SHAPING OF THE T CELL REPERTOIRE BY SELF AND FOREIGN ANTIGENS

by

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Abstract

T cells are an indispensable component of the immune system. Any perturbation in T cell function may have severe consequences such as immune deficiency, autoimmunity or development of malignancies. T cell receptors and the signals transduced by them are critical for many aspects of T cell biology. A diverse yet tolerant TCR repertoire is essential for the proper functioning of the immune system. Herein, we investigated several factors that regulate T cell immune responses, autoimmunity and the TCR repertoire.

We investigated whether co-delivery of autoantigen insulin along the natural killer T cell antigen αGalCer within novel liposomes could prevent T1D in non-obese diabetic mice. We found that subcutaneous injection of such liposomes could potently stimulate NKT cells to activate other immune cell types. Further, their subcutaneous administration reduced the frequency of islet-reactive T cells and conferred protection against diabetes. These experiments suggest that using liposomes to co-deliver autoantigens along with αGalCer may prove to be a valuable for the prevention of autoimmune diseases.

Next, we examined whether the expression of two types of TCRs by individual CD8 T cells could contribute to autoreactivity and autoimmunity. We compared the reactivity against the model autoantigen OVA between CD8 T cells capable of expressing two types of TCRs (bi-allelic, TCR$\alpha^{+/+}$) versus those restricted to a single TCR (mono-allelic, TCR$\alpha^{+/-}$). Bi-allelic CD8 T cells exhibited increased proliferative capacity and autoreactivity relative to mono-allelic CD8 T cells. Altogether, our results suggest that dual TCR-expressing CD8 T cells evade tolerance and participate in the pathogenesis of autoimmunity.

Lastly, we studied the role of MUC2 mucin in regulating CD8 T cell tolerance against oral antigens. Wild type and MUC2-deficient (*Muc2*$^{-/-}$) mice were orally gavaged with

ovalbumin (OVA) to examine the impact of dietary antigens on CD8 T cells. We found that oral administration resulted in rapid systemic dissemination of OVA in the blood and tissues of *Muc2*$^{-/-}$ mice, and culminated in the deletion of OVA-specific thymocytes. Consequently, our findings suggest that MUC2-deficiency results the shaping the TCR repertoire of developing thymocytes by intestinal luminal antigens.

Lay Summary

We studied whether a novel therapeutic agent called α-GalCer liposome can protect against type 1 diabetes (T1D). We found that under the skin injection using α-GalCer liposome can activate the immune system and protect against T1D. We also studied an escape mechanism of diabetes -causing T cells, which play major roles in causing T1D. We showed T cells that express two T cell receptors (TCR) can escape protective mechanisms that the body use to prevent T cells from attacking self-tissue. Finally, we studied the role of Muc2 mucus, an integral part of the intestinal mucus layer on food tolerance. We found that the intestine becomes leaky in the absence of Muc2 mucus, allowing food particles to alter the development of the immune system.

Preface

Contributions:

A version of CHAPTER 3 is being prepared for submission. Professor Priatel provided supervision for the project. Dr. I-Fang Lee and Mr. Jason Hung conducted all the NOD mice experiments with support from Ms. Nicole J. Leung, Dr. Derek L. Lai and Mr. Mitsu Komba (Chapter 3.2.5-3.2.6). I conducted all the C57BL/6 mice experiments, and analyzed the data (Chapter 3.2.1-3.2.4). The liposomal therapeutics were produced by REGiMMUNE Inc.

A manuscript describing a technique for the *in vivo* measurement of gut permeability in mice is being prepared for submission. I developed the technique under the guidance and supervision of professor Priatel. Dr. Vallance provided advice and technical support regarding mouse gut permeability (Chapter 2.13).

Professor Priatel provided supervision and guidance for all the experiments performed in Chapter 4. I performed all the experiments and analyzed the data.

A version of Chapter 5 is also being prepared for submission. Professor Priatel provided supervision of the project. Professor Vallance provided mice and technical support regarding gut permeability while Ms. Caixia Ma offered technical support regarding oral gavage of mice. I conducted all the experiments, and analyzed the data.

Ethics approval:

All animal experiments were conducted following protocols (A15-0043 and A14-0303) approved by the Animal Care Committee at the University of British Columbia in conjunction with the Canadian Council on Animal Care.

Figures approval:

Publishers have granted permission for the usage of already published figures in my thesis.

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List of Symbols

α	alpha
β	beta
γ	gamma
ζ	zeta
θ	theta
κ	kappa
μ	micron
Mb	megabase
M	molar
%	percent
Gene$^{-/-}$	genetic deletion of “gene”

List of Abbreviations

Ab	antibody
AD	autoimmune diabetes
Ag	antigen
AIRE	autoimmune regulator
αGalCer	alpha-galatosylceramide
APC	antigen presenting cell
B6	C57BL/6
BB	biobreeding
BCR	B cell receptor
CD	Crohn's disease
CD1	cluster of differentiation 1
CDR	complementarity determining region
CFA	complete Freund's adjuvant
CFSE	carboxyfluorescein succinimidyl ester
CFU	colony forming unit
Ci	Curie
CLR	C-type lectin receptors
cMOP	common monocyte progenitor
cDC-P	classical DC-restricted progenitors
CTL	cytotoxic T lymphocyte
CTLA-4	cytotoxic T-lymphocyte-associated protein 4
DAMP	damage-associated molecular pattern

DC	dendritic cell
DN	double negative
DNA-PK	DNA dependent protein kinase
DP	double positive
ER	endoplasmic reticulum
FBS	fetal bovine serum
Flt3	FMS like tyrosine kinase 3
FOXP3	forkhead box P3
GM-CSF	granulocyte macrophage colony stimulating factor
GFP	green fluorescent protein
GRB2	Growth factor receptor-bound protein 2
HLA	human leukocyte antigen
HSC	hematopoietic stem cell
IBD	inflammatory bowel disease
ICOS	inducible costimulator
IEC	intestinal epithelial cell
IEL	intraepithelial lymphocyte
IFN	interferon
iGb3	isoglobotriosylceramide
ILC	innate lymphoid cell
IMDM	Iscove's Modified Dulbecco's Media
iNOS	inducible nitric oxide synthase
i.p.	intraperitoneal

IPEX	immunodysregulation polyendocrinopathy enteropathy X-linked syndrome
IP-FCM	immunoprecipitation flow cytometry
ILC	innate lymphoid cells
ITAM	immunoreceptor tyrosine-base activation motif
ITSM	immunoreceptor tyrosine-based switch motifs
i.v.	intravenous
KIR	killer-cell immunoglobulin-like receptor
LAT	linker for activation of T cells
LCK	lymphocyte-specific protein tyrosine kinase
LM	Listeria monocytogenes
LPS	lipopolysaccharide
M-CSFR	macrophage colony stimulating factor receptor
MDP	macrophage and DC progenitor
MG	myasthenia gravis
MPS	monoculear phagocyte system
MAPK	mitogen-activated protein kinases
MDP	muramyl dipeptide
MFI	mean fluorescence intensity
MHC	major histocompatibility complex
MLN	mesenteric lymph node
MUC	mucin
MyD88	Myeloid differentiation primary response gene 88

NETs	neutrophil extracellular traps
NLR	NOD like receptors
NOD2	Nucleotide-binding oligomerization domain-containing 2
NOD	Non-obese diabetic
NK cell	natural killer cell
NKT cell	natural killer T cell
NLR	nucleotide-binding oligomerization domain (NOD)-like receptor
OVA	ovalbumin
PAMP	pathogen-associated molecular pattern
PBS	phosphate buffered saline
PKC	protein kinase C
PLZF	Promyelocytic leukaemia zinc finger
PMA	phorbol 12-myristate 13-acetate
pre-pDC-P	plasmacytoid DC-specific progenitors
PRR	pattern-recognition receptor
RA	rheumatoid arthritis
RAG	recombination-activating gene
RIP	rat insulin promoter
RBC	red blood cell
RLR	retinoic acid inducible gene 1 like receptors
RSS	recombination signal sequence
SCID	severe combined immunodeficient
s.c.	subcutaneous

SH2	Src homology 2
SHIP-1	Src homology 2 domain containing inositol polyphosphate phosphatase-1
SHP	Src homology 2 domain-containing phosphatase
SLE	systemic lupus erythematosus
SNP	single-nucleotide polymorphism
SP	single positive
SPF	specific-pathogen-free
STAT3	signal transducer and activator of transcription 3
T1D	type I diabetes
TAP	transporter associated with antigen processing
Tc	T cytotoxic
TCR	T cell receptor
TdT	terminal deoxynucleotidyl transferase
TEC	thymic epithelial cell
Tet	tetramer
TGF	transforming growth factor
Tfh	T follicular helper
Th	T helper
TLR	Toll-like receptor
TNF	tumor necrosis factor
Treg	T regulatory cell
TSA	tissue specific antigen
UC	ulcerative colitis

XRCC4	X-ray cross-complementing protein 4
ZAP70	zeta-chain-associated protein kinase 70

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Dedication

To my love ones, my mentors, and people who suffer from autoimmune diseases.

Chapter 1: INTRODUCTION

1.1 Immunity

Microflora is a term used to describe the ecosystem formed by the resident microbes that colonize various sites of the body. The human body is the home to approximately 38 trillion microbes, indicating a ratio of more than 1 microbe for every human cell given that the humans are comprised of an estimated 30 trillion cells [1, 2]. The majority of these microbial inhabitants reside either in the lumen of the intestine or the surface of the skin. Moreover, microbes have long established complex relationships co-evolving with their hosts over thousands of years. Microbes that colonize the gut have formed a complex ecosystem containing different communities and ecological niches. It is estimated that about 300-1000 different species of bacteria colonize the lumen of the human gut [3-5]. While some of these resident microbes act as commensals living together but not causing injury nor conferring any benefit to the host, some resident microbes have established a symbiotic relationship with humans by providing a range of benefits such as assisting in food digestion, and vitamin synthesis to the host in exchange for a protective, nutrient-rich environment [6, 7]. Further, some microbes have taken the concept of symbiosis even further by establishing intricate communication pathways with their host, influencing various vital host homeostatic processes such as sex hormone secretion [8], immune regulation and hematopoiesis [9-11].

Despite their contributions to host nutrition and homeostasis, microflora left unchecked by the immune system can cause serious or fatal opportunistic infections. In addition, many pathogenic microbial species are molecularly similar to the microflora and thus highlight the need for the host to discriminate harmful from harmless. Over the course of evolution, various life forms have evolved a sophisticated collection of defense mechanisms, collectively called the

immune system, to protect against microbial invasion. The vertebrate immune system is comprised of a collection of proteins, cells and structures, that are capable of differentiating self from non-self, normal from malignant. Together, they work in concert to respond appropriately to various insults and confer immunity towards infections and malignancies. In the following sections, the components of the innate and adaptive immune system and their contributions to host immunity will be described in detail.

1.1.1 Innate immune system

The immune system is often subdivided into two arms: the evolutionary ancient innate immune system found in all classes of plant and animal life and the more recently evolved adaptive immune system generally restricted to vertebrates. The innate immunity is the first line of defense against microbial invasion and can be further sub-divided into layers. The outermost layer is comprised of the physical barriers such as the skin and mucosal membranes. The innate immune system relies on a restricted set of germline-encoded receptors, called pattern recognition receptors (PRRs), to detect highly conserved pathogen-associated molecular patterns (PAMPs) present on the microbes.

PAMPs consist of a vast array of different molecules and are essential structures required for pathogen growth and virulence. Recognition of the microbial patterns by a variety of PRRs can lead to the release of pro-inflammatory cytokines and chemokines from various cell types, leading to the recruitment of phagocytes capable of engulfing and destroying the invaders. Further, phagocytes also play a critical role in recruiting and activating adaptive immune cells. In addition, a specialized subset of phagocytes, termed dendritic cells (DCs), process engulfed

microbes into peptide fragments that are then presented to the adaptive immune cells called T cells to elicit cell-mediated adaptive immune responses.

The complement system refers to PRRs found within the blood that recognize negatively charged moieties present on microbial surfaces. Upon recognition, they form covalent bonds with such moieties to coat the microbe, allowing enhanced uptake and destruction of the microbe. The activation of complement also initiates a protein cleavage cascade that ultimately leads to the assembly of a pore-forming protein complex, known as the membrane attack complex, on the surface of microbes to mediate their lysis.

1.1.2 Anatomical barriers

Anatomical barriers are a set of physical barriers found on the outer most layer of the body where there is constant contact with the resident microflora and environment. For non-absorptive surfaces such as the skin, epithelial cells, keratin and keratinocytes combine to form a protective barrier impervious to most pathogens and microflora. For absorptive surfaces such as the digestive tract and the respiratory tract, a layer of protective mucus segregates the resident microflora from the epithelial cells underneath the mucus layer. The light transmitting surface of the cornea is protected by a layer of tear film, which is comprised of lipids, mucus, water and a complex mixture of nutrients and anti-microbial proteins.

1.1.3 Mucin and the mucus barrier

The skin provides an effective physical barrier that protects the body from most infectious agents. However, a skin-type barrier is not suitable for internal surfaces such as the respiratory tract and the digestive tract. The lumen of the respiratory and digestive tract is lined

with a single layer of epithelial cells rather than keratin and keratinocytes to facilitate gas exchange and nutrient absorption. Instead, these absorptive epithelial surfaces, collectively known as the mucosa, is protected by a thin layer of mucus, which helps to keep the surface moist for nutrient absorption, and provides the barrier function needed to segregate the potentially infectious microflora from the vulnerable epithelial cell wall underneath.

Mucus is a gel-like substance comprised of mucins, water and a mixture of anti-microbial proteins. The primary constituent mucins, are a family of evolutionarily ancient proteins [12] carrying a proline, serine and threonine rich domain, and are heavily glycosylated with oligosaccharides. Its O-linked glycans are responsible for its gel-like properties and serves to attract and retain water molecules. In addition, mucus acts as a permeable barrier, protecting the epithelial lining underneath, and as a food source for gut microbiota [13, 14]. To date, 11 and 20 mucin genes have been identified in humans [15] and mice [16], respectively. Mucins are subdivided into two classes based on the presence of a transmembrane domain. Mucins that have a transmembrane domain are called transmembrane mucins and ones lacking this domain are called gel-forming mucins.

Transmembrane mucins are anchored to the apical surfaces of mucus membranes where they function to maintain surface moisture and provide lubrication for passing luminal contents [17]. Gel-forming mucins secreted by the goblet cells form the backbone of the protective mucus layer that lines the lumen of the mucosa. In addition, emerging evidence also suggest that mucins and the specialized cells which secrete them can also play roles in promoting immune tolerance and homeostasis. For example, goblet cells of the small intestine can capture low-molecular weight antigens from the intestinal lumen and deliver them to DCs in the lamina propria with tolerogenic properties [18]. Furthermore, recent work suggests that MUC2, the primary

constituent of secreted mucins of the gastrointestinal tract, switches intestinal DCs from a pro-inflammatory to a T regulatory cell-inducing phenotype [19]. Altogether, both of these reports indicate that mucins exert their immune suppressive properties through a population of tolerogenic DCs.

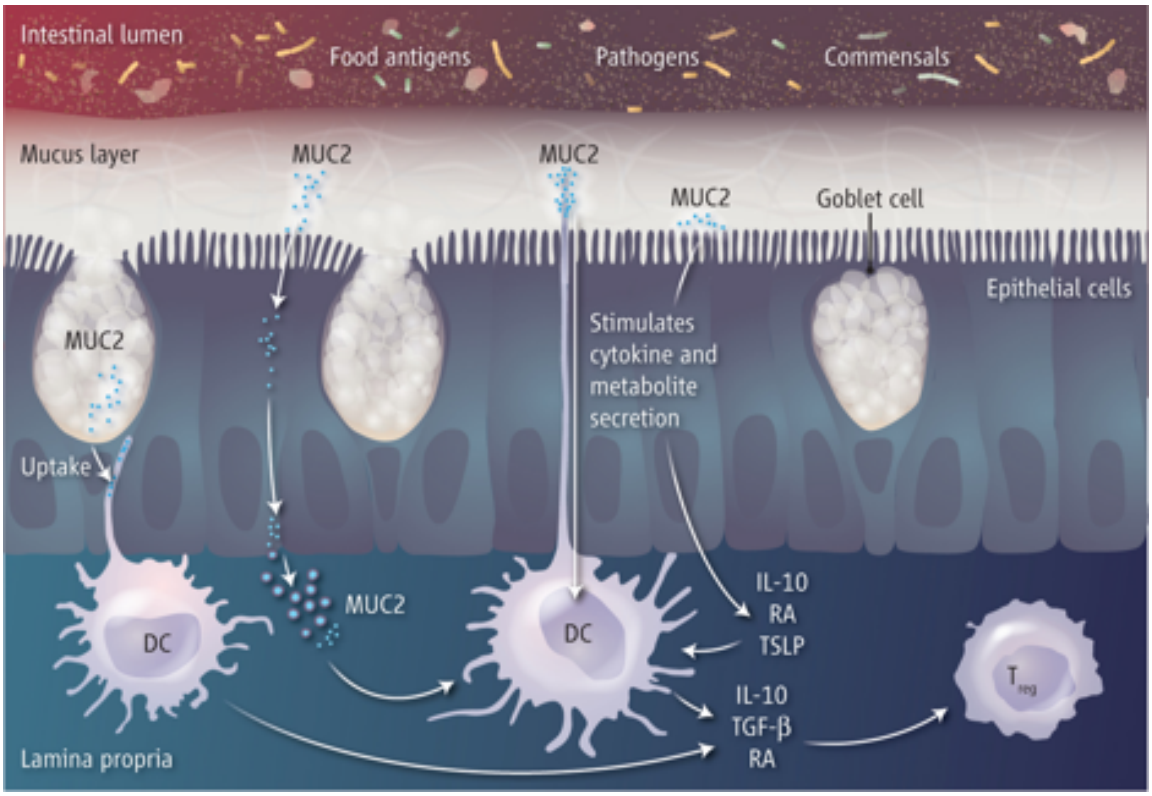

Figure 1-1 Conventional and immunological roles of the intestinal mucus layer.
The mucus layer that lines the lumen of the gastrointestinal tract plays several important roles. It provides a permeable barrier that segregates the content of the intestinal lumen from the intestinal epithelial cells while allowing nutrients to freely diffuse across the barrier. In addition, the intestinal mucus also serves as lubricant for luminal contents to pass through, reducing mechanical stress and the risk of damage to the intestinal epithelium. The heavily glycosylated mucus also serves as a food source for the microflora, which distracts the microflora and prevents them from invading the intestinal epithelium. Immunologically, mucus can act as a tolerogenic signal that can drive tolerogenic DC polarization and promote tolerance through the induction of T regulatory cells. Figure adapted from Belkaid *et al. Science* 2013, with permission from the American Association for the Advancement of Science [20].

1.1.4 Pattern recognition receptors

The pattern recognition receptors (PRRs) are a set of evolutionary ancient germline-encoded receptors found across all living multi-cellular life forms. PRRs can be found in the cytoplasm, on the cell surface as transmembrane proteins, or as secreted proteins in the plasma. They function to recognize surface features and repeating molecular patterns conserved across microbial species otherwise known as pathogen associated molecular patterns (PAMPs) and initiate a variety of responses [21-24]. Although each individual PRR can only recognize a certain type of PAMP, collectively PRRs are able to effectively detect invading microbes, and provide immunity through their combined coverage and their strategic placement.

PRRs can function in several ways; while some PRRs can act as opsonins after binding to microbial surface molecules to facilitate their destruction by either enhancing their uptake by phagocytes or activating the complement system [25], other PRRs can trigger signaling pathways upon binding to PAMPs and signal to activate inflammatory genes, and resulting in chemokine and cytokine secretion. There are four major classes of PRRs classified to date, namely toll-like receptors (TLRs), C-type lectin receptors (CLRs), retinoic acid inducible gene 1 like receptors (RLRs) and NOD-like receptors (NLRs).

The TLR family, the best characterized group of PRRs, consists of a group of transmembrane PRRs that function to detect the presence of microbes both outside of the cell and within the cell in endosomes/lysosomes. TLRs are primarily expressed in immune cells [26], especially in B cells, DCs, macrophages and neutrophils, which are known to take up and present antigens on the cell surface to activate the adaptive immune system [27-29]. With that being said, TLRs can also be found in epithelial cells of several anatomical sites (skin, intestine, urinary system), where there is a constant contact between self-tissue and resident microflora

[26, 30]. To date, there are 10 TLRs identified in humans (TLR1-10) and 13 identified in mice (TLR1-13), with each TLR exhibiting a specificity towards a certain type of microbial pattern/product [24]. Bacterial flagellin and profilin from toxoplasma can be detected by TLR5. Microbial cell wall/cell membrane derived macromolecules such as lipopeptides, lipoproteins, lipoteichoic acids, peptidoglycans, and lipo-polysacchride (LPS) are detected by TLR1, TLR2, TLR6, and TLR4 respectively whereas double stranded RNAs, single stranded RNAs, and unmethylated DNAs can be detected by TLR3, TLR7 and TLR9, respectively [24]. In addition, some PRRs can also respond to self-molecules released by damaged dying cells known as damage associated molecular pattern (DAMPs), and thus may play a role in the initiation of autoimmunity [31].

Recognition of PAMPs by TLRs can lead to the activation of several signaling pathways. The myeloid differentiation primary response gene 88 (MyD88) pathway is the primary pathway used by most TLRs (except TLR3) to transmit signal upon TLR engagement. MyD88 is an adaptor protein, which can be recruited to the TLR to facilitate the recruitment of IL-1R-associated kinase 4 (IRAK4) to the TLR and facilitate the recruitment of other IRAKs, leading to the degradation of inhibitor of κ, and activation of the transcription factor, nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB). The activation of NF-κB can then lead to the expression of various pro-inflammatory molecules and initiate an inflammatory response [24]. In addition, TLR can also signal in a MyD88 independent manner. The TIR-domain-containing adapter-inducing interferon-β (TRIF) is another adaptor protein, which is also recruited to the TLR upon TLR engagement. TRIF in turn recruits TANK binding kinase 1 (TBK1) and TBK1 activates interferon regulatory factor (IRF) transcription factor family members through phosphorylation, and results in the expression of inflammatory mediators [24].

The CLRs are a family of transmembrane receptors expressed on dendritic cells (DCs), which can recognize carbohydrates derived from microbial cell walls with their carbohydrate binding domains. But unlike the TLRs, CLR signaling may lead to different signals being sent downstream in a ligand-dependent manner. While signaling through some of the CLR family members results in the activation of NF-κB and secretion of pro-inflammatory cytokines, signaling through CLRs such as BDCA2, DCIR and MICL can antagonize TLR signaling thereby inhibiting the activation of DCs and affecting the outcome of T cell priming [32].

The RLR family consists of 3 family members; RIG-I, melanoma differentiation associated gene 5 (MDA5) and laboratory of genetics and physiology 2 (LGP2), which can be found in the cytoplasm to detect viral genomes [24, 33, 34]. RLRs upon recognition of viral nucleic acid transmit signal through IRF transcription factor family members, resulting in the expression of type 1 interferons and inflammatory cytokines.

The nucleotide-binding oligomerization domain (NOD) like receptors (NLRs) family is another class of cytoplasmic PRR, which can detect bacterial cell wall components and activate the NF-κB pathway. In addition, some NLR family members such as NACHT, LRR and PYD domains-containing protein 3 (NALP3) and NLR family CARD domain-containing protein 4 (NLRC4) also play a critical role in the assembly and activation of inflammasome [34].

1.1.5 Antigen-presentation using major histocompatibility complex molecules

Antigen-presentation is a cellular process that occurs in all nucleated cells wherein endogenously generated or exogenously acquired proteins are digested into peptides, loaded onto a set of specialized surface protein molecules called major histocompatibility complex (MHC) and presented on the cell surface as peptide-MHC complexes (pMHC) for T cells to survey using

their T cell receptor (TCR) [35]. Peptide-MHC complexes are the foundation upon which T cell immunity is built as virtually all facets of T cell biology, ranging from development, function and survival, revolve around interactions between TCR and the pMHC. As such, MHC genes are often associated with autoimmune diseases in genome-wide association studies[36], and mice lacking MHC molecules are devoid of T cells [37-39] and extremely susceptible to microbial infections [40-42].

Major histocompatibility complexes, otherwise known as human leukocyte antigens (HLA) in humans and H-2 antigens in mice, are a set of genes encoded in a large gene cluster found on chromosome 17 of mice and chromosome 6 of humans. The HLA/MHC gene cluster is approximately 3.6 Mb pairs long in humans [43], and 837 Kb long in Balb/c mice [44]. Despite being polymorphic, MHC molecules can be divided into two classes (MHC class I and MHC class II) based on their expression in tissues as well as their sources of peptide molecules [45-47].

MHC class I (MHC I) is expressed on all nucleated cells of the body, but can be found on platelets as well. The function of MHC I in the majority of nucleated cells except professional antigen presenting cells (APC), is to present cytosolic protein derived peptides, sizes between 8-10 amino acids long, to $CD8^+$ T cells [48]. The presentation of cytosolic peptides on MHC I molecules serves several purposes. In the thymus, peptides derived from self-proteins play an important role in thymocyte selection. In the peripheral tissues, self-peptide derived from the cytosol provide an important survival signal to CD8 T cells.

In the event of an undergoing infection, pathogen peptides derived from the cytosol presented on MHC I will provide CD8 T cells with activation signals to invoke a CD8 T cell mediated response. For cytosolic-derived peptides to be loaded onto MHC I, they must first be

degraded by the proteasome of the cytosol. The resulting peptide fragments with sizes ranging from 8 to 10 amino acids long, are then transported into the endoplasmic reticulum (ER) via a transporter protein called transporter associated with antigen presentation (TAP). Once loaded onto MHC I molecules, the pMHC complexes are then transported to the cell surface via the Golgi apparatus for antigen presentation [45, 49]. Although MHC I is largely responsible for presenting cytosolic-derived peptides on MHC I, professional antigen presenting cells such as dendritic cells can also present exogenous protein molecules on MHC I using a process known as cross-presentation. A detailed description of cross presentation can be found in section 1.1.6.

Professional antigen-presenting cells (APCs) are specialized immune cells with the capability to take up exogenous proteins, process and present them on MHC Class II molecules (MHC II). The ability to take up exogenous antigens, coupled with expression of many co-stimulatory molecules grants professional APCs the unrivaled capability in activating T cells compared to non-professional APCs [50-53]. Exogenous proteins acquired via phagocytosis, receptor-mediated endocytosis or macropinocytosis are processed and presented on MHC II molecules, which are expressed exclusively on professional APCs [45]. After being acquired, exogenous proteins are digested in endosomes via endosome acidification and lysosome fusion [54]. The digested content of the endosome is then fused with a vesicle containing newly synthesized MHC II molecules within which peptide fragments, ranging between 11-30 amino acids in size [55], are loaded onto MHC II molecules [56]. The vesicle containing newly synthesized pMHC II molecule is then transported to the cell surface via the Golgi apparatus to surveying CD4 T cells.

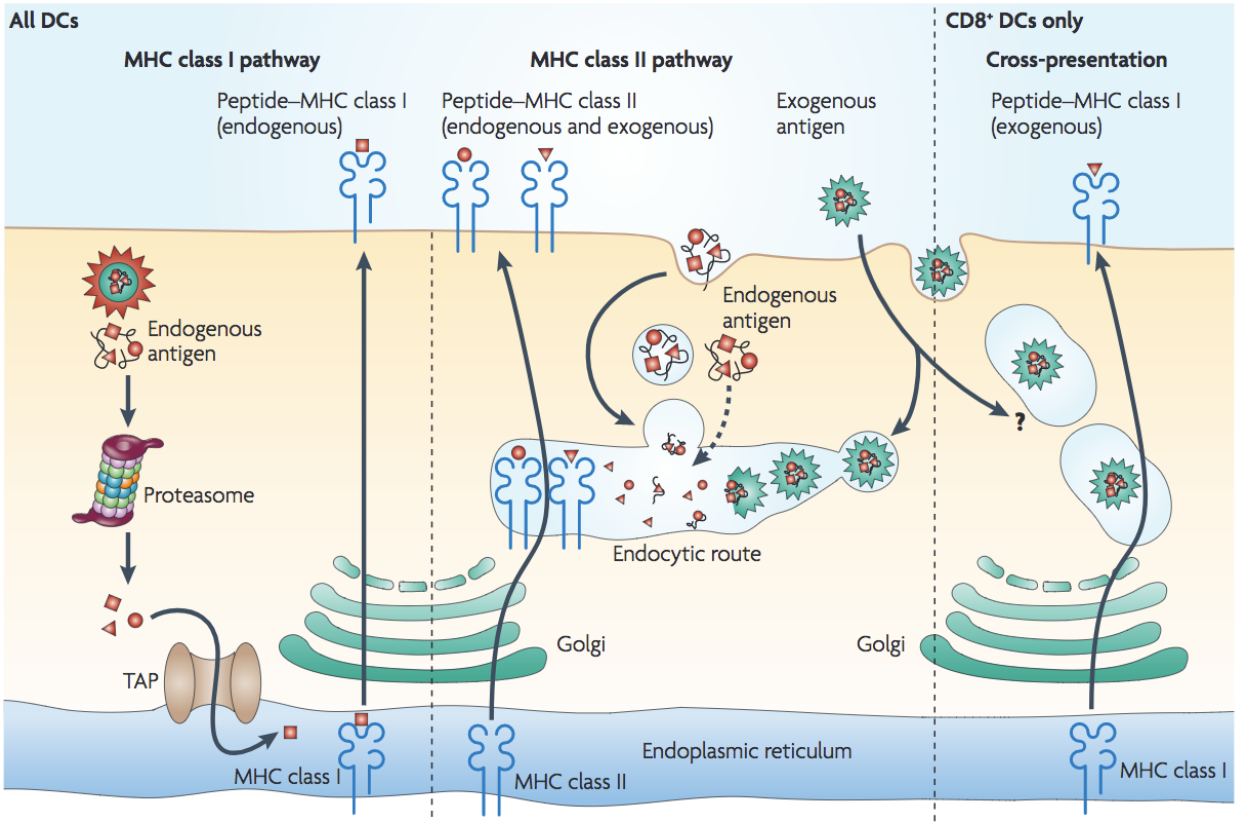

Figure 1-2 Pathways of antigen presentation by DCs on MHC class I and MHC class II. Some professional antigen presenting cells such as dendritic cells possess multiple antigen presentation pathways that are able to process and present both endogenous and exogenous proteins and present them on both MHC class I and MHC class II molecules. To present endogenous protein on MHC class I, proteins are first degraded in the cytosol by proteasomes, the peptides are then transported via the transporter-associated with antigen processing (TAP) protein into the endoplasmic reticulum where the peptides are loaded onto MHC class I molecules for surface presentation. For presentation of endogenous or exogenous proteins on MHC class II molecules, proteins are first acquired via endocytosis, macropinocytosis or autophagy, and digested into peptides within phagolysosomes. Vesicles containing newly synthesized MHC class II molecules then fuse with the peptide containing phagolysosomes allowing the loading of peptides onto MHC class II molecules. DCs also possess a specialized pathway by which exogenous antigens acquired by endocytosis or macropinocytosis can be presented on MHC class I molecules. Figure adapted from Villadangos *et al. Nature Reviews Immunology.* 2007 with permission from the Nature Publishing Groups [46].

1.1.6 Cross-presentation and cross dressing

Professional APCs are antigen presenting cells specialized in the presentation of exogenous antigens to T cells [57]. One of the unique features of professional APCs is the ability to present exogenously acquired antigens on MHC I molecules in addition to presenting exogenously acquired antigens on MHC II molecules. This process, known as cross-presentation or cross-priming, is found predominantly in DC populations [58-61], although some macrophage subtypes are thought to possess such capability as well [62]. The process of cross-presentation is thought to occur through two pathways: the cytosolic pathway and the vacuolar pathway [58, 63].

In the cytosolic pathway of cross-presentation, a protein transporter called SEC61 transports exogenous protein molecules within the endosome into the cytosol [64] where these proteins are degraded by proteasomes into peptide fragments and then transport into the ER lumen to be loaded on to MHC I for surface expression. In the vacuolar pathway, MHC I molecules containing vesicles are directed from the ER to fuse with the endolysosome. The MHC I molecules are then loaded with peptides derived from the digested endosomal content, and transported to the surface to the surveying CD8 T cells [65].

Cross-dressing is the third pathway in addition to classical antigen presentation and cross -presentation, by which professional APCs can present antigen to CD4/CD8 T cells. In cross-dressing mode of antigen presentation, DCs can nibble on another cell's membrane to acquire pMHC complexes from the donor cell for antigen presentation [66]. This process is thought to preferentially activate memory T cells to accelerate the T cell mediated antiviral response to confer immunity [67].

Although there is no definitive answer why cross-presentation and cross-dressing pathways exist. Based on our understanding of the frequencies of DCs and how valuable they are to the optimal functioning of the immune system, it is reasonable to speculate that the aforementioned pathways came to be so that DCs don't need to be infected by interacellular pathogens for them to present antigens to activate CD8 T cells.

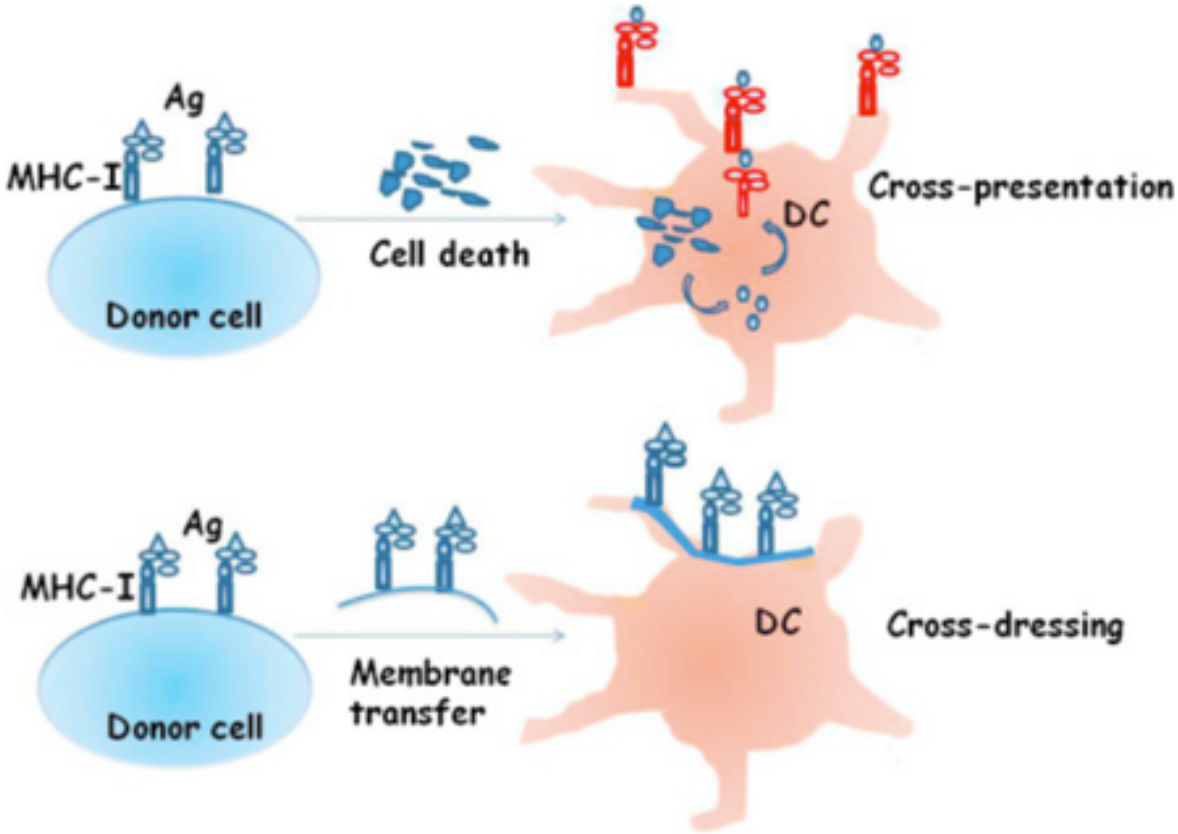

Figure 1-3 Specialized antigen presentation pathways of dendritic cells.
In addition to normal antigen presentation pathways, DCs also possess specialized antigen presentation pathways. In cross-presentation, exogenous antigens acquired via phagocytosis, or macropinocytosis can be processed within the phagolysosome and presented on MHC class I molecules. In cross-dressing, DCs can acquire MHC molecules via trogocytosis, or exosomes. These acquired MHC molecules are then presented on the cell surface of DCs. Figure adapted from Campana *et al. Immunology Letters*. 2015, with permission from European Federation of Immunological Societies [67].

1.1.7 Lipid antigen presentation using CD1 molecules

CD1 family molecules, consisting of 5 isoforms CD1a, CD1b, CD1c, CD1d and CD1e, are a family of surface glycoproteins that shares sequence and structural homology to MHC I molecules. CD1 family molecules can be classified into 3 major groups based on their amino acid sequence: group 1 is comprised of CD1a, CD1b, CD1c, group 2 CD1d, and group 3 CD1e [68]. CD1 family molecules are expressed primarily on professional APCs but can also be found on thymocytes [69]. All 5 isoforms of CD1 can be found in humans while rodents only express CD1d [68-70]. Unlike MHC I molecules, which present peptide antigen to activate conventional T cells, CD1 molecules present lipid antigens and activate a special subset of T cells known as NKT cells. CD1-mediated lipid presentation is essential for the development and function of NKT cells. A detailed description of lipid antigens, CD1 molecules and their role in NKT cell development will be presented in detail in section 1.1.10.8.

1.1.8 Innate immune cells

Cells from the innate system are the first line of defense against pathogens that have managed to get pass the anatomical barriers. The innate immune cells consist of neutrophils, polymorphonuclear cells, monocytes/macrophages, dendritic cells (DCs), natural killer (NK) cells, and innate lymphoid cells (ILCs). Innate immune cells can be found patrolling in circulation and at various anatomical sites and can be rapidly recruited to the site of infection by chemokines.

1.1.8.1 Mononuclear phagocyte system

The mononuclear phagocyte system (MPS) is comprised of a collection of bone marrow derived phagocytic cells, which are crucial not only in conferring innate and adaptive immunity but also in assisting in wound healing and tissue regeneration [71]. Although it is still a subject of debate, it is thought that the MPS consist of different populations of neutrophils, monocytes, macrophages and dendritic cells [72, 73]. While some of these mononuclear phagocytes are long lived tissue-resident phagocytes which provide various homeostatic functions such as dead cell clearance [71] and tolerance induction [73], some are short lived and have an average lifespan of 5 days [74]. Detailed descriptions of the definition and function of different components of the MPS can be found in sections 1.1.8.2 and 1.1.8.3.

1.1.8.2 Dendritic cells

Dendritic cells (DCs) were first discovered by Paul Langerhans [75] and subsequently characterized in mice by Nobel laureate Ralph Steinman in 1973 and subsequently named after their unique stellate morphology [76]. In a subsequent study, Steinman showed that DCs exhibit potent immune cell stimulating activity [77] and laid the foundation for the field of DC biology. Four decades since their discovery, DCs are now one of the most extensively studied immune cell types and are highly touted as the curative key to a plethora of conditions ranging from autoimmunity to cancer [78-80]. The DC's exquisite capability to stimulate the adaptive immune system to cause autoimmunity or cure malignancy is not the only reason why they received such focused attention from immunologists. Mounting evidence also show that in addition to priming the adaptive immune system to initiate inflammation, DCs can also orchestrate the regulatory

arm of the adaptive immune system and induce antigen-specific tolerance towards self or non-harmful antigens [73, 78, 81].

DCs and macrophages are derived from a population of bone marrow progenitor cells called macrophage and DC precursor (MDP) cells. In mice, MDPs can give rise to common monocyte progenitors (cMoP), classical DC restricted progenitors (cDC-P) and plasmacytoid DC specific progenitors (pre-pDC-P), which in turn can give rise to monocytes, cDCs and pDCs respectively [82]. Mouse monocytes are characterized by their expression of macrophage colony stimulating factor receptor (M-CSFR), Ly6C and CD11b, and can give rise to various macrophage populations during both the steady state and inflammation. In addition, they can be polarized by granulocyte macrophage colony stimulating factor (GM-CSF) to become a pro-inflammatory population of DCs known as TNF-α, iNOS producing DCs (TipDCs) [83]. It is thought that GM-CSF secreted by first responder cells such as NK cells [84], NKT cells [85], mast cells [86], fibroblasts, endothelial cells, and epithelial cells [87] during the early stages of inflammation lead to the differentiation of monocytes into this highly microbicidal population of DCs to facilitate the clearance of the source of inflammation [83].

Classical DCs and pDCs are generated from distinct progenitor cells under the guidance cytokine FMS-like tyrosine kinase 3 ligand (Flt3L), which signals through the receptor Flt3 on DCs and DC progenitors. Flt3 signaling is required for the generation and maintenance of cDC and pDC populations, as disruption in this pathway can drastically reduce the frequency and number of cDCs and pDCs [82]. cDCs can be further classified into several different subsets based on their location and surface molecule expression, which will be discussed in greater detail below.

Classical DCs can be found throughout the body both in lymphoid and non-lymphoid tissues. There are several cDC subsets found in the non-lymphoid tissues, namely the CD11b$^-$ CD11c$^+$ CD103$^+$ DCs (CD103$^+$ DCs), CD11b CD11c$^+$ DCs (CD11b$^+$ DCs), Langerhans cells (LCs), and tissue migratory DCs. The CD11b, F4/80 negative, CD103$^+$ DCs are found throughout the body's connective tissues, especially at sites where there is constant contact with the external environment. CD103$^+$ DCs are one of a few cDC populations that express TLR3 and TLR11, enabling them to detect double-stranded RNA derived from virions and profilin-like molecules derived from protozoans. In addition to PRRs, CD103$^+$ DCs also express high levels of apoptotic cell recognizing scavenging receptor CD36 and necrotic bodies detecting C-type lectin 9A (Clec9A), enabling them to clear dead or necrotic cells [88, 89]. When CD103$^+$ DCs become activated through their PRRs, they migrate to local draining lymph nodes, where they cross-present captured exogenous antigens to the T cells to initiate an adaptive T cell response. In the absence of inflammatory stimuli, CD103$^+$ cells migrate to local draining lymph node after capturing exogenous antigen, where they can either delete autoreactive T cells or induce T regulatory cells. CD103$^+$ and CD11b$^+$ CD103$^+$ DCs are major contributors of peripheral tolerance. Within the gut, CD103$^+$ DCs and CD11b$^+$ CD103$^+$ DCs (also known as mononuclear phagocytes) play a major role in T regulatory cell induction both within the thymus and in the gut [19, 73, 88, 90].

Plasmacytoid DCs represent the most recently discovered population of DCs with surface morphology resembling antibody-producing plasma cells. In steady state, pDCs express B220, a limited variety of PRRs (TLR7 and 9), low levels of CD11c, MHCII, costimulatory molecules, and can be found largely in circulation and in lymphoid tissues. Upon sensing viral/bacterial nucleic acids, pDCs produce can readily produce large amounts of IFN-α/β, TNF-α and acquire

the capacity to cross-present exogenous antigens [91, 92]. These potent cytokines coupled with cross-presentation capability make pDC a potent stimulator for T cell mediated antiviral responses.

Lymphoid tissue resident cDCs are generated from DC precursors in lymphoid tissues (lymph node, spleen, and thymus) and spend their entire life time in these tissues [88, 93]. In the gut, lymphoid resident cDC can be found in Peyer's patches, isolated lymphoid follicles, and cryptopatches [88, 93]. Lymphoid resident DCs can be classified into the $CD8^+$ subset and $CD11b^+$ subset. The $CD8^+$ subset of lymphoid resident DCs are similar to the $CD103^+$ non-lymphoid tissue resident DCs both developmentally and functionally: they are cross-presentation specialists generated from the same DC progenitor population and express similar PRRs and scavenging receptors. The $CD11b^+$ subpopulation of lymphoid tissue resident DCs are generated from a DC progenitor population under the influence of Flt3L and have comparable expression levels of CD11c and MHCII but do not express CD8, TLR3, TLR11, CD36 and Clec9a. A fraction of the $CD11b^+$ subset may express CD4, but the defining feature of the CD11b subset is their surface expression of signal regulatory protein alpha (Sirpα), also known as CD172a. The $CD11b^+$ DC subset express a vast array of PRRs but does not express TLR3 nor TLR11. This DC subset can participate in pathogen sensing, and antigen presentation to both $CD4^+$ and $CD8^+$ T cells just like the $CD8^+$ DC subsets. The $CD11b^+$ subset is also the DC subset known to constantly carry antigen from the peripheral tissues and migrate into the thymus and delete autoreactive T cells [90, 94].

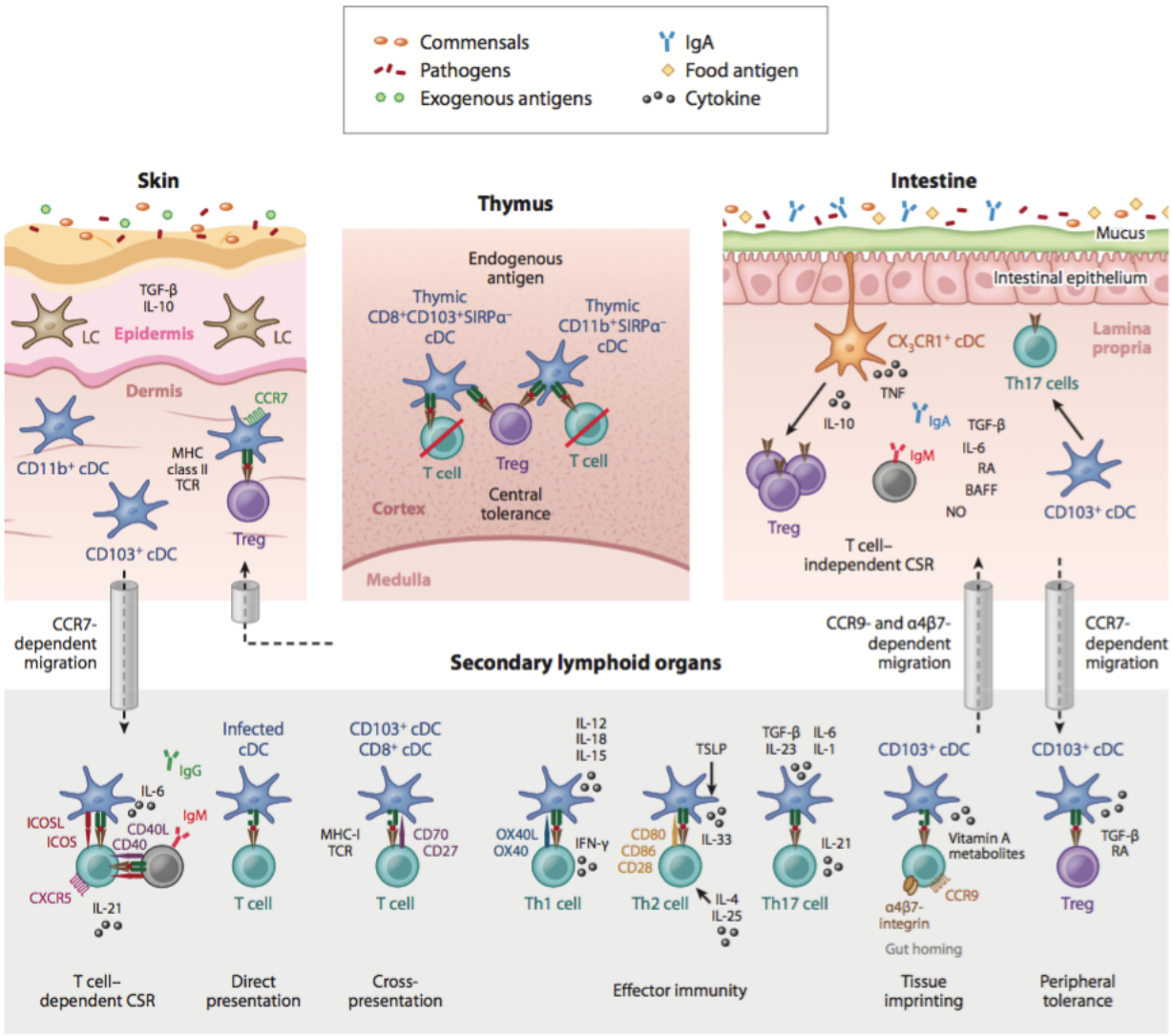

Figure 1-4 Control of innate and adaptive immune system by DCs.
DCs play indispensable roles in regulating adaptive immunity in both lymphoid and non-lymphoid tissues. DC subsets are not only important in capturing and presenting exogenous antigens to T cells to drive T cell responses, they can also capture non-harmful antigens such as commensal antigens, food antigens or environmental antigens and transport them to local draining lymph nodes in a CCR7-dependent manner to mediate peripheral tolerance. In addition to peripheral tolerance, the $CD11b^+$ DC subset from non-lymphoid tissues are known to carry antigens derived from peripheral sites and migrate to the thymus to mediate negative selection of developing thymocytes. Figure adapted from Merad *et al. Annual Review of Immunology*. 2013 with permission from Annual Reviews Immunology [88].

1.1.8.3 Macrophages

Macrophage, or big-eater in Greek, consist of a hetrogeneous population of monocyte-derived phagocytic cells. In steady state, macrophages are found throughout the body where they reside as tissue resident macrophages [71, 95]. Macrophages are classified by their surface expression of macrophage colony stimulating factor receptor (M-CSFR) [71] and adhesion molecules CD11b. Other surface markers such as F4/80 and Ly6C are used to further define macrophage populations in mice [73, 95]. Macrophages are known to play dual roles throughout the process of inflammation both as inflammation promoter and suppressor and are linked to the pathogenesis of autoimmunity [96] as well as failed tumor surveillance [97].

At the beginning of an inflammatory event, monocytes are recruited to the site via chemokines and differentiate into a pro-inflammatory phenotype known as M1 macrophages [98]. M1 macrophages become activated when their PRRs detect PAMPs or DAMPs, and in response secrete a variety of pro-inflammatory cytokines such as IL-12, TNF-α and super oxide radicals to initiate the inflammation cascade. These pro-inflammatory cytokines and mediators then exert their actions either through lymphocyte recruitment and differentiation or direct killing of the pathogens to facilitate the clearance of the source of inflammation. When the pathogen or source of inflammation is cleared, detection of apoptotic effector cells by M1 macrophages repolarize them into a healing phenotype known as M2 macrophages [99]. M2 macrophages can facilitate wound healing by secreting key immunoregulatory cytokines IL-10 and TGF-β to suppress immune cell function and promote tissue repair by stimulating the recruitment and differentiation of fibroblasts [100-102].

1.1.8.4 NK cells

Natural killer cells (NK cells) are a lineage of lymphoid cells that provide quick response to eliminate virally infected cells, stressed cells, or malignant cells using similar cytolytic mechanisms found in T cells but doing so without using rearranged antigen receptors like T cell receptor or B cell receptors [103-105]. Unlike T cells, which require priming and expansion phases to acquire cytolytic capacity, NK cells can readily lyse target cells that are stressed or lacking surface expression of MHC, earning the nickname "natural killer cells" [106].

The cytolytic activities of NK cells are regulated by a balance of activating (to kill) and inhibitory (not to kill) signals transduced by several families of NK cell surface receptors [107]. Activating receptors such as NKG2D and Ly49H can recognize stress molecules or virus encoded proteins on the cell surface and signal the NK cell to kill the target cell. Conversely, inhibitory receptors such as KIR or KLRG1 can recognize surface expression of MHC class I or E-cadherin [108] and signal to prevent the NK cell from killing the target cell. When the combined signal strength of the activating signal is greater than the inhibitory signal, NK cells can lyse target cell using cytolytic molecules granzyme and perforin as well as cytokines of the likes of interferon gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α). In addition to killing target cell, cytokines such as IFN-γ and granulocyte macrophage colony stimulating factor (GM-CSF) and CCL family chemokines secreted by NK cells can also help to recruit and activate innate and adaptive immune cells. Although initially NK cells were thought to mediate their cytolytic activity without the help of immunoglobulins [109], a later study showed that NK cells can also kill target cells coated with immunoglobulin [110]. This antigen dependent mode of killing called antigen-dependent cell-mediated cytotoxicity (ADCC) is mediated by an antibody

receptors CD16 (FCRγIIIA), which recognize the Fc region of the IgG antibody and signal through CD3ζ to mediate their killing activity [106].

Tumor immune surveillance is an arms race between the immune system and the tumor. Tumor-associated antigens generated by abnormal genetic recombination events during malignant transformation provide important epitopes for T cells to recognize. To evade T cell mediated tumor surveillance, tumor cells often evolve to downregulate surface expression of MHC molecules [111]. NK cells can provide protection against MHC downregulating tumors when T cells are rendered ineffective. Aside from killing of stressed, virally infected or malignant transformed cells, emerging evidences also show that NK cells may also participate in the pathogenesis of autoimmunity, contributing to the exacerbation or protection of diseases [112].

1.1.9 Adaptive immune system

The adaptive immune system is a more recent addition of the immune system found only in vertebrates. Unlike the innate immune system, adaptive immunity evolved to recognize a considerably larger number of non-self-molecules using their antigen receptors. Upon recognition of microbial antigens, the adaptive immune system can mount antigen specific immune responses directed against the microbial threat without harming healthy tissues. After the threat is neutralized, a small portion of antigen-specific adaptive immune cells differentiates to become memory cells.

The adaptive immune system is comprised of several types of highly specialized cells, which can respond to incoming microbial insults in conjunction with the innate immune system to confer long-lasting immunity. Two critical cellular components of the adaptive immune

system were first discovered by Cooper and Good in 1965 in chickens [113]. When Cooper performed thymectomy or surgery to remove the bursa of Fabricius in chickens, they found that cells derived from the thymus, which they named T cells, are important for skin graft rejection [114, 115], while cells derived from the bursa of Fabricius (which he named B cells) are important in producing antibodies, mediating what is now known as the humoral response [116].

Adaptive immunity, sometimes called acquired immunity, differs from the innate immunity in several ways. The most striking feature of the adaptive immune system is its remarkable specificity. Unlike the innate immune system, which relies on PRRs to recognize and respond to pathogens in a non-specific way, the adaptive immune system relies on a set of antigen receptors (T cell receptors on T cells, and B cell receptors on B cells) to confer antigen-specific immunity towards pathogens and or environmental irritants [117, 118]. Another unique aspect of the adaptive immune system that sets it apart from the innate immune system is its ability to form immunological memory after the initial encounter with a pathogen. The immunological memory allows the adaptive immune system to "remember" molecular patterns of a specific pathogen, enabling the adaptive immune system to respond the same pathogen in an even faster, more antigen specific, and in an even more rigorous manner with each successive encounter, even when the time in between the encounters is years apart. This unique feature of antigenic memory is the basis upon which modern day immunization protocols are built [119, 120].

1.1.10 T cell development

T cell development is a series of maturation steps that occur in the thymus where bone marrow-derived lymphoid progenitors develop into functional T cells expressing T cell defining

features such as CD3, CD4, CD8 and the TCR [121, 122]. It is also the site where nascent T cells receive thymic education, allowing them to differentiate self from non-self once they fully develop into mature T cells and exit the thymus. The importance of the thymus in T cell development is illustrated in DiGeorge syndrome patients [123], and in thymic epithelium deficient mice [124]. In both cases, the thymus is either missing (in patients with DiGeorge syndrome) or has serious defects (nude mice), and both result in T cell deficiency.

The thymus is comprised of two lateral lobes situated right above the heart within the thoracic cavity. Each of the lobes, surrounded by a capsule, is comprised of cortical and medullary areas marked by specialized stromal epithelial cells. When lymphoid progenitors enter into the subcapsular cortical area, they become committed to the T cell fate and lose their ability to differentiate into B cells or NK cells [125]. Starting from the cortical areas, the T cell progenitors receive signals from thymic epithelial cells (TECs) to begin the maturation processes, and migrate towards the medullary area as the developmental proceeds [125, 126]. During the early stages of T cell development, immature T cells lack the expression of TCRs as well as CD4/ CD8 co-receptors, and are termed double negative (DN) T cells. While both αβ and γδ T cells can arise from the DN population, the majority of the DN T cells go on to become αβ T cells.

The DN T cells can be further divided into four distinct developmental stages based on the surface expression of CD25 and CD44 (DN1: $CD25^-$ $CD44^+$; DN2: $CD25^+$ $CD44^+$; DN3: $CD25^+$ $CD44^-$; DN4: $CD25^-$ $CD44^-$), allowing researchers to chronicle key T cell developmental events which occur at each of the stages [127]. The first key event occurs between DN2 and DN4, where nascent T cells begin rearranging their TCRβ chain loci to produce a TCRβ chain. The TCRβ chain then pairs with a surrogate pre-TCRα chain to produce the pre-TCR [128]. The

newly formed pre-TCR is then tested against self-peptide:MHC complexes located on the thymic cortical epithelial cells (cTEC). Nascent thymocytes that are able to receive signal from their pre-TCR then progress to the double positive (DP) stage characterized by the expression of both the CD4 and CD8 co-receptors. During the DP stage, one of the TCRα chain loci is rearranged to produce a TCRα chain, replacing the pre-TCRα chain in pairing with the TCRβ chain to form an αβTCR. Thymocytes with newly arranged αβTCRs can then undergo positive or selection in the thymic cortex region but can also occur when the nascent thymocytes migrate into the medullary area. Negative selection of the αβ TCR expressing DP thymocytes against tissue specific antigens is thought to occur in the medullary area where they interact with AIRE expressing medullary thymic epithelial cells (mTEC) and thymic APCs bearing self-peptide loaded MHC I or II molecules. The DP thymocytes that survive selection then commit to either CD4 or CD8

single positive (SP) lineage depending on which class of MHC molecule the TCR can establish a functional interaction with.

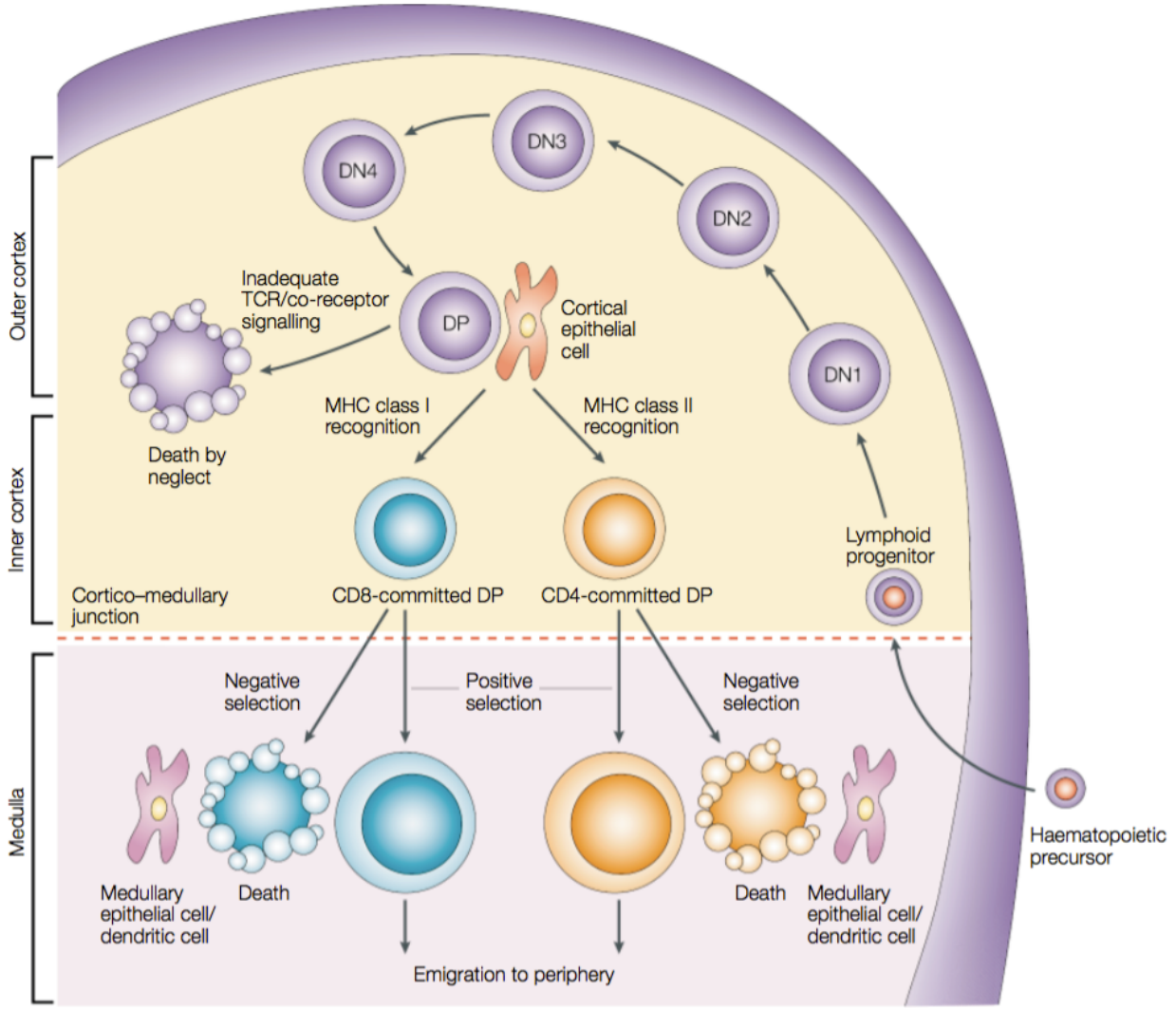

Figure 1-5 Overall schematic of T cell development and lineage commitment in the thymus. The goal of thymic development is to generate a functionally diverse, self-restricted yet self-tolerant T cell population through a series of developmental steps which occur within the thymus. To accomplish this goal, T cell progenitors first migrate into the thymus from the bone marrow and commit to the T cell lineage. At this point, these thymocyte progenitors express neither CD4 or CD8, and are called double negative thymocytes (DN, CD4- CD8-). DN thymocytes can be further subdivided into 4 developmental stages based on their surface expression of CD25 and CD44 (DN1, $CD44^+$ $CD25^-$; DN2, $CD44^+$ $CD25^+$; DN3 $CD44^-$ $CD25^+$; and DN4, $CD44^-$ $CD25^-$). During the DN stages, nascent thymocytes begin genetic rearrangement of the T cell receptor (TCR) genes to generate functional TCRα and TCRβ chains, which together form the TCR. One of the two TCRβ chain gene loci is first rearranged to produce a functional TCRβ chain. Upon successful TCRβ chain gene rearrangement, the newly

form TCRβ chain is then paired with a surrogate pre-TCRα chain, forming a pre-TCR complex. Thymocytes that can signal through the pre-TCR are then induced to undergo proliferation, expanding greatly in numbers and transition into the double-positive stage (DP, $CD4^+$ $CD8^+$). During this process, one of the TCRα chain gene loci is rearranged, producing a TCRα chain to replace the pre-TCRα chain, and pair with the TCRβ chain, forming a TCR. The newly formed TCR on the DP thymocytes then undergo positive and negative selection by interacting with self-peptide bearing MHC class I and MHC class II molecules expressed by thymic epithelial cells and thymic APCs. During positive and negative selection, thymocytes that are unable to interact with self-peptide-MHC molecules are instructed to die by apoptosis or undergo further TCR rearrangement. Thymocytes that interact too strongly with self-peptide-MHC molecules are instructed to die by apoptosis, undergo further TCR rearrangement or commit to T regulatory cell fate. Only thymocytes that are weakly reactive to self-peptide-MHC molecules can mature and transition into the CD4 or CD8 expressing single positive stage (SP, $CD4^+$ or $CD8^+$) and exit thymic development into circulation. Figure shown is adapted from Germain RN. *Nature Reviews Immunology*. 2002 with permission from the Nature Publishing group [125].

1.1.10.1 TCR signal transduction

The signal transduced by the TCR complex during TCR:pMHC interaction is essential for many aspects of T cell biology. During resting state, the weak tonic signal transduced by TCR self-peptide-MHC interaction is critical for T cell survival [129]. During T cell activation, TCR signaling play a major role in determining whether said T cell becomes anergized, activated, or dies by activation-induced cell death. TCR signaling also plays an indispensable role in cytoskeletal reorganization and polarization, enabling effector T cells to secrete cytokines and conduct contact-dependent killing of target cells [130].

The TCR lacks intrinsic kinase activity and relies on the recruitment of SRC kinase family members such as LCK and FYN to transduce signal during TCR:pMHC interaction [131]. Upon a strong interaction between the TCR and the pMHC molecule located on an APC, the TCR complex, comprised of TCR, the CD3 co-receptor, the CD3 associated ζ chain, is brought together with the CD4 or CD8 co-receptor, forming an immunological synapse. SRC kinase family member LCK [132], which is known to constitutively associate with the cytoplasmic tail of the CD4 or CD8 co-receptor [133, 134], then phosphorylate the immunoreceptor tyrosine-base

activation motifs (ITAMs) located on the cytoplasmic tails of the CD3 co-receptor components and the ζ chain, facilitating the recruitment of Zeta-chain-associated protein kinase 70 (ZAP70) to the TCR complex via its SH2 domain [135]. Recruited ZAP70 molecules are then phosphorylated and activated by LCK, allowing the activated ZAP70 molecules to recruit, and phosphorylate linker for activation of T cells (LAT) protein [136]. Phosphorylated LAT then acts as an adaptor protein to recruit key signaling molecules such as GRB2 [137], phospholipase Cγ (PLC-γ)[138], and GADS [139] to the TCR complex and initiate signal transduction down several major signaling pathways. The TCR signals transduced via the Ras and PLC-γ pathway lead to activation of the protein kinase Cθ (PKCθ) pathway [138], the PI3 kinase pathway, the AKT pathway [140], and calcium mobilization [141] resulting in T cell activation, cytokine secretion and T cell survival.

1.1.10.2 T cell mediated response

Nascent T cells that newly migrated from the thymus are called recent thymic emigrants. Recent thymic emigrants are phenotypically and functionally semi-mature and require approximately 3 week before they become naïve T cells [142]. Naïve T cells are functionally mature but antigen-inexperienced as they possess the potential to exert effector functions, but do not have the capability yet. To acquire effector function, naïve T cells must go through a series of activating steps during which signaling from multiple pathways converge to drive the proliferation and differentiation of naïve T cells into effector T cells. These series of events can be broken down into three phases: T cell priming phase [143, 144], contraction phase [145, 146], and memory forming phase [147, 148]. The T cell priming phase encompasses the first series of activating events which lead to T cell proliferation and differentiation. During this phase, signals

received by naïve T cells from various pathways converge into 3 major classes of signals: signal 1, signal 2 and signal 3. Only after receiving these three signals can naïve T cells proliferate and differentiate into effector T cells. Missing one or more signals can result in the naïve T cell becoming antigen unresponsive (anergy) [149-151].

Signal 1 is the signal received by a T cell through its TCR when the TCR can interact strongly with pMHC complexes on APCs. After emerging from thymic development, nascent T cells home to peripheral lymph nodes [152] where they survey the surface of APCs using their TCR for their cognate antigen in the form of pMHC complexes. APCs such as DCs, macrophages, B cells within lymph nodes are thought to be the primary sources of signal 1. In addition to professional APCs, activated epithelial cells and endothelial cells can also provide naïve T cells with signal 1 [153].

Signal 2 is the co-stimulatory signal received through the engagement of co-stimulatory molecules such as CD2, CD28, CD40 and inducible costimulator (ICOS) on the T cell surface with their respective partners on APCs [154] or CD4 T helper cells [155]. After detecting the presence of PAMPs and/or DAMPs through PRRs, APCs upregulate cell surface expression of co-stimulatory molecules such as CD80/86 [156] or CD48 [157]. These co-stimulatory molecules can then interact with CD28 or CD2 on T cell surface respectively, allowing additional activating signals to be sent into the T cell in conjunction with signal 1 and drive T cell proliferation. Upon receiving signal 1 and signal 2, a naïve T cell undergoes a rapid and robust phase of proliferation known as clonal expansion, which is critical for the control and clearance of the infection. During this stage, the number of antigen specific T cells can double every 4 to 6 hours, reaching a peak cellular concentration several orders of magnitude greater approximately a week after activation [158-160]. The clonal expansion phase is also the phase

where T cell fate is decided under the guidance of signal 3. Signal 3 is the signal received through cytokines. Cytokines secreted by APCs and other immune cells help direct the differentiation of these T cells into different effector cell fates in a context-dependent manner (a detailed description of how cytokines secreted by APCs can affect the differentiation of T cells can be found in sections 1.1.2, 1.1.9.6 and 1.1.9.7).

After clearing the infection, the effector T cell population begins to undergo apoptosis in a phase known as the contraction phase. During this phase between 90-95% of the effector T cells will die by apoptosis, the remaining 5-10% then transition to become memory T cells [144, 146]. Memory T cells are maintained by the joint actions of IL-7 and IL-15 [161] and can stay in circulation years after the infection to provide lasting protection against the same pathogen. Should these memory T cells encounter the same pMHC signature, memory T cells can readily exert effector function without having to go through the priming phase while undergoing clonal expansion to provide an effective T cell response against the infection [162-165].

1.1.10.3 TCR gene rearrangement and TCR repertoire diversity

A diverse TCR repertoire, defined as the number of different antigen specific TCRs that an organism possess, is the key to T cells' ability to respond to a seemingly endless number of antigens to confer immunity. A majority of the T cells within the body is comprised of T cells bearing the TCRαβ chains. The genetic rearrangement of the human αβTCR loci can produce approximately 1×10^{15} possible TCR αβ chain combinations, with 25×10^{6} different αβ TCRs being detectable in circulation at one time [166]. The astonishing TCR diversity is achieved by utilizing a set of specialized DNA recombination machinery comprised of recombination activated gene 1 and 2 (RAG1 and RAG2) [167-169], DNA-dependent protein kinase (DNA-PK)

[170], Artemis nuclease [171], terminal deoxynucleotidyl transferase (TdT) [172], X-ray repair cross-complementing protein 4 (XRCC4) [173], and DNA ligase 4 [174]. These enzymes are upregulated during early stages of T cell development and work in concert to randomly recombine segments of the TCRα/β chain loci into unique coding sequences, leading to their expression on the cell surface.

The TCRβ chain gene locus is organized into four regions: V (variable), D (diversity), J (joining) regions and C (constant) region. For human TCRβ chain gene, the Vβ region is comprised of approximately 47 different Vβ coding segments, the VD region is comprised of 2 Dβ coding segments, the J region is comprised of 13 Jβ coding and the C region is comprised of two Cβ coding segments [166]. During T cell development between stages DN2 to DP, recombinases RAG1/2 are expressed in developing T cells, which can recognize and bind to special DNA sequences called recombination signal sequences (RSS) located before and after each of the coding segments of Dβ, and Jβ and at the 3' end of the V segments. To rearrange the TCRβ chain loci the RAG1/2 recombinase first bind to the RSS sequences at the 3' end of a random D segment and the 5' end of a random J segment, and make cuts at the two sites. The excised intervening piece is ligated to form a piece of circular DNA and discarded. The D and J strand is joined by the combined action of DNA-PK, Artemis, XRCC4 and DNA-ligase 4.

Terminal deoxynucleotidyl transferase contributes to the diversity of the D and J junction by randomly adding in nucleotides between the D segment and the J segment, producing a new DNA sequence with high variability. The newly joined DJ segment is then joined with a random V segment using the same mechanism, producing a TCRβ chain gene comprised of randomly joined VDJ segments and a TCRβ chain with a unique amino acid sequence. A successful VDJ joining event allows the expressed TCRβ chain to pair with the pre-TCRα chain, forming the

pre-TCR. Signal transduced by the pre-TCR then stops the rearrangement process on the other TCRβ chain locus from happening in a process known as allelic exclusion. To date, it is thought that TCR signaling feedback inhibition and epigenetic mechanisms both contribute to the tight regulation of TCRβ allelic exclusion [169].

After the successful rearrangement and expression of the pre-TCR, the same genetic recombination machinery is used to randomly recombine the TCRα chain loci, which in humans is known to contain 42 Vα segment and 61Jα segments, producing a TCRα to pair with the TCRβ chain replacing the pre-TCRα chain. TCRα chain allelic exclusion is not as tightly regulated as the TCRβ chain, which may result in the simultaneous rearrangement of both TCRα chain loci, and the expression of two different TCRα chains on the same thymocyte [175, 176].

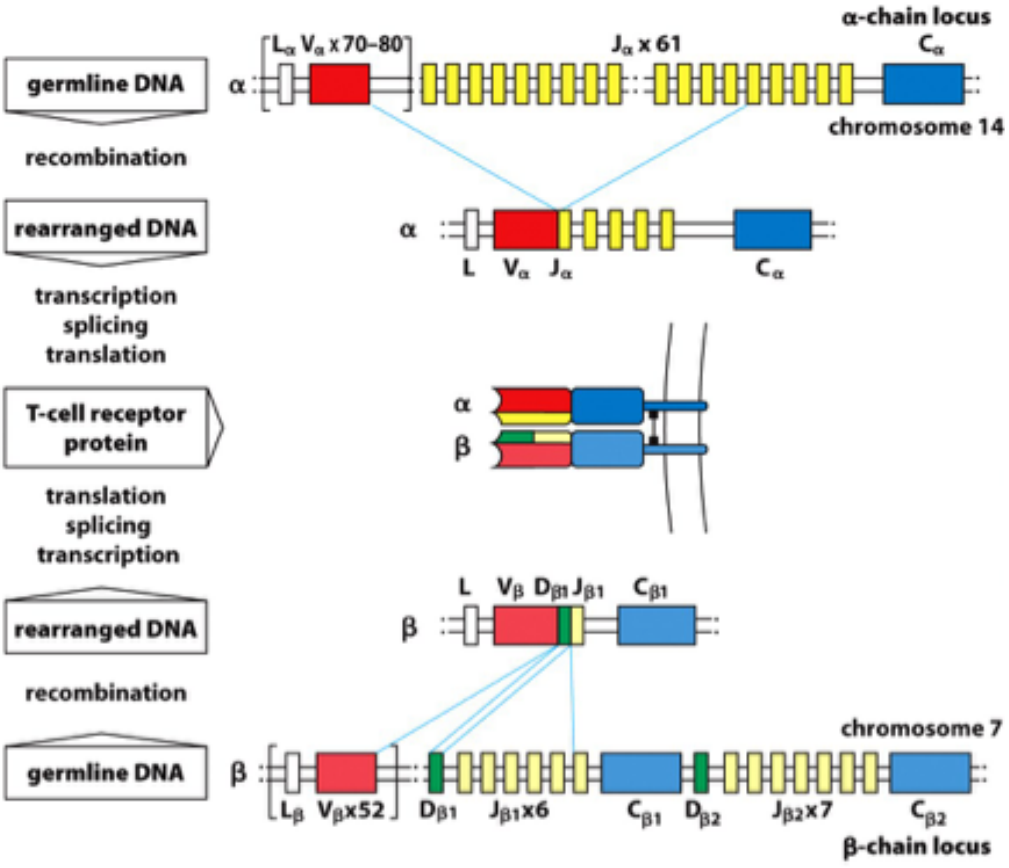

Figure 1-6 Overall schematic of TCRα and TCRβ chain gene rearrangement.
The TCRβ and TCRα genes are comprised of distinct gene segments, which are randomly selected and joined together by somatic recombination during thymic development to produce functional TCRβ and TCRα chains. The TCRβ chain gene cluster contain Vβ, Dβ, Jβ segments, whereas the TCRα chain gene cluster contain only Vα and Jα segments. During thymic development, random Dβ, and Jβ segments are joined together forming the DJ gene segment. The DJ gene segment is then joined together with a randomly selected Vβ segment forming a complete VDJ segment. The VDJ segment then is joined with the Cβ (constant) segment to produce a complete TCRβ chain gene. The TCRα chain gene cluster is arranged in similar ways except there is no Dα and Jα joining due to the lack of Dα clusters in the TCRα gene cluster. Randomly selected Vα and Jα segments are joined together to form a VJ segment, which is then joined with the Cα segment to form a complete TCRα gene segment. The TCRβ gene is arranged first during thymic development to produce a TCRβ chain, which then pairs with a TCRα chain to form an αβ TCR heterodimer. Figure reprinted with permission from Garland Science. Janeway. *Immunobiology: The immune system in health and disease.* Current Biology Publications.

1.1.10.4 Thymic selection and central tolerance

Through random rearrangement of TCR genes, the thymus can produce an incredibly diverse population of T cells with antigen receptors capable of responding to a wide spectrum of foreign antigens, and confer immunity. This random approach however, can also lead to the generation of T cells bearing non-functional TCRs or worse yet, autoreactive TCRs. To remedy these drawbacks, the immune system has evolved a collection of mechanisms called thymic selection that it relies upon to induce central tolerance. Central tolerance is described a series of events that occur during T cell development where developing T cells are educated to recognize MHC molecules and self-peptides, so that T cell response can be directed towards foreign threats and away from healthy tissues [177, 178]. Specialized self-peptide presenting thymic APCs, and the TCR signaling play indispensable roles during thymic selection [125, 179, 180].

Thymic selection can be subdivided into two parts: positive selection and negative selection. During positive selection, DP thymocytes, with newly rearranged TCRs emerged from the DN stages, and migrate to the cortical-medullary junction where they interact with cortical thymic epithelial cells (cTEC) [181]. cTECs are specialized thymic epithelial cells expressing self-peptide loaded MHC class I or class II molecules, which play an important role in testing the TCRs of the DP thymocytes for their ability to recognize self-peptide-MHC complexes. DP thymocytes with TCRs that are able to interact weakly with the self-peptide-MHC complexes receive survival signal through their TCR, and migrate into the medullary areas to undergo negative selection. DP thymocytes that are unable to interact with self-peptide-MHC complexes either undergo the genetic rearrangement process again to produce a new TCR and re-enter into positive/negative selection or die by apoptosis [125, 177, 181, 182]. In addition to the selection of DP T cells that are able to interact with self-MHC, the CD4/CD8 lineage commitment is also

determined during positive selection. DP thymocytes that are able to interact with self-peptide loaded MHC I molecules utilizing TCR and CD8 co-receptor commit to the CD8 lineage fate. Similarly, DP thymocytes that are able to interact with self-peptide loaded MHC II molecules utilizing TCR and CD4 co-receptor commit to the CD4 lineage fate [125, 183].

Negative selection, otherwise known as clonal deletion is perhaps the most crucial step of T cell development where DP or SP thymocytes with high affinity self-antigen specific TCRs are eliminated to induce central tolerance and prevent autoimmunity [125, 180, 184, 185]. Negative selection is mediated by APCs in the thymus including thymic DCs [78] and thymic epithelial cells both in the cortical and medullary compartments. Together, these APCs present self-peptides loaded on MHC I or II molecules to DP or SP thymocytes to mediate negative selection and induce central tolerance to ubiquitous self-antigens. The mTECs play a central role in mediating negative selection and tissue-specific antigen tolerance through the expression of a key transcription factor, autoimmune regulator (AIRE). AIRE is primarily expressed in the mTECs [186], which permits the etopic expression and presentation of tissue-specific antigens (TSA), such as insulin [187, 188] that are otherwise not expressed in the thymus epithelium [186, 189]. The ectopic expression and presentation of TSAs to nascent thymocytes not only allows the elimination of high affinity TSA-specific thymocytes but also promotes the generation of TSA-specific T regulatory cells [190, 191]. Thymic DCs, though lacking the expression of AIRE can also partake in TSA-mediated negative selection indirectly by picking up TSA-containing apoptotic bodies from dead mTECs, and presenting the TSA peptides on their own MHC molecules [192].

The affinity of a TCR for self-peptide loaded MHC molecules on TECs is a key determining factor in the outcome of negative selection. As the TCRs are randomly generated by

chance through genetic recombination, most of the thymocytes undergoing negative selection have TCRs that possess little to no affinity towards self-peptide-MHC molecules. Thymocytes that fail to receive signal through their TCRs during thymocyte selection are neglected and die by apoptosis. Thymocytes with high affinity self-peptide specific TCRs are thought to be autoreactive and may adopt several cell fates upon interacting with self-peptide-MHC molecules. For the most part, thymocytes with high affinity TCRs are instructed to die by apoptosis [193], while some upregulate genetic rearrangement machinery to produce a new TCR [182]. Some may enter into a state of antigen non-responsiveness known as anergy [194], and lastly some differentiate into regulatory T cells [195, 196]. These thymus-induced T regulatory cells (nTreg) then exit the thymus, and exert their suppressive function in the periphery on other self-antigen reactive cells and contribute to peripheral tolerance [180, 197]. In the end of negative selection, the only thymocytes that are permitted to exit the thymus as mature T cells are thymocytes with TCRs that possess weak affinity towards self-peptide-MHC molecules.

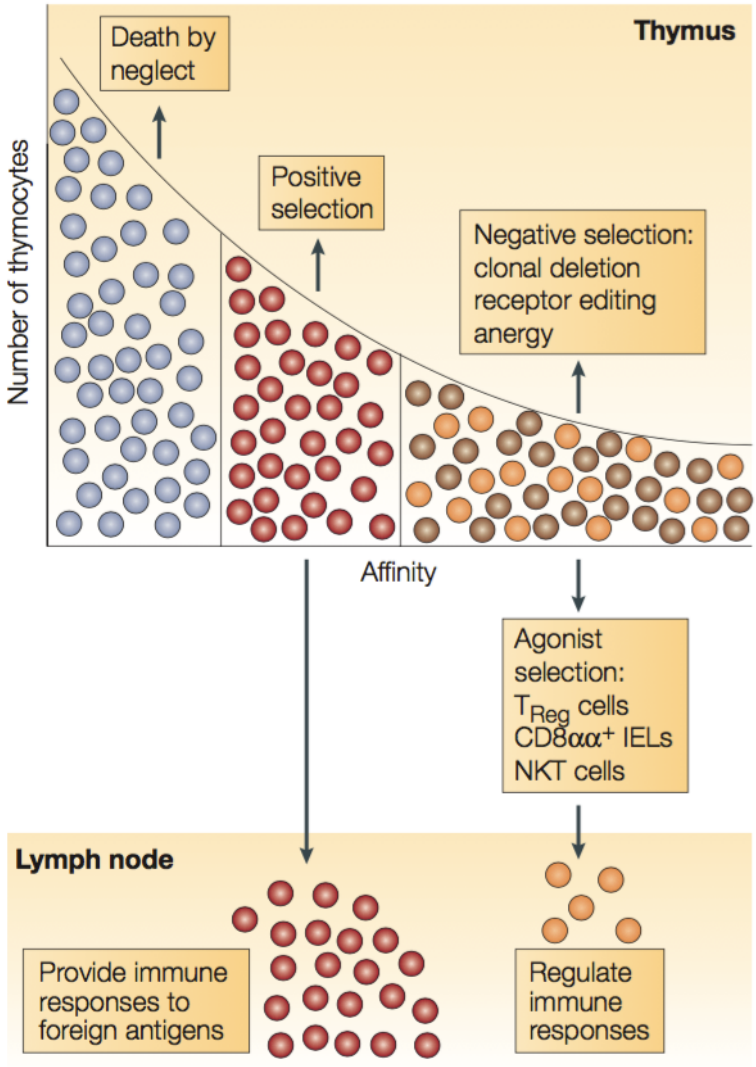

Figure 1-7 The affinity model of central tolerance.
The fate of developing thymocytes undergoing positive and negative selection is determined by the affinity of their TCR towards self-peptide-MHC molecules located on thymic APCs and thymic epithelial cells. The majority of thymocytes bear TCRs that possess little to no affinity towards self-peptide-MHC molecules. Since they cannot receive survival signal through their TCR, they die by neglect. Thymocytes that express TCRs which interact too strongly with self-peptide-MHC molecules are thought to be autoreactive, and are instructed to die by apoptosis (negative selection), undergo receptor editing, becoming anergic or differentiating into T regulatory cells. Some thymocytes that express strongly self-reactive TCRs are also known to differentiate into NKT cells or CD8αα^{+} intestinal epithelial lymphocytes (IELs). The molecular determinant which commit high affinity T cells to T regulatory cell fate instead of apoptosis, anergy, or receptor editing is currently undetermined. Figure adapted from Hogquist *et al. Nature Reviews Immunology.* 2005 with permission from Nature Publishing Group [180].

1.1.10.5 T cell subsets

αβT cells can be divided into two major subsets based on their expression of the CD4 or CD8 co-receptor. T cells that express the CD8 co-receptor are known as cytotoxic lymphocytes (CTL) or T cytotoxic (Tc) cells. CD8 T cells recognize peptide presented on MHC class I, and confer immunity against intracellular pathogens and malignant cells through cell:cell contact. CD4 T cells on the other hand, play a largely supportive role in the adaptive immune system, and are often referred to as T helper (Th) cells. Upon recognizing peptides presented on MHC class II molecules, they secrete important cytokines critical for the function and differentiation of other immune cell types [198].

1.1.10.6 CD4 T cells

CD4 T cells are termed "T helper" cells because of the critical supportive role they play to other immune cells [199]. $CD4^+$ T cells play important coordinating roles in the immune system to fine tune immune responses against different types of microbial pathogens, as well as malignant cells [200]. For phagocytic cells such as macrophages, IFN-γ produced by CD4 T cells upon recognition of microbial antigens are essential for the induction of the respiratory burst in macrophages, which is critical for the destruction of intracellular pathogens [201]. Furthermore, the activating signal sent by CD40 ligand (CD40L) expressed on activated CD4 T cells, to CD40 on APCs such as DCs macrophages and B cells help their antigen presentation and co-stimulation capacity [155]. For CD8 T cells, signals provided by CD40:CD40L interaction is essential for CTL priming [155].

In addition to coordinating immune responses, a subset of CD4 T cells also play an indispensable role in mediating antigen specific tolerance and tissue repair [196, 202, 203]. CD4

T cells can be further divided into several subsets based on the expression of their lineage determining transcription factors and the cytokines that they secrete upon TCR engagement.

Naïve CD4 T cells are CD4 T cells that have not seen their cognate antigens. They are characterized by their lack of the ability to secrete cytokine upon cognate antigen encounter. When a naïve CD4 T cell encounters its cognate antigen presented on the MHC II molecule of a professional APC for the first time, it starts to proliferate and differentiate into different effector CD4 T cell lineages by upregulating lineage determining transcription factors depending on local cytokine milieu. The resulting effector CD4 T cells, driven by their transcriptional program then secrete different sets of effector cytokines to coordinate different types of immune responses against a variety of pathogens. Cytokine support from effector CD4 T cells is crucial not only for the functioning of the antibody mediated humoral response, cell mediated cytotoxic response, but also for the activation of phagocytes, recruitment of innate lymphocytes and establishment of tolerance [200].

To date there are several CD4 T cell subsets that can be found *in vivo* or differentiated *in vitro* by supplementing the cell culture with cytokines [200]. T helper 1 (Th1) cells are one of the first CD4 Th lineages described [204, 205], and consequently one of the most well characterized Th subsets. The Th1 lineage is essential for clearance of various intracellular pathogens as well as malignant cells [206-208] through the action of signature cytokines IFN-γ, TNF-α, and IL-2 [206]. The Th1 transcriptional program is regulated by lineage-determining transcription factor Tbet [209] and cytokines IL-12 and IFN-γ [200]. When naïve CD4 T cells (Th0) receive signals through their TCR, Tbet is induced and results in the secretion of small amounts of IFN-γ. The secreted IFN-γ can act in an autocrine or paracrine manner on Th0 cells to further upregulate the expression of Tbet through IFN-γ receptor and STAT-1 signaling should the local concentration

of IFN-γ be greater than that of IL-4. Upregulated Tbet then in turn suppresses the expression of the Th2 lineage-determining transcription factor GATA-3 while driving the expression of more IFN-γ, thereby polarizing the Th0 cell to the Th1 cell lineage. IL-12 secreted from activated DCs can also assist in the polarization of Th1 cells through IL-12 receptor signaling, which activates transcription factor STAT-4 to promote IFN-γ secretion [210].

The IL-4 secreting Th2 lineage is the other early characterized Th cell lineage besides Th1 [204, 205]. Th2 cells, with their cytokines IL-4, IL-5 and IL-15, are central to humoral immunity, parasite expulsion and allergic response [200]. IL-4 secreted by Th0 cells as well as Th2 cells, polarized macrophages and DCs is critical for the differentiation of Th2 lineage. IL-4 signaling through the IL-4 receptor activates transcription factor STAT-6, which promotes the secretion of Th2 signature cytokines and Th2 lineage determining transcription factor GATA-3. GATA-3 in turn promotes the expression of Th2 cytokines, while suppressing the expression of IL-12 receptor β chain and STAT-4 to inhibit the Th1 transcription program [210, 211].

Regulatory CD4 T cells (Tregs) that express the master transcription factor FOXP3 are essential for peripheral tolerance to self [196] and environmental antigens [202]. The importance of this cell lineage is highlighted by findings that FOXP3-deficiency results in a fatal multi-organ autoimmunity in both human and mice [212, 213]. FOXP3-expressing Treg cells can be generated in the thymus or in the periphery [214-217]. The expression of high affinity IL-2 receptor CD25 is a key feature of Treg cells in addition to immune suppressive cytokines. CD25 is thought to act as a sponge to sequester IL-2 and prevent proliferation of other T cell subsets. Several cytokines are known to drive the differentiation of Treg cells. IL-2 (which signals through the IL-2 receptor) and STAT-5 are indispensable for the maintenance of Treg function

[218]. TGF-β which signals through the TGF-β receptor and activates Smad transcription factors, which also play an important role in promoting Treg polarization and function [219].

The IL-17 secreting Th17 lineage is associated with a variety of autoimmune conditions [138, 220-223] but also plays essential roles in the defense against microbial infections [224] and lymphocyte recruitment [225, 226]. The retinoic acid-related orphan receptor-γt (RORγt) is the lineage-determining transcription factor expressed by Th17 cells, which enables Th17 cells to secrete the signature cytokine IL-17 [227]. RORγt is induced by the combined action of TCR, IL-6, IL-21, and TGF-β signaling pathways through downstream transcription factors STAT3, AP-1 and NFAT [228-230], with IL-23 providing the signal to stabilize the expression of RORγt [219, 231]. Although Th17 cells have been shown as a stable lineage of cells, emerging evidence also suggest that Th17 is a plastic population, which may have transitioned from other T cell lineages [223, 232] and may continue to transition into other Th lineages depending on local cytokine milieu [233-236].

T follicular helper (Tfh) cells are a recently described CD4 Th population found within the B cell follicles of secondary lymphoid organs such as the spleen, lymph nodes, and Peyer's patches. Tfh cells express the chemokine receptor CXCR5, which allows them to home to the B cell follicles where they provide help to form B cell germinal centers through Tfh cytokines IL-4, IL-21 and the co-stimulatory molecule CD40 ligand (CD40L) [237, 238]. Unlike Th1 or Th2, which can be induced upon cytokine exposure, Tfh cell differentiation requires a multi-step, multifactoral program involving multiple co-stimulatory molecules and transcription factors. TCR signaling, with contributions from IL-6 and inducible costimulatory (ICOS) signaling pathways, plays an important role in inducing Tfh lineage determining transcription factor Bcl6, during the priming stage of Tfh development. Bcl6 expression then drives the expression of

CXCR5 while downregulating CCR7, allowing the newly primed Tfh cells to leave the T cell zone and migrate to the border of B cell follicles where they interact with antigen-presenting B cells and differentiate into Tfh cells [237].

CD4 T helper cells possess high degree of plasticity, capable of switching cytokine secretion profile by taking cues from local cytokines milieu of the micro-environment [239]. IL-6 and IL-21 are known to polarize Treg cells into Th17 cells [240, 241], and the resulting Th17 cells may be further polarized to Tfh cells [242] or Th1 cells [243] depending on the cytokine signal received. Furthermore, Th1 and Th2 cells can acquire Tfh phenotype and participate in the humoral response by activating and differentiating B cells into plasma cells [244]. These findings highlight the plasticity of Th cells and shed light on how differential contributions from these Th cell subsets may shape the course of an inflammatory response and dictate its outcome in a pathogen-dependent manner.

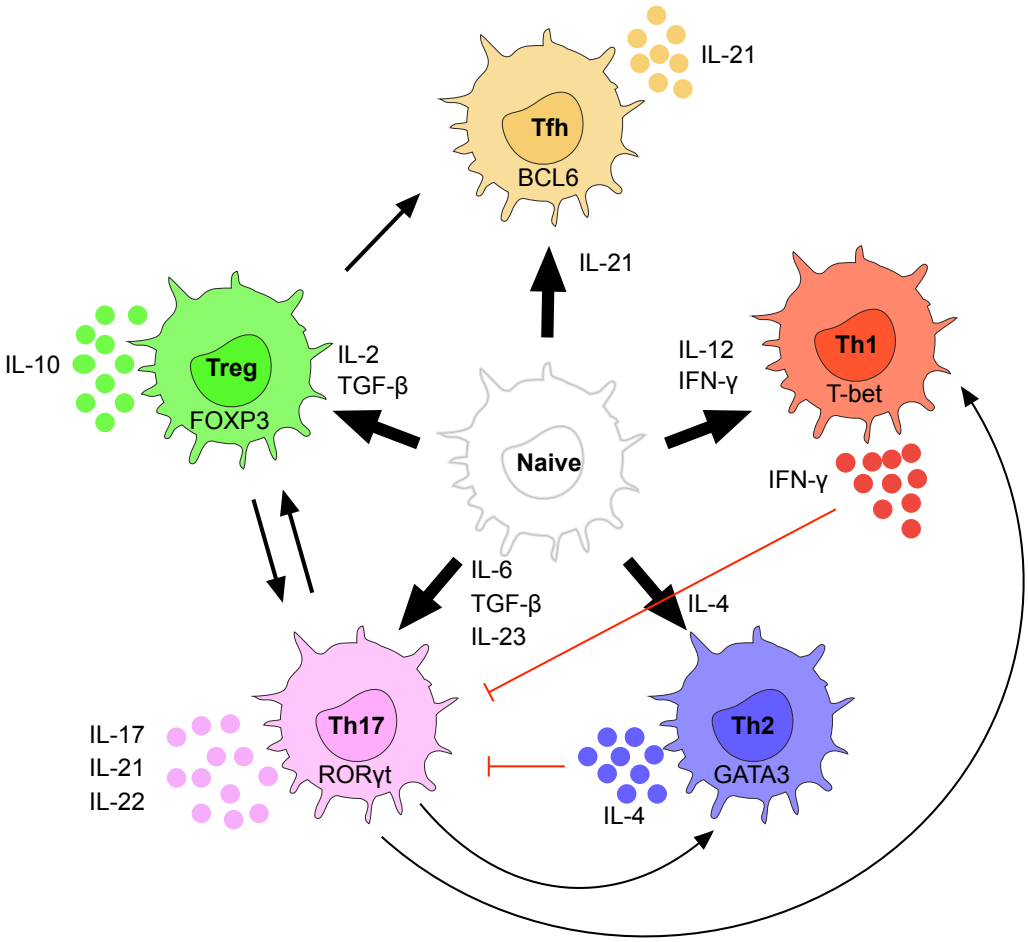

Figure 1-8 An overview of CD4 T cell differentiation and plasticity.
After receiving signal 1 and signal 2 from APCs, naïve CD4 T cells undergo clonal expansion to become effector T cells. During this expansion stage, the local cytokine milieu can influence the lineage commitment of differentiating CD4 T cells by signaling through their respective receptors to induce the expression of lineage-determining master transcription factors such as T-bet, GATA3, RORγt, FOXP3 and Bcl-6. These master transcription factors in turn initiate lineage-specific transcription programs, thereby differentiating these CD4 T cells into their respective lineages. Recent evidence suggest that this lineage commitment is not permanent, and can change as the local cytokine milieu changes.

1.1.10.7 CD8 T cells

CD8 T cells, otherwise known as CTL or Tc are important contributors in immunity and autoimmunity. In the aspect of conferring immunity, CTLs play a critical role in the control of a variety of intracellular pathogens [245, 246], and cancerous cells [247] through their ability to directly kill target cells on contact using secreted cytotoxic molecules and cytokines [248]. In the aspect of autoimmunity, CTLs are known as a major driver for T cell mediated tissue destruction in a variety of autoimmune conditions such as type 1 diabetes [249] and inflammatory bowel disease [250-252].

Analogous to its $CD4^+$ counterpart, naïve CD8 T cells, after receiving activating signals from APCs through their TCRs, can differentiate into several different effector subsets such as Tc1, Tc2, and Tc-17 [253] depending on the local cytokine milieu. IL-2 in combination with IL-12 and type 1 interferons is known to drive the expression of transcription factor T-bet to differentiate naïve CD8 T cells into the IFN-γ secreting CTLs [163, 209]. IL-4, the key cytokine for Th2 differentiation exert similar actions in naïve CD8 T cells, differentiating them *in vitro* in to a IL-4 secreting phenotype [254] possibly through the induction of key Th2 lineage determining transcription factor GATA3 [253]. An IL-17 secreting CD8 subset, known as Tc17 can be found *in vivo* after viral infection or generated *in vitro* using cytokines IL-6, TGF-β and IL-23 [253].

1.1.10.8 NKT cells

Natural killer T (NKT) cells are a NK cell marker-expressing αβ T cell subset that behave like innate immune cells [255] and play roles in autoimmunity [256-259], defense against microbial infections [260, 261] and tumor immune surveillance [262]. One of the defining

features of the NKT cells that sets them apart from conventional T cells is their TCR. Unlike conventional αβ T cells, which express a repertoire of highly variable TCR αβ chains recognizing peptides presented on MHC class I or II molecules, NKT cells express a set of semi-invariant αβ TCR comprised of an invariant α chain and a β chain derived from a limited set of β chain genes, which recognize lipid antigens presented on a MHC like molecule called CD1d [263, 264]. Also unlike conventional T cells, which require activation and proliferation to gain the ability to secrete cytokines, NKT cells possess a memory T cell like phenotype, which allows them to readily secrete a wide array of T cell cytokines such as IL-2, IL-4, IL-17 and IFN-γ upon TCR engagement [85].

NKT cells are generated in the thymus utilizing a process analogous to that of conventional αβ T cells, but using self-lipid loaded CD1d molecules for positive and negative selection instead of self-peptide loaded MHC molecules [265]. Differential expression of the transcription factor promyelocytic leukemia zinc finger (PLZF) induced by TCR signaling [266, 267], in conjunction with Th lineage determining transcription factors T-bet, GATA3 and RORγt, play an important role in NKT cell thymic development, polarizing the developing NKT cells to phenotypes analogous to Th cells [266, 268]. NKT1 cells, expressing Th1 transcription factor T-bet and secreting predominantly IFN-γ, preferentially home to the liver and spleen after emerging from the thymus. NKT2 cells, expressing Th2 transcription factor GATA3, and Th2 cytokine IL-4, preferentially home to the lung, whereas NKT3 cells, expressing Th17 transcription factor RORγt and IL-17, preferentially home to the peripheral lymph nodes [266].

To date, there are two types of NKT cells identified based on their TCRαβ chain genes usage: type I NKT cells are known as classical NKT cells, characterized by their usage of TCRα genes Vα14-Jα18 (mice), Vα24-Jα18 (humans), and TCRβ genes Vβ8.2, Vβ7, Vβ2 (mice),

Vβ11 (humans) as well as their affinity towards α-galatosylceramide (α-GalCer) loaded CD1d molecules. TCRαβ chain genes usage in type II NKT cells in contrast, is much more diverse than that of type I NKT cells, and does not possess high affinity TCR towards αGalCer loaded CD1d but instead towards self-lipid (sulfatides) loaded CD1d molecules [269, 270].

The first NKT cell ligand identified is a naturally occurring glycolipid called alpha galatosylceramide (α-GalCer). Although α-GalCer is derived from marine sponges, its structural similarity to cognate NKT cell ligands allows it to mimic their action and potently activate NKT cells [271]. Since the discovery of α-GalCer, several more physiologically relevant and possibly cognate type I NKT cell ligands have been identified. Isoglobotriosylceramide (iGB3), which is a naturally occurring self-lipid synthesized in LPS activated DCs [272, 273], and glycolipids isolated from *Sphingomonas* species [274, 275] all showed structural similarity to α-GalCer and exhibit NKT cell stimulatory activity.

The discovery of αGalCer allowed the identification, enumeration and study of type I NKT cells to delineate NKT cell contributions in immune responses. To date, emerging evidence suggest that NKT cells can orchestrate a variety of immune responses and confer protection through T cell cytokines IL-2, IL-4, IFN-γ, IL-10 and IL-17 (*Figure 1-9*). IFN-γ secreted by activated NKT cells is thought to be important in promoting maturation of dendritic cells, thereby enhancing their ability to prime CD4 and CD8 T cells [276, 277]. Furthermore, IFN-γ secreted by NKT cells is thought to activate NK cells, driving NK cell proliferation, enhancing NK cell cytolytic function [278, 279], and confer protection against type 1 diabetes [280-282] or contributing to tumor surveillance [283-285]. In addition to activation of immune cells through IFN-γ, NKT cells can also exert immune regulatory effects through a variety of avenues. Activated NKT cells can secrete IL-4, which is known to inhibit the polarization of Th1 cells,

and confer protection against Th1-mediated autoimmune diseases [286, 287]. IL-4, in combination with IL-13 and GM-CSF, which can also be secreted by NKT cells can promote tolerance through the induction of tolerogenic DCs [287]. Lastly, IL-2 and IL-10 secreted by NKT cells can also promote tolerance through the induction of Treg cells [288] and confer protection against Th1/Th2 mediated graft versus host disease [289].

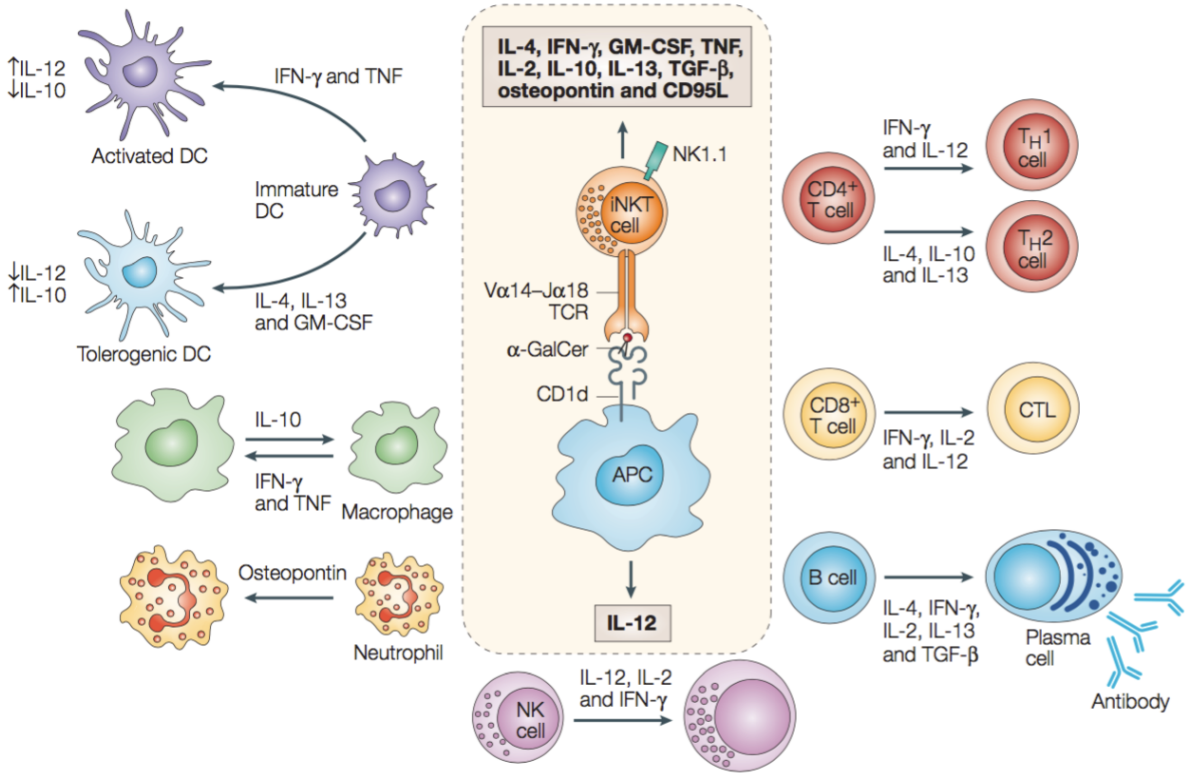

Figure 1-9 Modulation of innate and adaptive immune cells by activated NKT cells. NKT cells, once activated by lipid antigens bearing CD1d molecules on the surface of professional APCs, can rapidly produce a variety of immunomodulatory cytokines without the need of a priming step unlike the conventional T cells. The cytokines secreted by activated NKT cells include IL-4, IFN-γ, TNF, GM-CSF, IL-2, IL-10, TGF-β IL13, osteopontin, and CD95L. These cytokines are known to play major role in driving the differentiation of naïve CD4 T cells into Th1 or Th2 lineages, naïve CD8 T cells into CTLs, and naïve B cells into antibody-producing plasma cells. IFN-γ can also activate DCs and macrophages, increasing their antigen processing, presentation capabilities, and induce expression of iNOS. In addition to eliciting inflammatory immune responses from the innate and adaptive immune cells, cytokines such IL-2, IL-10 and TGF-β also play major roles in the differentiation and maintenance of T regulatory cells. Figure adapted from Van Kaer, *Nature Reviews Immunology*, 2004 with permission from Nature publishing group [287].

1.1.11 B cells

B cells received the name from the organ in which they were discovered in by Cooper in 1965 [113]. Five decades after their discovery, B cells are now known to participate in many different immunological processes ranging from antigen presentation to tolerance induction/autoimmune pathogenesis in addition to their well-known role as the provider of antibody mediated immunity (humoral immunity) [290-293]. B cells are generated from pluripotent stem cells and undergo a maturation process analogous to the T cells within the bone marrow of mammals. Similar to T cells, each of the B cell expresses an unique B cell receptor specific for an epitope, which is generated by the same genetic recombination machinery used to generate T cell receptor [294]. Utilizing their B cell receptors (BCR), B cells can recognize their cognate antigens in the form of a whole protein, unlike T cells, which recognize peptide fragments presented on MHC molecules on the cell surface of APCs [295].

Each BCR is comprised of a pair of duplicate heavy chains and a pair of duplicated light chains, both of which are generated via somatic genetic recombination from BCR gene clusters in a similar fashion to the TCR [296]. During the somatic recombination event, one randomly chosen D segment of the BCR heavy chain gene is joined with a randomly selected J cassette forming a unique D-J fragment. The DJ fragment is then joined with a randomly selected V fragment upstream of the DJ fragment forming a complete VDJ gene segment. There are two types of BCR light chain gene loci used by mammals to pair with the endogenously rearranged BCR heavy chain to generate a complete BCR, namely the kappa chain (κ) and the lambda (λ) chain. Both loci are rearranged in a similar fashion as the BCR heavy chain, with the only difference being that BCR light chain genes cassettes lack the D cassettes, hence a random V cassette is joined to a random J cassette to form a complete VJ gene fragment. Upon the

completion of BCR rearrangement, developing B cells receive survival signals in the form of cytokine IL-7 and c-Kit ligand from the bone marrow stromal cells and proceed further into their development and maturation [297, 298].

Developing B cells undergo a positive/negative selection process similar to that of T cells to ensure BCR functionality and to eliminate potentially autoreactive B cells based on the BCR affinity towards autoantigens [299, 300]. Developing B cells with BCRs that interact too strongly with self-proteins present on the cell surface within the bone marrow stroma are instructed to undergo further BCR rearrangement or to die by apoptosis. Developing B cells that interact too strongly with soluble proteins become anergized by these interactions, whereas B cells that recognize soluble antigen weakly become ignorant. These B cells then exit the bone marrow and migrate into the periphery as mature B cells. When an antigen naïve B cell encounters its cognate antigen in the periphery, the cross-linking of BCR molecules set off a chain of signaling events, which in combination with activating signals contributed from Tfh cells, drive proliferation, and in the process, differentiate these clonally expanded B cell into antibody-producing plasma cells [301, 302].

Antibodies, otherwise known as immunoglobulins, are a type of protein molecule produced by plasma cells essential for antibody-mediated immune responses. Structurally, an antibody is comprised of the same variable regions of the BCR, but instead of a membrane bound constant region, the antibody carries a soluble constant region, which allows the antibody to be secreted into the surrounding. There are several different classes of the constant regions known as isotypes, which not only dictates the location where the antibody will function but also how the antibody will function during a humoral response. While some isotypes can only bind and neutralize target antigen, some isotypes have additional functions such as enhancing antigen

uptake by phagocytes (opsonization), activating the complement system and recruiting cytotoxic lymphocytes to kill target cells. Below, the functions the isotypes will be discussed in greater detail.

There are several antibody isotypes produced by the mammalian B cells, namely IgM, IgD, IgA, IgG, and IgE [302]. IgM and IgD are two antibody isotypes found in the blood after the initial encounter with an antigen. IgM functions poorly as an opsonizing antibody but can effectively activate the complement system to destroy invading microbes. IgA is a dimeric antibody secreted into the mucosa to coat target micro-organisms, thereby preventing them from interacting with host cells. IgG is a type of monomeric high-affinity antibody that can be found in the blood and in breast milk. In addition to antigen neutralization, opsonization, and complement system activation, the Fc regions of IgG can be used by the cytotoxic lymphocytes to lyse target cells through a mechanism known as antibody-mediated cytotoxicity. Furthermore, IgG is the only isotype with the capability to cross the placenta, thereby providing protection to the fetus during gestation and early infancy. IgE is a monomeric antibody found in the blood and mucosa responsible for helminth and protozoan parasite expulsion. It is also the causal agent for allergic reactions.

As previously mentioned, IgM and IgD are low affinity antibodies, which also possess less functionality compared to IgG. This is because they have yet to undergo antibody fine-tuning processes such as isotype class switching and affinity maturation. Isotype switching is a genetic rearrangement process, which occurs in activated B cells where the gene segment which codes for a particular constant region is spliced out by DNA recombination and replaced by another constant region under the guidance of cytokine and CD40 signals provided by Tfh cells. In humans, exposure to Th2 cytokine IL-4 during B cell activation can result in isotype class

switching from IgM to IgG1, IgG4, and IgE while exposure to Th1 cytokine IFN-γ can result in class switching to IgG3. In mice, activated B cells can be directed to class switch to IgG1 and IgE by IL-4, whereas IFN-γ exposure can result in class switching to IgG3 [303, 304].

Affinity maturation is an antibody fine-tuning process, which takes place in activated B cells after each successive antigen encounter. During this process, random nucleotides are added or deleted from the BCR gene variable region to generate a new variable region. The newly edited BCR then undergoes another round of positive and negative selection to eliminate autoreactivity while selecting for higher affinity BCRs and thus higher affinity antibodies [305, 306]. After the clearance of invading pathogen, B cells undergo a contraction phase similar to T cells where the majority of the antibody producing plasma cells die by apoptosis. The remaining plasma cells then differentiate into long-live memory B cells, which can provide long term protection against the same pathogen [307-309].

A subset of B cells is also known to regulate immune responses. These regulatory B cells, otherwise known as Bregs, can exert regulatory function through several mechanisms. Similar to Treg cells, Breg cells have been reported to produce immunoregulatory cytokines such as IL-10 and TGF-β upon activation, and these immunoregulatory cytokines can even confer protective effect against EAE, IBD, and lupus. In addition, Bregs have also been shown to inhibiting T cell response through CD40/CD40L interaction or by downregulating TCR expression on effector T cells. Moreover, a subset of B cells can also influence the migration of DCs and macrophages by the expression of CXCL13 [310].

1.2 Peripheral tolerance

There are several flaws within the process of clonal deletion, which can limit its effectiveness in establishing immunological tolerance. The transcription factor AIRE allows ectopic expression and presentation of TSAs on MHC molecules to mediate clonal deletion of autoreactive thymocytes. However, the level of AIRE mediated expression of TSAs in mTECs may vary greatly between the TSAs, and for some, the expression may be very low, thus impact the efficacy of negative selection. Furthermore, it has been reported that only 1-3% of the mTECs actually express a particular TSA at a given time [311], leading to the possibility of autoreactive thymocytes escaping negative selection in a stochastic manner. Most importantly, clonal deletion can only eliminate autoreactive thymocytes specific for proteins encoded in the genome, thus having little to no effect in deleting thymocytes specific for environmental antigens derived from sources such as food particles, inhaled particles, or commensal microflora.

Negative selection operates on the principle of TCR affinity towards self-peptide-MHC molecules (pMHC), which allows efficient elimination or attenuation of autoreactive T cells bearing high affinity TCRs towards pMHCs but cannot effectively eliminate autoreactive T cells bearing low affinity TCRs towards pMHCs. Consequently, it has been reported that escaped low affinity autoreactive T cells can cause autoimmunity in genetically predisposed animals [312] or in otherwise healthy animals immunized with an autoantigen [249]. To compound this problem, negative selection also cannot effectively eliminate autoreactive T cells that bear two TCRs with distinct specificities. The dual TCR expressing T cells are generated during thymic development by incomplete allelic exclusion of the TCRα chain genes. It is thought that the less strict allelic exclusion process leads to the possibility of both TCRα chain gene loci being simultaneously rearranged, resulting in the generation of two distinct TCRα chains. Both TCR α chains can then

pair with the TCRβ chain, giving rise to two TCRs with distinct antigen specificities and affinities. Since thymic selection only requires one TCR with the right amount of affinity towards pMHC, autoreactive T cells bearing high affinity TCRs may escape negative selection if it is expressed at a lower level compared to the TCR with the right affinity [175, 176]. In order to restrain autoreactive T cells that escaped negative selection and confer tolerance to antigens that are not encoded in the genome, the immune system has evolved a series of extra-thymic mechanisms to induce peripheral tolerance [313-315].

One mechanism of peripheral tolerance is immunological ignorance. In immunological ignorance, autoreactive T cells are prevented from detecting their autoantigen, usually through physical sequestration [184]. There are several elaborate mechanisms tolerogenic APC can induce peripheral tolerance namely, induction of anergy, induction of extra-thymic deletion, extra-thymic induction of T regulatory cells. Immunological ignorance is defined as a state where antigen-specific T cells are not aware of their cognate antigen. Ignorance is achieved through the physical sequestration of autoreactive T cells from their cognate antigen either by anatomical location or through deprivation of inflammatory cytokines, co-stimulatory signals and chemokines necessary for the activation of T cells [184, 316, 317].

Anergy, defined as a state of antigen unresponsiveness, can be induced in several ways [318]. It has been reported that the engagement of TCR without a signaling contribution from the CD28:B7 co-stimulatory pathway can result in T cell anergy [149-151]. Cytotoxic lymphocyte associated-protein 4 (CTLA-4), a high affinity B7 receptor expressed on activated T cells and Tregs, which can compete with CD28 to bind and sequester B7 molecules [319], thereby denying T cells of the necessary CD28 co-stimulatory signal [214] and contributing to the induction of T cell anergy [320, 321]. However, other inhibitory receptors exist to suppress T cell activation

such as PD-1, BTLA, LAG3 and TIM3 [322, 323]. In addition, repeated strong engagement of TCR signaling by super-antigens, as well as antigen presentation from immature APCs are known to induce an anergy- like state in T cells [318]. Peripheral deletion of chronically stimulated antigen specific T cells is another avenue to induce peripheral tolerance. It has been reported that antigen specific T cells are deleted in the periphery after chronic antigen exposure, and the T cell deletion is mediated by the collaborative action of apoptosis regulators FAS and BIM [324].

Extrathymic induction of Tregs is another mechanism by which peripheral tolerance is achieved. Tregs play a critical role in the establishment of peripheral tolerance [196], and tolerance to environmental antigens [202]. Treg deficiency can lead to the development of fatal multi-organ autoimmunopathy in both human and experimental animals [212, 213]. Treg cells exert their function to suppress T cell response by secretion of immune suppressive cytokines, killing of effector T cells, induction of tolerogenic dendritic cells, metabolic disruption of T cell proliferation [217], and sequestration of B7 co-stimulatory molecules using CTLA-4 [214]. Treg cells can be generated in the thymus as well as in the peripheral tissues. Generally, extrathymic derived Treg cells also express the key lineage determining transcription factor FOXP-3, which in turn plays indispensable roles in various aspects of Treg function [214, 215].

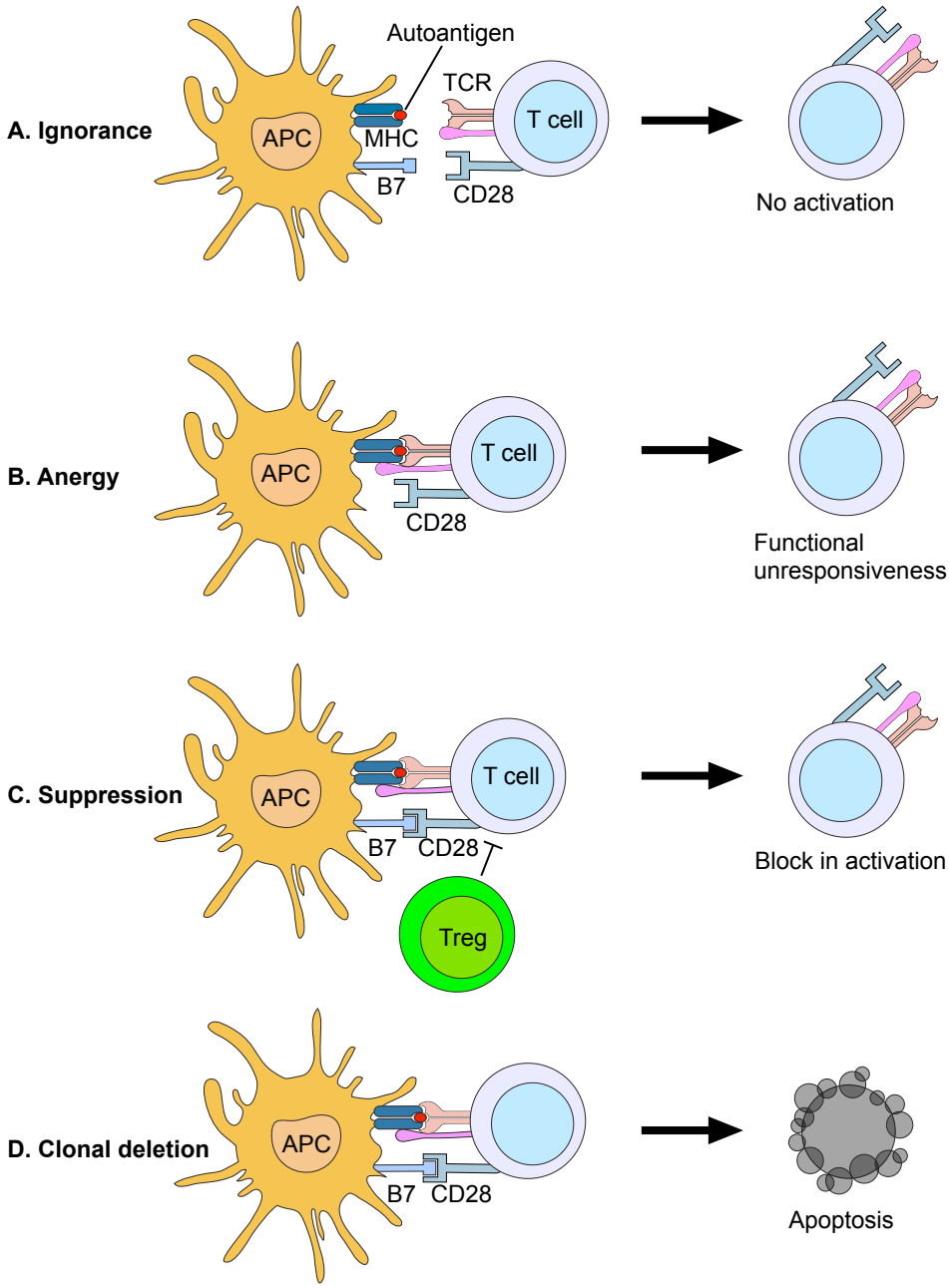

Figure 1-10 Mechanisms of peripheral tolerance.
Several extra-thymic mechanisms are known to confer protection against autoreactive T cells that managed to escape central tolerance. In T cell ignorance, autoreactive T cells fail to encounter their cognate autoantigen due to the scarcity of the autoantigen. T cell anergy, which is defined as a state of antigen unresponsiveness, which is thought to occur when a naïve autoreactive T cell engages its autoreactive TCR in the absence of signaling contribution from co-stimulatory molecules such as B7 (CD80/86). The activation and proliferation of naïve autoreactive T cells can also be prevented by autoantigen specific Tregs through cell-cell interaction and immunosuppressive cytokines. Naïve autoreactive T cells can undergo clonal deletion in the periphery through activation induced cell death.

1.3 Autoimmune diseases

Autoimmunity is a term used to describe a collection of scenarios in which the adaptive immune system is directed towards healthy host tissues, resulting in self-tissue destruction and morbidity. Any pathological conditions caused by autoimmune-mediated destruction of tissues are therefore termed autoimmune diseases. Autoimmune diseases can be characterized by the cell type (s) of the adaptive immune system mediating the destruction, and can be antibody mediated, T cell mediated or both. There are many types of human autoimmune diseases with the most common ones including type 1 diabetes (T1D), inflammatory bowel disease (IBD), Hashimoto thyroiditis, psoriasis, rheumatoid arthritis (RA), myasthenia gravis (MG), multiple sclerosis(MS), and systemic lupus erythematosus (SLE).

Although the precise etiology is unknown, experimental evidence suggests that autoimmune diseases are the result of a complex interplay between genetic factors, environmental factors and endogenous factors such as sex difference, age, and pregnancy (*Figure 1-11)*. The interaction between these factors then culminates in the activation of autoreactive lymphocytes and the destruction of healthy tissues. Regardless of the cause, it is widely believed that the pathogenic process is initiated in genetically susceptible individuals by a pro-inflammatory event usually in the form of a microbial infection [325, 326]. When such event occurs in healthy individuals, various immunoregulatory mechanisms work in concert to restrain the activation of autoreactive lymphocytes. In genetically susceptible individuals however, various genetic defects either enhance the activation of autoreactive lymphocytes or impede the proper regulation of the inflammatory response, resulting in the activation of autoreactive lymphocytes and the initiation of autoimmunity.

A complex signaling network, involving many cell types, enzymes, signaling molecules and transcription factors, regulates the immune system. Consequently, any perturbations to this intricate signaling network can potentially result in autoimmunity. In patients, genetic polymorphism in the antigen presentation pathway [327-329], and T cell regulation pathways [329, 330] have been implicated as contributors to autoimmune diseases such as T1D and IBD. Furthermore, similar genetic defects in the aforementioned pathways have also been identified in NOD mice, further corroborating the role of genetic factor in autoimmune diseases [331, 332]. Although research evidence indicate a strong association between certain genetic mutations and susceptibility to autoimmune diseases, T1D concordance studies showed that 78% of monozygotic twins both develop T1D [333], suggesting that other factors besides genetics influences the susceptibility to T1D.

As more evidence come into light, it is becoming increasingly clear that the immune system is constantly engaged in a complex cross-talk with the resident microflora, under the influence of the environment. Consequently, any changes in the environment could have downstream affects to the microflora and therefore the immune system. It has been shown that in humans, reduced sunlight exposure [334], reduced endotoxin exposure during childhood [335] or even mode of childbirth (vaginal vs C-section) [336] can all impact susceptibility to autoimmune diseases. While differences in microflora composition has been shown in NOD mice to affect disease outcome [337-339], there is currently no evidence linking UV exposure to IBD in mice. Taken together, these evidence suggest that environmental factors such as exposure to microflora can influence host susceptibility to T1D both in human and in mice.

The observation that most autoimmune diseases are more prevalent in woman than in men [340] has led to the postulation that gender difference can somehow affect autoimmune

disease susceptibility. Curiously, it has been shown in NOD mice that sex-hormones can influence the disease susceptibility through a complex interplay between the host immune system, the host microflora and sex hormone-secreting cells [8]. These findings however, have not been corroborated in human studies.

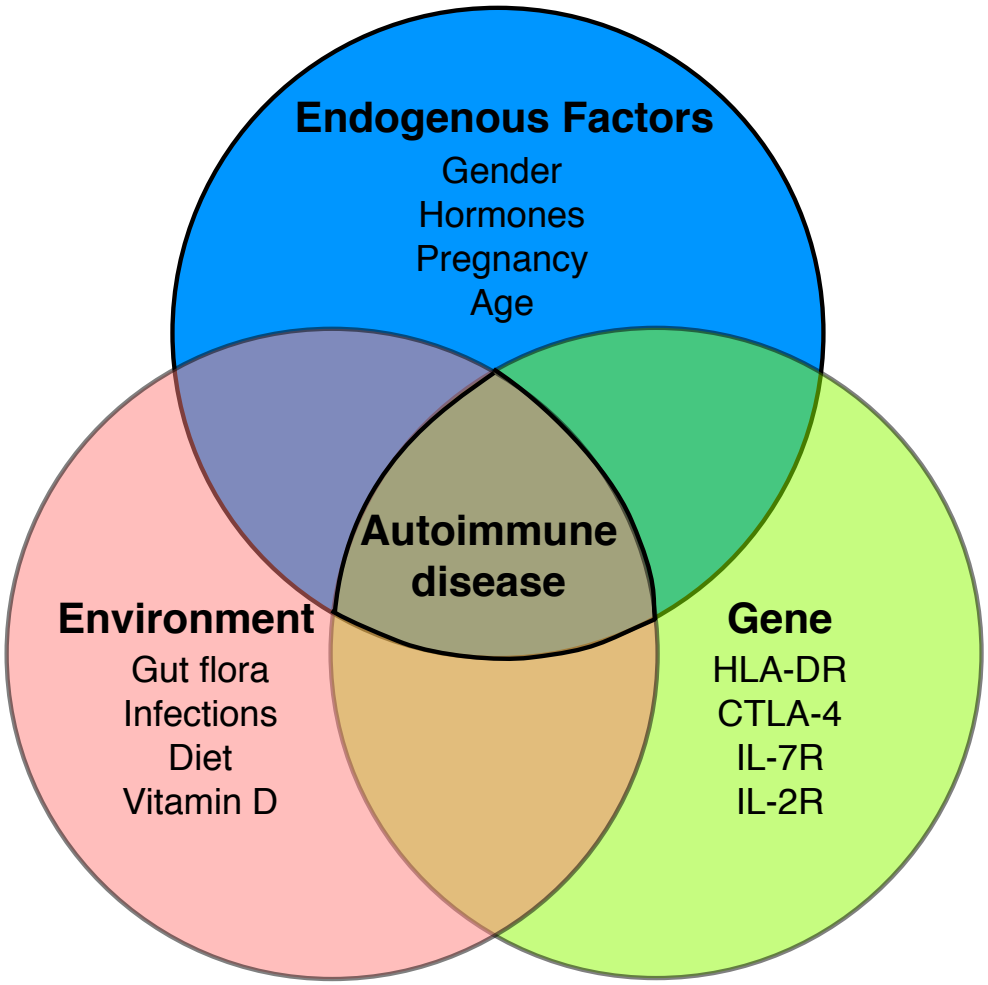

Figure 1-11 The etiology of autoimmune diseases.
The etiology of autoimmune diseases is largely unknown, however it is commonly believed that autoimmune diseases are the result of a complex interplay between genetic predispositions, environmental factor and endogenous factors. The figure above was adapted from a figure by Ermann and Fathman. *Nature Immunology.* 2001 [341].

1.3.1 Type 1 diabetes

Autoimmune diabetes, otherwise known as juvenile diabetes or type 1 diabetes in humans is a debilitating condition characterized by chronic immune mediated destruction of insulin producing β cells in the pancreas leading to uncontrolled blood glucose levels in affected individuals [342]. If left untreated, T1D can result in many serious complications such as heart disease, renal failure, blindness, amputation, or even death. According to reports from the Canadian Diabetes Association (now Diabetes Canada) published in 2009, more than 300,000 Canadians were diagnosed with T1D, and the rate of incidence in children under age of 14 was expected to increase 3% annually worldwide.

Currently, there is no cure for T1D. In order to minimize complications associated with dysregulated blood glucose levels, T1D patients must stringently control their daily carbohydrate intake, routinely monitor their blood glucose levels throughout the day and inject insulin as the need arises. Even with careful monitoring and treatment, the life expectancy of patients can still be shortened by as much as 15 years, and since glucose monitors and insulin cannot mimic the action of the β cells, patients may still experience episodes of hyper or hypoglycemia due to improper eating and or insulin dosing [343]. Although transplantation using donor pancreata, donor β cells, xenogeneic β cells, or even host-derived β cells may prove attractive options to restore euglycemia in T1D patients, the pre-existing autoimmune condition and the subsequent anti-graft immune response must first be addressed in order for the aforementioned treatments to become permanent solutions [344].

1.3.1.1 Etiology of type 1 diabetes

The real cause of T1D is unknown. However, results from decades of research suggest that T1D is caused by a complex interplay of genetic susceptibility, gut microflora dysbiosis, environmental factors, and infection by several viruses [343, 345-347]. In the most widely accepted model of T1D pathogenesis [348, 349], the vicious cycle is thought to begin when the β- cells in a susceptible individual are injured by a chemical or viral insult. This injurious event leads to β cell necrosis and the release of β cells specific antigens and other pro-inflammatory molecules into the surroundings, eliciting an inflammatory response. Genetic defects in multiple immune cell types prevent or impede with the resolution of the initial inflammatory event, resulting in the activation of the adaptive immune system and further destruction of the β cell to release more β cell antigens. Additional β cell antigens allow the adaptive immune system to generate more β cell specific T cells to target multiple epitopes, accelerating the rate of β cell destruction [343, 347]. This vicious cycle eventually results in insulin insufficiency and the symptoms of T1D then begin to manifest. In the following sections, the relative contribution of immune cell types and genetic predispositions to the pathogenesis of T1D will be discussed in detail.

1.3.1.2 Animal models for type 1 diabetes

The non-obese diabetic (NOD) mice and biobreeding (BB) rats are two of the most widely used animal models for the study of T1D [350, 351]. Both animal models have disease features such as insulitis, polyuria, and glycosuria, which closely resemble some of the symptoms of T1D; however, because T1D is a T cell driven disease and BB rats are lymphopenic [352], NOD mice became the tool of choice for researchers studying T1D [332,

352, 353]. The NOD mouse model shares many genetic similarities in disease susceptibility with T1D in humans in that many of the same genes are linked including MHC II and other immune regulatory loci [331, 332].

1.3.1.3 Role of innate immune system in type 1 diabetes

Type 1 diabetes is thought to be an adaptive immune system driven disease. However, because the adaptive immune system is intimately connected to the innate immune system, it is perhaps of no surprise that abnormalities in the innate immune system could culminate in the involvement of the adaptive immune system. Thus far, several phagocyte lineages and NK cells have been implicated in T1D pathogenesis.

Neutrophils are the most abundant phagocyte population of the innate immune system, which are amongst the first cell types recruited at the beginning of an inflammatory event by IL-8 and leukotriene B4 [354, 355]. Neutrophils are potent microbicidal phagocytes whose primary purpose is to ingest and destroy microbes through phagocytosis, anti-microbial products and neutrophil extracellular traps (NETs). These antimicrobial mechanisms although useful in the destruction of microbes, can also cause tissue damage or even provoke autoreactive T and B cell responses [356, 357]. In T1D patients, changes in circulating numbers of neutrophils are associated with disease progression [357]. In NOD mice, the inability of macrophages to clear apoptotic cells [358] in combination with NETs were shown to activate autoreactive T cells through a complex crosstalk between B cells and plasmacytoid dendritic cells [293].

DCs are a type of professional APC specialized in modulating and shaping T cell immunity both during T cell development and in peripheral tissues [90, 94, 359]. In T1D patients, changes in the frequency of circulating pDC were found at the time of diagnosis [360,

361]. In addition, reduced circulating cDC and NKT cell numbers at the time of diagnosis were found to correlate with reduced β-cell function [362]. Furthermore, a small scale pancreatic biopsy study of newly diagnosed T1D patients found that TNF-α and IL-1β secreting DCs and macrophages were infiltrating the islets [363]. In NOD mice, it has been shown that DCs were skewed towards the pro-inflammatory phenotype with the numbers of the immune regulatory $CD8^+$ cDCs being reduced in favor for other CD8- DC subsets [364-366]. Phenotypically, these $CD8^-$ DCs secrete IL-12, a key cytokine for Th1/CTL polarization, suggesting that they may play a role in activating β-cell specific autoreactive T cells [365, 367].

Macrophages are another phagocytic cell lineage known to influence the progression of T1D. In T1D patients, macrophages have been found in the islets during early and later stages of insulitis [368]. In NOD mice, macrophages infiltrating the islets were shown to exert deleterious effects on the β cells: Directly, inflammatory macrophages could cause β cell death directly through secreted cytokines such as TNF-α and IL-1β. Indirectly, inflammatory macrophages can skew T cell differentiation towards the disease-causing Th1/CTL phenotype through the secretion of IL-12 [369]. Taken together, these results suggest that DCs are involved in the pathogenesis of T1D both in humans and in mice.

NK cells have been implicated in both human and animal studies as a contributor of the pathogenesis of T1D, however the results seemed to suggest that NK cells may play dual roles both as promoter and protector to the disease [370]. In humans, reduced circulating NK cell numbers as well as impaired NK cell function were associated with T1D [370]. In animal models, although diabetes-prone BB rats possess increased frequency and activity of NK cells, depleting NKT cells did not show any protection against disease [371, 372]. In NOD mice, while some groups showed that depleting NK cells could prevent or delay the progression of T1D

[373-375], other groups have shown that decreased peripheral NK cell frequency and impaired NK cell function were associated with disease progression [376-378]. Interestingly, IFN-γ secreted by complete Freund's adjuvant stimulated NKT cell was shown to restore NK cell function and confer disease protection in NOD mice [282, 379]. Taken together these findings suggest that NK cells may protect or potentiate the disease in an organism-dependent manner.

1.3.1.4 Role of adaptive immune cells in type 1 diabetes

Type 1 diabetes (T1D) was identified as a T cell driven autoimmune disease a few decades ago. In earlier studies, T cells were seen infiltrating and destroying the islets in the pancreata of newly diagnosed patients, as well in patients with HLA matched pancreas allografts [380, 381]. After the discovery of the T cell specific immunosuppressant cyclosporine, it was subsequently shown that administration of cyclosporine was able to slow down the progress of allograft loss [381] or delay the progress of the disease [382]. A similar effect of disease retardation was also observed in patients treated with T cell depleting antibodies [383], further substantiating the notion that T1D is a T cell driven autoimmune disease.

Experimental evidence from both human studies and animal models largely support Th1 cells and their signature cytokines as the primary drivers of T1D with other Th cell subsets playing less prominent roles. In mice, ectopic expression of Th1 signature cytokine IFN-γ in the β-cells of BALB/c mice driven by the human insulin promoter was shown to cause diabetes [384] while inhibition of IFN-γ was shown to prevent disease in NOD mice [385, 386]. In humans, analyses of islet-reactive T cells from the peripheral blood of newly diagnosed T1D patient showed that they were strongly Th1 polarized, whereas islet reactive T cells from healthy controls were polarized towards a Treg phenotype [387, 388]. Functionally, Th1 cells are thought

to mediate β-cell destruction via direct and indirect mechanisms. Indirectly, Th1 cytokines such as IFN-γ can facilitate the destruction of β-cells by activating macrophages to increase their cytotoxicity and drive CD8 T cell proliferation and differentiation [389, 390]. Directly, Th1 cytokines IFN-γ and TNF-α can trigger cell death pathways leading to β-cell apoptosis [391].

The Th17 subset has recently gained increased recognition as another prominent player in the pathogenesis of autoimmune diseases [392], however their exact role remains to be elucidated [393]. Analysis of PBMCs from newly diagnosed T1D patients revealed an increase in the frequency of IL-17 producing CD4 or CD8 T cells [394, 395]. In addition to IL-17 producing T cells being found in the blood, a study also found elevated IL-17 in the pancreata of deceased T1D patients [396]. Taken together, these results suggest that Th17 may play a role in T1D, but the exact nature of such role remains undetermined. In studies with NOD mice, inhibition of Th17 delayed the onset of T1D [397-399]; however, some of these studies also show that the Th17 cells may not be as pathogenic as the Th1 cells, as conversion to a Th1 phenotype was required for pathogenesis [243, 400]. Interestingly, treatment of NOD mice with a blocking antibody that targets IL-12 p40, a subunit shared between IL-12 and IL-23, was shown to prevent disease [401]. Based on our understanding that IL-12 is a key cytokine required for Th1 polarization and IL-23 is important for the stabilization of Th17 differentiation, these data suggest that Th17 cells may be the first Th subset to arise in the disease course of T1D, which later transitioning into a Th1 phenotype and causing disease.

Tregs are another critical component of T cell tolerance as evident in human and mice where deficiency in Tregs leads to fatal multi-organ immunopathy [212, 213]. Treg-deficiency in NOD mice was observed to accelerate the progression of disease as well as increase the frequency of disease, whereas transfer of β-cell specific Treg cells slow the progression and

lowered the frequency of disease [402-404]. The role of Tregs in T1D patients remains murky, which is likely the result of a combination of confounding factors such as genetic heterogeneity, age of disease onset, disease progression, and limitation in the availability of Treg markers. Thus far, it has been shown that in the pancreatic lymph nodes of T1D patients, the demethylation of the Treg-specific demethylated region (TSDR) of the FOXP3 loci could result in the loss of FOXP3 expression and reduction in Treg function while favoring the expansion of Th17 cells [405]. IL-2 is a key cytokine not only in maintaining FOXP3 expression [406], but also for the survival and expansion of $FOXP3^+$ CD4 T cells [218]. In humans and NOD mice, genetic polymorphisms in the IL-2 signaling pathway have been associated with Treg dysfunction and T1D [407].

The appearance of insulin-specific autoantibodies which precedes hyperglycemia is not only an important clinical indicator of T1D but also an indicator of the breakdown of B cell tolerance, suggesting that B cells are involved in the pathogenesis of T1D. Although the importance of β-cell specific autoantibody to T1D pathogenesis remains poorly understood, depletion of B cells using anti-CD20 antibody was demonstrated to be effective in preserving β cell function after the initial diagnosis [408]. Similarly in NOD mice, B cell deficiency, as well as depletion of B cells using antibodies were shown to be protective against disease [409]. Although B cells are not as efficient in the priming of naïve T cells as DCs, *in vitro* studies done in mice showed that they possess the capacity to prime antigen-specific CD8 T cells, and confer protection against B cell lymphoma [410]. Moreover, in a NOD mouse study, B cells producing DNA autoantibodies was shown to engage in a complex cross talk with neutrophils and pDCs to prime β-cell reactive T cells, and cause disease [293]. Taken together, these data suggest that a breach in B cell tolerance results in BCR-mediated uptake and presentation of islet specific

antigens by B cells, resulting in the activation of autoreactive T cells. Activated autoreactive T cells then provide ligand and or cytokine assistance to B cells to drive B cell differentiation and autoantibody production in addition to causing T1D.

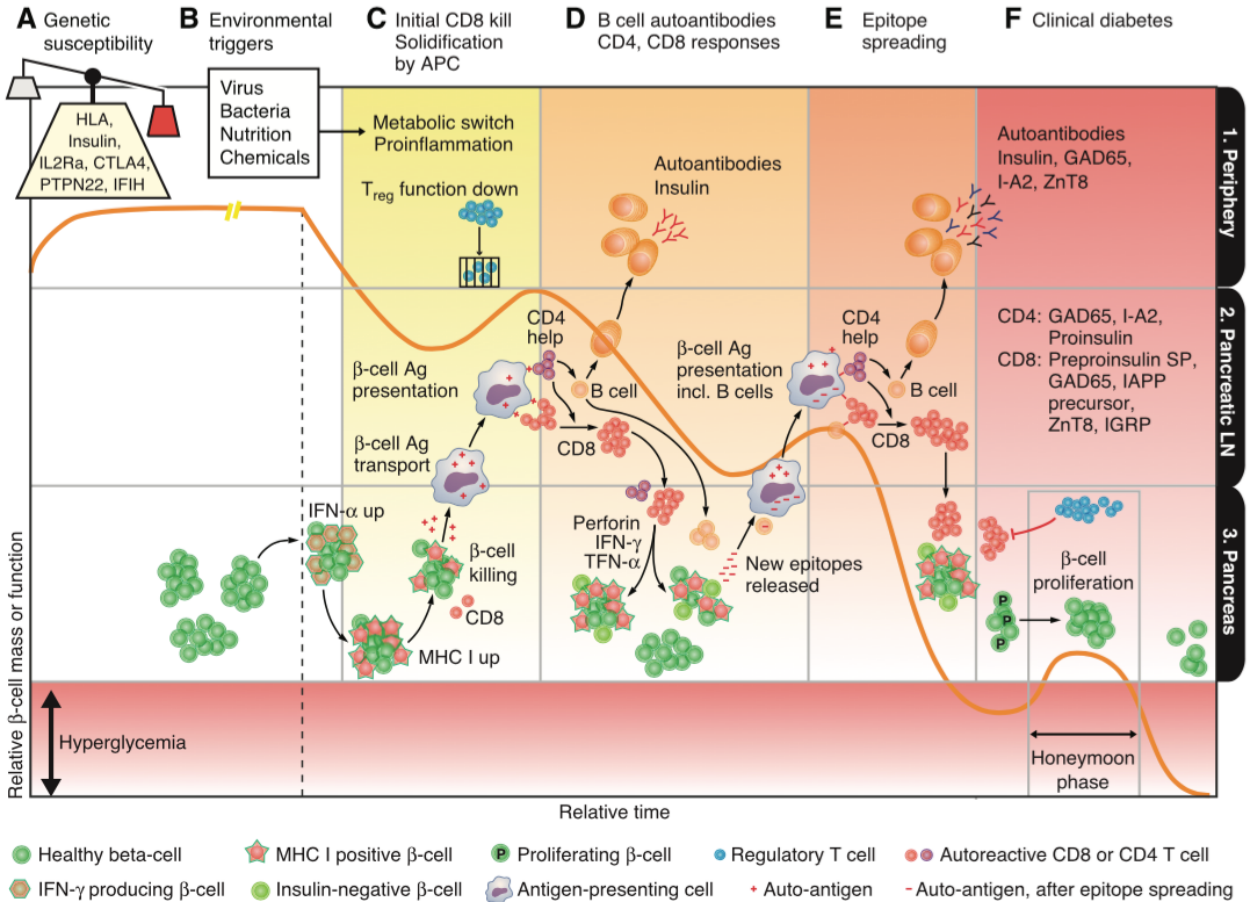

Figure 1-12 Pathogenesis of type 1 diabetes.
The orange line denotes β-cell mass and function. Letters on top denote different disease phases. It is thought that type 1 diabetes is precipitated in genetically susceptible individuals by environmental triggers. These events cause β-cells to release pro-inflammatory cytokines such as IFN-α and upregulate MHC class I. The upregulation of β-cell antigen presentation on MHC class I and the presence of pro-inflammatory cytokines in the micro-environment in combination with disease promoting MHC class I and defective Treg function cause CD8 T cell mediated killing of β-cells. This results in the release of β-cell specific antigens, especially insulin. Insulin is then picked up by the resident APCs and presented to the T cells in the pancreatic lymph node, resulting in the activation and proliferation of more β-cell antigen-specific autoreactive T and B cells in a process known as epitope spreading. These newly activated effector cells then infiltrate the islets, causing more β-cell death through perforin, IFN-γ, and TNF-α, leading to even more β-cell killing and antigen release in the form of a vicious cycle, ultimately results in insulin deficiency and T1D. The figure was adapted from Van Belle *et al. Physiology Review* 2011, with permission from the American Physiological Society [343].

1.3.2 Inflammatory bowel disease

Inflammatory bowel disease (IBD) is a term used to describe a collection of chronic relapsing inflammatory conditions involving the digestive tract. IBD is primarily comprised of two subtypes of conditions, Crohn's disease (CD) and ulcerative colitis (UC). The location and depth of the inflammation are two of the key diagnosis determine factors since patients from both disease populations share many common symptoms such as fever, fatigue, severe abdominal pain and rectal bleeding.

In CD patients, inflammatory lesions can be found throughout the digestive tract from mouth to anus, while in UC patients the location of the inflammatory lesions is restricted only to the colon and rectum. The depth of inflammation is another distinguishing factor between the two diseases with CD involving the whole intestinal wall while UC involves primarily the mucosa layer.

Currently, IBD is managed using a combination of anti-inflammatories, immunosuppressive drugs, antibiotics, and probiotics [411], while patients refractory for aforementioned treatments are treated with biological therapeutic agents such as anti-TNF-α antibodies [411, 412]. All current treatment options for IBD carry significant side effects and toxicities; it is therefore imperative to elucidate the disease-causing mechanisms in IBD so that new treatment strategies can be developed. The etiology of IBD is elusive; however it is widely believed that the disease is caused by a combination of environmental factors, immune dysregulation, genetic predisposition and microflora dysbiosis [330]. In the following sections, the relative contributions of several contributing factors to IBD susceptibility will be discussed in detail.

1.3.2.1 The role of intestinal barrier function in IBD

The mucus layer of the gastrointestinal tract, segregating the luminal contents of the intestine from the underlying epithelial layer, is the first line of defense against the commensal microflora and potentially harmful agents. As such, any perturbation that negatively impacts the integrity of this barrier could carry adverse consequences to the health of the host. It has long been documented that Crohn's disease patients exhibit increased intestinal permeability [413] and such an increase correlates positively with the chance of relapse [414, 415]. The relationship between the integrity of the intestinal barrier and IBD susceptibility was subsequently investigated in a mouse model in which *Muc2*, the primary constituent of the intestinal mucus layer was genetically disrupted. Studies done with *Muc2*$^{-/-}$ mice showed that in the absence of the protective mucus layer, increased contact between the epithelial cells and the intestinal luminal content can result in overt immune activation, leading to intestinal inflammation and the development of spontaneous colitis [416, 417]. In agreement with the mouse data, MUC1 expression was found to be decreased in Crohn's disease patients [418], and in patients with active ulcerative colitis the colonic mucus layer was seen penetrable to the colonic bacteria [419]. Taken together, existing data suggest that there is a link between defective intestinal barrier function and one's susceptibility to IBD.

1.3.2.2 Role of innate immunity in IBD

The innate immune system, which includes the mucus layer, is the first line of defense against the intestinal microflora, which is made of mostly harmless microorganisms. To avoid an excessive response towards the resident microflora while providing protection against microbes that can invade and cross the intestinal epithelial cell (IEC) layer, the PRRs on the IECs are

expressed in a strategic manner with the viral sensors such as TLR9 being expressed on the apical surface of the IECs and bacterial product-sensing PRRs such as TLR4 and TLR5 being expressed on the basal surface of the IECs. In addition to the polarized placement of PRRs, the signal transduced by PRRs is polarized as well. A TLR9 signal transduced from the apical surface of the IEC results in the inhibition of NF-κB signaling while TLR9 signaling from the basal lateral surface promotes the activation of the NF-κB signaling. The polarized PRR signaling is an important regulator for various IEC homeostatic processes such as mucus production, anti-microbial peptide production, tight junction maintenance and IEC regeneration [420, 421].

NOD2, a PRR that recognizes muramyl dipeptide found in all gram-negative bacteria and gram positive bacteria, was identified in multiple genome wide association studies as a IBD susceptibility gene [420, 421]. Normally, NOD2 is recruited to the basal lateral surface of the IEC upon muramyl dipeptide detection, and transduce a signal to activate the NF- κB pathway. In Crohn's disease patients, mutated NOD2 protein was found to have lost the ability to be recruited to the basal lateral surface of the IEC in response to the ligand. It is thought that this loss of function in NOD2 impairs efficient bacterial clearance, leading to chronic inflammation and IBD [422, 423].

Macrophages and DCs are two indispensable APC populations for modulating immunity and maintaining homeostasis in the gut [73]. To avoid excessive responses towards resident microflora while conferring protection against pathogens that can invade the IEC, macrophages evolve a strategy similar to that of IECs. In the mucosa of healthy individuals, macrophages exhibit a phenotype which is heightened for the secretion of inflammatory cytokines upon PAMP encounter, but their phagocytic and bactericidal activities remained unchanged [424]. In patients

with IBD, it was observed by one group of investigators that lamina propria macrophages exhibited heightened ability to secrete inflammatory cytokines (IL-6, IL-23, TNF-α) and mononuclear cells from the same site exhibited increased IFN-γ secretion [425]. In contrast to this report, another group of investigators found that macrophages isolated from IBD patients exhibit impaired cytokine secretion (IL-1β, IL-6, IL-8, TNF-α) upon LPS stimulation [424]. These seemingly dichotomous findings perhaps could be the result of differences in ethnicity as well as disease progression.

DCs in the intestinal mucosa contribute greatly to anti-microbial immunity and maintenance of intestinal homeostasis. In mouse IBD models, existing data suggest that intestinal homeostasis is maintained by the $CD103^+$ DCs, which are important for promoting tolerance by promoting the generation of Treg cells, whereas $CX3CR1^+$ mononuclear phagocytes are important for promoting intestinal inflammation through the secretion of Th-17 stabilizing cytokine IL-23 and TNF-α [426]. Relatively less is known about human DC subsets with regards to the pathogenesis of IBD compared to mice. Under normal conditions, the IEC-derived thymic stromal lymphopoietin polarizes the intestinal DCs to the nonpathogenic Th2 like phenotype, whereas in IBD patients, lymphopoietin production in IECs was reduced to low to non-detectable levels [427]. Furthermore, DCs from IBD patients can secrete more IL-6, IL-12, and express higher levels of CD40 [428]. Taken together, these findings suggest that in IBD patients there is a shift in intestinal DC polarization from a tolerogenic Th2 like phenotype to a pro-inflammatory phenotype, which may promote IBD pathogenesis.

1.3.2.3 Role of adaptive immunity in IBD

The pathogenicity of T cells in causing IBD was originally demonstrated in an article describing the technique of T cell transfer colitis. In this report, the authors showed that adoptive transfer of naïve CD4 T cells into SCID mice caused Th1 polarization of the transferred CD4 T cells and resulted in severe colitis. Subsequent TNF-α neutralization was shown to ameliorate the disease and that colitis could also be prevented by the inhibition of IFN-γ or the administration of IL-10 [429]. Upon further investigation, it was revealed that the administration of antibiotics [430] or adoptive transfer of Tregs [431] could also abrogate the disease, indicating that a portion of the CD4 T cells transferred are reactive towards the microflora and that Tregs play an important role in suppressing these microflora-reactive CD4 T cells to prevent them from causing disease.

With the identification of Th17 and Treg cells, there is mounting evidence that intestinal homeostasis is the result of a complex interplay between Th1, Th2, Th17 and Treg subsets, and that dysregulated Th1/Th17 response towards the microflora can result in IBD [330, 432]. The role of Th17 cells in human IBD pathogenesis is unclear. Although increased IL-17 levels were found in the sera and mucosa of IBD patients [433] and that genetic polymorphism in the IL-23 receptor was identified as one of the strongest association with IBD [434], a clinical trial using IL-17A neutralizing antibody in patients with moderate to severe Crohn's disease not only showed no effect, but even exacerbated disease in some patients [435]. A clinical trial using an IL-12/23 neutralizing antibody showed benefit in patients with moderate to severe IBD patients refractory to anti TNF-α treatment, but did not induce remission [436].

1.4 Research aims

1.4.1 Evaluating the immunomodulatory effects of αGalCer liposome

Aim (Chapter 3): Novel liposome therapy prevents type 1 diabetes in non-obese diabetic mice.

T1D is an autoimmune disease caused by T cell mediated destruction of insulin producing β-cells in the pancreas, which results in hyperglycemia, and lifelong insulin dependency [342]. It is well established that the activation and expansion of insulin specific T cells plays a key role in the progression of this disease, and thus stopping insulin specific T cells in an autoantigen-specific manner could potentially stop the progression of the disease [343]. In this Chapter, we explored the possibility of achieving autoantigen specific immunosuppression by harnessing the immunomodulatory potential of NKT cells through a novel liposome reagent. Alpha galatosylceramide (αGalCer) is a naturally occurring glycolipid known for its capacity to stimulate NKT cells and confer protection against autoimmune diseases by activating and expanding T regulatory cells and NK cells through their cytokine production [287]. Despite its immunomodulatory potential, several issues hinder the translation of αGalCer into clinical use [437, 438]. To address these issues, αGalCer was embedded in liposomes to increase solubility and reduce toxicity. In addition, the use of liposomes also allows the co-delivery of autoantigen insulin and αGalCer to antigen presenting cells. ***We postulated that co-delivery of insulin and αGalCer to APCs would allow the simultaneous presentation of the autoantigen and αGalCer by the same APCs thereby bringing together NKT cells and insulin specific T cells to achieve antigen-specific immune regulation.*** To study the immunomodulatory effects of αGalCer

liposomes, we compared the level of cell activation exhibited by different immune cell subsets from C57BL/6 mice subcutaneously injected with liposome or aqueous αGalCer.

This work lays the foundation for the translation of αGalCer liposome into clinical use.

1.4.2 The role of dual TCR expressing T cells in promoting autoimmunity

Aim (Chapter 4): The role of dual-TCR expressing T cells in promoting autoimmunity

The thymus generates a pool of mature T cells expressing a highly diverse TCR repertoire through the somatic recombination of TCRα and TCRβ chain genes [169]. Upon random assortment and pairing of TCRα and TCRβ chains, newly developing T cells that express strongly autoreactive T cell receptors are deleted through a process called negative selection to maintain self-tolerance. However, some autoreactive T cells escape negative selection in the thymus to cause autoimmune diseases. Although tight allelic exclusion limits thymocytes to expressing a single TCRβ chain, rearrangement of the TCRα chain continues partially unabated by successful rearrangement of one TCRα chain gene, enabling thymocytes to express up to two TCRα chains and thus two TCRs. [175, 176]. ***Here, we postulate that pathogenic autoreactive CD8 T cells escape central tolerance through expression of a second, benign TCR that mediates positive selection***. To determine the role of dual TCR expressing CD8 T cells in autoreactivity and autoimmunity, we have compared CD8 T cell autoreactivity against the model autoantigen ovalbumin between T cells capable of expressing dual TCRs (*TCR*$\alpha^{+/+}$) versus T cells expressing a single type of TCR (*TCR*$\alpha^{+/-}$).

This work will provide valuable insight into the escape mechanisms exploited by pathogenic autoreactive CD8 T cells to cause autoimmune diseases.

1.4.3 The role of Mucus in T cell homeostasis and tolerance

Aim (Chapter 5): The role of MUC2 in T cell homeostasis and tolerance.

A thin layer of mucus functions to segregate contents of the intestinal lumen from the intestinal epithelium. MUC2 is the primary constituent of intestinal mucin and plays critical roles in the maintenance of immune cell homeostasis and protection against bacterial infection. The importance of mucus to the intestinal barrier is supported by findings that relapse in IBD patients is associated with decreased mucus secretion and increased intestinal permeability [330, 414, 415]. In addition, MUC2 has also been reported to promote immune tolerance to the gut microbiome through the induction of T regulatory cells [19]. **Here, we set out to investigate *our hypothesis that intestinal mucus is essential for maintaining T cell tolerance towards intestinal luminal antigens and preventing disease caused by constitutive T cell activation.*** To test this hypothesis, wild-type and MUC2-deficient (*Muc2*$^{-/-}$) were orally gavaged with the model antigen ovalbumin (OVA) and the elicited OVA-specific T cell responses monitored at various times post-treatment.

This work provides important insights into how intestinal luminal antigens can affect TCR repertoire. Further, these results yield new insights into how T cell tolerance against intestinal luminal antigens are established and may lead to novel treatment strategies against allergies and autoimmune diseases.

Chapter 2: MATERIALS AND METHODS

2.1 Mice

Six week old non-obese diabetic (NOD) mice [350], C57BL/6 mice, B6.SJL-Ptprca Pep3b/BoyJ (CD45.1) mice, C57BL/6-Tg(Ins2-TFRC/OVA)296Wehi/Wehi mice [439] and B6.129S2-TCRatm1Mom/J (TCR$\alpha^{-/-}$) mice [440] were purchased from the Jackson laboratory. The *Muc2*$^{-/-}$ line [417] was provided by Dr. Bruce Vallance, University of British Columbia. The Vβ5 TCRβ transgenic mice [441] is a generous gift from Dr. Pamela Fink, University of Washington. We generated the Vβ5 × RIP-mOVA line by crossing the Vβ5 TCRβ transgenic line with the C57BL/6-Tg (Ins2-TFRC/OVA)296Wehi/WehiJ line. The Vβ5 × RIP-mOVA line was then crossed with TCR$\alpha^{-/-}$ line to generate the Vβ5 × RIP-mOVA TCR$\alpha^{+/-}$ line. The Vβ5 *Muc2*$^{-/-}$ line was created by backcrossing the *Muc2*$^{-/-}$ line [417] with the Vβ5 line. The Vβ5 Nur77-GFP *Muc2*$^{-/-}$ line was generated by crossing Nur77 GFP reporter line [442]. Most of the experiments use mice with age less than 16 weeks, with exception of the NOD mice. NOD mice were treated with liposomes twice weekly starting from week 4 for five weeks and followed until week 30. All mice were housed, bred and crossed in a specific pathogen-free and *Helicobacter*-free animal facility at BC Children's Hospital Research Institute. All experiments were conducted in accordance with the protocols and guidelines approved by the Animal Care Committee at University of British Columbia and Canadian Council on Animal Care.

2.2 Administration of Alpha galatosylceramide

Synthetic alpha galatosylceramide (αGalCer), KRN7000 (Enzo Life Sciences) was solubilized according to manufacturer's specifications. αGalCer embedded liposomes with or without insulin were obtained from Dr. Omar Duramad of RegImmune Inc (San Francisco, CA). Mice aged 6-10 weeks were injected with approximately1 μg of KRN7000 either dissolved in PBS or encapsulated within liposomes in absence or presence of human insulin via s.c., i.p. or i.v. injection.

2.3 Administration of ovalbumin

Six to ten weeks old *Muc2*$^{-/-}$ or C57BL/6 mice were gavaged with PBS alone or 1 mg of grade III chicken ovalbumin (Sigma Aldrich) dissolved in 100 μL of sterile PBS.

2.4 Immunization with ovalbumin-expressing *Listeria monocytogenes*

Recombinant *Listeria monocytogenes* expressing ovalbumin (Lm-OVA) was obtained from Dr. Hao Shen, University of Pennsylvania school of medicine [443]. A frozen stock was used to inoculate brain heart infusion broth (BD Biosciences) to generate a log phase culture. Cell numbers were determined by plate counting and the culture was diluted down to 20,000 CFU/mL to generate the working culture. Mice aged 6-10 weeks were each immunized with 2,000 CFU of the Lm-OVA working culture diluted in 100 μL of PBS via tail vein injections.

2.5 Identification of H-2K^{b} OVA-specific CD8 T cells

The following protocols were consulted for identification of OVA-specific CD8 T cells [184, 249]. 3×10^6 of single cell suspension from lymphoid organs were first stained with fixable

viability dye eFluor 780 (eBioscience) followed by Fc blocking with 5 μg/mL of anti-CD16/32 antibody (eBioscience) in 100 μL of FACS buffer on ice for 15 minutes. After viability stain and Fc block, samples were stained with a mixture of phycoerythrin-labeled and brilliant violet 421-labeled $OVA_{257-264}$-H-$2K^b$ tetramer for 30 minutes on ice. Samples were spun down after tetramer stain and stained with anti CD8 APC clone 53-6.7 (eBioscience) and other surface markers for 15 minutes on ice. Samples were then washed twice with FACS buffer prior to analysis. All stored samples were stored on ice in the dark, and acquired within 1 hour after the tetramer stain.

2.6 Magnetic isolation of immune cells

$CD11c^+$ DCs and CD8 T cells were enriched using magnetic based cell separation (Miltenyi Biotec). Single cell suspensions were prepared from thymi or pooled lymphoid organs of mice. For $CD11c^+$ DC isolation, isolated thymi were first digested in 5 mL of 1 mg/mL collagenase supplemented IMDM for 30 minutes in 37°C (Collagenase type IV, 186 units/mg, Worthington Biochemical Corporation). After collagenase digestion, single cell suspensions were prepared from the digested thymi by pushing the digested tissue through 40 μm nylon mesh cell strainers (Falcon) with the pistons of 1 mL syringes (BD). The resulting single cell suspension were then washed twice with 5mL of complete IMDM media (Life Technologies) to remove collagenase, and then treated with RBC lysis buffer (ACK lysis buffer: 150 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA, pH 7.3) for 1 minute to remove red blood cells. 5×10^6 thymocytes from the prepared single cell suspension were then labeled with 100 μL of biotinylated anti-CD11c antibodies (clone N418, eBioscience, final concentration 1 μg/mL) on ice for 15 minutes. The antibody

labeled thymocytes were then washed twice with 10 mL of FACS buffer and suspended in 90 μL of MACS buffer (0.5% BSA, 2 mM EDTA in PBS). 10 μL of the streptavidin-microbeads where then added to the thymocyte suspension and the samples were then incubated in 4 °C for 18 minutes. After the incubation, the suspensions were washed once with 500 μL MACS buffer and suspended in 500 μL of MACS buffer for Miltenyi MS column separation according to manufacturer's specifications (Miltenyi Biotech).

To isolate $CD8^+$ T cells, single cell suspensions were prepared from pooled lymph nodes by pushing the lymph nodes through 40 μm nylon mesh cell strainers (Falcon) with the pistons of 1 mL syringes (BD). The lymph node cell suspensions were then treated with RBC lysis buffer for 1 minute to remove RBCs. After RBC lysis, 1×10^7 lymph node cells were suspended in 95 μL of MACS buffer, and 5 μL of anti CD8α microbeads were added (Miltenyi Biotech). The mixture was then incubated for 18 minutes at 4 °C followed by one 500 μL wash with MACS buffer before being suspended in 500 μL of MACS buffer for Miltenyi MS column separation according to manufacturer's specifications (Miltenyi Biotech).

2.7 *In vitro* CD8 T cell expansion

Aliquots of 500 μL sterile PBS containing 10 μg/mL of purified anti-mouse CD3ε antibody (145-2C11) and 2 μg/mL anti-mouse CD28 (37.51) were used to label the wells of a 12 well flat-bottom plate (Corning). The plate was incubated for 1 hour at 37°C. After the incubation, 1,000 mL of lymphocytes suspended in complete Iscove's Modified Dulbecco's Medium, IMDM (Life technologies) were plated into each well after the wells were washed twice with FACS buffer. The plate was then incubated in a CO_2 incubator at 37°C for 3 days. On the 3rd day following

plating, cells were counted and suspended in complete IMDM supplemented with 100 Units/mL of recombinant IL-2 at 500×10^3 cells/mL. Cells were further expanded for an additional 2-3 days, before they were collected for experiments.

2.8 Fluorescence labeling of immune cells

Lymphocyte suspensions were washed twice with PBS and suspended at 2×10^7 cells/mL. For labeling with cell proliferation dye eFluor 450, the cell suspensions were combined with equal volume of 20 μM eFluor 450 dye (eBioscience) diluted in PBS. For CFSE labeling, cell suspensions were combined with equal volume of 1 μM CFSE diluted in PBS. The cell suspension-dye mixtures were incubated in a CO_2 incubator at 37°C for 10 minutes in the dark. Following the incubation, cells were spun down in a centrifuge and cell pellet suspended in 2 mL fetal bovine serum. The cell suspensions were incubated in the dark on ice for 5 minutes followed by two washes using 5 mL of complete IMDM media each time.

2.9 Antigen dependent fluorescence dilution assay

CD8 T cells were first isolated and fluorescently labeled with proliferation tracker eFluor 450 or CFSE. 20×10^3 fluorescence-labeled CD8 T cells were co-cultured with 1×10^6 BoyJ splenocytes in the presence of varying concentration of the $OVA_{257\text{-}264}$ peptide and incubated in a 96 well U-bottom plate for 4 days in a CO_2 incubator at 37°C. On day 4, cell samples were FC blocked with 5 μg/mL of anti-CD16/32 (eBioscience) in 100 μL of FACS buffer, stained with eFluor 780 viability dye (eBioscience), surface markers and acquired using LSRII or Fortessa flow cytometers (BD Biosciences).

2.10 Intracellular cytokine staining

To assess antigen sensitivity of effector CD8 T cells, 1×10^6 *in vitro* expanded CD8 T cells were co-cultured with 1×10^6 BoyJ splenocytes and varying concentrations of $OVA_{257-264}$ peptide in the presence of Golgiplug (BD Biosciences) for 6 hours in a 96 well U-bottom plate (BD/Falcon). To assess the frequency and avidity of effector CD8 T cells *ex-vivo*, 3×10^6 cells from lymphoid organs were co-cultured with varying concentration of the $OVA_{257-264}$ peptide in the presence of Golgiplug (BD Biosciences) for 6 hours. Following the incubation in a CO_2 incubator at 37°C, the cell culture were FC-blocked with 5 μg/mL of anti-CD16/32 (eBioscience) in 100 μL FACS buffer, stained with eFluor 780 viability dye (eBioscience) and surface markers on ice for 15 minutes. Cells were then fixed with BD Cytofix/Cytoperm solution (BD Biosciences) for 10 minutes on ice in the dark followed by staining with anti-cytokine antibodies suspended in 1 mL of BD Perm/Wash buffer for 30 minutes in the dark at room temperature. After cytokine staining, cells were washed once with 1× BD Perm/Wash buffer followed by two additional washes with FACS buffer before data acquisition. Stained samples were stored at 4°C in the dark, and acquired within 24 hours after the staining procedure was completed. LSRII and Fortessa (BD Biosciences) were used to acquire the data.

2.11 Thymidine incorporation assay

One hundred thousand magnetically purified CD8 T cells were co-incubated with 1×10^6 irradiated splenocytes (2,000 rd, Rad source RS-2000 X-ray irradiator) and various concentrations (From 10^{-5} M to 10^{-12} M) of the $OVA_{257-264}$ peptide for 3 days. Cell culture was

then pulsed with 1 μCi of 3H thymidine for 16 hours and radioactive incorporation measured by a Microbeta Trilux counter (Perkin Elmer).

2.12 Immunoprecipitation-flow cytometry

Antibodies were conjugated to carboxylate-modified latex (CML) beads using the carbodiimide method [444, 445]. Briefly, 5 μm CML beads (Life Technologies) were enumerated on a hemacytometer. Eighteen million CML beads were then washed twice in 1 mL sterile PBS and activated with carbodiimide. Following activation, CML beads were incubated with 5 μg of goat-anti ovalbumin polyclonal antibody (MP Biomedicals) for 4 hours. After incubation, CML beads were then BSA-blocked, washed and stored in accordance to the protocol [444]. To detect the presence of OVA in mouse blood, 10 μL aliquots of blood samples were collected using Heparinized Micro-hematocrit capillary tubes (Fisher) following tail poke. Blood samples were mixed with PBS containing 0.1% TWEEN 20, and 50 mM of EDTA for RBC lysis. Following RBC lysis, samples were topped up to 100 μL with PBS and each sample was probed with 50,000 CML beads suspended in 5 μL of FACS buffer. Samples were incubated in a 96 well U-bottom plate (BD/Falcon) overnight on a plate shaker at 4 °C. OVA molecules captured by the CML beads were then detected using 100 μL of of polyclonal rabbit anti-ovalbumin antibody (Biodesign International) suspended in FACS buffer at a final concentration of 10 μg/mL followed by secondary antibody labeling using 100 μL PE-conjugated goat anti-rabbit IgG secondary antibody (Jackson Immunoresearch Laboratories) at a final concentration of 0.5 μg/mL. Data was acquired using BD Fortessa flow cytometer in conjunction with BD FACSDiva software (BD Biosciences), and analyzed as described above.

2.13 Flow cytometry

Antibodies (clone name) against CD3 (145-2C11), CD4 (GK1.5), CD5 (53-7.3), CD8 (53-6.7), CD19 (ebio1D3), TCRβ (H57-597), Vβ5 (MR9-4), CD11b (M1/70), CD11c (N418), CD24 (M1/69), CD28 (37.51), CD44 (IM7), CD45.1 (A20), CD45.2 (104), CD62L (MEL-14), CD69 (H1.2F3), CD90.1 (HISS1), CD90.2 (53-2.1) $H2K^b$-SIINFEKL (eBio25-D1.16), F4/80 (BM8), MHCII (M5/144.15.2), NK1.1 (PK136), IFN-γ (XMG-1.2), TNF-α (MP6-XT22), and fixable viability dye eFluor 780 were purchased from eBioscience. Rabbit anti-chicken ovalbumin antibody was a generous gift from Dr. Kenneth Harder who purchased the Ab from Biodesign international. Goat anti-chicken ovalbumin antibody was purchased from MP Biomedicals and PE-conjugated goat anti-rabbit antibody was purchased from Jackson Immunoresearch Laboratories. Alpha galatosylceramide loaded CD1d tetramer was used to detect NKT cells was obtained from the NIH tetramer core at Emory University. Flow cytometry data were collected using LSRII and Fortessa in conjunction with BD FACS Diva software (BD Biosciences). Flow cytometry data was analyzed using FlowJo 8.6 (Tree Star).

2.14 Statistical analyses

Statistical significance was determined by unpaired, two-tailed student *t* tests unless otherwise specified. In section 3.2.5, a Dunnett's test was used to determine the statistical significance of splenic Treg cell frequencies. In section 3.2.6, Gehan-Breslow-Wilcoxon tests were used to determine whether the differences in the treatment groups were statistically significant. All statistical analyses were performed using Prism6 (GraphPad).

Chapter 3: NOVEL LIPOSOME THERAPY PREVENTS AUTOMMUNE DIABETES IN NON-OBESE DIABETIC MICE

3.1 Introduction

Type 1 diabetes is an autoimmune-mediated disease in which the insulin producing β-cells of the pancreatic islets are targeted and destroyed by the immune system resulting in hyperglycemia and lifelong insulin dependency [342]. The etiology of T1D is believed to be a complex combination of environmental and genetic factors that culminate in the activation of autoreactive T cells that infiltrate pancreatic islets and destroy the β-cells. Although the precise factors responsible for initiating pathogenesis remained unknown, defects in T and B lymphocyte selection (central tolerance), regulatory lymphocyte function (peripheral tolerance), antigen presentation, as well as other immune cells underlie the development of T1D.

Investigation of the non-obese diabetic (NOD) mice revealed that NKT cells play critical roles in the development of T1D. NKT cells are a distinct lineage of highly conserved T cells that bridge innate and adaptive immunity by receiving signals from antigen-presenting cells (APCs) and delivering signals that can efficiently prime and expand antigen-specific T cells [255, 269]. Unlike conventional T cells, NKT cells express an invariant αβ TCR that recognizes endogenous and exogenous lipid antigens presented on the non-classical MHC molecule, CD1d [255, 264, 446]. Upon strong interaction between NKT cell TCR and lipid antigens:CD1d complex, NKT cells can become activated and exert their immunomodulatory function. The most powerful activator of NKT cells described to date is a non-mammalian derived lipid antigen alpha galactosylceramide (αGalCer) [287]. NKT cells activated by αGalCer can rapidly produce copious quantities of immunomodulatory cytokines, including IL-2, IL-4, IL-10 and IFN-γ [85].

IL-2 is a key cytokine for both the survival and expansion of $FOXP3^+$ CD4 T cells [218]. In addition, NKT cells may also modulate self-reactive cytotoxic $CD8^+$ T lymphocytes (CTLs) by promoting the maturation of tolerogenic APCs or through the regulation of NK cell activity [280, 379]. DCs tolerized by NKT cells can then promote the generation of T regulatory cells to promote tolerance [287]. Thus, NKT cells possess multiple mechanisms by which they may tolerize self-reactive immune cells.

The transcription factor forkhead box p3 (FOXP3) expressing T regulatory (Treg) cells, are one of the key peripheral tolerogenic mechanisms responsible for suppressing autoimmune reactions against tissue-specific antigens through several mechanisms [202]. In both humans and mice, Treg deficiency results in the development of a fatal autoimmune condition involving multiple organs including the islets of Langerhans [212, 213]. In T1D patients, defects in Treg phenotype and suppressive function have been observed [447, 448]. In NOD mice, adoptive transfer of islet-specific Treg cells or administration of IL-2 have been shown to confer protection against T1D [449]. These findings suggest that Treg cells play a critical role in suppressing β-cell reactive T cells to confer protection against T1D.

It has been shown that NKT cells can protect NOD mice from T1D [450, 451]. Conversely, NKT cell deficiency can result in the exacerbation of the disease [452]. The protective effects of NKT cells can be harnessed by repeated intraperitoneal (i.p.) or daily intravenous (i.v.) plus i.p. injections of aqueous αGalCer in wild-type NOD mice, but not NKT-cell deficient *CD1d*$^{-/-}$ mice [450-452]. The mechanism of protection shown with the aforementioned studies was the skewing of T cell polarization away from the IFN-γ secreting Th1 phenotype towards the IL-4 secreting Th2 phenotype. Interestingly, NKT cells activated with complete Freund's adjuvant (CFA) was also shown to be protective against T1D, but the

mechanism of protection requires the actions of Th1 cytokine IFN-γ and NK cells [280, 282, 379]. Moreover, NKT cells can also confer protection through Treg cells. Taken together, current experimental evidence suggest that NKT cells possess therapeutic potential against T1D in NOD mice through several T cell mediated mechanisms.

Despite NKT cell initial promising results, disease protection provided by NKT cells in the aforementioned studies was dependent on continuous, life-long administration of αGalCer, and the protection conferred by them was largely restricted to a 4 week delay in disease progression. To complicate the matter further, in some studies treatment of αGalCer resulted in immune activation rather than suppression. Furthermore, αGalCer treatment in certain strains of mice have resulted in pathologies such as livery injury, spontaneous abortion and arteriosclerosis [287]. To reduce the unwanted side effects while facilitating differential cell targeting of αGalCer to antigen presenting cells, αGalCer was encapsulated in liposomes, and the resulting liposomal αGalCer has been found to be especially immune dampening, thus demonstrating a potentially viable option for αGalCer to be used as an immunosuppressive agent to treat autoimmune diseases such as T1D [289, 453, 454].

Mice possess two insulin genes, insulin 1 and insulin 2, which differ by two amino acids at the positions 9 and 29 on the insulin B chain. Curiously, breeding insulin 2 knockout gene onto the NOD background greatly accelerates T1D, while breeding insulin 1 knockout gene onto the NOD background prevents the disease [455]. These findings suggest that two amino acids difference between insulin 1 and insulin 2 drive autoimmunity in NOD mice. It is now known that in NOD mice, most islet-infiltrating CD4 T cells are reactive to insulin with more than 90% of the reactivity directed against insulin B:9-23. Furthermore, a segment of insulin B:9-23, namely insulin B:15-23 can also be recognized by CD8 T cells. These observations led to the

generation of a NOD mouse strain in which the amino acid on position 16 of the insulin B chain was changed from tyrosine to alanine. The resulting NOD strain was diabetes free [455], suggesting that stopping the priming of insulin B:9-23-specific T cells not only prevents the initiation of the disease but also T cell autoimmunity against other β-cell-specific antigens [456]. Consequently, these experimental results suggest that insulin B:9-23 may be a suitable target for future tolerogenic treatment strategies against T1D.

In this study, we examined whether co-delivery of αGalCer and insulin encapsulated within liposomes would be effective in activating the NKT cells and could ameliorate T1D in NOD mice. We found that αGalCer liposome, administered subcutaneously can potently activate NKT cells in a CD1d dependent manner. Consequently, NKT cells from the subcutaneous liposomal αGalCer treated animals were found to produce copious amounts of cytokines and could activate other immune cell subsets such as NK cells, B cells and T cells through cytokine secretion. In addition to activating effector cells, we also showed that subcutaneous liposomal αGalCer induced rapid expansion and activation of splenic APC subsets. Upon closer examination of the splenic APC subsets, we found that encapsulation of αGalCer into liposomes enhanced uptake and presentation of αGalCer by splenic APCs. Further, we found that subcutaneous administration of liposomes containing αGalCer plus insulin reduced the frequency of islet reactive T cells and conferred protection against T1D.

3.2 Results

3.2.1 Subcutaneous injection of liposome-embedded αGalCer results in rapid and potent immune activation of splenic NKT cells

Previous studies have administered αGalCer either intravenously or intraperitoneally to modulate immunity *in vivo* [278, 289, 454]. Here, we sought to determine whether delivering αGalCer encapsulated within liposomes could activate NKT cells upon subcutaneous injection given the greater potential for translation to the clinic with this route of administration. To visualize TCR signaling within NKT cells, we utilized a transgenic reporter mouse line in which GFP expression is controlled by the promoter of the immediate early gene *Nr4a1* (Nur77) locus [442]. In T cells of this reporter mouse, GFP expression is upregulated upon TCR-induced activation but not by inflammatory stimuli and the level of GFP intensity is reflective of overall TCR signaling strength. To test whether liposomes containing αGalCer can activate NKT cells, cohorts of $Nur77^{GFP}$ mice were subcutaneously injected with αGalCer liposomes (Lp) or sterile PBS near the tail base and the frequency and activation status of splenic NKT cells were assessed 12 or 24 h later. Strikingly, the subcutaneous injection of liposomes containing αGalCer resulted in a decreased frequency of detectable splenic NKT cells at 24 h post-treatment relative to PBS-injected or untreated naïve mice (*Figure 3-1* and data not shown; 0.19 ± 0.054 % vs 2.58 ± 0.38 %, $P < 0.01$). Further, the reduced proportion of splenic NKT cells at 24 h post treatment with liposomal αGalCer was associated with a 3-fold increase GFP expression relative to mice injected with PBS (*Figure 3-1*). The observed decreases in splenic NKT cell frequencies upon liposomal αGalCer treatment may be the consequence of TCR downregulation, activation-induced cell death, NKT cell mobilization or increase in other splenic cell populations [457].

Taken altogether, these results indicate that subcutaneous administration of αGalCer containing liposomes potently activates NKT cells through direct TCR stimulation.

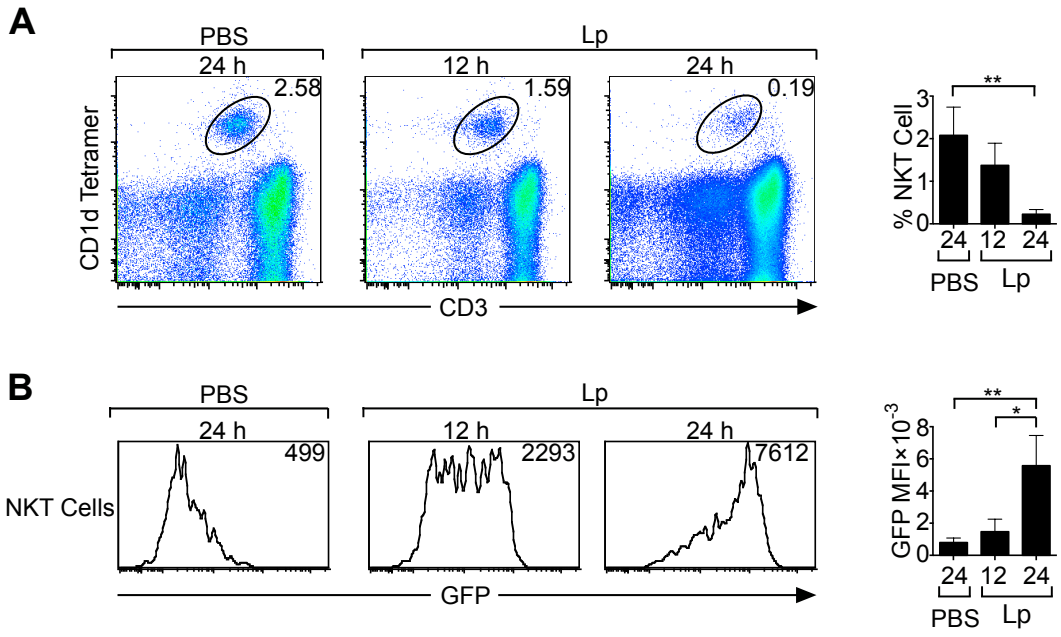

Figure 3-1 Subcutaneous injection of liposome-embedded αGalCer results in rapid and potent immune activation of splenic NKT cells.
Cohorts of TCR signaling reporter Nur77^{GFP} mice were injected s.c. near the base of the tail with saline (PBS) or liposome-encapsulated αGalCer (Lp). (***A***) NKT cells were discriminated from the splenic population that is CD19^{-}, on the basis of αGalCer/murine CD1d tetramer and CD3 antibody staining. At 12 and 24 h post-treatment, the frequencies of splenic NKT cells are presented in the upper right hand corner as a bar graph (n=3). (***B***) Histogram plots of GFP expression in NKT cells are shown in the lower left corner. The cumulative mean fluorescence intensity values are shown as a bar graph on the lower left. Error bars represent the SEM and single or double asterisks signify p values of less than 0.03 and 0.01 respectively (n=3). Data shown is representative of 2 separate experiments.

3.2.2 Subcutaneous administration of liposome-encapsulated αGalCer causes rapid CD1d-dependent activation of multiple immune cell types

NKT cells secrete copious amounts of cytokines rapidly upon intravenous injection of aqueous αGalCer which in turn activates other immune cells such as NK cells, B cells, CD4 T cells and CD8 T cells [278]. Consequently, we next sought to determine whether subcutaneous injection of liposomal αGalCer can induce NKT cells to activate other immune cell subsets. Cohorts of wild type (C57BL/6) or NKT cell-deficient (*CD1d*$^{-/-}$) C57BL/6 mice were subcutaneously injected with PBS, liposomal encapsulated αGalCer (Lp, ~1μg) or aqueous αGalCer (Aq, ~1μg) and activation of splenic subsets assessed at 24 h post-treatment. Delivery of αGalCer in liposomal resulted in substantial increases in CD69 levels by NKT cells, NK cells and B cells while CD8 and CD4 T cells exhibited smaller rises versus PBS-treated mice (*Figure 3-2A*, NKT cells, 2.5 ± 0.034-fold, $p < 0.0005$; NK cells, 4.6 ± 0.78-fold, $p < 0.004$; B cells, 4.8 ± 0.65-fold, $p < 0.001$; CD4 T cells, 1.7 ± 0.12-fold, $p < 0.002$; CD8 T cells, 2.9 ± 0.48-fold, $p < 0.02$). In addition to an activated surface phenotype, liposomal αGalCer induced NKT cells and NK cells to secrete IFN-γ as compared to PBS treated mice (*Figure 3-2A*, NKT cells, 2.5 ± 0.21-fold, $p < 0.0004$; NK cells, 2.0 ± 0.19-fold, $p < 0.003$). Despite similar amounts of αGalCer in aqueous or liposome-derived and shared route of administration, direct comparisons between these cohorts is complicated because of possible variances in activities between these two formulations. Lastly, the lack of immune activation observed in *CD1d*$^{-/-}$ mice upon administration of liposomal αGalCer demonstrates that its activity is dependent on the presence and function of NKT cells (*Figure 3-2C*). Taken together, these results showed that

subcutaneous delivery of liposomal αGalCer treatment is effective at activating NKT cells and modulating the functions of other immune cell subsets.

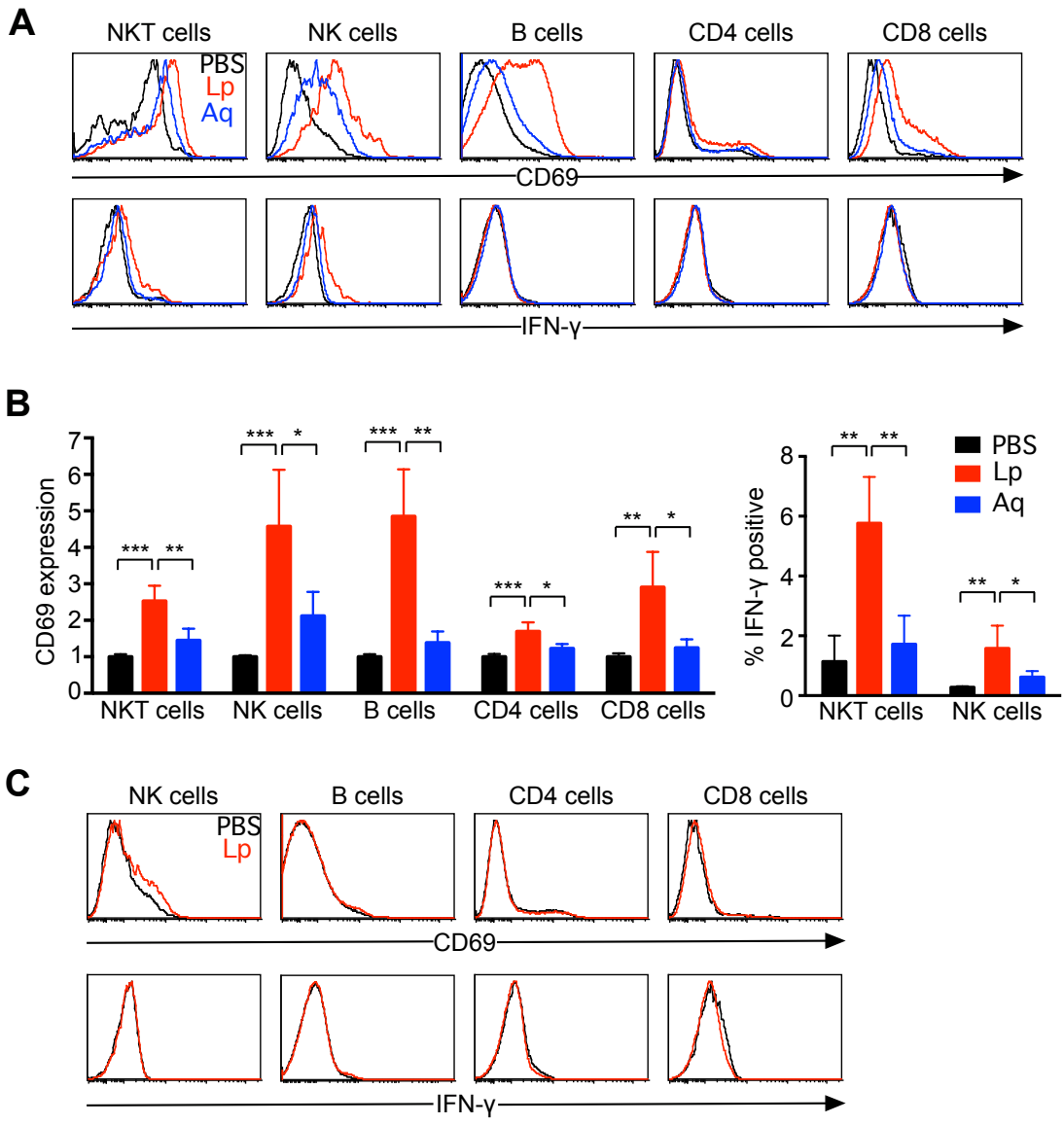

Figure 3-2 Subcutaneous administration of liposome-encapsulated αGalCer causes rapid CD1d-dependent immune cell activation.
(***A***) Wild type C57BL/6 mice were injected subcutaneously near the base of the tail with PBS, aqueous αGalCer (Aq, ~1μg) or liposome-encapsulated αGalCer (Lp, ~1μg). At 24 h post treatment, spleens were rapidly immerced in ice-cold media and processed under ice cold conditions to preserve cytokine for staining. Subsequently, single-cell suspensions were labeled for cell surface markers, fixed and stained intracellularly with cytokines-specific antibodies. (***B***) Cumulative data of A are shown as bar graphs normalized to PBS MFI, error bars represent the SEM and single, double or triple asterisks signify p values of less than 0.04, 0.008 and 0.0002. (***C***) *CD1d*$^{-/-}$ mice on the C57BL/6 background (n=3) were injected s.c. with PBS or liposome-encapsulated αGalCer (Lp, ~1μg). At 24 h post treatment, spleens were treated and stained the same as ***A***. Results representative of 3 separate experiments.

3.2.3 Subcutaneous injection of liposome-embedded αGalCer activates splenic antigen presenting cell subsets

Liposomes can facilitate target-specific delivery of their drug payload both passively and actively. In active targeting, a ligand-specific for a target cell surface molecule is incorporated into the lipid bi-layer of the liposomes, allowing the ligand to bind to target cell thus brining the liposome close to cell target for uptake. Passive targeting takes advantage of the tendency of DCs and macrophages to randomly take up, and process particles of certain sizes (200-400 nm) for antigen presentation [458, 459]. In this part of the study, we sought to investigate whether encapsulation of αGalCer in a liposome would impact its uptake by DCs. To test our hypothesis, we stained splenocytes from liposomal or aqueous αGalCer treated C57BL/6 mice with surface markers to delineate different population of splenic APCs and assess αGalCer presentation on CD1d molecules by these splenic APC populations. We found that subcutaneous treatment using liposomal or aqueous αGalCer resulted in a expansion of splenic APC populations. The splenic $CD11c^{low}$ $CD317^{+}$ population in particular saw the most significant expansion, having expanded 10 and 11 fold in liposomal αGalCer and aqueous αGalCer treated mice relative to the PBS controls, respectively (*Figure 3-3A&B*). In addition, liposomal αGalCer treatment was found to expand the CD11c intermediate-high, $CD317^{+}$ population by approximately 1.5 fold compared to PBS treated controls (*Figure 3-3A&B*). Interestingly, liposomal αGalCer and aqueous αGalCer treatment resulted in cell number decrease in the splenic $CD11c^{int}$ $CD317^{-}$ and $CD11c^{hi}$ $CD317^{-}$ populations (*Figure 3-3 A&B*). Since CD317 can become upregulated in myeloid cells following exposure to IFN-γ [460], and that splenic NKT cells and NK cells produced IFN-γ upon αGalCer

treatment (*Figure 3-2A&B*), our data suggest that αGalCer treatment can induce activation of splenic APC populations, possibly through the action of IFN-γ.

Another hallmarks of APC activation by cytokines or inflammatory stimuli is the upregulation of MHC class I and II molecules [153]. The upregulation of MHC molecules allows the APCs to better present peptide antigens to T cells for T cell activation. We analyzed surface expression of MHC II molecules in splenic APC populations and found that αGalCer treatment resulted in changes of MHC II expression in the splenic APC subsets. In the $CD11c^{low}$ $CD317^{+}$ population, which expands the most after αGalCer treatment, MHC II expression was downregulated (*Figure 3-3C&D*, MHC II MFI Lp 957 ± 43.2 vs PBS 551 ± 22.2, $p < 0.006$; Aq 430 ± 21.2 vs PBS 551 ± 22.2, $p < 0.008$). In the $CD11c^{int}$ $CD317^{+}$ subpopulation, αGalCer treatment induced MHC II upregulation compared to PBS treated controls (*Figure 3-3C&D*, MHC II MFI Lp 1,489 ± 136.7 vs PBS 957 ± 43.2, $p < 0.01$; Aq 1,301 ± 111.7 vs PBS 957 ± 43.2, $p < 0.03$). Both liposomal and aqueous αGalCer could induce MHC II upregulation with samples from liposomal αGalCer treated mice having MHC II MFI approximately 3 times higher than that of PBS treated mice, while samples from aqueous αGalCer treated mice exhibited MHC II MFI approximately 2 times higher than PBS treated mice (*Figure 3-3C&D*). Taken together these results showed that αGalCer treatment can activate certain splenic APC populations.

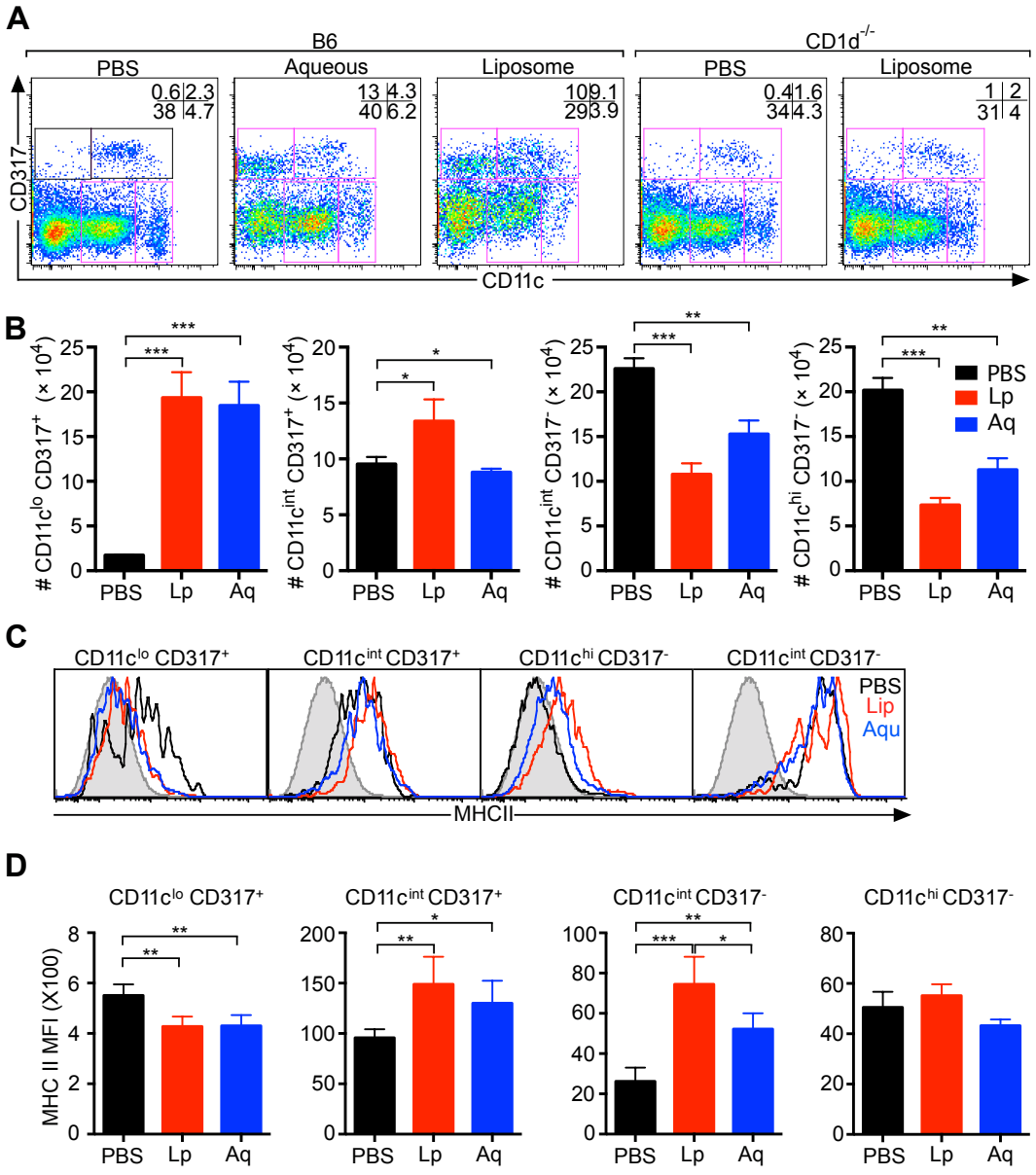

Figure 3-3 Subcutaneous injection of liposome-embedded αGalCer induces the frequency increase of splenic antigen presenting cell subsets.
Wild type or *CD1d*$^{-/-}$ (n=2) C57BL/6 mice (n=4) were injected s.c. near the base of the tail with PBS, aqueous αGalCer or liposome-encapsulated αGalCer. At 24 h post treatment, splenic single cell suspensions were stained with fluorchrome-conjugated antibodies specific for CD3, CD19, CD11c, CD317, MHC Class II and CD11b. (***A***) After the exclusion of B cells and T cells (CD19^{+}CD3^{+} events), antigen-presenting cell subsets were discriminated by expression of CD317 and CD11c. (***B***) Cumulative data is shown for the proportion of the indicated immune cell subsets. Error bars represent the SEM. Shaded histograms represent staining of CD1d-deficient splenic subsets. (***C***) Expression of surface MHC class II and CD11b by the indicated

antigen presenting cell subsets. (***D***) Cumulative results of ***C***, single, double or triple asterisks denote p values of less than 0.04, 0.008, and 0.0007 respectively.

3.2.4 Lipid-antigen presentation by antigen-presenting cell populations upon s.c. injection of liposome-embedded or aqueous αGalCer

We next sought to determine whether encapsulation of αGalCer would enhance its uptake by antigen presenting cells. To this end, we analyzed splenic APC sub-populations (defined by CD11c and CD317, as shown in *Figure 3-3A*) of αGalCer or PBS treated mice for surface expression of CD1d and αGalCer : CD1d molecules. Our analyses found that αGalCer treatment resulted in the upregulation of CD1d in several splenic APC subpopulations. In the $CD11c^{-}$ $CD317^{+}$ population, treatment of αGalCer induced upregulation of CD1d as indicated by the increase in MFI (*Figure 3-4B&C*, CD1d MFI Lp 1,266 ± 102.5 vs PBS 944 ± 68.1, $p < 0.0396$; Aq 1,400 ± 59.5 vs PBS 943.5 ± 68.2, $p < 0.002$). In the $CD11c^{int}$ $CD317^{+}$ population, both liposomal and aqueous αGalCer induced upregulation of CD1d (*Figure 3-4A*, Lp 1,558 ± 134.6 vs PBS 628.3 ± 21.0, $p < 0.0005$; Aq 1,097 ± 106.2 vs PBS 628± 21.0, $p < 0.005$). Liposomal αGalCer treatment resulted in a 2.5 fold increase in CD1d expression compared to PBS treated controls, and aqueous αGalCer treatment resulted in a 1.7 fold increase (*Figure 3-4*). In the $CD11c^{int}$ $CD317^{-}$ population, αGalCer treatment resulted in minor CD1d upregulation, although samples from liposomal αGalCer treated mice showed slightly higher CD1d expression, the difference was not statistically significant (*Figure 3-4A*, Lp 543 ± 15.2 vs PBS 464 ± 7.09, $p < 0.003$; Aq 515 ± 8.65 vs PBS 464 ± 7.09, $p < 0.004$). We next analyzed αGalCer presentation on CD1d molecules in the APC subsets by using an antibody specific for αGalCer:CD1d complexes. We found that liposomal encapsulation of αGalCer allowed αGalCer uptake and

presentation by the splenic CD11c^{int} CD317^{+} population (*Figure 3-4B*, CD1d: αGalCer MFI Lp 50.0 ± 3.33 vs PBS 2.87 ± 0.86, $p < 0.0001$). Taken together these resutls suggest that subcutaneous αGalCer treatment can result in the upregulation of CD1d and presentation of αGalCer in several splenic DC subsets.

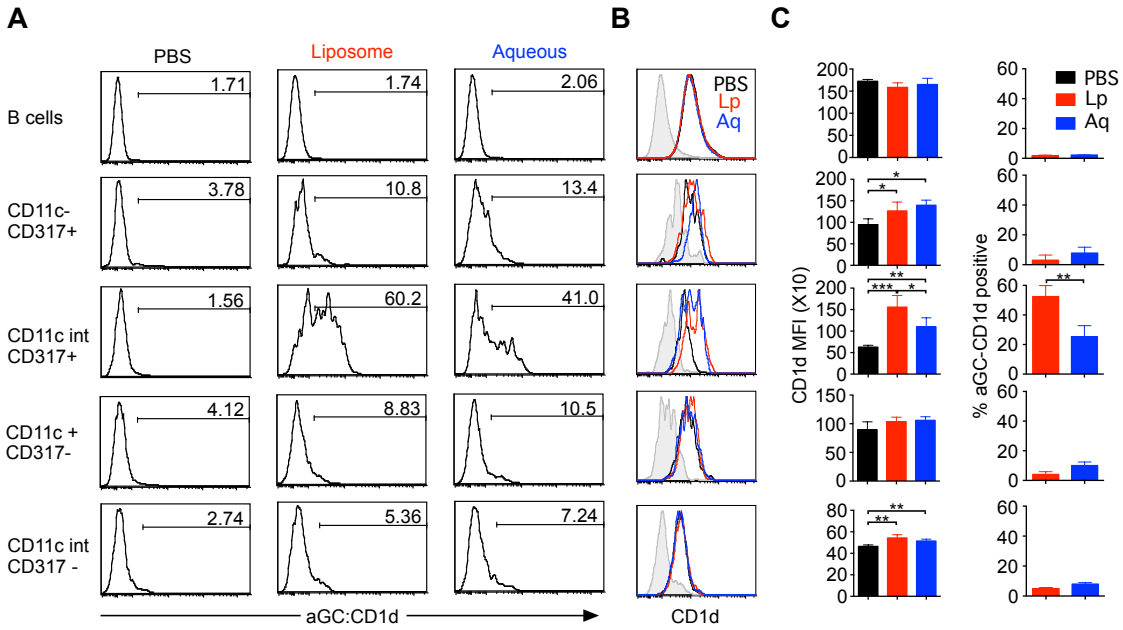

Figure 3-4 Lipid-antigen presentation by antigen-presenting cell populations upon s.c. injection of liposome-embedded or aqueous αGalCer.
Wild type (n=4) or *CD1d*$^{-/-}$ C57BL/6 mice (n=4) were injected s.c. near the base of the tail with PBS, aqueous αGalCer or liposome-encapsulated αGalCer. At 24 h post treatment, splenic single cell suspensions were stained with fluorchrome-conjugated antibodies specific for CD3, CD19, CD11c, CD317, MHC Class II, CD1d and anti αGalCer:CD1d antibodies. (***A***) CD1d levels are shown for indicated the antigen-presenting cells. Shaded histograms represent staining of CD1d-deficient splenic subsets. (***B***) Detection of αGalCer presentation using αGalCer:CD1d complex antibodies. (***C***) Cumulative data are shown as bar graphs with error bars representing the SEM (*: $p<0.05$, **$p<0.005$, ***$p<0.0005$).

3.2.5 Co-delivery of αGalCer and insulin reduces the frequency of circulating autoreactive CD8 T cells but does not impact insulitis

Repeated injection of αGalCer via the intraperitoneal and intravenous route was reported by multiple research groups to ameliorate a variety of autoimmune disorders including T1D

[289, 453, 454]. After observing the effects of subcutaneous liposomal αGalCer in activating the immune cell subsets of C57BL/6 mice we investigated whether liposomal αGalCer could modulate the immune system and prevent T1D in NOD mice and whether the inclusion of autoantigen insulin would provide additional benefit in curtailing autoimmune responses from the autoreactive T cells. To investigate our hypothesis, we injected cohorts of four-week old female NOD mice with liposomal αGalCer, or liposomal αGalCer with insulin via the intraperitoneal, intravenous or subcutaneous routes. Treated animals were followed for eleven-weeks, and every week blood and tissue samples were taken for enumeration of autoreactive T cells and T regulatory cells. Liposomal αGalCer-insulin treatment lowered the frequency of circulating β-cell-specific CD8 T cells (identified by NRPV7 tetramer staining and CD8, *Figure 3-5B*) on week 8 (*Figure 3-5A,B&C*, frequencies of β-cell-specific CD8 T cells in the blood αGalCer-insulin i.p. 0.525 ± 0.0640 vs no treatment 1.26 ± 0.144, $p < 0.0003$; αGalCer-insulin i.v. 0.649 ± 0.111 vs no treatment, $p < 0.005$; αGalCer-insulin s.c. 0.438 ± 0.0603 vs no treatment, $p < 0.0002$). One of the mechanisms of immune regulation by αGalCer treatment was the expansion of T regulatory cells [289]. We next analyzed spleen samples from 16 week old treated NOD mice to enumerate T regulatory cell frequency. Surprisingly, we found that in most groups of αGalCer-treated mice, the treatment had resulted in a noticeable decrease in splenic T regulatory cell frequency compared to no treatment controls (*Figure 3-5D&E*, splenic T regulatory cell frequency αGalCer i.p. 5.70 ± 0.540 vs no treatment 10.5 ± 1.43, $p < 0.02$; αGalCer-insulin i.p. 5.43 ± 0.326 vs no treatment, $p < 0.03$; αGalCer-insulin s.c. 5.63 ± 0.260 vs no treatment, $p < 0.02$). Lastly we analyzed histological sections of pancreata from liposome treated mice (five sections per pancreas, cut approximately 100 microns apart and a least 100

islets scored in a blinded fashion). Our results showed that regardless of route of administration, αGalCer-insulin treatment did not ameliorate the degree of insulitis compared to the non-treated controls (*Figure 3-5G*).

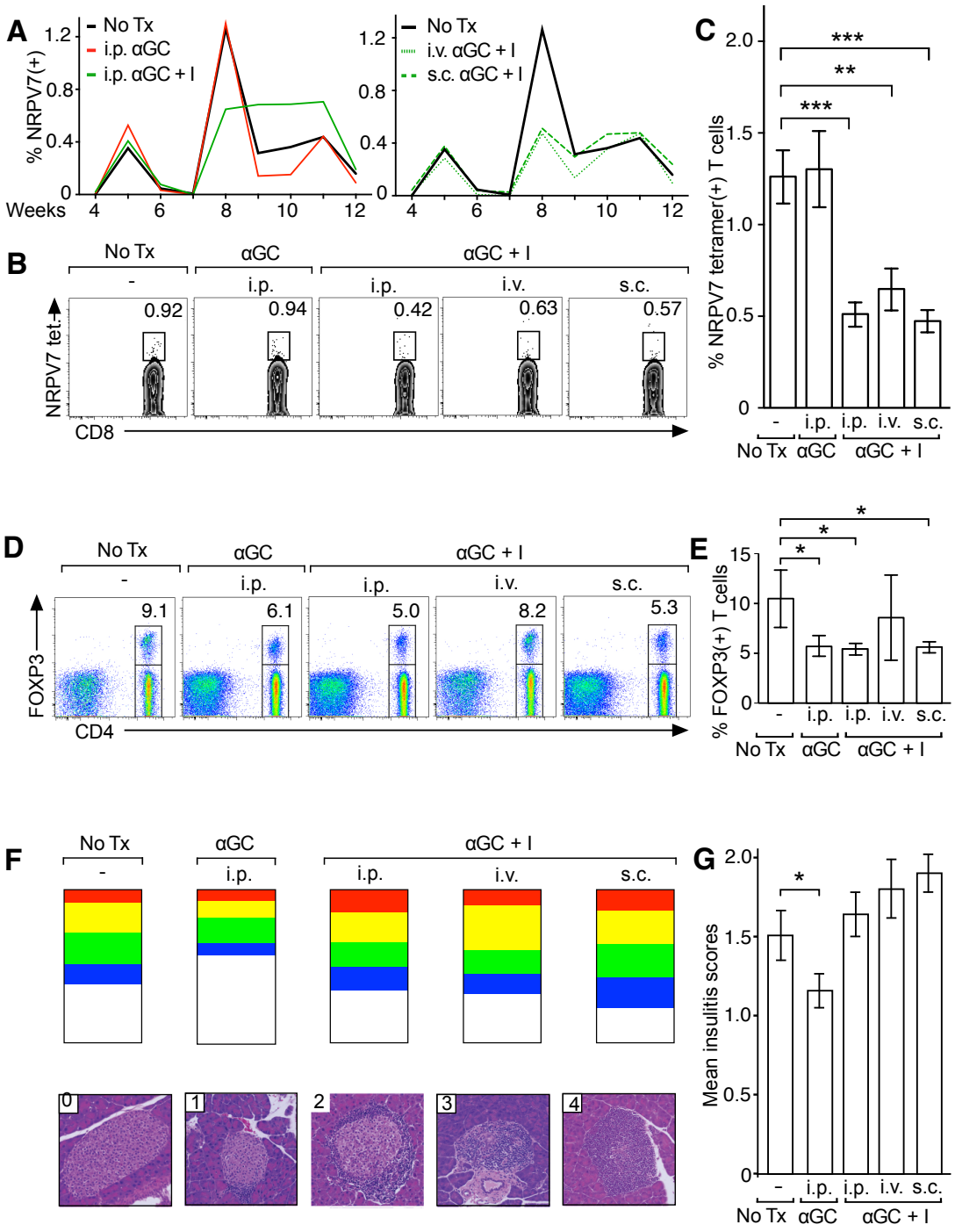

Figure 3-5 Co-delivery of αGalCer and insulin reduces the frequency of circulating autoreactive CD8 T cells but does not impact insulitis.

Cohorts of four-week old female NOD mice (8 per group) were left untreated (No Tx) or injected with liposomes embedded with αGalCer alone (αGC; i.p.) or in tandem with insulin (αGC + I; i.p., i.v. or s.c.) semi-weekly for 5 weeks. (***A***) Peripheral blood from treated and untreated mice

was drawn weekly and the mean frequency of NRPV7-K^d tetramer$^+$ $CD8^+CD3^+$ T cells, measured via flow cytometry, plotted versus age of mice (in weeks). (***B***) Peripheral blood mononuclear cells were electronically gated on $CD3^+CD8^+$ T cells and frequency of $NRPV7^+$ CD8 T cells is presented in the top right corner of representative zebra plots. (***C***) Mean $NRPV7^+$ CD8 T cells per treatment group at 8 weeks of age is shown. Statistical significance was determined using a Dunnett's test and double and triple asterisks represent p values of less than 0.005 and 0.0005 respectively. (***D***) Representative density plots indicating the frequency of $FOXP3^+$ cells among splenic CD4 T cells (top right corner) in untreated and liposome-injected NOD mice at 16 weeks of age. (***E***) Cumulative data indicating the frequency of $FOXP3^+$ cells among splenic CD4 T cells in 16 weeks-old mice is shown. Errors bars represent the SEM and statistical significance assessed using a Dunnett's test. Single asterisk (*) indicates a p value of less than 0.05. (***F***) Histological analyses of pancreata from naive and liposome-treated NOD mice (top panel). Mean ages (weeks) ± STD (n = 8) per group are as follows: No Tx, 20.4 ± 8.1; αGalCer (i.p), 28 ± 2.9; αGalCer + I (i.p.), 27.3 ± 4.6; αGalCer + I (i.v.), 19.0 ± 7.6 and αGalCer + I (s.c.), 28.0 ± 3.9. Five sections per pancreas were cut approximately 100 microns apart and at least 100 islets scored in a blinded fashion. Insulitis was scored on a four-tier scale as described (bottom panel): 0, no infiltration; 1, peri-insulitis; 2, < 50% infiltration; 3, > 50% infiltration; 4, complete infiltration or breakdown of islet morphology. (***G***) Mean insulitis scores are shown with error bars representing the SEM and a single asterisk indicating a p value of less than 0.05.

3.2.6 Subcutaneous injection of liposomes bearing aGalCer and insulin protect NOD mice from type 1 diabetes

Continuous treatment of female NOD mice starting from early life with aqueous αGalCer injected intraperitoneally has been shown to confer protection against T1D in NOD mice [450, 451], however the dosage used and the frequency of use of αGalCer in these studies raised concerns of toxicity and the route of administration may prove to be limiting in its potential for translation to clinical use. To address these concerns, we treated cohorts of female NOD mice for 5 weeks starting from week 4 with liposomal αGalCer-insulin semi-weekly via i.p., i.v., or s.c. with 1 μg of αGalCer per injection. We then monitored the treated and untreated animals for incidences of diabetes for 21 weeks post treatment. We found that regardless of the presence of insulin, liposomal αGalCer injected intraperitoneally delayed the onset of diabetes but ultimately did not prevent type 1 diabetes. Although αGalCer alone injected i.p. trends towards

significance, the results were not statistically significant (*Figure 3-6A*). Surprisingly, liposomal αGalCer-insulin injected intravenously did not confer any protection against type 1 diabetes. The treatment group that was found effective in preventing type 1 diabetes was the subcutaneous liposomal αGalCer-insulin treated group (*Figure 3-6B*).

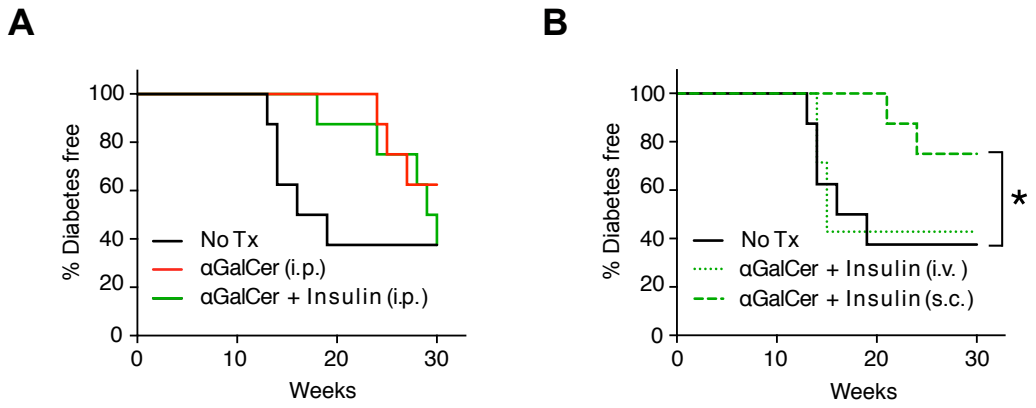

Figure 3-6 Subcutaneous injection of liposomes bearing αGalCer and insulin protect NOD mice from type 1 diabetes.
Cohorts of four-week old female NOD mice (8 per group) were left untreated or injected with liposomes embedded with αGalCer alone or in tandem with insulin semi-weekly for 5 weeks. Subsequently, blood glucose was monitored weekly for 21 weeks post-treatment and mice considered diabetic if registering two consecutive readings of 25 mmol/L or higher. (***A***) Survival curves are shown for NOD mice left untreated (No Tx, black line) or injected intraperitoneally (i.p.) semi-weekly for a 5-week period with liposomes carrying either αGalCer (αGC, red line) or αGalCer plus insulin (αGC + I, green line). (***B***) Survival curves are presented for NOD mice receiving semi-weekly injections of αGalCer and insulin-embedded liposomes intravenously (i.v., dotted green line) or subcutaneously (s.c., dashed green line). For reference, untreated mice injected are again shown as a solid black line. Statistical significance was determined using Gehan-Breslow-Wilcoxon tests and a single asterisk represents a p value equal to 0.044.

3.3 Discussion

NKT cells play an important role in conferring protection against T1D as their absence or defectiveness promote the disease [256-258, 452] and their activation confer protection against the disease [379, 450, 451]. Repeated, life-long injection of aqueous αGalCer, a glycolipid known to elicit robust NKT cell activation has been shown to protect against the disease however, the dosage, length of treatment as well as the routes of administration hinder the translation of αGalCer into clinical use. In this study, we showed that a single subcutaneous injection of liposomal αGalCer could potently activate immune cell subsets in a NKT cell/CD1d dependent manner, and that subcutaneous administration of liposomal αGalCer with insulin for a period of 5 weeks could confer long-term protection against T1D in NOD mice. Although the results of this study may serve as a proof of concept for future liposomal αGalCer agents aimed at achieving antigen-specific tolerance induction, the immunological mechanism that conferred protection in this study remains undefined. Herein, we will propose several mechanisms based on our data and the existing literature to chart the direction for future research.

Upon activation, NKT cells can rapidly secrete a variety of different cytokines to aid the activation of other immune cell subsets, many of which are known to confer protection against T1D and other autoimmune diseases [287]. Our previous work showed that complete Freund's adjuvant (CFA) could activate NKT cells in a CD1d-dependent manner. NKT cells were essential for NK cell mediated protection against T1D and IFN-γ secreted by NK cells played a pivotal role in conferring disease protection [280, 282, 379]. Interestingly, in this study we found that subcutaneous liposomal αGalCer treatment could rapidly activate splenic NK cells and induce IFN-γ secretion in a NKT cell/CD1d dependent manner, reminiscent of the results from our previous studies. In addition, activated splenic NKT cells readily secrete IFN-γ, a cytokine

important for NK cell activation and function, which also played a central role in NK cell mediated protection of T1D [278-280, 379]. Taken together, these results suggest that subcutaneous liposomal αGalCer-insulin treatment confers protection through the actions of NK cells.

Another piece of evidence that suggests the involvement of NK cells in conferring disease protection is the observed reduction in frequency of circulating β-cell-specific CD8 T cells in liposomal αGalCer-insulin treated mice on week 8 (*Figure 3-5A, B &C*). In our previous study, we showed that NK cells in CFA treated NOD/SCID mice reduced the number of islet infiltrating β-cell-specific CD8 T cells [280]. Although the experimental conditions between this study and the previous are different in many aspects, the reduction of circulating autoreactive CD8 T cells in the liposomal αGalCer-insulin treated mice may provide clues to the underlying mechanism of T1D protection conferred by the liposomal αGalCer-insulin treatment.

Plasmacytoid dendritic cells (pDCs) are a CD317 expressing DC subset known to induce tolerance in several inflammatory disorders primarily through T regulatory cells induction [461], and lower pDC frequency has been associated with T1D [462-464]. In the context of T1D, it has been reported that intraperitoneal aqueous αGalCer treatment could protect against T1D in NOD mice through pDC mediated induction and expansion of T regulatory cells [465]. In our study, treatment of liposomal or aqueous αGalCer resulted in an increase in number of two splenic $CD317^+$ APC subsets. The splenic $CD11c^{lo}$ $CD317^+$ population saw the largest amount of increase, having expanded in frequency 10 and 11 fold in liposomal αGalCer and aqueous αGalCer treated mice, respectively (*Figure 3-3*). This subset, characterized by their low MHCII expression best suits the pDC population described by Beaudoin *et al.* which were responsible for inducing β-cell-specific T regulatory cells after αGalCer treatment [465]. Unexpectedly, our

results showed decreased splenic T regulatory cell frequency and the degree of insulitis after liposomal αGalCer-insulin seemed to increase (*Figure3-5D-G*). One possible explanation is that splenic T regulatory cells were being mobilized to infiltrate the islets causing increased insulitis but instead of causing β-cell destruction, these T regulatory cells protected the β-cells from autoimmune destruction.

In addition to the $CD11c^{lo}$ $CD317^{+}$ subset, we also found that liposomal αGalCer treatment could expand the $CD11c^{int}$ $CD317^{+}$ population of APCs. This population of DCs expressed high levels of MHCII and CD1d after liposomal αGalCer treatment and also showed elevated surface staining of αGalCer-CD1d complex compare to PBS, indicating that encapsulation of αGalCer in liposomes allows uptake of αGalCer by this particular population of DCs. A previous report showed that this population of DCs could stimulate T cell proliferation upon activation by CpG, and appeared to exhibit antiviral activity [466]. Whether this pDC population could induce tolerance by activating NKT cells would be of great interest to examine in the future.

Achieving antigen-specific tolerance induction is one of the most important goals of any immune modulatory therapy; in this study we demonstrated the possibility of achieving such a goal through this novel liposomal αGalCer therapy. The proposed mechanism of protection conferred by our liposomal αGalCer therapy is illustrated in (*Figure 3-7*).

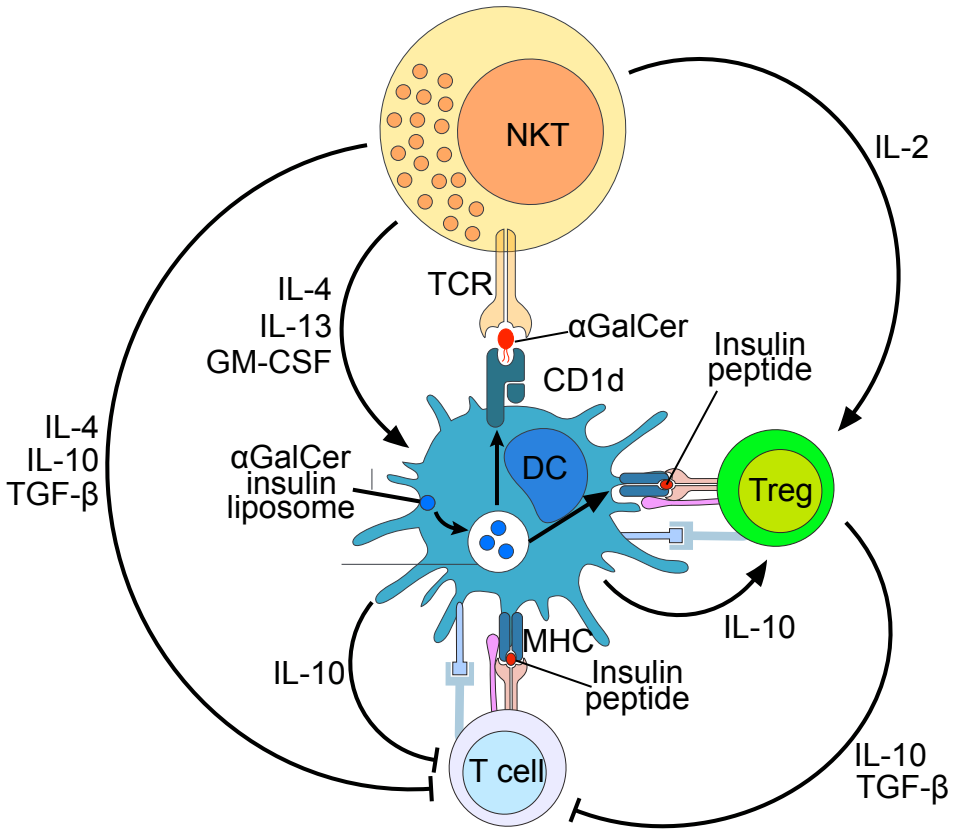

Figure 3-7 Proposed mechanism of protection against T1D by insulin containing αGalCer liposome treatment.
Uptake and processing of insulin containing αGalCer liposomes by DCs can lead to the presentation of αGalCer on CD1d molecules and insulin peptides on MHC molecules. αGalCer activated NKT cells can secrete tolerogenic cytokines such as IL-10 and TGF-β which can directly suppress insulin reactive T cells, thereby preventing them from acquiring effector function. Cytokines IL4, IL-13, secreted by NKT cells can polarize DCs into tolerogenic DCs, which in turn, can suppress insulin reactive T cells through IL-10 secretion or Treg induction. Insulin reactive Treg cells induced by the joint effort of NKT cells and tolerogenic DCs can also exert their suppressive effect on insulin reactive T cells, preventing them from acquiring effector function.

Chapter 4: DUAL TCR-EXPRESSING CD8 T CELLS ARE ASSOCIATED WITH ISLET AUTOREACTIVITY

4.1 Introduction

Type 1 diabetes is an autoimmune disease that culminates in T cell-mediated destruction of pancreatic β cells, and has been purported to result from defective central or peripheral T cell tolerance. Central tolerance, also known as negative selection, plays a critical function in eliminating autoreactive cells from developing T cells expressing newly rearranged T cell receptors. A key element for negative selection to be effective at deleting pathogenic autoreactive T cells is the expression and presentation of tissue-restricted antigens by medullary thymic epithelial cells (mTECs). The importance of negative selection in preventing autoimmunity is highlighted by the findings that mice and humans with impaired thymic tissue restricted antigen expression caused by mutations in the gene encoding AIRE result in a broad spectrum of autoimmune diseases. However, negative selection does not completely purge pathogenic autoreactive T cells from the T cell repertoire so peripheral tolerance mechanisms, such as regulatory T cells, are also necessary to preserve immunological tolerance.

One mechanism by which autoreactive T cells may escape negative selection is through the expression of two different TCRs. Unlike the TCRβ chain genes, which are subject to allelic exclusion, TCRα chain genes undergo rearrangement simultaneously on both chromosomes until the developing T cell has been positively selected. Hence, the successful rearrangement of both TCRα chain genes can generate a T cell expressing two distinct TCRs that utilize the same TCRβ chain gene. Moreover, about 10-30% of αβ T cells have been found to possess two productively rearranged TCRα alleles [176, 467-469]. Therefore, dual TCR expression may pose

an autoimmune threat by enabling a developing T cell to use a primary "benign" TCR for thymocyte differentiation, navigating positive and negative selection, and subsequently, express a secondary "pathogenic" TCR to cause self-destruction.

Many studies have used αβ TCR transgenic mice expressing a single "monoclonal" TCR to study central and peripheral tolerance. However, these experiments differ from "natural" circumstances in that these T cells express a TCR with same high affinity for a self-antigen, and thus do not permit the study of T cells possessing a range of TCRs with lower affinities for the specific self-antigen. By contrast, investigations using non-TCR transgenic models are challenging because of the very low frequency of T cells that recognize any given antigen. To more closely resemble "normal" T cell physiology, it would be ideal to track a polyclonal CD8 T cell repertoire with a range of avidities for their cognate antigen. Using such an elegant model system, Zehn and Bevan showed that low avidity T cells for a tissue restricted antigen are capable of escaping central and peripheral tolerance mechanisms and cause disease [249]. Consequently, we have adapted the double transgenic model previously used by Zehn and Bevan to determine whether dual-TCR expressing CD8 T cells play a role in autoreactivity and autoimmunity.

In our study, double transgenic mice that express the OT-I TCRβ chain (here in, Vβ5 TCR$\alpha^{+/+}$) and membrane-bound ovalbumin (OVA) controlled by rat insulin promoter (Rip-mOVA) were compared to mice bearing these same transgenes but heterozygous for the TCRα mutation (TCR$\alpha^{+/-}$) and consequently capable of only expressing a single type of TCR per T cell. Previous studies have shown that CD8 T cells from Vβ5 TCR$\alpha^{+/+}$ mice have a semi-diverse TCR repertoire through rearrangement of endogenous TCRα genes and skewed towards the recognition of the Ova (SIINFEKL)-H2-K^b MHC class I molecules [470]. Further, ectopic OVA

expression driven by the Rip-mOVA transgene has been found in the pancreatic β cells, proximal tubules of the kidney and mTECs [192, 471] and is capable of inducing thymic deletion of OVA-specific TCR transgenic CD8 and CD4 T cells [192, 472].

The question of whether dual TCR-expressing T cells play a pathogenic role in autoimmune diseases remains controversial: some groups showed that they promote autoimmunity [473, 474] while others suggest that they do not cause disease [175, 469]. Here, we find that dual TCR-expressing CD8 T cells are intrinsically more autoreactive through comparison of CD8 T cells capable of expressing two types of TCRs (bi-allelic, $TCR\alpha^{+/+}$) versus those restricted to a single TCR (mono-allelic, $TCR\alpha^{+/-}$) against the model autoantigen OVA. Bi-allelic CD8 T cells exhibited increased proliferative capacity relative to mono-allelic CD8 T cells upon activation with antigen. In addition, a greater frequency of bi-allelic CD8 T cell effectors secreted IFN-γ upon antigen stimulation compared to mono-allelic CD8 T cell effectors generated upon *in vitro* polyclonal activation. Altogether, our results suggest that dual TCR-expressing CD8 T cells evade central and peripheral tolerance and participate in the pathogenesis of autoimmunity.

4.2 Results

4.2.1 Tetramer stain does not discriminate antigen reactivity between CD8 T cells from the mono-allelic or bi-allelic mice.

The Vβ5 transgenic mice have a naturally higher frequency of $OVA_{257-264}$-K^b -specific CD8 T cells in peripheral tissues due to the expression of the transgenic OT-I TCRβ chain and can be quantified using MHC class I tetramer staining [249, 470]. The OT-I transgenic line is largely considered a “monoclonal” line because almost all the CD8 T cells express the same

TCR and thus have the same affinity towards the H-2K^b-OVA tetramer. By contrast, wild type C57BL/6 mice are considered "polyclonal" because all T cells express endogenously rearranged TCRα and β chains, therefore having very few CD8 T cells specific to H-2K^b-OVA tetramer (*Figure 4-1*). As shown in *Figure 4-1*, tetramer staining of Vβ5 splenocytes revealed a population of high affinity OVA-specific CD8 T cells, which stained brightly with the OVA tetramer. In addition to the tetramer bright population, the Vβ5 mice also harbor a population of OVA-specific T cells that possess lower affinity towards OVA. In the Vβ5 × RIP-mOVA mice, the ectopic expression of OVA within the thymus results in the loss of tetramer bright cells, the low affinity population however remained unchanged. Using tetramer staining, we assessed the frequency of OVA-specific CD8 T cells in the mono-allelic Vβ5 × RIP-mOVA mice to determine whether genetic disruption of one TCRα chain gene would result in any alteration to the CD8 T cell repertoire. Tetramer staining of peripheral cells from mono-allelic Vβ5 × RIP-mOVA mice revealed that the frequency of tetramer high cells and tetramer intermediate cells in these mice were comparable to that of their bi-allelic counterpart, suggesting that the disruption of one TCRα chain loci did not have any effect detectable by tetramer staining (*Figure 4-1*).

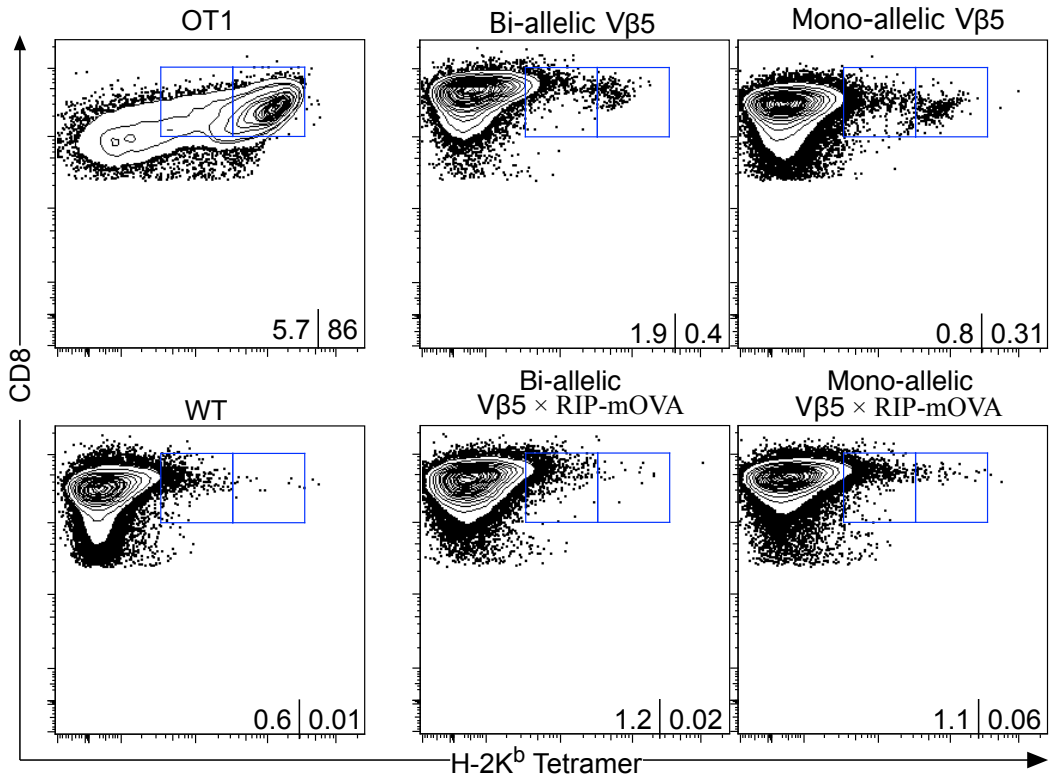

Figure 4-1 Tetramer staining does not discriminate antigen reactivity between CD8 T cells from the mono-allelic or bi-allelic mice.
Splenocytes of bi or monoallelic Vβ5, and Vβ5 × RIP-mOVA mice were stained with anti-CD8 and H-2K^b-OVA tetramer(s). The representative plots shown are gated on the total CD8$^+$ T cells. Tetramer positive cells were gated based on their staining intensity into tetramer high or tetramer intermediate populations. The data shown are representative of at least 5 independent experiments.

4.2.2 Bi-allelic Vβ5 × RIP-mOVA CD8 T cells have increased overall antigen reactivity to model antigen in an auto-antigen dependent manner

After observing that tetramer staining could not discriminate antigen reactivity between mono-allelic and bi-allelic CD8 T cells, we tested our hypothesis using the gold standard assay for T cell antigen reactivity, namely antigen receptor driven proliferation assay. One of the hallmarks of T cell activation is the rapid proliferative phase that follows. The proliferative capacity of these expanding T cells can be tracked by radioactive nucleotide incorporation, and the amount of radioactive nucleotide incorporated can serve as a surrogate indicator for T cell antigen reactivity [475]. We assessed the overall antigen reactivity of bi or mono-allelic CD8 T cells isolated from Vβ5 × RIP-mOVA mice using radioactive thymidine incorporation assay. Our results showed that CD8 T cells from the mono-allelic Vβ5 × RIP-mOVA mice exhibited reduced antigen reactivity compared to those from the bi-allelic Vβ5 × RIP-mOVA mice across different $OVA_{257-264}$ peptide concentrations. On average, bi-allelic CD8 T cells incorporated 2 to 3 times more radioactive thymidine compared to mono-allelic CD8 T in response to peptide concentrations ranging from 1×10^{-5} M to 1×10^{-6} M (*Figure 4-2A*). When the same assay was performed on CD8 T cells from bi or mono-allelic Vβ5 mice, the amounts of radioactive thymidine incorporated were comparable between the two genotypes, across all $OVA_{257-264}$ peptide concentrations. These results suggest that CD8 T cells from the bi-allelic Vβ5 × RIP-mOVA mice could incorporate more radioactive nucleotides, compared to those of the mono-allelic Vβ5 × RIP-mOVA mice in an autoantigen dependent manner.

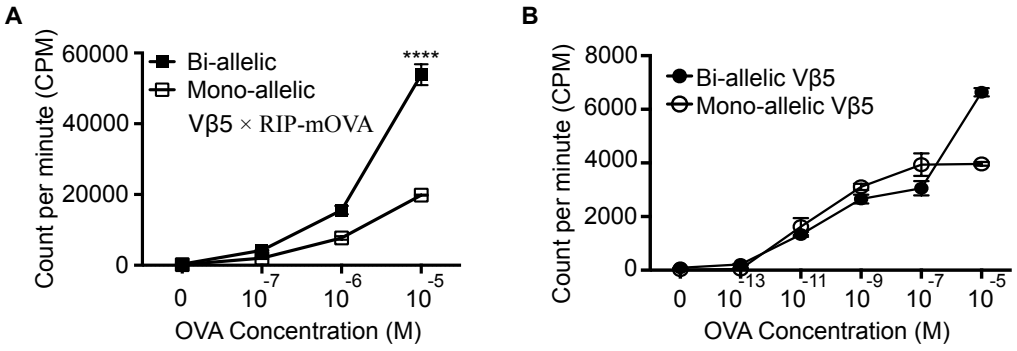

Figure 4-2 Bi-allelic Vβ5 × RIP-mOVA CD8 T cells have increased overall antigen reactivity to model antigen in an auto-antigen dependent manner.
CD8 T cells were isolated from pooled lymphocytes isolated from cervical, axillary, inguinal, and MLN of bi-allelic Vβ5 × RIP-mOVA mice, mono-allelic Vβ5 × RIP-mOVA mice, bi-allelic Vβ5 mice, and mono-allelic Vβ5 mice using magnetic separation columns. 1×10^5 CD8 T cells were co-cultured with 1×10^6 irradiated B6 splenocytes and varying concentrations of $OVA_{257-267}$ peptide. The cell culture was expanded for 3 days before being pulsed with 1μ Ci of tritiated thymidine, and harvested 16 hours later. Radioactivity incorporation was quantitated using scintillation counting. Quadruple asterisks denotes p value of <0.0001. Data representative of 3 separate experiments.

4.2.3 Bi-allelic Vβ5 × RIP-mOVA CD8 T cells have increased autoreactivity

We observed that there was a difference in overall autoantigen reactivity between the CD8 T cells from the bi-allelic Vβ5 × RIP-mOVA mice compared to the mono-allelic Vβ5 × RIP-mOVA mice. To study whether single or dual TCR expression would have functional effects on autoreactive T cells, we first expanded CD8 T cells from the peripheral lymph nodes of bi or mono-allelic Vβ5 × RIP-mOVA mice *in vitro* using plate bound anti-CD3/CD28 antibodies in combination with IL-2 for 5 days. This resulted in the unbiased expansion of the CD8 T cells population, and their acquisition of effector function such as IFN-γ secretion capability. After expansion, we co-cultured these effector T cells with splenocytes in combination with $OVA_{257-264}$ peptides and measured the amount of IFN-γ produced by these effector cells. It has been shown by previous investigators that the amount of IFN-γ secreted by the effector cells after cognate peptide stimulation is proportional to the avidity of the said effector cells, therefore allowing one to measure autoantigen reactivity of T cells that are weakly autoreactive [249].

Without peptide stimulation, about 1% of the bi or mono-allelic effector CD8 T cells produced IFN-γ. After being stimulated with varying concentrations of the $OVA_{257-264}$ peptide, 5-8 times more bi-allelic Vβ5 × RIP-mOVA effector cells were able to produce IFN-γ compared to mono-allelic effector cells in response to different peptide concentrations (*Figure 4-3A&B*). The observed decrease cytokine production from the mono-allelic Vβ5 × RIP-mOVA effectors was likely due to a decrease in their autoreactivity because mono-allelic Vβ5 × RIP-mOVA effector cells stimulated with a strong pan T cell stimulation in the form of PMA/ionomycin resulted in comparable IFN-γ production to bi-allelic Vβ5 × RIP-mOVA effector cells.

To assess whether the observed differences in autoreactivity exhibited by the bi or mono - allelic Vβ5 × RIP-mOVA T cells were autoantigen dependent, we expanded effector cells from pooled lymph node cells of bi or mono-allelic Vβ5 mice, and stimulated them with different concentrations of the $OVA_{257-264}$ peptide or PMA/Ionomycin. Similar to bi or mono-allelic Vβ5 × RIP-mOVA effector cells, bi or mono-allelic Vβ5 effector cells produced little IFN-γ without peptide stimulation. Upon PMA/Ionomycin stimulation, both the bi and mono-allelic Vβ5 effector cells produced comparable amounts of IFN-γ. When stimulated with different concentrations of the $OVA_{257-264}$ peptide, a similar proportion of the bi or mono-allelic Vβ5 effector cells were able to respond to peptide stimulation and produced IFN-γ at similar rates (*Figure 4-3 C&D*), suggesting that without thymic expression of autoantigen, bi and mono-allelic Vβ5 effector cells exhibit similar activity towards this antigen. Taken together our results suggest that when autoantigen is present during thymic selection, the CD8 T cell population within the dual TCR expressing Vβ5 × RIP-mOVA mice contains more autoreactive CD8 T cells, whereas when the autoantigen is absent, single or dual TCR expression have no effect on the abundance on autoreactive CD8 T cells.

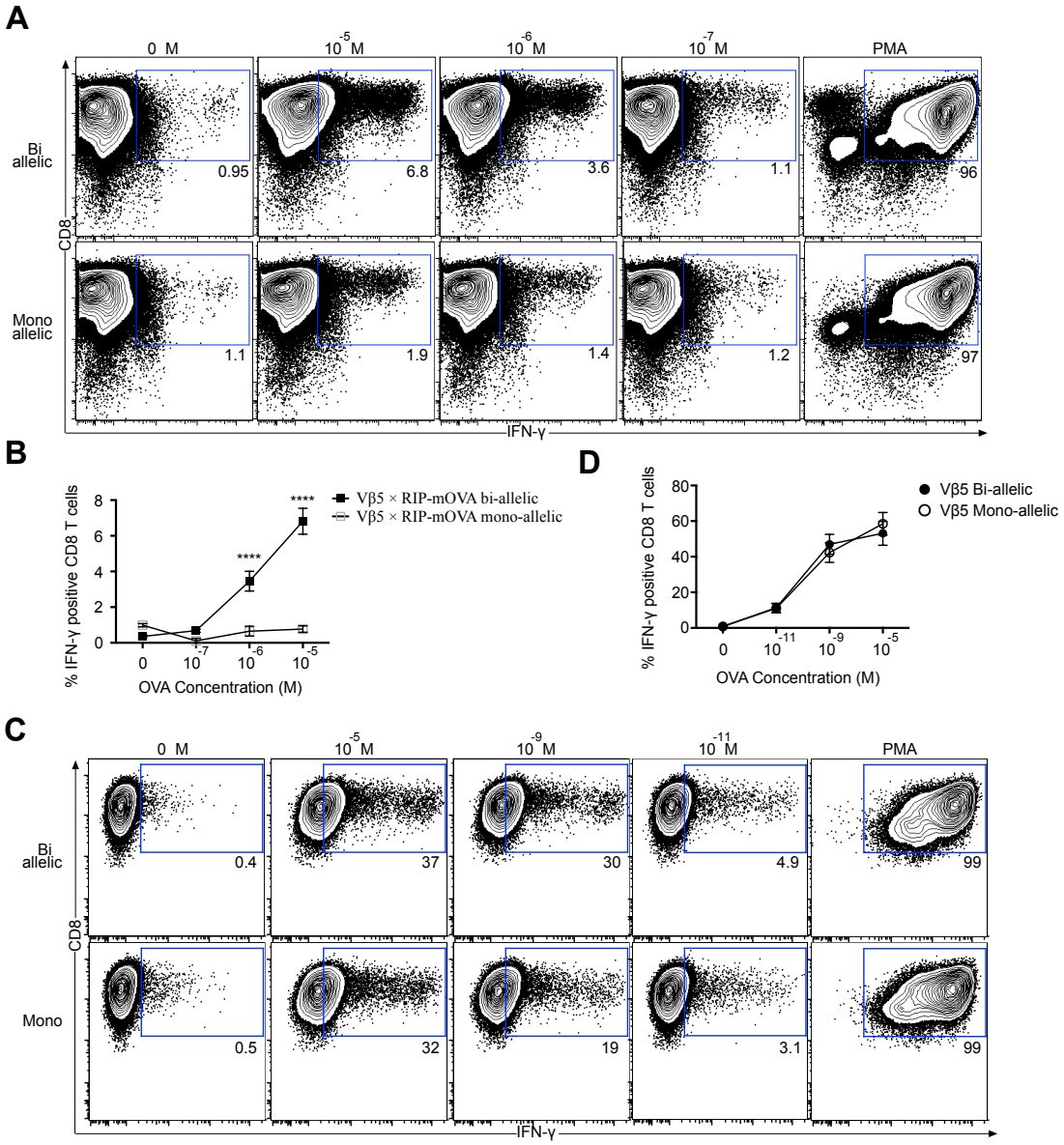

Figure 4-3 Bi-allelic Vβ5 × RIP-mOVA CD8 T cells have increased autoreactivity.
Pooled lymph node cells from bi or mono-allelic Vβ5 × RIP-mOVA mice and Vβ5 mice were expanded using plate-bound anti-CD3 and anti-CD28 antibodies. 6 days later, cells were co-cultured with congenic splenocytes as APCs and indicated concentrations of $OVA_{257-264}$ peptide, no peptide, or PMA ionomycin. The cell culture was then analyzed for IFN-γ production using intracellular cytokine staining. ***(A)*** Representative plots gated on Vβ5 × RIP-mOVA CD8 effectors cells that produce IFN-γ in response to the $OVA_{257-264}$ peptide are shown. Results shown is representative of at least 3 separate experiments (***B***) Cumulative peptide dose-response of expanded CD8 T cells shown in *A* ****: $p < 0.0001$. ***(C)*** Representative plots gated on Vβ5 CD8 effectors cells that produce IFN-γ in response to $OVA_{257-264}$ peptide are shown. ***(D)*** Cumulative peptide dose-response of expanded CD8 T cells shown in *C*. Data representative of 3 separate experiments.

4.2.4 Bi-allelic Vβ5 × RIP-mOVA mice have increased blood glucose levels after Lm-OVA infection but failed to develop type 1 diabetes

We sought to determine whether the difference in autoreactivity observed between the bi or mono-allelic CD8 T cells would translate into difference in T1D susceptibility *in vivo.* Previously it has been shown that bi-allelic Vβ5 × RIP-mOVA mice do not spontaneously develop T1D, but will develop T1D only when immunized with ovalbumin expressing *Listeria monocytogenes* (Lm-OVA) [249]. We immunized cohorts of bi or mono-allelic Vβ5 × RIP-mOVA mice, age 6-10 weeks with Lm-OVA intravenously and monitored their blood glucose levels every other day to determine whether the immunization would result in T1D. We found that although Lm-OVA immunized mice did not develop T1D, the blood glucose level in bi-allelic Vβ5 × RIP-mOVA mice became elevated 8 days after immunization but returned to normal by day 10. Such effect was not observed in mono-allelic Vβ5 × RIP-mOVA mice (*Figure 4-4*; day 8 blood glucose 11.2 ± 1.23 mmol/dL vs 8.08 ± 0.598 mmol/dL, $p<0.005$). These results suggest that although the immunization did not cause T1D, the treatment did result in a transient elevation of blood glucose levels in bi-allelic Vβ5 × RIP-mOVA mice on day 8 post infection.

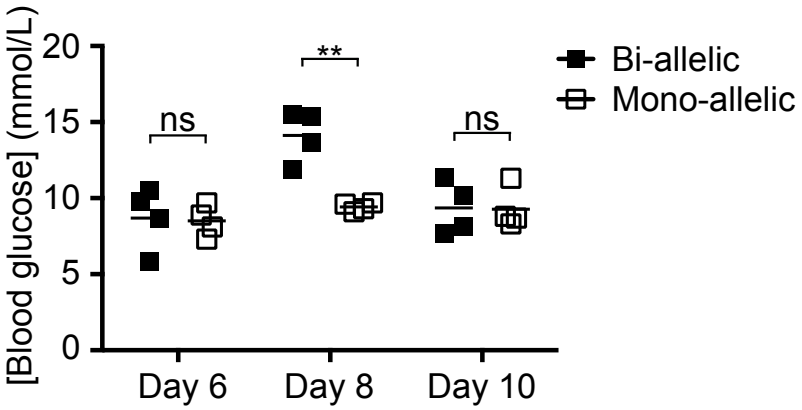

Figure 4-4 Bi-allelic Vβ5 × RIP-mOVA mice have increased blood glucose levels after Lm-OVA infection, but did not develop diabetes.
Cohorts of bi or mono-allelic Vβ5 × RIP-mOVA mice (6-10 weeks, n=8 of each group) were immunized with Lm-OVA intravenously. Blood glucose were monitored from day 6 to day 10. An animal with two consecutive blood glucose readings higher than 15 mmol/L is considered diabetic. Double asterisk denotes p value of less than 0.005.

4.3 Discussion

In the RIP-mOVA mice, the autoantigen OVA is expressed in the thymi, pancreatic β-cells, renal proximal tubules, and in the testis of male mice [471]. When the RIP-mOVA mice are crossed to the Vβ5 TCR transgenic mice, high affinity OVA-specific CD8 T cells are efficiently deleted by both central and peripheral tolerance mechanisms, thereby preventing them from causing autoimmunity in the peripheral tissues. In central tolerance, the deletion of high OVA affinity thymocytes are carried out by the AIRE expressing mTEC cells, which ectopically express the OVA transgene and present the $OVA_{257-264}$ peptide to mediate negative selection. This process is extremely effective in eliminating high OVA affinity thymocytes as previously shown by tetramer staining and IFN-γ assay. However, thymocytes with lower affinity towards OVA could escape negative selection and enter into circulation as evident by tetramer staining on the Vβ5 × RIP-mOVA lymphocytes and IFN-γ assay. These low OVA affinity CD8 T cells were demonstrated to be diabetogenic when activated by a strong Th1 response stimulator expressing the autoantigen [249]. Whether these low OVA affinity CD8 T cells that are bi-allelic play a role in T1D is currently unknown.

Dual TCR expressing T cells are thought to be generated during thymic development, where the simultaneous rearrangement of both TCRα chain gene loci result in the expression of two TCRα chains, which subsequently pair with the TCRβ chain to produce two TCRs with distinct specificities. It is estimated that about 10-30% of all T cells possess two functional rearranged TCRα chains in both humans and mice [474]. Some have proposed that these dual TCR expressing thymocytes may utilize their secondary TCRs to by-pass negative selection and cause disease in the periphery [473, 474, 476, 477], others postulated that dual TCR expression merely promote positive selection and might not be disease causing [175, 469]. To investigate

the question whether dual TCR expression could allow autoreactive thymocytes to by-pass negative selection we generated mono-allelic Vβ5 × RIP-mOVA mice by crossing bi-alleic Vβ5 × RIP-mOVA mice with TCRα$^{-/-}$ mice. The resulting F1 generation possess only one functional TCRα chain gene cassette, and therefore are only able to rearrange and express one TCRα chain and one TCR per T cell (mon-allelic). We then assessed CD8 T cells from these single TCR expressing mice for their autoreactivity against the auto antigen OVA, and compared the results to the Vβ5 × RIP-mOVA TCRα^{WT} mice to determine whether single TCR expression could alter the outcome of negative selection and impact CD8 T cell autoreactivity.

Tetramer staining was one of the methods previously used to assess the frequency of autoreactive CD8 T cells in the Vβ5 × RIP-mOVA mice. Our tetramer staining results showed that approximately 0.1% of all CD8 T cells in the bi-allelic Vβ5 × RIP-mOVA mice were high OVA affinity cells (OVA^{hi}), and approximately 1.6% were intermediate OVA affinity cells (OVA^{int}). In the bi-allelic Vβ5 mice, approximately 1% of all CD8 T cells were OVA^{hi} cells and approximately 1.6% were OVA^{int} cells. These results were comparable with published results, and showed that central tolerance is efficient in eliminating high OVA affinity cells. Surprisingly, in the mono-allelic Vβ5 × RIP-mOVA mice and Vβ5 mice, the frequencies of OVA^{hi} and OVA^{int} cells were comparable with their bi-allelic expressing counterpart. These results suggested that either single tetramer staining was insufficient in resolving the differences in autoreactive CD8 T cell frequencies or dual TCR expression had not impacted the frequency or reactivity of autoreactive cells.

We investigated whether there was a difference in autoreactivity between bi or mono-allelic Vβ5 × RIP-mOVA CD8 T cells by measuring the rate of their autoantigen dependent proliferation and functional avidity. Our autoantigen dependent proliferation assay showed that

the bi-allelic Vβ5 × RIP-mOVA CD8 T cell population possess more proliferative capability compared to the mono-allelic Vβ5 × RIP-mOVA CD8 T cells. When the same assay was performed on bi or mono-allelic Vβ5 CD8 T cells, the difference was not observed. This finding suggested that when the autoantigen OVA is expressed in the thymus to mediate negative selection of high OVA affinity thymocytes, dual TCR expression allowed the thymocytes to retain higher autoreactivity as indicated by their increased rate of proliferation. Indeed, the increase in proliferation rate could indicate that bi-allelic Vβ5 × RIP-mOVA CD8 T cells possessed increased autoreactivity since T cell proliferation is closely associated with cell activation; and by the same token, the lack of proliferation seen in the mono-allelic Vβ5 × RIP-mOVA CD8 T cells could suggest that they did possess less autoreactivity. However, it could not be ruled out that an increased rate of activation induced cell death (AICD) in the single TCR expressing CD8 T cells upon the exposure to $OVA_{257-264}$ peptide was the cause of decreased capacity to proliferate.

To eliminate such possibility, we first activated and expanded lymphocytes collected from peripheral lymph nodes of bi or mono-allelic Vβ5 × RIP-mOVA mice with plate bound anti-CD3, anti-CD28 antibodies to activate these T cells and differentiate them into effector cells. This process allowed the unbiased expansion of all T cells, and should there be any AICD during the activation and expansion, it would happen to all T cells in an autoantigen independent manner. After 6 days of expansion, the effector cells were then co-cultured with congenic APCs and various concentrations of the $OVA_{257-264}$ peptide. The amount of IFN-γ produced by the effector cells in response to the $OVA_{257-264}$ peptide was then measured using intracellular cytokine staining as an indicator of T cell autoreactivity. Our results showed that more bi-allelic Vβ5 × RIP-mOVA effector cells were able to produce IFN-γ compared to mono-allelic Vβ5 ×

RIP-mOVA effector cells when stimulated with different concentrations of the $OVA_{257-264}$ peptide. The difference observed was not due to cell viability and was auto-antigen dependent. Almost all the effector cells of both genotypes could produce similar amounts of IFN-γ when stimulated with pan T cell stimulator PMA and ionomycin. When bi or mono-allelic Vβ5 effectors were stimulated with the $OVA_{257-264}$ peptide, both genotypes were able to respond to similar concentrations of $OVA_{257-264}$ peptide and produced IFN-γ.

Central tolerance was effective in eliminating high affinity autoreactive thymocytes in the Vβ5 × RIP-mOVA mice, however lower affinity thymocytes could escape negative selection and enter the circulation. Despite possessing a higher frequency and number of low OVA affinity CD8 T cells, Vβ5 × RIP-mOVA mice were shown to remain diabetes-free until they were inoculated with OVA expressing *Listeria monocytogenes* (Lm-OVA). We used LM-OVA infection to assess whether the difference in autoreactivity observed in IFN-γ and proliferation assays could translate into protection against type 1 diabetes. Despite Lm-OVA being able to elevate blood glucose levels in bi-allelic Vβ5 × RIP-mOVA mice more than mono-allelic Vβ5 × RIP-mOVA mice day 8 post inoculation, the elevation did not persist for more than 24 hours, and returned to normal levels by day 10. This increase in blood glucose level could be an indication that activated autoreactive CD8 T cells were infiltrating the islets in the bi-allelic Vβ5 × RIP-mOVA mice, but were unable to cause disease.

The inability of autoreactive CD8 T cells to destroy OVA bearing β-cells after Lm-OVA immunization could be the result of suboptimal T cell activation or that not all the β-cells in these transgenic animals express OVA. In our preliminary experiment (data not shown), *ex-vivo* PMA/ionomycin stimulation of splenocytes from LM-OVA immunized animals revealed that they have vastly more IFN-γ producing CD8 T cells compared to unimmunized ones. This

indicates that immunization with Lm-OVA had caused OVA-specific CD8 T cells in the immunized animals to differentiate into effector cells. Taken together with the results from *ex-vivo* $OVA_{257-264}$ peptide stimulation of the same splenocytes, in which bi-allelic Vβ5 × RIP-mOVA CD8 T cells produced more IFN-γ compared to mono-allelic Vβ5 × RIP-mOVA CD8 T cells, the results suggest that Lm-OVA immunization activated OVA-specific CD8 T cells and drove their differentiation into effector cells. To determine how much OVA is being expressed in the Vβ5 × RIP-mOVA mice, pancreatic sections of these mice can be stained with antibodies specific for β-cell markers, insulin and OVA to determine the extent of OVA expression within the β-cells.

In this report, we showed that bi-allelic CD8 T cell populations are more autoreactive compared to mono-allelic ones and the difference is autoantigen dependent. These results provide valuable insight into the escape mechanisms of disease causing autoreactive CD8 T cells.

Chapter 5: MUCUS DEFICIENCY RESULTS IN NEGATIVE SELECTION OF T CELLS RESTRICTED TO ORAL ANTIGENS

5.1 Introduction

The lumen of the gastrointestinal tract is a complex environment comprised of food particles, a large surface area of absorptive mucosal epithelial cells and billions of enteric micro-organisms. The mucosal epithelial cell lining of the gastrointestinal tract is covered by a thin layer of mucus that functions to segregate contents of the intestinal lumen from the intestinal epithelium to prevent infection [478]. Besides contributing barrier function, the mucus layer also lubricates the gastrointestinal tract facilitating intestinal contents to pass without damaging intestinal epithelium. Mucus is a gel-like substance that is rich in mucins, a family of heavily glycosylated proteins, that help retain water and yield its viscous properties [479]. The sugar molecules attached to mucins may serve as a food source for resident microbes and distract microbes from invading the intestinal epithelium [480]. In addition, mucus also contains a diverse number of antimicrobial molecules including anti-microbial peptides and immunoglobulins specific for microbial surface molecules. Defective mucus production has been often associated with intestinal inflammation and heightened susceptibility to the development of IBD [413-415]. Together, the multiple components of mucus are critical for host health by helping support barrier function and preventing infection by gut microbes.

Mucin-2 (MUC2) is the major secretory mucin constituent of the intestinal mucus layer of both humans and mice. MUC2 is hyperglycosylated (80% glycan by weight) and thought to function as a structural scaffold of the mucus shielding the intestine. Goblet cells of the small and large intestine are responsible for synthesizing MUC2 and releasing it into the intestinal

lumen to help form the mucus layer. The importance of MUC2 to intestinal physiology is underscored by phenotype of MUC2-deficient mice that includes the spontaneous development of colitis and/or colorectal cancer [416]. Although the precise mechanisms that lead to intestinal disorders in *Muc2*$^{-/-}$ mice remain unclear, MUC2 deficiency may impact the localization of gut microbiota and dietary antigens, disrupt homeostasis of the intestinal epithelium and alter immune cell activation.

MUC2 may have additional functions besides acting as a physical barrier. Recent work suggests that MUC2 mediate critical roles in restraining immune responses towards gut antigens. Specifically, Shan *et al.,* show that treatment of DCs with MUC2 quenches their responses to microbe-derived signals while enhancing their capacity to stimulate the expansion of regulatory T cells [19]. In addition, MUC2 was also found to stimulate epithelial cells to secrete factors that promote regulatory functions by DCs [19]. Consequently, MUC2 may play important immune dampening roles to gut antigens by limiting exposure of luminal contents to intestinal epithelium and by polarizing DCs to a regulatory phenotype and function.

Here, we investigated whether intestinal MUC2 is essential for maintaining T cell tolerance towards intestinal luminal antigens and preventing disease caused by constitutive T cell activation. To test this hypothesis, wild type and MUC2-deficient (*Muc2*$^{-/-}$) were gavaged with the model antigen ovalbumin (OVA) and the elicited OVA-specific T cell responses monitored at various times post-treatment. Significantly, the oral administration of OVA resulted in antigen being rapidly disseminated in the blood and lymphoid tissues of *Muc2*$^{-/-}$ but not wild type mice. Given the systemic spread of antigen in *Muc2*$^{-/-}$ mice, we next sought to investigate if dietary antigens impact the selection of developing T cells in the thymus of *Muc2*$^{-/-}$ mice. Remarkably, we found that gavage of *Muc2*$^{-/-}$ mice lead to presentation of OVA by thymic dendritic cells and

the deletion of OVA-specific thymocytes whereas T cell development in wild type mice appeared unaffected. Importantly, our findings demonstrate that a leaky gut may result in intestinal luminal antigens shaping the T cell repertoire of developing thymocytes.

5.2 Results

5.2.1 Peripheral T cells of *Muc2*$^{-/-}$ mice acquired antigen experience after oral antigens exposure but lacked effector function

MUC2 contribute to intestinal homeostasis by providing barrier function as well as inducing Treg cells [19]. Treg cells maintain intestinal homeostasis by suppressing the priming of naïve luminal antigens-specific T cells patrolling within the gut. In the absence of Treg cells, naïve luminal antigen-specific T cells can become activated after detecting their cognate antigens and cause T cell driven colitis [429]. Although Treg cells are critical in providing protection against colitis, it is currently unknown how Treg cells and conventional cells patrolling the gut would behave in the absence of MUC2. One possible scenario is that without the tolerizing signal provided by MUC2, gut patrolling DCs would become pro-inflammatory and in turn polarize naive luminal antigen-specific T cells to Th1 phenotype and cause disease. Another possible scenario is that, without the barrier function provided by MUC2, the intestinal epithelium becomes damaged due to prolonged contact with the luminal content. The disrupted intestinal epithelial barrier could lead to increased permeability and antigen availability [416, 417, 481] resulting in T cell anergy or even death.

To investigate how the absence of MUC2 impacts antigen-specific T cells in the absence of MUC2, we first generated a transgenic *Muc2*$^{-/-}$ mouse line, which harbors a transgenic TCRβ chain (Vβ5) specific for chicken ovalbumin amino acids 257-264 ($OVA_{257-264}$ peptide) presented

on the MHC I molecule $H2K^b$. We then orally gavaged these TCR transgenic *Muc2*$^{-/-}$ mice to ovalbumin (OVA) and assessed $OVA_{257-264}$ -specific CD8 T cell expansion and antigen experience in spleen a week later using tetramer and flow cytometry. Our results showed that in WT Vβ5 mice, splenic CD8 T cells underwent a 4 fold expansion 1 week after oral OVA protein gavage (*Figure 5-1A&B*). CD44, a marker of Ag-experienced T cells was also observed in the mesenteric lymph nodes of the same animals with WT CD8 T cells expanded 1.3 fold compared to CD8 T cells from *Muc2*$^{-/-}$ animals (*Figure 5-1A&B*). The antigen experience marker CD44 was also elevated 2.2 fold in the splenic OVA-Ag tetramer positive reactive CD8 T cells in OVA gavaged WT mice relative to *Muc2*$^{-/-}$ mice (*Figure 5-1C*). MLN OVA reactive CD8 T cells also showed a similar trend in CD44 upregulation, having upregulated 1.8 fold in WT animals relative to those from *Muc2*$^{-/-}$ (*Figure 5-1C*). Surprisingly, peripheral OVA-reactive CD8 T cells from the OVA treated Vβ5 *Muc2*$^{-/-}$ mouse failed to expand and did not acquire antigen experience as indicated by CD44 expression (*Figure 5-1A&B*). After observing that OVA treated Vβ5 *Muc2*$^{-/-}$ spleen cells did not expand nor upregulate CD44 after OVA gavage, we stimulated the same peripheral cell samples *ex-vivo* with 10^{-9} M of the $OVA_{257-264}$ peptide to determine whether these tetramer high CD44 high CD8 T cells could produce IFN-γ. We found that splenic CD8 T cells from the treated Vβ5 WT mice could produce IFN-γ in response to the peptide challenge but cells from the treated Vβ5 *Muc2*$^{-/-}$ mice could not (*Figure 5-1D&E*; WT 2.76 ± 0.91 *vs Muc2*$^{-/-}$ 0.70 ± 0.24, $p < 0.04$). Similar results were also observed for MLN CD8 T cells (*Figure 5-1D&E*; WT 0.44 ± 0.0.14 *vs Muc2*$^{-/-}$ 0.15 ± 0.04, $p < 0.05$). Taken together, these results suggest that CD8 T cells within the peripheral tissues of *Muc2*$^{-/-}$ mice failed to differentiate into effector cells after OVA gavage. There are several possibilities, namely defective APCs and T cells due to excessive PAMP exposure, altered APC and T cell trafficking

patterns, reduced antigen availability, and T cell anergy, which may cause the phenotype observed.

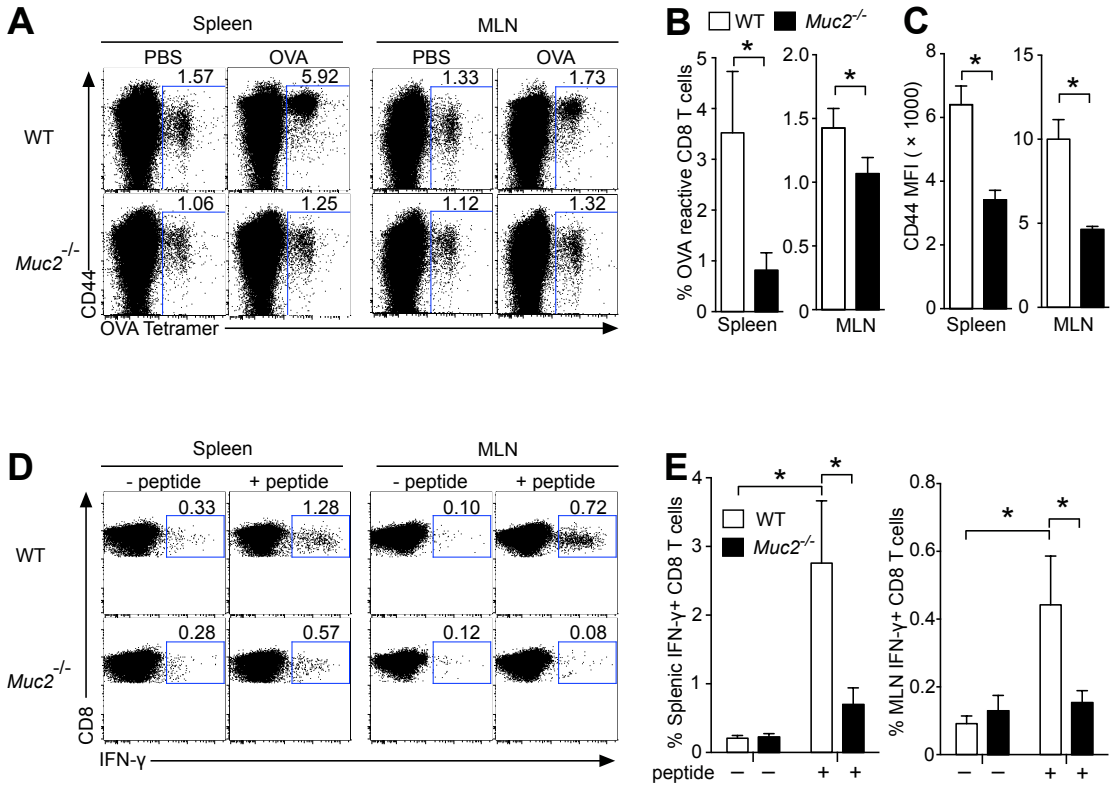

Figure 5-1 *Muc2*$^{-/-}$ mice mount reduced CD8 T cell responses towards dietary antigens. Wild type or *Muc2*$^{-/-}$ Vβ5 mice (n=4) were gavaged with 1 mg of OVA. One week after treatment, spleens and mesenteric lymph nodes were harvested for CD44 and H-2K^{b}-OVA tetramer staining or *ex-vivo* $OVA_{257-264}$ peptide stimulation followed by intracellular cytokine staining. ***(A)*** Spleens and mesenteric lymph nodes of PBS or OVA-gavaged WT and *Muc2*$^{-/-}$ Vβ5 mice were first gated on CD8 T cells, and the frequency of $OVA_{257-264}$-reactive CD8 T cells within the CD8 gate was quantified. Bar graphs represent mean ± SEM, n=4. ***(B)*** Cumulative data is shown for WT and *Muc2*$^{-/-}$ Vβ5 mice gavaged with ovalbumin. ***(C)*** CD44 levels on $OVA_{257-264}$-reactive CD8 T cells are shown for mice orally gavaged with ovalbumin. ***(D&E)*** Frequency of IFN-γ-producing CD8 T cells with or without $OVA_{257-264}$ peptide stimulation are shown for mice orally gavaged with OVA (protein). Bar graphs represent mean ± SEM. Single asterisk denotes p values of < 0.05.

5.2.2 Design of a sensitive assay to detect ovalbumin

In the previous section, we observed that splenic $OVA_{254-264}$ -specific CD8 T cells from the Vβ5 *Muc2*$^{-/-}$ mice failed to expand after oral antigen gavage. Reduced antigen availability and T cell anergy due to excessive antigen exposure were two of the probable causes for such phenotype. Therefore we hypothesize that mucus deficiency may result in vastly increased antigen availability in *Muc2*$^{-/-}$ mice following OVA gavage. To test our hypothesis, we developed a high sensitivity, flow cytometry based assay to measure the concentration of OVA in the blood following OVA gavage. First, we covalently linked an ovalbumin-specific polyclonal antibody to carboxylate modified latex (CML) beads via the carbodiimide method [444]. We then validated the CML beads by using them to immunoprecipitate serially diluted ovalbumin. We then added another ovalbumin-specific polyclonal antibody and a fluorescence-conjugated secondary antibody to detect bead-bound ovalbumin using flow cytometry. Our results showed the fluorescence intensity of the bead-bound OVA correlate positively with the concentration of the ovalbumin in a dosage-dependent manner, suggesting that the assay is OVA- specific, and sensitive from 10^{-5} g/mL to 10^{-11} g/mL of ovalbumin (*Figure 5-2A&B)*.

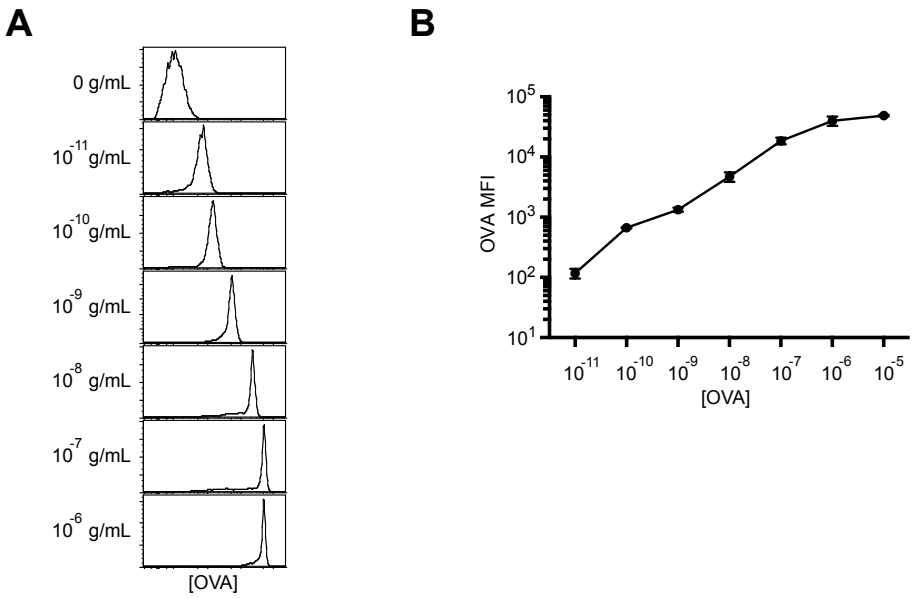

Figure 5-2 Cytometric bead assay reliably detects low concentrations of ovalbumin. Ovalbumin was first suspended in sterile PBS before being titrated into indicated concentrations using FACS buffer. 80 µL aliquots of the serially titrated ovalbumin were then combined with 10 µL of 0.5% Tween 20 PBS inside the wells of a 96-well U-bottom plate in triplicates. The ovalbumin solutions were then probed with CML beads. (***A***) Representative histograms of PBS titrated ovalbumin. (***B***) A titration curve was plotted based on the MFI numbers of ***A***. Error bars denote mean ± SEM. Data representative of at least 4 separate experiments.

5.2.3 Oral antigens are rapidly disseminated into the blood of *Muc2*$^{-/-}$ mice

To determine which fraction of the blood contains ovalbumin, we first fractionated the blood samples into cellular and plasma fraction by centrifugation, and probed the fractions using CML beads to determine which fraction contains OVA. We found that the majority of the blood borne OVA was present in the plasma fraction of the OVA gavaged mice, approximately 20 times higher than that of the cellular fraction (*Figure 5-3 A&B*) with the treated *Muc2*$^{-/-}$ showing approximately 70 times higher plasma OVA concentration compared to WT mice (*Figure 5-3 A,C,D*). OVA was also detectable within the cellular faction of the *Muc2*$^{-/-}$ mice albeit in lower concentrations (*Figure 5-3 A&D*). OVA was not detectable using IP-FCM within the cellular fraction of the WT treated mice (*Figure 5-2 A&D*; *Muc2*$^{-/-}$ 1,005 ± 259 vs WT 180 ± 28, $p < 0.01$). We next sought to measure plasma OVA concentration over a period of 72 hours to

determine whether the plasma OVA concentration changed over time. We found that plasma OVA concentration of treated *Muc2*$^{-/-}$ mice began to rise 1 hour after gavage, reaching the peak concentration approximately 3-6 hours post gavage and began to slowly decline but remained detectable even after 72 hours (*Figure 5-3 A&C*). In the OVA treated WT mice, the plasma OVA concentration peaked approximately 1 hour after treatment and began to decline, but remained detectable after 24 hours (F*igure 5-3 A&C*). Taken together our data showed that in *Muc2*$^{-/-}$ mice, oral antigens are rapidly dispersed following gavage, with the majority of antigens being located in the plasma fraction.

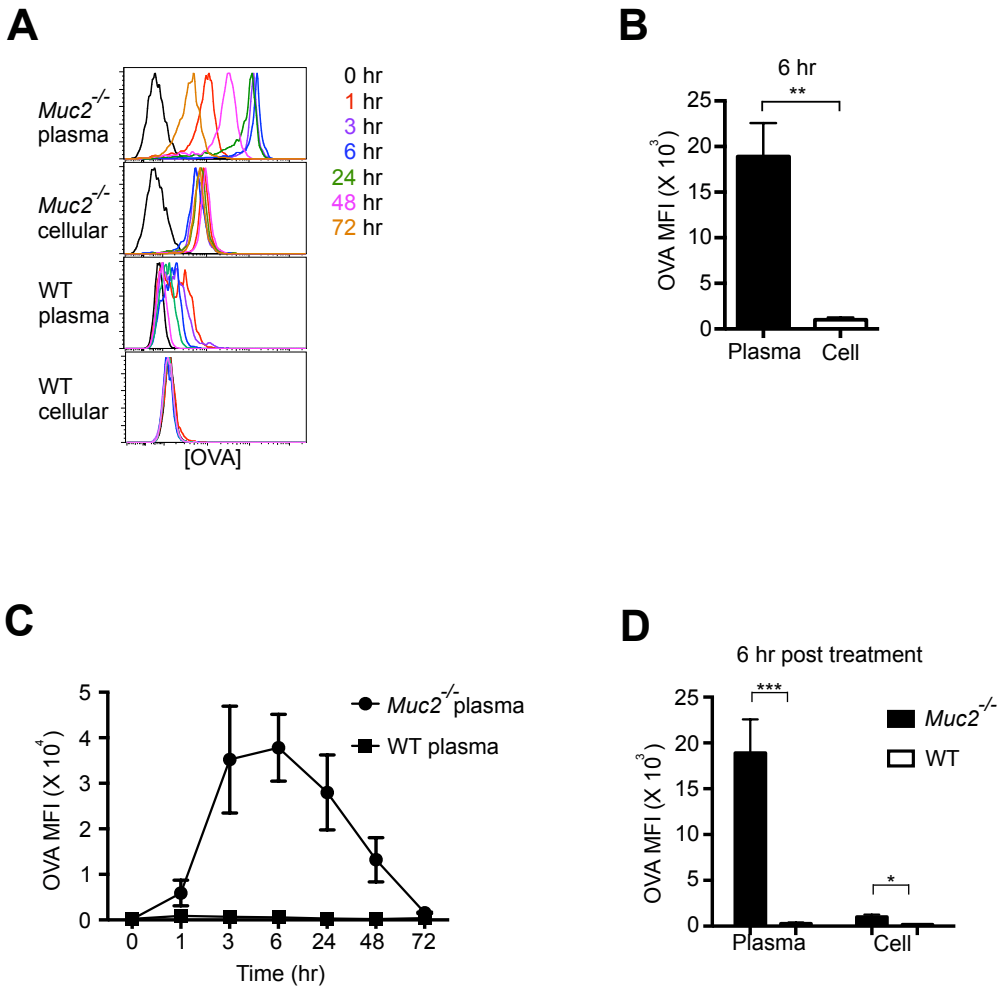

Figure 5-3 Oral antigens are rapidly disseminated into the blood of *Muc2*$^{-/-}$ mice. Cohorts of 5 week-old *Muc2*$^{-/-}$ mice were gavaged with OVA or PBS. 10 µL of blood was collected at the indicated time points and OVA was detected IP-FCM. (***A***) Representative histograms of blood-borne OVA concentrations in the plasma and cellular fractions of WT or *Muc2*$^{-/-}$ mice treated with OVA, measured using IP-FCM. (***B***) Cumulative data of OVA concentrations within different fractions of OVA treated *Muc2*$^{-/-}$ mice 6 hours post treatment. Error bars represent SEM. **:0.001 (***C***) Cumulative result plasma OVA concentration of OVA treated *Muc2*$^{-/-}$ or WT mice. (***D***) Cumulative results (n=5) of OVA concentration in the plasma or cellular fraction of treated *Muc2*$^{-/-}$ mice, 6 hours after gavage. *: $p < 0.01$; ***: $p < 0.0009$.

5.2.4 Peripheral T cells of *Muc2*$^{-/-}$ mice get highly activated upon exposure to oral antigens

Thus far, we found that oral antigen-specific CD8 T cells in the peripheral lymphoid tissues of OVA treated *Muc2*$^{-/-}$ mice became anergic and that disrupted intestinal barrier in *Muc2*$^{-/-}$ mice resulted in heighten systemic dissemination of oral antigens. Based on these observations we hypothesize that antigen-specific CD8 T cells within the Vβ5 *Muc2*$^{-/-}$ mice were being anergized by high levels of oral antigens.

To investigate our hypothesis, we crossed Vβ5 *Muc2*$^{-/-}$ mice to a strain of GFP reporter mice (Nur77^{GFP} mice) in which the GFP expression is induced only upon TCR activation, independent of other inflammatory stimuli [249, 442]. First, we gavaged WT or Muc2-/- Vβ5 Nur77^{GFP} mice with OVA. Sixteen hours later we measured OVA-specific CD8 T cell activation in spleen and MLN by identifying and enumerating $OVA_{254-264}$ -specific CD8 T cells using two-tetramer staining, which increases the tetramer staining resolution [184]. We then assessed the degree of antigen-specific T cell activation and Nur77 expression by assessing GFP fluorescence (*Figure 5-4A&B)*.

We found that OVA gavage did not alter the frequency of $OVA_{254-264}$ -specific CD8 T cells in the tissues examined, however GFP expression in the tetramer positive CD8 T cells isolated form the MLN of Vβ5 *Muc2*$^{-/-}$ mouse was higher compared to those from the Vβ5 *Muc2*^{WT} mouse (*Figure 5-4A*). We next examined the Nur77-reporter expression within the $OVA_{254-264}$ -specific CD8 T cells isolated from the spleen. Our results showed that GFP expression in the tetramer positive CD8 T cells isolated from the spleen of Vβ5 *Muc2*$^{-/-}$ mouse was higher than those of the Vβ5 *Muc2*^{WT} mouse, and surprisingly even higher than CD8 T cells from the MLN of the same mouse (*Figure 5-4B*). Taken together, our preliminary results suggest

that OVA-specific CD8 T cells in the MLN (proximal to the location of the oral antigens) as well as spleen (distal to the location of the oral antigens) were being activated by high levels of the oral antigen within the plasma.

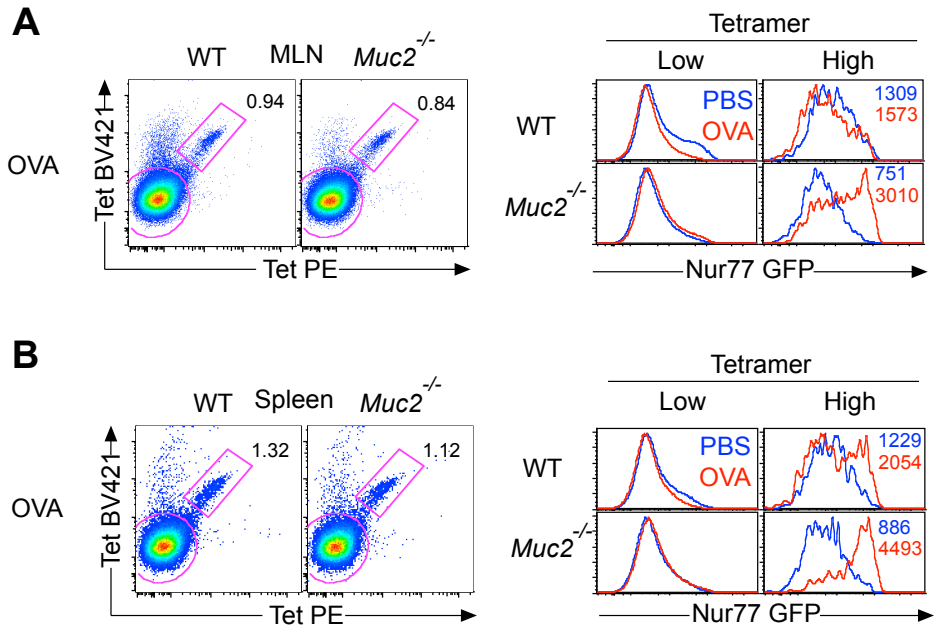

Figure 5-4 Peripheral antigen-specific T cells in *Muc2*$^{-/-}$ mice become highly activated after oral antigen exposure.
Mesenteric lymph nodes and spleens were harvested 16 hours following OVA gavage and the frequency of $OVA_{254-264}$ specific $Nur77^{GFP}$ expressing Vβ5 *Muc2*^{WT} and Vβ5 *Muc2*$^{-/-}$ CD8 T cells were analyzed. MLN and spleens were harvested and stained with surface markers and tetramers to enumerate OVA-specific CD8 T cells. CD8 T cells were gated based on their tetramer staining intensity into tetramer^{high} and tetramer^{low} subpopulations, and the GFP expression within these two populations was examined. (***A***) The frequencies of OVA-specific CD8 T cells in the MLN and the level of GFP expression in the gated populations. (***B***) The frequencies of OVA-specific CD8 T cells in the spleen and the level of GFP fluorescence in the gated populations. Data shown above is preliminary with n of 1.

5.2.5 Treatment of *Muc2*$^{-/-}$ mice with oral antigen results in the depletion of DP thymocytes and alteration of T cell receptor repertoire

Peripheral T cells respond to their cognate antigens by undergoing rapid expansion and in the process, acquire effector functions to eliminate target cells bearing the antigens [143-148]. These processes are central for T cell mediated responses, which confer protection against various types of microbial infections. Unlike peripheral T cells, developing thymocytes do not proliferate upon encountering their cognate antigens presented by thymic APCs, instead they die by apoptosis, become anergic, undergo TCR editing, or differentiate into T regulatory cells so that the host is protected from autoimmunity [180]. The systemic dispersal of oral antigens in *Muc2*$^{-/-}$ mice after gavage led us to hypothesize that T cell development was being affected.

To investigate our hypothesis, we gavaged cohorts of WT or *Muc2*$^{-/-}$ Vβ5 mice with OVA and analyzed their thymocyte populations. In WT Vβ5 mice, OVA gavage did not affect numbers or frequencies of different thymocyte populations (*Figure Figure 5-5A&C)*. In Vβ5 *Muc2*$^{-/-}$ mice, we noticed that OVA gavage caused a 2-fold reduction in total thymocyte numbers compared to PBS treated Vβ5 *Muc2*$^{-/-}$ mice (*Figure 5-5A&C*). Upon closer examinations, we found that OVA treatment of Vβ5 *Muc2*$^{-/-}$ mice caused an approximately 1.5 fold reduction in double positive (DP) thymocyte frequency and 1.8 fold reduction in DP numbers compared to PBS treated mice (*Figure 5-5A&C*). Compared to treated Vβ5 *Muc2*$^{-/-}$ mice, the frequency and number of DP thymocytes in OVA treated *Muc2*$^{-/-}$ mice remained unchanged (*Figure 5-5B*), suggesting that the observed loss of DP thymocytes in the OVA treated Vβ5 *Muc2*$^{-/-}$ mice was antigen dependent.

We next sought to determine whether the DP thymocytes lost in the OVA treated Vβ5 *Muc2*$^{-/-}$ mice were thymocytes with high affinities to the OVA tetramer. The detection of

antigen-specific DP thymocytes is challenging, because they stain strongly with MHC tetramers in a CD8- (but not specific peptide-MHC) dependent fashion [482, 483]. To increase our staining resolution, we stained thymocytes from PBS or OVA treated Vβ5 *Muc2*$^{-/-}$ mice using dual-tetramer staining, and found that OVA gavage resulted in the loss of high OVA affinity cells in the thymus of Vβ5 *Muc2*$^{-/-}$ mice (*Figure 5-5C&D*). Taken together, our results suggest that systemic dissemination of oral antigen resulted in antigen-dependent loss of double positive thymocytes, particularly thymocytes with high affinity TCRs towards the oral antigen OVA.

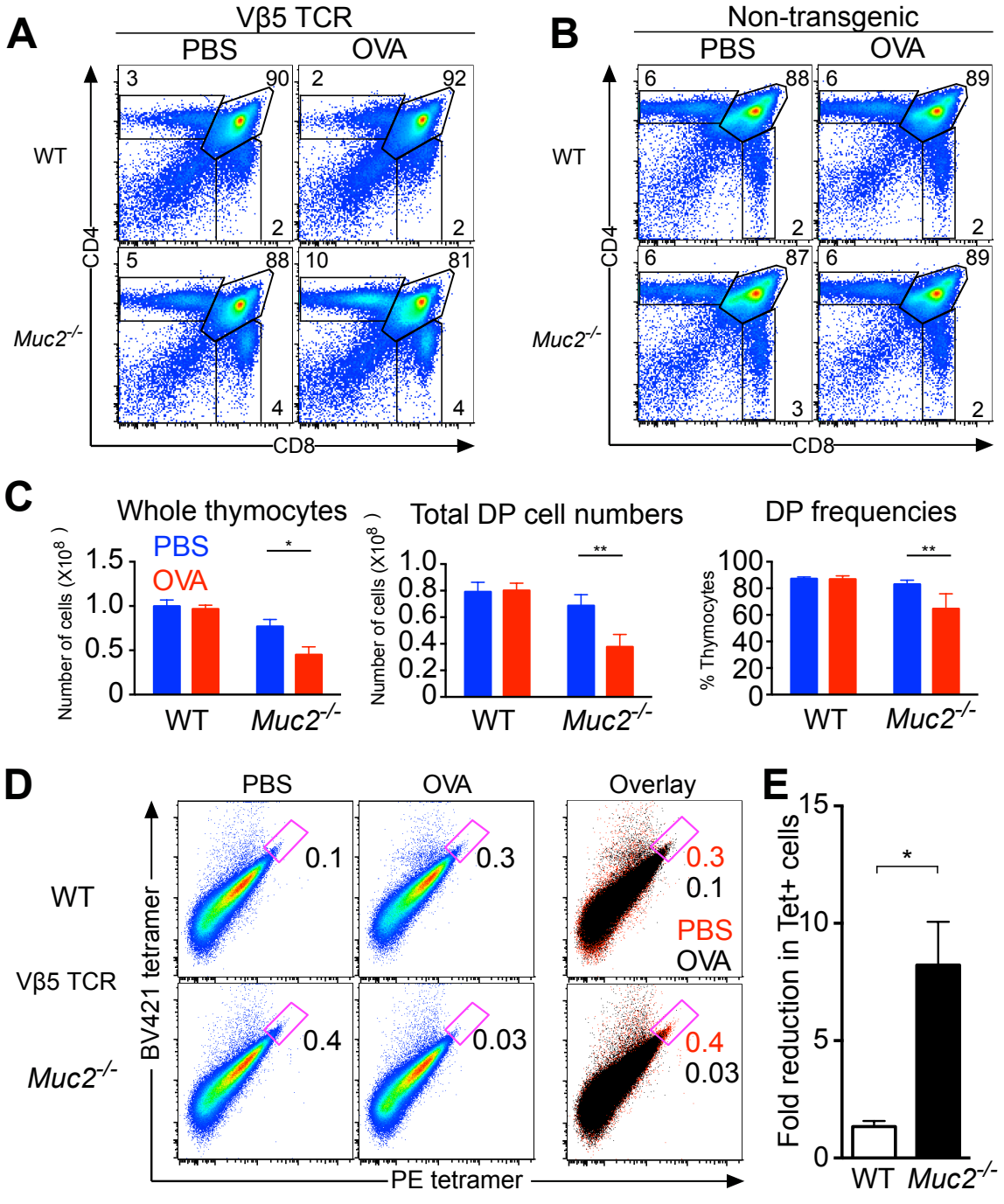

Figure 5-5 Treatment of *Muc2*$^{-/-}$ mice with oral antigen results in the depletion of DP thymocytes and alteration of T cell receptor repertoire.

WT or *Muc2*$^{-/-}$ Vβ5 mice were orally gavaged with OVA or PBS. 1 day post treatment, thymi were collected and stained for thymic differentiation markers. (***A***) The proportions of thymocyte populations in OVA-gavaged WT or *Muc2*$^{-/-}$ Vβ5 mice. Data shown is representative of at least 5

separate experiments. (***B***) The proportions of thymocyte populations in treated WT or *Muc2*$^{-/-}$ mice. (***C***) The frequency and number of CD4^{+} CD8^{+} DP cells was decreased in OVA treated Vβ5 *Muc2*$^{-/-}$ mice but not in the OVA treated WT mice or PBS treated Vβ5 *Muc2*$^{-/-}$ mice. Bar graphs shown represent cumulative data from 5 separate experiments. Single asterisks denote p values less than 0.05. (***D***) The frequency of tetramer positive DP cells and tetramer staining intensity in OVA treated *Muc2*$^{-/-}$ Vβ5 mice compared to that of the PBS treated Vβ5 *Muc2*$^{-/-}$ mice. (***E***) Cumulative results of ***D***, bar graph represents mean ± SEM with n of 3, single asterisk denotes p values less than 0.05.

5.2.6 Dietary antigens alter thymocyte differentiation markers in *Muc2*$^{-/-}$ mice

We next sought to determine whether there is additional evidence in addition to changes in thymic population and thymocyte repertoire that would indicate that the systemic dissemination of oral antigen in Vβ5 *Muc2*$^{-/-}$ mice resulted in the alteration of thymic development. Several T cell specific markers and activation markers are commonly used to study the process of thymic development. TCR expression on thymocytes is known to increase as the thymocytes mature from the DN stage towards the CD4/CD8 SP stage [125]. CD69 is an acute activation marker, which becomes upregulated not only in activated mature lymphocytes but also in thymocytes populations that are undergoing the process of thymocyte selection [125]. We stained thymocytes from OVA or PBS treated Vβ5 *Muc2*$^{-/-}$ mice with antibodies to discriminate between different thymocyte populations and assessed TCRβ and CD69 expression levels on these thymocyte subsets. We found that the OVA gavage resulted in an approximately 1.5 fold increase in TCRβ and CD69 expression in whole thymocytes of Vβ5 *Muc2*$^{-/-}$ mice but not in the non TCR transgenic *Muc2*$^{-/-}$ mice (*Figure 5-6 A&B*). Within the thymocyte subpopulations, we also observed an approximately 1.5 fold increase in TCRβ and CD69 staining in the DP and CD8^{+} single positive populations, but not in the CD4^{+} SP populations (*Figure 5-6 A&B*).

Taken together, these results suggest that OVA treatment resulted in an increase in thymic TCRβ and CD69 staining in Vβ5 *Muc2*$^{-/-}$ mice, and that these increases were antigen

dependent and Muc2-deficiency dependent. During thymocyte development, TCRβ expression has been known to increase as thymocytes mature from the DP stage towards the CD4/CD8 SP stage [125]. The increase of TCRβ staining observed in the DP thymocyte population of OVA treated Vβ5 *Muc2*$^{-/-}$ mice could mean that there was an increase in maturation marker expression in DP thymocytes or that there was a loss in the TCRβ^{low} population of less mature DP thymocytes. Taken together with the findings shown in Figure 5-6C, these results suggest that the increase in TCRβ and CD69 staining we observed was more likely the result of a loss in the TCRβ^{low} population of DP thymocytes.

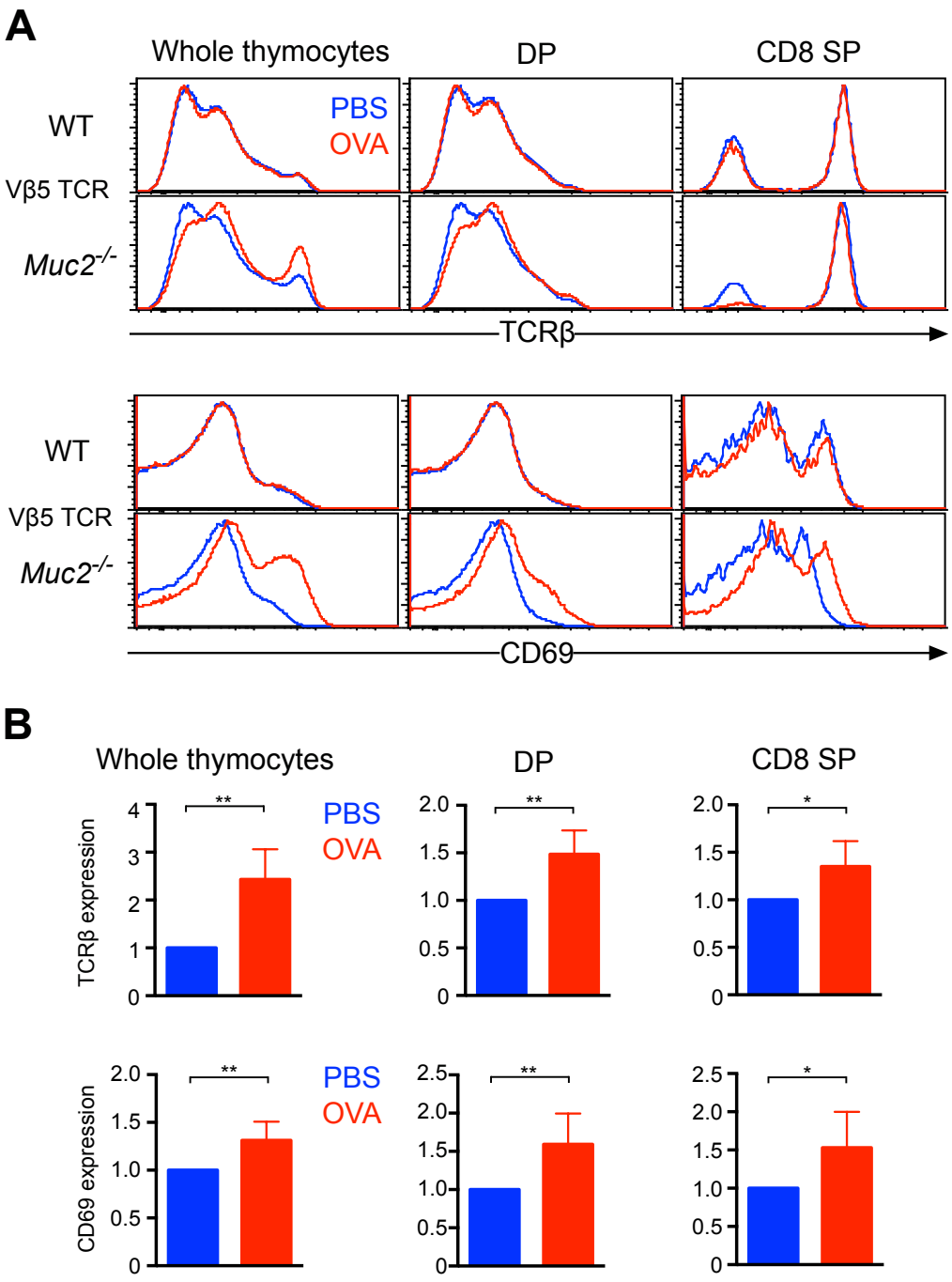

Figure 5-6 Dietary antigens alter thymocyte differentiation markers in Vβ5 *Muc2*$^{-/-}$ mice. Vβ5 *Muc2*^{WT} or Vβ5 *Muc2*$^{-/-}$ mice (n=5) were orally gavaged with OVA or PBS. 1 day post treatment, thymi were collected and stained for thymic differentiation and activation markers. (***A***) Maturation marker staining of indicated thymocyte populations (***B***) Cumulative fold expression of TCRβ and CD69 in OVA treated Vβ5 *Muc2*$^{-/-}$ mice normalized to PBS treated Vβ5 *Muc2*$^{-/-}$ mice. *: $p < 0.0133$, **: $p < 0.0073$, ***: $p < 0.0009$. Data representative of at least 5 separate experiments.

5.2.7 Dietary antigens induce TCR signaling in developing thymocytes

The frequency of OVA-specific CD8 T cells in the peripheral lymphoid tissues of Vβ5 TCR mice is about 1-2 % of the CD8 T cell population as determined by tetramer staining. While the frequency of OVA-specific CD8 T cells in Vβ5 TCR mice is much lower than that of OT-1 TCR mice, the elevated frequency of OVA-specific T cells in the peripheral tissues presents a potential caveat to the interpretation of our results as it is a possibility that cytokines secreted by peripheral OVA-specific T cells could affect thymic development, causing DP thymocyte loss and CD69 upregulation independent of TCR engagement. To ensure that the observed changes in the DP thymocyte population is caused by TCR engagement, we gavaged WT or *Muc2*$^{-/-}$ Vβ5 Nur77^{GFP} mice, and analyzed the thymic populations 1 day later. We found that OVA gavage resulted in the decrease of DP frequency and numbers, and simultaneous upregulation of GFP and CD69 in the DP and CD8 SP populations (*Figure 5-7*). These results indicate that the loss of DP thymocytes and the upregulation of CD69 was due to TCR activation in the thymus and not as the result of cytokines secreted by peripheral T cells.

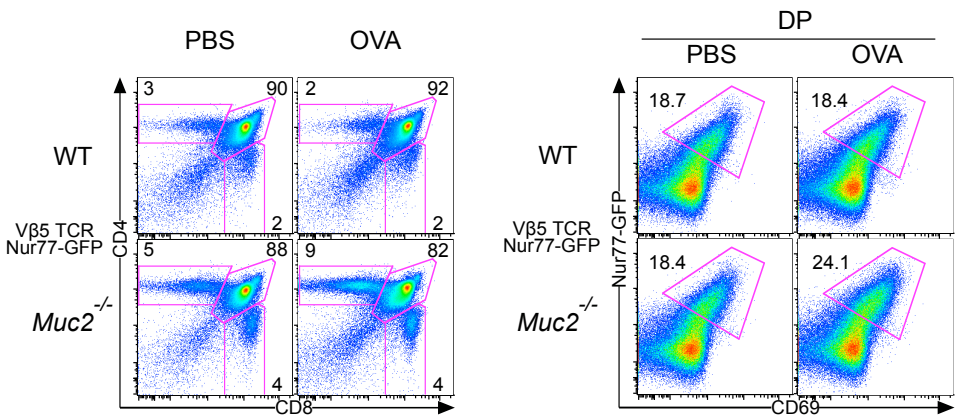

Figure 5-7 Dietary antigen induce TCR signaling in developing thymocytes.
WT or *Muc2*$^{-/-}$ Vβ5 Nur77^{GFP} mice (n=1) were orally gavaged with OVA. Thymi were collected 24 hours later and stained with thymocyte developmental markers for the analysis of thymocyte activation and maturation.

5.2.8 Thymic dendritic cells of treated *Muc2*$^{-/-}$ mice take up and present OVA on MHC class I molecules following treatment.

DCs are known to participate in the negative selection of developing thymocytes by capturing and presenting antigens acquired from the dying cells in the thymus as well as antigen acquired from peripheral tissues. After observing that OVA gavage caused negative selection of DP thymocytes in Vβ5 *Muc2*$^{-/-}$ mice we sought to determine whether thymic DCs were responsible for the capture and presentation of blood borne OVA. To determine whether thymic DCs play a role in capturing and presenting blood born OVA CD11c^{+} cells were isolated from the thymi of OVA gavaged Vβ5 *Muc2*$^{-/-}$ or Vβ5 *Muc2*^{WT} mice using magnetic cell isolation. These isolated DCs were then stained with DC/macrophage surface markers and an antibody specific for OVA$_{257-264}$ presented on H2K^{b}, followed by intracellular staining for OVA. If our hypothesis is true, thymic DCs isolated from the OVA treated Vβ5 *Muc2*$^{-/-}$ mice should stain

positive for OVA in the intracellular stain and positive for $OVA_{257-264}$:H-$2K^b$ on the surface, indicating DC OVA uptake and presentation.

Our preliminary staining results showed there was an in increase in intracellular OVA staining, surface $OVA_{257-264}$:$H2K^b$ staining, and intracellular OVA, $OVA_{257-264}$:$H2K^b$ double staining in DCs isolated from the OVA treated Vβ5 *Muc2*$^{-/-}$ mice. This increase in OVA staining was not observed in DCs isolated from the thymi of PBS treated Vβ5 *Muc2*$^{-/-}$ mice, nor OVA treated Vβ5 *Muc2*^{WT} mice, suggesting that blood borne OVA in the OVA treated Vβ5 *Muc2*$^{-/-}$ mice was picked up, processed, and presented by thymic DCs to mediate negative selection of thymocytes that possess high affinity TCRs towards OVA (*Figure 5-8*).

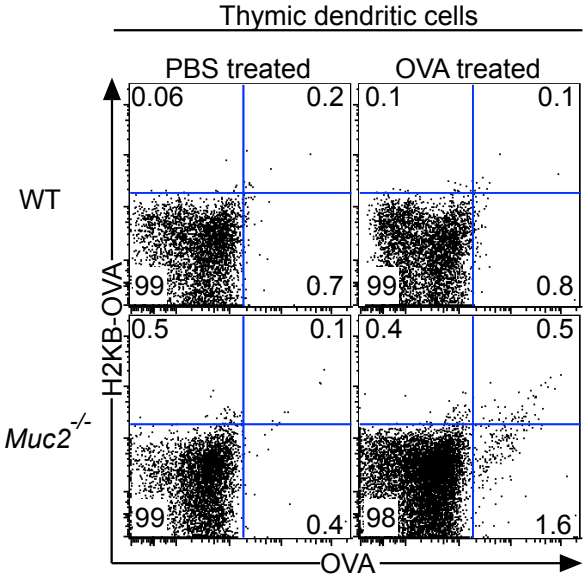

Figure 5-8 Thymic dendritic cells of treated *Muc2*$^{-/-}$ mice take up and present OVA on MHC I molecules following treatment.
Thymic dendritic cells were isolated from the thymocyte suspensions of OVA or PBS treated mice (n=2) using CD11c antibody labeling and magnetic cell isolation. Isolated DCs were then stained with dendritic cell surface markers and antibodies which recognize OVA and $OVA_{257-264}$ presented on $H2K^b$ molecules. Dot plots shown were gated on $CD11c^+$ cells.

5.3 Discussion

The intestinal mucus layer is essential for gut homeostasis [479]. It provides a selectively permeable barrier for nutrient absorption, lubricates the gastrointestinal tract to reduce mechanical stress, separates the luminal content from the intestinal epithelium to prevent microbial invasion, and promotes intestinal homeostasis through immunological ignorance and T regulatory cells induction [19, 478, 480]. The absence of intestinal mucus barrier or defects in its integrity has been associated with increased chance of IBD relapse or increased susceptibility to autoimmune diseases [414, 415]. In this report, we demonstrated the importance of the intestinal mucus barrier in preventing the negative selection of developing thymocytes specific for the luminal antigens.

Clonal deletion of autoreactive T cells (also known as negative selection) is a tolerogenic mechanism utilized by both central and peripheral tolerance to eliminate high avidity autoreactive T cells and prevent autoimmunity [125, 484]. In central tolerance, negative selection is mediated by thymic epithelial cells (TECs) and thymic dendritic cells. While cortical thymic epithelium (cTEC) can induce clonal deletion of thymocytes with high avidity towards ubiquitous self-antigens, deletion of thymocytes specific for tissue restricted antigens is carried out by medullary TECs (mTECs) through ectopic gene expression mediated by the transcription factor autoimmune regulator (AIRE) [125, 180]. Dendritic cells can further augment the antigen converge provided by mTECs and mediate thymic negative selection by either processing and presenting antigens from dying thymocytes to delete ubiquitous antigen-specific thymocytes or by transporting tissue-specific antigens from peripheral tissues to the thymus to delete tissue-specific thymocytes [90]. In the peripheral tissues, dendritic cells can mediate clonal deletion by

taking up and presenting self-antigens or innocuous antigens such as food antigens in local draining lymph nodes [90, 484].

Oral administration of ovalbumin to mice harboring OVA-specific T cells is known to induce activation of said T cells in the MLN [485] however, there is no clear evidence on the effect of a single dose of ovalbumin on splenic OVA-specific T cells. We found that a single 1 mg dose without any adjuvant could robustly activate and expand OVA-specific CD8 T cells in the spleen and MLN of the WT animals but not in the *Muc2*$^{-/-}$ animals. Furthermore, peripheral OVA-specific CD8 T cells in the *Muc2*$^{-/-}$ mice failed to upregulate CD44, and could not produce IFN-γ in response to $OVA_{257-264}$ peptide challenge. These results suggest that either the oral antigen somehow became unavailable in the Vβ5 *Muc2*$^{-/-}$ mice or CD8 T cells within the Vβ5 *Muc2*$^{-/-}$ mice became anergized by excessive exposure to the oral antigens.

CD69 expression on the OVA-specific T cells has been used as one of the gold standards to measure the extent of acute T cell activation. While this method of measuring T cell activation may be adequate in mice with an intact intestinal mucus layer, it may yield false positive results in the intestinal mucus deficient *Muc2*$^{-/-}$ mice, as heightened exposure to the intestinal microbiota may result in upregulation of cytokines and increased amounts of inflammatory stimuli, both of which are known to trigger CD69 upregulation in a TCR independent manner [442]. To remedy this problem, we crossed a mouse line expressing a GFP reporter specific only to TCR signaling [442] to our *Muc2*$^{-/-}$ mice expressing an OVA-specific transgenic TCR. Using this transgenic line, we found that in the MLNs of OVA treated *Muc2*$^{-/-}$ mice, GFP expression in OVA-specific CD8 T cells became upregulated following OVA treatment, indicating that these T cell became activated upon encountering their cognate antigen and that OVA is being presented in the MLNs of treated animals. To our surprise, we found that the GFP signal in the splenic OVA-specific

$CD8^+$ population also became upregulated upon OVA treatment in both $Muc2^{WT}$ or $Muc2^{-/-}$ mice with $Muc2^{-/-}$ mice showing higher level of GFP expression which may also suggest higher antigen availability in the spleens of treated $Muc2^{-/-}$ mice.

The increased GFP signal observed in the splenic CD8 T cells of $Muc2^{-/-}$ mice raised the question whether the oral antigen was dispersed systemically and whether there are any changes in the intestinal permeability of $Muc2^{-/-}$ mice. Oral administration of FITC labeled dextran is a common method used to assess intestinal permeability [486]. While the assay can the detect changes of intestinal permeability as well as the location of the breach, treated animals would have to be sacrificed at a given time point for the assay making it impossible to continuously track the concentration of FITC dextran in the blood over a given period of time. To remedy this problem, we developed a flow cytometry based assay which allows for continuous tracking of blood ovalbumin concentrations after gavage without the need to sacrifice the animal. Using this assay, we found that although the majority of the oral antigen was found in the plasma fraction of OVA treated $Muc2^{-/-}$ mice, some could also be detected in the cellular fraction. It is unclear how OVA present within each fraction contribute to the activation splenic OVA-specific CD8 T cells.

In $Muc2^{-/-}$ mice, the plasma OVA concentration rose sharply after OVA treatment, reaching peak concentrations 3-6 hours after treatment and remained detectable after 72 hours, whereas in treated WT mice plasma OVA concentration rose slightly above background 1 hour after gavage and quickly dissipated into the background within 24 hours. The rapid and prolonged appearance of oral antigens in the plasma of $Muc2^{-/-}$ mice suggests that their intestinal permeability may be drastically higher than that of wild type mice.

One of the hallmarks of oral tolerance towards food antigens is that the lack of immune response towards the food antigen systemically [485]. While this phenomenon has been known

for years, current evidence suggest that the food antigens are transported and presented in the lamina propria and lymphatic tissues restricted to the gut associated lymphoid tissues (GALT), which does not explain the observed systemic effect. Curiously systemic dissemination of food antigens through blood after ingestion had been observed in both human and mice, however the effect of these oral antigens on thymic development remained unknown.

In this study, we demonstrated that under the conditions of mucus deficiency and increased intestinal permeability, oral antigens OVA could rapidly disseminate systemically through the blood. The blood borne OVA could then be picked up by DCs in the thymus to mediate negative selection of thymocytes expressing high OVA affinity TCRs. We arrived at this conclusion based on the observations that only in the OVA treated *Muc2*$^{-/-}$ mice were we able to detect a rapid increase in blood OVA concentration; thymic CD11c^{+} cells were able to pick up and present blood borne OVA to developing thymocytes, as indicated by the increase in internal OVA as well as cell surface $OVA_{257-264}$:$H2K^{b}$ staining; blood borne OVA presented by thymic CD11c^{+} cells could trigger TCR signaling in high OVA affinity DP thymocytes, evident by the simultaneous upregulation of GFP and CD69 in DP thymocytes of OVA treated Vβ5 Nur77^{GFP} *Muc2*$^{-/-}$ mouse and increased TCRβ, CD69 staining in the DP thymocytes of OVA treated Vβ5 *Muc2*$^{-/-}$ mice; the engagement of TCRs in the high OVA affinity DP thymocyte population resulted in a decrease of OVA tetramer staining intensity, frequency and numbers of DP thymocytes in the OVA treated Vβ5 *Muc2*$^{-/-}$ mice.

The systemic effect of oral tolerance has been known for years, however the tolerogenic mechanisms describe thus far are largely mechanisms of peripheral tolerance with little mention of the contribution from central tolerance [485]. One possible reasons why oral antigen could not affect thymic development in previous studies is that the intestinal mucus barrier was intact,

thereby limiting the amount of oral antigens that were able to cross the intestinal mucosa to affect thymic development. In a report by Baba *et al.* intravenous administration of a high dose (200 μg) of $OVA_{323-339}$ peptide could potently delete OV- specific DP thymocytes [487]. In our study using *Muc2*$^{-/-}$ mice, we observed a peak plasma OVA concentration several magnitudes lower than that of 1×10^{-4} g/mL (standard curve data not shown, assuming each mouse has a 2 mL total blood volume) with plasma OVA concentrations in treated WT animals being even lower. It is possible that under normal conditions, short term oral antigen exposures were not able to affect T cell development and alter TCR repertoire because the systemic oral antigen concentrations were simply not high enough even if the thymocytes express a transgenic TCR specific for the oral antigens. Whether long term exposure to oral antigens would have any effect on the outcomes of T cell development and selection is perhaps the most important question that needs to be investigated.

The mucosa of the gastro-intestinal tract is constantly in contact with a large number of diverse antigens. We showed that a compromised mucosal barrier could lead to systemic dispersal of a 45KDa food protein, which in turn altered the composition of thymocyte TCR repertoire. Although the experimental setup is highly artificial as mucus deficiency and elevated frequency of food antigen-specific thymocytes are two conditions not found in humans or WT animals, our findings never the less raise an important question: If a 45KDa protein can cross a compromised mucosa barrier, be dispersed through blood in high concentrations and cause antigen-specific negative selection of thymocytes, would microbial proteins and environmental antigens be able to alter thymocyte repertoire as well? Could systemic dispersal of microbial and environmental antigens through the gut cause immunodeficiency and tolerance? Our findings, summarized in (*Figure 5-9*) shed new light on how intestinal permeability could affect

thymocytes selection, and may offer new insights into how tolerance towards environmental and food antigens is established and maintained.

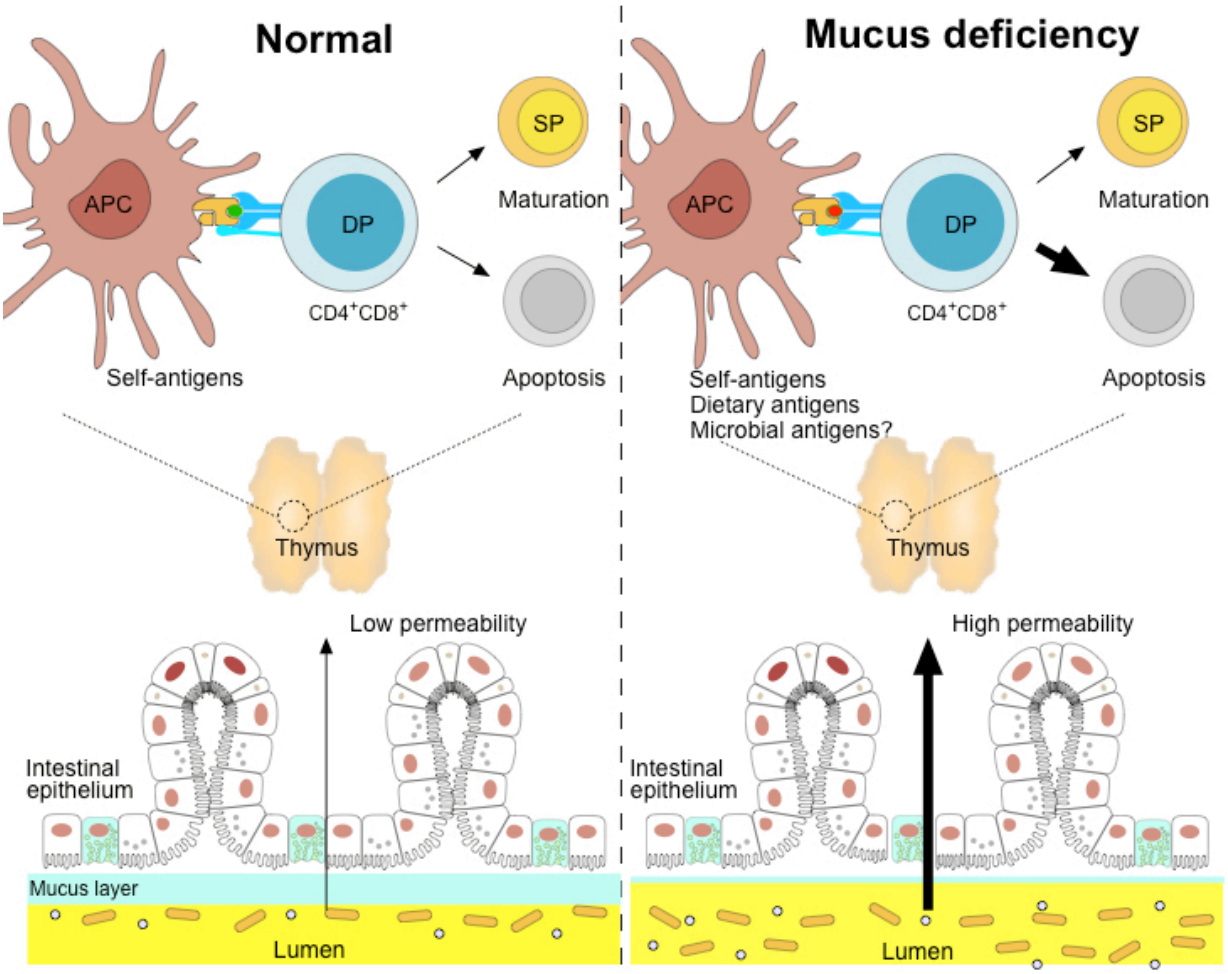

Figure 5-9 MUC2 deficiency increases intestinal permeability and alter T cell development and T cell repertoire.
Muc2 plays an important role in barrier function serving to segregate the intestinal luminal contents from the intestinal epithalamium. Under normal conditions, low intestinal permeability allows little luminal antigens to enter circulation. Developing T cells are not selection against luminal antigens. Under conditions of mucus deficiency, dietary antigens (and perhaps microbial antigens) enter the circulation and skew T cell development.

CHAPTER 6: CONCLUSIONS AND IMPLICATIONS

6.1 Chapter 3 conclusions and implications

Autoreactive T cells play a central role the pathogenesis of many autoimmune diseases including type 1 diabetes[488]. Consequently, blunting autoreactive T cell insult on healthy tissue is key to the success of immunomodulatory therapies aimed at stopping the progression of these diseases. Although the field of immunomodulatory therapy against autoreactive T cells has gained significant advancement over last seventy years, current immunomodulatory therapeutics are still largely restricted to a blanket approach, which targets all T cells indiscriminately. The unspecific nature of these therapeutics, while useful in suppressing autoreactive T cells, may also lead to opportunistic infections or development of malignancies. Previous studies have shown that αGalCer can protect NOD mice from T1D through NKT cell-mediated mechanisms [450-452]. By incorporating αGalCer into the lipid bilayer of liposomes, our collaborators created a drug delivery platform which allows for simultaneous delivery of αGalCer and an autoantigen to APCs, which can potentially achieve antigen-specific suppression of autoreactive T cells by harnessing the tolerogenic effects of NKT cells.

In Chapter 3 of this thesis, we studied whether liposomal αGalCer can activate NKT cells and whether inclusion of the autoantigen insulin in this new formulation would be effective in preventing T1D in mice. We show that liposomal αGalCer when injected subcutaneously into C57BL/6 mice, can potently activate splenic NKT cells, and through NKT cells other immune cell types such as NK cells, B cells, and T cells. Other than the cell types mentioned, we also find that subcutaneous liposomal αGalCer treatment results in the activation and expansion of splenic $CD11c^{int}$ $CD317^{+}$ antigen presenting cells, which in turn present αGalCer on cell surface

CD1d molecules. Most importantly, subcutaneous αGalCer liposome encapsulated insulin treatment to NOD mice can reduce the frequency of circulating β cell-reactive CD8 T cells, and prevent type 1 diabetes. Taken together, our results show that the subcutaneous αGalCer liposome treatment can potently activate NKT cells in C57BL/6 mice, and when insulin is included within the liposomes, the liposomal αGalCer treatment can prevent T1D in NOD mice.

Although our results demonstrate the feasibility of using liposomal αGalCer encapsulated with autoantigen to prevent autoimmunity, our results also raised several questions. First, we are not certain whether the $CD317^+$ APC subpopulation which was expanded by αGalCer treatment are indeed bona fide plasmacytoid DCs, as CD317 can also be upregulated after DC activation. Second, we are not certain what type of immune cells are causing insulitis in αGalCer liposome treated NOD mice despite our data showing a decrease in circulating autoreactive CD8 T cell and splenic Treg cells frequencies. Lastly, can we change the autoantigen encapsulated within these αGalCer liposomes, and use this αGalCer liposome platform to prevent or even treat other autoimmune conditions in which the autoantigens are known? Answering these questions will not only help us better understand how αGalCer liposomes confer protection against T1D but also provide us with more options to prevent or treat other forms of autoimmunity.

6.2 Chapter 4 conclusions and implications

The expression of two different αβ TCRs with distinct specificities in a single T cell (bi-allelic T cells) is the result of a lack of TCRα chain allelic exclusion during T cell development. The role of bi-allelic T cells in autoimmune diseases is a subject of contention: while some researchers postulate that the expression of secondary TCRs may result in autoreactive T cells

escaping central tolerance [473, 474], others suggest the secondary TCRs do not cause autoimmunity [175, 469]. In Chapter 4 of this thesis, we investigated whether the expression of two TCRs with distinct specificities by individual CD8 T cells could contribute to increased autoreactivity and autoimmunity. We showed that naïve CD8 T cells capable of expressing two types of TCRs (bi-allelic) can proliferate more when activated with the model autoantigen versus CD8 T cells restricted to only one TCR (mono-allelic). In addition, a greater frequency of bi-allelic CD8 effector T cells can secrete IFN-γ upon autoantigen stimulation compared to mono-allelic CD8 effector T cells. Taken together, these results suggest dual TCR expression can increase CD8 T cell autoreactivity *in vitro*. Unfortunately, we could not definitively demonstrate that bi-allelic TCR expressing CD8 T cells constitute a greater risk for autoimmunity *in vivo* relative to mono-allelic TCR expressing CD8 T cells as we could not induce diabetes in Vβ5 × RIP-mOVA mice.

The reason that we were not able to induce fulminant T1D in Vβ5 × RIP-mOVA mice through infection with Lm-OVA as has been previously described is not immediately clear [249]. Despite using the same genotype of mice, route of Lm-OVA administration and Lm-OVA dosage as Zehn et al. immunizing bi-allelic Vβ5 × RIP-mOVA mice with Lm-OVA resulted in a transient increase in blood glucose on day 8 in some bi-allelic Vβ5 × RIP-mOVA mice, which returned to normal by day 10. There are several plausible explanations: (1) The RIP-mOVA strain we used in our experiments may not be the exact RIP-mOVA strain used by *Zehn* et al. As there might have been multiple founder transgenic mice made with slightly different OVA expressions, the animal we used might have different OVA expression compared to what was published. (2) OVA is not expressed well enough in a sufficient number of β-cells to cause diabetes. (3) OVA is expressed more strongly in the thymus, resulting in more efficient deletion

of autoreactive T cells. (4) Differences in microbiome and other environmental factors are known to influence the outcome of T1D in NOD mice [8, 489], it is therefore possible that one or more differences in these factors altered the disease course in our mice.

6.3 Chapter 5 conclusions and implications

The surface of the intestinal lumen is lined with a layer of mucus comprised primarily of MUC2 mucin. In healthy individuals, MUC2 is an important contributor to gut homeostasis [19]. It not only separates the microbe-rich luminal content from the epithelial layer to prevent unnecessary immune activation but also functions to induce Treg cells [19]. Although it has been shown that in IBD patients and *Muc2*$^{-/-}$ mice, a disrupted mucus barrier can result in excessive immune activation and IBD [416], whether MUC2 deficiency can contribute to dysregulated T cell response remains unknown. On one hand, increased T cell exposure to luminal antigens and PAMPs in the absence of regulatory influence from MUC2 may indeed result in excessive pro-inflammatory T cell activation. On the other hand, increased exposure to luminal antigens or perhaps hyperactivation of APCs may also result in activation induced T cell death or anergy.

In Chapter 5, we investigated the role of MUC2 mucin on T cell tolerance against oral antigens to shed light on how mucus deficiency can influence CD8 T cell repertoire and function. We orally gavaged wild-type and MUC2-deficient (*Muc2*$^{-/-}$) mice with the model antigen chicken ovalbumin (OVA) and monitored OVA-specific T cell responses at various time points after treatment. Our results show that contrary to our hypothesis, peripheral CD8 T cells from *Muc2*$^{-/-}$ mice respond more poorly to the model dietary antigen than CD8 T cells from wild type mice. Upon closer examination, we find that MUC2-deficiency is associated with drastically increased intestinal permeability, which results in rapid dissemination of oral antigens

throughout the *Muc2*$^{-/-}$ mice. We show that much of the oral antigen is located within the plasma fraction of treated *Muc2*$^{-/-}$ mice and that the increased availability of oral antigens results in their uptake by thymic DCs and activation of OVA-specific thymocytes. Importantly, we show that OVA gavage can mediate antigen-specific negative selection of OVA-specific thymocytes in *Muc2*$^{-/-}$ mice. Taken together, our results suggest that MUC2-deficiency results in a rapid increase of a dietary antigen within the peripheral blood that in turn shapes the TCR repertoire of developing thymocytes.

Results from our study shed light on the importance of intestinal barrier integrity to central tolerance towards luminal antigens and raised several important questions pertaining to the establishment and maintenance of oral tolerance and tolerance towards luminal antigens. Is central tolerance involved in both processes? If so, when is central tolerance invoked? What about leaky gut and other diseases? Does this process happen in these situations or normally during ontogeny (early life)? Does increased gut permeability impact the generation of microbe-specific T cells and cause immune deficiency in IBD patients? Studies that aim to answer these questions may not only show us why food allergies and autoimmune diseases are on the rise but also how to better treat patients afflicted with food allergies or IBD.

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