ShcA and Shcbp1 in T cell development and function

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Abstract

T cell development and function critically depend upon productive signaling via the pre T cell receptor (preTCR) during early development and the T cell receptor (TCR) at later stages of development and activation. Propagation of signals downstream of the preTCR and TCR requires adapter proteins, including the adapter protein ShcA, to help assemble signaling complexes. Furthermore, I found that ShcA dependent preTCR signaling leads to the upregulation of *Shcbp1*, a poorly understood protein that binds to the adapter protein ShcA and is elevated in activated lymphocytes. Additionally*, Shcbp1* expression tightly correlates with proliferative stages of T cell development. When I generated and analyzed mice deficient in Shcbp1 expression, unexpectedly, loss of *Shcbp1* did not have an obvious effect during T cell development *in vivo*. *Shcbp1* is also upregulated in activated T cells and in the context of autoimmunity. I found that in a mouse model of multiple sclerosis, *Shcbp1* deficient mice had reduced disease severity and improved survival, and this effect was T cell intrinsic. Thus, Shcbp1 is dispensable for T cell development, but influences $CD4^+$ T cell effector function in autoimmunity.

Previously, our laboratory has shown that ShcA is required for progression through the β-selection checkpoint. However, the role of ShcA in signaling via the TCR at the DP stage of development and in late T cell development has not been addressed. I found that ShcA is required for late T cell development and progression from the DP to SP stage of thymocyte development (done in collaboration with Dr. Trampont). Mice expressing a dominant negative ShcA transgene from the DN4/DP stage of development exhibit peripheral lymphopenia and develop attenuated disease in the EAE model of MS. Therefore, ShcA contributes to progression through positive selection checkpoints and the maintenance of peripheral T cell numbers.

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List of Abbreviations

Ab	Antibody
Ag	Antigen
AIDS	Acquired immunodeficiency syndrome
ALL	Acute lymphoblastic leukaemia
ANOVA	Analysis of variance
APC	Antigen presenting cell
APL	Altered peptide ligand
AUC	Area under the curve
AurB	Aurora B
BCR	B cell receptor
BME	β-mercaptoethanol
bp	Base-pairs
BrdU	5-Bromo-2-Deoxyuridine
BSA	Bovine Serum Albumin
cDNA	Complimentary DNA
CFSE	Carboxy-Fluorescein DIacetate Succcinimidyl Ester
CH	Collagen Homology
CLP	Common Lymphoid Progenitor
CMJ	Corticomedullary junction
CNS	Central nervous system
CS	Central spindlin complex
CSF	Cerebral spinal fluid
C-terminal	Carboxyl-terminus
CTLA4	Cytotoxic T-Lymphocyte Antigen 4

CXCR4	C-X-C chemokine receptor type 4
DAPI	4',6-diamidino-2-phenylindole
DCIS	Ductal carcinoma *in situ*
DNA	Deoxyribonucleic Acid
DN	Double Negative
DP	Douple Positive
EAE	Experimental autoimmune encephalomyelitis
EBV	Ebstein-Barr virus
EGF	Epidermal growth factor
Egr	Early growth response
ELISA	Enzyme-linked immunosorbent assay
ETP	Early thymic progenitors
F	Phenylalanine
FACS	Fluorescence-Activated Cell Sorter
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
FITC	Fluorescein Isothiocyanate
Fl	Floxed
Grb2	Growth factor receptor-bound protein 2
GWAS	Genome-wide association studies
eGFP	enhanced-Green fluorescent protein
HCC	Human hepatocellular carcinoma
H&E	Hematoxylin and eosin stain
HHV-6	Human herpesvirus 6
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen

HSC	Hematopoietic Stem cell
IB	Immunoblot
Ig	Immunoglobulin
IDC	Invasive ductal carcinoma
IFN	Interferons
IF	Immunofluorescent
IHC	Immunohistochemistry
IV injection	Intravenous injection
IL	Interleukin
IMDM	Iscove's Modified Dulbecco's Medium
IP	Immunoprecipitation
IP injection	Intraperitoneal injection
JC virus	John Cunningham virus
kDa	Kilodalton
LCMV	Lymphocytic choriomeningitis virus
Lin	Lineage
LMPP	Lymphoid primed multipotential progenitor
LN	Lymph node
MHC	Major histocompatibility complex
mLin41	mouse Lin41
ml	milli-liter
MOG_{35-55}	Myelin Oligodendrocyte Glycoprotein Peptide Fragment 35-55
mPAL	murine Protein of Activated Lymphocytes
MPP	Multipotent progenitor cells
MRI	Magnetic resonance imaging
mRNA	Messenger RNA

MS	Multiple Sclerosis
MV	Measles virus
Nesd	Nessun Dorma
OVA	Ovalbumin
Pav-KLP	Pavarotti kinesin-like protein
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PE	Phycoerythrin
PerCp	Peridinin chlorophyll
PecLD	Pectin lyase-like domain
PI(3)K	Phosphoinositide 3-kinase
PMA	Phorbol 12-myristate 13-acetate
pMHC	peptide-MHC
PML	Progressive multifocal leukoencephalopathy
PPMS	Primary progressive multiple sclerosis
preTCR	pre T cell receptor
PRMS	Progressive relapsing MS
PTB	Phosphotyrosine-binding domain
PyV-MT	Polyoma virus middle T antigen
RBC	Red blood cell
RT-PCR	Quantitative reverse transciptase PCR
Rag	Recombination-activating gene
RNA	Ribonucleic Acid
RRMS	Relapsing remitting multiple sclerosis
RT	Room Temperature
SARS	Severe Acute Respiratory Syndrome

SEM	Standard error of the mean
Ser	Serine
SH2	Src homology 2
Shcbp1	Shc SH2-domain binding protein 1
SL-TBI	Sub-lethal total body irradiation
S1P	Sphingosine-1-phosphate
SP	Single Positive
SPMS	Secondary progressive multiple sclerosis
T-ALL	T-cell acute lymphoblastic leukemia
TCR	T cell receptor
TGFβ	Transforming growth factor β
Thr	Threonine
μl	micro-liter
μg	micro-gram
WCL	Whole cell lysate
WT	Wild-type
w/v	Weight/volume
Y	Tyrosine

Chapter I

Introduction

T cell development and activation are highly regulated processes. The proper execution of development and T cell activation is important for achieving a competent immune system, while defects can lead to uncleared infections, autoimmunity, and cancer[1]. Therefore, it is important to understand the molecular mechanisms involved in T cell development and activation.

T cell development and function critically depend upon productive signaling via the pre T cell receptor (preTCR) during early development and T cell receptor at later stages of development and activation[2-6]. Propagation of signals downstream of the preTCR and TCR require adapter proteins to help assemble signaling complexes[7]. ShcA is an adapter protein expressed in thymocytes that has an essential and non-redundant role in T cell development[8,9]. Furthermore, Shc SH2-domain binding protein-1 (Shcbp1) (also called murine Protein of Activated Lymphocytes, mPAL) is a relatively poorly understood protein that binds to the adapter protein ShcA and is elevated in activated lymphocytes. Previous studies have linked Shcbp1 to proliferation, which suggested the hypothesis that Shcbp1 may regulate T cell signaling and proliferation during development and activation[10].

In this introduction, I will provide a review of four topics relevant to my main thesis projects: (1) T cell development; (2) the autoimmune disease multiple sclerosis (MS) and the mouse model experimental autoimmune encephalomyelitis (EAE); (3) the adapter protein ShcA; and (4) a review of the current literature relevant to Shcbp1.

Although these topics are somewhat disparate, this introduction is expected to provide the necessary background and context for Chapter III and Chapter IV of this thesis.

1: T cell development

1.1. *Anatomy and Function of the thymus*

The thymus is a bi-lobed structure that is the primary site for T cell lymphopoiesis[11,12]. The thymic microenvironment provides the necessary signals to induce T lineage commitment in thymic precursors as well as support further differentiation, proliferation, selection, and survival[11]. The thymic microenvironment contains thymocytes, specialized epithelial cells, macrophages, fibroblasts, and dendritic cells. The thymus contains a highly cellular outer cortex with cortical epithelial cells and a large number of thymocytes, an inner medulla with medullary thymic epithelial cells and fewer thymocytes, and a transitional cortico-medullary junction containing primarily blood vessels[11] **(Figure 1.1B)**. Migration of maturing thymocytes through distinct regions and microenvironments is key for normal T cell development[13,14].

1.2. *Overview of T cell development*

T cell development in the thymus is a highly regulated process that involves the coordinated expression of cell surface receptors and multiple selection processes. The process of developing a functional T cell in the thymus takes approximately two weeks; during this time, the immature thymocytes migrate through different regions of the thymus as they develop and the different thymic environments provide distinct signals and cues[14]. Expression and productive signaling through the preTCR and TCR are essential for proper T cell development and progression through the developmental

checkpoints. Development proceeds from the most immature $CD4^-CD8^-$ double negative (DN) stage (which can further be subdivided into DN1-DN4 based on the expression of CD25, CD44, and CD117), thorough the $CD4^+CD8^+$ double positive (DP) stage, to the $CD4^+$ or $CD8^+$ single positive (SP) stage[2,15] **(Figure 1.1A)**. T cell development can be roughly divided into four main steps: (1) The colonization of the thymus and commitment of precursors to the T cell lineage; (2) $\alpha\beta$ or $\gamma\delta$ lineage commitment and the β-selection checkpoint; (3) Acquisition of immunological recognition and tolerance via positive and negative selection, and (4) Emigration of mature $CD4^+$ and $CD8^+$ SP thymocytes out of the thymus[16]. As the focus of this work is on $\alpha\beta$ T cell development, I will focus the next sections on the development of $\alpha\beta$ T cells.

The first step of T cell development is the colonization and seeding of the thymus by early thymic progenitors (ETP) and the commitment of ETPs to the T cell lineage[14]. Unlike lineages that develop in the bone marrow, the thymus has no known self-renewing potential and is seeded periodically by progenitors either from the bone marrow or the fetal liver during embryonic life[16,17]. ETPs enter the thymus via post-capillary venules at the corticomedullary junction (CMJ)[14]. Colonization of the thymus via ETPs involves redundant signals mediated by CCL25/CCR9, CCL19/CCL21/CCR7, and P-selectin/PSGL-1[16,18-20]. The ETP are uncommitted cells that retain myeloid and B cell but not the megakaryocyte-erythroid lineage potential[17,21]. However, the environment within the thymus promotes T lineage commitment and restrains myeloid and B cell development[22]. T lineage commitment occurs during the DN2 stage of development and requires the interaction of the Notch 1 and 2 receptors on developing thymocytes with the Notch ligands Delta-like ligand 1 and 4 expressed by thymic epithelial cells[16,23]. Cooperation of Notch with other transcription factors including Runx1, E-box proteins,

GATA-3, and TCF-1 drives the expression of T cell specific genes and T cell commitment[1,24].

The second major step of T cell development involves the commitment of DN thymocytes to either αβ or γδ T cell lineages and TCR gene rearrangements[16]. During the DN2/DN3 stage of development, thymocytes fully commit to the T cell lineage and initiate TCR gene rearrangement via recombination activating gene (Rag)-mediated recombination[16]. For αβ T cell differentiation, a properly rearranged TCRβ chain associates with an invariant preTα chain and the accessory cell surface CD3 complex to form the preTCR[3]. Proper preTCR expression and signaling allows DN3 thymocytes to proceed through a developmental checkpoint termed the 'β-selection checkpoint'. Signaling through the preTCR triggers a series of events including further differentiation into DN4 and DP thymocytes, proliferation, survival, migration inwards towards the medulla, and allelic exclusion at the β-chain loci[3,25] **(Figure 1.2)**. Progression through the β-selection checkpoint requires cooperation and integration of at least three signaling pathways: the preTCR, Notch, and the chemokine receptor CXCR4[4,26-28]. In fact, there is an absolute requirement for the preTCR and Notch in thymocyte development and proliferation[4,29]. Additionally, Notch promotes cell survival of DN3 thymocytes during β-selection via regulating cellular metabolism, glucose uptake, and cell size[26]. CXCR4 regulates the localization of immature thymocytes and is also a co-stimulator of the preTCR during β-selection facilitating optimal preTCR induced survival and proliferation[28]. DN3 thymocytes can be subdivided into pre-β-selection DN3 thymocytes (denoted DN3E or DN3a) and post-β-selection thymocytes (DN3L or DN3b) based on cell size (forward-side scatter) or CD27/CD28 expression, respectively[30-32] **(Figure 2.1)**.

The process of αβ versus γδ T cell commitment also occurs during the DN stage of development. Unlike, αβ T cell development, during γδ T cell development there is no known involvement of the preTCR or another invariant chain, rather the DN3 checkpoint assesses signaling via the complete γδTCR[16,23,33]. Although the precise events involved in αβ versus γδ T cell lineage commitment are not fully understood, previous research has suggested that stronger signals promote γδ T cell development[16]. Signaling via the γδTCR results in stronger signaling than the αβTCR, perhaps since it is expressed at higher levels or can engage intrathymic ligands[33]. Regardless, divergence of αβ and γδ T cell lineages results in distinct transcriptional programs and developmental outcomes.

The third major step of T cell development involves acquisition of immunological recognition and tolerance via positive and negative selection as well as CD4 or CD8 lineage commitment[16,34]. After the β-selection checkpoint, thymocytes initiate rearrangement of the α-chain of the TCR to generate a functional αβTCR[16]. The fate of each thymocyte critically depends upon the quality, duration, and strength of signaling through the αβTCR[2]. Positive selection ensures that a functional αβTCR repertoire is generated that is MHC-restricted; thymocytes that are able to productively interact with pMHC and receive an intermediate level of activation are positively selected, while thymocytes that receive very weak or no TCR activation undergo death by neglect[2,35]. Negative selection is crucial for central tolerance and to prevent generation of auto-reactive T cells; thymocytes that receive very strong TCR activation undergo activation-induced apoptosis[36]. Furthermore, within positively selected thymocytes, stronger and more persistent signaling via MHC class II-restricted TCRs promotes the CD4-lineage, while intermittent signaling via MHC class I-restricted TCRs promotes development of the CD8-lineage cells[35,37,38]. Signaling through the TCR and the process

of positive and negative selection is crucial for the maturation of DP thymocytes to CD4 or CD8 SP thymocytes.

The last stage of thymocyte development involves the emigration of mature CD4 and CD8 SP thymocytes from the thymus to the peripheral circulation. Thymocytes exit the thymus at the medulla, likely through post-capillary venules[14]. Emigration of thymocytes requires signaling via sphingosine-1-phosphate (S1P)/S1P1 receptor[39,40]. The S1P1 receptor is expressed on mature SP thymocytes and S1P is found in high concentrations in the peripheral circulation[40]. However, T cell development is not completed within the thymus, and recent thymic emigrants (RTEs) undergo further maturation post-thymically[41,42]. RTEs express lower levels of maturation markers and have a proliferative defect as compared to the pool of mature peripheral T cells[42]. However, RTEs undergo further development and contribute to the peripheral T cell compartment as naïve T cells[42].

1.3. *Proliferation during T cell development*

Proliferation is precisely regulated during T cell development and is absolutely required for normal thymopoiesis[11,29]. Maturing thymocytes undergo stages of active proliferation followed by temporary withdrawal from the cell cycle but maintain the ability to develop into a thymocyte and T cell with proliferative capacity[11]. DN2 thymocytes are characterized by active proliferation, which allows expansion of the rare thymic seeding progenitors[11]. Thymocytes withdraw from the cell cycle at the DN3 stage of development, during which the thymocytes initiate rearrangement of the β-chain of the TCR. Productive rearrangement of the β-chain and signaling through the preTCR allows the DN3 thymocytes to enter the cell cycle and undergo a proliferative burst of 6-8 cycles

immediately following β-selection. On the other hand, thymocytes that have failed to generate a functional β-chain undergo cell death[3,11,25,29]. The preTCR and Notch signaling are both crucial for proliferation during β-selection. Mice deficient in any component of the preTCR or downstream signaling components (such as pre-Tα, Rag1, Rag2, or ShcA) or Notch signaling have small thymi and impaired thymic proliferation[4,8,23,28,43]. The post-β-selection DN3b/DN3L and DN4 thymocytes are highly proliferative; this proliferation expands the population of thymocytes that have generated a functional β-chain, allowing for increased TCR diversity as these thymocytes can generate and pair with different TCRα chains[25]. In fact, the transition from DN3a to DN3b thymocytes represents one of the most prominent transcriptional shifts during T cell development, and 48% of the genes upregulated in DN3a thymocytes are related to proliferation[44]. After this proliferative burst, thymocytes undergo an abrupt transcriptional shutdown (especially genes involved in proliferation, metabolism, and other housekeeping functions)[44]. This transcriptional shutdown (during transition from DP blastic to DP small thymocytes) occurs immediately prior to the positive and negative selection checkpoints during which the majority of thymocytes undergo apoptosis, and this shutdown is thought to reduce the cellular energy cost[44]. Signaling through the TCR is required for positive and negative selection; however, unlike β-selection, this process occurs in the absence of extensive proliferation[44].

Additionally, recent studies have shown that proliferation is actually key for further development of DN thymocytes into DP thymocytes[29]. Knockout of genes required for thymocyte proliferation, such as Myc, E47, and Ccnd3, also leads to impaired thymocyte development in mouse models[29,45,46]. Additionally, elegant studies using the *ex vivo* T cell development assay, OP9-DL1 co-culture, demonstrated that

thymocytes were unable to develop into DP thymocytes in the presence of inhibitors that blocked proliferation[29]. In fact, although Notch and the preTCR are required for both proliferation and differentiation, differentiation could be rescued in the absence of Notch signaling via ectopic activation of proliferation[4,29]. Further studies showed that thymocytes acquire the ability to differentiate only after they have undergone multiple divisions, and thymocytes that had undergone 4-5 divisions were most capable of efficient development into DP thymocytes[29]. These studies collectively demonstrate that differentiation and proliferation are inextricably linked during T cell development. However, why proliferation during β-selection is required for further differentiation is not clear; perhaps proliferation triggers epigenetic changes or there is a requirement for specific proteins in T cell development that are activated during proliferation[29].

1.4. *T cell leukemias and lymphomas*

Although proliferation is key for normal development, its dysregulation can lead to cell transformation and the development of T cell leukemias and lymphomas. Many oncogenes and oncogenetic pathways involved in T cell transformation are also required for normal T cell development[1,34]. In fact, more than 50% of all T-cell acute lymphoblastic leukemia (T-ALL) cases have activating mutations in Notch, a key component in T cell commitment as well as proliferation during β-selection[1]. The majority of T cell leukemias and lymphomas arise during the intermediate stage of T cell development (DN/DP), during which thymocytes are generally highly proliferative[1,34,47,48]. Furthermore, the prognosis of leukemias arising from immature thymocyte subsets is generally worse than lymphomas arising from more mature thymocytes[47,48]. Additionally, the preTCR is required for leukemic transformation in mice and is expressed by nearly all human

T-ALL[1,49]. The close relationship between the process of T cell development, proliferation, and pathological transformation further highlights the importance of understanding the mechanisms controlling thymic development and proliferation[34].

2: Autoimmunity and Multiple Sclerosis

2.1. *Autoimmunity*

T cell development in the thymus generates a repertoire of T cells capable of protecting against infection and transformation, yet tolerant to avoid self-recognition and autoimmunity. T cells expressing a TCR that strongly recognizes self-peptide or MHC are deleted during negative selection and this 'central tolerance' leads to a T cell repertoire that is mostly depleted of T cells that recognize self-antigens[50]. However, despite the stringent checkpoints during T cell development, auto-reactive T cells are present in the periphery of healthy individuals[50,51]. Therefore, the regulation of T cell activation is critical for both the optimal function of adaptive immunity as well as the prevention of autoimmunity resulting from excessive stimulation. The development of autoimmunity is a complex process that involves genetic susceptibility in addition to environmental triggers/insults[50,51]. In the next section, I will focus on the human disease multiple sclerosis (MS) and experimental autoimmune encephalomyelitis (EAE), which is a mouse model of MS. The EAE model was utilized to study peripheral T cell function in Chapter III and IV of this thesis.

2.2. *Background on Multiple Sclerosis*

Multiple sclerosis (MS) is a chronic inflammatory demyelinating disease of the central nervous system (CNS) that is characterized by multifocal areas of myelin loss

with relative preservation of axons during early disease[51-53]. As the disease progresses, there is axonal damage and loss, widespread microglia activation, loss of brain volume, and extensive neurodegeneration leading to progressive disability[52,53]. The diagnosis of MS is a clinical diagnosis that relies on clinical history, MRI imaging, and laboratory tests. The diagnosis requires that there is a 'dissemination of CNS lesions in space and time', meaning that an individual must have two or more lesions acquired during two or more distinct clinical attacks to be diagnosed with MS[52,54]. MS affects more than 2.5 million individuals worldwide and is the most common cause of disability in young adults (excluding trauma) and leads to extensive morbidity and mortality[51,55]. The symptoms of MS are also diverse and depend on where the inflammatory lesions and demyelination occurs within the CNS and include visual, motor, sensory, cognitive, and autonomic dysfunction[54,55].

MS is a heterogeneous disease, both in its clinical presentation and in immunopathogenetic findings[52-54]. The most common form of MS is relapsing-remitting MS (RRMS), which affects 85-90% of individuals with MS and is characterized by periods of exacerbations followed by remission with complete or partial resolution of symptoms[51,54]. However, most individuals with RRMS eventually develop secondary progressive MS (SPMS), in which the disease is characterized by continual progression with occasional plateau or remission[54]. Other individuals develop progressive MS from initial diagnosis and these forms of MS are called primary progressive MS (PPMS) and progressive relapsing MS (PRMS)[54]. In addition to these four main subtypes of MS, there are also several rare and more severe variants of MS including Marburg-type and Balo's Concentric sclerosis[51].

In addition to the heterogeneity of clinical presentation in MS, there is also heterogeneity in the pathological findings. Acute plaques can be classified into four different patterns based on the type of cells present, the pattern of myelin protein loss, complement activation, and oligodendrocyte destruction[52,53]. These findings further highlight that no one mechanism can explain the development of multiple sclerosis and that the immune activation, target of injury, and the process of demyelination are likely variable[52,53]. However, despite the clinical and immunopathological heterogeneity, there is extensive data that demonstrate the crucial role of the immune system in the pathogenesis of MS[51,56].

2.3. *Role of $CD4^+$ T cells in Multiple Sclerosis*

$CD4^+$ T cells have a crucial role in the initiation and development of multiple sclerosis. In patients with MS, myelin specific $CD4^+$ T cells are present in the acute lesions and in the cerebral spinal fluid (CSF). In brief, the current model to explain the induction of MS is that myelin specific T cells are activated in the periphery, migrate to the CNS, and are re-activated by antigen presenting cells (APCs) in the CNS leading to inflammation and demyelination[51,56]. In support of this model, previous studies have shown that myelin specific $CD4^+$ T cells can be found in individuals with MS as well as healthy controls. However, myelin specific T cells in individuals with MS have an activated/memory phenotype while they remain naïve in healthy individuals[51]. One hypothesis is that myelin specific T cells are activated via molecular mimicry and cross react with microbial epitopes[55]. In fact, individuals with MS have a higher frequency of degenerate TCRs, and relapses in RRMS can be triggered by infection. Several pathogens have been identified as potential triggers of relapses including EBV and

HHV-6; the percentage of individuals with MS that are seropositive for EBV is 100% although it is also quite high (90%) within the general population[55]. Additional insights into the role of T cells in MS have been elucidated by clinical trials and therapies used to treat MS. Immunotherapy for MS using an altered peptide ligand (APL) resulted in exacerbation of symptoms in some individuals and inadvertently showed that myelin specific T cells can undergo a 2000-fold expansion during an exacerbation of MS[55,57,58]. Furthermore, the importance of T cell and other mononuclear cell migration into the CNS during MS was highlighted by the use of Natalizumab, which blocked extravasation of immune cells into the CNS by disrupting the interaction of $\alpha 4\beta 1$ on lymphocytes with VCAM-1 on inflamed endothelial cells[55,59]. Natalizumab significantly reduced the number of relapses and prevented disease progression[55]. Unfortunately, this clinical trial also highlighted the importance of normal immune surveillance of the CNS as some individuals developed progressive multifocal leukoencephalopathy (PML), a devastating disease of the CNS caused by the JC virus[60].

Additionally, many genetic loci identified as conferring susceptibility to MS have been linked to the immune system and to $CD4^+$ effector T cell differentiation, activation, and proliferation[61,62]. In fact, certain HLA class II genes as well as the TCR have been strongly linked to increased susceptibility for MS[51]. Recently, new loci have been identified through GWAS collaborations and these studies have shown that immunologically relevant genes are significantly over-represented. Variants of genes conferring susceptibility to MS have been identified in the cytokine/chemokine pathways (IL2, IL2RA, IL12, IL7, CCR4, etc), co-stimulation (CD86, CTLA-4, CD40, etc), and signal transduction (Stat3, Tyk2, etc)[61,62]. Although recently the role of the CNS and CNS specific factors in the pathogenesis of MS have been investigated, surprisingly, there

was a relative absence of genes involved in non-immune mediated neurodegeneration[61,62]. Therefore, these studies have further highlighted the importance of the immune system, and in particular $CD4^+$ T cells, in the pathogenesis of MS.

The crucial role of $CD4^+$ T cells in MS has also been demonstrated via the EAE mouse model. The EAE model reproduces many of the clinical, pathological, and immunological aspects of the human disease MS, including infiltration of auto-reactive T cells into the CNS causing inflammation and demyelination[54,63]. EAE is induced in animals via immunization with myelin antigens in adjuvant and results in ascending flaccid paralysis[64]. EAE is a $CD4^+$ T cell driven disease; EAE can be induced in animals passively via transfer of activated myelin specific $CD4^+$ T cells and mice lacking T cells are completely resistant to the induction of EAE[51,55,65]. Additionally, transgenic TCR mice that express a TCR specific for Myelin Oligodendrocyte Glycoprotein Peptide Fragment 35-55 (MOG_{35-55}) develop spontaneous EAE (~4%) or optic neuritis (~40%), a clinical finding in MS.[66] Recent studies has shown that T_H17 $CD4^+$ T cells are involved in the pathogenesis of EAE (as well as MS) and mice deficient in genes required for T_H17 differentiation are resistant to EAE[67,68]. Collectively, these findings in human MS as well as the mouse EAE have demonstrated that $CD4^+$ T cells are an important component of the pathogenesis of multiple sclerosis.

3: The adapter protein ShcA

3.1. *Overview of ShcA*

Adapter proteins are molecules that do not have any intrinsic catalytic activity, but contain protein-protein and protein-lipid interaction domains and are crucial for propagating signals from activated receptors[69,70]. Through protein-protein and protein-

lipid interaction domains, adapter proteins bring additional molecules into close proximity to the activated receptor including other adapter proteins, kinases, and phosphatases[7,71].

The adapter protein ShcA is a ubiquitously expressed adapter protein with three different isoforms: p46, p52, and p66[7] **(Figure 1.3A)**. Alternative translation initiation sites leads to the generation of the p46 and p52 isoforms while alternative splice sites leads to the generation of p66[71] **(Figure 1.3A)**. ShcA is a modular protein with multiple protein-protein interaction domains with an N terminal PTB domain, a central collagen homology (CH) domain rich in glycine and proline residues, and a C-terminal SH2 domain **(Figure 1.3A)**[7,71,72]. In fact, ShcA was first identified via a search for novel SH2 sequences in a human cDNA library[73]. The p66 isoform of ShcA also contains an additional N-terminal CH2 domain **(Figure 1.3A**)[71]. ShcA is an essential gene in the mouse as *ShcA* deficient mice die at embryonic day 11.5 due to profound embryonic cardiovascular defects[74] and mice expressing ShcA mutants are embryonically lethal or are born at reduced ratios with developmental defects[75].

ShcA lies downstream of many activated receptors, including the growth factor receptors, GPCRs, integrins, TCR, BCR, and the preTCR, and is tyrosine phosphorylated on three critical tyrosine residues within the central collagen homology domain - Y239, Y240, and Y317 in humans and Y239, Y240, and Y313 in mice[71,76,77] **(Figure 1.3A)**. Phosphorylation of ShcA on these tyrosine residues creates a binding motif for the SH2 domain of the Grb2 adapter[71,78,79]. Grb2, in turn, recruits other proteins through its SH3 domain including Sos1 and Gab1 to activate the Ras-Erk MAP kinase pathway and the phosphatidylinositol-3-OH kinase PI(3)K/Akt pathway[78,80,81].

Recently, a study was published that investigated the regulation of EGF signaling by ShcA and the kinetics of protein interactions with ShcA after EGF stimulation. After

stimulation, ShcA is rapidly tyrosine phosphorylated on Y239/Y240 and Y313, and this tyrosine phosphorylation is followed by a wave of Serine/Threonine phosphorylation[82]. ShcA interacts with numerous proteins after EGF signaling: Cluster 1 proteins bind to ShcA maximally at 1-2 minutes, Cluster 2 proteins bind maximally at 2-5 minutes, and Cluster 3 proteins bind maximally at 15-20 minutes after stimulation[82]. The Cluster 1 proteins are primarily involved in stimulating the Ras-Erk MAPK and Akt pathways to promote mitogenic and survival pathways, and association of these proteins with ShcA required Grb2 binding[72,82]. In fact, activation of the Ras-Erk and Akt pathways also leads to Ser/Thr phosphorylation of ShcA and represents a kinetics feedback loop[82]. Cluster 2 and 3 proteins include regulators of cytoskeletal organization, cell migration, tumor suppressors, and inhibitors of mitogenic signaling. Interaction and recruitment of these proteins to ShcA switches the EGF signaling from signals driving proliferation and Erk-MAPK and Akt pathway activation to cell migration and termination of signaling[72,82]. Lastly, there is one protein, Shcbp1, which does not belong to any cluster and binds maximally before stimulation, and it was posited to be a negative regulator of EGF signaling[10,82]. Shcbp1 will be further discussed in the next section, and is the focus of Chapter III of this thesis. Therefore, ShcA plays a role in regulating the temporal outcome of EGF signaling, however the relevance of these findings to the role of ShcA downstream of other receptors and in other cell types is not known. Previous studies have shown that the role of ShcA is context dependent (cell type and receptor).

3.2. *ShcA in T cell development*

Thymocytes and T cells contain several adapter proteins including ShcA, SLP-76, GADS, and LAT, which are required for efficient signaling through the preTCR

and TCR[8,83-85]. Despite the numerous adapter proteins present in thymocytes, ShcA has a crucial and non-redundant role in T cell development that cannot be rescued by the expression of other adapter proteins[8]. ShcA is expressed from the earliest stages of T cell development and is rapidly tyrosine phosphorylated following preTCR and TCR engagement and relays signaling essential for development[8,71,76]. ShcA can be phosphorylated by the tyrosine kinases Lck, Syk, and ZAP-70, although there is a preference for Y239/Y240 phosphorylation by ZAP-70 and Y317 phosphorylation by Lck[79]. Additionally, it is likely that the p46 and p52 isoforms of ShcA are the isoforms crucial for T cell development as the p66 isoform is not expressed or is lowly expressed in hematopoietic cells[7] **(Figure 1.3B)**.

To study the role of ShcA in T cell development *in vivo*, our laboratory has used several genetically modified mouse strains: a conditional ShcA deficient mouse line as well as transgenic mouse lines expressing either wild-type or mutant ShcA[8,9] **(Figure 1.4)**. To conditionally delete ShcA in thymocytes, we used a *Shc*$^{fl/fl}$ mouse line in which exons one and two of ShcA are flanked by loxP sites[8]. Importantly, Cre-mediated recombination results in the ablation of all three isoforms of ShcA. In a complementary genetic approach, our laboratory has also generated transgenic mouse lines that allow conditional expression of Flag-tagged wild-type or mutant ShcA transgenes via a lox-STOP-lox sequence between the EF-1α promoter and the cDNA encoding the human ShcA transgene[7,8] **(Figure 1.4B)**. Of note, ShcA is highly conserved and mouse and human ShcA share 94% amino acid identity[8]. The mutant ShcA transgenic mouse lines encode ShcA cDNAs in which the crucial tyrosine residues within the CH1 domain were mutated to phenylalanine and therefore cannot be phosphorylated: ShcF_{317} (ShcF), Shc$F_{239}F_{240}$ (ShcFF), and Shc$F_{239}F_{240}F_{317}$ (ShcFFF)[9] **(Figure 1.4C)**. In order to study the

role of ShcA in early thymic development, these mouse lines were crossed to the *Lck*-Cre transgenic mouse line (originally generated in Dr. Chris Wilson's laboratory), which expresses the Cre recombinase from the DN2/DN3 stage of development via the *Lck* proximal promoter[86] **(Figure 1.4A)**. In the *Lck*-Cre^{+}/*Shc*$^{fl/fl}$ mice, the loss of ShcA expression led to a significant reduction in the number of thymocytes, a block in progression through β-selection, and a relative accumulation of thymocytes at the DN3 stage of development. The *Lck*-Cre^{+}/ShcFF and *Lck*-Cre^{+}/ShcFFF mice had an even more profound impairment in T cell development compared to the *Lck*-Cre^{+}/*Shc*$^{fl/fl}$ mice, and, in fact, the ShcFF and ShcFFF transgenes act as dominant negative proteins[8,9] **(Figure 1.5)**. There were no defects found in T cell development in the in the *Lck*-Cre^{+}/ShcA and negligible effects in the *Lck*-Cre^{+}/*ShcF317* mice which suggests that the Y_{239}/Y_{240} are sufficient for the functional requirements of ShcA in thymic development[9].

Recent investigations in our lab have found a differential requirement for ShcA in the events downstream of β-selection. The β-selection checkpoint triggers proliferation, differentiation, migration, survival, and allelic exclusion[3]. ShcA is required for proliferation, differentiation, and migration during β-selection, but is dispensable for survival and allelic exclusion at the β-locus (Trampont *et al*, manuscript submitted) **(Figure 1.2)**. However, previous studies have shown that proliferation is required for differentiation during thymic development; whether there is an independent requirement for ShcA in differentiation, or the block in differentiation is due to impaired proliferation is not known[29]. In fact, genetic mouse models that have impaired thymic proliferation and cellularity generally also have impaired thymic differentiation[29]. The differential

requirement for ShcA in the events downstream of the preTCR during β-selection further demonstrates the ability of adapter proteins to organize signaling networks[82].

Work by a former post-doctoral fellow in our laboratory (Paul Trampont) has further defined the role of ShcA in proliferation during thymic development. *In vivo* BrdU pulse labeling studies revealed that thymocytes expressing the ShcFF or ShcFFF transgenes have a reduction in proliferation of the highly proliferative DN3L and DN4 populations (Trampont *et al*, manuscript submitted). The impaired proliferation of thymocytes lacking ShcA or expressing ShcA dominant negative transgenes may be partially explained by the requirement for ShcA in optimal activation of the Ras-Erk MAPK pathway in DN thymocytes[9]. Studies have shown that the Ras-Erk MAPK pathway is required for proliferation during the β-selection checkpoint, and ShcA is required for approximately 70% of Erk activation in DN thymocytes[9,87]. In fact, activation of Erk kinases in DN thymocytes contributes to the induction of early growth response factor 1 (Egr1) and Egr3, and these factors promote cell growth and differentiation during thymic development[87]. As expected *Lck*-Cre^{+}/ShcFFF mice have reduced expression of Egr1 and Egr3, and ectopic expression of Egr1 and Egr3 can partially rescue the proliferative defect in the presence of the ShcFFF transgene (Paul Trampont *et al*, manuscript submitted). However, ShcA also promotes proliferation in DN thymocytes independent of the Ras-Erk MAPK pathway. ShcA is required for optimal c-Myc expression (Trampont *et al*, manuscript submitted) and c-Myc has been identified as a major regulator of DN proliferation independent of the Ras-Erk MAPK pathway[45]. Therefore, ShcA may regulate thymocyte proliferation and differentiation both through Erk dependent and independent mechanisms. The potential role of Shcbp1 as a

mediator of ShcA dependent proliferation during β-selection is addressed in Chapter III of this thesis.

ShcA is also required for migration of thymocytes during β-selection. DN3 thymocytes are primarily localized within the subcapsular zone of the thymus, but after β-selection, DN4 thymocytes migrate from the subcapsular zone back towards the cortex and to the medulla[11,88]. The chemokine receptor CXCR4 mediates migration inwards from the subcapsular zone. However, SDF-1α/CXCR4 signaling is not only important for the localization of thymocytes, but it also acts as a co-stimulator of the preTCR during thymocyte development and *Lck*-Cre^{+}/*CXCR4*$^{fl/fl}$ mice have impaired thymic differentiation and survival during early thymic development[28]. Importantly, ShcA is required downstream of SDF-1α/CXCR4 signaling[28,89,90]. ShcA is phosphorylated in thymocytes via SDF-1α stimulation and DN3 thymocytes isolated from *Lck*-Cre^{+}/*Shc*$^{fl/fl}$ and *Lck*-Cre^{+}/ShcFFF mice have impaired CXCR4 mediated migration[28,89]. Therefore, ShcA is required for optimal signaling through the preTCR as well as CXCR4 mediated migration.

Collectively, these data demonstrate the requirement for ShcA in early thymic development and progression through the β-selection checkpoint[8,9,28]. However, the role of ShcA beyond the β-selection checkpoint and in late T cell development has not been investigated. The role of ShcA in late T cell development and signaling through the TCR is addressed in Chapter IV of this thesis.

3.3. *ShcA in Cancer*

ShcA is considered a proto-oncogene, since it is able to induce a transformed phenotype when overexpressed in certain cell types, and is involved in transformation

driven by oncogenes such as BCR-Abl and polyoma virus middle T[72,91]. ShcA acts downstream of growth factors and hormone receptors, and ShcA mediated signaling has been implicated in various stages of tumorigenesis (especially in breast cancer) including initiation, survival, angiogenesis, and migration/invasion[72,92-94]. Furthermore, ShcA is constitutively phosphorylated in several different cancer cell lines and hyperphosphorylation of ShcA in breast tumors predicts poor prognosis[92]. Additionally, *in vivo* studies in mouse models of breast cancer have supported the role of ShcA as an oncogene; ablation of ShcA specifically in mammary-tissue prevented Neu-mediated tumor development and expression of the ShcFFF transgene also attenuated PyV MT-mediated tumor development[72,95].

4: Shc SH2-domain binding protein-1

Shc SH2-domain binding protein-1**,** Shcbp1, is a poorly understood protein that is a binding partner of the adapter protein ShcA. When I first started this project (June, 2011), there was only one paper on Shcbp1, which was the original cloning paper that was published in 1999. However, over the last three years, several papers have been published that have begun to identify Shcbp1 in different contexts. In this section, I will review the relevant literature on Shcbp1.

4.1. *Identification of Shcbp1*

Shcbp1 was first identified in a yeast-two hybrid screen designed to find new binding partners of the adapter protein ShcA and was isolated from both T-cell and 11.5 day-old mouse embryo libraries. Shcbp1 was initially named *murine Protein of Activated Lymphocytes* (mPAL), due to upregulation of its expression during T cell activation[10].

Shcbp1 is an evolutionarily conserved protein, with human SHCBP1 sharing 78% identity with mouse Shcbp1, and 23% identity with the Drosophila melanogaster homolog Nessun Dorma[10]. Recently, in unbiased screening assays, Shcbp1 has been identified in a diverse array of biological functions with links to proliferation and differentiation, including embryonic development, growth factor signaling, cytokinesis, spermatogenesis, tumorigenesis, and viral infections[82,96-101].

4.2. *Structure of Shcbp1 and binding to ShcA*

Shcbp1 was first identified by its ability to bind to the Shc SH2 domain. SH2 domains generally bind to phosphorylated tyrosine residues, and Shcbp1 contains 23 tyrosine residues, many of which are found in consensus binding motifs for SH2 domains[10]. However, Shcbp1 binds the Shc SH2 domain through a tyrosine-phosphorylation independent mechanism[10]. Although SH2 domains generally bind to phosphotyrosine residues, serine/theronine phosphorylation can also mediate SH2 binding in some proteins[102]. Further studies demonstrated that the interaction of Shcbp1 with the SH2 domain of ShcA also does not require serine/threonine phosphorylation[10]. Therefore, Shcbp1 binds ShcA in a phosphotyrosine-independent mechanism.

Although the structure of Shcbp1 has not been solved, sequence analysis in the Drosophila homolog and mammalian SHCBP1 identified two potential domains: a C-terminal pectin lyase-like domain (PecLD) and a highly conserved region in the N-terminus that has been named Nesd homology domain (NHD)[98] **(Figure 1.6A)**. Pectin lyase-like domains are characterized by a series of parallel β-strands and are found in enzymes from bacterial plant pathogens that are used to digest sugars in the cell wall of plants[103] **(Figure 1.6B)**. Additionally, studies in Drosophila demonstrated that the

Drosophila homolog of Shcbp1 has a high binding affinity for β-galactosides *in vitro*[98]. However, whether Shcbp1 binds to carbohydrates *in vitro* or *in vivo* and the functional importance of this potential interaction is not known. Carbohydrate modifications are often found on extracellular proteins, but Shcbp1 is predicted to be a cytoplasmic protein and does not contain a secretion signal or transmembrane domain[10,98]. Of note, glycosylated proteins can also be found in the cytoplasm and nucleus (O-GlucNAc modified proteins), and it is possible that Shcbp1 may interact with these intracellular glycosylated proteins[98,104]. The Nesd homology domain is the most highly conserved motif and has been shown to be required for Shcbp1 interaction with the central spindlin complex as further detailed below[98].

Recent studies have identified two different post-translational modifications of Shcbp1: phosphorylation on serine 634 (S634) and ubiquitination[96,99]. Shcbp1 is phosphorylated during mitosis on S634 by the Aurora B kinase, and this modification changes the binding of Shcbp1 to members of the central spindlin complex, as discussed further below[96]. Shcbp1 is ubiquitinated by the E2 ubiquitin ligase mouse Lin 41 (mLin41), and this modification may increase the stability of Shcbp1[99].

4.3. *Expression of Shcbp1 correlates with proliferation*

In gene expression databases, Shcbp1 expression appears to correlate well with actively proliferating cells. In the mouse, Shcbp1 is highly expressed during embryonic development and in the thymus, testis, and spleen, but is absent from the brain, liver, and skeletal muscle[10]. Shcbp1 is also highly elevated in actively proliferating cell lines, but is downregulated in terminally differentiated post-mitotic cell lines (P19 cells induced to differentiated into neuronal or muscle cells by retinoic acid or DMSO, respectively)

and in serum starved cell lines[10]. Further studies showed that Shcbp1 is low or absent in cells in the G0/G1 stages of the cell cycle, but elevated in cells committed to the cell cycle or actively cycling[10]. In contrast, the expression of ShcA does not change and remains constant throughout the cell cycle[10].

Shcbp1 is also expressed in actively proliferating cells of the immune system. As its original name implies, murine Protein of Activated Lymphocytes, Shcbp1 is elevated in anti-CD3 stimulated T cells, while it is expressed at low levels in naïve resting T cells[10]. Furthermore, Shcbp1 protein is upregulated in T cells from *CTLA4*-deficient mice compared to T cells from wild-type mice[10]; *CTLA4*-deficient mice have high lymphoproliferation and lethal autoimmunity[105,106]. Therefore, the expression of Shcbp1 appears to correlate with rapidly proliferating cells, both within the immune system as well as other tissues, and I hypothesized that Shcbp1 is likely required for optimal proliferation. The role of Shcbp1 in $CD4^+$ T cell development and function and the autoimmune disease EAE will be addressed in Chapter III of this thesis.

4.4. *Role of Shcbp1 in cytokinesis and spermatogenesis*

In a screen designed to identify new binding partners of the central spindlin (CS) complex in Drosophila, the Drosophila homolog of Shcbp1 was identified as binding to the kinesin 6 protein, Pavarotti kinesin-like protein (Pav-KLP) (MKLP in mammals), and the Rho family GTPase-activating protein RacGAP50C/Tum (RacGAP1 in mammals). Due to its binding to Pavarotti-KLP, the protein was named Nessun Dorma after the opera singer Pavarotti's most famous aria, "Nessun Dorma" ("None shall sleep")[98]. Nessun Dorma (Nesd) was found to localize to the midbody of the cleavage furrow during late telophase[98]. These findings were also confirmed in the mammalian system

using human SHCBP1 in HeLa cells[96,98]. However, unexpectedly, knockdown of Nessun Dorma and SHCBP1 using RNAi did not impair cytokinesis during mitosis in fly and human cultured cell lines, respectively[96,98].

To further analyze the potential role of Nessun Dorma, the authors generated two mutant fly lines; *Nesd*^{1} mutant fly line contained a transposable P-element inserted 53 nucleotides upstream of the ATG start codon of *Nessun Dorma* (not a null mutant) and the *Df(2L)ED1306* mutant fly line that had a deficiency that completely deleted the *Nesd* genomic region (null mutant)[98]. Homozygous *Nesd*^{1} flies were viable, however 50% of males were sterile. On the other hand, *Nesd*^{1}*/Df* mutant flies were semi-lethal (only 9% *Nesd*^{1}*/Df* flies obtained from a cross with an expected yield of 50%) and 80% of the surviving males were sterile[98]. Furthermore, the sterility was due to defects in cytokinesis during meiosis[98]. Interestingly, the requirement for certain proteins during cytokinesis is variable based on whether the cells are undergoing meiosis or mitosis as well as the specific cell type[107]. Of note, the embryonic lethality was not solely due to impaired cytokinesis and Nessun Dorma likely has additional roles in embryogenesis independent of cytokinesis, as rescue of the defect in cytokinesis via overexpression of its binding partner Pav-KLP did not rescue the lethality defect[98]. Collectively, these results demonstrated that *Nessum Dorma* is an essential gene in the fly as deficient flies exhibit partial sterility in males and partial lethality[98].

Recently, SHCBP1 was also identified as a protein that regulates cytokinetic furrow ingression via preventing Rac inactivation. Furrow ingression requires Rho activation and Rac inactivation, and RacGAP1 inactivates Rac1 to promote furrow ingression[96]. Studies in HeLA cell lines demonstrated that SHCBP1 prevents premature furrow ingression by binding and suppressing RacGAP1 GAP activity and, by extension,

the inactivation of Rac1[96]. The negative regulation of RacGAP1 by SHCBP1 is removed by Aurora B (AurB) phosphorylation of SHCBP1 during mitosis, which prevents SHCBP1 binding to RacGAP1. Overexpressing a mutant of SHCBP1 that cannot be serine phosphorylated on serine 634 (SHCBP1 S634A) led to an increase in the number of multinucleated cells as compared to overexpressing WT SHCBP1[96]. Collectively, these results suggest that SHCBP1 regulates cytokinesis through its interaction with the centralspindlin complex; *Nessun Dorma* deficient flies exhibit cytokinesis defects during spermatogenesis, while overexpression of SHCBP1 mutants leads to cytokinesis defects in HeLA cells[96,98].

Collectively, these studies have demonstrated that Shcbp1 is involved in cytokinesis. Studies in flies deficient for the Drosophila homolog *Nessun Dorma*, demonstrated that *Nessun Dorma* was required for cytokinesis during meiosis[98]. Additionally, Shcbp1L deficient mice (a protein related to Shcbp1) have defects in meiosis during spermatogenesis and have reduced fertility[108]. However, recent studies overexpressing SHCBP1 in HeLA cells have shown that SHCBP1 may also be required for proper cytokinesis during mitosis[96]. Although there are still a lot of unanswered questions, these studies clearly point to a role of *SHCBP1* during cytokinesis.

4.5. *Shcbp1 as a regulator of growth factor signaling*

This past year, murine Shcbp1 was shown to be a positive regulator of fibroblast growth factor signaling (FGF) in neural progenitor cells. Shcbp1 was identified in an unbiased yeast-two hybrid screening assay designed to find new binding partners of the E3 ubiquitin ligase mLin41[99]. *mLin41*-deficient mice are embryonically lethal due to failure of neural tube closure and decreased proliferation and premature differentiation of

neural progenitor cells. Furthermore, *mLin41*-deficient neuroepithelial cells are hyposensitive to FGF signaling, and the authors suggest that this may be due to a decrease in the expression of Shcbp1[99]. The authors demonstrated that Shcbp1 protein is decreased in the neuroepithelial cells from *mLin41* deficient mice. In fact, mLin41 ubiquitinates Shcbp1 and promotes its stability. Knockdown of *Shcbp1* in a neural-epithelial cell line led to a slight reduction in Erk and Akt phosphorylation after FGF stimulation[99]. Importantly, ShcA is known to act downstream of the FGF receptor mediating activation of the Erk/Akt pathways[71]. Collectively, this data suggests that Shcbp1 may be a positive regulator of FGF induced proliferation and the neural tube defects in *mLin41*-deficient mice may be partly due to reduction in Shcbp1 stability[99].

Although the findings of this study are very interesting and potentially relevant, there are several limitations of this study in regards to Shcbp1. First, the decreased expression of Shcbp1 may simply be a reflection of the reduced proliferative capacity of the *mLin41*-deficient cells[10,99]. Second, the evidence suggesting that Shcbp1 might be a positive regulator of FGF signaling was not sufficiently supported by data (slightly reduced Akt and Erk phosphorylation) and there was no evidence presented that decreased Shcbp1 expression alone led to proliferative defects or premature differentiation *in vivo*[99]. Therefore, it is likely that mLin41 mediates the temporal regulation of neural progenitor cell proliferation versus differentiation via a Shcbp1 independent mechanism[99].

Recently, Shcbp1 was also identified as a potential regulator of signaling downstream of epidermal growth factor (EGF) signaling[82]. The authors investigated the kinetics of protein interactions with the adapter protein ShcA after EGF signaling. Shcbp1 was identified as the only protein that binds maximally to ShcA prior to

stimulation, but was found to be displaced from ShcA after EGF stimulation[82]. As Shcbp1 binds to the SH2 domain of ShcA, it is possible that Shcbp1 and phosphorylated tyrosine residues bind competitively to the SH2 domain[10,82]. These findings bring up the intriguing possibility that Shcbp1 may act as a negative regulator of EGF signaling[82] and this possibility is discussed in Chapter V of this thesis. However, the relevance of these findings to other cell types or receptors is not known, as ShcA dependent signaling is variable based on the specific context of signaling.

4.6. *Elevation of Shcbp1 in human cancers*

Shcbp1 expression correlates with highly proliferative cells, and *SHCBP1* is upregulated in human cancer cell lines and in primary human tumors[10,97,100]. In breast tumors of young women (who typically have more aggressive cancer, shorter disease free survival, and poorer prognosis), *SHCBP1* was shown to be upregulated in ductal carcinoma *in situ* (DCIS), as well as in the invasive ductal carcinoma (IDC) compared to aged-matched healthy controls or adjacent tumor free tissue[97]. Cross-species comparative genomic analysis also verified that the upregulation of *SHCBP1* was conserved between mouse and humans, as *Shcbp1* was also upregulated in DCIS and IDC in a SV40-induced mouse model of breast cancer[97,109]. Additionally, the upregulation of *SHCBP1* had prognostic significance for recurrence free survival and perhaps for distant metastasis free survival (depending on the database analyzed)[97]. In humans, *SHCBP1* is also located in a chromosomal region that has genomic copy number alterations associated with invasive breast tumors[97]. Interestingly, *Shcbp1* is also upregulated in mammary tissue of lactating mice as compared to the mammary tissue of non-pregnant/lactating mice, suggesting upregulation of *Shcbp1* also occurs during

normal physiological proliferation in the mammary gland[110]. Therefore, it is possible that SHCBP1 may have a role in both normal and pathogenic proliferation of the mammary gland and that SHCBP1 may have prognostic and/or therapeutic value for the management of breast cancer[97].

SHCBP1 was also found to be upregulated in human hepatocellular carcinoma (HCC) samples via RT-PCR and western blotting as compared to healthy non-HCC liver samples[100]. Furthermore, knockdown of *SHCBP1* in HCC cell lines reduced cell proliferation and colony formation while over-expression promoted cell proliferation and colony formation[100]. Collectively, these two studies suggest that not only is *SHCBP1* upregulated in human tumors, but its upregulation may have important prognostic value and influence tumor progression, proliferation and invasiveness[97,100].

Microarray data have also shown upregulation of *SHCBP1* in certain leukemia / lymphoma in both humans and mice[110,111]. In peripheral T cell lymphomas, *SHCBP1* expression was higher in leukemic cells compared to both resting and activated peripheral T cells[110]. Additionally, the bone marrow of patients with acute lymphoblastic leukemia showed higher expression of *SHCBP1* compared to cells isolated from the thymus of healthy controls[110]. In mouse models, *Shcbp1* is upregulated in a mouse model of Burkitt's lymphoma[111]. Collectively, this data suggests that the expression of *SHCBP1* in proliferating cells in the immune system is potentially relevant for these leukemias/lymphomas.

Shcbp1 in viral disease

SHCBP1 has been linked to viral disease in both human studies as well as in animal models. In gene expression databases, *SHCBP1* is upregulated in the lymph

node (LN) of patients with acute HIV infection as compared to uninfected, healthy controls[110,112]. Furthermore, activated $CD4^+$ T cells from HIV infected individuals have higher expression of *SHCBP1* than activated $CD4^+$ T cells from healthy controls[113]. Shcbp1 has also been linked to viral disease in mouse models of viral infection[110]. In SARS infection, *Shcbp1* expression was found to be elevated in the lungs from mice infected with SARS both 4 and 7 days post infection as compared to lungs from mock-infected mice. In LCMV infection, *Shcbp1* is elevated in antigen specific $CD8^+$ T cells (H2-DbGP33 tetramer positive) from LCMV infected mice as compared to naïve $CD8^+$ T cells isolated from healthy controls[110,114,115]. Furthermore, *Shcbp1* is upregulated approximately 20-fold in mice during the acute infection (day 6 post-infection), but is not elevated in $CD8^+$ T cells after the infection is cleared (day 15 and day 30 post-infection)[114,115]. Interestingly, *Shcbp1* remains elevated at these time-points in $CD8^+$ T cells isolated from mice infected with a mutant LCMV that establishes chronic infection and cannot be cleared[110,114,115]. Therefore, *Shcbp1* is elevated during the primary immune response in the context of infection and the kinetics of upregulation correlate with time-points in which T cells were actively proliferating.

Recently, SHCBP1 has been identified as a host protein that is exploited by the measles virus (MV) and is required for efficient MV growth[101]. SHCBP1 was identified in a yeast-two hybrid screen designed to identify cellular host proteins that interact with the MV C protein[101]. The MV C protein is known to down-regulate viral RNA synthesis to evade detection by the host cytosolic RNA sensors (RIG-I and MDA5) and prevent host IFN production[101]. However, the MV C protein does not bind any subunits of the MV RNA-dependent RNA polymerase, and therefore must regulate MV viral RNA synthesis through its interaction with host proteins; SHCBP1 was identified as a protein that may

regulate MV viral RNA synthesis[101]. Additionally, SHCBP1 was found to be induced in lung carcinoma cell lines (A549/hSLAM) after interferon stimulation as well as during infection with the measles virus[101]. Further analysis showed that SHCBP1 is required for efficient MV RNA synthesis through its interaction with the MV N protein of the RNA-dependent RNA polymerase. The authors proposed the following model for MV subversion of the host via SHCBP1: At the early stages of MV replication, a small amount of type I interferons are generated, which induces the expression of SHCBP1. The induced SHCBP1 interacts with the RNA-dependent RNA polymerase complex to promote optimal RNA synthesis[101]. However, to prevent induction of the immune response and massive production of interferons, the MV down-regulates viral RNA synthesis. One proposed mechanism of the down-regulation of viral RNA synthesis is the binding of the MV C protein to SHCBP1 which prevents SHCBP1 binding to MV N protein[101]. Although in this study the authors demonstrated that SHCBP1 was subverted by the measles virus and was required for optimal growth and viral RNA synthesis, their findings bring up the intriguing question of what the normal role of SHCBP1 might be during the anti-viral response, and why the host would upregulate SHCBP1 in response to interferons or viral infection. Often pathogens evolve mechanisms to subvert the normal immune response to allow for more efficient replication and colonization. Collectively, the expression of SHCBP1 in the content of viral infections suggests that SHCBP1 may have a role in the anti-viral immune response, and this question is addressed in Chapter III of this thesis.

5. Brief summary of the following chapters

In Chapter II, I will outline the experimental procedures and methods utilized throughout the rest of this thesis. The focus of Chapter III is to investigate the role of Shcbp1 during T cell development and activation. While Shcbp1 has been linked to proliferation in different *in vitro* settings and in Drosophila, the potential *in vivo* role of Shcbp1 in mammals has not been elucidated. Given the expression of Shcbp1 in the thymus and the requirement of ShcA in T cell development, I hypothesized that Shcbp1 may be involved in the proliferation that occurs during T cell development or during proliferation induced in peripheral T cells during an immune response. The testing of this hypothesis is detailed in Chapter III.

Another focus of my graduate work was on the role of ShcA phosphorylation in late stages of T cell development, a project that I completed in collaboration with a former post-doctoral fellow in our laboratory, Dr. Paul Trampont. Previously, we have shown that ShcA is required for productive signaling through the preTCR and that deletion of ShcA or expression of a dominant negative ShcA transgene leads to a developmental block at the β-selection checkpoint in early T cell development. To address the role of ShcA in late T cell development, we generated *CD4*-Cre/ShcFFF transgenic mice, and the analysis is presented in Chapter IV of this thesis.

Lastly, in chapter V, I will discuss the major findings, significance, and future directions of my main thesis projects. This section will focus on future directions based on my preliminary data as well as published studies. Additionally, in the appendix, I will present data on the expression of Shcbp1 in B cells and the analysis of B cell development in the *Shcbp1*-deficient mice.

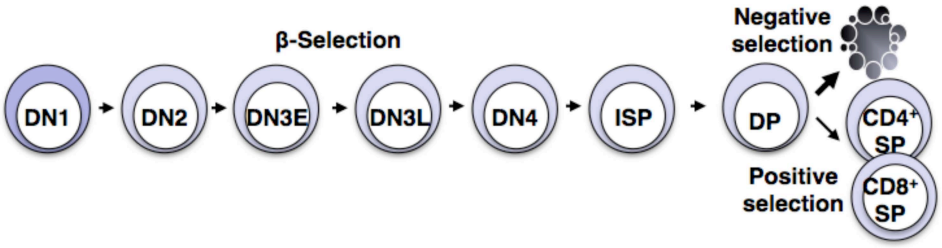
Figure 1.1: T cell development

(A) Schematic of T cell development and the different thymic subsets

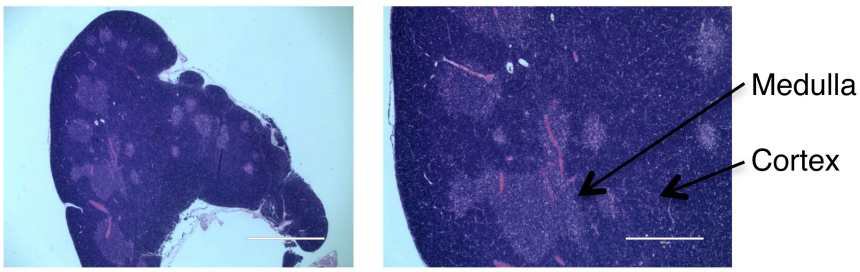
(B) H&E staining of paraffin imbedded thymic sections from a 4-6 week old C57BL/6 mouse. The cortex and the medulla of the thymus are clearly indicated; the cortex appears darker due to its greater cellularity. The cortico-medullary junction is the area between the cortex and medulla.

(C) Left, cell surface staining of CD4 and CD8 of thymocytes and gating for DN, DP, CD4SP, and CD8SP thymic subsets. Right, cell surface staining for CD25 and CD44 on DN thymocytes (gated on CD4-, CD8-, $B220^-$ $Gr1^-$ $Ter119^-$ $CD11b^-$ $CD11c^-$) and gating for DN1, DN2, DN3, and DN4 thymic subsets.

A


β-Selection
DN1
DN2
DN3E
DN3L
DN4
ISP
DP
Negative selection
CD4+ SP
Positive selection
CD8+ SP

B


Medulla
Cortex

C

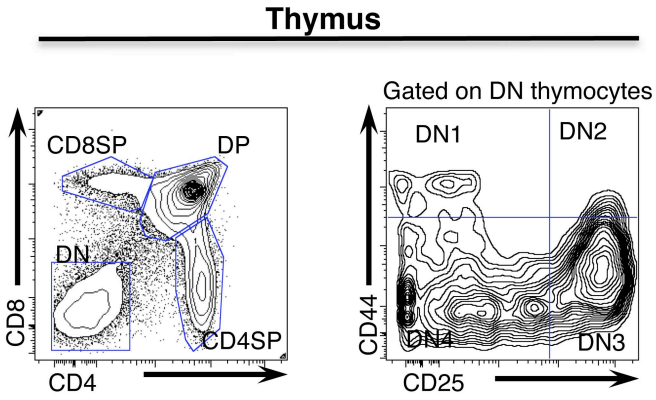

Thymus
CD8SP
DP
DN
CD4SP
CD8
CD4
Gated on DN thymocytes
DN1
DN2
DN4
DN3
CD44
CD25

Figure 1.2: The requirement for ShcA during preTCR signaling

Diagram of preTCR signaling and the events triggered by β-selection. Additionally, this diagram shows the differential requirement for ShcA during β-seleciton; ShcA is required for differentiation, proliferation, and migration but is dispensable for survival and allelic exclusion.

Diagram based on experiments performed by Paul Trampont.

Pre-TCR/CD3 signaling complex

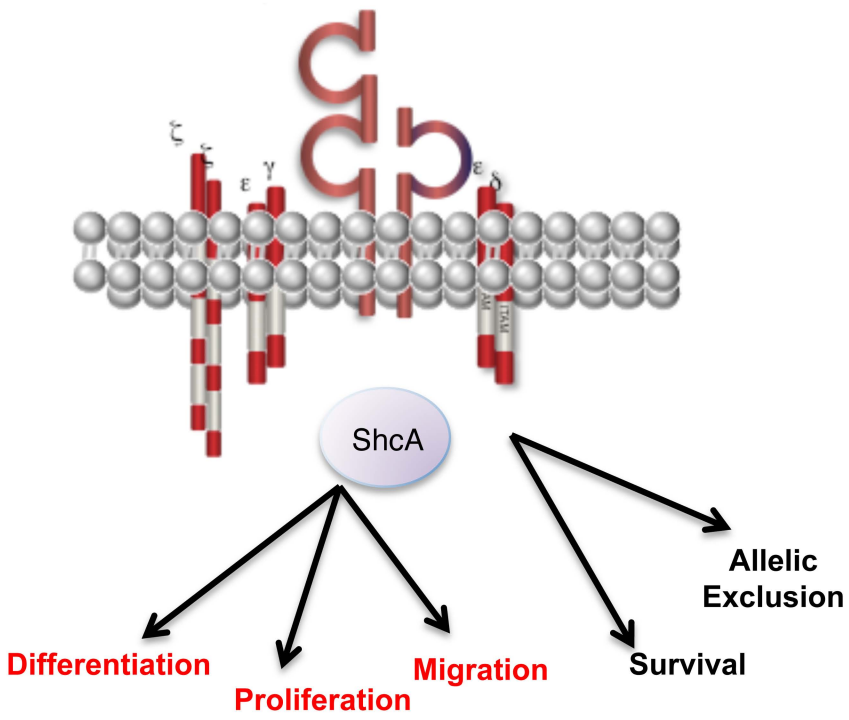
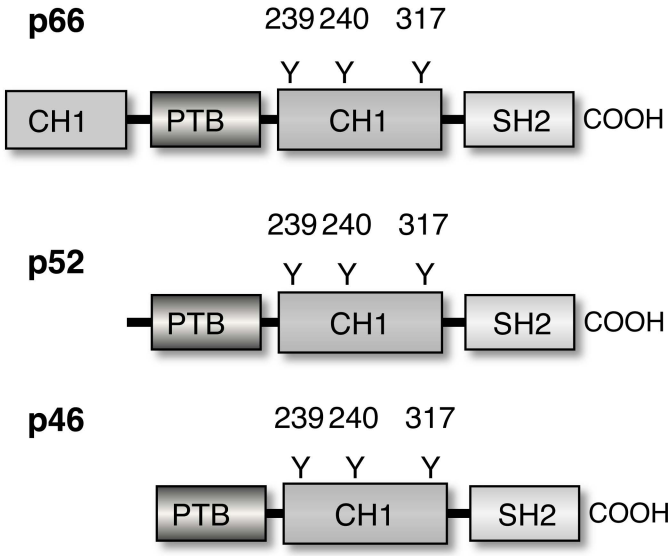


Figure 1.3: The adapter protein ShcA

(A) Schematic detailing the three different isoforms of ShcA, p66, p52, and p46. Diagram shows the different protein domains of ShcA and the three crucial tyrosine residues within the CH1 domain (modified from Trampont et *al*, manuscript in submission).

(B) Immunoblot for ShcA in the thymus, spleen, and lymph node. The p52 and p46 isoforms of ShcA are present in these immune compartments, but the p66 isoform is largely absent from immune cells.

A


p66
239 240 317
Y Y Y
CH1
PTB
CH1
SH2
COOH
p52
239 240 317
Y Y Y
PTB
CH1
SH2
COOH
p46
239 240 317
Y Y Y
PTB
CH1
SH2
COOH

B

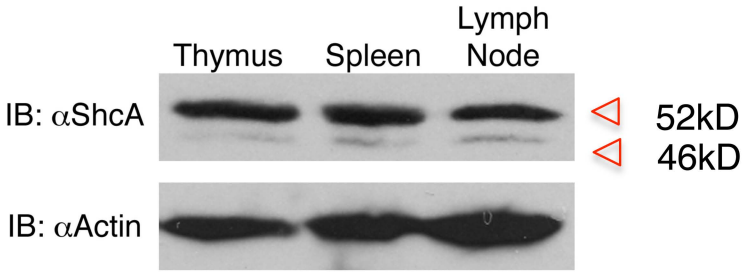

Thymus
Spleen
Lymph Node
IB: αShcA
IB: αActin
52kD
46kD

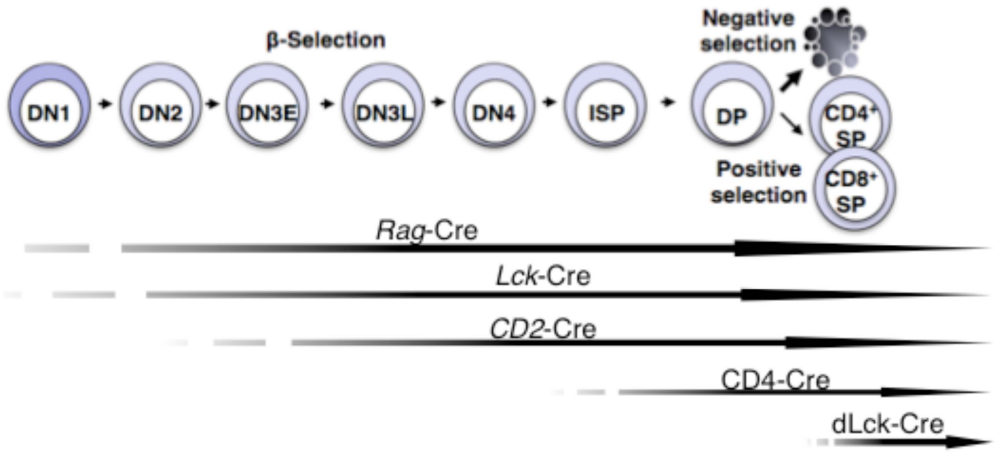
Figure 1.4: Mouse models used to study ShcA in thymic development

(A) Transgenic cre mouse lines used to study T cell development and indications of when cre is expressed during T cell development.

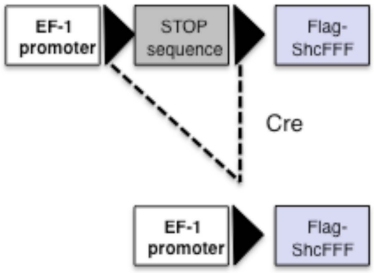
(B) Schematic detailing expression of the ShcFFF transgene via a lox-STOP-lox sequence between the EF-1α promoter and the cDNA encoding the human ShcFFF transgene.

(C) Transgenic ShcA mouse lines that are specifically expressed via a lox-STOP-lox sequence. In addition to ShcWT, there are transgenic mouse lines that express dominant negative ShcA transgenes in which the crucial tyrosine residues in the CH1 domain were mutated to phenylalanine (modified from Trampont et *al*, manuscript in submission)

A


β-Selection
Negative selection
DN1
DN2
DN3E
DN3L
DN4
ISP
DP
CD4+ SP
CD8+ SP
Positive selection
Rag-Cre
Lck-Cre
CD2-Cre
CD4-Cre
dLck-Cre

B


EF-1 promoter
STOP sequence
Flag-ShcFFF
Cre
EF-1 promoter
Flag-ShcFFF

C

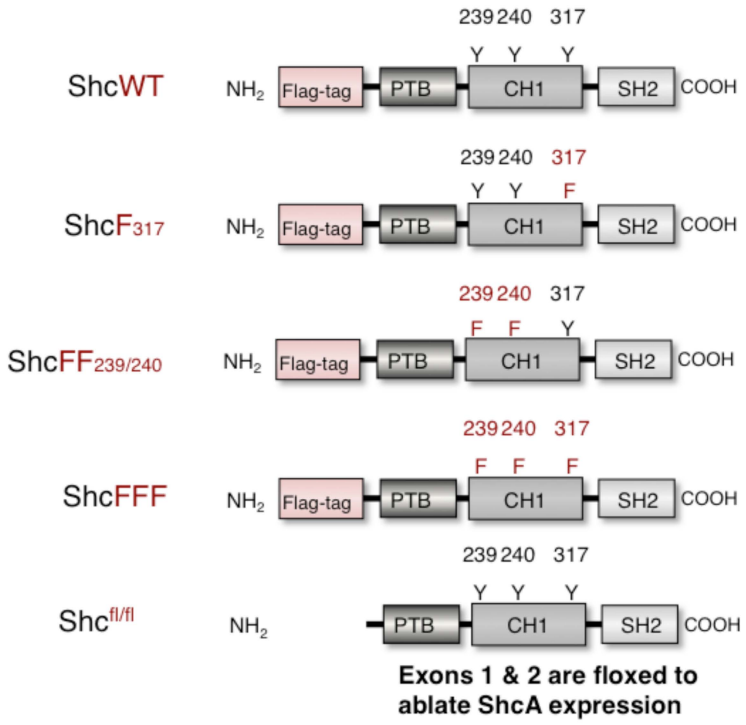

ShcWT
239 240 317
Y Y Y
NH2 Flag-tag PTB CH1 SH2 COOH
ShcF317
239 240 317
Y Y F
NH2 Flag-tag PTB CH1 SH2 COOH
ShcFF239/240
239 240 317
F F Y
NH2 Flag-tag PTB CH1 SH2 COOH
ShcFFF
239 240 317
F F F
NH2 Flag-tag PTB CH1 SH2 COOH
Shcfl/fl
239 240 317
Y Y Y
NH2 PTB CH1 SH2 COOH
Exons 1 & 2 are floxed to ablate ShcA expression

Figure 1.5: Impaired thymic development in *Lck*-Cre^{+}/ShcFFF and *Lck*-Cre^{+}/ShcFF mice

(A) Left, representative picture of control and *Lck*-Cre^{+}/ShcFFF mice. Right, cell surface staining for CD4 and CD8 and cell surface staining for CD25 and CD44 after gating on DN thymocytes. *Lck*-Cre^{+}/ShcFFF have reduced thymic cellularity and an accumulation of DN3 thymocytes, demonstrating the requirement for phosphorylation of the three tyroine residues within the CH1 domain during T cell development.

(B) Left, representative picture of control and *Lck*-Cre^{+}/ShcFF mice. Right, cell surface staining for CD4 and CD8 and cell surface staining for CD25 and CD44 after gating on DN thymocytes. *Lck*-Cre^{+}/ShcFF have reduced thymic cellularity and an accumulation of DN3 thymocytes, demonstrating the requirement for tyrosine 239/240 phosphorylation during T cell development.

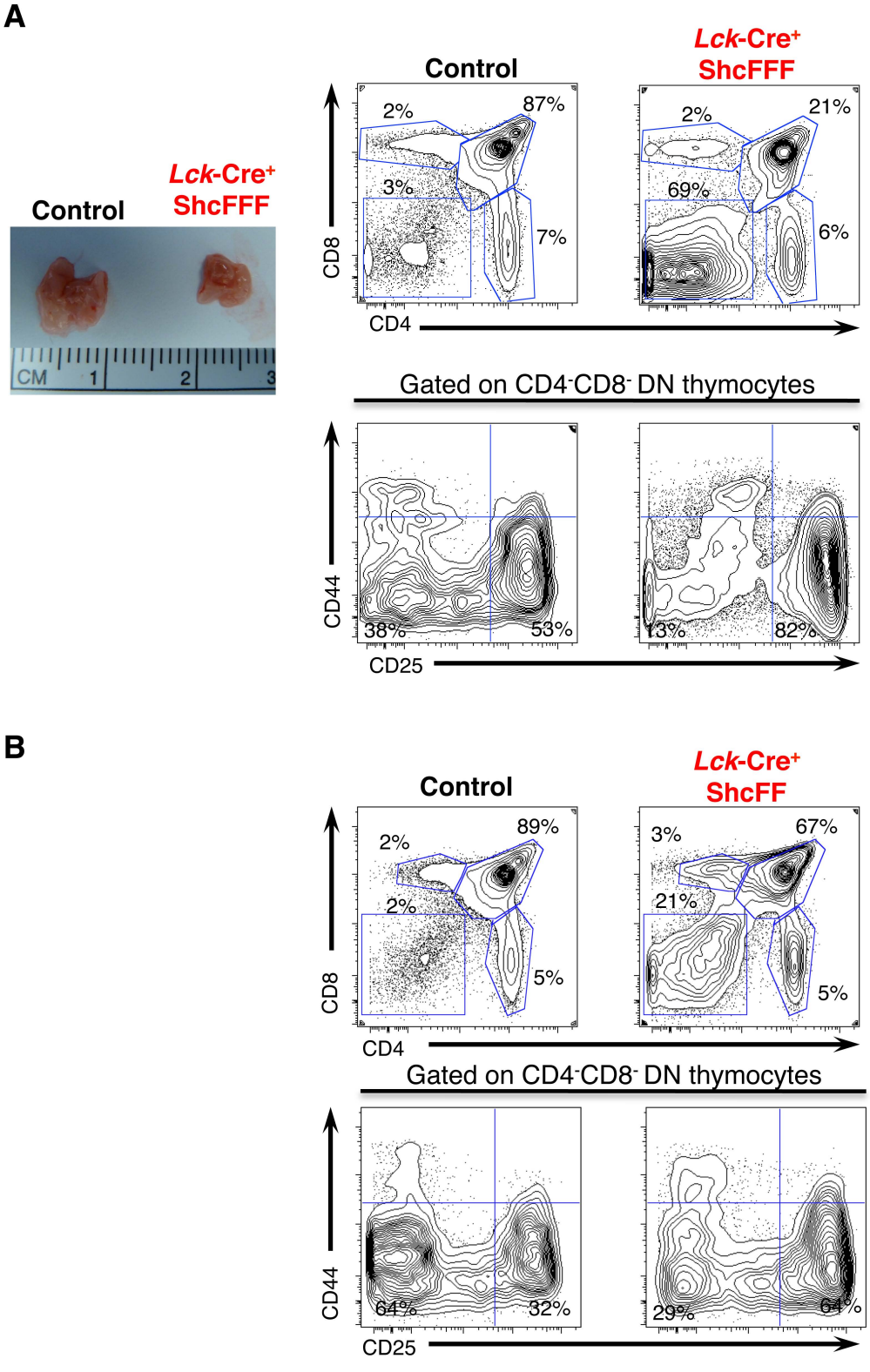

A
Control
Lck-Cre+ ShcFFF
CM
1
2
3
Control
Lck-Cre+ ShcFFF
2%
87%
3%
7%
2%
21%
69%
6%
CD8
CD4
Gated on CD4-CD8- DN thymocytes
38%
53%
13%
82%
CD44
CD25
B
Control
Lck-Cre+ ShcFF
2%
89%
2%
5%
3%
67%
21%
5%
CD8
CD4
Gated on CD4-CD8- DN thymocytes
64%
32%
29%
64%
CD44
CD25

Figure 1.6: The structure of Shcbp1 and the pectin lyase-like domain

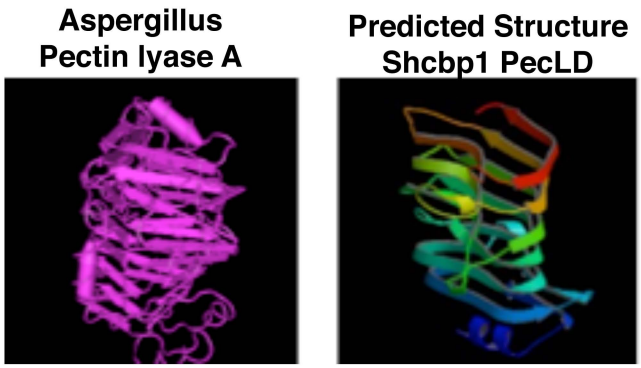
(A) Diagram of the structure of Shcbp1 indicating the two predicted domains of Shcbp1, the Nessum Dorma homology domain (Nesd) and the pectin lyase-like domain (PecLD).

(B) Crystal structure of aspergillus pectin lyase A as well as the predicted structure of the Shcbp1 pectin lyase-like domain. Pectin lyase-like domains are characterized by a series of parallel β-strands. (Figure modified from http://genome.ucsc.edu)

A


N-terminal
Nesd
PecLD
C-terminal

B


Aspergillus
Pectin lyase A
Predicted Structure
Shcbp1 PecLD

Chapter II

Materials and Methods

1. Mice

JM8.A2 embryonic stem cells (C57BL/6 origin) carrying *loxP* sites flanking exons 4-6 of the *Shcbp1* locus were obtained from EUCOMM and injected into C57BL/6 blastocysts by the University of Virginia Gene Targeting and Transgenic Core Facility[116,117]. The resulting chimeric mice were mated to C57BL/6J mice for germline transmission and the resulting progeny were screened for *Shcbp1* targeting via genotyping for the neomycin targeting cassette. Further crossing to a flippase transgenic mouse to remove the neomycin targeting cassette resulted in mice with floxed *Shcbp1* allele[118]. The *Shcbp1*$^{fl/fl}$ mouse was subsequently crossed to the *Lck*-Cre transgenic mouse, *Rag*-Cre transgenic mouse, and *EIIA*-Cre transgenic mouse to generate mice with *Shcbp1* conditionally deleted and *Shcbp1* null mice *(Shcbp1*$^{-/-}$ mice*),* respectively[86,119,120].

All mice used were on the C57BL/6J background unless otherwise noted. C57BL/6J, *Rag1* deficient, *TCR*α deficient, *Lck*-Cre, *EIIA*-Cre, *CD4*-Cre, OT-I, OT-II, DO11.10, H-Y and flippase transgenic mice were purchased from the Jackson Laboratories or Taconic[6,86,119,121-125]. The *Lck*-Cre^{+}/ShcFFF and *Lck*-Cre^{+}/ShcFF mouse lines have been previously described[8,9]. The *Rag*-Cre (mixed background) transgenic mouse line and the DO11.10 TCR transgenic (BALB/c) were kindly provided by the Bender and Lorenz laboratories, respectively, at the University of Virginia[123]. The LckF505 transgenic mouse line and the Rag2p-eGFP mouse line were kindly provided by Dr. Roger Perlmutter's laboratory and Dr. Pamela Fink's laboratory, respectively, and have been previously described[126-128]. Mice were bred and housed under specific

pathogen-free environment and all animal experiments were approved by the University of Virginia Animal Care and Use Committee.

2. Mouse Genotyping

Genotyping was performed on tail DNA as well as sorted cell populations. Tail DNA and sorted cell populations were digested in DirectPCR lysis reagent (Viagen) with 0.25ug/uL Proteinase K. Genotyping was performed using 1ul of 10mM primer dilution in a 25μl PCR reaction using the following primers written 5' to 3':

2.1 **Shcbp1 wild-type, floxed, and deleted loci**

The following primers are used in the same reaction to generate the following bands to detect *Shcbp1* wild-type (191 bp), *Shcbp1* floxed (329 bp), and *Shcbp1* deleted (250 bp):

Shcbp1 floxed/wild-type reverse (CCACTTGCCCAGGTCAAACTGTAAAA)

Shcbp1 Universal forward (ATCCTCTTGGAGATCAATCAAATGTTTGTG),

Shcbp1 Deleted reverse (GAGAGGAGATGTATGTATTTGTTGAACTGATGG).

2.2 **Universal Cre**

The universal Cre genotyping was used to detect Cre from the *Lck*-Cre, *EIIA*-Cre, *CD4*-Cre, and *Rag*-Cre transgenic mice lines. The Cre transgene was maintained as a single copy in all experiments. Additionally, *Rag*-Cre disrupts the normal *Rag* loci and therefore homozygosity disrupts T and B cell development and homozygosity of the *Lck*-Cre leads to impairment in T cell development[120,129]. The *EIIA*-Cre was used to generate the *Shcbp1* deficient mouse and the Cre was subsequently removed via breeding to C57BL/6J mice. The following primers were used to detect Cre (217 bp):

246- Cre (ATA ATC GCG AAC ATC TTC AGG T)

29-Cre (CCA ATT TAC TGA CCG TAC ACC A)

2.3. ShcA transgenes

Genotyping for the ShcA transgenes can be used in the same reaction with the universal Cre primers to detect the ShcA transgene (382 bp) and Cre (217 bp). The following primers are used to detect the ShcA transgenes (ShcFFF and ShcFF)[8,9]:

4140/Shc (ACC CAT GTA CCG AAC CAA GTA G)

3758/Shc (GTG GTT TGT CCA AAC TCA TCA A)

2.4. Rag1

The following primers were used in the same reaction to detect the WT (474 bp) or mutant *Rag1* (530 bp) loci[121]:

Wild-type forward oIMR1746 (GAG GTT CCG CTA CGA CTC TG)

Common reverse oIMR3104 (CCG GAC AAG TTT TTC ATC GT)

Mutant forward oIMR8162 (TGG ATG TGG AAT GTG TGC GAG)

2.5. Neomycin

The following primers were used to detect the neomycin-targeting cassette and used to detect germ-line transmission from the *Shcbp1* chimeric mice.

IMR13 p53 Neo forward (CTTGGGTGGAGAGGCTATTC)

IMR14 p53 Neo reverse (AGGTGAGATGACAGGAGATC)

2.6. Flippase

The following primers were used to detect the flippase transgene. The flippase transgene was used to remove the neomycin targeting cassette and generated the *Shcbp1* floxed mouse. The flippase transgene was subsequently removed via breeding of the *Shcbp1* floxed mouse to a C57BL/6J mouse[118].

FLP forward (CACTGATATTGTAAGTAGTTTGC)

FLP reverse (CTAGTGCGAAGTAGTGATCAGG)

2.7. Rag2-eGFP

Rag2-eGFP transgenic mice express enhanced GFP (eGFP) under the *Rag2* promoter and the transgene does not interfere with normal T or B cell development. The following primers can be used in the same reaction to detect the Rag2-eGFP transgene (245 bp) as well as an internal positive control (200 bp)[127]. Additionally, 'genotyping' of these mice can be performed via flow cytometry.

Transgene forward oIMR8583 (GGGGCAGGAGGTGTCTTAT)

Transgene reverse oIMR8564 (GTTTACGTCGCCGTCCAGT)

Internal positive control forward (CAA ATG TTG CTT GTC TGG TG)

Internal positive control reverse (GTC AGT CGA GTG CAC AGT TT)

2.8. DO11.10

The DO11.10 transgenic mice express a MHC class II-restricted T cell receptor transgene that reacts to ovalbumin (OVA) peptide specifically $OVA_{323-339}$ peptide[123]. The transgenic TCR is expressed in the majority of CD4 T cells. The following primers are used in the same reaction to detect the TCR transgene (290 bp) as well as an internal positive control (200 bp)[123].

Transgene oIMR0786 (CAG GAG GGA TCC AGT GCC AGC)

Transgene oIMR0787 (TGG CTC TAC AGT GAG TTT GGT)

Internal positive control oIMR8744 (CAA ATG TTG CTT GTC TGG TG)

Internal positive control oIMR8745 (GTC AGT CGA GTG CAC AGT TT)

2.9. OT-II

The OT-II transgenic mouse line expresses a MHC class II-restricted TCR transgene that reacts to ovalbumin. The following primers can be used in the same reaction to detect the TCR transgene (500 bp) as well as an internal positive control (160 bp)[122].

Transgene IMR1825 (GCT GCT GCA CAG ACC TAC T)

Transgene IMR1826 (CAG CTC ACC TAA CAC GAG GA)

Internal positive control IMR1880 (AAA GGG AGA AAA AGC TCT CC)

Internal positive control IMR1881 (ACA CAG CAG GTT CTG GGT TC)

2.10. H-Y

The H-Y transgenic mouse line expresses the MHC class I-restricted TCR transgene that reacts to the male specific antigen H-Y. In female H-Y transgenic mice, positive selection leads to generation of CD8 SP thymocytes, while in male H-Y transgenic mice, negative selection leads to a decrease in overall thymic cellularity and very few DP and SP thymocytes. The following primers can be used to detect the H-Y transgene[124].

5'HY-betachain **(**GAC ATT GAG CTG TAA TCA GAC)

HY-betachain3' **(**ACA GCG TTT CTG GAC TGT TAT CAC C)

2.11. OT-I

The OT-I transgenic mouse expresses the MHC class I-restricted TCR specific for OVA antigen[125]. The following primers can be used to detect the OT-I transgene (300 bp).

OT-I IMR0675 **(**AAG GTG GAG AGA GAC AAA GGA TTC)

OT-I IMR0676 (TTG AGA GCT GTC TCC)

2.12. STOP cassette PCR

The STOP cassette PCR is a non-quantitative PCR strategy to detect the deletion of the STOP sequence in ShcFFF transgenic mice crossed to transgenic Cre mouse lines. Cre-mediated removal is detected by 800bp product while the presence of the STOP cassette generates a 511bp product. Presence of two bands represents heterogeneity of STOP cassette removal due to incomplete deletion of the STOP cassette or cells that have escaped Cre mediated deletion[9](Trampont *et al*, manuscript in submission). The following primers can be used to detect the STOP cassette and deletion of the STOP cassette in the same reaction:

4329/ ShcTg (TGG AGA CGG TGA GAG TGA TT)

2106/EF1 (TGA GTC ACC CAC ACA AAG GA)

2617/STOP (TTC TTC GGT GGA AAC AAC GG)

3. Flow Cytometry

Thymocytes, splenocytes, and lymphocytes were isolated from 4- to 6-week old mice (littermates) for analysis of T cell development and activation. DP and DN compartments were analyzed by staining thymocytes with antibodies specific for CD4, CD8, CD3, Thy1.2, CD25, CD44, TCRβ, and TCR$\gamma\delta$ as well as lineage markers (CD11b, CD11c, B220, GR1, Ly6G, and Ter119) at 1:100 as described previously[28]. Our gating strategy for the DN compartment is shown in **Figure 2.1**. Splenocytes and lymphocytes were stained with antibodies specific for CD3, CD4, CD8, FoxP3, as well as the activation markers CD62L, CD44, CD25, and CD69. Absolute numbers were determined via enumeration with the hemocytometer followed by flow cytometry analysis or via

inclusion of sephrotec counting beads. Viability and apoptosis were evaluated by staining with Annexin V and 7AAD (Invitrogen), according to manufacturer's instructions.

Early hematopoietic development, as well as B cell development, were performed as described previously by staining for the following markers: HSP/LMPP (CD127, CD117, Sca1, CD135, lineage (B220, CD3, CD11b, CD19, DX5, Gr1, Ly6C, Ter119)); CLP (CD19, B220, CD127, CD135, lineage (CD3, CD11b, DX5, Gr1, Ly6C, Ter119)); B cell development (Ly6C, NK1.1, B220, IgM, CD43, CD19). Viability was determined via staining with DAPI[130].

All antibodies used were obtained from eBioscience unless otherwise noted. FACSCanto (BD Biosciences) was used for flow cytometry and results were analyzed by FlowJo software (TreeStar Inc.).

4. T cell stimulation and proliferation

For anti-CD3/anti-CD28 stimulation, $8x10^4$ $CD4^+$ MACS selected T cells (Miltenyi Biotec) were stimulated with anti-CD3/anti-CD28 beads (Dynabeads, Life Technologies) according to the manufacturer's protocol. To assess proliferation via CFSE dilution, T cells were stained with 5μM CFSE (Molecular Probes) prior to stimulation[131]. OVA peptide stimulation was performed by plating 40,000 DO11.10 TCR transgenic $CD4^+$ T cells along with 80,000 mitomycin (Sigma)-treated antigen presenting cells and the indicated concentration of $OVA_{(323-339)}$ peptide (AnaSpec). Stimulations were also performed by culturing cells with 50 ng/ml PMA (phorbol 12-myristate 13-acetate; Calbiochem) along with 500 ng/ml ionomycin (Calbiochem). All stimulations were performed in 200ml RPMI 1640 medium (supplemented with 10% FBS, 50μM β-Mercaptoethanol, 2-mM L-glutamine, and 1% pennicillin/streptomycin) in round bottom

96-well plates and cultured at 5% CO2 at 37°C. Alternatively, T cell stimulations were performed in 24-well plates (Costar) using 2μg/ml plate-bound anti-CD3 (BD Pharmingen) and either 1μg/ml soluble anti-CD28 (BD Pharmingen) or 100 U/ml IL-2 (Peprotech).

In vivo T cell stimulation was performed by intraperitoneal injection of 100 μg of anti-CD3. $CD4^+$ T cells were harvested from the spleen and lymph nodes 24 hours after injection. For *in vivo* model of preTCR signaling, *Rag1*-deficient mice were injected with 100μg of anti-CD3 intraperitoneally and the thymus was collected 24 hours after injection[132].

5. T cell skewing

T_H17 skewing was performed by selecting naïve $CD4^+$ $CD62L^+$ T cells from spleens and lymph nodes of 4-week old mice (Miltenyi Biotec). For T_H17 skewing, naïve T cells were skewed for 4 days on 1μg/ml anti-CD3 and 2μg/ml anti-CD28 coated plates along with 0.3ng/ml TGF-β1 (R&D Systems), 20ng/ml IL-6 (R&D Systems), 10ng/ml IL-23 (eBioscience), 10μg/ml anti-IL4 (eBioscience), and 10μg/ml anti-IFNγ (eBioscience) in IMDM supplemented with 10% FBS (Atlanta Biologics), 50 μM β-Mercaptoethanol (Sigma), 2-mM L-glutamine, non-essential amino acids, 1 mM sodium pyruvate, and 10 mM Hepes (Thermo Scientific). After 4 days, cells were collected for analysis. Cells analyzed by intracellular cytokine staining were stimulated with 50 ng/ml PMA and 1 μM Ionomycin along with GolgiStop (BD Pharmingen) for 6 hours prior to staining. Intracellular staining for IL-17A and IFNy was performed by fixing the cells in 4% paraformaldehyde followed by permeabilization with 0.1% Saponin. Cells analyzed by

ELISA were collected and re-stimulated on plates coated with 1 μg/ml anti-CD3 and the supernatant was collected after 24 hours.

T_H1 skewing was performed by culturing naïve $CD4^+$ $CD62L^+$ T cells on 1μg/ml anti-CD3 and 2 μg/ml anti-CD28 coated plates along 100U/ml IL-2 (Peprotech), 10ng/ml IL-12 (Ebiosciences), and 10 μg/ml anti-IL4 (eBioscience) in complete RPMI on 24-well plates (Costar). On day 4, three wells were combined into a 6-well plate and supplemented with 2ml of additional complete RPMI with 100U/ml IL2. Analysis was performed on day 7 via intracellular staining for IFN as described above. Cells analyzed via ELISA were collected on day 7 and restimulated on plates coated with 1 μg/ml anti-CD3 and the supernatant was collected after 24 hours for IFNγ ELISA.

For T_H2 skewing, naïve T cells were skewed for by culturing on 1μg/ml anti-CD3 and 2μg/ml anti-CD28 coated plates along with 100U/ml IL-2 (Peprotech), 10ng/ml IL-4 (Ebiosciences), and 10 μg/ml anti-IFNγ (eBioscience) in complete RPMI. On day 4, each well was expanded into a 5-well plate and supplemented with 2ml of additionally complete RPMI with 100U/ml IL2. Cells were collected on day 7 and restimulated on plates coated with 1 μg/ml anti-CD3 and the supernatant was collected after 24 hours for IL4 ELISA.

6. Experimental Autoimmune Encephalomyelitis

EAE immunization was performed as previously described[64]. In brief, 10 week-old female or male mice were immunized subcutaneously into the upper and lower back with 200μg MOG_{35-55} peptide (CS Bio Co), emulsified in equal volume of complete Freund's adjuvant (Sigma) supplemented with heat killed M. tuberculosis (clone H37RA) (Difco) for a total of 400μg H37RA per mouse. Mice received 200ng of pertussis toxin

(List Biologicals) intraperitoneally on day 0 and 1 after immunization. Two investigators, blinded to the genotype of mice, independently analyzed the mice daily on a 5-point scale: 0-no clinical signs; 1-paralyzed tail; 2-mild hindlimb paresis; 3-severe hindlimb paresis; 4-hindlimb paralysis; 5 quadriplegia/moribund **(Figure 2.2)**. Brain and spinal cord leukocytes were isolated on day 28 post-injection using Percoll (GE Healthcare) gradient centrifugation, according to published protocols. Isolated cells were identified by staining for CD4, CD45, CD11b, and B220. For histological analysis, mice were perfused with 4% paraformaldehyde and the sacral, lumbar, thoracic, and cervical parts of the spinal cord were fixed in 4% paraformaldehyde and imbedded in paraffin.

EAE induction in *Rag1*$^{-/-}$ mice was achieved by retro-orbital injection of 2x10^6 CD4$^+$ selected T cells from either *Shcbp1*$^{+/+}$ or *Shcbp1*$^{-/-}$ mice. One week after CD4$^+$ T cell transfer, mice were immunized for EAE and scored as described above.

7. Influenza Infection

Mouse adapted Type A influenza virus (IAV), A/PR/8/34 (H1N1), was grown in day 10 chicken embryo allantoic cavities[133]. Mice were infected intranasally (in 50 μl) either with 350 egg infectious doses (EID) for sublethal infection or with 3500 EID for lethal infection after a mild anaesthetization. IAV-infected mice were monitored daily for weight loss and survival over time. Adaptive immune response to IAV was measured by the standard tetramer staining for NP- and PA-specific epitopes for IAV-specific lung CD8$^+$ T cells and by ELISA for IAV-specific antibody titers in circulation[133].

8. IgG Elisa

Total IgG elisas were performed by coating wells with unlabeled mouse IgG (Southern Biotech 1030-01) overnight at 4 degrees followed by washing and blocking with 1% BSA in PBS. A standard curve (IgG mouse unlabelled, Southern Biotech 0107-01) or serum was incubated for 2 hours at room temperature. After washing, the detection antibody was added (Goat anti-mouse IgG HRP, Southern Biotech 1030-05) for 1 hour followed by developing using the TMB reagent. The reaction was stopped via addition of 2N H_2SO_4 and read at 450nM using a FlexStation 3 Microplate Reader (Molecular Devices).

9. Quantitative PCR

Total RNA was extracted from thymocytes and selected $CD4^+$ T cells using a QIAshredder and RNeasy kit (Qiagen) followed by reverse transcription using the SuperScript III (Invitrogen) kit. Quantitative PCR was performed using the TaqMan Gene Expression assays (Applied Biosystems) on a StepOnePlus system (Applied Biosystems). TaqMan gene expression probes were used for gene analysis of mouse *Shcbp1*, *ShcA*, *HPRT*, *Shcbp1-L*, *Fbox10*, *Fbox11* and *IL-2*. Each sample was performed in duplicate, target transcripts were normalized to *HPRT* mRNA as an internal control gene, and the relative expression of each target gene was calculated using the comparative cycling method with StepOne v2.1 software (Applied Biosystems).

10. Immunohistochemistry and Immunofluorescence

For detection of Shcbp1 *in vivo*, thymi were fixed by immersion in 10% neutral buffered formalin (Fisher) and embedded in paraffin blocks. Sections were processed for immunohistochemistry using standard techniques. Briefly, Shcbp1 staining was

performed using a purified Shcbp1 polyclonal antibody (Schmandt et *al*) followed by amplification using the Vectastain ABC kit (Vector laboratories); the peroxidase detection was performed using the DAB peroxidase substrate kit (Vector Laboratories). Images were acquired on an Olympus SZX12 low magnification microscope equipped with a Olympus DP70 digital camera.

For detection of Shcbp1 via immunofluorescence, thymi were embedded in OCT (Torrance) and frozen at -80^{0}C. Frozen sections were cut to 4μm in thickness and fixed in 4% paraformadehyde followed by permeabilization in 0.1% Triton. Frozen sections were rehydrated with PBS, blocked for 60 minutes with 5% (vol/vol) goat serum, and stained overnight at 4^{0}C with Shcbp1 specific antibody (Schmandt et *al*). Slides were washed with PBS and incubated with appropriate secondary antibody at 1:300. Slides were further stained with fluorophore-conjugated antibodies specific for CD25, CD4, CD8, or Ki67 (BD) as well as Hoechst (Molecular Probes), and mounted with Prolong Gold antifade reagent (Molecular Probes). Slides were viewed using the Axio Imager 2 with Apotome (Zeiss) and AxioVision software was used for analysis.

11. Immunoblotting and Immunoprecipitation

Immunoblotting of primary murine tissues was performed by lysing cells in ice-cold RIPA buffer containing protease inhibitors (Calbiochem), followed by analysis via SDS-PAGE and immunoblotting. To detect Shcbp1, the following antibodies were used: Shcbp1 rabbit polyclonal (Schmandt *et al*), PAL N17 (Santa Cruz), PAL K20 (Santa Cruz), Shcbp1 C-terminal (Abgent), and Shcbp1 (ProteinTech Group) **(Figure 2.3)**. Immunoblotting for actin (Sigma) was performed on the same blot as a loading control. Quantification was performed using NIH Image-J software.

Immunoprecipitation was performed by lysing primary murine thymocytes or the SCB29 and SCIET27 cell lines[134]. Lysates were incubated with either 4 μg anti-Shcbp1 (PAL N17, Santa Cruz) or 4 μg normal goat IgG (Santa Cruz) overnight, followed by 2 hours incubation with protein A/G beads (Santa Cruz). Beads were washed and eluted by boiling in SDS sample buffer containing β-Mercaptoethanol and analyzed via SDS-PAGE and immunoblotting for Shcbp1 (Santa-Cruz) and ShcA (BD Biosciences).

12. Migration

Migration of thymocytes was performed using the transwell system (Costar)[28]. Briefly, thymocytes were isolated from 4-6 week old mice and cells were re-suspended in RPMI with 0.5% BSA at 10^7 cells/ml. One million thymocytes (100μl) were placed in the upper chamber of a transwell with 5uM pore size (Costar). Migration medium (RPMI 0.5% BSA) or varying concentrations of the indicated cytokine in 600μl of migration medium were placed in the bottom well. An additional well was loaded with 500μl of migration medium and 100μl of cells, and this well represent 'input' or 100% migration. Cells were allowed to migrate for 3 hours in the 37^{o}C incubator. After migration, the cells in the bottom chamber as well as the input chamber were collected, the bottom chambers were rinsed with ice-cold PBS and the cells were stained with cell surface markers. The cells were enumerated and analyzed via flow cytometry and the percentage of migration was determined via comparison to the 'input' chamber.

13. Cell Culture

Primary murine T cells were cultured as described previously[28]. The cell lines SCIET27 and SCB29 have been described previously and were cultured in IMDM

supplemented with 10% FBS, 50μM β-Mercaptoethanol, 2-mM L-glutamine, and antibiotics at 5% CO_2 and 37°C[134].

14. *In vivo* and *Ex vivo* survival assays

For *in vivo* model of survival and apoptosis, 4 to 6-week old mice were injected intraperitoneally with 250μg dexamethasone (Calbiochem). Thymocytes were collected 5 hours post dexamethasone injection and stained with Annexin-V, 7AAD (Invitrogen), and antibodies specific for CD4, CD8, and CD3 and analyzed on a FACSCanto flow cytometer[135]. *Ex vivo* survival assays were performed by incubating thymocytes in complete RPMI at 37°C for the indicated times.

Thymocytes isolated from DO11.10 mice were incubated with the A20 B cell line along with OVA peptide. After 8 and 20 hours, thymocytes were stained with CD4, CD8, Annexin V, and 7AAD, according to manufacturer's instructions.

15. Statistical analysis

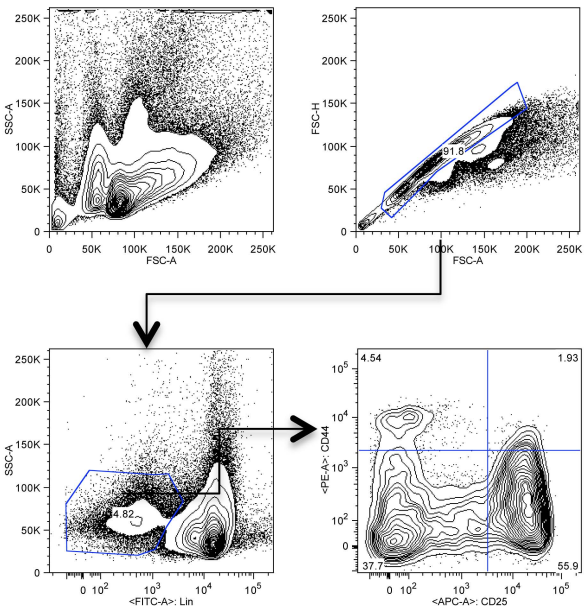
Statistical comparisons were determined by student's two-tailed t-test or a two-way ANOVA (clinical scores for EAE) using GraphPad Prism version 4.0. Result with a *p*-value lower than 0.05 was considered significant.

Figure 2.1: Gating strategy for the DN compartment in T cell development

(A) Gating strategy for the DN1-DN4 thymic compartments. The lineage gate includes CD4, CD8 Gr-1, Ter119, B220, and CD11b.

(B) Gating strategy for DN3E vs DN3L or DN3a vs DN3b. DN3E vs DN3L subsets are distinguished via forward scatter within the DN3 compartment. DN3a vs DN3b subsets are distinguished via upregulation of CD27 or CD28 (CD28 staining shown).

A



Lineage Gate
CD4
CD8
Gr-1
Ter119
B220
CD11b

B

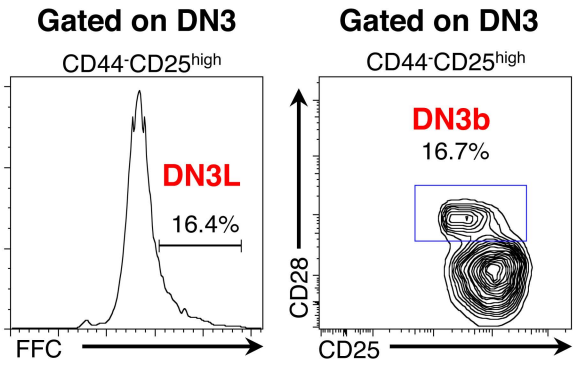
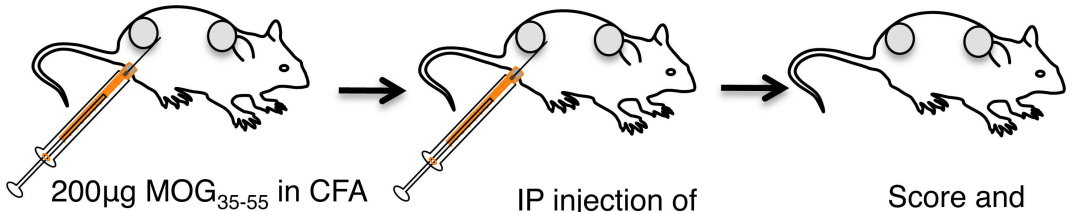


Figure 2.2: Experimental Autoimmune Encephalomyelitis immunization and scoring

(A) Schematic depicts immunization protocol for optimal EAE induction in C57BL/6 mice.

(B) Chart outlines scoring criteria during EAE. (Modified from Current protocols in neuroscience)

A



200µg MOG_{35-55} in CFA supplemented with H37RA injected subcutaneously into upper and lower back

IP injection of 400µg PTx

IP injection of 400µg PTx

Score and weigh daily

B

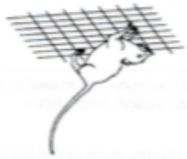
Score	Mouse Posture	
0		Normal
0.5		Weak Tail
1.0		Paralyzed Tail
2		Mild hindlimb dysfunction
3		Severe hindlimb dysfunction
4		Hindlimb paralysis
4.5		Hindlimb paralysis + Forelimb dysfunciton
5		Dead/Moribund

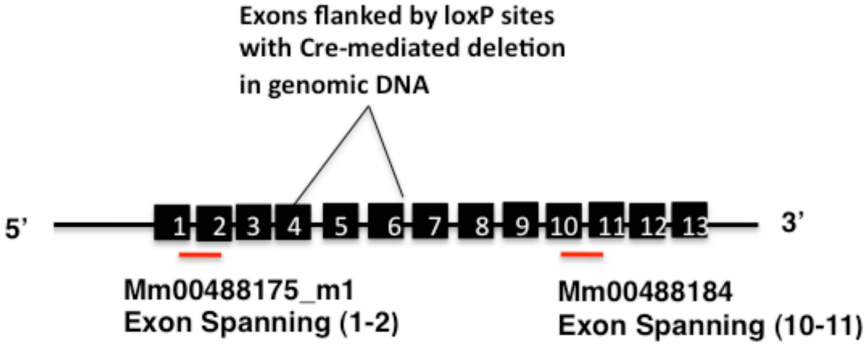
Figure 2.3: Detecting deletion of Shcbp1 via RT-PCR and immunoblotting

(A) Diagram depicting Shcbp1 mRNA, the exons flanked by loxP sites, and the RT-PCR taqman probes used to detect deletion.

(B) Diagram depicting Shcbp1 protein and the antibodies used to detect Shcbp1 via immunoblotting.

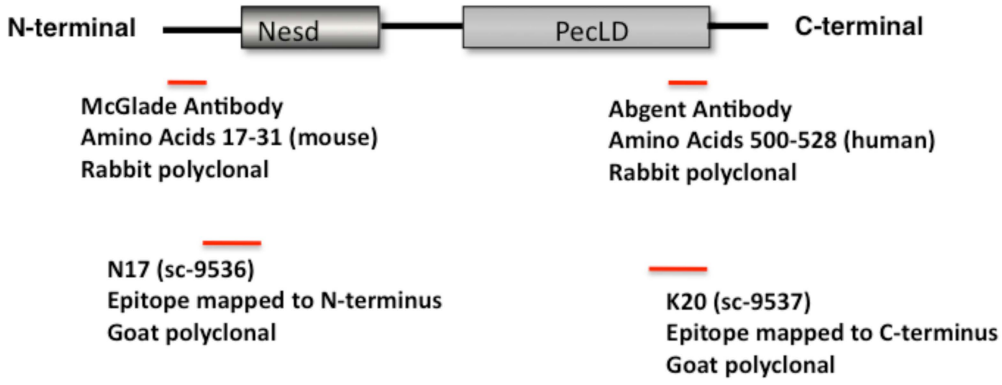
A

mRNA



B

Protein



Chapter III

Unexpected phenotype of mice lacking Shcbp1, a protein upregulated during T cell proliferation

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1. Abstract

T cell development and activation are highly regulated processes, and their proper execution is important for a competent immune system. Shc SH2-domain binding protein-1 (Shcbp1) is an evolutionarily conserved protein that binds to the adaptor protein ShcA. Studies in Drosophila and in cell lines have linked Shcbp1 to cell proliferation, embryonic development, growth factor signaling, and tumorigenesis. In this chapter, we demonstrate that Shcbp1 expression is strikingly upregulated during the β-selection checkpoint in thymocytes, and that its expression tightly correlates with proliferative stages of T cell development. To evaluate the role for Shcbp1 during thymic selection and T cell function *in vivo*, we generated mice with global and conditional deletion of *Shcbp1.* Surprisingly, the loss of Shcbp1 expression did not have an obvious effect during T cell development. However, in a mouse model of experimental autoimmune encephalomyelitis (EAE), which depends on $CD4^+$ T cell function and mimics multiple features of the human disease multiple sclerosis, *Shcbp1* deficient mice

had reduced disease severity and improved survival, and this effect was T cell intrinsic. Furthermore, although Shcbp1 is also upregulated in the context of viral infection, it appears dispensable for the primary immune response during influenza infection. These data suggest that despite the striking upregulation of Shcbp1 during T cell proliferation, loss of Shcbp1 does not directly affect T cell development, but regulates $CD4^+$ T cell effector function *in vivo* in the context of autoimmunity.

2. Introduction

Shcbp1 was first identified in a yeast-two hybrid screen designed to find new binding partners of the adapter protein ShcA, a critical regulator of T cell development[7,8,10]. Shcbp1 was initially named *murine Protein of Activated Lymphocytes* (mPAL), due to upregulation of its expression during T cell activation[10]. Shcbp1 is an evolutionarily conserved protein, with human SHCBP1 sharing 78% identity with mouse Shcbp1 and 23% identity with the Drosophila melanogaster homolog Nessun Dorma[98]. Nessun Dorma is an essential gene, as flies lacking Nessun Dorma exhibit partial lethality and defects in spermatogenesis, leading to infertility[98].

Recently, in unbiased screening assays, Shcbp1 has been identified in different contexts. These studies in mammalian cell lines and via genetic studies in Drosophila have implicated Shcbp1 in a diverse array of biological functions with links to proliferation and differentiation, including embryonic development, growth factor signaling, cytokinesis, spermatogenesis, tumorigenesis, and viral infections[10,82,96-101]. In gene expression databases, *Shcbp1* expression also appears to correlate well with actively proliferating cells of the immune system, including developing thymocytes[110,136]. From a human health perspective, the proliferation that occurs during development is of interest since T cell leukemia and lymphoma often arise in the thymus during proliferative developmental stages[1,34,47,48]. Thus, the correlation between Shcbp1 and proliferation is potentially relevant for these lymphomas.

Shcbp1 binds ShcA, an adaptor protein that functions as a critical regulator of T cell development[7,8,10]. ShcA is phosphorylated downstream of the preTCR during β-selection, and relays signals essential for development[7,9,28]. In the absence of ShcA,

there is a block in progression through the β-selection checkpoint, with impaired differentiation and reduced numbers of total thymocytes[8,9].

While Shcbp1 has been linked to proliferation in different *in vitro* settings and in Drosophila, the *in vivo* function of Shcbp1 in mammals has not been elucidated. Given the expression of Shcbp1 in the thymus and the requirement of ShcA in T cell development, we hypothesized that Shcbp1 may be involved in the proliferation that occurs during T cell development or activation. To address these questions, we generated global and conditional *Shcbp1* deficient mice. We analyzed T cell development and proliferation via flow cytometry and microscopy. Furthermore, we analyzed the role of Shcbp1 in $CD4^+$ T cells using the $CD4^+$ T cell-drive EAE model, which mimics many of the clinical and immunological aspects of the human disease multiple sclerosis (MS).

3. Results

3.1. *Shcbp1 is highly expressed in the thymus*

To determine the expression of Shcbp1 in different immune compartments, we analyzed lysates from the bone marrow, thymus, lymph node, and spleen of naïve C57BL/6J mice by immunoblotting. Expression of Shcbp1 was highest in the thymus compared to the other immune tissues **(Figure 3.1A)**. During development, thymocytes undergo stages of rapid proliferation. Within the bone marrow, Shcbp1 was expressed at low levels, which is likely since the bone marrow primarily consists of endothelial cells and other stromal components. In fact, the expression of Shcbp1 within the proliferating hematopoietic cells in the bone marrow is quite high **(Figure A.1B)**. Furthermore, the expression of Shcbp1 was low in the spleen and lymph node, and splenocytes and lymphocytes are quiescent and not actively cycling in naïve animals.

To further examine the expression of Shcbp1 in the thymus, we analyzed the expression of Shcbp1 in the native architecture of the thymus, via immunohistochemistry and immunofluorescence analysis of thymic sections. Using immunohistochemistry, we found that Shcbp1 expression was most evident in the highly cellular thymic outer cortex, which contains the $CD4^-CD8^-$ double negative (DN) thymocytes, as well as the $CD4^+CD8^+$ double positive (DP) thymocytes[11,14] **(Figure 3.1B)**. When we co-stained for CD25, a cell surface marker expressed on DN2 and DN3 thymocytes, most of the $CD25^+$ thymocytes did not express Shcbp1. Since most $CD25^+$ thymocytes are non-proliferating DN3a thymocytes, this suggests that Shcbp1 is likely not expressed in the non-cycling or slowly cycling DN3a thymocytes[44] **(Figure 3.1C)**. However, CD25 is also expressed on the highly proliferative DN3b and DN2 thymocytes, and a subset of these $CD25^+$ thymocytes co-expressed Shcbp1 **(Figure 3.1C)**. We further examined the correlation

between Shcbp1 expression and thymocyte proliferation by co-staining for Shcbp1 and the proliferation marker Ki67. The cells expressing Shcbp1 also co-stained for Ki67 **(Figure 3.1D)**. These data suggested that Shcbp1 is expressed in the thymus within the actively proliferating thymic populations.

3.2. *Expression of Shcbp1 during T cell development tightly correlates with proliferative stages and is induced by preTCR signaling*

The increased expression of Shcbp1 in proliferating thymic subsets suggested that Shcbp1 might be up-regulated by preTCR signaling. To test this *in vivo*, we injected the antibody specific for CD3 (anti-CD3) into *Rag1*$^{-/-}$ mice[132]. Thymocytes from *Rag1* deficient mice are arrested at the DN3 stage of T cell development, but express low levels of CD3, and crosslinking CD3 is sufficient to induce proliferation and differentiation of the arrested DN3 thymocytes[132] **(Figure 3.2A)**. Twenty-four hours after anti-CD3 injection, approximately 40% of the *Rag1* deficient thymocytes downregulated CD25 and differentiated into DN4 thymocytes **(Figure 3.2B)**. Shcbp1 was upregulated in thymocytes from anti-CD3 injected *Rag1*$^{-/-}$ mice, compared to the PBS injected *Rag1*$^{-/-}$ mice, as determined by RT-PCR and immunoblotting for Shcbp1 **(Figure 3.2C)**. Importantly, the expression of Shcbp1 is likely even higher in the thymocytes that differentiated into DN4 thymocytes, and we performed analysis on the total thymocyte population so we are likely underestimating the upregulation. Collectively, this data demonstrated that Shcbp1 is inducible after preTCR signaling in DN3 thymocytes.

We also examined the expression profile of *Shcbp1* in the different thymic subsets using data from the publicly available Immunological Genome Project Database

(www.immgen.org)[136]. The Immunological Genome Project Database provides post-normalized values and mathematic modeling suggests that a gene with a normalized value less than 47 has a ≥95% probability of being a silent gene while a normalized value greater than 120 has a ≥95% probability of having true expression[136]. Again, *Shcbp1* expression highly correlated with thymocyte subsets known to be proliferative. Within the DN compartment, *Shcbp1* expression was increased in the highly proliferative DN2, DN3b, and DN4 compartments, but was low/not expressed in the non-cycling or slowly cycling DN3a compartment[136] **(Figure 3.2D)**. Previous studies have shown that β-selection represents a major transcriptional shift during thymocyte development, with 48% of the genes upregulated being related to proliferation[44]. We noted about a 6-fold increase in *Shcbp1* between the DN3a and DN3b compartment **(Figure 3.2D)**. With respect to later thymic developmental stages, *Shcbp1* expression was low in the noncycling DP and SP compartments[136] **(Figure 3.2D)**. TCR signaling is also required in another thymic development checkpoint, namely positive selection at the DP stage. However, unlike β-selection, positive selection occurs in the absence of extensive proliferation[44]. Interestingly, there was no upregulation of *Shcbp1* in the transition from small DP to the positively-selected $CD69^+$ DP thymocytes[136] **(Figure 3.2D)**. Collectively, these data suggest that *Shcbp1* expression in the thymus tightly correlates with the proliferative state of thymocytes and that *Shcbp1* is induced during preTCR signaling in DN thymocytes but not TCR signaling in DP thymocytes.

3.3. *Shcbp1 expression is regulated by ShcA signaling in the thymus*

Shcbp1 was initially identified as a binding partner of ShcA, an adapter protein that relays signals downstream of many receptors including the TCR and the preTCR[8,10].

Although ShcA binds to Shcbp1 in activated T cells[10], whether this interaction also occurs in the thymus was not known. Using the the preTCR$^-$ SCIET27 murine thymocyte cell line, preTCR$^+$ SCB29 murine thymocyte cell line, and primary murine thymocytes, we found that ShcA binds to Shcbp1 in thymocytes **(Figure 3.3A, B, C)**. Importantly, the expression of Shcbp1 is elevated in cell lines, likely since cell lines contain continuously proliferating cells.

We next tested whether signaling via ShcA was required for Shcbp1 upregulation. I used transgenic mice with thymic expression of dominant negative forms of ShcA that cannot be phosphorylated on critical tyrosine residues (denoted Shc$F_{239}F_{240}F_{317}$ (ShcFFF) and Shc$F_{239}F_{240}$ (ShcFF)). Thymocytes expressing the ShcFFF or ShcFF transgene have a block in T cell development at the DN3 stage of development [8,9] along with a proliferative defect **(Figure 1.4A, B**). We found that Shcbp1 expression was significantly reduced in thymocytes expressing the mutant ShcA transgenes compared to control thymocytes **(Figure 3.3D, E)**. DNA microarray analysis of DN4 thymocytes expressing ShcFFF also showed that Shcbp1 expression was reduced 16-fold compared to control DN4 thymocytes. To test whether ShcA was required for Shcbp1 expression in the thymus, we crossed *Lck*-Cre$^+$/ShcFFF transgenic mice to *Rag1*$^{-/-}$ mice. In the *in vivo* model of anti-CD3-induced preTCR signaling, Shcbp1 was not upregulated to the same extent in *Rag1* deficient thymocytes expressing the ShcFFF transgene **(Figure 3.3F)**. These data suggest that preTCR-induced upregulation of Shcbp1 requires optimal ShcA-mediated signaling.

3.4. *Generation of mice with conditional and global deletion of Shcbp1*

The striking expression of Shcbp1 within the proliferating populations of thymocytes and its upregulation in response to preTCR signaling suggested that Shcbp1 likely played a role in regulation of T cell development. To test this *in vivo,* we generated *Shcbp1* deficient mice. Since Shcbp1 is highly expressed in the embryo, and previous studies in Drosophila demonstrated that loss of the Shcbp1 homolog caused partial lethality[98], we chose to use the conditional knockout approach.

JM8.A2 embryonic stem cells (C57BL/6N orgin) with exons 4-6 of the *Shcbp1* locus flanked by *loxP* sites were obtained from EUCOMM and used to generate the *Shcbp1* floxed mouse[116] **(Figure 3.4A)**. Cre-mediated deletion of these exons is designed to cause frame-shift mutations and the generation of multiple STOP codons, leading to ablation of protein expression. Importantly, the JM8.A2 embryonic stem cells are derived from C57BL/6N blastocysts, which eliminates the requirement for extensive backcrossing to obtain a pure background. Additionally, the dominant agouti coat color marker was introduced into the JM8 ES cells to generate the JM8.A2 ES cells, which permits assessment of germline transmission via coat color since C57BL/6J mice (black coat) are frequently used for blastocyst injection. The *Shcbp1* targeted JM8.A2 ES cells were injected into C57BL/6J blastocysts by the UVA Gene Targeting and Transgenic Core Facility. The resulting chimeric progeny were mated to C57BL/6J mice for germline transmission, and we obtained agouti pups and germline transmission from one chimeric mouse. Crossing of the *Shcbp1* targetted mice to a flippase transgenic mouse to remove the neomycin cassette resulted in mice with floxed *Shcbp1* allele **(Figure 3.4A, B)**.

To obtain deletion of Shcbp1, we crossed the floxed Shcbp1 mice with different Cre-expressing transgenic mice. First, we crossed the $Shcbp1^{fl/fl}$ mice with the

ubiquitously Cre expressing *EIIA*-Cre line to generate *Shcbp1*$^{-/-}$ mice[119] **(Figure 3.4A, C)**. Surprisingly, the *Shcbp1*$^{-/-}$ mice were viable, born in normal ratios, and grossly normal **(Figure 3.5)**. One possible reason for the lack of an obvious phenotype is the incomplete deletion of the *Shcbp1* locus or continued protein expression. We addressed this via several approaches. Shcbp1 expression by both mRNA and protein analysis was reduced in a dose dependent manner in the thymus of *Shcbp1*$^{+/-}$ *and Shcbp1*$^{-/-}$ mice compared to *Shcbp1*$^{+/+}$ animals **(Figure 3.4D, E)**. It is noteworthy that in the *Shcbp1*$^{-/-}$ mice, *ShcA* expression was unchanged as ShcA is required for both embryonic and T cell development[8,9,74,75] **(Figure 3.4D)**. Loss of Shcbp1 expression was also confirmed by immunofluorescence **(Figure 3.4F)**. From these data, we concluded that we successfully generated mice with global deletion of Shcbp1.

Next, we also generated mice with conditional deletion of *Shcbp1* to determine whether specific loss of Shcbp1 in developing thymocytes causes impairment in thymic development or T cell function. We crossed the *Shcbp1*$^{fl/fl}$ mice to the *Lck*-Cre transgenic mouse line and the *Rag*-Cre transgenic mouse line. The *Lck*-Cre transgenic mouse line expresses Cre under the *lck*-proximal promoter from the DN1/DN2 stages of thymocyte development[86] while the *Rag*-Cre transgenic mouse line expresses Cre during early hematopoiesis in the bone marrow (Tim Bender, personal communication) **(Figure 1.3A)**. Next, we confirmed deletion of Shcbp1 in thymocytes and peripheral T cells using several different techniques **(Figure 3.6)**. First via genotyping DNA isolated from the thymus as well as splenocytes (T cells versus non-T cells), we found that Shcbp1 was deleted at the genetic level in thymocytes and CD4$^+$ T cells from *Lck*-Cre$^+$/*Shcbp1*$^{fl/fl}$ mice, but not in non-T cell splenocytes **(Figure 3.6A, B)**. Furthermore, Shcbp1 mRNA and protein were reduced in a dose dependent manner in *Lck*-Cre$^+$/*Shcbp1*$^{fl/wt}$ and

Lck-Cre^{+}/*Shcbp1*$^{fl/fl}$ mice using two different RT-PCR primers and four different Shcbp1 antibodies **(Figure 2.3A, B, 3.6C-E)**. Deletion of exons 4-6 of *Shcbp1* did not lead to a detectable truncated protein in thymocytes, as immunoblotting with antibodies to the N or C-terminus of Shcbp1 did not identify a band that might represent a truncated protein **(Figure 3.6D)**. Lastly, we also verified deletion of Shcbp1 via immunohistochemistry and found reduced staining in *Lck*-Cre^{+}/*Shcbp1*$^{fl/fl}$ mice **(Figure 3.6F)**. Importantly, this finding also verified the specificity of the Shcbp1 staining via immunohistochemistry in **Figure 3.1B**. Deletion of Shcbp1 in the *Rag*-Cre^{+}/*Shcbp1*$^{fl/fl}$ mice was also verified via western blotting and RT-PCR **(Figure 3.11 A, B)**.

3.5. *Loss of Shcbp1 does not lead to an obvious impairment in T cell development*

Given the high Shcbp1 expression in proliferating thymocytes and its upregulation by preTCR signaling, we hypothesized that Shcbp1 would be required during thymocyte development. Much to our surprise, mice with a global deletion of Shcbp1 contained normal thymic appearance, cellularity and normal ratios of DN, DP, as well as CD4 and CD8 SP thymocytes **(Figure 3.7A-C)**. In further analysis of the DN compartment, the fraction and absolute number of DN1, DN2, DN3, and DN4 subsets were largely unchanged **(Figure 3.7B, lower panel**). Additionally, TCRβ, CD3, and TCR$\gamma\delta$ expression on thymocytes was comparable between *Shcbp1*$^{+/+}$ and *Shcbp1*$^{-/-}$ mice **(Figure 3.7D, E)**.

To rule out potential compensatory effects in the global knockout mice and to directly test whether loss of Shcbp1 in developing thymocytes causes an effect, we also analyzed T cell development in both the *Lck*-Cre^{+}/*Shcbp1*$^{fl/fl}$ and the *Rag*-Cre^{+}/*Shcbp1*$^{fl/fl}$ mouse lines[86,120]. The *Lck*-Cre^{+}/*Shcbp1*$^{fl/fl}$ and *Rag*-Cre^{+}/*Shcbp1*$^{fl/fl}$ mice also showed

normal percentages and numbers of thymic compartment subsets compared to littermate controls in 4-6 week old mice **(Figure 3.8A-D, 3.11)**. Consistent with the normal thymocyte composition, the overall structure and organization of the thymus appeared normal, as shown by the H&E staining of thymic cortex and medulla **(Figure 3.8B)**. We also evaluated T cell development in two-week old mice (since certain phenotypes with ShcA are more evident in younger mice)[137], and observed no obvious differences **(Figure 3.9)**. Loss of Shcbp1 did not alter thymocyte survival, as determined by staining for Annexin V and 7AAD in thymocytes freshly isolated from mice, *ex vivo* survival assays, and an *in vivo* dexamethasone injection survival/apoptosis assay **(Figure 3.10)**. Collectively, these data suggested that, despite a remarkable upregulation of Shcbp1 upon preTCR signaling and Shcbp1 correlation with proliferative stages of thymic development, Shcbp1 appears dispensable for T cell development.

One possible reason for the lack of Shcbp1 requirement during thymocyte development is that another protein closely related to Shcbp1 might compensate for its loss. Shcbp1 contains a pectin lyase-like domain (PecLD), which is characterized by a series of parallel β-strands found in enzymes from certain bacterial plant pathogens that digest sugars in the plant wall[98,103]. Although the role of this domain in mammalian intracellular proteins is unknown, a few other mammalian proteins also contain PecLD sequences including Shcbp1-L, Fbox10, and Fbox11. Additionally, Shcbp1L has been recently identified as having an important role in spermatogenesis, and it was suggested that Shcbp1 may partially compensate for the loss of Shcbp1L[108]. When we assessed the expression of genes coding these proteins via RT-PCR, there was no compensatory upregulation of these genes in cells lacking *Shcbp1* **(Figure 3.8G)**. Further, the

transcript levels of Shcbp1-L and Fbox10 were barely detectable within the thymus. Therefore, other PecLD-containing proteins such as Shcbp1-L, Fbox10, and Fbox11 do not appear to compensate for the loss of Shcbp1.

3.6. *Peripheral T cell compartment is normal in Shcbp1 deficient mice*

Since T cell development also involves the migration of thymocytes out of the thymus and ShcA is required for CXCR4 mediated thymocyte migration[42,88], we next investigated the peripheral T cell compartment. In mice with global or conditional deletion of *Shcbp1*, there were no obvious differences in the fraction or absolute numbers of $CD4^+$ and $CD8^+$ T cells in the spleen or lymph nodes **(Figure 3.7F-H, 3.8E,F, 3.11D)**. There were also no differences in the expression of the regulatory T cell transcription factor FOXP3 or a panel of markers found on activated or memory cells **(Figure 3.7I, Figure 3.13B)**. These data suggested that Shcbp1 was not required for the development or maintenance of the peripheral T cell compartment.

3.7. *Shcbp1 is upregulated by stimulation in peripheral $CD4^+$ T cells*

To test whether Shcbp1 is upregulated during activation in peripheral T cells, we performed anti-CD3/anti-CD28 stimulation of $CD4^+$ T cells sorted from the lymph nodes. Shcbp1 was upregulated 24 hours after anti-CD3/anti-CD28 stimulation **(Figure 3.12A)**. We also confirmed Shcbp1 protein upregulation by immunoblotting **(Figure 3.12B)**. To test whether Shcbp1 was upregulated after T cell activation *in vivo*, we injected anti-CD3 into C57BL/6J mice and found ~20-fold increase in Shcbp1 transcript and protein levels in $CD4^+$ T cells at 24 hours after injection **(Figure 3.12C)**. We further examined whether Shcbp1 was induced in $CD4^+$ T cells activated by stimuli that bypass the TCR and found

comparable *Shcbp1* upregulation after PMA and ionomycin stimulation **(Figure 3.12D)**. To determine whether Shcbp1 is induced by physiological, antigen-specific, TCR stimulation, we used DO11.10 TCR transgenic mice, which express a transgenic TCR that recognizes a peptide containing amino acids 323 through 339 of ovalbumin ($OVA_{(323-339)}$)[123]. Activation of DO11.10 $CD4^+$ T cells with two different doses of $OVA_{(323-339)}$ showed that *Shcbp1* was induced by antigen-specific TCR signaling **(Figure 3.12E)**. Additionally, we confirmed that Shcbp1 binds to ShcA in activated splenocytes **(Figure 3.12F)**. Collectively, these results demonstrate that Shcbp1 was induced by antigen-specific, antibody-mediated, and chemical T cell stimulation.

To determine whether Shcbp1 was required for T cell proliferation, we labeled *Shcbp1*$^{+/+}$ and *Shcbp1*$^{-/-}$ $CD4^+$ T cells with the cytosolic dye CFSE and stimulated with anti-CD3/anti-CD28 for various times (24 hours, 48 hours, 72 hours). However, we observed no discernible differences in the proliferation of *Shcbp1*$^{+/+}$ and *Shcbp1*$^{-/-}$ T cells as monitored by the CFSE dilution **(Figure 3.13A)**. Further, T cells deficient in *Shcbp1* upregulated markers of T cell activation and became blastic by 24-hours after stimulation **(Figure 3.13A, B)**. These data suggest that Shcbp1 was likely dispensable for *ex vivo* anti-CD3/anti-CD28 mediated T cell proliferation and activation, as measured under these assay conditions.

3.8. *Shcbp1 is upregulated in Experimental Autoimmune Encephalomyelitis*

Previous research has shown that Shcbp1 protein is upregulated in T cells from *CTLA4*-deficient mice compared to T cells from wild-type mice[10]. *CTLA4*-deficient mice have high lymphoproliferation and lethal autoimmunity[105,106]. To test whether Shcbp1 might play a role *in vivo* in T cell effector responses in the context of autoimmune

disease, we chose to use the CD4 T cell driven experimental autoimmune encephalomyelitis (EAE) model. The EAE model reproduces many of the clinical, pathological, and immunological aspects of the human disease multiple sclerosis (MS), including infiltration of autoreactive T cells into the central nervous system (CNS), causing inflammation and demyelination[52,54,63]. Moreover, many genetic loci identified as conferring susceptibility to MS are linked to CD4 effector T cell differentiation and function[61,62]. We first analyzed the expression of Shcbp1 in the spinal cords of mice immunized with MOG_{35-55} peptide to induce EAE. *Shcbp1* expression was upregulated in mononuclear cells from the spinal cord and brain of animals subjected to EAE (compared to healthy controls) as analyzed via RT-PCR **(Figure 3.14A)**. Shcbp1 protein expression was also detectable by immunohistochemistry in the spinal cords of EAE mice, especially in the areas of cellular immune infiltration, while it was mostly absent in healthy control animals **(Figure 3.14B)**.

3.9. *Loss of Shcbp1 affects disease severity in $CD4^+$ T cell driven autoimmune disease*

We next analyzed disease severity in *Shcbp1*$^{-/-}$ and control mice after MOG_{35-55} peptide injection to induce disease. Two independent investigators (myself and Sanja Arandjelovic), blinded to the genotype of the mice, monitored clinical scores for a period of 28 days after peptide immunization. *Shcbp1* deficiency consistently resulted in a reduction of disease severity along with improved survival in both male and female mice subjected to EAE **(Figure 3.15A, B)** (n=3 independent experiments with a total of n=18, 19 male and n= 17, 12 female *Shcbp1*$^{+/+}$ and *Shcbp1*$^{-/-}$ mice, respectively). *Shcbp1* deficient mice exhibited a lower maximum score and reduced overall disease index (area under the curve) (Table 1). On day 28 of the disease, we also examined the composition

of the immune infiltrate in the CNS by analyzing the cells isolated from the brain and spinal cord of *Shcbp1*$^{+/+}$ *and Shcbp1*$^{-/-}$ mice via flow cytometry. *Shcbp1*$^{-/-}$ mice displayed a trend towards overall fewer mononuclear cells isolated from the brain and spinal cord compared to *Shcbp1*$^{+/+}$ mice **(Figure 3.15C)**. Although the overall percentage of each cell type was not significantly altered, *Shcbp1*$^{-/-}$ mice had fewer CD4^{+} T cells, B cells, and macrophages **(Figure 3.15C)**. Histological analysis by H&E staining of the spinal cords confirmed that *Shcbp1* deficient mice had fewer loci of immune infiltration **(Figure 3.15D)**. Importantly, *Shcbp1*$^{-/-}$ mice also had significantly fewer CD3^{+} T cells in their spinal cords, as detected by immunohistochemistry **(Figure 3.15D, E)**. Together, these data suggested that Shcbp1 is upregulated in the CNS tissue in the EAE model and that loss of Shcbp1 expression attenuates disease severity.

3.10. *Shcbp1 expression in CD4 T cells contributes to disease severity in EAE*

Rag1$^{-/-}$ mice are resistant to EAE due to the lack of mature T cells, and their engraftment with T cells isolated from the secondary lymphoid organs of wild type mice has been demonstrated to be sufficient for disease development[138]. To directly test whether T cell specific expression of Shcbp1 contributes to disease severity, we next transferred *Shcbp1*-deficient or control T cells into *Rag1*$^{-/-}$ mice, and induced disease one week after transfer. The *Rag1*$^{-/-}$ deficient mice that received *Shcbp1* deficient CD4^{+} T cells had overall reduced disease severity along with improved survival, compared to *Rag1*$^{-/-}$ mice that received wild-type CD4^{+} T cells **(Figure 3.16A, B)**. As an additional control, we immunized mice that received no transfer of CD4^{+} T cells, and confirmed that these mice did not develop disease. Furthermore, we immunized *Lck*-Cre^{+}/*Shcbp1*$^{fl/fl}$ and

Lck-Cre^{+}/*Shcbp1*$^{wt/wt}$ mice to induce EAE, and observed reduced disease severity in *Lck*-Cre^{+}/*Shcbp1*$^{fl/fl}$ mice (we did not observe any mortality in this experiment as the *Lck*-Cre transgene alone leads to a significant impairment in T cell development and the peripheral T cell compartment) **(Figure 3.16C)**. Based on these observations, we concluded that Shcbp1 expression in CD4^{+} T cells contributes to disease severity in the EAE model.

3.11. *Shcbp1 is induced in T cells by conditions present in MS and EAE*

In multiple sclerosis and EAE, there is inappropriate T cell activation as well as abnormal IL-2 and T_H17 skewing conditions within the cerebrospinal fluid (CSF) and serum[67,139,140]. Therefore, we next determined whether the conditions likely present in the CSF in MS and EAE were capable of upregulating Shcbp1 expression in CD4^{+} T cells. We first evaluated how the kinetics of Shcbp1 upregulation **(Figure 3.12A)** correlated with upregulation of IL-2 after anti-CD3/anti-CD28 stimulation. *IL-2* was upregulated rapidly after stimulation (in both wild-type and *Shcbp1*-deficient T cells), with maximal induction around 12 hours post-stimulation, while *Shcbp1* upregulation occurred with much delayed kinetics **(Figure 3.16A)**. Since *IL-2* was upregulated prior to *Shcbp1* induction, we also tested whether IL-2 could induce *Shcbp1*. IL-2 stimulation induced *Shcbp1* expression, suggesting that Shcbp1 is a novel IL-2 responsive gene **(Figure 3.16B)**. We also found that Shcbp1 was upregulated after culturing CD4^{+} T cells in T_H1 or T_H17 skewing conditions **(Figure 3.16C, D)**. However, Shcbp1 was not required for *ex vivo* T_H1 or T_H17 skewing as determine by intracellular IL17 and IFNγ staining or CCR6 mediated migration of T_H17 cells **(Figure 3.16E, F)**. Therefore, conditions that are

present and therapeutically relevant in multiple sclerosis and EAE were capable of inducing Shcbp1 expression in $CD4^+$ T cells *ex vivo*.

3.12. *Shcbp1 is induced in peripheral $CD8^+$ T cells*

Next, we investigated whether Shcbp1 upregulation was specific to $CD4^+$ T cells or whether it was also upregulated in $CD8^+$ T cells. We found that the expression of *Shcbp1* in $CD8^+$ T cells was upregulated *ex vivo* after anti-CD3/anti-CD28 stimulation. In a time course of anti-CD3/anti-CD28 stimulation, we found that Shcbp1 was upregulated 10 to 15-fold after 24 hours of stimulation and remained elevated after 48 and 72 hours of stimulation **(Figure 3.18A)**. To test whether Shcbp1 was upregulated after $CD8^+$ T cell stimulation *in vivo*, we injected anti-CD3 into C57BL/6J mice and found ~12-fold increase in Shcbp1 transcript and protein levels in $CD8^+$ T cells isolated from the spleen at 24 hours after injection **(Figure 3.18B)**. Therefore, we concluded that Shcbp1 is upregulated in $CD8^+$ T cells after activation via anti-CD3/anti-CD28 both *in vivo* and *ex vivo*.

3.13. *Shcbp1 is induced in peripheral $CD8^+$ T cells during the primary immune response to infection*

To determine whether Shcbp1 is induced in $CD8^+$ T cells by physiological, antigen-specific, TCR stimulation and during the primary immune response, we investigated the expression of *Shcbp1* in the context of *in vivo* infection. Data from the Immunological Genome Project Database also showed that *Shcbp1* was induced in $CD8^+$ T cells *in vivo* during both bacterial and viral infection[136]. OT-I $CD8^+$ T cells, which express a MHC class I-restricted transgenic TCR specific for ovalbumin[125], were transferred to congenic C57BL/6 mice and the mice were then infected with either

Listeria monocytogenes-OVA (LM-OVA) or *Vesicular Stomatitis Virus*-OVA (VSV-OVA), and the expression of *Shcbp1* in the OT-I CD8 T cells was analyzed via microarray at various time-points after infection[136]. *Shcbp1* was upregulated with kinetics that correspond to the time-points during which the $CD8^+$ T cells were proliferative[136]. In the LM-OVA infection, *Shcbp1* was upregulated in $CD8^+$ T cells by 24 hours with maximal upregulation at 48 hours. *Shcbp1* remained elevated at day 6, but returned to basal expression by day 8 **(Figure 3.18C)**; importantly, in wild-type C57BL/6 mice *Listeria* is generally cleared around day 7[136,141]. Furthermore, in VSV-OVA infection, Shcbp1 was induced at day 5 and day 6 and returns to basal expression by day 8[136] **(Figure 3.18C)**.

3.14. *Shcbp1 is dispensable for $CD8^+$ T cell function in influenza infection*

Shcbp1 has been linked to viral disease both in human studies as well as in animal models[101,110,112-114]. Previous studies have shown that Shcbp1 was induced in the lung carcinoma cell lines (A549/hSLAM cells) after interferon stimulation as well as during infection with the measles virus[101]. Although in this study the authors demonstrated that Shcbp1 was subverted by the measles virus and was required for viral synthesis[101], their findings bring up the intriguing question of the relevance of Shcbp1 upregulation and what the normal function of Shcbp1 is during the anti-viral immune response. Additionally, *SHCBP1* has been found to be upregulated via microarray analysis in human viral diseases, including in $CD4^+$ T cells isolated from HIV infected individuals as compared to healthy controls[110,112,113]. Given these previous findings and the upregulation of *Shcbp1* in $CD8^+$ T cells after anti-CD3/anti-CD28 stimulation, we hypothesized that Shcbp1 may be required during the anti-viral immune response.

To evaluate the role of Shcbp1 in $CD8^+$ T cell function *in vivo*, we used the mouse model of the *influenza* virus infection in collaboration with the Braciale laboratory at UVA (Taeg Kim). First, we analyzed the expression of *Shcbp1* after intranasal PR8 influenza infection in $CD8^+$ T cells isolated from the mediastinal lymph nodes. After administration of the influenza virus, viral epitopes-specific $CD8^+$ T cells become activated, proliferate, and differentiate to effector cells in the mediastinal lymph nodes, before migrating to the infected lung[142]. We found that Shcbp1 was up-regulated ~10-fold in both the $CD8^+$ and $CD4^+$ T cells isolated from the mediastinal lymph nodes on day 5 after infection, as compared to $CD8^+$ or $CD4^+$ T cells isolated from the inguinal lymph nodes of the same animal **(Figure 3.18D**). Therefore, Shcbp1 is induced specifically in both $CD4^+$ and $CD8^+$ T cells within the mediastinal lymph nodes during influenza infection.

To investigate the *Shcbp1* function in $CD8^+$ T cells *in vivo*, *Shcbp1* deficient and control mice were infected with either high (3500 EID) or low dose (350 EID) PR8 influenza virus and the weight and survival of animals was monitored for 21 days after infection. Surprisingly, despite induction of Shcbp1 expression during the influenza infection, we found no differences in the survival, weight-loss, or recovery between *Shcbp1* deficient and wild type animals (**Figure 3.19A, B**). The humoral immune response to influenza is crucial for the clearance of the virus as well as for the protection from re-infection[143]. In agreement with the overall disease parameters listed above, *Shcbp1*$^{-/-}$ mice also generated a normal humoral response to influenza infection at day 35, with equivalent amounts of total, as well as influenza specific, IgG-type immunoglobulins **(Figure 3.19D)**. Since minor defects in $CD8^+$ T cell function may not be recognized during the acute phase of the influenza viral infection, we also analyzed

$CD8^+$ T cell tetramer staining in mice 35 days post infection with the low-dose (LD_{10}) PR8 influenza. However, we observed no significant differences in the percentages of NP^+ or PA^+ tetramers stained $CD8^+$ T cells isolated from the blood of *Shcbp1*$^{-/-}$ mice and the control C57BL/6 mice **(Figure 3.19C)**. Collectively, these data suggest that Shcbp1 deficient mice are fully competent in the response to the influenza viral infection. Furthermore, the *Shcbp1* deficient animals also generated normal levels of flu specific IgG, which indicates that $CD4^+$ T cell help to B cells and antibody production were also unaffected.

4. Discussion

Recently, several laboratories have identified Shcbp1 through unbiased screening techniques and have linked Shcbp1 to diverse biological functions including embryonic development, cytokinesis, spermatogenesis, growth factor signaling, neuronal development, viral responses, and tumorigenesis[10,96-101]. Remarkably, many of these processes have links to proliferation. While these data were suggestive, none of the previous studies evaluated the *in vivo* requirement for Shcbp1. By generating mouse strains with conditional and global deletion of *Shcbp1,* we have carefully evaluated the *in vivo* requirement for Shcbp1 in T cell development as well as in an autoimmune disease model and in the context of viral infection.

Although the primary objective of our work was to determine the function of Shcbp1 in the T lymphocyte lineage, a significant unexpected finding was that Shcbp1 is dispensable for embryonic development in mice. Since previous studies have shown that deletion of the Drosophila homolog Nessun Dorma resulted in partial lethality and Shcbp1 is highly expressed in the embryo[10,98], it was possible that Shcbp1 may also be required for embryonic development in the mammalian system[99]. Additionally, Shcbp1 is highly expressed in the testis and ovaries, and male Drosophila lacking Nessun Dorma are not fertile[10,98]. However, our findings did not support a role for Shcbp1 in mouse spermatogenesis or oogenesis *in vivo,* as both male and female *Shcbp1*$^{-/-}$ mice are fertile. Thus, surprisingly, *Shcbp1* is not an essential gene in the mouse, despite significant similarity between the mouse and fly proteins, and these mice do not show compensatory upregulation of proteins with known similarity.

Given the high expression of Shcbp1 in the thymus and activated T cells, we also determined the function of Shcbp1 in the T lymphocyte lineage. Proliferation during T cell development is tightly regulated and thymocytes undergo stages of active proliferation followed by temporary withdrawal from the cell cycle[11]. Shcbp1 expression tightly correlated with actively proliferating thymic subsets and was upregulated via optimal ShcA-mediated preTCR signaling. However, despite the striking correlation of Shcbp1 and proliferative stages of thymocyte development, there was no apparent defect in thymocyte development *in vivo* in the absence of Shcbp1.

Interestingly, we found that Shcbp1 plays a role in $CD4^+$ T cells in the context of the autoimmune EAE model of multiple sclerosis. Shcbp1 expression was induced in the brain and spinal cords of mice immunized for EAE and Shcbp1 expression was co-localized with immune infiltrates of the spinal cord. Previous studies have shown that cytokines, including IL2 and the T_H17 skewing cytokines (IL23, IL6, TGFβ), are abnormally present in the CSF of some patients with multiple sclerosis as well as in mice immunized for EAE[67,68,139,140]. Further, mutations within the IL2 promoter and IL2-receptor confer susceptibility to MS[62,144,145], and targeting the IL2 signaling pathway via IL2-receptor blocking antibodies is therapeutically beneficial in humans [59]. Similarly, *IL2*-deficient mice are resistant to EAE induction[146]. Additionally, multiple components of the T_H17 cell differentiation pathway (IL6, STAT3, IL21) are located near loci that have been identified to confer susceptibility to MS[62] and targeting components of T_H17 cell differentiation and function has also been shown to confer resistance or lead to attenuated disease in EAE[147,148]. Therefore, Shcbp1 is induced in T cells under conditions that are likely present and functionally relevant in the inflammatory

environment of the CNS during MS/EAE. Furthermore, we found that mice deficient in Shcbp1 had overall reduced EAE disease severity as well as markedly improved survival compared to wild-type control mice, likely due to impairment in the *in vivo* effector function of the $CD4^+$ T cells.

In contrast, we found no defect in the $CD8^+$ anti-viral immune response to influenza infection, although *Shcbp1* was induced in T cells isolated from the mediastinal lymph nodes after influenza infection. We found this data surprising, as previous findings suggested that *Shcbp1* was induced by the interferon response and has been linked to viral infections in both humans and mice[101,110,112]. Furthermore, we also used the model of influenza infection in the *Shcbp1* deficient mice to investigate B cell function, and we found that there was no difference in total IgG or in flu specific IgG. Therefore, Shcbp1 is dispensable for the antiviral immune response in the context of influenza infection.

The current treatment approaches for multiple sclerosis and many other autoimmune diseases involve the use of immunosuppressant drugs that broadly suppress the immune system with many adverse side-effects including susceptibility to infections and cancers[54,63]. Recent reports have highlighted the potential benefits of new therapies that specifically target the encephalitogenic T cells during EAE and, by extension, multiple sclerosis[149]. Given the expression of Shcbp1 in inflammatory lesions within the spinal cord as well as reduced disease severity in *Shcbp1* deficient mice, Shcbp1 may represent a therapeutic target for autoimmune disease such as multiple sclerosis. Additionally, our findings suggest that targeting Shcbp1 may have an additional benefit of minimal side effects, since loss of Shcbp1 does not appear to

directly affect development or proliferation of $CD4^+$ T cells and does not lead to impairment of the antiviral immune response in the context of influenza.

5. Acknowledgment of co-author contributions

I would like to thank Dr. Sanja Arandjelovic for providing mentorship during all stages of this project. Specifically, she was instrumental for helping me learn how to perform the EAE mouse model. Additionally, she helped me to score all of my EAE experiments. This project would not have been possible without her helpful insights and assistance. I would also like to thank Dr. Paul Trampont for his initial findings on the expression of *Shcbp1* in DN3 thymocytes from *Lck*-Cre/ShcFFF mice and for teaching me how to analyze thymic development. Lastly, I would like to thank Dr. Taeg Kim for his assistance with the influenza model.

Figure 3.1: Expression of Shcbp1 in the thymus

(A) Immunoblotting of Shcbp1 in the indicated tissues of a naïve C57BL/6J mouse (representative of n=3 mice).

(B) Left, immunohistochemistry of Shcbp1 in paraffin embedded thymic tissue from the C57BL/6J mouse (representative of n=4 mice). Right, secondary antibody control staining.

(C) Immunofluorescence of Shcbp1 and CD25 in frozen thymic tissue from the C57BL/6J mouse (representative of n=2 mice).

(D) Immunofluorescence of Shcbp1 and Ki67 in frozen thymic tissue from the C57BL/6J mouse (representative of n=2 mice).

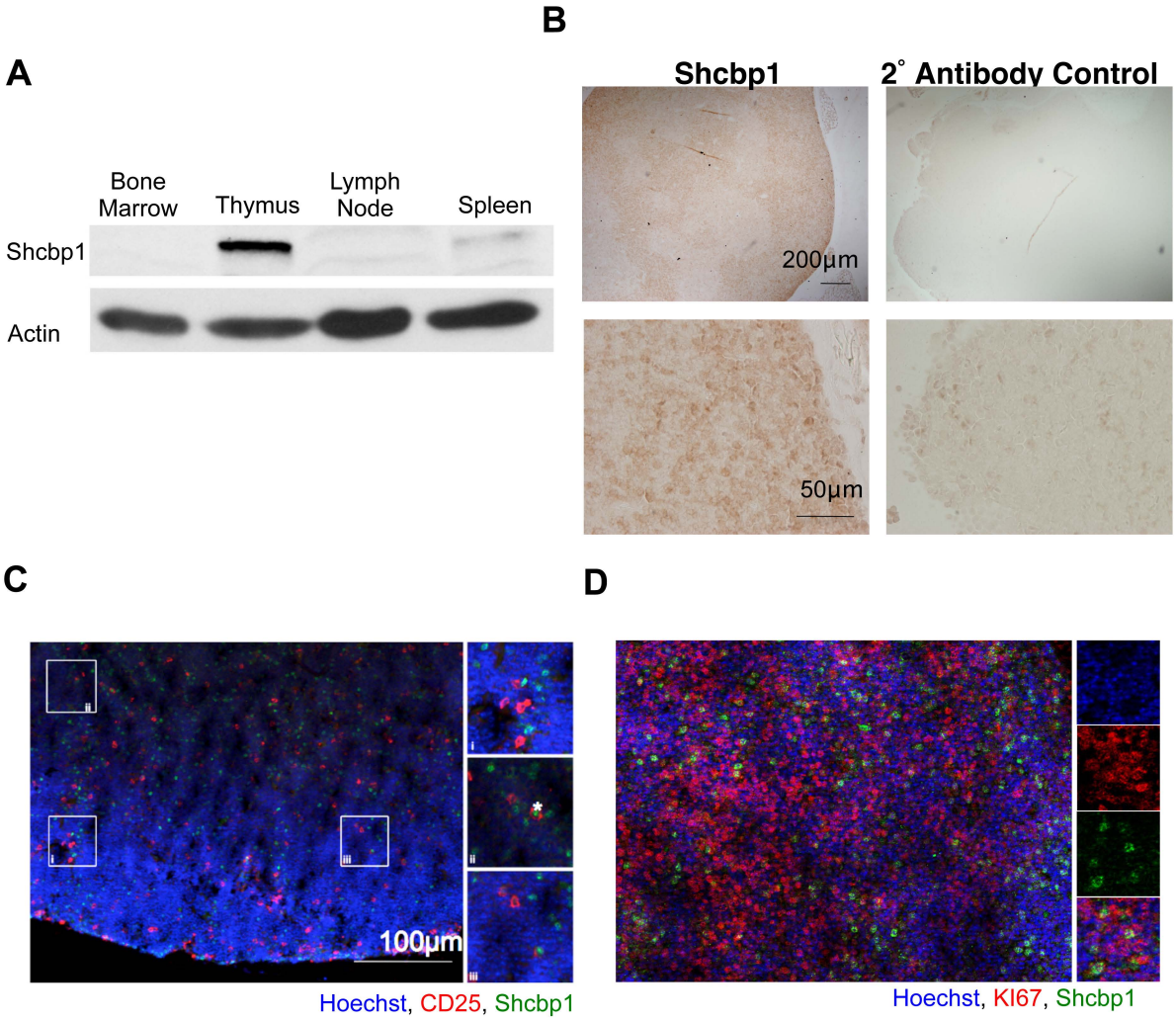

A
Bone Marrow
Thymus
Lymph Node
Spleen
Shcbp1
Actin
B
Shcbp1
2° Antibody Control
200μm
50μm
C
100μm
Hoechst, CD25, Shcbp1
D
Hoechst, KI67, Shcbp1

Figure 3.2: Expression of Shcbp1 during T cell development tightly correlates with proliferative stages and is induced by preTCR signaling

(A) Flow cytometry for CD25 and CD44 of thymocytes isolated from *Rag1* deficient mice injected with either PBS or 100μg of anti-CD3 antibody for 24 hours (Representative of n=5 mice of each treatment).

(B) Quantification of the percentage of DN4 thymocytes from Rag1 deficient mice injected with either PBS or 100μg of anti-CD3 antibody for 24 hours (n=5 mice of each treatment).

(C) Left, *Shcbp1* mRNA expression in thymocytes from *Rag1*$^{-/-}$ mice injected with either PBS or 100μg of anti-CD3 antibody for 24 hours (n=6 mice of each treatment, $p<0.0001$). Right, representative immunoblot of Shcbp1 with quantification.

(D) *Shcbp1* mRNA expression analyzed by microarray in electronically sorted thymic subsets (data curated from the Immgen Database). Normalized expression value lower than 47 represents a gene that has a ≥95% of being a silent gene, while normalized value greater than 120 represents a gene that has a ≥95% probability of having true expression.

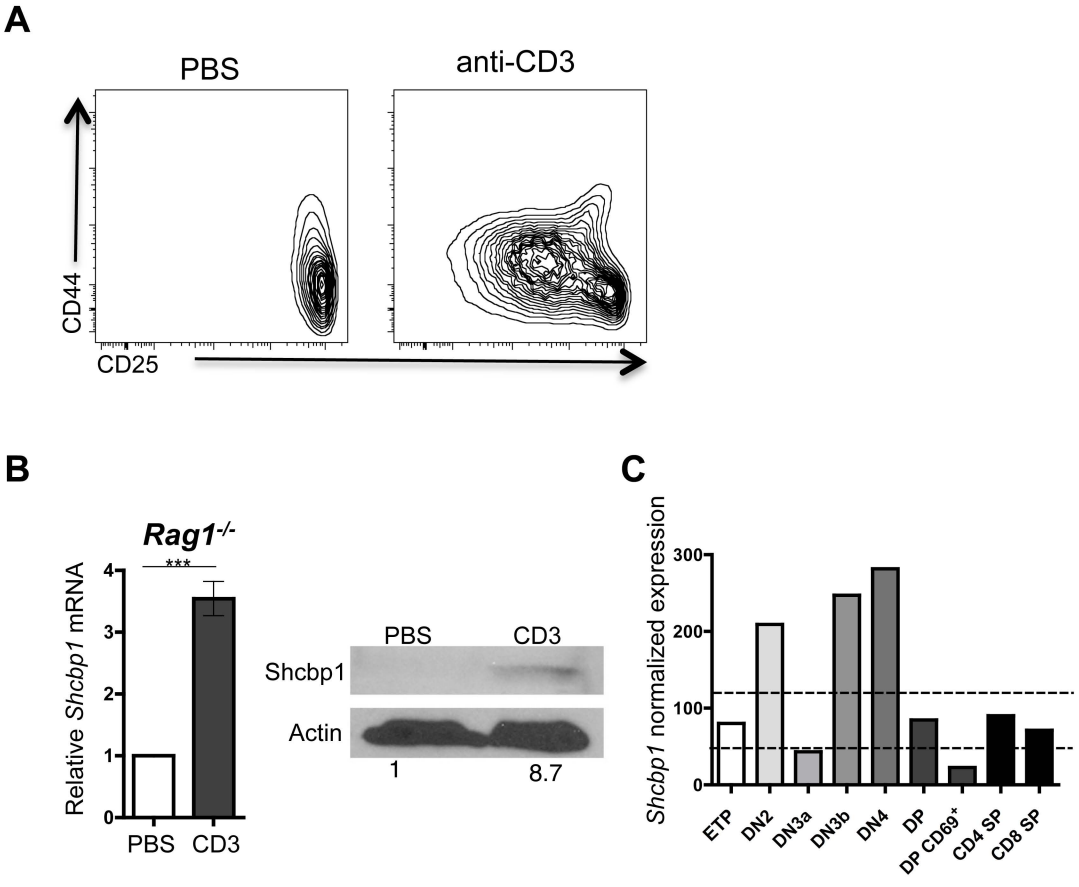

A
PBS
anti-CD3
CD44
CD25
B
Rag1-/-
***
Relative Shcbp1 mRNA
PBS
CD3
PBS
CD3
Shcbp1
Actin
1
8.7
C
Shcbp1 normalized expression
ETP
DN2
DN3a
DN3b
DN4
DP
DP CD69+
CD4 SP
CD8 SP

Figure 3.3: Shcbp1 expression is regulated downstream of ShcA signaling in the thymus

(A) Immunoprecipitation of Shcbp1 and immunoblotting for ShcA and Shcbp1 in the preTCR^{-} murine thymocyte cell line SCIET27 (representative of n=3 experiments).

(B) Immunoprecipitation of Shcbp1 and immunoblotting for ShcA and Shcbp1 in the preTCR^{+} murine thymocyte cell line SCB29 (representative of n=3 experiments).

(C) Immunoprecipitation of Shcbp1 and immunoblotting for ShcA and Shcbp1 in primary murine thymocytes (representative of n=2 experiments).

(D) Shcbp1 expression in thymocytes expressing the ShcFFF transgene analyzed by immunoblotting in total thymocytes with quantification (n=9 control and n=5 *Lck*-Cre^{+}/ShcFFF, $p<0.0001$).

(E) Immunoblotting of Shcbp1 expression in thymocytes expressing the ShcFFF transgene, along with quantification (n=4 control, n=3 *Lck*-Cre^{+}/ShcFF, $p=0.0003$).

(F) *Shcbp1* mRNA expression in thymocytes from *Rag1*$^{-/-}$ or *Lck*-Cre^{+}/ShcFFF *Rag1*$^{-/-}$ mice injected with either PBS or 100μg of anti-CD3 for 24 hours (n=3 mice of each treatment, $p=0.07$).

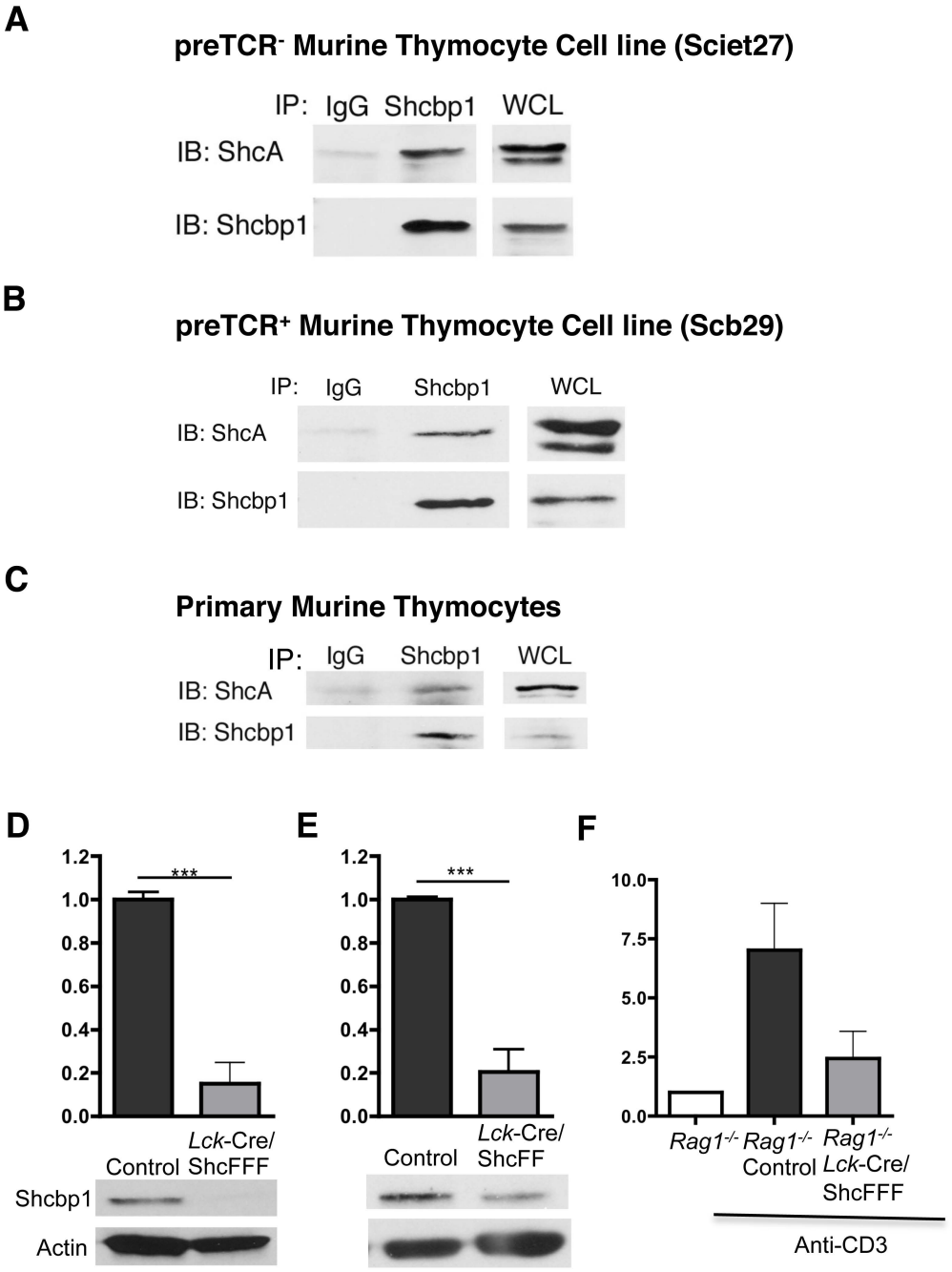

A
preTCR- Murine Thymocyte Cell line (Sciet27)
IP: IgG Shcbp1 WCL
IB: ShcA
IB: Shcbp1
B
preTCR+ Murine Thymocyte Cell line (Scb29)
IP: IgG Shcbp1 WCL
IB: ShcA
IB: Shcbp1
C
Primary Murine Thymocytes
IP: IgG Shcbp1 WCL
IB: ShcA
IB: Shcbp1
D
1.2
1.0
0.8
0.6
0.4
0.2
0.0
***
Lck-Cre/
Control ShcFFF
Shcbp1
Actin
E
1.2
1.0
0.8
0.6
0.4
0.2
0.0
***
Control
Lck-Cre/
ShcFF
F
10.0
7.5
5.0
2.5
0.0
Rag1-/-
Rag1-/-
Control
Rag1-/-
Lck-Cre/
ShcFFF
Anti-CD3

Figure 3.4: Generation of mice with global deletion of *Shcbp1*

(A) Schematic detailing the generation of mice carrying global deletion of *Shcbp1* and the strategy for conditional deletion of *Shcbp1* exons 4-6 and the neomycin targeting cassette.

(B) Identification of mice with a floxed or WT *Shcbp1* loci (*Shcbp1*$^{wt/wt}$, *Shcbp1*$^{fl/wt}$, and *Shcbp1*$^{fl/fl}$) assessed by genotyping PCR in tail DNA.

(C) Genotyping PCR for *Shcbp1* WT and *Shcbp1* deleted loci in tail DNA from mice with the indicated genotypes.

(D) Left, *Shcbp1* mRNA levels in thymocytes isolated from *Shcbp1*$^{+/+}$, *Shcbp1*$^{+/-}$, and *Shcbp1*$^{-/-}$ mice normalized to *HPRT* and to control *Shcbp1*$^{+/+}$ mice (n=2-5 mice per genotype). Right, *ShcA* mRNA levels in thymocytes isolated from mice of the indicated genotype normalized to *HPRT* and to control *Shcbp1*$^{+/+}$ mice (n=2-5 mice per genotype).

(E) Immunoblotting of Shcbp1 in total thymocytes from mice with the indicated genotypes (representative of n=3 experiments).

(F) Immunofluorescence of thymi from wild-type and *Shcbp1*$^{-/-}$ mice with staining for Hoechst and Shcbp1 (representative of n=2 experiments).

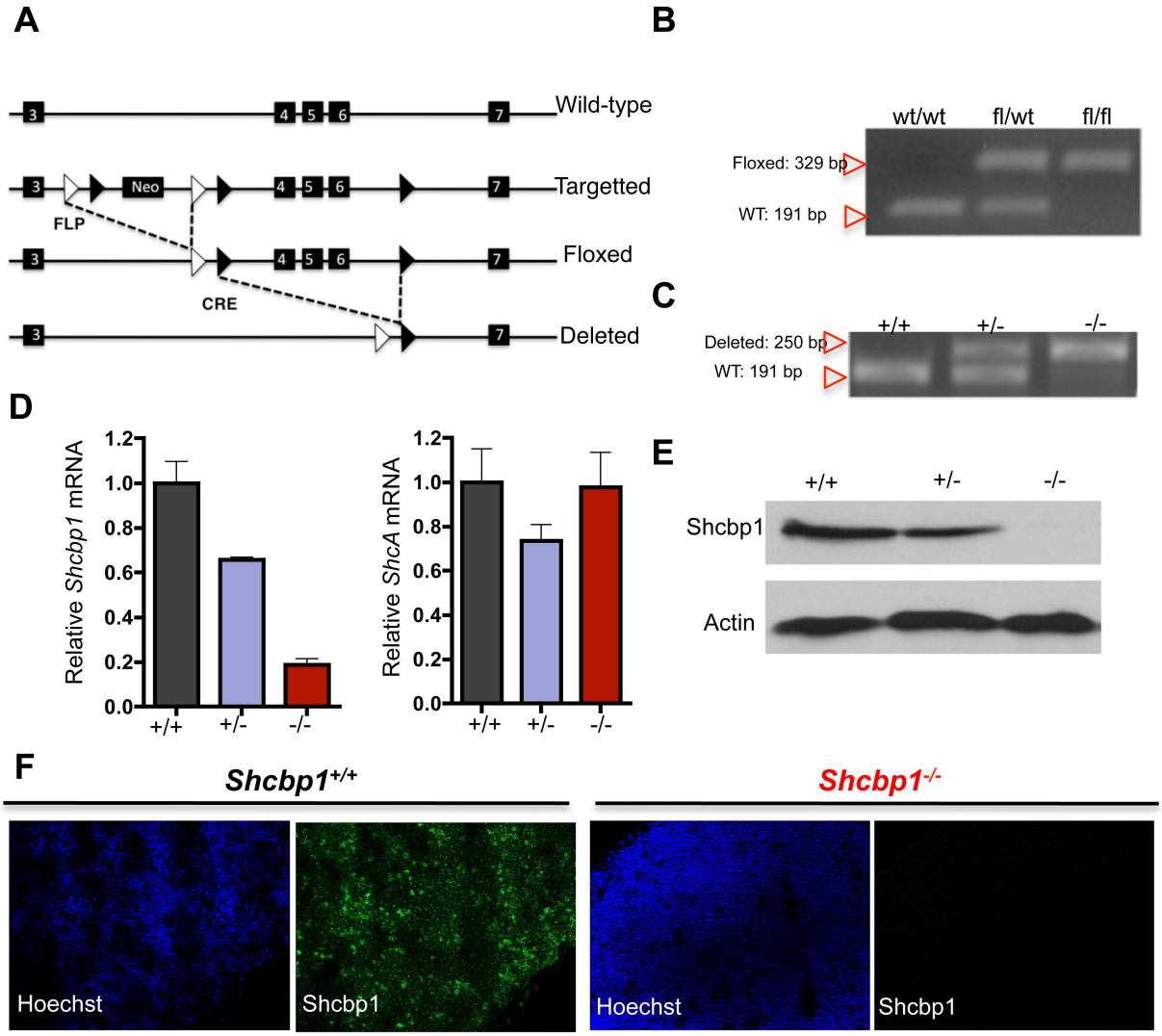

A
Wild-type
Targetted
Floxed
Deleted
Neo
FLP
CRE
B
wt/wt
fl/wt
fl/fl
Floxed: 329 bp
WT: 191 bp
C
+/+
+/-
-/-
Deleted: 250 bp
WT: 191 bp
D
Relative Shcbp1 mRNA
Relative ShcA mRNA
E
Shcbp1
Actin
F
Shcbp1+/+
Shcbp1-/-
Hoechst
Shcbp1
Hoechst
Shcbp1

Figure 3.5: Viability, gross development, and fertility are normal in *Shcbp1* deficient mice

(A) Chart of mice born with the indicated genotypes from a cross of *Shcbp1*$^{+/-}$ to *Shcbp1*$^{+/-}$.

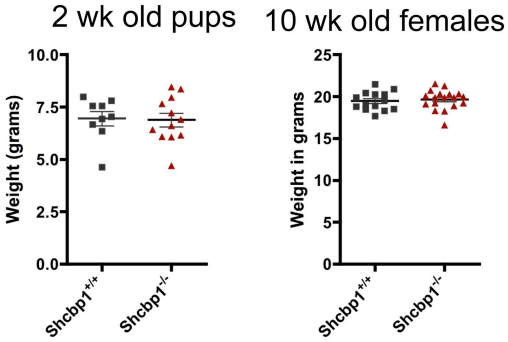
(B) Weight of 2-week old pups and 10-week old female mice of *Shcbp1*$^{+/+}$ and *Shcbp1*$^{-/-}$ mice.

(C) Chart of the percentage fertile male and female *Shcbp1*$^{+/+}$ and *Shcbp1*$^{-/-}$ mice.

A

Genotype	Shcbp1$^{+/+}$	Shcbp1$^{+/-}$	Shcbp1$^{-/-}$
Pups born with genotype (+/- X +/-)	23% (91/396)	51.5% (204/396)	25.5% (101/396)

B



C

Genotype	Shcbp1$^{+/+}$	Shcbp1$^{-/-}$
Males Percent Fertile	83% (5/6)	100% (7/7)
Females Percent Fertile	100% (7/7)	100% (10/10)

Figure 3.6: Generation of *Lck*-Cre^{+}/*Shcbp1*$^{fl/fl}$ mice

(A) PCR for *Shcbp1* WT, *Shcbp1* floxed, and *Shcbp1* deleted loci in thymic DNA from mice with the indicated genotypes.

(B) PCR for *Shcbp1* WT, *Shcbp1* floxed, and *Shcbp1* deleted loci in DNA from sorted T cells and non-T splenocytes.

(C) *Shcbp1* mRNA in mice with indicated genotypes normalized to *HPRT* and to control mice (n>3 mice per genotype).

(D) Immunoblots of Shcbp1 from mice with the indicated genotypes. Immunoblot demonstrates that there is no truncation product in *Lck*-Cre^{+}/*Shcbp1*$^{fl/fl}$ mice. There is a non-specific band at approximately 120kD and a potential Shcbp1 doublet at 150kD.

(E) Immunoblots of Shcbp1 from mice with the indicated genotypes using the indicated Shcbp1 antibodies.

(F) Immunohistochemistry of Shcbp1 in thymic tissue from wild type and *Shcbp1*-deficient mice (representative of n=3 experiments).

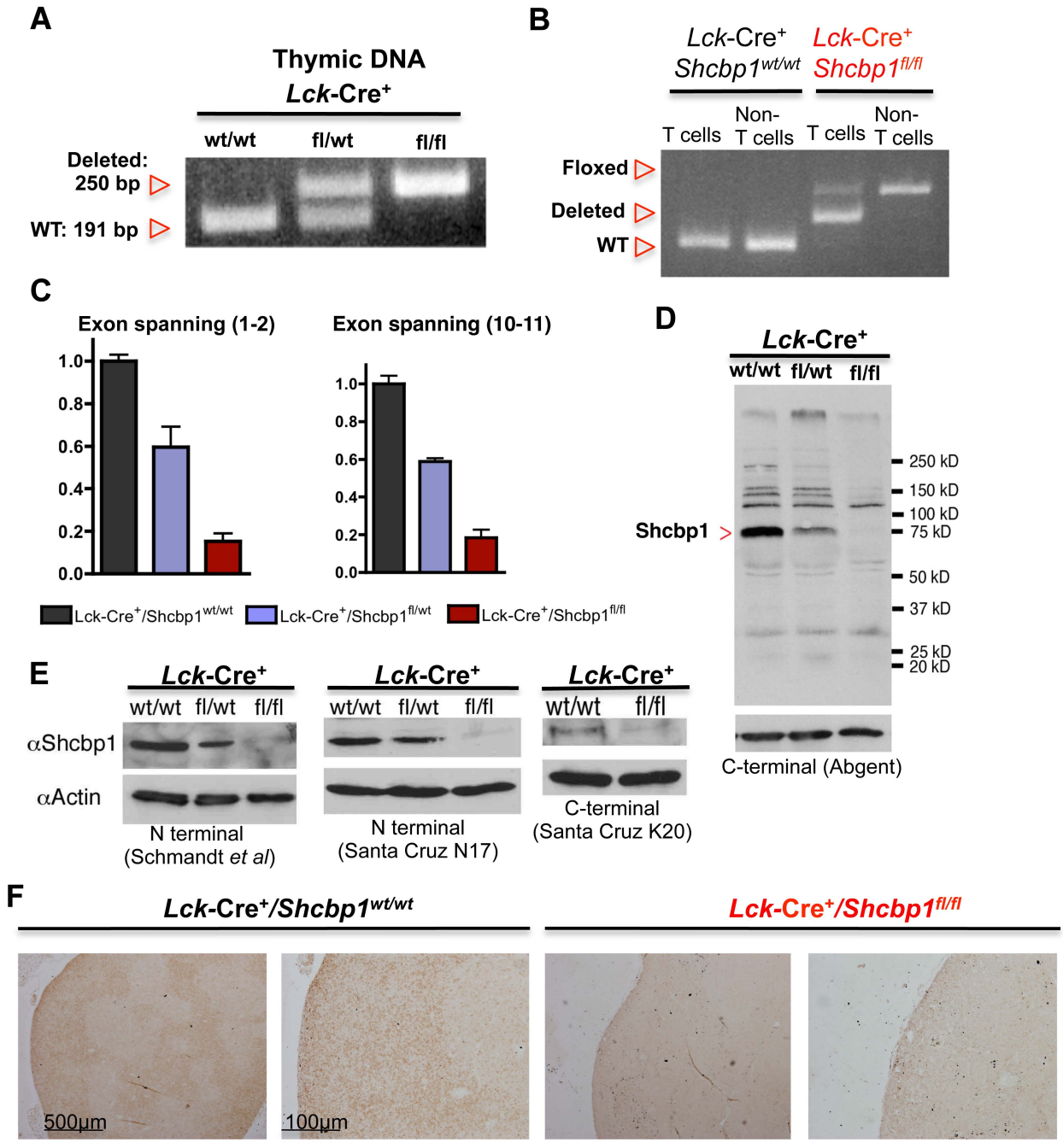

A
Thymic DNA
Lck-Cre+
wt/wt
fl/wt
fl/fl
Deleted: 250 bp
WT: 191 bp
B
Lck-Cre+ Shcbp1wt/wt
Lck-Cre+ Shcbp1fl/fl
T cells
Non-T cells
T cells
Non-T cells
Floxed
Deleted
WT
C
Exon spanning (1-2)
Exon spanning (10-11)
1.0
0.8
0.6
0.4
0.2
0.0
Lck-Cre+/Shcbp1wt/wt
Lck-Cre+/Shcbp1fl/wt
Lck-Cre+/Shcbp1fl/fl
D
Lck-Cre+
wt/wt fl/wt fl/fl
Shcbp1
250 kD
150 kD
100 kD
75 kD
50 kD
37 kD
25 kD
20 kD
C-terminal (Abgent)
E
Lck-Cre+
wt/wt fl/wt fl/fl
αShcbp1
αActin
N terminal (Schmandt et al)
N terminal (Santa Cruz N17)
C-terminal (Santa Cruz K20)
F
Lck-Cre+/Shcbp1wt/wt
Lck-Cre+/Shcbp1fl/fl
500μm
100μm

Figure 3.7: Loss of Shcbp1 does not impair T cell development

(A) Representative picture of the thymus from *Shcbp1*$^{+/+}$ and *Shcbp1*$^{-/-}$ mice.

(B) Flow cytometry of thymi isolated from 4-to-6 week old *Shcbp1*$^{+/+}$ and *Shcbp1*$^{-/-}$ mice. Top panel is the surface marker expression of CD4 and CD8. Bottom panel shows surface marker expression of CD44 and CD25 gated on DN thymocytes (CD4^{-} CD8^{-} B220^{-} Gr1^{-} Ter119^{-} CD11b^{-} CD11c^{-}). Data presented are representative of n=4-6 mice per genotype of age-matched littermate controls.

(C) Total cellularity and absolute number of thymic subsets in 4-to 6-week-old *Shcbp1*$^{+/+}$ and *Shcbp1*$^{-/-}$ mice (n=4-6 mice of each genotype).

(D) Staining for TCRβ on different thymic subsets from *Shcbp1* WT and deficient thymocytes (n=2 mice per genotype).

(E) Staining for TCRβ and TCRγδ on thymocyte isolated from WT and *Shcbp1*-deficient mice (n=2 mice per genotype).

(F) Flow cytometry for CD4 and CD8 of splenocytes isolated from 4-to-6 week old *Shcbp1*$^{+/+}$ and *Shcpb1*$^{-/-}$ mice (n>3 mice per genotype).

(G) Flow cytometry for CD4 and CD8 of lymphocytes isolated from 4-to-6 week old *Shcbp1*$^{+/+}$ and *Shcpb1*$^{-/-}$ mice (n>3 mice per genotype).

(H) Absolute numbers of CD4^{+} and CD8^{+} T cells in the spleen of *Shcbp1*$^{+/+}$ and *Shcbp1*$^{-/-}$ mice (n>3 mice per genotype).

(I) Intracellular staining for *Foxp3* in CD4^{+} T cells from *Shcbp1* WT and deficient mice (n=2 mice per genotype).

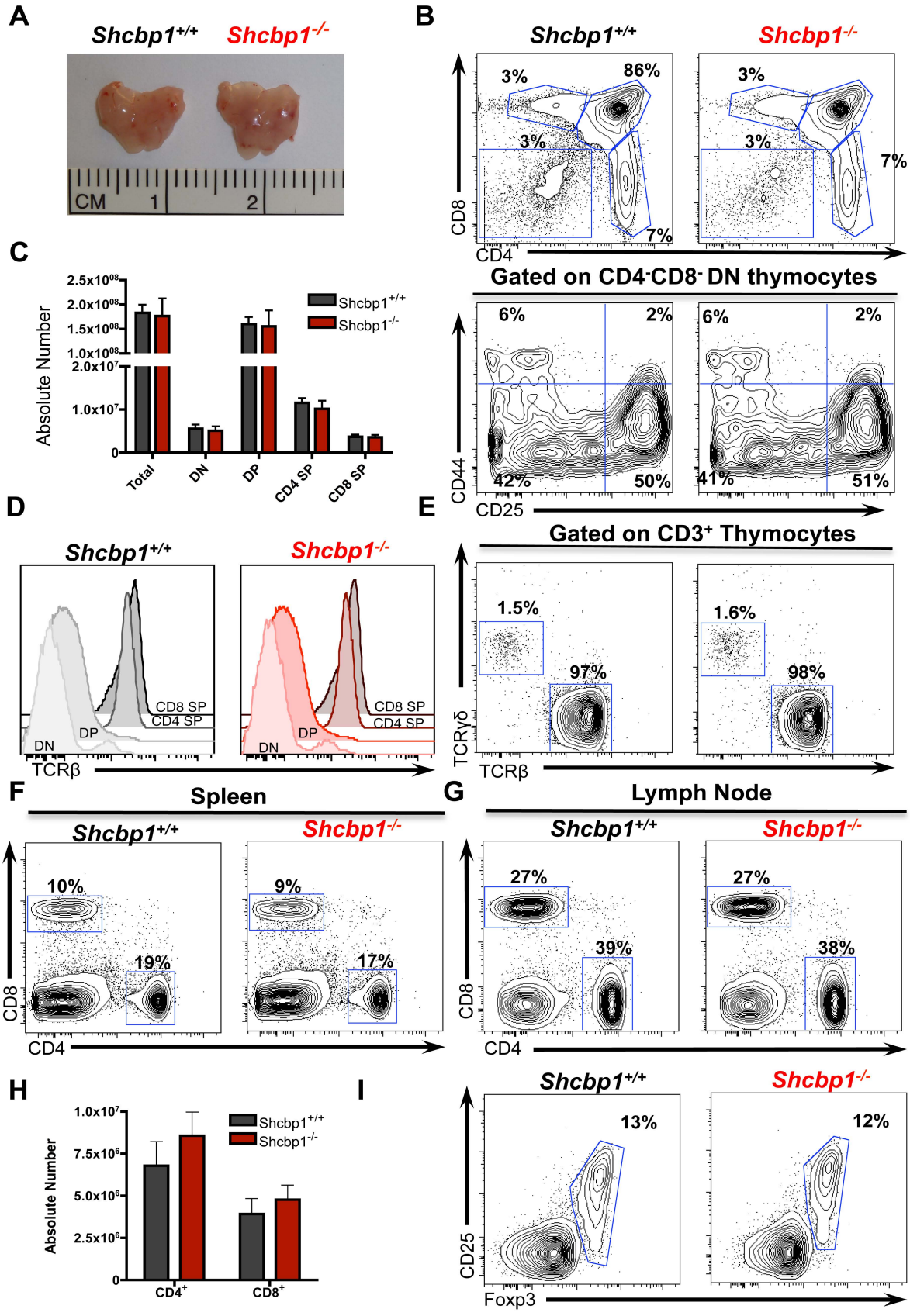

A
Shcbp1+/+
Shcbp1-/-
CM
1
2
B
Shcbp1+/+
Shcbp1-/-
3%
86%
3%
7%
3%
3%
7%
CD8
CD4
Gated on CD4-CD8- DN thymocytes
6%
2%
42%
50%
6%
2%
41%
51%
CD44
CD25
C
Absolute Number
2.5x10^08
2.0x10^08
1.5x10^08
1.0x10^08
2.0x10^07
1.0x10^07
0
Shcbp1+/+
Shcbp1-/-
Total
DN
DP
CD4 SP
CD8 SP
D
Shcbp1+/+
Shcbp1-/-
CD8 SP
CD4 SP
DP
DN
TCRβ
E
Gated on CD3+ Thymocytes
1.5%
97%
1.6%
98%
TCRγδ
TCRβ
F
Spleen
Shcbp1+/+
Shcbp1-/-
10%
19%
9%
17%
CD8
CD4
G
Lymph Node
Shcbp1+/+
Shcbp1-/-
27%
39%
27%
38%
CD8
CD4
H
Absolute Number
1.0x10^7
7.5x10^6
5.0x10^6
2.5x10^6
0
Shcbp1+/+
Shcbp1-/-
CD4+
CD8+
I
Shcbp1+/+
Shcbp1-/-
13%
12%
CD25
Foxp3

Figure 3.8: Thymic development is normal in *Lck*-Cre^{+}/*Shcbp1*$^{fl/fl}$ mice

(A) Representative picture of the thymus from *Lck*-Cre^{+}/*Shcbp1*$^{wt/wt}$ and *Lck*-Cre^{+}/*Shcbp1*$^{fl/fl}$ mice.

(B) H&E staining of paraffin imbedded thymic sections from *Lck*-Cre^{+}/*Shcbp1*$^{wt/wt}$ and *Lck*-Cre^{+}/*Shcbp1*$^{fl/fl}$ mice (representative of n=2 mice per genotype).

(C) Flow cytometry of thymi isolated from *Lck*-Cre^{+}/*Shcbp1*$^{wt/wt}$ and *Lck*-Cre^{+}/*Shcbp1*$^{fl/fl}$ with analysis of cell surface markers CD4 and CD8 (representative of n=11 mice per genotype of age-matched littermate controls).

(D) Total cellularity and absolute number of thymic subsets in 4-to 6-week-old *Lck*-Cre^{+}/*Shcbp1*$^{wt/wt}$ and *Lck*-Cre^{+}/*Shcbp1*$^{fl/fl}$ mice (n=11 mice of each genotype with age-matched littermate controls).

(E) Flow cytometry for cell surface markers CD4 and CD8 in spleens isolated from 4-to-6-week old *Lck*-Cre^{+}/*Shcbp1*$^{wt/wt}$ and *Lck*-Cre^{+}/*Shcbp1*$^{fl/fl}$ mice (n=11 mice of each genotype with age-matched littermate controls).

(F) Absolute numbers of splenic CD4^{+} and CD8^{+} T cells as well as total splenocytes from *Lck*-Cre^{+}/*Shcbp1*$^{wt/wt}$ and *Lck*-Cre^{+}/*Shcbp1*$^{fl/fl}$ mice (n=11 mice of each genotype).

(G) mRNA levels of indicated genes in WT and thymocytes lacking Shcbp1 (n=5, 6 mice)

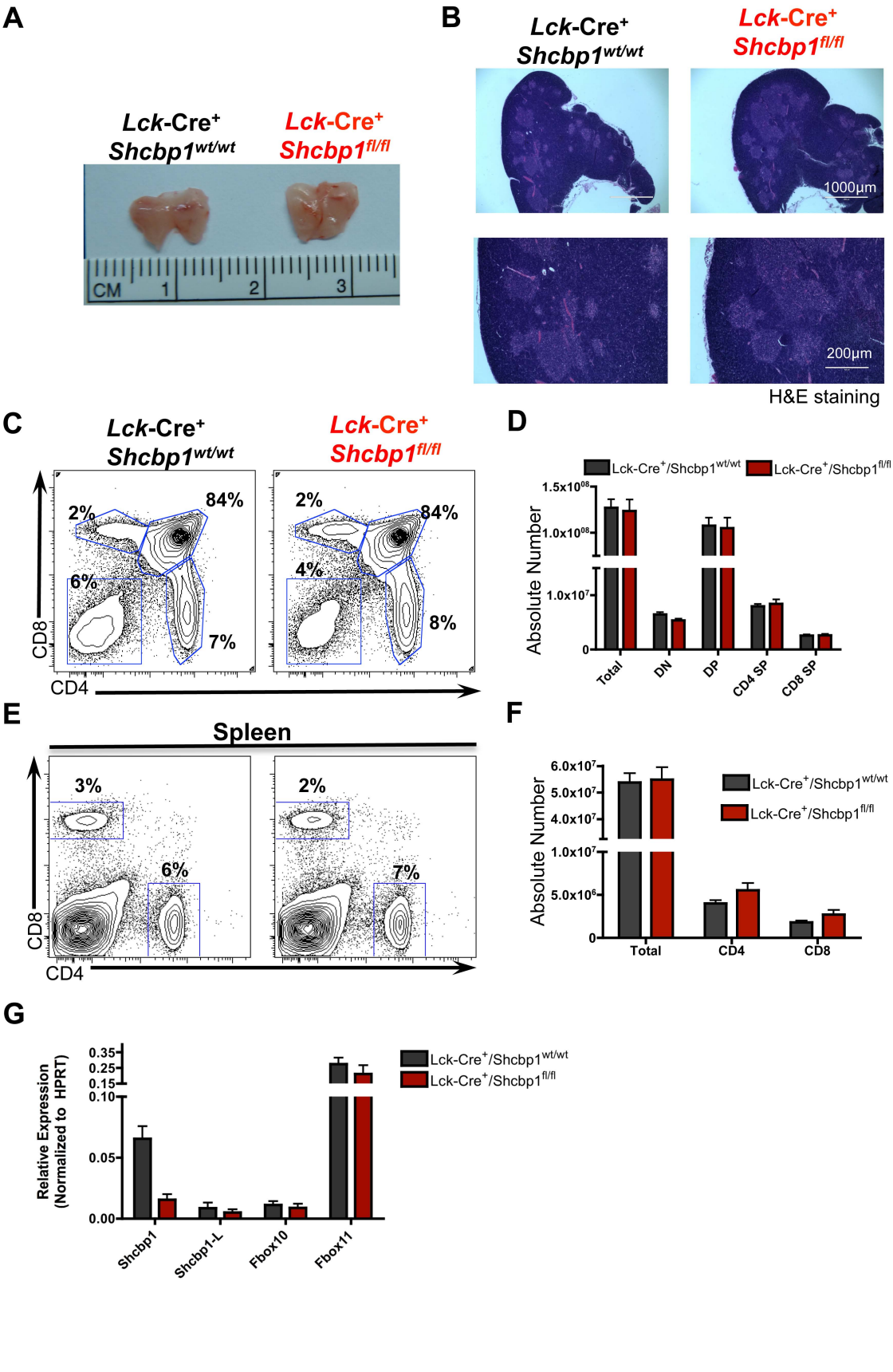
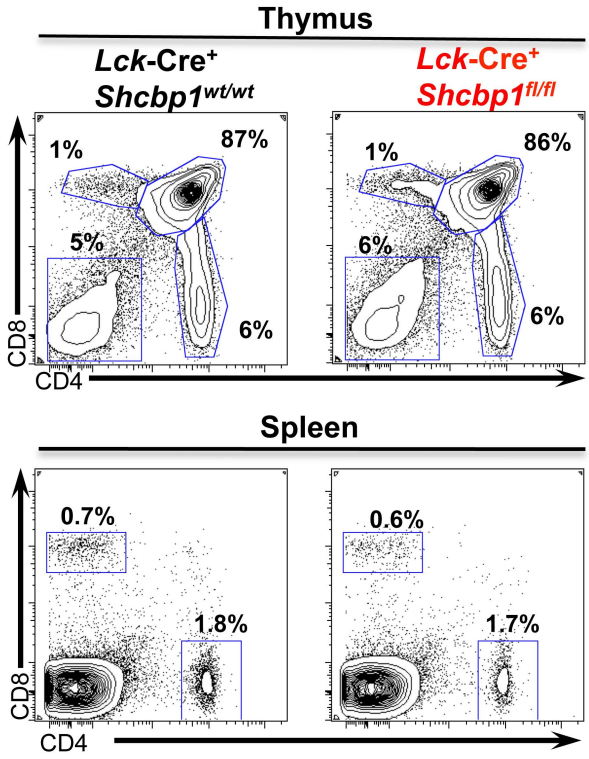

A
Lck-Cre+ Shcbp1wt/wt
Lck-Cre+ Shcbp1fl/fl
CM 1 2 3
B
Lck-Cre+ Shcbp1wt/wt
Lck-Cre+ Shcbp1fl/fl
1000μm
200μm
H&E staining
C
Lck-Cre+ Shcbp1wt/wt
Lck-Cre+ Shcbp1fl/fl
2%
84%
6%
7%
2%
84%
4%
8%
CD8
CD4
D
Lck-Cre+/Shcbp1wt/wt
Lck-Cre+/Shcbp1fl/fl
Absolute Number
1.5x10^08
1.0x10^08
1.0x10^7
0
Total
DN
DP
CD4 SP
CD8 SP
E
Spleen
3%
6%
2%
7%
CD8
CD4
F
Absolute Number
6.0x10^7
5.0x10^7
4.0x10^7
1.0x10^7
5.0x10^6
0
Lck-Cre+/Shcbp1wt/wt
Lck-Cre+/Shcbp1fl/fl
Total
CD4
CD8
G
Relative Expression (Normalized to HPRT)
0.35
0.25
0.15
0.10
0.05
0.00
Lck-Cre+/Shcbp1wt/wt
Lck-Cre+/Shcbp1fl/fl
Shcbp1
Shcbp1-L
Fbox10
Fbox11

Figure 3.9: Thymic development in 2-week old *Lck*-Cre^{+}/*Shcbp1*$^{fl/fl}$ mice

(A) Flow cytometry for CD4 and CD8 of thymi (top) and spleen (bottom) isolated from 2 week old *Lck*-Cre^{+}/*Shcbp1*$^{wt/wt}$ and *Lck*-Cre^{+}/*Shcbp1*$^{fl/fl}$ mice (n=3-5 mice per genotype).

(B) Absolute numbers of thymic subsets from two-week old *Lck*-Cre^{+}/*Shcbp1*$^{wt/wt}$ and *Lck*-Cre^{+}/*Shcbp1*$^{fl/fl}$ mice (n=3-5 mice of each genotype).

A


Thymus
Lck-Cre+ Shcbp1wt/wt
Lck-Cre+ Shcbp1fl/fl
1%
87%
5%
6%
1%
86%
6%
6%
CD8
CD4
Spleen
0.7%
1.8%
0.6%
1.7%
CD8
CD4

B

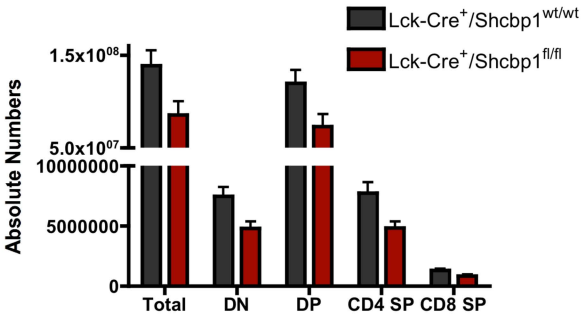

Lck-Cre+/Shcbp1wt/wt
Lck-Cre+/Shcbp1fl/fl
Absolute Numbers
1.5x10^08
5.0x10^07
10000000
5000000
0
Total
DN
DP
CD4 SP
CD8 SP

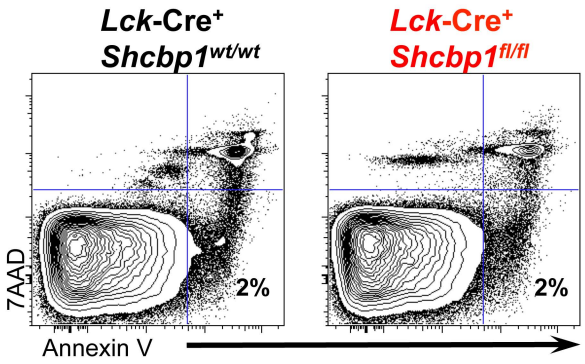
Figure 3.10: No apparent defect in survival in *Lck*-Cre^{+}/Shcbp1$^{fl/fl}$ mice

(A) Annexin V and 7AAD staining of freshly isolated thymocytes (n=2 mice per genotype)

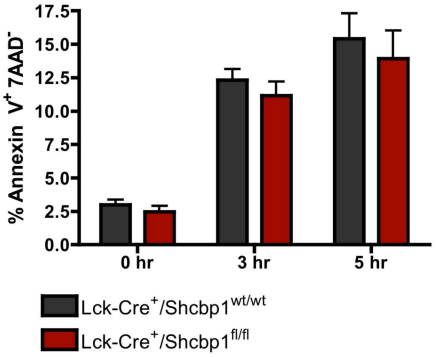
(B) Quantification of flow cytometric analysis of Annexin V and 7AAD in thymocytes freshly isolated or incubated at 37^{o} for the indicated time (n>3 mice per genotype).

(C) Left, schematic of the *in vivo* model of thymic survival and apoptosis. Right, percentage of Annexin V^{+} 7AAD^{-} thymocytes after injection with either PBS or 250μg dexamethasone (n=4 mice of each genotype).

A


Lck-Cre+
Shcbp1wt/wt
Lck-Cre+
Shcbp1fl/fl
7AAD
2%
2%
Annexin V

B


% Annexin V+ 7AAD-
17.5
15.0
12.5
10.0
7.5
5.0
2.5
0.0
0 hr
3 hr
5 hr
Lck-Cre+/Shcbp1wt/wt
Lck-Cre+/Shcbp1fl/fl

C

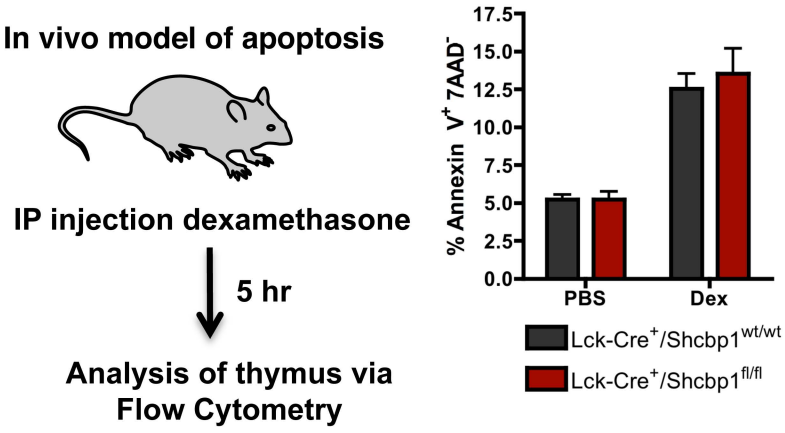

In vivo model of apoptosis
IP injection dexamethasone
5 hr
Analysis of thymus via
Flow Cytometry
% Annexin V+ 7AAD-
17.5
15.0
12.5
10.0
7.5
5.0
2.5
0.0
PBS
Dex
Lck-Cre+/Shcbp1wt/wt
Lck-Cre+/Shcbp1fl/fl

Figure 3.11: Generation and analysis of T cell development in the *Rag*-Cre^{+}/*Shcbp1*$^{fl/fl}$ mice.

(A) *Shcbp1* mRNA levels in thymocytes from *Rag*-Cre^{+}/*Shcbp1*$^{wt/wt}$ and *Rag*-Cre^{+}/*Shcbp1*$^{fl/fl}$ mice normalized to *HPRT* and to control (n>3 mice per genotype)

(B) Immunoblotting of Shcbp1 in total thymocytes (n=2 experiments).

(C) Flow cytometric analysis of thymi isolated from 4-to-6 week old *Rag*-Cre^{+}/*Shcbp1*$^{wt/wt}$ and *Rag*-Cre^{+}/*Shcbp1*$^{fl/fl}$ mice. Top panel shows surface marker expression of CD4 and CD8. Bottom panel depicts surface marker expression of CD44 and CD25 gated on DN thymocytes (CD4^{-} CD8^{-} B220^{-} Gr1^{-} Ter119^{-} CD11b^{-} CD11c). Left, total cellularity and absolute number of thymic subsets in 4-to 6-week-old *Rag*-Cre^{+}/*Shcbp1*$^{wt/wt}$ and *Rag*-Cre^{+}/*Shcbp1*$^{fl/fl}$ mice (n=4-6 mice of each genotype with age-matched littermate controls).

(D) Flow cytometric analysis for cell surface markers CD4 and CD8 in spleens isolated from 4-6 week old *Rag*-Cre^{+}/*Shcbp1*$^{wt/wt}$ and *Rag*-Cre^{+}/*Shcbp1*$^{fl/fl}$ mice (representative of n=3-6 mice of each genotype, littermate controls).

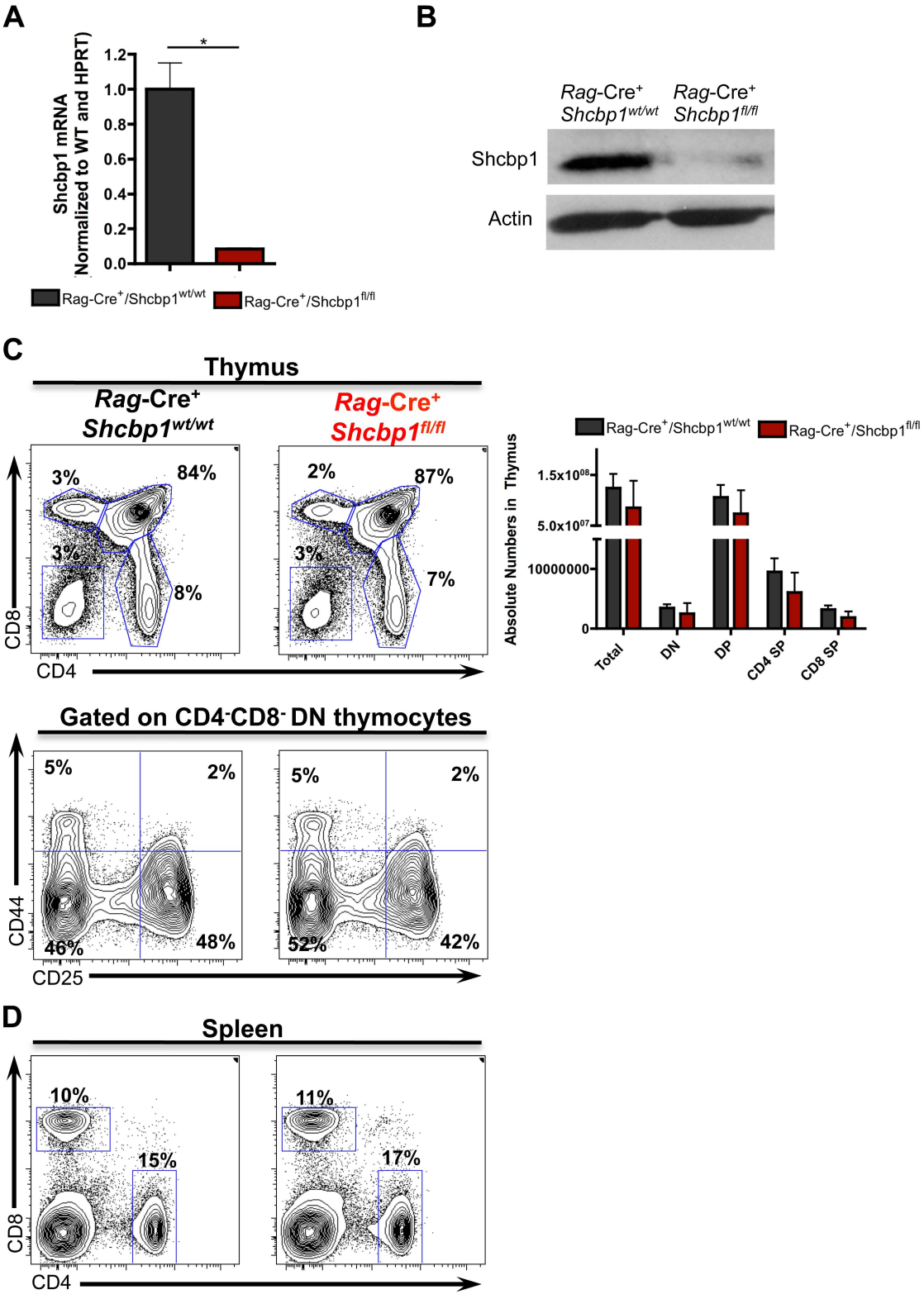

A
Shcbp1 mRNA Normalized to WT and HPRT)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
Rag-Cre+/Shcbp1wt/wt
Rag-Cre+/Shcbp1fl/fl
B
Rag-Cre+ Shcbp1wt/wt
Rag-Cre+ Shcbp1fl/fl
Shcbp1
Actin
C
Thymus
Rag-Cre+ Shcbp1wt/wt
Rag-Cre+ Shcbp1fl/fl
3%
84%
3%
8%
2%
87%
3%
7%
CD8
CD4
Rag-Cre+/Shcbp1wt/wt
Rag-Cre+/Shcbp1fl/fl
Absolute Numbers in Thymus
1.5x10^08
5.0x10^07
10000000
0
Total
DN
DP
CD4 SP
CD8 SP
Gated on CD4-CD8- DN thymocytes
5%
2%
46%
48%
5%
2%
52%
42%
CD44
CD25
D
Spleen
10%
15%
11%
17%
CD8
CD4

Figure 3.12: Shcbp1 is upregulated by stimulation in peripheral CD4 T cells

(A) RT-PCR for *Shcbp1* mRNA expression in *ex vivo* anti-CD3/anti-CD28 stimulated $CD4^+$ T cells for the indicated times (normalized to *HPRT* and unstimulated $CD4^+$ T cells, n=4 for $Shcbp1^{+/+}$ and n=2 for $Shcbp1^{-/-}$).

(B) Immunoblotting of Shcbp1 from total splenocytes ($Shcbp1^{+/+}$ or $Shcbp1^{-/-}$) stimulated with plate-bound anti-CD3/anti-CD28 for 72 hours (representative of n=3 experiments).

(C) Top, *Shcbp1* mRNA expression in $CD4^+$ T cells isolated from wild-type mice injected with either PBS or 100μg of anit-CD3 for 24 hours (n=6 mice of each treatment, p=0.0012). Bottom, representative immunoblot for Shcbp1.

(D) *Shcbp1* mRNA in CD4 T cells stimulated with PMA/Ionomycin for the indicated times (normalized to *HPRT* and unstimulated $CD4^+$ T cells) (n=3, * $p<0.02$).

(E) Left, RT-PCR for *Shcbp1* in $CD4^+$ T cells isolated from DO11.10 mice stimulated with $OVA_{(323-339)}$ for the indicated times (normalized to *HPRT* and unstimulated $CD4^+$ T cells, n=3 experiments, *$p<0.03$). Right, representative immunoblot for Shcbp1 (n=2).

(F) Immunoprecipitation of Shcbp1 and immunoblotting of ShcA and Shcbp1 in primary murine splenocytes activated with plate bound anti-CD3/anti-CD28 for 72 hours (representative of n=2 experiments).

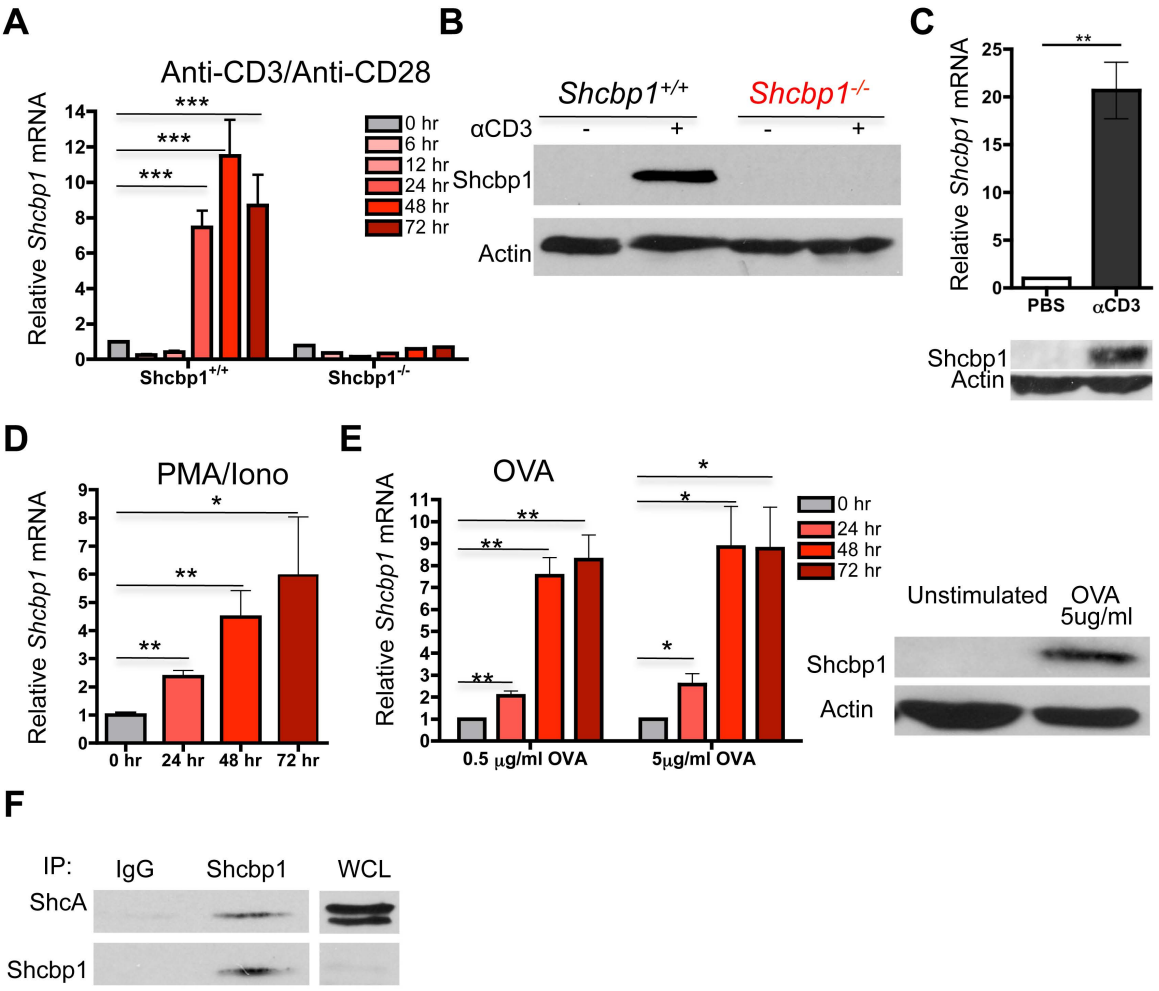
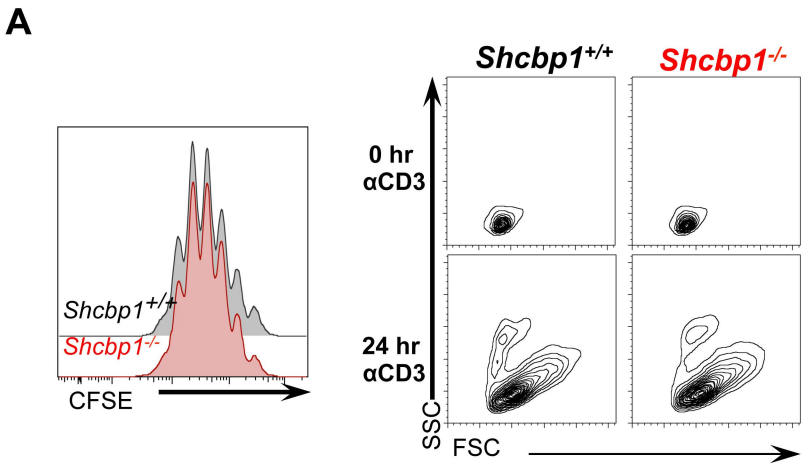

A
Anti-CD3/Anti-CD28
Relative Shcbp1 mRNA
0 hr
6 hr
12 hr
24 hr
48 hr
72 hr
Shcbp1+/+
Shcbp1-/-
B
Shcbp1+/+
Shcbp1-/-
αCD3
Shcbp1
Actin
C
Relative Shcbp1 mRNA
PBS
αCD3
Shcbp1
Actin
D
PMA/Iono
Relative Shcbp1 mRNA
0 hr
24 hr
48 hr
72 hr
E
OVA
Relative Shcbp1 mRNA
0 hr
24 hr
48 hr
72 hr
0.5 μg/ml OVA
5μg/ml OVA
Unstimulated
OVA 5ug/ml
Shcbp1
Actin
F
IP:
IgG
Shcbp1
WCL
ShcA
Shcbp1

Figure 3.13: Proliferation and activation of *Shcbp1* deficient T cells is normal

(A) Flow cytometry of $CD4^+$ T cells isolated from *Shcbp1*$^{+/+}$ and *Shcbp1*$^{-/-}$ mice, stained with CFSE, and activated with anti-CD3/anti-CD28 for 72 hours (representative of n=3). Left, representative plot of forward scatter and side-scatter in naïve or stimulated $CD4^+$ T cells.

(B) Flow cytometry for cell surface markers (CD44, CD62L, CD25, and CD69, CD4) of $CD4^+$ T cells isolated from *Shcbp1*$^{+/+}$ and *Shcbp1*$^{-/-}$ mice after 24-hour stimulation with anti-CD3/ anti-CD28 (n=3 mice of each genotype).


A
Shcbp1+/+
Shcbp1-/-
0 hr αCD3
24 hr αCD3
SSC
FSC
Shcbp1+/+
Shcbp1-/-
CFSE

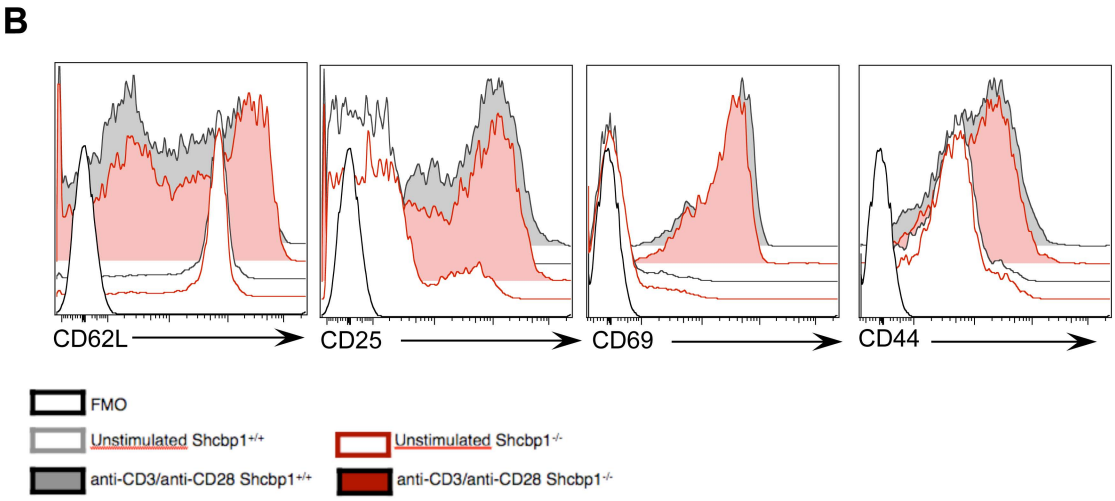

B
CD62L
CD25
CD69
CD44
FMO
Unstimulated Shcbp1+/+
Unstimulated Shcbp1-/-
anti-CD3/anti-CD28 Shcbp1+/+
anti-CD3/anti-CD28 Shcbp1-/-

Figure 3.14: Shcbp1 is upregulated in the spinal cords of mice immunized for EAE

(A) *Shcbp1* mRNA expression in mononuclear cells isolated from the brain and spinal cords of healthy controls or from mice immunized with $MOG_{(35-55)}$ on day 28 after disease induction (Normalized to *HPRT* and healthy controls) (n=3 mice of each condition, pooled).

(B) Shcbp1 expression assessed by immunohistochemistry in spinal cords isolated from healthy controls or *Shcbp1*$^{+/+}$ and *Shcbp1*$^{-/-}$ mice on day 28 after immunization for EAE.

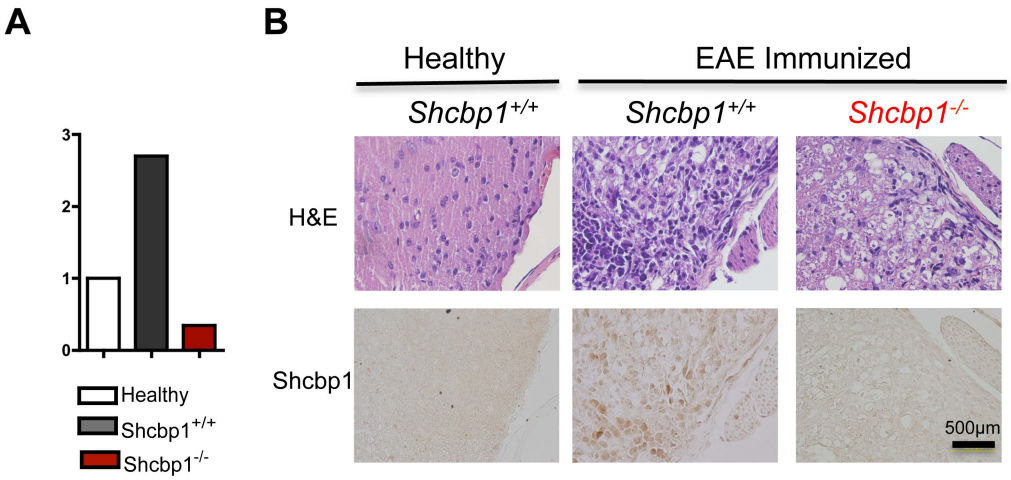

A
3
2
1
0
Healthy
Shcbp1+/+
Shcbp1-/-
B
Healthy
EAE Immunized
Shcbp1+/+
Shcbp1+/+
Shcbp1-/-
H&E
Shcbp1
500μm

Figure 3.15: Loss of Shcbp1 affects disease severity in $CD4^+$ T cell driven autoimmune disease

(A) Left, mean clinical scores of EAE in male *Shcbp1*$^{+/+}$ and *Shcbp1*$^{-/-}$ mice. Middle, percent survival of male *Shcbp1*$^{+/+}$ and *Shcbp1*$^{-/-}$ mice. Right, disease index (area under the curve) plotted for each mouse individually. Compiled data with n= 18-19 mice of *Shcbp1*$^{+/+}$ and *Shcbp1*$^{-/-}$ genotype, respectively.

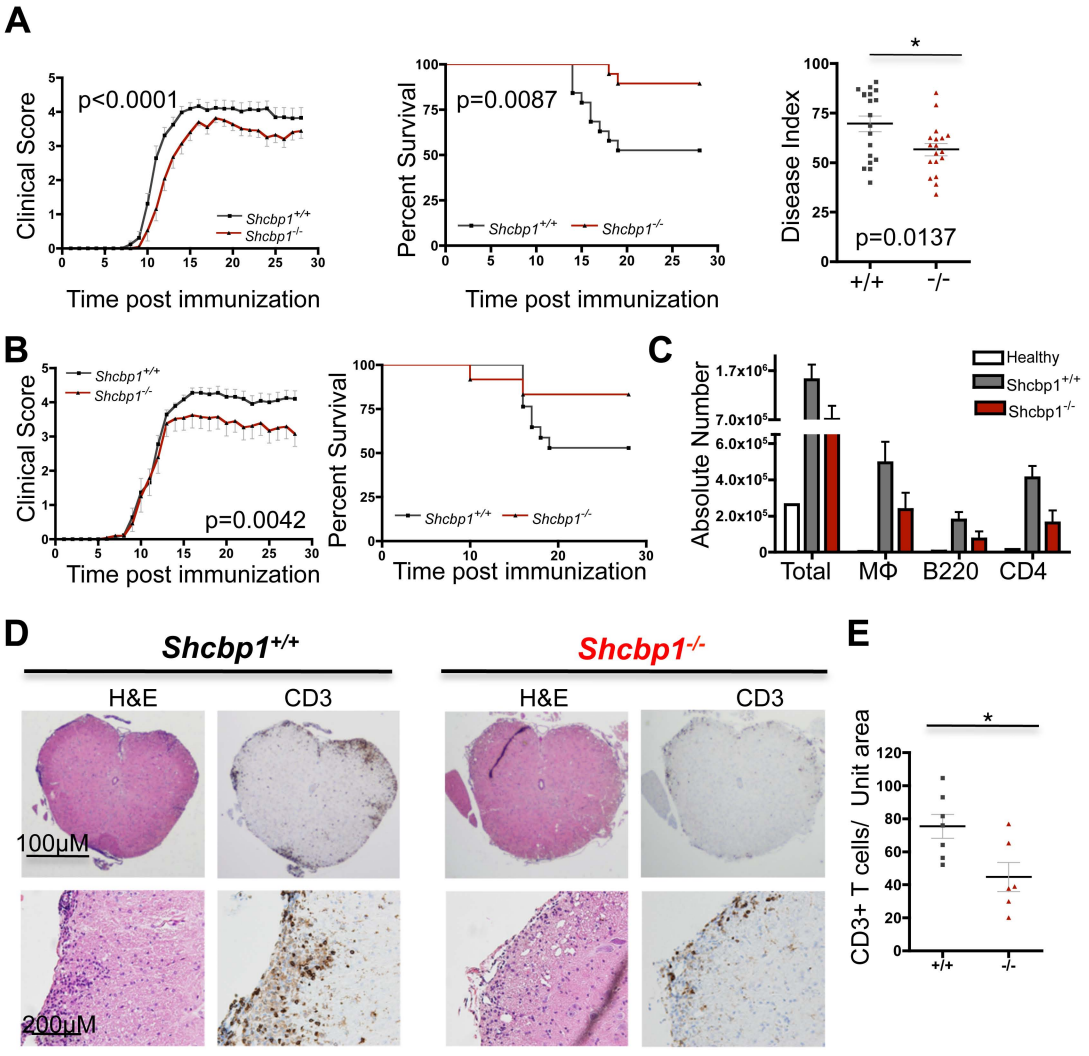
(B) Left, mean clinical scores of EAE in female *Shcbp1*$^{+/+}$ and *Shcbp1*$^{-/-}$ mice. Right, percent survival of female *Shcbp1*$^{+/+}$ and *Shcbp1*$^{-/-}$ mice (n= 17, 12 *Shcbp1*$^{+/+}$ and *Shcbp1*$^{-/-}$ mice respectively).

(C) Left, absolute numbers of total cells, $CD4^+$ T cells, B cells, and macrophages isolated from the spinal cords and brain of *Shcbp1*$^{+/+}$ and *Shcbp1*$^{-/-}$ mice on day 28 after immunization (n=3 mice of each genotype).

(D) Immunohistochemistry for CD3 and H&E staining on sacral spinal cord sections from *Shcbp1*$^{+/+}$ and *Shcbp1*$^{-/-}$ mice on day 28 after immunization with EAE (representative of n=7 *Shcbp1*$^{+/+}$ and n=6 *Shcbp1*$^{-/-}$ mice, respectively).

(E) Quantification of number of $CD3^+$ cells from the sacral spinal cord sections (n=7 and n=6 for *Shcbp1*$^{+/+}$ and *Shcbp1*$^{-/-}$ mice, respectively, p=0.02).

(F) Table of the incidence, day of onset, maximum score, disease index, and mortality for wild-type and *Shcbp1* deficient mice.



Genotype	Incidence	Day of Onset	Mean Max	Disease Index	Mortality
***Shcbp1*$^{+/+}$ (Males)**	100% (19/19)	10.4 +/- 1.2	4.5+/- 0.56	69.6 +/- 17.2	47.4% (9/19)
***Shcbp1*$^{-/-}$ (Males)**	100% (18/18)	11.8 +/- 1.5**	4.1 +/- 0.45*	56.6 +/- 13.0*	11.1%** (2/18)
***Shcbp1*$^{+/+}$ (Females)**	100% (17/17)	10.5 +/- 1.2	4.5+/- 0.56	70.0 +/- 13.9	47% (8/17)
***Shcbp1*$^{-/-}$ (Females)**	100% (12/12)	11.5 +/- 3.3	3.9 +/- 1.0	59.0 +/- 21.9	16.6% (2/12)

Figure 3.16: Shcbp1 expression specifically in T cells contributes to EAE disease severity.

(A) Survival curves of *Rag1*$^{-/-}$ mice after EAE induction one-week post transfer with CD4^{+} T cells isolated from either *Shcbp1*$^{-/-}$ or *Shcbp1*$^{+/+}$ mice (n=7, 8).

(B) Clinical scores of *Rag1*$^{-/-}$ mice after EAE induction one-week post transfer with CD4^{+} T cells isolated from either *Shcbp1*$^{-/-}$ or *Shcbp1*$^{+/+}$ mice (n=7, 8).

(C) Clinical scores of *Lck*-Cre^{+}/*Shcbp1*$^{wt/wt}$ and *Lck*-Cre^{+}/*Shcbp1*$^{fl/fl}$ mice after EAE induction (n=4,8).

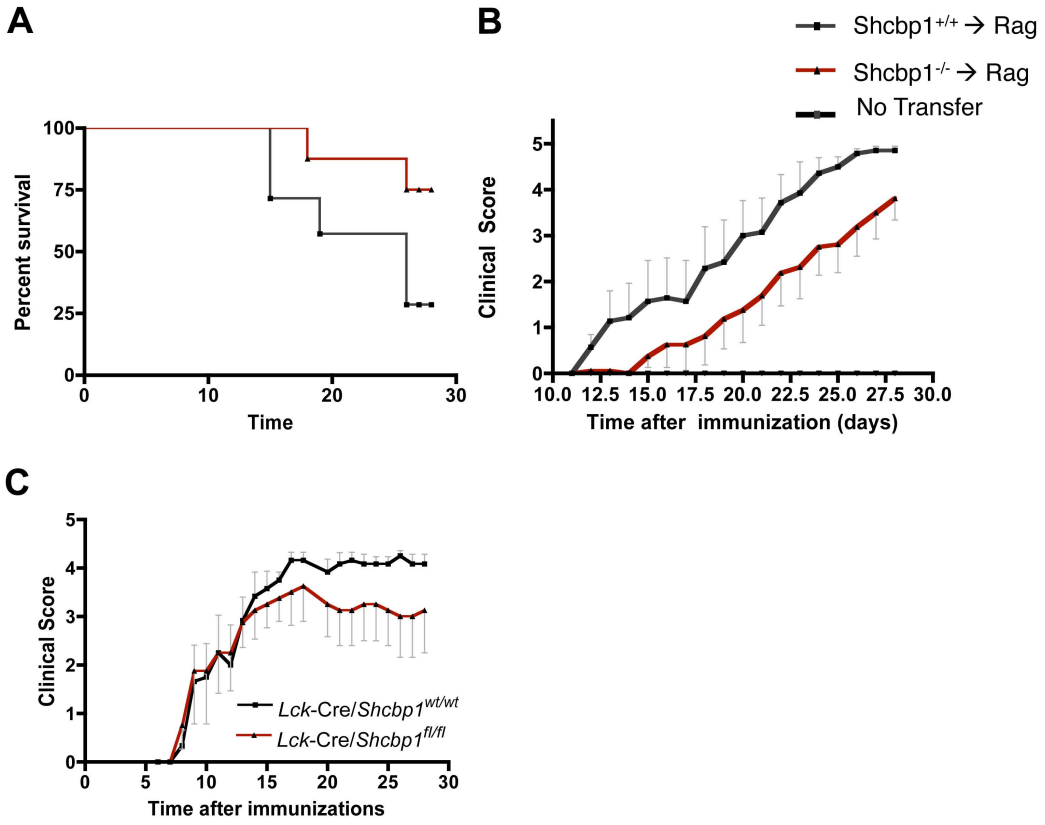

A
Percent survival
100
75
50
25
0
0
10
20
30
Time
B
Shcbp1+/+ → Rag
Shcbp1-/- → Rag
No Transfer
Clinical Score
5
4
3
2
1
0
10.0 12.5 15.0 17.5 20.0 22.5 25.0 27.5 30.0
Time after immunization (days)
C
Clinical Score
5
4
3
2
1
0
0
5
10
15
20
25
30
Time after immunizations
Lck-Cre/Shcbp1wt/wt
Lck-Cre/Shcbp1fl/fl

Figure 3.17: Shcpb1 is induced in CD4 T cells under conditions present during EAE/MS

(A) Relative expression of *ShcA, Shcbp1*, and *IL2* in *Shcbp1*$^{+/+}$ T cells (left) or *Shcbp1*$^{-/-}$ T cells (right) after activation with anti-CD3/anti-CD28. Normalized to *HPRT* and unstimulated cells (n=2-3 experiments)

(B) Upregulation of *Shcbp1* in CD4^{+} T cells stimulated with IL2 (normalized to *HPRT* and unstimulated cells) (n=3, p=0.01).

(C) Left, RT-PCR and right, immunoblotting for Shcbp1 in naïve and T_H17 skewed cells from *Shcbp1*$^{+/+}$ and *Shcbp1*$^{-/-}$ mice (representative of n=2 experiments).

(D) RT-PCR for *Shcbp1* in naïve or *ex vivo* T_H1 skewed CD4^{+} T cells (normalized to *HPRT* and unstimulated CD4^{+} T cells) (n=2 mice of each genotype).

(E) Intracellular staining for IL17-A from *Shcbp1*$^{+/+}$ *and Shcbp1*$^{-/-}$ mice after T_H17 skewing (representative of n=4 mice of each genotype).

(F) Intracellular staining for IFNγ in CD4^{+} T cells from *Shcbp1*$^{+/+}$ *and Shcbp1*$^{-/-}$ mice after T_H1 skewing (representative of n= 2 mice of each genotype).

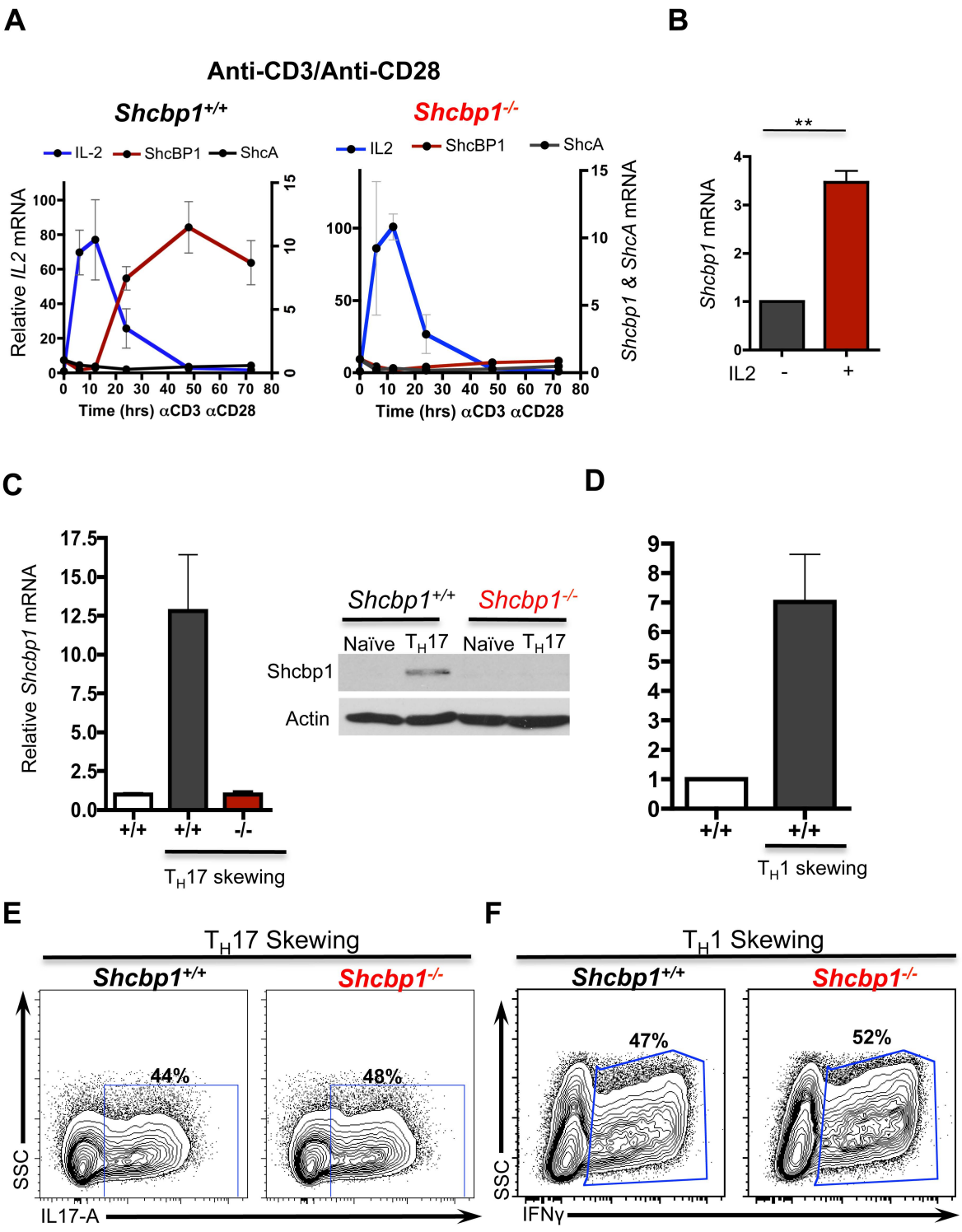

A
Anti-CD3/Anti-CD28
Shcbp1+/+
Shcbp1-/-
IL-2
ShcBP1
ShcA
IL2
Relative IL2 mRNA
Shcbp1 & ShcA mRNA
Time (hrs) αCD3 αCD28
B
**
Shcbp1 mRNA
IL2
C
Relative Shcbp1 mRNA
Shcbp1
Actin
Naïve
TH17
TH17 skewing
D
TH1 skewing
E
TH17 Skewing
44%
48%
SSC
IL17-A
F
TH1 Skewing
47%
52%
IFNγ

Figure 3.18: Shcbp1 is upregulated in activated $CD8^+$ T cells

(A) RT-PCR for *Shcbp1* mRNA expression in *ex vivo* anti-CD3/anti-CD28 stimulated $CD8^+$ T cells for the indicated times (normalized to *HPRT* and unstimulated $CD4^+$ T cells).

(B) Left, *Shcbp1* mRNA expression in $CD8^+$ T cells isolated from wild-type mice injected with either PBS or 100μg of anit-CD3 for 24 hours (n= mice of each treatment, $p<0.0001$). Right, representative immunoblot for Shcbp1.

(C) *Shcbp1* mRNA expression analyzed by microarray in electronically sorted OT-I CD8 T cells. OT-I T cells were transferred to wild-type mice, and then mice were infected for indicated times with either Listeria-OVA or VSV-OVA (data curated from the Immgen Database).

(D) *Shcbp1* mRNA expression in $CD4^+$ or $CD8^+$ T cells isolated from the mediastinal lymph nodes of mice on day 5 after influenza infection (normalized to HPRT and $CD4^+$ or $CD8^+$ T cells isolated from the inguinal lymph node of the same animal, n=5 mice).

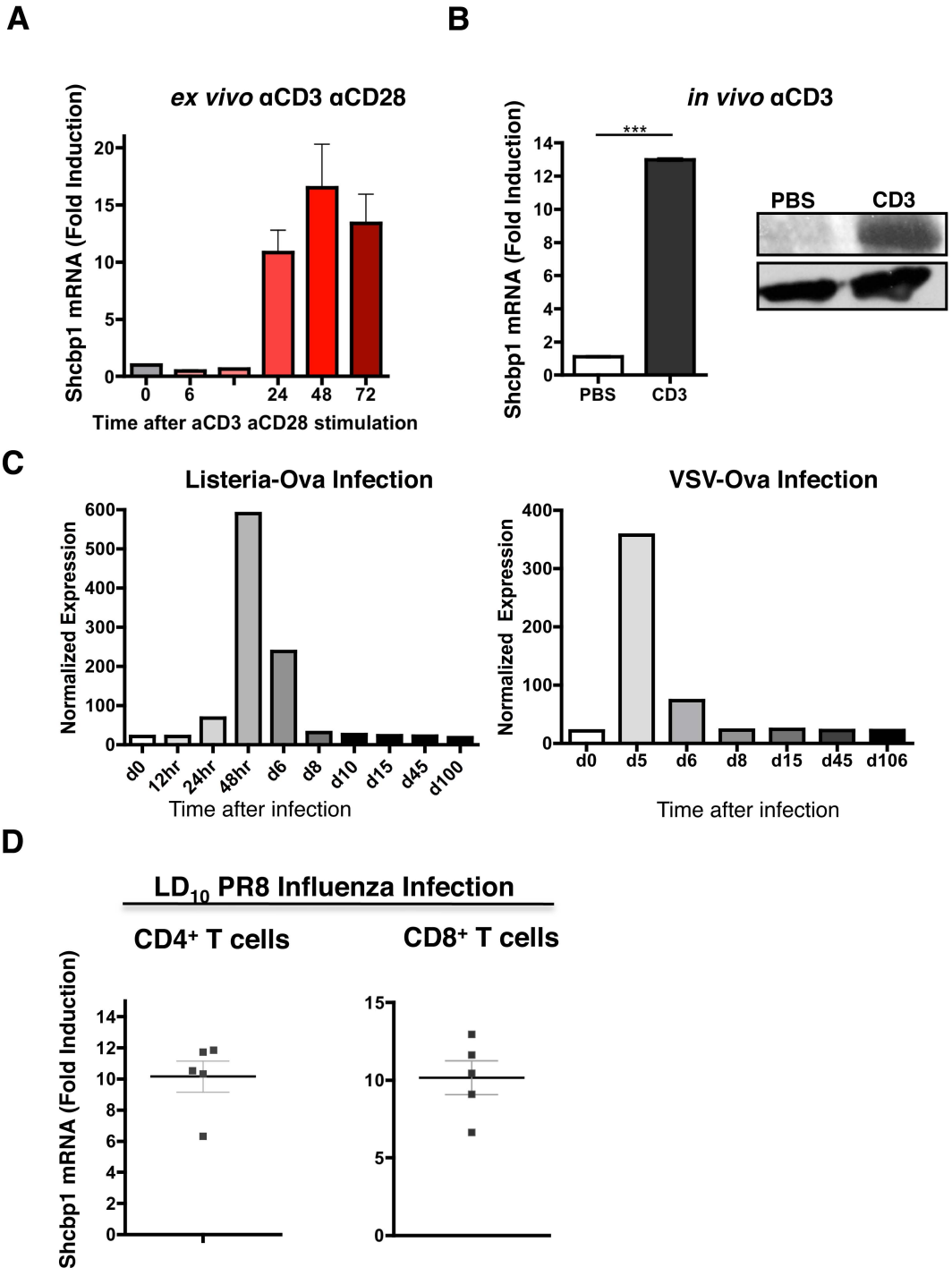

A
ex vivo αCD3 αCD28
Shcbp1 mRNA (Fold Induction)
0
5
10
15
20
0
6
24
48
72
Time after aCD3 aCD28 stimulation
B
in vivo αCD3
***
Shcbp1 mRNA (Fold Induction)
0
2
4
6
8
10
12
14
PBS
CD3
PBS
CD3
C
Listeria-Ova Infection
Normalized Expression
0
100
200
300
400
500
600
d0
12hr
24hr
48hr
d6
d8
d10
d15
d45
d100
Time after infection
VSV-Ova Infection
Normalized Expression
0
100
200
300
400
d0
d5
d6
d8
d15
d45
d106
Time after infection
D
LD10 PR8 Influenza Infection
CD4+ T cells
CD8+ T cells
Shcbp1 mRNA (Fold Induction)
0
2
4
6
8
10
12
14
0
5
10
15

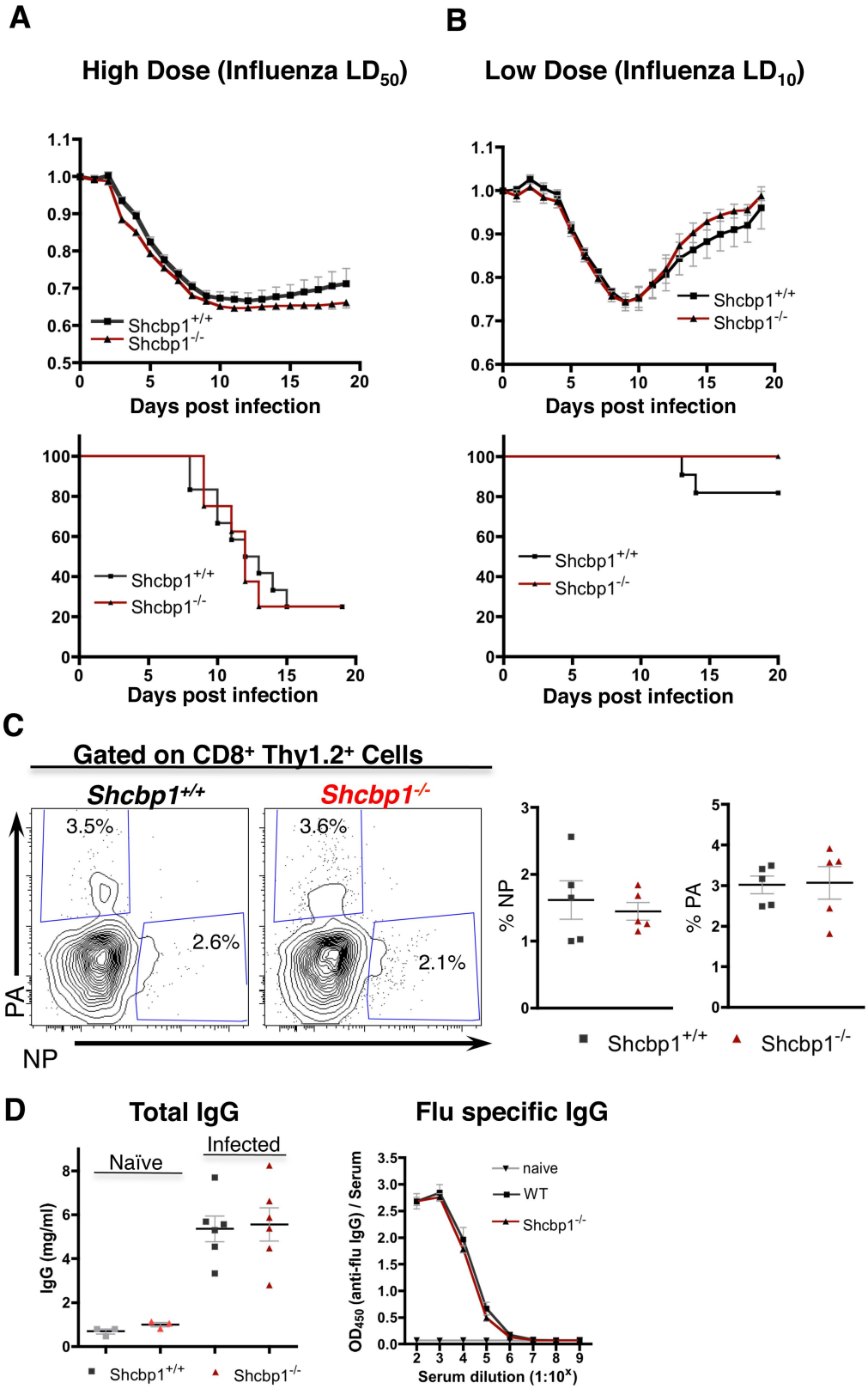
Figure 3.19: Shcbp1 is dispensable for CD8 T cell function in influenza infection

(A) Weight loss and survival curves of *Shcbp1*$^{+/+}$ and *Shcbp1*$^{-/-}$ infected intranasally with high dose (LD_{50}) influenza PR8 virus.

(B) Weight loss and survival curves of *Shcbp1*$^{+/+}$ and *Shcbp1*$^{-/-}$ infected intranasally with low dose (LD_{10}) influenza PR8 virus.

(C) Left, cell surface staining of PA and NP specific tetramers in gated $CD8^+$ $Thy1.2^+$ T cells isolated from the blood *Shcbp1*$^{+/+}$ and *Shcbp1*$^{-/-}$ mice on day 35 post-infection with low dose (LD_{10}) PR8 infection. Right, quantification of percentage NP or PA positive of $CD8^+$ T cells.

(D) Left, elisa for total IgG of naïve or mice infected with low dose PR8 infection (day 35 post-infection) of $Shcbp1^{+/+}$ and $Shcbp1^{-/-}$ mice (n=3-6 mice per condition). Right, elisa for flu-specific IgG antibodies from mice infected with low dose PR8 infection (day 35 post-infection).


A
High Dose (Influenza LD50)
Shcbp1+/+
Shcbp1-/-
Days post infection
B
Low Dose (Influenza LD10)
Shcbp1+/+
Shcbp1-/-
Days post infection
C
Gated on CD8+ Thy1.2+ Cells
Shcbp1+/+
Shcbp1-/-
3.5%
2.6%
3.6%
2.1%
PA
NP
% NP
% PA
Shcbp1+/+
Shcbp1-/-
D
Total IgG
Naïve
Infected
IgG (mg/ml)
Shcbp1+/+
Shcbp1-/-
Flu specific IgG
naive
WT
Shcbp1-/-
OD450 (anti-flu IgG) / Serum
Serum dilution (1:10x)

Chapter IV

ShcA phosphorylation is required for late T cell development

I completed this project in collaboration with a fellow post-doctoral fellow in our laboratory, Dr. Paul Trampont, who generated and performed the initial analysis of the CD4-Cre$^+$/ShcFFF transgenic mouse line and all of the experiments using the transgenic TCR mouse lines. This work has been submitted for publication and is currently under revisions in the Journal of Immunology: Buckley, Monica W., Trampont, Paul C., Arandjelovic, Sanja, Juncadella, Ignacio J., and Ravichandran, Kodi S. ShcA regulates late stages of T cell development and peripheral CD4 T cell numbers.

1. Abstract

T cell development in the thymus is a highly regulated process that critically depends upon productive signaling via the pre T cell Receptor (preTCR) at the β-selection stage, and via the TCR for selection from the CD4$^+$CD8$^+$ double positive stage to the CD4 or CD8 single positive stage. ShcA is an adapter protein expressed in thymocytes, and our laboratory has previously shown that ShcA is required for productive signaling through the preTCR with impaired signaling via ShcA leading to a developmental block at the β-selection checkpoint. However, due to the requirement for ShcA to progress beyond the β-selection checkpoint, the role of ShcA in subsequent stages of T cell development has not been addressed. In this chapter, I describe the generation a transgenic mouse line that specifically expresses a phosphorylation-defective dominant negative ShcA transgene (ShcFFF) in late T cell development (*CD4*-Cre$^+$/ShcFFF mice). Thymocytes in *CD4*-Cre$^+$/ShcFFF mice progressed normally

through the β-selection checkpoint, yet displayed a significant reduction in the number and percentage of single positive (SP) CD4 thymocytes. Furthermore, *CD4*-Cre^{+}/ShcFFF mice, when bred with transgenic TCR mouse strains, had impaired signaling through the transgenic TCRs. Consistent with defective progression to the single positive stage, the *CD4*-Cre^{+}/ShcFFF mice have a significant peripheral lymphopenia. Moreover, these *CD4*-Cre^{+}/ShcFFF mice develop attenuated disease in CD4^{+} T cell dependent experimental autoimmune encephalomyelitis (EAE), a mouse model of multiple sclerosis (MS). Collectively, these data identify an important role for the adapter protein ShcA in later stages of thymic T cell development and in peripheral T cell-dependent events.

2. Introduction

T cell development in the thymus is a highly regulated process that involves the coordinated expression of cell surface receptors and multiple selection steps. Development proceeds from the most immature $CD4^-CD8^-$ double-negative (DN) stage to the $CD4^+CD8^+$ double positive (DP) stage, and then the $CD4^+$ or $CD8^+$ single positive (SP) stage[2,15]. Expression and productive signaling through the preTCR and TCR are essential for proper T cell development and progression through developmental checkpoints[2-6]. Signaling via the preTCR allows thymocytes to progress through the first developmental checkpoint, the β-selection checkpoint, and undergo further differentiation to the DP stages of development[3]. The quality of TCR signaling at the DP stage controls progression through the next checkpoints that involve positive and negative selection of DP thymocytes leading to single positive CD4 or CD8 thymocytes[2,35,38]. The duration and strength of TCR signaling determines the fate of each thymocyte; thymocytes that receive intermediate TCR activation are generally positively selected, while thymocytes that receive strong TCR activation or very weak/no TCR activation undergo apoptosis[2]. Furthermore, stronger and more persistent signaling via MHC class II-restricted TCRs promotes the CD4-lineage commitment, while intermittent signaling via MHC class I-restricted TCRs is linked to the development of the CD8-lineage cells[35,38].

T cell development and function are impaired by the disruption of genes encoding components of the preTCR/TCR as well as disruption of many downstream signaling molecules, including adapter proteins[2,69]. During T cell development, ShcA is essential for progression through the β-selection checkpoint[7-9,28]. In developing thymocytes, ShcA is phosphorylated on three conserved tyrosine residues within the CH1 domain, and links receptor activation to the Ras-MAPK pathway[7-9]. Additionally, ShcA is required for

productive signaling through the preTCR; thymocytes either lacking the expression of ShcA (*Lck*-Cre^{+}/*Shc1*$^{fl/fl}$) or expressing the phosphorylation-defective ShcA transgene (*Lck*-Cre^{+}/ShcFFF) from the DN2/DN3 stage, have a developmental block at the DN3 stage of development[8,9]. Furthermore, in mature T cells, ShcA is phosphorylated after CD3 stimulation, and *in vitro* studies have shown that ShcA affects functions such as IL2 production[76,81,150].

While previous studies have highlighted the requirement for ShcA in the DN to DP transition[7-9,28], the nearly complete block in development at the β-selection checkpoint in the *Lck*-Cre^{+}/ShcFFF transgenic mouse line has precluded us from using this mouse line to study the role of ShcA in subsequent developmental stages. In this work, we used *CD4*-Cre-mediated expression of the phosphorylation-defective ShcFFF transgene, where the crucial ShcA tyrosine residues within the CH1 domain were mutated to phenylalanine[8]. The *CD4*-Cre transgenic mouse line expresses the Cre recombinase from the late DN4/early DP stage, allowing normal progression at the β-selection checkpoint[86]. We now find that ShcA function is important during DP to SP transition. *CD4*-Cre^{+}/ShcFFF mice have reduced thymic cellularity with fewer SP thymocytes, and display impaired positive selection in mice expressing either endogenous or transgenic TCRs. *CD4*-Cre^{+}/ShcFFF mice also have alterations in the peripheral T cell compartment, with an overall lymphopenia and skewing of the CD4:CD8 T cell ratio. Peripheral lymphopenia leads to a functional defect in immunity, as *CD4*-Cre^{+}/ShcFFF mice develop attenuated disease in the CD4^{+} T cell driven experimental autoimmune encephalomyelitis (EAE) model of multiple sclerosis (MS).

3. Results

3.1. Generation of CD4-Cre$^+$/ShcFFF transgenic mice

We have previously used *Lck*-Cre$^+$/ShcFFF and *Lck*-Cre$^+$/*Shc$^{fl/fl}$* transgenic mice to demonstrate an absolute requirement for ShcA in β-selection[7-9,28]. To determine whether ShcA phosphorylation is required in later stages of T cell development, we crossed our dominant negative ShcFFF transgenic mouse line to the *CD4*-Cre transgenic mouse line[86] **(Figure 4.1A,C)**. The ShcFFF transgenic construct allows specific expression of a phosphorylation defective ShcFFF transgene downstream of the ubiquitous EF-1α promoter; however, to keep basal expression of the ShcFFF transgene silent and to allow Cre-mediated transgene expression, a floxed STOP cassette has been inserted between the promoter the ShcFFF coding sequence[8,9] **(Figure 4.1C)**. To confirm the fidelity of the *CD4*-Cre transgenic mouse line, we first crossed the *CD4*-Cre mouse line to the Rosa26$^{STOP\text{-}EYFP}$ and analyzed eYFP expression in the different thymic subsets. We found that eYFP is expressed in DN4, DP, CD4 SP, and CD8 SP but not in DN3 thymocytes and importantly, as expected, the *CD4*-Cre affects both the CD4 and CD8-lineages[129] **(Figure 4.1C)**. Therefore, we expected that the *CD4*-Cre$^+$/ShcFFF mice would allow us to bypass the requirement for ShcA phosphorylation during β-selection, yet facilitate investigation of ShcA function in late T cell development.

3.2. *CD4-Cre$^+$/ShcFFF mice have defects in T cell development*

We first observed that *CD4*-Cre$^+$/ShcFFF mice have altered T cell development with a decrease in overall thymic cellularity and a striking reduction in the percentage of the CD4 SP compartment with no changes in the percentages of the CD8 SP compartment **(Figure 4.2A, B)**. *CD4*-Cre$^+$/ShcFFF mice showed a reduction in the

absolute number of the DP, $CD4^+$ SP, and $CD8^+$ SP thymocytes **(Figure 4.2A, Table I)**. Although there was a slight decrease in the absolute number of DP thymocytes, the defect in the SP compartment was more profound than the defect in the DP compartment, suggesting that the reduction in SP thymocytes is likely not entirely due to the reduced numbers of DP thymocytes.

The development of the CD4-lineage is often more sensitive to alterations in TCR signaling as CD4-lineage commitment requires stronger and more sustained signaling than CD8-lineage commitment[35,38,151-153]. We noted that in the *CD4*-Cre^+/ShcFFF mice, the development of $CD4^+$ SP thymocytes was more affected than $CD8^+$ SP thymocytes, leading to an alteration in the ratio of $CD4^+$ to $CD8^+$ SP thymocytes **(Figure 4.2B)**. Moreover, the phenotypic maturation of $CD4^+$ SP and $CD8^+$ SP thymocytes also appeared to be impaired in the *CD4*-Cre^+/ShcFFF mice based on cell surface staining for CD24 and CD5[154-156] **(Figure 4.2C, D)**. Both $CD4^+$ and $CD8^+$ SP thymocytes displayed a reduced percentage of mature $CD24^{lo}$ SP thymocytes and an alteration in the ratio of mature ($CD24^{lo}$) to immature ($CD24^{hi}$) SP thymocytes **(Figure 4.2C, D)**. In mature T cells, CD24 is thought to be involved in T cell activation/costimulation, but in developing thymocytes CD24 ligation plays a role in the induction of apoptosis in the absence of TCR signaling. Additionally, expression of the maturation and adhesion molecule CD5 was decreased in the $CD8^+$ SP thymocytes[156] **(Figure 4.2C, D)**. Importantly, in addition to being a maturation marker, CD5 attenuates TCR signaling and is involved in the processes of positive and negative selection. Collectively, the impairment in $CD4^+$ and $CD8^+$ SP compartments, with a more profound defect in the $CD4^+$ SP compartment, suggests that the *CD4*-Cre^+/ShcFFF mice have a defect in TCR signaling and the transition from DP to SP stages of thymocyte development.

To further understand the specific developmental stages affected in the *CD4*-Cre^{+}/ShcFFF mice, we subdivided thymocytes into five developmental stages based on the expression of TCRβ and CD69[151-153]. Populations 1 (TCRβ^{lo}CD69^{lo}) and 2 (TCRβ^{int}CD69^{lo}) represent the most immature pre-positive selection DN and DP thymocytes[151]. *CD4*-Cre^{+}/ShcFFF mice have a slight increase in the percentages of both of these subsets **(Figure 4.3)**. Population 3 (TCR^{int}CD69^{int}) has partially up-regulated CD69 and is thought to be undergoing positive selection[157]. We found a slight reduction in this population in the *CD4*-Cre^{+}/ShcFFF mice. Additionally, *CD4*-Cre^{+}/ShcFFF mice had a significant reduction in the composition of post-positive selection thymocytes (population 4, TCR^{hi}CD69^{hi}) and mature thymocytes (population 5, TCR^{hi}CD69^{lo}) **(Figure 4.3)**. Collectively, these data suggested that *CD4*-Cre^{+}/ShcFFF mice have deficiencies in late T cell development, possibly due to altered positive selection.

3.3. *Signaling though MHC class II-restricted TCRs is impaired in CD4-Cre^{+}/ShcFFF mice*

Developmental defects in positive selection are often better revealed in mice carrying a transgenic TCR, since the normal TCR repertoire may undergo compensatory changes that can mask defects in thymocyte selection[152,158,159]. Therefore, we crossed the *CD4*-Cre^{+}/ShcFFF mice to two different MHC class II-restricted TCR transgenic lines: OT-II and DO11.10. The developmental defects due to ShcFFF expression were more pronounced in these TCR transgenic backgrounds **(Table I)**. *CD4*-Cre^{+}/ShcFFF mice expressing either the OT-II or the DO11.10 transgenic TCR had an overall reduced thymic cellularity and decreased numbers in the DP and CD4 SP thymic compartments **(Table I)**. Additionally, staining with antibodies specific for the transgenic TCRs

demonstrated a specific reduction in thymocytes expressing the transgenic TCRs **(Figure 4.4A, B)**, with fewer $CD4^+$ SP transgenic TCR^{hi} cells.

Previously, we have demonstrated that the block in early T cell development in mice expressing the ShcFFF transgene is essentially complete such that any DP, SP, or peripheral T cells observed in the *Lck*-Cre^+/ShcFFF mice arise due to incomplete Cre-mediated recombination at the *loxP* sites and, in turn, fail to express the ShcFFF transgene (i.e. essentially wild type cells)[7-9]. To determine whether peripheral T cells in the DO11.10/*CD4*-Cre^+/ShcFFF mice were similar escapees without Cre-mediated deletion of the STOP cassette, we assessed the expression of the transgene-encoded, flagged tagged ShcFFF protein by immunoblotting. We were able to detect expression of Flag-ShcFFF in the lysates from the thymus, but not lysates of peripheral $CD4^+$ T cells **(Figure 4.4C)**. This suggested that in DO11.10/*CD4*-Cre^+/ShcFFF mice, ShcA phosphorylation is required for the development of $CD4^+$ T cells and that the peripheral T cells detected in these animals likely do not express the ShcFFF transgene or do so at low levels.

DO11.10 mice have a MHC class II-restricted TCR that recognizes the OVA peptide, and it has been shown that *in vivo* intraperitoneal injection of OVA peptide or the *ex vivo* treatment of thymocytes with the peptide leads to apoptosis of DP thymocytes[123,160]. To test a potential defect in signaling through the transgenic TCR in the DO11.10/*CD4*-Cre^+/ShcFFF triple transgenic mice, we assessed peptide-induced apoptosis of DP thymocytes from these mice. In an *ex vivo* assay, thymocytes from DO11.10/*CD4*-Cre^+/ShcFFF mice had reduced apoptosis of DP thymocytes after 8 hours and, in turn, increased recovery of DP thymocytes after 20 hours **(Figure 4.4D)**,

suggestive of reduced signaling via the transgenic TCR in these thymocytes. Collectively, these data suggest a defect in signaling through MHC class II-restricted TCRs in the *CD4*-Cre$^+$/ShcFFF mice. These transgenic TCR studies further demonstrated a crucial role for ShcA phosphorylation during the DP to SP stage of development, since even the enhanced signaling through the transgenic TCR failed to rescue the developmental defects in *CD4*-Cre$^+$/ShcFFF mice.

3.4. ShcA phosphorylation less profound role in CD8-lineage development

Next, we investigated whether ShcA phosphorylation is required for CD8-lineage commitment and positive selection. In *CD4*-Cre$^+$/ShcFFF mice, we found a less profound defect in the development of CD8$^+$ compared to CD4$^+$ SP thymocytes. To further address this in the context of transgenic TCRs, we crossed our *CD4*-Cre$^+$/ShcFFF mice to MHC class I-restricted mouse lines: OT-I and H-Y transgenic TCR lines. We noted a decrease in overall thymic cellularity in the *CD4*-Cre$^+$/ShcFFF mice expressing either the H-Y (female mice) or OT-I transgenic TCR, with a decrease in DP and CD8$^+$ SP thymocytes **(Table I)** with fewer transgenic TCR^{hi} CD8 SP thymocytes **(Figure 4.5)**.

To assess negative selection in thymocytes expressing the ShcFFF transgene, we analyzed thymocyte development in male H-Y/*CD4*-Cre$^+$/ShcFFF mice. The H-Y transgenic mouse line encodes an MHC class I-restricted transgenic TCR that recognizes a male antigen[124]. While in female H-Y transgenic mice, positive selection leads to the generation of CD8$^+$ SP thymocytes, in the male H-Y transgenic mice, negative selection leads to a decrease in overall thymic cellularity and very few DP and SP thymocytes[124]. Negative selection appeared normal in the H-Y/*CD4*-Cre$^+$/ShcFFF mice; these mice have a greatly reduced thymic cellularity due to negative selection and

a decrease in the expression of CD4 and CD8 comparable to control male H-Y mice without ShcFFF **(Figure 4.5D)**. Collectively, these data suggest that the CD8-T cell lineage is less affected than the CD4-lineage, both in the context of endogenous and transgenic TCRs.

3.5. CD4-Cre$^+$/ShcFFF mice do not display obvious defects within the DP thymocyte subset

Defects in TCR signaling and positive selection often lead to a relative accumulation and increased percentage of DP thymocytes. However, we found that there was a slight reduction in the absolute number of DP thymocytes in the *CD4*-Cre$^+$/ShcFFF mice **(Table I)**. Therefore, we next assessed whether there was an additional defect within the DP compartment. First, we crossed the transgenic *CD4*-Cre$^+$/ShcFFF mouse line to the *TCR*α deficient mouse line. Previous studies have shown that thymocytes from *TCR*α deficient mice can undergo normal DN —> DP transition, but are blocked at the DP stage due to their inability to generate the $\alpha\beta$TCR and undergo positive selection[6]. We found that in the *TCR*α deficient *CD4*-Cre$^+$/ShcFFF mice there was only a slight difference in the total cellularity compared to control *TCR*α deficient mice **(Figure 4.6B)**. This suggested that the reduced thymic cellularity in the *CD4*-Cre$^+$/ShcFFF mice is not reflective of a major defect in DP thymocytes, and is mainly due to defects in the DP to SP transition. Furthermore, no defects in the survival of DP thymocytes expressing the ShcFFF transgene was revealed by Annexin V and 7AAD staining of freshly isolated thymocytes, or thymocytes undergoing spontaneous and anti-CD3 mediated apoptosis under *ex vivo* conditions **(Figure 4.6C)**. Based on

these data, we conclude that the primary defect in the *CD4*-Cre$^+$/ShcFFF mice is the impaired DP to SP transition.

3.6. ShcA is downstream of p56^{lck} in DP thymocytes

The protein tyrosine kinase p56^{lck} (Lck) in an important signaling molecule in T cells and signals downstream of both the preTCR and the TCR[7]. Lck activity is required for preTCR and TCR signaling, as the constitutively active LckF505 transgene can rescue thymic development defects resulting from the absence of either the preTCR or TCR[161-163]. Previous studies have shown that during TCR stimulation, Lck phosphorylates ShcA on the same three crucial tyrosine residues that are mutated in the ShcFFF transgene[7]. To determine whether ShcA acts downstream of Lck and whether a constitutively active version of Lck (LckF505) can bypass the need for ShcA mediated signaling, we crossed *CD4*-Cre$^+$/ShcFFF mice to the LckF505 transgenic mouse line. Despite the known augmented signaling in the LckF505 context, LckF505/*CD4*-Cre$^+$/ShcFFF mice displayed significantly impaired T cell development compared to control mice expressing LckF505 alone, which suggests that LckF505 transgene is unable to rescue T cell development in the context of impaired ShcA signaling **(Figure 4.6A, Table I)**. These data suggest that ShcA acts downstream of Lck in DP thymocytes and that ShcA phosphorylation is necessary for Lck mediated downstream signaling.

3.7. *CD4-Cre$^+$/ShcFFF mice display defects in thymic organization and cell trafficking*

Next, we performed H&E staining on thymic sections and found the thymic architecture considerably altered in *CD4*-Cre$^+$/ShcFFF mice, with reduction in the area

representing both the cortex and the medulla **(Figure 4.7A, B)**. Furthermore, the medulla was fragmented and disorganized, as is often seen in mice with defects in positive selection[151,164], and the cortex to medulla area ratio was higher in the *CD4*-Cre$^+$/ShcFFF mice (7.9) compared to control (3.6) **(Figure 4.7A, B)**.

Previous studies have shown that ShcA acts downstream of CXCR4 and is required for CXCR4-mediated migration in T cells and thymocytes[28,89]. After positive selection, thymocytes upregulate the CCR7 receptor and migrate from the cortex towards the medulla via a gradient of CCL19 and CCL21 expressed by medullary epithelial cells[165,166]. We assessed the migration of DP thymocytes from *CD4*-Cre$^+$/ShcFFF mice. Interestingly, the thymocytes from *CD4*-Cre$^+$/ShcFFF mice had reduced migration toward the CCR7 ligands CCL19 and CCL21 **(Figure 4.7D, E)**. Notably, this was not due to altered CCR7 expression on these thymocytes, as the post-positively selected TCR^{hi} DP thymocytes from *CD4*-Cre$^+$/ShcFFF mice had expression of CCR7 comparable to control mice **(Figure 4.7C)**. These findings suggest that the thymic architecture is likely altered due to both defects in positive selection and migration of post-positive selection DP thymocytes. It is noteworthy that the defect in CCR7 migration is likely underestimated in the above assay as many of the post-positively selected DP thymocytes likely represent 'escapees' that still retain the STOP cassette and do not express the ShcFFF transgene.

3.8. *CD4-Cre$^+$/ShcFFF mice have a reduced peripheral T cell compartment*

Next, we investigated the peripheral T cell compartment in *CD4*-Cre$^+$/ShcFFF mice. The *CD4*-Cre$^+$/ShcFFF mice had a significant lymphopenia and reduction of both the number and percentage of CD4$^+$ and CD8$^+$ T cells in the lymph nodes and spleen

(Figure 4.8A). Additionally, while the CD4 to CD8 ratio is 2:1 in wild type mice, this ratio in the *CD4*-Cre^{+}/ShcFFF mice was roughly 1:1 **(Figure 4.8B)**. The lymphopenia did not appear to be due to improper accumulation of T cells in non-lymphoid peripheral tissues, as we also saw reduced numbers of T cells in other tissues such as the lung and blood (data not shown). As has been seen previously in lymphopenic conditions, we also found a slight increase in the percentage of the 'memory like' CD62L^{lo}CD44^{hi} CD4^{+} T cells, which is often the result of homeostatic proliferation of T cells **(Figure 4.8C)**. We next investigated whether the peripheral T cells in the *CD4*-Cre^{+}/ShcFFF mice were Cre 'escapees' by assessing the deletion of the STOP sequence. Although this PCR based approach was non-quantitative, we were able to detect some excision of the STOP cassette in peripheral T cells from *CD4*-Cre^{+}/ShcFFF **(Figure 4.1E)**. However, we were unable to detect the Flag-tagged ShcFFF transgene-encoded protein by western blotting **(Figure 4.1F)**. Thus, while some peripheral T cells might have undergone Cre-mediated excision of the STOP cassette, most of them likely do not express the ShcFFF transgene. Additionally, we did not observe a proliferative defect in the peripheral T cells from *CD4*-Cre^{+}/ShcFFF mice after anti-CD3/anti-CD28 stimulation *ex vivo*, which is likely because many of these T cells do not express the ShcFFF transgene **(Figure 4.8D)**.

A similar impairment was seen in peripheral T cells in the *CD4*-Cre^{+}/ShcFFF mice on the background of MHC class I and class-II restricted transgenic TCRs, with reduced splenic cellularity and reduction in CD8^{+} or CD4^{+} peripheral T cells **(Table I)**. In the OT-I/*CD4*-Cre^{+}/ShcFFF mice, we found a substantial reduction in the numbers of CD8^{+} T cells as well as reduction in the percentage of cells staining for the OT-I transgenic TCR (data not shown).

Since the *CD4*-Cre$^+$/ShcFFF mice have an overall reduction in thymic cellularity with a further reduction in CD4$^+$ SP thymocytes, initially we thought it is not too surprising that the *CD4*-Cre$^+$/ShcFFF mice have a defect in the peripheral T cell compartment. Many genetic mouse models with disruption in genes required for positive selection, such as *Themis* and *Tespa* deficient mice, also have defects in the peripheral T cell compartment[151-153,164]. However, we found that the defect in peripheral T cell compartment appeared to be disproportionate and could not be exclusively due to the reduced thymic cellularity **(Table I)**. We hypothesized that the *CD4*-Cre$^+$/ShcFFF mice have an additional defect in the emigration of the CD4$^+$ SP and CD8$^+$ SP thymocytes from the thymus into the periphery. Previously, it has been shown that the Rag2 eGFP transgenic mice (which express enhanced GFP under the expression of the Rag2 promoter) can be used to identify recent thymic emigrants (RTE) in the periphery. Furthermore, thymocytes from Rag2-eGFP mice express GFP in the late DN and DP stages of development, but the GFP expression briefly persists in peripheral T cells and, therefore, GFP$^+$ T cells in the periphery represent the RTEs[41,42,126] **(Figure 4.9B)**. To identify recent thymic emigrants in the context of impaired ShcA signaling, we crossed the *CD4*-Cre$^+$/ShcFFF mice to the Rag2-eGFP transgenic mice **(Figure 4.9A)**. We calculated the emigration ratio for the Rag2-eGFP/*CD4*-Cre$^+$/ShcFFF mice and Rag2-eGFP control mice by dividing the absolute number of GFP$^+$ CD4$^+$ or CD8$^+$ splenocytes by the GFP$^+$ CD4$^+$ SP or GFP$^+$ CD8$^+$ SP thymocytes **(Figure 4.9A)**. The emigration ratio for both CD4$^+$ and CD8$^+$ T cells was reduced in the *CD4*-Cre$^+$/ShcFFF mice, and therefore the *CD4*-Cre$^+$/ShcFFF mice appeared to have an additional defect in the emigration of the mature SP thymocytes into the periphery **(Figure 4.9C)**. However, we did not observe an accumulation of these SP subsets in the thymus, which is often

found in mice with defects in thymic emigration[39] most likely a consequence of the concurrent defects in positive selection and a reduction in the numbers of $CD4^+$ and $CD8^+$ SP thymocytes.

3.9. *Lymphopenia persists in adult CD4-Cre$^+$/ShcFFF mice and these mice display attenuated disease in the experimental autoimmune encephalomyelitis (EAE) model*

T cell developmental defects are often more striking in younger animals and certain phenotypes in ShcA mutant mice (in the context of brain) are only present in young mice[137]. Therefore, we asked whether the peripheral lymphopenia is transient or if it persist in adult *CD4*-Cre^+/ShcFFF mice. When we analyzed 10 to 12 week old *CD4*-Cre^+/ShcFFF mice, the reduced numbers of $CD4^+$ and $CD8^+$ T cells in the spleen and lymph nodes still remained **(Figure 4.10A, B)**. Next, we subcutaneously immunized the *CD4*-Cre^+/ShcFFF mice with $MOG_{(35-55)}$ and collected the draining lymph nodes on day 8 after immunization. In the *CD4*-Cre^+/ShcFFF mice, we found reduced number and percentage of the $CD4^+$ and $CD8^+$ T cells after immunization with $MOG_{(35-55)}$ **(Figure 4.10C)**. Therefore, the peripheral lymphopenia in the *CD4*-Cre^+/ShcFFF mice persists with age, as well as after immunization.

To test whether the lymphopenia of the *CD4*-Cre^+/ShcFFF mice leads to attenuated disease in the context of autoimmunity, we chose to use the $CD4^+$ T cell driven experimental autoimmune encephalomyelitis (EAE) model[63]. In MS and EAE there is infiltration of autoreactive T cells into the central nervous system (CNS), which leads to inflammation, demyelination and progressive disability[54,63]. Furthermore, 8 days after

immunization with the $MOG_{(35-55)}$ peptide, *CD4*-Cre^+/ShcFFF mice have reduced numbers of $CD4^+$ and $CD8^+$ T cells in the draining lymph nodes **(Figure 4.10B)**. We immunized *CD4*-Cre^+/ShcFFF and control mice with $MOG_{(35-55)}$ to induce EAE, and monitored disease severity and weight loss over a period of 28 days after peptide immunization. We found that *CD4*-Cre^+/ShcFFF mice developed strikingly attenuated disease with a significant reduction in disease severity, had better maintenance of body weight, and improved survival **(Figure 4.11A)** (in 3 independent experiments with a total of n=16 mice per genotype, $p<0.0001$ for clinical scores by 2-way ANOVA). Additionally, the *CD4*-Cre^+/ShcFFF had a lower incidence of disease with 25% of *CD4*-Cre^+/ShcFFF mice never exhibiting any signs of disease (defined as score of 1 or greater) and a lower overall maximum score and disease index (area under the curve) **(Figure 4.11B)**. Of the *CD4*-Cre^+/ShcFFF mice that exhibit signs of disease, the day of onset was significantly delayed from an average of 10.1 days for control mice to an average of 14.1 days for *CD4*-Cre^+/ShcFFF mice **(Figure 4.11B)**. Additionally, using a sub-optimal EAE induction, we found that the *CD4*-Cre^+/ShcFFF develop attenuated disease as compared as compared to control mice **(Figure 4.11C)**.

At chronic stage of the disease (day 28), we assessed the composition of the immune infiltrate in the CNS by analyzing the cells isolated from the brains and spinal cords of *CD4*-Cre^+/ShcFFF and control mice. *CD4*-Cre^+/ShcFFF mice had overall fewer mononuclear cells isolated from the brain and spinal cords **(Figure 4.12A-C)**. There was a decrease in the percentage of $CD4^+$ and $B220^+$ lymphocytes in the *CD4*-Cre^+/ShcFFF mice compared to control mice **(Figure 4.12A-C)**. Overall, the *CD4*-Cre^+/ShcFFF mice had a trend of reduced total numbers of $CD4^+$ T cells, B cells, macrophages, and microglia in the brain and spinal cord at the chronic stage of the disease **(Figure 4.12B-**

C). Histological analysis by H&E staining and immunohistochemistry confirmed that the *CD4*-Cre^{+}/ShcFFF mice had fewer loci of immune infiltration and fewer CD3^{+} T cells in the spinal cords **(Figure 4.12D)**. Collectively, these data demonstrated that the lymphopenia present in the *CD4*-Cre^{+}/ShcFFF mice leads to a net functional deficit in CD4^{+} T cells manifested as attenuated disease in the EAE model.

4. Discussion

In this study, we demonstrate a requirement for ShcA function in late T cell development, beyond the previously reported requirement for ShcA in preTCR signaling and progression through the β-selection checkpoint[8,9]. To bypass the requirement for ShcA in early T cell development, we generated the *CD4*-Cre$^+$/ShcFFF transgenic mice, which express the ShcFFF transgene from the DN4/DP stage of development[86]. We find that the *CD4*-Cre$^+$/ShcFFF transgenic mice show two notable defects in late T cell development: a defect in the absolute number and percentage of CD4$^+$ SP thymocytes, and a persistent peripheral lymphopenia.

Productive signaling through the TCR in DP thymocytes is required for positive selection and the development of mature SP thymocytes. Additionally, sustained signaling through the TCR is essential for the development of CD4$^+$ SP thymocytes, and the CD4$^+$ SP compartment is more sensitive to defects in TCR signaling than the CD8$^+$ SP compartment[2]. Through several approaches, we find that impaired ShcA mediated signaling affects the development of mature CD4$^+$ SP thymocytes. We also found that the impairment in positive selection and development of CD4$^+$ SP thymocytes due to impaired ShcA signaling is more profound than the development of CD8$^+$ SP thymocytes. To overcome the issue that developmental defects in positive selection can be masked by compensatory changes in the TCR repertoire, we also analyzed the *CD4*-Cre$^+$/ShcFFF mice in the context of expressing a MHC class II-restricted transgenic TCR and confirmed the defect in positive selection. Furthermore, antigenic-peptide induced signaling via the transgenic TCR in the DP thymocytes was also decreased in DO11.10/*CD4*-Cre$^+$/ShcFFF transgenic mice. Collectively, these data demonstrated that

ShcA phosphorylation is required for optimal signaling through the TCR in DP thymocytes to progress further through maturation.

The *CD4*-Cre$^+$/ShcFFF mice also had impairment in the peripheral T cell compartment, with reduced numbers and an alteration in the ratio of CD4$^+$ to CD8$^+$ T cells. Peripheral lymphopenia can be partially explained by the defects in the thymic CD4$^+$ and CD8$^+$ SP compartments. However, we found that the defect in the peripheral compartment is disproportionate to the thymic defect. Our data suggests that the peripheral compartment in *CD4*-Cre$^+$/ShcFFF mice may also be partially altered due to impaired emigration of thymocytes into the periphery. Despite extensive investigation, we were unable to find a definitive explanation for the disproportionate peripheral defect; there was no significant defect in *ex vivo* S1P mediated migration of CD4$^+$CD62L^{hi} SP thymocytes, and we did not observe an apparent survival or proliferative defect in peripheral T cells. However, the peripheral lymphopenia in *CD4*-Cre$^+$/ShcFFF mice caused functional impairments, as *CD4*-Cre$^+$/ShcFFF mice develop attenuated disease in the CD4$^+$ T cell drive EAE mouse model. Furthermore, the defects in positive selection may have led to changes in the TCR repertoire and V segment usage, which may have also contributed to the attenuated disease in the EAE model.

Development of $\alpha\beta$ T cells critically depends upon productive signaling through the preTCR in DN thymocytes and the TCR in DP thymocytes. Disruption of the components of the preTCR and TCR, or of downstream signaling molecules, leads to impairment in T cell development and results in the block of developmental progression. Along with our previous studies on the essential role of the adapter protein ShcA via the preTCR, and the studies presented here on the role of ShcA in signaling via the αβTCR,

these data suggest that ShcA critically contributes to progression through the β-selection and positive-selection checkpoints.

5. Acknowledgment of co-author contributions

I would like to thank Dr. Paul Trampont for initiating this project and providing feedback/suggestions. Dr. Paul Trampont generated the *CD4*-Cre/ShcFFF transgenic mouse line, performed the initial experiments characterizing T cell development, and generated all of the data on the TCR transgenic mouse lines. This project would not have been possible without his extensive contributions. I would also like to thank Dr. Sanja Arandjelovic for helping me perform the EAE experiments (scoring, histology, mononuclear cell preparations) and Dr. Ignacio Juncadella for performing the initial EAE experiments for this project.

Figure 4.1: Generation of *CD4*-Cre/ShcFFF transgenic mouse line

(A) Schematic of T cell development and the expression of Cre in the *CD4*-Cre transgenic mouse line

(B) Expression of eYFP in the indicated thymic subset from *CD4*-Cre transgenic mice crossed to the Rosa26$^{STOP\text{-}EYFP}$ transgenic reporter mouse line.

(C) Schematic detailing expression of the ShcFFF transgene via a lox-STOP-lox sequence between the EF-1α promoter and the cDNA encoding the human ShcFFF transgene.

(D) Representative genotyping PCR for the ShcFFF transgene (382 bp) and Cre (217 bp) from genomic DNA.

(E) Non-quantitative PCR strategy to detect deletion of the STOP cassette in genomic DNA isolated from either the thymus or peripheral T cells. Removal of the STOP cassette via Cre is detected by an 800bp product while the presence of the STOP cassette results in a 511 bp PCR product.

(F) Immunoblot for ShcA in thymocytes and splenocytes from mice with the indicated genotypes in either whole cell lysates or after immunoprecipitation with Flag.

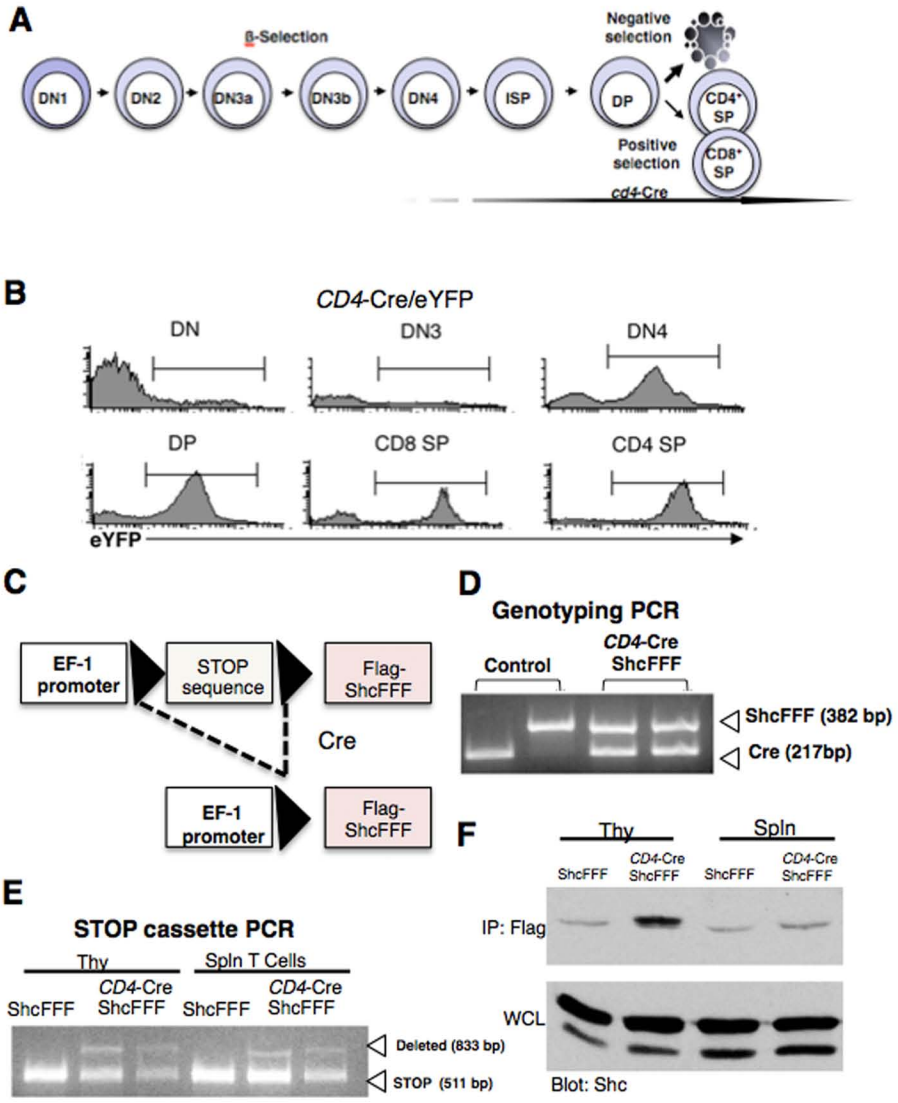

A
β-Selection
Negative selection
DN1
DN2
DN3a
DN3b
DN4
ISP
DP
CD4+ SP
CD8+ SP
Positive selection
cd4-Cre
B
CD4-Cre/eYFP
DN
DN3
DN4
DP
CD8 SP
CD4 SP
eYFP
C
EF-1 promoter
STOP sequence
Flag-ShcFFF
Cre
EF-1 promoter
Flag-ShcFFF
D
Genotyping PCR
Control
CD4-Cre ShcFFF
ShcFFF (382 bp)
Cre (217bp)
E
STOP cassette PCR
Thy
Spln T Cells
ShcFFF
CD4-Cre ShcFFF
ShcFFF
CD4-Cre ShcFFF
Deleted (833 bp)
STOP (511 bp)
F
Thy
Spln
ShcFFF
CD4-Cre ShcFFF
ShcFFF
CD4-Cre ShcFFF
IP: Flag
WCL
Blot: Shc

Figure 4.2: Decreased cellularity and defect in late thymic development in CD4-Cre/ShcFFF mice

(A) Left, flow cytometry of thymi isolated from 4-to-6 week old *CD4-Cre/*ShcFFF or control mice mice. Top panel is surface marker expression of CD4 and CD8. Bottom panel is surface marker expression of CD44 and CD25 gated on DN thymocytes ($CD4^-$ $CD8^-$ $B220^-$ $Gr1^-$ $Ter119^-$ $CD11b^-$ $CD11c^-$) Right, total cellularity and absolute numbers of the thymus and thymic subsets (n>12 mice per genotype, age-matched littermate controls).

(B) Cell surface expression of HSA, TCRβ, and CD5 on DP, CD4 SP, and CD8 SP thymocytes isolated from 4-to-6 week old *CD4*-Cre/ShcFFF or control mice (representative of n>5 mice per genotype).

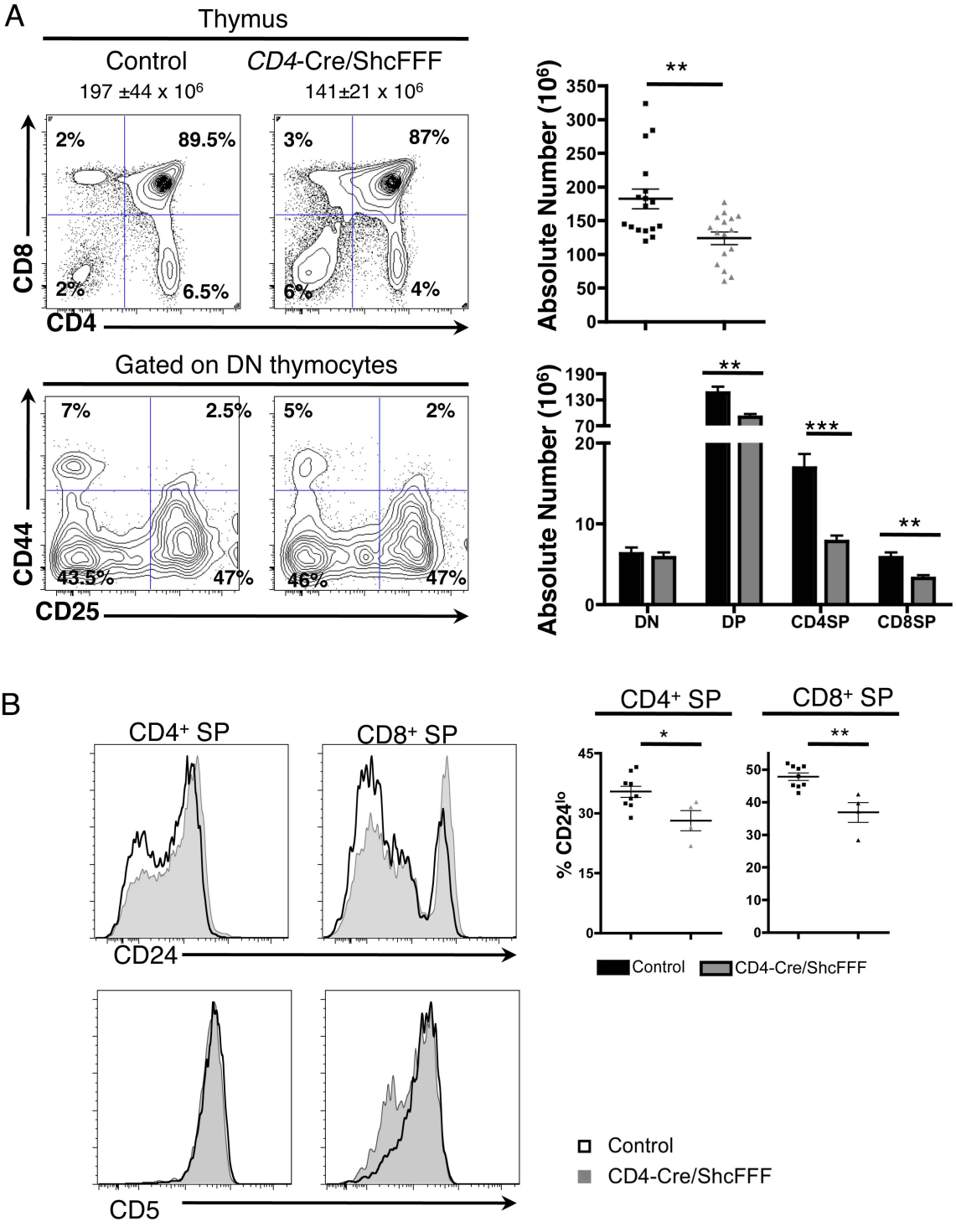

A
Thymus
Control
CD4-Cre/ShcFFF
197 ±44 x 10^6
141±21 x 10^6
2%
89.5%
2%
6.5%
3%
87%
6%
4%
CD8
CD4
Gated on DN thymocytes
7%
2.5%
43.5%
47%
5%
2%
46%
47%
CD44
CD25
Absolute Number (10^6)
DN
DP
CD4SP
CD8SP
B
CD4+ SP
CD8+ SP
CD24
CD5
% CD24lo
Control
CD4-Cre/ShcFFF

Figure 4.3: Defect in the DP to SP transition and positive selection in the *CD4*-Cre/ShcFFF mice

(A) Left, surface expression of TCRβ and CD69 on total thymocytes from control or *CD4*-Cre/ShcFFF mice to identify thymocytes of different maturity and the process of positive selection (n>5 mice per genotype). Right, quantification of post-positively selected population 4 and population 5.

(B) Cell surface staining for CD4 and CD8 in thymic subsets from control or *CD4*-Cre/ShcFFF mice.

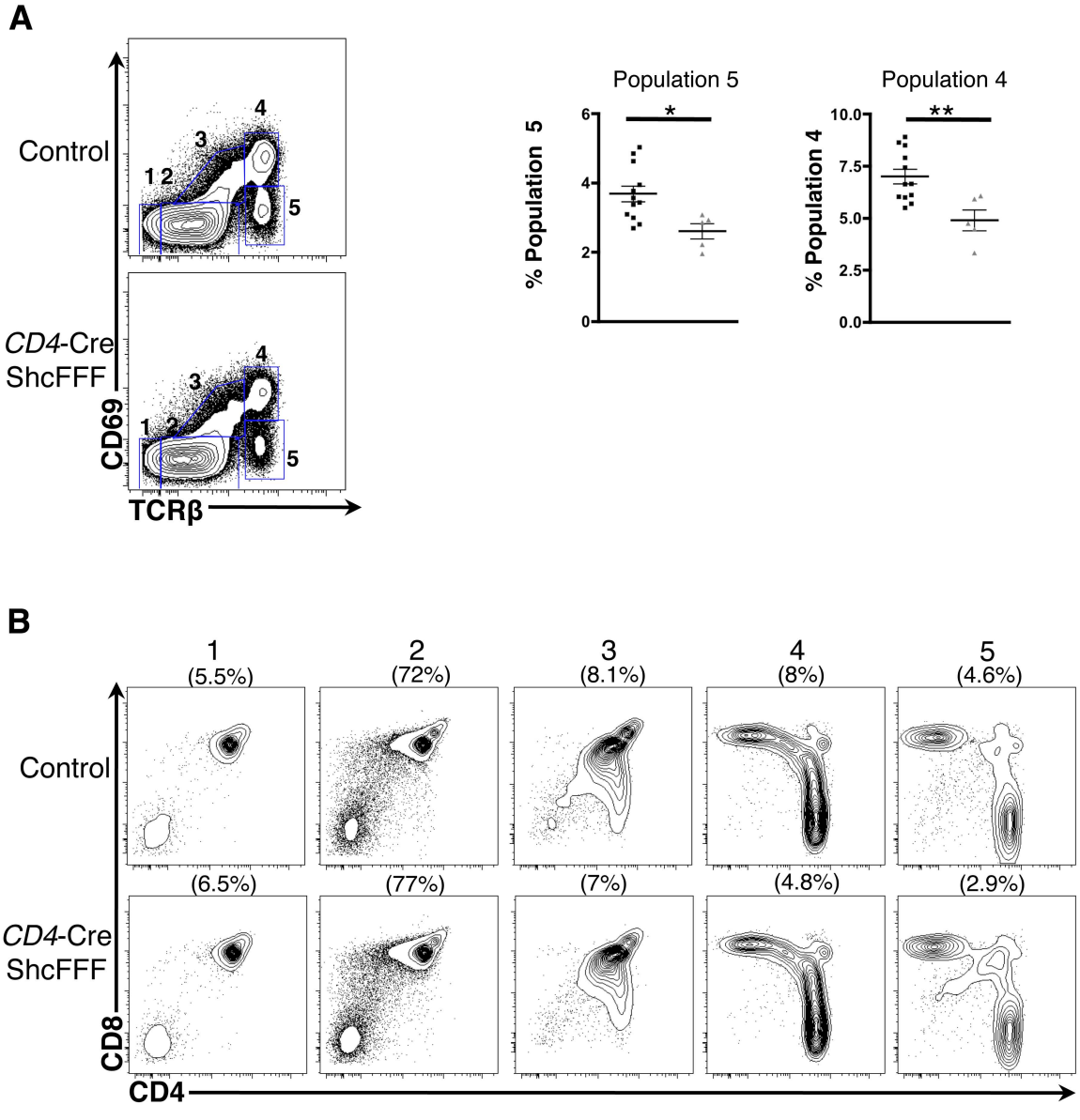

A
Control
CD4-Cre ShcFFF
CD69
TCRβ
1
2
3
4
5
Population 5
Population 4
% Population 5
% Population 4
*
**
B
(5.5%)
(72%)
(8.1%)
(8%)
(4.6%)
(6.5%)
(77%)
(7%)
(4.8%)
(2.9%)
CD8
CD4

Figure 4.4: Signaling through MHC class II-restricted transgenic TCRs is impaired in *CD4*-Cre/ShcFFF mice

(A) Top panel is surface marker expression of CD4 and CD8 in control and *CD4*-Cre/ShcFFF mice on the DO11.10 transgenic background. Bottom panel is cell surface expression of KJ1.26 to recognize the DO11.10 transgenic TCR (n=9 mice per genotype).

(B) Top panel is surface marker expression of CD4 and CD8 in control and *CD4*-Cre/ShcFFF mice on the OT-II transgenic background. Bottom panel is cell surface expression of Vβ5.1/2 to recognize the OT-II transgenic TCR (n=8, 10 mice per genotype).

(C) Immunoblot for ShcA in thymocytes and splenocytes from DO11.10 transgenic mice with the indicated genotypes in either whole cell lysates or after immunoprecipitation with Flag.

(D) Left panel is percent annexin V^+ DP thymocytes after incubation with the A20 B cell line and the indicated concentration of OVA peptide for 8 hours. Right panel is percent recovery of DP thymocytes after incubation for 20 hours.

Experiments performed by Paul Trampont

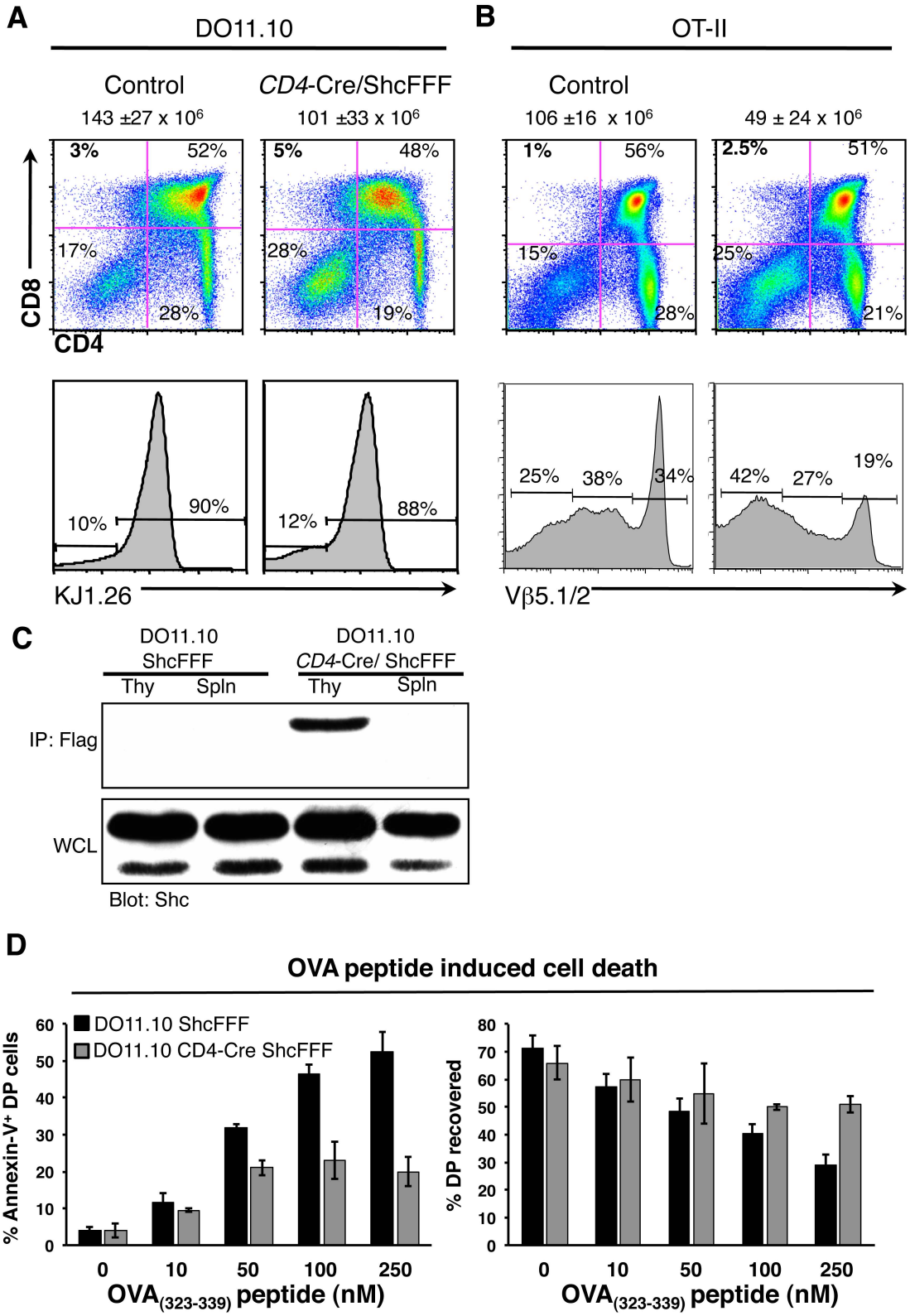

A
DO11.10
Control
143 ±27 x 10^6
CD4-Cre/ShcFFF
101 ±33 x 10^6
3%
52%
17%
28%
5%
48%
28%
19%
CD8
CD4
10%
90%
12%
88%
KJ1.26
B
OT-II
Control
106 ±16 x 10^6
49 ± 24 x 10^6
1%
56%
15%
28%
2.5%
51%
25%
21%
25%
38%
34%
42%
27%
19%
Vβ5.1/2
C
DO11.10 ShcFFF
DO11.10 CD4-Cre/ ShcFFF
Thy
Spln
Thy
Spln
IP: Flag
WCL
Blot: Shc
D
OVA peptide induced cell death
DO11.10 ShcFFF
DO11.10 CD4-Cre ShcFFF
% Annexin-V+ DP cells
0
10
20
30
40
50
60
0
10
50
100
250
OVA(323-339) peptide (nM)
% DP recovered
0
10
20
30
40
50
60
70
80
0
10
50
100
250
OVA(323-339) peptide (nM)

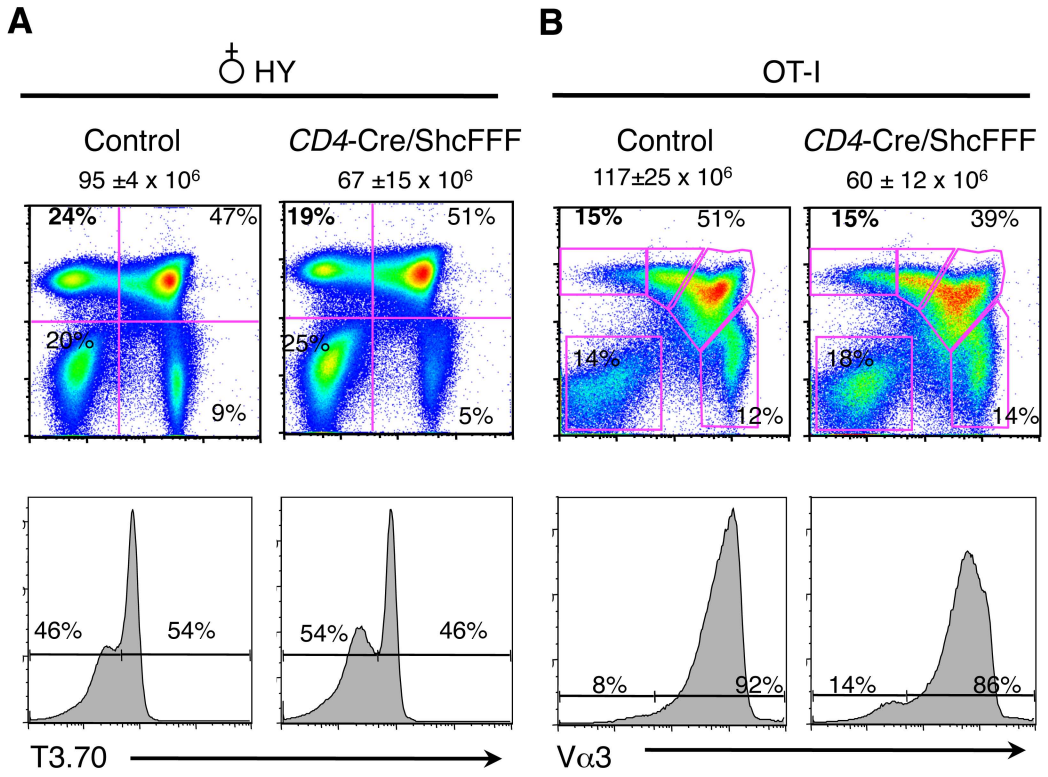
Figure 4.5: ShcA phosphorylation in CD8-lineage development

A) Top panel is surface marker expression of CD4 and CD8 in control and *CD4*-Cre/ShcFFF mice on the HY transgenic background. Bottom panel is cell surface expression of T3.70 to recognize the HY transgenic TCRs. (n=6-8 mice per genotype)

B) Top panel is surface marker expression of CD4 and CD8 in control and *CD4*-Cre/ShcFFF mice on the OT-I transgenic background. Bottom panel is cell surface expression of Vα3 to recognize the OT-I transgenic TCRs. (n=2 mice per genotype)

C) Surface marker expression of CD4 and CD8 in male control and *CD4*-Cre/ShcFFF mice on the H-Y transgenic TCR background.

Experiments performed by Paul Trampont


A
HY
Control
95 ±4 x 10^6
CD4-Cre/ShcFFF
67 ±15 x 10^6
24%
47%
20%
9%
19%
51%
25%
5%
46%
54%
54%
46%
T3.70
B
OT-I
Control
117±25 x 10^6
CD4-Cre/ShcFFF
60 ± 12 x 10^6
15%
51%
14%
12%
15%
39%
18%
14%
8%
92%
14%
86%
Vα3

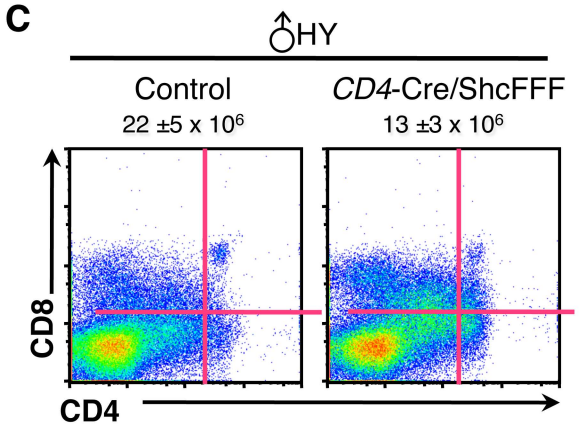

C
HY
Control
22 ±5 x 10^6
CD4-Cre/ShcFFF
13 ±3 x 10^6
CD8
CD4

Figure 4.6: *CD4*-Cre/ShcFFF thymocytes are impaired in DP to SP transition with minor impairment in DP compartment

(A) Surface marker expression of CD4 and CD8 in control and *CD4*-Cre/ShcFFF mice on the LckF505 transgenic background (n=4 mice per genotype).

(B) Cell surface staining for CD4 and CD8 on thymocytes isolated from TCR$\alpha^{-/-}$ control or TCR$\alpha^{-/-}$ *CD4*-Cre/ShcFFF mice.

(C) Left panel, quantification of flow cytometry analysis of Annexin V^- and $7AAD^-$ (percent survival) in DP thymocytes incubated at 37^0 for the indicated time. Right panel, quantification of flow cytometry analysis of of Annexin V^- and $7AAD^-$ (percent survival) in indicated thymic subset after incubation with anti-CD3 for 8 hours.

Experiments performed by Paul Trampont

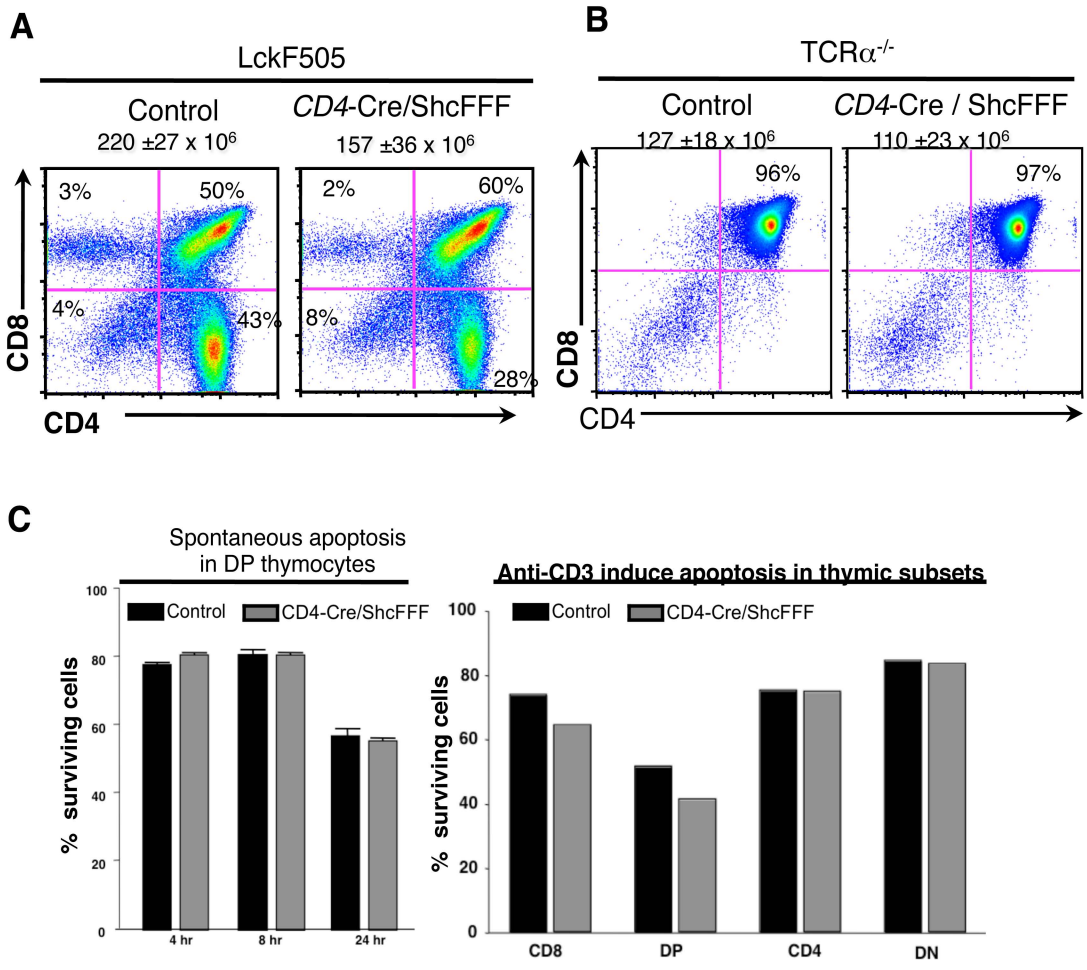

A
LckF505
Control
220 ±27 x 10^6
CD4-Cre/ShcFFF
157 ±36 x 10^6
3%
50%
4%
43%
2%
60%
8%
28%
CD8
CD4
B
TCRα-/-
Control
127 ±18 x 10^6
CD4-Cre / ShcFFF
110 ±23 x 10^6
96%
97%
CD8
CD4
C
Spontaneous apoptosis in DP thymocytes
Control
CD4-Cre/ShcFFF
% surviving cells
4 hr
8 hr
24 hr
Anti-CD3 induce apoptosis in thymic subsets
CD8
DP
CD4
DN

Figure 4.7: Defect in thymic organization and cell trafficking in *CD4*-Cre/ShcFFF mice

(A) Representative H&E staining of thymi isolated from *CD4*-Cre/ShcFFF or control mice. R-value is calculated as the area of the cortex divided by the area of the medulla (Courtesy of Paul Trampont)

(B) Area of the cortex and medulla of thymi isolated from *CD4*-Cre/ShcFFF or control mice (Courtesy of Paul Trampont).

(C) Surface staining for CCR7 on $CD4^+$ $CD8^+$ DP $CD3^{Hi}$ thymocytes isolated from *CD4*-Cre/ShcFFF or control mice (n=2 mice of each genotype).

(D) Migration of $CD4^+$ $CD8^+$ DP $CD3^{Hi}$ thymocytes to CCL19 and CCL21 from *CD4*-Cre/ShcFFF or control mice with representative plot of CD3 expression of gated $CD4^+$ $CD8^+$ DP thymocytes after migration to CCL19 and CCL21.

(E) Quantification of migration of $CD4^+$ $CD8^+$ DP $CD3^{Hi}$ thymocytes from *CD4*-Cre/ShcFFF or control mice to CCL19 and CCL21 (normalized to control migration from each experiment, n>4 mice per genotype)

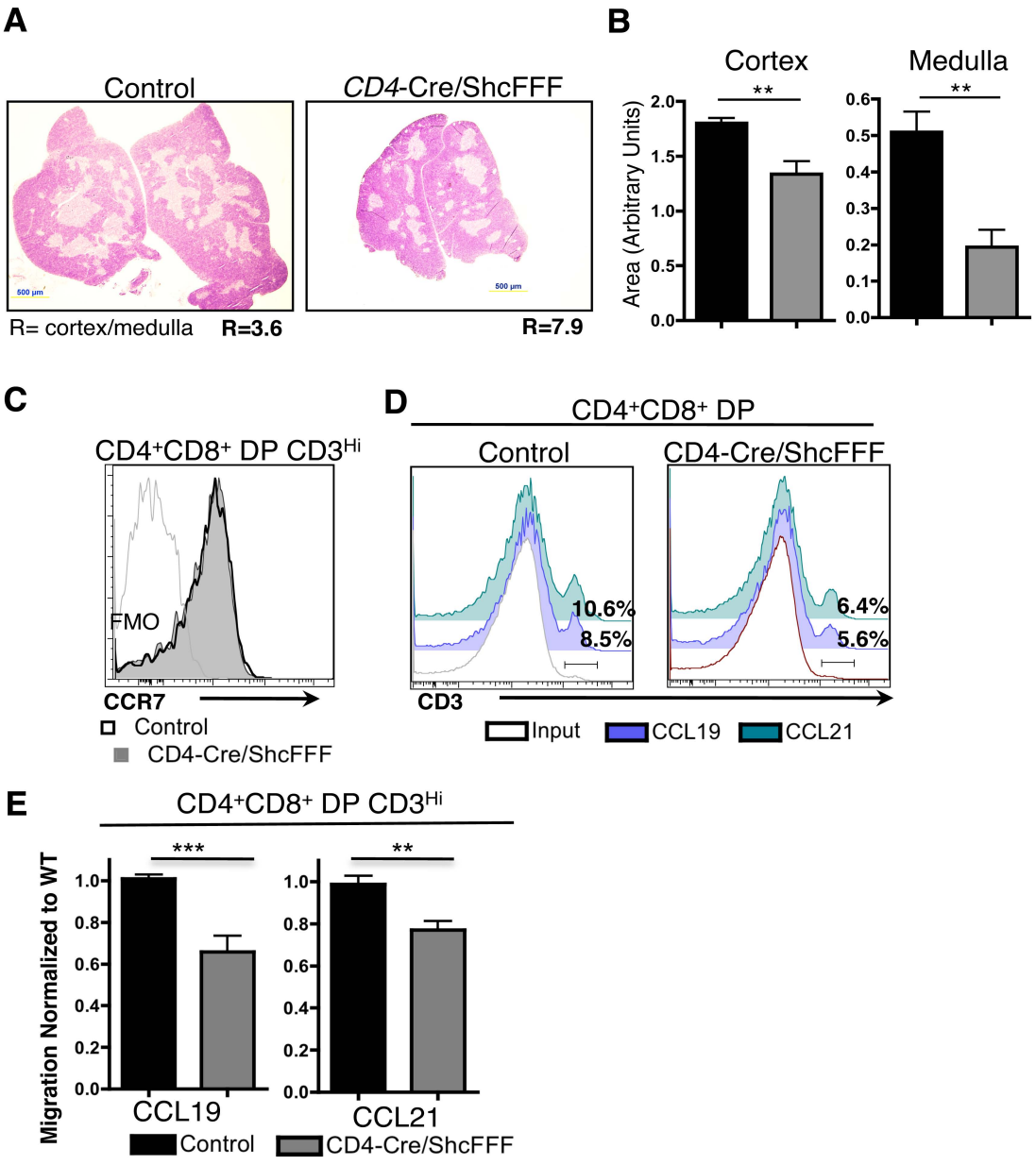

A
Control
CD4-Cre/ShcFFF
R= cortex/medulla R=3.6
R=7.9
B
Cortex
Medulla
Area (Arbitrary Units)
C
CD4+CD8+ DP CD3Hi
FMO
CCR7
Control
CD4-Cre/ShcFFF
D
CD4+CD8+ DP
Control
CD4-Cre/ShcFFF
10.6%
8.5%
6.4%
5.6%
CD3
Input
CCL19
CCL21
E
CD4+CD8+ DP CD3Hi
Migration Normalized to WT
CCL19
CCL21
Control
CD4-Cre/ShcFFF

Figure 4.8: Peripheral T cell compartment in *CD4*-Cre/ShcFFF mice

(A) Left, CD4 and CD8 cell surface staining of cells isolated from the lymph node or spleen of *CD4*-Cre/ShcFFF or control mice. Right, total cellularity and absolute numbers of CD4 and CD8 T cells from the spleen (n>12 mice of each genotype).

(B) Percentage of CD4 T cells and CD8 T cells in the spleen from control or *CD4*-Cre/ShcFFF mice (n>18 mice per genotype)

(C) Cell surface staining for CD62L and CD44 in CD4 splenocytes isolated from control or *CD4*-Cre/ShcFFF mice (n>4 mice per genotype).

(D) Left, flow cytometry of CD4 T cells isolated from control or *CD4*-Cre/ShcFFF mice, stained with CFSE, and activated with anti-CD3/anti-CD28 for 72 hours. Right, representative plot of forward scatter and side scatter in naïve or stimulated CD4 T cells (representative of n>3 experiments).

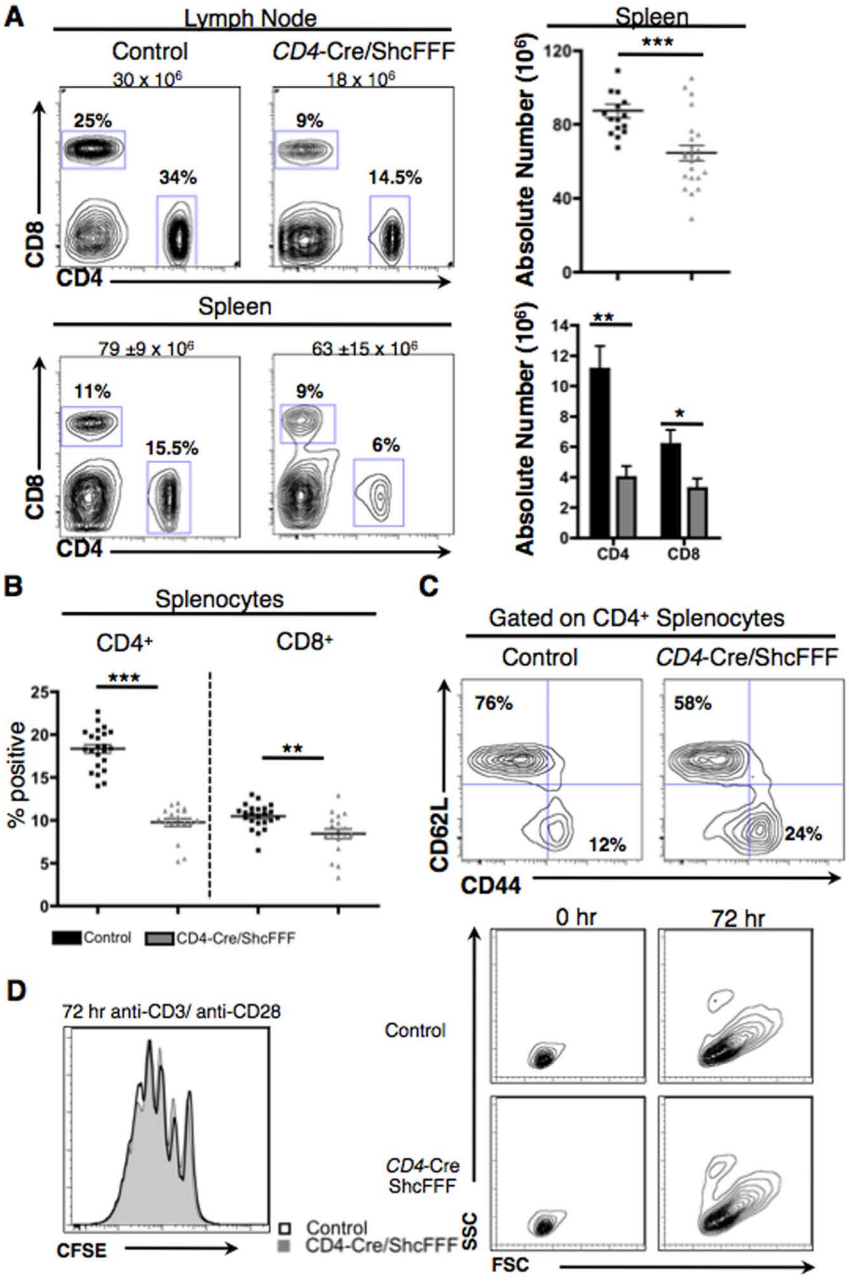

A
Lymph Node
Control
CD4-Cre/ShcFFF
30 x 10^6
18 x 10^6
25%
34%
9%
14.5%
CD8
CD4
Spleen
79 ±9 x 10^6
63 ±15 x 10^6
11%
15.5%
9%
6%
Spleen
***
Absolute Number (10^6)
**
*
CD4
CD8
B
Splenocytes
CD4+
CD8+
***
**
% positive
Control
CD4-Cre/ShcFFF
C
Gated on CD4+ Splenocytes
Control
CD4-Cre/ShcFFF
76%
12%
58%
24%
CD62L
CD44
D
72 hr anti-CD3/ anti-CD28
CFSE
Control
CD4-Cre/ShcFFF
0 hr
72 hr
Control
CD4-Cre ShcFFF
SSC
FSC

Figure 4.9: CD4-Cre/ShcFFF mice have a additional impairment in the peripheral compartment independent of thymic development

(A) Schematic detailing generating of *CD4*-Cre/ShcFFF expressing eGFP under the Rag2 promoter to identify recent thymic emigrants.

(B) Flow cytometry for GFP in the indicated cell subsets from the spleen or thymus of Rag2p-eGFP control or Rag2p-eGFP *CD4*-Cre/ShcFFF mice.

(C) Quantification of the emigration ratio (GFP^+ CD4 or CD8 Splenocytes / GFP^+ CD4 or CD8 SP Thymocytes) from control or *CD4*-Cre/ShcFFF mice.

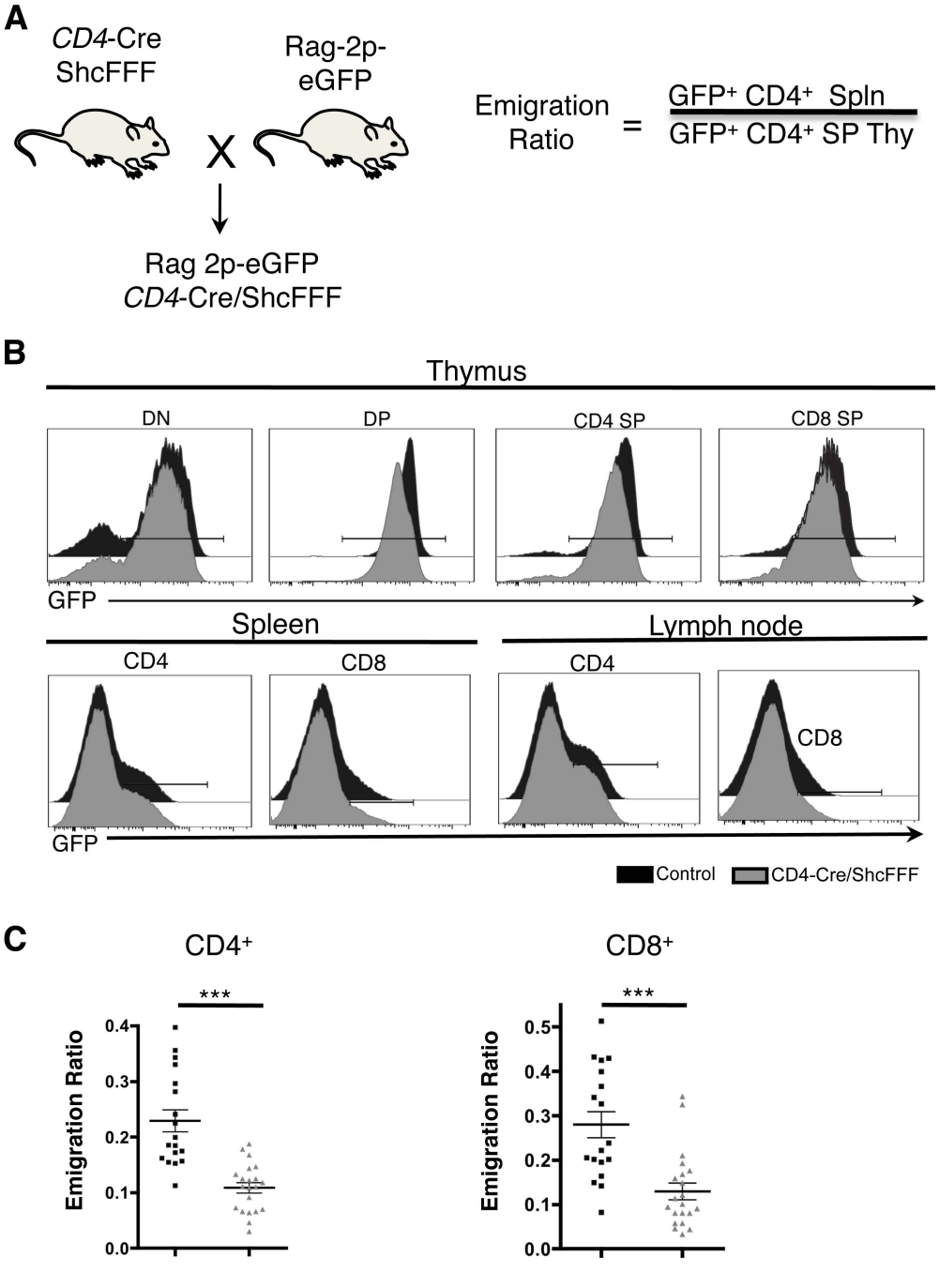

A
CD4-Cre ShcFFF
Rag-2p-eGFP
X
Rag 2p-eGFP CD4-Cre/ShcFFF
Emigration Ratio = GFP+ CD4+ Spln / GFP+ CD4+ SP Thy
B
Thymus
DN
DP
CD4 SP
CD8 SP
GFP
Spleen
Lymph node
CD4
CD8
CD4
CD8
GFP
Control
CD4-Cre/ShcFFF
C
CD4+
CD8+
***
***
Emigration Ratio
Emigration Ratio
0.0
0.1
0.2
0.3
0.4
0.5

Figure 4.10: Peripheral lymphopenia in 10 week old *CD4*-Cre/ShcFFF mice and after EAE immunization

(A) Left, cell surface expression of CD4 and CD8 isolated from the spleen or lymph node isolated from 10-week old *CD4*-Cre/ShcFFF or control mice. Right, absolute numbers of CD4 and CD8 T cells (n=10 mice of each genotype).

(B) Left, cell surface expression of CD4 and CD8 isolated from the draining lymph node of *CD4*-Cre/ShcFFF or control mice on day 8 after $MOG_{(35-55)}$ immunization (n=3, 5 of *CD4*-Cre/ShcFFF or control mice respectively).

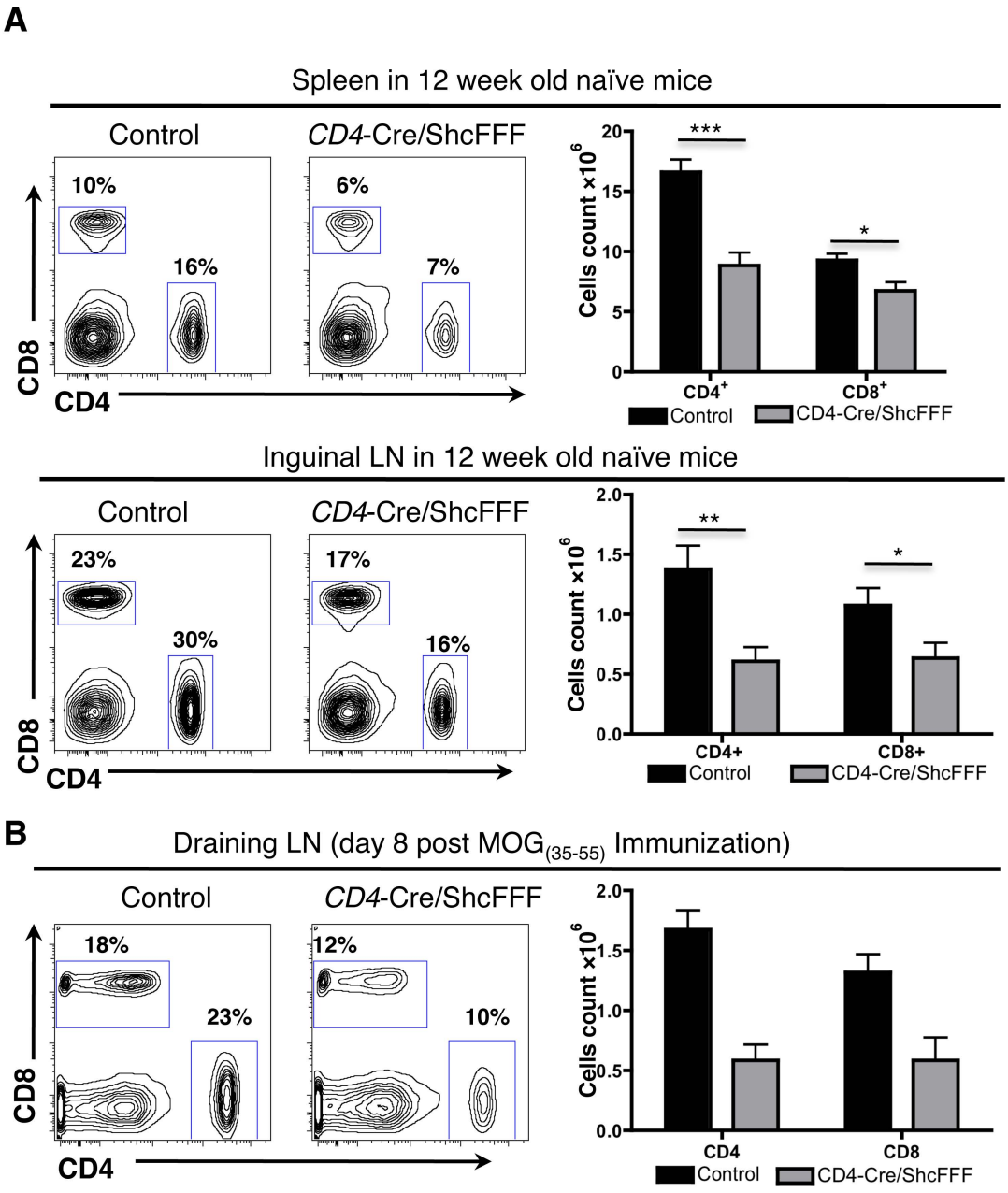

A
Spleen in 12 week old naïve mice
Control
CD4-Cre/ShcFFF
10%
16%
6%
7%
CD8
CD4
Cells count ×10^6
***
*
CD4+
CD8+
Control
CD4-Cre/ShcFFF
Inguinal LN in 12 week old naïve mice
23%
30%
17%
16%
**
*
B
Draining LN (day 8 post MOG(35-55) Immunization)
18%
23%
12%
10%
CD4
CD8

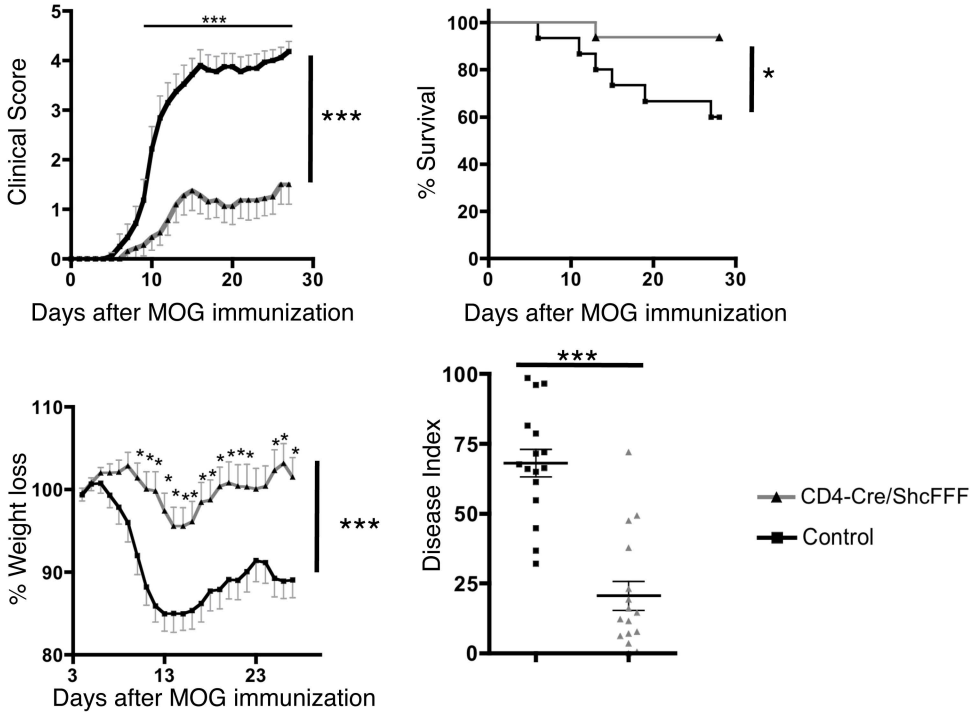
Figure 4.11: *CD4*-Cre/ShcFFF mice are mostly resistant to EAE

(A) (A) Mean clinical scores, survival, weight loss, and disease index of *CD4*-Cre/ShcFFF and control mice immunized for EAE. Compiled data of 3 experiments with a total of n=16 mice of each genotype.

(B) Table of incidence, day of onset, mean maximum score, disease index, and mortality of control and *CD4*-Cre/ShcFFF mice.

(C) Left, schematic of induction of suboptimal EAE. Right, mean clinical scores of *CD4*-Cre/ShcFFF and control mice immunized sub-optimally for EAE, plot is representative of n=3 experiments.

A

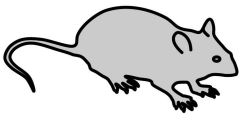


B

Genotype	Incidence	Day of Onset	Mean Max	Disease Index	Mortality
Control	100% (16/16)	10.1 +/- 3.0	4.5+/- .45	68.1 +/- 19.8	37.5% (6/16)
CD4-Cre/ ShcFFF	75% (12/16)	14.1 +/- 5.4	2.4 +/- 1.6	20.5 +/- 20.6	6.3% (1/16)

C

Sub-optimal EAE Induction



75µg $MOG_{(35-55)}$ in CFA subcutaneously
IP injection of 200ηg PTx on day 0

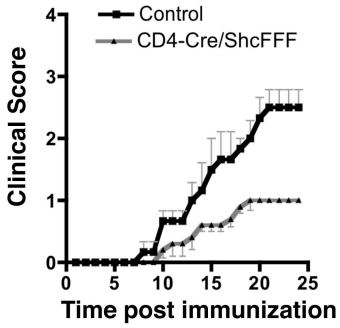


Figure 4.12: *CD4*-Cre/ShcFFF mice have fewer immune infiltrates in spinal cord after EAE immunization

(A) (A) Cell surface staining of CD45, CD4, CD11b, and B220 on cells isolated from the spinal cord and brain of *CD4*-Cre/ShcFFF or control mice on day 28 after MOG immunization (n=3 mice of each genotype).

(B) Percentage of $CD4^+$ and $B220^+$ cells from $CD45^+$ cells isolated from the spinal cord and brain of *CD4*-Cre/ShcFFF or control mice on day 28 after MOG immunization (n=3 mice of each genotype).

(C) Absolute numbers of $CD4^+$, $B220^+$, macrophages, and microglia isolated from the spinal cord and brain of *CD4*-Cre/ShcFFF or control mice on day 28 after MOG immunization (n=3 mice of each genotype).

(D) Left, immunohistochemistry for CD3 and H&E staining on spinal cord sections from CD4-Cre/ShcFFF or control mice on day 28 after immunization with EAE. Right, quantification of number of $CD3^+$ T cells from spinal cord sections (n=6 mice of each genotype).

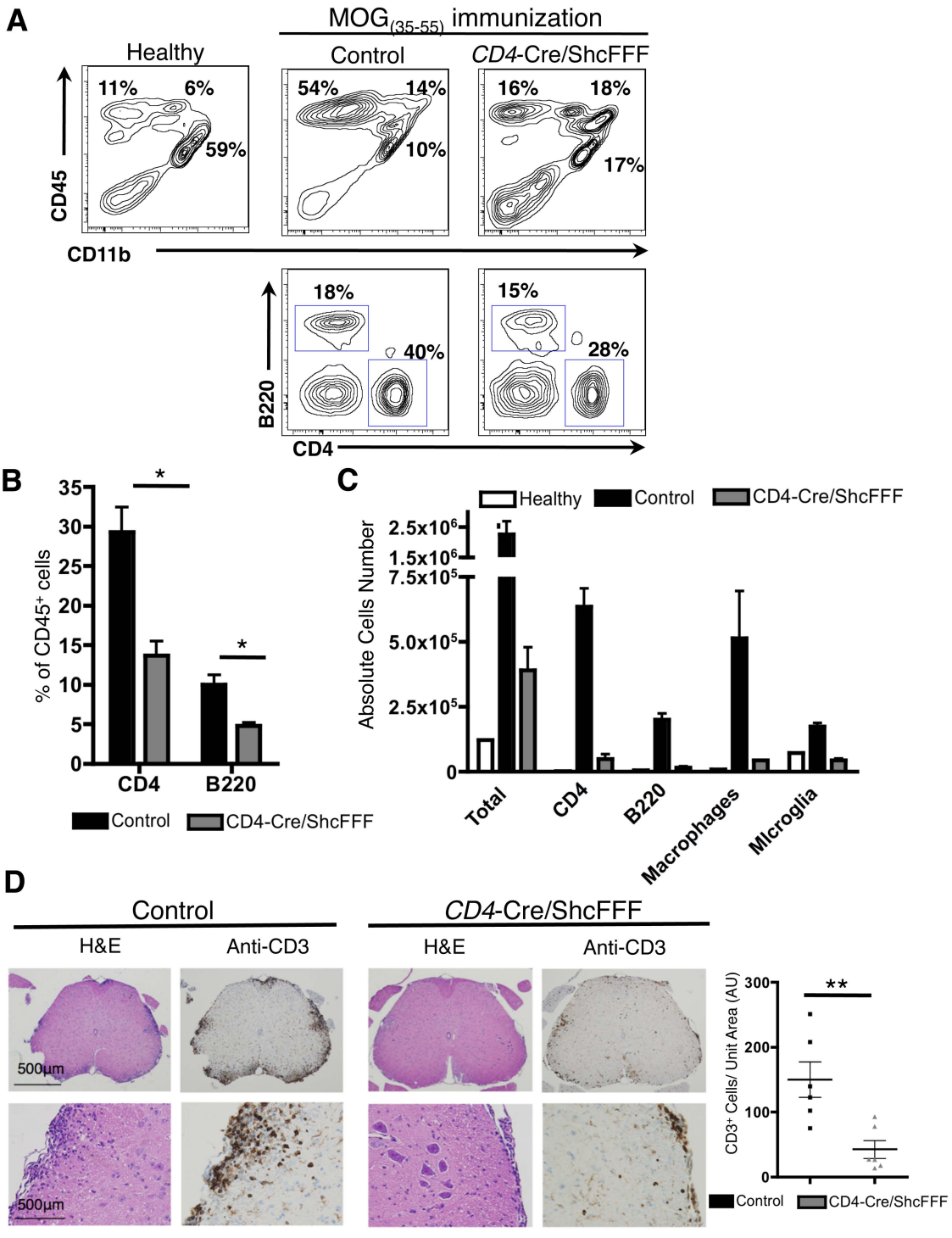

A
MOG(35-55) immunization
Healthy
Control
CD4-Cre/ShcFFF
11%
6%
59%
54%
14%
10%
16%
18%
17%
CD45
CD11b
18%
40%
15%
28%
B220
CD4
B
% of CD45+ cells
35
30
25
20
15
10
5
0
*
*
CD4
B220
Control
CD4-Cre/ShcFFF
C
Healthy
Control
CD4-Cre/ShcFFF
Absolute Cells Number
2.5x10^6
1.5x10^6
7.5x10^5
5.0x10^5
2.5x10^5
0
Total
CD4
B220
Macrophages
Mlcroglia
D
Control
CD4-Cre/ShcFFF
H&E
Anti-CD3
H&E
Anti-CD3
500μm
500μm
CD3+ Cells/ Unit Area (AU)
300
200
100
0
**
Control
CD4-Cre/ShcFFF

Table I: Cellularity in *CD4*-Cre/ShcFFF transgenic mouse lines

Absolute numbers (± standard deviations) of T cell subsets. Thymi and spleen from mice of the indicated genotypes were analyzed by flow cytometry and numbers were determined based on their fractions within the total population. For the transgenic TCR setting, the cells were first gated on cells expressing the transgenic TCR before estimating the specific subsets. C/S refers to *CD4*-Cre/ShcFFF transgenic mouse line.

* refers to n=4 mice analyzed

			DO11.10		OT-II		H-Y		OT-I		dLckF505	
	Littermate control (n=12)	*CD4*-Cre/ ShcFFF (n=12)	Control (n=9)	C/S (n=9)	Control (n=8)	C/S (n=10)	Control (n=6)	C/S (n=8)	Control (n=2)	C/S (n=2)	Control (n=4)	C/S (n=4)
Thymocytes	191 ± 67	119 ± 35	143 ± 27	101 ± 33	106 ± 16	49 ± 24	95 ± 4	67 ± 15	117 ± 25	60 ± 12	220 ± 27	157 ± 36
DN CD4-CD8-	6.3 ± 2.6	5.8 ± 2.2	24 ± 11	23 ± 7	17 ± 6	12 ± 5	19 ± 2	17 ± 6	15 ± 3.5	10 ± 2	7 ± 1	6.6 ± 2
DP CD4+CD8+	146 ± 47.9	90±24.8	74 ± 23	46 ± 20	61 ± 6	27 ± 14	45 ± 3	34 ± 5	57 ± 12	20 ± 4	108 ± 21	97 ± 30
CD4SP	16.9 ± 6	7.8 ± 2.7	42 ± 12	23 ± 8	30 ± 4	11 ± 5	-	-	-	-	110 ± 10	52 ± 12
CD8SP	5.8 ± 2.1	3.3 ± 1.4	-	-	-	-	23 ± 6	12 ± 3	19 ± 3	10 ± 1.5	6.2 ± 1.8	5.5 ± 1.2
Splenocytes	84 ± 11.7	72 ± 17.5	87.5 ± 16	69 ± 17	59* ± 2	54* ± 6	71 ± 18	59 ± 12	87 ± 21	100 ± 29	88 ± 13	70 ± 10
CD4SP	8.9 ± 2.4	3.2 ± 1.8	8.5 ± 1.6	6 ± 2	5 ± 0.2	4 ± 0.4	-	-	-	-	12 ± 1.1	7.7 ± 1
CD8SP	5 ± 1.8	1.7 ± 1.7	-	-	-	-	5 ± 1.2	4.4 ± 2	3.6 ± 1.8	1 ± 0.8	2.1 ± 0.1	1.9 ± 0.5

Chapter V

Summary and Future Directions

1. *Unexpected phenotype of mice lacking Shcbp1, a protein upregulated during T cell proliferation*

1.1. Summary

Shcbp1 is an evolutionarily conserved protein that binds to the adapter protein ShcA. Studies in Drosophila and in cell lines have strongly linked Shcbp1 to cell proliferation, embryonic development, growth factor signaling, tumorigenesis, and the antiviral immune response[10,96,98-101]. However, very little was known about the function of Shcbp1 or the *in vivo* role of Shcbp1 in mammals when I started this project. To study the role of Shcbp1 in T cell development and activation, we generated mice deficient in *Shcbp1* expression either globally or conditionally. Initially, we hypothesized that Shcbp1 was required for the proliferation that occurs during β-selection or during the primary immune response.

First, we investigated the expression of Shcbp1 in the different immune compartments. Shcbp1 is highly expressed in the thymus compared to other immune tissues **(Figure 3.1A)**. Furthermore, the expression of Shcbp1 correlates with the highly proliferative thymic subsets and is upregulated via preTCR signaling **(Figure 3.2)**. Optimal signaling through the preTCR and progression through the β-selection checkpoint requires ShcA, and we found that Shcbp1 induction during preTCR signaling is dependent on signaling through ShcA **(Figure 3.3)**. Therefore, Shcbp1 is expressed in highly proliferative thymic subsets and its induction requires ShcA-mediated signaling[136].

To gain insight into Shcbp1 function, we generated a mouse line deficient in *Shcbp1* to test whether Shcbp1 was required during thymic development **(Figure 3.4,**

3.6). However, we found that Shcbp1 was dispensable for T cell development *in vivo* after investigating several parameters of T cell development including proliferation, survival, migration, and developmental progression **(Figure 3.7, 3.8)**.

We next investigated the role of Shcbp1 in mature T cell activation. We found that *Shcbp1* was induced in $CD4^+$ and $CD8^+$ T cells after anti-CD3/anti-CD28 stimulation *ex vivo* and *in vivo* **(Figure 3.12)**. Furthermore, antigen stimulation and chemical stimulation (PMA/Ionomycin) also leads to upregulation of *Shcbp1* **(Figure 3.12)**. Previous research has shown that Shcbp1 is also upregulated in $CD4^+$ T cells isolated from *CTLA4* deficient[10], which exhibit massive lymphoproliferation leading to lethal autoimmunity[105,106]. Furthermore, we found that *Shcbp1* was induced in immune infiltrates in the spinal cord of mice immunized for experimental autoimmune encephalomyelitis (EAE) and upregulated in $CD4^+$ T cells after IL2 stimulation or T_H17 skewing **(Figure 3.14, 3.17)**. Additionally, Shcbp1 contributes to disease severity in the EAE model as *Shcbp1*-deficient mice develop attenuated disease **(Figure 3.15)**. By transferring *Shcbp1*-defficient T cells into *Rag1* deficient mice, we demonstrated that the attenuated EAE disease phenotype was T cell intrinsic **(Figure 3.16)**.

Lastly, we also investigated the role of Shcbp1 in the context of influenza infection. We found that *Shcbp1* was induced in both $CD4^+$ and $CD8^+$ T cells isolated from the mediastinal lymph node 5 days after intranasal infection with PR8 influenza virus **(Figure 3.18)**. However, surprisingly, *Shcbp1*-deficient mice have a normal immune response to influenza infection with no differences in survival, weight loss, influenza specific $CD8^+$ T cells, or influenza specific antibodies **(Figure 3.19)**. Therefore, although Shcbp1 contributes to pathogenic CD4 T cell activation in the context of EAE, it

appears dispensable for T cell development, proliferation, and the anti-viral immune response.

1.2. Future Directions

Can other pectin-lyase like domain containing proteins compensate for the loss of Shcbp1? Specifically, can Fbox11 compensate for Shcbp1 in the thymus?

Although Shcbp1 is highly expressed in the thymus and correlates with highly proliferative thymic subsets, we found that Shcbp1 is dispensable for T cell development *in vivo* **(Figure 3.7, 3.8)**. Shcbp1 contains a pectin lyase-like domain, which is composed of parallel beta-helix repeats and is a motif found on proteins that bind carbohydrate residues[98]. In the mouse, there are a few other proteins that also contain pectin lyase-like domains including Shcbp1-L, Fbox10, and Fbox11. Furthermore, a recent work has suggested that Shcbp1L may have overlapping functions with Shcbp1 during spermatogenesis[108]. To address whether these proteins compensated for the loss of *Shcbp1*, we performed RT-PCR on control or *Shcbp1*-deficient thymocytes and found that there was no compensatory upregulation and that *Shcbp1L* and *Fbox10* are likely not expressed in the thymus **(Figure 3.8G)**. However, *Fbox11* is highly expressed in the thymus, and it is possible that Fbox11 may compensate for the loss of Shcbp1. Unlike *Shcbp1*, the expression of *Fbox11* does not change during thymic development and its expression does not correlate with highly proliferative thymic subsets[136]. Fbox11 is a ubiquitin ligase that has been shown to interact with proteins involved in regulation of the cell cycle (DCAF) and also regulates cell survival (targets BCL6 for degradation)[167]. However, it is not known whether Fbox11 plays a role in thymic development.

To test this hypothesis, it would be necessary to knockdown the expression of *Fbox11* in both wild-type and in *Shcbp1* deficient thymocytes and perform *ex vivo* T cell development assays. In order to knockdown the expression of Fbox11, we could generate a lentiviral construct expressing an shRNA targeting Fbox11 using the pSicoR vector. The pSicoR vector encodes an IRES-GFP, which allows tracking of cells expressing the shRNA. We used this system to generate a control lentivirus that targets luciferase as well as a lentivirus to knockdown *Shcbp1*, and we observed >70% knockdown after transduction and GFP selection in murine thymocyte cell lines (data not shown). To test whether knockdown of *Fbox11* effects T cell development (in either control or *Shcbp1*-deficient thymocytes), we could transduce DN2 thymocytes with the control or the Fbox11 shRNA lentivirus and perform the OP9-DL1 coculture assay to asses developmental progression, survival, and proliferation. OP9-DL1 is a stromal cell line that ectopically expresses the Notch ligand Delta-like 1 and with the addition of IL7 and Flt3 can support T cell development from immature precursors *in vitro*[168,169].

Could Shcbp1 act as a negative regulator of preTCR signaling?

Another possibility is that Shcbp1 may act as a negative regulator of preTCR signaling. Previous research has shown that negative regulators of preTCR and TCR signaling often do not have obvious phenotypes during T cell development. For example, SHP1 has been shown to be a negative regulator of the TCR, however deletion of SHP1 in late T cell development using the *CD4*-Cre did not interfere with normal T cell development and positive selection[170]. Additionally, a previous proteomics-based study in Rat-2 cells has shown that Shcbp1 binds ShcA maximally in unstimulated cells, but is displaced from ShcA after EGF stimulation[82]. To test this hypothesis, the OP9-DL1 coculture system could be quite useful. The MigR1 vector allows bicistronic expression

of the gene of interest along with eGFP and has been previous used in our laboratory (Trampont *et al*, manuscript in submission). Furthermore, we have already generated a MigR1 vector containing human *Shcbp1*. After generating retroviral particles, we could transduce DN2 thymocytes and plate the thymocytes on the OP9-DL1 monolayer along with Flt3 and IL7. We could then use flow cytometry to determine whether over-expressing Shcbp1 leads to an impairment of T cell developmental progression, proliferation, or survival.

Do Shcbp1 deficient mice have impaired thymic regeneration and repair?

Although we did not observe any differences in T cell development in the absence of Shcbp1, it is possible that *Shcbp1*-deficient mice may have a defect in thymic regeneration and repair. Although the thymus is remarkably resilient to damage, the thymus can be damaged due to stress, steroid treatments, immunodepletion, infection, and pregnancy[171]. Thymic regeneration is crucial to recover a fully competent adaptive immune response. Additionally, previous research has shown that genes and proteins required for thymic regeneration and repair are often dispensable for normal thymopoiesis[171].

To study whether Shcbp1 is required for thymic regeneration, we could use a sublethal total body irradiation (SL-TBI). Mice that have undergone SL-TBI exhibit extensive thymic damage with partial recovery 1 week after irradiation and full recovery approximately 8 weeks after irradiation[171]. Therefore, after SL-TBI on control and *Shcbp1*-deficient mice, we could analyze the total numbers of thymocytes at various time-points after irradiation. As thymic epithelial cells and innate lymphoid cells have been shown to be crucial for thymic regeneration[171], these experiments may also be

performed using the *Lck*-Cre^{+}/*Shcbp1*$^{fl/fl}$ mice to determine whether any effect is T cell intrinisic. Although Shcbp1 is not required for proliferation during thymic development, perhaps the massive thymic regeneration and proliferation that occurs in this model may uncover a role for Shcbp1 in thymic proliferation/regeneration.

Unraveling the role of Shcbp1 in T cell effector function in the context of autoimmunity?

Previous research has shown that *Shcbp1* is upregulated in activated T cells[10] and T cells isolated from *CTLA4* deficient mice exhibit lethal autoimmunity[105,106]. Furthermore, we found that Shcbp1 was induced in spinal cord lesions from mice that were immunized to induce EAE **(Figure 3.14)**. Our studies demonstrated that *Shcbp1* expression in the CD4^{+} T cell compartment contributes to disease severity in the context of autoimmune EAE **(Figure 3.15)(Figure 3.16)**. Therefore, we hypothesize that Shcbp1 is required for optimal CD4^{+} T cell effector functions in the context of autoimmunity.

To further test the requirement for Shcbp1 in CD4^{+} T cells in EAE, we could first analyze the T cell responses during the initiation stage of EAE. We would immunize control or *Shcbp1* deficient mice for EAE and isolate the spleen, draining lymph nodes, and mononuclear cells from the CNS on day 8 after immunization. At this time-point, most of the MOG specific CD4^{+} T cells are still within the secondary lymphoid organs and have not migrated to the CNS (and therefore most of the mice do not exhibit signs of disease at this time-point). This system would allow the study of different effector functions of T cells: activation, cytokine secretion, proliferation, and migration. Although we did not detect any impairment in T cell development, proliferation, skewing, or

effector functions *ex vivo*, it is possible that there may be minor defects *in vivo* that leads to the reduced disease severity in the EAE model.

First, we would closely analyze the absolute numbers and percentages of T cells in the spleen, draining lymph node, and CNS. If we find differences in the absolute numbers of T cells, this would indicate that *Shcbp1* deficient T cells might have a defect in T cell activation and proliferation *in vivo*. To further evaluate whether there was a proliferative defect, we could isolate $CD4^+$ T cells from the spleen after immunization and perform MOG specific proliferation assays. Although we did not find a proliferative defect after anti-CD3/anti-CD28 stimulation **(Figure 3.13)**, it is possible that this more physiological antigen specific stimulation may reveal a proliferative defect.

Next, we could investigate IL17 skewing and cytokine secretion *in vivo*. Although we did not defect any defects in T_H17 skewing *ex vivo* **(Figure 3.17E,F)**, it is possible that there are defects in T_H17 skewing *in vivo*. After EAE immunization, we would perform PMA/Ionomycin restimulation and intracellular staining for IL17 and IFNγ and closely evaluate the percentage of IL17 or IFNγ positive $CD4^+$ T cells. Additionally, we would restimulate splenocytes with MOG_{35-55} for 5 days and collect the supernatants for IL17 to analyze via ELISA. Collectively, these experiments would address whether there is a defect in T_H17 skewing *in vivo*.

Lastly, we would use this model to study whether there were any migratory defects of $CD4^+$ T cells to the CNS. In EAE, $CD4^+$ T cells migrate to the CNS via CCR6/CCL20 mediated migration[172]. We found that there were fewer CD3 positive cells in the spinal cords of *Shcbp1* deficient mice immunized for EAE but we did not observe any defects in CCL20 mediated migration of *Shcbp1* deficient T_H17 T cells *ex vivo* (data not shown). To address whether there was a migratory defect in *Shcbp1* deficient T cells

in the context of EAE, we could analyze the numbers of $CD4^+$ T cells in both the draining lymph node, spleen, and within the CNS on day 8 after immunization. If equivalent numbers of $CD4^+$ T cells are seen within the spleen/lymph node but a decrease in the numbers of $CD4^+$ T cells in the spinal cord and brain, it would be suggestive of a migratory defect. These above experiments may provide more insight into the potential role of Shcbp1 in CD4 T cell effector responses in the context of EAE. After this initial experiment, we would follow up with appropriate experiments as needed.

Furthermore, other models of autoimmunity could be used to determine whether Shcbp1 contributes to disease severity in other autoimmunity models. Previously, it has been shown that *CTLA4* deficient mice exhibit lethal autoimmunity, and the T cells isolated from these mice have increased expression of Shcbp1[10]. Therefore, we could cross the *Shcbp1* deficient mice to the *CTLA4* deficient mice to determine whether loss of Shcbp1 attenuates the severity of autoimmunity and leads to prolonged survival. Other models of autoimmunity including the pristane model or systemic lupus erythematosus (SLE) or crossing to the autoimmune prone *FcγR2B* deficient mice could be used to further investigate the role of Shcbp1 in autoimmunity (see Appendix).

Does Shcbp1 play a role in tumorigenesis?

Recent studies have shown that *Shcbp1* is highly expressed in tumors and may contribute to tumor growth and progression. In breast cancer tumors from young women, who typically have more aggressive tumors and worse prognosis, *SHCBP1* was found to be upregulated in ductal carcinoma *in situ* and invasive ductal carcinoma[97]. Additionally, SHCBP1 is upregulated in HCC tumor samples as compared to adjacent tumor free tissues, and knockdown of *SHCBP1* in HCC cell lines reduced cell proliferation and

colony formation[100]. Furthermore, *SHCBP1* is also upregulated in leukemic cells as compared to normal immune cells[110,111]. Therefore, I hypothesize that Shcbp1 may be involved in tumor proliferation and invasion.

To study the potential role of Shcbp1 in tumorigenesis, we could utilize a genetic mouse model that leads to the generation of tumors via the expression of the Kras-G12D point mutation[173]. The $Kras^{LSL\text{-}G12D}$ transgenic mouse line carries a lox-stop-lox sequence, which allows the specific expression of the oncogenic Kras-G12D protein via Cre-mediated recombination and leads to the generation of tumors within that cell type[173]. Previous research has shown that crossing this mouse line to the *Lck*-Cre transgenic mouse line leads to the emergence of T cell leukemia in the transgenic mice with 100% penetrance and average development of T-ALL 180 days after birth[174]. Therefore, we could cross the *Lck*-Cre^{+}/$Shcbp1^{fl/fl}$ mouse line to the $Kras^{LSL\text{-}G12D}$ transgenic mouse line to determine whether Shcbp1 is involved in the development or pathogenesis of T-ALL. We would monitor for the emergence of T-ALL via flow cytometry analysis of the peripheral blood (T-ALL can be recognized by the presence of leukemic blasts on blood smear and immature $CD4^{+}$ $CD8^{+}$ T cells in the peripheral blood)[174]. From these studies, we would determine whether Shcbp1 plays a role in the emergence or pathogenesis of T-ALL. Furthermore, this same model could be used to study the role of Shcbp1 in other tumors, including breast cancer.

Could Shcbp1 play a role in spermatogenesis? Do Shcbp1L and Shcbp1 have overlapping functions during spermatogenesis?

Previous studies have shown that the Drosophila homolog of Shcbp1, Nessun Dorma, is required for cytokinesis during spermatogenesis and Nessun Dorma-

deficient male drosophila are partially infertile[98]. Furthermore, Shcbp1L was recently identified as a testicular-predominantly-expressed protein that is involved in maintenance of the meiotic spindle during spermatogenesis. *Shcbp1L* deficient male mice are fertile and have no obvious defects, but close analysis demonstrated that they have lower sperm counts, an increase in the number of abnormal spermatocytes, and a slightly reduced litter size when crossed to wild-type female mice[108]. Our studies indicate that *Shcbp1* deficient male mice are fertile **(Figure 3.5C)**, although we did not thoroughly analyze spermatogenesis and it is possible that *Shcbp1*-deficient mice may have subtle defects in spermatogenesis. Given these previous studies, we hypothesize that Shcbp1 may be involved in spermatogenesis and Shcbp1L and Shcbp1 may have overlapping functions during spermatogenesis.

To follow up, we could closely analyze for any defect in spermatogenesis in male *Shcbp1* deficient mice. We could set up crosses of *Shcbp1* deficient male mice to wild-type control female mice and evaluate the numbers of pups per litter. Furthermore, we would perform histological analysis of the testis via H&E and PAS staining, as previously described, to evaluate the overall structure and numbers of spermatids[108]. Since previous research has shown that *Shcbp1L* deficient mice have defects in spermatogenesis and Shcbp1L shares 35% similarity to Shcbp1, we would next cross the *Shcbp1L* deficient mouse line to the *Shcbp1* deficient mouse line. It is possible that Shcbp1 and Shcbp1L have overlapping functions during spermatogenesis, and male mice deficient in both Shcbp1L and Shcbp1 may be infertile. These experiments would evaluate the potential for a role of Shcbp1 in spermatogenesis.

2. ShcA phosphorylation is required for late T cell development

2.1. Summary

T cell development in the thymus is a highly regulated process that critically depends upon productive signaling via the preTCR at the β-selection stage and via the TCR during positive and negative selection. Propagation of signals downstream of the preTCR and TCR requires adapter proteins to help assemble signaling complexes[2-6]. ShcA is an adapter protein that is expressed in thymocytes and is required for optimal preTCR signaling and progression through the β-selection checkpoint[7,8]. Additionally, we have found that ShcA phosphorylation is required for productive signaling through the TCR and later stages of T cell development **(Figure 4.2)**.

To investigate the role of ShcA in late T cell development, we generated *CD4*-Cre$^+$/ShcFFF transgenic mice, which express the phosphorylation defective ShcFFF transgene from the late DN4/early DP stage of thymocyte development **(Figure 4.1A)**. We found that *CD4*-Cre$^+$/ShcFFF transgenic mice have impaired thymic development with a reduction in the total number of thymocytes and a severe impairment in the absolute numbers and percentages of CD4$^+$ SP thymocytes **(Figure 4.2)**. Further analysis demonstrated that *CD4*-Cre$^+$/ShcFFF transgenic mice have a reduction in the percentage of post-positively selected TCR^{hi}CD69^{hi} and TCR^{hi}CD69^{lo} thymocytes **(Figure 4.3)**. Additionally, H&E staining demonstrated that the thymic architecture was considerably altered in *CD4*-Cre$^+$/ShcFFF transgenic mice with a disorganized and fragmented medulla **(Figure 4.7)**.

To further investigate the role of ShcA in positive selection, we crossed the *CD4*-Cre$^+$/ShcFFF transgenic mouse line to MHC class I-restricted and MHC class II-restricted transgenic TCR mouse lines. We found that the defects were more

pronounced on the transgenic TCR mouse lines, and that there was a defect in signaling via MHC class II-restricted transgenic TCRs **(Figure 4.4, 4.5)**. Therefore, the *CD4*-Cre^{+}/ShcFFF transgenic mouse line has a defect in positive selection and signaling via transgenic TCRs.

Next, we investigated the peripheral T cell compartment in *CD4*-Cre^{+}/ShcFFF transgenic mice. We found that *CD4*-Cre^{+}/ShcFFF transgenic mice have a significant peripheral lymphopenia, and this peripheral lymphopenia persists with age and after immunization **(Figure 4.8, Figure 4.10)**. Additionally, we found that the peripheral lymphopenia results in functional deficits as the *CD4*-Cre^{+}/ShcFFF mice develop attenuated disease in the EAE mouse model of MS **(Figure 4.11, 4.12)**.

Below, I have provided future directions for this project. The future directions are mainly focused on (1) investigating the mechanistic role of ShcA during positive selection and (2) further dissecting the role of ShcA in the peripheral T cell compartment. The use of the *CD4*-Cre/ShcFFF transgenic mouse line complicates the analysis of ShcA function in peripheral T cells as we have found that many of the peripheral T cells in the *CD4*-Cre/ShcFFF transgenic mice do not express the ShcFFF transgene encoded protein **(Figure 4.1)**. Therefore, the focus of these future directions in the peripheral T cell compartment will be on analyzing the role of ShcA in peripheral T cell functions via the use of two new genetic models: the distal *Lck*-Cre transgenic mouse line and the *CD4*-CreER^{T2} transgenic mouse line[175,176].

2.2. Future Directions

Further investigating the role of ShcA during positive selection

Our findings have shown that ShcA is required for optimal signaling via the TCR at the DP stage of development and *CD4*-Cre/ShcFFF transgenic mice have defects in positive selection. Previous research has shown that ShcA is required for optimal preTCR signaling and Erk activation in DN thymocytes[7-9]. Furthermore, Erk activation has an essential role during positive and negative selection, and positive selection requires sustained Erk activation[36, 185]. Therefore, we could test whether ShcA is required for optimal Erk activation in DP thymocytes via flow cytometry. We would isolate thymocytes from control and *CD4*-Cre/ShcFFF mice and perform intracellular staining for pErk and Erk in thymocytes. To further investigate the role of ShcA during positive selection and CD4 versus CD8 lineage commitment, we would next investigate the expression of transcription factors involved in these processes including GATA-3, Tox, Egr-1, c-Myb, ThPOK, and Runx3. Collectively, these experiments would provide more insight in the mechanistic requirement for ShcA during late T cell development.

Role of ShcA in the peripheral T cell compartment

To more fully explore the role of ShcA in peripheral T cells *in vivo* and *ex vivo*, we would generate two new transgenic mouse lines: distal *Lck*-Cre^{+}/ShcFFF and *CD4*-CreER^{T2}/ShcFFF[175,176]. The distal *Lck*-Cre transgenic mouse line expresses Cre under the *lck*-distal promoter from the SP stage of thymocyte development[176]. The *CD4*-CreER^{T2} transgenic mouse line expresses a tamoxifen inducible Cre recombinase under the control of the *CD4* gene promoter and can be inducibly activated via tamoxifen treatment both *in vivo* and *ex vivo*[175]. Importantly, using this model, only mature CD4^{+}

not $CD8^+$ T cells will express Cre[175]. Therefore, the distal *Lck*-Cre transgenic mouse line will allow us to investigate the role of ShcA beyond the positive selection checkpoint[176]. However, if ShcA signaling is required for thymic egress, in order to study the role of ShcA in peripheral $CD4^+$ T cell function we would use the inducible *CD4*-$CreER^{T2}$ transgenic mouse line.

Evaluate the role of ShcA in SP thymic development

First, we would evaluate thymic development in the distal *Lck*-Cre^+/ShcFFF transgenic mouse line. Since the distal *Lck*-Cre transgenic mouse line mediates Cre recombinase expression after positive selection in SP cells, this transgenic mouse line would allow us to investigate the role of ShcA in SP thymocytes without complications due to the role of ShcA in the DP to SP transition[176]. We would evaluate the absolute numbers and percentage of the $CD4^+$ and $CD8^+$ SP compartments. Furthermore, we would investigate the maturation of $CD4^+$ and $CD8^+$ SP thymocytes via cell surface staining for CD24, CD69, Qa2, and CD69. If we found that there was a decrease in the percentage or numbers of $CD4^+$ and $CD8^+$ SP thymocytes in distal Lck-Cre^+/ShcFFF transgenic mice, we would further investigate whether ShcA is required for SP thymocyte maturation and survival. However, if we found that there was an increase in the numbers and percentage of SP thymocytes, we would further investigate whether ShcA is required for thymic emigration as outlined below. Due to our previous findings, we hypothesize that ShcA is likely required for thymic emigration but dispensable for thymic survival *in vivo* **(Figure 4.6, Figure 4.9)**.

Is ShcA required for thymic egress?

Next, we would investigate whether ShcA is required for thymic emigration. Mature SP thymocytes exit the thymus at the medulla and emigration of thymocytes requires signaling via S1P receptor[39,40,177]. The S1P receptor is expressed on mature single positive thymocytes and the ligand S1P is found in high concentrations in the peripheral circulation[40]. Impairment in thymic emigration often leads to accumulation of SP thymocytes in the thymus as well as very few peripheral T cells in the blood, lymph nodes, and spleen[39]. Therefore, first we would closely evaluate the numbers and percentages of SP thymocytes in the thymus and peripheral T cells in the distal *Lck*-Cre^{+}/ShcFFF transgenic mice. Next, we would perform *ex vivo* S1P migration assays using the transwell assay[39]. We have already optimized the conditions for *ex vivo* S1P migration of CD62L^{HI} CD4 SP thymocytes and have found a very minor defect in S1P migration in *CD4*-Cre^{+}/ShcFFF transgenic mice (data not shown).

Is ShcA required for T cell proliferation?

We would further investigate the role of ShcA in peripheral T cell functions. In order to study peripheral T cell functions, we would use an inducible *CD4*-CreER^{T2}/ShcFFF transgenic mouse line. Additionally, if we find that the distal *Lck*-Cre^{+}/ShcFFF transgenic mouse line does not have any defects in thymic development and emigration, we could also use this transgenic mouse line to study the role of ShcA in peripheral T cell function *in vivo*.

First, we would use the *CD4*-CreER^{T2}/ShcFFF transgenic mouse line to study the role of ShcA in CD4^{+} T cell proliferation. CD4^{+} T cells isolated from the *CD4*-Cre^{+}/ShcFFF transgenic mice did not have a proliferate defect *ex vivo*, which is

likely because most of the $CD4^+$ T cells are Cre escapees and do not express the ShcFFF transgene **(Figure 4.8)**. We would use tamoxifen to inducibly express ShcFFF specifically in $CD4^+$ T cells *ex vivo* and perform anti-CD3/anti-CD28 proliferation assays. Furthermore, we could also administer tamoxifen *in vivo* to study the role of ShcA in T cell proliferation and function *in vivo*.

Is ShcA required for T cell skewing?

Next, we would investigate the role of ShcA in peripheral T cell skewing. Our previous studies have shown that *CD4*-Cre^+/ShcFFF transgenic mice have peripheral lymphopenia and develop attenuated disease in the T_H17 CD4 T cell driven EAE mouse model of MS. However, interestingly, we found that the *CD4*-Cre^+/ShcFFF transgenic mice develop exacerbated inflammation in a T_H2 driven model of airway inflammation (data not shown). Therefore, we hypothesize that ShcA may be involved in T cell skewing; ShcA may be required for T_H17 skewing but is likely dispensable for T_H2 skewing as *CD4*-Cre^+/ShcFFF transgenic mice develop exacerbated airway inflammation. To investigate the role of ShcA in T cell skewing, we would treat *CD4*-$CreER^{T2}$/ShcFFF $CD4^+$ T cells with tamoxifen and perform T cell skewing *ex vivo*.

Collectively, these experiments would provide insight into the role of ShcA in peripheral T cell functions. Depending on these initial experiments, we could plan experiments to test the role of ShcA in peripheral T cell functions *in vivo*. To test the role of ShcA in T_H17 cell *in vivo*, we could use the EAE model or colitis models (transfer of CD25 depleted $CD4^+$ T cells to *Rag1* deficient mice). Additionally, to test the role of ShcA in T_H2 cells *in vivo*, we could utilize the T_H2 driven model of airway inflammation.

Appendix I

Analysis of B cell development in Shcbp1 deficient mice

1. Abstract

B cell development is a highly regulated process that involves the coordinated expression of cell surface receptors and multiple selection processes. Productive signaling through cell surface receptors is essential for the progression of developing B cells through developmental checkpoints and the generation of mature B cells[178]. The adapter protein ShcA is required for early B lymphopoiesis; deletion of ShcA or expression of a dominant negative ShcFFF transgene leads to developmental defects in the transition from pre-pro B cells to pro-B cells[179]. Shcbp1 is a binding partner of ShcA and is expressed during B lymphopoiesis, and we hypothesized that Shcbp1 may be required during B cell lymphopoiesis.

2. Introduction

B cell development is a highly regulated process, and proper B cell development is essential for a normal humoral immune response[178]. Defects in B cell development, such as in individuals with X-linked agammaglobulinemia (XLA), leads to increased susceptibility to infections and the development of serious and even fatal infections[180]. Furthermore, dysregulation of B cell development and function can lead to the development of lymphoproliferative disorders, such as B-cell acute lymphoblastic leukemia (B-ALL), or autoimmunity[181]. Therefore, understanding the molecular mechanisms of B cell development is potentially relevant to human health.

2.1. *B cell development*

In the post-natal animal, B cell development occurs in the bone marrow. B cell development involves the coordinated expression of multiple cell surface receptors and proper signaling through these receptors is essential for progression through developmental checkpoints. This section will review the stages of B cell development.

2.2. *B cell development from HSCs to mature B cells*

B cells originate from hematopoietic stem cells (HSCs), which have the potential to develop into all the hematopoietic lineages and have self-renewing capacity. HSCs can develop into common lymphoid progenitors (CLPs), which still retain other lineage potential but are B-lineage specified[178]. Furthermore, CLPs are actively rearranging the Ig heavy chain through D_H-J_H recombination and also express the IL7 receptor, which is essential for B cell development. CLPs develop into pre-pro B cells, and pre-pro B cells continue D_H-J_H recombination and are B lineage committed[178]. Pre-pro B cells develop into pro B cells, and this transition requires signaling through the IL-7 receptor. Pro-B cells express the CD19 coreceptor and undergo V_H-DJ_H recombination. Proper rearrangement leads to the expression of the heavy chain of the BCR, which can be found in the cytoplasm of pro-B cells[178]. The Ig heavy chain binds to the surrogate light chain, forming the pre-BCR, and signaling occurs using the Igα/Igβ heterodimer[178,182].

Proper expression and signaling through the pre-BCR is essential for developmental progression, and this developmental checkpoint is similar to the β-selection checkpoint in T cell development[182]. Signaling through the pre-BCR leads to further development, proliferation, and survival[182]. In fact, disruption of any component of the pre-BCR or its signaling leads to developmental arrest. However, cells that

successfully proceed through the pre-BCR checkpoint become pre-B cells[182]. Early or large pre-B cells are rapidly proliferating cells, and this proliferation leads to expansion of cells expressing functional Ig heavy chains. Proliferation is shutdown as cells become late or small pre-B cells and initiate rearrangement of the light chain of the BCR[182]. Proper rearrangement of the Ig light chain leads to the expression of IgM on the cell surface. As in T cell development, B cell development continues in the periphery. Immature B cells exit the bone marrow and undergo further maturation within the spleen to become mature B cells, which express both IgM and IgD on the cell surface[178].

2.3. *The adapter protein ShcA in B cell development*

During B lymphopoiesis, optimal signaling through cell surface receptors is required for progression through developmental checkpoints. Adapter proteins are critical for propagating signals from activated receptors by acting as scaffolds and recruiting other proteins into the signaling complex. Adapter proteins are required at multiple stages during B cell development and function, and deletion of adapter proteins during B cell development often leads to defects in the pre-BCR checkpoint and the progression of pro-B cells to pre-B cells. The adapter protein ShcA is a ubiquitously expressed adapter protein that is required during early B cell development prior to the pre-BCR checkpoint[179].

The adapter protein ShcA is a ubiquitously expressed adapter that signals downstream of several different receptors including the BCR, TCR, cytokine receptors, chemokine receptors, and growth factor receptors[71]. After receptor activation, ShcA is phosphorylated on three critical tyrosine residues and ShcA dependent signaling leads to the activation of the MAPK cascade[71]. Using genetic models to specifically express

the dominant negative ShcFFF transgene or conditionally delete ShcA in CLPs (*Mb1*-Cre/ShcFFF or *Mb1*-Cre/*Shc*$^{fl/fl}$), our laboratory demonstrated that ShcA was required during early B lymphopoiesis and the pre-pro B cell to pro B cell transition[179]. Furthermore, ShcA phosphorylation is required for optimal IL7 signaling in pro B cells[179]. ShcA is phosphorylated downstream of the IL7-receptor, and IL7 signaling is crucial for proliferation, survival, and lineage commitment during early B cell development[179,183]. In pro-B cells expressing the ShcFFF transgene, there is impairment in cell survival with increased levels of apoptosis. Therefore, ShcA is required during early B lymphopoiesis and expression of the ShcFFF transgene disrupts optimal IL7 signaling[179].

3. Results

3.1. *Shcbp1 is expressed in during B cell lymphopoiesis*

In gene expression databases, *Shcbp1* also appears to correlate well with actively proliferating cells of the immune system. We examined the expression profile of *Shcbp1* in the different B cell subsets using data from the publicly available Immunological Genome Project Database[136]. *Shcbp1* is expressed during B cell lymphopoiesis and its expression closely correlates with stages during which developing B cells are highly proliferative[136]. *Shcbp1* is upregulated approximately 3-fold in the transition from pro-B cells to large pre-B cells **(Figure A.1)**. Large pre-B cells are highly proliferative, and this proliferation expands cells that have properly arranged the Ig heavy chain. This upregulation is similar to the upregulation of *Shcbp1* in the transition from DN3a to DN3b thymocytes. Additionally, the expression of *Shcbp1* is very low in mature B cells and peripheral B cells isolated from the lymph node. *Shcbp1* is also upregulated in germinal center B cells as compared to mature B cells, and germinal

center B cells are undergoing rapidly proliferation and differentiation within the germinal centers. In comparison, the expression of *ShcA* does not change appreciably during B cell development and activation **(Figure A.1)**. Therefore, the expression of *Shcbp1* seems to correlate with rapidly proliferating subsets during B cell development and during B cell activation[136].

3.2. *Loss of Shcbp1 does not lead to an obvious impairment in B cell development*

Given the expression of Shcbp1 during B lymphopoiesis, we hypothesized that Shcbp1 may be required for B cell development *in vivo*. Additionally, Shcbp1 is upregulated in cycling pre-B cells, and we hypothesized that Shcbp1 may be required for progression through the pre-BCR checkpoint and/or the rapid proliferation of pre-B cells. To investigate the role of Shcbp1 in B cell development *in vivo*, we used global *Shcbp1* deficient mice and analyzed B lymphopoiesis in the bone marrow using flow cytometry as detailed in **Figure A.2**. We analyzed the percentage and absolute numbers of the HSC, LMPP, CLP, pre-pro B, pro-B, pre-B, immature B cells, and mature B cells in the bone marrow **(Figure A.3)**. However, there was no striking impairment in B cell development in the *Shcbp1* deficient mice, and the absolute numbers and percentages of each subset were comparable **(Figure A.3)**. Importantly, the number and percentage of pre-B cells were unchanged, so Shcbp1 is likely not required for optimal signaling through the pre-BCR. Furthermore, there was no impairment in the progression of pre-pro B cells to pro-B cells and the total number of pro-B cells was largely unchanged. Of note, the transition from pre-pro to pro-B cells is impaired in *Mb1*-Cre/ShcFFF mice.

3.3. *Shcbp1 deficient mice have normal percentages of peripheral B cells and total IgG levels*

Next, we analyzed the absolute numbers and percentage of $B220^+$ $CD19^+$ B cells in the spleen. We found that the absolute numbers of splenocytes were unchanged in *Shcbp1* deficient mice and there was no difference in the percentage of $B220^+$ $CD19^+$ B cells **(Figure A.3)**. Furthermore, to investigate the function of B cells in a naïve mouse, we analyzed the total IgG levels of 10-week old *Shcbp1* deficient and wild-type mice. We found no differences in the levels of total IgG in naïve *Shcbp1* deficient and wild-type mice **(Figure A.3)**.

3.4. *Shcbp1 deficient mice generate normal IgG antibodies after influenza infection*

Furthermore, to investigate whether there was impairment in the function of B cells during the primary immune response in the context of infection, we investigated the humoral immune response during influenza infection. The humoral immune response is crucial for the clearance of the influenza virus as well as protection from re-infection. After infection with a low dose of PR8 influenza virus, we found that the *Shcbp1* deficient mice generated a normal humoral response, with equivalent levels of total and influenza specific IgG **(Figure 3.19)**. Therefore, the *Shcbp1* deficient mice have no defects in B cell development or the generation of specific antibodies after influenza infection.

4. Discussion and suggested future studies

Although Shcbp1 is highly expressed in proliferative subsets during B cell development and in germinal center B cells, it appears that Shcbp1 is dispensable for B

cell development *ex vivo* **(Figure A.3)**. Additionally, we found that *Shcbp1*-deficient mice generated a normal humoral response in the context of influenza infection **(Figure A.3)**. However, we have also shown that Shcbp1 contributes to disease severity in the context of autoimmunity **(Figure 3.15)**. Therefore, we hypothesized that Shcbp1 may also be required in B cell driven models of autoimmunity.

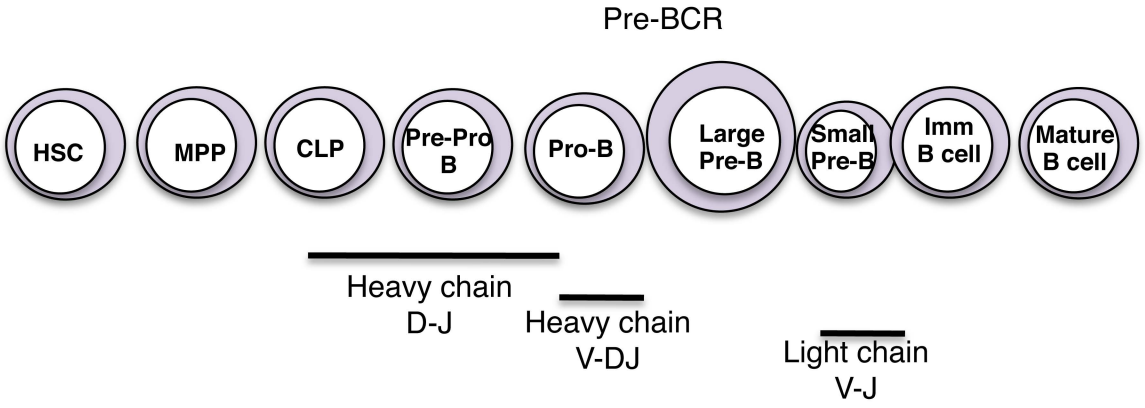
System lupus erythematosus (SLE) is an autoimmune disorder that leads to multi-organ inflammation and the generation of autoantibodies[184]. Intraperitoneal injection of pristane leads to chronic inflammation and the generation of a lupus-like disease in mice[184]. Unlike the EAE model, which is predominately dependent on T cells, the pristine induced model is dependent on the function of both T and B cells. Pristane-injected mice produce autoantibodies and can have signs of inflammation including glumeronephritis[184]. Therefore, to determine whether Shcbp1 contributes to disease severity in the pristane model, we could inject control and *Shcbp1* deficient mice with pristane and monitor for the development of autoreactive antibodies via ELISA. Furthermore, after approximately 6 months, we would evaluate for signs of autoimmunity via immunohistochemistry and immunofluorescence in target organs, including the kidneys. Collectively, this experiment would provide insight into the role of Shcbp1 in models of autoimmunity that are dependent on both T and B cells.

Figure A1: Shcbp1 expression in early hematopoiesis and during B cell development

(A) Schematic detailing the stages of B cell development

(B) *Shcbp1* mRNA expression analyzed by microarray in electronically sorted subsets (data curated from the Immgen Database).

A


Pre-BCR
HSC
MPP
CLP
Pre-Pro B
Pro-B
Large Pre-B
Small Pre-B
Imm B cell
Mature B cell
Heavy chain D-J
Heavy chain V-DJ
Light chain V-J

B

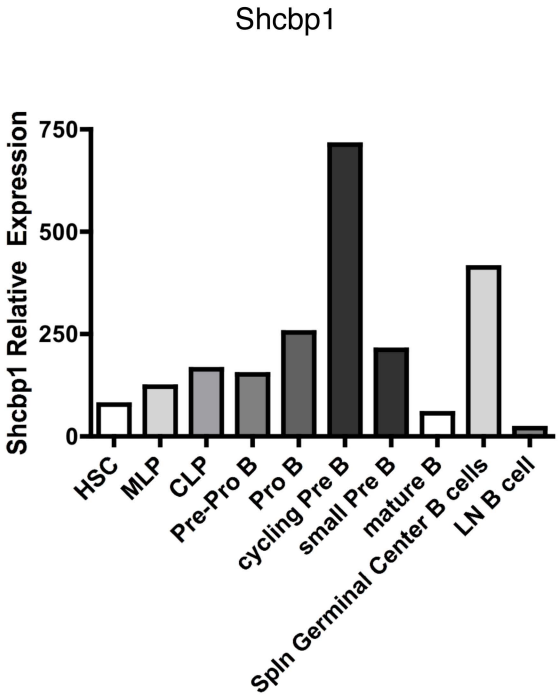
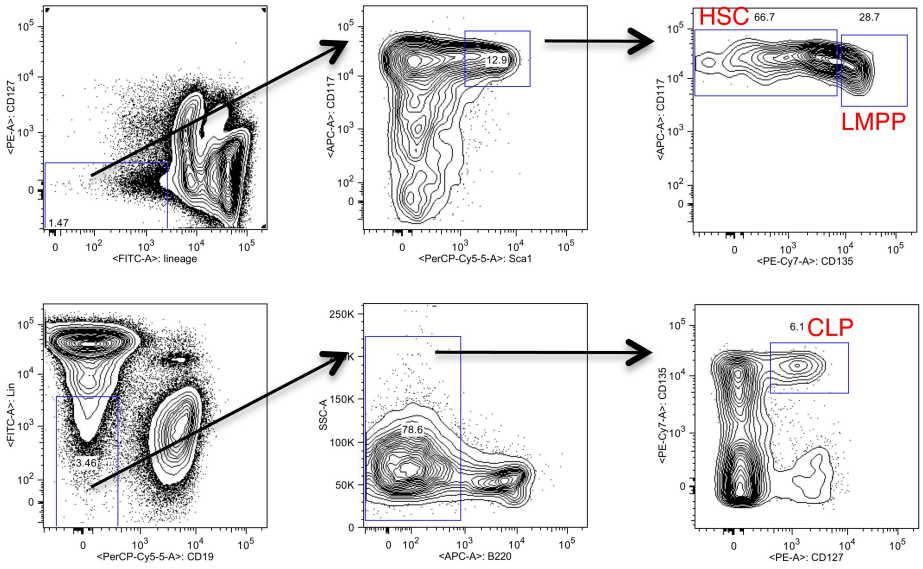

Shcbp1
Shcbp1 Relative Expression
750
500
250
0
HSC
MLP
CLP
Pre-Pro B
Pro B
cycling Pre B
small Pre B
mature B
Spln Germinal Center B cells
LN B cell

Figure A2: Gating strategy for early hematopoiesis and B cell development in the bone marrow

Gating strategy for HSCs, LMPPs, CLPs, Pre-B cells, Pre-Pro B cells, Pro-B cells, immature B cells and mature B cells in the bone marrow.

Early Lymphopoiesis: HSC/LMPP/CLP



B cell lymphopoiesis

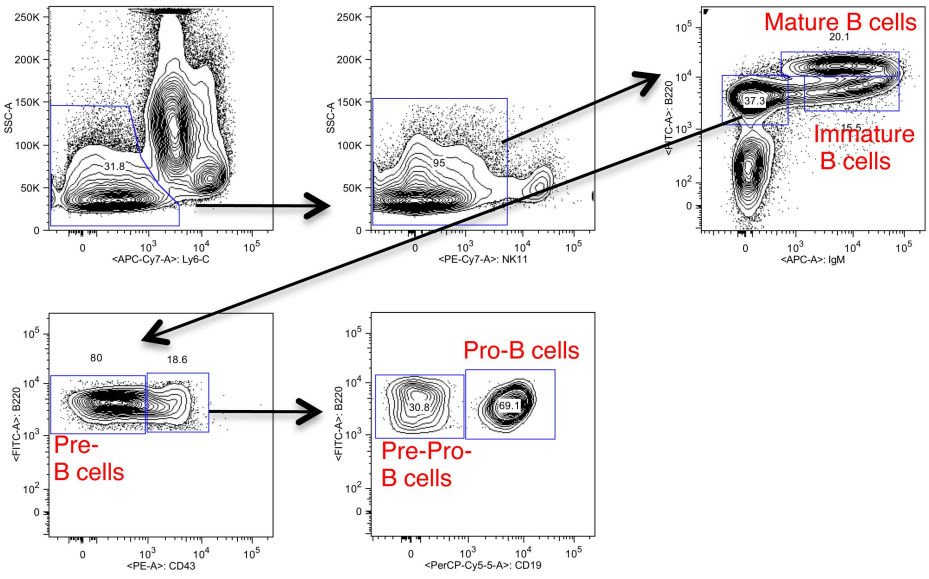


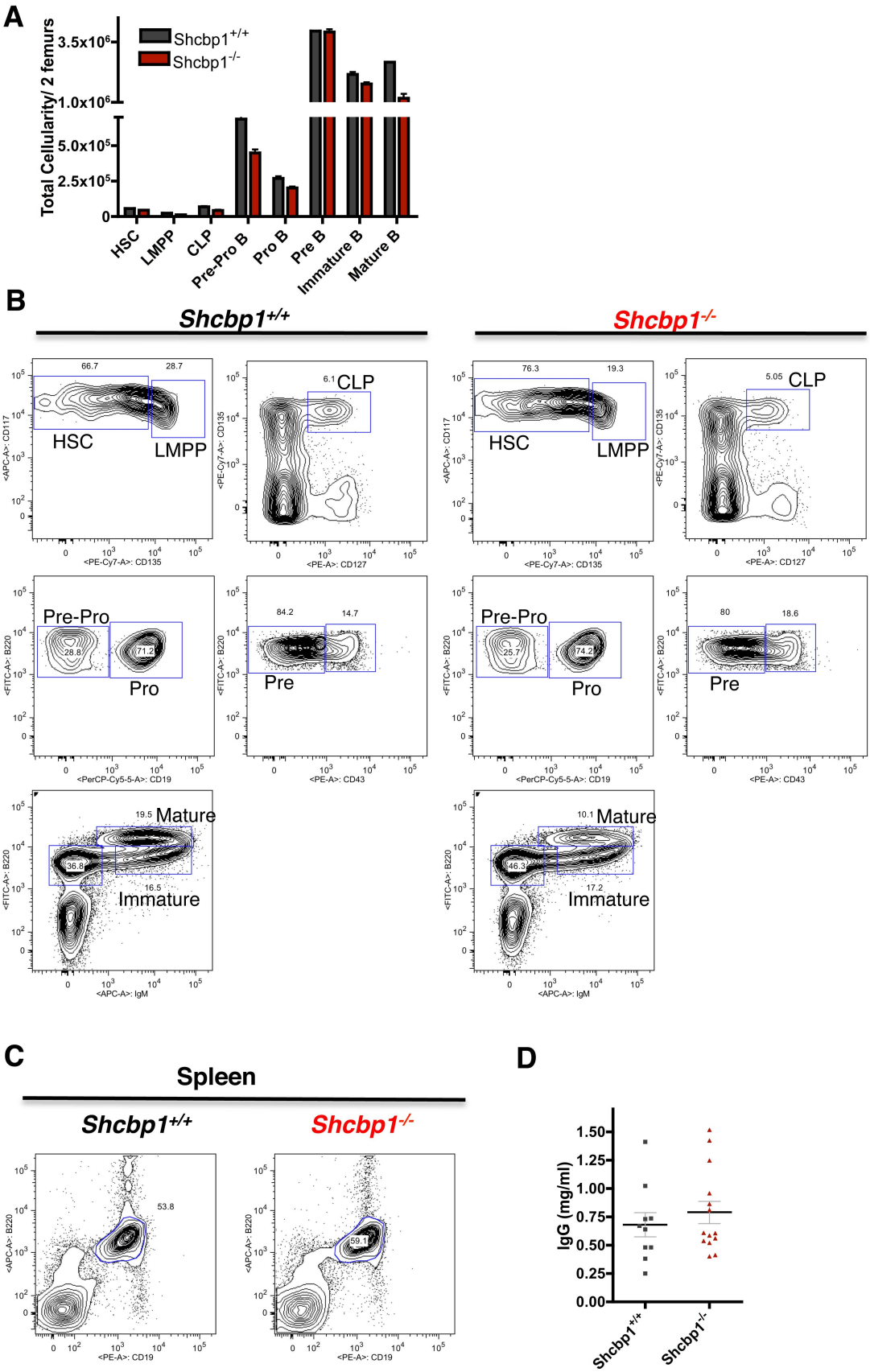
Figure A.3: B cell development appears normal in *Shcbp1* deficient mice

(A) Absolute numbers of subsets during B-lymphopoiesis in *Shcbp1* wild-type and deficient mice (n=2 mice of each genotype).

(B) Cell surface staining of subsets during B-lymphopoiesis in *Shcbp1* wild-type and deficient mice (n=2 mice of each genotype).

(C) Cell surface staining of CD19 and B220 in splenocytes isolated from *Shcbp1* wild-type and deficient mice.

(D) Quantification of total IgG in 10-week old wild-type and *Shcbp1* deficient mice (n>10 mice of each genotpye).


A
Total Cellularity/ 2 femurs
3.5x10⁶
1.0x10⁶
5.0x10⁵
2.5x10⁵
0
Shcbp1+/+
Shcbp1-/-
HSC
LMPP
CLP
Pre-Pro B
Pro B
Pre B
Immature B
Mature B
B
Shcbp1+/+
Shcbp1-/-
66.7
28.7
HSC
LMPP
6.1
CLP
76.3
19.3
HSC
LMPP
5.05
CLP
<APC-A>: CD117
<PE-Cy7-A>: CD135
<PE-A>: CD127
Pre-Pro
28.8
71.2
Pro
84.2
14.7
Pre
25.7
74.2
Pro
80
18.6
Pre
<FITC-A>: B220
<PerCP-Cy5-5-A>: CD19
<PE-A>: CD43
19.5
Mature
36.8
16.5
Immature
10.1
Mature
46.3
17.2
Immature
<APC-A>: IgM
C
Spleen
Shcbp1+/+
Shcbp1-/-
53.8
59.1
<APC-A>: B220
<PE-A>: CD19
D
IgG (mg/ml)
1.50
1.25
1.00
0.75
0.50
0.25
0.00
Shcbp1+/+
Shcbp1-/-

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