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EFFECTS OF PANCREATIC AMYLIN ON THE PERIPHERAL AND CNS IMMUNE RESPONSE IN A MODEL OF TYPE-2 DIABETIC BRAIN INJURY

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DISSERTATION

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in the
College of Medicine
at the University of Kentucky

By

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ABSTRACT OF DISSERTATION

EFFECTS OF PANCREATIC AMYLIN ON THE PERIPHERAL AND CNS IMMUNE RESPONSE IN A MODEL OF TYPE-2 DIABETIC BRAIN INJURY

Type-2 diabetes (T2D) is a metabolic disorder that increases the risk for cerebrovascular disease and dementia and is associated with the progression of Alzheimer's Disease; however, the mechanisms responsible for T2D-associated dementia are still poorly understood. T2D leads to alterations of the innate and adaptive immune response that contribute to its progression, but how these cells contribute to cognitive decline during T2D is unknown. As there are no approved therapeutics for T2D that modulate the immune response, it is important that we investigate how peripheral immune cells contribute to cognitive decline during T2D. Amylin is an amyloidogenic hormone synthesized and co-secreted with insulin from pancreatic ß-cells that has been shown to contribute to the development and progression of T2D, cognitive decline, and AD pathology. Amylin deposits in the brain microvasculature and leads to cerebral amylin vasculopathy. We have shown that inducing amylin dyshomeostasis in rats by pancreatic-specific hypersecretion of human amylin (HIP rats) leads to cerebral amylin vasculopathy, neuroinflammation, and neurological deficits. Here, we tested the hypothesis that neuroinflammation caused by hypersecretion of pancreatic amylin leads to a dysregulated peripheral immune response that alters the immune profiles of the brain. We used HIP rats to determine the relationship between neuroinflammation and the impact of amylin on the peripheral immune response and immune cell migration into the brain. Using RNA sequencing, we found that hypersecretion of pancreatic amylin leads to altered genes in the brain involved in neuroinflammation and immune cell signaling, such as antigen presentation, B cell development, and T cell responses. Immune profiling showed a decrease in splenic immune cells that, in turn, led to a decrease in immune cell migration to the brains of HIP rats. As well as alterations in the blood. We then found that amylin led to alterations in proteins responsible for immune cell migration into the brain, such as ICAM-1 and VCAM-1. To determine how amylin

acutely impacted the immune response, we injected wild-type (WT) rats with aggregated amylin. We found that amylin led to immune alterations in the spleen and blood but no changes in the brain. Next, we sought to determine a mechanism for the dysregulated immune response seen in HIP rats. To do this, we used mice that also hypersecrete pancreatic human amylin (HIP mice). In HIP mice, we were able to replicate our results seen in rats, showing a reduction in lymphocyte population in the spleen. Furthermore, we found that high levels of amylin are able to cause immune cell dysregulation by affecting hematopoiesis and B cell development, characterized by alteration of B cells and B cell progenitors in the spleen and bone marrow and an overall decrease of B cells in the brain, through the CXCL12/CXCR4 axis. These data show evidence for the role of pancreatic amylin and how it contributes to type-2 diabetic pathology by altering the peripheral and neuroimmune response. This gives rise to the potential for using immunotherapy to reverse and prevent the progression of type-2 diabetic cognitive decline.

KEYWORDS: Type-2 diabetes, pancreatic human amylin, immunity, neuroinflammation, B cells, CXCL12

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To my family, friends, and loving fiancé for their eternal support in this journey. Also, to the black and brown children coming up behind me, anything is possible.

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LIST OF ABBREVIATIONS

Abbreviation	Definition
T2D	Type 2 Diabetes
AD	Alzheimer's Disease
IR	Insulin resistance
PD	Parkinson's Disease
CNS	Central nervous system
IAPP	Islet amyloid polypeptide
PC2	Prohormone convertase 2
PC1/3	Prohormone convertase 1/3
CPE	Carboxypeptidase E
WT	Wild-type
AP	Area postrema
BBB	Blood-brain barrier
CTR	Calcitonin receptor
RAMPS	Receptor activity modifying proteins
WAF1	Wildtype p53-activated fragment 1
CIP1	CDK2-interacting protein
ROS	Reactive oxygen species
JNK	C-Jun N-terminal kinase
CHOP	C/EBP homologous protein
RAGE	Receptor for advanced glycation endproducts
ER	Endoplasmic reticulum
XBP-1	X-box binding protein 1
ATF4	Activating transcription factor 4
UCH-L1	Ubiquitin C-terminal hydrolase L1

HSP90	Heat shock protein 90
LCB	Long chain base
TNF-α	Tumor necrosis factor alpha
IL	Interleukin
LPS	Lipopolysaccharide
RNA	Ribonucleic acid
PPP	Pentose phosphate pathway
TCA	Tricarboxylic acid cycle
HIF-1α	Hypoxia-inducible factor 1
PFKB3	6-phosphofrcutose-2kinase/fructose-2, 6-biphosphotase 3
Aβ	Amyloid beta
HNE	4-hydroxynonenal
MDA	Malondialdehyde
UCD	UC Davis
NLRP3	NOD-like receptor family pyrin domain containing 3
AKO	Amylin-knockout
HFD	High-fat diet
FJC	Fluro-jade C
GLUT4	Glucose transporter type 4
MRI	Magnetic resonance imaging
CSF	Cerebrospinal fluid
APP	Amyloid precursor protein
PS1	Presenilin 1
fAD	Familial AD
NFT	Neurofibrillary tangles
AT	Adipose tissue

Tregs	Regulatory T cells
T_h17	T helper 17
IgM	Immunoglobulin M
IFN	Interferon
MCP-1	Monocyte chemoattractant protein-1
LTB4	Leukotriene B4
CX3CL1	Fractalkine
SEMA3E	Semaphorin 3L
MIF	Macrophage migration inhibitory factor
Fcy	Fc-gamma
NETs	Neutrophil extracellular traps
NK	Natural killer
LFA-3	Lymphocyte function associated antigen-3
PCR	Polymerase chain reaction
HbA1c	hemoglobin A1c
MHC II	Major histocompatibility complex class II
TCR	T cell receptor
STAT6	Signal transducer and activator of transcription 6
CTLA-4	Cytotoxic T-lymphocyte associated protein 4
BM	Bone marrow
HSC	Hematopoietic stem cell
SCA-1	Stem cell antigen-1
MPP	Multipotential progenitor
LMPP	Lymphoid primed multipotential progenitor
CLP	Common lymphoid progenitor
EBF	Early B cell factor

TCF3	Transcription factor 3
Pax5	Paired box 5
CXCL12	C-X-X motif chemokine 12
FLT3	Fms-related tyrosine kinase 3
CXCR4	C-X-C chemokine receptor type 4
GFP	Green-fluorescent protein
WNV	West Nile virus
CAMs	Cell adhesion molecules
NVU	Neurovascular unit
ZO-1	Zonula occludens-1
VE	Vascular endothelial
TREM1	Triggering receptor expressed on myeloid cells 1
GPCR	G-protein coupled receptor
PBGF	Pre-B cell growth factor
SDF-1α	Stromal cell-derived factor 1α
LESTR	Leukocyte-derived seven-transmembrane domain receptor
WHIM	Warts, hypogammaglobulinemia, immunodeficiency, myelokathexis
PBMCs	Peripheral blood mononuclear cells
SNPs	Single nucleotide polymorphisms
GLP-1	Glucagon-like peptide-1
DRG	Dorsal root ganglion
NIHSS	National Institutes of Health Stroke Scores
IL3	Innate lymphoid cell type 3
EPCs	Endothelial progenitor cells
OPCs	Oligodendrocyte precursor cells

RHP	Repetitive hypoxia reconditioning
MS	Multiple sclerosis
EAE	Experimental autoimmune encephalomyelitis
SN	Substantia nigra
TH	Tyrosine hydroxylase
T1D	Type 1 diabetes
NIH	National Institutes of Health
HBSS	Hanks balanced salt solution
EDTA	Ethylenediaminetetraacetic acid
RBCs	Red blood cells
RPMI	Roswell Park memorial institute
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
MEM	Minimal essential medium
NEAA	Non-essential amino acid
RT	Room temperature
PBS	Phosphate buffer saline
FMO	Fluorescence-minus-one
HSPCs	Hematopoietic stem and progenitor cells
HCL	Hydrochloric acid
EGTA	Ethylene glycol-bis(β-aminoethyl ether
BCA	Bicinchoninic acid
VCAM-1	Vascular cell adhesion molecule 1
ICAM-1	Intracellular adhesion molecule 1
HRP	Horseradish peroxidase
SDS_PAGE	Sodium dodecyl-sulfate polyacrylamide gel electrophoresis

IHC	Immunohistochemistry
QUIVER	Quantitative multiplex Immunohistochemistry with Visual colorimetric staining to Enhance Regional protein localization
DE	Differentially expressed
IPA	Ingenuity Pathway Analyses
GO	Gene Ontology
DAVID	Database for Annotation, Visualization, and Integrated Discovery
CT	Cycle threshold
Rt-PCR	real-time PCR
SEM	Standard area of mean
DEGs	Differentially expressed genes
RT1-S3	RT1 class Ib, locus S3
RT1-A1	RT1 class Ia, locus A1
RT1-Db2	Rt1 class II, locus Db2
RT1-DOa	RT1 class II, locus Doa
Lag3	Lymphocyte activating gene 3
NOD	Non-obese diabetic
SCI	Spinal cord injury
LT-HSCs	Long term-hematopoietic stem cells
ST-HSCs	Short term-hematopoietic stem cells
PD-1	Programmed cell death protein 1
PD-L1	Programmed cell death ligand 1
IGF-1	Insulin growth factor 1
G-CSF	Granulocyte-colony stimulating factor
FDA	Food and Drug Administration

CHAPTER 1: Introduction

1.1 Type 2 Diabetes, Amylin, and Cognitive Decline

Type-2 diabetes (T2D) is a metabolic disorder characterized by high glucose levels, leading to insulin resistance and pancreatic β cell dysfunction.[1] It is also a risk factor for many disorders, such as heart failure, stroke, and, more recently, dementia. Globally, T2D affects over 400 million people and is in the "top 10" for mortality,[2] making it a growing concern as studies have estimated that by 2040, there will be over 600 million individuals with T2D.[3] Dementia, also a global health concern, is characterized by a progressive decline in cognitive abilities, the most common form of dementia being Alzheimer's disease (AD).[4] AD affects more than 50 million people while costing healthcare more than $500 billion annually.[5] Studies have shown that T2D increases the risk for dementia and cognitive decline,[6, 7] suggesting that the increase in the number of individuals with T2D will result in an increase in individuals developing dementia. Although the mechanisms responsible for T2D-associated dementia are still mostly unknown, studies have shown that some mechanisms that contribute include hyperglycemia, insulin resistance (IR), inflammation, hypertension, and microvascular disease.[8]

In our lab, we focus on how amylin contributes to cognitive decline and AD pathology. Amylin is a pancreatic β-cell hormone important in regulating satiety.[9] In individuals with T2D, it aggregates, leading to pancreatic β-cell dysfunction and contributes to the development of T2D.[10, 11] Histologic studies from our lab and others have shown that amylin deposits in the small vessels of the brains of patients with T2D and dementia.[12, 13] In rats that hypersecrete human amylin in the pancreas (i.e., HIP

rats), amyloid-forming amylin leads to the accumulation of amylin deposition in the brain, neuroinflammation, defined by glial activation, and increases in inflammatory cytokines, leading to cognitive decline.[13-15] Together, these studies suggest a pathological function of amylin in the brain and links between T2D and amylin-induced cognitive decline.

As T2D leads to inflammation and immune dyshomeostasis,[16] the overlap of how these factors contribute to T2D-induced neuroinflammation and cognitive decline is still poorly understood. The peripheral immune response plays a significant role in the pathogenesis of T2D. We and other labs have shown that T2D leads to neuroinflammation and cognitive decline.[13, 14, 17, 18] However, it is currently unclear how the peripheral immune response contributes to these pathologies. As studies have begun to elucidate the role of the peripheral immune response and how it modulates neuroinflammation and subsequent cognitive decline in diseases such as AD and Parkinson's disease (PD),[19, 20] it is important to understand how the peripheral immune response is associated with T2D-induced cognitive decline to find immunotherapies for slowing disease progression caused by T2D.

The following Introduction will give a brief overview of the current knowledge surrounding T2D-induced cognitive decline in relation to amylin, including its role in pancreatic dysfunction and diabetic complications. This chapter will also discuss research on the peripheral and central nervous system (CNS) immune system during T2D and related mechanisms, specific chemokine roles in immune system development and activation during disease, peripheral inflammation as it relates to cognitive decline, as

well as what neuro-immune mechanisms have been identified known in other neurodegenerative diseases and mouse models of T2D.

1.2 Amylin: Roles in health and disease

1.2A Overview of amylin

Islet amyloid polypeptide (IAPP) or amylin is a hormone peptide co-secreted with insulin by the pancreatic β-cells[9]. It was discovered in 1901 independently by two researchers and was initially named "islet hyalinization", due to the hyaline material found in the pancreas.[21, 22] It wasn't until later that researchers confirmed that the hyaline substance shared the characteristics of amyloid,[23, 24] with the peptide for amylin first isolated from T2D patients in 1987.[25] It was later named IAPP due to its secretion from pancreatic β-cells and propensity to aggregate into insoluble amyloid fibers.[26-29] Amylin is a 37-amino acid long peptide derived from pre-pro-amylin, an 89-amino acid peptide that contains 22-amino acid N-terminal signal peptide, and two short flanking peptides. The signal peptide is removed in the endoplasmic reticulum to produce a 67-amino acid pro-amylin. Pro-amylin contains cleavage sites for endoproteases prohormone convertase 2 (PC2), prohormone convertase 1/3 (PC1/3), and by carboxypeptidase E (CPE) in the Golgi to produce mature amylin peptide. Pro-amylin is fully converted to mature amylin in the β-cells secretory vesicles where insulin is also stored.[9]

Studies have shown that amylin is expressed in different organs throughout the body. As well as being expressed in pancreatic β-cells, amylin is also expressed in pancreatic δ-cells in rats and mice.[9] Outside of the pancreas, it has been found in the rat, mouse, cat, and human gastrointestinal tract[30] as well as the kidney[31]. In the CNS, amylin is found in primary sensory neurons in mice and rats.[32] In chickens, amylin is

mainly expressed in the brain and the intestine, and smaller amounts are found in the pancreas.[33] While amylin is expressed in these different animal species, it does not form amyloid in rodents due to a lack of fibrollogenicity. This is mainly due to the differences in the IAPP20-29 region, where in rats/mice, there are three proline residues preventing it from being amyloidogenic.[34] Other species where amylin is amyloidogenic, in addition to humans, include cats, dogs, and monkeys.[9]

1.2B Amylin in Satiation

One of the first studied roles for amylin is its importance in controlling food uptake in the body, otherwise known as satiation. Through endocrine signaling, amylin regulates how much a person eats[35]. Studies have shown that there is an immediate increase of plasma amylin minutes after the meal begins and food is consumed, causing a loss of appetite.[36, 37] To determine how important amylin was in regulating appetite, studies were performed using exogenous amylin and amylin antagonism. When endogenous amylin was administered, it reduced eating by up to 50%, while amylin receptor antagonist AC187 reversed this action and increased eating and meal size.[38] To further show the importance of amylin in feeding, studies using global amylin-deficient mice were used in which the mice displayed overeating and adiposity compared to their wild-type (WT) counterparts. When these mice were given amylin endogenously, it reduced eating similarly to WT counterparts.[39]

The mechanisms underlying amylin's important role in satiety are mediated by the primary site for amylin receptors in the brain: the area postrema (AP), a circumventricular organ that lacks a blood-brain barrier (BBB).[40] The core binding site for amylin in the AP is the calcitonin receptor (CTR). The CTR is a heterodimer with two

subtypes (A and B) plus receptor activity modifying proteins (RAMPS). Amylin specifically binds to the CTR when co-expressed with one of the known RAMPS in the same cell, RAMP1 or 3.[41-45] For amylin to bind CTRs, RAMPs confer specificity to amylin by altering CTR pharmacology from calcitonin-preferring to amylin-preferring receptors. RAMPS then regulates the transport of the core receptors to the cell surface and their glycosylation state and, therefore, influences ligand specificity.[42, 46]

Studies confirmed that amylin activates neurons in the AP, and when amylin is exogenously administered into the AP, food intake is inhibited. [47, 48] The threshold at which amylin activates AP-localized neurons is near circulating blood levels, which is around 10^{-8} M. [47, 49] Furthermore, peripheral amylin induces the expression of c-Fos in the AP[50-52]; c-fos is a cellular activation marker that shows amylin activates cells within the AP. When AC187 is administered in the AP, the amylin-induced c-Fos expression is also inhibited. Additional studies show that lesioning the AP or injecting it with AC187 both block the effect of peripherally administered amylin to increase food intake, [38, 48, 49] and decrease c-fos expression. These studies suggest that functioning AP neurons are essential for amylin to mediate its effect on food intake.

1.2C Amylin and T2D

Although amylin's physiologic role is in satiety, its production also has pathological consequences. Multiple studies confirm that amylin is important in developing T2D. Individuals with T2D become insulin resistant, leading to pancreatic β-cell failure due to pancreatic β-cell exhaustion.[53] As a consequence of insulin resistance and to correct it, the pancreatic β-cells increase insulin production, leading to a decrease in β-cell mass and function and subsequent IR.[9, 54] In the pancreas, amylin is co-

secreted with insulin by β-cells.[29] As the pancreas over secretes insulin, more amylin is also produced, leading to amylin deposition in hearts[55] and kidneys[56] in patients with T2D and in brains.[13] Studies have shown that the increase of aggregated amylin in the pancreas contributes to β-cell dysfunction, further contributing to the pathogenesis of T2D through various mechanisms mentioned below:

1.2C.1 Apoptosis

Apoptosis is a form of programmed cell death used to regulate tissue homeostasis. It is characterized by cell shrinkage, chromatin condensation, internucleosomal DNA fragmentation, and disassembly into membrane-encircled bodies (i.e., apoptotic bodies).[57] In the case of T2D, there is excess apoptosis leading to increased cell death of pancreatic β-cells. Early studies in monkey-kidney COS (CV-1 simian-derived cells transformed by simian virus 40) cells transfected with the human amylin gene show that amylin is cytotoxic and causes an increase in cell death and apoptotic cells independent of insulin.[58, 59] Studies also showed that incubating pancreatic β-cells with amylin alone also caused an increase in cell death and apoptotic cells.[10, 60] Incubation of amylin induced the expression of apoptotic markers, including p-53, cyclin D1 (Bcl-1), and wildtype p53-activated fragment 1 (WAF1)/CDK2-interacting protein (CIP1), suggesting that the mechanism of cell death was due to apoptosis. Studies also showed that oxidative stress contributes to pancreatic β- cell apoptosis after amylin incubation as increased levels of reactive oxygen species (ROS) were correlated with apoptosis.[61, 62]

Although the signaling pathways responsible for amylin-induced β-cell apoptosis are not fully elucidated, studies suggest that the c-Jun N-terminal kinase (JNK) pathway is essential. The JNK pathway is a known pro-apoptotic stress pathway in pancreatic β-

cells. In-vitro studies show that after amylin incubation, c-Jun and JNK expression was increased in pancreatic β-cells[63-65] and mediated by caspases -1, -3, and -8, which are also pro-apoptotic.[64] Blocking these pathways and caspases significantly inhibited β-cell apoptosis. Rodent studies employing transgenic mice and rats that endogenously hypersecrete human amylin confirm its contribution to the apoptosis of pancreatic β-cells. Accumulation of amylin leads to β-cell mass and increased β-cell apoptosis and the induction of apoptotic pathways such as pathways such as the C/EBP homologoues protein (CHOP)[66] and receptor for advanced glycation endproducts (RAGE).[67] These studies confirm that the accumulation of amylin is an important mediator of apoptosis during the pathogenesis of T2D.

1.2C.2 Endoplasmic reticulum (ER) Stress

Another hallmark that contributes to pancreatic β-cell death during the pathogenesis of T2D is ER stress. The ER is responsible for the homeostatic control of proteins, specifically how misfolded proteins are targeted for degradation by the ubiquitin/proteasome system. The removal of misfolded proteins prevents protein accumulation that triggers ER stress.[68] In-vivo studies using mice that express human amylin in the pancreas showed that amylin causes an accumulation of polyubiquitinated proteins and associated ER stress markers such as X-box binding protein 1 (XBP-1), CHOP and activating transcription factor 4 (ATF-4),[69] although other studies report these markers are not changed in islets from human amylin transgenic mice.[66] Studies in HIP rats and in humans with T2D also show an accumulation of ubiquitinated proteins that were associated with a decreased expression of ubiquitin C-terminal hydrolase L1 (UCH-L1), a deubiquitinating protein that is abundant in β-cells.[70] UCH-L1 is required

for proteins to be targeted for degradation and released so that they can gain access to the proteasome and become monomerized. Deficiencies in UCH-L1 impair ubiquitin-dependent protein degradation and result in the accumulation of highly ubiquitinated proteins. Furthermore, studies show that amylin increases ER stress proteins in the heat shock protein 90 (HSP90) family, such as HSP90B1 and HSP90AA1 in human pancreatic islets.[71] Misfolded proteins need to be removed for cellular recovery during ER stress, lest accumulation of unfolded proteins impairs the proteasome system leading to the accumulation of ubiquitinated proteins and ER stress.

1.2C.3 Autophagy

Another mechanism that contributes to T2D pathology is impaired autophagy signaling. Autophagy is important in preventing cell death by removing irregular organelles and proteins. In-vitro studies show that amylin causes a formation of autophagosomes and changes of long chain base (LCB) I to II.[72] Studies in HIP rats show that in the pancreas of HIP rats, there are also increases of LC3-1 and 2 and p62, indicating an increase in autophagosomes and decreased p62 lysosomal degradation.[73] This was also shown in mice that are transgenic for human amylin. These results were also validated in-vitro using insulinoma cells. Loss of p62 made cells more vulnerable to amylin-induced cytotoxicity, and increased apoptosis marker caspase-3 and overexpression reversed these impacts and rescued cells. And increasing autophagy using rapamycin saved cells as well. Studies also show that when autophagy is decreased, T2D phenotypes are exacerbated in-vitro and in amylin mice, along with increased p62 inclusion, amylin oligomers, oxidative stress,[74-76] whereas an autophagy enhancer helps[77].

1.2C.4 Cytokine-mediated inflammation

Patients with T2D exhibit an increase of pro-inflammatory cytokines in both pre-diabetic and diabetic states. These cytokines include C- reactive protein, tumor necrosis factor alpha (TNF-α), interleukin-6 (IL-6), IL-1RA, and IL-1β,[78-80] though most studies focus on IL-1β as most inflammatory cytokines are IL-1-dependent and blocking IL-1 reduces inflammation.[81-83] In-vitro studies found that amylin can increase pro-inflammatory IL-1β in lipopolysaccharide (LPS)-stimulated monocytes and microglia. In addition to IL-1β, TNF-α, IL-6 and IL-8 were also induced in LPS-stimulated monocytes but no microglia.[84, 85] In human glioma cells, amylin stimulated the production of IL-1β, IL-6 and IL-8,[84] showing the potential importance of human amylin in causing inflammation. In bone marrow (BM)-derived dendritic cells and macrophages, amylin activated the inflammasome and IL-1β production again after LPS stimulation. This same study confirmed this in-vivo using mice that hypersecretion of pancreatic human amylin (HIP mice) to show that induction of IL-1β occurs directly in the pancreatic islets during accumulation of amylin.[86] In addition, more recent studies using ribonucleic acid (RNA)seq analysis of pancreatic islets from HIP mice identified increased gene expression for markers of inflammation similar to islets from humans with T2D.[87]

1.2C.5 Metabolism

At the cellular level, metabolic regulation is important to generate the energy needed for cellular processes. It is well known, however, that alterations in metabolism contribute to the development of T2D in humans[88, 89]. Early studies in our lab suggest that the accumulation of amylin causes alteration in several metabolites in the heart, liver, plasma, and brains of HIP rats. Some of the key pathways altered by human amylin were

the pentose phosphate pathway (PPP), the tricarboxylic acid cycle (TCA), and gluconeogenesis, establishing early how amylin causes alterations in metabolic functions[90]. Other labs have shown that amylin also activates the hypoxia-inducible factor 1 (HIF-1α)/6-phosphofrcutose-2kinase/fructose-2, 6-biphosphotase 3 (PFKB3) stress response pathway[91, 92]. HIF-1α is a stress response protein that has been shown to be an important regulator of metabolism in cancer, as well as foundational for hypoxia-induced signaling in peripheral and neurodegenerative disorders[93-95]. Studies have shown that in the pancreatic β-cells and failing hearts of patients with T2D, HIF-1α is decreased and increasing HIF-1α reverses the diabetic phenotype.[96, 97] PFKB3 is a master regulator of glycolysis[98] that recent studies found is important for glucose metabolism, mitochondrial metabolism, glycolipid synthesis, and the PPP.[91, 92] In the pancreatic β-cells of patients with T2D it is increased[91] and the silencing of PFKB3 reverses the diabetic phenotype in rodents.[99] These studies suggest that the HIF-1α/PFKB3 pathway has protective functions during the pathogenesis of T2D. Studies focusing on how amylin contributes to this pathway have found that in contrast to previous studies, nuclear expression of HIF-1α is increased as the same with PFKB3 in pancreatic β-cells from patients with T2D and in HIP rats.[91] Later studies also found that the accumulation of amylin in failing hearts in non-human primates was also associated with the upregulation of both HIF-1α and PFKB3.[92]. Silencing of HIF-1α and PFKB3 in pancreatic β-cells in these studies restored amylin-mediated metabolic dysfunction by restoring glycolysis and TCA function and apoptotic stress to pancreatic β-cells, showing the importance of this pathway in the pathogenesis of amylin mediated T2D. These

studies show that the HIF1-α/PFKB3 is important in T2D and in amylin-mediated T2D, but more studies need to be conducted as there are contradictory results.

1.2D Human amylin, cerebrovascular impairment, and cognitive decline

Studies from our lab found that even in the absence of amyloid beta (Aβ) pathology, amylin is still able to deposit in the brain and induce neurological deficits such as decreased exploratory drive and impaired rotarod in HIP rats. These rats also experience an increase in inflammation including TNF-α, IL-6, microglia activation, and a decrease in anti-inflammatory cytokines like IL-10[(14)]. HIP rats exhibit an increase in lipid peroxidation shown by increased 4-hydroxynonenal (HNE) and malondialdehyde (MDA) and IL-1β in neurons that were positive for amylin in the brains compared to control animals[(100)]. This was also confirmed in-vitro by incubating neurons with amylin, as they also increased lipid peroxidation and IL-1β production. Since HIP rats exhibit hyperglycemia, we wanted to confirm that the findings were due to increased amylin and not hyperglycemia. To do this we used UC Davis (UCD) T2D rats that establish hyperglycemia but only have non-amyloidogenic rodent amylin. Lipid peroxidation and IL-1β production levels were the same in UCD rats as compared to WT controls, confirming that human amylin was responsible for their upregulation. These data suggest that amylin is able to activate the NOD-like receptor family pyrin domain containing 3 (NRLP3) inflammasome pathway. Later studies using HIP rats show that at a later age, HIP rats still see increased neurological deficits and white matter injury shown by magnetic resonance imaging (MRI). Areas of neurological injury associated with cerebral microhemorrhages and astrocyte activation surrounding amylin deposition leading to a decrease in BBB integrity [(13)](**Fig 1.1**). These results were validated using amylin-

knockout (AKO) rats injected with amylin showing that these results were secondary to amylin and not T2D-related hyperglycemia.

As these studies focused on rats, although not many studies focus on brain pathology in HIP mice, we also show that mice similar phenotypes exist. When WT mice were also injected with amylin which induced an increase of lipid peroxidation and IL-1β production confirming that amylin secretion alone directly impacts the brain independent of hyperglycemia in a second animal species.[100] Mice that hypersecrete amylin in the pancreas and with a high-fat diet (HFD) display amylin accumulation in the brain starting at 6 months of age and increasing significantly by 12 months. At 12 months, these mice exhibit neural degeneration and aging in the hippocampus, as shown by increased fluro-jade C (FJC) positive cells and β-galactosidase staining.[101] They also showed reductions of glucose transporter type 4 (GLUT4) in the hippocampus as well as impaired cognitive deficits such as passive learning, memory, and social learning abilities[101].

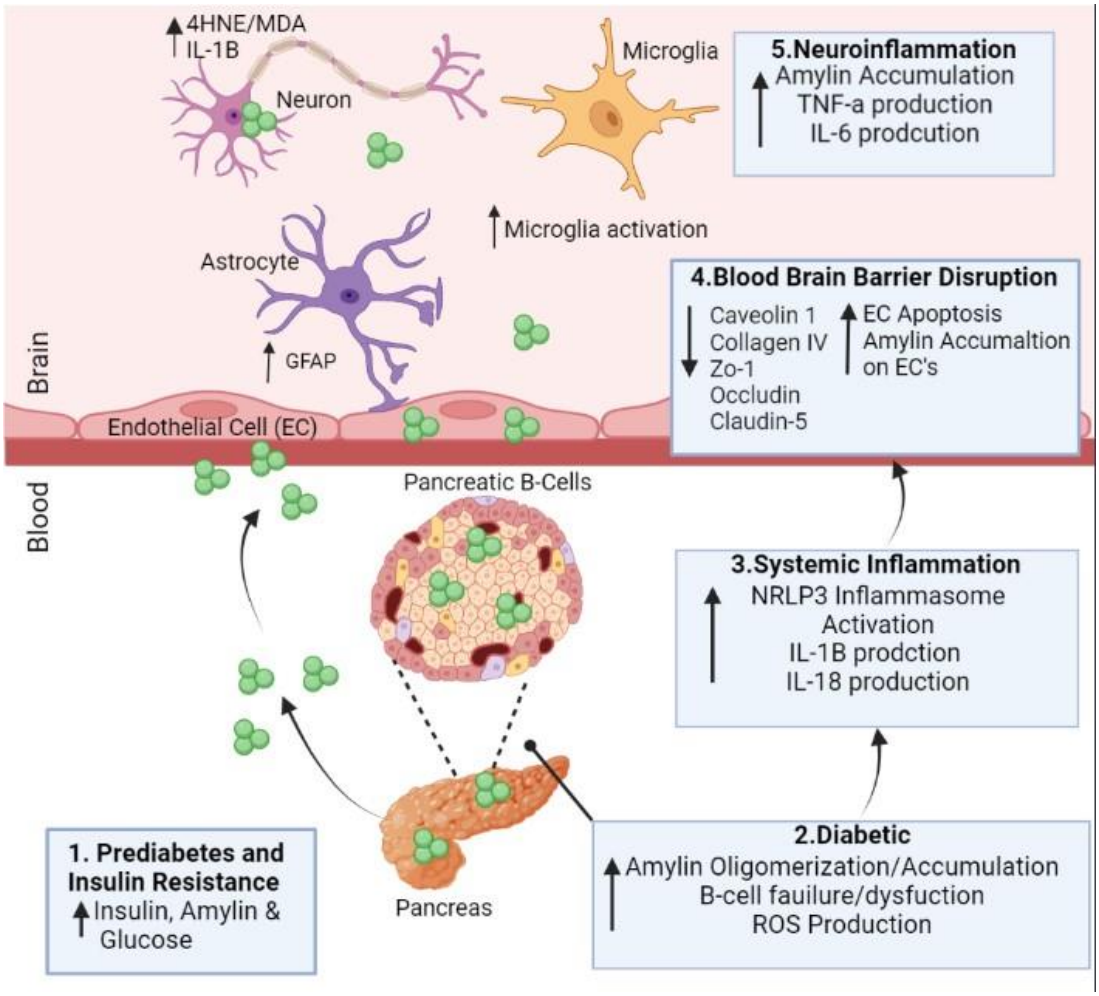

Figure 1.1: Mechanisms of neuroinflammation and cognitive decline due to the hypersecretion of pancreatic human amylin.

1.2E Amylin and Alzheimer's disease

Studies in our lab and others have shown that amylin also aggregates in the brain and is present in the cerebrospinal fluid (CSF) of AD patients.[12, 100, 102-105] Patients with T2D exhibit amylin deposits in blood vessels and perivascular spaces in the brain that are not typically found in patients without T2D. Furthermore, in patients with late-onset AD without diabetes, amylin deposition was also found in the brain, with mixed amylin and Aβ deposition in the plaques. Amylin-Aβ plaque formation in the brains of patients with AD was later confirmed[102, 103] in addition to identification of amylin cross-seeding with Aβ in the brains of HIP mice injected with Aβ.[103] Collectively, these were the first studies to show that circulating amylin from the pancreas can get into brain regions with ostensibly intact BBB potentially to contributing to cognitive decline in patients with AD and T2D.

Studies in our lab also show that in patients with AD, amylin deposits directly in neurons.[100] This neuronal deposition of amylin associated with an increase of peroxidative membrane injury in the pancreas of the patients and increased IL-1β levels surrounding amylin-positive neurons, highlighting an interplay of several of the aforementioned pathologic mechanisms. We also showed that amyloid precursor protein (APP)/presenilin 1 (PS1) rats that hypersecrete pancreatic human amylin (APP/PS1/HIP rat) display increased amylin- Aβ seeding in cerebral blood vessels as well as perivascular spaces, both associated with increased markers for inflammatory macrophages/microglia (e.g, CD68, CD11b).[106] Furthermore, individuals with familial AD (fAD) carrying the mutation for PS1 presented with higher amounts of amylin and greater $A\beta_{42}$ levels in the brains compared to cognitively normal controls.[106] This study

confirmed in APP/PS1/HIP rats that AD-like pathology is increased through amylin-Aβ cross seeding.

Studies also show that amylin can deposit into pericytes in patients with AD. Pericytes are mural cells that line the capillaries in the brain, which have shown to be important in BBB integrity and blood flow.[107] Loss of pericytes in mouse models of AD lead to accumulation of Aβ plaques, which was associated with neuronal loss and cognitive decline.[108] The accumulation amylin on pericytes was associated with altered morphology, decreased expression of pericyte markers, and pericyte cell death[104, 105]. Recent studies have also investigated the relationship between amylin and tau. Tau is a protein that becomes misfolded during AD similar to Aβ, that studies have also shown leads to neuroinflammation and contribute to cognitive decline.[109] As Aβ has been shown to interact with amylin so has tau. Including its interaction with amylin in the hippocampus of patients with AD and T2D it has also been shown to interact with amylin in the pancreas [110], suggesting that amylin may contribute to AD pathology by interacting with tau as well as Aβ.

While these studies focus on the interaction between amylin and Aβ in the brain, studies have shown that amylin is also deposited in the retina of patients with AD. Retinal amylin deposition correlated with pericyte numbers and decreased angiogenesis in the retina,[105] suggesting that amylin can prevent the formation of new vessels in the retina. Furthermore, they found that amylin levels in the retina were correlated with amylin levels in the hippocampus and later the same lab found that retinal amylin levels correlated with neurofibrillary tangles (NFT) and Aβ scores[105, 111] in patients with AD. These studies suggest that amylin is not only present in the retina but could potentially

contribute pathologically by binding with Aβ and impairing retinal function, which may in fact be a highly accessible biomarker for similar parenchymal pathology in AD.

1.3 Peripheral immune responses during T2D

As highlighted above, during T2D there is an array of immune-related complications that arise, which contribute to the pathogenesis of T2D-related secondary complications. Most studies focusing on peripheral immunity in T2D are focused on obesity-associated T2D. Thus, innate and adaptive immunity are generally studied in the context of adipose tissue (AT) and hyperglycemia. During obesity-related T2D, the environment shifts from a homeostatic to a pro-inflammatory environment due to a decrease in anti-inflammatory cell types such as regulatory T cells (Tregs), Immunoglobulin M (IgM)-producing B-1 cells, and M2 macrophages. These beneficial immune subsets are replaced with pro-inflammatory cell types including $CD8^+$ T cells, T helper 17 (T_h17) cells, antibody producing B-2 cells, and M1 macrophages.[112-115] These pro-inflammatory cells secrete pro-inflammatory cytokines (highlighted in Section 2.3.4), which further contribute to the progression of T2D through pancreatic β-cell dysfunction, glucose intolerance, and IR.

1.3A Innate immunity: Monocytes/macrophages

Macrophages are popular due to their accumulation in AT and pleotropic functions. Macrophages are often classified as either pro-inflammatory (i.e., M1) or anti-inflammatory (i.e., M2). M1 macrophages are induced by pro-inflammatory mediators such as LPS and interferon (IFN)-y and can secrete pro-inflammatory cytokines including IL-6, IL-1β, inducible (i)NOS, and TNF-α. M2 macrophages are induced by cytokines

(e.g., IL-4, IL-13) and secrete anti-inflammatory cytokines (e.g., IL-10, IL-1 decoy receptor, arginase) to play vital roles in tissue repair, angiogenesis, and resolution of inflammation.[116] However, it should be noted that studies have shown that this nomenclature should be avoided as macrophages are more complex than M1 and M2, being in most cases these markers in most cases overlap.[117] To address previous studies, we will keep in line with the M1 and M2 nomenclature as described by studies mentioned. Studies in humans focusing on macrophages have shown that macrophages in AT expressed higher levels of inflammatory genes such as IL1β, IL6, IL8, TNFA, and CCL3, displayed transcripts for T cell recruitment.[118] This confirms a pro-inflammatory phenotype during obesity and T2D, but also suggests macrophages may play a direct role in initiating the adaptive immune response. The same study suggested that there was a positive association between M1/M2 ratio and IR[118] but other studies in non-obese humans with and without T2D show no relationship.[119] Furthermore, other studies in patients with T2D show that $CD14^+$ macrophages in AT were associated with obesity and IR.[120] Weight loss following bariatric surgery in obese subjects decreased activated macrophages and proinflammatory T cells in AT[121] and peripheral blood,[122-124] suggesting the T2D-induced pro-inflammatory immune state can be modulated by lifestyle interventions.

Studies in animal models of T2D and obesity show that macrophages are the most abundant cell type in AT, with a variety of chemokines upregulated during the inflammatory response.[125] In general, 40%-60% of AT immune cells are macrophages in obese mice compared to 10-15% in lean mice.[126, 127] Control mice also exhibit an M2 phenotype while obese mice exhibit the pro-inflammatory M1 phenotype.[128] To recruit

macrophages into AT, adipocytes secrete chemokines that attract macrophages from the periphery. Chemokines are a class of proteins that induce chemotaxis of immune cells (detailed in Section 5). One major chemokine, monocyte chemoattractant protein-1 (MCP-1)[129], is increased in the AT of both obese mice and humans compared to controls, with MCP-1 upregulation secondary to the aforementioned upregulation of IL-1β, TNF-α, IL-8, IL-4, and IL-6. Another chemokine important in macrophage recruitment is leukotriene B4 (LTB4). Blocking LTB4 prevented the induction of IR in obese mice on HFD, that also included a significant decrease in AT-localized macrophages.[130] Fractalkine (CX3CL1) and its receptor CX3CR1 have also been implicated in macrophage recruitment as it is expressed in adipocytes and increased in the AT of humans.[131] Semaphorin 3L (SEMA3E) is also chemoattractant for macrophages, SEMA3E and its receptor plexinD1 is increased in obese mice and inhibition of this pathway resulted in decreased numbers of macrophages in the AT.[132] Finally, there is macrophage migration inhibitory factor (MIF), a proinflammatory chemokine that studies have shown is associated with impaired insulin signaling.[133] While these studies focus on macrophages accumulating in AT, other studies have suggested that macrophages from patients with T2D also exhibit dysfunction during T2D, including defects in complement and fc-gamma (Fcy) receptors that result in an inability to phagocytosis correctly.[134] Furthermore, studies have shown that macrophages cultured in hyperglycemic conditions also have reduced phagocytic abilities, suggesting a pro-inflammatory cell type unable to function in its canonical capacity.[135]

1.3B Innate immunity: Neutrophils

Neutrophils are leukocytes most known for their role for being immediately recruited to sites of infections and injury where they kill harmful substances through phagocytosis and the release of ROS and cytokines.[135] Furthermore, they are able to release chromatin filaments that form net/mesh like structures called neutrophil extracellular traps (NETs), that have antimicrobial properties and have been shown to be important in various disorders.[136] They are also important in regulating the immune response by being able to regulate natural killer (NK) cells, dendritic cells, and T cells.[137-139] While macrophages are the most studied in adaptive immunity during T2D, studies have shown that neutrophils have their importance as well.

Studies have shown that in mice on HFD, neutrophils migrate to AT as early as 3 days preceding macrophages migration,[140] showing they are the first responders during obesity induced T2D. Furthermore, studies have shown that neutrophil elastase, a proinflammatory proteinase secreted by neutrophils is increased in AT of diabetic mice and its deletion was able to reverse the diabetic phenotype and reduce inflammation,[141] providing evidence of neutrophils involvement during the pathogenesis of T2D. In patients with T2D, neutrophil counts are increased and positively associated with pancreatic β-cell function and IR.[142] Furthermore, studies have also suggested that high neutrophil counts can predict the incidence of T2D and could be a potential biomarker.[143] Neutrophil activation markers such as CD11b and CD66b are increased in patients with T2D,[144, 145] while activation markers such as lymphocyte function associated antigen-3 (LFA-3) is decreased.[146] Despite studies showing increased migration markers, studies using polymerase chain reaction (PCR) and RNA-seq have

suggested that in rodents[147] and human's[148] neutrophils have decreased migration capacity due to reduced levels of adhesion molecules, but more studies are needed to confirm this. Earlier studies have also suggested impairments in phagocytosis.[149, 150] These studies support the idea that neutrophils are important during the pathogenesis of T2D. In the case of neutrophil NETS, patients with T2D have increased amounts of NET markers that were positively associated with glycated hemoglobin A1c (HbA1c),[151] a marker for glucose control, and pro-inflammatory cytokines such as IL-6 and TNF-α,[152] suggesting a link between neutrophil NETs and T2D. Interestingly, neutrophils isolated from patients with T2D are able to form NETS without any stimulus,[153, 154] but fail to release NETS upon stimulation.[151, 153] Patients on diabetic medication such as metformin, saw decreased NET formation after 12 months, suggesting that in addition to neutrophils themselves, the NETs they form also contribute to T2D.[151]

1.3C Adaptive immunity: T helper cells

$CD4^+$ T cells recognize antigens presented by major histocompatibility complex class II (MHC II) molecules on various antigen-presenting cells including macrophages, dendritic cells, and B cells. Activation of these T helper cells cause them to migrate toward sites of injury/infection to help other cells activate and migrate to the site as well. $CD4^+$ T cells are characterized based on the cytokines they produce: T_h1 cells express IFN-γ; T_h17 cells express IL-17; T_h2 cells express IL-4 and IL-13; and Tregs are classified by the expression of the Foxp3 transcription factor, while also expressing IL-10. T_h1 and T_h17 cells are usually pro-inflammatory while Tregs and T_h2 cells are typically anti-inflammatory $CD4^+$ subsets.[151] As the cytokines mentioned above are used to classify them, they are able to secrete a wide range of cytokines depending on the

environment. Studies specifically focusing on the T cell receptor (TCR) during obesity suggest that AT cells undergo clonal expansion in response to antigens, though specific mechanisms and antigen targets that are unknown.[155] Differences also exist in the restriction of TCRs in $CD4^+$ and $CD8^+$ T cells depending on the different types of AT when comparing obese mice to controls.[156] Mice on HFD show that Tregs are decreased in AT but remain in contact with other lymphocytes and macrophages though treatments in obese mice with IL-2 therapeutically increased Tregs to reduce macrophage infiltration and decrease TNF-α expression.[113, 157]

Concerning specific $CD4^+$ T cell subsets, studies in humans show that increases in Th2 cells in AT and blood are inversely associated with IR,[158] while other studies show only an association between increasing obesity and $CD4^+$ T cells in AT.[159] As in the M1/M2 ratio for macrophages, T2D also induces an imbalance of the Th17/Treg ratio in patients.[160] It was also found that Th17 cells associated with the severity of diabetes and IR[161, 162] though counterintuitively, studies in humans show that the Treg marker FoxP3 was increased in obese humans compared to lean subjects;[159, 163] however, those study only show mRNA levels, not actual infiltration of Tregs in the AT. Preclinical experiments using mice on HFD show an increase of Th1 cells in AT associated with development of glucose intolerance and inflammation. This includes induction of IFN-y expressing $CD3^+$ and $CD4^+$ T in obese mice, though whole-body deletion of IFN-y improved IR and lowered macrophage infiltration.[157, 164]

The adoptive transfer of $CD4^+$ T cells into Rag1 mice (i.e., a mouse strain endogenously free of lymphocytes) on a HFD improved glucose intolerance and fasting insulin through a direct increase of Th2 cells, highlighting the anti-inflammatory capacity

of Th2 cells during T2D.[157] Furthermore, the same study found obese mice treated with an immunotherapeutic anti-CD3 antibody reduced Th1 cells over Tregs and reversed IR despite HFD. Lymphocyte transfer from signal transducer and activator of transcription 6 (STAT6) KO mice, which have normal Th1 but impaired Th2 signaling, did not reverse IR in Rag1 mice further supporting a protective role of Th2 cells and highlighting the potential for immune modulation as a treatment to counter T2D-induced inflammation.[157]

1.3D Adaptive immunity: cytotoxic T cells

$CD8^+$ T cells are essential for killing infected or foreign cells by direct cell-to-cell contact and the release of perforin and granzyme into the target cell, for the most part rendering them as a class of pro-inflammatory lymphocytes.[165] In obese humans, $CD8^+$ T cells are increased in AT, but this was not associated with systemic IR as was the $CD4^+$ T cell population.[158] Furthermore, in lean to moderately obese men, $CD8^+$ T cells in AT did not correlate with adiposity which is surprising given the amount of IFN-y and granzyme B that CD8+ T cells are producing are increased in AT.[159] $CD8^+$ T cell increases precede both reductions in $CD4^+$ T cells and Tregs and the increase in macrophages in AT while the loss of $CD8^+$ T cells decreases M1 (but not M2) macrophage infiltration, AT inflammation, and systemic IR in obese mice. Studies also show that adoptive transfer of $CD8^+$ T cells into CD8-deficient mice increased AT inflammation and IR in obese mice[166], but other studies show that blocking activation of T cells using anti-CD40L or cytotoxic T-lymphocyte associated protein 4 (CTLA-4)–Ig reduced activated $CD8^+$ T cells and inflammatory macrophages in AT but didn't improve insulin sensitivity.[167]

1.3E Adaptive immunity: B cells

1.3E.1 B cell development and function

B cells are antibody-producing lymphocytes that have also been shown to be important in various disorders including the pathogenesis of obesity and T2D. B cells are produced and develop in the BM from hematopoietic stem cells (HSC's), which are characterized by their expression of stem cell antigen-1 (sca-1) and receptor tyrosine kinase (c-kit), before migrating to the periphery. From HSCs they develop into multipotential progenitors (MPPs), and lymphoid primed multipotential progenitors (LMPPs), that give rise to other cells such as erythrocytes, megakaryocytes, and monocytes. In the BM, B cells are derived from common lymphoid progenitor (CLP) cells, which gives rise to leukocytes. The earliest stage of B cell development is called pre-pro B cells, in which they lose sca-1 and c-kit expression and gain B cell lineage markers such as B220 and CD43, but at this point are still negative for Ig. After pre-pro B cells, there is pro-B cells in which another B cell lineage marker is added such as CD19, next is pre-B cells in which they have all markers mentioned previously but lose CD43. Immature B cells are next, and this is the stage in which they gain IgM and leave the BM to mature in the spleen and become mature B cells that develop into transitional, marginal and follicular B cell (**Figure 1.2**).[(168)] There are a number of transcription factors that regulate early-stage B cell development, but some of the most impart ones are early b cell factor (EBF), transcription factor 3 (TCF3/E2A) and Paired box 5 (Pax5). The knockout of these transcription factors have shown to block stages of B cell development and also reduce early B cell survival.[(169)] There are also microenvironmental factors that influence B cell precursors such as C-X-X motif chemokine 12 (CXCL12), fms-related tyrosine

kinase 3 (FLT3), and IL-7. For example, the loss of CXCL12 and C-C-X-C chemokine receptor type 4 (CXCR4) resulted in a significant loss in B cell precursors such as pre-pro B cells. Studies have also shown that CXCL12 is important for allowing the migration of B cells in and out of the BM during development and maturity. FLT3L and IL-7 are also important for B cell precursors as the loss of them reduces pre-pro B cells and pro-B cells. In addition, simulation with these two leads to growth of pre-pro B cells.[168]

1.3E.2 B cells in T2D

Peripheral blood-borne B cells isolated from T2D patients exhibit a pro-inflammatory phenotype, characterized by increased amounts of IL-8, IL-6, and TNF-α production and decreased IL-10 secretion.[170] In-vivo studies in obese mice show confirm an increase of B cells in AT, specifically class-switched mature IgG^+ B cells,[156] while the transfer of antibodies produced by B cells such as IgG from obese mice to young mice on a HFD diet accelerated development of AT inflammation and IR.[120] Furthermore, B cell–knockout mice on HFD exhibit improved insulin sensitivity and glucose tolerance compared with WT mice on HFD, as well as a decreased proinflammatory cytokines in the serum associated with concomitant increases of Tregs in the AT and spleen.[170] Other studies corroborated these results showing that B cell deficiency in mice on HFD directly correlated with a reduction of TNF-α–producing M1 macrophages in AT.[156] Furthermore, the same study showed that when mice on HFD were treated with a B-cell depleting antibody, TNF-α–producing macrophages were decreased highlighting the intricate interplay of both the innate and adaptive immune systems in the ongoing immunopathological development of T2D.

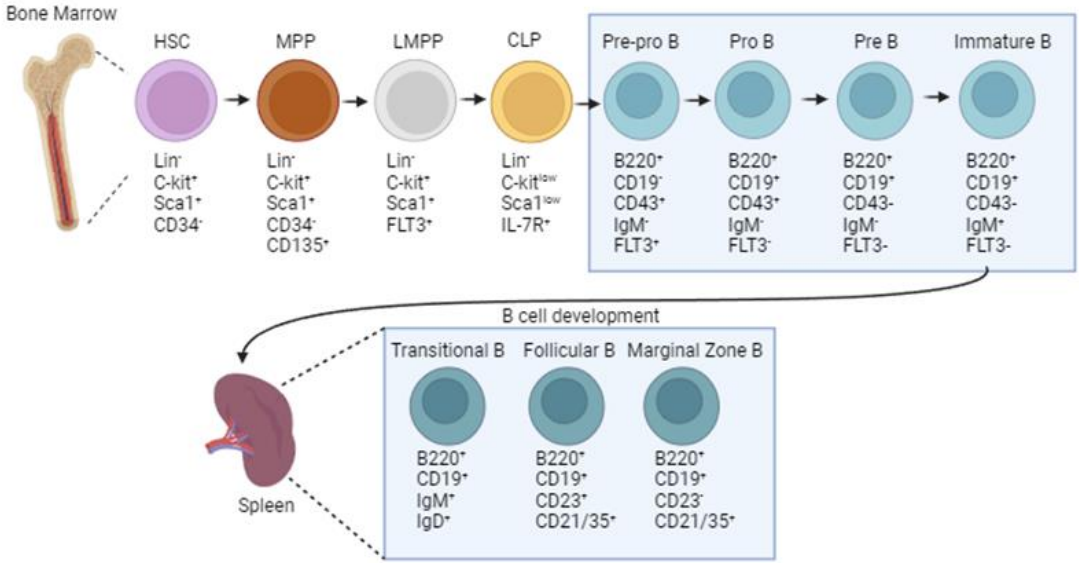

Figure 1.2: B cell development pathway in the bone marrow and spleen.

1.4 Neuroimmune responses during T2D

As described in Section 3, many T2D studies focus on the peripheral immune response in AT, though several studies in other models of neurodegeneration such as AD confirm the critical role that the peripheral immune cells play in the development and progression of cognitive decline.[171] In addition to peripheral immune cells, CNS cells such as microglia, astrocytes and neurons have also been shown to be important are also important during T2D. Mechanisms that allow immune cells to cross the BBB includes are also altered during T2D, which will be discussed in this section.

1.4A Infiltration of peripheral immune cells into the CNS

Although originally thought to be immune privileged, recent studies confirm that immune cells actively survey the brain during homeostasis through niches such as meninges, brain skull marrow, choroid plexus, and CSF – with surveillance important for development and cognitive function.[172] Even with the gaining attention on immune cells in the CNS, studies on T2D and how the peripheral immune response contributes to cognitive decline and neuroinflammation are still poorly understood. One study found that in mice on HFD, there was an increase of green-fluorescent protein (GFP) $CD45^+$ labeled peripheral immune cells in the brain.[173] This suggests that the diabetic phenotype can lead to increased immune cells in the brain, however the type of immune cells were not evaluated. A more recent study found that in the brains of aged rats on short term HFD, found an increase of $CD8^+$ T cells in the brain that was associated with memory deficits, and the depletion of $CD8^+$ reversed this phenotype.[174] However, it is important to note that in this study there was no hyperglycemia or IR. Although, these studies suggest an increase of immune cells to the CNS and diabetes is leads to the

increase of immune cells in the AT as mentioned in section 3.1, early studies also show that diabetes can also impair trans endothelial cell migration and adherence of leukocytes to venules,[175, 176] leaving immune cells unable to diapedeses into certain tissues in response to chemokine upregulation. A study focused on diabetes as a comorbidity shows similar results. For example, in diabetic mice infected with west nile virus (WNV) showed significant reductions of immune cell infiltrates into the brain as compared to controls, as well as decreased expression of cell adhesion molecules (CAMS) which help immune cells infiltrate into the brain.[177] However, in contrast, in diabetic mice that underwent a transient ischemic stroke induction showed significant increase in immune cells infiltrating the injured brains, suggesting an worsening of post-stroke severity for T2D mice,[178] an unfortunate consequence found in patient populations.[179] Although controversial, these studies provide a framework for peripheral-CNS interactions that may be occurring in the brain during the pathogenesis of T2D.

1.4B T2D and the BBB

The blood brain barrier is important for maintaining CNS homeostasis by preventing harmful substances and pathogens into the brain as well as regulating the transport of molecules in and out of the CNS. The BBB is comprised of various cells such as endothelial cells, pericytes and astrocytic endfeet, which come together to form the neurovascular unit (NVU). Under pathological conditions, the BBB leakage occurs leading to an influx of neurotoxic molecules and inflammatory cells that further degrade the permeability, eventually contributing to neuroinflammation and cognitive decline.[180] Studies have shown that in T2D, the BBB is compromised and is associated with neuroinflammation and cognitive decline.

In both mouse and rat models of T2D, studies have shown that there are decreases in tight junction proteins, which are located on endothelial cells, such as zonula occludens-1 (ZO-1), occludin, claudin 5, and vascular endothelial (VE)-cadherin.[181-183] Tight junction proteins are crucial for maintaining the integrity of the BBB by creating a high resistance barrier. BBB disruption in other studies was associated with cognitive decline in diabetic mice.[184, 185] Studies have also shown in diabetic mice that there is an increase of large plasma proteins such as albumin and fibrinogen in the brains, suggesting an increase in BBB permeability.[186] Although still understudied, contrasting studies exist on how pericytes are impacted during T2D, one study shows there is no difference in pericyte coverage,[186] while another shows significant decreases in pericyte coverage in the brains of diabetic mice.[183] More studies are needed to determine how they are impacted. Studies using radiolabeled and fluorescent tracer in diabetic mice and rats also show that BBB permeability is increased due to T2D.[183, 187] Using novel MRI techniques in the brains of diabetic rats' studies are able to confirm that BBB permeability is increased using a quantitative vascular biomarker,[188] providing more evidence to BBB disruption during T2D. MRI studies have also been used in diabetic monkeys showing that BBB permeability is increased and is associated with reductions in ZO-1 and increased IgG levels.[189] Similar findings were observed in patients with T2D patients using imaging techniques, showcasing increased BBB permeability.[190] While astrocytic endfeet are important for maintaining BBB homeostasis, currently to our knowledge there are no studies that have investigated how they are impacted during T2D.

1.4C T2D and other CNS cells

In addition to the BBB being disrupted, studies have shown that other cells of the CNS are altered during T2D such as neurons and microglia, which have also been shown to be important in neuroinflammation and cognitive decline. Multiple studies have shown that T2D leads to neuronal death and apoptosis,[191, 192] which in some cases were associated with cognitive decline,[17] increased markers of oxidative stress and neuroinflammation characterized by astrogliosis and increased in inflammatory marker such as IL-1β, TNF-α, IL-6.[18, 184] Furthermore, studies have shown using single cell sequencing that T2D leads to the alteration of genes important and neuronal maturation and metabolism.[193] These studies suggest that T2D leads to cognitive decline through damaging neurons and neuroinflammation. Microglia, which are the innate immune cells in the brain, have also been shown to be activated during T2D, shown by increases in Iba-1 co-localized with MHC II.[184] Other studies have shown that M1 markers for microglia are increased while M2 markers are decreased.[194] More recent studies have found that in diabetic mice microglia exhibited an increase in lipid droplets, which is a characteristic of aging, in the hippocampus. Furthermore, this increase in lipid droplets was associated with inflammatory markers such as NRLP3 and triggering receptor expressed on myeloid cells 1 (TREM1).[195] Interesting in the same study, when plasma from aged patients with T2D was incubated on human microglia it also increased the formation of lipid droplets and increased the expression of TREM1. These findings highlight the impacts of T2D on neurons and microglia, providing insight into potential mechanisms underlying cognitive decline.

1.5 CXCL12/CXCR4 axis

1.5A Overview of CXCL12/CXCR4

Chemokines or chemotactic cytokines are a type of signaling protein that is secreted and signals through the G-protein coupled receptor (GPCRs) family.[196] Chemokines are secreted by various cells, including immune cells, endothelial cells, fibroblasts, and cells of the CNS, and are most known for their role in the immune response, more specifically, orchestrating leukocyte migration and thereby directly contributing to homeostasis.[197] Leukocytes and non-leukocytic cells such as neurons, endothelial cells, astrocytes, epithelial cells, and mesenchymal cells express receptors for chemokines and contribute to maintaining homeostasis.[198] Although most studies focusing on chemokines consist of their roles in migration, studies have shown that they also play roles in development, inflammation, atherosclerosis, cancer, hematopoiesis, autoimmunity, and CNS development and disorders. Chemokines can bind to several receptors with similar affinities, and several receptors can bind to the same ligand. Chemokines are divided into four subfamilies based on molecular structures C-C, C-X-C, CX3-C and C.[199] One important chemokine-receptor axis is the CXCL12/CXCR4 axis contributes to pathological progression in multiple diseases including T2D.

CXCL12, a member of the CXC-chemokine family, was initially discovered as a pre-B cell growth factor (PBGF) and was found to be essential for embryogenesis and lymphopoiesis.[200] Later, due to its constitutive expression in BM stromal cells, its name was changed to stromal cell-derived factor 1α (SDF-1α).[201] CXCL12 is a highly conserved gene, having only one amino acid difference between murine, rat, and human homologs, making the actions of this chemokine very similar across species. Also, the

coding regions of the nucleotide sequences in humans and mice for CXCL12 genes are 99% identical, making it one of the highest conserved chemokines described to date.[202] CXCL12 is an 8-kDa protein that is present in either a monomeric or dimeric form. It is considered a homeostatic chemokine produced in multiple tissues such as the pancreas, lungs, kidneys, and the brain, as well as leukocytes and monocytes.[203] In addition to its roles in leukocyte migration, it is important in trafficking of hematopoietic cells, secondary lymphoid tissue architecture, B cell development and trafficking, and angiogenesis.[204, 205] For example, early studies focusing on the chemoattractant potential of CXCL12 showed its ability to induce the migration of both lymphocytes and monocytes in-vitro,[206] while loss of CXCL12 leads to deficits in hematopoiesis and nervous system development.[204, 207] Furthermore, mice lacking CXCL12 showed reductions in B cell progenitors in the BM, showing its importance in B cell lymphopoiesis.[204]

CXCL12 is the unique and specific chemokine for the receptor CXCR4,[208] originally known as leukocyte-derived seven-transmembrane domain receptor (LESTR) or Fusin.[209] CXCR4 is a 7 transmembrane-spanning GPCR.[210] and is expressed on the cell surface of most leukocytes and is also expressed on non-hematopoietic cells including endothelial cells, fibroblast, and other tissue in the lungs, kidney, liver, and brain, including glia.[203] CXCR4 is considered a homeostatic receptor that is expressed during development and in adult tissue. Deficiency of CXCR4 is embryonic lethal and mice exhibit defects in hematopoiesis in the nervous and cardiovascular during development.[211] Mutations in the CXCR4 gene in humans and mice lead to warts, hypogammaglobulinemia, immunodeficiency, myelokathexis (WHIM) syndrome, a

severe combined immunodeficiency disease characterized by an increase in susceptibility to viruses and infection.[212] Patients with WHIM syndrome suffer from neutropenia, B cell lymphopenia, and hypogammaglobulinemia highlighting the critical role of the CXCL12/CXCR4 axis during homeostasis and association with many diseases, including T2D, that will be discussed below.

1.5B CXCL12/CXCR4 and T2D

The CXCL12/CXCR4 axis is important in the pathogenesis of T2D. Patients with T2D have increased concentrations of CXCL12 compared to healthy controls,[213, 214] though their peripheral blood mononuclear cells (PBMCs) have decreased expression of CXCR4,[215] which has also been shown in pre-diabetic patients,[216] suggesting that patients with T2D have defective migration and homing potential due to loss of CXCR4. Furthermore, studies have shown that CXCL12 single nucleotide polymorphisms (SNPs) genotype influences the mobilization of progenitor cells during T2D.[217] In-vitro studies demonstrated that CXCL12 promotes survival among insulin-producing pancreatic β-cells in mice and that in response to initial injury, pancreatic β-cells upregulate CXCL12, which significantly contributes to their growth, survival, and overall viability through glucagon-like peptide-1 (GLP-1) signaling.[218, 219] Furthermore, the overexpression of CXCL12 in pancreatic β-cells increased viability by protecting them from necrotic death.[220] In-vivo studies using mice on HFD showed that in plasma and AT is an increase of CXCL12, that is associated with the accumulation of macrophages and the antagonism of CXCR4 reversed macrophage accumulation and pro-inflammatory cytokines.[221] Studies in obese patients confirmed that CXCR4 expression was increased

in the visceral AT.[222] These findings highlight the pivotal role of CXCL12/CXCR4 axis in protecting pancreatic β-cells during the early stages of T2D pathogenesis.

1.5B.1 Diabetic Wound Healing

As T2D proceeds, however, many organs are impacted leading to damage and an array of complications such as diabetic wound healing, neuropathy, and nephropathy, with the CXCL12/CXCR4 axis implicated in all co-morbidities. In diabetic wound healing, T2D patients have decreased levels of CXCL12 in both circulation and tissue biopsy samples[223] suggesting a link between the loss of a functioning CXCL12/CXCR4 axis and impaired healing. Overexpression of CXCL12 in wounds from diabetic mice lead to an increase in enhancing wound healing,[224] while inhibition of CXCL12 slowed the rate of wound healing, emphasizing the importance of the CXCL12/CXCR4 axis in diabetic wound healing.[225]

1.5B.2 Diabetic Neuropathy

During diabetic neuropathic pain, primary sensory nerves and dorsal root ganglions (DRGs) are undergo small fiber degeneration and hyperexcitability and as a consequence pain signals become abnormal.[226] Patients with diabetic neuropathy have increased CXCR4 expression in sural nerves and PBMCs[227, 228] while in diabetic rats, CXCR4 expression was also increased in DRG neurons.(*229*) In mice on HFD, there was decreased protein expression but increased mRNA levels of CXCR4 in DRG neurons, which associated with an increase in $CXCR4^+$ immune cells.[230] This same study showed that antagonizing CXCR4 reversed neuropathic pain. In addition, deletion of CXCR4 in DRG neurons prevented allodynia and small fiber degeneration in mice on HFD.[231]

Additionally, the CXCL12/CXCR4 axis contributed to neuron hyperexcitability, further supporting its involvement in diabetic neuropathy.

1.5B.3 Diabetic Nephropathy

Diabetic nephropathy is a condition where the kidneys gradually fail due to a decreased capacity for blood filtration. It usually starts with albuminuria, which is the presence of albumin in the urine, followed by a decline in the glomerular filtration rate, eventually leading to renal failure.[232] CXCL12 is increased in people with diabetic kidney disease, and it is positively associated with incidence of diabetic kidney disease and is an independent contributor to diabetic kidney disease.[233] Studies in diabetic mice, for example, show that CXCL12 was found in podocytes and was associated with glomerulosclerosis, and that CXCL12 inhibition reduced pathology, and increased podocyte numbers and prevented albuminuria.[234] Researchers from the same group suggested a new method to prevent glomerulosclerosis in diabetic mice by blocking the dual chemokines CCL2-CXCL12.[235] This approach inhibited glomerular leukocyte recruitment mediated by CCL2 and loss of podocytes caused by CXCL12. Taken together, these data suggest that in the early stages of T2D increased CXCL12 expression may be beneficial and promote survival. Its chronic expression may start to contribute to T2D progression making it harmful. Blocking CXCR4 during its chronic state may be more beneficial than blocking it during its early expression.

1.5C CXCL12/CXCR4 in CNS and CNS-related disorders

As the CXCL12/CXCR4 axis is important in the periphery during T2D, it is just as important in the CNS during health, and in other diseases and injuries. Both CXCL12

and CXCR4 are expressed in the developing and mature CNS and is important for the migration of neuronal progenitor cells forming complex cortical structures in the developing CNS. During development the CXCL12/CXCR4 axis also provides cues that regulate axon projection to form synaptic connections. After CNS development, the CXCL12/CXCR4 axis maintains adult neurogenesis and tissue repair by guiding neural progenitor cells and contributes to regulation of neurotransmission in mature neurons. As these studies have shown the importance of the CXCL12/CXCR4 axis during homeostasis, it is also important during pathological conditions in the CNS. *Importantly for this thesis,* studies focusing on the CXCL12/CXCR4 axis in the CNS during T2D are lacking so we will discuss what is known about the axis in other CNS disorders.

1.5C.1 Stroke

A stroke occurs when a blood vessel in the brain is blocked or burst leading to loss of blood flow and subsequent neuroinflammation, cognitive deficits and potential long-term disability.[236] In stroke patients, CXCL12 is increased in both plasma and serum,[237-240] however whether CXCL12 is helpful or determinantal during stroke is controversial. For example, multiple studies found that CXCL12 levels are positively associated with infarct and National Institutes of Health Stroke Scores (NIHSS),[237, 239, 241] while another found that CXCL12 levels were negatively associated with infarct and NIHHS.[238] Additionally, increased CXCL12 levels were seen in stroke patients experiencing recurrent strokes compared to patients that had a single stroke,[242] associating CXCL12 with an elevated stroke risk. There is also an increase of $CXCR4^{+}$ PBMCs in patients with stroke, which was inversely associated with Rankin scale score,[238] which measures the degree of disability in patients with strokes. Furthermore,

CXCL12 was increased in non-survivors and patients with unfavorable outcomes post-stroke.[243] Studies using post-mortem brains samples from stroke patients identified elevated CXCL12 levels in the peri-infarct[244] and increased CXCR4 expression in the infarct region.[245]

Mouse models of stroke focused on the CXCL12/CXCR4 axis further add controversy to whether the axis is beneficial or not. As the CXCL12/CXCR4 axis is important is most known for its role in immune cell migration in the CNS, studies have found that in mouse models of stroke, CXCL12 and CXCR4 is increased in the brain and associated with an increase of T cells in the brain.[245] Another study found that the CXLC12/CXCR4 axis is important in recruiting NK cells and innate lymphoid cell type 3 (IL3s) into the ischemic brain, which was associated with poor outcomes.[246] Removing CXCR4 expression in NK cells led to higher behavioral deficits due to the loss of NK cells migrating to the stroke lesion, suggesting that NK cells are neuroprotective post-stroke but also that the CXCL12/CXCR4 axis is important in recovery post-stroke. Other studies with similar finding show that the delivery of CXCL12-expressing endothelial progenitor cells (EPCs) post-stroke led to reduction of brain atrophy, enhance behavioral outcomes, increased blood vessel density, preserving myelin sheath integrity, and promoting neurogenesis, angiogenesis, and proliferation of oligodendrocyte precursor cells (OPCs).[247] Furthermore, using AAVs carrying CXCL12 reduce brain atrophy, increase neurogenesis, angiogenesis, protection from white matter injury, and repair damage through OPC proliferation.[248] Surprisingly, the same study found that AMD3100, a CXCR4 antagonist, reversed these effects, which is on par with other studies showing that AMD3100 treatment reduced EPCs, capillary density, cerebral

blood flow in the ischemic area, and resulted in worse behavioral outcomes post-stroke,[249] suggesting that the CLXC12/CXCR4 axis is needed for neuroprotection. Studies also show using repetitive hypoxia reconditioning (RHP) that CXCL12 signaling is required for post stroke protection and the blocking of CXCR4 reverse this protection.[250] While these studies suggest a determinantal impact of blocking CXCR4, other studies have shown that blocking is beneficial marked by improved behavioral function, reduced infarct size, reducing immune cell infiltration and suppressed inflammatory cytokines post-stroke.[245, 251] These studies suggest the CXCL12/CXCR4 is important during the pathogenesis of stroke, but they also add controversy to whether it is protective or harmful. This seems to depend on multiple factors such as when CXCL12 is increased or how it was delivered for therapeutic purposes, and this is the same for antagonisms. Human studies do provide a strong case for it being determinantal, but more studies are needed to fully elucidate its potential.

1.5C.2 Multiple Sclerosis

Multiple sclerosis (MS) is an autoimmune disorder that causes damage to myelin sheaths, leading to neuroinflammation, behavioral and cognitive deficits, and eventually death. In patients with MS, CXCL12 is increased in the CSF and CXCR4 expression is increased on PBMCs.[252-254] MRI showed that $CXCR4^+$ cells are increased in the brains of patients with MS in areas with activated microglia.[255] Additionally, CXCL12 levels were increased in astrocytes and endothelial cells, correlating with inflammation in MS lesions though other studies show this was not concomitantly linked to immune cell infiltration.[253] Histological studies in patients with MS identified a redistribution of CXCL12 from the parenchymal to the luminal side of cerebral vessels that associated

with infiltrating mononuclear cells expressing CXCR4.[256] This loss of abluminal polarity correlated with the severity of MS. Studies in the mouse model of MS, experimental autoimmune encephalomyelitis (EAE), also show increased CXCL12 expression in the brain produced by astrocytes, which was associated with myelin and neuronal repair processes.[257] In the spinal cords of mice with EAE, there was an increase of CXCR4 expression, along with increased $CXCR4^+$ T cells in lesion sites.[258] In this same study, inhibiting CXCL12 reduced EAE severity and immune cell infiltration. Other studies showed that CXCL12 was found on astrocytes and microglia, alongside increased CXCR4 expression on OPCs. Loss of CXCR4 hindered OPC maturation and impeded remyelination processes.[259] These studies emphasize the importance of the CXCL12/CXCR4 axis in immune cell migration and potentially how the axis impacts MS severity. Similar to the section in stroke the upregulation or antagonism of this axis can be both beneficial and detrimental.

1.5C.3 Parkinson's Disease

PD is a neurodegenerative disorder characterized by the abnormal deposition of α-synuclein in the brain also leading to neuroinflammation and cognitive behavioral deficits. In patients with PD, there is an increased expression of CXCR4 in the substantia nigra (SN), while CXCL12 and CXCR4 expression was increased in the SN in tyrosine hydroxylase (TH)-positive cells.[260] Patients with PD also had increased CXCL12 in the serum and plasma, with increased CXCR4 expression on PBMCs.[261] Postmortem studies in PD patient brains confirmed increased CXCL12 and CXCR4 in the SN, which co-localized with microglia,[262] suggesting a mechanism for microglia migration to SN during PD. More recent studies showed that CXCL12 is increased in the CSF and plasma

of patients with PD, accompanied by $CXCR4^+$ CD4 T cells, which was associated with neurodegeneration.[263, 264] As in the case with MS, CXCL12 colocalized with the cerebrovasculature and $CXCR4^+$ T cells were found in the meninges.[263] In mouse models of PD, CXCR4 – but not CXCL12 – was increased in a time-dependent manner[260] and CXCR4 knockout mice displayed improved coordination, reduced degeneration of dopaminergic neurons, decreased microglia and astrocyte activation, reduced BBB leakage, increased CD31 cells (endothelial cells), and decreased migration of peripheral immune cells.[265] As in-vitro studies demonstrated that α-synuclein upregulated CXCL12 levels in microglia,[262] it may be that prolonged neuronal upregulation of CXCL12 in PD-related brain recruits pathogenic $CXCR4^+$ cells that contribute to disease progression.

1.5C.4 Alzheimer's Disease

AD is the most common neurodegenerative disorder and is caused by the deposition of amyloid plaques and neurofibrillary tau tangles that, as with the aforementioned CNS diseases, leads to neuroinflammation and cognitive decline. Patients with early AD exhibit a decrease in plasma CXCL12 that was inversely associated with CSF tau levels and positively correlating with cognitive function suggesting a negative effect of CXCL12 on brain health in AD.[266] Although no significant differences were found in CSF CXCL12 levels compared to controls, it also showed a positive correlation within-patient to plasma CXCL12. Contrary reports in similar patients with AD identified an increase in CXCL12 in the CSF which was negatively associated with cognitive scores.[267] Post-mortem studies in patients with confirm a decrease in CXCL12 expression in the brain while CXCR4 expression is increased and associated with

markers of postsynaptic damage and microglia activation.[268] In mouse models of AD, both CXCL12 and CXCR4 expressions were increased in the brain, and CXCL12 colocalized with astrocytes, coinciding with cognitive deficits.[269] However, in the same study, cognitive deficits were also reported when using AMD3100 in non-transgenic mice, although studies using AMD3100 in AD mice showed improved cognitive function and decreased neuroinflammation[270] adding a mixed understanding of the interpretation of these findings. Intracerebroventricular injections of CXCL12 in AD mice reduced amyloid-beta deposits, increased microglia, and plaque-associated microglia[271] and pretreatment of neurons with CXCL12 increased dendrite length and reduced apoptosis in-vitro.[272] Although these studies are far from consistent in identifying benefit or injurious mechanisms of activation, they do show a potential role for the CXCL12/CXCR4 axis in the pathogenesis of AD.

1.6 Animal models of amylin-induced T2D

This introduction has looked at the known pathologic mechanisms contributing to T2D, including the role of amylin as a co-secreted factor that also induces neurovascular injury independent of T2D-mediated mechanisms. In addition, both the peripheral and CNS-localized immune responses have been reviewed, including the extensive role of CXCL12/CXCR4 axis in CNS injuries/diseases as this specific inflammatory mechanism has not been studied in amylin-induced T2D. This final section will highlight animal models of T2D.

1.6.A Mouse models of T2D

T2D is a multifaceted condition in humans, involving numerous risk factors, including genetic predisposition, age, obesity, sedentary lifestyle, and cardiovascular disease. Consequently, studying this disease in animal models becomes challenging. To gain insights into T2D development and possible treatments, various animal models have been developed. These models can be divided into groups 1) where their phenotype is derived spontaneously due to genetic manipulation, 2) diet/nutrition-induced, 3) chemically-induced, and 4) surgically-induced. Models that are spontaneous usually have deficits in the gene that encodes for the leptin or through selective breeding over generations. For example, the Goto-Kakizaki rat was generated by selectively inbreeding Wistar rats which have abnormal glucose tolerance over several generations. Leptin is a protein that is important in regulating food intake and energy expenditure. Deficits in the leptin gene lead to altered feeding, metabolism, and endocrine function leading to hyperphagia, decreased energy expenditure and obesity. Models that utilize deficits in the leptin gene include the db/db, ob/ob, Zucker fatty rat, and the JCR/A-cp rat. These animals exhibit overeating or hyperphagia, hyperglycemia, hyperinsulinemia, and IR due to mutations in the leptin gene. Other models such as the KK mouse, NZO mouse, Cohen rat and GK rats are also spontaneous but instead were created through selective inbreeding for traits such as body size, hyperglycemia, hyperphagia and obesity. Like the animals with leptin deficiency, these spontaneous lines develop the characteristics of T2D listed above. Models that chemically induce T2D use streptozotocin, though this method more closely mimics type 1 diabetes (T1D). Streptozotocin is an antibiotic derived from *Streptomyces achromogenes* and is a derivative of nitrosourea. It causes hyperglycemia

and hyperinsulinemia though exerting its cytotoxic effects in pancreatic β cells. Models that utilize dietary changes use HFD that model obesity, hyperglycemia and insulin resistance, and glucose intolerance typically using C57BL/6J mice due to their ability to develop leptin resistance after HFD. (review in [273])

Although these models are very useful in their ability to provide insights into the multifaceted components of T2D they lack an important component that is involved in the pathogenesis of T2D, namely amylin. As previously described in section 1.2A, amylin is a hormone co-secreted with insulin by the pancreatic β-cells. As insulin increases, so does amylin with multiple studies showing that amylin contributes to the β-cell dysfunction contributing to the pathogenesis of T2D. In our lab we employ both mouse and rat models of T2D in which human amylin is hypersecreted from the pancreases, which leads to hyperglycemia IR. The mouse model of human amylin expresses h-IAPP under the control of the insulin II promoter. The hemizygous mouse does not develop diabetic phenotype unless they have some type of stimulus such as HFD.[274] The homozygous line in male mice, however, develops T2D by around 8 weeks of age. Interestingly, in the female mice only ~20% of the females develop the diabetic phenotype by 30 weeks of age, *which is why this study uses only male mice with consistent T2D presentation*. In male mice there was increased blood glucose and amylin in the plasma along with pancreatic β-cell death and aggregates of IAPP. This mouse spontaneously dies starting ~16 weeks of age.[275] The h-IAPP transgenic rat (i.e., HIP rat) is our rat model of amylin-induced T2D. The homozygous rat rapidly develops diabetes and experiences decline in β-cell mass starting at ~2 months of age with no amyloid deposition in the brain.[276] The hemizygous HIP rat spontaneously develops

diabetes starting between 6-12 months and progressively shows the characteristics of human diabetes including progressive loss of pancreatic β-cells mass, increase β-cell apoptosis and increase amyloid deposits in the pancreas, hearts, kidneys, and brains. Throughout this thesis I will be employing the hemizygous HIP rat model and the homozygous HIP mouse model.

1.7 Conclusion

T2D contributes to cognitive decline and accelerated the progression to AD. During T2D the immune response becomes dysregulated and contributes to its progression in the periphery, but it is unknown how the peripheral immune response contributes to cognitive decline. We have shown that the accumulation of amylin is an important factor during T2D that leads to neuroinflammation, BBB disruption and cognitive decline. Despite the critical role of peripheral immune system during T2D, how T2D impacts immune cell migration into the CNS and the role they play in cognitive decline and neuroinflammation has yet to be evaluated. To investigate the role of the peripheral immune response contributes to amylin-induced cognitive decline we must identify how T2D impacts the peripheral immune response as well as immune cell migration. We must also determine the mechanisms responsible for immune cell migration during T2D and how amylin contributes. The overall goal of this thesis is to understand how the accumulation of pancreatic amylin alters the neuroimmune response during T2D. We hypothesize that the neuroinflammation associated with amylin deposition into brain will be characterized by infiltration of immune cells into the brain.

Chapter 2: Methods

2.1 Rats and Mice

Sprague-Dawley male rats that hypersecrete (three-fold) the pancreatic hormone human amylin in pancreatic β-cells (HIP rats) (n=18) and age-matched wild-type (WT) (n=19 rats were used in this study. Breeding pairs were purchased from Charles Rivers Laboratory. For experiments conducted on diabetic rats, we used HIP rats at ages 16-18 months.[13, 14] HIP rats that did not develop hyperglycemia or were found to have brain tumors during dissection were excluded from the study. For experiments conducted on injected rats, we used WT rats at the age of ~14 months. HIP mice that hypersecrete human amylin in the pancreas were purchased from Jackson Laboratory. Only diabetic male mice were a part of this study at ages 5-6 months. Mice that were not hyperglycemic were excluded from this study. All experiments conducted on animals complied with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee at the University of Kentucky. Rats and mice had ad libitum access to food and water. Rats were caged 2/cage or individually according to DLAR weight standards, in ventilated cages, and mice were caged 3-5 mice/cage. Mice and rats were on a 12-hour light cycle and were randomly assigned to groups when appropriate.

2.2 Mice and rat tissue collection

In all experiments, rats and mice were anesthetized with isoflurane, blood collected by heart puncture, and then transcardially perfused with hanks balanced salt solution (HBSS) (Thermo Fisher).

For plasma, blood was collected in 5mL ethylenediaminetetraacetic acid (EDTA)-coated tubes (Vitality Medical) for rats and 1.5mL microcentrifuge tube with .5M of EDTA for mice. Blood was then centrifuged at 1000 g for 10 min at 4C. Supernatant plasma and red blood cells (RBCs) was collected and snap-frozen in liquid nitrogen.

For flow cytometry, PBMCs from rats were isolated from blood by Ficoll-Paque PLUS density gradient centrifugation (cytiva –density 1.077 + 0.001 g/ml). In brief, 2 mL of blood was diluted with 3mL of HBSS, layered upon an equal amount of Ficoll-Paque plus, and centrifuged at 500 g for 30 min with brakes turned off. The buffy coat containing cells was collected and resuspended in EAE media (Roswell Park memorial institute [RPMI] supplemented with 10% fetal bovine serum, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid [HEPES], sodium pyruvate, minimal essential medium [MEM] non-essential amino acid [NEAA], penicillin-streptomycin, L-glutamine and beta-mercaptoethanol) until use.

For BM, single-cell suspensions were generated by using a 25-gauge syringe to flush out cells from dissected tibia and femur bones using EAE media. BM cells were then strained through a 70um filter to remove debris, and cells were then centrifuged to generate a pellet, and resuspended in EAE media for use. Upon dissection, the spleen was collected and homogenized with a plunger through a 70 µm

strainer and washed and centrifuged at 500 g for 5 min. The remaining pelleted was suspended in 5ml of RPMI, layered on an equal amount of rat-lympholyte (Cedarlane Labs) for rats and mouse-lympholyte (Cedarlane Labs) for mice, and spun at 1000g for 20 min at room temperature (RT) with the brakes turned off. The buffy coat containing splenocytes was then collected and used for flow cytometry. Extra cells were cryopreserved in freezing media for future *in-vitro* experiments.

The brain was dissected into hemispheres, and the cerebellum was removed. One hemisphere was used for flow cytometry, and the other for immunohistochemistry was fixed in 10% formalin. For the isolation of immune cells, brains were homogenized using dounce homogenizers. The remaining homogenate was made into a 30% percoll solution and centrifuged at 500g for 30 min at RT with no breaks. The debris layer was removed, and the cell pellet was washed with EAE media, strained through a 70µm filter to remove the remaining debris, then resuspended in EAE media until use. All cells were counted using a cellometer.

2.3 Flow cytometry

Cell suspensions isolated from BM, spleens, blood, and brains were washed with cold RPMI and then washed with phosphate buffer saline (PBS). Cells were stained with live/dead marker Ghost dye-Alexa Fluro 700 (Tonbo Biosciences) for 30 min at 4°C according to the manufacturer's instructions, then washed with FACS buffer. Fc receptors were blocked using CD16/32 (Thermo Fisher) for 5 min at RT. For rats, cells were stained for immune cell markers defined in **Table 2.1**. For mice, cells were stained for immune cell markers defined in **Table 2.2** and **Table 2.3.** After staining with antibody for 30 min, cells were washed twice and resuspended in FACS

buffer. BD Symphony A3 was used to acquire cell populations. Analysis was conducted with Flow v10.8 software. (FMO) controls were used to identify cell populations, and each experiment conducted had a single-stained control and negative. The gating strategy for general leukocytes in the spleen and blood of rats is outlined in **Fig. 2.1**. The gating strategy for general leukocytes in the brains of rats is outlined in **Fig. 2.2**. The gating strategy for general leukocytes in the spleens of mice is outlined in **Fig. 2.3**. The gating strategy outlined for hematopoietic stem and progenitor cells (HSPCs) is in the BM of mice is outlined in **Fig. 2.4**.

The gating strategy for B cell populations in the spleens of mice is outlined in **Fig. 2.5**. The gating strategy for B cell populations in the BM in mice is outlined in **Fig. 2.6**.

Table 2.1 - Antibodies used for rats		
Antibody	**Fluorochrome/Clone**	**Vender/Cat#**
Mouse Anti-rat CD45	APC/Cyanine 7/OX-1	BioLegend/202216
Mouse anti-rat CD3	BV605/IF4	BD Biosciences/563949
Mouse anti-rat CD45RA	APC/OX-33	BioLegend/202216
Mouse anti-rat CD4	V450/OX-35	BD Biosciences/561579
Mouse anti-rat CD8a	BUV395/OX-8	BD Biosciences/740257
Mouse anti-rat HIS48	FITC/HIS48	eBioscience/11-0570-82
Mouse anti-rat CD11b/c	BV510/OX-42	BD Biosciences/743978
Ghost Dye	Red 710	Cytek/13-0871-T100
Rabbit anti-rat CXCR4	Unconjugated	Invitrogen/PA1-224866
Goat anti-rabbit IgG Secondary	PE	Invitrogen/P-2771MP

Table 2.2 - Antibodies used for mice		
Antibody	**Fluorochrome/Clone**	**Vender/Cat#**
Rat Anti-mouse CD45	APC/Cyanine 7/30-F11	BD Biosciences/557659
Rat anti-mouse TCR beta	BV605/H57-597	BD Biosciences/562840
Rat anti-mouse CD19	APC/1D3	BD Biosciences/550992
Rat anti-mouse CD4	BUV395/GK1.5	BD Biosciences/563790
Rat anti-mouse CD8a	BV421/53-6.7	BD Biosciences/563898
Rat anti-mouse Ly6g	PE/1A8	BD Biosciences/551461
Rat anti-mouse CD11b	BV510/M1/70	BD Biosciences/562950
Ghost Dye	Red 710	Cytek/13-0871-T100
Mouse lineage	APC	BD Biosciences/558074
Rat anti-mouse CD117/c-kit	BV421/2B8	BD Bioscience/562609
Rat anti-mouse Ly6A/E/sca-1	PE/E13-161.7	BD Bioscience/553336
Rat anti-mouse CD34	FITC/RAM34	BD Bioscience/553733
Rat anti-mouse CD135	PE-CF594/A2F10.1	BD Bioscience/562537

Table 2.3 - Antibodies used for B cell panel in mice		
Antibody	**Fluorochrome/Clone**	**Vender/Cat#**
Rat Anti-mouse CD45	BUV805/30-F11	BD Biosciences/748370
Rat anti-mouse TCR beta	PE-Cy5/H57-597	Invitrogen/15-5961-82
Rat anti-mouse CD19	BV421/1D3	BD Biosciences/562701
Rat anti-mouse CD185/CXCR5	BUV737/2G8	BD Biosciences/741785
Rat anti-mouse CD23	BV605/B3B4	BioLegend/101637
Hamster anti-mouse CD27	BV711/LG.3A10	BD Biosciences/551461
Rat anti-mouse CD11b	BB515/M1/70	BD Biosciences/564454
Ghost Dye	Red 780	BD Biosciences/565388
Rat anti-mouse CD184/CXCR4	PE-Cy7/2B11	Invitrogen/25-999-182
Rat anti-mouse CD43	PerCP-Cy5.5/S7	BD Bioscience/562865
Rat anti-mouse CD45R/B220	PE/RA3-6B2	BD Bioscience/553090
Hamster anti-mouse CD11c	PE-CF594/N418	BD Bioscience/565591
Hamster anti-mouse CD80	BUV661/16-10A1	BD Bioscience/741515
Mouse anti-mouse IgD	R718/217-170	BD Biosciences/752167
Rat anti-mouse IgM	BV480/II/41	BD Biosciences/746681
Rat anti-mouse CD138	BUV395/281-2	BD Biosciences/740240
Rat anti-mouse CD21/35	BUV496/7E9	BD Biosciences/752913

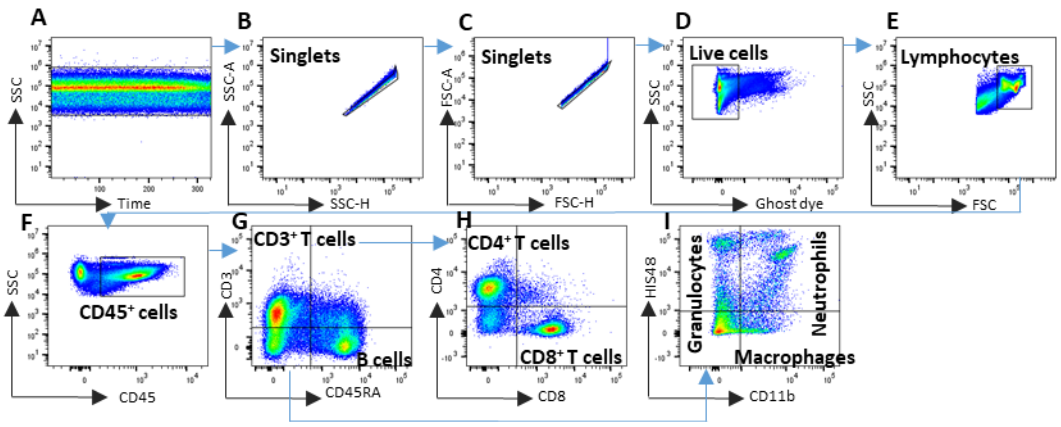

Figure 2.1: Gating strategy to identify lymphocytes in the spleen of rats. (A) Time gate on the flow of cells to visualize proper acquisition of cells. (B) Singlets on side scatter to exclude doublets. (C) Singlets on forward scatter to exclude doublets. (D) Ghost dye negative cells to gate on live cells. (E) Lymphocytes based on side scatter and forward scatter. (F) $CD45^+$ leukocytes. (G) $CD3^+$ T cells and $CD45RA/B220^+$ B cells. (H) $CD4^+$ and $CD8^+$ T cells derived from $CD3^+$ T cells. (I) $His48^+$ granulocytes, $Cd11b^+$ macrophages, $His48^+$ $Cd11b^+$ neutrophils.

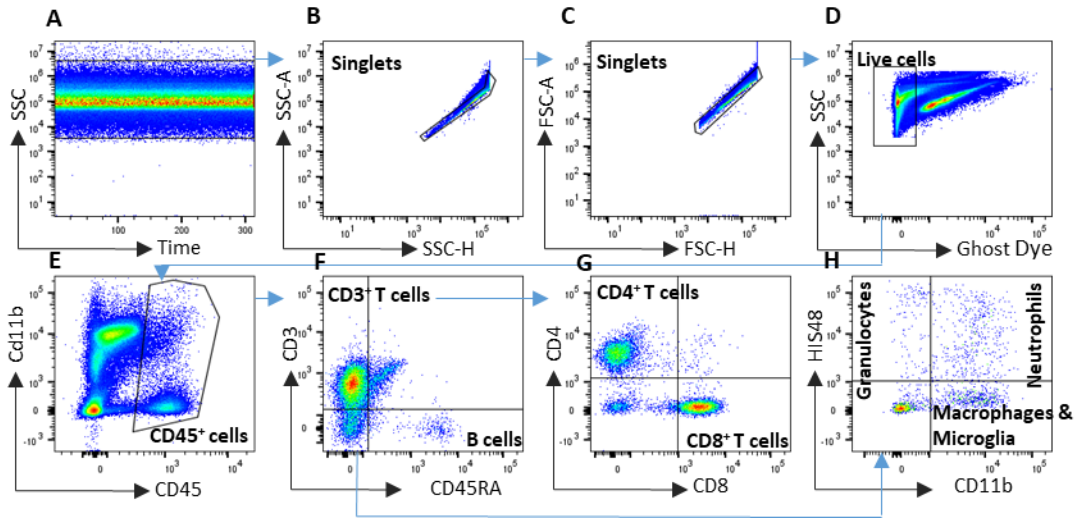

Figure 2.2: Gating strategy to identify lymphocytes in the brains of rats. (A) Time gate on the flow of cells to visualize proper acquisition of cells. (B) Singlets on side scatter to exclude doublets. (C) Singlets on forward scatter to exclude doublets. (D) Ghost dye negative cells to gate on live cells. (E) CD45+ leukocytes (F) $CD3^+$ T cells and $CD45RA/B220^+$ B cells. (G) $CD4^+$ and $CD8^+$ T cells derived from $CD3^+$ T cells. (H) $His48^+$ Granulocytes, $Cd11b^+$ macrophages/microglia, and $His48^+$ $Cd11b^+$ neutrophils.

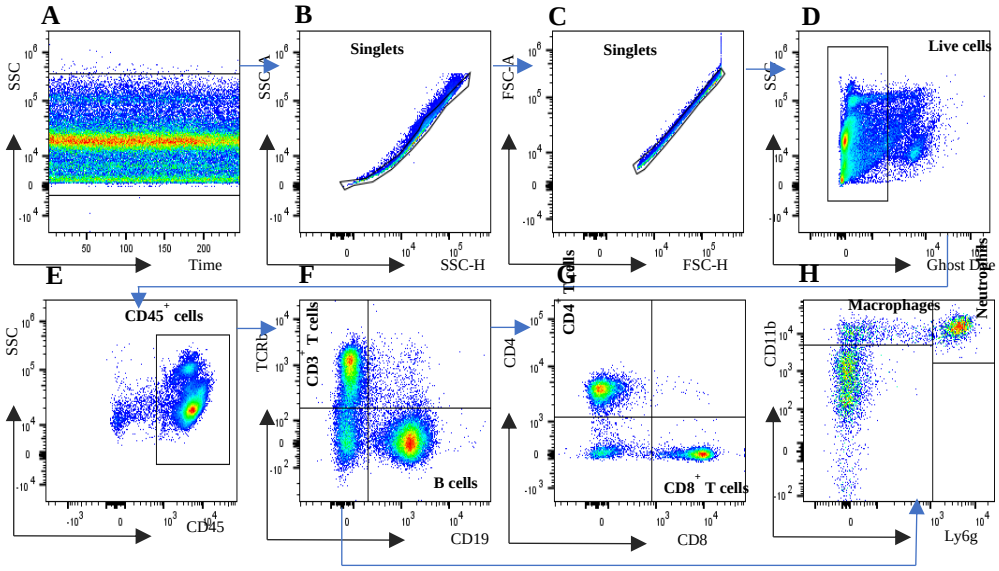

Figure 2.3: Gating strategy to identify lymphocytes in the spleens of mice. (A) Time gate on the flow of cells to visualize proper acquisition of cells. (B) Singlets on side scatter to exclude doublets. (C) Singlets on forward scatter properties to also gate our doublets. (D) Ghost dye negative cells to gate on live cells. (E) $CD45^+$ leukocytes (F) $TCR\beta^+$ T cells and $CD19^+$ B cells (G) $CD4^+$ and $CD8^+$ T cells derived from $TCR\beta^+$ T cells. (H) $Cd11b^+$ macrophages and $Cd11b^+$ $Ly6g^+$ neutrophils.

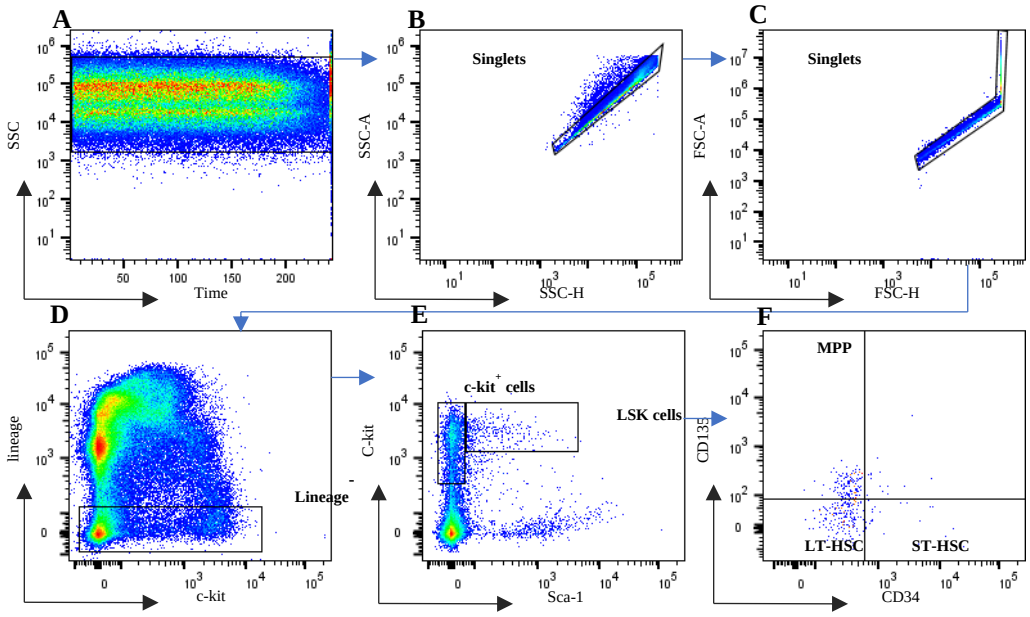

Figure 2.4: Gating strategy to identify HSPCs in the bone marrow of mice. (A) Time gate on the flow of cells to visualize proper acquisition of cells. (B) Singlets on side scatter to exclude doublets. (C) Singlets on forward scatter to exclude doublets. (D) C-kit^{+} and lineage^{-} cells. (E) C-kit^{+} and sca-1^{+} (LSK) cells. (F) CD135^{+} CD34^{-} cells are multipotent progenitors (MPPs), CD135^{-} CD34^{-} cells are long term-hematopoietic stem cells (LT-HSCs), and CD135^{-} CD34^{+} cells are short term-hematopoietic stem cells (ST-HSCs).

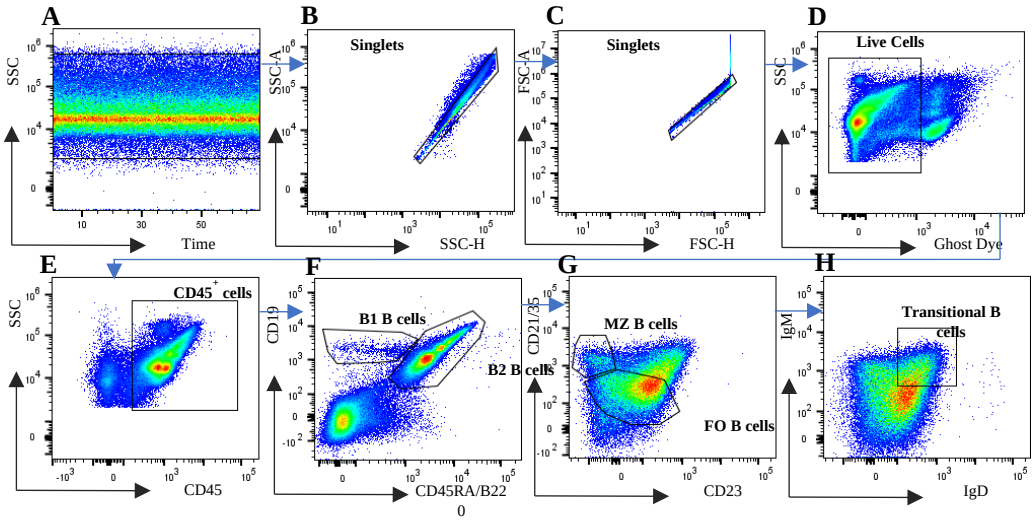

Figure 2.5: Gating strategy to identify B cell populations in the spleens of mice.

(A) Time gate on the flow of cells to visualize proper acquisition of cells. (B) Singlets on side scatter exclude doublets. (C) Singlets on forward scatter properties to exclude doublets. (D) Ghost dye negative cells to gate on live cells. (E) $CD45^+$ leukocytes. (F) $CD19^+$ $CD45RA/B220^-$ (B1 B cells) and $CD19^+$ $CD45RA/B220^+$ B cells (B2 B cells). (G) $CD21/35^+$ $CD23^-$ marginal zone (MZ) B cells and CD21/35 intermediate and $CD23^+$ Follicular (FO) B cells. (H) IgM^+ IgD^+ Transitional B cells.

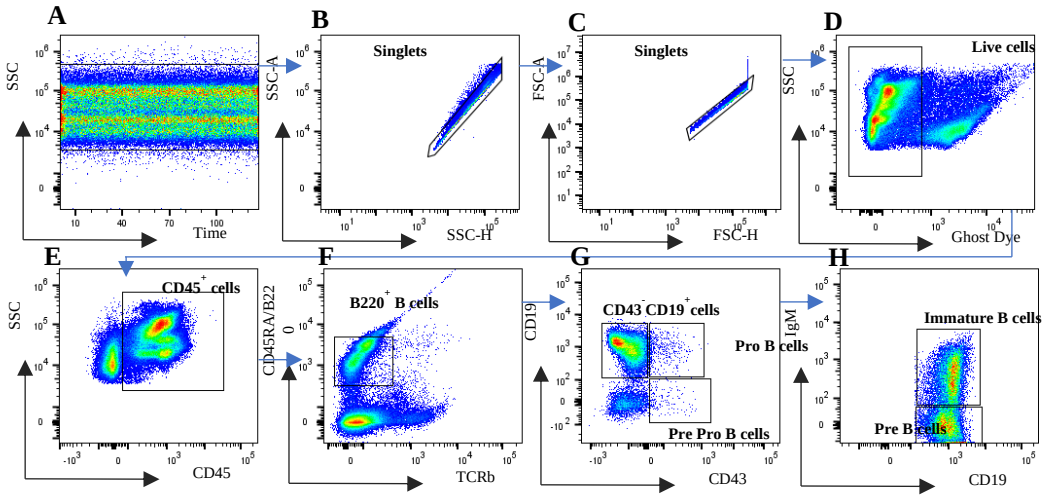

Figure 2.6: Gating strategy to identify B cell populations in the bone marrow of mice. (A) Time gate on the flow of cells to visualize proper acquisition of cells. (B) Singlets on side scatter to exclude doublets. (C) Singlets on forward scatter properties to exclude doublets. (D) Ghost dye negative cells to gate on live cells. (E) $CD45^+$ leukocytes (F) $CD45RA/B220^+$ $TCR\beta^-$ B cells. (G) $CD19^+$ $CD43^+$ cells are pro B cells, $CD19^-$ $CD43^+$ cells are pre pro B cells, and $CD19^+$ $CD43^-$ B cells. (H) IgM^+ $CD19^+$ cells are immature B cells and IgM^- and $CD19^+$ cells are pre B cells.

2.4 Tissue homogenization

Frozen rat brain samples were homogenized with 1% Triton buffer (25 times tissue volume) containing 20 mM Tris-hydrochloric acid (HCl), 150 mM NaCl, 1 mM EDTA, 1 mM ethylene glycol-bis (β-aminoethyl ether) (EGTA), 1% Triton X-100 (v/v), 1% (v/v) protease and phosphatase inhibitors, pH 7.5. The homogenates were left on ice for 15 min. The homogenates were centrifuged at 22,000 × *g* for 15 min at 4 °C. The supernatant (Triton-soluble fraction) was separated from the pellet and used for western blot experiments.

2.5 Western blot

Western blot analysis was performed on brain tissue homogenate from rats as reported recently. Total protein levels were estimated using a bicinchoninic acid (BCA) kit (Thermo Fisher). Anti-vascular cell adhesion molecule 1 or (VCAM-1) (1:500, MA5-31965, Thermo Fisher), anti-intracellular adhesion molecule 1 or (ICAM-1) (1:250; MA5407, Thermo Fisher), mouse anti-β actin (1:10,000; clone BA3R; MA5-15739; Thermo-Fisher), anti-rabbit IgG horseradish peroxidase (HRP) conjugated (1:30,000; NA934VS; GE Healthcare) and anti-mouse IgG HRP conjugated (1:20,000; NXA931; GE Healthcare) were primary antibodies. 50 µg of protein from tissue homogenate were loaded on sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels. Equal loading in Western blot experiments was verified by re-probing with a monoclonal anti-β actin antibody (raised in mouse, clone BA3R, Thermo Scientific; 1:2000). Protein levels were compared by densitometric analysis using ImageJ software.

2.6 Immunohistochemistry (IHC)

For IHC, an adapted Quantitative multiplex Immunohistochemistry with Visual colorimetric staining to Enhance Regional protein localization (QUIVER) protocol was used to repeatedly stain a single section of tissue.[277] Antibodies CXCL12 (1:100), were used with a soluble chromogenic substrate (ImmPACT AMEC Red Substrate kit (Vector Laboratories). After the first round of staining, tissue was digitized using a Zeiss Axio Scan Z.1 slide scanner to capture and digitize complete slide images at 20x magnification. Tissue was then chemically destained then serially stained for neuronal marker protein (Neun 1:250) with a permanent chromogen (DAB Substrate Kit, Peroxidase (HRP) (3,3'-diaminobenzidine) (SK-4100), and immediately co-labeled with IBA1 (1:1000, Synaptic Systems, RRID AB_2493179) and (ImmPACT AMEC Red Substrate kit (Vector Laboratories). Images were subsequently scanned, registered, deconvolved, and consolidated into a single pseudo-color image using the HALO software (Indica Labs, version 3.6).

For the quantification of cell number and colocalization for each marker, HALO software utilized the Object Colocalization algorithm to assess the overall cell number for each marker as well as number of cells colocalized in the cortex. To establish a ROI for each rat, brightfield images for each rat were used to outline the cortical region of similar size in each animal. Each brightfield image was registered, deconvolved and merged to make a single pseudo-fluorescent image. This was achieved by separating the chromogenic stain from each round of staining using the HALO deconvolution algorithm. This algorithm uses color selection and thresholding to create a single-channel image. Then using the HALO Serial Registration module,

the multiple rounds of staining were merged into a pseudo-fluorescent image. After merging images, the fluorescent Object Colocalization algorithm was used to automatically detect each marker. Parameters for marker detection were determined based on stain intensity, signal to noise ratio, and relative cell size-including processes, then applied to all sections. To ensure that only cell bodies were being counted and not aggregates of intersecting processes, a larger minimum size threshold was set (30um-10,000um). After setting detection parameters, previously outlined cortical grey matter in each section was analyzed. Results presented are based on number or cells per mm^2 in each section.

2.7 Amylin Aggregation and injection

Lyophilized amidated human amylin peptide (Anaspec #AS-60254-1) was dissolved in PBS (pH 7.4) to the concentration of 50 μM. The mixture was incubated at 37 °C for 72 h with occasional shaking to allow amylin to form aggregates. Aggregated human amylin solution was injected into 14 months old male WT rats via tail vein (80 μg/kg) (n = 6/group), 1x daily via tail vein for 1week. There was also rats that were injected 2x daily for 2 weeks with the same concentration of human amylin. For a total of n=28 rats. The first two groups of rats were inbred from WT colonies at the University of Kentucky, while the third and fourth group of rats were purchased from Envigo.

2.8 RNAseq analysis

Total RNA was extracted from the cerebral cortex using plastic dounce homogenizers from HIP and WT rats (n = 10 males/group) using Qiagen's RNeasy Mini Kit (Ref # 74104). Omega Bioservices performed RNAseq library preparations

and sequencing using the Illumina HiSeq 2500. RNAseq data were analyzed using PartekFlow software (Partek, MO) as previously published.[15] Briefly, RNAseq fastq files were imported, aligned to rat reference genome (Rattus norvegicus-rn7) and quantified at gene level using Ensembl105 annotation. RNAseq read counts were normalized and further analyzed for gene differential expression between HIP and WT groups using DESeq2 algorism. Between WT and HIP rats, RNAseq revealed 403 genes differentially expressed (DE) genes that had P-values ≤ 0.05. These DEGs were analyzed using Ingenuity Pathway Analyses software (IPA, Qiagen) and further for enrichment in Gene Ontology (GO) biological processes of these DE genes using NIHs Database for Annotation, Visualization and Integrated Discovery (DAVID) tool.

2.9 Rt-PCR

RNA was isolated from the brain tissue of WT and HIP rats using Qiagen's RNeasy kit. RNA concentration was quantified using a Nanodrop. RNA was then reverse transcribed to cDNA using an iScript cDNA synthesis kit (Bio-Rad), followed by quantitative real-time PCR (rt-PCR) to amplify target gene primers. Primers used for genes in this study are identified in **Table 2.4**. Results from samples were compared relative to the standard curve to calculate cycle threshold (CT) in each sample using Bio-Rad CFX Maestro and re-PCR (Manager software version 3) software. Quantification of the PCR data was analyzed using the 2^(-ΔΔCT) method and was normalized to the GAPDH signal.

Table 2.4 - qPCR Gene Primers		
Gene	**Primer Sequence**	**Product size**
Lag3	F: 5'-GCA GGG CCT GTG AAG CC -3' R: 5'- GCC CTG AAG ACA CAA CTG GA-3'	107bp
RT1-A1	F: 5'-CTT TTG GGA AGG AGC AGA ATA CCAC -3' R: 5'- TCT CTG GGA AAG TGG CTC AAG-3'	71bp
RT1-Doa	F: 5'- CGT TGT TAA AGC CAC CCA ACC-3' R: 5'- GGG GAC CCA GGA AGCT CAT TA-3'	87bp
RT1-DB2	F: 5'- GTG ACT TCT ACC CTG GCA CC-3' R: 5'-AGA GAT CAG GTC GGT GGA CA -3'	89bp
RT1-S3	F: 5′-GCT GTG GTA ATG CCT TTC TGG -3′ R: 5′-ATC TCA GGG CGA GAG GTT CA -3′	112bp

2.10 Statistical Analysis

All individuals performing experiments were blinded to experimental conditions. Data are presented as mean ± standard area of mean (SEM). For studies comparing WT and HIP animals, student's t-test was used. Power analyses were performed to determine the appropriate number of rats for injection experiments and rats were randomly assigned to each group. Statistical analysis of differences between groups was performed in Prism 8.1.2. Differences were considered statistically significant when $P < 0.05$. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$.

Chapter 3: The neuroimmune axis in T2D

3.1 Introduction

T2D increases the risk for cognitive decline and the progression of dementia-related disorders such as AD. Although it is not fully understood, some of the mechanisms responsible include hyperglycemia, insulinemia (high amounts of insulin in the blood), and neuroinflammation. Diabetes occurs in about 5-10% of the population, while T2D is the most prevalent. Patients with T2D have exhibit brain atrophy which has been linked to cognitive decline, which may suggest a more rapid cognitive decline in individuals with T2D.

The immune response plays a significant role in the pathogenesis of T2D, with studies confirming that T2D is associated with a dysregulated immune response secondary to hyperglycemia and insulinemia[134]. While most studies focus on how T2D leads to immune activation,[278] studies also show that T2D is characterized by impaired chemotaxis, leukocyte adhesion to venules, and trans-endothelial migration by leukocytes which also contribute to disease progression.[175, 176, 279, 280] While immune cells are most known for their role in the periphery, they are also crucial in the maintenance and repair of the brain throughout life.[281] Originally thought to be immune privileged, immune cells actively survey the brain during homeostasis through CNS niches such as the meninges, brain skull marrow, the choroid plexus, and CSF and play essential roles in neurodevelopment and cognition.[171, 282] In fact, mice that lack germline endogenous T cells demonstrated impaired cognitive performance, while mice that lack both B and T cells showed impaired neurogenesis, highlighting the importance of immune cells in the maintenance of a healthy brain.[283,

[284] Immune cells are also important during neurodegenerative disorders, and often with a negative impact as they can contribute to the pro-inflammatory environment characterized by neuronal loss and cognitive decline.[263, 284-289] However, in the case of T2D-induced dementia, it is unknown how immune cells contribute to neurodegeneration, neuroinflammation, and changes in dementia/cognition.

Amylin (or islet amyloid polypeptide) is a hormone peptide co-secreted with insulin by the pancreatic β-cells.[9] Amylin crosses the BBB [290, 291] and binds to receptors on neurons in the feeding centers to regulate satiety.[292, 293] Amylin also has amyloidogenic properties;[34] in patients with obesity or pre-diabetic insulin resistance, amylin is hypersecreted and forms amyloid in the pancreatic β-cells contributing to pancreatic β-cell dysfunction in individuals with T2D. Amyloid aggregates and incorporates into cell membranes, altering β-cell viability and function.[11, 69] Amylin deposits in the cerebrovessels of patients with T2D and dementia[12, 13] and rats that hypersecrete human amylin in the pancreas (HIP rat) exhibit accumulation of amylin deposition in the brain, as well as neuroinflammation and cognitive deficits.[13-15] Together, these studies suggest a pathological function of amylin in the brain and links between T2D and amylin-induced dementia and cognitive decline, though identification of neuroinflammatory cues originating in the brain are necessary for understanding the role of the immune system in pathogenesis, as well as to develop novel immunotherapies.

One possible mediator of a peripheral-to-brain immune response in T2D is through the chemokine CXCL12 or SDF-1α and its receptor CXCR4. CXCL12/CXCR4 are expressed in a wide range of tissues in the periphery and CNS

and expressed by multiple immune cells including T cells, B cells, and macrophages.[203, 294, 295] The CXCL12/CXCR4 axis is essential in multiple physiological processes such as leukocyte chemotaxis, cell survival, proliferation, hematopoiesis, and inflammation.[296-300] It has also been shown to be important in various disease states, including cancer, diabetes, pathological pain, MS, stroke, and neurodegeneration.[245, 250, 300-304] The CXCL12/CXCR4 axis is important in B cell lymphopoiesis as loss of either CXCL12 or CXCR4 impairs B cell development. In patients with T2D, CXCL12 is increased in the serum[305] though the number of $CXCR4^+$ PBMCs is decreased,[215] supporting a link between the CXCL12/CXCR4 dysregulation and the evolving pathogenesis of T2D. In mouse models of T2D, blocking CXCR4 using AMD3100, a CXCR4-specific antagonist, reduced M1 macrophage phenotype while also improving insulin sensitivity.[221] But contrasting studies suggest that CXCL12 can also show anti-inflammatory properties through polarizing T cells to a Tregs (i.e., anti-inflammatory)[306] and polarizing macrophages to an M2 phenotype[307]; however, these results were shown in non-diabetic models.

Studies in other CNS diseases highlight the importance of the CXCL12/CXCR4 axis in neuroinflammation and leukocyte migration, showing elevated brain levels of CXCL12 and/or CXCR4 during MS, stroke, Parkinson's disease and AD.[245, 300, 308, 309] In autoimmune disease, CXCL12 is expressed in inflamed tissues in the CNS such as the endothelium and astrocytes and recruits $CXCR4^+$ leukocytes. Whether CXCL12/CXCR4 contributes to injury or repair is highly dependent on the CNS disease being studied. For instance, blockade of CXCR4 using AMD3100 during autoimmune disease increased migration of $CD45^+$ leukocytes

from the perivascular spaces into the parenchyma and correlated with worsened outcomes,[300] while the same CXCR4 blockade in experimental stroke reduced the infiltration of T cells into the brain, leading to improved outcomes.[245, 250] Finally, in neurodegenerative models of PD and AD, CXCR4 blockade ameliorated neuroinflammation through modulating migrating microglia and reducing inflammatory cytokines, leading to improved cognitive outcomes.[270, 308]

These studies suggest that the CXCL12/CXCR4 axis is essential in regulating leukocyte migration and neuroinflammation. However, little is known about the relationship between the accumulation of amylin and the CXCL12/CXCR4 axis. Here, we tested the hypothesis that the accumulation of amyloid-forming amylin leads to a dysregulated peripheral immune response that alters immune profiles in the T2D brain. Using both transgenic rat and mouse models of T2D that hypersecrete human pancreatic amylin (HIP rat and mice), we found that the accumulation of amylin decreased immune cell populations in the BM, spleen, and brain, concomitant with changes in proteins responsible for immune cell migration in the brain. We also found that the accumulation of pancreatic amylin leads to specific defects in B cell development within the BM, a previously undescribed phenomenon that may be critical for co-morbidities and pathologies secondary to the development of T2D.

3.2 Results in HIP rats

3.2.1 Hypersecretion of pancreatic human amylin alters genes important for neuroinflammation and immune signaling in the brains of HIP rats.

To understand how the hypersecretion of pancreatic human amylin impacts the brain, we employed RNA-sequencing in the brains of WT rats that express non-amyloidogenic rat amylin and HIP rats (n=10/group). RNA seq analysis identified 408 differentially expressed genes (DEGs) when comparing WT and HIP rats. Hypersecretion of pancreatic human amylin led to a range of upregulated and downregulated genes shown by volcano plot (**Fig. 3.1A**) and hierarchal clustering of DEGs (**Figure 3.1B**). When DEGs were annotated in the IPA software, multiple pathways were enriched, such as neuroinflammation, neurovascular coupling, T and B cell signaling, and neuronal signaling (**Fig. 3.1C**). Furthermore, when DEGs were annotated based on GO using the DAVID software, biological pathways relating to the immune response, hypoxia, and neuronal development were enriched (**Fig 3.1D**). There were also enriched pathways in the molecular function, such as death receptor activity, misfolded protein, and cell-to-cell adhesion (**Fig 3.1E**). To validate genes in the brains of HIP rats, we targeted genes in immune related pathways that were known to be important in the peripheral adaptive immune response. The genes RT1 class Ib, locus S3 (RT1-S3) and RT1 class Ia, locus A1 (RT1-A1) are genes in important in MHC I. The genes Rt1 class II, locus Db2 (RT1-Db2) and RT1 class II, locus Doa (RT1-DOa) are genes important in MHC II. MHC molecules are expressed on the surface of peripheral immune cells and immune cells in the brain such as astrocytes and microglia. They are important for the binding of antigens and presenting them to

different cells for recognition to elicit an immune response. Lymphocyte activating gene 3 (Lag3) is an inhibitory receptor that is expressed on peripheral immune cells and glia, its most known role is currently expressed on exhausted immune cells. We conducted rt-PCR on these genes, but we did not see a significant difference in these genes when comparing WT and HIP rats (**Fig. 3.2**).

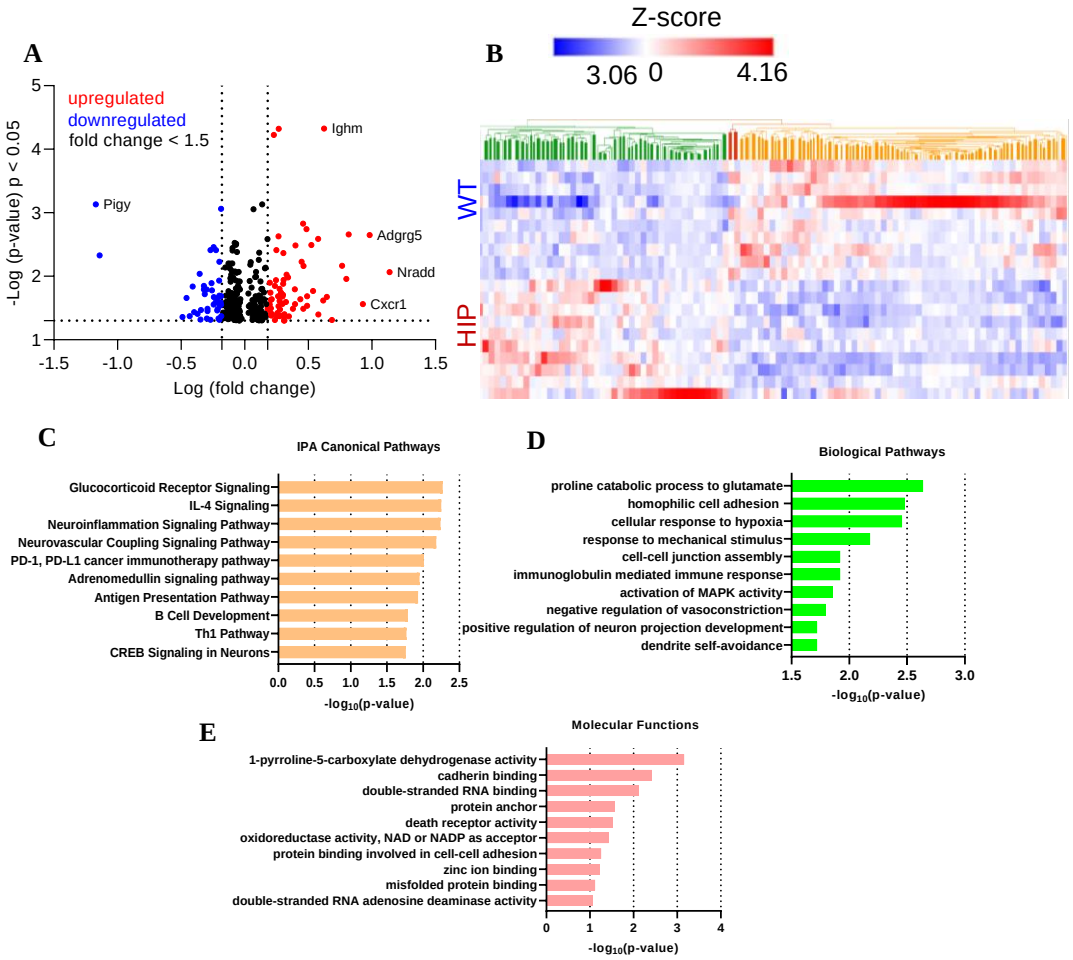

Figure 3.1: Neuroinflammation and immune related pathways induced by the hypersecretion of pancreatic human amylin in the brains of HIP rats. (A) Volcano plot showing the Log_{10} (p-value) vs. Log_{10} (fold change) of DEGs in brains of WT and HIP rats. Each dot represents a gene. Red dots indicate upregulation, and blue represents downregulation. (B) Hierarchical clustering of DEGs in brains of WT vs HIP rats. (C) Top 10 canonical pathways identified by IPA software of DEGs in brains of rats WT vs HIP. (D) Top 10 biological pathways from GO identified by DAVID software. (E) Top 10 molecular function pathways identified by DAVID software.

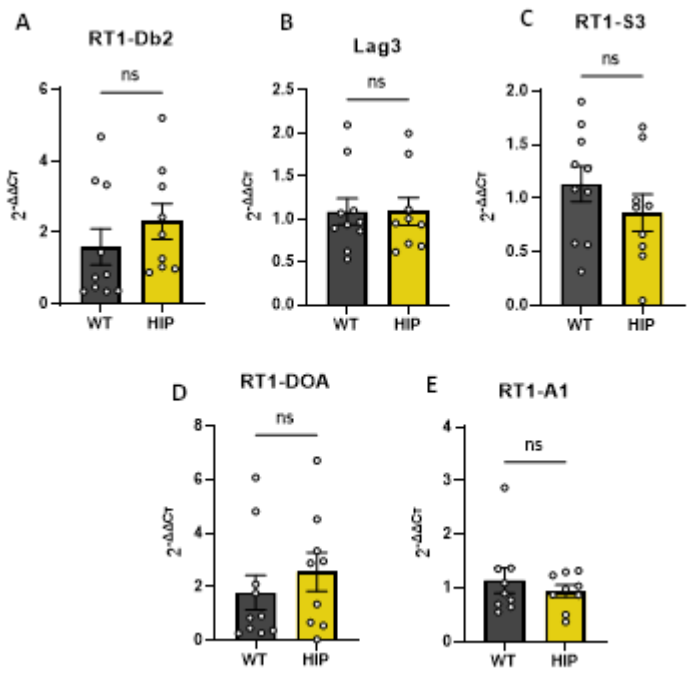

Figure 3.2: Impact of the hypersecretion of pancreatic human amylin on adaptive immunity related genes in the brains of HIP rats. Rt-PCR was used to quantify mRNA expression of immune-related genes identified by RNA-sequencing (A) RT1-Db2, (B) Lag3, (C) RT1-S3, (D) RT1-DOA, (E) and RT1-A1 in the brains of WT and HIP rats (n=8-10/group). Data are presented as means ± SEM.

3.2.2 Hypersecretion of pancreatic amylin leads to a dysregulated peripheral immune response in rats

As six of the top 10 canonical pathways identified were immune/inflammation-related, we next wanted to determine the impact of the hypersecretion of pancreatic human amylin on the immune system. To this end, we conducted flow cytometry on cells from the spleen and blood from WT and HIP rats. Although T2D is characterized by immune dysfunction, few studies focus on how increased concentrations of human amylin in the blood impact peripheral immune cell populations. In the spleens of HIP rats, we found no significant difference between the proportion of $CD45^+$ immune cell subsets, $CD45RA^+$ B cells, $CD4^+$ T cells, $CD8^+$ T cells, $CD3^+$ DN T cells, and macrophages and neutrophils compared to WT counterparts (**Fig. 3.3A**). When we looked at cells/gram of $CD45^+$ leukocytes in the spleen there were no changes when compared to WT counterparts; however, there was a trend towards a decrease ($p = 0.0615$). Among the $CD45^+$ subpopulations, we found a significant decrease in $CD3^+$ T cells, $CD4^+$ T cells, $CD8^+$ T cells, B cells, macrophages, and neutrophils in the spleens of HIP rats (**Fig. 3.3B**). For circulating populations in the blood, there was no significant difference in the percent of total $CD45^+$ cells in HIP rats but again for subpopulations we identified a significant decrease in $CD3^+$ and $CD8^+$ T cells though $CD4^+$ T cells significantly increased. No significant change was observed in B cells, monocytes, and neutrophils in HIP rats compared to WT counterparts (**Fig.3.4**).

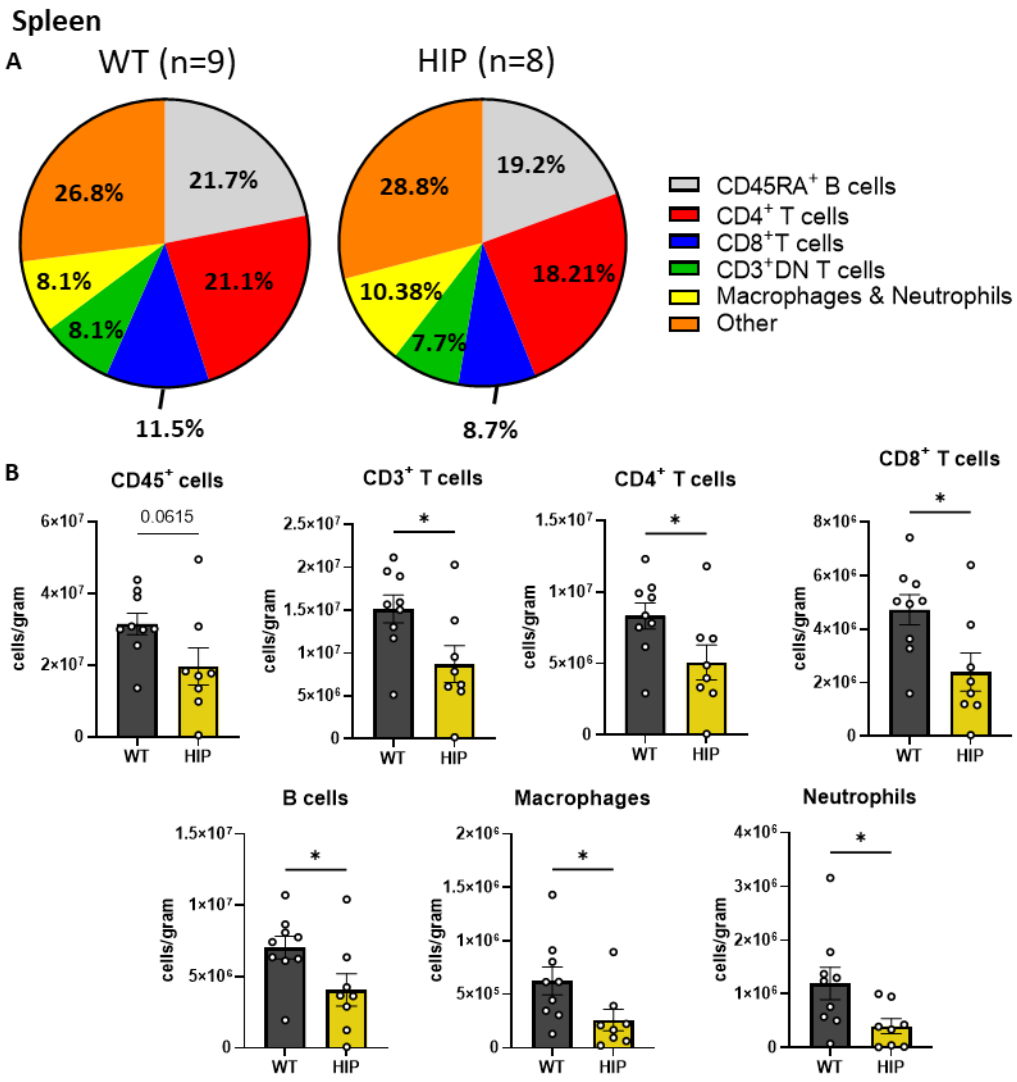

Figure 3.3: Hypersecretion of pancreatic human amylin leads to splenic lymphopenia. (A) Proportion of $CD45^+$ cell immune cell subsets ($CD45RA^+$ B cells, $CD4^+$ T cells, $CD8^+$ T cells, $CD3^+$ $CD4^-CD8^-$ (DN) T cells, and macrophages and neutrophils) in the spleens of WT and HIP rats (n = 8-9/group). (B) Cells/grams of immune cell subsets ($CD45^+$ cells, $CD3^+$ T cells, $CD4^+$ T cells, $CD8^+$ T cells, B cells, macrophages, and neutrophils) in the spleens of WT and HIP rats (n = 8-9/ group). *p < 0.05, **p < 0.01 as determined using unpaired two-tailed Students t-test. Data are presented as means ± SEM.

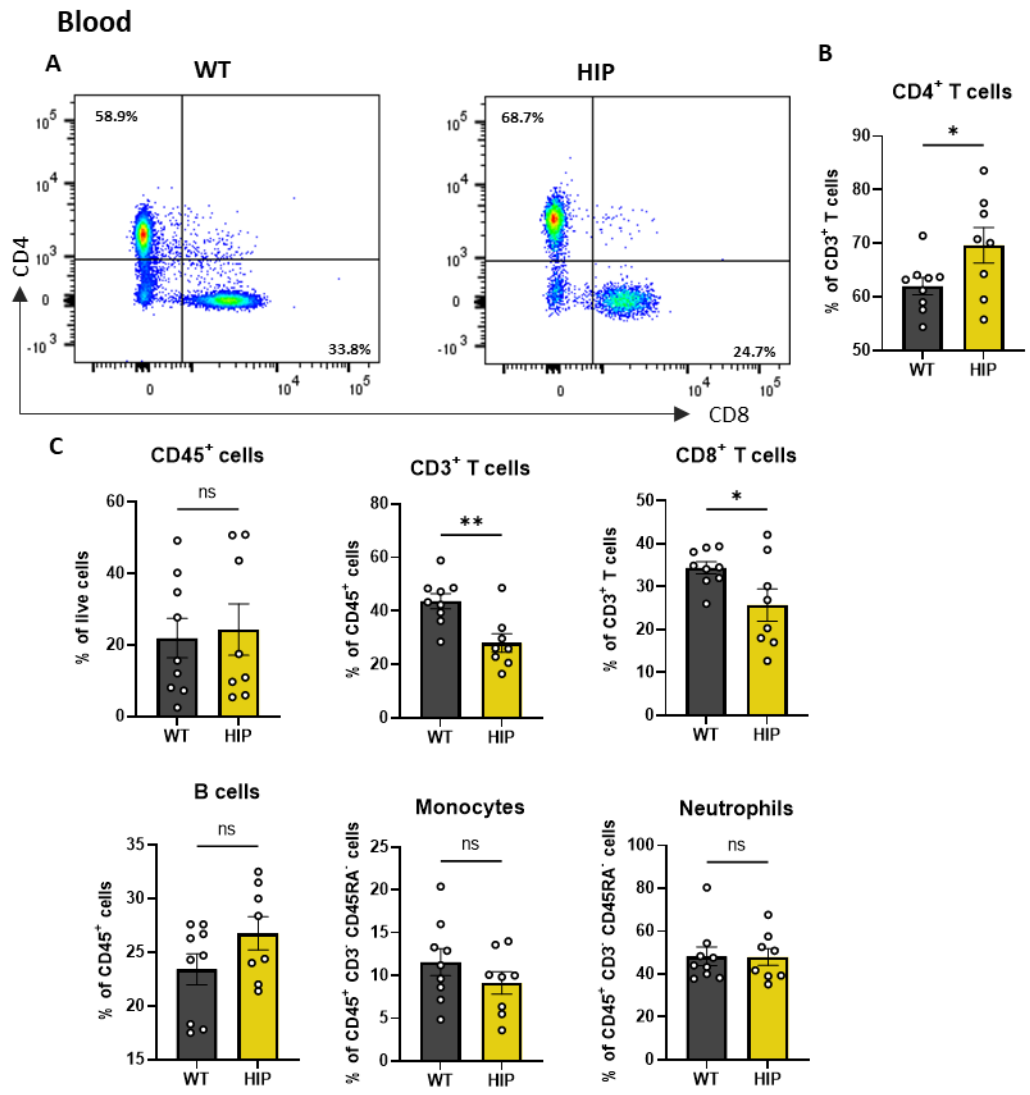

Figure 3.4: Hypersecretion of pancreatic human amylin leads to alterations in circulating T cells. (A) Representative flow cytometry analyses of $CD4^+$ and $CD8^+$ T cells. (B) Percent of $CD4^+$T cells in the blood of WT and HIP rats. (C) Percent of immune cell immune subsets ($CD45^+$ cells, $CD3^+$ T cells, $CD8^+$ T cells, B cells, macrophages and neutrophils) in the blood of WT and HIP rats (n = 8-9/group). *$p < 0.05$, **$p < 0.01$ as determined using unpaired two-tailed Students t-test. Data are presented as means ± SEM.

3.2.3 Hypersecretion of human amylin leads to deficits in immune cells migration into the CNS

We used flow cytometry to determine if the hypersecetion of pancreatic human amylin decreased immune cell infiltration into the CNS similar to peripheral suppression of immune populations. Prior studies from our lab found that there is amylin accumulation in the brains of HIP rats' concomitant with neuroinflammation characterized by glial activation and BBB dysfunction, with cognitive decline starting at 10-12 months and exacerbated with aging.[13-15] It is yet to be determined how these pathologies are associated with innate and adaptive peripheral immune populations. In the brains of HIP rats, we found no significant difference between the proportion of $CD45^+$ immune cell subsets, $CD45RA^+$ B cells, $CD4^+$ T cells, $CD8^+$ T cells, $CD3^+$ DN T cells, and macrophages and neutrophils compared to WT counterparts (**Fig. 3.5A**). However, we identified a significant decrease of $CD45^+$ immune cells in the brains of HIP rats that also exhibited depleted peripheral populations described above. Among $CD45^+$ subsets, we saw a significant decrease in $CD3^+$ T cells, $CD4^+$ T cells, $CD8^+$ T cells, and neutrophils in the cortical hemispheres. As with the circulating profiles, we found no significant changes with B cells and macrophages/microglia, although they were trending toward a decrease in number ($p=.0928$) and ($p = 0.0502$), respectively (**Fig. 3.5B**)

3.2.4 Hypersecretion of pancreatic human alters CXCR4 expression on $CD4^+$ T cells in the spleen

Studies in T2D have shown that the CXCL12/CXCR4 axis is activated shown by increased plasma CXCL12 levels and decreased CXCR4 expression on

PBMCs.[213-215] Furthermore, patients with pre-diabetes and T2D exhibit a decreased expression of CXCR4 on PBMCs.[216] These studies suggests a potential deficit in the ability of immune cells to migrate and home to where they need to be potentially contributing to the pathogenies of T2D. To understand how the hypersecretion of pancreatic human amylin impacts immune cells homing mechanisms, we first used flow cytometry to determine the percent of CXCR4-expressing cells in the spleens of HIP rats. We found a significant increase in the percentage of $CD4^+$ T cells expressing CXCR4 in HIP rats compared to WT controls (**Fig. 3.6A&B**). We did not, however, see any differences in the percentage of $CD45^+$ cells, $CD3^+$ T cells, $CD8^+$ T cells, B cells, neutrophils or macrophages expressing CXCR4 (**Fig.3.6C**) suggesting that the hypersecretion of pancreatic amylin may not be the mechanism responsible for the lack of immune cell migrating to the brain.

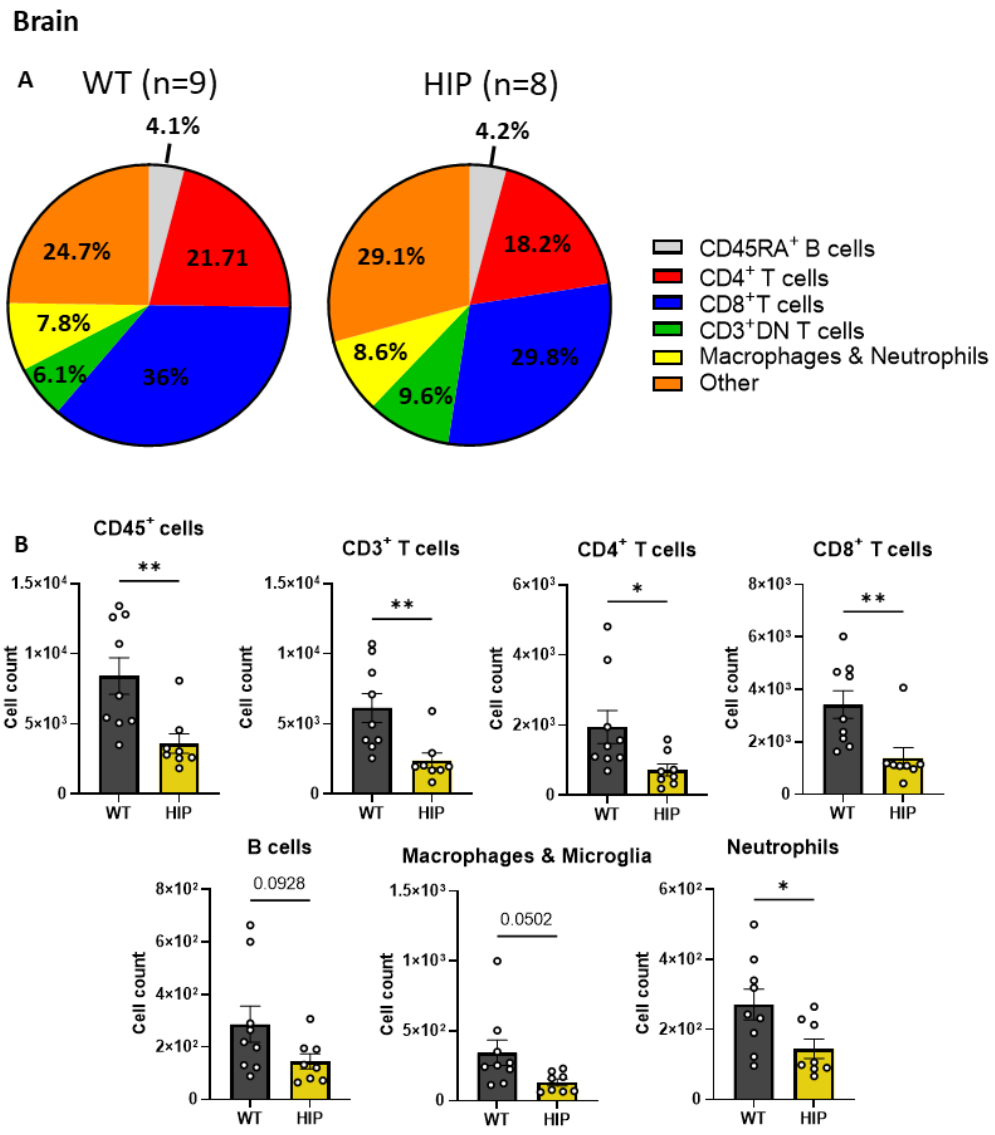

Figure 3.5: Hypersecretion of pancreatic human amylin leads to decreases in immune cell migration into the brain of HIP rats. (A) Proportion of $CD45^+$ cell immune cell subsets ($CD45RA^+$ B cells, $CD4^+$ T cells, $CD8^+$ T cells, $CD3^+$ $CD4^-CD8^-$ (DN) T cells, and macrophages and neutrophils) in the brains of WT and HIP rats (n = 8-9/group). (B) Total cell count of immune cells ($CD45^+$ cells, $CD3^+$ T cells, $CD4^+$ T cells, $CD8^+$ T cells, B cells, macrophages, and neutrophils) in the brain of WT and HIP rats (n = 8-9/ group). *$p < 0.05$, **$p < 0.01$ as determined using unpaired two-tailed Students t-test. Data are means $\pm$ SEM.

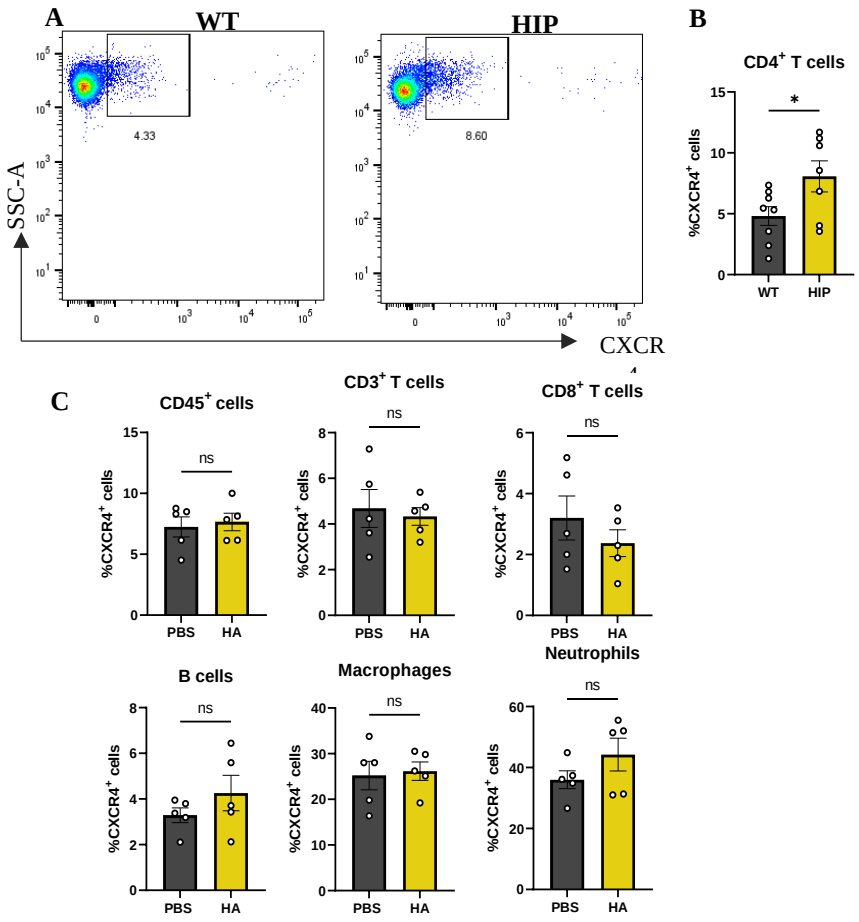

Figure 3.6: Hypersecretion of pancreatic human amylin leads to an increase of CXCR4$^+$ CD4$^+$ T cells. (A) Representative flow cytometry analyses of splenic CXCR4$^+$ CD4$^+$ T cells. (B) Percent of CXCR4 CD4$^+$ T cells in the spleens of WT and HIP rats (n = 8-9/ group). (C) Percent of CXCR4$^+$ immune cell subsets (CD45$^+$ cells, CD3$^+$ T cells, CD8$^+$ T cells, B cells, macrophages, and neutrophils) in the spleens of WT and HIP rats (n = 8-9/ group). *$p < 0.05$ as determined using unpaired two-tailed Students t-test. Data are means $\pm$ SEM.

3.2.5 Hypersecretion of pancreatic human amylin does not alter CXCL12 in the brain but alters CAMs

CXCL12 is the chemokine that attracts CXCR4-expressing cells, and studies have shown that CXCL12 expression in the brain is associated with an increase of immune cells in the CNS, for subpopulations either expressing CXCR4 or not.[253] To determine if the decrease in immune cells in the HIP rat brains caused by hypersecretion of pancreatic human amylin is secondary to disruptions in the CXCL12/CXCR4 axis, we used IHC to determine the expression of CXCL12 in the brain, including co-localization to $NeuN^+$ neurons and $Iba1^+$ microglia. We found no significant differences in the expression of CXCL12, NeuN, or Iba1 protein within the cortex. Furthermore, we found no differences in the percent of $CXCL12^+$ neurons or microglia in the presence of human amylin (**Fig.3.7**).

CAMS are cell surface molecules on cerebral venules that are important in mediating the interaction between circulating immune cells and the endothelium.[310] Recent studies in T2D show that soluble levels of circulating CAMs are altered,[311, 312] but it is unknown how brain vessel-specific levels are altered by amylin. Since we observed no differences in CXCL12 expression, we determined if changes in CAM expression was another mechanism responsible for decreased immune cell migration into the brain. Studies have shown that VCAM-1 and ICAM-1 are important for binding, crawling and diapedesis across the endothelium.[313-315] Specifically, VCAM-1 is involved in leukocyte adhesion to the BBB while ICAM-1 is more important for diapedesis, although it is important to mention they both can mediate both binding and entry. Western blot analysis of VCAM-1 and ICAM-1 shows that in the brains of HIP

rats, there was a significant increase in the expression of VCAM-1, while in ICAM-1, there was a significant decrease in ICAM-1 expression compared to WT controls (**Fig.3.8**).

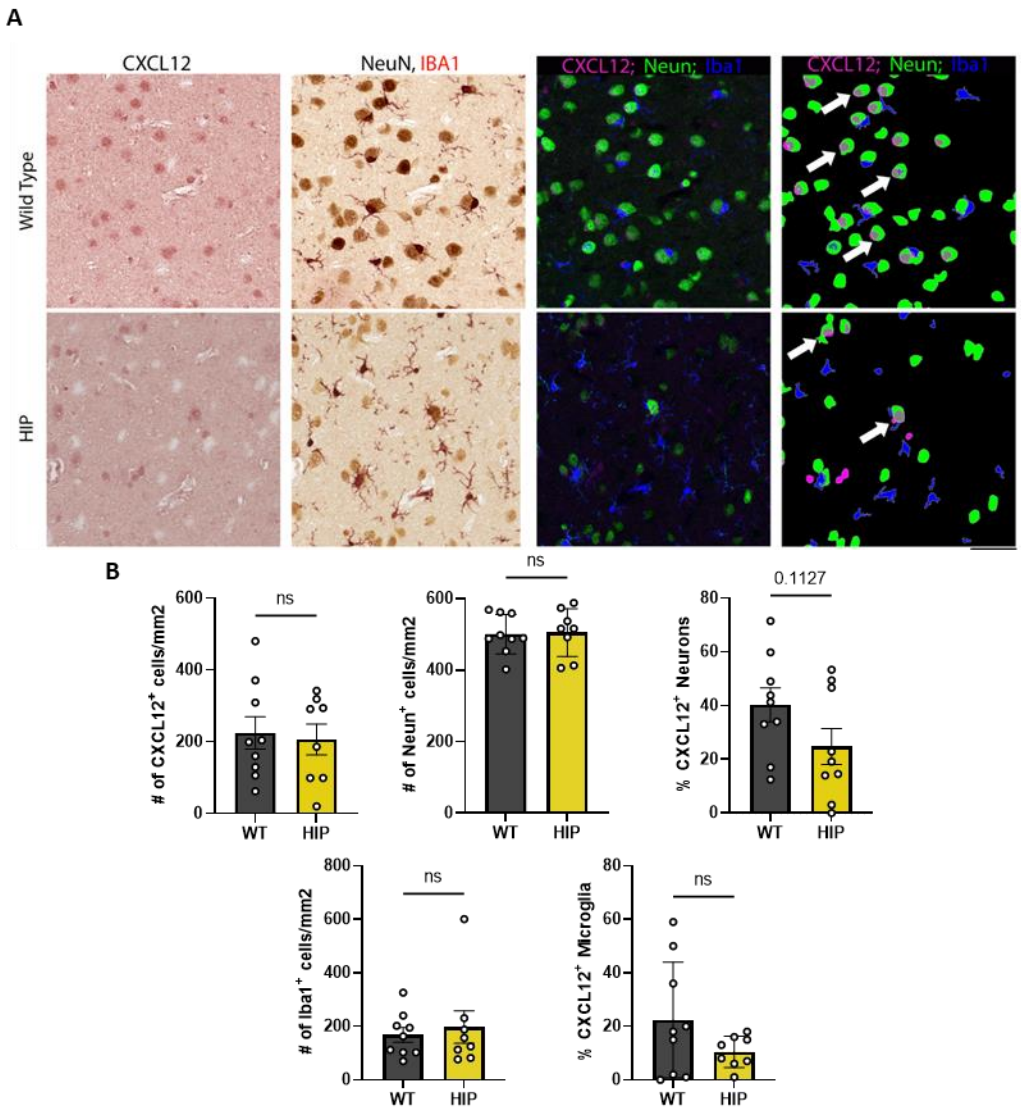

Figure 3.7: Impact of hypersecretion of pancreatic amylin on CXCL12 expression. (A) Immunohistochemistry analysis of CXCL12 in the brains of WT and HIP rats and co-localization with neurons and microglia. Arrows show co-localization of CXCL12 with neurons. (B) Number of $CXCL12^+$ cells, number of $NeuN^+$ cells, percent of $CXCL12^+$ neurons, number of $Iba1^+$ cells, and percent of $CXCL12^+$ microglia in brains of WT and HIP rats (n = 8-9/ group). Data are means $\pm$ SEM

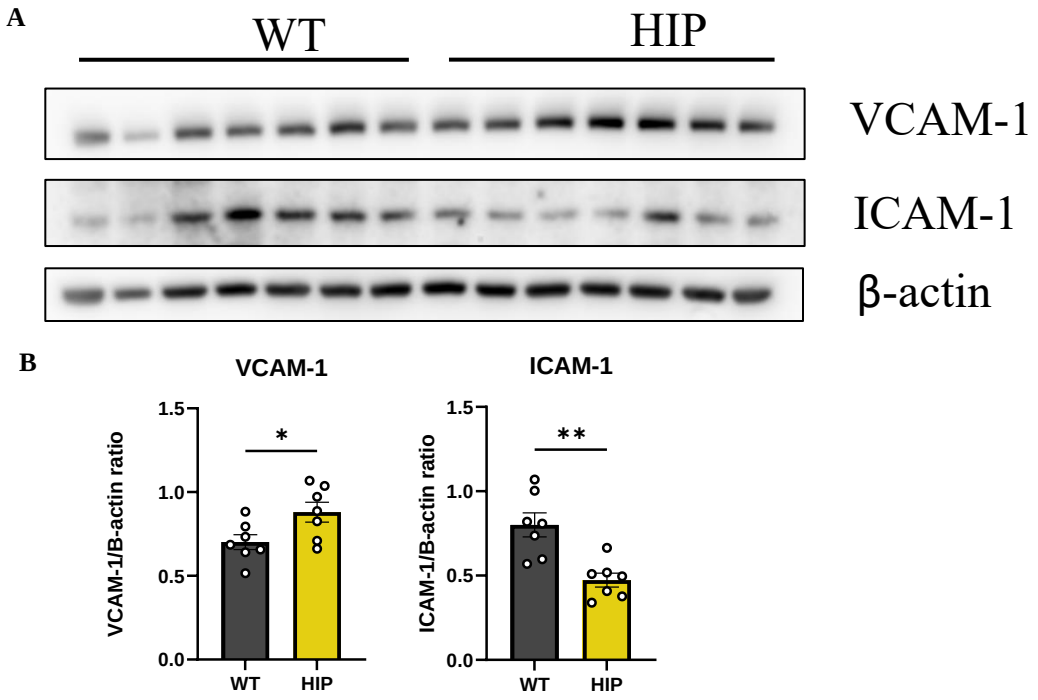

Figure 3.8: Hypersecretion of pancreatic human amylin leads to alterations of proteins responsible for immune cell migration. (A) Representation of protein levels using western blot of VCAM-1 and ICAM-1. (B) Analysis of VCAM-1 and I-CAM-1 expression from brain tissue in WT and HIP rats (n =7/ group). Changes in protein expression were quantified to the ratio of B-actin. *$p < 0.05$, **$p < 0.01$ as determined using unpaired two-tailed Students t-test. Data are means $\pm$ SEM.

3.2.6 Aggregated human leads to an altered acute immune response in WT rats

Previous studies in non-obese diabetic (NOD) mice suggest that treatment with human amylin induced Treg proliferation.[316] Considering these studies show that human amylin can cause changes in T cell populations, we wanted to determine the acute immune response to human amylin in WT rats. We conducted flow cytometry on WT rats intravenously injected with PBS or aggregated human amylin, as published previously over a 1 and 2 week period.[13, 15] After 1 week of injections with human amylin we saw no significant differences in the proportion of $CD45^+$ immune cells subsets or in the cells/grams of $CD45^+$ leukocytes and $CD45^+$ immune cell subpopulations in the spleens of rats injected with human amylin (**Fig. 3.9**). When we looked at circulating $CD45^+$ populations in the blood of injected rats we also saw no significant differences after 1 week of injections (**Fig. 3.10**). We next looked at whether two weeks of injections would elicit an immune response to human amylin. After 2 weeks of injections with we saw no differences in the proportion of $CD45^+$ immune cells subsets in the spleens of injected rats (**Fig. 3.11A**). However, we did see a significant increase in total splenic counts for $CD45^+$ immune cells, with specific increases in $CD4^+$ T cells while there was no change in other cell types, although there was a trend for an increase in general $CD3^+$ T cells ($p=0.0702$; **Fig. 3.11B**). Surprisingly, among the circulating $CD45^+$ cells in the blood of the same animals there was a significant increase of $CD4^+$ T cells with a concomitant significant decrease in $CD8^+$ T cells. As with the HIP rats under chronic human amylin exposure, there was no change to $CD3^+$ T cells, B cells, neutrophils, and monocytes compared to PBS injected (**Fig. 3.12**).

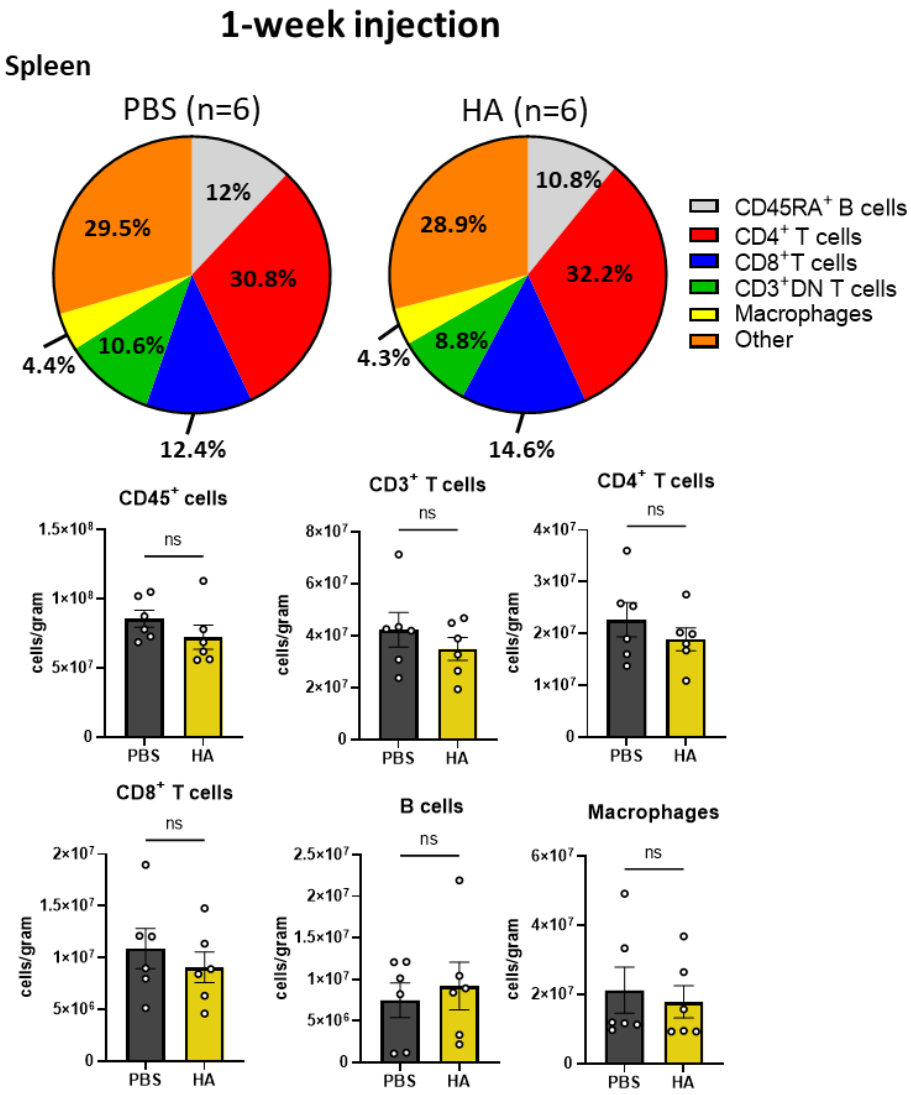

Fig. 3.9: Aggregated human amylin does not induce splenic leukocyte changes after 1 week of injections. (A) Proportion of $CD45^+$ cell immune cell subsets ($CD45RA^+$ B cells, $CD4^+$ T cells, $CD8^+$ T cells, $CD3^+$ $CD4^-CD8^-$ (DN) T cells, and macrophages and neutrophils) in the spleens of WT rats injected with PBS or aggregated human amylin (HA) (n = 6/group). (B) Total cell count of immune cell subsets ($CD45^+$ cells, $CD3^+$ T cells, $CD4^+$ T cells, $CD8^+$ T cells, B cells, macrophages, and neutrophils) in the spleens of WT rats injected with PBS or aggregated human amylin (HA) (n = 6/ group).

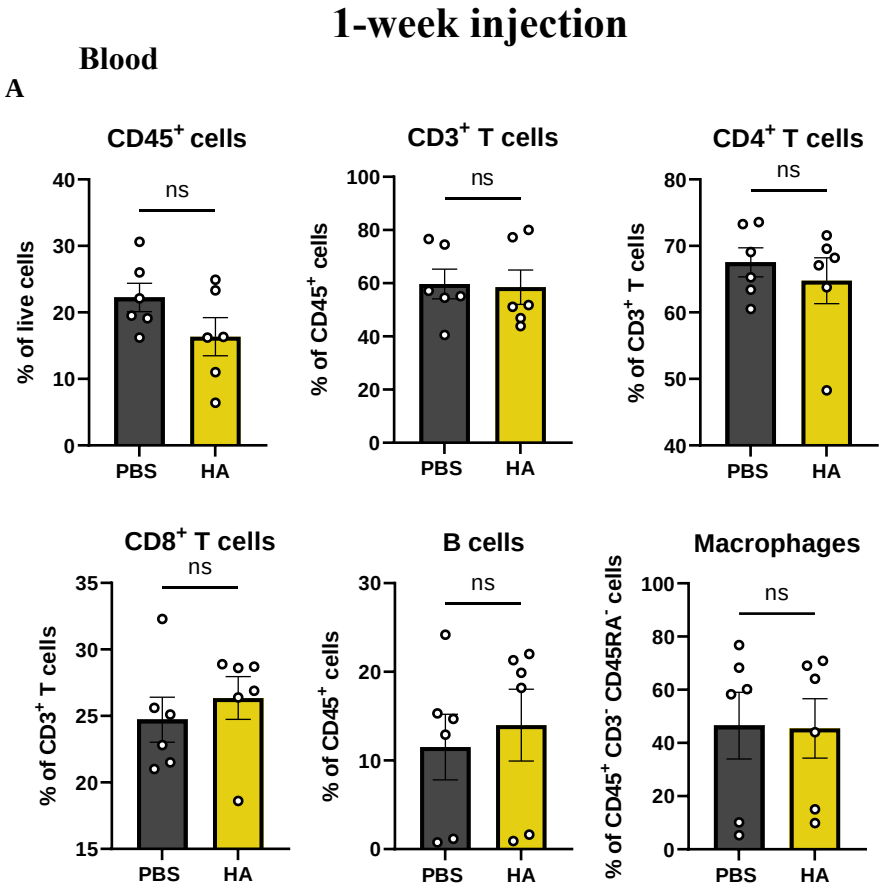

Fig. 3.10: Aggregated human amylin does not induce circulating leukocyte changes after 1 week of injections. (A) Percent of immune cell subsets ($CD45^+$ cells, $CD3^+$ T cells, $CD4^+$ T cells, $CD8^+$ T cells, B cells, macrophages, and neutrophils) in the blood of WT rats injected with PBS or aggregated human amylin (HA) (n = 6/ group).

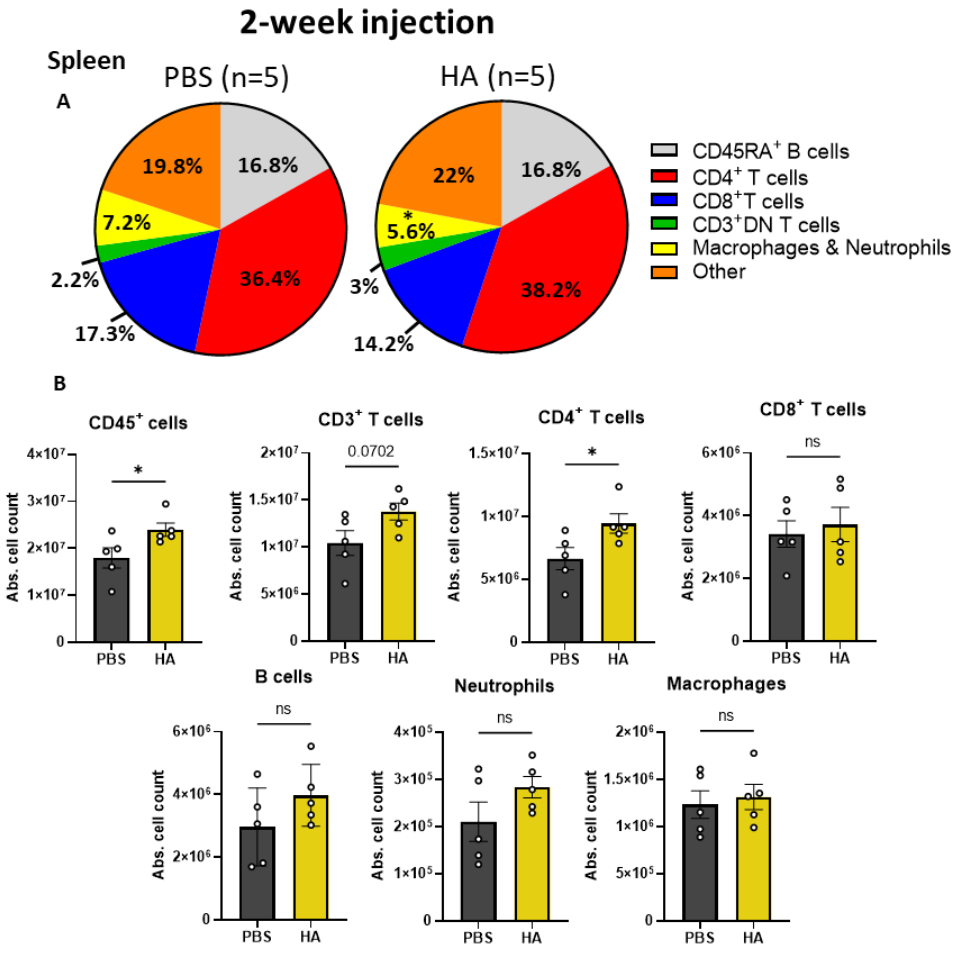

Fig. 3.11: Aggregated human amylin induces acute proliferation of CD4+ T cells in the spleen after 2 weeks of injections. (A) Proportion of $CD45^+$ cell immune cell subsets ($CD45RA^+$ B cells, $CD4^+$ T cells, $CD8^+$ T cells, $CD3^+$ $CD4^-CD8^-$ (DN) T cells, and macrophages and neutrophils) in the spleens of WT rats injected with PBS or aggregated human amylin (HA) (n = 6/group). (B) Total cell count of immune cell subsets ($CD45^+$ cells, $CD3^+$ T cells, $CD4^+$ T cells, $CD8^+$ T cells, B cells, macrophages, and neutrophils) in the spleens of WT rats injected with PBS or aggregated human amylin (HA) (n = 6/ group). *$p < 0.05$ as determined using unpaired two-tailed Students t-test. Data are means ± SEM.

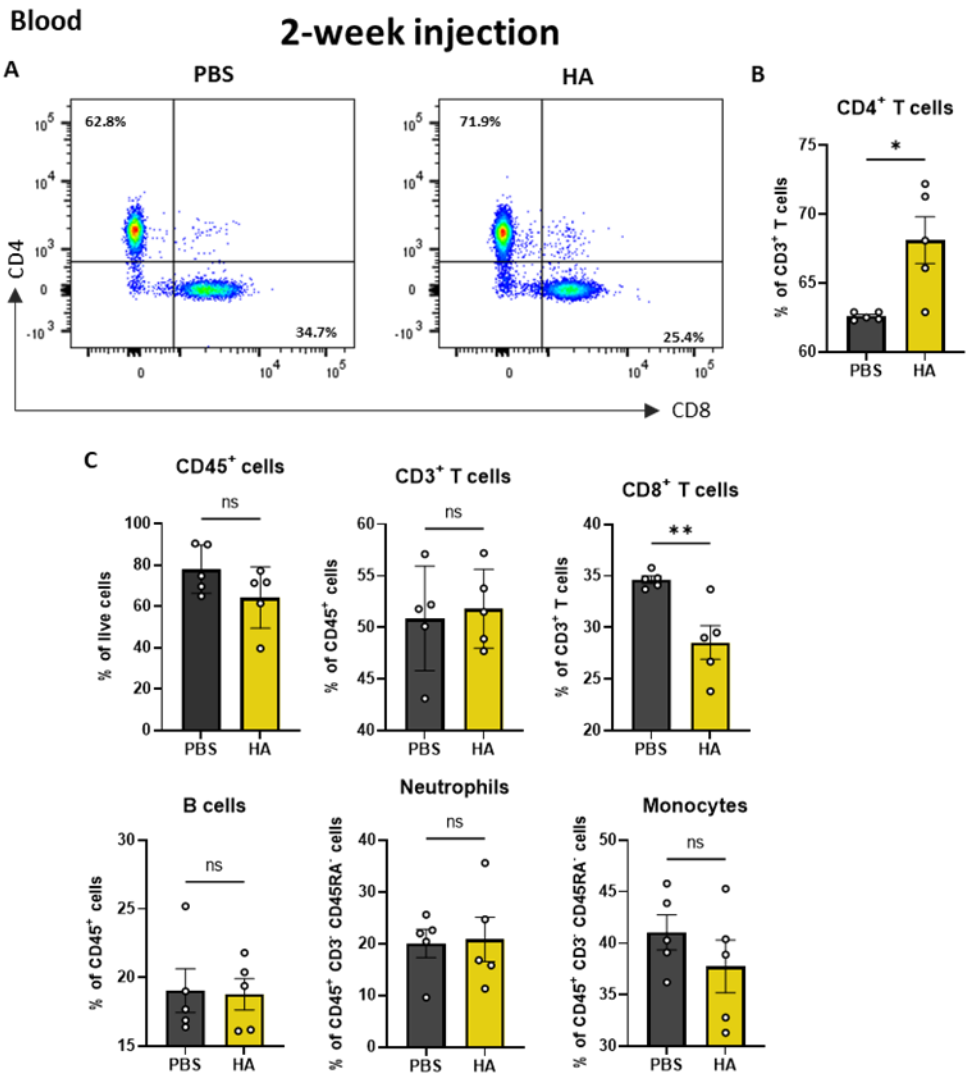

Fig. 3.12: Aggregated human amylin induces acute proliferation of CD4+ T cells in the blood after 2 weeks of injections. (A) Proportion of $CD45^+$ cell immune cell subsets ($CD45RA^+$ B cells, $CD4^+$ T cells, $CD8^+$ T cells, $CD3^+$ $CD4^-CD8^-$ (DN) T cells, and macrophages and neutrophils) in the blood of WT rats injected with PBS or aggregated human amylin (HA) (n = 6/group). (B) Total cell count of immune cell subsets ($CD45^+$ cells, $CD3^+$ T cells, $CD4^+$ T cells, $CD8^+$ T cells, B cells, macrophages, and neutrophils) in the blood of WT rats injected with PBS or aggregated human

amylin (HA) (n = 6/ group). *$p < 0.05$ as determined using unpaired two-tailed Students t-test. Data are means $\pm$ SEM**.**

3.2.7 Aggregated human immune does not induce lymphocyte cell migration into the brain

Previous studies have shown that intravenous injection with human amylin into AKO was able to injure endothelial cells and induce BBB dysfunction.[13] Since our prior work showed that aggregated human amylin opens the BBB, and we identified a proliferation of splenic $CD4^+$ T cells and a decrease in $CD8^+$ T cells, we wanted to know if that was reflected in the brains of these same animals. Flow cytometry showed that after 1 week of acute human amylin injections we saw no difference in the proportion of $CD45^+$ immune cells subsets or in the total cell count of $CD45^+$leukocytes and $CD45^+$ immune cell subpopulations in the brains of injected rats (**Fig. 3.13**). When we looked at the two week injects, in which did elicit a peripheral immune response, we also saw no difference in the proportion of $CD45^+$ immune cells subsets or in the total cell count of $CD45^+$ leukocytes and $CD45^+$ immune cell subpopulations in the brains of injected rats. (**Fig.3.14**), confirming that CNS-related changes secondary to human amylin require a longer exposure time as seen in the HIP rats.

3.2.8 Aggregated human amylin alters the CXCR4 expression in the periphery but not CXCL12 in the brain

Since aggregated human amylin led to increases of $CD4^+$ T cells into the spleen, we wanted to know if the CXCL12/CXCR4 axis was initiated with acute exposure. We isolated splenocytes from WT rats injected with PBS or aggregated human amylin and conducted flow cytometry to determine the amount of CXCR4-expressing immune cells. We found no significant differences in $CD45^+$ general

leukocytes, nor changes in $CD3^+$ T cells, $CD4^+$ T cells, $CD8^+$ T cells, B cells, neutrophils, or macrophages CXCR4 expression (**Fig. 3.15**). We also wanted to determine if the aggregated human amylin impacted the CXCL12/CXCR4 axis in the brains of injected rats. IHC was used to determine the expression of CXCL12 in the brains of WT rats injected with PBS and aggregated human amylin for colocalization similar to the HIP rats. We found again no significant differences in the number of CXCL12-expressing cells, neuronal, or microglial populations. Furthermore, we found no differences in the percent of CXCL12 expressing neurons or microglia in rats injected with PBS or aggregated human amylin (**Fig. 3.16**).

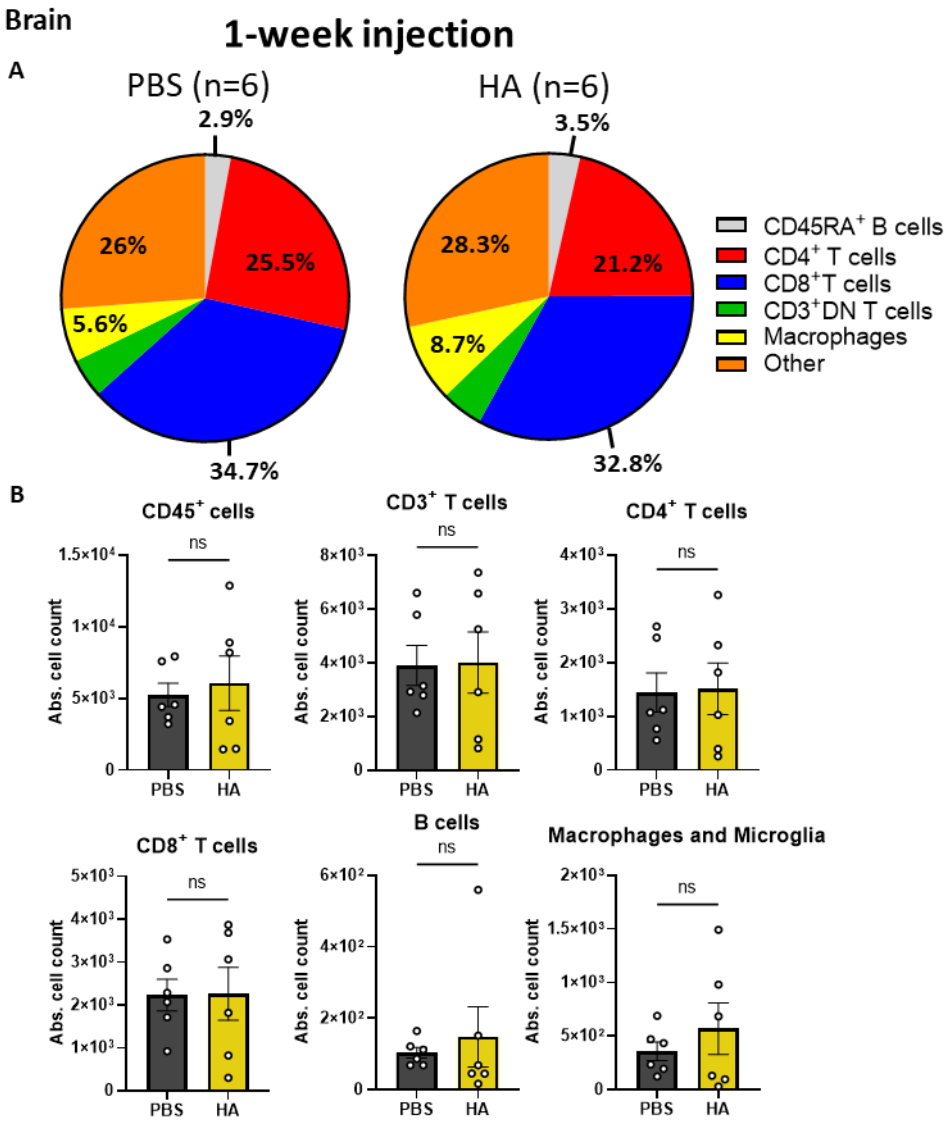

Fig. 3.13: Aggregated human amylin does impact immune cell migration after 1 week of injections. (A) Proportion of $CD45^+$ cell immune cell subsets ($CD45RA^+$ B cells, $CD4^+$ T cells, $CD8^+$ T cells, $CD3^+$ $CD4^-CD8^-$ (DN) T cells, and macrophages and neutrophils) in the brains of WT rats injected with PBS or aggregated human amylin (HA) (n = 6/group). (B) Total cell count of immune cell subsets ($CD45^+$ cells, $CD3^+$ T cells, $CD4^+$ T cells, $CD8^+$ T cells, B cells, macrophages, and neutrophils) in

the brains of WT rats injected with PBS or aggregated human amylin (HA) (n = 6/ group).

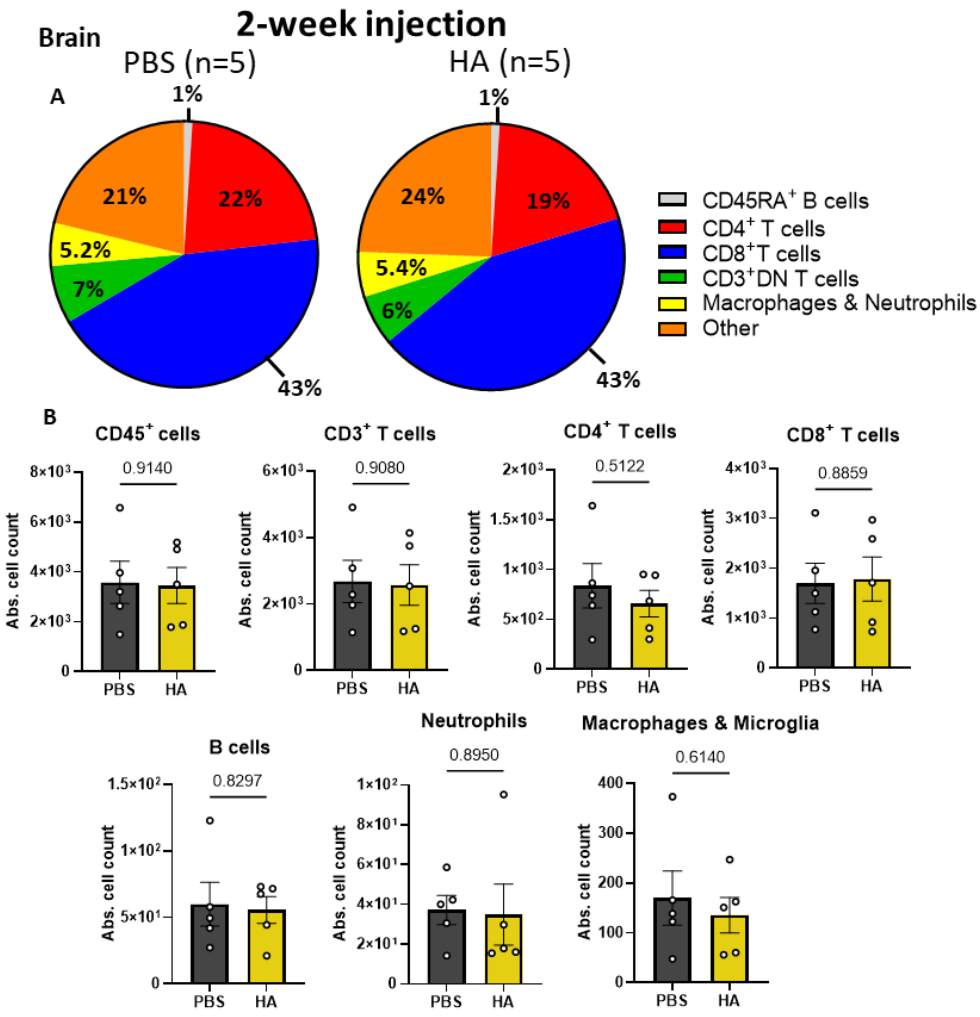

Fig. 3.14: Aggregated human amylin does not impact immune cell migration after 2 weeks of injections. (A) Proportion of $CD45^+$ cell immune cell subsets ($CD45RA^+$ B cells, $CD4^+$ T cells, $CD8^+$ T cells, $CD3^+$ $CD4^-CD8^-$ (DN) T cells, and macrophages and neutrophils) in the blood of WT rats injected with PBS or aggregated human amylin (HA) (n = 6/group). (B) Total cell count of immune cell subsets ($CD45^+$ cells, $CD3^+$ T cells, $CD4^+$ T cells, $CD8^+$ T cells, B cells, macrophages, and neutrophils) in the blood of WT rats injected with PBS or aggregated human amylin (HA) (n = 6/ group). *$p < 0.05$ as determined using unpaired two-tailed Students t-test. Data are means $\pm$ SEM**.**

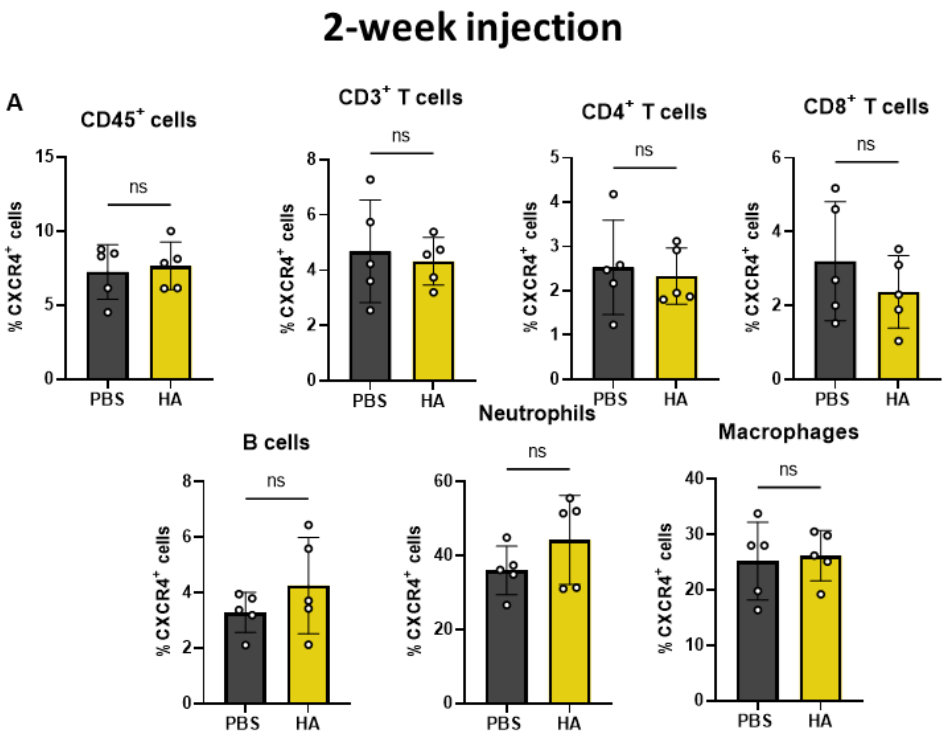

Figure 3.15: Aggregated human amylin does not change CXCR4 expression on splenocytes. (A) Percent of CXCR4$^+$ immune cell subsets (CD45$^+$ cells, CD3$^+$ T cells, CD4$^+$ T cells. CD8$^+$ T cells, B cells, macrophages, and neutrophils) in the spleens of WT rats injected with either PBS or HA (n = 5/ group). Data are means $\pm$ SEM

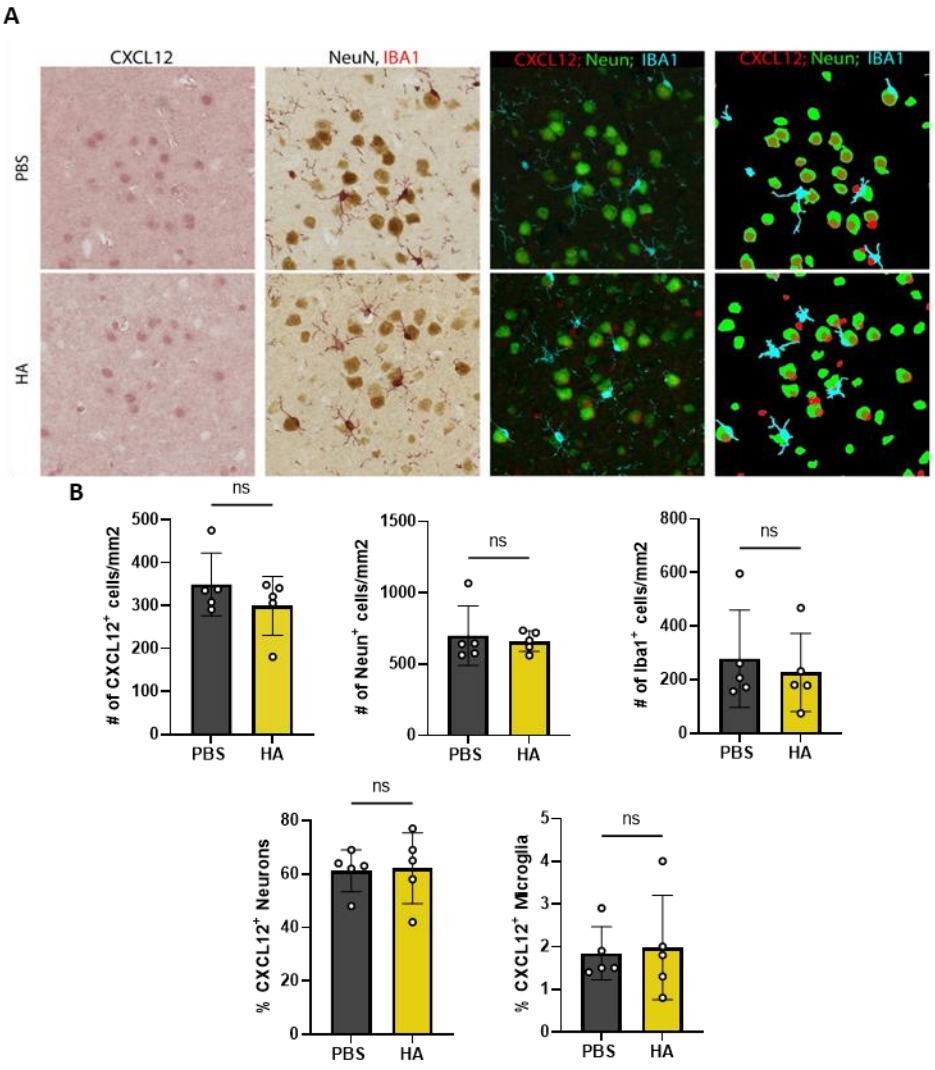

Figure 3.16: Aggregated human does not lead to acute changes in CXCL12 expression in the brain. (A) Immunohistochemistry analysis of CXCL12 in the brains of WT rats injected with PBS or aggregated human amylin and co-localization with neurons and microglia. Arrows show co-localization of CXCL12 with neurons. (B) Number of $CXCL12^+$ cells, number of $Nuen^+$ cells, percent of $CXCL12^+$ neurons, number of $Iba1^+$ cells, and percent of $CXCL12^+$ microglia in brains of WT rats injected with PBS or aggregated human amylin (n = 5/ group). Data are means $\pm$ SEM.

3.3 Results in HIP mice

Our studies in HIP rats show a dysregulated immune response due to the hypersecretion of pancreatic human amylin. As many tools to study the immune response are limited in rats, we expanded these studies to include a HIP mouse model. While this mouse model also expresses pancreatic human amylin, there are a few caveats not found in the rat model. In homozygous conditions, mice typically only last 5-7 months of age, leaving us unable to age the animals to have equivalent long-term chronic human amylin in the circulation.[275] However, they still develop the diabetic phenotype, characterized by hyperglycemia, and have amylin deposits in the pancreas. The advantage, however, is that more antibodies are available in mice for flow cytometry, allowing us to better phenotype the immune response.

3.3.1 Hypersecretion of pancreatic human amylin leads to a dysregulated peripheral immune response in mice similar to HIP rats

For these studies, we used male HIP mice (age ~6 months). We showed that in HIP rats, there was lymphopenia in the spleens of HIP rats (**Fig. 3.3**); when we looked at the proportion of $CD45^+$ immune cell subsets, we saw significant increases in $CD19^+$ B cells and a significant decrease in CD4+ T cells in HIP mice compared to WT controls (**Fig. 3.17A**). When we looked at the total count of immune cells in spleens of HIP mice, we were able to replicate the results seen in HIP rats, showing that there is a significant decrease in total cell count among $CD45^+$ cells. Among $CD45^+$ subpopulations, we also confirmed a decrease in total $CD3^+$ T cells, $CD4^+$ T cells, $CD8^+$ T cells, B cells, macrophages, and neutrophils in HIP mice compared to WT controls (**Fig. 3.17B**).

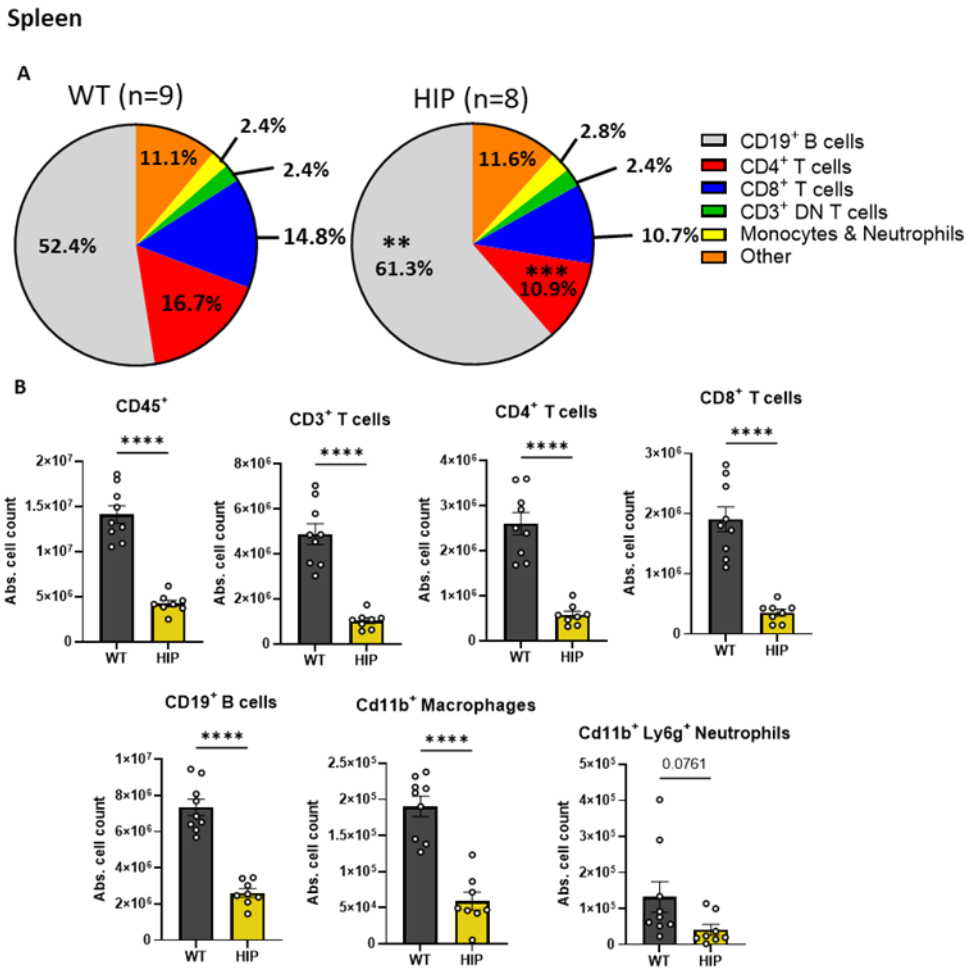

Figure 3.17: Hypersecretion of pancreatic human amylin leads to lymphopenia and alterations of the frequency of immune cells in the spleens of HIP mice. (A) Proportion of $CD45^+$ immune cell subsets ($CD19^+$ B cells, $CD4^+$ T cells, $CD8^+$ T cells, $CD3^+$ T cells, monocytes & neutrophils, and other cells include $CD3^+$ $CD19^+$ cells and $CD45^+$ $CD3^-$ $Cd19^-$ $Ly6g^-$ $CD11b^-$ cells) in the spleens of WT and HIP mice. (B) Total cell count of immune cell subsets ($CD45^+$ cells, $CD3^+$ T cells, $CD4^+$ T cells, $CD8^+$ T cells, B cells, macrophages, and neutrophils) in the spleens of WT and HIP mice. (n = 8-9/ group) ****$p < 0.0001$ as determined using unpaired two-tailed Students t-test. Data are presented as means ± SEM.

3.3.2 Hypersecretion of pancreatic human amylin decreases B cell frequency in the brains of mice.

Next, we wanted to know if cells were significantly decreased in the brains of HIP mice, similar to what was seen in HIP rats (**Fig. 3.5B**) Surprisingly, in the proportion of $CD45^+$ immune cells subsets, we found that $CD19^+$ B cells were significantly decreased in the brains of HIP mice, with no changes to other populations (**Fig. 3.18A&B**). When we looked at the total cell counts there were no significant differences in $CD45^+$ immune cells and CD45+ immune subsets such as $CD3^+$ T cells, $CD4^+$ T cells, $CD8^+$ T cells, macrophages/microglia, and neutrophils (**Fig. 3.18C**).

3.3.3 Hypersecretion of pancreatic human amylin leads to bone marrow failure

Studies in models such as spinal cord injury (SCI) and WHIM syndrome show that lymphopenia is usually associated with BM dysfunction and hematopoiesis. To determine if the reduced splenocyte counts with chronic human amylin expression is a true lymphopenia secondary to BM dysfunction, we conducted flow cytometry on immune cells in the BM of WT and HIP mice. We found that in proportion of $CD45^+$ immune cell subsets there was a significant increase in monocytes and neutrophils and $CD4^+$ T cells in HIP mice compared to controls, suggesting a sequestration of immune cells due to the hypersecretion of pancreatic human amylin. Interestingly, B cells remained significantly decreased similar to the spleen and brain. (**Fig. 3.19A&B**). When we looked at the total cell counts, we again found that B cells were significantly decreased, with no changes in other immune cell populations in the BM (**Fig 3.19C**). These data suggest there is a B cell-specific lymphopenia in HIP mice.

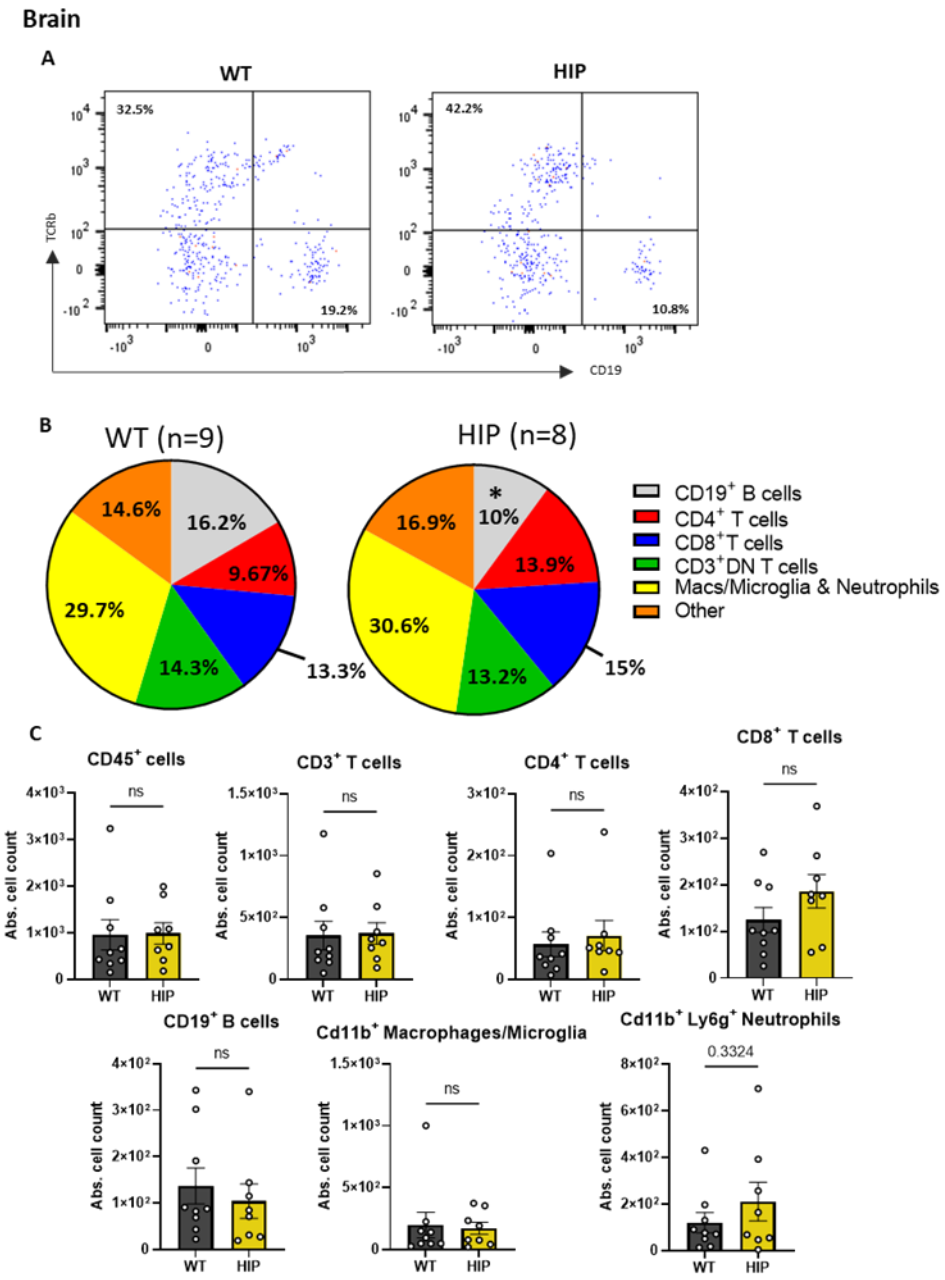

Figure 3.18: Hypersecretion of pancreatic human amylin leads to decreased B cell migration into the brain. (A) Representative flow cytometry analyses of $CD19^+$ B cells in the brains of HIP mice. (B) Proportion of $CD45^+$ immune cell subsets ($CD19^+$ B cells, $CD4^+$ T cells, $CD8^+$ T cells, $CD3^+$ T cells, monocytes & neutrophils, and other cells include $CD3^+$ $CD19^+$ cells and $CD45^+$ $CD3^-$ $Cd19^-$ $Ly6g^-$ $CD11b^-$ cells)

in the brains of WT and HIP mice. (C) Total cell count of immune cell subsets ($CD45^+$ cells, $CD3^+$ T cells, $CD4^+$ T cells, $CD8^+$ T cells, B cells, macrophages, and neutrophils) in the brains of WT and HIP mice. (n = 8-9/ group) *$p < 0.05$ as determined using unpaired two-tailed Students t-test. Bar graphs show the absolute number of. Data are means $\pm$ SEM.

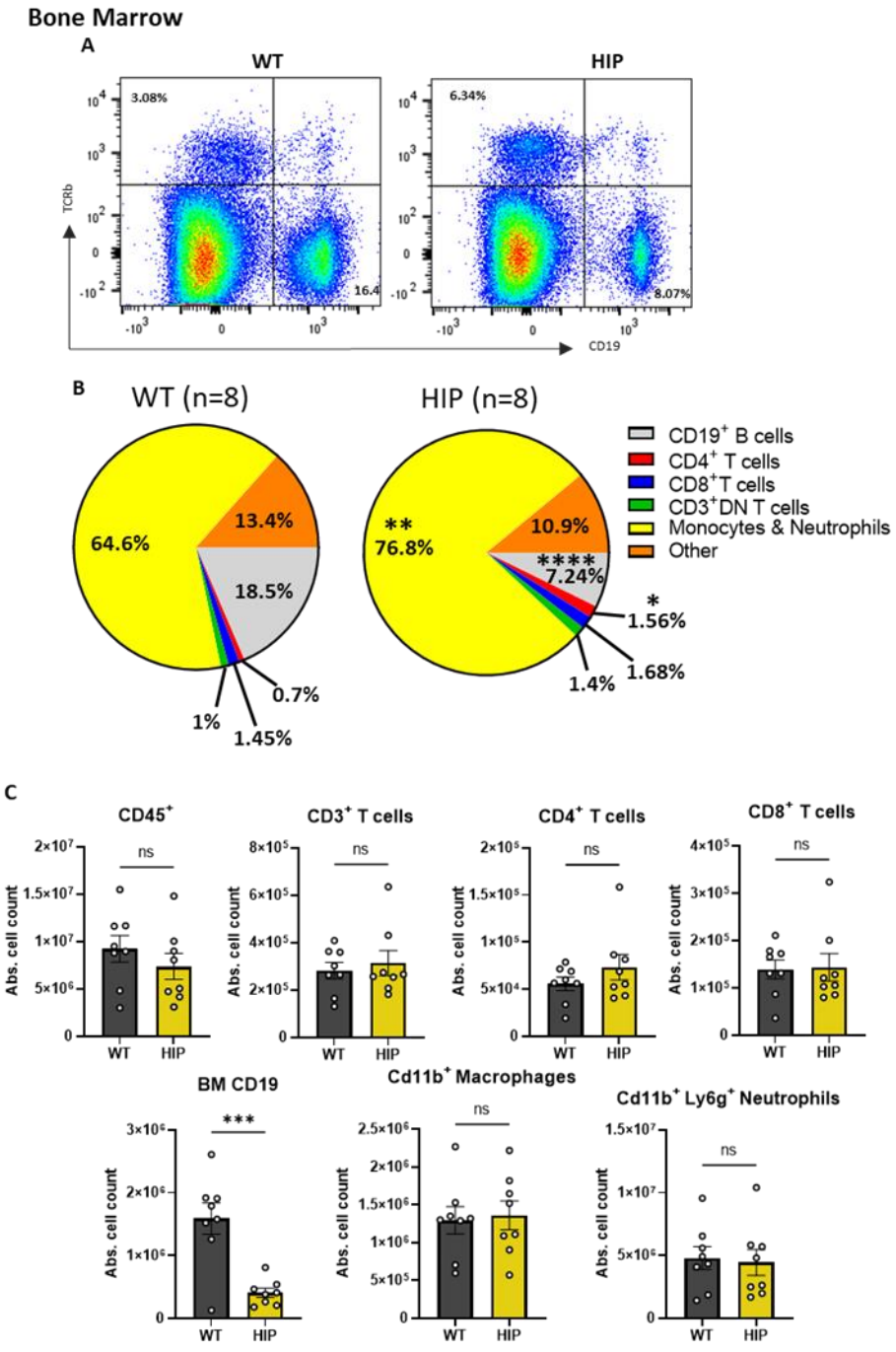

Figure 3.19: Hypersecretion of pancreatic human amylin leads to sequestration of immune cells in the bone marrow except B cells. (A) Representative flow cytometry analyses of TCRβ$^+$ T cells and CD19$^+$ B cells in the BM of WT and HIP mice. (B) Proportion of CD45$^+$ immune cell subsets (CD19$^+$ B cells, CD4$^+$ T cells,

$CD8^+$ T cells, $CD3^+$ T cells, monocytes & neutrophils, and other cells include $CD3^+$ $CD19^+$ cells and $CD45^+$ $CD3^-$ $Cd19^-$ $Ly6g^-$ $CD11b^-$ cells) in the BM of WT and HIP mice. (C) Total cell count of immune cell subsets ($CD45^+$ cells, $CD3^+$ T cells, $CD4^+$ T cells, $CD8^+$ T cells, macrophages, and neutrophils) in the BM of WT and HIP mice (n = 8-9/ group). *$p < 0.05$, and **$p < 0.01$ as determined using unpaired two-tailed Students t-test. Data are presented as means $\pm$ SEM.

3.3.4 Hypersecretion of pancreatic human amylin leads to deficits in hematopoiesis

Studies have shown that diabetes can alter hematopoiesis, but it is unknown how the hypersecretion of pancreatic human amylin contributes to this dysfunction. We wanted to determine if defects in hematopoiesis contributed to the decreased immune cells in the periphery in HIP mice, or decreased representation of B cells within the BM. Using flow cytometry on cells from the BM, we looked for HSPCS. We found that in the BM, there was a significant increase in MPPs (**Fig. 3.14A&B**) and a significant decrease in long term-hematopoietic stem cells (LT-HSCs) cells in HIP mice compared to WT controls. We did not see any difference in c-kit$^+$ sca-1$^+$ (LSK) cells, markers on early developing hematopoietic stem cells, and short term-hematopoietic stem cells. (ST-HSCs) cells (**Fig. 3.14A&C**).

3.3.5 Direct evidence of dysregulation of B cell development in HIP mice

The marked decrease in the B cell populations seen in the spleen, brain, and BM led us to hypothesize that these decreases resulted from defects in B cell development. B cell development occurs in the BM and is initiated from the hematopoietic stem cells that differentiate into common lymphoid progenitors, then follow the order of pre-pro B cells, pro-B cells, pre-B cells, and immature B cells. When we look at the proportion of B220$^+$ B cells in the BM we found that there is a significant increase in pre-pro and immature B cells, while there was a decrease in pre B cells in HIP mice compared to controls (**Fig. 3.21A**). When we looked at the total count of developing B cells, we found that in the BM of HIP mice, there was a significant decrease in total B cells, pre-pro B cells, Pro B cells, pre B cells, and

immature B cells compared to WT mice (**Fig. 3.21B**). Furthermore, maturation of B cells continues in lymphoid organs such as the spleen, so we also looked at developing B cell populations in the spleen. We found that in the proportion of B cells in the spleens of HIP mice, there was significant decreases in follicular B cells and an increase in marginal zone B cells (**Fig. 3.22A**). When we looked at the total count for these populations, there was a significant decrease in total B cells, transitional B cells and follicular B cells with no changes to marginal B cells compared to WT counterparts (**Fig. 3.22B**). These data suggest that the hypersecretion of pancreatic human amylin leads to B cell development deficits.

3.3.6 Hypersecretion of pancreatic human leads to alters the CXCL12/CXCR4 axis on B cells in mice

The CXCL12/CXCR4 axis is also important in B cell lymphopoiesis and we wanted to determine if the expression of CXCR4 expression on these B cell progenitors was altered in HIP mice. Using flow cytometry, we found that the expression of CXCR4 was increased on these developing B cell populations in the BM, such as pro B, pre-B, and immature B cells, with no changes in expression on pre pro B cells (**Fig. 3.16A**). There were also increases of CXCR4 on developing B cells in the spleen, such as marginal B cells with no changes in the follicular B cells and transitional B cells, although transitional B cell CXCR4 expression was trending towards an increase ($p = 0.0753$) (**Fig. 3.16B**).

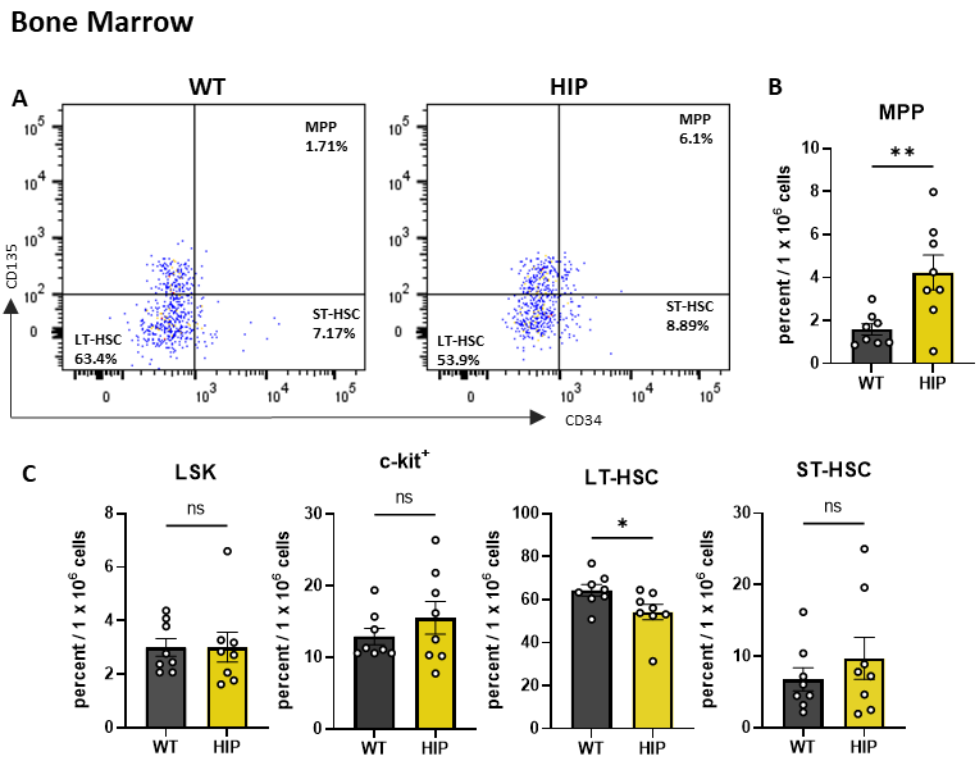

Figure 3.20: Hypersecretion of pancreatic amylin of HSPCs in the bone marrow of HIP mice. (A) Representative flow cytometry analyses of MPPs, LT-HSCs, and ST-HSCs in the BM of WT and HIP mice. (B) Percent of MPP cells in the bone marrow of WT and HIP rats (n = 8-9/ group). (C) Percent of HSPCs such as LSK cells, c-kit^{+} cells. LT-HSCs and ST-HSCs in the bone marrow of WT and HIP rats (n = 8-9/ group). *$p < 0.05$, **$p < 0.01$ as determined using unpaired two-tailed Students t-test. Data are means $\pm$ SEM.

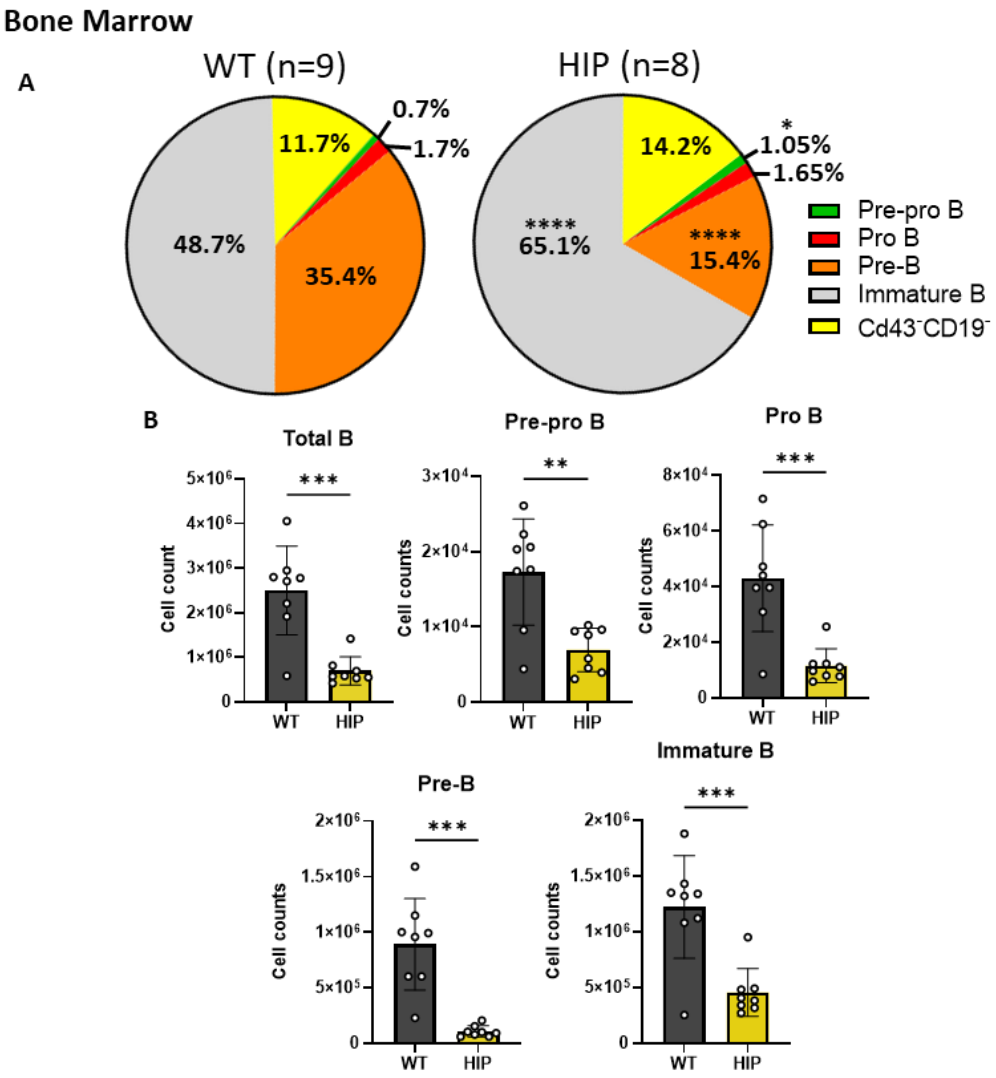

Figure 3.21: Hypersecretion of pancreatic human amylin impairs B cell lymphopoiesis in the bone marrow of HIP mice. (A) Proportion of developing $B220^{+}$ B cells such as pre-pro B cells, pro B cells, pre B cells, and immature B cells in the BM of WT and HIP mice (n=8/group). (B) The total cell counts of developing B cells such pre-pro B cells, pro B cells, pre B cells, and immature B (n=8/group). **p < 0.01, ***p < .001, and ****p < 0.0001 as determine using unpaired two-tailed Students t-test. Data are presented as means ± SEM.

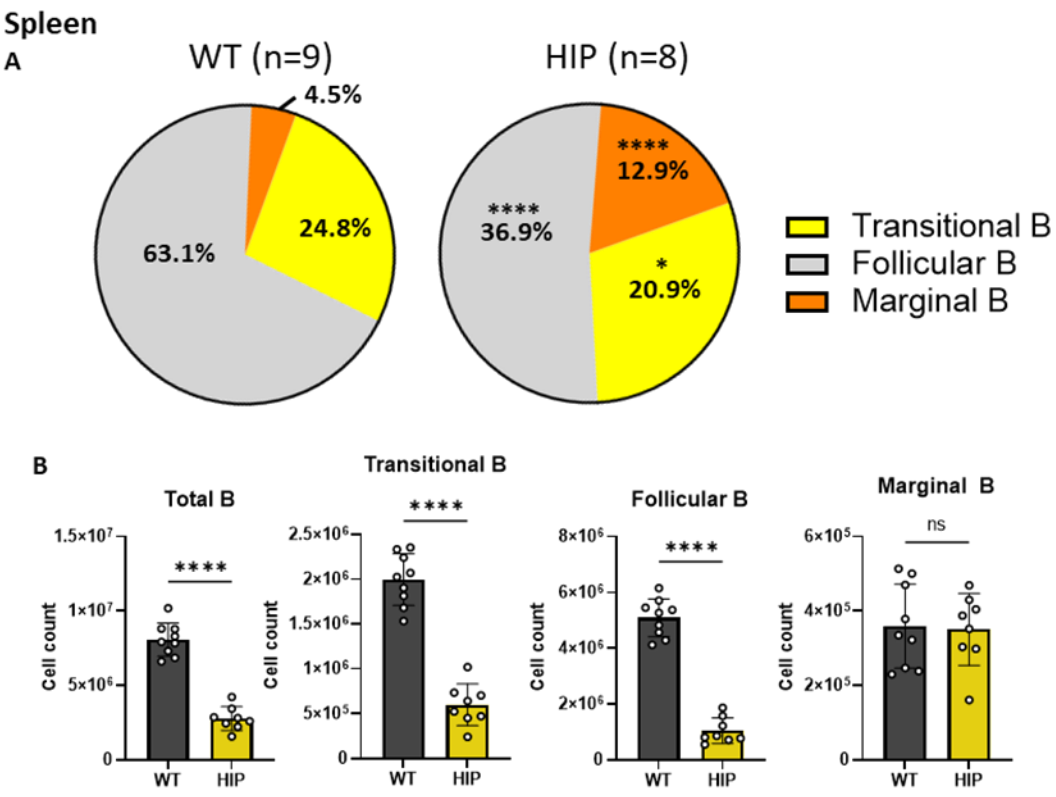

Figure 3.22: Hypersecretion of pancreatic human amylin impairs B cell lymphopoiesis in the spleens of HIP mice. (A) Proportion of developing $B220^{+}CD19^{+}$ B cells such as transitional, follicular and marginal zone B cells in the spleens of WT and HIP mice. (B) The total cell counts of developing B cells such transitional, follicular and marginal zone B cells in the spleens of WT and HIP mice (n=8-9/group). **p < 0.01, ***p < .001, and ****p < 0.0001 as determine using unpaired two-tailed Students t-test. Data are presented as means ± SEM.

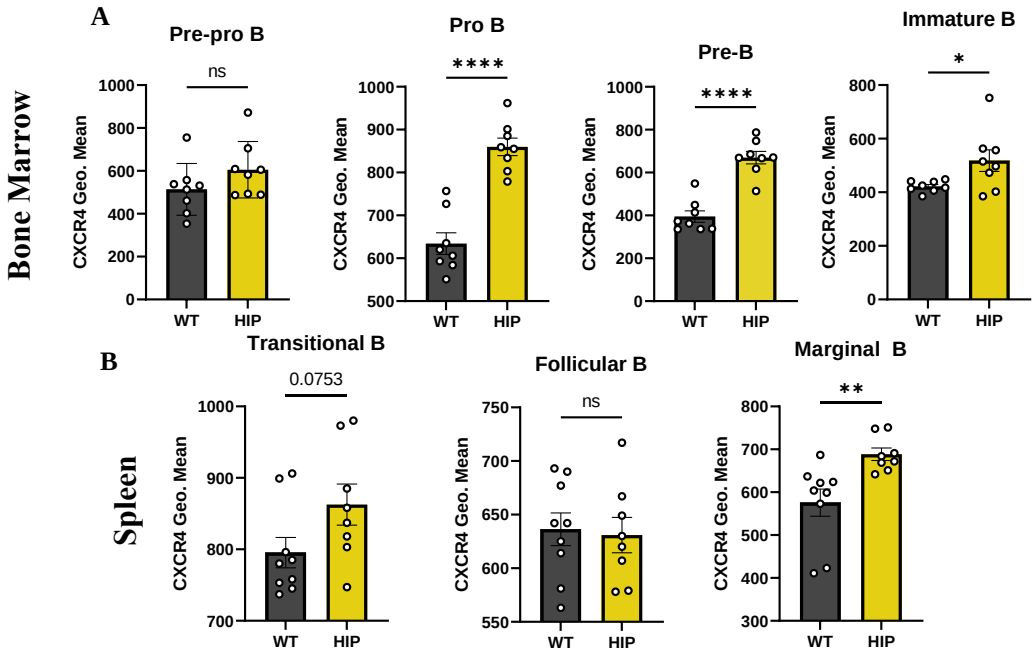

Figure 3.23: Hypersecretion of pancreatic human amylin leads to increased expression of CXCR4 on developing B cells HIP mice. (A) Geometrical mean of CXCR4 on developing B cells such as, pre-pro B cells, pro B cells, pre B cells and immature B cells in the BM of WT and HIP mice (n=8/group). (B) The geometrical mean of CXCR4 on developing B cells such as transitional B cells, follicular B cells, and marginal B cells in the spleen of WT and HIP mice (n=8/group). *p < 0.05, **p < 0.01, and ****p < 0.0001 as determine using unpaired two-tailed Students t-test. Data are presented as means ± SEM.

Chapter 4: Conclusion & Discussion

4.1 Conclusion

Previous studies show that the hypersecretion of pancreatic human amylin leads to neuroinflammation and cognitive decline in rats (reviewed in Chapter 1), and T2D is well known for causing a disturbance in the immune response in both humans and animal models[157-159]. However, how human amylin contributes to this immune dyshomeostasis is poorly understood. In this current chapter, we observed that the hypersecretion of pancreatic human amylin led to alterations in genes responsible for neuroinflammation, neurovascular coupling, and the immune response in the brains of HIP rats. These data were the first indication that human amylin played a role in altering the neuroimmune axis. We then found that in both rats and mice, the hypersecretion of pancreatic amylin led to lymphopenia in the spleen, followed by alterations of T cell subsets in the circulation. This peripheral lymphopenia led to a decrease in immune cells that migrated to the brain in rats but not mice, though mice did exhibit lower parenchymal B cell frequency.

We next sought to understand mechanisms contributing to change in immune cell distribution with chronic human amylin exposure. First, using IHC, we observed the CXCL12/CXCR4 axis was not responsible for this reduced migration into the brain in HIP rats. We confirmed that acute injections with human amylin were able to elicit an immune response but also did not impact immune cell migration into the brain, nor did it activate the CXCR4 axis. In moving to HIP mice to capitalize on additional immunophenotyping tools, we found that the hypersecretion of pancreatic human amylin in mice led to the sequestration of immune cells in the BM associated

with B cell development and hematopoiesis deficits, which led to a decrease in B cells in the BM, spleen, and brain that was also associated with defects in the CXCL12/CXCR4 axis within the BM. Collectively, our studies suggest a role for lymphopenia and BM failure as a contributing factor to altered neuroinflammation caused by the hypersecretion of pancreatic human amylin, an immunophenotype occurring in a model of T2D-mediated cognitive decline.

4.2 The implications of pancreatic human amylin on altered gene expression in the brains of HIP rats

Previous studies in the lab show that in the brains of 12-month-old HIP rats, there is an increase of genes important for M1 microglia/macrophages, which were associated with an increase of inflammatory cytokines, such as TNF-α and IL-6, and a decrease in the anti-inflammatory cytokine IL-10; all changes associated with cognitive decline.[14] This led us to question: what genes in the brain were associated with the accumulation of pancreatic human amylin? We now demonstrate using RNA-seq that the accumulation of pancreatic human amylin alters genes responsible for neuronal signaling, neuroinflammation, and the immune response, the latter two important for protection and tissue repair in response to CNS injury.[19] Chronic neuroinflammation is associated with neurodegeneration mediated by the activation of resident immune cells of the CNS as well as peripheral immune cells, further contributing to the development of neurodegenerative disorders and cognitive decline.[317, 318] This finding provides additional evidence for the role of pancreatic human amylin in contributing to neuroinflammation and related pathological signaling in the brain.

RNA seq analysis highlighted several specific pathways related to immune signaling due to the accumulation of pancreatic amylin, such as antigen presentation, B cell development, the Th1 pathway, and the PD-1, PD-L1 pathway. However, it is important to note we did not see any differences in validation using rt-PCR. Antigen presentation in the CNS is mostly carried out by microglia, which express MHC II in response to injury, allowing them to present antigens to $CD4^+$ and $CD8^+$ T cells.[319] Studies in mice on HFD have shown that upregulation of MHC II on microglia is associated with poor cognition.[320] Furthermore, MHC II is upregulated on microglia in patients with AD and is also associated with cognition.[321, 322] Another pathway highlighted was B cell development, which was interesting because it is currently unknown what the roles of B cells are in the diabetic brain while studies in AD are controversial for whether the deletion of B cells is beneficial [323] or detrimental.[324] However, the peripheral aspects of how pancreatic human amylin impacts B cells will be discussed later in this chapter. Th1 was another immune pathway highlighted. As discussed in Chapter 1, Th1 are mainly IFN-y producing T-cells that are increased in mice on HFD,[157, 164] but their role in the brain during T2D is mostly unknown. Our data suggest a potential role for IFN-y during the progression of T2D and the accumulation of pancreatic human amylin. In the brain studies have suggested that IFN-y is necessary for CNS immune surveillance and repair by infiltrating T cells,[325] suggesting a compensatory mechanism to recruit T cells to the brain. The final major immune-related pathway activated by human amylin was the programmed cell death protein 1 (PD-1) and programmed cell death ligand 1 (PD-L1) pathway. PD1 and PD-L1 are mainly expressed on activated immune cells and are important in the immune checkpoint pathway by

regulating the immune response through suppression. In the periphery, they are mainly associated with cancer and exhausted T cells.[326] In the brain, they are expressed on the endothelium and glial cells and are associated with neuroinflammation [327] but have not previously been associated with T2D. As such, these findings further provide evidence for the detrimental role of the hypersecretion of pancreatic human amylin; they are also the initial evidence for the hypothesis that pancreatic human amylin disturbs the neuroimmune axis during the progression of T2D and T2D-induced cognitive decline.

4.3 The implications of pancreatic human amylin on the peripheral immune response in HIP rats.

Studies have shown that in both patients with T2D and mouse models of T2D, immune cells including $CD4^+$ T cells, $CD8^+$ T cells, B cells, macrophages, and neutrophils are increased in the AT and circulation.[126, 140, 156, 158] However, studies have also shown that T2D can also be associated with lymphopenia.[328] In our studies we found the latter: the hypersecretion of pancreatic human amylin in rats decreased T and B cell populations and myeloid cells in the spleen of HIP rats. Although most studies attribute the dysregulation of the immune response to hyperglycemia, individuals with T2D also have increased amounts of amylin in circulation, which has been ignored as a potential contributing factor to the immune response. As aggregated human amylin is well known to cause apoptosis in various cell types,[59, 60] immune cells could be undergoing apoptosis due to the increased amount of amylin in circulation in patients with T2D and HIP rats from our studies.

An example of this is the decrease in $CD8^+$ T cells: Studies in mice on HFD show that pro-inflammatory $CD8^+$ T cells are increased in the AT preceding $CD4^+$ T cell

alterations, and $CD8^+$ T cells are also associated with IR and obesity as blocking $CD8^+$ T cells improved diabetic phenotypes.[166] As the only difference between our study and others is the accumulation of pancreatic amylin, this suggests a potential role of pancreatic amylin in $CD8^+$ T cell survival. It is currently unknown how amylin impacts immune cells in general; $CD8^+$ T cells could be more sensitive to amylin than other immune cell subsets in circulation. This finding was further supported in our experiments where rats injected with aggregated human amylin for 2 weeks showed a decrease in $CD8^+$ T cells compared to PBS-injected rats (**Fig. 3.12**). The same could be true for B cells, neutrophils, and macrophages as they were also found to be decreased in the spleens of HIP rats with chronic amylin exposure.

When we looked in the circulation, contrary to what we saw in the spleen, we saw an increase in $CD4^+$ T cells, however the decrease in $CD8^+$ T cells in HIP rats remained. $CD4^+$ T cells are increased in patients with T2D and mouse models of T2D and are usually pro-inflammatory T cells, such as Th1 and Th17 cells. In particular, Th1 and Th17 cells have been reported to be associated with insulin resistance and severity of T2D by contributing to the chronic pro-inflammatory environment.[157, 161, 162] This finding of increased $CD4^+$ T cells in circulation was also supported in rats injected with aggregated human amylin, showing an increase in $CD4^+$ T cells in the spleen and blood. These findings suggest that pancreatic human amylin potentially plays a role in the proliferation of $CD4^+$ T cells, but further studies need to determine the phenotype of these cells.

4.4 The implications of pancreatic human amylin on the peripheral immune cell migration to the brain in HIP rats.

Our study found that similar to what was seen in the spleen of HIP rats, there was a decrease in immune cells in the brains of HIP rats. Studies have shown that T2D is characterized by decreased leukocyte adherence and transendothelial migration,[175] preventing immune cells from reaching their target tissue or environment. However, one study showed that HFD can lead to an increase in immune cells in the brain, ostensibly through an upregulation of these same mechanisms.[173] Due to the scarce amount of studies focused on how diabetes impacts immune cell migration into the brain and, furthermore, how pancreatic amylin contributes to this, we relied on AD models and models that have used diabetes as a co-morbidity for insight into how immune cells are altered in the brain under conditions of neuroinflammation and cognitive decline as well as T2D. In mouse models of AD, studies show there is increase in inflammatory T cells that contribute to neuroinflammation and cognitive decline,[317, 318] suggesting that in neurodegenerative diseases there is increase of immune cell infiltration into the brain. In contrast, studies using diabetic mice infected with WNV show that diabetes decreased the recruitment of $CD45^+$ immune cells and specifically $CD8^+$ T cells in the brain.[177] This study suggested that there is a defect in immune cell migration into the CNS due to T2D. However, contradicting studies have shown that in diabetic mice that received an ischemic stroke, immune cells in the brain increase, such as $CD4^+$ T cells, $CD8^+$ T cells, B cells, and neutrophils.[178] In light of these AD-related studies and due to our prior work showing neuroinflammation in HIP rats, we hypothesized that there would be an increase of immune cells into the brain, which turned out untrue. This suggests that the peripheral

immune response is important and critical to the immune response reflected in the brain. Meaning that the peripheral environment – including lymphopenia secondary to chronic human amylin exposure – takes precedent over brain-localized neuroinflammation and general parenchymal immune responses.

This next led us to want to understand a potential mechanism for the reduced immune cells into the brain. The study in WNV showed that there was a decrease in the CAMs (ICAM-1 and VCAM-1), which was associated with a decrease in immune cell migration.[177] As mentioned in Chapter 2, ICAM and VCAMs are important in leukocyte migration across the BBB. However, in most studies,[177, 329] their expression levels are usually expressed in the same degree, for example, if VCAM is down, then ICAM is down, as also shown in the study in WNV and T2D. However, in our study, VCAM-1 was increased while ICAM-1 was decreased. As they both are important in the overall process of leukocyte migration across the BBB, studies have suggested that ICAM-1 is the main contributor to the diapedesis across the endothelium, while VCAM-1 is more important in the adhesion of T cells to the endothelium.[313-315] These studies suggest that during the accumulation of pancreatic human amylin, immune cells may have the ability to adhere to the BBB, but due to the loss of ICAM-1, they cannot diapedese, providing a potential mechanism for decreased immune cells in the brain. However, no other studies have investigated the relationship between immune cell migration and CAM expression during T2D, showing a need for more studies focusing on how T2D impacts immune cell migration into the CNS. Our study suggests a specific role for pancreatic human amylin in reducing immune cell migration into the CNS though altering CAM expression in the

brain, potentially contributing to neuroinflammation and cognitive deficits as previously reported.

The CXCL12/CXCR4 axis is implicated in many physiological processes in various conditions, including T2D, with one of the most-studied mechanisms being control over immune cell migration. In fact, studies in patients with T2D show that there is a decrease in expression of CXCR4 on PBMCs.[215] In our study, we found that $CD4^+$ T cells have an increased expression of CXCR4 in the spleens, which suggests that immune cells' surface expression of CXCR4 may differ once cells reach their target organ vs. expression levels in the circulation. Furthermore, it suggests that pancreatic human amylin is able to modulate migration proteins on immune cells in the periphery. No studies to date have shown how T2D or amylin impacts CXCL12 expression in the brain; in our study, we did not identify differences between CXCL12 expression. When rats were injected with aggregated human amylin, we also did not see any acute differences in CXCR4 expression in splenocytes or CXCL12 expression in the brain, suggesting that acute amylin aggregation does not induce changes in the CXCL12/CXCR4 axis.

4.5 The implications of pancreatic human amylin on lymphopenia and bone marrow failure in HIP mice

When we employed HIP mice to get a better understanding of the peripheral immune response secondary to pancreatic human amylin, we found similar results to HIP rats with decreases in splenic immune cell subsets, further suggesting that the accumulation of pancreatic human amylin induces lymphopenia. Studies in diseases such as SCI and WHIM syndrome have shown that lymphopenia is usually associated with BM failure, which can lead to the sequestration of HSPCs and immune cells in the

BM.[212, 330] Mature immune cells develop in the BM from a pool of HSPCs through a process called hematopoiesis.[331] Under homeostatic conditions, this process is well regulated through the CXCL12-CXCR4 axis, and under pathological conditions, CXCL12 and CXCR4 are increased, leading to impairment of hematopoiesis and immune cell mobilization. Both mouse and human studies show that T2D impairs HSPC mobilization,[332] and we identified specific decreases in HSPCs such as LT-HSC, as well as increases in MPP. We also found sequestration of immune cells such as $CD4^+$ T cells, $CD8^+$ T cells, and macrophages in the BM of HIP mice, suggesting that the accumulation of pancreatic amylin is associated with BM dysfunction. However, this did not extend to B cells as they were still decreased as they were in the spleen, which was also seen in the brains of HIP mice, providing the first pieces of evidence that the hypersecretion of pancreatic human amylin may lead directly to B cell defects.

4.6 The implications of pancreatic human amylin on B lymphopoiesis in HIP mice

B cell lymphopoiesis is a well-controlled process that occurs in the BM, and studies have shown that the CXCL12/CXCR4 axis is essential in both B cell development and mobilization from the BM.[332, 333] We found that pancreatic hypersecretion of amylin leads to a defect in B cell development in the BM and spleen characterized by a decrease in developmental B cell populations. Furthermore, these B cell populations had an increase of CXCR4 expression, which will tether these cells in the BM environment that is expressing CXCL12, suggesting that the hypersecretion of pancreatic human amylin leads to defects in B cell lymphopoiesis through the CXCL12/CXCR4 axis. To date, there are no studies showing that T2D or amylogenic disorders impair B cell development. Studies have shown that disorders in thyroid hormones in mice deficient in

growth hormones, including prolactin, insulin growth factor 1 (IGF-1), thyrotropin, and thyroxine, can impair B lymphopoiesis.[334] As pancreatic human amylin is a hormone, it could be added to the list of hormones impairing B lymphopoiesis. In the context of T2D, the current understanding is that B cells are major drivers of the pathogenesis of T2D, contributing to the inflammatory response and insulin resistance.[156] This study adds an interesting contradiction, showing that discrete hypersecretion of pancreatic human amylin yields fewer B cells though it is still associated with T2D. B cell-deficient mice on HFD show improved IR and decreased inflammatory cytokines. Although there are fewer B cells, they could still be contributing to the inflammatory milieu as we currently don't know their inflammatory phenotype but should investigate in future studies.

4.7 Targeting hematopoiesis, lymphopenia, and bone marrow failure

HSPCs are essential for producing and replenishing different types of blood cells, which include immune cells in the BM. Under homeostatic conditions, blood cells go through coordinated differentiation and mobilization to maintain normal levels of circulating immune cells.[331] In conditions such as aging,[335] SCI,[330] WHIM syndrome,[212] and T2D,[336] these processes are impaired, leading to BM failure characterized by sequestration of immune cells, hematopoietic dysfunction, and lymphopenia. In patients with T2D, HSPCs are reduced by up to 40% and are associated with poor mobilization of HSPCs.[337] However, it is mostly unknown how disorders of hematopoiesis and BM failure impact immune cell migration into the brain, neuroinflammation, and cognitive decline. As we see defects of hematopoiesis, lymphopenia, and BM failure in animals that have been shown to have neuroinflammation and cognitive decline,[13, 14] we believe that harnessing therapies

geared toward these symptoms may be a promising therapeutic to human amylin-induced neuropathology.

To this end, hematopoietic stem cell transplantation is a process in which healthy hematopoietic stem cells are administered to patients with BM dysfunction.[338] It is a common procedure to treat blood disorders and cancers, in which over 60,00 are performed annually.[339] Once administered, they are accompanied by a mobilizing agent, allowing the cells to escape the BM and circulate. One of the most common and well-known mobilizing agents is granulocyte-colony stimulating factor (G-CSF). C-GSF induces mobilization by decreasing CXCL12 and upregulating CXCR4.[340] Most transplants that are accompanied by C-GSF are successful. However, studies have shown that in patients with T2D, there are still patients who exhibit low mobilization even after administration with C-GSF due to dysfunction of mesenchymal and perivascular neural cell dysfunction in the BM, possible again related to elevated amylin levels disrupting BM function but not generally investigated.[336, 341, 342]

AS HSPCs are regulated by the CXCL12/CXCR4 axis, inhibitors of CXCR4 have emerged as a therapeutic candidate to overcome immune deficiency disorders due to BM failure. AMD3100, an Food and Drug Administration (FDA)-approved CXCR4 antagonist, induces a rapid mobilization of HSPCS.[299] Studies in SCI have shown that the administration of AMD310 was able to release HSPCS and leukocytes from the BM.[330] In patients with WHIM syndrome, ADM3100 reverses immunodeficiency safely[343] while studies in T1D confirm that AMD3100 is able to induce the mobilization of HSPCS.[336] Although studies are lacking in T2D, AMD3100 has been beneficial in diabetic wound healing through mobilizing endothelial progenitor cells that promote

healing to the site of injury.[344] In other models of neurodegeneration, AMD3100 is neuroprotective by reducing neuroinflammation, BBB dysfunction, cognitive decline, and reduced immune cell migration.[245] Studies in AD have also confirmed that AMD3100 reduces cognitive decline through increased recruitment of BM-derived microglia cells in the brain.[270] Together, these studies 1) show a need for increased investigation in patients with T2D who are experiencing immunodeficiency due to BM failure; and 2) a potential future direction for therapeutics to counter lymphopenia and hematopoiesis deficits, which in turn could alleviate neuroinflammation and cognitive decline caused by the hypersecretion of pancreatic human amylin.

4.8 Targeting Inflammation & Immunotherapies

Immunomodulatory biologics as therapeutics have gained traction in both animal studies and clinical trials over the years.[345] However, in the case of T2D, some of these therapeutics have not been considered, and if so, they don't focus on how it affects the immune response, neuroinflammation, and cognitive decline that T2D can lead to. As discussed in Chapter 1, in T2D there is an increase in inflammatory cytokines such as TNF-α, IL-1β, and IL-6, with pancreatic human amylin specifically increasing IL-1, TNF-α, and IL-6 in monocytes, macrophages, and dendritic cells.[78-80] In the brains of HIP rats, there is an increase of IL-1β in neurons, increased pro-inflammatory cytokines such as TNF-α and IL-6, and a decrease in anti-inflammatory cytokine IL-10.[14, 100] These cytokines are released by both immune and non-immune cells and contribute to the immune response, neuroinflammation, and cognitive decline, suggesting that inhibiting pro-inflammatory cytokines, administration of anti-inflammatory cytokines, and/or modulating immune cell phenotypes are all promising therapeutic targets.

IL-1β is one of the most studied pro-inflammatory cytokines regarding T2D. In animal models and patients with T2D, antagonism of IL-1 reduces hyperglycemia and inflammation,[82, 83] and although amyloid-forming amylin was not considered in these models, they show promise for IL-1 as a potential therapeutic. Surprisingly, IL-1β is also important in hematopoiesis. Studies in aging found that the BM environment is characterized by increases in IL-1β and is associated with BM failure.[346] They also show that chronic injections with IL-1β are able to induce BM dysfunction, and blocking of IL-1β reversed BM dysfunction.[346] As we and others have shown, T2D and the hypersecretion of pancreatic amylin led to increases in IL-1β; this could be a potential contributing factor to BM dysfunction and lymphopenia seen in our studies. Furthermore, studies in AD that block IL-1β using NRLP3 inhibitors rescue learning and memory deficits and reduce neuroinflammation and Aβ plaques.[347] Together these studies suggest that IL-1β inhibitors are an attractive therapeutic option to reverse BM failure, which may result in reversing lymphopenia and help immune cells migrate to the brain, potentially leading to neuroprotection. Future studies should focus on whether the blockade of IL-1β in HIP animals leads to reversing BM failure and if this is associated with reversing B lymphopoieses, as no studies have explored this connection. As these studies suggest that IL-1β blockade will reverse BM failure, it should be explored if it also reverses lymphopenia, increases immune cell migration to the brain, and is associated with improvements in cognitive decline and neuroinflammation in HIP animals.

Another immunotherapy shown to reduce IL-1β levels is PD-1/PD-L1 inhibitors. Currently, PD-1/PD-L1 inhibitors are FDA-approved for cancer and have been successful in increasing anti-tumor immunity.[348] Confirming potential efficacy in T2D, studies in

patients with T2D show that $CD8^+$ T cells expressing PD-1 have impaired bioenergetics and cytokine production.[349] Furthermore, our RNAseq results discussed in Chapter 3 show that the PD-1/PD-L1 pathways are enriched in HIP rats' brains. However, as of yet no studies have considered these inhibitors as therapeutics for patients with T2D. This may be due to concerns that drugs in this category have been known to increase blood sugar and, in some cases, can lead to T1D, but the rate of this was lower than 3% and nonexistent in other drugs.[350] Also, studies have shown that the use of these inhibitors can lead to severe lymphopenia.[351] Studies in AD found that the use of PD-1 and PD-L1 inhibitors led to an increase in cognition and a decrease in amylin plaques in the brain.[352] They also found that systemic T cells were increased, which was associated with an increase of monocyte-derived macrophages in the brain, suggesting the beneficial effects of both circulating T cells and the migration of macrophages in the brain. Furthermore, as mentioned, the inhibitor reduced brain levels of IL-1β. Due to the risk of lymphopenia associated with PD-1/PD-L1 inhibitors, this drug may not be the best for our model since the hypersecretion of pancreatic human amylin already leads to lymphopenia. However, it is still unknown how this drug impacts BM dysfunction and B lymphopoiesis. The upregulation of the PD-1/PD-L1 pathway in HIP brains and studies in AD showing its ability to decrease IL-1β and improve cognitive decline suggests it is a potential therapeutic candidate, but the side effects make it controversial.

Another cytokine impacted by the accumulation of pancreatic human amylin is IL-10. IL-10 is a cytokine with anti-inflammatory properties known for its role in preventing inflammation. It is secreted by multiple cell types, the most prominent producers being Th2 cells and Tregs.[353] In patients with T2D, IL-10 is decreased and

mutations in the IL-10 gene is associated with T2D.[354] In the brains of HIP rats, IL-10 is decreased and is associated with neuroinflammation and cognitive decline.[14] These studies provide a rationale for IL-10 as a potential therapeutic target. Multiple studies show that the administration of IL-10 is beneficial in autoimmune disorders, inflammatory diseases, cancer, and CNS disorders.[355-357] One of the mechanisms of action behind IL-10 therapy is the increase of Tregs. Tregs are known anti-inflammatory immunosuppressants, with their increase's protective in many CNS diseases.[356, 357] T regs are also important in hematopoiesis and B cell development. Mice deficient in Tregs lead to B cell development and hematopoiesis deficits, which were rescued by transplantation of normal BM cells.[358, 359] Furthermore, studies have shown they are important for suppressing pathogenic Th17 cells, which are increased in T2D.[360] Unfortunately, studies in animal models and humans with T2D have shown that Tregs are decreased limiting their benefit during chronic neuroinflammation.[113, 361] Though studies in AD have shown that IL-10 therapy exacerbated AD-increased plaque burden and behavior deficits,[362] IL-10 decreased neuroinflammation, BBB leakage, infarct volume while increasing IL-10^{+} Tregs in the brain in TBI and stroke.[356, 357] This provides a rationale, however, that administration of IL-10 may be enough to prevent peripheral inflammation and increase Tregs, which could reverse BM failure and deficits in B lymphopoieses in HIP animals.

Another potential therapeutic that can increase Tregs is IL-2. IL-2 is a cytokine produced by immune cells that plays a critical role in the modulation of the immune system, including for Treg survival and growth.[363] IL-2 therapies shown promise in patients with cancer, but in high doses cause adverse reactions, which caused studies to

focus on low dose IL-2 treatments.[364] In the CNS, IL-2 therapy decreased Aβ plaque coverage and rescued memory deficits through the expansion of Tregs.[365] Furthermore, astrocyte-targeted IL-2 gene therapy was neuroprotective in stroke and TBI by increasing brain-localized Tregs.[366] While studies in T1D found that low-dose IL-2 therapy is a promising therapeutic target through increased Tregs, [367, 368] this relationship is poorly understood in T2D with only one study showing that IL-2 was increased in patients with T2D.[369] However, the ability of IL-2 to expand Tregs similar to IL-10 makes it a potential therapeutic to reduce inflammation, reverse BM failure, and deficits in B lymphopoiesis in HIP animals.

4.9 Lifestyle choices as a therapeutic

Although therapeutics are important in helping individuals with T2D, one therapy that shouldn't be ignored is lifestyle intervention(s). Lifestyle factors such as smoking, drinking, physical activity and diet play a critical role in the management and prevention of T2D.[370] For example, T2D studies in mice and humans show that exercise is able to reverse insulin resistance, hyperglycemia, and associated complications.[371, 372] Studies have also shown that exercise can improve hematopoiesis, but it is unknown how this impact B cell development.[373, 374] Furthermore, in patients with T2D physical activity slows cognitive decline.[375] Studies have also shown that in patients with T2D that a healthier diet such as a Mediterranean diet or high fiber diet is also beneficial in management.[376, 377] Although it is unknown how exercise and diet play in preventing the accumulation of amylin, we can suggest that it would also be protective in these circumstances.

4.10 Sex-based differences

Sex differences exist in both the immune response and during the development of T2D, but this is still understudied specifically for the immune response during T2D. Regarding T2D, men are diagnosed with T2D more than women and are diagnosed at an earlier age than women attributed to the fact that estrogen is protective from T2D.[378] Hormone replacement therapy reduces T2D incidence in postmenopausal women and enhances insulin sensitivity in women with T2D.[379] In HIP rats, females have significantly lower amounts of plasma amylin than male HIP rats and develop hyperglycemia much later than male HIP rats,[13] contributing to the idea that estrogen is protective during T2D, but it is currently unknown how estrogen impacts amylin aggregation.

Regarding hematopoiesis and BM function studies not many studies have shown the sexual dimorphism that may exist. One study did show that HSPCs divide more frequently, while they found no differences in the amount of HSPC progenitor cells in the BM.[380] However, studies do show that HSPCs can be regulated by sex hormones. For example, estradiol has been found to regulate dendritic cell differentiation in BM cells.[381] Another study found that the deletion of estrogen receptors was able to decrease developing B cell populations with no change to HPSCs.[382] These data suggest that sexual dimorphism could play important roles in hematopoiesis and B cell development.

Generally, females have higher $CD4^+$ T cells and CD4/CD8 ratios than males.[383] Following in-vitro stimulation, females have more activated $CD4^+$ and $CD8^+$ T cells than men, while men have higher Th17 cells and fewer Th1 cells than women.[384] Studies show that treatment of $CD4^+$ T cells with estrogen decreased Th17 cells and increased the

proportion of Tregs,[385] a generally anti-inflammatory effect of estrogen. These studies highlight how the immune response is sex-dependent and give insight into why men and women develop and progress through diseases differently. Women with impaired glucose tolerance exhibit increases in B-1 B cells,[386] which is an anti-inflammatory B cell subset while studies in obesity show there are sex differences in Tregs in AT that was regulated by sex hormones such as estrogen and androgen.[387] These findings highlight the influence of sexual dimorphism in immune cell function inside and outside of the context of T2D.

When it comes to CNS disorders, there are also known sex differences that occur. For example, women are more likely to develop AD and MS[388, 389] than males, while males develop PD at higher rates than women.[390] Studies focused on T2D-induced cognitive decline show that women are at increased risk for cognitive decline compared to males.[391] These studies further add to the notion that sex differences need to be included due to the fact that after menopause, women also become more likely to develop disorders.[392] Future studies in HIP animals should focus on how these sex differences impact the accumulation of pancreatic human amylin, including the time course of neuropathology, and the impact on the neuroimmune axis response.

4.11 Technical & Experimental Limitations

Following the completion of these studies, we acknowledge that the experiments as designed have limitations. The use of animals that hypersecrete human amylin provides more physiological relevance when studying the pathogenesis of T2D. In humans, amylin is co-secreted with insulin from the pancreas and contributes to the pathogenesis of T2D,[9] making this model unique and not just another hyperglycemic or

obesity model. However, since the hypersecretion of pancreatic amylin in this model also leads to hyperglycemia, it is difficult to determine the exact role that human amylin plays independently. In this model, hyperglycemia and insulinemia are accompanied by the hypersecretion of amylin, which all contribute to the pathology in these animals. Thus, we are not able to delineate which pathology is only caused by the hypersecretion of human amylin. Therefore, in the future, it may be necessary to compare HIP animals to animals that are only hyperglycemia and insulinemia to gain insight into which pathologies are human amylin-causing.

Despite clear sex-based differences in both the immune system and T2D pathology, we used only male rats in our experiments. As mentioned in earlier sections, female HIP rats do not express human amylin to the same magnitude as HIP males, developing hyperglycemia much later in life and making it hard to study the impact on the accumulation of human amylin.[13] However, as mentioned, post-menopause, the severity of T2D increases, and associated diabetic complications increase as well.[378] Although female HIP rats may need more time to develop the same pathologies as males, studies should be conducted to determine how they differ at those time points to determine what the accumulation of pancreatic human amylin leads to in post-menopause females.

Another limitation of our study is our inability to validate immune genes from our RNAseq experiment. Commonly after RNAseq rt-PCR is used to validate genes and ensure the results are true. In the genes we chose to validate, we did not see any significant differences between WT and HIP rats. This does not automatically suggest that the results from this experiment are invalid. It could suggest that there were problems

during rt-PCR as many critical steps during rt-PCR can impact accuracy as well as interpretation of results, such as the quality of the mRNA and amplification efficiency.[393] Furthermore, for the genes we chose to validate, the primers used were uncommon, and we could not find published primers for our species, so we had to create our own primers using BLAST. This could lead to using primers different from the gene encoded during RNA-seq experiments. Finally, the expression level of the genes we chose to validate was low in the brain, which could lead to inconsistent and inaccurate results. For future studies, well-established and published genes should be used for the validation of RNA-seq, particularly in HIP mice.

Our human amylin injection experiments also had their limitations. We initially started the injections with WT rats bred from our current in-house colonies. To repeat this study, rats of similar age were purchased from Envigo. It was important to control for age which impacts spleen size and thus the number of cells from each animal, risking consistent results for flow cytometry analysis. However, purchased aged rats are usually breeders, meaning, in some cases, rats' age is determined by weight. In this case, the rats we ordered are aged by weight; in some cases, you may not be getting the correct age, and even if all rats are not equal, our rats at age 12 could be around 800 grams, while that same age at another vivarium could be 500 grams, which could contribute to how they respond to amylin and their immune response. Studies have shown that rodents from different vendors with the same genotype have significantly different variations in the microbiome,[394] which suggests that their immune response to insults would be different as well. Though we confirmed with the vendor that all rats ordered were the same age as our in-house rats, they were still significantly smaller leading to differences. Furthermore,

as injections took place during different times, the human amylin ordered was more than likely from different batches, which could contribute to different effects seen in injected animals. Therefore, in the future, for experiments of this nature, rats should either be all bred in-house or all purchased from the same vendor for the same weight/age, and the same batch for amylin should be used in all animals to prevent variability.

Finally, our study started in HIP rats, which allowed us to understand how long-term accumulation of pancreatic amylin impacts the neuroimmune response. However, for flow cytometry, the availability of antibodies is limited in rats. Therefore, we switched to mice to better understand the immune response though there are some drawbacks to using mice. For example, HIP mice do not survive as long as HIP rats.[395] Meaning, pathology seen in HIP mice may be indicative of what happens early on in HIP rats. Furthermore, HIP rats are heterozygous for the human amylin gene while the mice are homozygous, so there may be differences in levels of secretion, but this was necessary due to the fact that heterozygous mice do not spontaneously develop diabetes. Heterozygous mice need a stimulus such as HFD, which turns the model into an obesity model of T2D. Therefore, to compare the two, HIP rats need to be used at earlier stages to determine if the immune response develops the same as HIP mice.

4.12 Remaining questions and concluding remarks

4.12A What are the phenotypes of the T and B cells?

The studies in this thesis show that there are alterations in T cell and B cell populations. As mentioned in Chapter 1, many types of T cells (e.g., Th1, Th2, Th17, T regs) are altered during T2D. We did not phenotype for these cells, leaving us unable to

determine how pancreatic human amylin alters these cell types. Although we can suggest which of these cell types may be altered in our model, surprising results such as $CD8^+$ T cells being decreased in HIP rats and rats injected with human amylin suggest that human amylin can impact certain cell types very differently. One of the key cells that would be important to monitor is Tregs. As mentioned, they have functions in hematopoiesis, B lymphopoiesis, and neuroinflammation and could add another potential mechanism explaining the pathology seen in HIP animals.[13, 14] The same for B cells; although we were able to look for different types of B cells, we don't know their inflammatory profile. B cells could express both pro-and anti-inflammatory cytokines that are important during the pathogenesis of T2D, and it could be important to note how the accumulation of pancreatic humans alters cytokine production from all responding subsets.

4.12B What is the impact of the hypersecretion of pancreatic human amylin on skull bone marrow and meningeal lymphatics?

The meninges surround the CNS and harbor various immune cells that survey the CNS borders to maintain homeostasis, and it is important during neuroinflammation, tissue repair, and neuronal activity.[396, 397] The meninges also have lymphatic vessels that are important for draining solutes from the CSF to cervical lymph nodes.[398] In addition to roles in regulating CSF, these lymphatics are important in regulating the immune response in meninges.[399] Studies have shown that loss of meningeal lymphatics leads to cognitive impairment and drainage capacity, and in AD models leads to the accumulation of Aβ plaques.[400] While these studies identify the importance of meningeal lymphatics in neuroinflammation and cognitive decline, it is mostly unknown how T2D or how the accumulation of pancreatic amylin impacts meningeal lymphatics. It may be possible that

in HIP rats there is disruption of meningeal lymphatics that alter the clearance of human amylin from the brain. Furthermore, the meninges are connected to the skull marrow, which harbors immature B cells in a novel BM niche[401, 402]. In the meninges, B cells represent the main immune cell type, are phenotypically similar to BM B cells, and can develop in the skull marrow similar to the BM.[403] Studies in MS have shown that defects that occur in the BM are almost identical in the skull marrow and contribute to pathology in the brain.[404] These studies suggest that the deficits in hematopoiesis and B cell development may also be simultaneously occurring in the skull BM in HIP animals. Future, studies should focus on the hypersecretion of pancreatic human amylin impacts meningeal lymphatics and the immune response that occurs within.

4.13 Thesis Summary: The major findings in this work and their implications in the field

T2D leads to neuroinflammation, thus leading to cognitive decline and increasing the risk for developing AD.[6] Pancreatic human amylin has been shown to be a contributing factor to neuroinflammation and cognitive decline during T2D.[7] However, to date there are no drug treatments available that are focused on reducing neuroinflammation or pathology caused by the accumulation of pancreatic human amylin, nor are there any immune therapies targeted at reducing neuroinflammation or T2D induced pathology. As immune cells are now recognized to have both neurotoxic[317] and neuroprotective properties,[366] immune biologics should be considered for T2D as it is clearly characterized by a dysregulated immune response.

Previous studies showed that in diabetic HIP rats there was an increase in neuroinflammation, BBB dysfunction and cognitive decline.[13] Given that studies in

neurodegeneration have shown that these pathologies are associated with an increase of immune cells into the brain, we hypothesized that in HIP rats there would be a dysregulated neuroimmune response, which would lead to an increase in immune cells that was associated with neuroinflammation and cognitive decline as previously reported. Instead, we discovered that the hypersecretion of pancreatic human amylin actually led to splenic lymphopenia that translated to a reduction of immune cells in the brain. We found that this decreased immune cell migration into the brain was associated with altered CAM expressions which are essential for allowing immune cells across the BBB. Lastly, we discovered that the hypersecretion of pancreatic human amylin can lead to BM failure characterized by the sequestration of immune cells and deficits in B lymphopoiesis and hematopoiesis, which is mediated by the CXCL12/CXCR4 axis. These data are the first to demonstrate an amylin-mediated role in the peripheral and CNS immune response during the pathogenesis of T2D, which could potentially contribute to neuroinflammation and cognitive decline.

Our studies highlight novel therapeutic targets for T2D – because if the immune deficits revealed can be reversed it may in turn reduce neuroinflammation and cognitive decline. Future studies might consider 1.) Identifying therapeutic compounds that can reverse lymphopenia and B cell development deficits; and 2.) how these cells can be modulated to be neuroprotective. Overall, the studies conducted in this thesis highlight the importance of holistically investigating the neuroimmune axis, from development through recruitment to the parenchyma, as they are critical in contribution to the development of diabetic cognitive decline.

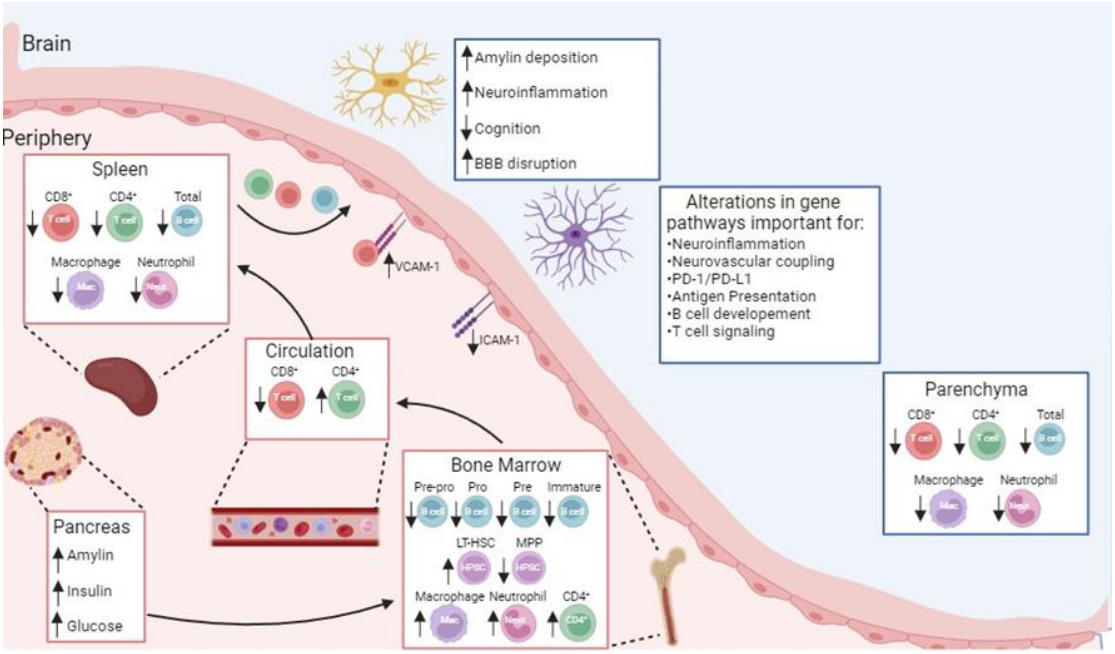

Figure 4.1: Mechanisms of peripheral and CNS immune alterations due to hypersecretion of pancreatic human amylin as it related to neuroinflammation and cognitve decline.

The hypersecretion of pancreatic human amylin leads to increase in insulin, as it is co-secreted with amylin leading to hypergycemia. We have shown this leads to alterations in immune populations in the bone marrow, circulation and spleen. This in turn leads to a decrease in immune cell populations migrating into the brain due to alteration in CAMS. These alteration potentially contribute to neuroinflammation, plaque deposition, BBB disruption and cognitive decline.

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Professional Publications

Nirmal Verma[#], Gopal Viswanathan Velmurugan#, **Edric Winford**, Han Ly, Deepak Kotiya, Gopal Viswanathan Velmurugan, Noah Leibold, Laura Radulescu, Sanda Despa, Kuey C. Chen, Linda J Van Eldik[5], Peter T. Nelson[5], Donna M. Wilcock, Gregory A. Jicha, David K. Powel, Jeffrey H Walton, Manuel F. Navedo, Matthew A. Nystoriak, Claire Troakes, Henrik Zetterberg, John Hardy, Tammaryn Lashley, Andrew J. Murray, Ann M Stowe, Larry B.Goldstein, Geert Jan Biessels, and Florin Despa. "Inflammation and reduced cerebral AB clearance induced by pancreatic amylin." *Communication Biology*. 2022. #-co-first author.

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