Chronic viral infection impacts cell fate decisions in the thymus

By

Stefanie Valbon Fernandes Garcia

Department of Microbiology and Immunology, McGill University, Montreal

August 2020

A thesis submitted to McGill University in partial fulfilment of the requirement of the degree of Master of Science.

Table of Contents

Acknowledgements

I would first like to thank my supervisor and mentor Dr. Martin Richer, for accepting me into his laboratory and for all the help during the development of my project and my scientific career. To my mentor Dr. Stephanie Condotta for her help when designing and performing experimental procedures and for sharpening my presentation skills. I wish to express my sincere gratitude to both, for not only providing me with instrumental guidance and mentorship since the start but most importantly for your continuous advice and trust. I will be forever thankful for your teaching as it has truly sparked the love for scientific research that is now present inside of me.

I would like to thank the current and past members of the Richer Lab, for providing me with an excellent environment to learn and develop my scientific skills. I would like to pay special regards to Ryan Pardy for his constant support and willingness to help through every single step, since day one. I am very grateful for your friendship, which I am sure will last a long time.

To the members of my M.Sc. committee, Dr. Heather Melichar and Dr. Judith Mandl, for all your insights and guidance. By pushing me out of my comfort zone, you have helped me gain self-confidence while expanding my critical thinking abilities. I am very fortunate and truly thankful for all your mentorship.

Thank you to Camille Stegen and Julien Leconte for your exceptional assistance, during the execution and troubleshooting flow cytometry sorting experiments.

I would like to show my utmost gratification to my loving parents, Selma Valbon and André Garcia, because without their love and care I would never have reached this milestone. Thank you for nourishing my curiosity from a young age. Muito obrigada por estarem sempre ao meu lado, e por tudo o seu amor. To my brothers, Rafael and Vinícius Valbon, for their constant support and friendship, even if from far away. Thank you for helping me see the scientist that is present inside of me. To Kévin Millar, for staying by my side through the easy and challenging times. Merci de tout arrêter et être attentif lorsque je veux parler de mes recherches et experiences. J'apprécie vraiment tous tes conseils et encouragements.

Lastly, I would like to acknowledge all the animals used in this study and staffs that helped ensured their wellbeing. I would like to recognize the invaluable role that each animal has provided for the development of this project. I would also like to appreciate their immense contribution to all scientific research, for allowing us to advance our scientific knowledge and for helping us improve people's health.

Abstract (English)

The thymus is a primary lymphoid organ with a distinct organization to provide cues for the generation of a functional and self-tolerant T cell repertoire. Environmental stresses, such as infections, can lead to thymic involution, which is marked by a decrease in tissue mass and changes in thymic architecture. However, the impact of chronic viral infections on thymocyte development remains poorly understood. Herein, we show that in addition to thymic involution, chronic (LCMV Clone (Cl) 13), but not acute (LCMV Armstrong), viral infection leads to a double negative (DN)1 developmental block which was not fully restored as late as 20 days post-infection. This block was marked by an increase in $B220^{+}$ cells with a pre-pro B cell phenotype, which further developed to the more mature pro B cell stage at later time points. This transition correlated with increased expression of *Tnfsf13b* (BAFF encoding gene) in the thymus, a cytokine involved in B cell survival. At the DN1 stage, Notch1 signaling is crucial for T cell lineage commitment, since the abrogation of this signal promotes thymic B cells generation. We further show that chronic viral infection leads to an increase in Lunatic Fringe, a protein capable of modifying the interaction between Notch1 receptor and its ligand. Moreover, this phenotype was dependent on type I interferon, as a less severe DN1 block and a complete abrogation of the generation of B cell-like cells in the thymus was observed following LCMV Cl13 infection in mice lacking the type I interferon receptor. Together, our data highlight a novel impact of chronic viral infection on thymocyte development. Long term impairment of T cell development and redirection to a B cell lineage may lead to a decrease in available to T cells to counter infections and the generation of autoreactive B cells ultimately causing autoimmune diseases.

Abstract (French)

Le thymus est un organe lymphoïde primaire avec une organisation distincte pour fournir des signaux pour la génération d'un répertoire de cellules T fonctionnel et auto-tolérant. Les stress environnementaux, tels que les infections, peuvent entraîner une involution thymique, qui est marquée par une diminution de la masse tissulaire et des changements dans l'architecture thymique. Cependant, l'impact des infections virales chroniques sur le développement des thymocytes reste mal connu. Ici, nous montrons qu'en plus de l'involution thymique, une infection virale chronique (LCMV Clone (Cl) 13), mais pas une infection aiguë (LCMV Armstrong), cause un bloc développemental au stage double négatif (DN) 1 qui n'est pas complètement restauré aussi tard que 20 jours après l'infection. Ce bloc est marqué par une augmentation des cellules $B220^{+}$ avec un phénotype de cellule B pre-pro, qui s'est ensuite développé vers le stade plus mature de cellule pro B. Cette transition était en corrélation avec une expression accrue de *Tnfsf13b* (gène codant BAFF) dans le thymus, une cytokine impliquée dans la survie des lymphocytes B. Au stage DN1, la signalisation Notch1 est cruciale pour l'engagement de la lignée des cellules T, car l'abrogation de ce signal favorise la génération de cellules B thymiques. Nous montrons en outre que l'infection virale chronique conduit à une augmentation de Lunatic Fringe, une protéine capable de modifier l'interaction entre le récepteur Notch1 et son ligand. De plus, ce phénotype dépendait de l'interféron de type I, car un bloc DN1 moins sévère et une abrogation complète de la génération de cellules de type B dans le thymus ont été observés à la suite d'une infection par LCMV Cl13 chez des souris déficientes pour le récepteur de l'interféron de type I. Ensemble, nos données mettent en évidence un nouvel impact de l'infection virale chronique sur le développement des thymocytes. Une altération à long terme du développement des lymphocytes T et une redirection vers une lignée de lymphocytes B peuvent conduire à une réduction dans le nombre de cellules T en périphérie et à la génération de lymphocytes B autoréactifs provoquant finalement des maladies auto-immunes.

List of Abbreviations

AIDS:	acquired immunodeficiency syndrome
AIRE:	autoimmune regulator
ATI:	acute thymic involution
BAFF:	B cell-activating factor of the tumor necrosis factor family
BrdU:	5-bromo-2'-deoxyuridine
CCR7:	C-C Motif chemokine receptor type 7
CCR9:	C-C Motif Chemokine Receptor 9
CD:	cluster of differentiation
CSL:	CBF-1, Suppressor of Hairless, Lag-2
cTEC:	cortical thymic epithelial cells
CXCL12:	C-X-C motif chemokine 12
CXCR4:	C-X-C Motif Chemokine Receptor 4
DC:	dendritic cells
Dll1:	delta-like ligand 1
Dll4:	delta-like ligand 4
DN:	double negative
DNA:	deoxyribonucleic acid
DP:	double positive
dpi:	days post infection
eIF2α:	eukaryotic initiation factor 2α
ETP:	early thymic progenitor
FACS:	fluorescence-activated cell sorting
Fig:	Figure
Flt3:	fms-like tyrosine kinase 3
FTOC:	fetal thymus organ culture
GlcNAc:	N-Acetylglucosamine
gMFI:	geometric mean fluorescence intensity
GP:	glycoprotein
HCV:	hepatitis C virus
Hes1:	hairy and enhancer of split 1
HEV:	high endothelial venules
HIV:	human immunodeficient virus
i.p.:	intraperitoneal
i.v.:	intravenous
ICN:	intracellular domain of Notch1
IFN:	interferon
IFN-I:	type I interferon
IFNAR:	interferon α/β receptor
IL:	interleukin
IRF:	IFN regulatory factor
ISGs:	interferon stimulating genes
Jag1:	jagged ligand 1
Jag2:	jagged ligand 2
JAK:	janus Kinase
LCK:	lymphocyte-specific protein tyrosine kinase

LCMV:	Lymphocytic Choriomeningitis virus
LCMV Arm:	Lymphocytic Choriomeningitis virus Armstrong strain
LCMV Cl13:	Lymphocytic Choriomeningitis virus Clone 13 strain
Lfng:	lunatic fringe
LPS:	lipopolysaccharide
M-CSF:	macrophage colony-stimulating factor
MHC-I:	major histocompatibility complex class I
MHC-II:	major histocompatibility complex calls II
MHC:	major histocompatibility complex
mRNA:	messenger ribonucleic acid
mTEC:	medullary thymic epithelial cells
nAChR:	nicotinic acetylcholine receptor
NK:	natural killers
NP:	nucleoprotein
NR3C1:	nuclear receptor subfamily 3 group C member 1
PAMP:	pattern-associated molecular patterns
PFU:	plaque-forming units
PRR:	pattern recognition receptor
pMHC:	peptide major histocompatibility complex
RAG:	recombination activating gene
RBC:	red blood cells
RBPJ:	recombination signal binding protein for immunoglobulin kappa J region
RLR:	RIG-I-like receptors
RNA:	ribonucleic acid
RT-qPCR:	reverse transcription quantitative polymerase chain reaction
SCF:	stem cell factor
SIV:	Simian immunodeficiency virus
SP:	single positive
STAT:	signal transducer and activator of transcription
TCR:	T cell receptor
TEC:	thymic epithelial cells
TLR:	Toll-like receptor
TN:	triple negative
Tnfsf13b:	tumor necrosis factor ligand superfamily member 13B
TRA:	tissue-restricted self-antigen
TYK2:	tyrosine kinase 2
WT:	wild-type

Contribution of Authors

For this project, I have planned, performed and analyzed all the experiments presented. Dr. Stephanie A. Condotta prepared and tested viral titers for all the LCMV stocks used in the experiments, as well as provided guidance for the analysis of the experiments. The culture and maintenance of the B16-Blue cell line, as well as guidance for the execution of this assay was provided by Ryan D. Pardy. Dr. Martin J. Richer helped design, plan and execution the experiments and edited this thesis.

List of Figures

Chapter I: Literature Review and General Introduction

This chapter was written by SFV and edited by MJR. The section "IFN-I: production, function and signaling" was written by SFV and modified from a previously published section in the following review[1]: Pardy, R.D., Valbon, S.F. & Richer, M.J. Running interference: Interplay between Zika virus and the host interferon response. *Cytokine* **119**, 7-15 (2019).

Adaptive immune system

Our immune system is a highly regulated and interconnected system which provides us with protection against microorganisms and cancerous cells. The immune system can be divided into two main components, the innate and the adaptive system; although innate immunity is critical during the early onset of viral infection, the adaptive immunity provides a more specific and robust response against the pathogen. This occurs because the adaptive immunity is characterized by the ability of cells to recognize and respond to specific antigens. These cells are known as T and B lymphocytes[2]. During their development, they generate a vast diversity of antigen-specific receptors which is then expressed on their surface. The human body is composed of around $2x10^{12}$ lymphocytes[3], with a vast diversity of receptor specificity. Together, these cells enable the immune system to respond to virtually any antigen it encounters during the host's lifetime. Nevertheless, the ability of lymphocytes to generate this diverse receptor repertoire must be coupled with the lack of reactivity to self-antigens[4]. Recognition of self-antigen can be extremely detrimental to the host, since this can lead to the establishment of autoimmune diseases. Therefore, lymphocyte development must be tightly regulated to generate cells that can react to foreign antigens while preventing the generation of self-reactive lymphocytes that could ultimately lead to immunopathology. Although both T and B cells are part of the adaptive immune system, they are generated at a different location within our body. B cell development starts in the bone marrow, while T cell development occurs mostly in the thymus[4].

T cell development

The thymus is crucial for the development of T cells as it provides signals and cues required for the generation of a functional and self-tolerant T cell repertoire. The thymus is composed of two lobes which are further divided into small lobules with 3 distinct regions: the medulla, the cortex and the corticomedullary region. Cells present in this organ originate from both non-

hematopoietic and hematopoietic lineages[5]. The former generates the stromal part of the organ, which is composed of epithelial, endothelial and mesenchymal cells. On the other hand, the hematopoietic cells are bone marrow-derived cells including thymocytes, dendritic cells (DCs), natural killer (NK) cells and a small population of B cells[5]. The interaction of thymocytes with stromal cells is essential for the development of T cells, but it is also crucial for the maturation of the epithelial cells. Therefore, the constant interaction between the hematopoietic and non-hematopoietic cells is essential for the proper function of the thymus, elucidating the importance of an organized thymic network for the proper development of T cells[6].

In order for T cells to be generated, bone marrow-derived progenitors cells enter the thymus via the high endothelial venule (HEV) present in the corticomedullary region. At this stage, cells lack the expression of cluster of differentiation (CD)4 and CD8 co-receptors and are termed double negative (DN) cells. The DN stage can be further subdivided into 4 groups, DN1 to DN4, based on the expression of CD44 and CD25 surface molecules[5, 7]. Although most DN1 cells seed the DN2 population for further thymocyte development, the DN1 stage is heterogeneous and it has been shown to give rise to other cell types, such as B cells, NK cells, macrophages and dendritic cells (DC)[8]. Once committed to the T cell lineage (α/β T cells), DN cells migrate towards the cortex and start the rearrangement of the T cell receptor (TCR). Thymocyte development continues during the double positive (DP) stage, in which cells are characterized by the expression of both CD4 and CD8 co-receptors[9]. Following successful rearrangement and expression of a functional TCR, DP cells move towards the medulla and become single positive (SP) cells, expressing either CD4 or CD8[9]. Self-tolerant SP cells migrate towards the corticomedullary region prior to their release into the periphery through blood vessels present in this location[9, 10].

One of the essential characteristics of T cells is their ability to generate TCRs with very diverse specificities. This is possible due to somatic DNA recombination, also known as gene rearrangement[2]. The TCR is composed of two chains with a variable and a constant region. The variable region makes up the antigen-binding site and the constant region is essential for effector and signalling functions[4]. The former is further divided into 2 or 3 types of gene segments, known as variable (V), diversity (D) and joining (J) segments[2]. The potential to generate a high diversity of antigen-binding sites occurs due to the presence of many copies of each segment in the germline genome. Each copy of the segment is separated by a spacer recombination signal sequence which is recognized by recombination activating gene (RAG)-1/2 complex[2]. This complex is able to nick

the DNA, ultimately ligating different copies of each segment, giving rise to the high protein diversity. As previously mentioned, the TCR is composed of two chains and can be classified as α/β TCR or a γ/δ TCR. Although similar, the TCR structure between these two subsets is not identical. Moreover, the minority of T cells that possess a γ/δ TCR undergo a distinct development pathway in the thymus[9]. For the purpose of this thesis, we will be analyzing the development of α/β T cells, and therefore whenever mentioning T cells, we will be referring to α/β T cells, unless otherwise indicated.

TCR recombination begins at the DN2 stage ($CD44^+$ $CD25^+$) where cells rearrange the β-chain starting with D to J segments, followed by V to DJ segments. At the DN3 stage ($CD44^-$ $CD25^+$) cells express a pre-TCR complex, which is composed of the rearranged β-chain, an invariant pre-TCR α-chain and CD3 signalling molecules[11]. The pre-TCR complex enforces β-selection, which is marked by the suppression of further β-chain rearrangement, and allelic exclusion. These processes ensure that each cell expresses a single β-chain specificity[5]. DN3 cells that do not undergo β-selection die via apoptosis[12]. Signalling through the pre-TCR complex allows for survival and proliferation[5]. Proliferation at this stage is essential to increase TCR diversity and further thymocyte development since a unique β-chain can be further paired to many different rearranged α-chains[13]. Next, cells down-regulate the expression of CD25, transiently becoming DN4 cells ($CD44^-$ $CD25^-$), prior to the upregulation of both CD4 and CD8 co-receptors. The alpha-chain, which is composed of only 2 segments (V and J), is rearranged at the DP stage to produce the α/β TCR[2]. Cells then migrate towards the medulla, become SP cells and are exported to the periphery where they can respond to invading pathogens[10].

Although a broad TCR repertoire is essential to provide host protection, thymocytes undergo important checkpoints to ensure the removal of any cells expressing a dysfunctional, or a highly self-reactive TCR[4]. Positive selection occurs at the DP stages, and it ensures the survival of cells expressing a functional TCR. In order for the TCR to signal, it needs to recognize a peptide bond to a major histocompatibility complex (MHC), known as peptide MHC (pMHC) complex. In the thymus, cortical thymic epithelial cells (cTECs) express MHC bound to self-peptide. Low affinity interaction of DP TCR to self-pMHC complex provides survival signals allowing their differentiation into SP cells. However, if this interaction is unsuccessful or too strong, DP cells die by neglect (or lack of survival signals)[14]. Positive selection ensures that the TCR is able to successfully recognize self-MHC while preventing strong interaction with self-peptides. There are

two classes of MHC, class I (MHC-I) and class II (MHC-II). Although cTECs can express both classes, DP cells that recognize peptide in the context of MHC-I become SP CD8 T cells and MHC-II become SP CD4 T cells[4]. SP cells undergo a second checkpoint known as negative selection, in which cells recognizing self-antigen with high affinity are removed[9]. Medullary thymic epithelial cells (mTECs) express a transcription factor known as autoimmune regulator (AIRE). This molecule allows the transcription of a wide range of tissue-restricted self-antigen (TRA) that otherwise would not be express in the thymus[14]. This ensures that SP cells can test their self-reactivity to ultimately all the proteins present in our body. Therefore, negative selection is crucial to allow for the generation of a self-tolerant T cell repertoire. Only around 2% of DP thymocytes can survive these checkpoints which are essential to prevent immunopathology[4].

Regulated migration of thymocytes through the different compartments of the thymic environment allows for specific signals to be delivered at the required developmental stage. This migration is mainly orchestrated by chemokine gradients. Chemokines are small molecules (8-10 kDa) capable of inducing chemotaxis, which is characterized by the movement of cells towards stimuli. There are 4 different families of chemokines and different members act through specific receptors present at the cellular surface[15]. In the thymus, chemokines can be either secreted or anchored in the extracellular matrix. Bone marrow progenitor cells express both CCR7 and CCR9 receptors which are important for the entrance of these cells into the thymus[5]. Although double knock-out mouse studies ($CCR7^-$ $CCR9^-$) showed a 100-fold decrease of progenitor cells in the thymus, no decrease in the thymic output was observed. This observation was coupled with an increase in the proliferation of developing thymocytes, suggesting that mechanisms are put in place to ensure that a decrease in progenitor numbers does not affect the overall thymic output[16]. DN1 and DN2 cells are also known to express CXCR4, which binds to CXCL12 expressed on cTECs. Although studies have been somewhat contradictory, it is thought that the expression of CXCR4 might aid the migration of DN towards the cortical regions of the thymus[5]. Following positive selection, DP cells upregulate the expression of CCR7 before their maturation into SP cells. SP cells expressing CCR7 migrate towards the medulla where ligands for this receptor are found[5]. Together, these observations highlight the importance of thymic architecture and the precise orchestration of thymocyte migration patterns for successful T cell generation.

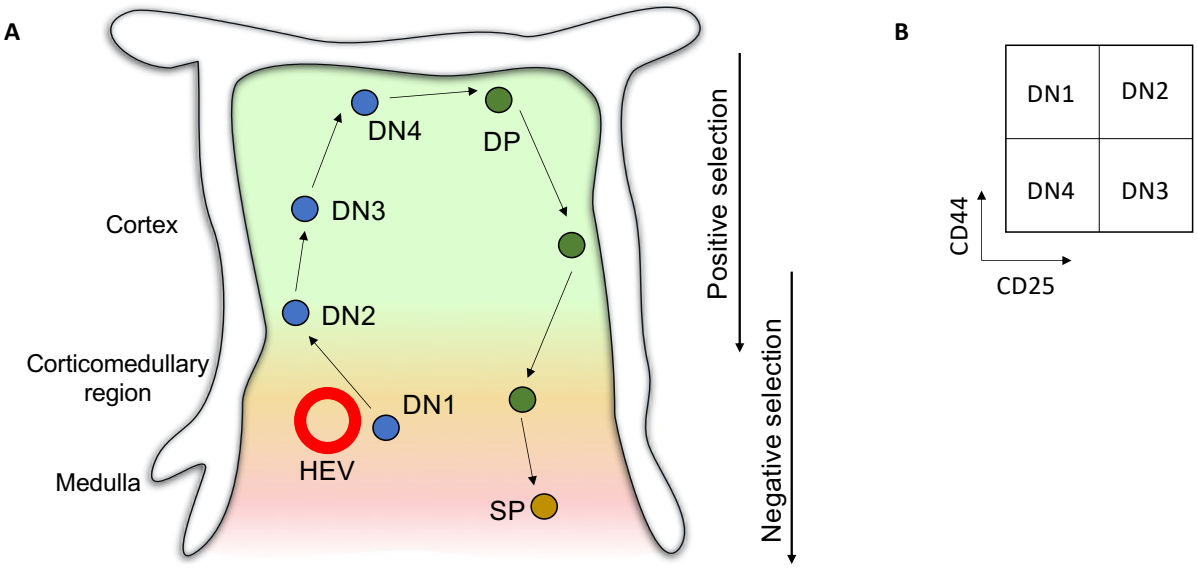

Figure I-1: Representation of T cell development in the thymus. (A) Bone marrow progenitor cells enter the thymus via the high endothelial venules (HEV). Development starts at the double negative (DN) stage where cells lack the expression of CD4 and CD8 co-receptors. This group can be subdivided into DN1 to DN4. Cells up-regulate the expression of CD4 and CD8 as they become double positive (DP). At the end of development, cells will either downregulate the expression of CD4 or CD8 becoming single positive (SP) cells. (B) Representation of flow cytometry gating of the DN subgroups. Cell are gating as following: $CD44^{+}$ $CD25^{-}$ (DN1), $CD44^{+}$ $CD25^{+}$ (DN2), $CD44^{-}$ $CD25^{+}$ (DN3) and $CD44^{-}$ $CD25^{-}$ (DN4).

Thymic B cells

Although the majority of bone marrow-derived progenitor cells entering the thymus fate into the T cell lineage, studies have shown that a few progenitor subsets retain the ability to generate B cells in this environment. The most immature DN development stage, the DN1, is more heterogenous than first thought, and therefore classifying this population using only the expression of CD44 and lack of CD25 can be somewhat misleading[17]. The expression of c-kit (CD117), a protein tyrosine kinase receptor for the stem cell factor (SCF), was shown to be differentially regulated in the DN population (also known, at that time, as triple negative (TN) due to the lack of expression of CD4, CD8 and CD3)[18]. Therefore, the expression of c-kit, together with CD24, was used to further subdivide the DN1 population into 5 different groups[17]. Although all 5 populations are known to be able to give rise to T cells, they do so with different proliferative capacities[17]. It is thought that each progenitor entering the thymus undergoes around 20 serial divisions, giving rise to 1 million cells in this organ. This high proliferative capacity is one of the hallmarks of DN1 cells. Out of the five groups analyzed, only DN1a (c-kit$^+$ CD24$^-$) and DN1b (c-kit$^+$ CD24^{lo}) showed the expected proliferative capacity of canonical T cell development. DN1a was observed to give rise to DN1b and together fate into the DN2 lineage. Moreover, these were the only two groups that could home to the thymus and give rise to detectable levels of T cells following intravenous injection[17]. Therefore, the DN1a and DN1b are believed to be part of the classical early thymic progenitor (ETP) cells which are characterized by CD3$^-$ CD4$^-$ CD8$^-$ CD25$^-$ CD44$^+$ c-kit$^+$ and are T cell lineage restricted[17]. DN1c (c-kit$^+$ CD24$^+$) is the only group that express c-kit and still retain the ability to generate B cells. On the other hand, the two remaining groups lack the expression of c-kit, however only DN1d (c-kit$^-$ CD24$^+$), and not DN1e (c-kit$^-$ CD24$^-$), have B cell generation capacity[17]. The view that bone marrow progenitor cells entering the thymus are not yet committed to a T cell lineage has been recently challenged. Studies using an *Rbpj*-inducible mouse model system suggest that the earliest bone-marrow progenitor cells entering the thymus are already committed to the T cell lineage[19]. It is important to note that the ability of each DN1 group to give rise to other cell types was mostly analyzed using the OP9 *in vitro* model of T cell development (explained below). Therefore, although a cell subset has the ability to generate B cells in culture, it doesn't mean that this cell will follow this route *in vivo*.

Thymic B cells were first identified in 1987 by Isaacson and colleagues, when thymic sections of patients undergoing heart surgery were analyzed. This study was the first one showing

that B cells are present in the thymic medulla under physiological conditions[20]. Thymic B cells were later observed in murine models, and their origin and function have since been the subject of extensive investigation[21]. It is now well accepted that naturally occurring thymic B cells are physiologically distinct from peripheral B cells. The former appears at the fetal stage day 14 which is before the generation of bone marrow B cells[22]. Studies using parabiosis and intravenous injections (i.v.) further elucidated that the splenic B cell compartment has no, or very minimal, contribution in the generation of thymic B cells[23, 24]. In addition, naturally occurring thymic B cells are also known to have a highly activated phenotype, expressing high levels of CD80, CD86, CD69, CD40 and MHC-II[25]. Although they are shown to be activated, they have low proliferation and antibody production capacity in response to lipopolysaccharides (LPS) *in vitro* when compared to splenic B cells[26]. Mature thymic B cells are only found in the medullary region of the thymus, mostly at the corticomedullary region. However, precursors ($B220^{lo}$ $CD43^{hi}$ IgM^{-}) are thought to also inhabit the cortical region, as B220 signals can be found throughout this region[23]. Together, these observations emphasize the differences between thymic and peripheral B cells.

Many mouse models have been used to understand the pathways in which thymic B cells are generated. An increase in thymic B cells is observed in $TCR\beta^{-/-}$ and $CD3^{-/-}$ mice, suggesting that T and B cell generation in the thymus might be the result of competition to different thymic niches[23, 27]. It is important to note, however, that although an increase in thymic B cells is observed in these mouse models, this increase in not proportional to the massive decrease in T cell frequency, illustrating the small niche for B cell generation in the thymus[25]. Therefore, under normal conditions, the thymus house a very small proportion of thymic B cells which are thought to be generated from a small population of DN1 cells.

Notch Signaling during T vs B cell fate decision

It is well accepted that early bone marrow progenitor cells, require Notch signalling to fate into the T cell lineage. As previously mentioned, whether this signalling is received in the bone marrow or the thymus is still the topic of debate. In the thymus, the Notch1 receptor expressed on the surface of DN1 cells interacts with ligands expressed by thymic epithelial cells (TECs). There are two families of Notch ligands expressed in the thymic microenvironment which are known as Delta-like ligands (Dll1, Dll4) and Jagged ligands (Jag1, Jag2)[28]. Following binding, the Notch receptor changes its conformation allowing for proteolytic cleavage of the intracellular and

extracellular domain to occur. The intracellular domain of Notch1 (ICN) undergoes different processing mechanisms, ultimately entering into the nucleus and binding to the transcription factor CSL (or the mouse analogue RBP-J)[29, 30]. This interaction recruits the transcription machinery and leads to the activation of different downstream target genes. At the early stage, these changes allow DN1 cell proliferation and for further thymocyte development, by acting on a gene target known as *Hes1*[31].

Multiple studies targeting different steps of the Notch signalling pathway have highlighted its importance on the T vs B cell fate decision. A study using conditional *Notch1*$^{-/-}$ mice showed that the absence of Notch1 leads to a DN1 block and a fate switch from T to B cell lineage[32, 33, 34]. The deletion of the RBP-J domain in mice also leads to a block in T cell development coupled with an increase in thymic B cells[35]. Moreover, transduction experiments conducted to ectopically express a constitutively activated form of Notch1 in hematopoietic-progenitor cells show the development of immature T cells in the bone marrow[36]. Together, these models illustrate the requirement of Notch signalling for T vs B cell fate decision. However, although genetically engineered models leading to the abrogation of Notch signalling increases the generation of thymic B cells, these are phenotypically different than naturally occurring thymic B cells. Analysis of the thymic B cells present in the conditional *Notch1*$^{-/-}$ mice model shows that these cells have a more immature phenotype and express markers, such as AA4, which are not usually present in naturally occurring thymic B cells. Moreover, in this knockout study, thymic B cells show a 10-fold increase in 5-bromo-2'-deoxyuridine (BrdU) incorporation compared to naturally occurring thymic B cells[34]. A study using *Cpa*-cre mice, in which Notch1 deletion occurs at the DN1-DN2 transition, reported higher levels of B cells in the thymus. Similar to the conditional *Notch1*$^{-/-}$ mice, these cells presented with a different phenotype (AA4^{+} CD19^{lo} B220^{lo}) than those of naturally occurring thymic B cells[37]. Additionally, Notch1 deletion induced by *Cpa*-cre is not 100% efficient. In this model, B cells present in the thymus with a similar phenotype to naturally occurring thymic B cells were shown to have retained a copy of Notch1[37]. Together, these results suggest that naturally occurring thymic B cells might require Notch1 signalling, while the abrogation of this signal leads to the generation of thymic B cells albeit with a distinct phenotype.

In summary, the adult thymus is composed of naturally occurring thymic B cells which are known to be different from splenic B cells. Bone marrow progenitor cells entering the thymus require Notch signalling to fate into the DN2 lineage for further T cell development. Mouse models

which are able to abrogate Notch1 signalling in the thymus shows an increase in the generation of thymic B cells. However, these cells possess a different, more immature, phenotype than naturally occurring thymic B cells. Lastly, all of these studies have analyzed the ability of DN1 cells to generate T cells under physiological conditions, and the possibility of a B cell fate switch under pathological conditions has not yet been elucidated.

Fringe proteins

Fringe protein were first identified in *Drosophila*, for having a crucial role during wing development. Modifications performed at the extracellular portion of the Notch receptor by fringe proteins lead to the modulation of receptor binding affinity to each family of Notch ligands. This regulation allowed for the proper development of wings in *Drosophila*[38, 39]. Fringe proteins are β 1,3 N-acetylglucosaminyl (GlcNAc) transferases which act within Golgi apparatus to add GlcNAc to O-fucose residues present on the extracellular potion of Notch receptors[40]. This modification increases the binding affinity of Notch to Delta-like ligands, while decreasing affinity to Jagged ligands. Therefore, fringe proteins play a crucial role in the regulation of Notch signaling.

In vertebrates, the fringe family of protein is composed of 3 members: Maniac, Radical and Lunatic (Lfng) Fringe. It has been shown that both Lunatic and Maniac Fringe receptor modification inhibit binding to Jag1 and enhance binding to Dll1, while Radical Fringe receptor modification enhance binding affinity to both ligands[40]. Due to the important role of Notch in the fate of DN1 cells, studies have been done to understand the role of fringe modification in the cell fate decision within the thymus. A study using a mouse strain to overexpress Lfng under the proximal LCK promoter, observed that the ectopic expression of this protein by DP cells impaired proper T cell development[41]. Under normal conditions, DP cells do not express Lfng, however, overexpression of Lfng in this model transform DP cells into "supercompetitors"[41]. This occurs, because Lfng modification is able to increase Notch1 affinity to Dll1, in which DP cells wouldn't otherwise bind. Therefore, this phenotype decrease the Dll1 niche availability to DN1 cells, resulting in a DN1 developmental block and an increase in the generation of thymic B cells[41]. Hence, this study illustrates how the modification of the extracellular portion of Notch1 by Lfng can impact the T vs B cell fate decision.

The OP9 System

The analysis of T cell generation using *in vitro* cultures provides researchers with the ability to study thymocyte development in a controlled environment while allowing experimental manipulations. As a result of the highly complex thymic environment, it was first thought that the generation of T cells *in vitro* could not be obtained from a monolayer culture[7]. Therefore, a model providing the essential cells and structures to allow T cell development *in vitro* known as fetal thymus organ culture (FTOC) was developed. In this model, fetal thymic lobes were treated with deoxyguanosine to deplete thymocytes and were maintained in a cell culture dish[42]. T cell development from the population of interest was analyzed by co-culturing progenitor cells on the thymic lobes.

Although FTOC is great for the analysis of T cell development, a thymus-independent model using a monolayer culture would allow for a simpler experimental layout while increasing cellular yield[7]. A clue that helped researchers generate a new model for T cell development *ex vivo*, came from the study of B cell development. Researchers were able to analyze the generation of B cell *in vitro* using the OP9 cell culture. OP9 is a bone marrow-derived preadipocyte cell line that originated from the *op/op* mouse line which lacks the expression of macrophage colony-stimulating factor (M-CSF). M-CSF allows for the proliferation and generation of macrophages from hematopoietic stem cells while interfering with lymphopoiesis[7]. Therefore, OP9 cultures, lacking this important factor, allow the development of B cells *in vitro*. As previously mentioned, Notch signalling is essential for the T-vs-B cell fate decision and although OP9 cells are known to express ligands from the Jagged-family, they lack the expression of delta-like ligands. Hence, Zúñiga-Pflücker and colleagues suggested that the missing factor preventing OP9 cells from generating T cell *in vitro* was delta-like ligands[43]. Using retrovirus transduction techniques to induce the expression of Dll1, they noticed that OP9 cells (now OP9-DL1) supported T cell development *in vitro* while preventing the generation of B cells[43]. This system is now widely used to analyze T cell development in a very controlled environment since it has a high cellular yield and allow for easy experimental manipulations. Although the OP9-DL1 system is great for the generation of large numbers of T cells, it is important to notice that it does have a few caveats. The OP9 cell line does not express MHC-II nor AIRE, therefore this *in vitro* model of T cell development does not take into account the effects of positive or negative selection[7].

Thymic Involution

Although the thymus is a crucial organ for the generation and maturation of T cells, it is well established that under some physiological and pathological conditions the thymus undergoes profound involution. Thymic involution, or thymic atrophy, is marked by a decrease in organ size and changes in thymic architecture ultimately impacting T cell development[44, 45]. This process can be further divided into two categories: chronic, or age-induced, and acute thymic involution. The former has been shown to start at an early age but peaking at the mid-phase of life (around 30-40 years old for humans and 9-12 months for mice) and it is marked by the substitution from lymphoid to adipose tissue[44, 45, 46]. This is an evolutionarily conserved mechanism that seems to be present amongst most vertebrates that possess a thymus, with the exception of some shark species[45, 47]. On the other hand, acute thymic involution (ATI) occurs in response to environmental stresses. ATI is different from chronic involution since the thymus is rapidly restored to its original size following the removal of the insult. ATI can occur following different stresses such as pregnancy, malnutrition, infections, sepsis, transplant rejection and cancer[44]. Although different conditions can lead to thymic involution, the mechanisms and consequences of this process in each situation are distinct.

During infections, the mechanisms of thymic involution can be broadly divided by the usage of two pathways: infection sensing pathways and inflammation-mediated pathways. The former occurs following the recognition of pattern associated molecular patterns (PAMPs) by TECs, which then direct thymic involution. The latter occurs due to byproducts of infection, such as the production and accumulation of cytokines, and can result in thymocyte apoptosis. Bacterial, parasite and fungal infections have been shown to lead to ATI, however, this thesis will focus on the consequences of ATI following viral infection.

Viruses such as the human immunodeficient virus (HIV), simian immunodeficiency virus (SIV), influenza virus, mouse hepatitis virus, human cytomegalovirus, measles virus and lymphocytic choriomeningitis virus (LCMV), to name a few, have been demonstrated to lead to thymic involution[48]. Different mechanisms underlining thymic involution have been observed and are thought to be virus specific. As previously mentioned, the main characteristic of ATI in a decrease in thymocyte cell count. One of the mechanisms observed during thymic involution is an increase in DP cell apoptosis triggered by a systemic rise in glucocorticoid levels[49]. This steroid hormone crosses the cellular membrane and binds to the NR3C1 receptor expressed in DP cells

ultimately leading to apoptosis. Another mechanism proposed is the targeting of DP cells by mature antigen-specific CD8 T cells. Contrary to what was first thought, many different viruses can infect the thymus[48]. Infected cells located in this organ present viral antigen to antigen-specific CD8 T cells which migrate from the periphery into the thymus[50, 51]. These cytotoxic cells can remove infected cells and lead to thymic depletion[50]. It is also important to note that infection of the thymus microenvironment can lead to tolerance to the ongoing infection. Due to the very regulated nature of the thymus to prevent the generation of auto-reactive T cells, presentation of viral antigens in this environment can affect thymic education[48]. Therefore, persistent infection in this environment can have long term impairment immunity to the ongoing pathogen.

While the striking decrease in DP cell count in the thymus has been the focus of many studies, little is known about the consequences of this process at earlier stages in T cell development and whether these infections have long lasting consequences for T cell development. LCMV Cl13 and *Salmonella* Typhimurium have been shown to not only impact the DP stage but also the DN stage. Both infections lead to a DN1 developmental block, however, the causes and consequences of this phenotype are yet to be described[50, 52]. The work presented in this thesis will concentrate on the mechanisms and consequences of this DN1 developmental block during the course of chronic viral infection.

Chronic Viral Infection

As mentioned, our immune system has evolved to provide us with specific mechanisms to recognize and clear viral infections. Although many viruses are characterized by their ability to replicate in the host leading to a brief period of symptoms prior to their removal by the immune system; some viruses have evolved to establish chronic infections. Chronic viral infections are characterized by the ability of the virus to persist in the host. While viruses have evolved different methods to allow their persistence in the host, a common feature is their ability to evade or dampen the immune response.

Chronic viral infections are still a major global health concern. The world health organization estimates that 257 million people are currently infected with hepatitis B virus, 71 million people with hepatitis C virus (HCV) and 37.9 million with the HIV[53, 54, 55]. These are a few of the globally relevant viruses characterized by persistent infection. Major research effort has been put to analyze the interaction between the immune system and the virus after the

establishment of chronicity. However, early onset mechanisms provided by the virus are crucial to allow for viral persistence. It has been shown that as early as 18 hours post-infection, there is an increase in pro-inflammatory cytokines in the serum of host infected by chronic compared to acute virus[56]. Moreover, an increase in anti-inflammatory cytokines at early onset has also being observed[57, 58]. The consequences of the difference in cytokine milieu are still not fully understood, however different mouse models of infection are currently used to investigate this impact. Therefore, although chronic viral infections are still highly prevalent in our society, its early impact on the immune system is still not fully understood.

Lymphocytic Choriomeningitis Virus

LCMV has been used for several years by immunologists as a tool to analyze the impact of virus infection on the host immune system. LCMV is a common mouse pathogen that was first identified by Charles Armstrong during an outbreak of encephalitis in St. Louis in 1933[59]. Although LCMV was not the cause of the outbreak, the isolation of LCMV by Dr. Armstrong was pivotal for the progress of immunology, particularly the study of T cell responses. LCMV is a natural mouse pathogen of the Arenaviridae family. It is composed of two negative single-strain RNA segments (known as L and S)[60]. These segments are ambisense, marked by protein-coding regions in the positive and negative sense, which are separated by an intergenic region[61]. The S segment codes for the nucleoprotein (NP) and the glycoprotein (GP-C) which undergo proteolytic cleavage generating GP-1 and GP-2[62, 63, 64]. The glycoproteins are present in the virus envelope and GP-1 is thought to interact with α-dystroglycan receptor present on the target cell allowing for viral entry[65]. The L segment encodes the RNA-dependent-RNA polymerase and a small Z polypeptide[66, 67]. Together, these proteins assist during viral entry, replication and exit from the target cell.

Infection with the LCMV strain isolated by Dr. Armstrong (LCMV Arm) gives rise to acute infection in mice, with a robust CD8 T cell response. However, in 1984 Rafi Ahmed isolated a new strain of LCMV known as Clone 13 (LCMV Cl13) which led to the establishment of a chronic viral infection[68, 69]. LCMV Cl13 encodes 2 functional mutations compared to LCMV Arm. The first mutation (F260L) is present at the GP-1 coding region leading to increased affinity to the entry receptor. This modified the cellular tropisms, allowing for LCMV Cl13 to infect $CD11c^{+}$ cells more efficiently than LCMV Arm[70, 71, 72]. The second mutation (K079Q) is present at the

RNA-dependent-RNA polymerase coding region resulting in an increase in viral replication of LCMV Cl13 compared to LCMV Arm[73]. Although LCMV Arm is cleared at around 5-8 days post-infection (dpi), LCMV Cl13 can persist as late as 80 dpi in specific organs[74]. Both functional mutations are shown to help in this process, however the mutation in the RNA-dependent-RNA polymerase coding region alone, but not at the GP-1 alone, was shown to lead to the establishment of chronicity[73]. Moreover, LCMV Cl13 induces a distinct cytokine milieu compared to LCMV Arm, such as increased levels and sustained expression of type I interferon (IFN-I) and Interleukin (IL)-10[56, 57]. Hence, LCMV Arm and Cl13 viral strains are great tools to analyze the effects of acute and chronic viral infection in the immune system.

IFN-I: production, signaling and function

Viral interference, the ability of an infected cell to become resistant to re-infection, is a concept that has been described for decades. However, it was in 1957 that Isaacs and Lindenmann first identified a secreted molecule capable of decreasing virus production following re-challenge. They hypothesized that this protein was capable of aborting (or interfering with) viral replication and it was therefore coined as interferon (IFN)[75]. Since this discovery, the IFNs have been expanded to a super-family of cytokines with three distinct classes (types I, II and III) which were categorized based on their structure, receptor utilization and biological function. The type I IFN class consists of 13 or 14 IFN-α subtypes, in humans and mice respectively, and a single subtype for IFN-β, as well as other, less studied subtypes such as IFN-ε, IFN-τ, IFN-κ, IFN-ω, IFN-δ and IFN-ζ. The type II IFN family consists of just one subtype, IFN-γ[76]. In 2003, three new IFN-like cytokines IFN-λ1, -λ2, and -λ3 (IL-29, IL-28A and IL-28B respectively) were identified and together with a more recently discovered cytokine, IFN-λ4, constitute the type III IFN class[77, 78, 79].The IFN response is initiated following the detection of broadly conserved microbial molecular domains, termed PAMPs, by three groups of cellular sensors: Toll-like receptors (TLRs), RIG-I-like receptors (RLRs) and DNA sensors. Once a sensor has been triggered, this initiates a signaling cascade that leads to the production of IFNs and activation of the innate immune system[80].

Following expression, IFNs bind to their cognate receptors, which triggers a signaling cascade that induces changes in gene expression and provides an early line of defense against viral infections. IFN receptors are class II helical cytokine receptors, however each family utilizes a different receptor dimer[81]. Type I IFNs signal through a heterodimeric receptor composed of

interferon α/β receptor subunit (IRNAR)1 and IFNAR2, which are low and high affinity, respectively. IFNAR2 exists in three different isoforms, however only IFNAR2c is able to signal through the Janus Kinase (JAK)-STAT pathway leading to an antiviral response. IFNAR2c possesses a long transmembrane domain and the full intracellular domain which is required for adapter binding and correct signal transduction[81]. During the canonical pathway, cytokine engagement and receptor dimerization allows the transphosphorylation and activation of receptor-associated kinases JAK1 (associated with IFNAR2) and tyrosine kinase 2 (TYK2; associated with IFNAR1). After activation, the receptor-associated kinases phosphorylate several tyrosine residues on both receptor subunits, creating a docking site. STAT2 then translocates to this docking site on the receptor and is phosphorylated by TYK2, allowing for the recruitment and binding of STAT1. Phosphorylated STAT1 and STAT2 dimerize, dissociate from the receptor, and associate with IRF9 (forming the IFN-stimulated gene factor 3 [ISGF3] complex) as they translocate to the nucleus. This complex is able to bind to interferon stimulating genes (ISG) promoters and induce the activation of several hundred ISGs, ultimately ensuring the establishment of an antiviral state in the cell[82].

Once bound to the ISG promoters, each class of IFN induces the expression of a unique and partially overlapping set of genes. This gene expression pattern can be further modulated depending on the cell type, IFN dose and timing. ISGs can target different steps of the viral life cycle such as entry, uncoating, transcription, translation, assembly and egress, and may have broad or restricted activity against different virus groups[83]. Some ISGs, such as MX1, OAS1, EIF2AK2 (which encodes protein kinase R [PKR]), TRIM5, ZC3HAV1 (which encodes zinc finger antiviral protein [ZAP]), APOBEC3G, and IFITM3 are considered to be individually very potent at blocking viral infection. However, it is hypothesized that the expression of multiple modest ISGs may be more advantageous, since high expression of a single, highly potent ISG could result in immunopathology[83]. ISGs exert their antiviral effects via a broad range of mechanisms. For example, the activation of 2′-5′-oligoadenylate synthetase 1 (OAS1) occurs following binding of viral dsRNA and leads to the synthesis of 2′-5′-oligoadenylates. This substrate activates latent ribonuclease (RNase L) which indiscriminately degrades all RNA (host or viral) in the infected cell[84]. Another well-studied ISG is PKR, which is activated by autophosphorylation following binding of dsRNA. Once activated, PKR phosphorylates eukaryotic initiation factor 2α (eIF2α), which inhibits viral RNA and most host mRNA translation[84]. Several other IGSs have been

identified and further analysis of their antiviral activity was conducted via overexpression screen studies, in which the activity of 380 genes were compared between several viruses, such as hepatitis C virus, yellow fever virus, West Nile virus, chikungunya virus, Venezuelan equine encephalitis virus and human immunodeficiency virus type-1[85]. There are also several ISGs that are not able to directly induce an antiviral response, but instead act to enhance pattern recognition receptor (PRR) pathways. These ISGs support post-translational modifications of genes downstream of PAMP recognition, which ultimately enhances the IFN response[86]. Together, the global change in gene expression allows the cell to create an environment where viral replication is abrogated, decreasing the chances of virus dissemination.

IFN-I and chronic viral infections

IFN-I is crucial for the establishment of an anti-viral state and for providing an adequate immune response against acute viruses. However, this family of cytokine is also one of the central drivers of inflammation in chronic viral infections[87]. Sustained IFN-I is a key contributor to immune dysregulation and disease progression in infections such as HIV, SIV and HCV[88, 89, 90]. IFN-I has shown to be important for the persistence of LCMV Cl13 infection, since following IFN-I signaling blockage viral clearance was accelerated[56]. IFN-I was also demonstrated to be one of the main factors responsible for the development of acquired immunodeficiency syndrome (AIDS) following SIV infection. Studies have shown that in natural monkey models of SIV infection, animals do not develop AIDS and are marked by a low IFN-I signature even in the presence of virus replication. However, in non-natural monkey model of SIV infection, animals exhibit a high and persistent release of IFN-I which contribute to the establishment of AIDS[89]. These studies illustrate the role of sustained IFN-I release in chronic viral infections for the development of pathogenesis.

Sustained IFN-I is coupled with an increase production of ISG altering the ability of many cell types to promote viral clearance. IFN-I signaling can increase the expression of PD-L1 and IL-10 impairing CD8 T cells function[56, 91]. IFN-I can also modulate the development, maturation and stimulatory capacities of DC and macrophages[87]. Moreover, IFN-I during chronic viral infections can alter splenic architecture and hinder lymphocyte migration within this organ[56]. Therefore, although IFN-I signaling is crucial for the establishment of an antiviral state, high and persistent levels of this cytokine impair proper immune responses.

The most studied members of the IFN-I family are IFNα and IFNβ, and although they can both contribute for the establishments of chronicity, each member utilize a different molecular mechanism. IFNα is produced following both LCMV Arm and LCMV Cl13 infection, albeit with a 3-fold increase following the chronic version of the virus[92]. On the other hand, IFNβ production is very robust following LCMV Cl13 infection while present in minimal levels following LCMV Arm infection[92]. Studies have demonstrated that LCMV Cl13 viral clearance can occur following the block of IFNβ, but not IFNα. Although IFNα is associated with early dissemination of the virus, IFNβ was the main drive determining splenic structure dysregulation and improper lymphocyte migrate and T cell anti-viral responses[93]. Therefore, analysis of each member of the IFN-I separately is essential to understand their mechanisms during the establishment of chronic viral infections.

Hypothesis and Rationale

The thymus is a complex organ with intrinsic mechanisms to ensure the development of T cells; however, thymic involution following infections leads to a drastic change in its structure possibly impairing thymocyte development and the mechanisms required to prevent the generation of autoreactive lymphocytes. Viral infections were shown to lead to thymic involution, however, the full mechanisms and consequences of this phenotype are still to be uncovered. Moreover, acute and chronic viral infections are known to lead to the establishment of a different cytokine milieu within the host. If these differences are also observed within the thymic microenvironment, they could further impact the generation of T cells. Hence, *we hypothesize that thymic involution following chronic viral infection leads to thymocyte development defects resulting in the generation of autoreactive lymphocytes.*

Chapter II: Chronic viral infection impacts cell fate decision in the thymus

Methods

Mice and pathogens

C57BL/6 wildtype (WT) and *Ifnar*$^{-/-}$ mice were originally purchased from Charles River and provided by Dr. J. Fritz (McGill University), respectively, and bred in house. Both male and female mice at 6-12wks old were used for experiments. LCMV Armstrong was provided by Dr. J. Harty (University of Iowa) and LCMV Cl13 by Dr. T. Watts (University of Toronto) from a strain originally propagated by Dr. M. Oldstone (The Scripps Research Institute). Viruses were propagated as previously described[94] and viral titers were determined by plaque assay using Vero cell line[69]. Mice were infected with $2x10^5$ plaque-forming units (PFU) of LCMV Arm (injection i.p. route) or with $2x10^6$ PFU of LCMV Cl13 (infection i.v. route). Infected mice were housed in the biocontainment level 2 and monitored at appropriate time points. All animal procedures were carried out following the Canadian Council on Animal Care and were approved by the McGill University Animal Care Committee.

Flow cytometry

Thymus samples were harvested at indicated time points and processed into a single-cell suspension. One ml of 1x Vitalyse buffer (Cedarlane, BioE) was added to lyse red blood cells (RBC), samples were washed and stained with antibodies in an appropriate combination of fluorochromes. The following antibodies were used for surface stain: B220 (clone RA3-6B2; BioLegend), CD3 (clone 145-2C11; BioLegend), CD4 (clone GK1.5; BioLegend), CD8 (clone 53-6.7; BioLegend), CD19 (clone 6D5; BioLegend), CD25 (clone 3C7; BioLegend), CD24 (clone M1/69; BioLegend), CD43 (clone S11; BioLegend), CD44 (clone IM7 ; BioLegend), c-kit (clone ACK2; BioLegend). For intracellular protein quantification, cells were stained for Lfng rabbit anti-mouse primary antibody (Abcam) and donkey anti-rabbit IgG secondary antibody (clone poly4064, BioLegend) in perm/wash Buffer (eBioscience). For nuclear staining, cells were stained with Ki-67 (clone 16A8, Biolegend) or isotype control IgG2a (clone RTK2758; Biolegend) using the eBioscience Foxp3/transcription factor staining buffer set (ThermoFisher) as per manufacture instructions. Samples were analyzed with a BD LSRFortessa Flow Cytometer (BD Biosciences) and FlowJo software (Tree Star).

RNA extraction and Gene expression analysis

RNA was extracted from the single-cell suspension (RBC lysed) of thymus samples using the TRIzol RNA isolation reagent according to manufacturer's instructions (Invitrogen). Reverse transcription (RT) of the RNA was performed using the Maxima H Minus cDNA Synthesis Master Mix (Thermo Scientific). Quantitative polymerase chain reaction (qPCR) was performed using the Bioline SensiFAST cDNA Synthesis kit (FroggaBio) and the following primers (Integrated DNA Technologies; 5' – 3'): *Lfng* forward (AAG CTG ACC CAT CCA GGT TC) *Lfng* reverse (TCC CTG AAG AAC ACG ACT GC), *Tnfsf13b* forward (GGC AGG TAC TAC GAC CAT CTC), *Tnfsf13b* reverse (TGG GCC TTT TCT CAC AGA AGT), *tbp* forward (TGG AAT TGT ACC GCA GCT TCA), *tbp* reverse (ACT GCA GCA AAT GCG TTG GG). Expression of each gene was normalized to TATA-binding protein (tbp) as an internal control and calculated using the ΔΔCt method comparing to the mean of the control group[95].

BrdU treatment and quantification

C57BL/6 WT mice were treated with 2 mg of BrdU (injection i.p. route) in PBS on the day of infection. Mice were also provided with 0.8 mg/ml of BrdU in the drinking water for 8 days. BrdU water was refreshed every 2-3 days. Thymus was harvested at 8 dpi and single-cell suspension prepared and RBC lysed with 1 ml of 1x Vitalyse buffer (Cedarlane, BioE) per organ. After surface staining, proliferation assay using the Phase-Flow BrdU cell proliferation kit (BioLegend) was performed as per manufacturer instructions.

B16 cell culture

B16-Blue cell line was provided by Dr. M. Divangahi (McGill University) and cultured in RPMI 1640 supplemented with 10% heat-inactivated FBS, 0.5% Penicillin-Streptomycin, 1% L-glutamine and 100 μg/mL Normocin (InvivoGen). After the third passage, 100ug/ml of Zeocin (InvivoGen) selection antibiotic was added to the culture media.

IFN-α/β quantification

C57BL/6 WT mice infected mice were euthanized at 1 and 2 days post-infection. Serum was collected via cardiac puncture, and thymus was harvested and homogenized. Murine bioactive IFN-α and IFN-β levels were measured using the B16-blue IFN-α/β reporter cell line (InvivoGen).

Briefly, 20 µl of sample plus 180 µl of B16 cells (75,000 cells/well) were incubated overnight at 37ºC and 5% CO_2. Next, 20 ul of B16 cell supernatant plus 180 µl of QUANTI-Blue medium (InvivoGen) were incubated at 37ºC and 5% CO_2. Readings were taken at OD655 nm starting at 1h post-incubation.

FACS Sort of DN1 cells

Thymus from C57BL/6 WT mice infected mice were collected, single-cell suspension prepared and RBC lysed using 1 ml of 1x Vitalyse buffer (Cedarlane, BioE) per organ. Cells were stained with Zombie Aqua Fixable Viability Kit (BioLegend) to assess live vs dead status of the cells. Cells were stained with surface antibodies mentioned and FACS sorted as following: Zombie $Aqua^-$ $CD3^-$ $CD4^-$ $CD8^-$ $CD44^+$ $CD25^-$ $B220^-$ $CD19^-$.

OP9-DL1 cell culture and ex vivo co-culture

OP9-DL1 cell line was provided by Dr. J. C. Zuniga-Pflücker (University of Toronto) and cultured in OP9-media (MEM media supplemented with 15% heat-inactivated FBS, 1% Penicillin-Streptomycin, 2 mM L-Glutamine, 14.3 µM of β-Mercaptoethanol) as previously described[96]. Co-culture experiments were prepared as previously described[97]. Briefly, $4x10^4$ of OP9-DL1 cells/well were added to a 6 well-plate and incubated overnight at 37ºC, 5% CO_2. FACS sorted DN1 cells (1,000 cell/well) were added to the plate in OP9 media supplemented with 1 ng/ml of murine IL-6, 1 ng/ml of murine IL-7, 25 ng/ml of murine IL-15 and 5 ng/ml human Flt3 ligand. Co-culture was incubated at 37ºC, 5% CO_2 and thymocyte development was analyzed 7-8 days post-co-culture by flow cytometry. Supplemented OP9 media was refreshed every 3-4 days. For IFN-β *ex vivo* treatment samples, 100 ng/well of murine IFN-β was added at 1 and 2 days post-co-culture.

Statistical analysis

Data were analyzed using GraphPad Prism software. The specific test used for each graph is described in the designated figure legend. P values less than 0.05 were considered statistically significant.

Results

Chronic, but not acute, LCMV infection leads to thymic involution and a DN1 developmental block

In order to analyze the consequences of early-stage acute versus chronic viral infection on thymocyte development, we infected C57BL/6 WT mice with either LCMV Arm ($2x10^5$ PFU i.p., acute infection) or LCMV Cl13 ($2x10^6$ PFU i.v., chronic infection) viral strain and harvested the thymus at 5 or 8 days post-infection (dpi). We observed that LCMV Cl13, but not LCMV Arm, resulted in thymic involution which was marked by a decrease of 20-fold in cell number at 8 dpi (Fig. II-1 A). While this phenotype was mostly dictated by the previously described reduction in DP cell numbers (Fig II-1 B-D)[50], we were interested in further analyzing any possible defects in the earlier stage of development, the DN. Our data showed a developmental block occurring at the DN1 stage (Fig. II-2 A-B), where more than 80% of the DN cells of mice infected with LCMV Cl13 at 8 dpi were present in this stage as opposed to around 35% in LCMV Arm infected mice. However, this did not result in a significant increase in cell number of the DN1 population, compared to uninfected mice, due to the massive decrease in organ size at this time point (Fig. II-2 C). While the DN1 cells are known to feed into the DN2 population for further thymocyte development, studies have shown that the DN1 stage is heterogeneous and can give rise to B cells in the thymus as a result of Notch signaling abrogation[17, 34]. Since thymic involution is marked by a disrupted thymic architecture[48], likely impairing signals received by thymocytes during development, we decided to analyze whether LCMV Cl13 infection would lead to an increased generation of B cells in the thymus. Although mature B cells are known to express both B220 and CD19 proteins on their surface, earlier B cells progenitors are known to express B220 prior to the expression of CD19. Surprisingly, LCMV Cl13 infection lead to an increase by 3.1-fold in the frequency of the $B220^+$ $CD19^-$ population, compared to LCMV Arm infection at a similar time point (8 dpi) (Fig. II-2 D-E). A trend towards increased numbers of $B220^+$ $CD19^-$ population was also observed in LCMV Cl13 compared to LCMV Arm infection, even with the 20-fold decrease in overall cell counts, however, this trend did not reach statistical significance (Fig. II-2 F). These results suggest that thymic involution following LCMV Cl13 infection might change the signals received by DN1 cells abrogating further thymocyte development and diverting the developmental pathway towards the generation of other cell types.

Due to the fact that thymic involution is marked by an increase in cell death, we decided to analyze whether the increase in the frequency of the $B220^+$ $CD19^-$ population was accompanied by an increase in proliferation, in order to determine whether this population was actively expanding or simply more resistant to cell death. Thus, we analyzed the expression of Ki-67, a marker of cell-cycle entry, and BrdU incorporation. BrdU is a thymidine analog commonly used in proliferation assays as it is incorporated into the DNA during replication. Therefore, increased BrdU incorporation, which can be measured by flow cytometry, correlates with higher cellular proliferation[98]. When comparing proliferation status of $B220^+$ $CD19^-$ and $B220^+$ $CD19^+$ populations 8 days post-LCMV Cl13 infection, the former had a significant increase in the frequency of cells expressing Ki-67 (Fig. II-2 G-H). We also analyzed the geometric mean fluorescence intensity (gMFI) of Ki-67, which correlates with protein expression on a per cell basis. We observed an increase in Ki-67 gMFI in $B220^+$ $CD19^-$ compared to mature ($B220^+$ $CD19^+$) B cells (Fig. II-2 I). Furthermore, the $B220^+$ $CD19^-$ population showed an increase in the frequency of cells that incorporated BrdU (Fig. II-2 J-K), and increased BrdU incorporation on a per cell basis (Fig. II-2 L). Taken together, these data show that $B220^+$ $CD19^-$ cells are entering cell cycle and undergoing proliferation to a higher extend than mature B cells. Hence, the increase in the frequency of $B220^+$ $CD19^-$ population following LCMV Cl13 infection may not be simply due to a decrease in the frequency of other cell types but rather a result of active cell proliferation leading to an expansion of this population.

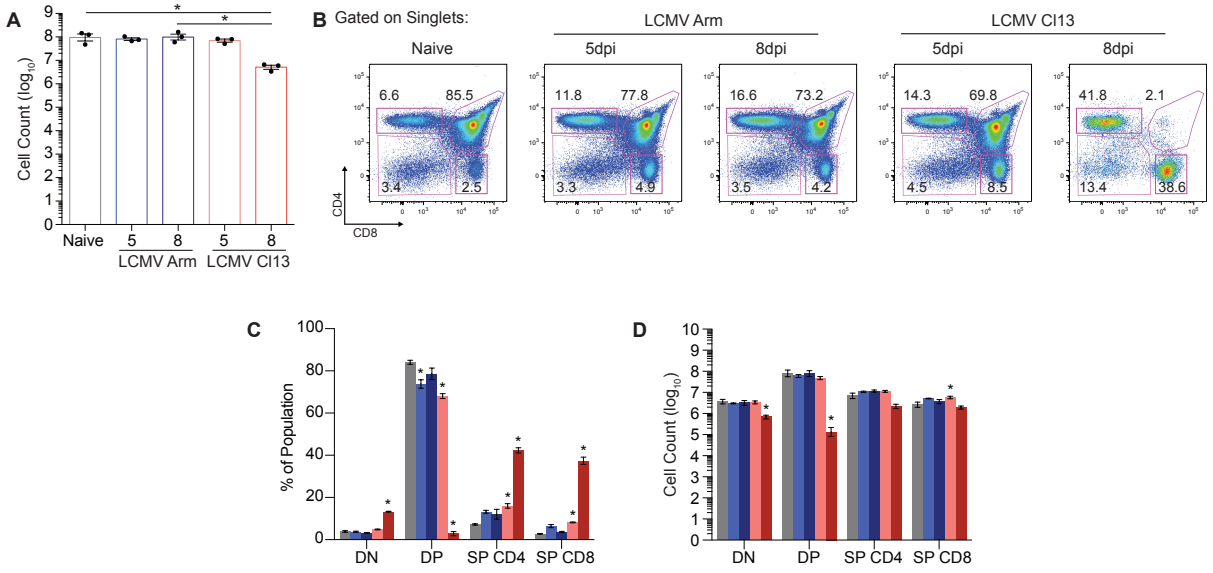

Figure II-1: LCMV Cl13, but not LCMV Arm, leads to thymic involution marked by a decrease in the DP thymocyte population. Thymus from naïve and infected C57BL/6 WT mice were analyzed 5 and 8 days post-LCMV Arm and LCMV Cl13 infection. (A) Thymus cell count. (B) Representative FACS plots thymocyte populations gated on singlets. (C) Frequency and (D) number of DN ($CD4^-$ $CD8^-$), DP ($CD4^+$ $CD8^+$), SP CD4 ($CD4^+$ $CD8^-$) and SP CD8 ($CD4^-$ $CD8^+$) populations. Data analyzed by 1-way ANOVA with Tukey's post-test for multiple comparisons. Data represent 3 mice per group and are representative of at least 2 independent experiments. For (A) * $p < 0.05$. For (C-D) statistics show comparison with naïve samples, *$p < 0.05$.

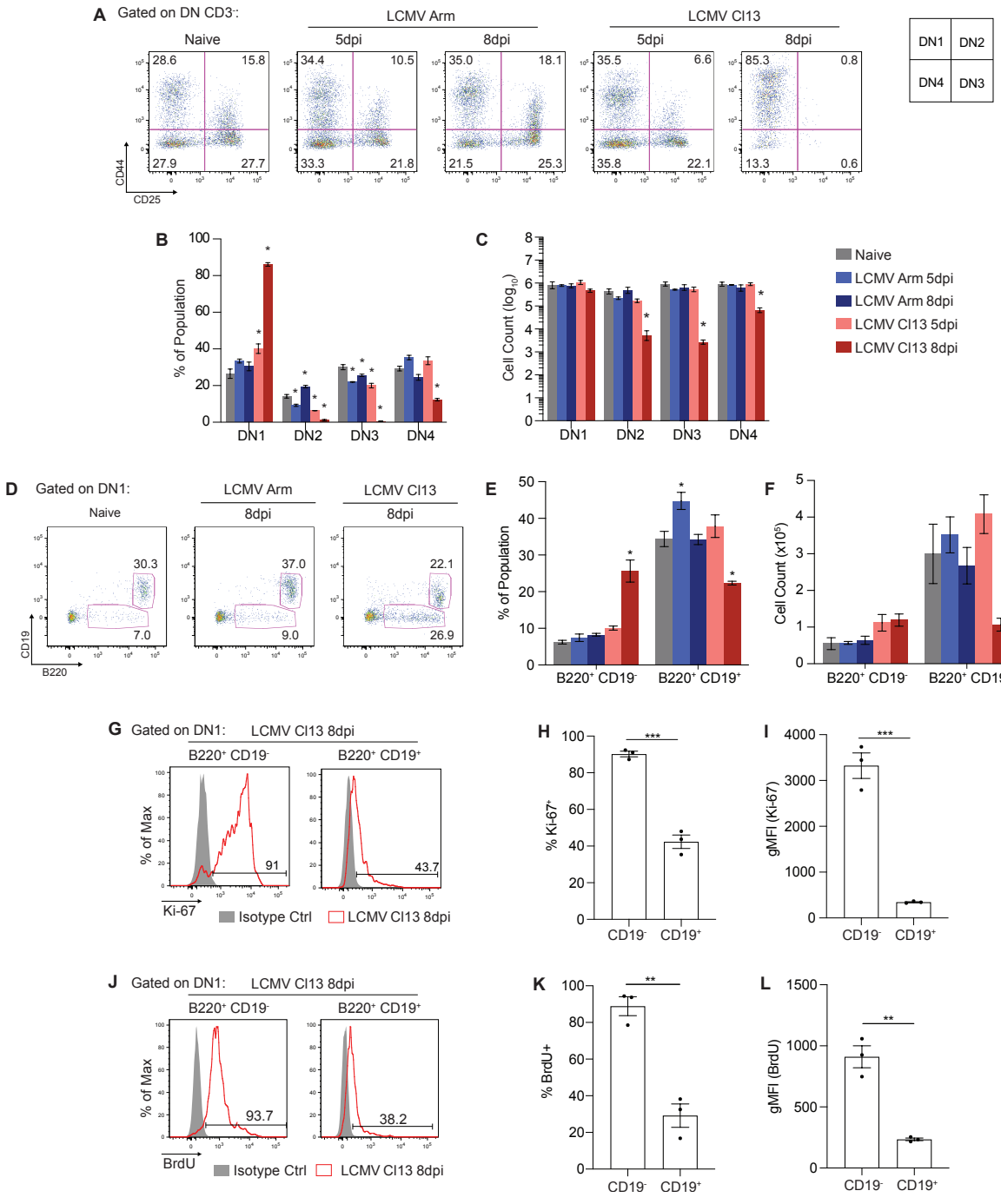
A
Gated on DN CD3-:
LCMV Arm
LCMV Cl13
Naive
5dpi
8dpi
5dpi
8dpi
28.6
15.8
27.9
27.7
34.4
10.5
33.3
21.8
35.0
18.1
21.5
25.3
35.5
6.6
35.8
22.1
85.3
0.8
13.3
0.6
CD44
CD25
DN1
DN2
DN4
DN3
B
% of Population
DN1
DN2
DN3
DN4
C
Cell Count (log10)
Naive
LCMV Arm 5dpi
LCMV Arm 8dpi
LCMV Cl13 5dpi
LCMV Cl13 8dpi
D
Gated on DN1:
Naive
LCMV Arm
8dpi
LCMV Cl13
8dpi
30.3
7.0
37.0
9.0
22.1
26.9
CD19
B220
E
% of Population
B220+ CD19-
B220+ CD19+
F
Cell Count (x10^5)
B220+ CD19-
B220+ CD19+
G
Gated on DN1:
LCMV Cl13 8dpi
B220+ CD19-
B220+ CD19+
% of Max
91
43.7
Ki-67
Isotype Ctrl
LCMV Cl13 8dpi
H
% Ki-67+
***
CD19-
CD19+
I
gMFI (Ki-67)
***
CD19-
CD19+
J
Gated on DN1:
LCMV Cl13 8dpi
B220+ CD19-
B220+ CD19+
% of Max
93.7
38.2
BrdU
Isotype Ctrl
LCMV Cl13 8dpi
K
% BrdU+
**
CD19-
CD19+
L
gMFI (BrdU)
**
CD19-
CD19+

Figure II-2: LCMV Cl13 infection promotes a DN1 block and the generation of a B220$^+$ CD19$^-$ population. Thymus from naïve and infected C57BL/6 WT mice were analyzed 5 and 8 days post-LCMV Arm and LCMV Cl13 infection. (A) Representative FACS plots gated on DN CD3$^-$ population. (B) Frequency and (C) number of DN1 (CD44$^+$ CD25$^-$), DN2 (CD44$^+$ CD25$^+$), DN3 (CD44$^-$ CD25$^+$) and DN4 (CD44$^-$ CD25$^-$) populations. (D) Representative FACS plots gated on the DN1 population. (E) Frequency and (F) number of B220$^+$ CD19$^-$ and B220$^+$ CD19$^+$ populations. C57BL/6 WT mice treated with 2 mg BrdU i.p. and infected with LCMV Cl13 on the same day. Mice provided with 0.8 mg/mL of BrdU in the drinking water from day 0 to 8 dpi. Thymus from infected mice were analyzed at 8 dpi. (G) Representative of Ki-67 histogram expression for B220$^+$ CD19$^-$ and B220$^+$ CD19$^-$ populations gated on DN1. (H) Frequency of Ki-67$^+$ cells. (I) gMFI of Ki-67 expression on B220$^+$ CD19$^-$ and B220$^+$ CD19$^-$ populations. (B-F) Data analyzed by 1-way ANOVA with Tukey's post-test for multiple comparisons. Statistics show comparison with naïve samples, * $p < 0.05$. Data represent 3 mice per group and are representative of at least 2 independent experiments. (H-L) Data analyzed by student's t-test for statistical analysis, ** $p < 0.01$, *** $p < 0.001$. Data represent 3 mice per group performed once.

The defect on thymocyte development induced by LCMV Cl13 infection persists even following the restoration of thymus involution

In order to analyze whether the disruption in thymocyte development following LCMV Cl13 infection was sustained, we performed a longitudinal analysis of the thymus at various dpi. Following LCMV Cl13 infection, the DN1 developmental block peaked at 8 dpi, but both frequency and number of this population remained elevated, compared to naïve, even after the overall thymic cell numbers were restored to their pre-infection levels (at 20 dpi) (Fig. II-3 A-D). It is important to note that although the DN1 development block was observed at 8, 15 and 20 dpi, the DN2 and DN3 developmental stages started to be repopulated, albeit not to pre-infection levels, at 15 dpi, and the DN4 at 20 dpi (Fig. II-3 A, C). This suggests that an early signal is promoting the DN1 developmental defect observed starting at 7 dpi. However, this signal is probably absent at 15 dpi allowing for the gradual repopulation of the DN groups. In order to investigate whether the DN1 block was marked by a sustained generation of $B220^{+}$ $CD19^{-}$ cells, we analyzed this cell population at each time point. Both frequency and number of $B220^{+}$ $CD19^{-}$ population were elevated, compared to naïve samples, starting at 8 dpi. This significant increase was sustained even following the restoration of thymic involution (at 20 dpi) (Fig. II-3 E-G). These data suggest that the consequences of thymic involution following chronic viral infection is long-lasting and present even after the thymus return to its original size, indicating the possibility of a long-term impairment in host immunity.

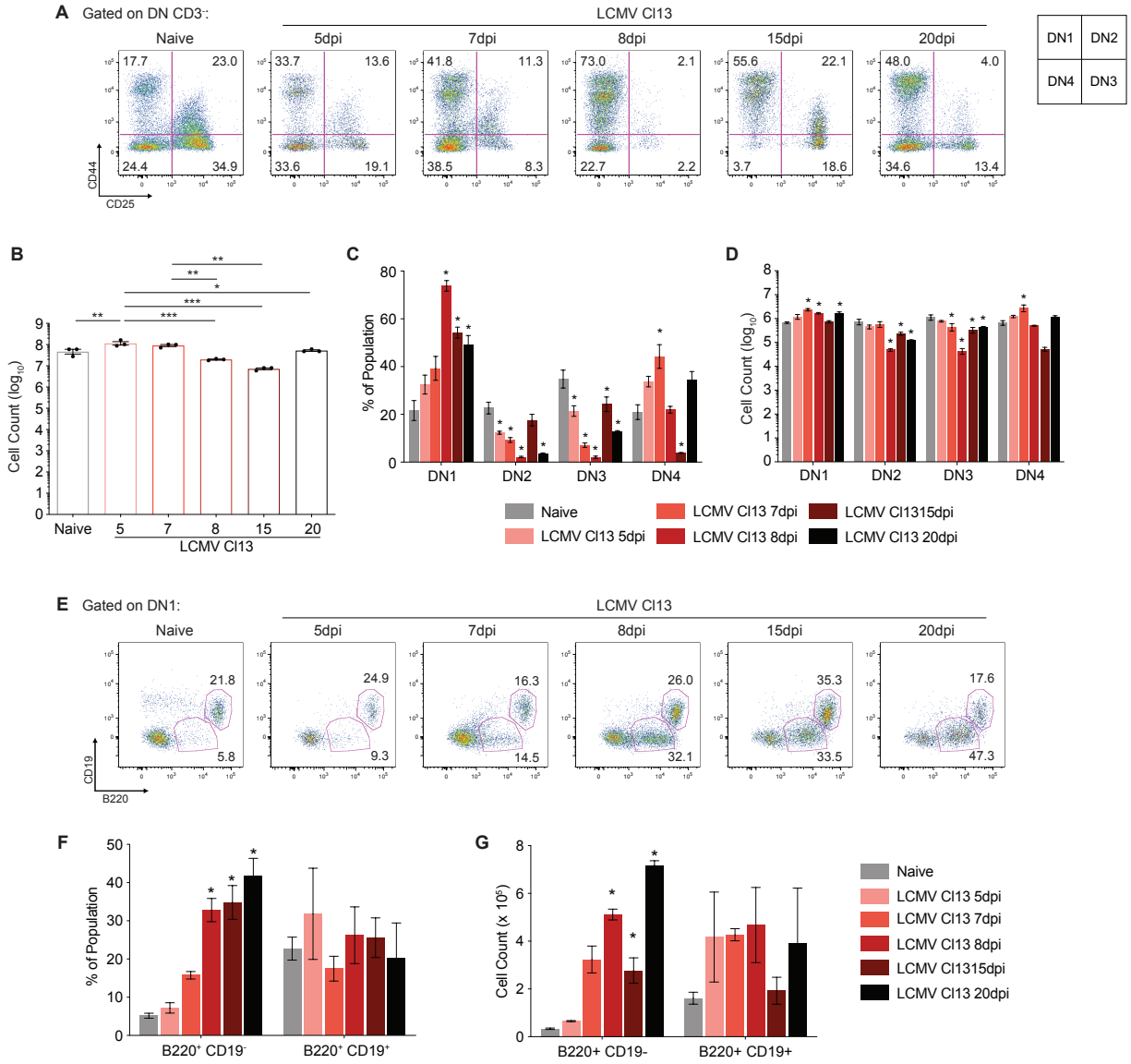

Figure II-3: LCMV Cl13 leads to a DN1 developmental block which is marked by a sustained increase of a B220$^+$ CD19$^-$ population. Thymus from naïve and infected C57BL/6 WT mice were analyzed 5, 7, 8, 15 and 20 days post LCMV Cl13 infection. (A) Representative FACS plots gated on DN CD3$^-$ population. (B) Thymus cell count. (C) Frequency and (D) number of DN1 (CD44$^+$ CD25$^-$), DN2 (CD44$^+$ CD25$^+$), DN3 (CD44$^-$ CD25$^+$) and DN4 (CD44$^-$ CD25$^-$) populations. (E) Representative FACS plots gated on the DN1 population. (F) Frequency and (G) number of B220$^+$ CD19$^-$ and B220$^+$ CD19$^+$ populations. Data analyzed by 1-way ANOVA with Tukey's post-test for multiple comparisons. For (A) * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. For (C-G) statistics show comparison with naïve samples, * $p < 0.05$. Data represent 3 mice per group and are representative of at least 2 independent experiments.

The B cell-like population matures from pre-pro to pro B cell stage while the thymus increases B cell survival and developmental signals

Chronic viral infection with LCMV Cl13 leads to the sustained generation of a population at the DN1 stage expressing B220 protein which is not usually not found within the thymus. During canonical development of B cells, progenitors express B220 before the expression of CD19. Therefore, we decided to analyze whether the $B220^{+}$ $CD19^{-}$ population, which from now on will be referred to as B cell-like cells, are the progenitor of B cells maturing in the thymus. First, we analyzed the gMFI of both B220 and CD19 at different time points following LCMV Cl13 infection. Although we labeled B cell-like cells as $CD19^{-}$, due to the lack of expression of this protein at 8 dpi, an increase in CD19 gMFI at later time points would suggest that B cell-like cells can mature within the thymus. Gating on the B-cell-like cell population we noticed a significant increase in B220 gMFI at 8, 15 and 20 dpi when comparing to cells at 7 dpi (Fig. II-4 A). While B cell-like cells lack the expression of CD19 at 7 and 8 dpi, a significant increase in expression of this protein is observed at later time points (15 and 20 dpi), albeit with lower expression than mature thymic B cells (Fig. II-4 B). These data suggest that B cell-like cells may be maturing in the thymus as they acquire the expression of CD19. In the canonical B cell development, early B cell progenitors can be further divided into 3 developmental stages based on the expression of CD43 and CD24 surface proteins. These stages are known as pre-pro ($CD43^{hi}$ $CD24^{lo}$), pro ($CD43^{hi}$ $CD24^{hi}$) and pre ($CD43^{lo}$ $CD24^{hi}$) B cells. While pre-pro B cells lack the expression of CD19, this protein is present at the pro B cell stage and it is maintained by all the following developmental stages. Since B cell-like cells are gaining expression of CD19 at later time points we asked whether these cells would also demonstrate a more mature B cell phenotype. Our data show that B cell-like cells matured from the pre-pro B cell to the pro B cell stage starting at 15 dpi (Fig. II-4 C-D). Taken together, our data suggest that the development defect caused by LCMV Cl13 infection leads to the generation of B cell-like cells that progressively mature in the thymus starting at 15 dpi.

In order to undergo proper development, B cells generated in the bone marrow receive multiple signals which promote survival and maturation. One of these signals is the B cell-activating factor of the tumor necrosis factor family (BAFF), a cytokine released by myeloid cells with an important role in the survival of mature naïve B cells[99]. Although signals required for B

cell development within the thymus are likely distinct to those in the bone marrow, pathologies associated with an increase in thymic B cells are marked by high BAFF expression in circulation[100]. To determine whether BAFF expression in the thymic microenvironment was altered following infection, mRNA extracted from a single cell suspension of the thymus at different time points following LCMV Arm and LCMV Cl13 infection were analyzed by RT-qPCR. The mRNA expression of *Tnfsf13b* (BAFF encoding gene) was significantly increased following LCMV Cl13 but not following LCMV Arm infection compared to uninfected control mice (Fig. II-4 E). A 10- and a 15-fold increase was observed at 8 and 15 dpi respectively, while levels at 20 dpi were not significantly different from those of naïve mice (Fig. II-4 E). Taken together, these data suggest that early stages of chronic infection lead to an increase in *Tnfsf13b* mRNA expression and that these signals may contribute to B cell-like cell maturation within the thymus.

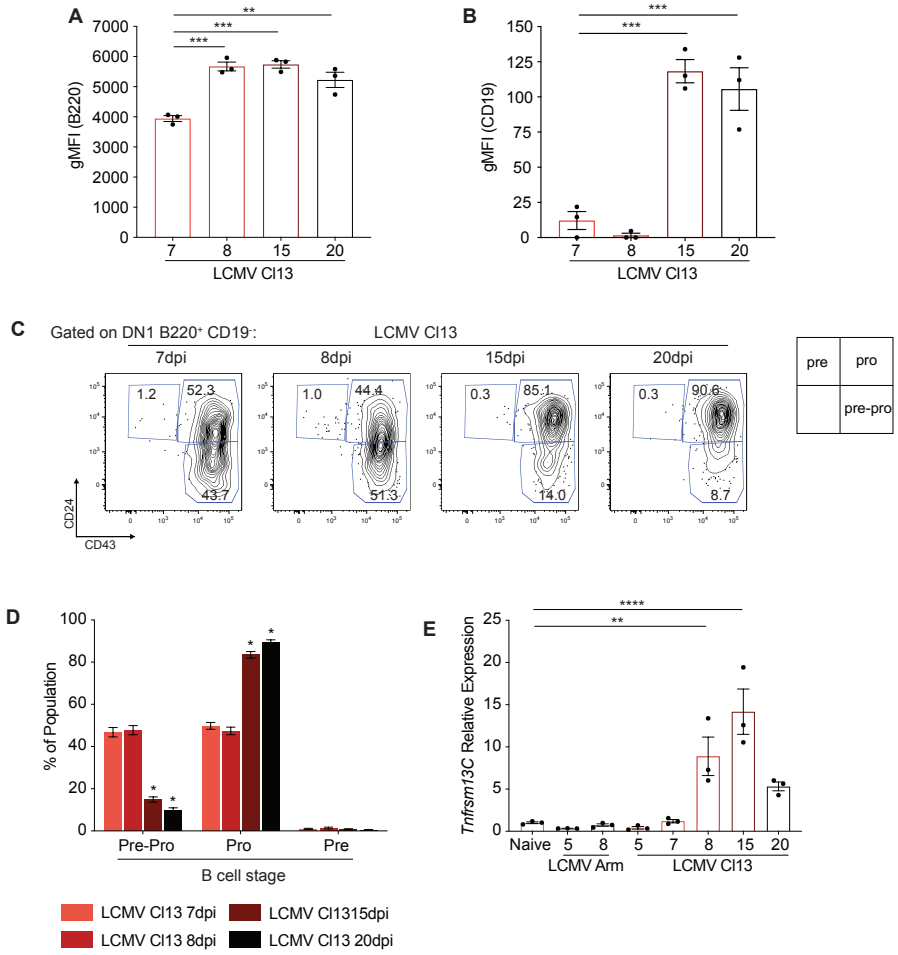

Figure II-4: B cell-like cells maturation within the thymus correlates with increased BAFF expression. Thymus from naïve and infected C57BL/6 WT mice were analyzed 5, 7, 8, 15 and 20 days post-LCMV Arm and LCMV Cl13 infection. Analysis of (A) B220 and (B) CD19 gMFI of the $B220^{+}$ $CD19^{-}$ population. (C) Representative FACS plots gated on $B220^{+}$ $CD19^{-}$ population. (D) Frequency of pre-pro ($CD24^{lo}$ $CD43^{hi}$), pro ($CD24^{hi}$ $CD43^{hi}$) and pre ($CD24^{hi}$ $CD43^{lo}$) B cell stages on the $B220^{+}$ $CD19^{-}$ population. (G) RNA extracted from a single cell suspension of thymus from naïve and LCMV Arm or LCMV Cl13 infected C57BL/6 WT mice. *Tnfsf13b* (BAFF encoding gene) mRNA expression fold change relative to *tbp*, analyzed by RT-qPCR. Data analyzed by 1-way ANOVA with Tukey's post-test for multiple comparisons. For (A, B, E) ** $p < 0.01$, *** $p < 0.001$. For (D) statistics show comparison with LCMV Cl13 7 dpi samples, * $p < 0.05$. Data represent 3 mice per group and are representative of at least 2 independent experiments.

B cell-like cell progenitors are distinct from those of naturally occurring thymic B cells previously reported

It has been previously demonstrated that the DN1 developmental stage is heterogeneous and it can be further divided into 5 different groups[17]. Using the surface expression of CD24 and c-kit proteins, the DN1 group is further divided into DN1a to DN1e. Each group have specific characteristic and are described to give rise to different cell populations. As previously mentioned, the DN1a and DN1b groups are thought to be part of the ETP cells giving rise to DN2 cells for further thymocyte development[17]. On the other hand, although DN1d can also give rise to DN2 cells, this group is known to have the ability to give rise to thymic B cells[17]. To further characterize the B cell-like cells generated in mice infected with LCMV Cl13, we decided to further distinguish its presence in each DN1 subgroup. Single-cell suspension from the longitudinal study of LCMV Cl13 infected mice were analyzed by flow cytometry. Surprisingly, B cell-like cells showed a dynamic DN1 subgroup phenotype (Fig. II-5 A). At 7 dpi, the frequency of B cell-like was almost equally present in the DN1b, DN1d and DN1e groups. At 8 dpi, the majority of B cell-like cells were present at the DN1b groups, which based on the literature, this group does not supposed to possess the ability to generate B cells. However, at 15 and 20 dpi, almost all B cell-like cells showed a DN1d phenotype (Fig. II-5 B-C). This data suggest that the B cell-like cells present in our model might be different than the thymic B cells which are generated under homeostatic conditions. Although pre-pro B cells (Fig. II-4 C-D) were shown to have a DN1b phenotype, maturation to the pro B cell stage accompanied the acquisition of a DN1d phenotype. Together, these data further support the hypothesis of the maturation of thymic B cells within the thymus during the LCMV Cl13 infection.

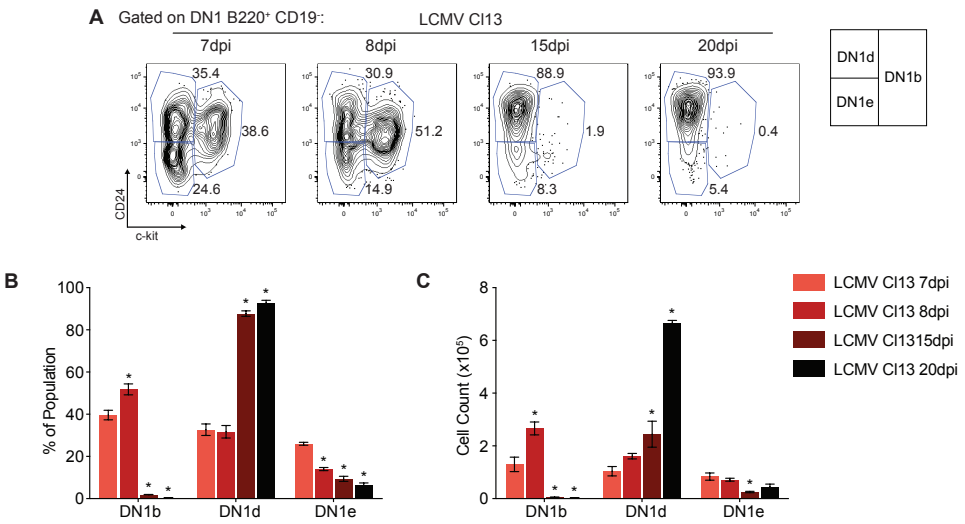

Figure II-5: B cell-like cells generated following LCMV Cl13 infection show a dynamic DN1 phenotype. Thymus from naïve and infected C57BL/6 WT mice were analyzed 5, 7, 8, 15 and 20 days post-LCMV Cl13 infection. (A) Representative FACS plot gated on $B220^{+}$ $CD19^{-}$ population. (B) Frequency and (C) number of DN1b ($c\text{-}kit^{hi}$ $CD24^{hi}$), DN1d ($c\text{-}kit^{lo}$ $CD24^{hi}$) and DN1e ($C\text{-}kit^{lo}$ $CD24^{lo}$) populations. Data analyzed by 1-way ANOVA with Tukey's post-test for multiple comparisons. For (B-C) statistics show comparison with LCMV Cl13 7 dpi samples. Data represent 3 mice per group and are representative of at least 2 independent experiments.

Type I interferon signaling is essential for the generation of B cell-like cells in the thymus

Acute and chronic infections induce the establishment of a different cytokine milieu within the host[56, 57, 58]. Studies have shown that LCMV Cl13 induce the release of higher IFN-I levels in the serum at early time points (18-24 h) when compared to LCMV Arm[56]. However, whether this difference is also observed within the thymic microenvironment remains unknown. We used the B16-Blue™ IFN-α/β reporter cell line, which allows for the detection and quantification of bioactive murine IFN-I, to compare expression levels within the thymus and in the periphery following either infection[101]. Serum and thymus homogenate from WT mice infected with LCMV Cl13 or LCMV Arm were collected at 1 and 2 dpi. Our data supported the previous finding that LCMV Cl13 promotes the establishment of a cytokine milieu marked by significantly higher levels of IFN-I in the serum at 1 dpi when compared to LCMV Arm (Fig. II-6 A). This difference was not sustained within the serum at 2 dpi. However, the thymus microenvironment displayed not only a higher fold difference of IFN-I expression levels between LCMV Cl13 and LCVM Arm, but this difference was also sustained at 2 dpi (Fig. II-6 B). Therefore, high levels of IFN-I within the thymus induced by LCMV Cl13 infection may play a role in the dysregulation of thymocyte development.

To test whether high levels of IFN-I play a role in the thymocyte development defect observed following LCMV Cl13 infection, we used the *Ifnar*$^{-/-}$ mouse strain. The IFN-I family of cytokine signal through the heterodimeric receptor composed of IFNAR1 and IFNAR2[1]. The *Ifnar*$^{-/-}$ mice lack the expression of IFNAR1 subunit and therefore prevent IFN-I from signaling allowing us to determine the contribution of this cytokine family in our model. Thymus from naïve and LCMV Cl13 infected WT and *Ifnar*$^{-/-}$ mice were analyzed. Although LCMV Cl13 infection in *Ifnar*$^{-/-}$ mice still caused a partial decrease in cell count, albeit not significant compared to *Ifnar*$^{-/-}$ naive mice (Fig. II-6 C), only a partial DN1 block was observed in this group at 8 dpi. While WT infected mice show approximately 70% of DN cells present at the DN1 stage, only approximately 40% of *Ifnar*$^{-/-}$ DN cells were present in this group (Fig. II-6 D-E). Strikingly, the generation of B cell-like cells in *Ifnar*$^{-/-}$ LCMV Cl13 infected mice was completely abrogated (Fig. II-6 F-H). Moreover, no increase in *Tnfsf13b* mRNA expression levels was observed in *Ifnar*$^{-/-}$ mice infected with LCMV Cl13. Taken together, these data demonstrate that although IFN-I signaling might be

partially required for the establishment of the DN1 block, this family of cytokine is essential for the generation of B cell-like cells.

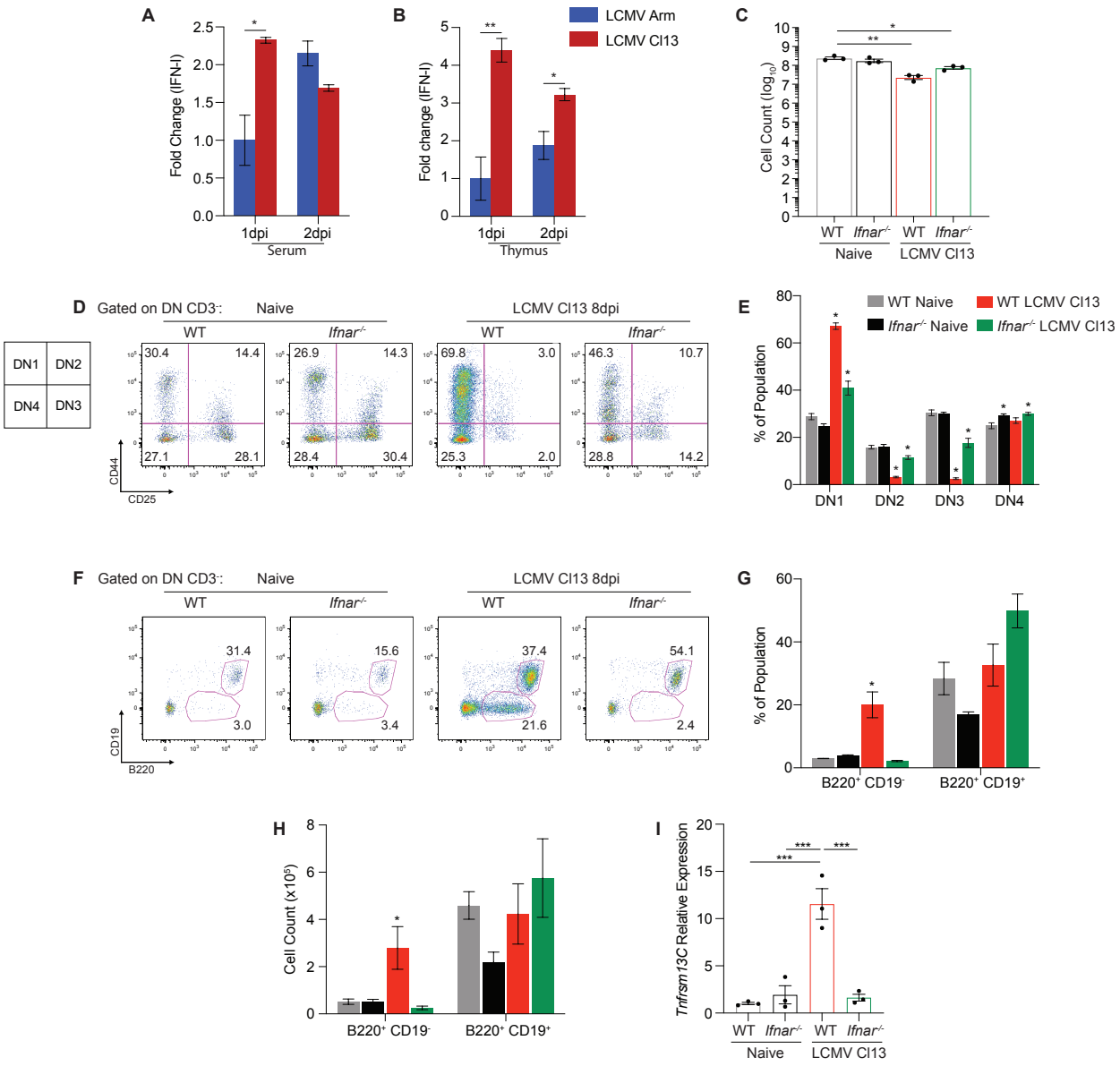
A
Fold Change (IFN-I)
1dpi
2dpi
Serum
B
LCMV Arm
LCMV Cl13
Fold change (IFN-I)
Thymus
C
Cell Count (log10)
WT
Ifnar-/-
Naive
LCMV Cl13
D
Gated on DN CD3-:
Naive
LCMV Cl13 8dpi
DN1
DN2
DN4
DN3
CD44
CD25
30.4
14.4
27.1
28.1
26.9
14.3
28.4
30.4
69.8
3.0
25.3
2.0
46.3
10.7
28.8
14.2
E
WT Naive
WT LCMV Cl13
Ifnar-/- Naive
Ifnar-/- LCMV Cl13
% of Population
DN1
DN2
DN3
DN4
F
Gated on DN CD3-:
Naive
LCMV Cl13 8dpi
CD19
B220
31.4
3.0
15.6
3.4
37.4
21.6
54.1
2.4
G
% of Population
B220+ CD19-
B220+ CD19+
H
Cell Count (x10^5)
B220+ CD19-
B220+ CD19+
I
Tnfrsm13C Relative Expression
WT
Ifnar-/-
Naive
LCMV Cl13

Figure II-6: B cell-like generation following LCMV Cl13 infection requires Type I interferon signaling. Infected C57BL/6 WT mice were analyzed 1 and 2 days post-LCMV Arm and LCMV Cl13 infection. Bioactive IFN-I levels in the (A) serum and (B) thymus homogenate were analyzed with the B16-BlueTM IFN-α/β reporter cell line. Thymus from naïve and infected C57BL/6 WT and *Ifnar*$^{-/-}$ mice were analyzed 8 days post-LCMV Cl13 infection. (C) Thymus cell count. (D) Representative FACS plots gated on DN CD3^{-} population. (E) Frequency of DN1 (CD44^{+} CD25^{-}), DN2 (CD44^{+} CD25^{+}), DN3 (CD44^{-} CD25^{+}) and DN4 (CD44^{-} CD25^{-}) populations. (F) Representative FACS plots gated on the DN1 population. (G) Frequency and (H) number of B220^{+} CD19^{-} and B220^{+} CD19^{-} populations. (I) RNA extracted from a single cell suspension of thymus from naïve and LCMV Cl13 infected C57BL/6 WT and *Ifnar*$^{-/-}$ mice. *Tnfsf13b* (BAFF encoding gene) mRNA expression fold change relative to *tbp*, analyzed by RT-qPCR. (A-B) Data analyzed by student's t-test for statistical analysis. Data represent 3 mice per group performed once. Data analyzed by 1-way ANOVA with Tukey's post-test for multiple comparisons. For (A-C, I) * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. (E-H) Statistics show comparison with WT naïve samples, * $p < 0.05$.

Lunatic Fringe mRNA and Protein expression patterns correlate with the DN1 developmental block

DN1 cells require Notch signaling in order to commit to the T cell lineage and continue thymocyte development[17, 34] . Since LCMV Cl13 infection induced a DN1 developmental block, we decided to analyze whether there were any changes in the regulators of Notch1 signaling. Lfng is a protein capable of modifying the extracellular portion of the Notch1 receptor leading to changes in affinity with its ligand. Moreover, increase expression of Lfng within the thymus was correlated with increased generation of thymic B cells[41]. Hence, we investigated whether the two strains of LCMV would modify the expression of Lfng within the thymus. RNA was extracted from a thymic single-cell suspension from mice infected with either LCMV Arm or LCMV Cl13 at different time points and *Lfng* mRNA expression levels were analyzed by RT-qPCR. Our data showed that LCMV Cl13, but not LCMV Arm, increased *Lfng* mRNA expression up to 8-folds at 8 dpi compared to uninfected mice (Fig. II-7 A). To determine whether this change in gene expression was sustained, we performed RT-qPCR from thymus samples collected from the longitudinal study following LCMV Cl13 infection. *Lfng* mRNA expression was significantly increase starting at 7 dpi, peaking at 8 dpi and remaining high even 15dpi (Fig. II-7 B). Expression levels returned to baseline at 20 dpi, suggesting that an initial signal might be sufficient to dysregulate Notch signaling to promote the generation of B cell-like cells. Next, we analyzed whether the increase in mRNA levels translated into an increase in protein expression within the thymus. Thus, single-cell suspensions of thymus from the LCMV Cl13 longitudinal study were analyzed by flow cytometry. Significantly increased Lfng gMFI was observed at 8 and 15 dpi, with levels returning to baseline at 20 dpi (Fig. II-7 C-D). The increase in Lfng gMFI was observed in all populations analyzed (DN, DP and SP cells) (data not shown). Our data suggest that this global increase of Lfng following LCMV Cl13 infection might be playing a role in the regulation of Notch1 signaling and ultimately in the generation of B cell-like cells.

Since IFN-I signaling is crucial for the generation of B cell-like cells, we next analyzed whether increased Lfng expression following LCMV Cl13 infection requires IFN-I signaling. We extracted mRNA from a single-cell suspension of the thymus of naïve and LCMV Cl13 infected WT and *Ifnar*$^{-/-}$ mice and analyzed *Lfng* levels by RT-qPCR. Although a similar increase of *Lfng* mRNA expression was observed in WT mice infected with LCMV Cl13 at 8 dpi, no increase was

observed in *Ifnar*$^{-/-}$ infected mice (Fig. II-7 E). Moreover, protein expression analyzed by flow cytometry showed significantly higher levels of Lfng in WT vs *Ifnar*$^{-/-}$ LCMV Cl13 infected mice (Fig. II-7 G). Taken together, these data suggest that IFN-I signaling is essential for the upregulation of Lfng. This protein which can modify the Notch1 receptor altering receptor-ligand affinity might ultimately diverge the commitment of T cells to B cells in our model.

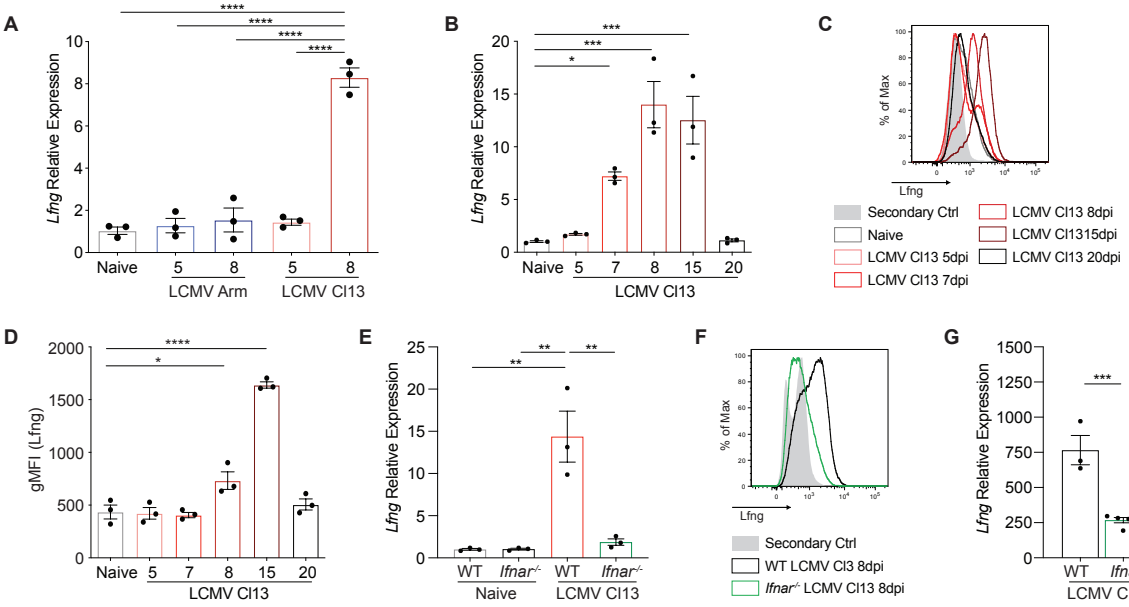

Figure II-7: The increase expression of Lunatic Fringe following LCMV Cl13 infection is dependent on IFN-I. A single-cell suspension of thymus from naïve and infected C57BL/6 WT mice were analyzed 5, 7, 8, 15 and 20 days post-LCMV Arm and LCMV Cl13 infection. (A-B) RNA extracted from single-cell suspension and *Lfng* mRNA expression fold change relative to *tbp* was analyzed by RT-qPCR. (C) Lfng expression analyzed by Flow cytometry. Lfng histogram expression gated on singlets. (D) gMFI of Lfng. (E-G) Single-cell suspension of thymus from naïve and infected C57BL/6 WT mice and *Ifnar*$^{-/-}$ were analyzed at 8 days post-LCMV Cl13 infection. (E) RNA extracted from single-cell suspension and *Lfng* mRNA expression fold change relative to *tbp* was analyzed by RT-qPCR. (F) Lfng expression analyzed by Flow cytometry. Lfng histogram expression gated on singlets. (G) gMFI of Lfng. (A-E) Data analyzed by 1-way ANOVA with Tukey's post-test for multiple comparisons. (G) Data analyzed by student's t-test for statistical analysis. Data represent at least 3 mice per group and are representative of at least 2 independent experiments.

LCMV Cl13 infection leads to intrinsic changes of the DN1 cells delaying their development ex vivo

Next, we decided to analyze whether the DN1 development block occurring following LCMV Cl13 infection was due to a DN1 cell-intrinsic or extrinsic mechanism. A previously published study has shown that the thymic niche occupied by DN1 cells can be masked by DP cells due to an increase of Notch1 receptor-ligand affinity caused by Lfng receptor modifications[41]. This phenotype was marked by a DN1 block and increased B cell numbers present in the thymus[41]. In this scenario, DN1 cells do not present with an intrinsic developmental defect and therefore an increase in ligand availability allows proper DN1 development. In order to test whether, in our model, the DN1 developmental block can be rescued by increasing the ligand availability, we used the well-established OP9-DL1 culture system[96]. In this system, all the signals required for thymocyte development, such as Notch1 ligand and cytokines, are provided in abundance to support proper thymocyte development. Therefore, any deviation or delay in the development of the DN1 cells harvested from LCMV Cl13 infected mice would suggest an intrinsic, and not extrinsic, defect. To test this hypothesis, WT mice were infected with LCMV Arm or LCMV Cl13 and the thymus was harvested at 2 dpi. DN1 cells were FACS sorted, plated on the OP9-DL1 system and thymocyte development was analyzed 7 days post-co-culture. We chose the 2 dpi time point since IFN-I was shown to be, at least partially, required for the establishment of the DN1 block, and at this time point LCMV Cl13 leads to significantly higher levels of IFN-I in the thymic microenvironment when compared to LCMV Arm infection. We observed that the DN1 cells harvested from LCMV Arm infected mice developed on the OP9-DL1 system and the majority of the cells were present in the DN3 developmental stage (Fig. II-8 A-B). However, DN1 cells harvested from LCMV Cl13 infected mice showed a delayed development in culture. Our data show a significant decrease in the frequency of cells were present at the DN3 stage. Moreover, although not statistically significant, there was a 4.5-fold increase in the frequency of cells retained at the DN1 stage, compared to DN1 cells harvested from LCMV Arm infected mice (Fig. II-8 A-B). Together these data support the hypothesis LCMV Cl13 infection is leading to intrinsic changes of the DN1 cells at an early time point, promoting a delayed development even in the presence of all signals required for development.

We hypothesized that the delay observed in thymocyte development of DN1 cells from LCMV Cl13 infected mice is a result of IFN-I signaling. Since IFN-I is present at higher levels in the thymic microenvironment of LCMV Cl13 infected mice compared to LCMV Arm, we decided to analyze whether treatment of LCMV Arm DN1 cells with IFN-I *ex vivo* would promote a delay in development. For that, we cultured DN1 sorted cells from LCMV Arm infected mice (2 dpi) in the OP9-DL1 system and provided a source of IFN-β (100ng/well) at 1 and 2 days post-co-culture. The time points were chosen to mimic the early release of IFN-I observed *in vivo*. Strikingly, IFN-β treatment completely abrogated the development of DN1 cells harvested from LCMV Arm infected mice (Fig. II-8 C). However, this experimental layout does not allow us to determine whether IFN-β was acting directly on the DN1 cells, or whether this treatment was abrogating the ability of the OP9-DL1 cells to support proper thymocyte development. For that, we analyzed the consequences of treating DN1 cells harvested from *Ifnar*$^{-/-}$ mice with IFN-β. In this scenario, IFN-β can signal only to the OP9-DL1 cells and not to the DN1 cells. Our data show that treatment with IFN-β did not change the development of *Ifnar*$^{-/-}$ DN1 cells compared to untreated, which indicated that IFN-β treatment does not alter the ability of OP9-DL1 cells to support proper thymocyte development (Fig. II-8 C). Taken together our data suggest that the increase in IFN-I levels provided by LCMV Cl13 infection act directly on the DN1 cells, promoting intrinsic changes and delaying their development.

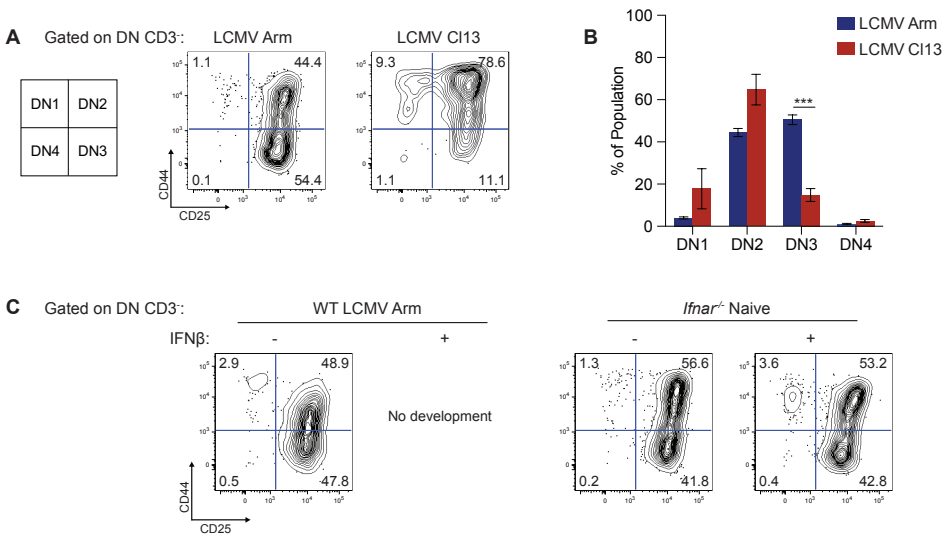

Figure II-8: LCMV Cl13 infection leads to intrinsic changes to the DN1 cells delaying their development *ex vivo*. DN1 cells FACS sorted from 2 days infected C57BL/6 WT mice, or naïve *Ifnar*$^{-/-}$ mice, plated in co-culture with OP9-DL1 for 8 days. (A) Representative FACS plot of thymocyte development. (B) Frequency of DN1 (CD44^{+} CD25^{-}), DN2 (CD44^{+} CD25^{+}), DN3 (CD44^{-} CD25^{+}) and DN4 (CD44^{-} CD25^{-}) populations. (C) Representative FACS plots from cells treated at 1- and 2-days post-co-culture with 100ng IFNβ. Data analyzed by student's t-test for statistical analysis. *** $p < 0.001$. Data represent 3 mice per group (A-B) or 1 mouse per group (C) performed once.

Discussion

Chronic viral infections still pose a tremendous burden in today's society and although a lot of effort has been directed at understanding its consequences during the chronic phase, less is known about its effect at an early time point. Here we show that chronic, but not acute, LCMV infection leads to thymic involution, and although the thymus size is restored at 20 dpi, other consequences of this infection are observed in the thymus beyond this time point. As previously mentioned, each viral infection leading to thymic involution affect the development of thymocytes differently, however a common phenotype is the massive increase in DP cell death[48]. In this study, we show that this phenotype is also accompanied by a DN1 developmental block, which has been previously reported in some infections[50, 52], but not yet characterized. This immature DN substage is known to be heterogenous and to possess the ability to give rise to B cells if Notch signaling is abrogated[17, 32]. LCMV Cl13 infection led to the generation of a $B220^{+}$ $CD19^{-}$ cell population, here referred to as B cell-like cells, which not only underwent active proliferation but also matured from the pre-pro to the pro B cell stage within the thymus. Although the DN1 block was partially dependent on IFN-I signaling, this family of cytokine was essential for the generation of B cell-like cells. One of the possible mechanisms dictating the switch in cell fate could be explained by the change in Notch1 receptor-ligand affinity. It has been well characterized, that this interaction can be modulated by fringe proteins[40]. LCMV Cl13 infection led to the upregulation of Lfng expression, in a IFN-I dependent matter, suggesting that a global change in ligand affinity might result in the generation of B cell-like cells. Moreover, LCMV Cl13 infection and IFN-β led to intrinsic changes in the DN1 population delaying their development *ex vivo,* an environment in which Notch ligand was not a limiting factor. Together, our study suggests that chronic viral infection lead to a block in T cell developmental coupled with changes in cellular fate. Therefore, the early effects of chronic infection within the thymus might pose long term consequences to the host.

Although the thymus is essential for the proper development of T cells during early life, it also plays a crucial role in adults with chronic viral infections. Studies have shown that during polyomavirus chronic infections, the release of newly formed thymocytes is essential to replenish exhausted cells and to promote antiviral immunity[102, 103]. Therefore, a block in thymocyte development could impact viral clearance. The DN1 block observed following LCMV Cl13 infection could lead to a decrease in thymic output, as the DN4 subgroup replenishment occurs

only at 20 dpi. It has been previously shown that LCMV Cl13, but not LCMV Arm, infection leads to irreversible splenic atrophy. This phenotype was marked by a decrease in the overall numbers of T cells, among other cell types, at 60 dpi when compared to pre-infection levels[104]. This study, however, did not analyze the impact of the thymus for the establishment of this phenotype. Hence, further studies have to be done to analyze possible changes in thymic output levels and its consequences during LCMV Cl13 infection at different time points.

LCMV Cl13 infection has been shown to impact thymic structure[50]. However, although an intact thymic structure is crucial for the proper development of thymocytes, our data show that this phenotype might not be playing a role, or at least is unlikely to be the only factor, in the DN1 block. If the disruption of the thymic structure was required to cause the developmental block, DN1 cells harvested from LCMV Cl13 and LCMV Arm infected mice, would develop similarly in the OP9-DL1 co-culture system where all the proper cues for development are provided. However, our data demonstrated otherwise as the development of thymocytes from LCMV Cl13 infected mice remains impaired on the OP9-DL1 co-culture system. Therefore, LCMV Cl13 infection is leading to intrinsic changes in the DN1 population. There are a few possibilities that could lead to this phenotype, and Notch signalling abrogation is one of them. Although it has been shown that Lfng increases the affinity of Notch to Dll1 binding[41], no studies have presented a threshold to this increase in affinity. It could be possible that Lfng is modifying the extracellular portion of the Notch receptor in a way that is leading to an excessively strong interaction, preventing the proper signaling from occurring. DN1 cells also require IL-7 and FMS-like tyrosine kinase 3 (Flt3) signaling for proper development. Therefore, another possibility leading to the DN1 intrinsic defect could be due to changes in IL-7 and Flt3 receptor expression by these cells. By making DN1 cells hyposensitive to IL-7 and/or to Flt3 ligand thymocyte development could be delayed.

It is also important to note that *in vivo*, the DN1 block is at least partially dependent on IFN-I signalling and IFN-β treatment *ex vivo* completely blocked the development of DN1 cells by acting directly on these cells. In line with this, we also have preliminary data demonstrating that the establishment of the DN1 block might not be directly linked to virus presence but rather could be due to the consequences of infection, such as IFN-I release. We analyzed a mouse model of sepsis (cecal ligation and puncture, CLP) which is known to lead to an increase production of pro-inflammatory cytokines[105]. The thymus phenotype of the CLP mice showed thymic involution,

a massive decrease in the DP population and a DN1 developmental block marked by an increase in thymic B cell numbers (data not shown). These data further suggest that the intrinsic changes in the DN1 population might not be directly linked to the presence of the virus within the thymus, but rather to a change in the cytokine milieu within this environment. Further studies have to be done to analyze the changes in gene expression following IFN-I signaling within the DN1 cells. These data would further help us tease out the mechanism leading to the DN1 development blocked *in vivo*.

Due to the immature and heterogeneous nature of the DN1 population, a block at this stage can lead to consequences beyond the decrease in thymic output. It is well established that the DN1 cell population can give rise to B cells in the thymus if Notch signaling is abrogated[25]. Although it is well accepted that the majority of thymic B cells are generated within the thymus during normal conditions[25], less is known about their development under pathological conditions. Here we show that LCMV Cl13 infection led to an increase in B cell-like cells, which show a low, but progressively increasing, CD19 gMFI expression. Using our experimental model, it was impossible to track B cell-like cells past pro B cell stage, since pre B cells express high levels of CD19, therefore, blending into the naturally occurring thymic B cell population. However, it is important to note that a study has suggested that pro B cells might not be able to continue their development to pre B cell, due to their hyposensitivity to IL-7 cytokine. Pro B cells were shown to express low expression of CD127 (α chain of the IL-7 receptor) preventing further maturation[106]. Therefore, further studies are required to analyze whether B cell-like cells generated in our models are able to continue their development within this organ.

Although the abrogation of Notch signaling in different models can lead to an increase in the generation of thymic B cells, these cells possess a different phenotype than the naturally occurring thymic B cells[25]. As previously mentioned, these cells show a more immature phenotype, an increased proliferative capacity and the expression of distinct surface proteins, when compared to naturally occurring thymic B cells[25]. In our model, B cell-like cells possess a high proliferative capacity (shown by the increase in BrdU incorporation) as well as an immature phenotype ($B220^{lo}$ $CD19^{lo}$). Moreover, these cells appear to develop from the DN1b group, which has been previously suggested to lack the ability to generate B cells[17]. Together, our data suggest that B cell-like cells generated following LCMV Cl13 infection are distinct from naturally occurring thymic B cells. Due to the immature phenotype similarities to $Notch1^{-/-}$ generated thymic

B cells[37], B cell-like cells generation could be linked to a block in the Notch signaling. However, further analysis of their phenotype is crucial to further validate this claim.

Many autoimmune diseases have been linked to chronic viral infections[107], however, it is still unknown whether the consequences of thymic involution, or misdirected fate decision in the thymus, is playing a role in this phenotype. Assuming that B cell-like cells can continue their maturation in the thymus, these cells could be associated with the underlining cause of B cell dictated autoimmune diseases. Since the thymus is not programmed to generate B cells, B cell-like cells might not be properly educated resulting in their self-reactivity. One autoimmune pathology which has been associated with autoreactive thymic B cells is called myasthenia gravis (MG). MG is marked by the accumulation of autoreactive B cells targeting components at the muscle membrane within the neuromuscular junction such as the Nicotinic acetylcholine receptor (nAChR)[108]. This results in varying degrees of skeletal muscle weakness, which can be life-threatening when targeting muscles such as the diaphragm. MG has been associated with viral infections and it possesses an IFN-I signature[109]. The prevailing dogma is that the autoreactive thymic B cells present in MG patients, are migrating B cells generated in the periphery[110]. However, the studies have not tested out the possibility of an *in situ* generation of these cells. MG might be the consequence of B cell-like cells generated following chronic viral infections. Therefore further understanding of this mechanism is essential to allow us to further understand the link between chronic viral infections and the development of autoimmune pathologies. As such, the LCMV infection model may provide a great system to study the role of thymic B cells in autoimmune pathologies that have been associated with various viral infections.

Chapter III: Conclusions and future directions

The thymic architecture is crucial for proper thymocyte development, infections not only promote thymic involution impairing such structure but also change the cytokine milieu modulating the expression of key proteins[48, 74]. Herein, we demonstrate that the acute and chronic strains of LCMV have different effects on the thymus. LCMV Cl13 promotes thymic involution leading to a profound DN1 developmental block which is marked by the generation of a $B220^{+}$ $CD19^{-}$ population (here called B cell-like cells). Our data suggest that these cells not only mature in the thymus but might also receive survival signals allowing them to remain in this environment even after the thymic involution is restored. We have observed that IFN-I is essential to generate B cell-like cells in the thymus as the impairment of IFN-I signaling (in *Ifnar*$^{-/-}$ mice) completely abrogates the generation of these cells. Moreover, IFN-I signaling might be leading to intrinsic changes in the DN1 population which delay their development in an environment where Notch ligand and cytokine availabilities are not limited. B cells generated in the bone marrow undergo important selection checkpoints to prevent their auto-reactivity. However, since the thymus is not programmed to generate B cells, B cell-like cells might not undergo such regulation potentially resulting in the generation of auto-reactivity, ultimately promoting immune pathologies.

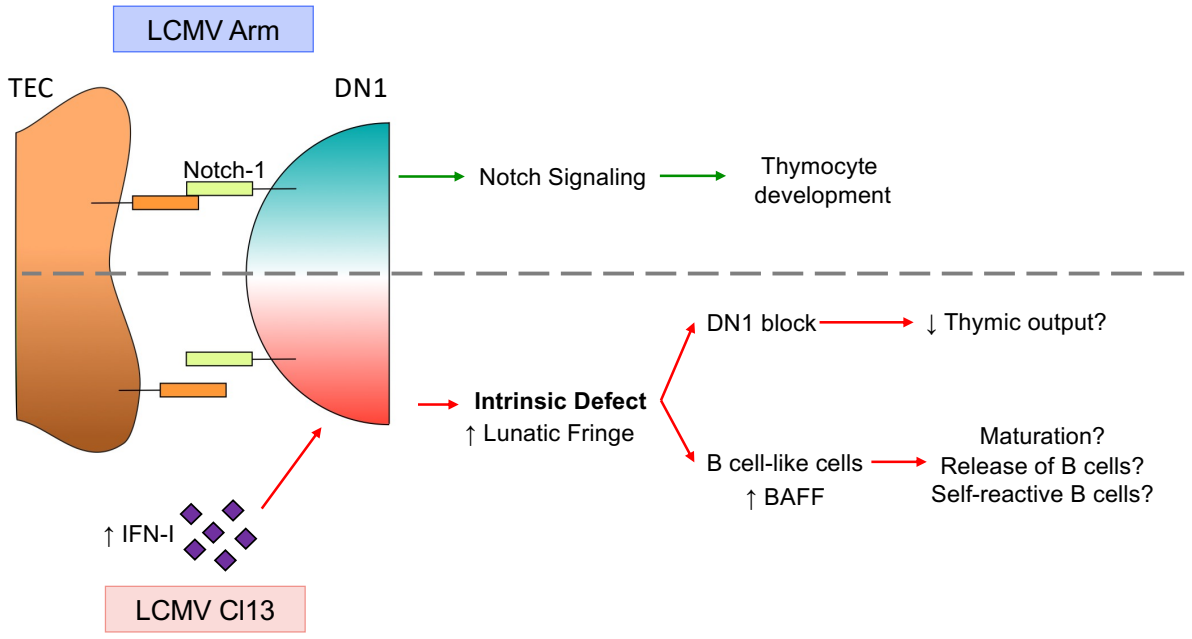

Figure III-1: Working model of the effects of LCMV Cl13 viral infection on the development of T cells within the thymus.

The next steps for these project can be divided into 3 categories: (1) the determination of the impact of IFN-I in thymocyte development and in the DN1 block; (2) the further analysis of B cell-like cells phenotype, maturation, location and function and (3) the further characterization of the role of Notch signaling and Lfng in the generation of B cell-like cells.

First, we would like to dissect the requirement of IFN-I for the establishment of the DN1 developmental defect. We would like to determine whether IFN-β treatment *ex vivo* is blocking DN1 cells from further development or whether it is promoting cell death. For this, we will increase the number of DN1 cells initially plated, as well as, use different doses of IFN-β treatment in the OP9-DL1 co-culture experiments. We will also analyze DN1 cell death and proliferation by flow cytometry at different time points, such as 1, 2 and 3 days post-IFN-β treatment. In order to analyze whether IFN-β treatment is only affecting DN1 cells, we will also plate, treat and analyze the development of DN2, DN3 and DN4 separately in OP9-DL1 co-culture. By treating these cells with IFN-β and tracking their development *ex vivo* we can determine whether this cytokine is having a specific effect on DN1 cells, or globally, in all DN populations. All the experiments previously mentioned will also be performed using IFN-α, to further understand the role of each cytokine in our model. *In vivo*, we will analyze the role of IFN-I by providing IFN-α and IFN-β intrathymically, and also by blocking their effect using blocking antibodies. To begin to elucidate the mechanism dictating the DN1 block by IFN-I we will perform RNA-seq analysis of the DN1 population in various conditions. WT and *Ifnar*$^{-/-}$ mice will be infected, or not, with LCMV Cl13 and thymus will be harvested at 2 dpi. RNA-seq will be performed in CD19^{-} DN1 FACS sorted cells. Comparison of gene expression between the groups will help us determine the mechanism is which IFN-I is able to block DN1 cell development. In order to rule out the requirement of viral infection within the thymus for the establishment of the DN1 developmental block, we will also perform polyinosinic-polycytidylic acid (poly I:C) intrathymic treatment and analyze thymocyte development at different time points. Moreover, we are currently setting up bone marrow chimera mice to analyze the requirement of IFN-I within the hematopoietic and non-hematopoietic compartment for the establishment of the DN1 block. We have transferred WT bone marrow cells into lethally irradiated *Ifnar*$^{-/-}$ mice and *Ifnar*$^{-/-}$ bone marrow cells into lethally irradiated WT host. LCMV Cl13 infection in each cohort, plus the appropriated cohort controls, will allow us to observe the effect of IFN-I in each cell population. Together, these experiments will help us to further understand the effects of IFN-I signaling on thymocyte development.

Second, we would like to further characterize the phenotype, maturation, location and function of B cell-like cells. We will use intrathymic adoptive transfer approaches to determine whether DN1 cells are switching to a B cell fate, or whether B cell-like cells are being generated from a different progenitor outside the thymus and migrating into this organ early during infection. For this, we will transfer DN1 cells intrathymically and track their maturation (using CD45 congenic markers) following LCMV Cl13 infection. This experimental setting will also allow us to determine whether B cell-like cells are maturing past the pro B cell stage and whether they are expressing a BCR. DNA extraction of B cell-like cells at the pro B cell stage (15-20 dpi) will be performed to analyze BCR recombination. Next, we would like to determine the requirement of BAFF within the thymus microenvironment for B cell-like cell maturation and survival. For this, we will use BAFF blocking antibodies delivered intrathymically at different time points following LCMV Cl13 infection. Moreover, we would like to determine whether B cell-like cells are retained within the thymus at later time points (60-80 dpi) or if these cells are being released into the periphery. This will be done by intrathymically injecting congenically marked DN1 cells in the thymus prior to LCMV Cl13 infection. At later time points, thymus, spleen and lymph nodes will be harvested to analyzed for the presence of adaptively transferred cells. We will also perform single-cell RNA seq in the B cell-like cell population at different time points, to have a better grasp of the population phenotype, diversity and to compare them with the canonical thymic B cell population.

Third, we would like to determine the requirement of Notch signaling and Lfng modification for the generation of B cell-like cells. Using confocal microscopy, we will determine the location of $B220^{+}$ cells. Naturally occurring thymic B cells are shown to be only present in the medullary region of the thymus, while B cell progenitors are shown to be present in the cortical region[23]. We would also like to determine whether B cell-like cells generation is abrogated once Notch1 signaling is activated, by utilizing the RBPJ-inducible system previously described[19]. This system allow for the activation of Notch1 signaling while bypassing the requirement of ligand binding. For this experiment, we will use intrathymic adoptive transfer approaches of RBJP-inducible, and control, DN1 cells into WT mice, prior to the infection with LCMV Cl13. We will then induce the production of RBPJ and track cellular development by harvesting the thymus at different time points. This approach will allow us to determine the requirement of Notch signaling in the generation of B cell-like cells, by bypassing the need for ligand binding. We will also analyze

the location of cells expressing Lfng, by confocal microscopy. Lfng expression is naturally present closer to the corticomedullary junction, expression of this protein deep into the cortex is shown to decrease Notch ligand availability and increase thymic B cell generation[111]. Together, these experiments will allow us to determine the need for Notch and Lfng in the generation of B cell-like cells.

Taken together, these experiments will allow us to further understand the defect and the consequences of LCMV Cl13 in the development of T cell. Understanding this process is crucial, since an impairment on the T cell pool can lead to a diminished resistance to infection and cancer. Moreover the generation of autoreactive lymphocytes following chronic viral infection could help us explain why some autoimmune disorders are shown to be liked to viral infections.

References

1. Pardy, R.D., Valbon, S.F. & Richer, M.J. Running interference: Interplay between Zika virus and the host interferon response. *Cytokine* **119**, 7-15 (2019).

2. Jung, D. & Alt, F.W. Unraveling V(D)J recombination; insights into gene regulation. *Cell* **116**, 299-311 (2004).

3. Alberts, B. *et al. Molecular Biology of the Cell* Fourth Edition edn, 2002.

4. K., M. *Janeway's Immunobiology*, 8th Edition edn, 2012.

5. Dzhagalov, I. & Phee, H. How to find your way through the thymus: a practical guide for aspiring T cells. *Cell Mol Life Sci* **69**, 663-682 (2012).

6. Klug, D.B. *et al.* Interdependence of cortical thymic epithelial cell differentiation and T-lineage commitment. *Proc Natl Acad Sci U S A* **95**, 11822-11827 (1998).

7. Zuniga-Pflucker, J.C. T-cell development made simple. *Nat Rev Immunol* **4**, 67-72 (2004).

8. Ciofani, M. & Zuniga-Pflucker, J.C. The thymus as an inductive site for T lymphopoiesis. *Annu Rev Cell Dev Biol* **23**, 463-493 (2007).

9. Shah, D.K. & Zuniga-Pflucker, J.C. An overview of the intrathymic intricacies of T cell development. *J Immunol* **192**, 4017-4023 (2014).

10. Zachariah, M.A. & Cyster, J.G. Neural crest-derived pericytes promote egress of mature thymocytes at the corticomedullary junction. *Science* **328**, 1129-1135 (2010).

11. Hayday, A.C. & Pennington, D.J. Key factors in the organized chaos of early T cell development. *Nat Immunol* **8**, 137-144 (2007).

12. Michie, A.M. & Zuniga-Pflucker, J.C. Regulation of thymocyte differentiation: pre-TCR signals and beta-selection. *Semin Immunol* **14**, 311-323 (2002).

13. Kreslavsky, T. *et al.* beta-Selection-induced proliferation is required for alphabeta T cell differentiation. *Immunity* **37**, 840-853 (2012).

14. Anderson, G. & Takahama, Y. Thymic epithelial cells: working class heroes for T cell development and repertoire selection. *Trends Immunol* **33**, 256-263 (2012).

15. Z., M. & D., L.A. *Middlenton's Allergy* 8th Edition edn, vol. Volume 1, 2014.

16. Zlotoff, D.A. *et al.* CCR7 and CCR9 together recruit hematopoietic progenitors to the adult thymus. *Blood* **115**, 1897-1905 (2010).

17. Porritt, H.E. *et al.* Heterogeneity among DN1 prothymocytes reveals multiple progenitors with different capacities to generate T cell and non-T cell lineages. *Immunity* **20**, 735-745 (2004).

18. Godfrey, D.I., Zlotnik, A. & Suda, T. Phenotypic and functional characterization of c-kit expression during intrathymic T cell development. *J Immunol* **149**, 2281-2285 (1992).

19. Chen, E.L.Y., Thompson, P.K. & Zuniga-Pflucker, J.C. RBPJ-dependent Notch signaling initiates the T cell program in a subset of thymus-seeding progenitors. *Nat Immunol* **20**, 1456-1468 (2019).

20. Isaacson, P.G., Norton, A.J. & Addis, B.J. The human thymus contains a novel population of B lymphocytes. *Lancet* **2**, 1488-1491 (1987).

21. Miyama-Inaba, M. *et al.* Unusual phenotype of B cells in the thymus of normal mice. *J Exp Med* **168**, 811-816 (1988).

22. Nango, K. *et al.* Ontogeny of thymic B cells in normal mice. *Cell Immunol* **133**, 109-115 (1991).

23. Akashi, K., Richie, L.I., Miyamoto, T., Carr, W.H. & Weissman, I.L. B lymphopoiesis in the thymus. *J Immunol* **164**, 5221-5226 (2000).

24. Perera, J., Meng, L., Meng, F. & Huang, H. Autoreactive thymic B cells are efficient antigen-presenting cells of cognate self-antigens for T cell negative selection. *Proc Natl Acad Sci U S A* **110**, 17011-17016 (2013).

25. Perera, J. & Huang, H. The development and function of thymic B cells. *Cell Mol Life Sci* **72**, 2657-2663 (2015).

26. Inaba, M. *et al.* Functional analyses of thymic CD5+ B cells. Responsiveness to major histocompatibility complex class II-restricted T blasts but not to lipopolysaccharide or anti-IgM plus interleukin 4. *J Exp Med* **171**, 321-326 (1990).

27. Ceredig, R. The ontogeny of B cells in the thymus of normal, CD3 epsilon knockout (KO), RAG-2 KO and IL-7 transgenic mice. *Int Immunol* **14**, 87-99 (2002).

28. Mohtashami, M. *et al.* Direct comparison of Dll1- and Dll4-mediated Notch activation levels shows differential lymphomyeloid lineage commitment outcomes. *J Immunol* **185**, 867-876 (2010).

29. Kovall, R.A. & Blacklow, S.C. Mechanistic insights into Notch receptor signaling from structural and biochemical studies. *Curr Top Dev Biol* **92**, 31-71 (2010).

30. Osborne, B.A. & Minter, L.M. Notch signalling during peripheral T-cell activation and differentiation. *Nat Rev Immunol* **7**, 64-75 (2007).

31. Tomita, K. *et al.* The bHLH gene Hes1 is essential for expansion of early T cell precursors. *Genes Dev* **13**, 1203-1210 (1999).

32. Radtke, F. *et al.* Deficient T cell fate specification in mice with an induced inactivation of Notch1. *Immunity* **10**, 547-558 (1999).

33. Tan, J.B., Visan, I., Yuan, J.S. & Guidos, C.J. Requirement for Notch1 signals at sequential early stages of intrathymic T cell development. *Nat Immunol* **6**, 671-679 (2005).

34. Wilson, A., MacDonald, H.R. & Radtke, F. Notch 1-deficient common lymphoid precursors adopt a B cell fate in the thymus. *J Exp Med* **194**, 1003-1012 (2001).

35. Han, H. *et al.* Inducible gene knockout of transcription factor recombination signal binding protein-J reveals its essential role in T versus B lineage decision. *Int Immunol* **14**, 637-645 (2002).

36. Pui, J.C. *et al.* Notch1 expression in early lymphopoiesis influences B versus T lineage determination. *Immunity* **11**, 299-308 (1999).

37. Feyerabend, T.B. *et al.* Deletion of Notch1 converts pro-T cells to dendritic cells and promotes thymic B cells by cell-extrinsic and cell-intrinsic mechanisms. *Immunity* **30**, 67-79 (2009).

38. Irvine, K.D. & Wieschaus, E. fringe, a Boundary-specific signaling molecule, mediates interactions between dorsal and ventral cells during Drosophila wing development. *Cell* **79**, 595-606 (1994).

39. Panin, V.M., Papayannopoulos, V., Wilson, R. & Irvine, K.D. Fringe modulates Notch-ligand interactions. *Nature* **387**, 908-912 (1997).

40. Kakuda, S. & Haltiwanger, R.S. Deciphering the Fringe-Mediated Notch Code: Identification of Activating and Inhibiting Sites Allowing Discrimination between Ligands. *Dev Cell* **40**, 193-201 (2017).

41. Visan, I. *et al.* Regulation of T lymphopoiesis by Notch1 and Lunatic fringe-mediated competition for intrathymic niches. *Nat Immunol* **7**, 634-643 (2006).

42. Jenkinson, E.J., Franchi, L.L., Kingston, R. & Owen, J.J. Effect of deoxyguanosine on lymphopoiesis in the developing thymus rudiment in vitro: application in the production of chimeric thymus rudiments. *Eur J Immunol* **12**, 583-587 (1982).

43. Schmitt, T.M. & Zuniga-Pflucker, J.C. Induction of T cell development from hematopoietic progenitor cells by delta-like-1 in vitro. *Immunity* **17**, 749-756 (2002).

44. Ansari, A.R. & Liu, H. Acute Thymic Involution and Mechanisms for Recovery. *Arch Immunol Ther Exp (Warsz)* **65**, 401-420 (2017).

45. Shanley, D.P., Aw, D., Manley, N.R. & Palmer, D.B. An evolutionary perspective on the mechanisms of immunosenescence. *Trends Immunol* **30**, 374-381 (2009).

46. Dooley, J. & Liston, A. Molecular control over thymic involution: from cytokines and microRNA to aging and adipose tissue. *Eur J Immunol* **42**, 1073-1079 (2012).

47. Zakharova, L.A. Evolution of adaptive immunity. *Izv Akad Nauk Ser Biol*, 143-154 (2009).

48. Nunes-Alves, C., Nobrega, C., Behar, S.M. & Correia-Neves, M. Tolerance has its limits: how the thymus copes with infection. *Trends Immunol* **34**, 502-510 (2013).

49. Tarcic, N., Ovadia, H., Weiss, D.W. & Weidenfeld, J. Restraint stress-induced thymic involution and cell apoptosis are dependent on endogenous glucocorticoids. *J Neuroimmunol* **82**, 40-46 (1998).

50. Elsaesser, H.J. *et al.* Chronic virus infection drives CD8 T cell-mediated thymic destruction and impaired negative selection. *Proc Natl Acad Sci U S A* **117**, 5420-5429 (2020).

51. Nobrega, C. *et al.* T cells home to the thymus and control infection. *J Immunol* **190**, 1646-1658 (2013).

52. Majumdar, S. *et al.* Differential susceptibility and maturation of thymocyte subsets during Salmonella Typhimurium infection: insights on the roles of glucocorticoids and Interferon-gamma. *Sci Rep* **7**, 40793 (2017).

53. Hepatitis B. 2019 July 18 2019 [cited 2020]Available from: https://www.who.int/news-room/fact-sheets/detail/hepatitis-b

54. Hepatitis C. 2019 July 9 2019 [cited 2020]Available from: https://www.who.int/news-room/fact-sheets/detail/hepatitis-c

55. HIV/AIDS. 2019 November 15 2019 [cited 2020]Available from: https://www.who.int/news-room/fact-sheets/detail/hiv-aids

56. Teijaro, J.R. *et al.* Persistent LCMV infection is controlled by blockade of type I interferon signaling. *Science* **340**, 207-211 (2013).

57. Brooks, D.G. *et al.* Interleukin-10 determines viral clearance or persistence in vivo. *Nat Med* **12**, 1301-1309 (2006).

58. Ng, C.T. & Oldstone, M.B. IL-10: achieving balance during persistent viral infection. *Curr Top Microbiol Immunol* **380**, 129-144 (2014).

59. Beeman, E.A. Charles Armstrong, M.D.: A Biography. (2007).

60. Lee, K.J., Novella, I.S., Teng, M.N., Oldstone, M.B. & de La Torre, J.C. NP and L proteins of lymphocytic choriomeningitis virus (LCMV) are sufficient for efficient transcription and replication of LCMV genomic RNA analogs. *J Virol* **74**, 3470-3477 (2000).

61. Auperin, D.D., Romanowski, V., Galinski, M. & Bishop, D.H. Sequencing studies of pichinde arenavirus S RNA indicate a novel coding strategy, an ambisense viral S RNA. *J Virol* **52**, 897-904 (1984).

62. Buchmeier, M.J. & Oldstone, M.B. Protein structure of lymphocytic choriomeningitis virus: evidence for a cell-associated precursor of the virion glycopeptides. *Virology* **99**, 111-120 (1979).

63. Buchmeier, M.J., Elder, J.H. & Oldstone, M.B. Protein structure of lymphocytic choriomeningitis virus: identification of the virus structural and cell associated polypeptides. *Virology* **89**, 133-145 (1978).

64. Riviere, Y. *et al.* The S RNA segment of lymphocytic choriomeningitis virus codes for the nucleoprotein and glycoproteins 1 and 2. *J Virol* **53**, 966-968 (1985).

65. Cao, W. *et al.* Identification of alpha-dystroglycan as a receptor for lymphocytic choriomeningitis virus and Lassa fever virus. *Science* **282**, 2079-2081 (1998).

66. Salvato, M., Shimomaye, E. & Oldstone, M.B. The primary structure of the lymphocytic choriomeningitis virus L gene encodes a putative RNA polymerase. *Virology* **169**, 377-384 (1989).

67. Salvato, M.S. & Shimomaye, E.M. The completed sequence of lymphocytic choriomeningitis virus reveals a unique RNA structure and a gene for a zinc finger protein. *Virology* **173**, 1-10 (1989).

68. Ahmed, R. & Oldstone, M.B. Organ-specific selection of viral variants during chronic infection. *J Exp Med* **167**, 1719-1724 (1988).

69. Ahmed, R., Salmi, A., Butler, L.D., Chiller, J.M. & Oldstone, M.B. Selection of genetic variants of lymphocytic choriomeningitis virus in spleens of persistently infected mice. Role in suppression of cytotoxic T lymphocyte response and viral persistence. *J Exp Med* **160**, 521-540 (1984).

70. Kunz, S., Sevilla, N., McGavern, D.B., Campbell, K.P. & Oldstone, M.B. Molecular analysis of the interaction of LCMV with its cellular receptor [alpha]-dystroglycan. *J Cell Biol* **155**, 301-310 (2001).

71. Sevilla, N. *et al.* Immunosuppression and resultant viral persistence by specific viral targeting of dendritic cells. *J Exp Med* **192**, 1249-1260 (2000).

72. Smelt, S.C. *et al.* Differences in affinity of binding of lymphocytic choriomeningitis virus strains to the cellular receptor alpha-dystroglycan correlate with viral tropism and disease kinetics. *J Virol* **75**, 448-457 (2001).

73. Bergthaler, A. *et al.* Viral replicative capacity is the primary determinant of lymphocytic choriomeningitis virus persistence and immunosuppression. *Proc Natl Acad Sci U S A* **107**, 21641-21646 (2010).

74. Wherry, E.J., Blattman, J.N., Murali-Krishna, K., van der Most, R. & Ahmed, R. Viral persistence alters CD8 T-cell immunodominance and tissue distribution and results in distinct stages of functional impairment. *J Virol* **77**, 4911-4927 (2003).

75. Isaacs, A. & Lindenmann, J. Virus interference. I. The interferon. By A. Isaacs and J. Lindenmann, 1957. *J Interferon Res* **7**, 429-438 (1987).

76. McNab, F., Mayer-Barber, K., Sher, A., Wack, A. & O'Garra, A. Type I interferons in infectious disease. *Nat Rev Immunol* **15**, 87-103 (2015).

77. Kotenko, S.V. *et al.* IFN-lambdas mediate antiviral protection through a distinct class II cytokine receptor complex. *Nat Immunol* **4**, 69-77 (2003).

78. Prokunina-Olsson, L. *et al.* A variant upstream of IFNL3 (IL28B) creating a new interferon gene IFNL4 is associated with impaired clearance of hepatitis C virus. *Nat Genet* **45**, 164-171 (2013).

79. Sheppard, P. *et al.* IL-28, IL-29 and their class II cytokine receptor IL-28R. *Nat Immunol* **4**, 63-68 (2003).

80. Levy, D.E., Marie, I.J. & Durbin, J.E. Induction and function of type I and III interferon in response to viral infection. *Curr Opin Virol* **1**, 476-486 (2011).

81. de Weerd, N.A. & Nguyen, T. The interferons and their receptors--distribution and regulation. *Immunol Cell Biol* **90**, 483-491 (2012).

82. Majoros, A. *et al.* Canonical and Non-Canonical Aspects of JAK-STAT Signaling: Lessons from Interferons for Cytokine Responses. *Front Immunol* **8**, 29 (2017).

83. Schoggins, J.W. & Rice, C.M. Interferon-stimulated genes and their antiviral effector functions. *Curr Opin Virol* **1**, 519-525 (2011).

84. Stark, G.R., Kerr, I.M., Williams, B.R., Silverman, R.H. & Schreiber, R.D. How cells respond to interferons. *Annu Rev Biochem* **67**, 227-264 (1998).

85. Schoggins, J.W. *et al.* A diverse range of gene products are effectors of the type I interferon antiviral response. *Nature* **472**, 481-485 (2011).

86. Schneider, W.M., Chevillotte, M.D. & Rice, C.M. Interferon-stimulated genes: a complex web of host defenses. *Annu Rev Immunol* **32**, 513-545 (2014).

87. Snell, L.M., McGaha, T.L. & Brooks, D.G. Type I Interferon in Chronic Virus Infection and Cancer. *Trends Immunol* **38**, 542-557 (2017).

88. Sedaghat, A.R. *et al.* Chronic CD4+ T-cell activation and depletion in human immunodeficiency virus type 1 infection: type I interferon-mediated disruption of T-cell dynamics. *J Virol* **82**, 1870-1883 (2008).

89. Harris, L.D. *et al.* Downregulation of robust acute type I interferon responses distinguishes nonpathogenic simian immunodeficiency virus (SIV) infection of natural hosts from pathogenic SIV infection of rhesus macaques. *J Virol* **84**, 7886-7891 (2010).

90. Bolen, C.R. *et al.* The blood transcriptional signature of chronic hepatitis C virus is consistent with an ongoing interferon-mediated antiviral response. *J Interferon Cytokine Res* **33**, 15-23 (2013).

91. Smith, L.K. *et al.* Interleukin-10 Directly Inhibits CD8(+) T Cell Function by Enhancing N-Glycan Branching to Decrease Antigen Sensitivity. *Immunity* **48**, 299-312 e295 (2018).

92. Virgin, H.W., Wherry, E.J. & Ahmed, R. Redefining chronic viral infection. *Cell* **138**, 30-50 (2009).

93. Ng, C.T. *et al.* Blockade of interferon Beta, but not interferon alpha, signaling controls persistent viral infection. *Cell Host Microbe* **17**, 653-661 (2015).

94. Slifka, M.K. & Whitton, J.L. Functional avidity maturation of CD8(+) T cells without selection of higher affinity TCR. *Nat Immunol* **2**, 711-717 (2001).

95. Livak, K.J. & Schmittgen, T.D. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* **25**, 402-408 (2001).

96. Holmes, R. & Zuniga-Pflucker, J.C. The OP9-DL1 system: generation of T-lymphocytes from embryonic or hematopoietic stem cells in vitro. *Cold Spring Harb Protoc* **2009**, pdb prot5156 (2009).

97. Schmitt, T.M., Ciofani, M., Petrie, H.T. & Zuniga-Pflucker, J.C. Maintenance of T cell specification and differentiation requires recurrent notch receptor-ligand interactions. *J Exp Med* **200**, 469-479 (2004).

98. Cavanagh, B.L., Walker, T., Norazit, A. & Meedeniya, A.C. Thymidine analogues for tracking DNA synthesis. *Molecules* **16**, 7980-7993 (2011).

99. Sakai, J. & Akkoyunlu, M. The Role of BAFF System Molecules in Host Response to Pathogens. *Clin Microbiol Rev* **30**, 991-1014 (2017).

100. Ragheb, S. & Lisak, R.P. B-cell-activating factor and autoimmune myasthenia gravis. *Autoimmune Dis* **2011**, 939520 (2011).

101. Rees, P.A. & Lowy, R.J. Measuring type I interferon using reporter gene assays based on readily available cell lines. *J Immunol Methods* **461**, 63-72 (2018).

102. Vezys, V. *et al.* Continuous recruitment of naive T cells contributes to heterogeneity of antiviral CD8 T cells during persistent infection. *J Exp Med* **203**, 2263-2269 (2006).

103. Wilson, J.J. *et al.* CD8 T cells recruited early in mouse polyomavirus infection undergo exhaustion. *J Immunol* **188**, 4340-4348 (2012).

104. Mbanwi, A.N., Wang, C., Geddes, K., Philpott, D.J. & Watts, T.H. Irreversible splenic atrophy following chronic LCMV infection is associated with compromised immunity in mice. *Eur J Immunol* **47**, 94-106 (2017).

105. Rittirsch, D., Huber-Lang, M.S., Flierl, M.A. & Ward, P.A. Immunodesign of experimental sepsis by cecal ligation and puncture. *Nat Protoc* **4**, 31-36 (2009).

106. Hashimoto, Y., Montecino-Rodriguez, E., Leathers, H., Stephan, R.P. & Dorshkind, K. B-cell development in the thymus is limited by inhibitory signals from the thymic microenvironment. *Blood* **100**, 3504-3511 (2002).

107. Smatti, M.K. *et al.* Viruses and Autoimmunity: A Review on the Potential Interaction and Molecular Mechanisms. *Viruses* **11** (2019).

108. Berrih-Aknin, S. & Le Panse, R. Myasthenia gravis: a comprehensive review of immune dysregulation and etiological mechanisms. *J Autoimmun* **52**, 90-100 (2014).

109. Cufi, P. *et al.* Central role of interferon-beta in thymic events leading to myasthenia gravis. *J Autoimmun* **52**, 44-52 (2014).

110. Berrih-Aknin, S. Role of the thymus in autoimmune myasthenia gravis. *Clinical and Experimental Neuroimmunology* **7**, 226-237 (2016).

111. Visan, I., Yuan, J.S., Tan, J.B., Cretegny, K. & Guidos, C.J. Regulation of intrathymic T-cell development by Lunatic Fringe- Notch1 interactions. *Immunol Rev* **209**, 76-94 (2006).