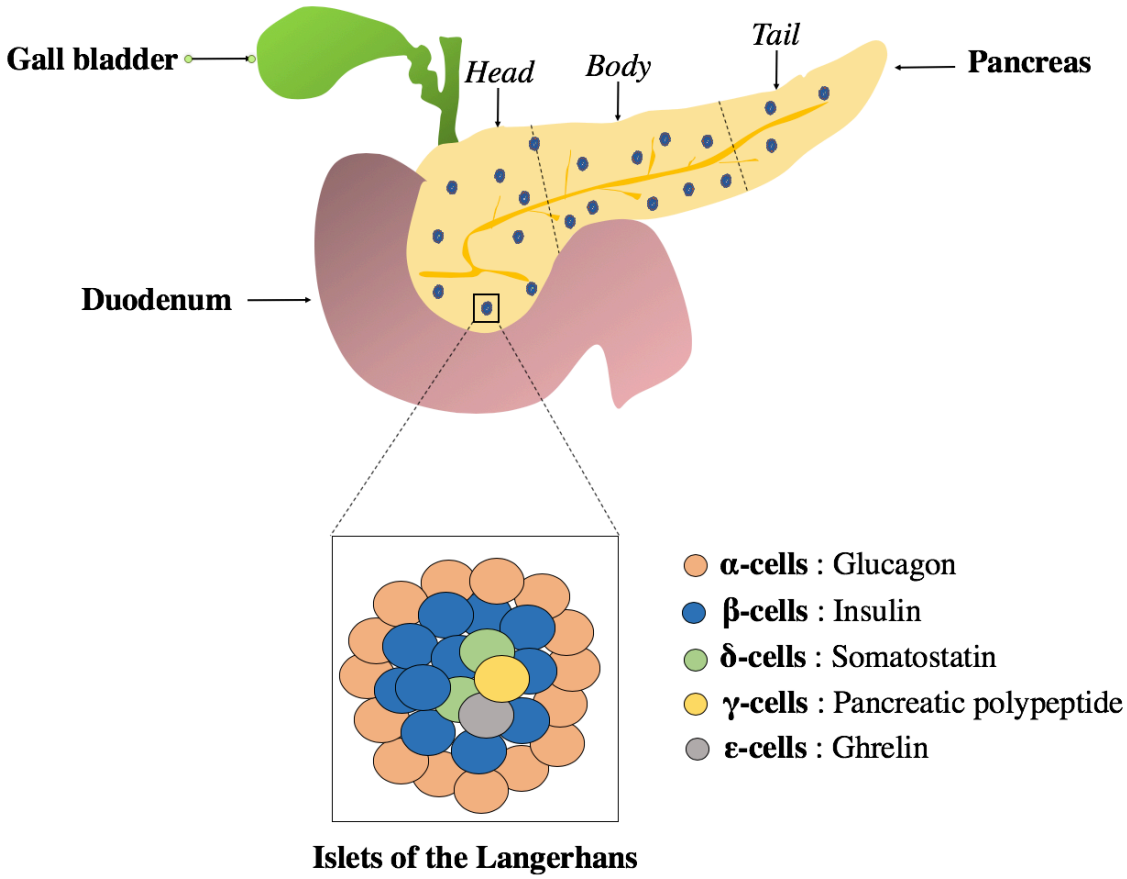


Figure 1: Pancreatic islet structure and function

1.2 Overview of Diabetes:

Diabetes is a global epidemic affecting over 500 million people worldwide and is expected to increase by 50% by 2030 (Zheng et al., 2018b). Most of these individuals suffer one of the two major forms of diabetes; type 1 diabetes (T1D) or type 2 diabetes (T2D). T1D accounts for ~10% of diabetes cases and has characteristic autoimmune destruction of the insulin-secreting β-cells of the pancreas. The most prevalent form of diabetes is T2D, accounting for ~90% of diabetes cases. Although genetic predisposition determines individual susceptibility to T2D to a certain extent, an unhealthy diet and a sedentary lifestyle are important factors that contribute to this epidemic. More uncommon types of diabetes and insulin resistance include single-gene disorders like MODY (Mature Onset Diabetes of the Young), gestational diabetes, and drug-induced diabetes, causing severe defects in insulin action and β-cell function (American Diabetes Association, 2010).

The World Health Organization criteria for the diagnosis of diabetes mellitus include the typical symptoms like polydipsia, polyphagia, and polyuria along with either random plasma glucose > 11.1 mmol/l or fasting plasma glucose > 7.0 mmol/l. Alternatively, other diagnostic criteria are HbA1C level > 6.5% or two-hour plasma glucose > 11.1 mmol/l after a 75 g glucose load (Pippitt et al., 2016). The rise in blood glucose levels occurs either due to insufficient insulin production or insulin resistance due to the dysfunction or death of β-cells. Understanding the underlying mechanisms and the factors involved in this dysfunction of the β-cells is crucial for designing innovative diagnostic tools and as well as identifying novel therapeutic targets for diabetes.

1.3 Glucose and insulin homeostasis:

Glucose is an essential substrate for energy production that is essential for all cellular processes. There are three sources through which the glucose enters the circulation. Firstly, upon food consumption, the carbohydrates are broken down, and through intestinal absorption, the glucose subsequently enters the bloodstream. Secondly, through hepatic activation, the stored glycogen is broken down to release glucose through the process called glycogenolysis. Finally, in conditions like fasting, through gluconeogenesis, glucose can be made from lactate and amino acids to meet the energy demands (Aronoff et al., 2004). Alterations in blood glucose levels can have severe consequences. The condition when the blood glucose levels are low or high as compared to the standard level is known as hypoglycemia or hyperglycemia, respectively. To avoid hypoglycemia or hyperglycemia, the body has innate mechanisms to maintain the blood glucose levels within a physiological range between 4 and 7 mmol/L by regulation of different hormones in response to nutritional, hormonal and neural stimuli (Giugliano et al., 2008; Leclercq-Meyer et al., 1979; Quesada et al., 2008; Weickert, 2012; Yeung et al., 2010). In diabetes, glucose homeostasis is disrupted due to an imbalance in these hormones.

As discussed earlier, the two critical hormones that are produced for maintaining glucose homeostasis are glucagon and insulin. The α-cells of the pancreas produce and secrete glucagon that has a critical role in promoting blood glucose levels. Glucagon is a 29-amino-acid peptide cleaved from proglucagon. Major processes like glycogenolysis and gluconeogenesis are partly under the control of glucagon. Glucagon promotes glycogenolysis during the first 8–12 hours of fasting, while for more extended periods of

fasting, it promotes gluconeogenesis. These processes restore blood glucose levels by the release of glucose from the liver (Janah et al., 2019).

On the other hand, when glucose levels rise in the blood, insulin is secreted by the β-cells of the pancreatic islets of Langerhans to restore the normal levels of glucose. Banting and Macleod received a Nobel prize in 1923 for the discovery of insulin, which changed the course of diabetes therapeutics (Quianzon and Cheikh, 2012). The insulin biosynthesis begins with the *preproinsulin* gene on chromosome 11 in humans that encodes the 110-amino acid precursor, preproinsulin. Preproinsulin contains a hydrophobic N-terminal signal peptide that interacts with cytosolic signal recognition particles (SRPs) (Egea et al., 2005). These SRPs facilitate translocation of preproinsulin from the rough endoplasmic reticulum (ER) to the lumen via a peptide conducting channel (Chan et al., 1976; Lomedico et al., 1977). The N-terminal signal peptide is cleaved in this channel to form a product called proinsulin, which undergoes folding and transports from the ER to the Golgi apparatus, where it enters secretary vesicles. Here, proinsulin is further cleaved to generate mature insulin and C-peptide (Munro and Pelham, 1987). The mature insulin is stored in the secretory granules, ready to be secreted in response to β-cell stimulus (Nishi et al., 1990). The secreted insulin is a 51 amino acid protein with a molecular weight of 5.8 kDa (Figure 2).

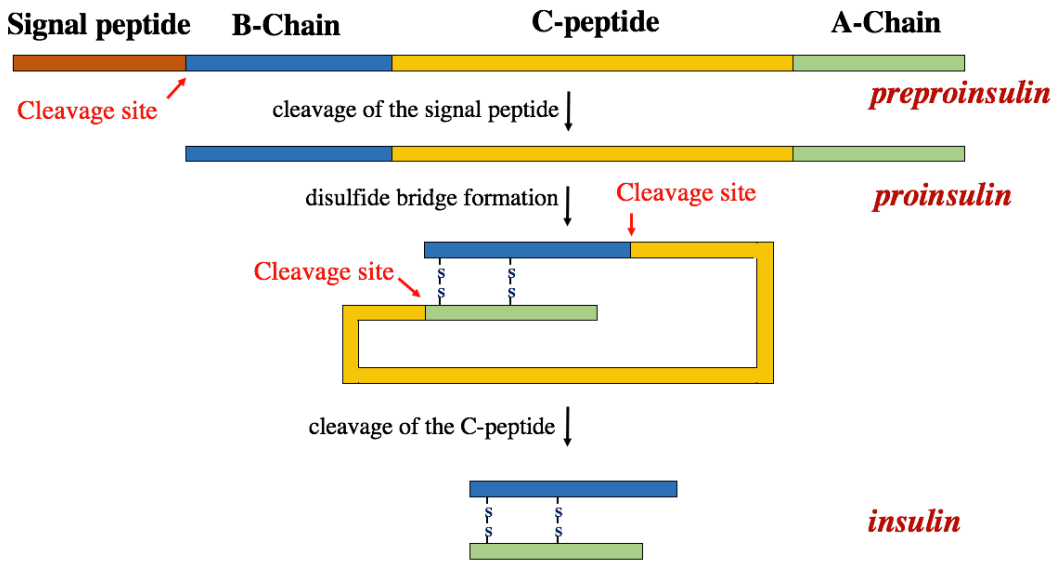


Figure 2: Biosynthesis of insulin

Insulin biosynthesis in the β-cells is primarily controlled by glucose metabolism, which is a critical physiological event that stimulates *insulin* gene transcription and mRNA translation (Poitout et al., 2006). In rodent pancreatic β–cells, GLUT2 is the major glucose transporter, whereas GLUT1 is a pre-dominant transporter in human β-cells. These glucose transporters are localized to the plasma membrane and act as major glucose sensors (De Vos et al., 1995). GLUT2 is a low-affinity glucose transporter that enables a high rate of glucose influx into β-cells. Glucose enters β-cells through GLUT2 transporters and is phosphorylated by glucokinase, which effectively traps it in the cell and prevents its movement back across the plasma membrane (Suckale and Solimena, 2008). This phosphorylation is the rate-limiting step in β-cell glycolysis. Glucose is then subsequently converted to pyruvate through glycolysis. Due to the relatively lower expression of the enzyme pyruvate dehydrogenase, β-cells do not convert pyruvate to lactate (Iynedjian, 1993). Hence, all the glucose entering the β-cells gets converted to pyruvate, which is further oxidized through the Krebs cycle to produce ATP in the mitochondria (Matschinsky, 1996). This glucose metabolism leads to an increase of ATP:ADP ratio, which promotes closure of the ATP-sensitive potassium (K_{ATP}) channels. Usually, at sub-stimulatory concentrations of glucose, these channels are open, allowing a free flow of K^+. At high glucose concentrations, once the K_{ATP} channel closes due to reduced efflux of K^+ ions, there is an increase in the membrane potential. This, in turn, leads to depolarization of the membrane resulting in opening up of the voltage-gated Ca^{2+} channels (Keahey et al., 1989; Ohta et al., 1993; Straub et al., 2004). This opening of the Ca^{2+} channels triggers an influx of Ca^{2+} that induces the fusion of insulin-containing secretory granules to the plasma membrane ultimately leading to the exocytosis of insulin granules (MacDonald and

Wheeler, 2003; Miki et al., 1999; Tarasov et al., 2004). Once enough insulin is produced and released to meet physiological demands, these Ca^{2+} channels close, which leads to repolarization of the membrane due to reduced cytoplasmic Ca^{2+} levels. This membrane repolarization results in the opening up of the K_{ATP} channel that brings the cell back to the normal unstimulated physiological state (Fridlyand et al., 2013; Jacobson and Philipson, 2007).

Although glucose is the primary stimulant for insulin secretion, other nutritional factors and hormones also contribute to insulin secretion. The upregulation of amino acid levels due to high protein intake promotes insulin secretion (Carr et al., 2008; Karamanlis et al., 2007; Nuttall et al., 1985). Interestingly, there are differences observed in the levels of insulin secretion when there is the administration of individual amino acids as opposed to the synergistic effect of a mix of amino acids (Straub et al., 2004). Amino acids have also been shown to have roles in glycolysis, Krebs cycle, and glucose sensitivity in the β-cells (Gannon and Nuttall, 2010; Prentki et al., 2013). Free fatty acid metabolism has also been implicated in enhanced insulin secretion (Boland et al., 2017; Carr et al., 2008; Chen et al., 1994; Nuttall et al., 1985; Prentki and Madiraju, 2012; Stein et al., 1997). In the case of insulin resistance, adipose tissue responds with continuous lipolysis leading to increased plasma levels of free fatty acids (Kashyap et al., 2004). Finally, hormones like catecholamines and somatostatin inhibit insulin secretion (Bellin (2), 2016).

Once insulin gets secreted, it enters the bloodstream through the pervasive islet vasculature and travels until it reaches the cell and binds to its receptors on the cell surface. This binding, in turn, leads to the activation of various signaling cascades (Section 1.4) (Vargas and Carrillo Sepulveda, 2019). One of the primary effects of activating these

cascades is the translocation of glucose transporter 4 (GLUT4) to the plasma membrane. GLUT4 is a major transporter through which glucose is taken up by the cells. Once GLUT4 gets transported to the membrane, it leads to a massive influx of glucose in the cells, thus reducing blood glucose levels (Furtado et al., 2002; Leto and Saltiel, 2012; Olson, 2012).

Insulin regulates glucose homeostasis at a variety of sites (Figure 3). The majority of insulin-mediated glucose disposal takes place in skeletal muscles (Bellin (2), 2016). By activation of glycogen synthase, insulin also promotes glucose storage in the form of glycogen in the muscles and liver (Honka et al., 2018). In the liver, insulin also prevents glucose output by inhibiting gluconeogenic enzymes and glycogen metabolizing enzymes. Insulin also promotes glucose storage in the form of fat in adipose tissues and in liver by increasing lipid synthesis. On the other hand, insulin also prevents lipolysis of the triglycerides in the adipose tissues (Buczkowska and Jarosz-Chobot, 2001; Dimitriadis et al., 2011; Girard, 2006). Hence, impaired insulin function is associated with hyperglycemia and dyslipidemia.

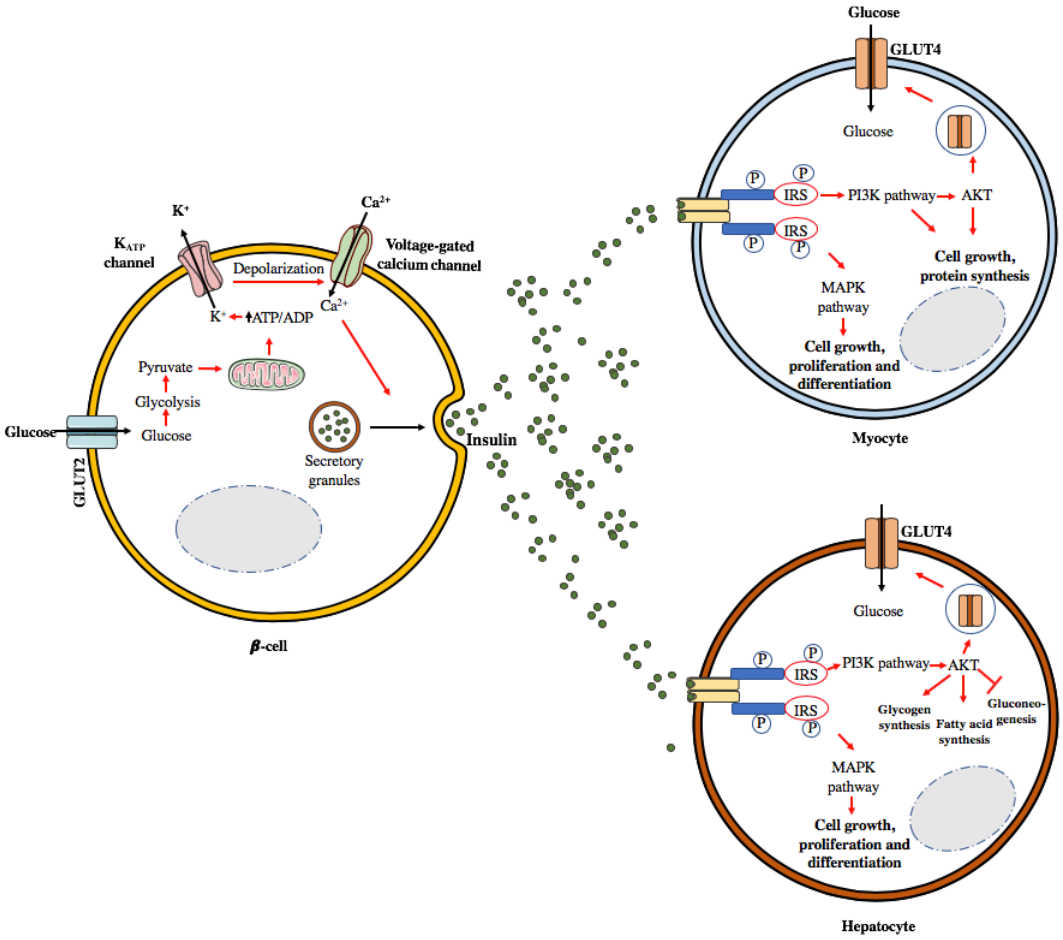


Figure 3: Glucose-stimulated insulin secretion and effects on different cell types

1.4 The insulin signaling cascade:

The insulin receptor is a heterotetrameric protein that belongs to the receptor tyrosine kinases (RTKs) family. It is composed of two extracellular α-subunits and two transmembrane β-subunits. The α-subunits inhibit the protein tyrosine kinase activity intrinsic to the β-subunits. Insulin binds to the α-subunits and induces a conformation change, that activates the intrinsic β-subunit kinase activity, resulting in autophosphorylation on intracellular tyrosine residues and allowing it to bind to ATP (Fu et al., 2013; Lee and Pilch, 1994; Scapin et al., 2018). It further catalyzes the phosphorylation of proteins known as insulin receptor substrates (IRS) (Ebina et al., 1985). IRS phosphorylation triggers the activation of multiple major pathways like phosphatidylinositol-3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) pathways. PI3K mediates most of the metabolic effects of insulin, whereas MAPK has a significant role in growth and differentiation.

IRS proteins have N-terminal pleckstrin-homology (PH) and phosphotyrosine-binding (PTB) domains and several tyrosine phosphorylation sites that enable binding of effector molecules. IRS1 and IRS2 are two proteins that are widely distributed and play discrete yet shared roles in glucose homeostasis (Burks and White, 2001; Lavin et al., 2016; White, 2002). The depletion of IRS1 in mice leads to growth retardation and insulin resistance, while IRS2 depletion leads to reduced β-cell mass (Araki et al., 1994; Kubota et al., 2000; Tamemoto et al., 1994). Double knockout of both IRS1 and IRS2 is lethal at an early embryonic stage. (Withers et al., 1999)

PI3K is an enzyme that consists of a regulatory subunit (p85) and a catalytic subunit (p110) (Wymann and Pirola, 1998). The regulatory subunit has an SH2 domain that

interacts with specific phosphotyrosine motifs on IRS proteins. This interaction enables the recruitment of PI3K to the plasma membrane and release of the catalytic subunit, which converts phosphatidylinositol-4,5-bisphosphate (PIP2) to phosphatidylinositol-3,4,5-triphosphate (PIP3). PIP3 then binds to proteins with PH domains allowing their activation at the plasma membrane (Metz and Houghton, 2011; Whelan et al., 2010). A critical target of the PIP3 is the cyclic adenosine monophosphate (cAMP) dependent protein kinase, including PI-dependent kinase 1(PDK1) and protein kinase B (PKB or also known as AKT) (Hemmings and Restuccia, 2012). PDK-1 gets activated upon binding to membrane-bound PIP3, allowing it to phosphorylate its targets at serine or threonine residues. Insulin-induced AKT activation requires its phosphorylation at the Thr-308 and Ser-473. PDK-1 phosphorylates AKT at Thr-308 inducing the start of its activation sequence while Ser 473 gets phosphorylated by mammalian target of rapamycin complex 2 (mTORC2) (Asano et al., 2007; Molinaro et al., 2019; Shepherd et al., 1998). The complete activation of AKT activates many downstream targets that ultimately exert insulin-mediated effects. AKT phosphorylates mTORC1 that regulates various metabolic pathways, growth, and protein synthesis (Dan et al., 2014; Heras-Sandoval et al., 2014). AKT also phosphorylates and deactivates glycogen synthase kinase 3 (GSK3), thereby preventing inhibition of glycogen synthase which promotes glycogen synthesis (Cross et al., 1995). Finally, AKT phosphorylates and inhibits the Rab-GTPase-activating protein, which promotes cytoskeletal re-organization required for GLUT4 translocation to the plasma membrane essential for glucose uptake into the cells (González-Sánchez and Serrano-Ríos, 2007; Petersen and Shulman, 2018; Wang et al., 1999).

The MAPK pathway is the other major downstream signaling pathway activated by insulin signaling. First, the adaptor protein growth factor receptor-bound protein 2 (Grb2) binds to the Src homology-2-domain-containing (SHC) and IRS, and forms a complex with the SOS (son of sevenless) protein, a guanyl nucleotide exchange factor (Skolnik et al., 1993). This interaction, in turn, activates the cascade of serine/threonine kinases Raf/MEK/ERK1/2. Activated ERK1/2 translocates to the nucleus and phosphorylates an array of transcription factors and various MAPKs to regulate many vital processes, including cell proliferation and differentiation (Avruch, 2007; Williamson et al., 2003). Subsequently, all these pathways act synergistically to maintain glucose homeostasis. In conclusion, insulin regulates many crucial pathways, and thus impairment in insulin secretion or alteration in the signaling cascade due to β-cell dysfunction or death can have severe consequences.

1.5 Islet β-cell dysfunction and its consequences:

The defining characteristic of diabetes is chronic hyperglycemia due to β-cell insufficiency and/or dysfunction. Hyperglycemia can have devastating consequences due to its associated complications affecting different organs. Most diabetes-related deaths are indirectly due to these diabetes-associated pathologies. Microvascular and macrovascular complications are commonly associated with hyperglycemia (Chowdhury et al., 2014; Papatheodorou et al., 2018). Macrovascular complications include cardiovascular diseases, while microvascular complications include neuropathy, retinopathy, and nephropathy (Chawla et al., 2016). Individuals with diabetes have a higher risk of suffering cardiovascular complications, including atherosclerosis, heart attack, and stroke (Resnick and Howard, 2002). High circulating glucose levels can damage the nerves that control the heart by generation of advanced glycation end-products (ASE). The AGE promote a state of chronic inflammation, which ultimately leads to the cardiovascular complications mentioned above (Zhang, 2014). Similarly, if peripheral nerves are damaged, it is termed as neuropathy. Diabetic neuropathy can be sensory, focal, or autonomic and can subsequently lead to numbness or severe pain in the affected region (Bansal et al., 2006). Hyperglycemia can also damage blood capillaries, which can lead to several microvascular complications. High glucose concentrations can promote the nonenzymatic formation of advanced glycosylated end products (AGEs), which are believed to cause damage to the nerves and blood vessels (Forbes and Cooper, 2013; Singh et al., 2014; Vlassara and Uribarri, 2014). The kidneys contain millions of capillaries that filter the blood. When these capillaries are injured by high glucose, it can lead to diabetic nephropathy. Diabetic nephropathy can damage the filtering system, and patients can need kidney transplants

(Lim, 2014; Nazar, 2014; Sulaiman, 2019). In diabetes, the blood capillaries of the eyes can also be severely affected, which can lead to retinopathy. Retinopathy can lead to glaucoma, which often necessitates cataract operation. In severe cases, diabetic retinopathy results in blindness (Duh et al.; Nentwich and Ulbig, 2015; Wang and Lo, 2018). These major complications are the reasons for morbidity associated with diabetes.

According to the International Diabetes Federation, diabetes currently accounts for approximately 5 million deaths annually worldwide (IDF Diabetes Atlas Group, 2015). Due to genetic pre-dispositions and unhealthy lifestyles, this epidemic is spreading rapidly and has already affected the majority of the world population. In 2017, more than 500 million individuals were reported to have diabetes globally, and this prevalence is expected to increase by 50% in the next 20 years (Kharroubi and Darwish, 2015). In the United States (U.S.) itself, over 30 million people have diabetes. More staggeringly, around 100 million individuals are in the pre-diabetic state and are at high risk of developing diabetes in the U.S. Diabetes has an economic impact, costing 327 billion dollars annually to the U.S. healthcare system. More importantly, diabetes being a chronic disorder, it leads to several co-morbidities. Annually, diabetes accounts for over 250,000 deaths in the U.S. (American Diabetes Association, 2018; Rowley et al., 2017).

1.6 Type 1 diabetes pathophysiology:

Diabetes is classified into two major types; type 1 diabetes (T1D) and type 2 diabetes (T2D). T1D is typically characterized by autoimmune destruction of the pancreatic β-cells, which ultimately leads to insulin deficiency, hyperglycemia, and a tendency toward ketoacidosis (Kahanovitz et al., 2017; Todd, 2010). T1D accounts for 5-10% of the diabetes cases globally (Maahs et al., 2010). T1D can be detected at any age, but in a majority of the cases, it is usually diagnosed in children and adolescents, and hence, it was earlier referred to as juvenile diabetes (Gale, 2005). T1D pathogenesis is closely associated with several elements, including genetic, environmental, and immunologic factors (Paschou et al., 2017).

Genetic factors: Contrary to the notion that T1D is hereditary, usually it is found to occur in individuals with no family history of T1D. However, the risk of developing the disease dramatically increases in the relatives of people with T1D, e.g., as high as 50% in the monozygotic twins (Beyan et al., 2012; Concannon et al., 2009; Redondo et al., 1999). More than 50 candidate genes that are associated with the risk of developing T1D have been identified (Barrett et al., 2009; Cooper et al., 2008; Størling and Pociot, 2017). The location of the majority of these genes is within the major histocompatibility complex (MHC) region, also known as HLA (human leukocyte antigen) located on chromosome 6. MHC is a cluster of genes that code for cell surface proteins essential for antigen recognition by the adaptive immune system. MHC molecules bind to the antigens and display them to the T-lymphocytes. There are two classes of HLA gene loci; Class I is associated with antigens that are recognized by cytotoxic T-lymphocytes (CD8+ T-cells) while Class II is associated with antigens recognized by helper T-lymphocytes (CD4+ T-

cells) (de Almeida and Holoshitz, 2011; Trowsdale, 2011; Wieczorek et al., 2017). Unsurprisingly, mutations in these HLA gene loci can alter the ability of the immune system to recognize the appropriate antigens. In TID, more than 90% of the cases have either HLA-DR3, DQB1*0201 or HLA-DR4 DQB1*0302 haplotypes suggesting the importance of these regions in increasing the susceptibility of the individuals to develop this autoimmune disease (Baschal et al., 2007; Khalil et al., 1990; Paschou et al., 2014; Pugliese et al., 1995; Rowe et al., 1994).

Apart from HLA, polymorphisms in the *insulin* gene on chromosome 11 have been shown to increase the genetic pre-disposition for TID (Aly et al., 2006). The variable number of tandem repeats (VNTRs) in the promoter region of the *insulin* gene have been associated with the risk of developing T1D (Barratt et al., 2004; Mein et al., 1998). Moreover, shorter VNTRs in the insulin promoter are associated with susceptibility while the longer forms are associated with protection against T1D (Bennett et al., 1995; Pugliese et al., 1997; Vafiadis et al., 1997). Finally, *CTLA-4* is another dominant non-HLA gene associated with the risk of developing T1D. Activated T-cells express CTLA-4 that has a critical role in transmitting an inhibitory signal to other T-cells, thus attenuating maladaptive inflammation (Anjos and Polychronakos, 2004; Scalapino and Daikh, 2008). However, in T1D, due to polymorphisms in *CTLA-4*, there is excessive stimulation and proliferation of T-cells that eventually leads to an imbalance of the immune response and attack on the β-cells (Chistiakov et al., 2001; Kavvoura and Ioannidis, 2005; Padma-Malini et al., 2018; Qu et al., 2009).

Environmental factors: Apart from genetics, multiple environmental factors are also known to play a critical role in the pathogenesis and susceptibility to T1D. The major

environmental factors are viruses and nutrition (Paschou et al., 2017). Several studies report that the incidence of T1D increases when children suffer from congenital rubella syndrome in which there is exposure to rubella viruses during fetal development (Devendra et al., 2004; Forrest et al., 1971; Menser et al., 1978). Other prime viral candidates for T1D are the enteroviruses, a genus of the viruses which infect their host via the intestine (Bergamin and Dib, 2015; Filippi and von Herrath, 2008). Enterovirus infection in pregnant mothers leads to elevated antibodies directed against the virus, and their children later often go on to develop T1D (Hyöty et al., 1995). Interestingly, it has been observed that the onset of diabetes-associated autoantibodies coincides with enterovirus infection in many affected children and their siblings (Hober and Sauter, 2010; Lönnrot et al., 2000). The Coxsackievirus B virus is the most common strain of enterovirus found in patients with T1D. Several studies have demonstrated that blood samples of T1D patients contain RNA of the Coxsackievirus B virus, which stimulates a strong immune response (Andréoletti et al., 1998; de Beeck and Eizirik, 2016; Clements et al., 1995). When the Coxsackievirus B virus infects the pancreatic islets, it leads to inflammation-mediated by natural killer (NK) cells (Dotta et al., 2007). Coxsackievirus B has also been shown to exert its effects by molecular mimicry that induces immune responses against autoantigens that resemble the viral antigens (Richardson and Morgan, 2018). The P2C protein of the Coxsackievirus B virus has significant amino acid sequence similarity to the β-cell protein glutamic acid decarboxylase (GAD65), and it has been shown that T-cells can react to both the proteins and trigger an immune attack (Honeyman et al., 1998; Schloot et al., 2001). Poliovirus has been shown to infect β-cells via the αvβ3 integrins on the cell surface (Ylipaasto et al., 2004). In response to this infection, there is an immune attack on the β-cells.

The other critical environmental factor associated with T1D pathogenesis is nutrition, which encompasses both diet and gut microbiota (Rewers and Ludvigsson, 2016). Children consuming cow-milk are at high risk of islet autoimmunity (Verge et al., 1994; Virtanen et al., 1994, 2000). As shown by one study, there was a relatively higher cellular and humoral response to cow's milk as compared to the breast-milk (Gottlieb, 2000). This risk of autoimmunity is attributed to the specific region of albumin protein found in the milk called ABBOS, which resembles p69 protein found on the surface of β-cells (Karlsson and Ludvigsson, 2000). Other dietary factors that promote risk of T1D include introduction of cereals in an infant's diet, deficiency of Vitamin D or inadequate intake of omega-3 fatty acids by pregnant mothers (Frederiksen et al., 2013; Norris et al., 2003, 2007; Raab et al., 2014; Sørensen et al., 2012a, 2012b; Virtanen et al., 2010). Finally, many studies have identified the gut microbiota as factors crucial for T1D development and progression. Unsurprisingly, the gut flora of T1D patients differs significantly from that found in healthy individuals (Han et al., 2018; Zheng et al., 2018a). The abundance of *Lactobacillus, Bifidobacterium, and Prevotella* is found to be lower in children suffering from T1D as compared to healthy children. On the other hand, the levels of Clostridium, Bacteroides, and Veillonella were higher in children suffering from T1D as compared to healthy children. Interestingly, other than abundance, diversity of the gut microbiota is also related to the development of T1D (Murri et al., 2013). A study showed that the microbiome of healthy children was more diverse when compared to children with T1D (Giongo et al., 2011). The gut microbiota is known to play substantial roles in glucose and lipid metabolism (Gérard and Vidal, 2019; van Olden et al., 2015). Moreover, they can modulate immune response as well as lead to systemic inflammation, further increasing the

disease risk (Brown et al., 2011; Wen et al., 2008). Although there are several association studies of the gut microbiota and T1D development, whether the microbiota is a cause or the effect of the disease, is still highly debated.

Immunological factors: The genetic and environmental factors discussed above, contribute to the destruction of β-cells that is mediated by the immunological factors. Indeed, the immune system faces a continuous barrage of antigenic challenges, and as such, it has a crucial duty to restrict its responses to non-self-antigens. The process by which the immune system is desensitized to self is referred to as immunological tolerance. The disruption of this process will likely make the immune system self-reactive, leading to autoimmunity (Luo et al., 2016; Romagnani, 2006). In T1D, the immune system, consisting of macrophages, dendritic cells, T-cells, and B-cells, mounts a concerted attack upon the pancreatic β-cells (Szablewski, 2014). First, the local macrophages and dendritic cells surveil the pancreas and then activate autoreactive T-cells in the pancreatic lymph nodes. These T-cells then promote β-cell destruction by two mechanisms, one direct and the other indirect. In the direct process, the cytotoxic T-cells release perforins and granzymes upon antigen recognition, which kill the targeted cell. In the indirect process, the activated T-cells release several pro-inflammatory cytokines that include IL-1 (interleukin-1), TNF-α (tumor necrosis factor-α) and INF-γ (interferon-γ) (Fatima et al., 2016; Graham et al., 2012; Grunnet and Mandrup-Poulsen, 2011; Thomas et al., 2010, 2013; Trivedi et al., 2016). Each of these factors trigger apoptosis in the targeted β-cells; however, there is evidence of necrosis and necroptosis as well (Templin et al., 2018; Tomita, 2017, 2006; Wilcox et al., 2016). A distinct population of T-cells, called regulatory T-cells (Tregs), is critical for limiting and attenuating the inflammatory effects of cytotoxic T-cells (Corthay,

2009). Predictably, the magnitude and the functional capacity of this T-cell population is severely affected in T1D (Hull et al., 2017; Tan et al., 2014). B-cells are responsible for the generation of antibodies, and in T1D, they generate "autoantibodies" directed against several β-cell proteins. The principal autoantibodies that are detected in approximately 60-70% T1D cases include those that are directed against the glutamic acid decarboxylase 65 kilodalton protein (GAD65), tyrosine phosphatase-related islet antigen 2 (IA-2), insulin (IAA) and zinc transporter (ZnT8) (Cheng et al., 2018; Yu et al., 2017). All these genetic, environmental, and immunological factors ultimately contribute to an almost permanent loss of β-cell mass (Figure 4). However, the events leading to the initiation and progression of the disease take place over a long time, and hence, there may be a long latency period between autoantibody detection and when T1D symptoms appear (Atkinson, 2012).

While the ongoing immune attack destroys β-cells, those that still survive can compensate for the loss by increasing their function and keep the glucose levels in check, upto a point. However, once the β-cell mass falls below a critical threshold, which is an estimated loss of 85% β-cell mass (Klinke, 2008), symptomatic hyperglycemia (i.e., blood glucose levels > 200mg/dL) is detected. Apart from the classic symptoms of diabetes, T1D patients also show the presence of autoantibodies and ketoacidosis (Kahanovitz et al., 2017).

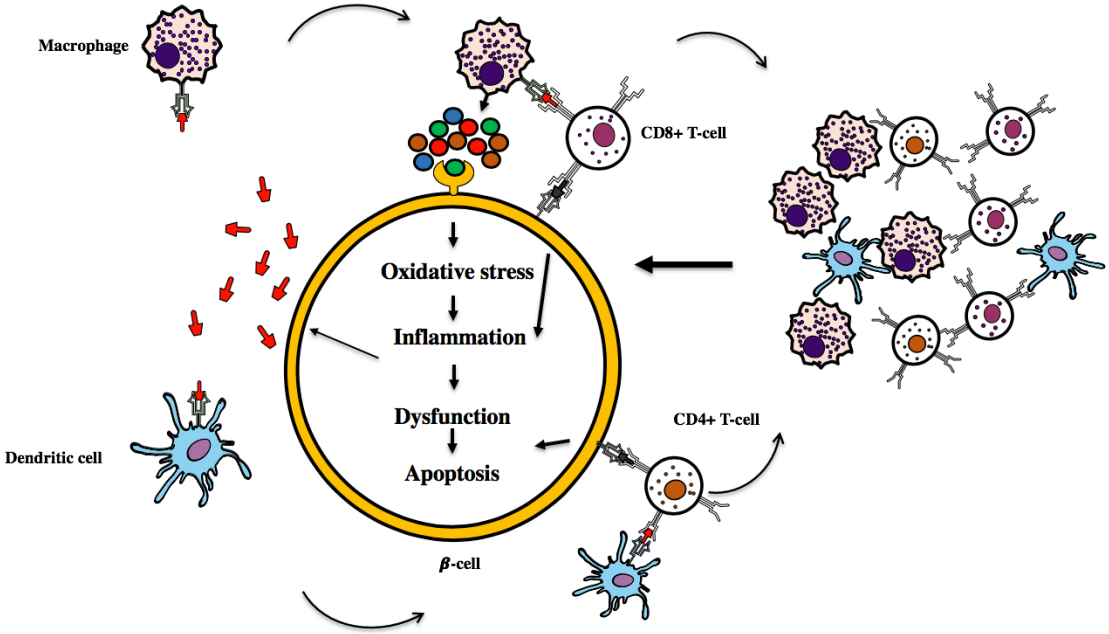


Figure 4: Pathophysiology of type 1 diabetes

The current management of T1D is the exogenous administration of insulin, dietary control, and strict monitoring of glycemic levels (Atkinson et al., 2014; Otto-Buczkowska and Jainta, 2017). Although this insulin administration is effective in managing the symptoms, it is not a permanent cure. There is a dire need to diagnose diabetes in advance and also to identify novel therapeutic strategies that could effectively and permanently cure this dreadful disease. Detecting biomarkers like autoantibodies and other associated proteins that indicate the early stages of the condition could significantly improve the diagnosis and provide a window for the treatment before the autoimmune attack is in full force. Moreover, the role of the immune system is undoubtedly critical in T1D pathogenesis and could serve as a potential site of intervention. Indeed, some current approaches towards the treatment are designed to suppress the immune system; while this has shown promise, it has not been particularly successful. However, if rather than global suppression, the immune system could be precisely modulated to prevent it from reaching or attacking the β-cells, this approach could be a more effective component of a cure for T1D.

1.7 Type 2 diabetes pathophysiology:

T2D is the predominant form of diabetes mellitus, accounting for 90-95% of cases of diagnosed diabetes. As with T1D, it is a metabolic disorder characterized by chronic hyperglycemia, lack of insulin production, and eventual pancreatic β-cell failure (Taylor, 2013). However, in contrast to T1D, T2D is also characterized by insulin resistance. In addition, although the immune cells contribute to the disease progression, T2D is not characterized by an autoimmune attack. Globally, more than 400 million are estimated to have T2D, and this number is expected to increase steeply in the next few years (Harding et al., 2019; Zheng et al., 2018b). T2D has several fatal comorbidities associated with it making it the seventh leading cause of death in the U.S. (Stokes and Preston, 2017). The cardiovascular complication is the most frequent comorbidity observed in T2D patients (Einarson et al., 2018). The prevalence of this disease varies drastically but usually directly correlated with the economic status of the nation affecting a majority of low to middle-income countries (Hu, 2011).

The key factors that contribute to the pathophysiology of T2D are genetic predisposition and lifestyle (Figure 5). Genetics has been shown to play a crucial role in susceptibility to the development of T2D. Genetic analyses in family members and twin studies of T2D patients shows 70% variability associated with genetic factors (Lyssenko and Laakso, 2013). Moreover, the disease development risk is shown to be almost three-fold if a sibling suffers from T2D (Medici et al., 1999). The genetic architecture of T2D differs significantly from T1D. Whereas in T1D, only a few genetic loci have a significant effect on disease susceptibility, in T2D there are more than 100 genes that have shown to have polymorphisms (Flannick and Florez, 2016; Fuchsberger et al., 2016). Overall, these

genetic loci can be categorized into four types: genes previously associated with T2D by gene-wide association studies (GWAS), genes associated with vascular T2D complications, genes associated with metabolic diseases, and genes associated with telomere stability and aging (Montesanto et al., 2018). The genes most significantly associated with the development of T2D include *TCF7L2, PPARG, FTO, KCNJ11, NOTCH2, WFS1, CDKAL1, IGF2BP2, SLC30A8, JAZF1, and HHEX*. These genes encode for proteins involved in numerous pathways including regulation of glucose homeostasis, insulin signaling, calcium regulation and transcription (McCarthy, 2010; Olokoba et al., 2012). Interestingly, predisposition to diabetes is increased in those suffering from certain other genetic disorders, including metabolic syndrome, Cushing's syndrome, Klinefelter's syndrome, Turner's syndrome, and Down's syndrome (Olokoba et al., 2012; Robinson and Kessling, 1992).

Another leading risk factor for developing T2D is lifestyle. More than 55% of individuals with T2D are obese in the U.S. (Centers for Disease Control and Prevention (CDC), 2004; Tobias and Manson, 2016). Diet is a critical component of the lifestyle; consumption of high-calorie dietary components that promote obesity, including meat, non-fermented dairy products, sugar-sweetened beverages, and refined grains, significantly increase the risk of development and progression of T2D (Aune et al., 2013; Barnard et al., 2014; Brouwer-Brolsma et al., 2018; Della Pepa et al., 2018; Forouhi, 2015; Kalergis et al., 2013; Liu et al., 2017; Malik et al., 2010; Mari-Sanchis et al., 2016; Papier et al., 2017).

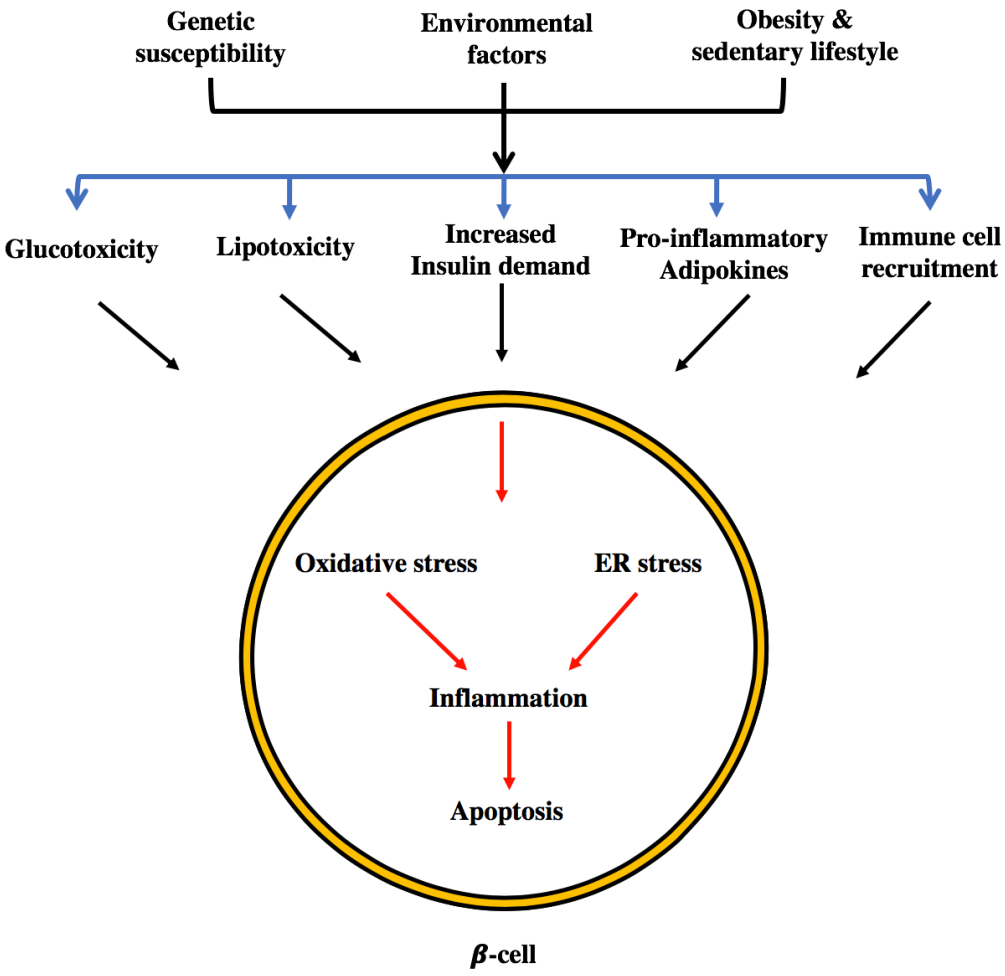


Figure 5: Pathophysiology of type 2 diabetes

Obesity is defined as abnormal or excessive fat accumulation in adipose tissue that leads to impairment of health (Ofei, 2005). This excessive fat can be distributed abdominally, centrally or peripherally around the body, and each distribution has several health implications. The degree of adiposity in an individual can be classified based on the calculated body mass index (BMI). According to the World Health Organization (WHO) standards, a BMI of 18.5–24.9 kg/m^2 is an ideal weight, and 25–29.9 kg/m^2 overweight or pre-obese. Individuals with a BMI higher than 30 kg/m^2 are considered obese and are at the highest risk of developing T2D (Scherer and Hill, 2016, 2000).

Several studies have demonstrated that obesity induces insulin resistance, which is a reduced response of target cells to insulin leading to abnormalities in glucose uptake, metabolism, or storage (Wilcox, 2005). Specifically, adipocytes and skeletal muscle cells are the most profoundly affected, where there is decreased insulin-stimulated glucose transport and metabolism, whereas hepatocytes have impaired suppression of glucose production (Reaven, 1995; Picarel-Blanchot et al., 1996). These effects can be attributed to impaired insulin signaling as well as downregulation of the GLUT4 transporter that actively responds to insulin (Olson, 2012). Typically, insulin binding to its receptors on the cell surface induces receptor phosphorylation and tyrosine kinase activities, which further phosphorylate the downstream insulin receptor substrates (IRS) that ultimately exert the effects of insulin. However, in obesity, all these processes are severely downregulated, which drives increased insulin demand to compensate. Additionally, there are tissue-specific abnormalities associated with obesity. These include reduced IRS-1 protein expression in adipose tissues and downregulated PI3K activity associated with both IRS proteins in skeletal muscles; these effectively lower the activity of circulating insulin

(Fernandez-Twinn et al., 2014; Kim et al., 1999; Rondinone et al., 1997; Standaert et al., 2002). Studies in mice demonstrate that obesity is associated with suppression of the forkhead transcription factor 1 (Foxo1) (Zhang et al., 2016). Foxo1 promotes the expression of several key enzymes of the gluconeogenesis pathway (Schmoll et al., 2000; Yeagley et al., 2001). In hepatocytes, insulin signaling phosphorylates Foxo1, limiting its levels in the cytoplasm (Gross et al., 2009). However, studies in these obese mice also show that there is an upregulation of Foxo1 that is insensitive to insulin regulation, which promotes gluconeogenesis in the liver (Qu et al., 2006; Titchenell et al., 2016).

Adipose tissue itself is one of the critical factors that mediates the effects of obesity. The primary role of adipocytes is to store fat; however, in a setting of obesity, the capacity of adipocytes to store these lipids is reduced severely, leading to ectopic fat accumulation and lipotoxicity in the liver and muscle. This lipotoxicity is also considered to be a significant factor contributing to insulin resistance (Lotta et al., 2017; Yazıcı and Sezer, 2017). There are several mechanisms through which adipocytes and lipotoxicity may cause insulin resistance and result in β-cell damage. First, obesity-mediated lipotoxicity is associated with the upregulation of c-Jun amino-terminal kinase (JNK) pathway activity, which leads to decreased insulin activity (Lee et al., 2003; Prada et al., 2005). Furthermore, the obesity-mediated insulin resistance drives an increased insulin demand that exacerbates endoplasmic reticulum (ER) stress in the β-cells (Boden, 2009; Cnop et al., 2012). Lipotoxicity is also associated with mitochondrial dysfunction that leads to oxidative stress by excessive production of reactive oxygen species (ROS), which are extremely deleterious to β-cells (Schrauwen and Hesselink, 2004; Schrauwen et al., 2010). Both, oxidative stress and ER stress, are known to promote pancreatic inflammation (Hasnain et al., 2016). In

addition to their primary role in fat storage, adipocytes also release signaling factors called adipokines that have several roles. These include regulating hormonal and lymphocyte homeostasis, blood pressure, lipid and glucose metabolism, and inflammation (Rabe et al., 2008). However, in obesity, there is a dysregulated release of these adipokines that contributes to the development and progression of T2D. Some of the critical adipokines are adiponectin, leptin, TNF-α, and IL-6. Adiponectin, the most abundantly secreted adipokine, binds to its receptors AdipoR1 and AdipoR2, which subsequently activate pathways like AMPK and PPAR-α; which in turn promotes increased insulin sensitivity. Unsurprisingly, the expression of both adiponectin and its receptors are downregulated in obesity, thus further promoting insulin resistance (Hotta et al., 2001; Rasmussen et al., 2006; Yamauchi and Kadowaki, 2013; Yang et al., 2002). Next, leptin is a hormone that has a vital role in regulating food intake and energy homeostasis (Park and Ahima, 2015; Triantafyllou et al., 2016). Leptin binds to receptors in the hypothalamus and signals via Janus kinase (JAK)-signal transducers and activators of transcription (STAT) and IRS/phosphoinositide-3 kinase (PI3K) signaling pathways. This represses orexigenic pathways and induces anorexigenic pathways, overall, leading to decreased food intake and increased energy expenditure (Prodi and Obici, 2006). In skeletal muscles and liver, leptin activates PI3K and AMPK pathways that regulate glucose metabolism and insulin sensitivity (Kahn et al., 2005; Minokoshi et al., 2002). Furthermore, leptin signaling protects β-cells from lipid accumulation in a state of overnutrition, which preserves β-cell function (Morioka et al., 2007). Predictably, T2D patients show reduced leptin levels (Bandaru and Shankar, 2011; Wauters et al., 2003). TNF-α and IL-6 are pro-inflammatory adipokines that are involved in adipose inflammation (Makki et al., 2013; Shi et al., 2014).

Once they are released systemically, they exert their effects on many cell types, including immune cells and the pancreatic cells. In the immune cells, they promote pro-inflammatory cellular phenotypes, which in turn promote systemic inflammation (Goyal et al., 2012; Hossain et al., 2010; Liu et al., 2007; Mirza et al., 2012). With similarity to T1D pathogenesis, as these pro-inflammatory immune cells infiltrate the pancreatic islets, they inflict severe damage upon β-cells (Shu et al., 2012; Xia et al., 2017; Zhou et al., 2018). Within the pancreatic β-cells, TNF-α and IL-6 signal through nuclear factor kappa beta (NF-κB) and JAK-STAT pathways, respectively, to promote oxidative stress and further attract pro-inflammatory immune cells (Chen et al., 2015; Turner et al., 2014). The pancreatic inflammation ultimately leads to hyperglycemia secondary to β-cell dysfunction and apoptotic death (Collier et al., 2011; Donath et al., 2009).

The diagnosis of diabetes is based on criteria formalized by the American Diabetes Association (ADA) and the World Health Organization (WHO). An individual is diagnosed as having diabetes if they present with one of the following conditions: (1) a fasting plasma glucose reading on two separate occasions of 126 mg/dL (7.0 mmol/L) or greater; (2) a plasma glucose of 200 mg/dL (11.1 mmol/L) or greater in a 2-hour oral glucose tolerance test; or (3) HbA1c level greater than 6.5% associated with symptoms like polyuria, polydipsia, polyphagia and weight loss (Cox and Edelman, 2009). Fasting blood glucose levels ranging between 100-126 mg/dL or HbA1c levels between 6-6.5% are considered to be indicative of a pre-diabetic state (Committee*, 2009; Cox and Edelman, 2009). Since the diagnostic criteria for both T1D and T2D are quite similar, other factors—like obesity and age—and other tests like the presence of specific autoantibodies, are used to help distinguish between the types of diabetes.

Since lifestyle is a strong factor driving T2D, the management of this disease primarily focusses on changes in lifestyle, such as diet, physical activity supplemented with drugs that help restore normal glycemic levels (Nyenwe et al., 2011). Several studies show that shifting to a healthy diet like a high-fiber-low-calorie diet leads to improvement in glucose levels (Kaline et al., 2007; Miranda and Horwitz, 1978; Wolfram and Ismail-Beigi, 2011). Importantly, any form of physical activity has been shown to prevent hyperglycemia (Colberg et al., 2010; Hamasaki, 2016; Yanai et al., 2018). In addition to these physical and dietary changes, avoiding unhealthy habits like smoking and drinking alcohol is essential (Shi et al., 2013). Along with a healthy lifestyle, individuals with T2D are prescribed medications based on the underlying pathology. These medications can be categorized into four major types based on their mode of action: i) improving insulin sensitivity (e.g., Metformin, Rosiglitazone), ii) stimulating pancreatic insulin release (e.g., Repaglinide, Nateglinide, Glipizide), iii) blocking carbohydrate breakdown in the stomach (e.g., Acarbose, Miglitol), and iv) inhibiting glucose reabsorption by the kidneys (e.g., Canagliflozin, Dapagliflozin) (Lorenzati et al., 2010). Additional medications or surgeries may be prescribed in advanced stages of T2D, depending on comorbidities and complications that may be present. In summary, most current treatments are focused on reducing the symptoms associated with diabetes. Hence, the development of novel drugs and therapeutic approaches will be necessary to deliver a permanent cure. It will require extensive research and a functional understanding of the different mechanisms that contribute to T2D pathology. Determining specific yet efficacious targets are the key to developing efficient therapeutics that will truly cure T2D.

1.8 The role of cellular stresses in β-cell dysfunction:

Diabetes results from dysfunction or death of pancreatic β-cells. Several studies attribute this to factors that promote cellular stress within the β-cells. The two major forms of cellular stress associated with β-cell damage are ER stress and oxidative stress. ER stress refers to an imbalance between the demand for protein folding and the capacity of the ER for protein folding (Schröder and Kaufman, 2005). In response to triggering by glucose sensation, the β-cells respond with a proportional synthesis of insulin. After translation, preproinsulin peptide enters the ER, where it is folded and cleaved, forming proinsulin (Liu et al., 2014a). β-cells are specialized secretory cells that have a well-developed ER and several mechanisms in place to handle the enormous biosynthetic load (Papa, 2012). However, in diabetes, the systemic insulin demand exceeds pancreatic capacity due to insulin resistance or diminished functional β-cell mass; this generates ER stress in the β-cells. Proinsulin is highly susceptible to misfolding due to high demand (Sun et al., 2015). Most of the misfolded proinsulin is not recognized by the insulin processing machinery, which leads to the aberrant accumulation and release of proinsulin from the stressed β-cells, which is seen in diabetes (Hasnain et al., 2016). To mitigate the ER stress, cells activate the unfolded protein response (UPR). Disruption in ER homeostasis triggers three conserved UPR pathways that: i) slow global protein synthesis; ii) increased synthesis of chaperones that aid protein folding; and iii) activate ER-associated degradation (ERAD) to degrade irreparably misfolded proteins (Pandey et al., 2019). Three major UPR mediate this response: PERK (PKR-like Endoplasmic Reticulum Kinase), IRE1α (Inositol Requiring Enzyme1α), and ATF6 (Activating Transcription Factor 6). Under normal conditions, these UPR sensors are associated with a chaperone BiP (Binding

immunoglobulin Protein), which renders them inactive. As ER stress builds, a conformational change in BiP results in its dissociation from the UPR proteins. The first sensor, PERK, phosphorylates eukaryotic translation initiation factor 2 alpha (eIF2α), and this, in turn, globally inhibits the translation of proteins. When the ER stress is chronic, PERK promotes translation of ATF4 (Activating Transcription Factor 4), which activates genes like C/EBP-homologous protein (CHOP) that promote apoptosis. The second sensor, IRE1α, is a protein with and endoribonuclease domain that splices the mRNA transcript for X-box-binding protein 1 (XBP1). This spliced form of XBP1 acts like a transcription factor for genes encoding chaperones and other proteins related to the ER-associated degradation (ERAD) pathway. IRE1α also catalyzes the degradation of membrane-associated mRNAs that encode secretory proteins through a process termed as RIDD (regulated IRE1 dependent decay). RIDD induces intrinsic apoptosis by activation of the c-Jun N-terminal kinase (JNK) pathway. The third sensor, ATF6, has an autocatalytic site that is cleaved by site 1/2 proteases (S1P and S2P) upon translocation to the Golgi. The cleaved form of ATF6 acts as a transcription factor for genes encoding chaperones, ERAD proteins, and XBP1. All three of these UPR proteins maintain ER homeostasis; however, under conditions of chronic ER stress, they induce apoptosis (Almanza et al., 2019; Cnop et al., 2012; Lin et al., 2008; Sano and Reed, 2013; Sundar Rajan et al., 2007).

Various studies have explored the role of ER stress in diabetes. Murine studies have shown the crucial roles of ER stress and UPR genes in diabetes. In mice, depletion of any of the UPR proteins (PERK, IRE-1 α, and ATF6) individually is enough to induce diabetes (Tsuchiya et al., 2018; Usui et al., 2012; Zhang et al., 2002). In T2D cases, there is a substantial increase in the size of ER as compared to non-diabetic controls. When islets

from diabetic donors were cultured in the presence of 11mM glucose, BiP and XBP1 expression are significantly increased. In contrast, islets isolated from non-diabetic donors did not show this response (Marchetti et al., 2007). This result suggests that healthy islets handle the glucose-mediated stress, while the ERAD pathway was activated in T2D islets due to the dysfunction of the β-cells. Furthermore, the immunostaining of pancreas tissue sections isolated from donors with T2D shows increased levels of both CHOP and BiP (Laybutt et al., 2007). It has also been observed that CHOP expression is increased in the β-cells of obese individuals (Huang et al., 2007). Strikingly, T1D studies report the same trends; islets isolated from individuals with T1D showed increased levels of both CHOP and BIP proteins (Marhfour et al., 2012). Together, these studies demonstrate that ER stress has an active role in the pathogenesis of T1D and T2D. However, β-cell dysfunction and death are not exclusively attributed to ER stress and suggested to be in conjunction with other cellular stresses, including oxidative stress and inflammatory responses.

Oxidative stress is an imbalance between the generation of damaging free radical molecules and the cellular antioxidant responses that mitigate these agents (Betteridge, 2000). Free radicals are short-lived reactive chemical units containing one or more unpaired electrons. These radicals are highly unstable and reactive, and they induce damage by oxidation of cellular molecules. The free radicals that lead to oxidative stress are reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Asmat et al., 2016; Bansal and Bilaspuri, 2010; Dröge, 2002). The ROS include peroxide (H_2O_2), superoxide ($^{\bullet}O^{-}_{2}$), singlet oxygen (O_2), and hydroxyl radical ($^{\bullet}OH$) (Lau et al., 2008). The RNS are generated when nitric oxide ($^{\bullet}NO$) reacts with the ROS. The RNS majorly include peroxynitrite ($ONOO^{-}$), nitrogen dioxide ($^{\bullet}NO_2$), and dinitrogen trioxide (N_2O_3) (Guzik et

al., 2002; O'Donnell et al., 1999; Pacher et al., 2007). Excessive generation of ROS and RNS leads to deleterious cellular oxidative stress. These highly reactive radicals are produced by both endogenous and exogenous mechanisms (Pham-Huy et al., 2008). Several endogenous factors produce ROS and RNS. The generation of adenosine triphosphate (ATP), the cellular currency of energy, occurs in mitochondria via a process called oxidative phosphorylation. This process involves the movement of electrons along an electron transport chain, which releases ROS as by-products (Finkel and Holbrook, 2000; Starkov, 2008). Peroxisomes are another major ROS source. The peroxisomal enzymes contained therein, such as acyl-CoA oxidases, urate oxidase, and xanthine oxidase, catalyze the breakdown of fatty acids. H_2O_2 is a by-product of these reactions, and it quickly diffuses through the cell membrane and can cause widespread molecular damage (De Duve and Baudhuin, 1966; del Río and López-Huertas, 2016; Schrader and Fahimi, 2006). Additionally, the activities of certain ER-resident enzymes, including cytochrome P450 and B5 enzymes and diamine oxidase, can generate ROS. Other endogenous producers of free radicals include enzymes like NADPH oxidase, nitric oxide synthases and lipoxygenases, $FMNH_2$, $FADH_2$, cytochrome P450, and riboflavin (Cheeseman and Slater, 1993; Halliwell and Gutteridge, 2015). The exogenous factors that lead to the generation of the free radicals include radiations, chemicals, carcinogens, smoking, alcohol consumption, pollution, and drugs (Dröge, 2002; Pham-Huy et al., 2008). Importantly, ROS are not only generated as an unintended by-product. For instance, certain immune cells, including macrophages, dendritic cells, and neutrophils generate ROS to use as a tool to eliminate pathogens (Bogdan et al., 2000; Matsue et al., 2003).

Oxidative stress exerts adverse effects on a range of cellular targets and machinery, including nucleic acids, proteins, and lipids (Bokov et al., 2004). DNA is susceptible to oxidation that can result in single-stranded breaks along with the formation of 8-hydroxyguanosine, which is a relatively stable oxidation product used as a measure of oxidative DNA damage within the whole body (Williams et al., 1998; Wu et al., 2004). The DNA damage is associated with cellular senescence, apoptosis, and the development of cancers. Lipid oxidation by enzymes like lipoxygenases and cyclooxygenases can alter the structural integrity and fluidity of the cellular membranes, which can be damaging due to increased cellular permeability. Moreover, lipid oxidation generates radicals that can further damage DNA and proteins (Barrera, 2012; Kwiecien et al., 2014; Landar et al., 2006). Protein oxidation can lead to altered structure and function that can have severe consequences for the cells including alteration of enzyme activity, loss of protein function, protein aggregation, and increased immunogenicity (Berlett and Stadtman, 1997; Celi and Gabai, 2015; Wall et al., 2012). Thus, oxidative stressors can have multiple molecular targets that directly inflict widespread damage to cells. However, ROS are also associated with the activation of several signaling pathways. Increased ROS can activate mammalian target of rapamycin complex1 (mTORC1), a protein complex downstream of AKT signaling that promotes apoptosis (Krakauer, 2015). In addition, ROS can activate MAPK, ERK, and JNK pathways, which can promote cellular senescence and trigger apoptosis in β-cells (Son et al., 2011). ROS also has shown to influence the pentose phosphate pathway, diverting this metabolic pathway from glycolysis into lipid oxidation, which can elevate insulin resistance (Dong et al., 2016). Importantly, ROS can trigger inflammation through

NF-κB, thereby promoting the production of pro-inflammatory cytokines TNF-α and IL-6 (Chelombitko, 2018; Mittal et al., 2014).

Cells have multiple mechanisms to counter these oxidative stresses. For regulating this 'reducing and oxidizing' (redox) homeostasis, the cells employ an antioxidant system comprised of enzymes like catalase, superoxide dismutase (SOD), glutathione peroxidase, and glutathione-S-transferase (Birben et al., 2012). These convert the toxic ROS to non-toxic metabolites, thus protecting the cells. Catalase is a tetrameric enzyme that reduces H_2O_2 to water and associated with the peroxisome (Glorieux and Calderon, 2017). SODs are major superoxide scavengers that are located on the mitochondrial and extracellular matrix (Fukai and Ushio-Fukai, 2011). Glutathione peroxidases catalyze the conversion of H_2O_2 to water and are located in the cytoplasm and mitochondria (Lubos et al., 2011). Lastly, the glutathione-S-transferases inactivate secondary metabolites, such as unsaturated aldehydes, epoxides, and hydroperoxides (Hayes et al., 2005).

Additionally, cells also employ multiple non-enzymatic antioxidants like Vitamin C, Vitamin E, and β-carotene. Vitamin C scavenges free radicals, thus providing an intracellular and extracellular aqueous-phase antioxidant environment (Padayatty et al., 2003). Vitamin E donates an electron to peroxyl radical, which is produced during lipid peroxidation, thus converting toxic radicals to water molecules (Niki, 2015). β-carotene can react with a variety of reactive species, including peroxyl, hydroxyl, and superoxide radicals to reduce them to non-toxic metabolites (El-Agamey et al., 2004).

There is much evidence implicating oxidative stress in the pathogenesis of both major forms of diabetes. In the hyperglycemic environment of diabetes, many factors contribute to the increased free radical formation, such as enhanced protein glycation,

glucose oxidation, and lipid peroxidation (Maritim et al., 2003). As introduced above, these free radicals damage cellular machinery; and in the β-cell thereby diminish insulin release. Moreover, due to their exceptionally high biosynthetic load and unusually low expression of antioxidant enzymes, the β-cells are particularly susceptible to oxidative damage (Lenzen, 2008). Predictably, individuals with T2D have higher oxidative stress as tested by serum total antioxidant status (TAS) and total oxidant status (TOS) (Kurban et al., 2011; Monnier et al., 2006). Hyperglycemia has been shown to lead to increased intracellular levels of advanced glycation end products that are associated with ROS generation (Di Naso et al., 2011; Wolf and Ziyadeh, 2007). Studies have also shown that hyperinsulinemia also promotes the generation of free radicals by an NADPH-dependent mechanism (Ceolotto et al., 2004). Furthermore, in pre-diabetic individuals, there is an increased level of 8-hydroxyguanosine, which indicates the oxidative damage to DNA preceding the clinical development of T2D (Styskal et al., 2012). Along with the upregulation of oxidative stress markers, there is also a reduction in the levels of antioxidants in T2D patients. Vitamin C and glutathione levels were significantly downregulated in T2D patients (Chou and Tseng, 2017; Pouvreau et al., 2018).

Oxidative stress is involved in the pathogenesis of T1D as well. Murine studies show that oxidative stress is higher in islets and vascular tissues of the T1D mouse model (the non-obese diabetic mice) as compared to littermate controls. Moreover, infiltrating immune cells demonstrate aberrant cytokine production that ultimately amplifies the oxidative stress in the β-cells in T1D (Haskins et al., 2003). Even in humans with T1D, markers of oxidative stress like lipid peroxides are upregulated, while levels of antioxidants like superoxide dismutase and glutathione peroxidase are reduced (Francescato et al., 2014;

McGrowder et al., 2013). All these studies suggest that like ER stress, oxidative stress is a critical factor involved in both the major forms of diabetes (Figure 6). Importantly, oxidative stress and ER stress are intertwined, and each can activate the other and contribute to diabetic development and progression (Cao and Kaufman, 2014; Hasnain et al., 2016). These stress factors are capable of damaging the β-cells themselves; however, studies show that they primarily mediate their effects by activating inflammation that amplifies the damage to the cells, as detailed in section 1.9 (Hasnain, 2018; Maamoun et al., 2019; Zhang, 2010).

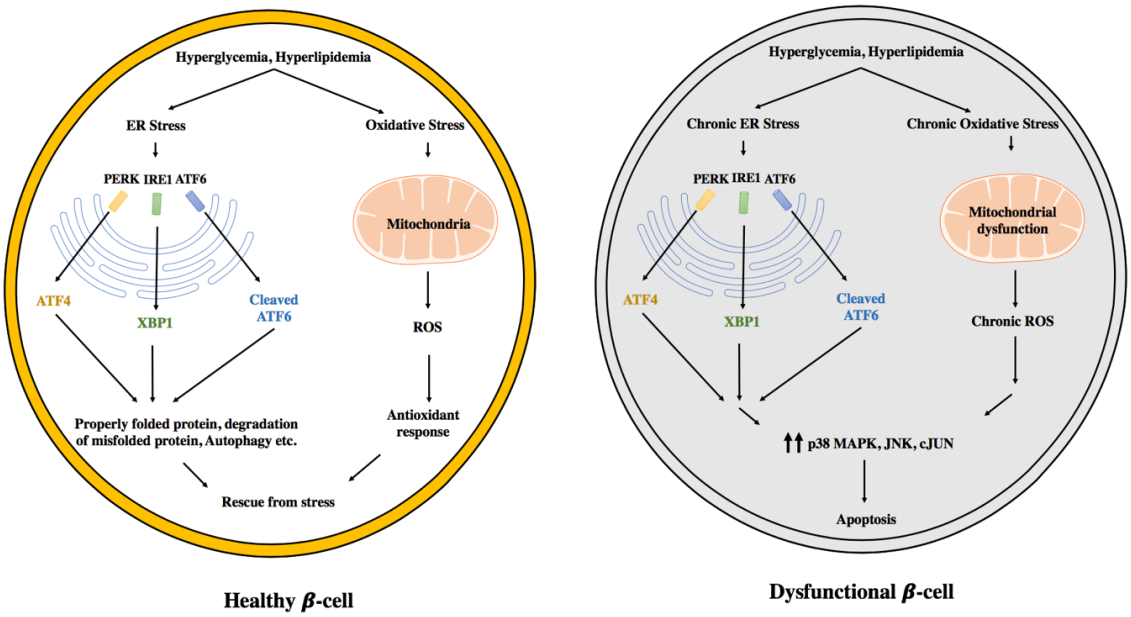


Figure 6: Cellular stress in the β-cells

1.9 Shared roles of inflammation in the pathogenesis of T1D and T2D:

Inflammation is a critical response of the body to various disruptive stimuli, including invasion of pathogens, dysfunctional cells, and injured tissues. It involves immune cells that ultimately clear the pathogens, remove the damaged cells, and start the repair of the injured tissues, as well as molecular mediators that amplify and resolve the response (Chen et al., 2017). Inflammation was initially considered to be only a part of the response to infectious diseases; but, later on, it has been associated with responses against non-infectious diseases as well (Hunter, 2012). When inflammation is limited and appropriate, it prevents disorders; however, maladaptive inflammation is often a trigger for the development and progression of the disease, including diabetes (Aroor et al., 2013).

The role of inflammation in T1D is well established. T1D is considered an autoimmune disorder in which the islets are inflamed by significant numbers of infiltrating immune cells, a condition known as insulitis (Tsalamandris et al., 2019). The development of T1D is dependent on T-cells, a class of immune cells that recognize non-self and regulate local and systemic responses. In the murine model of T1D, there is a failure of the central as well as peripheral tolerance, which results in the development of autoreactive T-cells that target antigens associated with the β-cell (Anderson and Bluestone, 2005). Moreover, studies show that by the adoptive transfer of T-cells from diabetic mice, there is an inflammatory response, which ultimately leads to peri-insulitis that is followed by the development of diabetes (Berry and Waldner, 2013; De Leenheer and Wong, 2016). Similar observations were obtained from the studies derived from the pancreas sections of T1D human samples. Severe defects in the regulatory Tregs accompanied by the participation of CD4+ and CD8+ T-cells in the attack of several β-cell autoantigens

contribute to the pathogenesis of T1D (Richardson et al., 2014). (Arif et al., 2014; Willcox et al., 2009).

Macrophages are another critical phagocytic immune cell type that promotes inflammation via the release of pro-inflammatory cytokines, and which can induce ROS in the β-cells (Tan et al., 2016). Additionally, dendritic cells, mast cells, and natural killer (NK) cells are also inflammatory immune cells detected in the immune infiltrate that contribute to the onset of autoimmunity (Lehuen et al., 2010). The major pro-inflammatory cytokines released by these immune cells include TNF-α, IL-1β, and IFN-γ. The receptors for these cytokines are found on β-cells and together these signaling pathways promote the induction of inducible nitric oxide synthase (iNOS) and ROS generation by the production of nitric oxide (Feuerer et al., 2009; Grunnet and Mandrup-Poulsen, 2011; Xiao et al., 2014a). Interestingly, the pro-inflammatory cytokines signaling can also trigger the NF-κB pathway, which feeds back to further promote the production of pro-inflammatory cytokines within β-cells. The autocrine signaling on the β-cells by these released cytokines can explain the exacerbated inflammation in T1D. The data supporting the mechanisms described above suggest that inflammatory responses are critical for the development and progression of T1D. In human studies, it has been well established that more than 90% of individuals with T1D exhibit the presence of specific autoantibodies that act as a trigger for autoimmune destruction of β-cells (Bingley, 2010). Moreover, in addition to autoantibodies, there are other inflammatory markers as well, which synergistically contribute to an inflammatory environment that promotes β-cell injury. First, genetic studies from subjects with T1D show an upregulated expression of inflammatory markers, including *IFIH1* and *TLR7/TLR8,* which are associated with immune responses to viral

infections (Barrett et al., 2009; Virgin and Todd, 2011). Next, subjects with T1D showed significantly higher expression of the inflammatory proteins IL-1α, IL-12p40, CCL2, CCL3, CCL4, TNF-α and IL-1β as compared to healthy individuals (Chen et al., 2014; Gordin et al., 2008; Hussain et al., 1998). Finally, a proteomics analysis of serum collected from individuals with T1D shows a protein signature that indicates the elevation of complement activation and inflammatory responses (Dogan et al., 2006; Zhang et al., 2013).

These and other studies indicate that inflammatory mechanisms are strongly associated with T1D pathogenesis. Confoundingly, anti-cytokine therapeutic approaches have not yet been successful in human trials. While anti-TNF-α showed some degree of β-cell protection, neither anti-IL-1β nor anti-IL-1R showed significant protection (Mastrandrea et al., 2009; Moran et al., 2013). Because inflammation plays a critical role in T1D pathogenesis, one explanation for this is that ideal targets have not been identified. Thus, for more effective T1D treatments, and for a lasting cure, novel targets of inflammation must be uncovered in order to counter the auto-immune attack of the β-cells.

T2D is characterized by insulin insufficiency and insulin resistance. Among the factors contributing to this disorder are oxidative stress, ER stress, glucotoxicity, lipotoxicity and amyloid deposition in the pancreas (Harding and Ron, 2002; Hull et al., 2004; Prentki and Nolan, 2006; Robertson et al., 2004; Weir and Bonner-Weir, 2004). Interestingly, each of these factors is associated with induction or exacerbation of inflammation (Donath et al., 2003, 2008; Ehses et al., 2009; Hotamisligil and Erbay, 2008; Masters et al., 2010). There is significant evidence that the immune system is not only critical in T1D, but also has a substantial role in T2D pathogenesis. As a result, T2D is

increasingly being viewed as an inflammatory condition, and immune system dysfunction is a focus of research in T2D pathogenesis. Interestingly, pancreatic sections from T2D patients show fibrosis, which is a marker of chronic inflammation. An in-depth cytokine profiling shows that there is a significant upregulation of pro-inflammatory cytokines, including TNF-α, IL-1β, IL-12, IL-6, IL-8, and IFN-γ in the T2D patients (Randeria et al., 2019; Spranger et al., 2003). Moreover, studies with mammalian models of T2D (high fat diet-fed mice, db/db mice, GK rat) as well as pancreatic sections of human T2D cases demonstrated that there is a presence of insulitis in the pancreatic islets, which is dominated by macrophages (Böni-Schnetzler et al., 2008; Donath et al., 2009; Ehses et al., 2007). Even before the onset of the clinical symptoms of T2D, the serum levels of IL-1β and IL-1R are higher in obese and pre-diabetic individuals (Carstensen et al., 2010; Meier et al., 2002). As discussed above, obesity is a major risk factor associated with T2D pathogenesis. The pro-inflammatory adipokines (TNF-α, IL-1β, and IL-6) that are released from adipose tissues in the setting of obesity contribute to a state of inflammation that ultimately exacerbates the pathogenesis of T2D. These adipokines activate the innate immune system and contribute to insulin resistance (Freitas Lima et al., 2015). Other factors contributing to inflammation in T2D include circulating free fatty acids, lipid acting enzymes like lipoxygenases and cyclooxygenases, and lipid mediators like eicosanoids, prostaglandins, and hepoxilins (Khan et al., 2014; Titos and Clària, 2013). Most of the well-known factors that contribute to inflammation mediate their effects via activation of two signaling pathways; IκB kinase-β (IKKβ) and c-Jun N-terminal kinase (JNK). The first of these, IKKβ, activates NF-κB, which is a transcription factor that induces the expression of the pro-inflammatory cytokines in the liver, adipose tissue, and muscles, which promotes

insulin resistance (Arkan et al., 2005; Cai et al., 2005). NF-κB is also activated in the islet β-cells in response to excessive glucose and pro-inflammatory cytokines (Maedler et al., 2002). Importantly, the depletion of NF-κB has been shown to have protective effects on the β-cells (Eldor et al., 2006). The other major pathway that gets activated is JNK, a kinase that, in turn, activates transcription factors such as ELK1 (ETS Like-1 protein), ATF2 (activating transcription factor 2), and c-Jun. JNK activation has different effects depending on the cell type. In adipose tissues and muscles, JNK directly phosphorylates IRS-1 and IRS-2. This results in reduced PI3K-AKT signaling in response to insulin and increased insulin resistance. In macrophages, JNK activation promotes a pro-inflammatory phenotype. In the liver, JNK stimulates ketogenesis and reduces fatty acid oxidation that leads to fatty liver and insulin resistance (Donath and Shoelson, 2011; Solinas and Becattini, 2016). These cytokines also activate circulating immune cells and affect the vasculature of the cardiac muscles, skeletal muscles, retina, and kidney; this further contributes to other diabetes-associated complications (King, 2008). Another source of the pro-inflammatory cytokine is the human islet amyloid polypeptide (hIAPP). hIAPP promotes the secretion of IL-1β from the islet infiltrating macrophages via activation of inflammasomes (Eguchi and Nagai, 2017). Inflammasomes are cytosolic multiprotein oligomers which are responsible for the activation of inflammatory responses (Broz and Dixit, 2016; Mariathasan et al., 2004). The secreted IL-1β exerts its effects on β-cells via the activation of NF-κB pathway to induce apoptosis (Westwell-Roper et al., 2013, 2014). On the other hand, inflammasome activation within the β-cells itself promotes apoptosis via activation of caspase-1 (Montane and Novials, 2016; Wali et al., 2013). In summary,

multiple factors promote inflammation and contribute to T2D pathogenesis via common downstream pathways (Figure 7).

Subjects with T2D show improved glycemic control and insulin sensitivity upon blocking NF-κB signaling using the non-steroidal anti-inflammatory drug, salsalate; however, these studies were performed over a limited duration, and hence the long-term effects of NF-κB inhibition are unclear. Although promising, the chronic inhibition of this critical transcription factor may be complicated as it will drive more widespread immune suppression, rendering susceptibility to other infections and diseases. (Fleischman et al., 2008; Goldfine et al., 2013). Other approaches have focused on inhibiting the signaling of pro-inflammatory cytokines like TNF-α and IL-1β. Whereas TNF-α receptor antagonists failed to improve blood glucose, IL-1β receptor antagonists showed a temporary improvement in T2D patients, which faded after 39 weeks of trial (Dominguez et al., 2005; Larsen et al., 2009; Paquot et al., 2000). These studies suggest that targeting inflammation can indeed improve the symptoms of T2D, though more effective strategies are needed.

In conclusion, inflammation seems to be the common factor in the pathogenesis of both the major forms of diabetes. To date, therapeutic approaches focused around inhibiting the pro-inflammatory cytokines have not been hugely successful; however, targeting inflammation definitely shows immense potential for treating diabetes. One of the strategies could be identifying novel targets of inflammation that could modulate the inflammatory environment or control the recruitment of immune cells that mediate the dysfunction or damage to the β-cells.

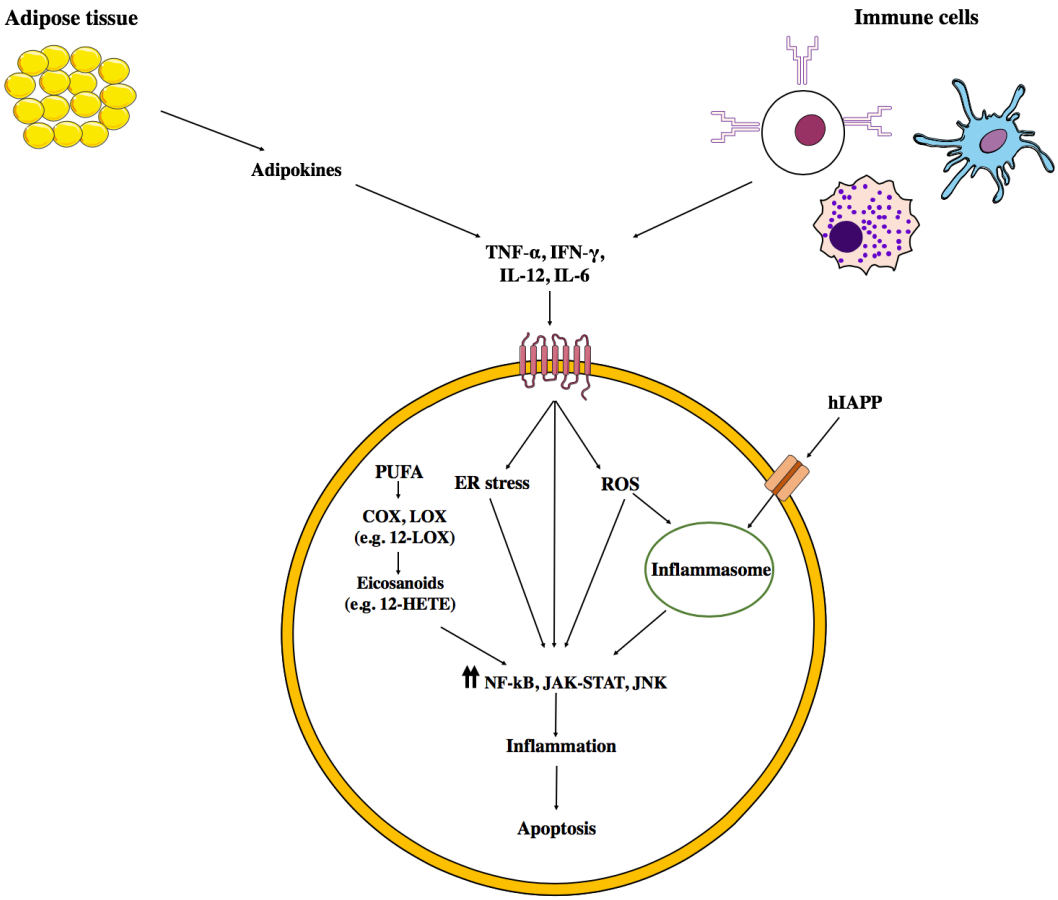


Figure 7: Activation of inflammatory signaling pathways in β-cells

1.10 Macrophage function in islet inflammation:

Macrophages are immune cells that are derived from myeloid progenitor cells. They have a critical role in innate immune responses, including pathogen recognition and elimination, detection of damaged or dead cells and their elimination, and regulating tissue repair (Gordon and Martinez-Pomares, 2017). Macrophages are highly plastic cells that can differentiate into different subtypes dependent on microenvironmental stimuli in a process called polarization. Classically, undifferentiated macrophages (M0 phenotype) get activated, or polarized, to pro-inflammatory (M1) or anti-inflammatory (M2) phenotypes. This nomenclature is analogous to the two subtypes of T-helper (Th) cells (Th1 and Th2) that have similar properties and activation states (Mantovani et al., 2004). M2 macrophages have been further subcategorized into M2a, M2b, M2c, and M2d types based on differences in their mode of activation and their ultimate function (Yao et al., 2019). However, it is important to understand macrophages often co-express markers of both polarization states at varying levels, and this is dependent on the stimuli. In other words, macrophage polarization could best be considered to be a spectrum of activation states/phenotypes, rather than three discrete states (Murray, 2017; Xue et al., 2014). However, a major factor that differentiates macrophages at opposite ends of the M1/M2 is their metabolism of arginine; while M1 macrophages metabolize arginine via NOS2 to produce nitric oxide, the M2 macrophages metabolize arginine via ARG1 to produce polyamines that are necessary for collagen synthesis and cellular proliferation (Mantovani et al., 2013). For clarity in this thesis, I have used the simpler, more classical differentiation of these macrophage phenotypes, i.e., M1 and M2 (Figure 8).

Specifically, M1 macrophages are activated by pro-inflammatory stimuli like IFN-γ and TNF-α or bacterial lipopolysaccharide (LPS) and are involved in Th1 responses. Upon their stimulation, M1 macrophages express typical markers like TLR-2 (Toll-like receptor-2), TLR-4, CD80 (Cluster of differentiation 80), CD86, iNOS, and MHC-II. They release cytokines and chemokines like TNF-α, IL-1α, IL-1β, IL-6, IL-12, CXCL9 (CXC chemokine ligand 9), and CXCL10 that usually leads to pro-inflammatory environment which attracts and activates more unpolarized (M0) macrophages promoting inflammation. The key proteins and pathways that contribute to M1 polarization include NF-κB, STAT1 (Signal Transducer and Activator of Transcription 1), STAT5, IRF3 (Interferon Regulatory Factor 3) and IRF5 signaling. The major roles of M1 macrophages include promoting inflammation, tissue injury, and killing the microbes and tumor cells (Martinez and Gordon, 2014; Porta et al., 2015; Wang et al., 2014b).

On the other hand, M2 macrophages are activated by anti-inflammatory stimuli like IL-4 and IL-13 and are involved in Th2 responses. M2 macrophages express markers like Arg-1 (Arginase 1), CD206, CD163, CD209, FIZZ1 (Resistin-like molecule alpha or found in inflammatory zone protein 1), and CHI3L1/2 (Chitinase-3-like protein-1 or Ym1/2). These macrophage release cytokines and chemokines like IL-10, TGF-β (Tumor growth factor-β), CCL1 [Chemokine (C-C motif) ligands 1], CCL17, CCL18, CCL22, and CCL24 which promote an anti-inflammatory environment, attract more M0 macrophages, and promote their polarization toward the M2 phenotype. STAT6, IRF4, PPARδ (peroxisome proliferator-activated receptor delta), and PPARγ are the key proteins and pathways that promote M2 polarization. The critical roles that M2 are involved in include anti-

inflammatory responses, infection prevention, phagocytosis, and assistance in tissue remodeling and repair (Mantovani et al., 2004, 2013; Mulder et al., 2014; Murray, 2017).

However, an imbalance in these activation states of macrophages has been associated with several diseases, including not only diabetes, but also atherosclerosis, tumor, asthma, sepsis, rheumatoid arthritis, and obesity (Atri et al., 2018; Liu et al., 2014b; Parisi et al., 2018). Germane to diabetes pathogenesis, macrophages have been implicated in β-cell development as well as their dysfunction and death. Studies have demonstrated that during early murine pancreatic development, macrophages are recruited to the branching ductal epithelial tissue. Specifically, they are localized to sites where the Islets of Langerhans bud from the ducts (Geutskens et al., 2005). Early macrophages are critical for normal islet development, as their loss in CSF1 (colony-stimulating factor 1) knockout mice results in a severe deficiency in β-cell mass in the developing and adult pancreas (Banaei-Bouchareb et al., 2004). Moreover, these mutants show abnormal islet morphogenesis after birth and impaired proliferation of islet cells. Furthermore, the injection of exogenous CSF-1 rescues this phenotype, restoring the β-cell mass in accord with increasing macrophages (Geutskens et al., 2005). These studies show a critical role of macrophages in the initial development of β-cells as well as maintenance of the morphology and the β-cell mass.

However, macrophages can also be deleterious to β-cells, contributing to the pathogenesis of diabetes. As discussed earlier, T1D is characterized by inflammation and is associated β-cell destruction. Macrophages are crucial immune cells that locally to regulate inflammation depending on their different polarization states (Davis Frank M. and Gallagher Katherine A., 2019). Importantly, macrophages are among the first innate

immune cells to infiltrate damaged or infected tissues, where they further activate adaptive immune cells. Thus macrophages represent a critical link between the innate and adaptive immune systems (Underhill et al., 1999). As such, macrophages are positioned to be major factors in the pathogenesis of T1D. In the murine T1D NOD model, macrophages, together with T-cells, are among the first cells to infiltrate the islets. These macrophages present β-cell autoantigens via MHC-II (Herold et al., 2013; O'Reilly et al., 1991). Interestingly, clodronate-mediated depletion of macrophages is associated with reduced insulitis and inflammation in NOD mice and protection from the development of diabetes. (Calderon et al., 2006; Jun et al., 1999). Recent studies report that islet resident macrophages initiate the cascade of events that ultimately damage the β-cells. In accordance, a study showed that genetic depletion of islet resident macrophages by CSF-1R knockout protects the NOD mice from diabetes development (Carrero et al., 2017). Concordantly, while CD8+ T-cells are the predominant cell type found in islet infiltrates in T1D patients, the macrophages make up a significant cellular fraction, as compared to other cell types; suggesting that T-cells and macrophages are key players in the death of pancreatic β-cells (Willcox et al., 2009).

As discussed above, macrophages release pro-inflammatory cytokines that increase oxidative stress and apoptosis in the β-cells via the activation of NF-κB and STAT pathways. Furthermore, monocytes and macrophages from T1D patients also promote the proliferation of Th17 cells, a set of pro-inflammatory T-helper cells, by release of IL-1β and IL-6, thereby promoting the pro-inflammatory and pro-apoptotic responses (Emamaullee et al., 2009; Shao et al., 2012). The neutralization of IL-17, a major effector cytokine release by Th17, protected NOD mice from the development of diabetes

(Emamaullee et al., 2009). Together these studies have demonstrated that macrophages are key players that contribute to diabetes pathogenesis not only by themselves but also by activating cytotoxic cells of the adaptive immune system, exacerbating inflammation, and generating a milieu that is conducive for maximal β-cell damage.

The role of the immune system has been studied less extensively in T2D than in T1D. However, recent work demonstrates that immune cells have critical roles in the development and progression of T2D as well (Grossmann et al., 2015; Shu et al., 2012; Zhou et al., 2018). Specifically, macrophages infiltrate the islets in large numbers and promote inflammation, both in T2D models and in human subjects with T2D and pre-diabetes (Ehses et al., 2007; Niu et al., 2016; Walker et al., 2018). In the former, for example, high-fat diet (HFD)-fed mice develop obesity-induced T2D. Moreover, the state of increased free fatty acids promoted the release of chemokines from β-cells that could attract M1 macrophages, driving their accumulation in the islets in large numbers. Furthermore, depletion of M1 macrophages protected these mice from lipotoxicity mediated β-cell dysfunction (Eguchi et al., 2012). Similarly, HFD is also associated with increased levels of the receptors TLR2 and TLR4 in humans and mammalian models. Both TLRs are associated with M1 macrophages that stimulate the release of IL-1β and IL-6, cytokines that foster β-cell dysfunction (Nackiewicz et al., 2014). Macrophages are also critical components of the inflammation in adipose tissue that results from obesity. These ultimately contribute to the release of pro-inflammatory adipokines that cause β-cell insult (Appari et al., 2018; Lumeng et al., 2007a). The role of macrophages in adipose is certain to be complex, as studies have established that acute macrophage accumulation in the

adipose tissues promotes insulin sensitivity, but chronic adipose inflammation leads to insulin resistance (Lumeng et al., 2007b; Wernstedt Asterholm et al., 2014).

M1 polarized macrophages exert deleterious effects due to their pro-inflammatory contributions in disease pathogenesis. However, macrophages are highly dynamic cells that also have an anti-inflammatory M2 state, which can promote tissue remodeling and repair (Arnold et al., 2007; Duffield et al., 2005; Lee et al., 2011; Lucas et al., 2010). M2 macrophages may release growth factors including vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), epidermal growth factor (EGF) and transforming growth factor-beta (TGF-β) that promote tissue regeneration and repair (Mantovani et al., 2013; Stefater et al., 2011). The role of M2 macrophages has also been studied specifically in pancreatic tissue repair. In the human pancreas, the infiltration of M2 macrophages has been associated with increased β-cell proliferation (In't Veld et al., 2010). Furthermore, in murine models, macrophages promote islet angiogenesis and protect against islet loss in certain models of chronic inflammation and exocrine pancreas degeneration. The depletion of macrophages in a model of pancreatitis leads to diabetes due to loss of endocrine cells, thus, emphasizing the critical role of these cells in the maintenance of the endocrine pancreas during the chronic inflammatory state (Tessem et al., 2008). Macrophages recruited to the site of β-cell injury release different growth factors (VEFG, PDGF, EGF, and TGF-β) that promote β-cell proliferation as well as β-cell regeneration (Brissova et al., 2014; Riley et al., 2015; Van Gassen et al., 2015). Additionally, TGF-β release by M2 macrophages promotes β-cell proliferation by activation of SMAD7, which increases the levels of cell-cycle activators CyclinD1 and CyclinD2 (Xiao et al., 2014b).

In conclusion, the interactions between macrophages and β-cells are quite dynamic. Dependent on the M1 or M2 activation state of the macrophages; it can either be deleterious or beneficial. These differences in the functionality of the macrophages certainly makes them interesting targets for the treatment of both the major forms of diabetes. The global depletion of macrophages likely would not be a feasible approach, as it can lead to severe immunosuppression and to the risk of secondary infections. However, two major alternative strategies for targeting the macrophages could include : i) identifying targets that could skew the polarization of the macrophages towards M2 phenotype to promote their anti-inflammatory and protective effects on β-cells, and ii) identifying targets that could prevent macrophage infiltration in the islets and thus could prevent the vicious self-reinforcing cycle of pancreatic islet inflammation altogether.

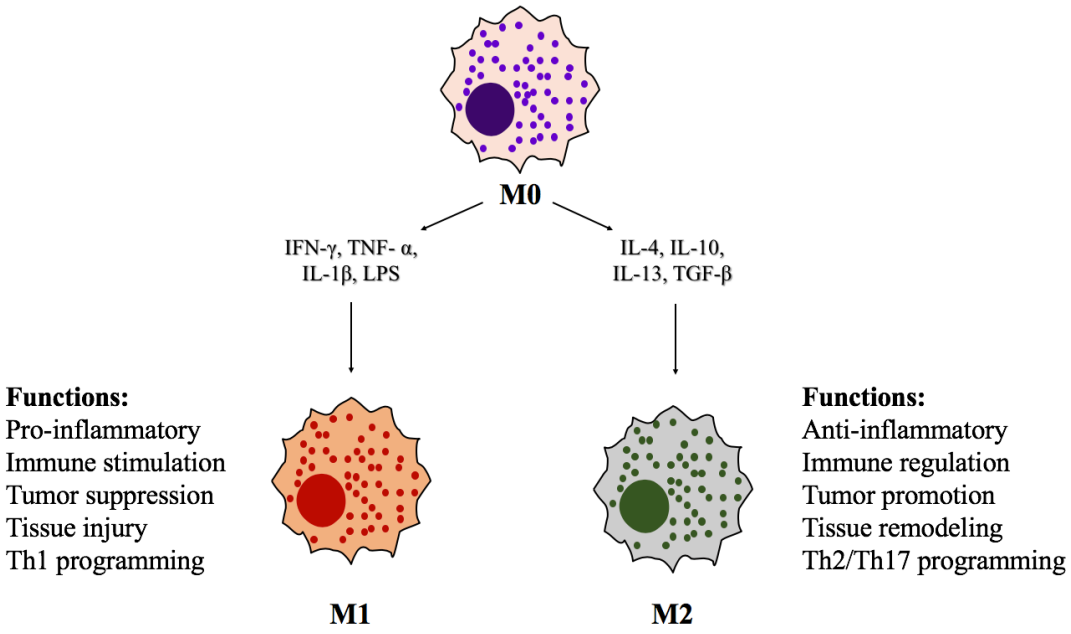


Figure 8: Macrophage polarization

1.11 The role of 12-lipoxygenase in inflammation:

Lipoxygenases (LOXs) are a set of enzymes that catalyze the oxygenation of cellular polyunsaturated fatty acids (PUFAs) to form metabolites that function in autocrine, paracrine, or endocrine pathways (Imig and Hye Khan, 2015; Piomelli, 1993). These are non-heme containing enzymes that begin the stereospecific insertion of molecular oxygen to a cis,cis-1,4-pentadiene moiety of the PUFA through a free radical mechanism. For complete activation of these LOX enzymes, the iron in the LOX active site is oxidized from the inactive ferrous (Fe^{2+}) state to the active ferric (Fe^{3+}) state. Consequently, LOX activity is regulated by the cellular hydroperoxides, the heterolytic cleavage of which provides the necessary electron acceptor to reduce the active-site iron (Haeggström and Funk, 2011). Following the acquisition of a substrate, the LOX extracts allylic hydrogen, forming a carbon-centered radical and reducing the active-site iron back to the ferrous state. This radical then reacts with molecular oxygen, forming a peroxy-radical that oxidizes the iron, forming a peroxy-anion. Finally, the peroxy-anion is protonated, creating fatty acid hydroperoxide (Glickman and Klinman, 1996). However, these intermediate products are unstable and toxic to the cells. Hence, cellular machinery utilizes glutathione peroxidases to form stable products that mediate the effects of PUFA metabolism (Brütsch et al., 2015; Czapski et al., 2016).

The LOX enzymes are involved in the metabolism of multiple PUFA substrates, including arachidonic acid (AA), dihomo-γ-linoleic acid (DLA), α-linolenic acid (ALA) docosahexaenoic acid (DHA), and eicosatetraenoic acid (EPA) (Figure 9) (Ikei et al., 2012). The nomenclature of LOXs is based on the location at which they insert the oxygen on their substrate. Thus, 12-LOX catalyzes the oxygenation of the 12^{th} carbon of its

substrate (Ding et al., 2003; Yamamoto, 1992). 12-LOX has been known to exert the majority of its known effects via arachidonic acid metabolism. 12-lipoxygenase (12-LOX) converts arachidonic acid to 12-hydroperoxy-eicosatetraenoate (12-HPETE), which subsequently gets reduced to a more stable 12-hydroxy-eicosatetraenoate (12-HETE) by glutathione peroxidase (Davies and Guo, 2014; Dobrian et al., 2011; Haeggström and Funk, 2011).

In mice, there are seven functional LOX genes (*Alox5, Alox12, Alox12b, Alox15, Alox15b, Aloxe3, Aloxe12*), three or four functional genes in zebrafish (*alox5a, alox5b, alox12*, and a possible *alox15* orthologue) and six functional genes in humans (*ALOX5, ALOX12, ALOX12B, ALOX15, ALOX15B, and ALOXE3*). The genomic distribution of the LOX genes is varied across species. In mice, *Alox5* is located on chromosome 6, while all others are found on chromosome 11 (Yamamoto, 1992). Similarly, in humans, ALOX5 is located on chromosome 10, while the rest of the LOX genes are clustered in chromosome 17 (Krieg et al., 2001). In zebrafish, *alox12* is located on chromosome 7, *alox5a* on chromosome 13, while *alox5b* on chromosome 15. These genes encode for different lipoxygenases; however, in this study, I have focused on the genes that encode for 12-lipoxygenase, as both 12-LOX and 12-HETE have been implicated in the pathogenesis of different diseases, including diabetes, by promoting oxidative stress and inflammation. However, 12-LOX also acts on other substrates, like linoleic acid and docosahexaenoic acid, to generate other eicosanoids that are involved in regulating inflammation (Ackermann et al., 2017).

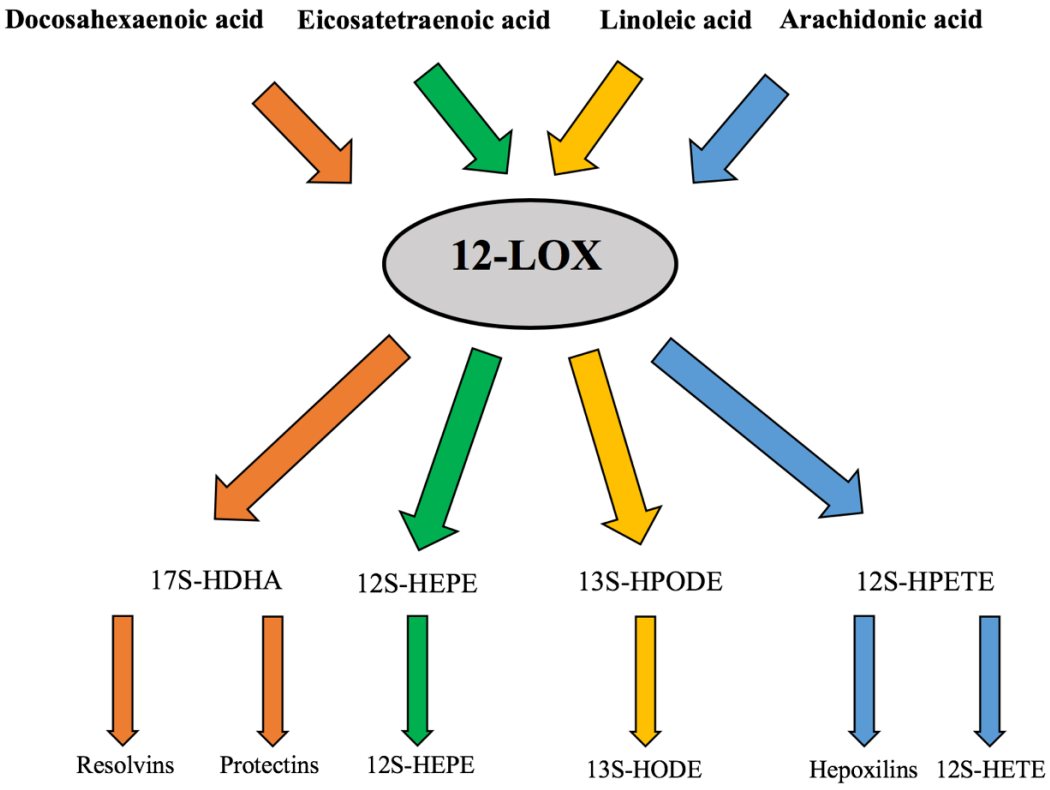


Figure 9: Substrates and products of 12-LOX

12-HETE, the product of 12-LOX resulting from arachidonic acid metabolism, is a lipid molecule that can easily transit through cell membranes and induce its effects. Intracellularly, 12-HETE generation promotes oxidative stress while extracellularly via interaction with the orphan G protein-coupled receptor 31 (GPR31). 12-HETE can impact a variety of signaling pathways (Guo et al., 2011; Porro et al., 2014). To study the effects of 12-LOX and 12-HETE, it is important to understand the differences in the regulation of 12-LOX and the production of 12-HETE amongst different species in order to appropriate animal models for elucidating disease mechanisms. The mouse enzyme, encoded by the gene *Alox15*, is typically referred to as "12/15-lipoxygenase" because it produces a ~6:1 ratio of 12-HETE:15-HETE from arachidonic acid. Interestingly, while the orthologous mouse gene *Alox12* encodes 12-lipoxygenase, the levels of 12-HETE produced by 12-lipoxygenase is lower than that produced by 12/15-lipoxygenase (Conteh et al., 2019). Hence, the focus of this thesis project was only the *Alox15* encoding lipoxygenase enzyme. Based on their tissue distribution and products generated, the mouse 12/15-lipoxygenase enzyme encoded by *Alox15*, and zebrafish 12-lipoxygenase enzyme encoded by *alox12* are the functional homologs of the human 12-lipoxygenase enzyme that is encoded by *ALOX12* (Haas et al., 2011; Jisaka et al., 2000). Henceforth, I will be referring all these enzyme homologs as 12-LOX.

12-LOX activity and 12-HETE levels have been linked to the pathogenesis of both of the major forms of diabetes. In the T1D mouse model (NOD mice), genetic knockout of *Alox15* shows protective effects against the development of diabetes. The NOD mice normally show peak levels of 12-LOX expression during the insulitis stage; however, in the absence of 12-LOX, knockouts showed reduced insulitis and maintenance of β-cell

mass (Green-Mitchell et al., 2013). Furthermore, knockout of *Alox15* in mice on a C57BL/6 background also protects them from hyperglycemia and β-cell loss following a diabetogenic multiple low dose streptozotocin (MLD-STZ) treatment (Bleich et al., 1999). Interestingly, protection from diabetes post-MLD-STZ was also seen in mice with a pancreas-specific knockout of *Alox15* (Tersey et al., 2014). These data suggest that the effects of 12-LOX in T1D diabetes pathogenesis are, at least, partly intrinsic to islets. Mechanistically, it appears to be an inflammation-mediated effect of 12-LOX that contributes to the β-cell insult. Concordant studies with human islets, murine islets, and the rodent β-cell lines each demonstrate that treatment with pro-inflammatory cytokines increases levels of 12-LOX and 12-HETE (Chen et al., 2005; Ma et al., 2010). Moreover, islet dysfunction is likely to be mediated at least in part by the actions of 12-HETE, as human islets treated with 12-HETE show reduced glucose-stimulated insulin secretion at lower concentrations (1nM) and induction n of cell death at higher concentrations (100nM) (Ma et al., 2010).

The role of 12-LOX has also been studied in the context of T2D and obesity. For example, when *Alox15* null mice are fed high-fat diet (45% or 60% kcal from saturated fat), they do not show impaired glucose or insulin tolerance whereas wild-type (WT) mice did. Moreover, the levels of circulating pro-inflammatory cytokines were lower in the *Alox15* mutant mice. Also, the pancreatic islets of these *Alox15* mutant mice did not exhibit hyperplasia, as is seen in WT mice fed with a high-fat diet. Finally, the *Alox15* null mice showed a reduced macrophage-mediated inflammation of adipose tissues (Nunemaker et al., 2008; Sears et al., 2009). Another study has demonstrated that 12-LOX levels are elevated under conditions of hyperglycemia (Natarajan et al., 1993).

Mechanistically, 12-HETE exerts its detrimental effects by the activation of NADPH oxidase-1 (NOX-1), which promotes oxidative stress in studies of both mouse and human islets (Weaver et al., 2012). This oxidative stress response to 12-HETE was further corroborated by studies in pancreas-specific *Alox15* knockout mice that were fed a high-fat diet. These mice showed elevated levels of the antioxidant enzymes, superoxide dismutase, and glutathione peroxidase, which are crucial for ameliorating oxidative stress (Tersey et al., 2014). Altogether, these studies establish the crucial roles that 12-LOX plays in the pathogenesis of both the major forms of diabetes. These studies have also propelled the field towards the development of small-molecule inhibitors against 12-LOX as a therapeutic approach. Out of several inhibitors, ML351 and ML355 have been shown to be potent and selective lipoxygenase inhibitors. More specifically, ML355 shows specificity for human 12-LOX, whereas ML351 shows higher specificity for murine 12-LOX (Adili et al., 2017; Luci et al., 2010; Rai et al., 2010). Recently, our lab demonstrated the utility of these inhibitors for T1D pathogenesis studies. In NOD mice, the administration of ML351 during the prediabetic phase prevented glycemic deterioration, reduced β-cell oxidative stress, and increased the fraction of anti-inflammatory immune cells in insulitis (Hernandez-Perez et al., 2017). Importantly, the treatment of human islets with pro-inflammatory cytokines, together with ML355, showed suppression of ROS generation as compared to vehicle-treated islets. These studies suggest that, indeed, these enzymes can be targeted specifically and efficaciously with these inhibitors.

The exact molecular mechanisms by which 12-LOX promotes β-cell dysfunction and death remain unclear. However, based on the positive association of stress and inflammation markers in the presence of 12-LOX or 12-HETE, some mechanisms working

with the β-cells themselves are possible. On the other hand, some studies have reported that these same markers could be detected at higher levels due to the presence of immune cells, including macrophages, which are well known to mediate these effects. Both 12-LOX and macrophages are factors that promote inflammation. Hence, I hypothesized that 12-LOX is a crucial factor in the pathogenesis of diabetes, whose effects are not specific only to β-cells but also in macrophages where it is highly expressed (Wuest et al., 2012). Thus, in addition to regulating β-cell function and eliciting oxidative responses, 12-LOX is likely influencing macrophage activity to promote pancreatic islet inflammation in parallel.

1.12 Model systems for studying diabetes:

Diabetes is a systemic disorder characterized by hyperglycemia that occurs due to pancreatic insulin deficiency and insulin resistance. Many studies have been conducted on cultured cell lines derived from β-cells, like Ins1 (rat insulinoma cells) or Min6 (mouse insulinoma cells); these cells share properties with β-cells, including a high insulin content and responsiveness to glucose (Asfari et al., 1992; Miyazaki et al., 1990). Although these cell lines have provided valuable insights into β-cell function, confoundingly, they are immortalized, highly proliferative, and cultured out of context; as such, the cell lines have many physiological properties that differ from endogenous β-cells. This is further reflected in differences of gene and protein expression relative to endogenous β-cells (Skelin et al., 2010). Similarly, countless studies have been performed with *in vitro* cultured islets isolated from mouse, rat, or human donors. Cultured islet studies have several advantages over cell culture studies. Firstly, these circumvent the intrinsic differences observed due to the transformation of cells. Secondly, primary tissues are better models to test some pharmacological properties of chemicals and drugs, as it can reflect the direct effects of the respective treatments (Goldbard, 2006). Finally, since β-cells in the islets remain closely associated with other islet cell types, and thus maintain any potentially important physiological paracrine interactions that collectively influence glucose metabolism (Göke, 2008). Specifically, using human islets is likely to provide a better picture of the responses of human β-cells to various stimuli and treatments that will be directly relevant from a clinical perspective. However, using cultured islets still has important limitations. First, and most importantly, the major disadvantage of *in vitro* studies with islets is the absence of complex physiological setting in which islets function. Islets are highly vascularized and

respond to different microenvironmental factors like growth factors, hormones, and cytokines (Aamodt and Powers, 2017). Moreover, there is also a crosstalk of the islets cells with other cell types, including endothelial cells and immune cells, which is a critical component of islet health and responses (Shirakawa et al., 2017). Furthermore, in diabetes pathogenesis, there are other complex influencing factors. For example, as described above, there are environmental factors that can increase the risk of T1D, and obesity-induced adipokine release which influences the development of T2D. It is extremely challenging to mimic all these complex factors that contribute to disease development in an *in vitro* setting, thus making *in vivo* studies critical for studying diabetes holistically.

Animal models have long played a critical role in the study of diabetes, for exploration and characterization of pathogenesis as well as identification of novel therapeutic targets. Various animal models have been developed for both T1D and T2D that induce hyperglycemia by mimicking human pathology. The major characteristics of T1D include autoimmune destruction of β-cells that contribute to hyperglycemia. In animal models, this hyperglycemia is commonly achieved by either the ablation of β-cells or by breeding animals that spontaneously develop diabetes. For ablation studies, diabetes is usually induced around 5–7 days prior to the start of the experiment to ensure stable hyperglycemia. In the ablation models of T1D, most β-cells are destroyed, resulting in lower insulin production that ultimately leads to leading to hyperglycemia. Two major chemical compounds that are commonly used to induce diabetes are streptozotocin (STZ) and alloxan. STZ and alloxan have a chemical structure analogous to glucose and thus are able to be transported into the β-cell specifically by the GLUT2 transporter. This confers a high degree of β-cell-specific uptake of these compounds (Damasceno et al., 2014;

Szkudelski, 2001). Once STZ enters the β-cell, it is transported to the nucleus where it causes DNA alkylation. The generation of free radicals due to STZ can cause DNA damage and apoptosis of β-cells. STZ is administered as a single high dose (100-200 mg/kg) or multiple low doses (20-55 mg/kg) depending on the study interests (Dekel et al., 2009; Wang and Gleichmann, 1998). Similarly, alloxan uptake by the β-cells causes the generation of free radicals that mediate the diabetic effects. Alloxan is reduced to dialuric acid and then re-oxidized back to alloxan, creating a redox cycle that generates highly reactive hydroxyl radicals that cause fragmentation of DNA. Alloxan is administered in mice at a dose concentration of 50-200 mg/kg. Although there is a destruction of β-cells by chemical methods, it is not mediated by the immune cells, as seen in humans with T1D.

The two widely utilized mammalian models of spontaneous diabetes development include non-obese diabetic (NOD) mice and Biobreeding (BB) rats. Firstly, NOD mice have polymorphisms in several genes that have also been observed in human T1D patients, like *MHC-II* and *CTLA-4* (Hanafusa et al., 1994; Serreze and Leiter, 1994). NOD mice develop insulitis around 4-5 weeks of age. In this pre-diabetic stage, cytotoxic CD8+ T-cells, CD4+ T-cells, and macrophages are the predominant immune cell types that constitute the infiltrate, with lower proportions of B-cells and NK cells (Anderson and Bluestone, 2005; Yoon and Jun, 2001). Although these infiltrated immune cells wreak havoc on β-cells at this stage, hyperglycemia is not seen until most β-cell have lost function at around 10-14 weeks. NOD mice are not only useful for testing drugs or chemical treatments, but also, they can be genetically modified; by performing genetic knockouts or knockins. The possibility of the generation of 'humanized' mice, provide a sophisticated system for modeling human T1D pathology in a physiological context (King et al., 2008;

Niens et al., 2011; Yang and Santamaria, 2003). Secondly, BB rats are another extensively used mammalian model of T1D. These rats have mutations in the MHC-II *RT1u* haplotype and the *Gimap5* gene. Although these two genetic loci are not directly similar to the human loci, their physiological effects of the mutations reproduce aspects of the human T1D pathology (Colle et al., 1981; Yale et al., 1985). BB rats develop diabetes at the age of 8-16 weeks with a characteristic development of insulitis; however, there is a major difference in the composition of the infiltrate, as compared to NOD mice. Due to mutation in the *Gimap5* gene, there is severe T-cell lymphopenia, and the immune infiltrate in these rats is dominated by macrophages, B-cells, and NK cells (Mordes et al., 2004). BB rats have been extremely valuable in elucidating more about the genetics of T1D (Wallis et al., 2009).

T2D animals have characteristic insulin resistance and relative insulin deficiency due to β-cell insufficiency. Many animal models are based on obesity, as that is a major factor contributing to T2D. Three major T2D models are high-fat diet (HFD) fed mice, leptin-deficient (ob/ob) mice, and leptin receptor-deficient (db/db) mice (Rees and Alcolado, 2005). First, in the HFD model, the normal chow diet (26% protein, 63% carbohydrate, and 11% fat) is exchanged for a diet where the fraction of calories from fat is increased substantially (40-60% from saturated fat). As a result of this, HFD-fed mice develop obesity, hyperinsulinemia, and impaired glucose homeostasis. The weight gain is often associated with hyperglycemia, insulin resistance, and impaired glucose tolerance due to the dysfunction of β-cells (Winzell and Ahrén, 2004). HFD models may be used in the context of transgenic or genetic knockout backgrounds. These can often show a normal phenotype under unchallenged conditions; however, in the presence of the HFD, when the

β-cells are forced to function at a higher level, the role of the gene or protein might become evident. The other two commonly used animal models of obesity-induced T2D, target the leptin signaling system. Leptin is a hormone that primarily induces satiety, hence lack of leptin signaling promotes polyphagia, which is a hallmark of diabetes; and this ultimately leads to obesity (Wang et al., 2014a). The two models based in leptin signaling are leptin-deficient (ob/ob) mice and leptin receptor-deficient (db/db) mice. The ob/ob mice have a mutated leptin protein that leads to the pathology (Zhang et al., 1994). These mice show increased weight, which accompanies hyperinsulinemia within two weeks of age. By four weeks of age, ob/ob mice develop hyperglycemia and hyperlipidemia that stays above limits for the rest of their lives (Lindström, 2007). Alternately, the db/db mice are characterized by a mutation in the leptin receptor (Chen et al., 1996). As with ob/ob mice, the db/db mice develop obesity and hyperinsulinemia at 2-4 weeks of age, while after 4-8 weeks, they show hyperglycemia. In contrast, db/db mice develop ketosis after a few months and have a relatively shorter lifespan as compared to ob/ob mice (Srinivasan and Ramarao, 2007).

There are rodent models of non-obese T2D as well, of which GK rats are the most widely used animal models. Goto–Kakizaki (GK) rats were generated by selective repetitive breeding of rats that exhibited the most reduced glucose tolerance (Goto et al., 1976). These animals are lean models of T2D, which is seen in ~20% of T2D human cases as well (George et al., 2015). They have impaired glucose tolerance and defective insulin secretion in response to glucose due to abnormal β-cell mass and function (Ostenson and Efendic, 2007; Portha et al., 2001). GK rats have been utilized as an invaluable tool for

studying β-cell dysfunction as well as diabetic complications (Dolz et al., 2011; King, 2012; Movassat et al., 2007; Okada et al., 2010).

Although these rodent models have proved to be excellent animal models for diabetes research, they have several important limitations that need to be considered. Currently, the majority of β-cell and islet studies in rodents depend on pancreas sections or isolated islets to analyze the cellular aspects of their pathology, often retrospectively. However, factors like free radicals and ROS are extremely transient and difficult to measure in an *ex vivo* setting. Moreover, there are particular physiological features, like immune infiltration and responses of β-cells to different stimuli that cannot be easily determined by staining the pancreatic sections. Advances in microscopic methods, like intravital imaging, have made it possible to visualize the islet and address some cellular physiology questions *in vivo,* thus circumventing some of these limitations (Reissaus et al., 2019). However, these present other challenges, including the difficulties of surgery to place imaging windows, the lower survival rate of the mice over a longer period, dependency on biosensors to mark the β-cells and the proteins of interest, and the overall expense of the experiment. Nevertheless, it represents a productive method to study islet physiology and responses *in vivo* in a mammalian model. There are other limitations of the existing rodent models as well. Due to the metabolic complexity of diabetes, the power of experiments is often lower, and thus, the experiments are repeated several times for accuracy and detection of a meaningful difference. Hence, there is a constant need for mice, which is hindered due to fecundity and maturation time. Wildtype mice generally produce litters of 1-10 pups and can only bear approximately 4-6 litters in their lifetime. Moreover, it takes about 2-3 months to have a new batch of pups, which require genotyping if they

are transgenic. In the case of transgenic mice, the homozygosity of alleles is often preferred, which further reduces the number of usable mice per litter. Furthermore, the development of transgenic mice is extremely challenging as injections of the RNA or DNA for transgenesis require extraction of mouse embryos by sacrificing the mouse as *in vivo* manipulation of these embryos is almost impossible. Next, to keep the embryos alive after fertilizing or injecting them, they need to be transplanted into another female mouse. All this processing takes a long time to develop a transgenic mouse, and several generations of breeding are necessary to establish a stable transgenic colony. Finally, the development or purchase of the transgenic mice, along with their maintenance, is expensive.

An interesting alternative to the rodent models for *in vivo* studies of diabetes pathogenesis is the zebrafish. Zebrafish (*Danio rerio*) share 70% genetic similarity with the human genome, and in regard to metabolic disorders, they share 84% similarity to humans (Barbazuk et al., 2000; Bradford et al., 2017; Howe et al., 2013). Zebrafish serve as an attractive animal model system that offers several advantages over other mammalian models. Critically, zebrafish have a high degree of genetic, anatomical, and physiological similarities to humans. Zebrafish have the same key organs that are important for regulation of energy homeostasis and metabolism as in mammals, including digestive organs, adipose tissues, and skeletal muscle. Thus, key processes like including regulation of appetite, insulin regulation, and lipid storage are well conserved. This characteristic makes them a suitable animal model for studying a metabolic disorder like diabetes (Zang et al., 2018). In addition, zebrafish are very fecund, capable of producing many hundreds of embryos per breeding; this provides enough genetically matched and chronologically synchronized samples to perform experiments with substantial power quickly. Moreover, they have a

short egg-to-egg generation time, along with being easier and less expensive to maintain in large numbers. One of the major advantages is the ease of genetic manipulation that is required to produce transgenic lines with fluorescent protein tags. Injecting RNA or DNA, including plasmids or morpholinos, at the one-cell stage for generating transgenic lines or inducing genetic knockdown of a protein, is easier than with other vertebrate model systems (Chen and Ekker, 2004). Moreover, screening chemicals or drugs is immensely easier. Since the zebrafish have a quick developmental time, they reach the larval stage where most organ systems are present and functional by 3 days post-fertilization (3 dpf) (Kimmel et al., 1995; MacRae and Peterson, 2015). Finally, a significant advantage of zebrafish that trumps other ones is their high degree of optical transparency, which allows for *in vivo* visualization / live imaging of the organ system of interest (Bradford et al., 2017; Burke, 2016; Kalueff et al., 2014; Saleem and Kannan, 2018).

Zebrafish are relevant tools for diabetes research as the morphogenesis and cellular architecture of the pancreas has similarities with that of the other mammalian models (Kinkel and Prince, 2009; Tehrani and Lin, 2011). The exocrine pancreas is comprised of ductal cells and acinar cells, while the endocrine compartment has α-cells, β-cells, δ-cells, and ε-cells, with all cells performing conserved functions and releasing designated hormones (Argenton et al., 1999; Biemar et al., 2001). More importantly, other organs involved in metabolism and diabetes, including liver, adipose tissues, brain, and skeletal muscles, have conserved mechanisms with respect to development and function (Maddison and Chen, 2017). With all these conserved factors, different genetic manipulations and experimental conditions have been made to study T1D and T2D. For T1D, chemical and genetic approaches have been utilized. On administration of β-cell toxins like STZ and

alloxan in adult or larval zebrafish, there is a rapid loss of β-cells, induction of hyperglycemia, and reduced insulin secretion. Interestingly, persistent administration of STZ also leads to diabetes-associated complications including retinopathy, nephropathy, and impaired fin regeneration (Castañeda et al., 2017; Intine et al., 2013; Moss et al., 2009; Nam et al., 2015; Olsen et al., 2010). In terms of genetic models, the most commonly utilized and efficient method of inducing β-cell damage in the Metronidazole-Nitroreductase system (MTZ-NTR). In this hybrid chemical-genetic system, the zebrafish β-cells express NTR, and when these transgenic fish are treated with MTZ, The MTZ-NTR reaction induces β-cell-specific damage (Curado et al., 2007; Pisharath et al., 2007). This MTZ-NTR mediated ablation leads to complete β-cell depletion after 12-24 hours of MTZ treatment that results in severe hyperglycemia. Interestingly, after 48-72 hours of MTZ removal, the β-cell mass is functionally restored by regeneration (Ye et al., 2015). This property of restoration of β-cell mass in the zebrafish larvae adds to another advantage of the zebrafish model, where β-cell regeneration can be effectively studied. T2D studies have also been carried using zebrafish models. As discussed earlier, T2D is characterized by insulin resistance and insulin deficiency that ultimately promotes hyperglycemia. Submerging young or adult zebrafish chronically in 2% glucose solution for 2-4 weeks results in hyperglycemia and insulin resistance (Alvarez et al., 2010; Capiotti et al., 2014; Gleeson et al., 2007). Obesity is another hallmark of T2D. Adult zebrafish, when fed with high-calorie diet or overfeeding, become obese. These high-calorie diet options included 60 mg *Artemia*, or 20% corn oil, or 20% vegetable oil, or egg yolk powder (59% fat) fed over extended periods and they resulted into hypertriglyceridemia, hepatosteatosis, hyperglycemia and increase visceral fats (Landgraf et al., 2017; Meguro et al., 2015; Oka

et al., 2010; Vargas and Vásquez, 2017; Zang et al., 2017). Interestingly, in the overfeeding induced diabetes study, treatment with commonly used anti-diabetic drugs (metformin and glimepiride), there was a reduction in glycemic levels (Yoon et al., 2011). These data suggest that the zebrafish can be efficient tools used for modeling human T2DM, and also screening for novel anti-diabetic drugs. However, since they cannot mimic the complex physiology of the mammalian system, supplementary studies in the other rodent models are essential to increase the robustness of the findings.

Summarily, various cell culture methods and animal models have been developed to study diabetes. Every model has its respective advantages and disadvantages; however, if a mechanism is determined to be conserved across species, it would suggest that it is probably the same in humans as well.

1.13 Summary:

In summary, although a variety of factors contribute to its development and progression, inflammation of the pancreatic islets is an aspect that is shared between T1D and T2D pathogenesis. Studies in animal models, as well as human pancreatic sections and islets, reveal that maladaptive islet inflammation is pervasive in diabetes. Hence, uncovering mechanisms that can be manipulated to modulate inflammation could prove to be an efficient therapeutic strategy to treat this dreadful disease. Two critical mediators of inflammation in the context of diabetes include macrophages and 12-LOX. Macrophages are immune cells known to regulate inflammation by the release of pro- and anti-inflammatory signaling molecules. On the other hand, 12-LOX is an enzyme that catalyzes the production of the pro-inflammatory molecule 12-HETE. Both macrophages and 12-LOX have been shown to promote oxidative stress and apoptosis in β-cells via inflammatory mechanisms. Moreover, individual depletion of macrophages or 12-LOX has been shown to have protective effects against diabetes. However, whether 12-LOX plays a role within the macrophage, in the context of diabetes, is an open question. Hence, the objective of this thesis project is to understand how 12-LOX regulates macrophage-mediated inflammation in the pathogenesis of diabetes. For this study, I have utilized the strengths of mouse models and of transgenic zebrafish to studying these prospective roles of 12-LOX and macrophages in β-cell homeostasis. These studies provide novel mechanistic insights as well as therapeutic targets for the resolution of inflammation-induced damage in the β-cell. My long-term goal is to unravel novel targets for effectively treating both major forms of diabetes, which could potentially be extrapolated more generally to all inflammation-associated pathologies.

Chapter Two: Materials and Methods

2.1. Zebrafish studies:

2.1.1. Zebrafish maintenance and embryo collection:

All zebrafish experiments were approved by the Indiana University School of Medicine IACUC. Wild-type, *Tg(ins:NTR)* (ZDB-ALT-130930-5), *Tg(mpeg1:eGFP)* (ZDB-ALT-120117-1), *Tg(ins:NTR) ; Tg(mpeg1:eGFP)* and *Tg(ins:Kaede); Tg(ins:NTR)* zebrafish were maintained at 28.5°C in a recirculating aquaculture system enclosed in a cabinet and subjected to a 14-/10-hour light/dark cycle in accordance with institutional policies under IACUC oversight. Heterozygous outcrossed embryos bearing the transgenic allele were collected at spawning and maintained in a 28.5°C incubator in fish water-filled petri dishes. At 3 days-post fertilization (larval stage), the transgenic zebrafish larvae were genotyped by epifluorescence at 80 hpf using a Leica M205FA dissecting microscope.

2.1.2. Chemical treatments:

1-Phenyl-2-thiourea (PTU; Acros #207250250) supplementation at 0.003% was used to prevent pigmentation in all embryos after gastrulation stages. 7.5 mM Metronidazole (MTZ) (Sigma #095K093) was prepared in fish water (0.1% instant ocean salt, 0.0075% calcium sulfate) that was supplemented with PTU. Control treatments for MTZ used fish water alone. The larvae were treated with 12-LOX inhibitor (ML355) or the vehicle (DMSO) for 2 hours as a part of pre-treatment followed by 6 hours for the assays used to study the role of 12-LOX in different injury models.

2.1.3. Tailfin injury assay:

20 *Tg(mpeg1:eGFP)* larvae were collected from each pre-treatment (ML355 or DMSO) and washed with fish water supplemented with PTU. The larvae were then treated

with 0.01% tricaine (Sigma #A-5040) in fish water-PTU to restrict their movements in a 10 cm petri dish temporarily. With a sharp scalpel, the distal end of the tail fin was amputated. After amputation, the larvae were transferred back to their respective treatments in 12-well plates. After the treatments, the larvae were fixed and stained (section 2.1.8). The GFP-labelled macrophages that migrated to the injured tail were quantified under the confocal microscope (Zeiss LSM700).

2.1.4. β-cell injury assay:

20 *Tg(ins:NTR)* larvae from each pre-treatment (ML355 or DMSO) were washed with fish water-PTU and then treated with 7.5mM MTZ for 6 hours. After the treatment, the larvae were washed with fish water and then fixed and stained (section 2.1.8). The GFP-labelled macrophages that migrated to the islets in response to the β-cell injury were quantified under the confocal microscope (Zeiss LSM700).

2.1.5. β-cell regeneration assay:

10 *Tg (ins:Kaede) ; Tg(ins:NTR)* larvae were used in triplicate. These expressed a photo-convertible Kaede protein that, when exposed to UV light, the bright green fluorescing Kaede protein gets converted to a red fluorescent protein. For these studies, larvae were exposed to UV light for 3 minutes. After photo-conversion, the larvae were subjected to 24 hours of MTZ treatment, followed by 24 or 48 hours of recovery in regular fish water-PTU. After treatment, the larvae were washed with fish water and then fixed and stained (section 2.1.8). The number of pre-existing β-cells (characterized by both green and red fluorescence) and the neogenic β-cells (characterized by only green fluorescence) were quantified using a confocal microscope (Zeiss LSM700).

2.1.6. ROS measurement:

For the detection of β-cell ROS, primarily peroxides, I used CellROX green reagent (Invitrogen #C10444). 10 larvae were transferred to 1.5 ml microcentrifuge tubes, washed with fish water, then incubated in the dark for 1 hour at 28.5°C with 10 μM CellROX green diluted in fish water. Similarly, for detection of superoxide in pancreatic β-cells, 10 heterozygous *Tg(ins:NTR)* larvae were treated with 7.5 mM MTZ for 3 hours then placed in 5 μM dihydroethidium (Thermo Fisher #D1168) + 0.02% DMSO for 30 minutes in dark conditions. After the treatment with ROS detecting reagents, the larvae were then treated with 0.01% tricaine (Sigma #A-5040), mounted on glass-bottom petri dishes (Mattek #P35G-0-10-C) in 0.5% low melt agarose (Sigma #A9414), and imaged with a Zeiss LSM700 confocal microscope.

2.1.7. Macrophage depletion:

30 *Tg(ins:NTR) ; Tg(mpeg1:eGFP)* zebrafish larvae were treated with 0.01% tricaine. They were then mounted in 2.5% methylcellulose (EMS #18560) and injected trans-pericardially with 7-10 nL clodronate (SKU# CLD-8901). 24 hours after clodronate injection, the larvae were subjected to either the tailfin injury assay or the β-cell injury assay. After these treatments, the larvae were fixed and stained (section 2.1.8). The GFP-labelled macrophages that migrated to the injured sites were quantified using a confocal microscope (Zeiss LSM700).

2.1.8. Immunofluorescence and image collection

At the conclusion of each experiment, larvae were washed in fish water and then fixed with 3% formaldehyde in a PEM buffer (0.21 M PIPES, 1 mM MgSO4, 2 mM EGTA at pH 7) at 4°C overnight. Fixed larvae were washed with PBS and deyolked, and then

antibody staining was performed as previously described (Ye et al., 2015). The following concentrations of primary antibodies were used: 1:200 guinea pig anti-insulin (Invitrogen #180067), 1:200 chicken anti-GFP (Aves Labs #GFP-1020). Primary antibodies were detected with 1:500 dilutions of complementary Alexa-conjugated secondary antibodies (Jackson ImmunoResearch). DNA was stained with TO-PRO3 (Thermo Fisher #T3605) diluted 1:500. After staining, larvae were mounted on charged glass slides in VECTASHIELD (Vector Labs H-1000), and confocal imaging was performed with a Zeiss LSM700 microscope.

2.1.9. Total glucose assay:

10 larvae from each condition were collected in triplicates, washed with fish water after the treatments, and stored in 500 µL glucose assay buffer (Biovision) at -80°C. The larvae in the glucose assay buffer were then homogenized with a tissue homogenizer. The tubes were centrifuged at 5000 r.p.m. for 5 minutes at room temperature and the supernatant was used in duplicate for the glucose assay performed in the 96-well plate. 50 µL of substrate-enzyme mix was added to the 50uL of the supernatant. The plate was stored at room temperature for 30 minutes in dark. The result of reaction was read in a spectraMax iD5 multi-mode microplate reader (Molecular Devices) at 405 nm to measure the glucose levels.

2.1.10. Gene expression analysis by quantitative PCR:

Quantitative PCR (qPCR) was performed to quantify the mRNA expression of different zebrafish genes. 60 larvae per condition (20 larvae in triplicates) were collected for RNA extraction using the RNeasy® Plus Micro Kit (Qiagen). The larvae were lysed in 350 µL of RLT buffer containing 10 µL of β-mercaptoethanol. The lysate was transferred

to the RNeasy MinElute spin column, which was centrifuged at full speed for 15 seconds. The flow-through was discarded, and the column was washed with 700 µL RW1 buffer followed by a wash using 500 µL of RPE buffer. Finally, 500 µL of 80% ethanol was added to the spin column and centrifuged at full speed for 2 minutes. For removing residual ethanol, the column was transferred to a fresh collection tube and spun at full speed for 2 minutes. RNA was collected in 14 µL of RNase-free water, and the concentration was measured using a Nanodrop (Thermo Scientific). 500 ng RNA was used for cDNA synthesis using the High-Capacity cDNA Reverse Transcription Kit (Thermofisher). The synthesized cDNA was diluted to a total volume of 120 µL. To quantify the gene expression using qPCR, 10 µL of the cDNA was used and the reaction was performed using the SsoFast™ EvaGreen® Supermix kit (Biorad). Each cDNA sample was run in triplicate in the Quantstudio 3 thermocycler (Applied Biosystems). The primers for *β-actin* and *cxcr3.2*, were purchased from Integrated DNA Technologies (Table 1). The average Ct value of the three replicates was calculated and normalized to *β-actin*.

Primer Name	**Sequence (5'-3')**
β-actin Forward	CGAGCAGGAGATGGGAACC
β-actin Reverse	CAACGGAAACGCTCATTGC
CXCR3.2 Forward	TGGTGGACATGCACTTTCGT
CXCR3.2 Reverse	GTCAGTCATCCGCAGAGCAT

Table 1: List of primers for qPCR (zebrafish)

2.2. Mouse studies:

2.2.1. Mouse maintenance:

All mouse experiments were approved by the Indiana University School of Medicine IACUC. The mice were maintained in pathogen-free conditions under a standard 12-hour light-dark cycle and provided unlimited access to water and standard rodent chow. *Alox15*$^{-/-}$ (12-LOX KO) mice were purchased from Jackson Laboratories and bred in the Indiana University School of Medicine animal facilities. For the experimental controls, I utilized wild-type (WT) littermates. The mice used in this study were 8- to 12-week old.

2.2.2. Peritoneal macrophage isolation, culture and treatment:

WT and 12-LOX KO mice were euthanized and immediately subjected to peritoneal macrophage isolation as described (Ray and Dittel, 2010). For isolation, ice-cold RPMI was injected into the peritoneal cavity. The mice were gently massaged near the peritoneum to dislodge any attached cells. The 25 g needle attached to a 5 mL syringe was inserted and the injected RPMI was pulled back to collect the peritoneal macrophages. These cells were centrifuged at 450 g for 5 minutes at 4°C. The supernatant was discarded, and the cell pellet was treated with 1mL RBC lysis using lysis buffer (eBioscienceTM #00-4333-57) for 1 minute to remove red blood cells. Immediately after 1 minute, 5mL of RPMI was added. The suspension was centrifuged at 450 g, for 5 minutes at 4°C, and the supernatant was discarded. The pellet was resuspended in 1 mL complete RPMI medium. Cells were counted using a hemocytometer and checked for viability using Trypan Blue. The peritoneal macrophages were then used for the polarization and migration assays.

2.2.3 Islet isolation, culture and treatment:

Mouse islets were isolated from both male and female WT mice by the IU Diabetes Center Islet and Physiology Core. Briefly, mice were sacrificed by cervical dislocation, and pancreata were inflated with 2.0 ml of collagenase. Pancreata were then incubated at 37°C for 15 minutes, followed by dissociation in Hank's Balanced Salt Solution (HBSS) and Bovine Serum Albumin (BSA). Islets were handpicked and allowed to recover overnight in complete medium (8 mM glucose RPMI) at 37°C before experimentation. The islets were then washed with RPMI and treated with either a pro-inflammatory cytokines (PIC) supplemented media (50 ng/mL TNF-α, 25 ng/mL IL-1β, 100 ng/mL IFN-γ) or media control for 24 hours. The supernatant was then used for the chemotaxis assay.

2.2.4. Polarization Assay:

Macrophages were seeded in 12-well plates for RNA studies ($1x10^6$ cells/condition) or 96-well plates for flow cytometry ($3x10^5$ cells/condition) in triplicates. They were then treated with PIC cocktail (TNF-α -50 ng/mL, IL-1β- 25 ng/mL, IFN-γ-100 ng/mL) or 10ng/mL IL4 or media control for 16 hours at 37°C. After 16 hours, the supernatant was collected and stored at -80°C for ELISA. The wells were washed with 1X PBS. The plate was centrifuged at 450 g, 5 min at 4°C. The supernatant was discarded, and then the wells washed again with 2% FBS. The cells were stained with F4/80 antibody (1:200) (Biolegend) at room temperature (RT) in the dark for 30 minutes, for staining the macrophage-specific surface antigen. The cells were washed with perm/wash (BD Pharmigen) The macrophages were then fixed and permeabilized using BD Cytofix/Cytoperm™ (BD Pharmigen), for the intracellular markers of polarization, including iNOS (1:100) (Biolegend) and CD206 (1:100) (Biolegend). The cells were then

washed with perm/wash, followed by PBS. The cells were collected in the collection tubes and subjected to flow cytometry to quantitatively determine the expression of different proteins (AttuneTM NxT Flow Cytometer).

2.2.5 In vitro chemotaxis assay:

A 96-well chemotaxis system (ChemoTx, 8 μm filter pore size; Neuro Probe) was loaded with triplicates of conditioned media from WT islets. Peritoneal macrophage suspension (0.5×10^5 cells) was placed on top of the filter above each well, and the chamber was incubated for 4 hours at 37°C. After incubation, non-migrated cells were washed while the filter side containing migrated cells was fixed with 4% paraformaldehyde (Thermofisher) and stained with Coomassie Blue (Thermofisher) to visualize the macrophages. The filter was mounted on the slides, and the number of migrated macrophages was quantified under LSM700 confocal microscope.

2.2.6. CXCR3 expression analysis by flow cytometry:

For polarization, the peritoneal macrophages from WT and 12-LOX KO mice were seeded in 12-well plates for RNA studies ($1x10^6$ cells/condition) or 96-well plates for flow cytometry ($3x10^5$ cells/condition) in triplicates. They were then treated with a PIC-supplemented media of or media-only control for 16 hours. After 16 hours, the supernatant was collected and stored at -80°C for ELISA. The wells were washed with 1X PBS. The plate was centrifuged at 450 g, 5 minutes at 4°C. The supernatant was discarded, and then the wells washed again with 2% FBS. The cells were stained with F4/80 (1:200) (Biolegend) and CXCR3 antibody (1:100) (Biologend) at room temperature (RT) in the dark for 30 minutes, for staining the macrophage-specific surface antigen. The cells were

then washed with PBS and collected in the collection tubes and subjected to flow cytometry to quantitatively determine the expression of CXCR3 (AttuneTM NxT Flow Cytometer).

2.2.7 ELISA:

The supernatant from the stimulated peritoneal macrophages was collected. Cytokine levels were measured by performing ELISA according to the manufacturer's instructions (eBiosciences). The result of reaction was read in a spectraMax iD5 multi-mode microplate reader (Molecular Devices)

2.2.8. Gene expression analysis by quantitative PCR:

qPCR was performed to quantify the mRNA expression of different macrophage genes. $3x10^5$ peritoneal macrophages ($1x10^5$ cells in triplicates) were cultured in different conditions for 16 hours at 37°C. These cultured cells were used for RNA extraction using the RNeasy® Plus Mini Kit (Qiagen). The cells were lysed in 350 µL of RLT buffer containing 10 µL of β-mercaptoethanol. The lysate was transferred to the RNeasy MinElute spin column, which was centrifuged at full speed (13000 g) for 15 seconds. The flow-through was discarded, and the column was washed with 700 µL RW1 buffer followed by a wash using 500 µL of RPE buffer. Finally, 500 µL of 80% ethanol was added to the spin column and centrifuged at full speed for 2 minutes. For removing residual ethanol, the column was transferred to a fresh collection tube and spun at full speed for 2 minutes. RNA was collected in 14 µL of RNase-free water, and the concentration was measured using a nanodrop (Thermo Scientific). 500 ng RNA was used for cDNA synthesis using the High-Capacity cDNA Reverse Transcription Kit (Thermofisher) The synthesized cDNA was diluted to a total volume of 120 µL. To quantify the gene expression using qPCR, 10 µL of the cDNA was used, and the reaction was performed using the SsoFast™ EvaGreen®

Supermix kit (Biorad). Each cDNA sample was run in triplicate in the Quantstudio 3 thermocycler (Applied Biosystems). The primers for *β-actin, cxcr3, il6, il12, il10 and tgf-β* were purchased from Integrated DNA Technologies (Table 2). The average Ct value of the three replicates was calculated and normalized to *β-actin*

Primer Name	**Sequence (5'-3')**
β-actin Forward	GGCACCACACCTTCTACAATG
β-actin Reverse	GGGGTGTTGAAGGTCTCAAAC
CXCR3 Forward	GCTGCTGTCCAGTGGGTTTT
CXCR3 Reverse	AGTTGATGTTGAACAAGGCGC
IL-6 Forward	TACCACTTCACAAGTCGGAGGC
IL-6 Reverse	CTGCAAGTGCATCATCGTTGTTC
IL-12 Forward	AAGCTCTGCATCCTGCTTCAC
IL-12 Reverse	GATAGCCCATCACCCTGTTGA
IL-10 Forward	CGGGAAGACAATAACTGCACCC
IL-10 Reverse	CGGTTAGCAGTATGTTGTCCAGC
TGF-β Forward	TTAGGAAGGACCTGGGTTGG
TGF-β Reverse	AGGGCAAGGACCTTGCTGTA

Table 2: List of primers for qPCR (mice)

2.3. Statistical Analysis:

The data are presented as mean ± standard error of the mean (SEM). The data analyses were performed using the GraphPad Prism 8 software package. Significant differences between the mean values were determined using Student's t-test, where two means were compared, and one-way analysis of variance (ANOVA) followed by post hoc Holm-Sidak test when more than two means were compared. The differences were considered statistically significant at $p<0.05$.

Chapter Three: An *in vivo* zebrafish model for interrogating oxidative stress and inflammation mediated pancreatic β-cell injury, response, and prevention

3.1 Introduction:

The generation of reactive oxygen species (ROS), including peroxides, superoxides, and oxygen radicals, results in oxidative stress. This oxidative stress results in cellular dysfunction and triggers regulated cell death under extreme circumstances (Kaneto et al., 2010). Cells of metabolically active tissues are predisposed to high levels of ROS production, and thus, metabolic diseases such as type 2 diabetes mellitus (T2D) are often associated with excessive ROS generation and the resulting oxidative stress (Yadav et al., 2016). T2D is characterized by chronic hyperglycemia resulting from the dysfunction of insulin-secreting pancreatic β-cells in the setting of overnutrition and obesity (Ogihara and Mirmira, 2010). This dysfunction may be driven in part by the generation of excessive ROS, which likely results from the low endogenous levels of antioxidant enzymes in β-cells (Ha et al., 2008). ROS diminishes the expression of insulin in β-cells, impairs glucose-stimulated insulin secretion, and promotes β-cell apoptosis (Hou et al., 2008; Robertson and Harmon, 2007). The availability of a vertebrate model to study factors that regulate ROS dynamics in the islet *in situ* would accelerate the discovery and testing of novel therapeutics for a variety of metabolic diseases, including T2D. The zebrafish, *Danio rerio*, is a robust model to interrogate the pathogenesis of metabolic disease and the efficacy of experimental therapeutics (Curado et al., 2007; MacRae and Peterson, 2015).

The oxygen-insensitive NAD(P)H nitroreductase (NTR, *NfsB*) enzyme, cloned from *E. coli*, has been harnessed to drive tissue-specific cell ablation in various transgenic zebrafish models. When NTR-expressing transgenic zebrafish lines, such as *Tg(ins:NTR)*,

are treated with the antibiotic metronidazole (MTZ), this prodrug gets metabolized into cytotoxins that are retained by NTR-expressing β-cells, rapidly inducing their death (Barros et al., 2008).While the precise mechanisms of MTZ-induced cell toxicity have not been characterized in NTR-expressing transgenic lines, existing research provides some insight: nitroreduction of MTZ may produce cytotoxic nitroradical metabolites, which can crosslink DNA (Felmer and Clark, 2004; Knox et al., 1988; Mathias et al., 2014). In this study, I used transgenic NTR+ zebrafish to demonstrate their suitability for modeling ROS generation, cellular responses to ROS, and pharmaceutical interventions in the β-cell *in vivo*.

3.2. Results:

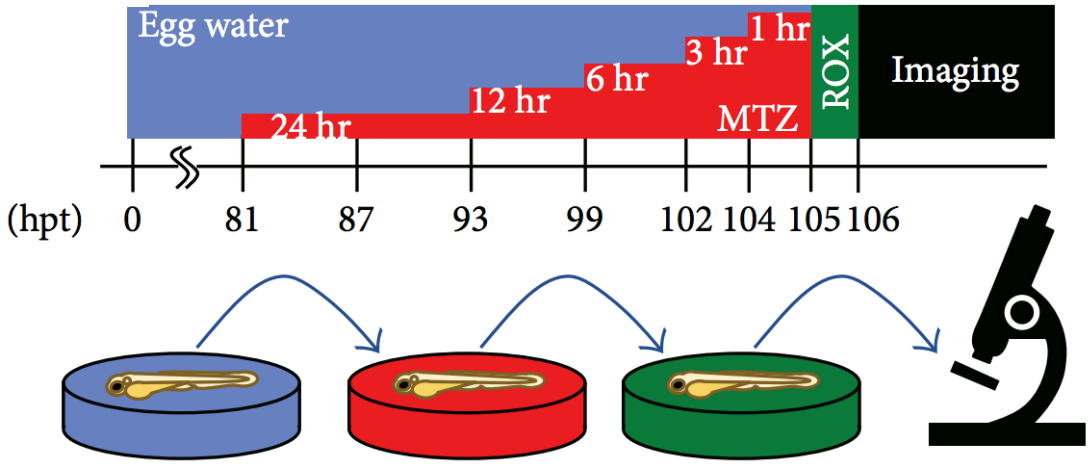
3.2.1. MTZ induces β-Cell ROS generation in an NTR- and dose-dependent manner:

The nitroreductase-metronidazole (NTR-MTZ) system has been widely implemented as a tool to efficiently ablate cells in a tissue-specific and temporally controllable manner (White and Mumm, 2013). However, the molecular mechanisms driving its induction of regulated cell death are not fully understood. To determine if ROS are generated in MTZ-treated NTR-expressing cells, I used *Tg(ins:NTR)* transgenic zebrafish that express insulin promoter-driven NTR in the pancreatic β-cells. I immersed the heterozygous transgenic larvae in a solution of MTZ for 0, 1, 3, 6, 12, or 24 hours, then stained with CellROX green to indicate ROS (Figure 10A). Incubation start times were staggered such that all larvae were at 106 hours post-fertilization (hpf) at analysis. Treatments were started with 7.5 mM MTZ, a dose that is known to be effective in ablating β-cells after 24-hour exposure (Ye et al., 2016). After 1 hour of treatment, ROS staining was observed specifically in β-cell nuclei, whereas adjacent islet cells were not stained (Figure 10B). With longer treatments, ROS levels increased by almost 4-fold relative to untreated controls, reaching peak intensity with 6 hours of treatment, followed by a decrease in intensity with 12 or 24 hours of treatment (Figure 10C). I attribute this diminished staining to the attrition of β-cells via regulated cell death mechanisms and their clearance by phagocytes (Figure 10D), as well as the neogenesis of β-cells that have not yet generated detectable ROS.

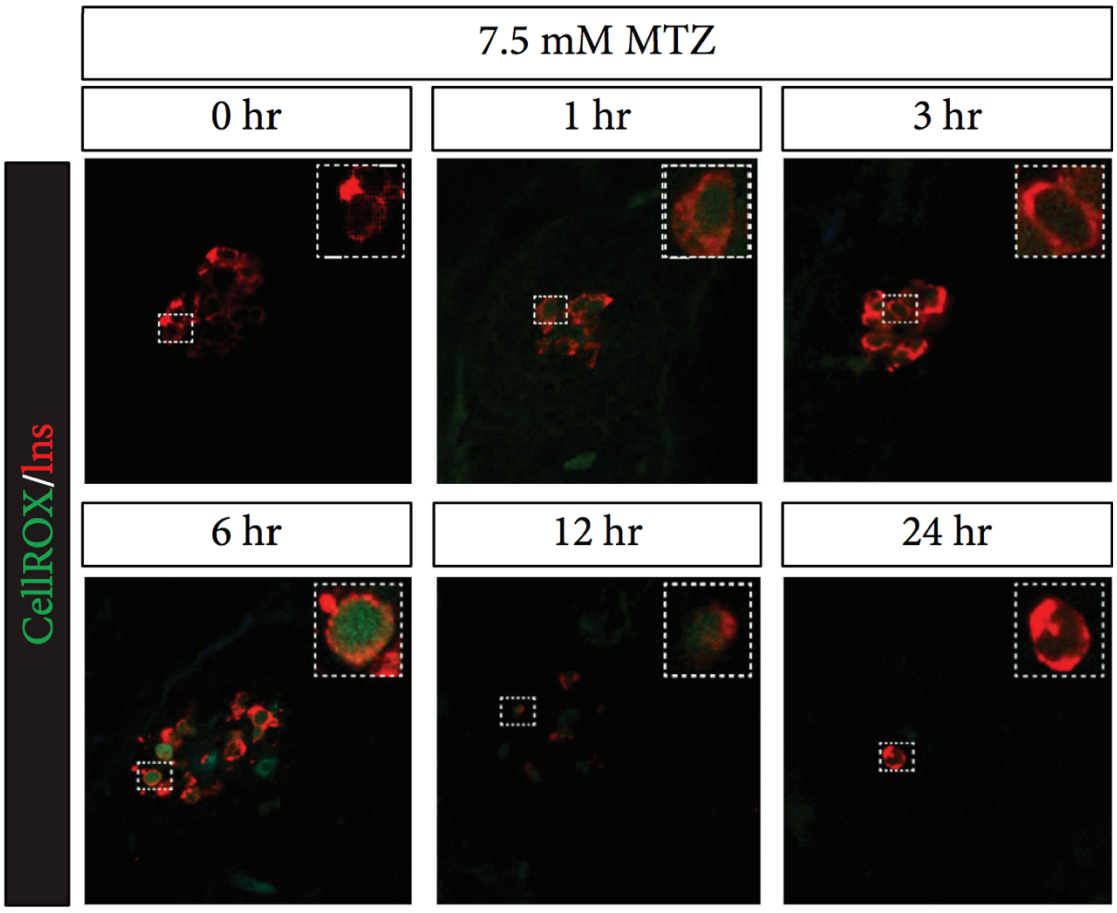
To further confirm that the CellROX green staining that I observed was truly representative of MTZ-induced cellular ROS and not artefactual (i.e., due to an interaction of CellROX green with the reduced nitroradical form of metronidazole), I next incubated

MTZ-treated transgenic larvae in 5 μM dihydroethidium (DHE). Upon oxidation of DHE to 2OH-ethidium by superoxides, this cell-permeant dye is excited at 405 nm and emits bright red fluorescence at 570 nm (Nazarewicz et al., 2013). In 106 hpf *Tg(ins:NTR)* larvae that were not treated with MTZ, I detected no specific pancreatic fluorescence in any sample (Figure 10E; n = 13). In contrast, transgenic larvae treated with 7.5 mM MTZ for 3 hours, a strong fluorescence in β-cells was observed in every case (Figure 10E; n = 14). These data indicate that superoxides are generated in transgenic β-cells in response to MTZ.

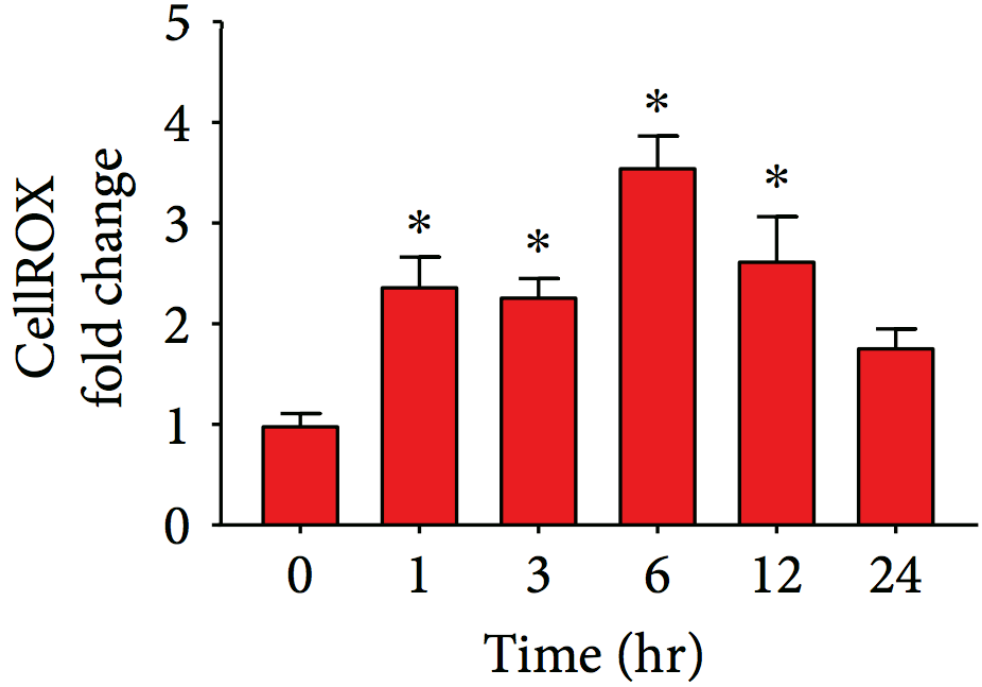
A.


Egg water
1 hr
3 hr
6 hr
12 hr
24 hr
MTZ
ROX
Imaging
(hpt)
0
81
87
93
99
102
104
105
106

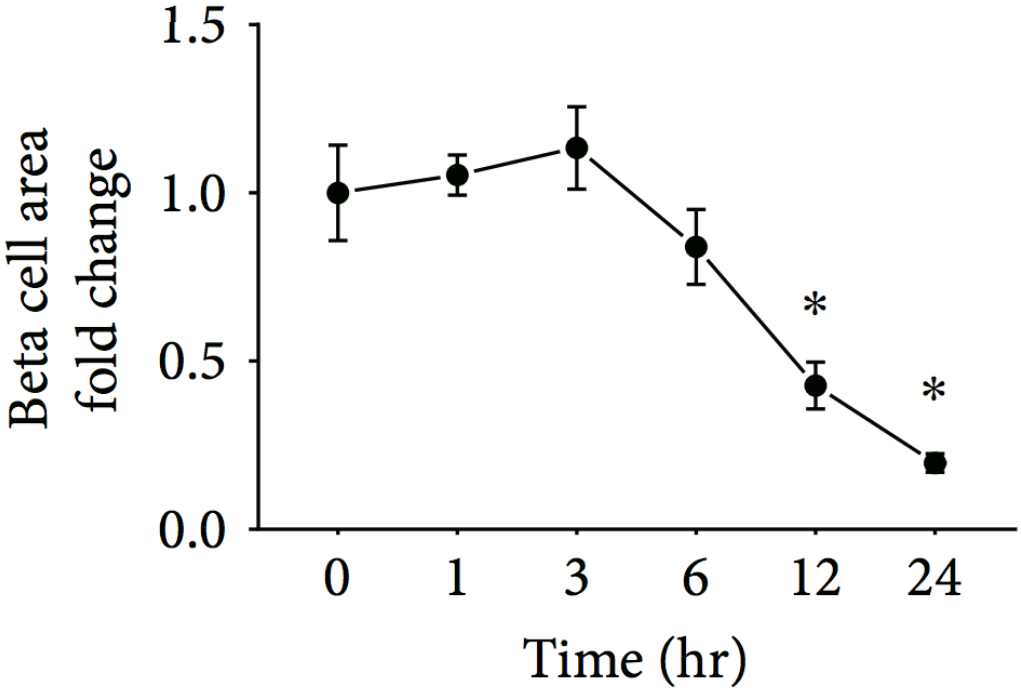
B.


7.5 mM MTZ
0 hr
1 hr
3 hr
6 hr
12 hr
24 hr
CellROX/Ins

C.


CellROX
fold change
5
4
3
2
1
0
*
*
*
*
0
1
3
6
12
24
Time (hr)

D.


Beta cell area
fold change
1.5
1.0
0.5
0.0
*
*
0
1
3
6
12
24
Time (hr)

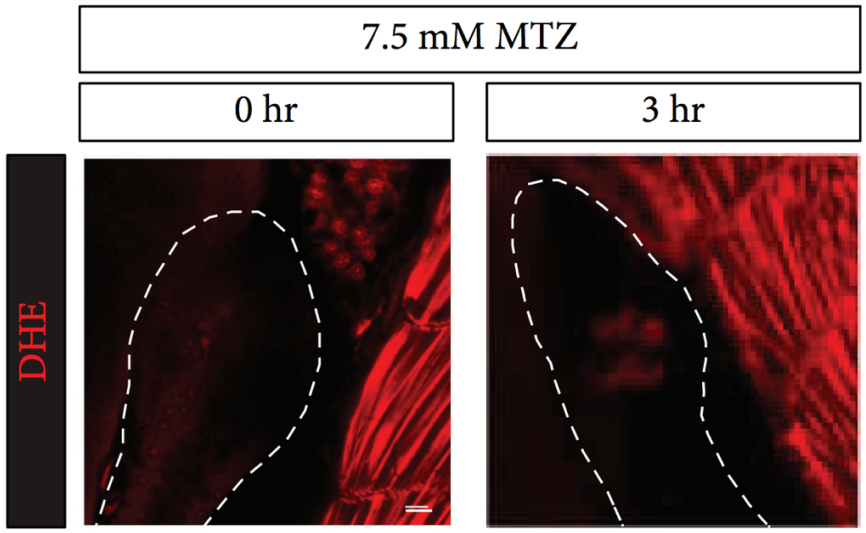
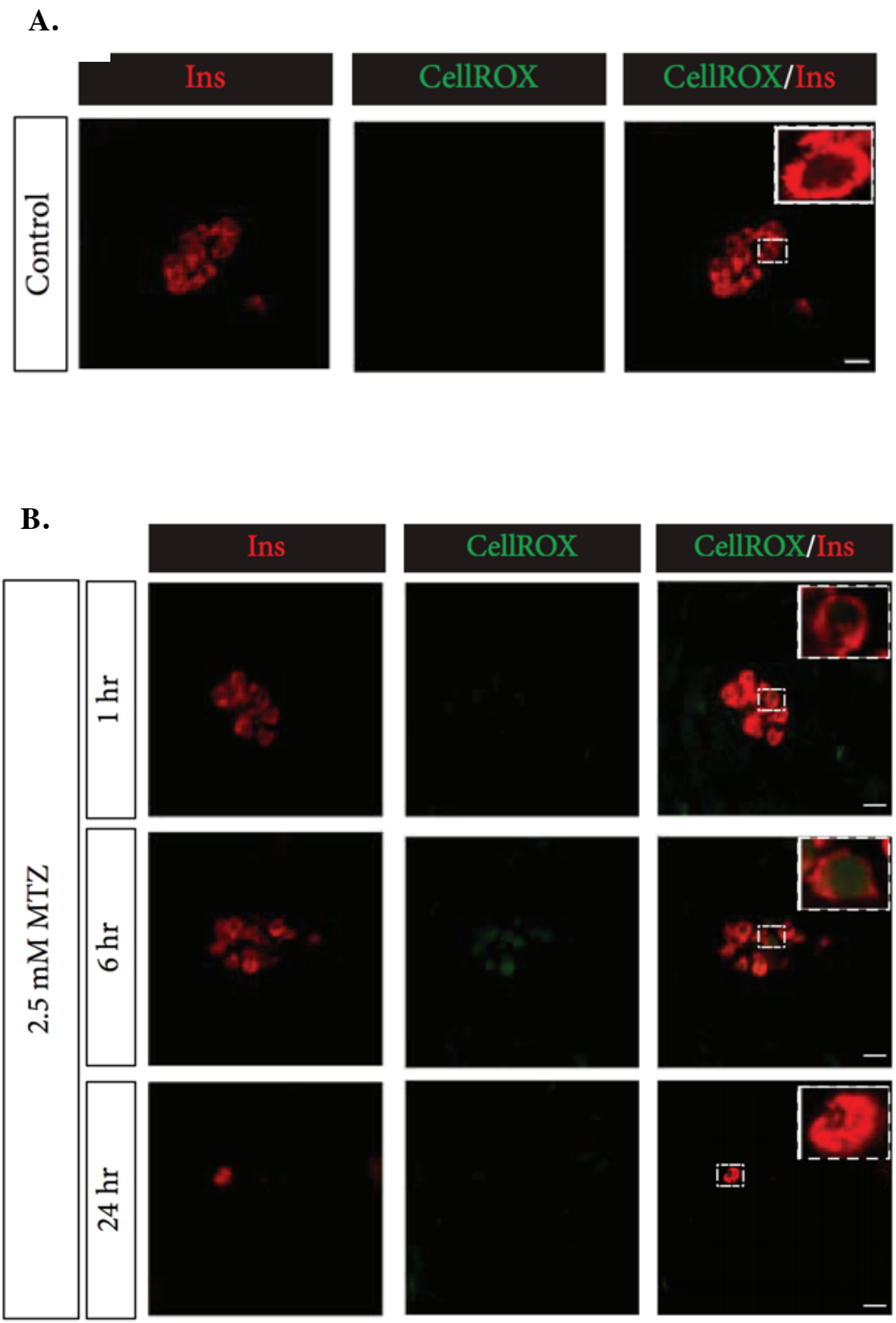
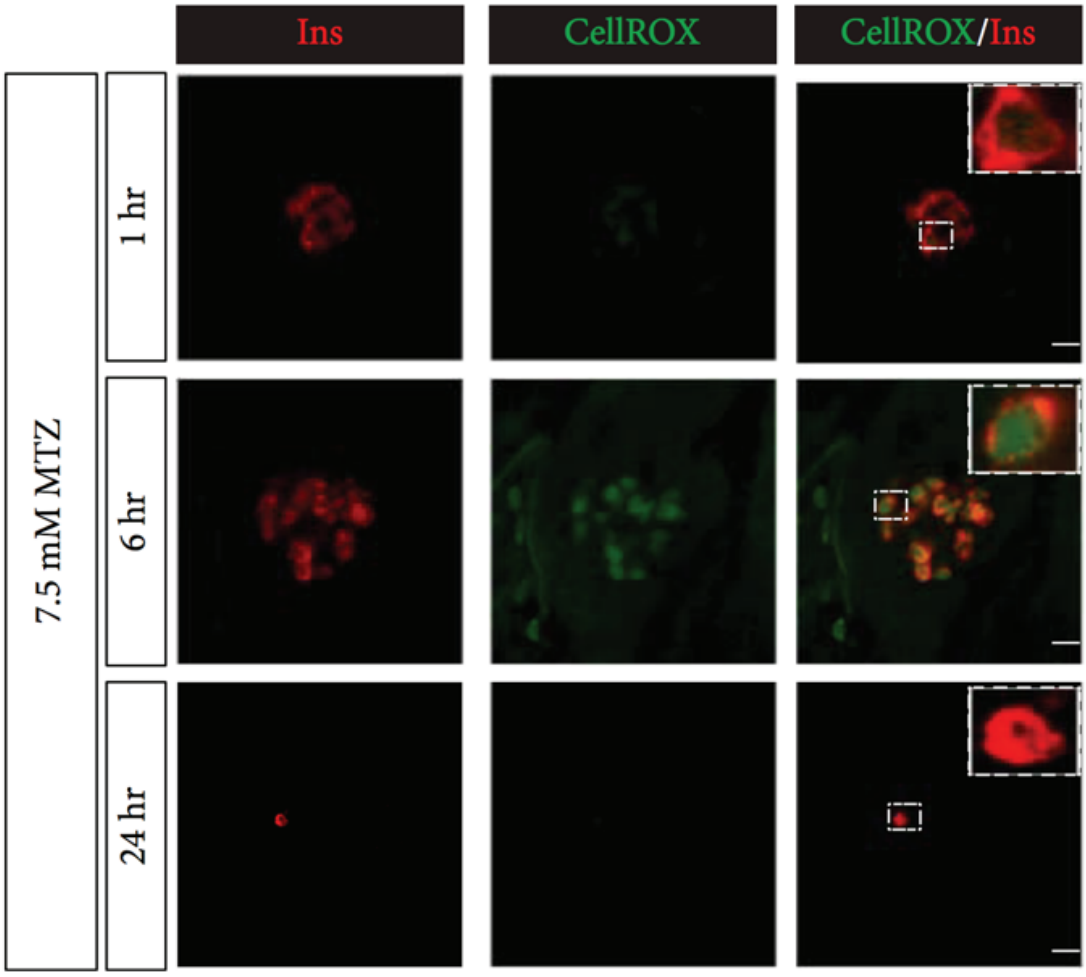


Figure 10: Time-dependent metronidazole induction of β-cell-specific ROS. (A) Schematic of MTZ treatments and imaging. Zebrafish (NTR+) larvae were treated with MTZ or vehicle for 0, 1, 3, 6, 12, or 24 hours with a "staggered start" such that all treatments were completed simultaneously; larvae were then incubated with CellROX green stain at 105 hpf and fixed at 106 hpf. (B) Representative immunofluorescence images of zebrafish pancreatic islets stained with insulin antibody and CellROX green after 7.5 mM MTZ treatments. Magnified insets (bounded by dashed boxes) highlight the dose-dependent increase in CellROX green signal in β-cells. (C) Quantification of CellROX green intensity in β-cells showing a significant increase in ROS generation after 1, 3, 6, and 12 hours of MTZ treatment as compared to vehicle-treated controls (n = 12). (D) MTZ treatment caused a significant decrease in β-cell area after 12 or 24 hours of treatment as compared to untreated controls. (E) Representative immunofluorescence images of zebrafish pancreatic islets treated for 3 hours with 0 or 7.5 mM MTZ and stained with 5 μM DHE. Dotted lines demarcate the boundaries of the pancreas. Graphed data are presented as mean ± SEM ($*p < 0.05$). Statistical significance was determined by one-way ANOVA followed by post hoc Holm-Sidak test. Scale bar indicates 10 μm.

Next, I hypothesized that the level of ROS generated in the NTR+ β-cells would be directly dependent on the concentration of MTZ present. To investigate if there is a dose-dependent relationship, I treated larvae with both low (2.5 mM) and high (7.5 mM) concentrations of MTZ using the same experimental paradigm indicated in Figure 10A. As expected, there was no ROS generation in the untreated control β-cells (Figure 11A). After 1 hour of treatment, the 2.5 mM dose did not show significant ROS generation, but 7.5 mM MTZ induced a > 4-fold increase in ROS levels as compared to untreated controls (Figures 11B-C). After 6 hours of treatment, 2.5 mM treatment did not result in significant ROS generation, while the 7.5 mM dose caused a nearly 6-fold rise in ROS levels relative to untreated controls. Consistent with previous observations, the measured ROS levels were no different from baseline with the 24-hour treatment of either the 2.5 mM or 7.5 mM MTZ. Despite the lower levels of ROS observed with 2.5 mM treatment, there was a dramatic reduction in the number of β-cells at the 24-hour time point (Figure 11D). Thus, even though induced ROS levels are lower with the 2.5 mM dose, this dose is sufficient to induce cell death over 24 hours.


A.
Ins
CellROX
CellROX/Ins
Control
B.
Ins
CellROX
CellROX/Ins
2.5 mM MTZ
1 hr
6 hr
24 hr

C.



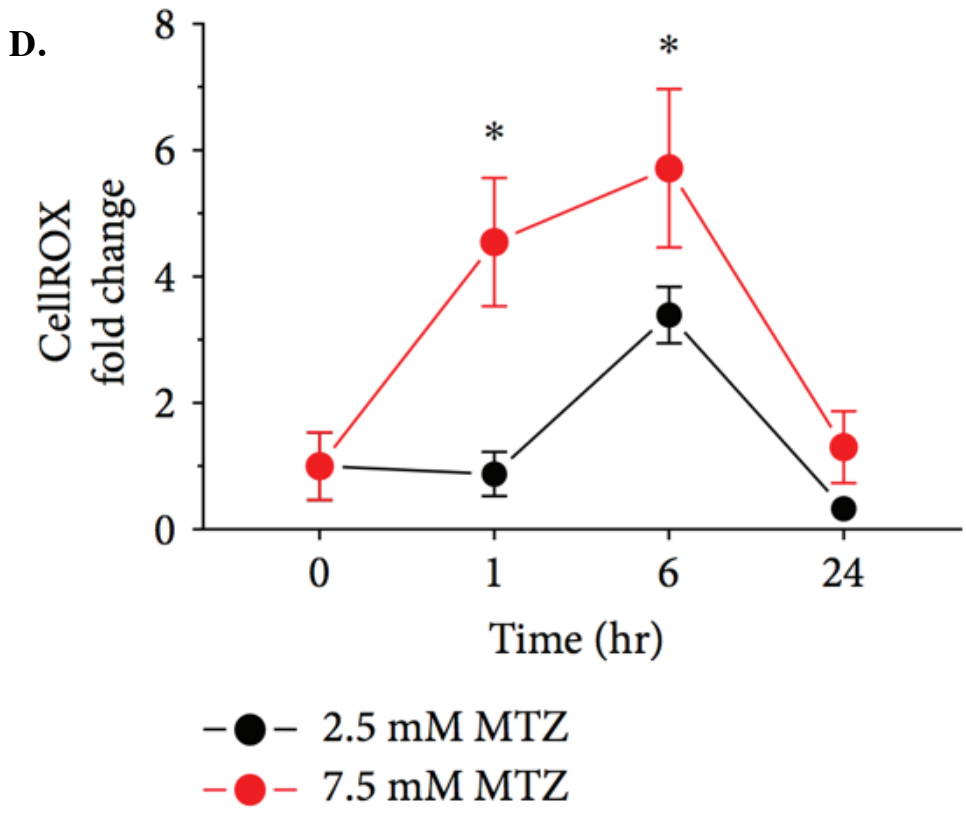


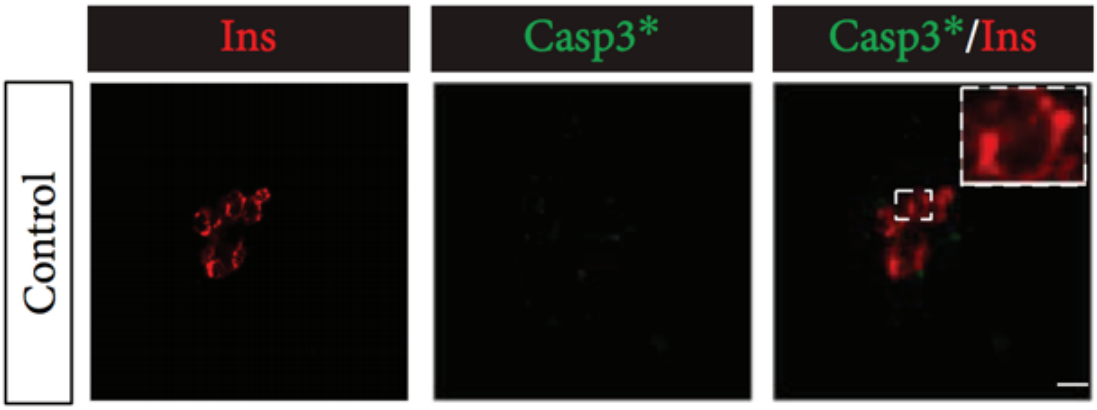
Figure 11: Metronidazole induces ROS generation in a dose-dependent manner. (A) Representative image of vehicle-treated zebrafish islets (n = 12) at 106 hpf. (B & C) Representative image of islets of zebrafish (NTR+) larvae (n = 12 per condition) treated with 2.5 mM and 7.5 mM MTZ at different time points. (D) Quantification of CellROX intensity shows a significant increase after 1 or 6 hours of treatment in the β-cells of 7.5 mM MTZ-treated larvae, as compared to untreated controls. Data are presented as mean ± SEM ($*p < 0.05$). Statistical significance was determined by one-way ANOVA followed by post hoc Holm-Sidak test. Scale bar indicates 10 μm.

3.2.2. MTZ-induced ROS generation leads to macrophage recruitment and β-Cell apoptosis:

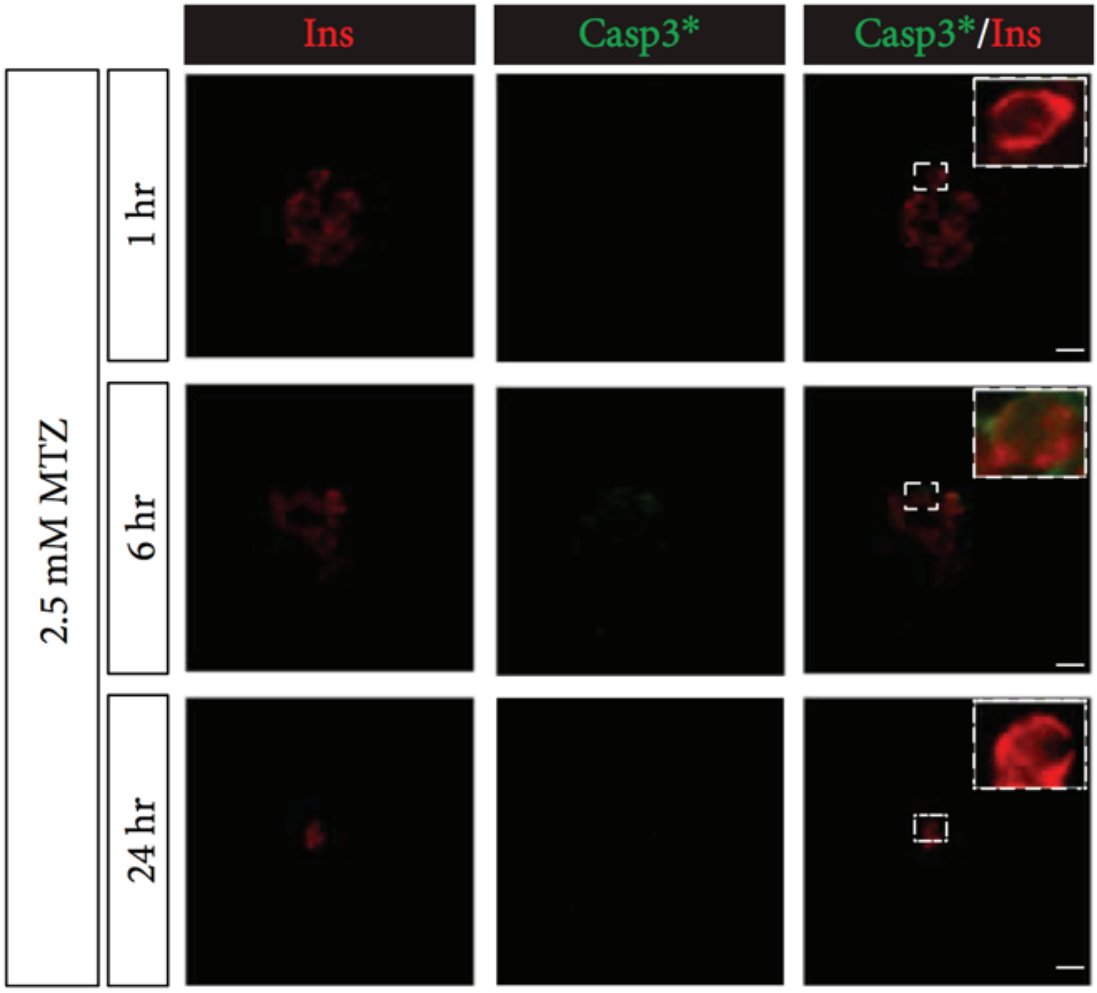
Many studies have correlated the production of ROS with the induction of apoptotic cell death (Circu and Aw, 2010; Nazarewicz et al., 2013; Simon et al., 2000). Therefore, to determine whether the generation of ROS correlates with the induction of β-cell apoptosis in this system, I analyzed cleaved caspase 3 (Casp3∗) in islet β-cells after following the same MTZ treatment paradigm shown in Figure 10A. Casp3∗ is the active form of caspase 3 and an indicator of the activated apoptotic pathway. I did not detect significant Casp3∗ staining in the untreated controls or with after 1-hour MTZ treatment (Figures 12A–C). However, after a 6-hour treatment, Casp3∗ was significantly increased with 7.5 mM MTZ, but not 2.5 mM relative to untreated controls, following a pattern similar to ROS generation (Figure 12B). As before, with 24 hours of treatment, almost all β-cells were ablated by both concentrations of MTZ (Figures 12B-D).

Evidence exists that shows ROS-injured β-cells release factors that attract immune cells (Gregory and Devitt, 2004). To test whether immune cells are attracted to MTZ-induced β-cell ROS generation, I utilized *Tg(mpeg1:eGFP)*+ zebrafish, in which macrophages fluoresce green. The first apparent macrophage located near a β-cell was seen after a 3-hour treatment, and peak infiltration at 6 hours post-MTZ treatment. Interestingly, this is the same time-point when the measurement of ROS staining is at its highest levels. Additionally, engulfment of β-cells by macrophages is distinct with a 12-hour MTZ treatment, a time point that is coincident with the observed drop in the β-cell area (Figure 13).

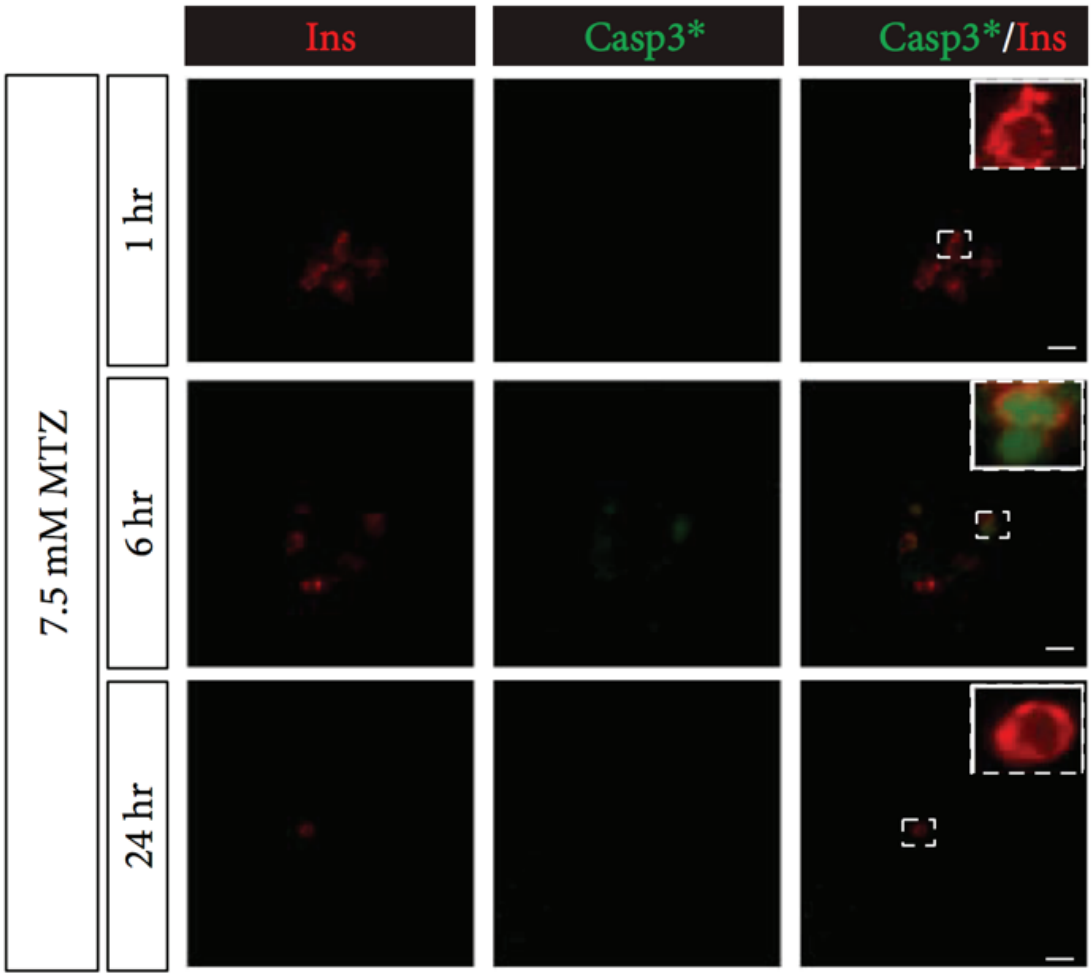
A.


Ins
Casp3*
Casp3*/Ins
Control

B.


Ins
Casp3*
Casp3*/Ins
2.5 mM MTZ
1 hr
6 hr
24 hr

C.



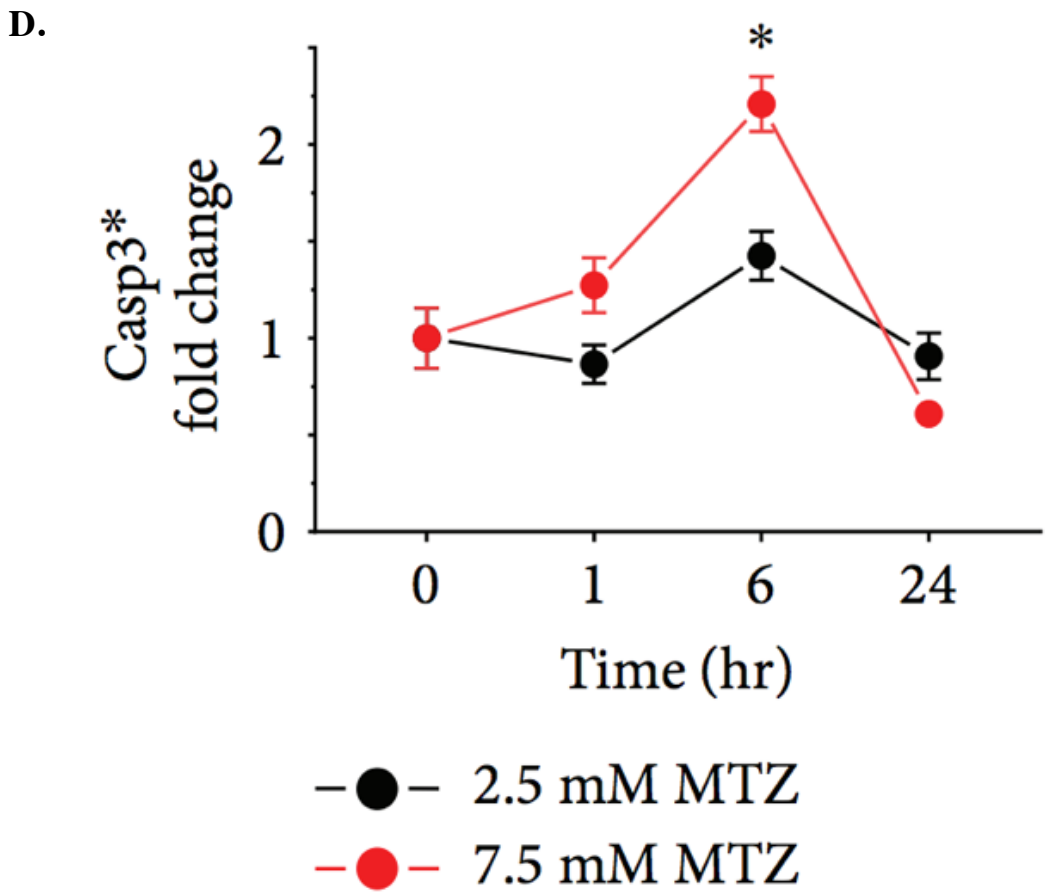


Figure 12: Metronidazole induces apoptosis signaling in β-cells. (A) Representative image of vehicle-treated zebrafish islets (n = 12) after fixing at 106 hpf. (B & C) Representative image of islets of zebrafish (NTR+) larvae (n = 12 per condition) treated with 2.5 mM or 7.5 mM MTZ at different time points and immune-stained for insulin and cleaved caspase 3 (Casp3∗). (D) Quantification of Casp3∗ intensity shows a significant increase after 6 hours of treatment in the β-cells of 7.5 mM MTZ-treated larvae compared to vehicle. Data are presented as mean ± SEM (∗$p < 0.05$). Statistical significance was determined by one-way ANOVA followed by post hoc Holm-Sidak test. Scale bar indicates 10 μm.

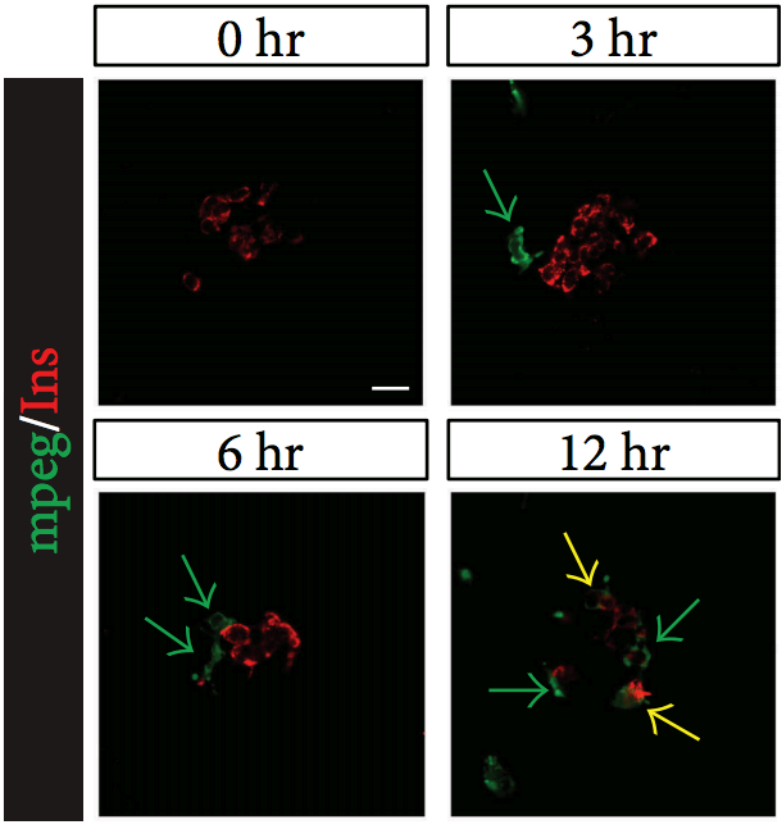
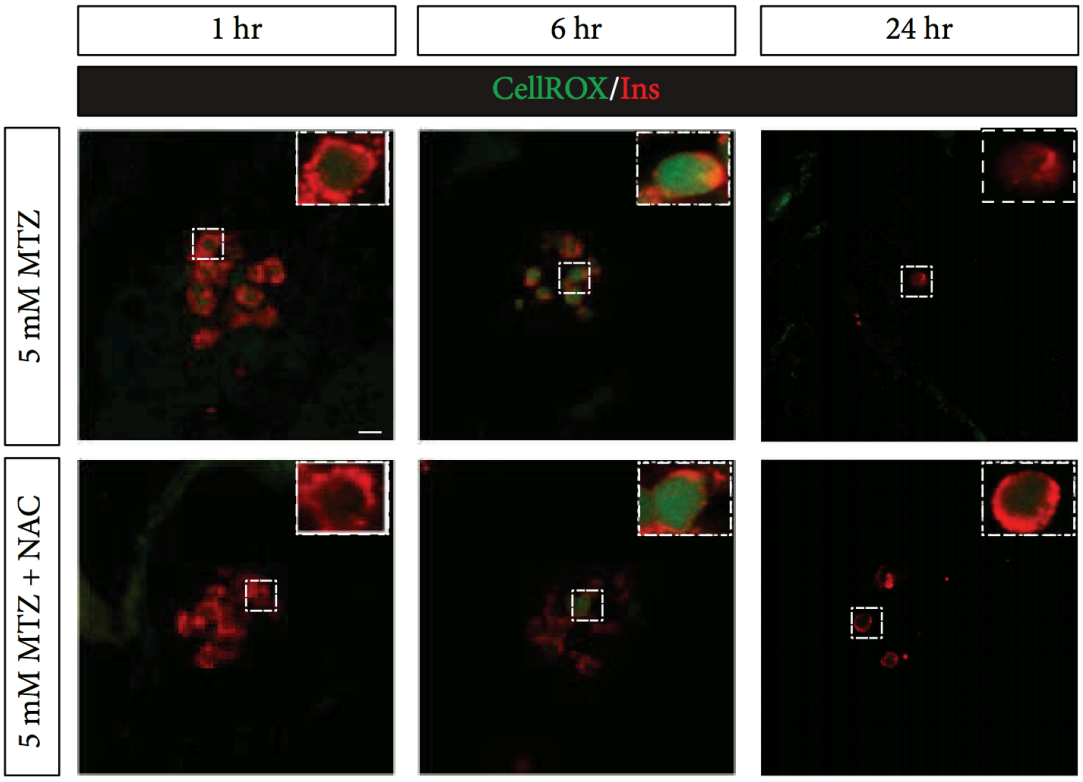


Figure 13: Macrophages infiltrate the islets in response to damage in the β-cells. Representative immunofluorescence images of zebrafish (mpeg+) islets (N = 6/condition) treated with 7.5 mM MTZ showing macrophage invasion into islets (green arrows) and their engulfment of β-cells (yellow arrows). Scale bar indicates 10 μm.

3.2.3. Antioxidants protect β-cells from MTZ-induced ROS generation:

Based on the data, I hypothesized that the generation of ROS in β-cells could be mitigated in this zebrafish model by the addition of small molecule antioxidants to the fish water. To test this hypothesis, I used the common antioxidant N-acetyl-L-cysteine (NAC). Zebrafish larvae were treated with an intermediate dose of 5 mM MTZ. This dose was chosen because it is strong enough to induce a rapid ROS response but would not overwhelm other treatments. I supplemented the MTZ treatments with 100 μM NAC and then measured the ROS intensity at multiple time points (Figures 14A-B). After either a 1- or 6-hour treatment of MTZ, NAC significantly reduced the levels of ROS staining in β-cells. Consistent with all other treatments, there was no significant difference with a 24-hour treatment, which again could be attributed to the ablation of nearly all β-cells in the presence of MTZ treatment alone (Figure 14B). Together, I conclude that MTZ drives the production of ROS in β-cells in the presence of NTR. Additionally, a known antioxidant was effective at mitigating this effect, suggesting that uncovering novel compounds can be efficient through screening approaches in this zebrafish system.


1 hr
6 hr
24 hr
CellROX/Ins
5 mM MTZ
5 mM MTZ + NAC

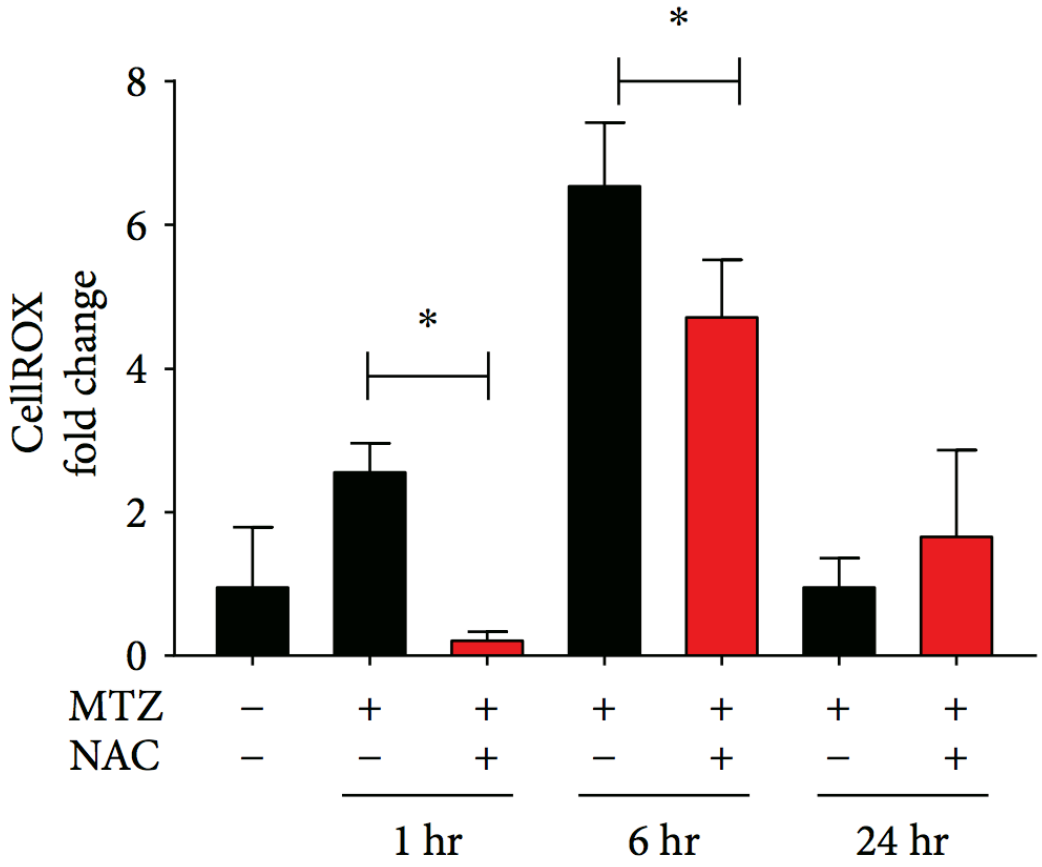


Figure 14: Antioxidant treatment protects from metronidazole-induced ROS generation in β-cells. Zebrafish larvae (n = 12 per condition) were treated with 5 mM metronidazole ± N-acetyl-L-cysteine (NAC) for 1, 6, or 24 hours followed by an assessment of ROS using CellROX green stain. (A) Representative images of islets of 106 hpf zebrafish (NTR+) larvae treated with 5 mM MTZ ± NAC at different time points. (B) Quantification of CellROX green intensity shows NAC-mediated protection from MTZ-induced ROS in β-cells after 1 or 6 hours of treatment. Data are presented as mean ± SEM ($*p < 0.05$). Statistical significance was determined by Student's t-test. Scale bar indicates 10 μm.

3.3. Discussion:

NTR (*NfsB*) is a type 1 oxygen-insensitive nitroreductase that catalyzes the full reduction of nitroaromatic compounds under anaerobic conditions (Whiteway et al., 1998). In anaerobes, MTZ serves as a prodrug that is metabolized by NTR to generate cytotoxic derivatives capable of blocking DNA synthesis and inducing DNA damage (Sisson et al., 2000). In this study, I found that MTZ also induces ROS generation in the presence of NTR. This ROS generation is consistent with the hypothesis that under aerobic conditions, as when expressed in mammalian cells, NTR might generate superoxide and derivative reactive oxygen species, potentially through a type 2-like "futile reduction cycle" (de Oliveira et al., 2010) (Figure 15).

As a β-cell ablation system, the relevance of MTZ-NTR to type 1 diabetes is evident. However, chronic ROS production and associated β-cell dysfunction are also critical to the pathology of type 2 diabetes even before the diminution of β-cell mass. Intriguingly, because the generation of ROS by NTR in this model is dependent on the dose of MTZ treatment, this provides a compelling opportunity to manipulate ROS under varied contexts. For instance, many other disease conditions like type 2 diabetes, atherosclerosis, diabetic neuropathy, and cancer arise as a result of chronic ROS generation in specific tissues (Lambeth, 2007). To model such cases, lower concentrations of MTZ could be used for generating persistent ROS conditions and studying the effects. Future studies will determine whether lower levels of ROS can be induced by MTZ treatments that are sufficient to impair β-cell function, but not to induce cell death.

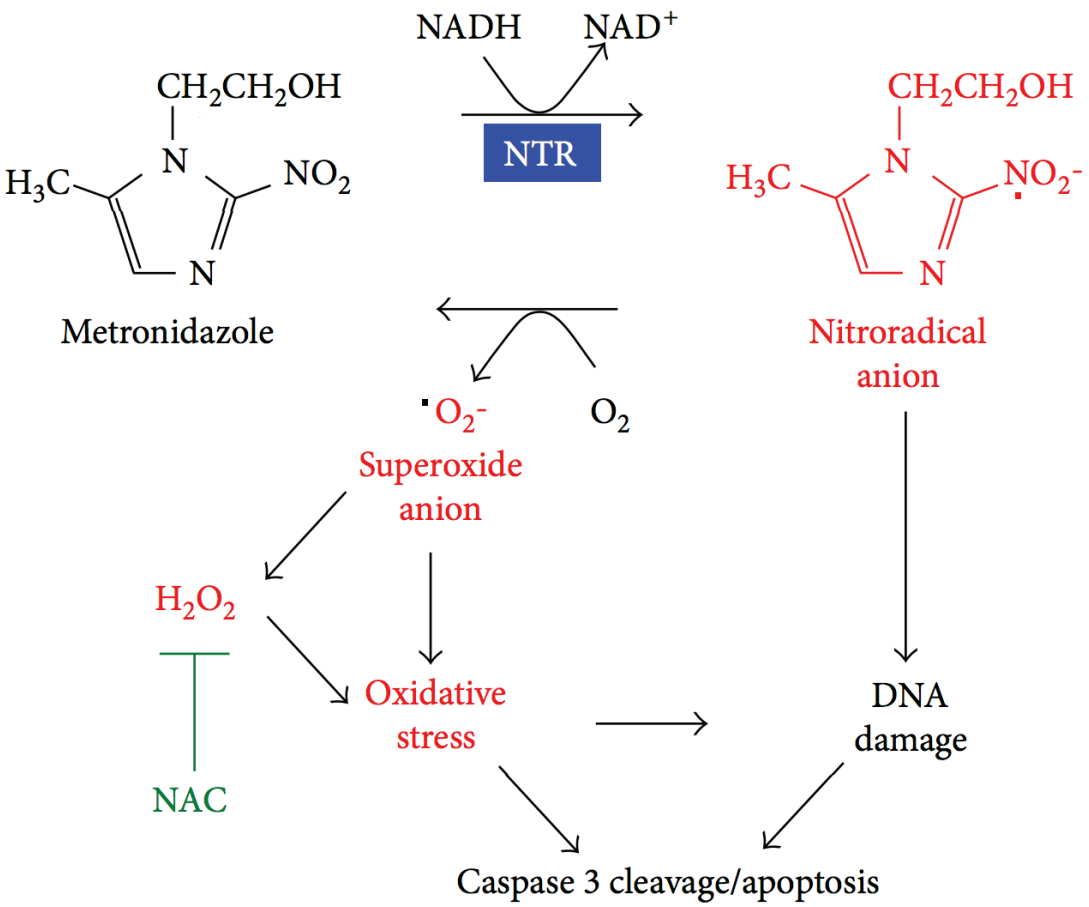


Figure 15: Proposed mechanism of MTZ-NTR-mediated cell ablation. In the aerobic setting of NTR-expressing eukaryotic cells, I propose that MTZ is reduced to a nitroradical anion by electron transfer from NADH, in a type 2-like mechanism. This radical may be cytotoxic and directly induces DNA damage and apoptosis. Alternately, this radical may regenerate back to metronidazole by electron transfer to O_2, concurrently forming superoxide anion and ROS derivatives. This, in turn, drives increased cellular-oxidative stress and triggering of regulated cell death.

In conclusion, zebrafish proves to be an outstanding model organism for studying ROS generation and ROS-related pathologies. Importantly, the physiological events that follow excessive ROS generation including infiltration of macrophages in response to injury as well as induction of apoptosis is conserved in zebrafish. This makes it a relevant *in vivo* model for visualization of oxidative stress and inflammation-mediated pathologies. Furthermore, the MTZ-NTR system seems to work exceptionally well for cell-specific ablation. However, with the added possibility of precisely modulating the ROS generation using MTZ dosing and antioxidants like NAC, this makes zebrafish a particularly flexible model. I exploited these strengths of this zebrafish model for our future studies.

Chapter Four: Depletion of macrophages restores the β-cell function after elimination of cellular stress

4.1 Introduction:

Macrophages are critical innate immune cells that regulate the initiation, progression, and resolution of inflammation (Liu et al., 2014b). Infiltration of macrophages in large numbers is associated with maladaptive inflammation that contributes to multiple disease pathologies (Atri et al., 2018). In diabetes, there is an infiltration of macrophages into the islet during the different phases of pathogenesis (Niu et al., 2016; Walker et al., 2018). Macrophages are major sources of pro-inflammatory cytokines that activate the signaling for programmed cell death in the β-cells. Moreover, macrophages are the primary phagocytes that engulf the damaged β-cells (Ward et al., 2018). In this study, I hypothesized that, under conditions of maladaptive inflammation, macrophages prematurely phagocytose partially damaged β-cells, contributing to the rapid loss of β-cell mass. To test this, I depleted the macrophages in *Tg(ins:NTR)* zebrafish larvae using clodronate liposome injections and measured β-cell function and death. Clodronate is an ATP-analogue that induces cell death. When phagocytes engulf liposomes containing clodronate, they are rapidly ablated (Moreno, 2018). As macrophages are the primary phagocytes in larval zebrafish, they become depleted explicitly due to the clodronate action. After macrophage depletion, I injured the β-cells using the MTZ-NTR system and assessed the functionality of the islets in the absence of phagocytes.

4.2. Results:

4.2.1. Clodronate injection depletes macrophages in the zebrafish larvae for at least three days:

To determine the effectiveness of clodronate liposomes in ablating macrophages in zebrafish, I trans-pericardially injected *Tg(mpeg1:eGFP)* zebrafish larvae with clodronate (5mg/mL) at age 3 days post-fertilization. I then performed a tail-injury assay at 24, 48, and 72-hour time points. In this assay, there is macrophage accumulation at the injured site. As shown in Figure 16A, 10 macrophages were visualized within 10 μm of the cut fin in control samples at 6 hours post-injury. However, in clodronate treated zebrafish, no macrophages were seen near the injured tailfin 24 hours after injections. This effect of clodronate persisted over 72 hours (Figure 16B).

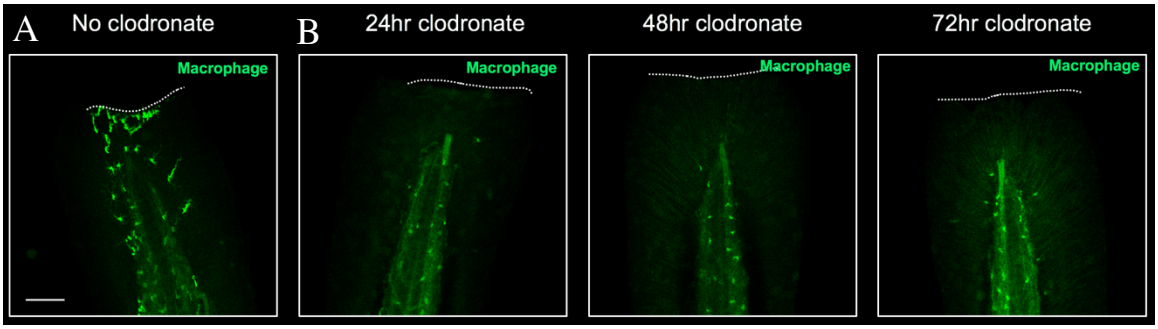
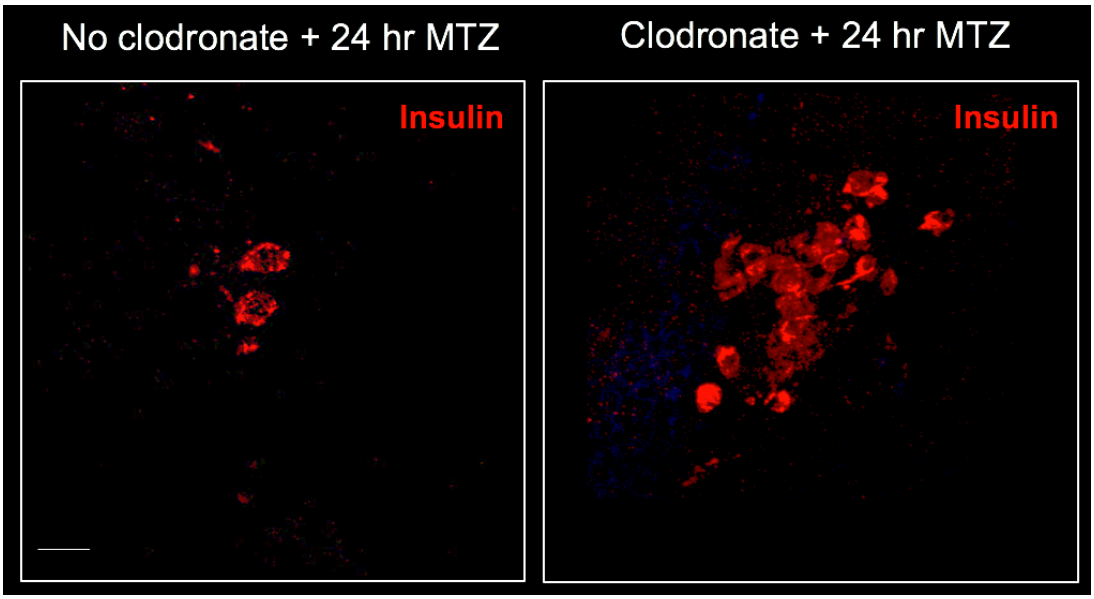


Figure 16: Clodronate treatment depletes macrophages from 24 to 72 hours after injection. (A) Representative image of a cut tailfin of a control zebrafish larva, which was not injected with clodronate. (B) Representative images of cut tailfins of clodronate-injected zebrafish larvae at 24 hours (left), 48 hours (middle), and 72 hours (right) after the injections, respectively. Scale bar indicates 100 μm.

4.2.2. β-cell mass is maintained after MTZ treatment in the absence of macrophages:

To assess the effects of macrophage depletion on β-cell mass under conditions of oxidative stress injury, I used larvae bearing the *ins:NTR* transgene that generates ROS in β-cells when treated with MTZ. I quantified the β-cell mass in the *Tg(ins:NTR)* larvae after 24-hour clodronate treatment followed by 24-hour MTZ treatment. In control samples that were not treated with clodronate, I observed near-complete ablation of β-cells suggesting that there was phagocytosis of β-cells by the macrophages (Figure 17A-left). By contrast, I found that the integrity of the islet was preserved in the clodronate-treated larvae (17A-right). Upon quantification of the β-cell mass before and after the MTZ treatment, I observed that 24 hours of clodronate treatment does not affect the β-cell mass (Figure 18B - 0 hr MTZ). More importantly, after 24 hours of MTZ treatment, the number of β-cells in the clodronate-injected samples were comparable to the controls (18 vs. 16.3, P=NS) (Figure 17B- 24 hr MTZ). These data emphasize that depletion of macrophages protects β-cell mass during ROS-mediated injury (Figure 18).

A.


No clodronate + 24 hr MTZ
Insulin
Clodronate + 24 hr MTZ
Insulin

B.

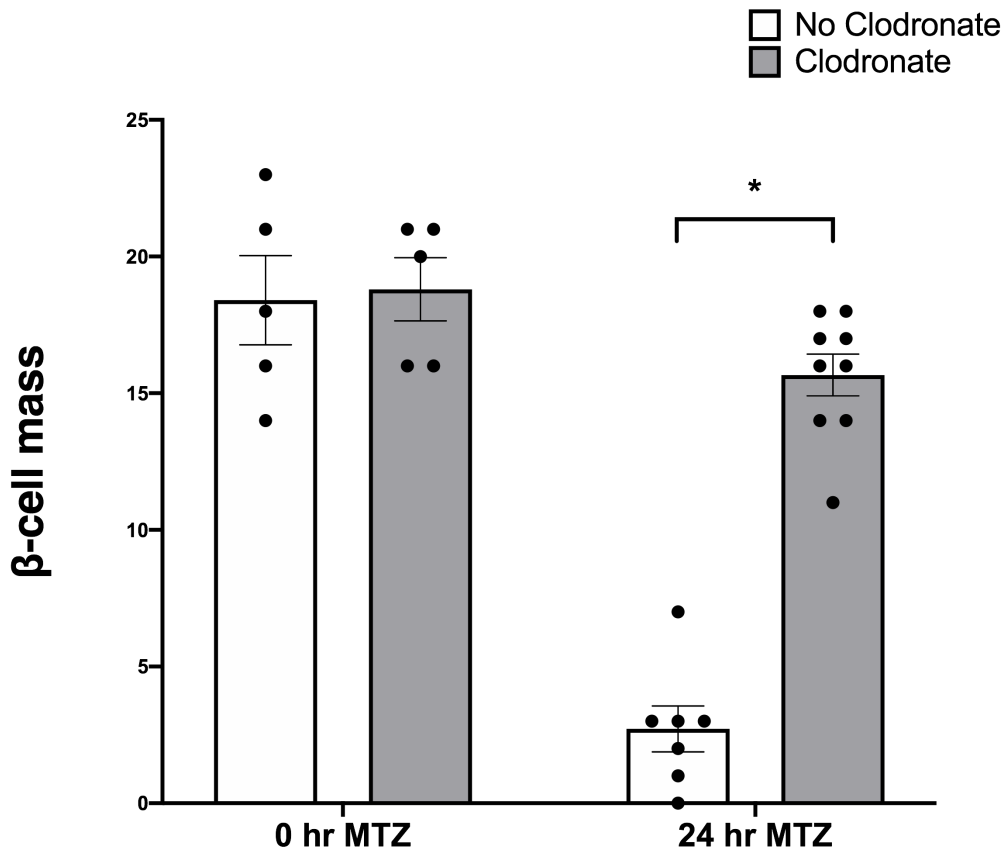


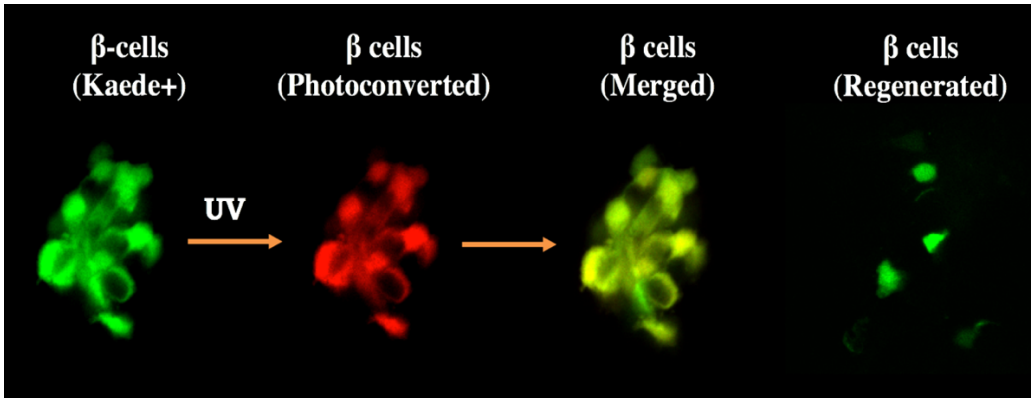
Figure 17: Macrophage depletion protects β-cell mass from ablation. (A) Representative images of islets zebrafish larva, which was not injected with clodronate (left) and which was injected with clodronate for 24 hours (right) after 24 hours of MTZ treatment. (B) Quantification of the β-cell mass in control and the clodronate injected larvae at 0 and 24 hours of MTZ treatment. Data are presented as mean ± SEM ($*p < 0.05$). Scale bar indicates 10 μm.

4.2.3. β-cell neogenesis is downregulated in clodronate-injected, MTZ-treated larvae:

I next asked whether the apparent preservation of β-cell mass was due to neogenesis. For this, I utilized the *ins:Kaede* transgene, which permits the temporal labeling of β-cells with green or red fluorescence. Kaede is a photoconvertible fluorescent protein, which emits green light in its native conformation; however, when exposed to UV light, it is converted to a conformation that emits red light. If a living cell continues to express the *Kaede* transgene, it will emit a yellow light (combination of red and green light). In contrast, any regenerated cell that begins expressing Kaede that has not been exposed to UV treatment will emit only green light. By exploiting this property of Kaede, it is possible to distinguish newly formed, green neogenic β-cells from pre-existing yellow β-cells (Figure 18A). In this study, I treated the clodronate injected and non-injected *Tg(ins:NTR);Tg(ins:Kaede)* larvae with MTZ for 24 hours, and then transferred them to regular fish water to eliminate the cellular stress and allow the β-cells to regenerate.

Upon quantification of the number of regenerated β-cells, the control larvae showed significant neogenesis of β-cells as compared to clodronate-injected larvae (3.5 vs. 1.8, $P<0.05$) (Figure 18B -24 hours regeneration). After 48 hours of recovery after MTZ removal, the difference in the neogenesis was even more significant in the clodronate-injected as compared to the non-injected control larvae (Figure 18B – 48 hours regeneration). Whereas the clodronate non-injected larvae at 48 hour of recovery showed more than 3-fold increase in the green neogenic β-cells relative to larvae with 24 hours of recovery (11.67 vs. 3.5, $P<0.05$), the clodronate injected larvae showed no significant difference in the number of neogenic β-cells (2.7 vs. 1.8, P=NS) (Figure 18B). These data indicate that very little β-cell neogenesis occurred in the clodronate-treated larvae.

A.


β-cells
(Kaede+)
UV
β cells
(Photoconverted)
β cells
(Merged)
β cells
(Regenerated)

B.

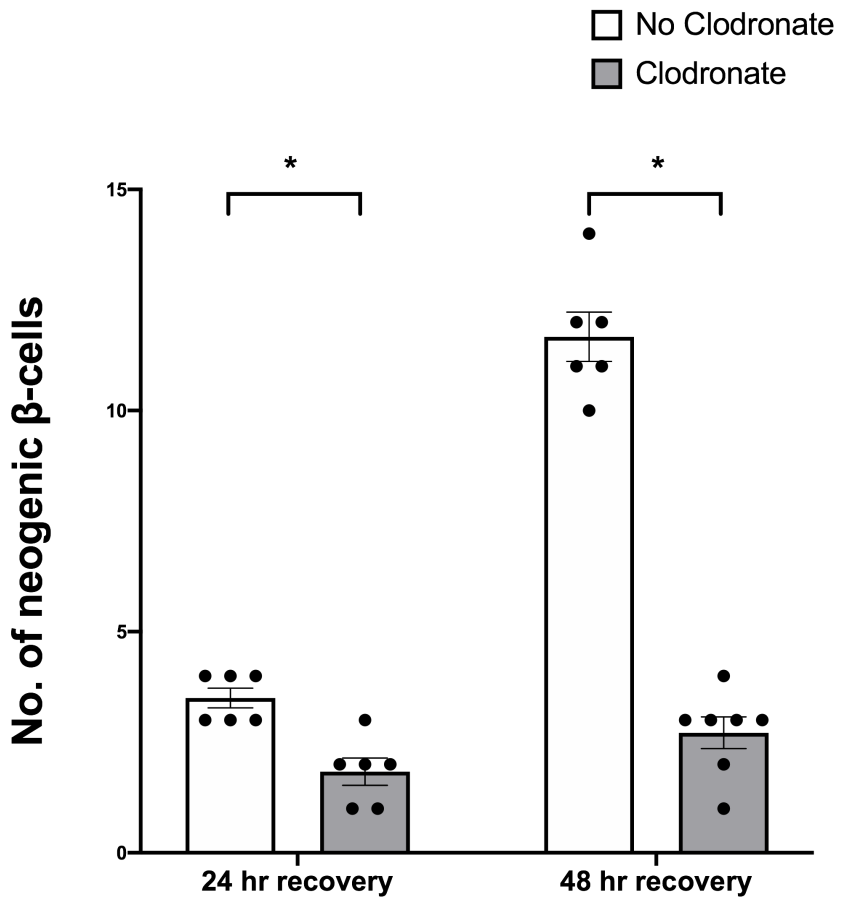


Figure 18: β-cell neogenesis is downregulated in clodronate-injected, MTZ-treated zebrafish. (A) Representative image of the islet from *Tg(ins:kaede)* zebrafish larvae. The β-cells express photoconvertible GFP (green). UV treatment converts GFP to RPF (red), making the photoconverted β-cells express both GFP and RFP (yellow). However, the regenerated β-cells express only the GFP as they are not photoconverted (green-first from the right) (B) Quantification of the regenerated β-cells mass in the controls and the clodronate-injected larvae after 24 hours and 48 hours of recovery in regular fish water. Data are presented as mean ± SEM ($*p < 0.05$).

4.2.4. *The surviving β-cell mass recovers function after ROS-injury in the absence of macrophages*:

The data above suggest that the β-cell mass observed in the larvae at the conclusion of MTZ and clodronate treatments is comprised almost entirely of pre-existing β-cells. Thus, I was compelled to determine if these pre-existing β-cells were functional with regard to glucose regulation, or if they were dead or otherwise defunct cells that had not been cleared due to lack of macrophages. To address this issue, I performed free glucose assays on pooled whole-zebrafish lysates at different stages of MTZ treatment and recovery.

I found that control samples that were not treated with MTZ showed constant free glucose levels over the course of the 4-day experiment (4 dpf-7 dpf) irrespective of the clodronate treatment (Figure 19 – purple and green line). However, in the 5 dpf zebrafish that were treated with MTZ for 24 hours, I found a steep increase in free glucose levels (Figure 19 – dark blue and red line). Next, at 6 dpf, after 24 hours of recovery after removal from the MTZ treatment, free glucose levels were still elevated (Figure 19 – dark blue and red line). However, at 7 dpf—48 hours following their removal from MTZ, the free glucose levels decreased substantially and trending towards returning to the levels similar to those seen in the control samples (Figure 19 – dark blue and red line vs. green and purple lines). Importantly, when the larvae were treated with MTZ after macrophage depletion via injection of clodronate, the free glucose levels were significantly lower than the MTZ-only group, although the overall trend of free glucose levels was still elevated, similar to control MTZ treatments (Figure 19 – At 6 dpf – dark blue vs. red line).

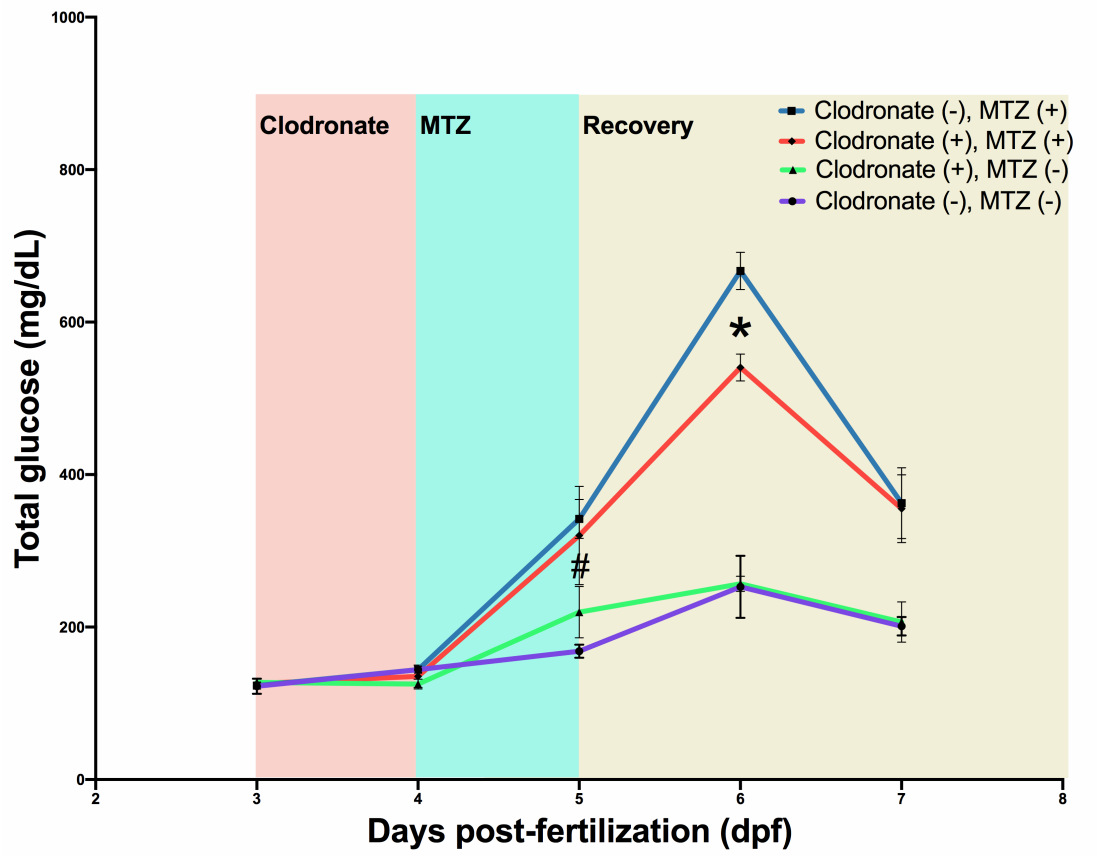


Figure 19: The surviving β-cell mass recovers glucoregulatory function after ROS-injury in the absence of macrophages. The graph represents free glucose measurements from whole-zebrafish lysates that were collected at different stages of MTZ treatment and recovery conditions as follows: MTZ-untreated and clodronate non-injected (purple), MTZ-untreated and clodronate-injected (green), MTZ-treated and clodronate non-injected (dark blue) and MTZ-treated and clodronate-injected (red). Clodronate treatment was done from 3 dpf-4 dpf (pink region), the MTZ treatments were done from 4 dpf-5 dpf (blue region), and the recovery phase in the fish water was done from 5 dpf-7 dpf (yellow region). Data are presented as mean ± SEM ($*p < 0.05$).

4.3. Discussion:

Macrophages have been implicated in the pathogenesis of both T1D and T2D. Evidence from several studies has indicated that macrophage depletion protects mouse models from the development of diabetes (Carrero et al., 2017; Jun et al., 1999). However, there are no studies that address the mechanisms by which the macrophages promote hyperglycemia in response to β-cell injury. In my study, I have used a zebrafish system to explore the immunological events that occur immediately after oxidative stress-mediated β-cell injury, and also during islet recovery, both in the presence and in the absence of macrophages.

Firstly, I identified the ideal conditions for ensuring the complete ablation of macrophages using clodronate injections i.e., 7-10 nL per embryo of 5 mg/mL clodronate for 24 hours. Importantly, I used this tailfin injury assay for the initial characterization of macrophage behavior since it is challenging to visualize deeply located macrophages that could remain hidden. Because macrophages are very sensitive to tissue injuries, macrophages that were not eliminated during the clodronate-induced phagocytosis would have been visible at the tail injury site. However, no macrophages were observed at the injury site, suggesting that I had achieved complete ablation, which persisted over 72 hours after the clodronate-injections. Since my experimental conditions did not exceed over 72 hours after the treatment, the persistence of the macrophage depletion by clodronate beyond 3 days of the treatment, still remains unexplored.

The role of macrophages in β-cell injury is widely studied in the context of T1D pathogenesis. The macrophages are one of the primary responders to β-cell stress as well as major sources of pro-inflammatory cytokines that ultimately cause β-cell destruction. I

wanted to determine the fate of these stressed β-cells in the absence of macrophages. Upon clodronate-mediated depletion of macrophages and after inducing stress by MTZ treatment, I observed near-complete protection of the β-cell mass. This data highlights one of the important yet unexplored aspects of macrophage function i.e., phagocytosis of damaged and dead cells. In the context of diabetes, macrophages are often studied for their roles as mediators of inflammation. However, their role in β-cell phagocytosis is not well studied. It is a crucial aspect, especially since the capacity of the β-cells to regenerate or replicate is extremely low (Kulkarni et al., 2012; Rankin and Kushner, 2009; Teta et al., 2005). Hence, the clearance of these cells leads to a state that is beyond repair. On the other hand, depletion of the macrophages protects this β-cell mass, suggesting that macrophages are active participants in the ablation of these insulin-producing cells of the islet in this model.

However, there are at least two possible explanations for the persistence of the islet β-cell mass. First, β-cells might persist because no macrophages are present to engulf the injured and dead β-cells. Second, the β-cells might rapidly regenerate because macrophages in the pancreas actively regulate β-cell neogenesis and replication (Nackiewicz et al., 2019; Xiao et al., 2014b), and that their depletion effectively de-represses new β-cell formation. Thus, to distinguish between these two possibilities, I used *Tg (ins:Kaede); Tg(ins:NTR),* which is a powerful tool to distinguishing the newly formed 'neogenic' β-cells from the pre-existing β-cells. In this experiment. I observed that, right from 24 hours of recovery from MTZ treatment, there were significantly higher neogenic β-cells in the controls as compared to the clodronate-injected larvae. This difference extended even further by 48 hours of recovery, especially since there was no difference in

the number of neogenic β-cells in the clodronate-injected samples from 24 to 48 hours of recovery. These data suggest that macrophages are critical factors for β-cell regeneration.

Finally, it was essential to determine whether the persisting β-cells were still functional, whether they were non-functional and temporarily dormant, but viable, or whether they were dead β-cells that were not cleared due to the absence of primary phagocytes. For this, I performed a colorimetric free glucose assay on pools of whole zebrafish larvae. This assay is based on a glucose oxidase enzyme-based method in which liberated glucose in the zebrafish lysates is specifically oxidized. This generates an intermediate product that reacts with a dye. The intensity of the resulting color is proportional to free glucose concentration and can be used as a surrogate measure of blood glucose in zebrafish larvae. With 24 hours of MTZ treatment, I observed a sharp increase in the free glucose levels that were measured in both the clodronate-injected and the non-injected larvae, as compared to the non-ablated controls. However, the free glucose levels in larvae treated with MTZ and clodronate was significantly lower than in those subjected to MTZ alone. The hyperglycemia seen in both cases could be attributed to a severe disruption of β-cell functions. In the clodronate non-injected, β-cell-ablated larvae, there are essentially no β-cells remaining to release insulin. On the other hand, in the clodronate-injected larvae, although there are β-cells, they are likely to be severely damaged. Importantly, the differences in the glucose levels suggest that, although injured, there was still some insulin-related function in the persisting β-cells. Importantly, after the larvae were removed from MTZ and transferred to fresh fish water, the exogenous source of β-cell oxidative stress was eliminated. In the clodronate-injected larvae, the injured but surviving β-cells, recovered from the ROS-mediated damage. The required recovery time

could explain a higher spike in the glucose levels at 24 hours in the recovery phase. However, it was still significantly lower than the clodronate non-injected larvae, in which there was a complete loss of β-cell mass with only about four β-cells evident at 24 hours of recovery. However, at 48 hours of recovery, there was a significant decrease in the glycemic levels, almost matching levels measured in the control larvae. This clodronate injected group suggests that 48 hours after the elimination of the stress inducer, the β-cells can recover their function. On the other hand, the non-injected group suggests that 12-14 functional β-cells can maintain normal glycemic levels as long as they are no more subjected to stress during the larval stage of the zebrafish.

In conclusion, my data show a significant new role for macrophages in β-cell homeostasis. Canonically, macrophages are known to infiltrate in large numbers and also release pro-inflammatory cytokines leading to maladaptive inflammation in the pancreas during diabetes pathogenesis. In this study, I show another aspect of macrophage function that might further exacerbate hyperglycemia. Here, I see that macrophages prematurely phagocytose injured β-cells that may still have the capacity to recover their function—if there was an abatement of the stress. Probably, that is the reason for the protection of the NOD mice model from the development of diabetes upon depletion of macrophages. However, depletion of macrophages would have drawbacks as a treatment strategy –it could weaken the immune system. Hence, I believe that if there is a more targeted prevention of macrophage infiltration in the pancreatic islets, this would be a better strategy to protect the β-cells from damage.

Chapter Five: 12-lipoxygenase regulates macrophage infiltration during islet inflammation

5.1 Introduction:

Lipoxygenases (LOX) are enzymes that catalyze the di-oxygenation of poly-unsaturated fatty acids. Specifically, 12-lipoxygenase (12-LOX) catalyzes the conversion of arachidonic acid to the eicosanoid 12-hydroxyeicosatetraenoic acid (12-HETE) (Ikei et al., 2012). Both 12-LOX and 12-HETE are implicated in pancreatic islet inflammation (Dobrian et al., 2019; Ma et al., 2010; Tersey et al., 2014). 12-LOX expression is upregulated in islets isolated from patients with either pre-diabetes or T2D (Tersey et al., 2015). Additionally, the treatment of human islets with pro-inflammatory cytokines significantly upregulates the gene expression of 12-LOX (Ma et al., 2010). With regard to enzyme function and pancreatic expression, mouse enzyme 12-LOX encoded by *Alox15*, and zebrafish enzyme 12-LOX encoded by *alox12*, are homologous to human 12-LOX encoded by the gene *ALOX12* (Dobrian et al., 2011; Haas et al., 2011; Kuhn et al., 2015). As seen in human islet studies, mutant mice lacking 12-LOX are protected from deterioration of islet dysfunction in the settings of either pro-inflammatory cytokine exposure, high-fat diet, or on the NOD background (Green-Mitchell et al., 2013). These data suggest a strong correlation between 12-LOX, the regulation of inflammation, and the pathogenesis of diabetes. However, studies that focus on the roles played by 12-LOX in immune cell-mediated inflammation in the context of diabetes are limited.

As discussed in previous chapters, the infiltration of macrophages into the pancreas promotes an inflammatory state due to the release of pro-inflammatory cytokines. This inflammatory environment causes damage to the β-cells of the pancreatic islets and

consequently promotes β-cell apoptosis (Kanter et al., 2012; Kraakman et al., 2014). This inflammation-mediated damage leads to a reduction in insulin secretion, which in turn promotes the progression of diabetes. Furthermore, the stress signals emitted from damaged β-cells further enhances macrophage infiltration and their associated phagocytosis activity. Since the regenerative capacity of β-cells is low, this phagocytic activity effectively leads to a permanent loss of β-cell mass, thus exacerbates diabetes pathophysiology. In this respect, several studies have suggested that depletion of macrophages, either islet-resident macrophages or a global deletion, can significantly slow or prevent the development of diabetes (Carrero et al., 2017; Jun et al., 1999). Practically, the impairment of macrophage infiltration into the islets would prevent the exacerbation of inflammation. Thus, by uncovering and manipulating the mechanisms that control macrophage migration, it will be possible to devise novel therapeutic strategies that would protect β-cell mass and thus halt or reverse diabetes progression.

12-LOX is expressed in macrophages at levels significantly higher than in other tissues (Wuest et al., 2012). Furthermore, in other contexts, there is evidence that 12-LOX promotes inflammation and cellular migration, and this could potentially extrapolate to macrophage migration and infiltration in the islets (Klampfl et al., 2012; Nie et al., 2000). However, there is no direct evidence of a role for 12-LOX in macrophage polarization or migration. In my studies, I investigated the roles of 12-LOX in macrophages using the strengths of both mouse and novel transgenic zebrafish models. I tested the hypothesis that 12-LOX promotes macrophage polarization to a pro-inflammatory (M1) phenotype, as well as migration, which together promotes damaging inflammation in the islets.

5.2. Results:

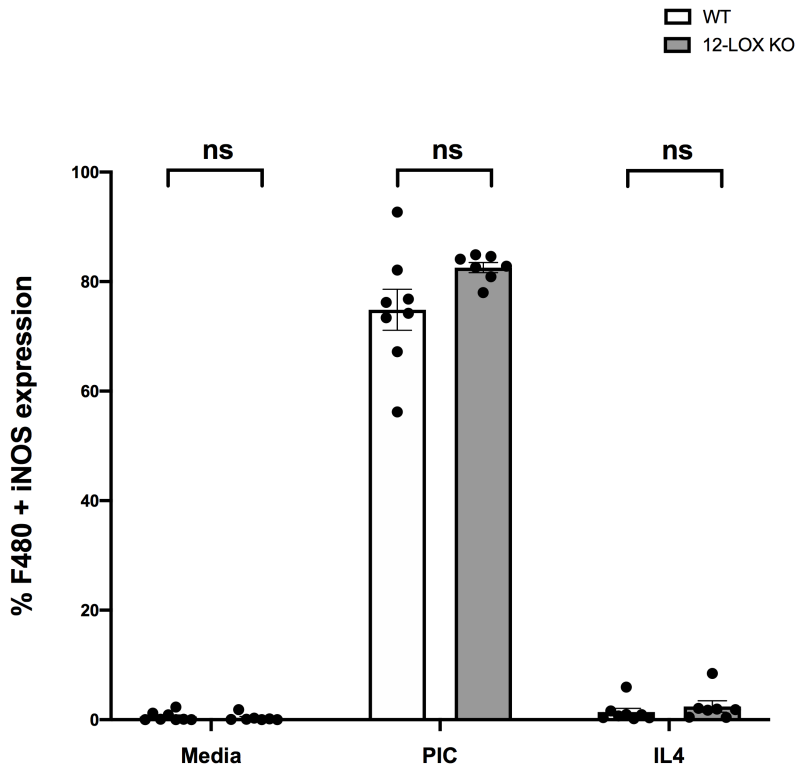
5.2.1. 12-LOX does not directly affect the polarization of the macrophages:

Macrophages become polarized towards either a pro-inflammatory (M1) or an anti-inflammatory (M2) phenotype based on the stimuli received from their environment (Liu et al., 2014b). As discussed above, 12-LOX has been associated with the induction and amplification of inflammation. Hence, I hypothesized that the depletion of 12-LOX would prevent polarization of the macrophages to the M1 phenotype, would promote M2 polarization, or both. Upon treatment with a pro-inflammatory cytokine cocktail, there was an upregulation in inducible nitric oxide synthase (iNOS) expression (Figure 20A). However, no significant difference in expression between WT and 12-LOX KO peritoneal macrophages was observed. I next tested whether loss of 12-LOX affected the levels of a major cytokine released by the M1 polarized macrophage, IL-6. Again, I observed no significant difference in the production of IL-6 between WT and 12-LOX KO (Figure 20B). Finally, I checked the gene expression levels of IL-6 and IL-12, two cytokines characteristically expressed in M1-polarized macrophages, to investigate polarization status at the transcriptional level. Consistent with my earlier observations, there was no difference detected in these markers of polarization (Figure 20C).

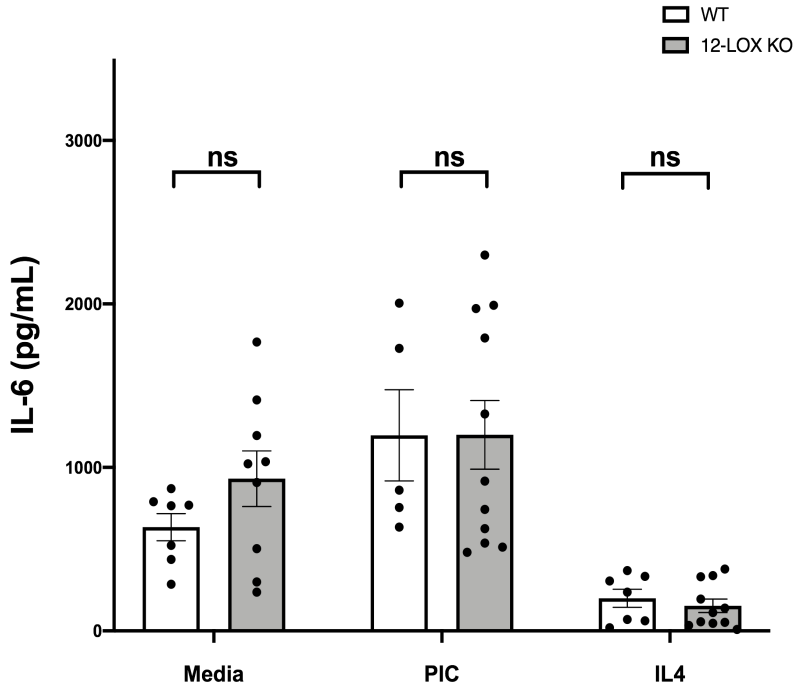
Next, I assessed whether loss of 12-LOX affected the M2 polarization of macrophages. Treatment with IL-4 promoted the expression of the mannose receptor (CD206) in the macrophages; however, both WT and 12-LOX KO macrophages expressed it similarly (Figure 20D). IL-4 treatment also promoted the release of the anti-inflammatory cytokine, IL-10. Although there was no significant difference in IL-10 production, there was a trend towards increased IL-10 release in 12-LOX KO macrophages (P=0.053),

suggesting that the depletion of 12-LOX might promote anti-inflammatory responses by the macrophages (Figure 20E). Finally, I measured the gene expression levels of two key markers of M2 polarization: IL-10 and TGF-β. Here again, I observed no significant difference in the transcript levels of the markers of M2 polarization (Figure 20F).

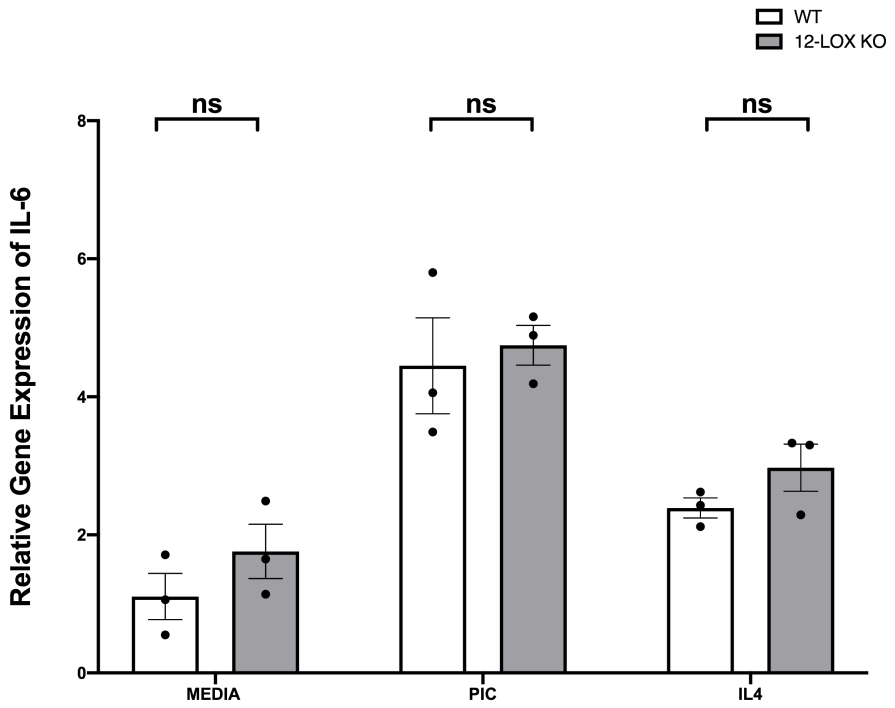
A.

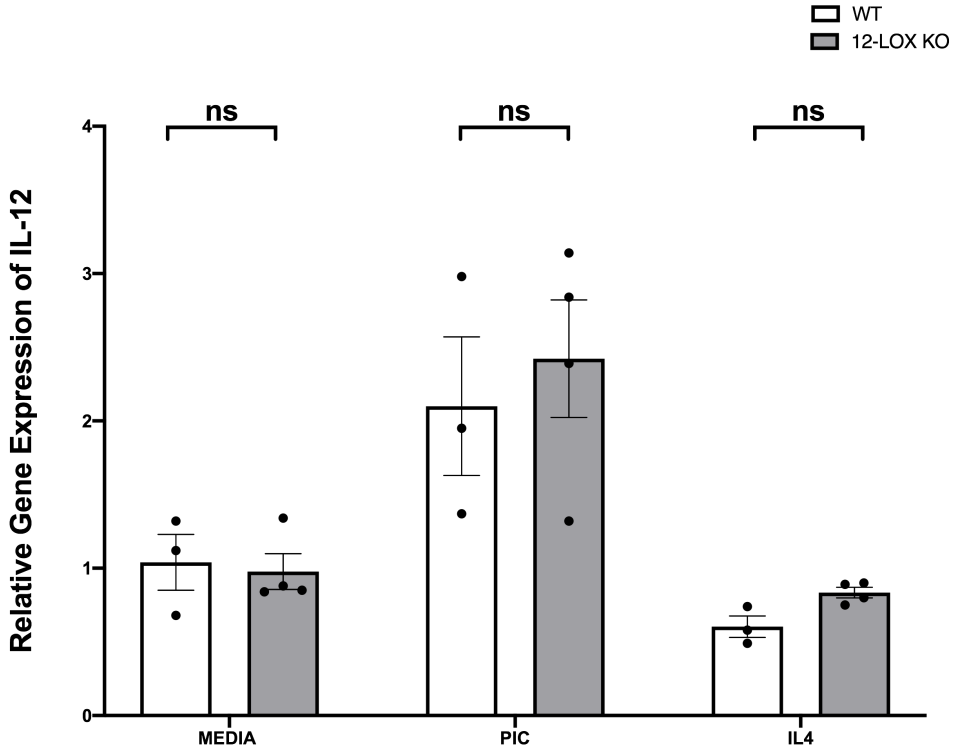

WT
12-LOX KO
ns
ns
ns
% F480 + iNOS expression
100
80
60
40
20
0
Media
PIC
IL4

B.

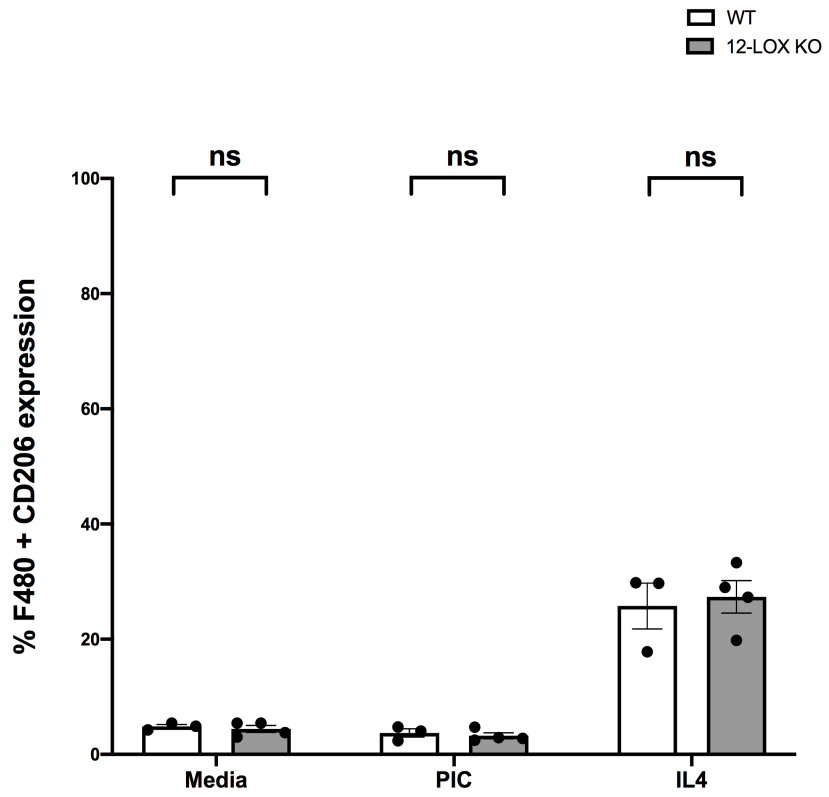

WT
12-LOX KO
ns
ns
ns
IL-6 (pg/mL)
3000
2000
1000
0
Media
PIC
IL4

C.

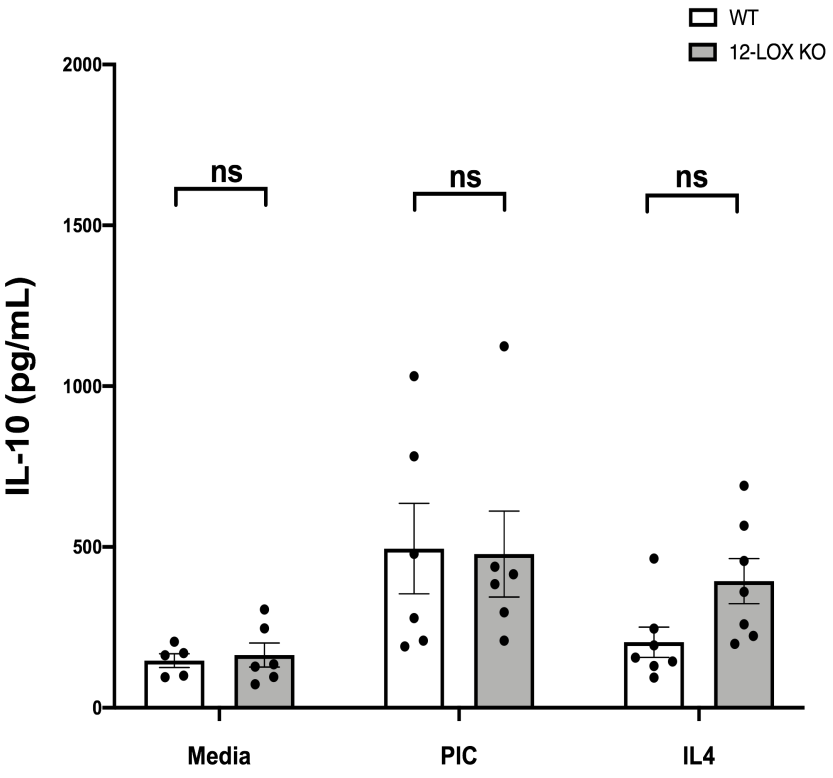

WT
12-LOX KO
ns
ns
ns
Relative Gene Expression of IL-6
8
6
4
2
0
MEDIA
PIC
IL4


WT
12-LOX KO
ns
ns
ns
Relative Gene Expression of IL-12
4
3
2
1
0
MEDIA
PIC
IL4

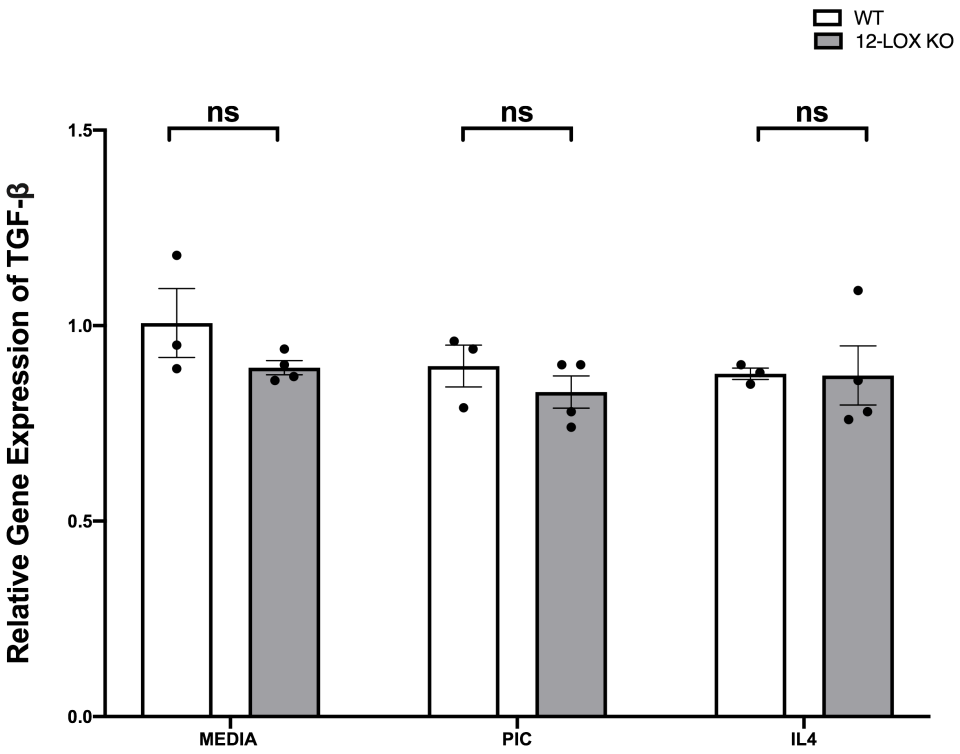
D.


WT
12-LOX KO
ns
ns
ns
% F480 + CD206 expression
100
80
60
40
20
0
Media
PIC
IL4

E.


WT
12-LOX KO
ns
ns
ns
IL-10 (pg/mL)
2000
1500
1000
500
0
Media
PIC
IL4

F.


WT
12-LOX KO
ns
ns
ns
Relative Gene Expression of TGF-β
1.5
1.0
0.5
0.0
MEDIA
PIC
IL4

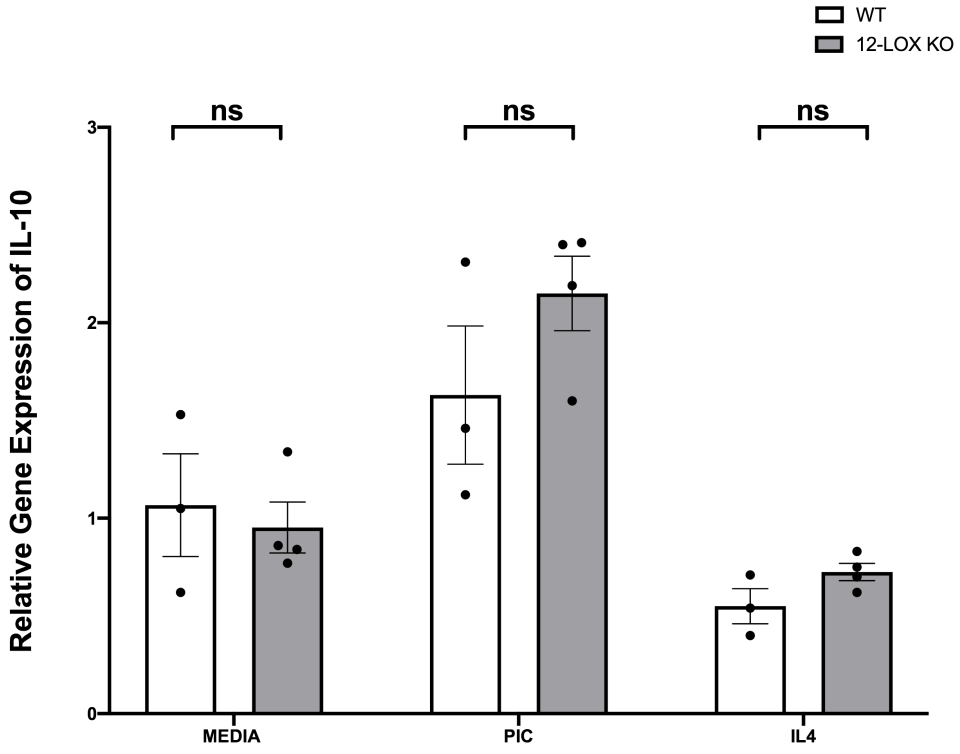
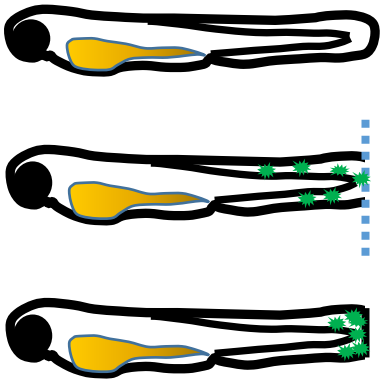

WT
12-LOX KO
ns
ns
ns
Relative Gene Expression of IL-10
3
2
1
0
MEDIA
PIC
IL4

Figure 20: 12-LOX does not directly affect the polarization of the macrophages. (A) Flow cytometry analysis of iNOS expression in WT and 12-LOX KO peritoneal macrophages. (B) ELISA for quantification of IL-6 production by the peritoneal macrophages. (C) qPCR for measuring gene expression of IL-6 (upper) and IL-12 (lower). (D) Flow cytometry analysis of CD206 expression in WT and 12-LOX KO peritoneal macrophages. (E) ELISA for quantification of IL-10 production by the peritoneal macrophages. (F) qPCR for measuring gene expression of IL-10 (upper) and TGF-β (lower) Data are presented as mean ±SEM.

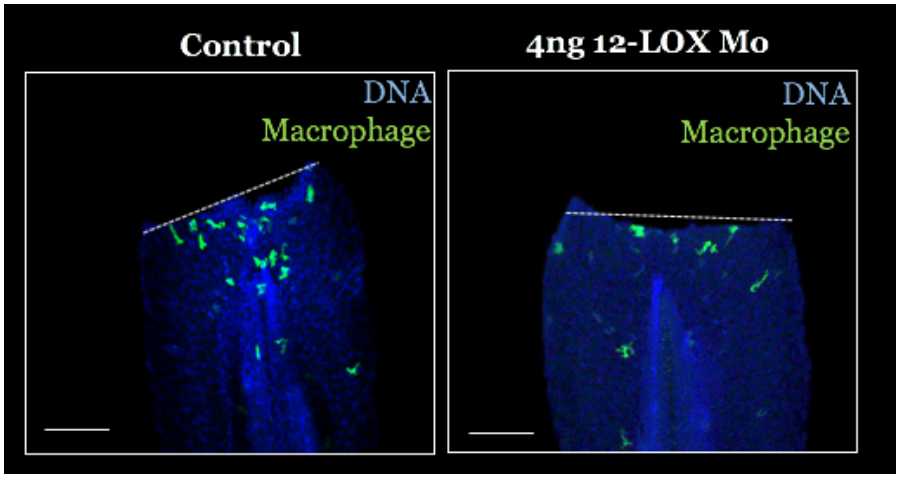
5.2.2. 12-LOX inhibition impairs migration of macrophages to the site of tailfin injury:

Zebrafish have been extensively utilized for studying inflammation because their optical transparency facilitates the visualization of immune cell infiltration *in vivo* and because they are amenable to genetic manipulation. To study the roles of 12-LOX in macrophage migration, I performed a tailfin injury assay, which is a well-established mechanical tissue injury model. In this assay, macrophages rapidly infiltrate to the injury site as part of an inflammatory response (Figure 21A). To reveal the functions of 12-LOX in the process of macrophage migration, I knocked-down 12-LOX protein levels by injecting a translation-blocking antisense morpholino at the one-cell embryonic stage. At 3 dpf (days post-fertilization), I performed the tailfin injury assay and quantified the number of macrophages that had migrated to the site of injury. I measured a significant reduction in the number of macrophages in the injured tailfins of morpholino-injected larvae as compared to the non-injected controls (Figures 21B-C). To further confirm this result using a second approach that may be therapeutically relevant, I performed an analogous experiment using the well-characterized small molecule ML355 (Luci et al., 2010) to inhibit 12-LOX activity. For this experiment, I pre-treated the larvae for 2 hours with either DMSO vehicle control or with 10 μM ML355, then performed the tailfin injury. The larvae were then returned to their respective drug/control treatments for an additional 6 hours. Similar to what I observed with the morpholino knockdowns, ML355 treatment prevented macrophage migration towards injured sites (Figures 21D-E).

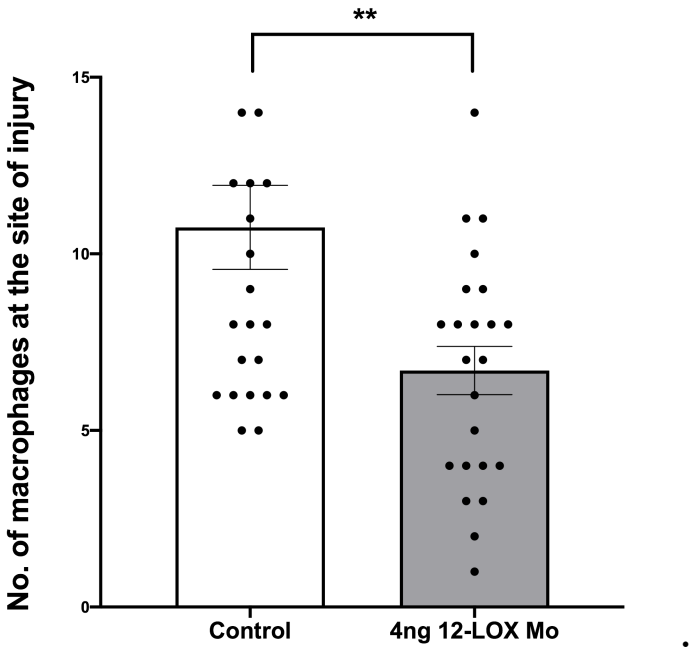
A.



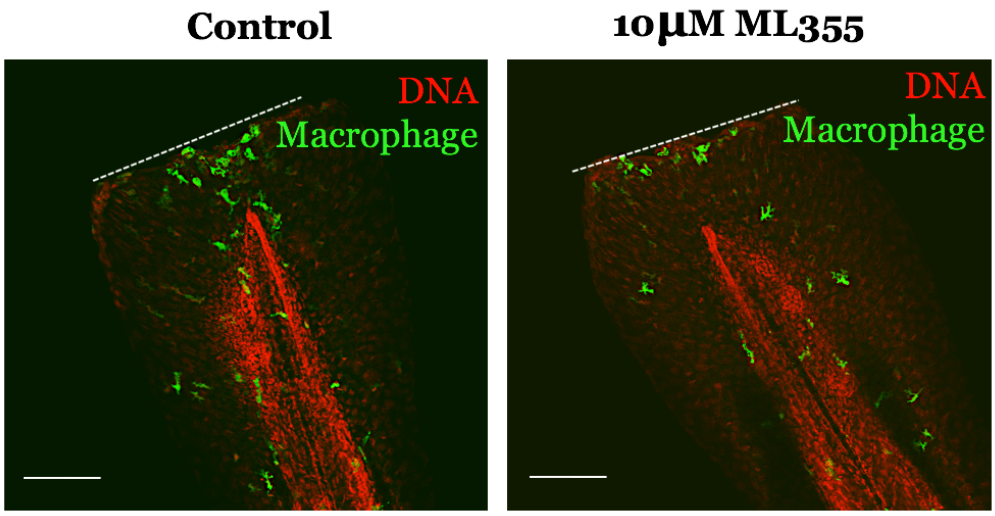
B.


Control
4ng 12-LOX Mo
DNA
Macrophage
DNA
Macrophage

C.


**
No. of macrophages at the site of injury
15
10
5
0
Control
4ng 12-LOX Mo

D.


Control
10µM ML355
DNA
Macrophage
DNA
Macrophage

E.

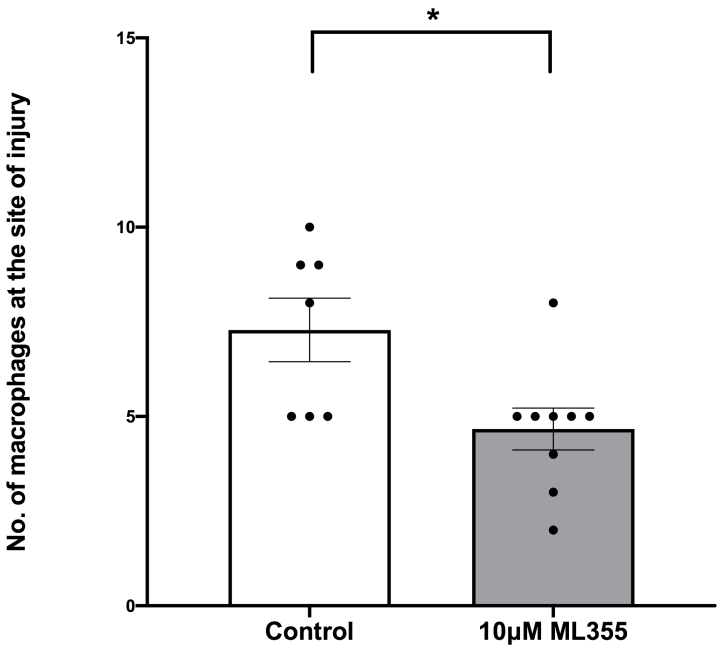
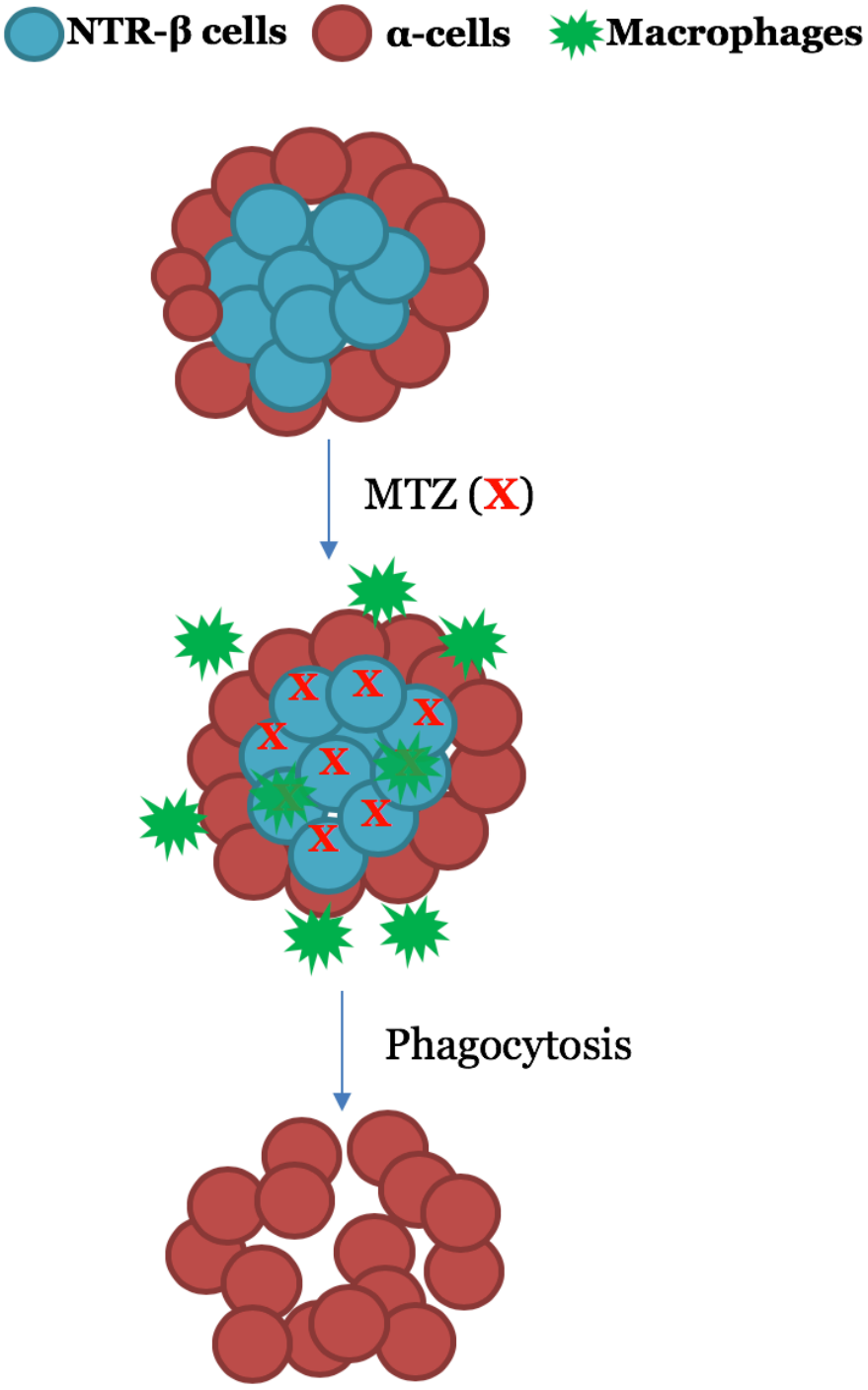

*
No. of macrophages at the site of injury
15
10
5
0
Control
10µM ML355

Figure 21: Inhibition of 12-LOX prevented the migration of macrophages to the tailfin injury site. (A) 4 dpf *Tg(mpeg:eGFP)* zebrafish larvae were subjected to tailfin injury for 6 hours. The GFP-labeled macrophages (green) accumulated at the injured site (B) Representative images of tailfins of zebrafish larvae with 4 ng *alox12* morpholino injected samples (right) or un-injected controls (left) (C) Quantification of the number of macrophages at the tailfin injury site shows a reduction with the knockdown of *alox12*. (D) Representative images of zebrafish tailfins in larvae treated with 10 μM ML355 (right) or DMSO vehicle control (left) (E) Quantification of the macrophage number at the tailfin injury site shows reduced inflammation after 6 hours of ML355 treatment. Data are presented as mean ± SEM ($*p < 0.05$). Scale bar indicates 100 μm.

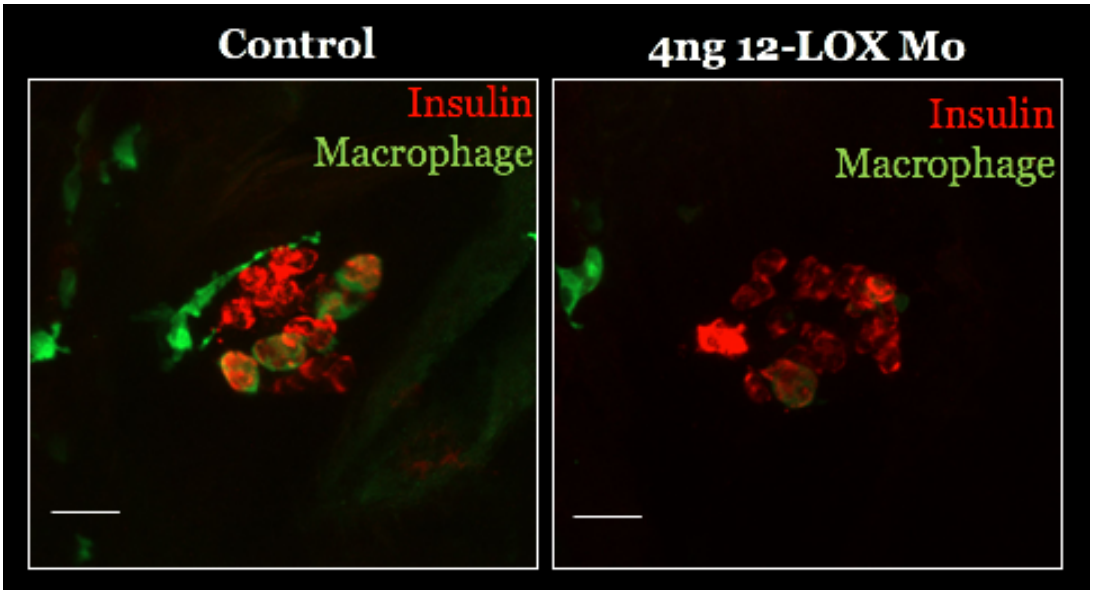
5.2.3. 12-LOX is required for migration of macrophages into the islet after β-cell injury:

Having uncovered a role for 12-LOX in macrophage migration in a mechanical injury context, I next investigated roles for 12-LOX during macrophage infiltration into pancreatic islets using a diabetes-relevant model system. In Chapter 3, I showed that macrophages associate with β-cells experiencing oxidative stress. Here, I have used the same chemical-genetic approach to drive oxidative damage in the β-cells (Figure 22A). 12-LOX was knocked down with morpholino or its activity was inhibited with 10 μM ML355 treatment. After oxidative β-cell injury, I measured a significant reduction in the number of macrophages that migrated to the injured islets in the *alox12* morpholino-injected larvae as compared to the non-injected controls (Figures 22B-C). These data suggest that 12-LOX has a role in macrophage migration. Similarly, when 12-LOX was inhibited with ML355, I observed similar effects wherein macrophage infiltration in the islets was reduced relative to DMSO-treated controls (Figures 22D-E)

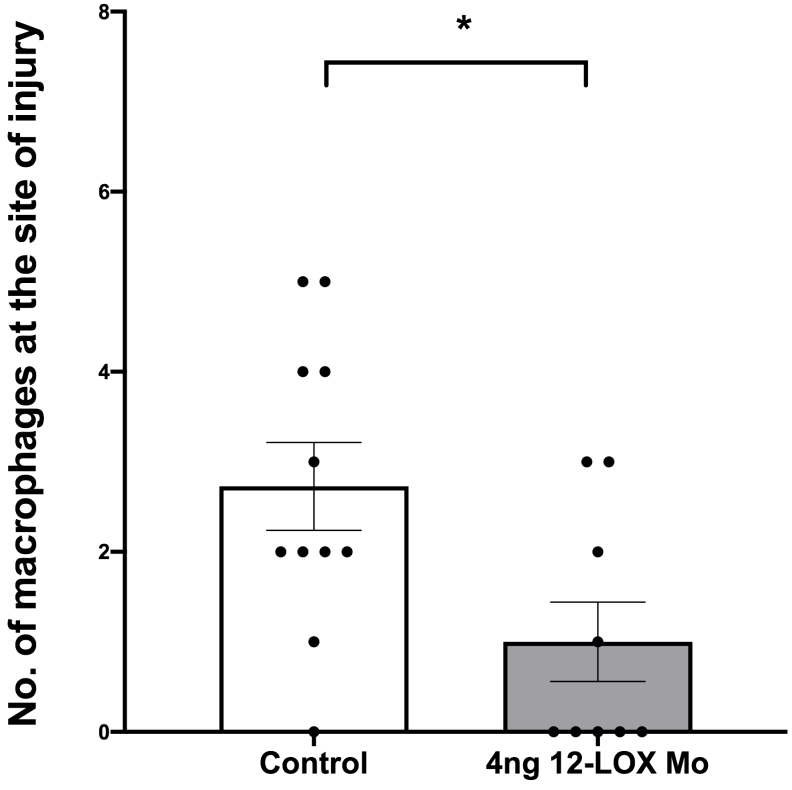
A.



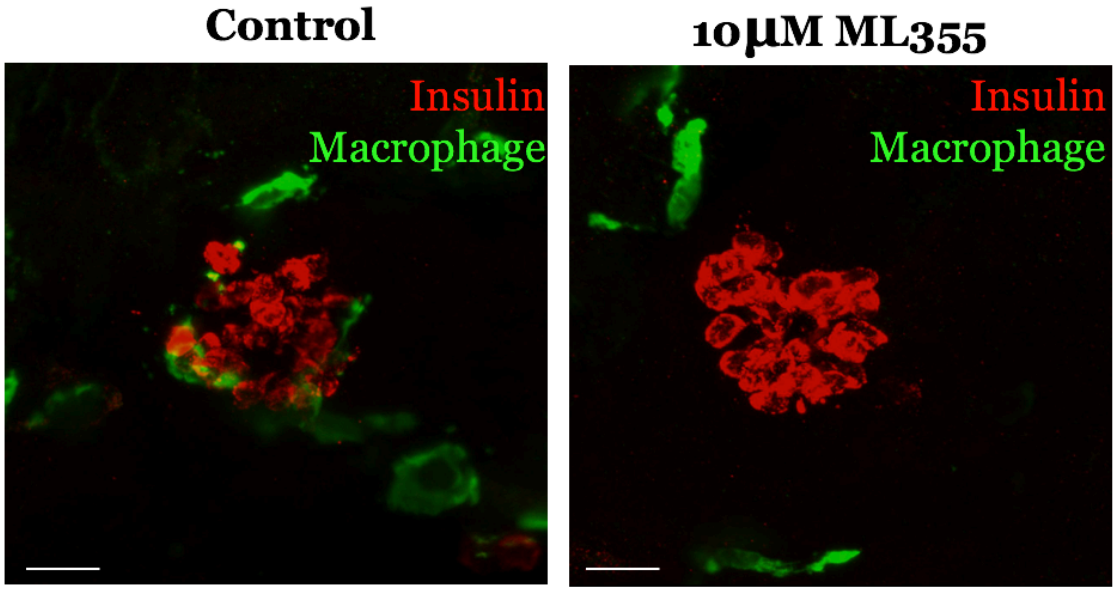
B.


Control
4ng 12-LOX Mo
Insulin
Macrophage
Insulin
Macrophage

C.


No. of macrophages at the site of injury
8
6
4
2
0
*
Control
4ng 12-LOX Mo

D.


Control
10µM ML355
Insulin
Macrophage
Insulin
Macrophage

E.

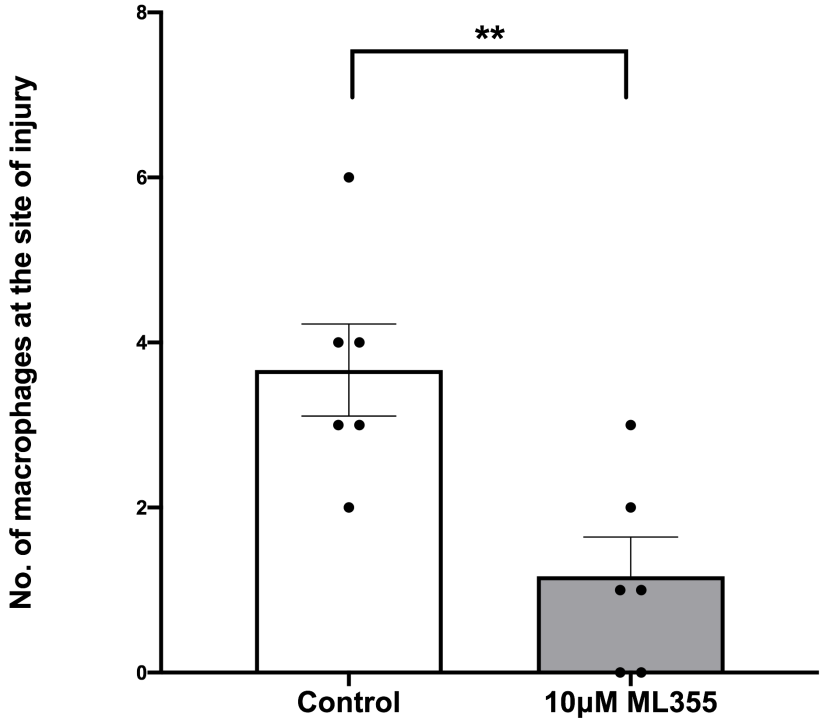
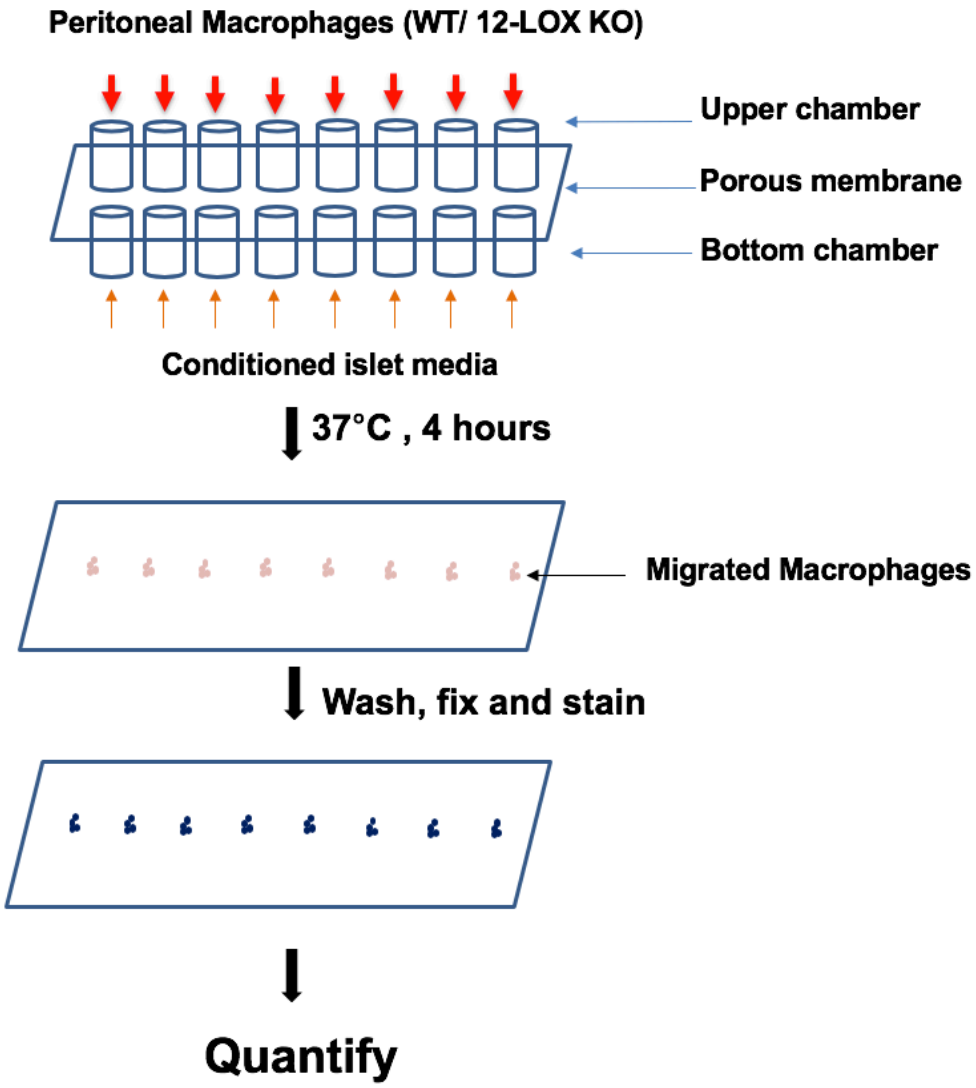

No. of macrophages at the site of injury
8
6
4
2
0
**
Control
10µM ML355

Figure 22: Inhibition of 12-LOX prevented migration of macrophages in the islet after β-cell injury. (A) 4dpf *Tg(mpeg:eGFP),Tg(ins:NTR)* zebrafish larvae were treated with 7.5 mM Metronidazole for 6 hours followed by sample processing and antibody staining. In response to β-cell injury, the macrophages infiltrate the islets and ultimately phagocytose the damaged β-cells. (B) Representative images of islets of zebrafish (NTR+) larvae treated with 7.5 mM MTZ with 4ng *alox12* morpholino injected samples (right), or un-injected controls (left) (C) Quantification of the number of macrophages at the injured islets shows protection from macrophage-mediated inflammation in β-cells by knockdown of *alox12*. (D) Representative images of islets of zebrafish (NTR+) larvae treated with 7.5 mM MTZ with 10 μM ML355 (right) or DMSO vehicle control (left). (E) Quantification of the number of macrophages at the injured islets shows protection from macrophage-mediated inflammation in β-cells after 6 hours of ML355 treatment. Data are presented as mean ± SEM ($*p < 0.05$). Scale bar indicates 40 μm.

5.2.4. Depletion of 12-LOX prevents macrophage chemotaxis in vitro:

Previous studies in my lab have demonstrated that loss of 12-LOX protects mice from developing diabetes in the NOD model (Hernandez-Perez et al., 2017). Based on this, I hypothesized that 12-LOX loss-of-function would prevent macrophage migration into the islets, and thus prevent any resulting inflammation-mediated damage to the β cells. To determine whether the role 12-LOX during macrophage migration into the zebrafish islets is conserved in mammals, I used the same 12-LOX knockout mouse model. I isolated peritoneal macrophages and pancreatic islets from wild-type (WT) and *Alox15* mutant mice. I then treated the islets with a pro-inflammatory cytokine cocktail for 24 hours and collected the conditioned media; this was utilized for the transwell macrophage migration assay. For this assay, I added WT or *Alox15* mutant macrophages to the upper chamber and measured their migration to the lower chamber in response to conditioned media from islets (Figure 23A). Consistent with the results from the zebrafish studies, I found a substantial reduction in the migration of the 12-LOX KO peritoneal macrophages as compared to the WT macrophages (Figure 23B).

A.


Peritoneal Macrophages (WT/ 12-LOX KO)
Upper chamber
Porous membrane
Bottom chamber
Conditioned islet media
37°C , 4 hours
Migrated Macrophages
Wash, fix and stain
Quantify

B.

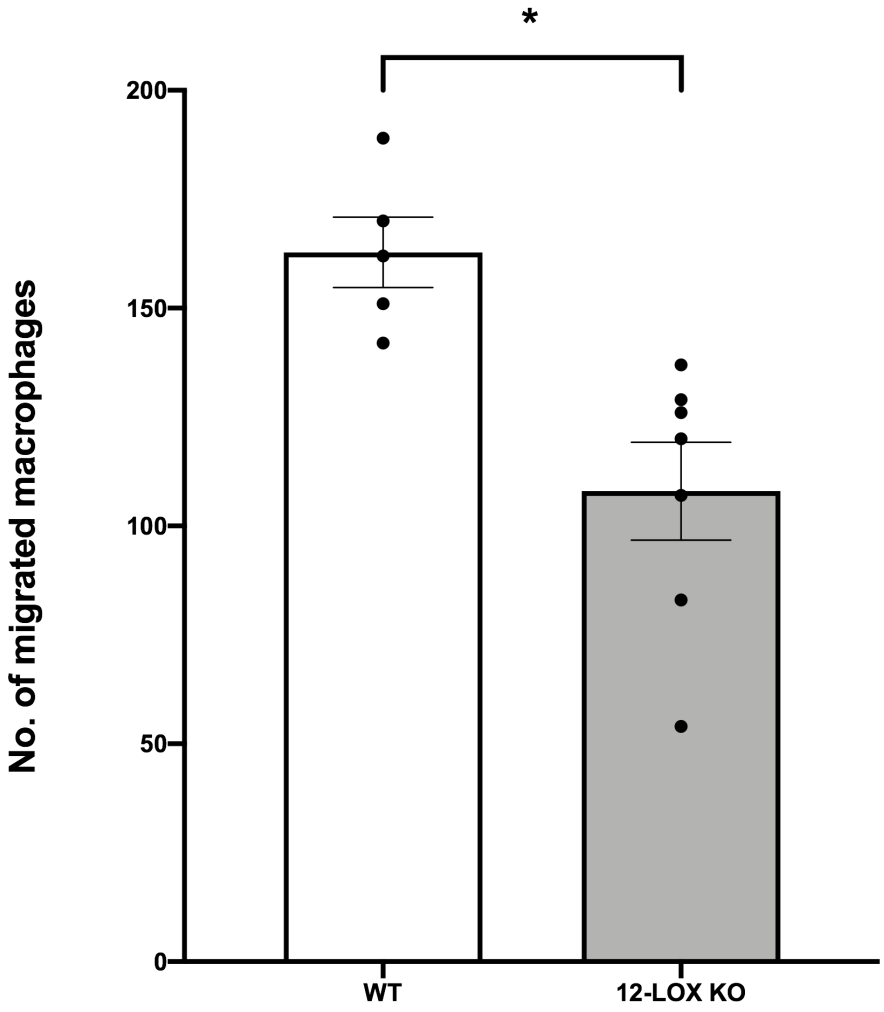
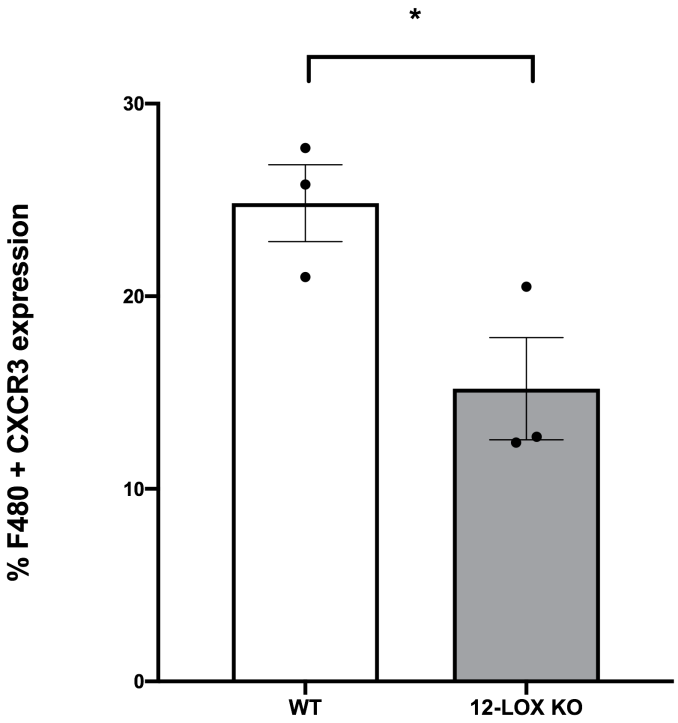


Figure 23: Depletion of 12-LOX prevents macrophage chemotaxis *in vitro*. (A) Transwell migration assay schematic- Conditioned islet media was loaded in the bottom chamber, and peritoneal macrophages isolated from WT or 12-LOX KO mice were loaded in the top chamber. The migrated macrophages were quantified after four hours. (B) Macrophage chemotaxis was impaired by the depletion of 12-LOX. Data are presented as mean ± SEM ($*p < 0.05$).

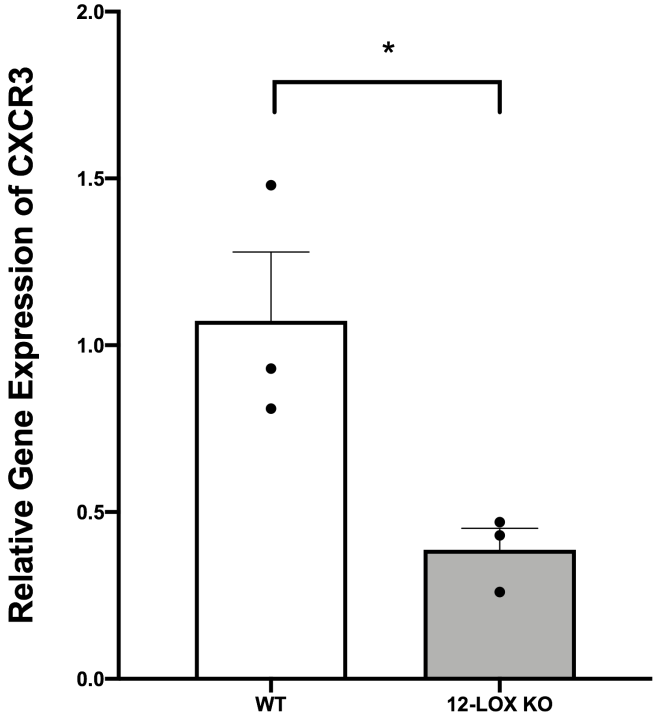
5.2.5. CXCR3 expression is downstream of 12-LOX in macrophages:

12-LOX has been implicated in regulating cellular migration in several different contexts. In some cancers, 12-LOX depletion substantially reduces the metastatic ability of cancer cells (Nie et al., 2003; Schneider and Pozzi, 2011; Zhong et al., 2018). Moreover, the depletion of 12-LOX prevents neutrophil migration by downregulating the chemokine receptor CXCR2 (Rossaint et al., 2012). Based on these studies, I measured the mRNA and protein expression levels of the cell surface receptor CXCR3 in peritoneal macrophages that were isolated from WT and 12-LOX KO mice. CXCR3 has been shown to have a critical role in the migration of macrophages. In 12-LOX KO macrophages, I observed significantly lower CXCR3 surface expression as compared to WT macrophages (Figure 24A). Moreover, gene expression of CXCR3 was significantly lower in 12-LOX KO macrophages compared to WT macrophages (Figure 24B). Finally, to determine whether 12-LOX depletion affects the CXCR3 receptor expression in the zebrafish as well. For this, I performed tailfin injury assay, on the *alox12* morpholino-injected and control (un-injected) larvae. I then bisected individual larvae and collected the bottom half of the larvae for analysis. In this portion of the larvae, the zebrafish CXCR3 orthologue CXCR3.2 is restricted to the macrophages. I pooled 25 larvae per condition and then extracted RNA and checked the expression of *cxcr3.2*. I observed a reduction in the levels of *cxcr3.2* in *alox12* morpholino-treated larvae. Consistent with the findings in mice studies, I found a significant reduction in the mRNA levels of *cxcr3.2* in the *alox12* morpholino-treated larvae compared to control larvae (Figure 24C).

A.


*
30
20
10
0
% F480 + CXCR3 expression
WT
12-LOX KO

B.


*
2.0
1.5
1.0
0.5
0.0
Relative Gene Expression of CXCR3
WT
12-LOX KO

C.

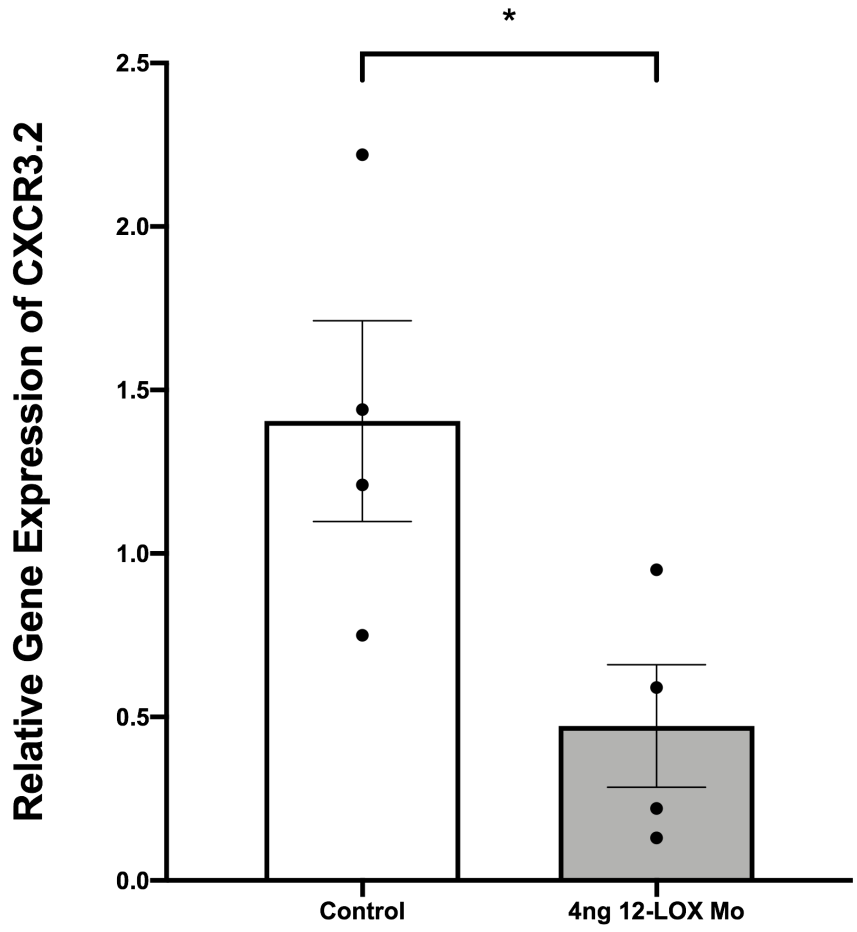


Figure 24: CXCR3 levels are downregulated in 12-LOX loss-of-function macrophages. (A) Flow cytometry analysis of CXCR3 expression in WT and 12-LOX KO macrophages. (B) qPCR for measuring gene expression of CXCR3. (C) 3dpf zebrafish larvae treated with *alox12* morpholino were subjected to tailfin injury and used for RNA extraction. The graph represents the gene expression of *cxcr3.2*. An individual point on the graph represents a clutch of 25 larvae pooled together.

5.3 Discussion:

Based on the specific PUFA substrate, LOX enzymes facilitate the production of metabolites called eicosanoids, which can be either pro-inflammatory or anti-inflammatory (Smith and Murphy, 2016). 12-lipoxygenase is implicated in oxidative stress and inflammation-mediated tissue damage. One of the major substrates of 12-LOX is arachidonic acid that leads to production to leukotrienes and 12-HETE. Both 12-LOX and its products have been associated with pancreatic inflammation. In this study, I sought to identify the roles of 12-LOX during macrophage-mediated inflammation, as this aspect has not been explored yet.

Two major factors define macrophage-mediated inflammation: polarization and directed migration. Macrophage polarization to a pro-inflammatory phenotype and infiltration in excessive numbers that lead to a pro-inflammatory milieu causes tissue damage and is associated with several pathologies like atherosclerosis, sepsis, osteoporosis, and diabetes. I attempted to elucidate the role of 12-LOX in these two major processes involved in macrophage-mediated inflammation. First, I checked the effect of depletion of 12-LOX, on the polarization of peritoneal macrophages in a mouse model. I used peritoneal macrophages as it has been shown that 12-LOX is only expressed in peritoneal macrophages at significant levels, while in other macrophage types—like bone marrow or tissue-resident macrophages—it is not detected (Sun and Funk, 1996). The polarization of macrophages can be tested by measuring the transcript or protein levels of the markers that contribute to or resolve inflammation. I observed that there was no difference in the expression of these markers between WT and 12-LOX KO peritoneal macrophages. These data suggest that 12-LOX might not have a direct role in the

polarization of macrophages. However, it remains possible that it could have synergistic effects together with other physiological mediators. They might also be indicative of polarization state rather than a cause of polarization of the macrophages. Furthermore, macrophage polarization studies such as this are typically performed *in vitro*. However, 12-LOX-mediated effect on the polarization might be an *in vivo* event, which requires more contextual information.

Maladaptive inflammation leads to excessive macrophage infiltration that, in turn, exacerbates the initial injury. Hence next, I investigated the actions of 12-LOX during macrophage migration. 12-LOX has been implicated in cellular migration in various other contexts. It has been widely studied in cancer metastasis, where it promotes the spread of colorectal cancer and pancreatic cancer cells (Nie et al., 2003; Schneider and Pozzi, 2011; Zhong et al., 2018). 12-LOX has also been associated with germ cell migration (Bromfield et al., 2017). In the context of immune cells, 12-LOX has been shown to promote neutrophil migration in an acute lung injury model (Rossaint et al., 2012). Based on this rationale, I used two *in vivo* zebrafish assays to test the hypothesis that 12-LOX also has a role in immune cell migration. In both injury models, tailfin injury and β-cell injury, I observed a significant reduction in the number of macrophages infiltrating into the injury sites when 12-LOX was inhibited with either morpholino injections or ML355 treatment. These data strongly suggest a migratory role of 12-LOX in macrophages. However, one caveat of this study is that I have globally inhibited 12-LOX, and the effect is not macrophage-specific. To focus specifically on the role in macrophage, I used peritoneal macrophages that were isolated from wild-type and 12-LOX knockout mice. I performed transwell migration assays, where these macrophages migrated in the context of media that was conditioned by

islet culture. Again, I found that peritoneal macrophages isolated from 12-LOX knockout mice show significantly less migration. These data establish a role for 12-LOX in macrophage migration, which is conserved between mice and zebrafish; this suggests that 12-LOX is part of a fundamental mechanism governing macrophage behavior, and as such, it is likely to be conserved in human macrophages as well.

The final question addressed in this study is the mechanism by which 12-LOX mediates its effects on macrophage migration. Both 12-LOX and 12-HETE have been shown to exert its effect on cell migration by direct inhibition of cell surface receptors. In 12-LOX knockout neutrophils, there is a significant reduction in CXCR2 expression levels. Similarly, I observed a significantly lower cell surface expression of CXCR3 levels in the macrophages isolated from 12-LOX knockout mice. Accordingly, I found a significantly lower expression of *CXCR3.2*, the zebrafish macrophage-specific isoform of CXCR3, in larvae injected with *alox12* morpholino. This strengthens the argument that even the mechanism of 12-LOX-mediated effects on macrophage migration is fundamental and conserved across species.

In conclusion, in this study, I have determined a role for 12-LOX in macrophage function as well as its mechanism. 12-LOX inhibition prevented macrophage infiltration into the islets, which could be exploited for diabetes therapeutics. As it is, various studies are focusing on preventing macrophage infiltration as a therapeutic measure for the treatment of these diseases. Macrophage-mediated inflammation has been thought to play a critical factor in diabetes pathogenesis. As discussed above, the current approaches to curb macrophages involve either depleting them or preventing the infiltration by targeting chemokine receptors. However, depleting macrophages causes weakening of the immune

system while targeting the receptors also inhibits other immune cell types as well that express the receptor, thus, having a similar effect on the immune system. Therefore, inhibition of 12-LOX could be a better strategy to prevent macrophage infiltration, and thus would suppress macrophage-mediated induction of inflammation and premature phagocytosis of injured, but reparable β-cells.

Chapter Six: Conclusion and future directions

6.1 Conclusion:

This thesis project provides insights and interventions into the inflammatory events that ultimately promote β-cell destruction resulting in diabetes. This work is based on the two key elements that regulate inflammation and contribute to β-cell damage; the macrophage and 12-lipoxygenase (12-LOX). Independent studies in our lab and others have shown that macrophages and 12-LOX can contribute to inflammatory damage to β-cells. More importantly, the depletion of either 12-LOX or macrophages protects rodent models from the development of diabetes. In my study, I established a link between these two potent mediators of diabetes pathogenesis. In this final chapter, I will be concluding with the major findings from the three studies, followed by potential future directions.

In the first study, I established a zebrafish model, *Tg(ins:NTR),* to effectively visualize ROS-mediated damage to the β-cells. In this model, I observed that the MTZ-NTR interaction induces ROS, which is followed by induction of apoptosis and macrophage infiltration, ultimately ablating the β-cells. Using this model, in the second study, I elucidated the role of macrophages in the conditions of β-cell stress by depleting these innate immune cells with clodronate injections in the *Tg(ins:NTR)* larvae. The depletion of macrophages protected the β-cell mass after MTZ treatment. Interestingly, these surviving β-cells regained their function upon the elimination of cellular stress. This study revealed a novel aspect of diabetes pathogenesis that an excessive infiltration of macrophages triggers premature phagocytosis of β-cells, leading to hyperglycemia. In the final study, I determined the role of 12-LOX in macrophage function in the context of islet inflammation. Although 12-LOX does not directly affect macrophage polarization, it

certainly promotes macrophage migration by upregulation of CXCR3 expression. Importantly, this role of 12-LOX in macrophage migration is conserved in zebrafish and mice.

Together, my studies illuminate a strong link between macrophage functions and diabetes pathogenesis that hinge on the activity of 12-LOX. Overall, based on the sum of data obtained from these studies, it can be inferred that excessive macrophage infiltration in the pancreatic islets in response to stressed β-cells exacerbates inflammation and promotes β-cell dysfunction and phagocytosis. Due to phagocytosis of β-cells, even if they are only partially damaged, there is a rapid loss of β-cell mass leading to hyperglycemia. Since the ability of the β-cells to regenerate is extremely low, this loss of β-cell mass is almost permanent. If there is macrophage depletion, the surviving β-cells can restore their function once cellular stress has been resolved. Since macrophage depletion is not a practical therapeutic approach, future research can instead focus on preventing or delaying their infiltration in the islets and thereby provide time for β-cells to recover. Based on the results from the last study, I found inhibition of 12-LOX to be effective at preventing macrophage infiltration. Importantly, I achieved inhibition of macrophage migration by using a pharmacologically relevant small molecule inhibitor of 12-LOX. One of the major advantages of specifically targeting 12-LOX is that this not only impairs macrophage infiltration, but also promotes a global reduction in oxidative stress and inflammation. This should create a physiological context that is conducive to the rapid recovery of the β-cells. In conclusion, my studies reveal exciting new roles for 12-LOX in macrophage function and provide potential therapeutic targets that can prevent inflammation-induced damage to the pancreatic islets.

6.2. Future Directions:

The data presented in this thesis study introduce a novel paradigm for determining the role of inflammation in diabetes pathogenesis and provide an interesting outlook on future interventions for diabetes. Still, much remains to be unraveled in terms of understanding the cellular and molecular mechanisms involved in the 12-LOX-diabetes pathogenesis axis. In the sections below, I have outlined a few future experimental directions that will further improve the understanding of this important axis, and which will surely inform the translation of these mechanisms from preclinical studies to human therapeutics.

6.2.1. Role of macrophages in β-cell neogenesis and function:

I established that the depletion of macrophages is beneficial for the survival and the recovery of function in ROS-injured β-cells. However, with regard to neogenesis, I did not observe any β cell regeneration in my studies where macrophages were depleted. It remains unclear, however, whether the lack of neogenesis was due to the maintenance of β-cell mass—rendering it unnecessary—or whether it was due to the absence of macrophages, which themselves might provide factors that promote regeneration. The depletion of macrophages before the beginning of the recovery phase can shed light on the role of these immune cells in promoting the neogenesis of the β-cells.

It is also essential to study these mechanisms in adult zebrafish, since, at the larval stages, the β-cells regenerative capacity is higher as compared to the adults (Matsuda, 2018). As such, the role of the macrophages could change with age as the complexity of their interactions is higher when the adaptive immune system gets involved. The next step

would be to translate these studies in rodent models as they are better disease models to match the physiological complexity of humans. The depletion of macrophages at different stages of development in the models of diabetes would highlight the role macrophages play in initiation as well as the resolution of diabetes. The two most important questions in these studies will be: i) Do the macrophages initiate the development of diabetes, or are they merely the factors that exacerbate the pathology? ii) Do the macrophages participate in β-cell neogenesis at the adult stage?

6.2.2. *Role of 12-LOX in vivo obesity zebrafish model of diabetes:*

I have looked at the effect of 12-LOX *in vivo* by utilizing the MTZ-NTR zebrafish model. Although this model efficiently induces hyperglycemia, it does not relate to obesity-induced diabetes, which is the characteristic of the most common form of diabetes (T2D). The *alox12* morpholino-treated fish can be placed on a high-fat diet and low-fat diet by immersing them in the egg yolk and egg white solution, respectively. The glycemic levels, as well as β-cell mass, can be tracked. It would be an efficient diabetes model for the *in vivo* visualization of the pathological effects on different organs apart from the pancreas, like liver and kidney, that get affected in conditions of hyperglycemia. Importantly, the effect of 12-LOX can be explored by easily performing 12-LOX knockdown or knockout. It is also easier to achieve β-cell- or macrophage-specific expression of 12-LOX in zebrafish as compared to the complex rodent models.

6.2.3. 12-LOX overexpression models for studying diabetes pathogenesis:

Most existing studies of 12-LOX in diabetes have focused on global or cell-specific loss-of-function, through depletion of the enzyme genetically or depletion of activity by small-molecule inhibitors. However, since excessive 12-HETE and 12-LOX expression are associated with diabetes, it will be informative to study whether 12-LOX overexpression can drive or exacerbate diabetes pathology. There are no studies that have examined the direct effects of global or cell-specific upregulation of 12-LOX in the wide array of diabetes models. It would be exciting to study the effect of macrophage-specific and β-cell-specific 12-LOX upregulation. In macrophage-specific upregulation, it would be interesting to see if it polarizes them to a pro-inflammatory phenotype and increases their migratory abilities. Similarly, in β-cell-specific upregulation, it would be curious to see if they promote oxidative stress and inflammation to trigger apoptosis.

6.2.*4. Exploring 12-LOX-12-HETE-gpr31 axis in diabetes pathogenesis:*

In diabetes pathogenesis, 12-LOX has been shown to exert its effect via its eicosanoid product 12-HETE. There is not much available information on the direct role of 12-HETE or its putative receptor gpr31, in the initiation and progression of the diabetes. My lab has developed different tools like gpr31 knockout as well as 12-LOX knockout zebrafish and mouse models to explore the role of this axis in diabetes pathogenesis. It will be interesting to explore how these factors individually regulate the β-cell activity and contribute to its dysfunction.

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(2010). Diagnosis and Classification of Diabetes Mellitus. Diabetes Care *33*, S62–S69.

Curriculum Vitae
Abhishek Anant Kulkarni

Education:

Indiana University (IUPUI), Indianapolis (2016-2020)

- Degree: Doctor of Philosophy (Ph.D.)
- Major: Biochemistry and Molecular Biology
- Minor: Bioinformatics

S.I.E.S College of Arts, Science and Commerce, University of Mumbai (2012-2014)

- Degree: Master of Science (M.Sc.)
- Major: Biotechnology

Kishinchand Chellaram (K.C.) College, University of Mumbai (2009-2012)

- Degree: Bachelor of Science (B.Sc.)
- Major: Biotechnology

Research experience:

- Graduate student under Dr. Raghavendra Mirmira working on the project titled "**Role of 12-LOX in macrophage activity in diabetes**", IUSM, Indiana, USA. (05/17-04/20)
- Graduate student under Dr. Mark Kaplan working on project titled "**Transcriptional Regulation in Th9 cells**", IUSM Indiana, USA. (03/17 - 05/17)
- Graduate student under Dr. Haitao Guo working on the project titled "**Molecular mechanisms of HBV replication**", IUSM, Indiana, USA. (09/16 - 12/17)
- Research assistant under the guidance of Dr. Indraneel Mittra working on the project titled "**miRNA as therapeutic targets in Breast Cancer**", Advanced Centre for Treatment, Research & Education in Cancer, Navi Mumbai, India. (02/16- 06/16)
- Research assistant under the guidance of Dr. Vikrant Bhor working on the project titled "**Studies on HIV-1 gp120 mediated α4β7 integrin dependent signaling in T-cells and its role in HIV pathogenesis**", National Institute for Research in Reproductive Health (NIRRH), Mumbai, India. (07/15- 12/15)
- Research assistant under the guidance of Dr. Ikram Khatkhatay on the project titled "**Effect of estrogen on the migration of primary monocytes**, National Institute for Research in Reproductive Health (NIRRH), Mumbai, India. (11/13- 04/14)
- Research fellow under the guidance of Dr. Suvarna Sharma working on the project titled "**A survey on awareness of HIV /AIDS amongst the educated working class**", Kishinchand Chellaram College, Mumbai, India. (07/10-02/11)

Skills:

- **Technical Skills**- Zebrafish and mice handling and experimentation, Cell culturing, qRT-PCR, Cell Isolation (PBMC, CD4 T-cells, Monocytes, Macrophages, Bone marrow cells), Biomolecules extraction (DNA, RNA, miRNA& Proteins), SDS-PAGE, Western blot, Flow cytometry, Immunofluorescence assay, Transwell migration assay, Recombinant DNA technology.
- **Software Skills**: R programming, Galaxy, Graphpad Prism 8.0, BLAST, CATH, SCOP, CLUSTALW, RasMol, Microsoft Office (Word, Excel, PowerPoint).

- **Management Skills**:
  - **Organizing head** of intercollegiate festival "BIOGENEi 2010", organized by Department of Biotechnology, K.C. College, Mumbai, India.
  - **Logistics head** of intercollegiate festival "OPERON 2012", organized by Department of Biotechnology, S.I.E.S. College, Mumbai, India.

Publications:

- **Abhishek A. Kulkarni**, Abass M. Conteh, , et al., "An In Vivo Zebrafish Model for Interrogating ROS-Mediated Pancreatic β-Cell Injury, Response, and Prevention", *Oxidative Medicine and Cellular Longevity*, vol. 2018, Article ID 1324739, 2018.
- Mr. Sameer Neve, **Mr. Abhishek Kulkarni,** Miss. Manjushree Aithal, 'Effect of Temperature Hike on Coastal Thermal Power Plants: A Case Study of Mundra, *International Journal of Innovative Research and Advanced Engineering*, Volume- 2, Issue – 6, Page No.180-184, ISSN 2349-2163, June 2015.
- **Abhishek Kulkarni**, Annie R. Piñeros, Marimar Hernandez-Perez, Sarah Tersey, Ryan Anderson and Raghavendra Mirmira, "12-lipoxygenase regulates macrophage migration during islet inflammation" (Manuscript in preparation)
- Marimar Hernandez-Perez, **Abhishek Kulkarni**, Niharika Samala, Cody Sorrell, Kimberly El, Isra Heider, Theodore R. Holman, Sarah A. Tersey, Raghavendra G. Mirmira, and Ryan M. Anderson, "A 12-Lipoxygenase-Gpr31 signaling axis is required for pancreatic organogenesis in the zebrafish" (Manuscript in preparation)
- Emily Anderson-Baucum, Annie R. Piñeros, Bernard Maier, **Abhishek Kulkarni**, Ryan Anderson, Sarah A. Tersey, Teresa L. Mastracci, Donalyn Scheuner, Raghavendra G. Mirmira and Carmella Evans-Molina, "Deoxyhypusine Synthase Promotes a Proinflammatory Macrophage Phenotype" (Manuscript in preparation)
- Annie R. Piñeros, **Abhishek Kulkarni**, Kara S. Orr, Christopher Reissaus, Marimar Hernandez-Perez, Marcia McDuffie, Jerry L. Nadler, Margaret A. Morris, Raghavendra G. Mirmira, Sarah A. Tersey, "Islet Proinflammatory Signaling is Required for Type 1 Diabetes Development in NOD mice" (Manuscript in preparation)

Presentations and conferences:

- Oral presentation titled "**Role of 12-LOX in macrophage migration in diabetes**" at Midwest Islet Club conference, Michigan, USA. (05/19)
- Presented a poster titled "**Role of 12-LOX in macrophage migration in diabetes**" at Wells Centre Symposium, Indiana, USA. (04/19)
- Presented a poster titled "**An In Vivo Zebrafish Model for Interrogating ROS-Mediated Pancreatic β-Cell Injury, Response, and Prevention**" at Midwest Islet Club conference, Missouri, USA. (05/18)
- Presented a poster titled "**An In Vivo Zebrafish Model for Interrogating ROS-Mediated Pancreatic β-Cell Injury, Response, and Prevention**" at ENDO 2018 conference, Chicago, USA. (03/18)
- Presented a poster titled "**Deoxyhypusine synthase activity is essential for the inflammatory response of macrophages to injury in Zebrafish**" at Annual Diabetes Symposium, Indiana, USA. (08/17)

Achievements:

- **People's choice award** for 3-minute thesis competition 2019 at I.U.P.U.I (12/19)
- Recipient of **DeVault Fellowship** 2019-2020 (07/19)
- Completed **teachers training** course at IU School of Medicine (05/19)
- Recipient of highly competitive "**Travel Grant**" from IU School of Medicine for Midwest Islet club conference (twice), Indiana, USA (05/18 & 04/19)
- **First** prize for poster presentation at the Annual Diabetes symposium, Indiana, USA (08/17)
- **First** rank in college during Semester IV of MS Biotechnology, Mumbai, India. (06/14)
- **First** position in college dissertation project, Mumbai, India. (06/14)
- Conferred the degree of Science Honor's in Biotechnology qualifying for the same with "**A**" grade (09/10)
- Certified as a '**Peer Educator**' at Red Ribbon Club- MDACS, Mumbai, India. (10/12)