THE ROLE OF MALT1 IN T CELL IMMUNITY

by

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Abstract

Individuals with combined immunodeficiency exhibit near-normal frequencies of T and B cells but suffer from severe recurrent infections related to defective cellular or humoral immunity. A child admitted to BC Children's Hospital displayed a novel clinical presentation of combined immunodeficiency with immune dysregulation. The patient was found to be homozygous for missense mutations in the *MALT1* gene inherited from her consanguineous parents. MALT1 plays critical roles in activating the NF-κB pathway upon T cell and B cell receptor stimulation. MALT1 functions by at least two different mechanisms: (1) as a scaffolding molecule bringing CARD11 and BCL-10 to form the CBM signalosome complex and (2) as a caspase-like protease cleaving substrates to regulate NF-κB signaling. Studies in *Malt1*$^{-/-}$ mice have revealed weakened T cell immunity whereas mice expressing MALT1 lacking paracaspase activity (but normal scaffolding function) are prone to fetal autoimmunity. In addition, patients with *MALT1* mutations shared similar clinical characteristics, experiencing chronic infections and gastrointestinal inflammation. Together, these findings raise questions regarding the nature of our patient's mutant MALT1 protein and the role it plays in her pro-inflammatory phenotype. Our data have shown that MALT1 is essential for IL-2 production in CD4 T cells despite the dispensable role in regulatory T cell development. In addition, we demonstrate that MALT1 plays a crucial role in effector function of Th1 and Th17 cells. Analysis of the patient's MALT1 scaffolding and paracaspase activity revealed that the patient's *MALT1* mutations act as a hypomorphic allele.

On the other hand, unlike our patient, *Malt1*$^{-/-}$ mice do not exhibit constitutive immune activation and severe intestinal inflammation. A limited pathogen exposure may be responsible for the discrepancy in phenotype of the patient and *Malt1*$^{-/-}$ mice. Hence, we introduced LCMV pathogen to *Malt1*$^{-/-}$ mice and assessed the role of MALT1 in anti-viral T cell immune responses. *Malt1*$^{-/-}$ mice exhibited severely impaired effector function of viral-specific CD4 T cells suggesting MALT1 is critical for the anti-viral immune responses in CD4 T cells. However, we found that MALT1 is dispensable for the generation and effector function of viral-specific CD8 T cells. Further, MALT1 alters the expression of T cell differentiation markers, whereas it does not modulate activation and inhibitory receptors upon viral infection. Collectively, our studies demonstrate that MALT1 plays a pivotal role in CD4 T cell immune responses, yet is not required for CD8 T cell anti-viral immunity.

Lay Summary

A child at BCCH presented with a novel primary immunodeficiency disease (inherited genetic disorder of the immune system) with immune dysregulation characterized by recurrent infections and severe intestinal inflammation. Genetic analysis revealed that the patient inherited homozygous mutations in the *MALT1* gene, which plays an important role in T cell function. T cells form an integral part of our immune system and are essential to fight off most infections. The finding that MALT1-deficiency is associated with immune activation and inflammatory disease indicate that MALT1 is essential in regulating immune responses. Thus, we hypothesize that MALT1 is a critical immune regulator required for T cell homeostasis. Consequently, our study will provide a greater understanding of how MALT1 functions in T cell immunity, and potentially support MALT1-targeted therapies to treat T cell-mediated immune disorders.

Preface

The hypothesis, research aims and experiment procedure were developed under the guidance and supervision of Dr. John Priatel. All cellular functional assays for primary human T cells including intracellular cytokine staining, activation and inhibitory marker staining, and immunoblot were conducted in Dr. Priatel's lab at the BC Children's Hospital Research Institute (BCCHR). Dr. Jacob Rozmus (Pathology and Laboratory Medicine, UBC) isolated the PBMC and performed direct *ex vivo* regulatory T cell staining, and flow cytometry data were analyzed by me shown in *Figure 2.3*. I also conducted all of the experiments and performed data analysis for *in vitro* mouse experiments.

Our lab collaborated with Dr. Stuart Turvey's group and published a paper in Nature Communications; 6:8777, 2016. Klein, T. et al. The paracaspase MALT1 cleaves HOIL1 reducing linear ubiquitination by LUBAC to dampen lymphocyte NF-κB signalling[1]. I am a coauthor on the study and performed T cell isolation and expansion, and assisted in experimental design and data interpretation. In addition, a version of Chapter 2 is currently in manuscript preparation.

BC Children's and Women's Research Ethics Board approved the protocols for PID research, Protocol Number H15-00641. All animal work was completed with approval from the Animal Care Committee at University of British Columbia, Protocol Number A14-0303 and A13-0116.

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List of Symbols

α	alpha
β	beta
γ	gamma
δ	delta
ε	epsilon
ζ	zeta
θ	theta
κ	kappa
μ	micron/mu
M	molar concentration
%	percent
$Gene^{-/-}$	genetic deletion of “gene”

List of Abbreviations

Ab	antibody
Ag	antigen
APC	antigen presenting cell
API2	apoptosis inhibitor protein 2
B6	C57BL/6J mouse
BCR	B cell receptor
BCL-10	B cell lymphoma-10
CARD11	caspase recruitment domain-containing protein 11
CBM	CARD11-BCL10-MALT1
CFSE	carboxyfluorescein succinimidyl ester
CFU	colony forming unit
CID	combined immunodeficiency disease
CLP	common lymphoid progenitor
CMP	common myeloid progenitor
CMV	Cytomegalovirus retinitis
cTEC	cortical thymic epithelial cell
CTL	cytotoxic T lymphocyte
CTLA-4	cytotoxic T-lymphocyte-associated protein 4
CXCR5	C-X-C motif receptor-5
d	days
DC	dendritic cell
DP	double positive
EAE	experimental autoimmune encephalitis
ER	endoplasmic reticulum
FBS	fetal bovine serum
FOXP3	forkhead box P3
GC	germinal center
GFP	green fluorescent protein
GMP	granulocyte and macrophage progenitor

h	hours
HSC	hematopoietic stem cell
HLA	human leukocyte antigen
HSCT	hematopoietic stem cell transplantation
IBD	inflammatory bowel disease
IFN	interferon
Ig	immunoglobulin
IKK	IκB kinase
i.p.	intraperitoneal
ITAM	immunoreceptor tyrosine-base activation motif
iTreg	induced regulatory T cell
i.v.	intravenous
KIR	killer cell immunoglobulin-like receptor
LAT	linker for activation of T cells
Lck	lymphocyte-specific protein tyrosine kinase
LCL	lymphoblastoid cell line
LCMV	lymphocytic choriomeningitis virus
MAPK	mitogen-activated protein kinase
MALT	mucosa-associated lymphoid tissue
MALT1	MALT lymphoma translocation gene 1
MDP	macrophage and dendritic cell progenitor
MFI	mean fluorescence intensity
MHC	major histocompatibility complex
min	minute
MLN	mesenteric lymph node
MS	multiple sclerosis
mTEC	medullary thymic epithelial cell
NFAT	nuclear factor of activated T cell
NF-κB	nuclear factor-kappa B
NK cell	natural killer cell
NKT cell	natural killer T cell

nTreg	natural regulatory T cell
PAMP	pathogen-associated molecular pattern
PBS	phosphate buffered saline
pDC	plasmacytoid dendritic cells
PID	primary immunodeficiency disease
PKC	protein kinase C
PMA	phorbol 12-myristate 13-acetate
PRR	pattern-recognition receptor
RA	rheumatoid arthritis
RAG	recombination-activating gene
RBC	red blood cell
RORγt	retinoic acid-related orphan receptor-γt
cDC	conventional dendritic cell
SCID	severe combined immunodeficiency disease
SP	single positive
STAT3	signal transducer and activator of transcription 3
T1D	type I diabetes
TAP	transporter associated with antigen processing
Tc	T cytotoxic cell
TCR	T cell receptor
Tet	tetramer
Tfh	T follicular helper cell
TGF	transforming growth factor
Th	T helper cell
TLR	Toll-like receptor
TNF	tumor necrosis factor
Treg	regulatory T cell
Wk	week
ZAP70	zeta-chain-associated protein

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Dedication

To my beloved family and friends for their endless support.

Chapter 1: Introduction

1.1 Immunity

All metazoans, including humans are surrounded and colonized by an enormous and diverse array of microbes, composed of both pathogenic and non-pathogenic microorganisms. Microbes contain a vast array of allergenic or toxic substances that are able to colonize our body including the skin, intestinal, and respiratory tract, which can be a constant challenge to the host[2]. However, not all microbes are harmful, some of them are beneficial to the host. For instance, gut microbiota, so called gut flora, play important roles in digesting nutrients and maintaining integrity of the intestinal mucosa necessary for barrier function[3]. Therefore, it is crucial that the host's immune system distinguishes dangerous pathogens from commensal microbes and the body's own healthy tissue. Further, the immune system utilizes multiple protective immune systems to combat the broad range of pathogenic challenges.

The immune system refers to multiple biological structures, groups of the cells and molecules that function to protect the host against disease. The host's immune defense system involves three lines of defense: 1. physical barriers; 2. innate immunity; and 3. adaptive immunity[2,3]. Each defense system interfaces with other immune mechanisms and works as a team to provide efficient protection to the host. Cells of the innate and adaptive immune systems recognize invading pathogens as "non-self", triggering an immune response to facilitate their destruction and elimination. Moreover, innate and adaptive immunity involves the activities of white blood cells (also called leukocytes), consisting of humoral and cell-mediated responses. Humoral immunity comprises substances secreted by cells and found in the humors (body fluids), such as antibodies, antimicrobial peptides and complement proteins. Cell-mediated immunity refers to cell-dependent immune activity and involves phagocytes, natural killer cells and T cells

and the cytokines they produce upon antigen recognition. Dysfunction of any of these mechanisms may result in specific vulnerability to infections by various pathogens.

1.2 Anatomical and physical barriers

Anatomical and physical barriers act as the first line of the defense system situated at the interface between the body and the outside world. These barriers include intact epithelium of skin, lung and intestinal tracts as well as mucus, saliva, tears, and stomach acid. Skin, a non-specific defense system made up of epithelial cells sealed together by tight junctions, forms a wall against the external environment and prevents microbes from entering the body. Shedding of old skin also provides protection by peeling away with adhered pathogens. At other body sites, tears and mucus membranes act as an alternate barrier where skin is not present. Mucus and antimicrobial proteins function to shield the epithelium of respiratory, gastrointestinal and urogenital tracts. In addition, antimicrobial enzymes and peptides serve to destabilize and breakdown bacterial cell walls helping to lyse and limit the growth of pathogens. Further, epithelial cilia help to limit interactions with pathogens by sweeping away overlaid mucus. The failure of one or more barriers may enable pathogens to invade the host and thus, require the prompt action of other host defense systems to limit infection.

1.3 Hematopoiesis and immune cell development

The innate and adaptive immune systems are composed of cells that are derived from hematopoietic stem cells (HSCs) found in the bone marrow (*Figure 1.1*). HSCs are long-lived and capable of both self-renewal and giving rise to multiple potent progenitors that generate various types of blood cells[4]. As HSCs differentiate, they lose their multi-potency and become

short-lived. HSCs differentiate into two major subtypes of stem cells yielding the myeloid and lymphoid lineages[5]. Myeloid cells are so called because they originate in the bone marrow whereas lymphoid cells mature and reside prominently in the lymphatic system. Hence, tightly controlled and continuous differentiation of HSCs is crucial for a proper functioning of the immune system[4].

Common myeloid progenitors (CMPs) generate the erythrocyte and megakaryocyte lineages as well as many types of leukocytes including mast cells, granulocytes, monocytes, macrophages and dendritic cells[6]. Erythrocytes (RBCs) are important to provide oxygen to tissues, and megakaryocytes form platelets that are essential for blood clotting. Granulocyte progenitor cells give rise to white blood cells that contain cytotoxic granules in their cytoplasm and are critical for innate immunity. Granulocytes are often called polymorphonuclear leukocytes due to their non-circular and often multi-lobed nucleus and classified into four types: mast cells, neutrophils, eosinophils and basophils. Mast cells fully differentiate within tissues, developing large granules containing histamine in their cytoplasm, and play important roles in the regulation of both allergy and inflammation[7]. Neutrophils are usually the first responders to bacterial or fungal infection, and migrate into the site of infection through the blood vessels[2]. Eosinophils and basophils comprise less than 5% and 1% of white blood cells respectively, and are known to play a role in asthma and allergic inflammation. Macrophage and dendritic progenitor (MDP) cells develop into monocytes that circulate in the blood and differentiate further into macrophages and dendritic cells (DCs) within tissues. Macrophages and DCs are both considered professional phagocytes, however, they have quite different immunological functions. Macrophages modulate innate immune responses by engulfing and digesting dead cells or pathogens. By contrast, the main function of DCs is to preserve and present the

'information' from dying or infected cells to stimulate the adaptive immune system rather than clear microbes and dying cells[2,8].

Common lymphoid progenitors (CLPs) give rise to lymphoid lineages as natural killer (NK) cells, B cells, and T cells[4]. NK cells play important roles in innate immunity by utilizing germline-encoded antigen receptors to recognize "infected" or "stressed" target cells. B and T cells are so called because of their site of maturation and function in adaptive immunity. B cells are named after the Bursa of Fabricius, a lymphoid organ in birds, where B cells were first discovered[184]. In mammals, the bone marrow is the site of B cell development and differentiation. Thymocytes differentiate into mature T lymphocytes in the thymus, giving T cells their name. The key feature of B and T cells that discriminate them from other immune cells is the derivation of their antigen receptors through somatic DNA rearrangement. When B cells encounter cognate antigen through their B cell receptor (BCR), they proliferate and mature into antibody-secreting memory B cells and plasma cells. Likewise, the recognition of cognate antigen by T cells via their T cell receptor (TCR) induces their expansion and differentiation into effector and memory T cells. Altogether, the activation, function and differentiation of lymphocytes is dependent on binding of cognate antigen to their antigen receptors.

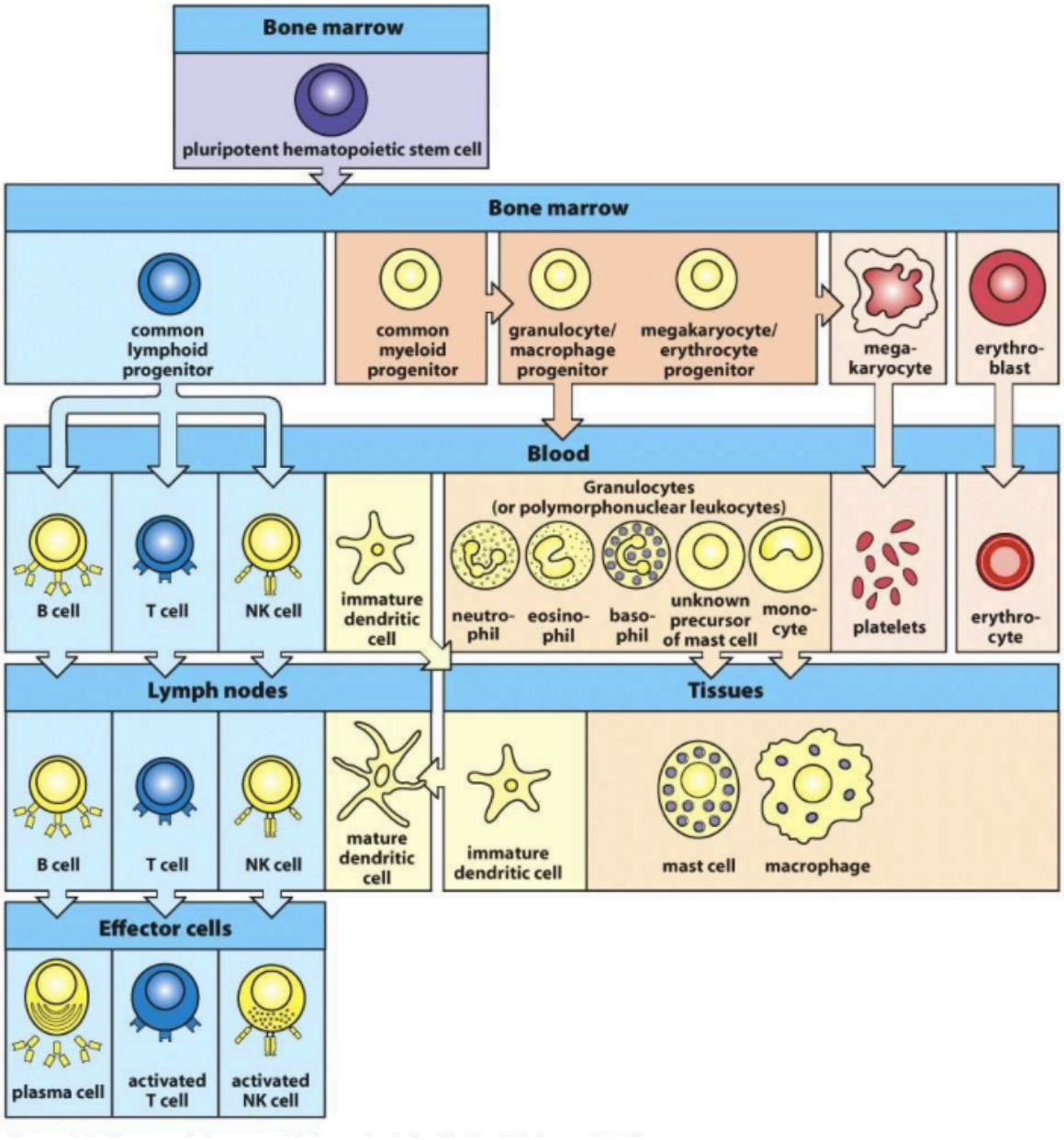

Figure 1. 1 The formation of various blood cell types from hematopoietic stem cells
Schematic diagram illustrates the differentiation of various blood cells arising from pluripotent hematopoietic stem cells (pHSCs). pHSCs generate common lymphoid progenitors (CLPs) and common myeloid progenitors (CMPs). CLPs give rise to lymphocyte lineages including B cells, T cells and NK cells. Subsequently, lymphocyte lineage cells can further differentiate into effector cells to mount immune responses against target cells. CMPs result in the formation of the myeloid lineage cells, including erythrocytes, megakaryocytes, granulocytes (neutrophils, eosinophils and basophils), mast cells and macrophages. Figure reprinted with permission from Garland Science (Kenneth Murphy. *Janeway's Immunobiology: An Introduction to Immunobioloy and Innate Immunity.* 8th Edition, 2012)

1.4 Innate immune system

The innate immune system is an evolutionary old defense system that is found in all classes of plant and animal life. The innate immune system, meaning 'in-born immunity', is present from birth (rather than acquire over time) and it is not dependent on previous exposure to pathogens. Innate immunity is continuously available to provide essential protection against invading pathogens and acts as the first line of defense. Innate immunity launches responses rapidly to harmful agents, yet it does not provide long-lasting immunity or immune memory to the host. The innate immune system is often subdivided into two components: humoral and cell-mediated immunity. Humoral immunity includes soluble proteins and biochemical molecules that are either persistently present in the body fluids (complement proteins), or released upon activation (cytokines)[3]. Complement proteins coat pathogens or dead cells to enable phagocytes to detect and destroy them. Cytokines are small proteins that are produced upon cell activation and mediate important cell signaling. Further, cytokines bridge humoral and cell-mediated immunity by recruiting immune cells to the site of infection.

Innate leukocytes include granulocytes (mast cells, basophils, eosinophils and neutrophils), NK cells and phagocytes (macrophages and DCs)[3]. These cells provide non-specific immune protection against pathogens or infected cells through recognition by a limited repertoire of germline-encoded receptors. The innate immune cells are activated through the activity of pattern recognition receptors (PRRs). PRRs are germline-encoded receptors that can recognize two classes of molecules: pathogen-associated molecule patterns (PAMPs) that are associated with microbial pathogens, and damage-associated molecular patterns (DAMPs) that are released from damaged, dying or dead cells[3]. One of the most well-studied family of PRRs is toll-like receptors (TLRs). TLRs are expressed on the membranes of leucocytes including DCs,

macrophages, NK cells, T cells and B cells as well as non-immune cells like epithelial cells, endothelial cells and fibroblasts. The interaction between TLRs and PAMPs or DAMPs activates immune cells to produce cytokines that triggers innate and adaptive immune responses. Although TLRs have a limited repertoire, they regulate immune responses and inflammation by detecting a vast range of microbe-associated antigens through recognition of conserved microbial elements[9,10].

1.4.1 Phagocytes

Phagocytes refer to the cells that protect the host by engulfing and digesting pathogens or dying cells[8]. Phagocytes include neutrophils, macrophages and DCs, and they express surface receptors that can recognize and bind to harmful substances. Neutrophils are the most abundant phagocytes in the immune system and capable of entering injured sites to initiate rapid immune responses[2]. Once neutrophils take up pathogens through phagocytosis, enzymes within lysosomes break down pathogens into smaller components that promotes the digestion of the target[8]. In addition, neutrophils recognize complement, cytokines and microbial pathogens through its receptors to amplify inflammatory reactions. A lack of neutrophils (neutropenia) is a serious risk factor for infection from normal gut resident bacteria resulting in colitis[11].

Macrophages are another crucial phagocyte that resides in almost all tissues of the body. They mediate non-specific immune protection and tissue homeostasis by engulfing cellular debris, dying cells, foreign agents and pathogens[12]. Besides phagocytosis, macrophages may also play an important role in initiating adaptive immune responses by presenting antigens to lymphocytes[12]. Macrophages can further modulate immune responses by producing either pro-inflammatory or anti-inflammatory cytokines. Moreover, macrophages that mediate pro-

inflammatory reactions are called M1 macrophages, whereas those that are anti-inflammatory and promote wound healing are called M2 macrophages[12,13]. Accordingly, macrophages play important roles in clearing cellular and microbial debris from tissue as well as bridging innate and adaptive immune system.

DCs are also classified as phagocytes but their main function is to process antigenic material and present this information to T cells. DCs play a critical role in the generation of adaptive immunity and will be discussed in more detail in Section 1.4.3.

1.4.2 Natural killer cells and innate lymphoid cells

Natural killer (NK) cells are cytotoxic lymphocytes that use germline-encoded receptors. They are derived from the same progenitor cell that gives rise to T or B lymphocytes[14]. The name 'natural killer' originates from their ability to spontaneously kill infected cells without a requirement for pre-activation. NK cells are prominent in the blood and lymphatic system and their numbers are dependent on steady production and differentiation from CLPs to NK cells[15]. Type I cytokines like IL-15 plays a key role at an early stage of NK cell development and promotes their maturation[16]. In addition, IL-15 promotes the expression of NK cell receptors and effector functions, like cytotoxicity and pro-inflammatory cytokine production, necessary to kill infected and stressed cells[17].

NK cells defend the host from infections by killing or inducing the apoptosis of infected cells. NK cells utilize a mechanism to distinguish infected from healthy cells based on the expression of various activation and inhibitory receptors[14,18]. The activation of NK cell killing involves recognition of major histocompatibility complex (MHC) molecules presented on the target cell surface[19]. For example, human NK cells express inhibitory receptors called killer cell

immunoglobulin-like receptors (KIRs) that recognize MHC class I molecules which are expressed by most healthy cells. Upon recognition of antigen ligation by a KIR, it sends inhibitory signal to the NK cell to suppress activation, ensuring tolerance and preservation of healthy cells. On the other hand, infected cells tend to lose their MHC class I expression to avoid recognition by cytotoxic T cells[20]. Consequently, when NK cells fail to "see" MHC class I molecules on a target cell, they become activated and attack the infected cells for 'missing-self' [14,17]. Furthermore, the importance of NK cells is underscored by findings that NK cell deficiency is associated with increased susceptibility to infectious diseases and malignancies[21].

Innate lymphoid cells (ILCs) are the most recently identified components of the innate immune system and resemble NK cells in having a common progenitor and in their developmental requirements. ILCs are CLP-derived lymphocytes, often residing at mucosal surfaces and share some traits with helper CD4 T cells[22] (CD4 T cell lineage will be described in Section 1.5.3.1). ILCs are divided into three subgroups based on expression of transcription factors that regulate their development, function and the cytokines they produce. ILCs are critical in maintaining tissue homeostasis and modulating immune responses towards pathogens[22,23]. Group 1 ILCs express the transcription factor T-bet and produce Th1 cytokines, such as IFN-γ and TNF-α but are weakly cytotoxic. Group 1 ILCs are critical in providing immunity to intracellular infection and regulating chronic inflammation. Group 2 ILCs express the transcription factor GATA3 and secrete Th2 cytokines IL-4, IL-5 and IL-13 necessary in controlling helminth infection and modulating allergic diseases. Lastly, Group 3 ILCs express the transcription factor RORγt and secrete IL-17 and IL-22 for protection against extracellular pathogens such as fungi by limiting fungal overgrowth[23,24]. Altogether, ILCs play critical roles in

tissue homeostasis, inflammation and the orchestration of adaptive immune responses necessary for protective immunity.

1.4.3 Dendritic cells and professional antigen-presenting cells

Dendritic cells (DCs) are considered professional phagocytes that play a key role in activating the adaptive immune system rather than serving as front-line defenders like macrophages and neutrophils[25]. They reside in the tissues that are in contact with the external surface such as the skin, respiratory and intestinal epithelium, constantly sampling environmental antigens[25]. Once they are activated by pathogens, DCs migrate into lymph nodes where they present antigens to T and B cells for priming of adaptive immunity[25,26]. DCs are often subclassified into either conventional DCs (cDCs) or plasmacytoid DCs (pDCs). The main role of cDCs is digesting microbes and generating antigenic peptides that can activate the adaptive immune system in response to microbial invasion. Besides antigen presentation function, pDCs play key roles in innate immunity by secreting large amounts of type I interferons (IFN-I), mainly IFN-α and IFN-β. Specifically, IFN-I are critical for protective immunity against bacterial and especially viral pathogens[27,28]. Moreover, IFN-I act by stimulating antigen presentation functions of DCs and macrophages as well as activating NK cells to elicit anti-viral responses.

Antigen presenting cells (APCs) function by processing antigens and displaying them on their surfaces in the context of self-MHC molecules for recognition by T cells[25] (will be discussed in details in Section 1.4.4). Almost all cell types except RBCs can serve as APCs by presenting endogenous antigens on their surface via MHC class I molecules. Macrophages, DCs and B cells are considered professional APCs because they are specialized in presenting antigens

to T cells. Professional APCs are able to present exogenous antigens through the expression of MHC class II molecules on their surface. Macrophages and DCs are very efficient at internalizing exogenous antigens through phagocytosis whereas B cells utilize BCR-mediated endocytosis to take up antigens. Professional APCs also express the costimulatory molecules CD80/CD86 that interact with CD28 on T cell surface to enhance T cell activation and differentiation necessary for productive responses[29]. The interaction between antigen presented on MHC molecule and naïve T cells that haven't encountered antigens is crucial for T cells differentiating into various effector T cells. In addition, APCs can secrete pro- and anti-inflammatory cytokines that can shape the appropriate type and magnitude of the T cell response. Collectively, professional APCs are key players regulating productive T cell immunity toward a variety of infections.

1.4.4 Major histocompatibility complex and recognition

The major histocompatibility complex (MHC) is a group of cell surface proteins that are essential for the adaptive immune system to recognize foreign antigens. MHC molecules are highly polymorphic and this diversity is responsible for graft rejection after transplantation due to mismatch of MHC between donor and recipient. In humans, MHC is also called the human leukocyte antigen (HLA) complex while mouse MHC is named the H-2 complex or simply H-2. The main function of MHC molecules is to present self and non-self antigen-derived peptides on the surface of cells for recognition by T cells. There are two main classes of MHC molecules, MHC class I and class II, and they function to present two different sources of antigens: MHC class I molecules present endogenous (intracellular) antigens whereas MHC class II molecules present exogenous (extracellular) antigens[19]. In general, the recognition of self-peptides in the

context of MHC molecules is thought to tolerize T cells towards self-antigens while the recognition of foreign-peptides associated with MHC molecules results in T cell activation, differentiation and the generation of antigen-specific effector T cells[19]. Consequently, antigen presentation by MHC molecules is essential for both self-tolerance and generating immunity toward foreign invaders.

MHC class I molecules are expressed by all nucleated cells and are especially important for T cell responses against viruses and other intracellular pathogens given that they classically present endogenously-derived antigens. For processing of antigens for MHC class I molecules (*Figure 1.2*), cytosolic antigens, derived from endogenous proteins and intracellular pathogens, are degraded by cytosolic and nuclear proteasomes into 8-10 amino acid peptides and subsequently, translocated into the endoplasmic reticulum (ER) by transporter associated with antigen processing (TAP)[30]. Within the ER, peptides are loaded into newly-synthesized MHC class I molecules and then shuttled to the cell surface for presentation to CD8 T cells[19,31].

MHC class II molecules are predominantly expressed by professional APCs. However, MHC class molecules are also found on some endothelial cells and thymic epithelial cells that are responsible for mediating CD4 T cell development in the thymus (see Section 1.5.2). MHC class II molecules are vitally critical for mounting T cell immunity against certain types of bacteria, fungi and parasites because they present antigens derived from exogenous sources [29]. For processing of antigens for MHC class II molecules (*Figure 1.2*), extracellular antigens are first phagocytosed by DCs and macrophages or BCR-mediated endocytosed by B cells and digested in lysosomes. Subsequently, 15-24 amino acid peptides are bound to antigen-binding groove of MHC class II molecules in the endosomal antigen-processing compartment. Finally,

peptide associated MHC class II molecule complexes are exported to the cell surface for the recognition by CD4 T cells[19,32].

Extracellular (exogenous) antigens can also be presented on MHC class I molecules through a process called "cross-presentation" that primarily occurs in DCs although other professional APCs like macrophages and B cells have been shown to have this capacity as well[33]. During cross-presentation, APCs take up extracellular antigens and these endocytosed proteins are transported out of endosome-like structures into cytoplasm by unknown means and processed by proteasomes for recognition by CD8 T cells[33]. Although the underlying mechanisms responsible for cross-presentation are unclear, the process is crucial to provide immune responses against tumors or viruses that do not infect professional APCs or interfere with antigen presentation upon infection[34].

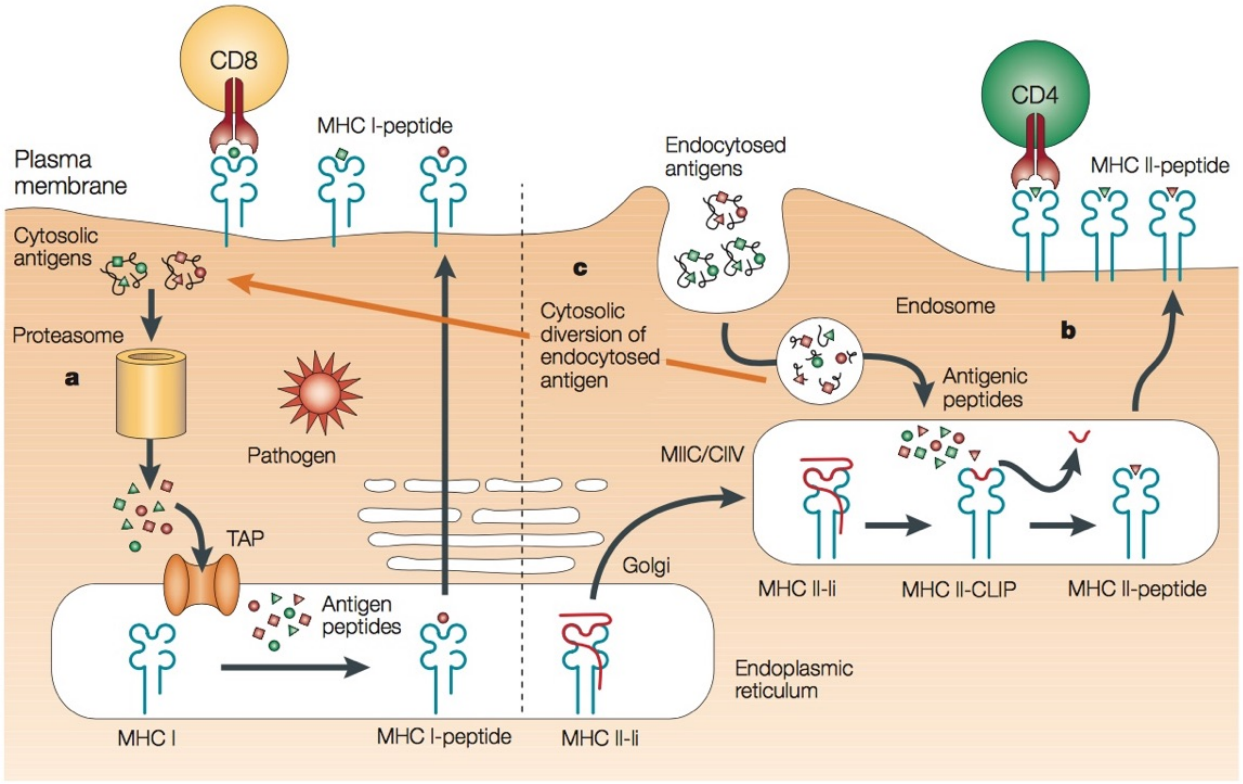

Figure 1. 2 Antigen processing pathways for MHC class I and class II molecules
a) Major histocompatibility complex (MHC) class I molecules classically present endogenous antigens. The antigens are degraded into peptides by proteasomes and then transported into endoplasmic reticulum (ER) via transporter associated with antigen processing (TAP) protein. Subsequently, peptides are loaded onto MHC class I molecules and presented to CD8 T cells. b) MHC class II molecules present exogenous antigens. In the ER, MHC class II molecules contain an invariant chain (Ii) to prevent binding to endogenous antigens. MHC class II-Ii complexes move to Golgi where the invariant chain is degraded leaving a small fragment known as CLIP (Class II-associated invariant chain peptide) that blocks binding of peptides to MHC class II cleft. Subsequently, the CLIP fragment is removed to allow endocytosed peptide antigens with higher affinities to be loaded onto MHC class II molecules. c) Professional antigen-presenting cells (APCs) are also able to present exogenous peptide antigens on MHC class I molecules to CD8 T cells through a process called cross-presentation. Cross-presentation is important for immune responses against extracellular pathogens that do not infect APCs. Figure reprinted from William R. Heath and Francis R. Carbone. *Nat Rev Immunol*. 2001 with permission from Nature Publishing Group[34].

1.5 Adaptive immune system

An adaptive immune system, also known as acquired immunity, is found in all vertebrates and composed of highly specialized cells that can destroy or prevent the growth of

pathogens. There are two major adaptive immune cells, T cells and B cells, and both are derived from progenitor lymphoid HSCs in the bone marrow. Upon maturation, T cells and B cells are exported to periphery and recirculate through the blood and lymphatic system. Newly exported B and T cells are considered naïve cells because they have not encountered cognate antigens for activation and differentiation. These naïve T cells and B cells can recognize new antigens that the immune system has not experienced. Upon antigen binding, they undergo differentiation and clonal expansion, triggering antigen-specific immune responses. Although, the adaptive immune system comes into play later than innate immunity, it provides stronger and long-lasting protection against pathogens. Moreover, one of the key features of the adaptive immune system is the development of immunological memory. Upon antigen activation, large number of clonally expanded effector cells are generated, and a fraction of these effector cells survive and develop into memory cells[35]. These memory cells can store the "information" from encountered antigens and enables faster and stronger immune response at the second encounter of the pathogen.

1.5.1 T cells

T cells (T lymphocytes) are a critical component of adaptive immune system providing the critical defense against invaders, such as viruses, bacteria and malignant cells. All T cells express TCRs on their surface that can recognize peptides in the context of self-MHC molecules. TCRs are heterodimers composed of α and β chain glycoproteins that each contain highly variable and constant regions. During early T cell development, TCRα and TCRβ genes undergo somatic recombination resulting in a vast diversity of TCR repertoires capable of recognizing an enormous number of antigens[36,37]. Once T cells successfully rearrange their TCR genes and mature, T cells become activated and differentiate upon binding their cognate antigen. Moreover,

T cells express markers CD4 or CD8 that act as co-receptors to binds to MHC class II and class I molecules on APCs respectively. These co-receptors play a critical role in enhancing the binding strength of TCR to peptide/MHC complex and promoting TCR signaling for T cell activation[38].

There are several subtypes of T cells serving different immunological functions. It includes helper (CD4) T cells, cytotoxic (CD8) T cells, regulatory T cells (Tregs) and memory T (Tmem) cells. Each T cell subtype expresses its own transcription factors and secretes cytokines to mediate immune responses. For instance, CD4 and CD8 T cells are able to produce pro-inflammatory cytokines that directly inhibit viral replication and increase MHC class I expression on APCs to enhance cytotoxic T cell activity. After clearance of pathogen, a fraction of activated effector cells differentiates into memory T cells. They serve an important function in host immunity by providing long-lasting protection against recurrent infections. Moreover, regulatory T cells are so called suppressor T cells because they secrete anti-inflammatory cytokines, such as IL-10 and TGF-β, to control immune activation and reduce inflammation. Tregs are crucial for immune tolerance and the prevention of autoimmune diseases. Accordingly, it is important to have the appropriate balance in each T cell type for proper immune homeostasis.

1.5.2 T cell development

T cell progenitors originate from the bone marrow and migrate to the thymus where immature T cells mature into functional naive T cells. In the thymus, immature T cells undergo serial differentiation and selection processes to shape a repertoire of CD4 or CD8 T cells (*Figure 1.3*). Early developing thymocytes do not express TCR, CD4 or CD8 co-receptors ($CD4^-$, $CD8^-$), thus they are called double negative (DN) thymocytes. Most DN thymocytes undergo four

different stages of differentiation based on CD44 and CD25 expression (DN1, $CD44^+CD25^-$; DN2, $CD44^+CD25^+$; DN3, $CD44^-CD25^+$; and DN4, $CD44^-CD25^-$)[36,37]. From the DN2 stage, DN thymocytes express pre-TCR bearing a rearranged TCRβ-chain and non-rearranged TCRα-chain[36,39]. When developing thymocytes transit from DN4 stage to double positive thymocytes (DP, $CD4^+CD8^+$), the pre-TCRα chain is replaced with the newly arranged TCRα-chain for the expression of mature TCRαβ complex[40]. Subsequently, TCRαβ DP cells interact with fibroblast-like thymic epithelial cells (TECs) expressing high levels of MHC class I and class II molecules presenting self-peptides. The interaction between TCRs and MHC molecules on TECs is crucial in determining the fate of DP cells (discussed in more detail in Section 1.5.2.2). DP thymocytes receiving appropriate level of TCR signaling differentiate into mature $CD4^+$ and $CD8^+$ single positive (SP) T cells and are exported to the periphery.

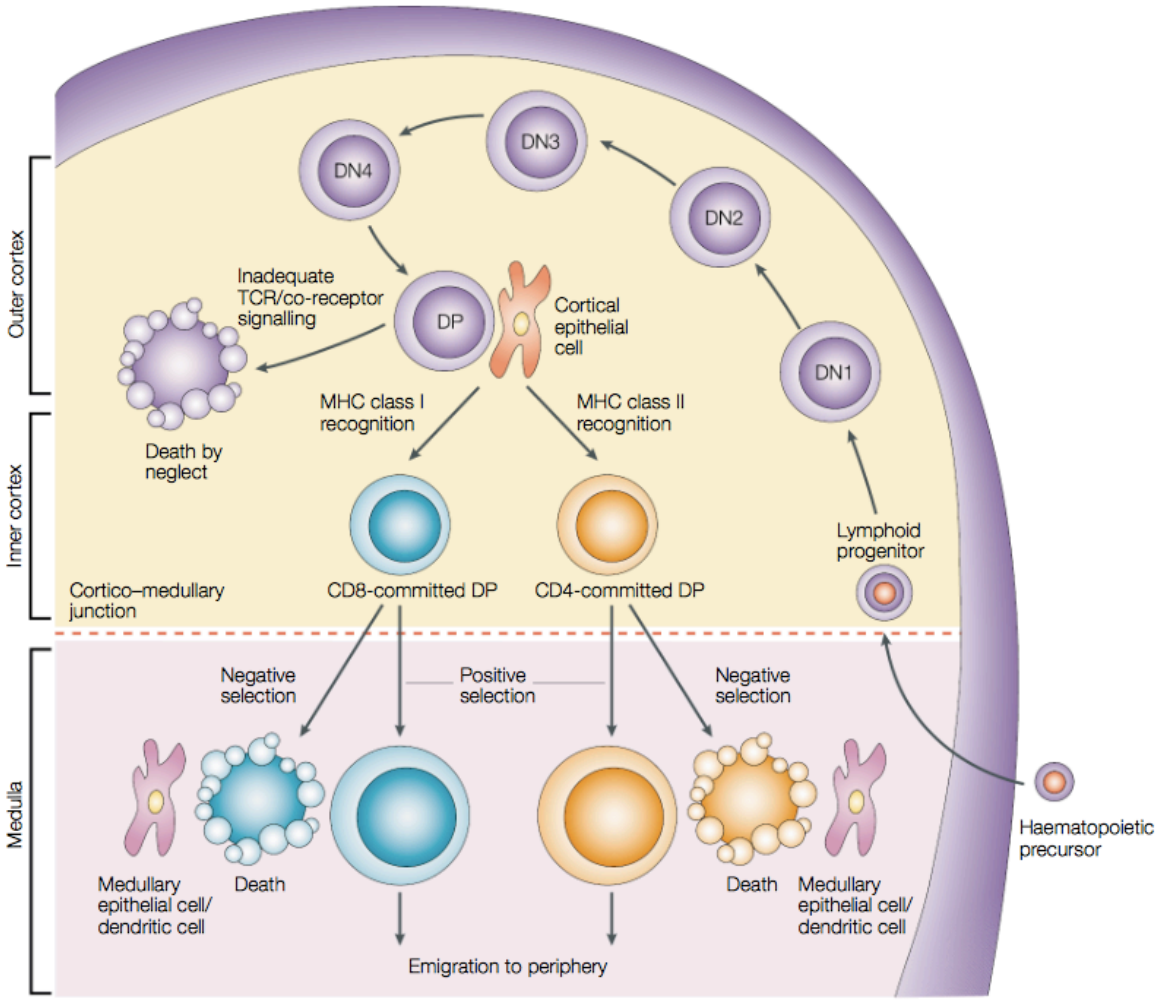

Figure 1. 3 The development of T cells in the thymus
Lymphoid progenitors from the bone marrow migrate to the thymus and commit to the T cell lineage. Thymocyte differentiation is delineated into four developmental stages based on the expression of CD4 and CD8 coreceptors. The most immature thymocytes are named double negative (DN) thymocytes as they do not express CD4 or CD8. The development of DN thymocytes is further discriminated into four stages on the bases of CD25 and CD44 expression (DN1, DN2, DN3 and DN4). At the DN2 stage, thymocytes that undergo productive rearrangements of TCRβ chain gene express a pre-TCR, resulting from pairing of the TCRβ chain with pre-TCRα, initiate massive proliferation and become double-positive $CD4^{+}CD8^{+}$ (DP) thymocytes. Upon successful TCRα chain rearrangement, DP thymocytes replace the pre-TCR with the complete αβ TCR and undergo thymocyte selection. DP thymocytes expressing TCRs that fail to interact with self-peptides in context of MHC class I and class II molecules or react very strongly die by neglect and negative selection respectively. By contrast, DP thymocytes expressing TCRs that interact weakly with self-peptides in context of MHC class I and class II molecules on the surface of cortical epithelial cells are positively selected. Positively selected DP thymocytes downregulate CD4 or CD8 and differentiate into mature CD4 or CD8 single-positive (SP) T cells and migrate to peripheral lymphoid organs. Figure reprinted from Ronald N. Germain. *Nat Rev Immunol*. 2002 with permission from Nature Publishing Group[12].

1.5.2.1 T cell receptor and TCR rearrangement

The TCR is responsible for recognizing antigen-derived peptides presented by MHC molecules on the surface of APCs. Consequently, the diversity of TCRs contain highly variable regions that are specific to its cognate antigens, which allows to recognize almost unlimited number of pathogens. There are two main lineages of T cells: those that are carrying αβ TCR (an α and a β chain) or γδ TCR (a γ and a δ chain). The majority of T cells (95%) carry αβ TCR chains while a smaller fraction of T cells (5%) have γδ TCRs[41]. In addition, a functional TCR complex is formed by non-covalent linkage to the CD3 complex, consisting of CD3εγ, CD3εδ, and CD3ζζ dimers[42,43]. Further, the association of the CD3 complex with TCR chains is required for it to deliver activation signals necessary to trigger T cell immune responses.

TCR diversity is critical for immune competency of the host, thus, establishing a highly variable TCR repertoire is vital for protective immunity against various pathogens. Both the TCRα and TCRβ subunits contain regions that are made highly variable, in part, through randomly rearranged gene segments (*Figure 1.4*). TCRα chain genes contain variable (Vα), Joining (Jα) and constant (Cα) segments and TCRβ chain genes contain variable (Vβ), diversity (Dβ), Joining (Jβ) and constant (Cβ) segments[40,44]. TCR recombination and rearrangement of V, (D), and J TCR genes are regulated by multiple enzymes, such as recombination-activating gene 1 and 2 (RAG-1 and RAG-2). The TCR- V, D and J gene segments are flanked by spacer recombination signal sequences (RSSs) where the V(D)J recombination occurs. RAG-1 and RAG-2 enzymes recognize these RSS regions and randomly break RSS DNA fragments. Then the repair enzyme, terminal deoxynucleotidyl transferase (TDT) repairs the breaks by randomly inserting and deleting nucleotides to form joined V(D)J gene segments. Subsequently, rearranged TCR genes are transcribed, joined to C regions through splicing , and finally, translated into

TCRα and TCRβ chains[35,40]. Through shuffling, recombination and TDT-induced junctional diversity, T cells establish TCR repertoire specificities for millions of different antigens.

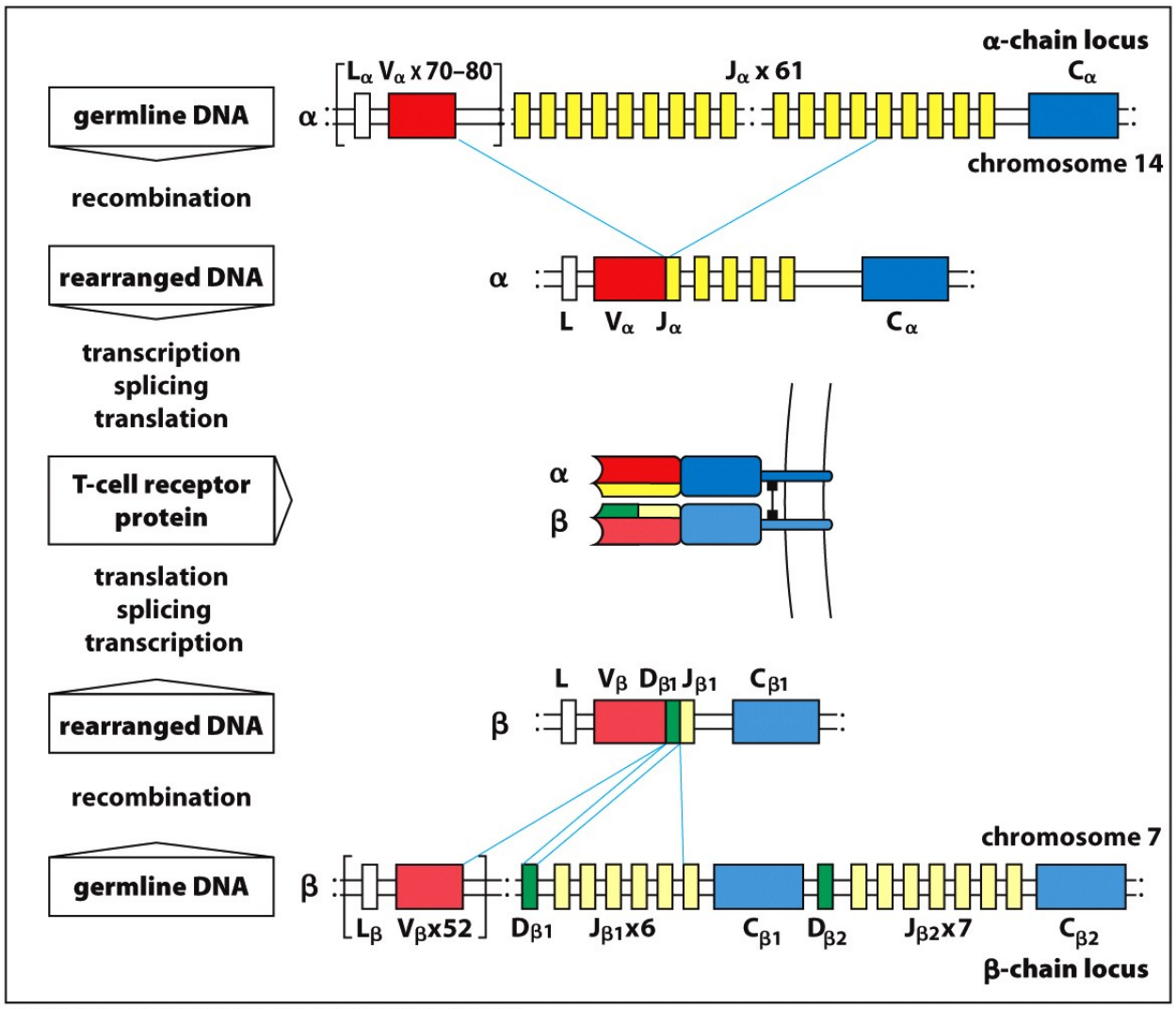

Figure 5.3 The Immune System, 3ed. (© Garland Science 2009)

Figure 1. 4 T cell receptor α and β chain gene rearrangement
Functional TCRs are heterodimers composed an α- and a β-chain. TCRα chain genes consist of variable (Vα), joining (Jα) and constant (Cα) segments and TCRβ chain genes contain Vβ, Jβ, (Dβ) and Cβ diversity segments. Rearrangement of these V, D and J gene segments results in the formation of a functional V(D)J region exon that is transcribed and spliced to join to the C region. Translation of TCR receptor α and β mRNAs produces TCR receptor α and β subunits that are subsequently paired to create the TCRαβ heterodimer. Figure reprinted with permission from Garland Science (Kenneth Murphy. *Janeway's Immunobiology: An Introduction to Immunobioloy and Innate Immunity.* 3rd Edition, 2009)

Both TCRα and TCRβ chain contain complementarity-determining regions (CDRs) consisting of hypervariable amino acid sequences and define TCR specificity[45]. There are three CDRs, called CDR1, CDR2 and CDR3, per TCR chain and they are the most variable regions of the TCR. CDRs form loops that engage amino acid residues of the peptides presented on MHC molecules. CDR3 is especially variable because it is associated with recombined V, and full D and J segments contains junction-induced diversity, whereas CDR1 and CDR2 regions are found in V regions. Altogether, each TCRα and TCRβ chain contains three CDRs and consequently, the mix and match of those six CDRs provides significant variability and thus, very diverse binding capacities.

1.5.2.2 Positive and negative selection

Upon productive rearrangements of their TCRα and TCRβ chain genes, DP thymocytes subsequently undergo positive and negative selection. Positive selection ensures that T cells are restricted to self-MHC molecules and that the appropriate co-receptor is expressed depending on whether the TCR recognizes MHC-class I or II molecules. Positive selection is mainly mediated by TECs situated in the thymic cortex and express MHC class I and MHC class II molecules that are loaded with self-peptides[46] (*Figure 1.5*). DP thymocytes that engage self-peptides in the context of MHC class I molecules commit to the CD8 T cell lineage, whereas DP thymocytes recognizing self-peptides bound to MHC class II molecules mature into the CD4 T cell lineage. When the TCR of DP thymocytes fails to interact with self-peptides presented by MHC molecules, they undergo programmed cell death, sometimes called “death by neglect”. However, the process of selecting T cells on the basis of self-reactivity poses the risk of autoimmunity.

Fortunately, there is a mechanism called negative selection in place to ensure that highly self-reactive (pathogenic) T cells don't escape to periphery.

Negative selection plays an essential role in development of T cell tolerance by eliminating T cells that are strongly reactive to self-antigens. This mechanism, also known as "clonal deletion", promotes apoptosis of DP and SP thymocytes that bear TCRs with high affinity for self-antigens[47]. Clonal deletion is necessary to prevent highly self-reactive T cells to mature and cause autoimmune diseases. Negative selection is efficiently performed by a variety of APCs in the thymus, especially medullary TECs and DCs. However, some highly self-reactive thymocytes escape negative selection and develop into regulatory CD4 T cells (Tregs). Tregs are important for peripheral tolerance through regulation of peripheral immune responses[48,49]. Altogether, T cells are educated to be self-MHC restricted and self-tolerant of self-peptides/MHC molecules through the process of positive and negative selection.

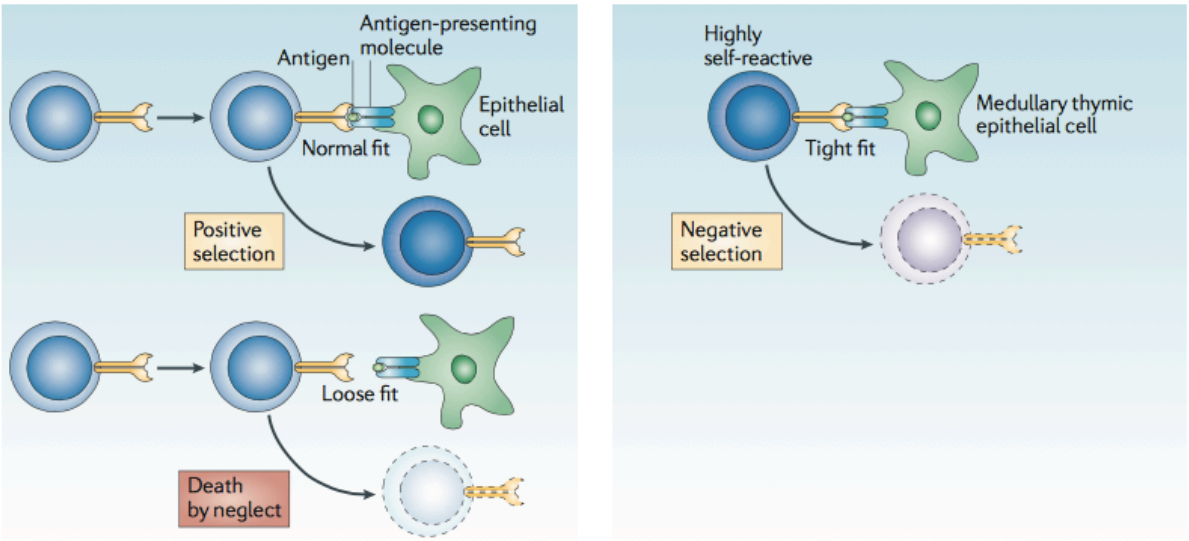

Figure 1. 5 The role of T cell receptor affinity for self-antigens in determining thymopoiesis The affinity of interaction between a thymocyte's T cell receptor (TCR) with self-peptides presented by MHC molecules is critical for thymopoiesis. Thymocytes expressing TCRs that can interact weakly with self-antigens on the surface of antigen presenting cells are positively selected and receive survival signaling (top left). Thymocytes that fail to interact with self-antigens die by neglect (bottom left). Positive selection is crucial to develop self-restricted T cells. Thymocytes expressing TCRs that interact strongly with self-antigens are negatively selected via programmed cell death (apoptosis), which is called negative selection. Negative selection (also known as clonal deletion) is important to eliminate pathogenic auto-reactive T cells (top right). Figure adapted from Thomas Boehm. *Nat Rev Immunol*. 2011 with permission from Nature Publishing Group[35].

1.5.3 T cell subsets

T cells can be divided into two main subpopulations through expression of co-receptors, CD4 or CD8. $CD4^+$ T cells recognize antigens presented on MHC class II molecules and are called helper T cells because they assist the function of other immune cells. CD8 T cells recognize cognate antigen in association of MHC class I molecules and are known as cytotoxic T cells because they can kill target cells when activated. Moreover, CD8 T cells play important roles in eliminating pathogen-infected cells and producing pro-inflammatory cytokines. In

addition, CD4 and CD8 T cell lineages can be further subclassified depending on transcription factor expression, cytokine profile and effector functions.

1.5.3.1 Helper T cells

CD4 T cells mediate immune responses against various pathogens through cell-cell interaction and cytokine secretion. For instance, CD4 T cells express CD40L, a membrane-bound molecule of TNF family, that interacts with CD40 on B cells. Moreover, CD40/CD40L interaction helps B cells differentiate into antibody-secreting plasma cells[50]. In addition, activated CD4 T cells produce cytokines that are important in promoting CD8 T cell activation, proliferation and cytotoxicity. CD4 T cells were first defined to belong within one of two subsets, T helper 1 (Th1) or T helper 2 (Th2) based on the expression of the cytokines IFN-γ or IL-4 respectively[51]. However, it has become clear that naïve (Th0) CD4 T cells can differentiate into several lineages upon antigen stimulation including Treg cell, T helper 17 (Th 17) cell and follicular helper T cell (Tfh) cell lineages (*Figure 1.6*). Each lineage possesses unique profile of transcription factors and exhibits distinct effector functions to combat different types of infections[50].

Th1 cells produce IFN-γ to help eradicate intracellular pathogens by inhibiting viral replication and inducing apoptosis of infected cells[52]. Th1 cell differentiation requires IL-12-induced expression of T-bet, a transcription factor that inhibits development of Th2 and Th17 cells. In addition to T-bet, STAT4 is another crucial IL-12-induced transcription factor that acts to promote Th1 cell function by enhancing IFN-γ production[51]. Further research suggests that T-bet requires STAT4 for complete Th1 fate determination in an IL-12 dependent manner[53]. Immunodeficient patients with defective Th1 cells display extremely high susceptibility to viral

infections[54]. Moreover, mice with IFN-γ deficiency are more susceptible and are unprotected from autoimmune diseases[55-57]. These findings highlight the protective role of Th1 cells.

Th2 cells produce IL-4, IL-5 and IL-13 that are capable of clearing extracellular pathogens such as helminthes and bacteria[51]. Th2 cell differentiation requires IL-4-induced expression of STAT6, which upregulates the expression of GATA3, the Th2 master transcription factor. GATA3 then promotes Th2 cytokine production by establishing an IL-4 positive feedback loop, and limiting Th1 differentiation by inhibiting the expression of T-bet[58]. Although Th2 cytokines are helpful for the elimination of extracellular pathogens, overproduction of IL-4 and IL-13 can promote allergic inflammatory diseases[59]. As a result, targeting Th2-cytokines may be a potential therapeutic strategy for allergies.

Th17 cells are crucial in controlling extracellular bacteria and fungi by producing inflammatory cytokines IL-17, IL-21 and IL-22. Th17 cell differentiation and cytokine production are mediated by their master transcription factor retinoic acid-related orphan receptor-γt (RORγt)[50,51]. Studies in IL-17R-deficient mice have shown that IL-17-deficiency results in high susceptibility to *Candida albicans* and *Klebsiella pneumonia* infections, highlighting the potent protective function of Th17 cells[60]. Recently, Th17 cells have been suggested to also play a role in controlling viral infections[61,62]. Hamada and colleagues reported that repeated influenza A challenges generate IL-17 secreting cells that protect against sublethal doses of virus[61]. Despite their protective effects, Th17 cells are a pathogenic risk factor in a number of autoimmune diseases, including multiple sclerosis (MS) and rheumatoid arthritis (RA)[63,64]. These studies suggest that regulation of the IL-17 pathway may be an effective therapeutic strategy for some forms of autoimmunity.

Tfh cells are a newly described Th lineage that are marked by expression of C-X-C motif receptor-5 (CXCR5), and play an important role in B cell maturation and humoral immunity[65,66]. They are found in the follicles of lymphoid tissues, where they enhance antigen-specific B cell development and the formation of germinal centers (GC). IL-21 secreted by Tfh cells promote B cell differentiation into antibody-secreting plasma cells upon pathogen invasion[66], and also the development of memory B cells[65]. Tfh differentiation is known to be driven by the cytokines IL-6 and IL-21, which upregulate the expression of CXCR5. The exact mechanism of Tfh cell differentiation, however, is still uncertain.

Tregs are essential in maintaining immune homeostasis by promoting tolerance towards self-antigens to protect against autoimmune diseases, including type 1 diabetes and inflammatory bowel disease (IBD)[67]. Tregs constitutively express CD25 and their master transcription factor forkhead transcription factor 3 (FOXP3)[50], and require IL-2 and TGF-β for their survival and differentiation. Mutations in *FOXP3* cause immune dysregulation polyendocrinopathy enteropathy X-linked syndrome (IPEX), resulting in severe systemic autoimmune disease[68]. Several studies indicate that Tregs or Treg-produced cytokines can be used to treat inflammatory diseases. For example, IL-10, which is produced by Tregs, can alleviate inflammatory diseases such as allergies and asthma[60]. Furthermore, Tregs can prevent graft-versus-host disease (GvHD) disease following transplantation by inducing tolerance against grafted cells or tissues[67,69-71]. Together, these findings emphasize that the suppressive function of Tregs is important for regulating the immune system.

Although, all Th subsets are considered fully differentiated CD4 T cells, recent findings suggest that there is 'plasticity' between Th lineages, particularly Treg and Th17 cells. For example, Tregs can potentially convert into pro-inflammatory Th17 cells in settings of

autoimmune diseases or organ transplantation, which can exacerbate the inflammatory conditions[50,51]. Immunologists have substantially improved our collective understanding Th cell biology, including their heterogeneity and plasticity. These new concepts will widen opportunities to target specific subsets of Th cells in order to treat T cell-mediated diseases or malignancies.

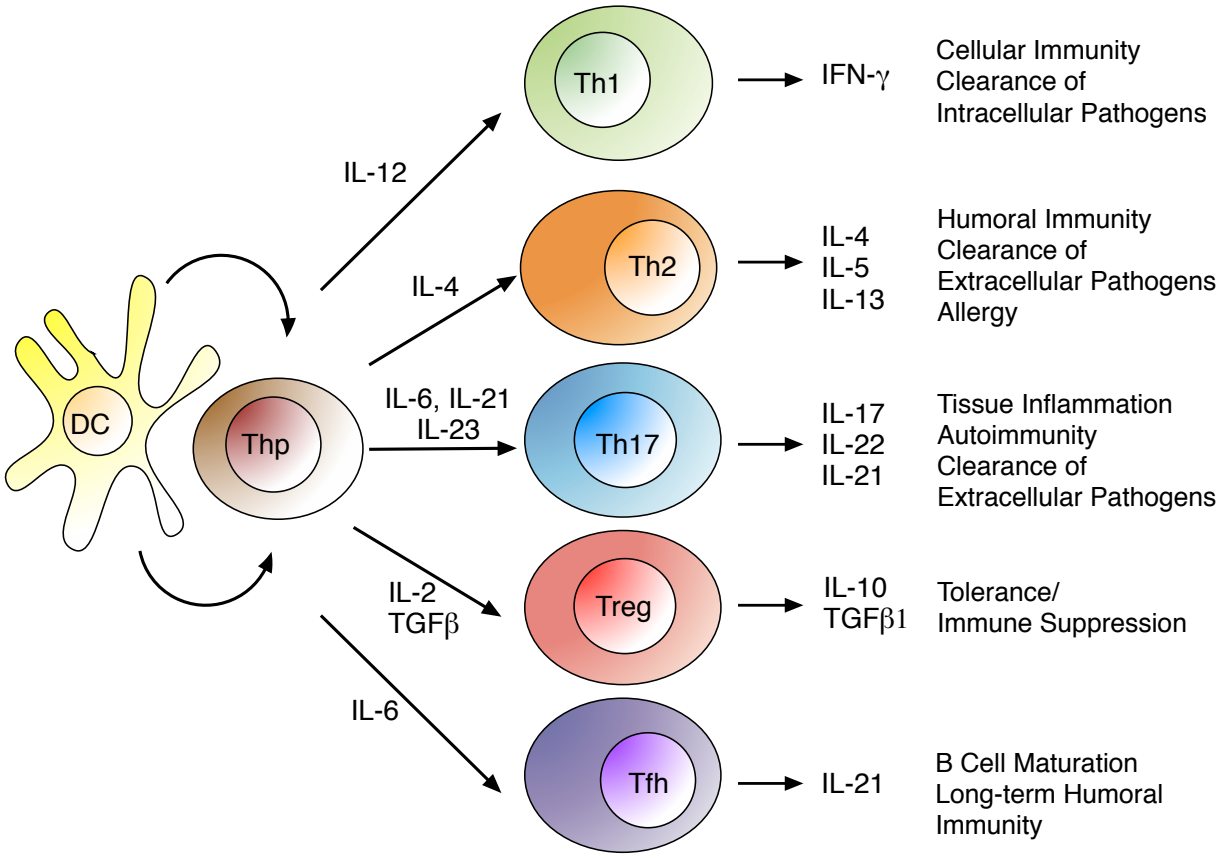

Figure 1. 6 Cytokines in regulating the differentiation of CD4 helper T cell lineages
Naïve CD4 T cells (Thp) can differentiated into Th1, Th2, Th17, regulatory T cell (Treg) and follicular helper T cell (Tfh) upon interaction with antigen presenting cells (APCs), such as dendritic cell. The differentiation of each lineage involves different cytokine and activation of its distinct transcription factor, which may favor one cell lineage and inhibit differentiation of other cell types. Recent findings suggested that there is 'plasticity' in Th cell lineages, which is one cell type is not fully committed to one lineage, but it can switch to other lineages depending on transcription factor activity and surrounding environment. Figure adapted from Anton M. Jetten, *NURSA*, 2009[72].

1.5.3.2 Cytotoxic T cells

CD8 T cells protect against pathogens and malignancies through direct killing of target cells and production of inflammatory cytokines[73]. Cytotoxic T (Tc) cells express TCRs that can recognize cognate antigens presented by MHC class I molecules on the surface of APCs. Upon antigen ligation, naïve CD8 T cells become activated and undergo massive differentiation into effector cells in a process known as clonal expansion. Clonally expanded CD8 T cells can then effectively eradicate pathogens or damaged cells by secreting inflammatory cytokines or inducing cell-mediated apoptosis. Clonal expansion is highly dependent on IL-2, a cytokine that is mostly produced by CD4 T cells[74]. CD8 T cells upregulate IL-2 receptor (CD25) upon activation to further promote their proliferation and function. Upon clearance of pathogens, the majority of effector CD8 T cells die by apoptosis, and a small fraction differentiate into long-lived memory T cells to provide faster and stronger responses against repeated antigen encounters[73,75].

Similar to CD4 T cells, CD8 T cells can be classified into several subpopulations based on the cytokines they produce and the transcription factor they express: T cytotoxic 1 (Tc1), T cytotoxic 2 (Tc2) and T cytotoxic 17 (Tc17) cells. Tc1 cells express the transcription factor T-bet, and produce inflammatory cytokines IFN-γ, TNF-α and granzyme to protect against intracellular pathogens[75]. Tc2 cells express the transcription factor GATA3, and secrete IL-4 and IL-5, which can help eliminate extracellular pathogens or promote allergic inflammation[76]. Tc17 cells secrete the inflammatory cytokine IL-17, and express the transcription factor RORγt. Similar to Th17 cells, Tc17 cell differentiation is induced by IL-6 and IL-21. Although Tc17 cells are important for the clearance of bacterial and fungal infections, they are thought to promote the development of autoimmune diseases, including Type 1 diabetes and MS[76,77].

Collectively, these Tc subsets are essential for the protection against various pathogens; however, their dysregulation can result in harmful inflammatory or autoimmune diseases.

1.5.4 T cell receptor signaling

A TCR complex is composed of the TCR itself, along with a CD4 or CD8 co-receptor, and a CD3 complex. This complex is formed upon the binding of the TCR to a peptide:MHC complex presented on the surface of an APC. The interaction between a TCR complex and a peptide:MHC molecule triggers a number of TCR signaling events that activate T cells, and regulate their function and survival. TCR signaling involves the SRC family kinase Lck (lymphocyte protein tyrosine kinase), a constitutively active kinase bound to the cytoplasmic domain of TCR co-receptors (*Figure 1.7*). Upon antigen binding, Lck phosphorylates immunoreceptor tyrosine-based activation motifs (ITAMs) of the TCR complex and promotes the recruitment of ZAP70 (Zeta-chain-associated protein kinase 70)[42]. Activated ZAP70 then phosphorylates linker of activation of T cells (LAT), which subsequently activates a number of signaling pathways, including mitogen-activated protein kinase (MAPK) pathways, protein kinase C-θ (PKC-θ)/nuclear factor-κB (NF-κB) pathway and calcium flux-mediated nuclear factor of activated T cell (NFAT) pathway[43,78]. Depending on cell type and status, TCR signaling can have different cellular effects, such as T cell proliferation, IL-2 production or apoptosis.

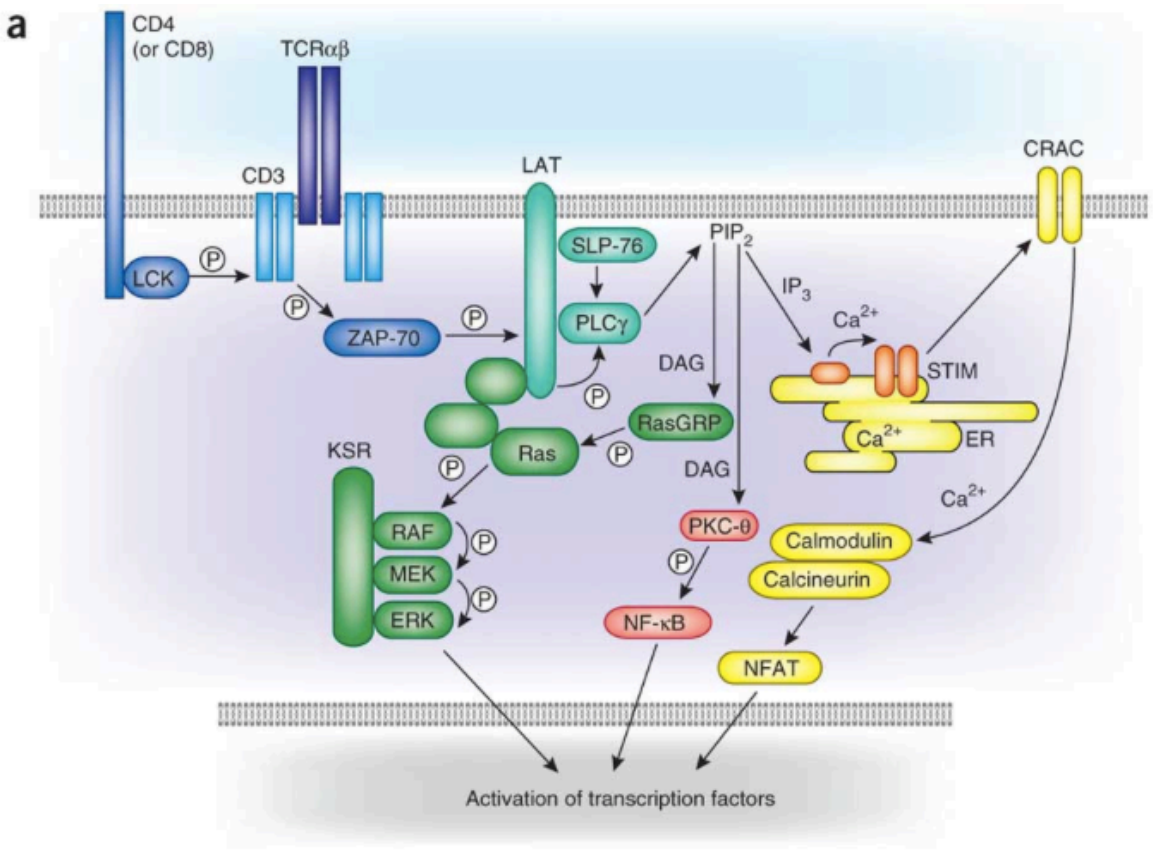

Figure 1. 7 T cell receptor signal transduction
T cell receptor (TCR) signal transduction is initiated by the interaction with MHC-peptides presented on antigen presenting cells (APCs). Upon TCR-CD3 complex activation, SRC kinase family Lck (lymphocyte protein tyrosine kinase) phosphorylates ZAP-70 (Zeta-chain-associated protein kinase 70), which subsequently phosphorylate and activate LAT (linker for activation of T cells). Upon phosphorylation of LAT, it recruits multiple adaptor proteins and activate three major downstream signaling pathways: It enhances the Ras-Erk, also known as MAPK (mitogen-activated protein kinase) pathway, the nuclear factor-κB (NF-κB) translocation, and calcium-flux mediated nuclear factor activated T cell (NFAT) signaling. These pathways lead transcription factor activation that are essential for T cell survival and differentiation. Figure reprinted from Gerald P. Morris and Paul M. Allen. *Nat Rev Immunol*. 2012 with permission from Nature Publishing Group[78].

1.5.5 B cells

B cells are another component of adaptive immunity that produce antibodies for humoral immunity. B cell progenitors originate and mature in the bone marrow. Similar to T cells, all B cells express B cell receptors (BCRs) on their surface, allowing them to bind cognate antigens. However, BCRs differ from TCRs as they directly bind to antigens presented on pathogens or cells rather than engaging MHC molecules expressed on APCs. Upon BCR stimulation, B cells are activated and differentiate into plasma cells that are capable of producing antibodies, which are secreted forms of BCRs. Secreted antibodies can destroy target cells through three main mechanisms: neutralization, opsonization and complement activation (will be discussed further in Section 1.5.5.2)[79]. In addition to antibody production, B cells act as professional APCs that can present both endogenous and exogenous antigens to T cells to initiate the other arm of adaptive immunity.

1.5.5.1 B cell receptor

Analogous to T cells, B cells undergo several developmental stages in the bone marrow in order to express functional surface BCRs. Successfully rearranged BCRs are composed of two heavy chains and two light chains, and each chain has variable (V) and constant (C) regions[80]. During B cell development, BCR genes undergo gene rearrangement by somatic recombination, which randomly rearranges V and C gene segments. This genetic recombination results in antigen specificity and diversity of BCRs[81]. Once B cells successfully rearrange their BCRs, they undergo selection processes to establish a proper repertoire of BCRs[82]. B cells that receive tonic levels of BCR signals from stromal cells survive, but B cells that do not receive BCR signaling or have strong affinities to self-antigens will die by apoptosis in a process known as clonal

deletion[80]. B cells that survive through these checkpoints leave the bone marrow as naive B cells, and can further differentiate in peripheral lymphoid organs, such as the spleen[81]. These selection mechanisms are crucial for the generation of BCR repertoires that can recognize a vast array of pathogens but are not self-reactive.

1.5.5.2 B cells in humoral immunity

In secondary lymphoid organs, naïve B cells can recognize antigens, and differentiate into antibody-secreting plasma cells[81,83]. Antibodies, also called as immunoglobulins (Igs), are Y-shaped glycosylated protein molecules expressed on the surface of B cells. Antibodies can be classified into five types depending on their heavy chain: IgM, IgD, IgG, IgA and IgE. Activated B cells may undergo somatic hypermutation, leading to more diverse antibody repertoires and stronger antibody affinities by inducing point mutations in the V regions of their BCR chains[83].

The B cell response requires CD4 T cell help in a cytokine- or contact-dependent manner[80,84]. Activated CD4 T cells secrete cytokines such as IL-4 and IL-21 to promote B cell proliferation and differentiation. Furthermore, B cells can present antigenic peptides on MHC class II molecules to CD4 T cells, stimulating them to produce more cytokines that subsequently promote B cell activation.

Antibodies provide humoral immune defense against pathogens in three principal ways. First, antibodies neutralize pathogens by binding or blocking key sites of the pathogens to decrease their infectivity. Second, antibodies bind and tag pathogens so that they can be recognized by phagocytes, such as macrophages or DCs. This process is called opsonization. Lastly, IgM and IgG can activate the complement system, which enhances opsonization or directly kills certain pathogens[80,85].

1.5.5.3 Antigen presentation

In addition to antibody production, B cells also serve as professional APCs to activate adaptive immune responses. BCRs capture antigens and internalize them into endosomal compartments, where the antigen is degraded into small peptides. These peptides are presented on the B cell surface through MHC molecules[80]. Like other professional APCs, B cells express both MHC class I and MHC class II molecules, presenting peptides derived from both intracellular and extracellular pathogens. Naïve T cells that recognize antigens presented by B cells receive activation signals and differentiate into effector T cells. Activated effector T cells, particularly CD4 T cells, can secrete cytokines that enhance antibody production and upregulate MHC molecule expression on B cells, including IL-10, IL-21 and IL-4[86]. Moreover, B cells express B7 and CD40 co-receptors that engage CD28/CTLA-4 and CD40L receptors on T cells, respectively. These receptor-mediated interactions between B cells and T cells modulate immune responses. The binding of B7-CD28 and CD40-CD40L delivers a stimulatory signal to enhance T cell activation, whereas B7-CTLA-4 interaction induces an inhibitory signal to dampen immune activation[86].

Although the ability of B cells to cross-present antigens is still unclear, B cells are able to cross-prime naïve CD8 T cells[19,32,80]. In this process, the binding of an antigen to a BCR initiates receptor-mediated endocytosis[87]. The internalized antigen is subsequently degraded into small peptides by lysosomes, and transported into the cytoplasm by an unknown mechanism. Peptides are then loaded onto MHC class I molecules for presentation to CD8 T cells[33]. It remains unclear whether B cell-dependent CD8 T cell activation is directly regulated by MHC class I-mediated antigen presentation or via indirect pathways such as activation of CD4 T cells[88].

1.6 Primary immunodeficiency diseases

Primary immunodeficiency diseases (PIDs) refer to a group of disorders characterized by absence or dysfunction of the immune system resulting from when either a critical immune component is missing or does not function properly[89]. Most PIDs result from an inherited genetic defect leading to increased susceptibility to infection, autoimmunity or malignancy[90]. PIDs are distinguished from secondary immunodeficiency diseases in that they are not caused by HIV infection, drug treatment, malnutrition, old age or environmental factors[91]. More than 130 different disorders have been classified as PIDs and this list has been growing[91,92]. PIDs are often considered as the equivalent of "human gene knockouts" and have provided a wealth of fundamental information about how various genes function in the human immune system.

PIDs are subdivided according to what arm of the immune system is afflicted: innate immune deficiency or adaptive immune deficiency. Innate immune deficiency refers to defects in innate immunity including epithelial barrier function, complement proteins, phagocytes and TLR signaling[89]. Disorders in the adaptive immune system often involve its primary immune cells, T and B cells. Dysfunction can predominantly occur either in B or T lymphocytes, but also both B and T cells, which is called combined immunodeficiency (CIDs)[92]. The most severe form of CIDs is called SCID, and frequently presents in infancy and is often characterized by loss of functional T and B lymphocytes[93]. SCID patients usually present with chronic diarrhea and severe recurrent infections within the first year of life[89,91]. Hypomorphic mutations in PID-causing genes can allow T cell and B cell development leading to a delayed onset of PIDs rather than complete loss of those lymphocytes. When this scenario happens, the PID patients can have normal number of T cells, yet defects in T cell function[90,92,93]. Moreover, PID are often associated with immune-dysregulation. PID patients commonly exhibit recurrent infections and

inflammation at internal organs, reflecting disruption in immune homeostasis[89,92]. For instance, immune dysregulation-polyendocrinopathy-enteropathy-X-linked (IPEX) syndrome is caused by mutations in the Treg transcription factor *FOXP3* gene[94]. Patients with IPEX syndrome present severe autoimmune enteropathy and dermatitis in early life, which is caused by impaired immune suppressive Treg function. Moreover, mutations in Fas, a molecule expressed on cells leading to apoptosis, can cause autoimmune lymphoproliferative syndrome (ALPS) [89,95]. ALPS is a heritable genetic disorder, often autosomal-negative mutations in Fas, interrupt signal pathway of apoptosis, resulting in accumulation of autoreactive lymphocytes and autoantibodies. These disorders highlight that PIDs are often associated with immune dysregulation, which cause other complications and exacerbate the symptoms in the patients.

PIDs are very complicated diseases and their symptoms may be highly variable. Hence, PID patients may require complex and personalized therapies. Moreover, PID treatments need to target the primary cause of disease, avoid potential complications like infections, and improve immune system function. Currently, a common therapy for PIDs is hematopoietic stem cell transplantation (HSCT) involving the introduction of healthy hematopoietic stem cells to replace the patient's immune system[91]. However, the procedure is risky because host's immune cells are killed off prior to the transplantation to make room for the grafted cells leaving the patients temporarily more vulnerable to infections. Bone marrow donors are usually selected from closely related family members due to higher chance of having a better HLA match and thus, lowering the incidence of graft-versus-host-disease (GvHD)[89]. Altogether, there are still many unsolved questions in the cause of PIDs and their treatment. Further, the investigation of PIDs will establish greater understanding of how genes function in the human immune system and may

have far-reaching implications for the development of novel therapies beyond immunodeficiency diseases.

1.7 MALT lymphoma and MALT1

MALT (mucosa-associated lymphoid tissue) lymphoma is a malignancy of the mucosa-associated lymphoid tissue caused by chronic infections[96]. It occurs most frequently in the stomach, although any mucosal sites can be affected. MALT lymphoma is triggered by repeated infections of pathogens such as *Helicobacter pylori (H. pylori)*, which induce chronic gastritis. Repeated infections in the mucosa layer can result in persistent activation and accumulation of lymphocytes in MALT that can lead to the development of MALT lymphoma[97]. The common recurrent chromosomal mutation found in MALT lymphoma patients is t(11;18)(q21;q21) translocation[98], where the *MALT1* (*MALT-lymphoma translocation gene 1*) gene was first discovered[97]. The translocation t(11;18)(q21;q21) leads to the fusion of MALT1 and API2 (apoptosis inhibitor protein 2), which enhances nuclear factor-κB (NF-κB) activation in lymphocytes[97,99]. Studies in MALT1-deficient mice have demonstrated that MALT1 contributes to Treg development, NF-κB signaling, IL-2 production and T cell activation[100,101]. Altogether, these findings suggest that MALT1 plays an important role in T cell immunity and malignancies.

1.7.1 MALT1 function in NF-kB signaling pathway

A number of studies have revealed that MALT1 is a key regulator of NF-κB signaling that is essential for lymphocyte survival, proliferation and inflammation[97,102]. NF-κB signaling can be classified into two pathways depending on the mechanism of activation: canonical and non-canonical pathways[103]. The canonical pathway is induced by most physiological stimuli,

including TCR, BCR and TNFR (TNF receptor) activation, and results in nuclear translocation of p50/p65 heterodimer. The non-canonical pathway is triggered by stimulation of specific TNFRs, including lymphotoxin β-receptor (LTβR) and BAFF (B cell activating factor) receptor, and leads to the nuclear translocation of p52/RelB complex[104]. MALT1 regulates both the canonical and non-canonical NF-κB pathways via two different mechanisms: i) as a signaling adaptor molecule and ii) as a paracaspase[97,93,99].

MALT1 can function as a scaffolding protein in the NF-κB pathway, where it binds to the adaptor protein, BCL-10 (B cell lymphoma-10)[79]. In response to antigen-induced TCR activation, MALT1 forms the CBM (CARD11-BCL10-MALT1) complex, which contains BCL-10 and CARD11 (caspase recruitment domain-containing protein 11) (*Figure 1.8*). In the canonical pathway, the CBM complex recruits E3 ubiquitin ligase, which subsequently ubiquitinates IKKγ (also known as NEMO: NF-κB essential modulator), a subunit of the IκB kinase (IKK) complex. This activates the IKK complex, resulting in the phosphorylation of IκB, and its ubiquitination and degradation. Degraded IκB releases p50/p65 heterodimer, which translocates into the nucleus and activates transcription of target genes[102,105].

Structural analysis of MALT1 has revealed that the carboxyl-terminal of MALT1 contains a proteolytic active site that is similar to that of caspase, hence classifying MALT1 as a "paracaspase"[106]. Using its paracaspase activity, MALT1 cleaves negative regulators of NF-κB activation, including A20 and RelB (transcription factor RelB), to further enhance NF-κB signaling[97,107,108]. A20, for example, is a deubiquitinating enzyme of the IKK complex that can be cleaved by MALT1 at a specific arginine residue, allowing NF-κB to successfully translocate into the nucleus[108]. Several recent studies suggest that MALT1 also has immunosuppressive functions, including our *Nature Communications* article describing the ability of MALT1 to

cleave HOIL-1 (heme-oxidized IRP2 ubiquitin ligase)[1,109]. HOIL-1 contains a linear ubiquitin E3 ligase that ubiquitinates IKKγ, leading to the activation of NF-κB signaling[1,110]. Our findings have demonstrated that MALT1-mediated HOIL-1 cleavage transiently dampens linear ubiquitination and acts as a brake on NF-κB signaling.

A better understanding of MALT1 function in immunity will improve medical diagnosis and clinical approaches for patients with *MALT1* mutations. There are a few reported cases of *MALT1* mutation-induced primary immunodeficiency diseases, and these patients share similar clinical phenotypes, including high susceptibility to bacterial, viral and fungal infections combined with severe gastrointestinal inflammation[93,111-113]. These findings suggest that MALT1 has a vital function in immune regulation, and that MALT1 is a potential therapeutic target.

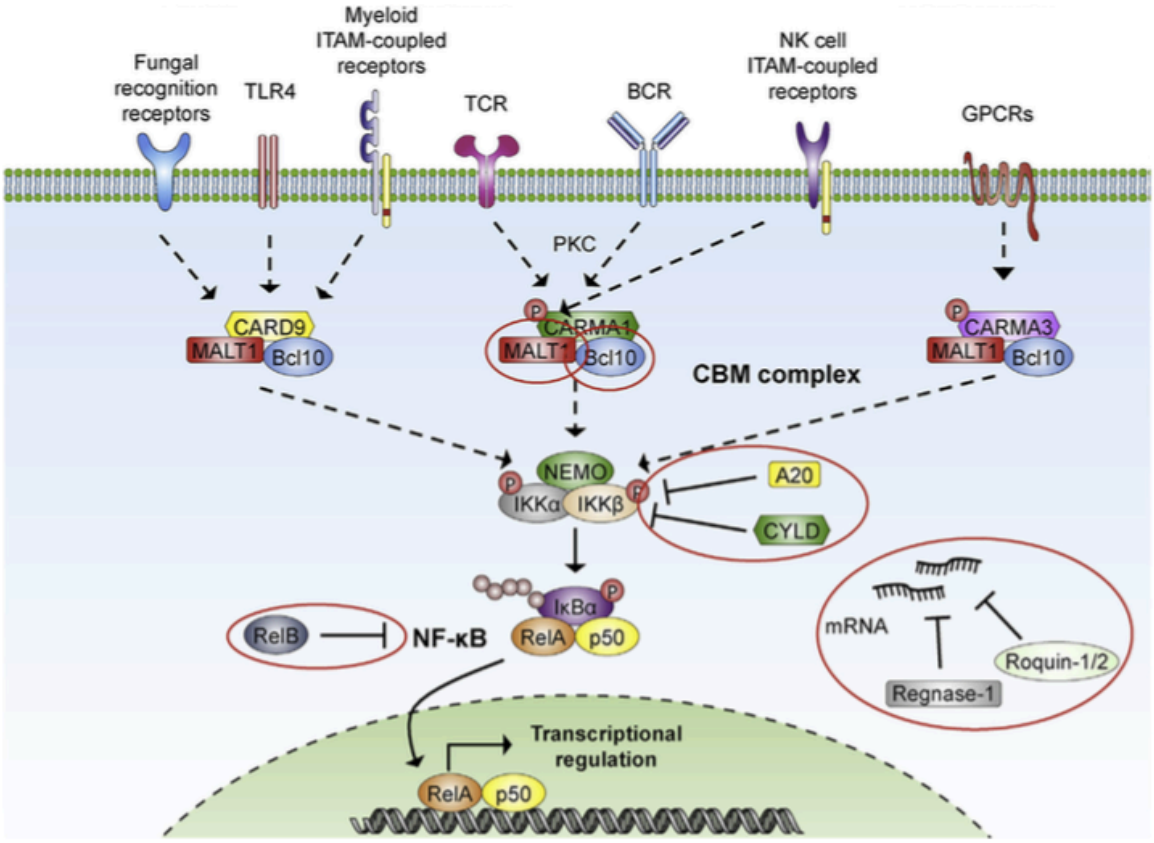

Figure 1. 8 MALT1's regulation of the canonical NF-κB signaling pathway
NK-κB signaling pathways can be activated through multiple receptor simulations including T cell- and B cell- receptor (TCR or BCR), Toll-like receptor 4 (TLR4) and G-protein couple receptors (GPCRs). Regardless the initiation of NF-κB signaling pathway, MALT1 (Mucosa-associated lymphoid tissue lymphoma translocation protein 1) plays a crucial role. MALT1 has two known functions. First it acts as a scaffolding protein that brings BCL-10 (B cell lymphoma-10) and CARD11 (caspase recruitment domain-containing protein 11) together to form CBM complex for signal transduction. Also, MALT1 has proteolytic activity that cleaves negative regulator for NF-κB signaling, such as A20, CYLD and RelB to enhance the activation. In addition, recent studies demonstrate the opposite function of MALT that it can act as a brake in NF-κB signaling pathway to dampen the immune responses. Figure reprinted from Janna Hachmann and Guy S. Salveson, *Biochimie*. 2016 with permission from Elsevier[97].

1.8 Rational and hypothesis

A child admitted at BC Children's Hospital displayed a novel presentation of immune dysregulation, and suffered with recurrent infections and severe inflammatory disease[112]. Whole-

exome sequencing revealed that the patient was found to have homozygous missense mutations in the *MALT1* gene. MALT1 is a key molecule in the NF-κB signaling pathway that regulates cell activation, proliferation and differentiation. MALT1 modulates TCR and BCR signaling through two different means: as a scaffolding adaptor molecule to form the CBM complex, and as a paracaspase cleaving substrates to regulate NF-κB signaling. Studies on *Malt1*$^{-/-}$ mice have revealed weakened T cell immunity and reduced NF-κB activation. By contrast, mice lacking MALT1 paracaspase activity but retaining MALT1 adaptor function are prone to fatal autoimmunity[114,97,115]. Consequently, these findings raise questions regarding the nature of our patient's mutant MALT1 protein and the role it plays in her proinflammatory phenotype. Interestingly, the mutation did not result in complete abrogation of MALT1 protein, unlike other *MALT1* mutant patients, suggesting that our patient's MALT1 may have residual function. Hence, we hypothesized that MALT1 is a critical component of immune functions in T cells and mutations in *MALT1* contribute to immune-dysregulation. To investigate our hypothesis, we characterized T cells from the patient (*MALT1*$^{mut/mut}$), patient's sibling (*MALT1*$^{WT/mut}$) and experimental control, and subsequently recruited controls (*MALT1*$^{WT/WT}$). Our following aims were to:

1. Characterize and determine the function of regulatory T cells.
2. Assess the function of conventional CD4 T cells.
3. Determine the activation and inhibitor marker upregulation upon TCR-induced simulation.
4. Investigate MALT1 scaffolding and paracaspase activity.

The *MALT1* mutant patient and MALT1-deficient mice share several immunological features including weakened NF-κB signaling, impaired T cell proliferation and reduced Treg populations. However, unlike our *MALT1*$^{mut/mut}$ patient, MALT1-deficient mice do not display constitutive immune activation or develop spontaneous inflammation. In addition, The *MALT1* patient experienced with recurrent viral infections, such as CMV, suggesting that MALT1 may play a role in protective immunity against viral pathogens[112]. The difference in phenotype of *MALT1* patient and *Malt1*$^{-/-}$ mice may due to differential exposure to pathogens. Laboratory mice are housed in specific pathogen-free "clean" facility while humans are exposed to a vast array of pathogens. Hence, we sought to test the immune system of *Malt1*$^{-/-}$ mice through infection with the murine viral pathogen lymphocytic choriomeningitis virus (LCMV). Altogether, we hypothesized that MALT1 plays a crucial role in anti-viral immunity. To study our hypothesis, I addressed the following specific aims:

1. Examine whether *Malt1*$^{-/-}$ mice are able to mount immune responses upon acute viral infection.
2. Enumerate the expansion of viral-specific T cells and assess the function of effector T cells.
3. Characterize T cell differentiation marker expression and measure activation and inhibitor marker upregulation.

To date, the exact role of MALT1 in T cell immune responses is still unclear; thus a greater understanding of MALT1 function in T cell immunity will provide invaluable knowledge of immunoregulation and potentially support MALT-targeted therapies to treat T cell-mediated inflammatory diseases.

Chapter 2: The function of MALT1 in human CD4 T cell immunity

2.1 Introduction

2.1.1 CBM complex associated primary immune deficiencies

The CARD11-BCL10-MALT1 (CBM) signalosome complex forms an essential link in immune cells between cell-surface antigen receptors and nuclear NF-κB activation. In T cells, the CBM complex is required for TCR-induced NF-κB activation and thus, plays an important role in regulating T cell immunity. Recently, a number of mutations have been identified in members of the CBM complex as a novel cause of primary immunodeficiency disease (PID). PID patients with CBM complex mutations share several clinical symptoms including severe gastrointestinal inflammation, eczema-associated skin diseases, recurrent infections and a failure to thrive. Moreover, these patients exhibit an immune phenotype associated with impaired NF-κB signaling and dysregulated B cell development as characteristic hallmarks. Together, these findings support the critical roles of CBM signalosome in normal immune system functioning necessary to prevent immune-related diseases.

Mutations in the CBM complex were first discovered in a patient suffering from recurrent pulmonary bacterial infection and pneumonia[93,116]. Whole-exome sequencing revealed that the patient had inherited homozygous mutations involving the deletion of exon 21 of *CARD11* from her consanguineous parents. Subsequently, several independent research groups have identified more PID patients attributed to CARD11-deficiency[93,117]. *CARD11* patients displayed T cell dysfunction including impaired Th1/ Th17 differentiation and a failure to proliferate *in vitro* upon anti-CD3/CD28 TCR stimulation[93,116]. Additionally, *CARD11* patients exhibited a reduction in the frequency of regulatory T cells, a phenomenon also observed in *Card11*$^{-/-}$

mice[118]. Together, these associations of *CARD11* mutations with PID indicate that CARD11 is essential for T cell immunity.

A child admitted to British Columbia Children's Hospital presented with a novel clinical presentation of combined immunodeficiency (CID) associated with immune dysregulation[112]. The patient suffered from numerous chronic bacterial (*Staphylococcus aureus*), viral (cytomegalovirus) and fungal (*Candida albicans*) infections. In addition, the patient exhibited severe gastrointestinal inflammation, low bone mineral density and delayed growth. The patient, a 15-year-old girl, was born to consanguineous parents of Kurdish descent. Whole-exome sequencing revealed that she had inherited an autosomal recessive trait, missense mutations in the *MALT1* gene (*MALT1*$^{mut/mut}$)[112]. The patient had a single putatively damaging variant in *MALT1* gene, a single base pair substitution (1739G>C), converting a tryptophan to serine (Trp580Ser) in an evolutionary-conserved C-terminal domain (*Figure 2.1*). The Trp580 position in MALT1 is predicted to reside in an alpha-helical linker domain with Trp's aromatic side chain interacting with the caspase-like (paracaspase) domain. Consequently, the Trp580Ser mutation in MALT1 may affect protein structure or paracaspase function.

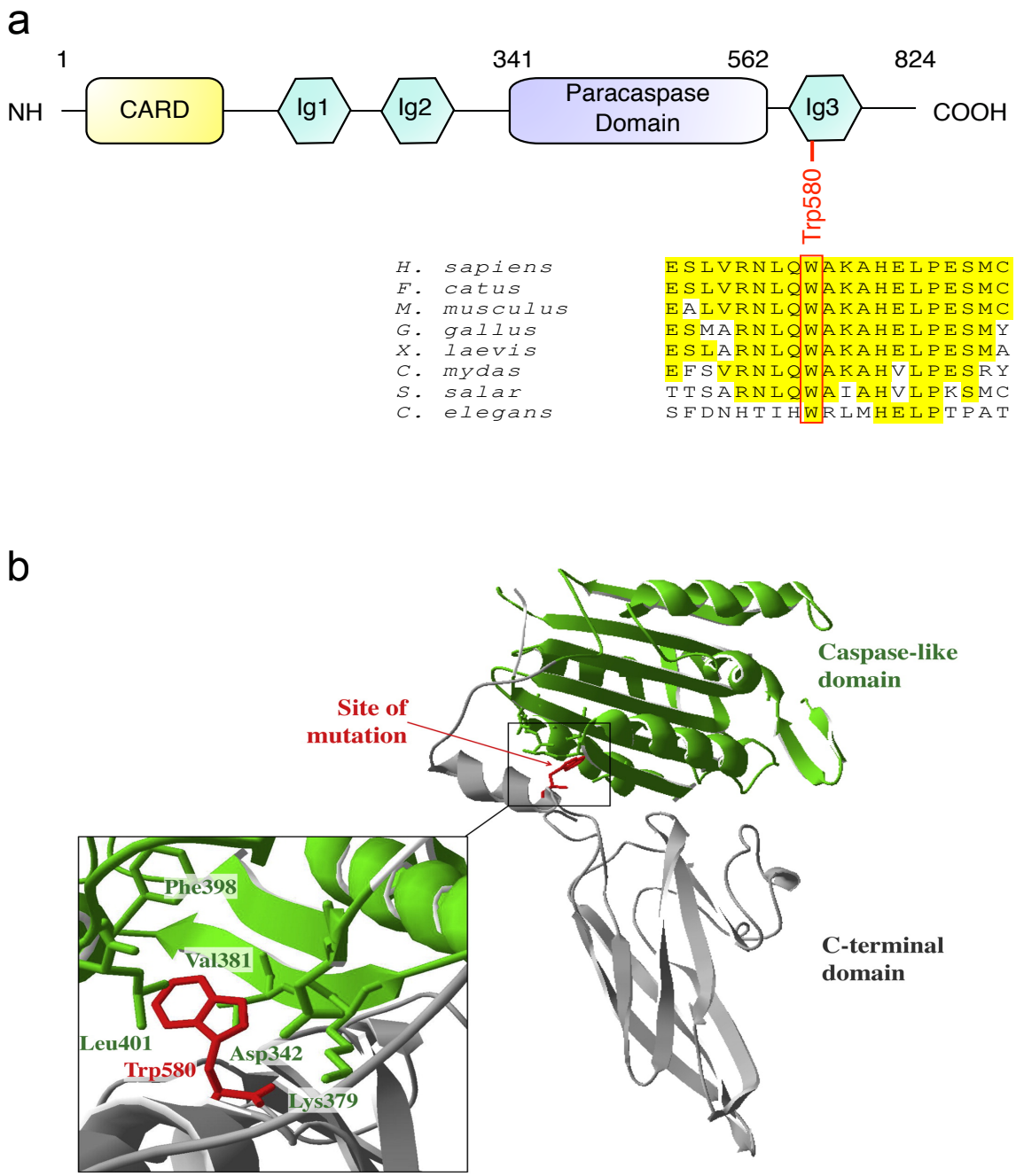

Figure 2. 1 The homozygous mutations occurred at conversed domain of *MALT1*
a) Domain structure of MALT1 and amino acid sequence of *Homo sapiens* and various species showing mutation site of *MALT1* is conserved throughout the species. b) Crystal structure of MALT1 showing side chain of Trp580 (red) in C-terminal domain interacts with caspase-like domain (green). Figure adapted from Margaret McKinnon, *et al.*, *J. Allergy Clin Immunol*. 2014 with permission from Elsevier[112]. CARD: caspase activation and recruitment domain; Ig: immunoglobulin-like domain; MALT1: mucosa-associated lymphoid tissue lymphoma translocation gene 1.

The *MALT1* mutations did not affect levels of RNA transcripts but did likely destabilize the protein since the patient's PBMC had reduced amounts of MALT1 protein relative to control[112]. Given the presence of protein, McKinnon et al interrogated the effects of homozygous *MALT1* mutations on MALT1 function. Significantly, the patient's mutant MALT1 was found to lack both paracaspase activity and scaffold function given that it failed to cleave BCL10, a known MALT1 substrate, and stably bind BCL10, respectively[112]. In addition, MALT1-dependent NF-κB-p65 phosphorylation was found to be defective in the patient's T cells and this deficit could be corrected by ectopic expression of wild type MALT1 transcript (*Figure 2.2*). By contrast, the parents and two siblings of the patient were heterozygous for the *MALT1* mutation, having protein levels and MALT1 function more closely resembling controls, and were phenotypically normal, showing no clinical symptoms. Altogether, these observations suggest that the mutated *MALT1* gene may represent a complete loss-of-function or, at least, a hypomorphic allele.

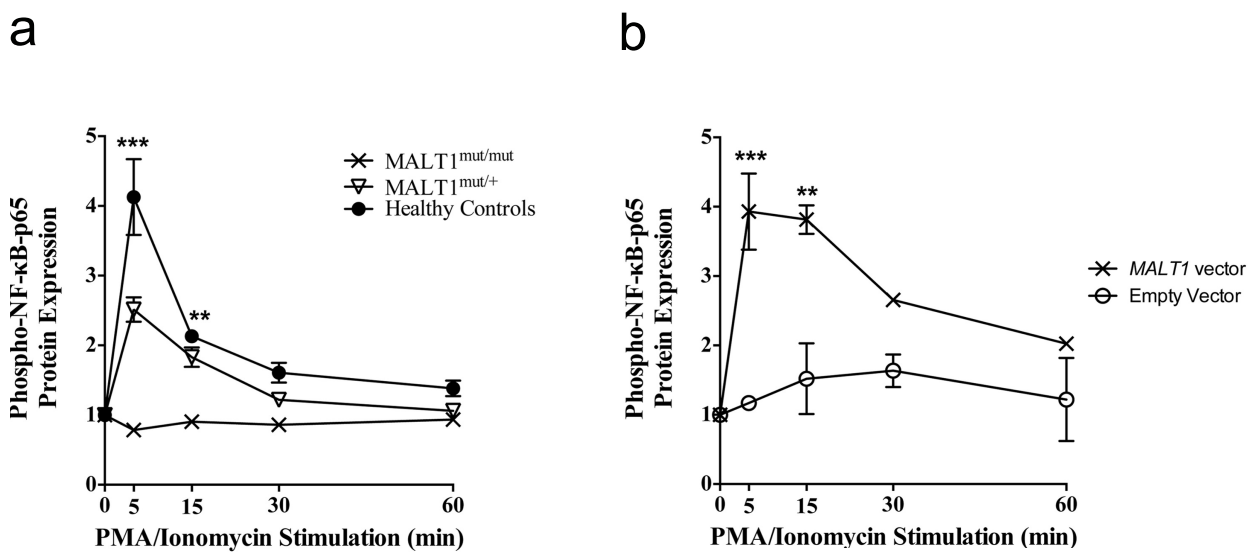

Figure 2. 2 Homozygous missense *MALT1* mutations abrogate NF-κB signaling
a) *MALT1*$^{mut/mut}$ CD3^{+} T cells presented a complete loss of NF-κB-p65 subunit phosphorylation upon P/I stimulation. b) *MALT1*$^{mut/mut}$ CD3^{+} T cells were transfected with a normal *MALT1* transcript and restored the ability to phosphorylate p65 subunit. The patient's CD3^{+} T cells transfected with an empty vector had no effect. Figure reprinted from Margaret McKinnon, *et al.*, *J. Allergy Clin Immunol*. 2014 with permission from Elsevier[112].

The key functioning of MALT1 in immune cells suggested that allogeneic hematopoietic stem cell transplantation (HSCT) might be a curative therapy for the severe morbidity associated with MALT1 deficiency. At 16 years of age, our BCCH MALT1 patient was transplanted with bone marrow from her HLA-matched sibling who is heterozygous for the *MALT1* mutation but has not showed any signs of immunodeficiency or disease[112,119]. At one year post-transplant, the patient has shown great clinical improvement including accelerated growth (showing significant rises in height and weight), increased bone density and resolution of severe dermatitis and enteropathy. Further, her PBMC now show normalization of NF-κB signaling and B cell numbers. Her successful transplantation (>95% of donor origin) preempted the ability to further recover primary white blood cells carrying homozygous *MALT1* mutations. Collectively, these

findings along with those by Punwani and colleagues support hematopoietic stem cell transplantation as a curative therapy for individuals afflicted with MALT1-deficiency[113].

To date, several PID patients have been identified with the root cause attributable to loss-of-function mutations in *MALT1* gene. The first two patients described with autosomal recessive *MALT1* mutations were reported in 2013[111]. They were siblings born to first-cousin parents and died from persistent infections and respiratory failure at age of 7 and 13.5 years, respectively. Both patients exhibited normal levels of *MALT1* mRNA, yet no MALT1 protein was detectable. Similar to our *MALT1* patient, these two individuals had normal T cell numbers, however, their T cells failed to proliferate and produce cytokines upon TCR stimulation *in vitro*[111].

Punwani and colleagues have also identified a case of MALT1-deficiency in a 9-month-old infant born to non-consanguineous parents with no family history of PID[113]. The infant patient had two non-synonymous *MALT1* variants (heterozygous *MALT1* mutations) resulting in loss of MALT1 protein. The infant patient suffered from severe intestinal inflammation, viral infection (especially cytomegalovirus), and erythroderma, a widespread reddening of skin due to inflammatory disease. The patient also exhibited activated, expanded CD4 and CD8 T cells that failed to proliferate upon TCR stimulation *in vitro*. At the age of 18 months, the patient received hematopoietic cell transplantation from 90% HLA matched unrelated donor[113]. Post-transplantation, the patient did not develop graft versus host disease (GvHD) and eczematous rash resolved within 4 weeks.

Most recently, two siblings (4-year-old boy, 7-year-old girl) born to distantly related parents were found to inherit homozygous missense (c. 550G>T) *MALT1* mutations. Both patients exhibited severe, recurrent and chronic infections suggestive of primary immunodeficiency[120]. The older sibling (P1) experienced fulminant intestinal inflammation and

severe dermatitis and low frequency of $CD25^{+}FOXP3^{+}$ regulatory CD4 T cells, accounting for authors' description of the disorder as an IPEX-like syndrome. The younger sibling (P2) also presented with similar disease phenotype, yet less severe than his sister. IPEX (polyendocrinopathy enteropathy X-linked syndrome) is a rare PID that is caused by mutations in FOXP3 resulting in the loss of regulatory CD4 T cells, autoimmune enteropathy and recurrent dermatitis[68]. Due to the similarity of phenotypes of these two patients to IPEX, the authors proposed that MALT1-deficiency should be considered as a potential cause of IPEX-like syndromes. Recently, the two patients underwent HSCT from two 100% HLA-matched unrelated donors leading to restoration of $CD25^{+}FOXP3^{+}$ regulatory CD4 T cell populations and resolution of infections, inflammatory skin and intestinal inflammation.

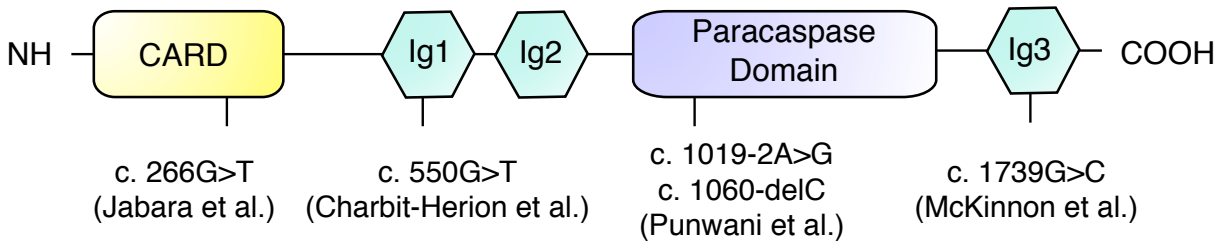

Figure 2. 3 Schematic presentation of MALT1 protein and localization of *MALT1* mutations in reported patients.
From left to right, c.266G>T mutation results in amino acid change from serine to isoleucine (p.S89I)[111]. c.550G>T variation substitute aspartic acid to tyrosine at amino-acid position 184 (p.D184Y)[120]. A maternally inherited splicer acceptor defect c.1019-2A>G and deletion of c.1060C result in frameshift within paracaspase domain and premature stop codon after 18 missense codons (p.Y353fs*18)[113]. c.1739G>C missense mutation converts tryptophan to serine at position 580 (p.W580S)[112].

Primary immunodeficiency diseases are a group of heterogeneous disorders in which one or more components of immune system are dysfunctional leading to increased susceptibility to

infection. Our MALT1 patient displayed a number of characteristics common to other MALT1 patients that may be useful for clinical diagnosis (*Table 1*)[111-113]. These hallmarks include: (1) abnormal B cell differentiation resulting in B cell lymphopenia associated with a lack of marginal zone and memory B cells, (2) normal T cell numbers but a failure to activate NF-kB upon TCR signaling, (3) defective T cell proliferation upon stimulation with T-cell receptor antibodies *in vitro*, and (4) inflammatory disease of the skin and intestine. Altogether, these features may be helpful to diagnose PID patients with CBM complex mutations, although more patients will need to be identified and characterized before firm conclusions can be drawn. In this chapter, we investigated the function of MALT1 in human T cell immunity using primary CD4 T cells from the BCCH patient, her afflicted sibling, and healthy controls. We specifically proposed to:

1. Characterize and determine the function of regulatory T cells.
2. Assess the function of conventional CD4 T cells.
3. Determine the activation and inhibitor marker upregulation upon TCR-induced simulation.
4. Investigate role of MALT1 T cell signaling.

Table 1. Summary of clinical and laboratory findings of MALT1-deficient patients.

	McKinnon et al[112]	Jabara et al[111]		Punwani et al[113]	Charbit-Henrion et al[120]	
		P1	P2		P1	P2
Mutation	Homozygous missense	Homozygous missense		Heterozygous non-synonymous	Homozygous missense	
MALT1 protein	Low	-		-	-	
Clinical phenotype						
Delayed growth	+	+	+	+	+	+
Eczema	+	-	-	+	+	+
Enteropathy	+	+	+	+	+	-
Bronchiectasis	+	+	+	-	ND	ND
Facial dysmorphia	+	-	-	-	+	+
High IgE	+	-	-	-	+	+
Chronic infections						
S. aureus	+	+	+	+	Bacteria*	
S. pneumoniae	+	+	+	-	Bacteria*	
C. albicans	+	+	+	+	Yeast*	
CMV	+	+	+	+	Virus*	
Immunodeficiency	+	+		+	+	
Tregs	+	Low		ND	Low	
Treatment	HSCT	Died		HSCT	HSCT	

Abbreviations: ND = not determined, HSCT = hematopoietic stem cell transplantation. *Charbit-Henrion et al. did not denote specific infections that patient experienced.

2.2 Materials and method

2.2.1 Cell preparation, PBMC isolation and primary T cell expansion

BC Children's Hospital Research Institute institutional review board approved the protocols for research (PID Study # H15-00641). The patient, her parents and two unaffected siblings were registered for the study. The parents provided written informed consent for both themselves and their children to participate in the study. PBMC from patient, sibling and control (denoted as experimental control) were stained with fluorescent-conjugated anti-CD4 (RPA-T4)

and anti-CD8 (HIT8a) antibodies (both from BD Biosciences) and sorted using a BD FACSAria flow cytometer (BD Biosciences) on the same day. In addition, healthy CD4 and CD8 T cells were prepared from a cohort of adult control PBMCs (n=5, control group) on a different day but in the exact same manner. Sorted CD4 and CD8 primary T cells (> 99% purity) were stimulated and expanded with feeder cells as described below.

To generate feeder cells, peripheral blood was collected from healthy adult donors. Healthy PBMCs were isolated from whole blood by Lymphoprep (StemCell Tech.) density gradient centrifugation at 2000 rpm for 20 min. After centrifugation, mononuclear cell layers containing PBMC were carefully transferred to a new falcon tube, washed, and resuspended in RPMI 1640 medium (HyClone) with 10% FBS, 1% NEAA and P/S (100 U/ml penicillin, 100 μg/ml streptomycin), and 2 mM GlutaMAX. Feeder PBMCs were subjected to gamma-irradiation using Gammacell 1000 elite irradiator (5000Gy) to prevent their proliferation. For T cell expansion, $2x10^6$ primary T cells from patient (*MALT1*$^{mut/mut}$), sibling (*MALT1*$^{mut/+}$) and controls (*MALT1*$^{+/+}$) were stimulated with $4x10^6$ of irradiated feeder cells (5000Gy), $4x10^5$ irradiated JY LCL (7500Gy), PHA (0.1ug/ml) and IL-2 (100U/mL) in 48 well plates. T cell cultures were incubated in 5% CO_2 at 37°C and T cell experiments were performed on day 7 post-stimulation.

2.2.2 Flow cytometry

Antibodies (clone name) against CD3ε (OKT3), CD4 (RPA-T4), CD8 (HIT8a), CD25 (BC96), CD69 (FN50), PD-1 (eBioJ105), TIM-3 (F38-2E2), FOXP3 (236A/E7), IFN-γ (4S.B3), TNF-α (MAb11), IL-2 (MQ1-17H12), IL-17 (BL168) were used to detect the subsets of human lymphocytes and function of T cells. Expanded T cells were washed with FACS buffer (PBS

with 2% FBS) prior to staining with surface markers. Antibodies were diluted in FACS buffer, incubated with samples for 15 minutes on ice and then washed with FACS buffer prior to data acquisition. For intracellular cytokine staining, T cells were starved in low-serum media (RPMI with 2% FBS) for 2 hours, followed by stimulation with CD3 (1ug/mL) and CD28 (5ug/mL) antibodies, T cell-expander CD3/CD28 beads (Dynabeads, Invitrogen), or PMA (100 ng/mL)/ ionomycin (1 ug/mL) (Sigma-Aldrich) in the presence of Golgi Plug (1:1000, BD Biosciences) for 4 h to trap cytokines intracellularly. Subsequently, cells were stained with antibodies for specific surface markers; then fixed (Cytofix, BD Biosciences) and permeabilized (BD Biosciences; eBioscience) and stained intracellularly with antibodies specific for cytokines. Data were acquired with either a BD LSRII benchtop or BD LSR FORTESSA cytometer using FACSDiVa software (BD Biosciences). Data was analyzed with FlowJo software (Tree Star).

2.2.3 Immunoblotting analysis

Primary antibodies against Erk (#9102s), p-Erk (#9101s), Jnk (#9252), p-Jnk (#9251s), GAPDH (#8884), β-actin (#8457) were purchased from Cell Signaling Technology and MALT1 (EP603Y) and BCL-10 (EP606Y) were acquired from Abcam. Activated T cells (Day 7 post-feeder cell stimulation) were starved for 2 at h 37°C in low-serum media (RPMI with 2% FBS) in the absence of IL-2. Starved cells were stimulated with PMA (50 ng/mL) and ionomycin (1 ug/mL) for 0, 10, and 30 min. After stimulations, cells were washed with ice-cold PBS and protein extracted using RIPA lysis buffer (0.5% Triton X100, 60 mM β-glycerophosphate, 20 mM MOPS pH 7.2, 5 mM EDTA, 5 mM EGTA, 1mM Na_3VO_4, 20 mM NaF, 1% Trasylol, and 1 mM PMSF). Subsequently, samples were sonicated (2 min), cleared by centrifugation and protein quantified using Pierce BCA Protein Assay Kit (Thermo Scientific) prior to Western

blotting.

Lysates (15-30 μg) were mixed with loading buffer (Laemmli buffer), heated at 95°C for 5 minutes, chilled briefly on ice, and loaded onto 10% SDS-PAGE gels, run at 100 V for approximately 90 min, transferred to methanol-activated PVDF membrane at 100V for 90 min on ice. Subsequently, the membrane was washed 3 times in TBST (0.1% Tween20) for 15 minutes. Next, the membrane was blotted in TBST containing 5% BSA with primary antibody overnight at 4° C. After incubation with primary antibody, the blot was washed three times with TBST for 15 min periods prior to detection with secondary antibodies conjugated to HRP (1:2000) for 1 h at room temperature. Blots were washed three times for 15 minutes and incubated with Luminata Cresecendo western HRP Substrate Reagent (Millipore, Cat#. WBLUR0100) and developed on X-ray film (Mandel Scientific, Ref. 3XA3C)

2.2.4 Statistical analysis

Statistical analyses were performed by using one-way analysis of variance (ANOVA) using post *t* tests. Statistical significance was determined as a p value of less than 0.05 (*), 0.01 (**) and 0.001 (***). All statistical analyses were executed using GraphPad Prism 6 (GraphPad Software, Inc.).

2.3 Results

2.3.1 MALT1 is critical for IL-2 production by CD4 T cells

IL-2 plays a key role in the priming and expansion of antigen-stimulated naive and effector T cells[121,122]. On the other hand, IL-2 is indispensable for the maintenance of regulatory T cells whose function is to suppress immune responses and prevent autoimmunity. Hence, we sought to assess whether the patient's homozygous *MALT1* mutations affect IL-2 secretion by CD4 T cells. To investigate this question, we stimulated the patient, sibling, and control CD4 T cells for 4 hr with the diacylglycerol analog PMA and calcium ionophore ionomycin, mimicking strong TCR stimulation (*Figure 2.4*). Strikingly, the patient CD4 T cells displayed significantly low frequency of IL-2-secreting CD4 T cells relative to sibling (2-fold, 21.2 ± 3.1 % *vs.* 44.8 ± 2.57 %, $p < 0.01$) and experimental control (2.7-fold, 56.33 ± 4.3 %, $p < 0.01$). In addition, we isolated CD4 T cells from five adults' PBMCs (control group) and measured IL-2 secretion to establish a baseline of normal responses. The patient exhibited significantly reduced IL-2 producing CD4 T cells compared to the control group (1.9-fold, 39.2 ± 2.1 %, $p < 0.01$). Together, these findings indicate that MALT1 plays a crucial role in the IL-2 production by human CD4 T cells.

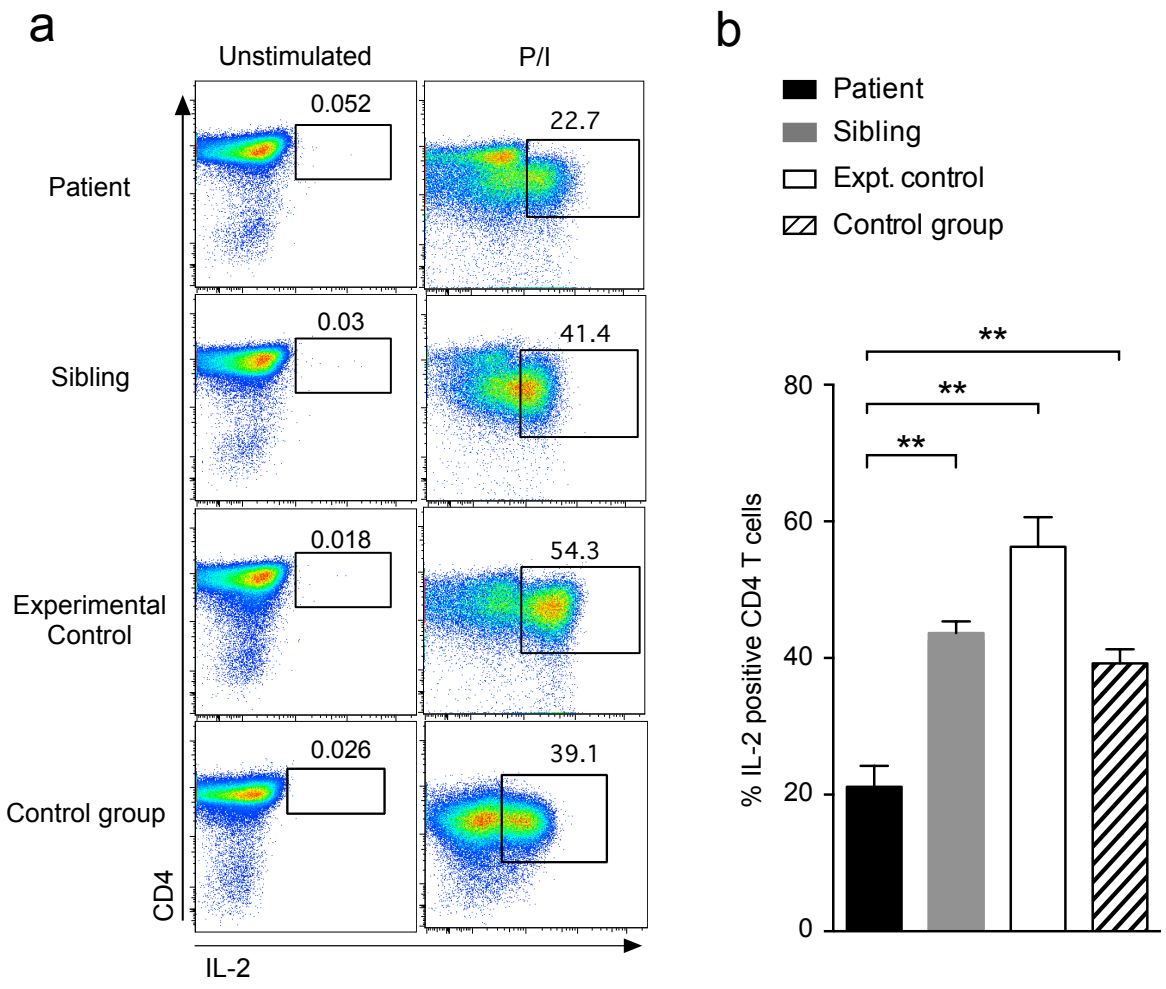

Figure 2. 4 Homozygous *MALT1* mutations affect IL-2 production in CD4 T cells. Expanded CD4 T cells from the patient, sibling, experimental control and control group were stimulated with P/I for 4 hr to measure IL-2 production. Cells from experimental control were prepared on the same day as patient and sibling cells, and control group (n=5) was recruited subsequently. a) Representative flow data of IL-2 secreting CD4 T cells from each sample. b) Cumulative frequency of IL-2 positive CD4 T cells upon P/I-stimulation. The data of IL-2 production are representative from 3 independent experiments. Bar graphs represent the mean ± SEM. Statistical significance was calculated using one-way ANOVA with post *t* test. (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$).

2.3.2 *MALT1* patient CD4 T cells show a normal frequency of $CD25^{+}FOXP3^{+}$ cells

IL-2 regulates immune responses by promoting Treg homeostasis and proliferation[122]. As patient CD4 T cells exhibited significantly reduced IL-2 production, we sought to investigate

whether the MALT1 patient possessed a normal frequency of regulatory T cells. Hence, we assessed the proportion of regulatory T cells among whole CD4 T cells from the patient, her heterozygous family members, and a group of control subjects (control group) using intracellular flow cytometry. Notably, the patient exhibited a similar frequency of regulatory T cells among whole CD4 T cells relative to the patient's family members in direct *ex vivo* analyses (*Figure 2.5*). Further, the patient and her unaffected family members displayed a similar frequency of Tregs compared to an independent group of recruited control subjects. However, we did observe a loss of a $FOXP3^{lo}CD25^{hi}$ population from the patient relative to her family members and controls, yet were unable to reproduce these *ex vivo* analyses due to the patient's successful HSCT. Together, these results indicate that the patient's homozygous *MALT1* mutations do not alter the frequency of regulatory T cells, however, an assessment of their function is no longer possible.

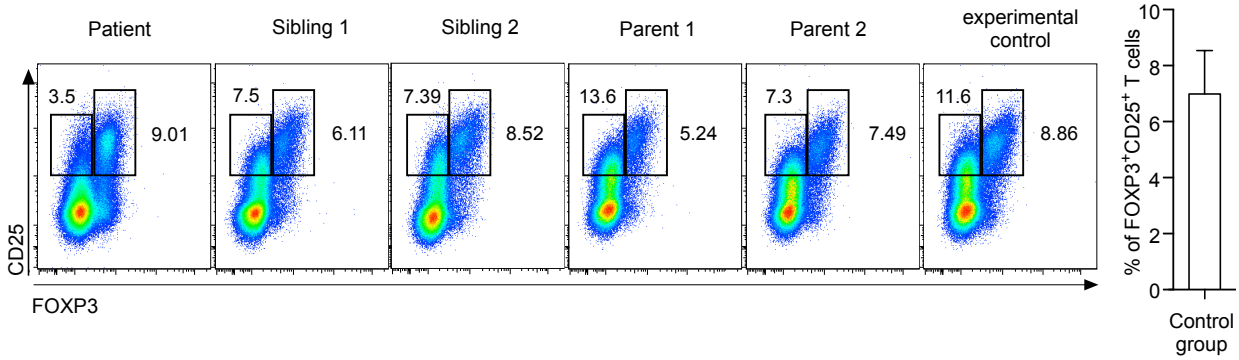

Figure 2. 5 *MALT1* patient possesses a normal frequency of regulatory CD4 T cells
Ex vivo PBMCs from the patient and heterozygous family members, siblings and parents, and controls were stained with anti-CD4, CD25 and FOXP3 antibodies and analyzed by flow cytometry. Flow plots are gated on CD4 T cells and bar graph presents the frequency of $CD25^{+}FOXP3^{+}$ within CD4 T cells from a cohort of control subjects (n=5).

2.3.3 *MALT1* mutations contribute to impaired effector CD4 T cell function

Chronic fungal, bacterial and viral infections were a constant challenge to the patient since infancy[112]. Hence, we investigated whether MALT1 plays a critical role in CD4 T cell differentiation by assessing the ability of patient's effector T cells to produce IFN-γ, IL-17 and TNF-α, a group, of cytokines crucial to limit infections by viruses, fungi and bacteria[52,123]. Activated CD4 T cells from the patient, sibling and controls were re-stimulated with P/I for 4 h prior to intracellular cytokine staining (*Figure 2.6*). Strikingly, the patient showed a decreased frequency of IFN-γ secreting CD4 T cells relative to sibling (5.9-fold, 2.52 ± 0.95 % *vs*. 14.9 ± 1.1 %, $p < 0.001$), experimental control (7.9-fold, 19.9 ± 1.1%, $p < 0.001$), and group of controls (4.8-fold, 12.2 ± 3.52 %, $p < 0.05$). In addition, the patient CD4 T cells also exhibited marked reduction in the frequency of IL-17 secreting cells relative to sibling (4.6-fold, 0.23 ± 0.07% *vs.* 1.06 ± 0.06 %, $p < 0.001$), experimental control (9-fold, 2.06 ± 0.16 %, $p < 0.01$), and a control group (3.5-fold, 0.81 ± 0.18 %, $p < 0.05$). Further, patient CD4 T cell displayed a modestly lower frequency of TNF-α secreting CD4 T cells than sibling (1.3-fold, 59 ± 3.28 % *vs*. 77.3 ± 4.89 %, $p < 0.05$), control (1.4-fold, 82.9 ± 2.59, $p < 0.01$) and the control group (1.5-fold, 90.3 ± 1.19 %, $p < 0.01$). Altogether, these findings suggest that MALT1 plays an important role in the differentiation of human CD4 T cells.

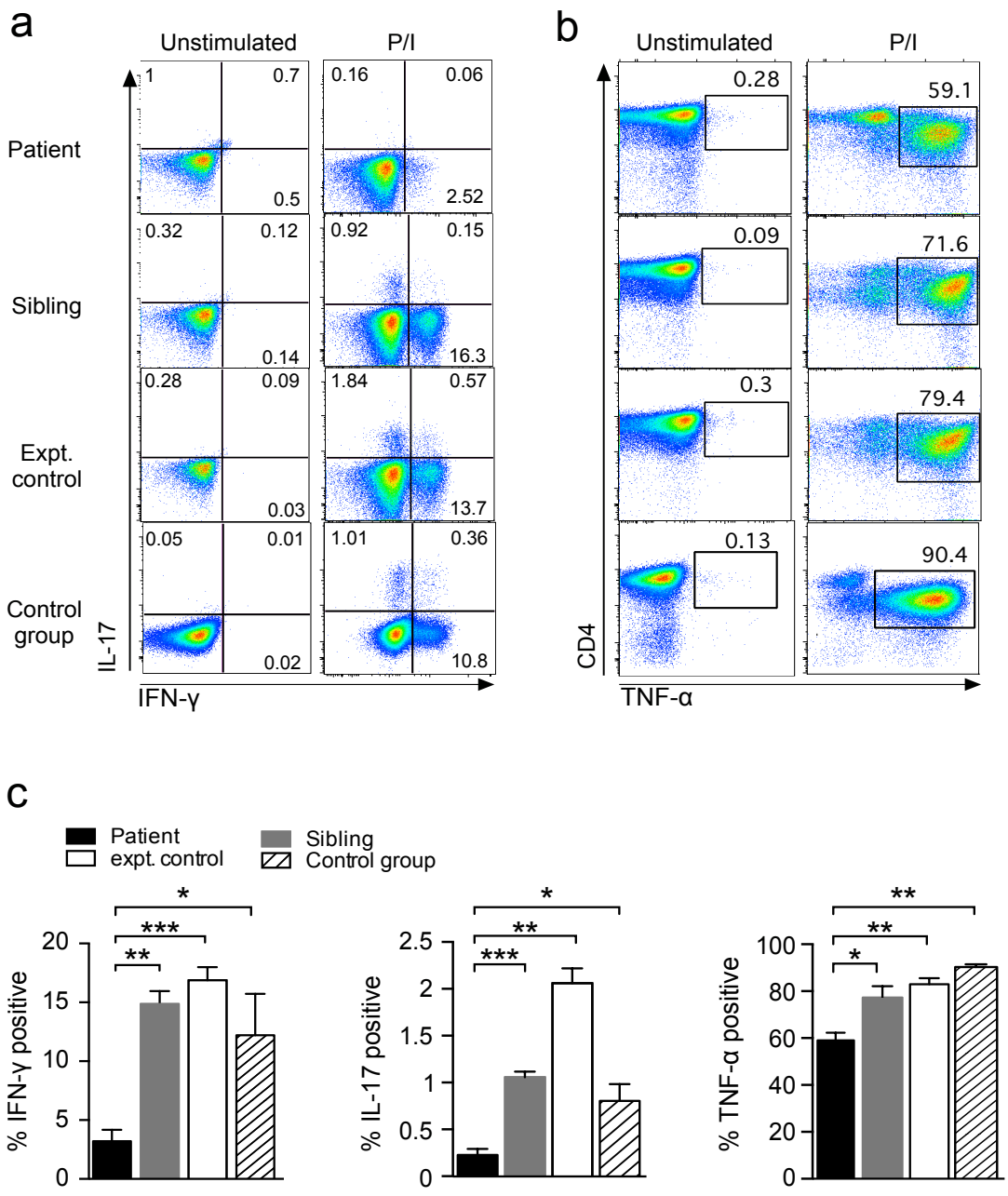

Figure 2. 6 MALT1 is important for the generation of IFN-γ-, TNF-α- and IL-17-secreting CD4 T cells

Expanded CD4 T cells from the patient, sibling, experimental control and independently recruited control subjects (n=5) were restimulated with P/I for 4 hr and analyzed for IFN-γ, TNF-α and IL-17 production. a) Representative flow plots of IFN-γ and IL-17 secreting CD4 T cells. b) Representative flow data of TNF-α secreting CD4 T cells. c) Cumulative data of (a), (b). Illustrated flow data are representative from 3 independent experiments. Bar graphs indicate the mean ± SEM. Statistical significance was calculated using one-way ANOVA with post *t* test (**p* < 0.05, ***p* < 0.01, and ****p* < 0.001).

2.3.4 MALT1 modulates CD25 levels on human CD4 T cells

T cells express both activation and inhibitory receptors to modulate T cell responses. For instance, T cells upregulate CD25 expression to facilitate responsiveness to IL-2, establishing a positive feedback loop upon TCR stimulation[124]. Alternatively, T cells can induce the expression of inhibitory receptors PD-1 and TIM-3 to apply a brake to immune responses during chronic infection, a phenomenon associated with prolonged immune activation called "T cell exhaustion" [125,126,127]. Given the importance of activation and inhibitory receptors on regulating the functional status of T cells, we sought to assess the expression of activation and inhibitory receptors on patient, sibling, and control CD4 T cells after stimulation. CD4 T cells were stimulated with CD3/CD28 beads for 24 h or 48 h prior to the assessment of CD25, PD-1 and TIM-3 expression (*Figure 2.7*). Significantly, the patient's CD4 T cells expressed reduced levels of CD25 relative to (1.3-fold) sibling and (1.5-fold) control CD4 T cells (MFI: 5445 ± 104.3 *vs.* 7091 ± 104.9, $p < 0.001$; 5445 ± 104.3 *vs.* 8187 ± 36, $p < 0.001$) after 24 h of stimulation. By contrast, all CD4 T cell samples showed comparable levels of PD-1 and TIM-3 expression. Together, these results suggest that MALT1 modulates CD25 expression by human CD4 T cells upon TCR stimulation.

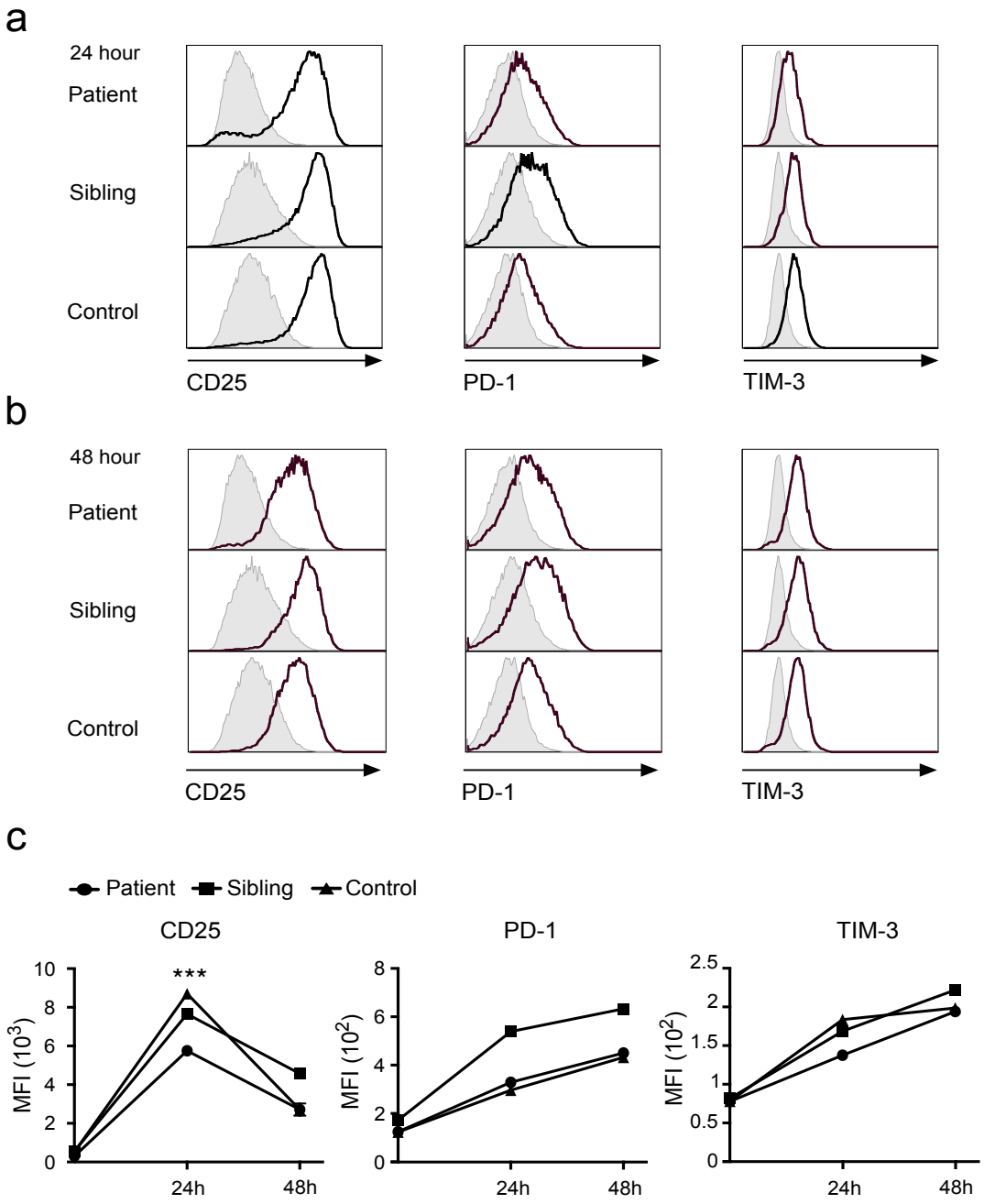

Figure 2. 7 MALT1 modulates CD25 expression upon TCR stimulation.
CD4 T cells from the patient, sibling and control were re-stimulated with CD3/CD28 beads for 24 h and 48 h and assessed activation and inhibitory receptor expression. a) and b) The expression of CD25, PD-1 and TIM-3 are presented in histograms upon 24 h and 48 h stimulation respectively. Gray shaded histogram represents background (unstimulated) for each marker. c) Liner graphs indicate the mean of triplicate cultures ± SEM. Statistical significance was calculated based on MFI difference upon stimulation (activated MFI – background MFI) using ANOVA with post *t* test.

2.3.5 Homozygous *MALT1* mutations are associated with diminished JNK activation and a failure to cleave HOIL-1

MALT1 plays crucial roles in regulating NF-κB signaling pathways upon TCR stimulation through scaffolding adaptor and paracaspase functions[97,103,99]. Studies in MALT1-deficient and MALT1 paracaspase-dead mice have demonstrated that MALT1 scaffolding function plays a key role in the regulation of mitogen-activated protein kinase (MAPK) pathways, especially JNK activation[100,101,115]. Importantly, the MAPK signaling pathways act as a bridge between cell surface antigen receptors and the cell nucleus, controlling cell metabolism, cell growth and apoptosis[128]. Hence, we sought to investigate whether the patient's homozygous *MALT1* mutations impact the scaffolding function of MALT1 to activate downstream MAPK pathways. To answer this question, patient, sibling, and control CD4 T cells were left untreated (unstimulated) or stimulated with P/I for 10 min or 30 min before detecting the phosphorylation of the JNK and ERK pathways (*Figure 2.8a*). As expected, control CD4 T cells strongly phosphorylated JNK at both 10 min and 30 min post-P/I stimulation. However, *MALT1* patient CD4 T cells exhibited weakened JNK phosphorylation at 10 min, and especially, at 30 min post-stimulation. The heterozygous *MALT1* sibling's CD4 T cells presented diminished p-JNK signalling compared to the control CD4 T cells. By contrast, *MALT1* patient CD4 T cells exhibited a modest reduction in ERK phosphorylation upon P/I stimulation as compared to sibling and control CD4 T cells. The expression of p-ERK between the sibling and control was comparable upon stimulation. Collectively, these findings indicate that the patient's MALT1 mutations selectively impact the activation of the JNK pathway.

Next, we investigated MALT1's paracaspase function that cleaves substrates to modulate NF-κB signaling pathways[97]. Interestingly, a newly identified MALT1 paracaspase substrate

called HOIL-1 is thought to suppress NF-κB activation and dampen immune responses upon its cleavage by MALT1[1,109]. To determine if the patient's mutated MALT1 possesses paracaspase function, patient, sibling and control CD4 T cells were stimulated with P/I for 10 min or 30 min and analyzed for MALT1, C-terminal HOIL-1 (C-HOIL-1) cleavage product and total HOIL-1 protein. First, an assessment of MALT1 expression revealed that our patient CD4 T cells exhibited modestly reduced MALT1 protein, normalizing to total GAPDH protein, as compared to the sibling and control, and are consistent with previous results finding that decreased MALT1 protein in patient's whole PBMC relative to controls[112]. Strikingly, P/I stimulation of control and sibling CD4 T cells resulted in a distinct C-HOIL-1 cleavage product whereas no such band was detected among the patient CD4 T cell sample (*Figure 2.8b*). Moreover, CD4 T cells from the sibling presented reduced C-HOIL-1 cleavage product only at 30 min post-stimulation compared to the control CD4 T cells. Together, these results indicate that the patient's mutant MALT1 protein exhibits diminished paracaspase activity relative to wild type MALT1 protein.

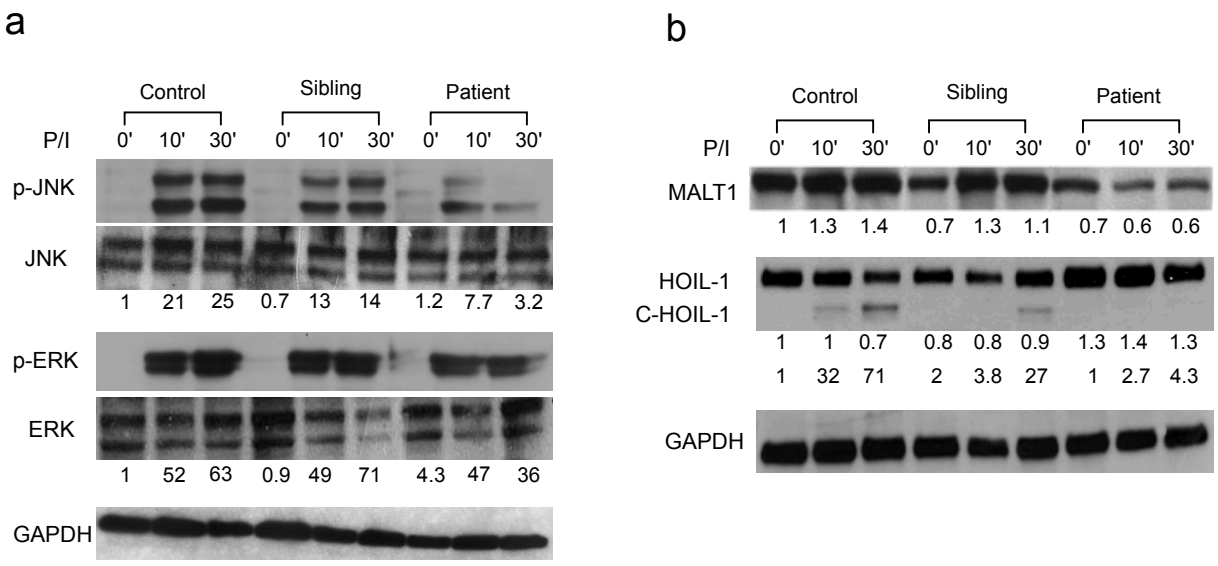

Figure 2. 8 MALT1 patient CD4 T cells exhibit defects in JNK activation and paracaspase cleavage of HOIL-1
Expanded CD4 T cells from the patient, sibling and control were stimulated for the indicated time points with P/I. a) After stimulation, CD4 T cells from each sample were stained with antibodies against p-JNK, JNK, p-ERK and ERK to assess MALT1 scaffolding activity. Unstimulated control CD4 T cells (p-JNK band/total-JNK band or p-ERK band/total-ERK band) were subjectively given score of 1 and band intensities were measured relative to the score of 1 using NIH ImageJ software. b) The control, sibling, and patient CD4 T cells were stained with antibodies against MALT1, HOIL-1 and GAPDH as a loading control to examine MALT1 paracaspase activity. Unstimulated control CD4 T cells (MALT1 band/ GAPDH band, HOIL-1 band/ GAPDH or C-HOIL-1 band/GAPDH band) were arbitrarily given a score of 1 and band intensities were calculated relative to the score of 1 using NIH ImageJ software.

2.4 Discussion

Whole-exome sequencing revealed that our BCCH patient, presenting with a novel type of combined immunodeficiency associated with immune dysregulation, had inherited homozygous missense mutations in the *MALT1* from her consanguineous parents. MALT1 is a critical regulator of NF-κB activation in lymphocytes and modulates antigen receptor signaling through its adaptor-like scaffolding and paracaspase functions[97]. Multiple lines of evidence pointed towards the homozygous *MALT1* mutations being causal in the patient's disease: (1) the

MALT1 mutation was the only damaging homozygous variant unique to the affected patient but not present in both parents and two healthy siblings, (2) the MALT1 mutation resulted in reduced levels of MALT1 protein along with loss of CBM-associated paracaspase proteolytic activity and scaffold function; and (3) the MALT1 mutation was associated with the failure of NF-kB p65 subunit phosphorylation in our patient's primary T cells that could be restored by ectopic expression of wild type but not mutant MALT1 protein[112]. Further corroboration has come from a number of independent reports identifying *MALT1* mutations as being responsible for a combined immunodeficiency disease consisting of chronic recurrent infections, severe dermatitis and fulminant inflammatory gastrointestinal disease[111,113,120]. Here, we further investigated MALT1 function in human T cell immunity using the patient primary T cells.

IL-2 plays important role in T cell differentiation and proliferation, and is mainly produced by CD4 T cells[121,122]. In healthy T cells, IL-2 binds to its receptor IL-2R and activates various signaling pathways including RAS/MAPK (mitogen-activated protein kinase) and Janus kinase/signal transduction and activators of transcription (JAK/STAT) pathways[129]. IL-2 also activates PI3K (phosphatidylinositol 3-kinase)/AKT signaling cascade, which activates NF-κB pathway via downstream signaling events, leading to transcription of the *IL-2* gene[130]. In addition, it has been shown that TCR and CD28 engagement induces NF-κB activation, which subsequently promotes IL-2 secretion in T cells[118]. These findings suggest that IL-2 production in T cells can be mediated by TCR activation-induced NF-κB signaling pathway. In this study, we observed that CD4 T cells in our patient exhibited impaired IL-2 secretion relative to her sibling's and control CD4 T cells upon P/I stimulation. Interestingly, this impairment has been reported in other patients with *MALT1* mutations[97,120,131], suggesting that defective IL-2 secretion is due to MALT1-deficiency. Since MALT1 is a key regulator of NF-κB activation, it is likely

that a reduction in MALT1 expression diminishes NF-κB signaling, which results in reduced IL-2 production in the patient's CD4 T cells. In addition, T cells from MALT1 paracaspase-dead mice exhibited significantly reduced IL-2 producing T cells, while *Malt1*$^{-/-}$ T cells presented complete abrogation of IL-2 secretion[120]. These findings suggest that IL-2 production in T cells is heavily dependent on MALT1 paracaspase activity and partially associated with MALT1 scaffolding function. Hence, it is likely that our patient's impaired MALT1 paracaspase function and reduced scaffolding activity may contribute to decreased IL-2 secretion in CD4 T cells in our patient. Taken together, our findings imply that MALT1 is essential for IL-2 production in CD4 T cells.

IL-2 is a potent growth factor for T cells, and is essential for maturation and maintenance of regulatory T cells (Tregs)[122,132]. Tregs play a pivotal role in immune homeostasis by suppressing excessive immune responses. Hence, a lack of IL-2 can result in deficiency in functional Tregs, leading to autoimmunity in mice and PID development in humans[133,134]. Strikingly, our patient displayed normal frequency of $CD4^+CD25^+FOXP3^+$ Tregs compared to the heterozygous family members and controls, despite diminished IL-2 production in CD4 T cells. This is contradictory to findings from other *MALT1* mutant patients, who exhibit considerably low Tregs counts (*Table 1*), and this discrepancy might be attributable to residual MALT1 protein and function in our patient. In addition, previous work has shown FOXP3 can be induced on $CD4^+CD25^-FOXP3^-$ T cells upon strong TCR stimulation, however, they lack suppressor function[135-137]. Consequently, constitutive immune activation in our MALT1 patient may induce FOXP3 expression in conventional CD4 T cells enabling them to masquerade as Tregs. More importantly though, our patient exhibited a severe inflammatory immune phenotype, including immunodeficiency, enteropathy and dermatitis, very similar to other described

MALT1-deficient patients despite having a normal proportion of $FOXP3^{+}CD25^{+}$ cells among their CD4 T cell population. Further, the suppressive capacity of our patient's Tregs was never assessed so it is unclear what role, if any, Treg dysfunction played in our MALT1 patient's phenotype.

To date, there are six reported patients with combined immunodeficiency attributable to MALT1-deficiency[93,120]. A key clinical phenotype shared among all *MALT1* patients is susceptibility to recurrent viral, bacterial and fungal infections, suggesting possible deficits in their effector T cell function. Among CD4 T cell lineages, Th1 cells secrete IFN-γ and TNF-α and provide protection against intracellular pathogens, inhibiting viral replication and bacterial growth[138]. Th17 cells play an important role in the clearance of fungal infections, including *C. albicans*[55,139,140,141]. Here, our patient CD4 T cells exhibited weakened Th1 and Th17 T cell function with significantly diminished IFN-γ, TNF-α, and IL-17 production. Further, it has been reported that activation of NF-κB plays an important role in IFN-γ and TNF-α production in T cells[142,143] and regulates Th17 differentiation[151,144,145]. Therefore, MALT1-deficiency in our patient weakens NF-κB signaling, which may lead to a reduction in Th1 and Th17 inflammatory cytokine secretion. In addition, T helper cell dysfunction can impact B cell differentiation, including defects in germinal center formation, antibody class switching, and B cell memory as shown in X-linked lymphoproliferative disease[146]. Consequently, aberrant T cell responses in MALT1 patients may contribute to the defective B cell differentiation and B cell lymphopenia observed in these patients. Taken together, *MALT1* mutations impair effector function of CD4 T cells, which in turn could be responsible for inefficient immune responses that result in recurrent and chronic infections.

CD25 (IL-2R) is expressed on activated and regulatory T cells, and is known to regulate T cell proliferation and differentiation. Hence, mutations in the *CD25* gene can result in immunodeficiency disorders associated with immune dysregulation including lymphoproliferation, autoimmune diseases or IPEX-like syndrome[147,148]. These findings highlight the important role of CD25 in normal immune function. Inhibitory receptors PD-1 and TIM-3 deliver negative signals to T cells to suppress T cell activation and function. During chronic viral infection, inhibitory receptors are induced on T cells to limit anti-viral immune responses[149,150]. Inhibition of PD-1 interacting with its ligand PD-L1 has been shown to restore anti-viral immune responses and improve viral control[126]. However, studies have shown that PD-1 deficiency and PD-1 blockade can lead to immunopathology or the development of autoimmune diseases[151]. Consequently, these receptors are necessary to act as an important "brake" on the immune system. Altogether, our findings suggest that while MALT1 modulates expression of the CD25 activation receptor, it does not appear to regulate the expression of inhibitory receptors on human CD4 T cells.

MALT1 plays critical roles in modulating immune responses through two different mechanisms: (1) as a scaffolding molecule for the formation of the CBM signalosome complex and (2) as a caspase-like protease (paracaspase) that cleaves substrates to regulate NF-κB signaling[97]. Our patient contrasted other described *MALT1* patients in having some MALT1 protein and consequently, possibly some residual MALT1 function (*Table 1*)[93]. To tease out the scaffolding and paracaspase function of MALT1 in our patient, we assessed the activation of MAPK, and the cleavage of HOIL-1. Studies on MALT1-deficient and MALT1 paracaspase-dead mice have demonstrated that JNK activation is dependent on MALT1 scaffold function[100,152]. Therefore, the finding that our patient CD4 T cells activate JNK, although to

lower levels than sibling or control CD4 T cells, suggests that mutated MALT1 protein retains some scaffolding activity. Furthermore, the sibling's CD4 T cells presented diminished p-JNK expression compared to the control, proposing that the missense mutations in *MALT1* alter its scaffolding function. In addition, we failed to detect the MALT1 cleavage product C-HOIL-1 in our patient's CD4 T cells, and reduced C-HOIL-1 expression was observed in the sibling's CD4 T cells. These results suggest that mutant MALT1 protein is severely defective in paracaspase function. Importantly, MALT1-mediated HOIL-1 cleavage negatively regulates NF-κB activation to suppress immune responses[1,109]. Moreover, the selective loss of MALT1 paracaspase activity with the retention of MALT1 scaffolding function has been shown to cause lethal autoimmunity in mice[101]. Altogether, our findings suggest that the patient has inherited hypomorphic rather than complete loss-of-function *MALT1* missense mutations that may have contributed to her disease phenotype.

Chapter 3: Anti-viral T cell responses in MALT1-deficient mice

3.1 Introduction

3.1.1 MALT1 scaffolding and paracaspase activities in immune cell function

MALT1 plays important roles in regulating immune receptor-induced NF-κB signaling that regulates lymphocyte activation, proliferation and differentation[102]. MALT1 performs its function through at least two different mechanisms: i) as a signaling adaptor molecule bringing CARD11 and BCL-10 together to form the CBM complex and ii) as a caspase-like protease (paracaspase) cleaving various substrates to modulate NF-κB activation[97,93,99]. Studies in MALT1-deficient (*Malt1*$^{-/-}$) mice have provided advanced knowledge of MALT1 function in the immune system. MALT1-deficient mice exhibit reduced frequency of Tregs, impaired cytokine production and defective antigen-receptor signaling upon TCR stimulation; yet *Malt1*$^{-/-}$ mice are otherwise healthy and have normal frequencies of T cells and B cells[114,97,115]. Paracaspase-dead mice (*Malt1*$^{PD/PD}$) have selectively impaired MALT1 paracaspase activity while retaining normal MALT1 scaffolding function. Interestingly, these mice present enlarged lymph nodes along with autoimmune disease and spontaneous multiorgan inflammation, suggesting that the paracaspase function of MALT1 is crucial in regulating immunity[100,131]. Moreover, two independent research groups have recently reported that *Malt1*$^{-/-}$ mice exhibit impaired Th17 (IL-17^{+}) cells, a subset of T cells that have been implicated in the pathogenesis of severe autoimmune diseases, including MS[126,153]. The lack of Th17 cells in *Malt1*$^{-/-}$ mice protects these mice from experimental autoimmune encephalomyelitis (EAE), a well-studied mouse model of human MS[131,154].

Malt1$^{-/-}$ mice have similar phenotypes to our *MALT1*$^{mut/mut}$ patient, including reduced production of the cytokines IL-2, IL-17 and IFN-γ. However, *Malt1*$^{-/-}$ mice do not present signs

of constitutive immune activation or intestinal inflammation. One hypothesis for this difference is that limited exposure of *Malt1*$^{-/-}$ mice to pathogens prevents the development of inflammatory disease. Humans encounter a vast array of microbes. On the contrary, laboratory animal facilities offer considerably 'clean' and specific-pathogen-free environments that limit pathogen exposure to mice, and do not represent real human living conditions. Studies have reported that the immune system of laboratory mice resemble those of human neonates rather than those of adults. Pet store-housed mice, on the other hand, have been suggested to exhibit immune responses more closely resembling those of adult humans[155]. These findings lead us to investigate the immune responses of *Malt1*$^{-/-}$ mice upon pathogen challenge with lymphocytic choriomeningitis virus (LCMV).

LCMV is one of the well-studied viruses in rodent model systems. This non-cytolytic virus does not directly damage cells or tissues[156,157], allowing researchers to study host immune responses against the virus separate from direct infection-mediated cellular damage. There are several well-characterized LCMV strains with varying viral persistency, including the Armstrong (acute infection) and Clone 13 (chronic infection) strains. The Armstrong strain is associated with robust immune responses, and can be cleared by day 8 after infection[131]. In contrast, Clone 13 leads to modest immune activation, and persists longer in the host, resulting in functional exhaustion of T cells. There are also several well-studied LCMV-specific immunodominant epitopes, which are useful for the investigation of virus-specific immune responses. Both acute and chronic LCMV strains share the same MHC-I and MCH-II restricted LCMV epitopes. MHC class I restricted epitopes include GP_{33-41}, $NP_{396-404}$ and $GP_{276-286}$, and MHC class II restricted epitopes are GP_{61-80} and $NP_{309-328}$[156,158]. Researchers can utilize tetramers conjugated to these specific epitopes to assess the frequencies of virus-specific CD4 and CD8 T cells upon LCMV

infection. In this report, we examined the immune responses of MALT1 deficient mice to acute LCMV infection. We specifically proposed to:

1. Examine whether *Malt1*$^{-/-}$ mice are able to mount immune responses upon acute viral infection.
2. Enumerate the expansion of viral-specific T cells and assess the function of effector T cells.
3. Characterize T cell differentiation marker expression and measure activation and inhibitor marker upregulation.

3.2 Materials and methods

3.2.1 Mice and LCMV infection

MALT1-deficeint mice were generated by Dr. Tak Mak's laboratory (University of Toronto) as described[114], and we were kindly provided breeder pairs by Dr. Laura Sly's laboratory (Department of Pediatrics, UBC). All mice were housed in a *Helicobacter*-free environment at the BC Children's Hospital Research Institute (BCCHR, UBC) animal facility. C57BL/6J (B6) mice were purchased from the Jackson Laboratory and bred within the BCCHR animal facility.

For viral infection experiments, LCMV Armstrong strain was kindly provided by Dr. Marc Horwitz (Department of Microbiology & Immunology, UBC). Ten to twelve-week-old sex- and age- matched wild type and *Malt1*$^{-/-}$ mice were injected i.p. with 200,000 plaque forming units (PFU) of LCMV Armstrong and euthanized at Day 8 post-infection.

3.2.2 Flow cytometry

Antibodies (clone) against CD16/32(93), CD3e (145-2C11), CD4 (GK1.5), CD8α (53-6.7), CD25 (PC61.5), CD44 (IM7), CD62L (MEL-14), CD69 (H1.2F3), CD127 (A7R34), KLRG1 (2F1), PD-1 (J43), LAG-3 (C9B7W), TIM-3 (8B.2C12), IL-2 (JES6-1A12), IFN-γ (XMG1.2), TNF-α (MP6-XT22), and FOXP3 (FJK-16s) were used to detect function and subsets of lymphocytes. $1x10^6$ - $2x10^6$ isolated splenocytes were stained with Fc blocker (CD16/32, eBioscience) on ice for 15 min followed by a 5-min wash with FACS buffer. Subsequently, cells were stained with surface marker antibodies for 15 min at 4°C, and then washed and resuspended in FACS buffer for data acquisition. Data were acquired with either a BD LSRII benchtop or BD LSR FORTESSA cytometer using FACSDiVa software (BD Bioscience). Data were analyzed with FlowJo (Tree Star, Ashland, OR) software.

To study virus-specific T cells, LCMV epitope-specific tetramers (GP_{33-41} and $NP_{396-404}$) were acquired from Fred Hutchinson Cancer Research Center (FHCRC). Isolated splenocytes from wild type and *Malt1*$^{-/-}$ mice were stained with Fc block followed by a 5-min wash with FACS buffer. A total of $1x10^6$- $2x10^6$ of splenocytes were stained with 0.3ug of LCMV tetramer with 0.5ug of CD8 antibody in 30uL of FACS buffer for 30 minutes at RT followed by surface staining.

LCMV peptides were synthesized at the University of British Columbia's Nucleic Acid Protein Service Unit. To assess function of LCMV-stimulated T cells via intracellular cytokine production, splenocytes were stimulated with GP33 (KAVYNFATC, 0.2ug/mL), NP396 (FQPQNGQFI, 0.2ug/mL), GP276 (SGVENPGGYCL, 0.2ug/mL), GP61 (GLKGPDIYKGVYQFKSVEFD, 2ug/mL) peptides or PMA (50ng/mL)/ionomycin (1ug/mL) (Sigma-Aldrich) for 4 h in the presence of Golgi Plug (1:1000, BD Bioscience) in MEM with

10% FBS at 37°C. Stimulated cells were washed with FACS buffer and incubated with Fc blocker for 15 mins on ice. Subsequently, samples were stained with surface markers, fixed, and made permeable using Fixation/Permeabilization Kit (BD Bioscience) following the manufacture's instructions. Permeabilized cells were stained with intracellular antibodies for 30 min at RT and washed with FACs buffer. Data were acquired with either a BD LSRII benchtop or BD LSR FORTESSA cytometer using FACSDiVa software (BD Bioscience). Data were analyzed with FlowJo (Tree Star, Ashland, OR) software.

3.2.3 LCMV plaque assays

Plaque assays were performed as described previously[159]. In brief, Vero cells were seeded into 6 well plates with complete EMEM (10% FBS, 1% L-Glu, 1% NEAA, 0.1% P/S). When cells reached at ~90% confluence, the media was aspirated and 1mL of serum free-EMEM added back to each well. LCMV-Armstrong was serially diluted in serum-free EMEM from 10^{-3} to 10^{-7} and 100ul of each dilution added to each well of a 6 well plate. The plates were incubated at 37°C incubator for 1 h and gently rocked once every 15 min. Followed by incubation, overlay with 4mL of a 1:1 mixture of 1% agarose and complete 2X MEM (10% FBS, 2 mM L-Glu, 100 U/mL P/S) solution, then plates were incubated at 37°C for 3 days. On day 3, a second overlay (1:1 mixture of 1% agarose and complete 2X MEM) with 1% Neutral Red dye in distilled H_2O (0.15mL/10mL of overlay) was performed. Plaques were counted after 48 hours of incubation at 37°C.

3.2.4 Statistical analysis

Statistical analyses were performed by using unpaired, two-tailed *t* tests. Statistical significance was determined as a p value of less than 0.05 (*), 0.01 (**) and 0.001 (***). All statistical analyses were executed using GraphPad Prism 6 (GraphPad Software, Inc., San Diego, CA, USA).

3.3 Results

3.3.1 MALT1-deficient CD8 T cells expand upon infection with LCMV

To investigate anti-viral immune responses in MALT1-deficient mice, we infected wild type and *Malt1*$^{-/-}$ mice with LCMV Armstrong strain ($LCMV_{Arm}$) and spleens were harvested on day 8 post-infection. As previously described, wild type mice presented no clinical symptoms and surprisingly *Malt1*$^{-/-}$ mice were also protected from $LCMV_{Arm}$ infection. Both wild type and *Malt1*$^{-/-}$ mice exhibited significantly increased numbers of total splenocytes relative to uninfected (naïve) mouse controls (*Figure 3.1a*; wild type: $12.5 \pm 0.58 \times 10^7$ *vs.* $8 \pm 0.24 \times 10^7$; *Malt1*$^{-/-}$ mice: $12.3 \pm 0.63 \times 10^7$ *vs.* $8.3 \pm 0.32 \times 10^7$, $p < 0.01$). Flow cytometric analysis revealed that wild type and MALT1-deficient mice possessed greatly (7-fold, 8.2-fold respectively) increased frequencies of splenic CD8 T cells upon LCMV infection relative to naïve uninfected mice (*Figure 3.1b,c,* $6.1 \pm 0.41 \times 10^7$ *vs*. $0.88 \pm 0.06 \times 10^7$, $p < 0.001$; $6.8 \pm 0.33 \times 10^7$ *vs*. $0.83 \pm 0.07 \times 10^7$, $p < 0.001$). Moreover, wild type mice presented a 1.6-fold reduced frequency of splenic CD4 T cells upon LCMV infection (*Figure 3.1c,* $0.81 \pm 0.04 \times 10^7$ *vs*. $1.27 \pm 0.06 \times 10^7$, $p < 0.01$) and *Malt1*$^{-/-}$ mice exhibited a modest 1.69-fold increase in splenic CD4 T cells upon infection relative to wild type mice (*Figure 3.1c*, $1.37 \pm 0.9 \times 10^7$ *vs.* $0.81 \pm 0.3 \times 10^7$, $p < 0.05$),

yet comparable CD8 T cell numbers. Together, these findings indicate that MALT1 is not required for CD8 T cell expansion upon acute viral infection.

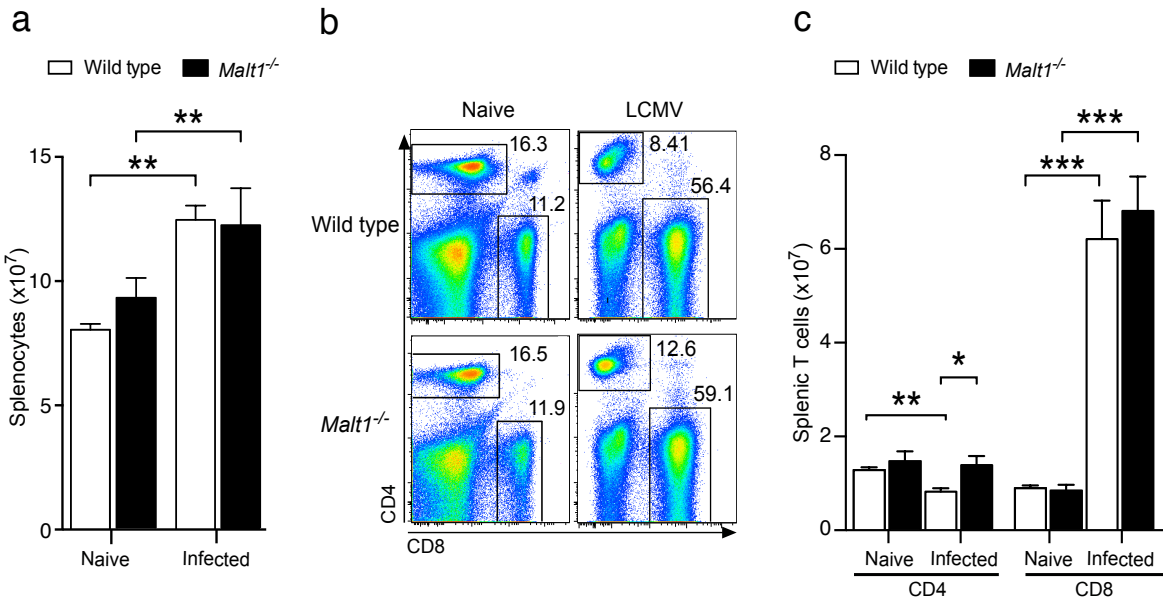

Figure 3. 1 MALT1-deficeint CD8 T cells expand upon infection with LCMV
Splenocytes from naïve and infected-wild type (n=3, n=4 respectively) and *Malt1*$^{-/-}$ (n=3, n=5 respectively) mice were isolated on day 8 post LCMV Armstrong infection. a) Absolute numbers of splenocytes from naïve- and infected-wild type and *Malt1*$^{-/-}$ mice. b) Representative flow data of CD4 and CD8 T cell composition of splenocytes from naïve and LCMV-infected wild type and *Malt1*$^{-/-}$ mice. c) Cumulative data of absolute number of CD4 and CD8 splenic T cells. Bar graphs represent the mean ± SEM. Statistical significance was calculated using two-tailed unpaired *t* test (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$).

3.3.2 MALT1 is crucial for generating viral-specific CD4 T cells upon LCMV infection

To first investigate the importance of MALT1 for mounting MHC class II-restricted T cell responses, we infected wild type and *Malt1*$^{-/-}$ mice with a sublethal dose of LCMV Armstrong. At Day 8 post-infection, viral-specific CD4 T cells were enumerated by stimulating splenocytes with major immunodominant LCMV-specific MHC class II H-2A^{b}-restricted GP61

peptide and assessing IL-2 production. Strikingly, the number of splenic *Malt1*$^{-/-}$ CD4 T cells capable of secreting IL-2 was greatly reduced (4.3-fold) relative to wild type CD4 T cells upon peptide stimulation (*Figure 3.2a, b*, $1.03 \pm 0.34 \times 10^5$ *vs.* $4.38 \pm 0.67 \times 10^5$, $p < 0.01$). In addition, we examined the potential of *Malt1*$^{-/-}$ CD4 T cells to secrete IL-2 independent of TCR specificity by stimulating with the diacylglycerol analog PMA and the calcium ionophore ionomycin. Again, *Malt1*$^{-/-}$ CD4 T cells exhibited a marked (3-fold) reduction in the frequency of IL-2 secreting effectors as compared to wild type ($3.8 \pm 0.77 \times 10^5$ *vs.* $11.5 \pm 1.22 \times 10^5$, $p < 0.001$). Together, these results indicate that MALT1 is critical for the generation of IL-2 producing effector CD4 T cells.

Next, we assessed the capacity of anti-viral CD4 T cells to secrete IFN-γ and TNF-α, two key inflammatory cytokines necessary to limit viral replication[52,138]. Upon GP61 peptide stimulation, *Malt1*$^{-/-}$ CD4 T cells displayed substantial decreased number of mono-functional (3.1-fold for IFN-γ^+ TNF-α^-, $7.96 \pm 0.92 \times 10^5$ *vs.* $2.5 \pm 0.72 \times 10^5$, $p < 0.01$; 3.6-fold for IFN-γ^- TNF-α^+, $1.8 \pm 0.5 \times 10^5$ *vs.* $6.5 \pm 0.81 \times 10^5$, $p < 0.01$) and dual-functional effectors (3.4-fold for IFN-γ^+TNF-α^+, $1.59 \pm 0.48 \times 10^5$ *vs.* $6.03 \pm 0.79 \times 10^5$, $p < 0.01$) relative to wild type mice (*Figure 3.2c,d*). Further, *Malt1*$^{-/-}$ CD4 T cells also exhibited marked reduction when activated with PMA and ionomycin compared to wild type CD4 T cells (*Figure 3.2c,d*, 3.1-fold for IFN-γ^+TNF-α^+, $3.5 \pm 0.77 \times 10^5$ *vs.* $11 \pm 1.4 \times 10^5$, $p < 0.01$; 2.4-fold for IFN-γ^+ TNF-α^-, $4.86 \pm 0.9 \times 10^5$ *vs.* 11.7 ± 1.45 %, $p < 0.01$; 6.5-fold for TNF-α^+ IFN-γ^-, 8.8 ± 1.31 % *vs.* 57.1 ± 4.97 %, $p < 0.001$). Collectively, our findings suggest that MALT1 plays crucial roles in generating functional viral-specific effector CD4 T cells.

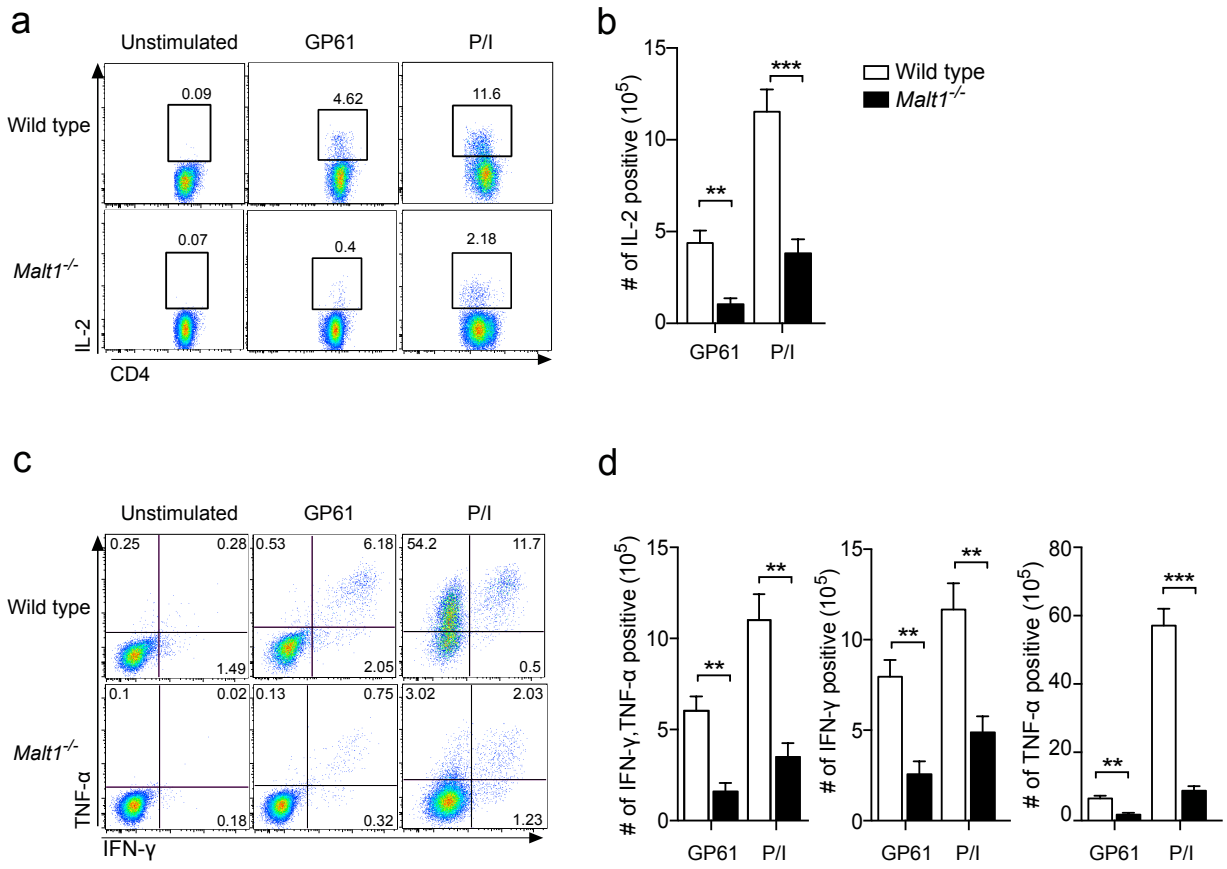

Figure 3. 2 MALT1 is critical for the generation of viral-specific CD4 T cells.
Splenic T cells from wild type (n=4) and *Malt*$^{-/-}$ mice (n=5) were stimulated with GP61 peptide or PMA/Ionomycin (P/I) for 4 hr on day 8 post-infection to assess viral-specific CD4 T cells. a) Representative CD4^{+}IL-2^{+} T cells from wild type and *Malt*$^{-/-}$ mice upon stimulation. b) Cumulative data of CD4^{+}IL-2^{+} T cells are presented in bar graphs, indicating the number of IL-2 producing CD4 T cells. c) Representative data of IFN-γ and TNF-α secreting CD4 T cells from wild type and *Malt*$^{-/-}$ mice. d) Cumulative data of IFN-γ and/or TNF-α producing CD4 T cells are presented in bar graphs, representing the frequency of cytokine producing CD4 T cells. Bar graphs represent the mean ± SEM. Statistical significance was calculated using two-tailed unpaired *t* test (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$).

3.3.3 MALT1 is not essential for the expansion of viral-specific CD8 T cells

Virus-specific effector CD8 T cells play critical roles through cytotoxicity and production of proinflammatory cytokines to facilitate the destruction of infected target cells and combat viral infections. Upon acute viral infection, virus-specific naïve CD8 T cells become activated

and undergo massive clonal expansion that is associated with the acquisition of effector functions like cytolytic machinery and capacity to produce proinflammatory cytokines[131].To investigate whether MALT1 is required for the generation of viral-specific CD8 T cells, we utilized two different LCMV tetramers of MHC class I molecules containing immunodominant viral peptides, H-2D^b-GP$_{33-41}$ and H-2D^b-NP$_{396-404}$, to directly count antigen-specific CD8 T cells of wild type and *Malt1*$^{-/-}$ mice as previously described[160]. At the peak of virus-specific T cell expansion (Day 8 post-infection), we found that the spleens of wild type and *Malt1*$^{-/-}$ mice exhibited similar numbers of NP396-H-2D^b-specific ($9.09 \pm 1.25 \times 10^6$ *vs*. $9.62 \pm 0.66 \times 10^6$ respectively) and GP33-H-2D^b-specific CD8 T cells ($3.61 \pm 0.45 \times 10^6$ *vs.* $4.01 \pm 0.54 \times 10^6$ respectively). Moreover, MALT1-deficient mice displayed comparable frequencies of LCMV-specific cells within whole splenic CD8 T cells relative to wild type mice. (*Figure 3.3c*, GP33, 5.88 ± 0.51 % *vs*. 5.87 ± 0.76 %; NP396, 14.3 ± 0.33 & *vs*. 14.7 ± 1.71 %). By contrast, naïve mice had few, if any, detectable LCMV-specific CD8 T cells. Consequently, these findings indicate that MALT1 is dispensable for the generation of viral-specific CD8 T cells upon acute viral infection.

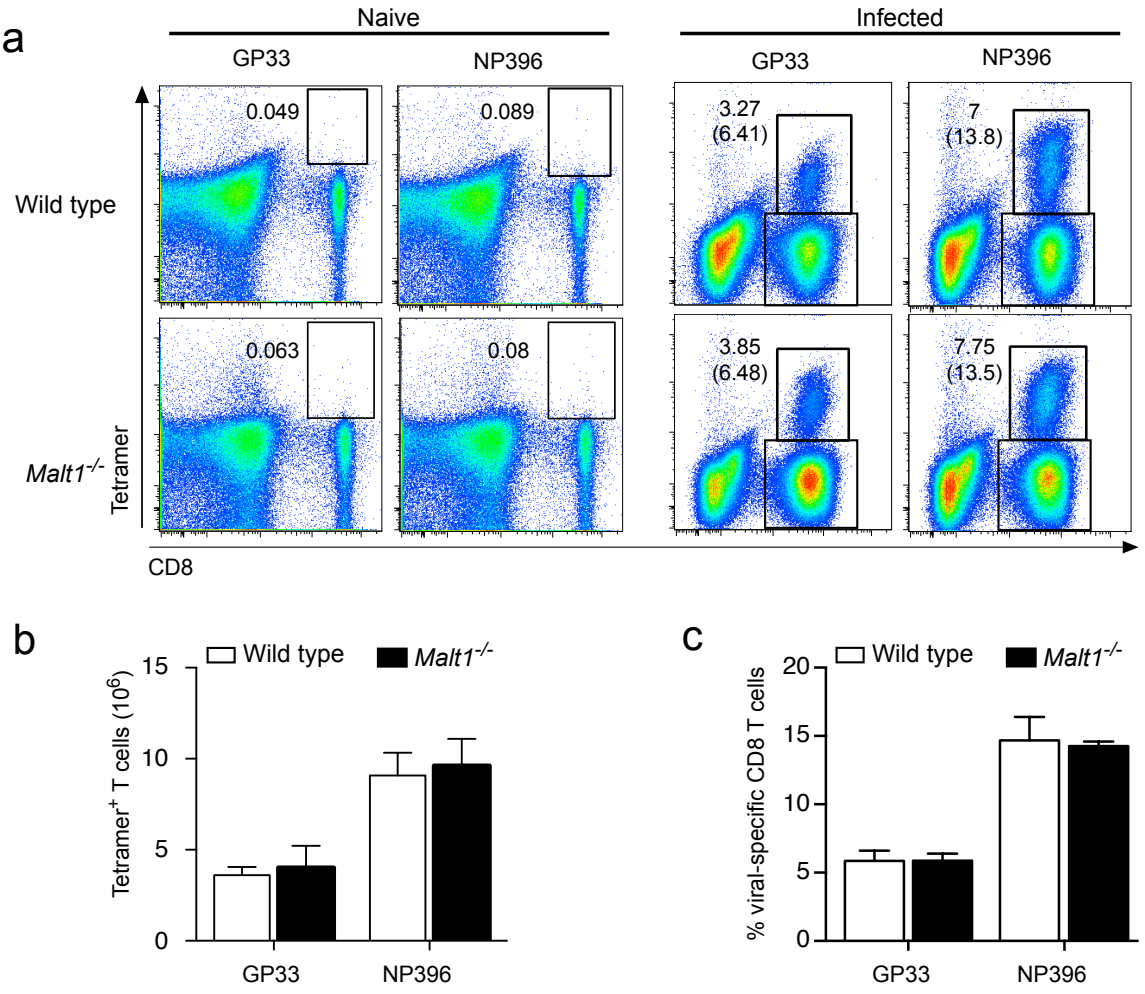

Figure 3. 3 MALT1 is not essential for the expansion of viral-specific CD8 T cells
Viral-specific T cells from wild type (n=4) and *Malt1*$^{-/-}$ (n=5) mice were determined using LCMV-specific epitope MHC class I restricted tetramers (GP33 and NP396). a) Representative tetramer positive splenic CD8 T cells from wild type and *Malt1*$^{-/-}$ mice on day 8 post-infection. Numbers in a bracket represent a frequency of viral-specific T cells within splenic CD8 T cells. b) Cumulative numbers of viral-specific CD8 T cells c) Cumulative % of tetramer positive cells within whole splenic CD8 T cells. Bar graphs indicate the mean ± SEM. Statistical significance was calculated using two-tailed unpaired *t* test (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$).

3.3.4 MALT1 modulates expression of T cell differentiation markers upon acute viral infection.

Next, we sought to investigate the role of MALT1 in T cell differentiation by assessing marker expression upon acute viral infection. Moreover, naïve and activated T cells express different surface markers enabling one to distinguish those two population and determine the cell status. For instance, naïve T cells display the cell adhesion molecule CD62L that controls homing of lymphocytes to enter secondary lymphoid tissues and this marker is downregulated upon activation[161]. CD44 is an activation marker that is express at high levels on proliferating T cells and memory T cells. Hence, naïve T cells exhibit a $CD62L^{hi}CD44^{lo}$ surface phenotype, whereas activated T cells are $CD62L^{lo}CD44^{hi}$ [161,162]. To characterize naïve and activated T cells in wild type and *Malt1*$^{-/-}$ mice upon infection, splenocytes from Day 8 post-LCMV infection were stained with CD62L and CD44 surface markers and analyzed with flow cytometry (*Figure 3.4a*). GP33- and NP396-viral-specific *Malt1*$^{-/-}$ CD8 T cells exhibited a heightened frequency of cells bearing a $CD62L^{hi}CD44^{hi}$ phenotype (4.6-fold, 4.1-fold respectively) relative to wild type (*Figure 3.4b*, $GP33^{+}$: 1.84 ± 0.15 % *vs*. 8.4 ± 0.99 % $<p=0.001$; $NP396^{+}$: 1.97 ± 0.12 % *vs*. 8.07 ± 1.24 % $<p=0.01$). Accordingly, these results suggest that MALT1 may regulate CD62L expression and T cell homing upon acute viral infection.

Subsequently, we characterized the expression of two markers, CD127 (IL-7Rα), a critical component for CD8 T cell memory, and KLRG1 (killer cell lectin-like receptor G1), a marker of terminally differentiated effector T cells, on LCMV-specific effector CD8 T cells at Day 8 post-infection[163]. Further, the expression of these two surface receptors have been used to discriminate effector CD8 T cells into two subsets called "memory precursor effector T cells (MPEC)" and "short-lived effector cells (SLEC)" as $CD127^{hi}KLRG1^{lo}$ and $KLRG1^{hi}CD127^{lo}$

respectively[164,165]. Significantly, we found that *Malt1*$^{-/-}$ GP33^{+} and NP396^{+} viral-specific CD8 T cells contained a substantial proportion of MPEC-like (CD127^{hi}KLRG1^{lo}) and CD127^{hi}KLRG1^{hi} populations relative to wild type CD8 T cell counterparts (*Figure 3.4c, 3.4d*, GP33^{+}: 12.1 $\pm$ 1 % $vs.$ 22.5 $\pm$ 1.76 % <p=0.01; NP396^{+}: 4.24 $\pm$ 0.51 % $vs.$ 16 $\pm$ 0.67 % <p=0.001; GP33^{+}: 7.3 $\pm$ 0.98 % $vs.$ 23.2 $\pm$ 1.39 % <p=0.001; NP396^{+}: 3.42 $\pm$ 0.37 % $vs.$ 9.14 $\pm$ 0.6 % <p=0.001 respectively). However, *Malt1*$^{-/-}$ tetramer positive CD8 T cells presented remarkably lower frequency of SLEC-like (KLRG1^{hi}CD127^{lo}) effector T cells than wild type (GP33^{+}: 54.5 $\pm$ 3.27 % $vs.$ 39 $\pm$ 2.85 % <p=0.01; NP396^{+}: 53.3 $\pm$ 3.86 % $vs.$ 35.7 $\pm$ 2.06 % <p=0.001). Together, these findings indicate that MALT1 may promote the differentiation or short-lived effector CD8 T cells.

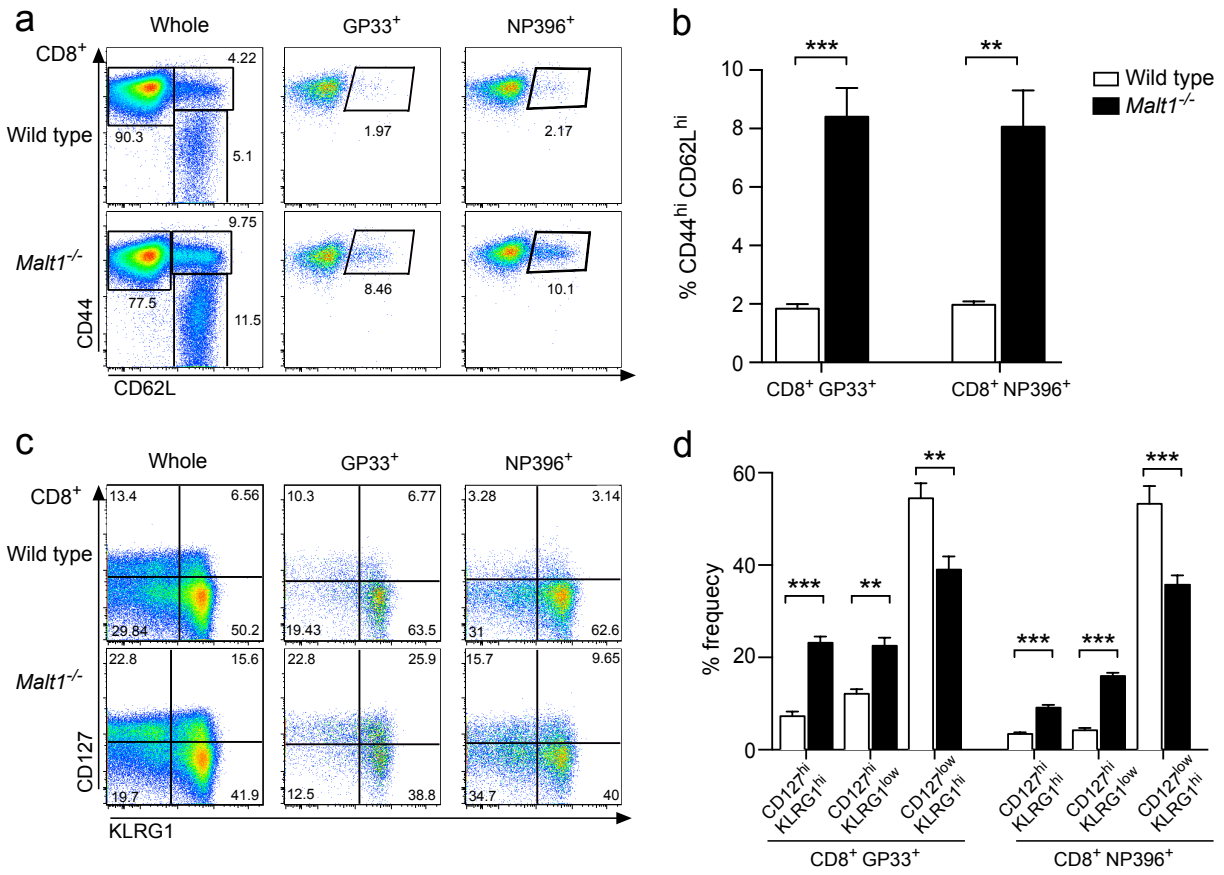

Figure 3. 4 MALT1 alters the expression of T cell differentiation marker
Phenotype of splenic and viral-specific CD8 T cells were determined by expression of CD44, CD62L, CD127 and KRLG1 surface markers at the surface of wild type and *Malt1*$^{-/-}$ splenocytes 8 days after $LCMV_{Arm}$ infection. a) Representative flow cytometry plots of CD44 and CD62L expression on total and viral-specific CD8 T cells from wild type and *Malt1*$^{-/-}$ spleen. b) Cumulative data presenting the frequency of $CD44^{hi}CD62L^{hi}$ viral-specific CD8 T cells. c) Representative flow cytometry data of CD127 and KLRG1 expression on total and viral-specific CD8 T cells from wild type and *Malt1*$^{-/-}$ mice. d) Cumulative data of CD127, KLRG1 presenting viral-specific CD8 T cells were shown in bar graphs. Bar graphs present the mean ± SEM and statistical significance was calculated using two-tailed unpaired *t* test (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$).

3.3.5 MALT1 is not required for the effector function of viral-specific CD8 T cells

Effector CD8 T cells generate cytotoxic T cell (CTL) responses through secreting inflammatory cytokines that are essential to eradicate infected or dying cells[131]. To address

whether MALT1-deficiency impacts effector function of viral-specific CD8 T cells, wild type and *Malt1*$^{-/-}$ splenocytes were stimulated with GP33 peptide, NP396 peptide, GP276 peptide or P/I for 4 hr and analyzed for IFN-γ^+TNF-α^+ dual-functional, IFN-γ^+ or TNF-α^+ mono-functional CD8 T cells. Surprisingly, *Malt1*$^{-/-}$ mice displayed an appreciably higher frequency of IFN-γ secreting viral-specific CD8 T cells upon GP33 and NP396 peptide stimulation relative to wild type mice (*Figure 3.5*, 1.7-fold for GP33, 13 ± 1.28 x 10^6 *vs*. 7.7 ± 1.23 x 10^6, $p < 0.01$; 1.5-fold for NP396, 10.7 ± 0.9 x 10^6 *vs*. 7.2 ± 1.02 x 10^6, $p < 0.01$), and exhibited a similar frequency of IFN-γ^+ CD8 T cells with GP276 peptide stimulation (*Figure 3.5*, 3.98 ± 0.56 x 10^6 *vs.* 3.2 ± 0.2 x 10^6). In addition, *Malt1*$^{-/-}$ mice presented comparable frequencies of IFN-γ^+TNF-α^+ dual-functional (*Figure 3.5*, GP33: 5.54 ± 0.6 x 10^6 vs. 4.13 ± 0.55 x 10^6; NP396: 3.54 ± 0.36 x 10^6 vs. 3.01 ± 0.44 x 10^6; GP276: 2.43 ± 0.43 x 10^6 vs. 2.23 ± 0.11 x 10^6) and TNF-α^+ mono-functional effector CD8 T cells relative to wild type mice upon all peptide stimulation. (*Figure 3.5*, GP33: 5.62 ± 0.6 x 10^6 vs. 4.27 ± 0.55 x 10^6; NP396: 3.65 ± 0.37 x 10^6 vs. 3.33 ± 0.44 x 10^6; GP276: 2.55 ± 0.43 x 10^6 vs. 2.4 ± 0.14 x 10^6). Interestingly, *Malt1*$^{-/-}$ mice showed considerable impairment in inflammatory cytokine production upon P/I stimulation compared to wild type mice (1.6-fold for IFN-γ^+TNF-α^+, 20.2 ± 1.53 x 10^6 *vs.* 33.1 ± 1.66 x 10^6, $p < 0.001$; 1.3-fold for IFN-γ^+, 30.7 ± 2.03 x 10^6 *vs.* 40,2 ± 2.79 x 10^6. $p < 0.01$; 1.8-fold for TNF-α^+, 21.2 ± 1.72 x 10^6 *vs*. 37 ± 1.5 x 10^6, $p < 0.001$). Collectively, our results suggest that MALT1 is not required for the CTL responses of viral-specific CD8 T cell function.

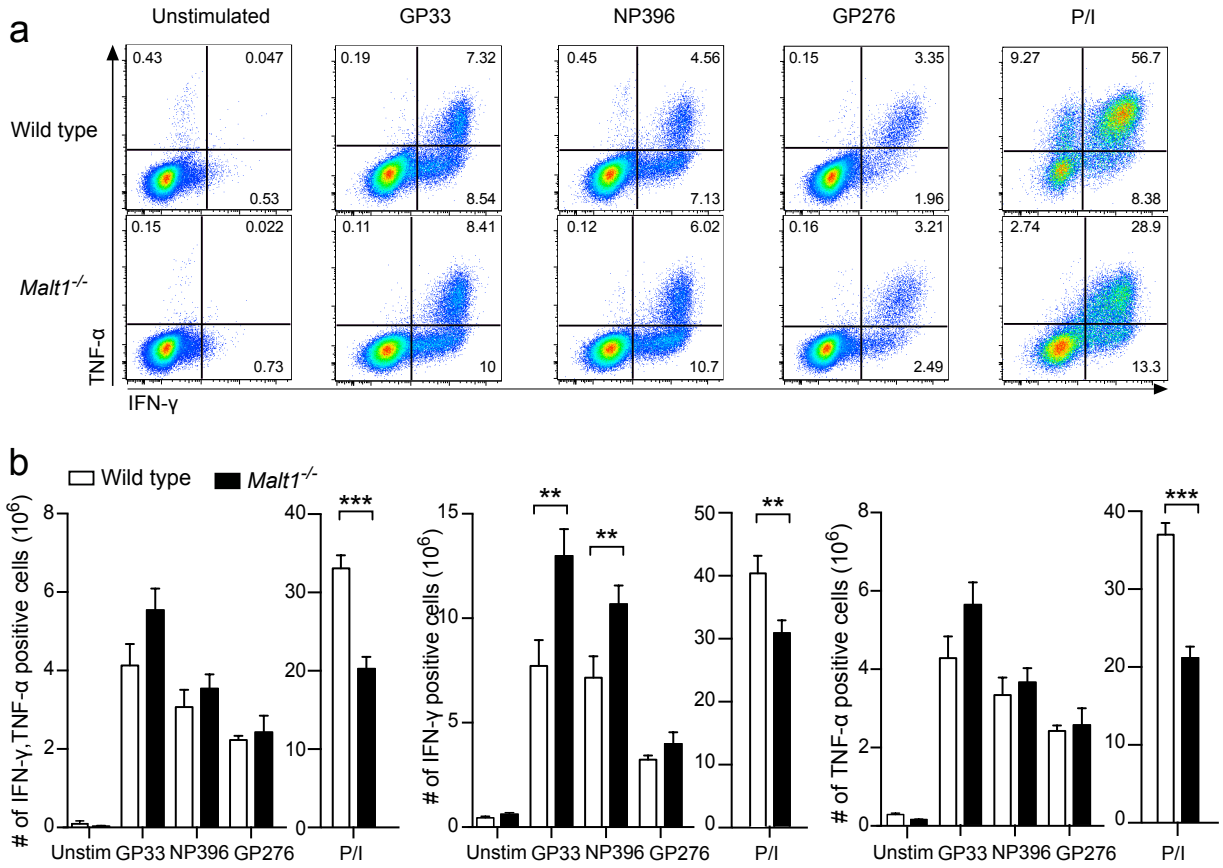

Figure 3. 5 MALT1 is not required for the effector function of viral-specific CD8 T cells. On day 8 post-infection with LCMV Armstrong, wild type (n=4) and *Malt1*$^{-/-}$ (n=5) splenocytes were stimulated for 4 h with MHC class I-presented LCMV-specific peptides (GP33, NP396 and GP276) or P/I, and analyzed for IFN-γ and TNF-α production. a) Representative flow cytometric plots of IFN-γ and TNF-α secreting splenic CD8 T cells upon peptides and P/I stimulation. b) Cumulative data shown in bar graphs, presenting the numbers of dual-functional (IFN-γ^+TNF-α^+) and mono-functional (IFN-γ^+ TNF-α^- or TNF-α^+IFN-γ^-) CD8 T cells. Bar graphs indicate the mean ± SEM and statistical significance was calculated using two-tailed unpaired *t* test (**p* < 0.05, ***p* < 0.01, and ****p* < 0.001).

3.3.6 MALT1-deficiency does not alter activation and inhibitory marker expression

The expression of T cell activation and inhibitory receptors plays crucial roles in regulating T cell immunity[166]. The overexpression of inhibitory receptors by viral-specific T cells during chronic infection has been shown to cause their functional impairment, also known as "T cell exhaustion"[126,127]. Moreover, the blockade of the inhibitory receptor PD-1 with its

ligand PD-L1 has been found to restore the capacity of viral-specific T cells to proliferate, secrete cytokines, kill infected targets and control viral replication[126]. Further, CD8 T cell exhaustion during chronic viral infection has been found to be coordinated by multiple inhibitory receptors including PD-1, LAG-3 and TIM-3[167]. On the other hand, findings that PD-L1-deficiency results in lethal immunopathology demonstrate that PD-1/PD-L1 signaling acts a protective brake on the immune system[126]. Altogether, these studies indicate that the aberrant function of inhibitory receptors may diminish or exaggerate T cell immunity.

To investigate whether MALT1 regulates the levels of activation or inhibitory receptors, splenic effector T cells from wild type and *Malt1*$^{-/-}$ mice at Day 8 post-LCMV infection were stained with CD69, PD-1, TIM-3 and LAG-3, and analyzed by flow cytometry. Whole splenic CD8 T cells from wild type and *Malt1*$^{-/-}$ mice expressed similar levels of CD69, PD-1, TIM-3 and LAG-3 upon acute LCMV infection (*Figure 3.6*). Further, MALT1-deficient GP33^{+} and NP396^{+} CD8 T cells also exhibited comparable levels of CD69, TIM-3 and LAG-3 relative to wild type viral-specific CD8 T cells. Together, these results suggest that MALT1 is not required for appropriate expression of activation and inhibitory markers on T cells following acute LCMV infection.

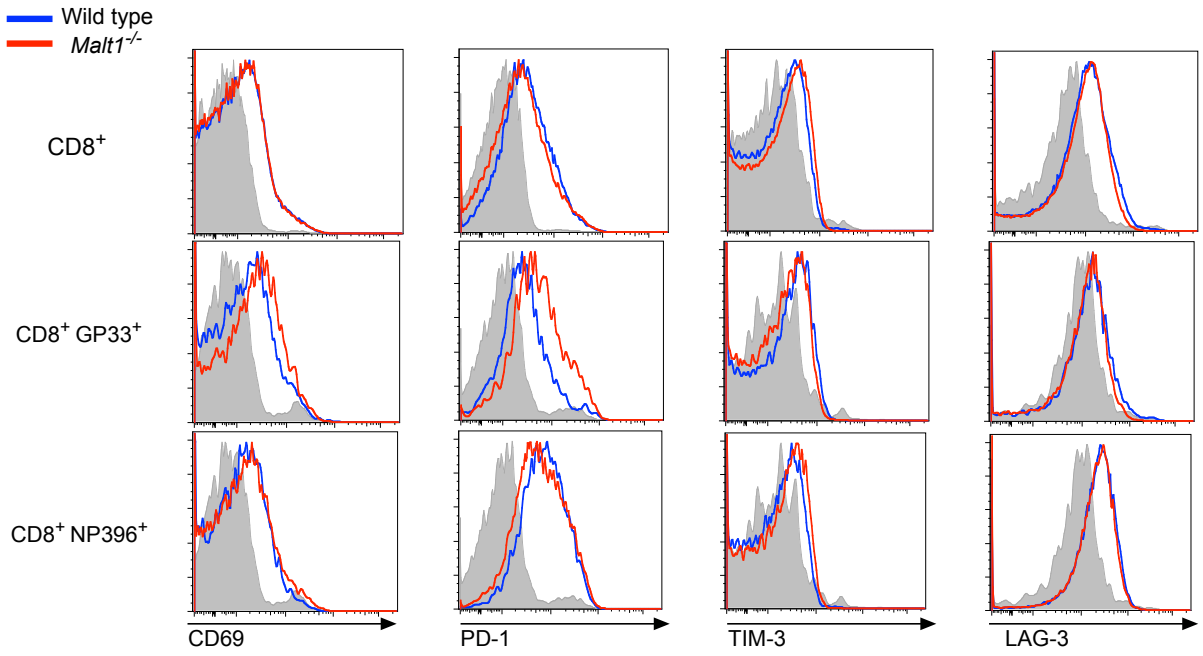

Figure 3. 6 MALT1 is dispensable for expression of activation and inhibitory receptors. On day 8 post-infection with LCMV Armstrong, whole splenic and viral-specific CD8 T cells from wild type and *Malt1*$^{-/-}$ mice were stained with activation (CD69) and inhibitory (PD-1, TIM-3 and LAG-3) surface receptors along with either GP33/D^{b}- or NP396/D^{b}-tetramers. Grey shade, blue line and red line represent FMO (background), wild type and *Malt1*$^{-/-}$ mice respectively.

3.4 Discussion

Mutations in *MALT1* result in a primary immunodeficiency disease associated with severe, recurrent infections and immune-dysregulation[112]. However, MALT1-deficient mice do not show signs of spontaneous inflammation, immune dysregulation or autoimmunity despite exhibiting a sharp reduction in Treg cells. One plausible explanation for some of the differences in immune phenotype between MALT1-deficient humans and *Malt1*$^{-/-}$ mice is the limited exposure of laboratory mice to pathogens[155]. Moreover, laboratory mice are housed under unusually clean conditions in specific pathogen-free facilities and many studies have shown that microbes play a major role in training and shaping the immune system[155]. Significantly, a recent

report demonstrated that simply co-housing laboratory mice with either pet store or feral mice (and their associated pathogens) shifted their blood cell gene expression patterns to more closely resemble adult rather than neonatal humans[155]. In this chapter, we infected *Malt1*$^{-/-}$ mice with the acute murine pathogen LCMV Armstrong and investigated their anti-viral T cell immunity relative to wild type mice.

Previous studies have established that CD4 T cells play important roles in viral immunity. CD4 T cells offer effector function against viral infection through production of cytokines, and also by promoting the expansion and differentiation of other immune cells[168-170]. We found that *Malt1*$^{-/-}$ CD4 T cells exhibited severely impaired IL-2 secretion upon LCMV infection compared to wild type CD4 T cells, and are consistent with finding that MALT1 paracaspase activity regulates IL-2 production[101]. In addition, *Malt1*$^{-/-}$ mice displayed greatly reduced numbers of whole and viral-specific (GP61-peptide reactive) CD4 T cell effectors capable of producing IFN-γ and TNF-α as compared to wild type mice. These findings suggest that either MALT1 is required for the generation of viral-specific CD4 T cells or that activated CD4 T cells require MALT1 for production of IFN-γ and TNF-α. Moreover, previous work has shown that MALT1-dependent NF-κB signaling promotes expression of these cytokines by T cells[97,100]. Collectively, our study demonstrates the indispensable roles of MALT1 in anti-viral CD4 T cell immune responses.

Intracellular pathogens like viruses trigger pathogen-specific naïve CD8 T cells to undergo massive clonal expansion and differentiation that is necessary to clear infection[158,171,172]. Upon LCMV infection, both wild type and *Malt1*$^{-/-}$ mice had greatly expanded splenic CD8 T cell populations and possessed similar numbers of viral-specific NP396- and GP33-reactive CD8 T cell effectors. In addition, MALT1-deficient mice displayed comparable or greater numbers of

dual-functional (IFN-γ^+TNF-α^+) and mono-functional (IFN-γ^+ or TNF-α^+) CD8 T cells relative to wild type mice upon stimulation with the LCMV-MHC class I-specific peptides GP33, NP396 and GP276. Consequently, these findings indicate that MALT1 is not required for the generation or the effector function of viral-specific CD8 T cells.

In response to viral infection, CD8 T cells undergo clonal expansion[131]. Simultaneously expanded CD8 T cells differentiate into memory precursor effector T cells (MPEC: CD127^{hi}KLRG1^{lo}) and short-lived effector cells (SLEC: KLRG1^{hi}CD127^{lo})[164,165,173]. MPECs become long-lived memory CD8 T cells, providing long term protection against repeated antigen challenge. On the other hand, SLEC are often described as terminally differentiated effector T cells and die by apoptosis. In this study, we found that viral-specific CD8 T cells from *Malt1*$^{-/-}$ mice exhibited increased MPEC populations and double positive effector (CD127^{hi}KLRG1^{hi}) T cells, but showed reduced numbers of SLECs. This phenotype may reflect the finding that IL-2 signaling is important for SLEC differentiation by regulating the transcription factor T-bet, which is important in normal cytotoxic T cell (Tc1) development[75,174]. Upon LCMV infection of IL-2R deficient mice, fewer KLRG1^{hi} and T-bet expressing CD8 T cells develop and CD8 T cell exhibit poor anti-viral immune responses[175]. Hence, it is possible that MALT1 deficiency contributes to impaired IL-2 production by helper CD4 T cells[176] or CD8 T cells themselves might be responsible for altering CD8 T cell differentiation; skewing towards MPEC phenotype, rather than the SLEC phenotype. Based on our findings, we propose that MALT1 may regulate the decisions of CD8 T cells to become MPECs or SLECs upon LCMV infection.

The expression of activation and inhibitory markers on T cells has been investigated and contrasted during acute and chronic LCMV infection[177,178]. Viral-specific CD8 T cells modestly upregulate activation and inhibitory receptors upon acute infection and downregulate these

markers upon viral clearance[177,178]. By contrast, chronic LCMV infection, is associated with high levels of inhibitory receptors (PD-1, TIM-3 and LAG-3) and results in dysfunctional viral-specific T cells, a phenomenon known as T cell exhaustion[168,126,127]. Inhibitory receptors are thought to be critical for immune regulation as PD-L1-deficient mice succumb to lethal immunopathology upon chronic LCMV infection[131]. However, our analyses did not observe noticeable differences in the expression of inhibitory receptors by whole splenic- or viral-specific CD8 T cells between wild type and *Malt1*$^{-/-}$ mice, suggesting that MALT1 does not contribute to the regulation of inhibitory receptors by CD8 T cells, at least, during acute viral-infection. Collectively, our findings from Chapter 3 have shown that MALT1 plays a crucial role in the function of anti-viral CD4 T cells but appears dispensable for the expansion and function of viral-specific CD8 T cells upon acute viral infection.

Chapter 4: Conclusion

4.1 Conclusions

MALT1 plays a critical role in signal transduction from antigen receptors on the surface of T and B cells to nuclear NF-κB activation, which is necessary for mediating lymphocyte activation, proliferation and differentiation[97]. MALT1 modulates immune responses through two functions: scaffolding and paracaspase activity. MALT1 act as a scaffolding molecule bringing CARD11 and BCL10 to form CBM complex, and as a paracaspase cleaving substrates to regulate NF-κB signaling. Recently, there are several reported cases of primary immunodeficiency patients with MALT1-deficiency, suggesting that *MALT1* mutations are causative of PID and that MALT1 plays key roles in immune system function[111-113].

Here, we investigated MALT1 function in T cell immunity using a *MALT1* mutant patient's primary T cells. We observed that our patient's CD4 T cells exhibited a normal frequency of Tregs, despite impaired abilities to produce IL-2. Defective IL-2 production in CD4 T cells may be due to diminished MALT1-mediated NF-κB signaling. Moreover, the crucial role of IL-2 in Treg maintenance and homeostasis suggests the possibility that the patient's Tregs may not be functional and that the patient's inflammatory disease may potentially be a consequence of impaired Treg function. However, further investigation of the function of our patient's Treg cells is not possible given that she underwent HSCT. In addition, we found that the patient T cells exhibited diminished IFN-γ, TNF-α and IL-17 secretion and such defects may contribute to chronic or recurrent viral, bacterial and fungal infections. Finally, our T cell signaling experiments revealed that our MALT1 patient T cells possessed greatly reduced MALT1 scaffolding and paracaspase activity, suggesting that her *MALT1* mutations may be acting as a hypomorphic alleles.

MALT1 patients and *Malt1*$^{-/-}$ mice share several immunological features including defective NF-κB signaling and T cell proliferation upon TCR stimulation[114,97,115]. However, *MALT1* patients experienced repeated viral infections, including CMV, whereas *Malt1*$^{-/-}$ mice do not present with obvious immune activation or spontaneous inflammation. The apparent discrepancy in phenotype between MALT1-deficient humans and mice might be due to the housing of these mice under specific pathogen-free conditions. Hence, wild type and *Malt1*$^{-/-}$ mice were infected with LCMV to examine the role of MALT1 in anti-viral T cell immunity. For control of LCMV infection, viral-specific CD8 T cells must undergo expansion and differentiation that is associated with the acquisition of effector functions. As virus-specific CD8 T cells proliferate, they form two subsets called "short-lived effector cells (SLEC)" and "memory precursor effector T cells (MPEC)" with the former required to combat the current infection while the latter is necessary for T cell memory to fight future infection. Interestingly, we found that MALT1 is dispensable for both formation and effector function of viral-specific CD8 T cells. However, *Malt1*$^{-/-}$ CD8 T cells exhibited alterations in the expression of L-selectin, a molecule important in lymphocyte trafficking, and the distribution of SLEC-like and MPEC-like cells. Consequently, further study may reveal some subtle phenotypes in *Malt1*$^{-/-}$ CD8 T cell differentiation.

Next, we examined viral-specific CD4 T cell responses by enumerating CD4 T cells specific for major immunodominant LCMV-specific MHC class II H-2A^{b}-restricted GP61-80 peptide. We observed that *Malt1*$^{-/-}$ mice displayed severely impaired helper CD4 T cell responses upon LCMV infection. Specifically, we found that *Malt1*$^{-/-}$ mice showed a severe decrease in the number and function of GP61-specific CD4 T cells relative to wild type mice that could secrete cytokines IL-2, IFN-γ and TNF-α. As CD4 T cells are important for the stability and function of

memory CD8 T cells[176], MALT1-deficiency could predispose to recurrent infections through defective CD4 T cell helper responses failing to sustain memory CD8 T cell responses. Consequently, our results suggest that MALT1 is indispensable for anti-viral CD4 T cell immune responses. Altogether, the findings presented in this thesis reveal the pivotal role that MALT1 plays in CD4 T cell immunity, and provide insight into how mutations in *MALT1* contribute to T cell-mediated immune dysregulation.

4.2 Future directions

In Chapter 2, we characterized the function of MALT1 in CD4 T cell immunity using primary CD4 T cells from the patient, sibling, and control individuals. One interesting finding was that the patient's CD4 T cell population contains a normal frequency of Tregs despite the impaired IL-2 production, raising the question of whether these Tregs are functional. To investigate the suppressive function of these T cells, we propose to analyze Cytotoxic T-lymphocyte antigen-4 (CTLA-4) expression. CTLA-4 is constitutively expressed on Tregs, and is upregulated on activated conventional T cells[179]. CTLA-4 is homologous to the T cell co-receptor CD28, and share the same ligands, CD80/86, which are expressed on APCs[180]. Binding of CD28 to CD80/86 delivers activation signals to T cells, which are important for cell proliferation and function. By contrast, CTLA-4 and CD80/86 engagement sends inhibitory signals to T cells, and suppresses immune activation. CTLA-4 has a greater affinity to CD80/86 compared to CD28, enabling them to outcompete CD28 for the same ligands[179]. CTLA-4 can also capture CD80/86 expressed on APCs via trans-endocytosis to further limit CD28-mediated T cell co-activation[181].

The inhibitory function of CTLA-4 contributes to the suppressive activity of Tregs. CTLA-4 deficiency in Tregs is strongly associated with primary immunodeficiency diseases such as IPEX syndrome[68,94]. CTLA-4 deficient mice develop lethal lymphoproliferative disorders at an early age, showing the crucial role of CTLA-4 in immune regulation[182,183]. Charbit-Henrion et al. reported that MALT1-deficiency is a potential cause of an IPEX-like syndrome because MALT1-deficient patients exhibit severe dermatitis and enteropathy, which are common phenotypes observed in IPEX patients[120]. Collectively, these findings suggest that investigation of CLTA-4 expression in our patient's T cells may elucidate the nature of her mutant MALT1 protein function and her inflammatory phenotype. Moreover, a better understanding of how MALT1 regulates CTLA-4 may reveal that MALT1 is a potential therapeutic target for immune dysfunctions. To understand the role of MALT1 in regulating CTLA-4 expression on T cells, we specifically propose to:

1. Assess the expression of CTLA-4 in patient's CD4 T cells.
2. Compare CTLA-4 expression in patient's CD4 T cells and MALT1 inhibitor (Mepazine)-treated control CD4 T cells.
3. Analyze CLTA-4 expression in *Malt1*$^{-/-}$ T cells.

In Chapter 3, we assessed the anti-viral immune responses of MALT1-deficient mice. Although *MALT1* mutant patients exhibit combined immunodeficiency diseases with immune dysregulation, *Malt1*$^{-/-}$ mice do not present a constitutive immune activation or immunodeficient phenotype[93,97]. The discrepancy in phenotype between *MALT1* mutant patients and *Malt1*$^{-/-}$ mice may due to a restricted pathogen exposure. Hence, we infected these mice with LCMV to investigate their immune responses against viral pathogen. We demonstrated that MALT1 plays

an important role in the anti-viral immune response of CD4 T cells. In contrast, MALT1 is dispensable for the expansion and effector function of viral-specific CD8 T cells. In addition, *MALT1* mutant patients have experienced repeated infections with various pathogens, suggesting impaired memory T cell development (*Table 1*). These findings suggest the need for future studies on viral clearance and memory T cell development. We propose to:

1. Measure viral loads at various times upon LCMV Armstrong infection
2. Assess memory T cell development upon long term exposure to LCMV Armstrong
3. Assess memory T cell function by analyzing cytokine production upon LCMV-specific peptide stimulation

LCMV Clone 13 is a chronic strain, and a variant from the parent strain Armstrong. Clone 13 causes mild, yet persistent cytotoxic T cell responses in the host leading to effector T cell exhaustion and unsuccessful virus control[156,158,177]. These findings, along with our patient's chronically infected phenotype, suggest that the immune response of *Malt1*$^{-/-}$ mice to chronic viral infection may reflect our *MALT1* mutant patient's immune system. This provides a suitable model for studying the immune responses of MALT1-deficient patients. We propose to introduce LCMV Clone 13 to *Malt1*$^{-/-}$ mice, and perform the following:

1. Measure the production of viral-specific T cells by LCMV-tetramer staining
2. Evaluate the functional status of viral-specific T cells by measuring cytokine secretion upon LCMV-specific peptide stimulation
3. Assess the development of memory T cells upon long-term exposure to LCMV Clone 13
4. Determine the viral loads at various time points post-infection

Furthering our understanding of MALT1 function in T cell immunity will advance our knowledge of immune regulation, and potentially suggest the development of therapies that target MALT1 for the treatment of T cell-mediated immune disorders.

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