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Role of CBL-family Ubiquitin Ligases as Critical Negative Regulators of T Cell Activation and Functions

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by

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Supervisor: Hamid Band, M.D., Ph.D.

Adaptive T cell immunity is essential for defense against foreign antigens and immune surveillance against cancer. Tight regulation of T cell activation is required to avoid autoimmunity to self-antigens or protracted inflammation after foreign antigens are cleared. Incomplete or inappropriate stimulation leads to an active shutdown of T cell activation called anergy. The Casitas B-lineage Lymphoma (CBL)-family of ubiquitin ligases (E3s) are essential negative regulators of T cell activation that impinge on thymic selection as well as anergy induction programs. Single gene studies show that CBL is critical during T cell development while CBL-B plays an essential role in peripheral T cells; however, a more severe inflammatory-autoimmune disease is observed upon T cell-specific deletion of CBL in CBL-B null mice indicating redundant roles of CBL proteins. Mutations in CBL-B have been linked with increased susceptibility in a number of autoimmune diseases, and CBL-B null mice exhibit constitutive tumor rejection. The mechanisms by which CBL proteins regulate T cells in autoimmunity and antitumor immunity are not fully understood. It was previously not feasible to test the functional redundancy of CBL proteins in specific populations of T cells using existing models because their generalized CBL-B deficiency leads to altered and/or enhanced function of all T cell subsets and other immune cells, including B cells, macrophages, mast cells, neutrophils, and NKT cells. Here, we generated the first CBL-B$^{flox/flox}$ mouse which allows conditional CBL and CBL-B deletion in a cell type-specific manner. By crossing this new

mouse strain with the previously generated $CBL^{flox/flox}$ mouse and to a CD4-Cre transgene, we obtained concurrent CBL and CBL-B double knockout (DKO) in all T cell subsets, with altered T cell development, widespread organ infiltration by immune cells and rapid lethality, consistent with a redundant functional role of CBL and CBL-B. Unexpectedly, CD4-Cre-induced deletion in a small fraction of hematopoietic stem cells led to expansion of certain non-T-cell lineages, suggesting caution in the use of CD4-Cre for T-cell-restricted gene deletion.

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I am sincerely grateful to Dr. Band for giving me the opportunity to carry out my graduate training in his laboratory. His support, mentoring, and patience have been instrumental in my becoming a better scientist. Thank you for believing in me and always being there to guide me throughout this chapter of my life. I would also like to thank the members of my supervisory committee, Dr. Joyce Solheim, Dr. Lee Mosley, and Dr. Karen Gould, for all of their advice and instruction. I would especially like to thank the CRGP program director, Dr. Solheim, whose door is always open to provide help and support for every student.

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LIST OF ABBREVIATIONS

ACK	Ammonium-chloride-potassium
APC	Antigen presenting cell
B6	C57BL/6J
BM	Bone marrow
CBC	Complete blood count
CBL	Casitas B-lineage Lymphoma
CNS	Central nervous system
DKO	Double knockout
DN	Double negative
DP	Double Positive
EAE	Experimental autoimmune encephalomyelitis
ELISA	Enzyme-linked immunosorbent assay
EtOH	Ethanol
ETP	Early thymic progenitor
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
FLPe	Enhanced FLP1 recombinase
GEF	Guanine nucleotide exchange factor
GFP	Green fluorescent protein
GWAS	Genome-wide association studies
H&E	Hematoxylin and Eosin
HSC	Hematopoietic stem cell
IL-2	Interleukin 2
INF-β	Interferon beta
LCK	Lymphocyte-specific protein tyrosine kinase

Lin^-	Lineage maker-negative
LK	Lin^-, $Sca\text{-}1^-$, $c\text{-}Kit^+$
LSK	Lin^-, $Sca\text{-}1^+$, $c\text{-}Kit^+$
LT-HSC	Long-term hematopoietic stem cell
mAb	Monoclonal antibody
MDS/MPN	Myelodysplastic syndrome/ myeloproliferative neoplasm
MHC	Major histocompatibility complex
MMMTV	Murine mammary tumor virus
MOG	Myelin oligodendrocyte glycoprotein
MPP	Multipotent progenitor
MS	Multiple sclerosis
Neo	Neomycin
NK	Natural killer
NKT	Natural killer-T
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
PI3K	Phosphoinositide 3-kinase
PLCγ	Phospholipase C gamma
PTK	Protein tyrosine kinase
qPCR	Quantitative polymerase chain reaction
RAG	Recombination-activation gene
RBC	Red blood cell
RPMI	Roswell Park Memorial Institute medium
RR-MS	Relapsing-remitting multiple sclerosis
RT	Room temperature
SP	Single positive

ST-HSC	Short-term hematopoietic stem cell
TCR	T cell receptor
Tg	Transgene
TKB	Tyrosine kinase-binding
TSP	Thymic seeding progenitor
Vav1	Vav guanine nucleotide exchange factor 1
WT	Wild type
Zap70	Zeta chain of T cell receptor associated protein kinase 70kDa

CHAPTER 1: INTRODUCTION

Parts of this chapter are derived from the following manuscript:

Benjamin Goetz*, Wei An*, Bhopal Mohapatra*, Neha Zutshi, Fany Iseka, Matthew D. Storck, Jane Meza, Yuri Sheinin, Vimla Band, Hamid Band. A novel *CBL-B*$^{flox/flox}$ mouse model allows tissue-selective fully conditional *CBL/CBL-B* double-knockout: CD4-Cre mediated *CBL/CBL-B* deletion occurs in both T-cells and hematopoietic stem cells. *Oncotarget, 7*(32), 51107-51123.

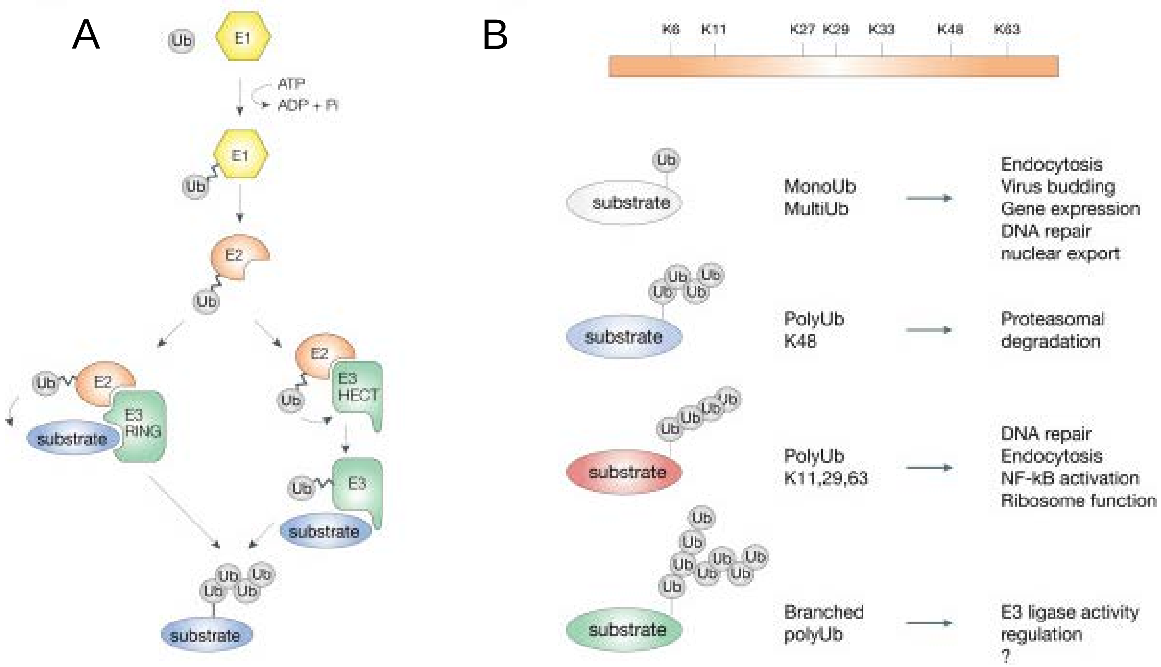
CBL family of E3 ubiquitin ligases

Review of the ubiquitination machinery

The ubiquitin system is a protein modification pathway in which ubiquitin, a 76-amino acid protein, is covalently attached to cellular proteins through an enzymatic cascade (Deshaies, Joazeiro 2009, Schulman, Harper 2009, MacGurn, Hsu et al. 2012). Ubiquitin can be attached in monoubiquitin and/or polyubiquitin chains to regulate target proteins through proteasomal degradation, lysosomal degradation, regulation of protein interactions, regulation of protein activity, or regulation of protein localization (Komander, Rape 2012). Ubiquitin modification affects many different cellular processes including cell growth and proliferation, signal transduction systems, endocytosis and downregulation of membrane proteins, degradation of abnormal proteins, development, apoptosis, and antigen processing (Komander, Rape 2012). Alterations in the ubiquitin system leads to a variety of human diseases including the onset and progression of cancer, autoimmunity and inflammatory disorders, neurodegenerative disorders (Parkinson's, Alzheimer's, and Huntington's diseases), and muscle wasting disorders (Popovic, Vucic et al. 2014). Moreover, further understanding of the ubiquitin system is needed for the development of new clinical therapies.

Ubiquitination involves the formation of an isopeptide bond between the C terminus of ubiquitin and a lysine in the target protein. This occurs through a multienzyme cascade involving ubiquitin-activating (E1), ubiquitin-conjugating (E2), and ubiquitin ligating (E3) enzymes (Figure 1.1). An E1 enzyme catalyzes the binding of the C-terminal glycine of ubiquitin to a cysteine residue of the E1 through a thiolester linkage in an ATP-dependent step. The activated E1-ubiquitin complex then binds to an E2 enzyme and transfers the activated ubiquitin to a cysteine residue on the E2. In the final step, the E2 enzyme binds to an E3 from one of two main classes: the catalytic

Figure 1.1. The ubiquitin pathway. A) Schematic representation of the ubiquitination process. A hierarchical set of three types of enzyme is required for substrate ubiquitination: ubiquitin-activating (E1), ubiquitin-conjugating (E2) and ubiquitin-protein ligase (E3) enzymes. The two major classes of E3 ligases are depicted. B) Schematic representation of the different Ub modifications with their functional roles. The question mark indicates that the functions of branched chains are largely unknown.


A
Ub
E1
ATP
ADP + Pi
E1
Ub
E2
Ub
Ub
E2
E3
RING
substrate
E2
E3
HECT
Ub
Ub
E3
substrate
Ub Ub
Ub Ub
substrate
B
K6 K11 K27 K29 K33 K48 K63
Ub
substrate
MonoUb
MultiUb
Endocytosis
Virus budding
Gene expression
DNA repair
nuclear export
substrate
PolyUb
K48
Proteasomal
degradation
substrate
PolyUb
K11,29,63
DNA repair
Endocytosis
NF-kB activation
Ribosome function
substrate
Branched
polyUb
E3 ligase activity
regulation
?

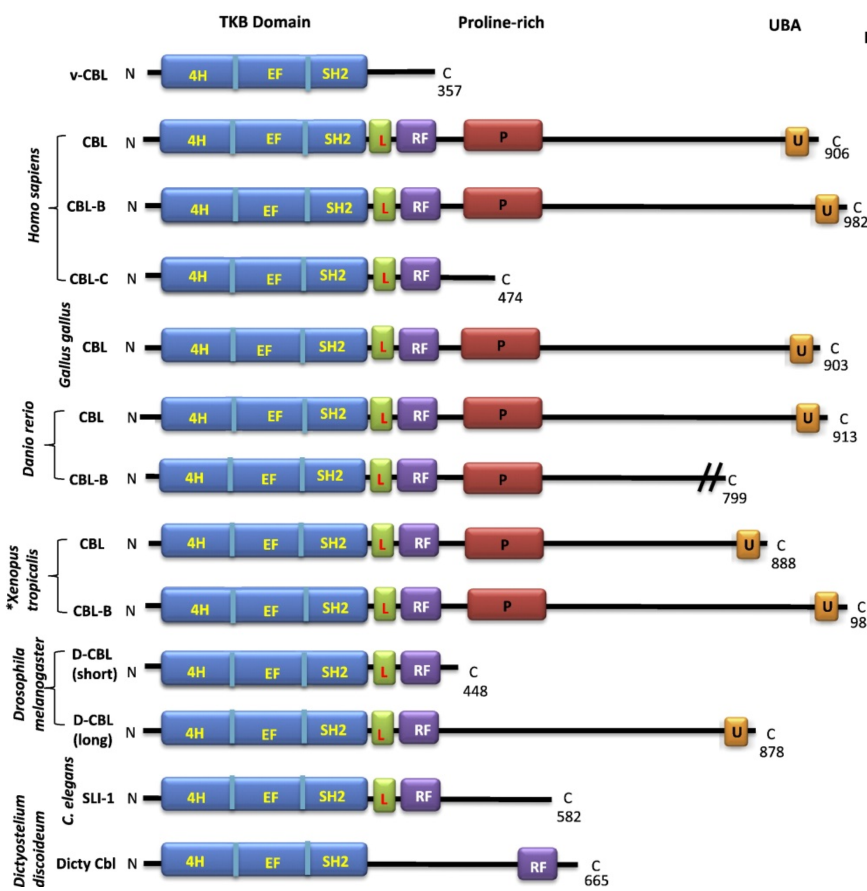
HECT-domain containing E3s or the non-catalytic RING finger domain (and related domains)-containing E3s. E2 enzymes bound to HECT domain-containing E3s transfer the activated ubiquitin to a cysteine on the E3 which then transfers the ubiquitin moiety to a lysine of the target protein through a isopeptide linkage; whereas, E2 enzymes bound to RING finger domain-containing E3s transfer the ubiquitin directly to the target protein with the E3 functioning as a scaffold. There are few known E1 enzymes, a larger family of E2s, and an even larger variety of E3 enzymes with the large number of E3s functioning to broaden the range and selectivity of target proteins regulated by ubiquitination modification (Komander, Rape 2012, Hershko, Ciechanover 1998, Deshaies, Joazeiro 2009, Schulman, Harper 2009).

Overview of the CBL family proteins

The CBL-family proteins (CBL, CBL-B, and CBL-C) (Figure 1.2) function as RING domain E3 ubiquitin ligases directed at protein tyrosine kinase (PTK) signaling pathways activated by stimulation through a number of cell surface receptors (Mohapatra, Ahmad et al. 2013). This function involves a highly conserved mechanism in which the N-terminal tyrosine kinase-binding (TKB) domain of CBL proteins binds to specific phosphotyrosine-containing motifs on receptor or non-receptor PTKs or adaptor proteins phosphorylated upon receptor-induced PTK activation (Thien, Blystad et al. 2005). Once recruited, CBL proteins are phosphorylated on an invariant tyrosine residue located within the linker region between the TKB domain and the RING finger domain; this phosphorylation event triggers intramolecular rearrangements that re-position the linker and RING finger domain for optimal binding of a ubiquitin conjugating enzyme (E2) and juxtapose the E2 closer to the TKB domain-bound PTKs for transfer of ubiquitin (Figure 1.3) (Dou, Buetow et al. 2012, Mohapatra, Ahmad et al. 2013). These mechanisms position CBL-family E3s as unique feedback negative regulators of activated PTKs. CBL and CBL-B, but not the epithelial-restricted CBL-C, also contain homologous C-terminal extensions that include an extensive proline-rich region for association with signaling proteins with SH3 domains such as SRC-family kinases, and specific tyrosine phosphorylation sites that help recruit SH2 domain-containing signaling intermediates, including PI3-kinase, RHO-family GTPase guanine nucleotide exchange factors (GEFs) of the VAV family and RAP1-GEF C3G (Figure 1.4) (Fang, Liu 2001, Tang, Subudhi et al. 2002, Zhang, Shao et al. 2003, Mohapatra, Ahmad et al. 2013). These mechanisms contribute to a coordinated program of negative regulation of PTK-coupled surface receptor signals that involve ubiquitination-dependent lysosomal targeting of receptors and their associated signaling proteins, proteasomal degradation of certain signaling intermediates, and degradation-independent negative regulation of certain signaling pathways (Rao, Ghosh et al. 2002, Rao, Miyake et al. 2002, Thien, Langdon 2001).

CBL/CBL-B double null mice are embryonic lethal, indicating the redundant but essential roles of CBL proteins during embryonic development. Aside from embryonic development, redundant functional roles of CBL and CBL-B have also emerged from a number of in vitro and genetic studies in other systems. Analyses of CBL null, CBL-B null and CBL/CBL-B double-null mouse embryonic fibroblasts (Duan, Miura et al. 2003, Ahmad, Mohapatra et al. 2014) and CBL and/or CBL-B knockdown in human mammary epithelial cells (Duan, Raja et al. 2011) and neural stem cells (Ferron, Pozo et al. 2010) in vitro showed that CBL and CBL-B function redundantly in negatively regulating EGF receptor traffic and signaling. Deletion of floxed CBL with murine mammary tumor virus (MMTV)-Cre on a CBL-B null background led to a myeloproliferative disease due to CBL deletion in hematopoietic stem cells (HSCs) and subsequent expansion of progenitors and myeloid progeny, but such a phenotype was not observed when CBL alone or CBL-B alone were deleted (Naramura, Nandwani et al. 2010, An, Nadeau et al. 2015). Using the same model, we have recently observed a redundant requirement of CBL and

Figure 1.2. Evolutionary conservation of the primary structure and domain organization of Cbl proteins. The comparison includes: the three human (*Homo sapiens*) Cbl proteins (Cbl or c-Cbl; Cbl-b; and Cbl-c, Cbl-3 or Cbl- SL) as representative mammalian Cbl proteins; Chicken (*Gallus gallus*) Cbl; Zebra fish (*Danio rerio*) Cbl; Frog (*Xenopus tropicalis*) Cbl; Fly (*Drosophila melanogaster*) long and short Cbl; Worm (*Caenorhabditis elegans*) *Cbl* (SLI-1); and Dicty (*Dictyostelium discoideum*) Cbl (Cbl-A). * *Xenopus tropicalis* was used for comparison rather than *Xenopus laevis*, as Cbl sequences in the databases for the latter species were partial. Domain designations: TKB, Tyrosine Kinase-Binding; 4H, four-helical bundle; SH2, Src-Homology 2; RF, RING Finger; L, Linker helical region; P, Proline-rich region; U, Ubiquitin-associated (UBA) domain; The amino acid sequences were compared to human Cbl and Cbl-b and are shown as two values separated by "/" under % identity and similarity for whole protein (or available partial sequence; shown with // across C-terminal end) and for the N-terminal domains (TKB, Linker and RF). The latter emphasizes the higher evolutionary conservation of the N-terminal domains that constitute the core PTK-directed E3 activity of Cbl proteins. V-Cbl corresponds to amino acids 1-357 of mouse Cbl that are present in viral Cbl oncogene. Dicty Cbl 4H region (inferred in UniProt) was confirmed using the YASARA structure program (www.yasara.org); however, a linker helical region has not been identified in Dicty Cbl, making it an exception in the entire Cbl protein family. N and C refer to amino and carboxyl termini.



	Overall		TKB + Linker + RF	
	Identity (%)	Similarity (%)	Identity (%)	Similarity (%)
v-CBL	97/83	98/90	97/83	98/90
Homo sapiens CBL	100/49	100/58	100/85	100/91
Homo sapiens CBL-B	70/100	78/100	81/100	88/100
Homo sapiens CBL-C	50/49	68/67	50/50	69/68
Gallus gallus CBL	84/72	89/80	98/83	98/90
Danio rerio CBL	69/81	77/88	90/82	92/89
Danio rerio CBL-B	79/63	84/71	92/95	96/97
**Xenopus tropicalis* CBL	75/49	82/59	94/82	96/90
**Xenopus tropicalis* CBL-B	69/77	77/84	80/95	86/98
Drosophila melanogaster D-CBL (short)	70/72	82/85	74/73	85/86
Drosophila melanogaster D-CBL (long)	67/62	79/76	74/73	86/86
C. elegans SLI-1	55/57	73/73	55/56	74/72
Dictyostelium discoideum Dicty Cbl	24/24	44/45	24/24	44/46

Figure 1.3. Domain architecture of Cbl proteins and major protein-protein interactions involving various domains/motifs. The N-terminal Tyrosine Kinase-Binding (TKB) domain binds to phosphotyrosine (pY)-containing sequence motifs in target proteins, that typically include activated receptor and non-receptor tyrosine kinases. The Linker region and the RING finger (RF) domain bind to ubiquitin conjugating enzymes (E2). The proline-rich motifs (Pro-rich) bind to SH3 domain containing signaling and endocytic proteins. Induced tyrosine phosphorylation sites (major sites at Y700, Y731 and Y774 are shown) recruit SH2 domain-containing signaling proteins. The Ubiquitin-associated (UBA) domain/leucine zipper near the C-terminus is involved in ubiquitin binding and dimerization. N and C refer to amino and carboxyl termini.

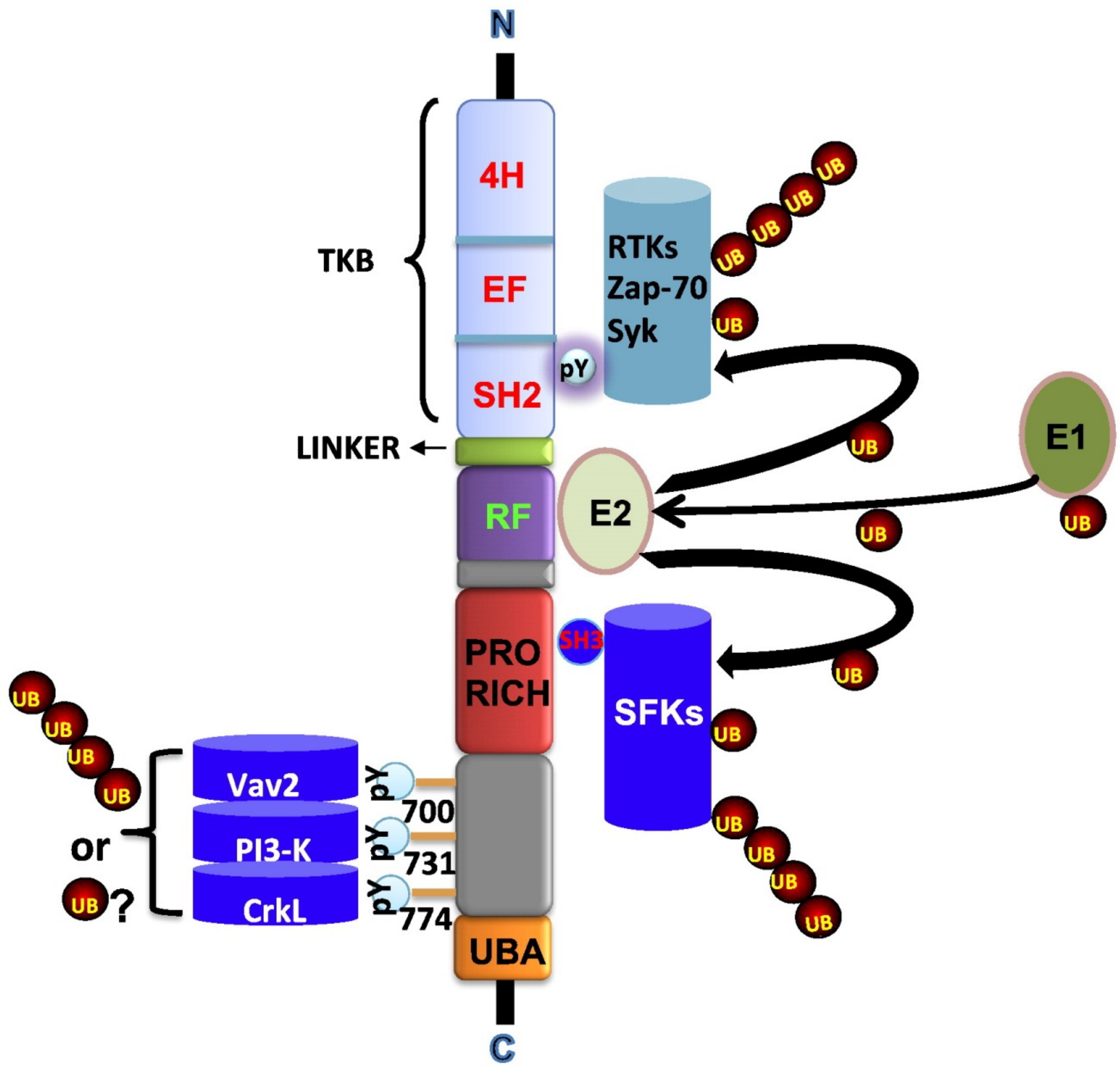
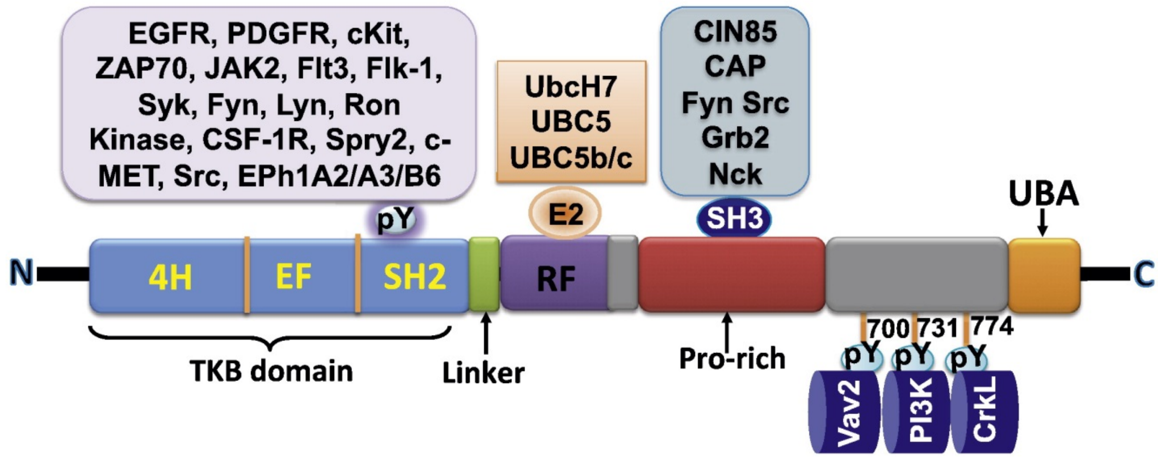

N
4H
EF
SH2
TKB
pY
LINKER
RF
E2
E1
RTKs
Zap-70
Syk
PRO
RICH
SH3
SFKs
Vav2
PI3-K
CrkL
or
?
pY
700
731
774
UBA
C
UB

Figure1.4. Schematic representation of the basic role of Cbl-family proteins as ubiquitin ligases (E3s) towards components of tyrosine kinase signaling pathways. Human Cbl is shown as a prototype of the family. The TKB domain, the proline-rich motifs and the induced tyrosine phosphorylation sites recruit targets for ubiquitin modification. The linker/RF-associated ubiquitin conjugating enzyme (E2) serves as an acceptor of activated ubiquitin from a ubiquitin-activating enzyme (E1) and transfers it to targets bound to various domain/motifs of Cbl to promote mono-ubiquitination (shown as a single UB subunit) or poly-ubiquitination (shown as four UB subunits).


EGFR, PDGFR, cKit, ZAP70, JAK2, Flt3, Flk-1, Syk, Fyn, Lyn, Ron Kinase, CSF-1R, Spry2, c-MET, Src, EPh1A2/A3/B6
UbcH7
UBC5
UBC5b/c
CIN85
CAP
Fyn Src
Grb2
Nck
UBA
pY
E2
SH3
N
4H
EF
SH2
RF
C
700
731
774
pY
pY
pY
Vav2
PI3K
CrkL
TKB domain
Linker
Pro-rich

CBL-B in mammary gland development (Mohapatra B, Zutshi N, An W et al. An essential role of CBL and CBL-B ubiquitin ligases in mammary stem cell maintenance. submitted).

T lymphocytes

Role in cell-mediated immunity

In the adaptive immune system, T cells play a central role in cell-mediated immunity against viral, bacterial, and parasitic infections and malignant cells in an antigen specific manner. T cell immunity can also lead to autoinflammation/autoimmunity through aberrant recognition of self-antigen. T cells originate in the bone marrow, where hematopoietic stem cell-derived progenitor cells migrate to the thymus and undergo further development into mature naïve T cells that express either co-receptor CD4 or CD8 followed by entry into the periphery (Germain 2002, Luckheeram, Zhou et al. 2012). Co-receptor CD4 interacts with major histocompatibility complex (MHC) class II and CD8 with MHC class I present on antigen-presenting cells (APCs) (Germain 2002). Naïve T cells migrate through the blood and secondary lymphoid tissues (lymph nodes and spleen) until activated by an APC (Pennock, White et al. 2013).

Activation of naïve T cells occurs though the T cell receptor (TCR) recognizing antigenic-peptide presented by APCs in the context of MHC and concurrent engagement of co-receptors (Smith-Garvin, Koretzky et al. 2009, Luckheeram, Zhou et al. 2012, Pennock, White et al. 2013). Following activation, T cells rapidly proliferate and migrate to sites where antigen is present. $CD4^+$ T cells (T helper cells), once activated, are capable of differentiating into multiple different subtypes, each of which can elicit different immune responses through the secretion of specific repertoires of cytokines (Zhu, Yamane et al. 2010, Luckheeram, Zhou et al. 2012, Pennock, White et al. 2013). $CD8^+$ T cells (cytotoxic T cells) destroy virus infected and malignant cells by delivery of cytotoxic granules to target antigen-expressing cells (Andersen, Schrama et al. 2006).

After antigen elimination, the majority of effector T cells die with the exception of a small population of memory T cells specific to the antigen (Pennock, White et al. 2013, Farber, Yudanin et al. 2014). Compared to naïve T cells, memory cells are more easily activated if challenged with the same antigen in the future and produce a more rapid immune response (Farber, Yudanin et al. 2014).

T cell development

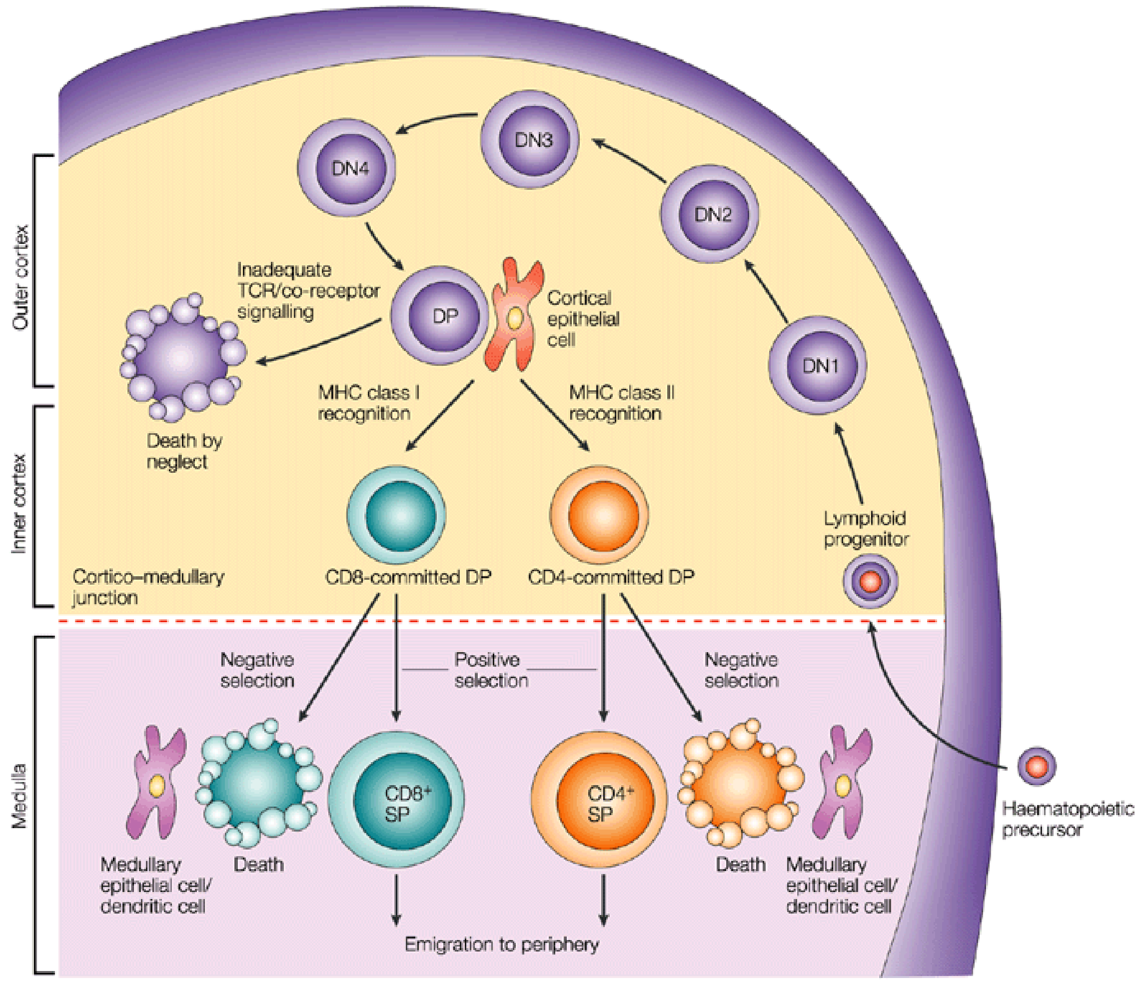
Thymic seeding progenitors (TSPs) generated from hematopoietic stem cells in the bone marrow migrate to the thymus where they undergo multiple stages of T cell maturation (Germain 2002). After encountering the thymic epithelium, cells progress to early thymic progenitors (ETPs) and enter the double negative (DN) stages of development which lack expression of CD4 and CD8 (Koch, Radtke 2011). DN1 thymocytes reside in the corticomedullary junction where they undergo proliferation before migrating deeper into the cortex for further differentiation into DN2 thymocytes (Porritt, Gordon et al. 2003, Petrie, Zuniga-Pflucker 2007). DN2 thymocytes upregulate genes involved in DNA rearrangement, such as recombination-activation genes (RAGs), before differentiating into DN3 thymocytes in the subcapsular zone where they undergo β-selection through rearrangement of the β loci to express a functional TCR- β chain (Taghon, Yui et al. 2006, Burtrum, Kim et al. 1996). A successfully rearranged TCRβ chain combines with CD3 chains and an invariant α chain to generate a functional pre-TCR. Cells that fail to undergo successful β chain rearrangement die (von Boehmer 2005). Developing thymocytes then mature to the DN4 stage and begin migrating back to the medulla. These cells undergo TCRα chain rearrangement and successful recombination leads to the expression of a mature αβ TCR, and thymocytes begin to express CD4 and CD8 leading to their progression to the double positive (DP) stage (Robey, Fowlkes 1994). Thymocytes with TCRs that interact with intermediate affinity to self-peptide-MHC complexes undergo positive selection; but, cells with poor affinity to self-peptide-MHC complexes die by neglect (Klein, Hinterberger et al. 2009). Thymocytes with a TCR that reacts with MHC class I commit to the CD8 single positive (SP) lineage, and those that interact with MHC class II commit to the CD4 SP lineage (Klein, Hinterberger et al. 2009). Single positive cells then migrate to the medulla where they undergo negative selection by apoptosis which eliminates SP thymocytes that possess a TCR with high affinity for self-antigens to prevent auto-reactive T cells from entering the periphery (Klein, Hinterberger et al. 2009). The resulting mature SP T cells then leave the thymus and enter the circulation (Figure 1.5).

T cell activation

Tight regulation of T cell activation and immunological tolerance are essential to allow the body to mount effective defense against foreign antigens and to provide immune surveillance against cancer without autoimmunity to self-antigens or protracted inflammatory sequels following infections. Most autoreactive T cells are eliminated by negative selection during thymocyte development; however, this process is not absolute, and some autoreactive T cells escape into the periphery (Baldwin, Trenchak et al. 1999). Such T cells are eliminated or kept in check through peripheral tolerance mechanisms (Redmond, Marincek et al. 2005). A key mechanism to prevent autoimmune consequences of peripheral T cell activation during immune responses is the imposition of a requirement for concurrent signals emanating from the TCR recognition of an antigen, presented by an antigen-presenting cell in the context of MHC, and those generated by the engagement of co-stimulatory molecules (Chen, Flies 2013). Engagement of the T cell receptor in the absence of co-stimulatory molecules results in cell intrinsic functional inactivation known as anergy (LaSalle, Hafler 1994). Co-stimulatory molecules on T cells, such as CD28, interact with their ligands expressed on

Figure1.5. Overall scheme of T-cell development in the thymus. Committed lymphoid progenitors arise in the bone marrow and migrate to the thymus. Early committed T cells lack expression of T-cell receptor (TCR), CD4 and CD8, and are termed double-negative (DN; no CD4 or CD8) thymocytes. DN thymocytes can be further subdivided into four stages of differentiation (DN1, $CD44^+CD25^-$; DN2, $CD44^+CD25^+$; DN3, $CD44^-CD25^+$; and DN4, $CD44^-CD25^-$). As cells progress through the DN2 to DN4 stages, they express the pre-TCR, which is composed of the non-rearranging pre-T chain and a rearranged TCR -chain. Successful pre-TCR expression leads to substantial cell proliferation during the DN4 to double positive (DP) transition and replacement of the pre-TCR -chain with a newly rearranged TCR -chain, which yields a complete TCR. The $-TCR^+CD4^+CD8^+$ (DP) thymocytes then interact with cortical epithelial cells that express a high density of MHC class I and class II molecules associated with self-peptides. The fate of the DP thymocytes depends on signalling that is mediated by interaction of the TCR with these self-peptide–MHC ligands. Too little signalling results in delayed apoptosis (death by neglect). Too much signaling can promote acute apoptosis (negative selection); this is most common in the medulla on encounter with strongly activating self-ligands on hematopoietic cells, particularly dendritic cells. The appropriate, intermediate level of TCR signaling initiates effective maturation (positive selection). Thymocytes that express TCRs that bind self-peptide–MHC-class-I complexes become $CD8^+$ T cells, whereas those that express TCRs that bind self-peptide–MHC-class-II ligands become $CD4^+$ T cells; these cells are then ready for export from the medulla to peripheral lymphoid sites. SP, single positive.

Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Immunology. Ronald N. Germain. T-cell development and the CD4-CD8 lineage decision. (2002). (http://www.nature.com/nri/index.html)



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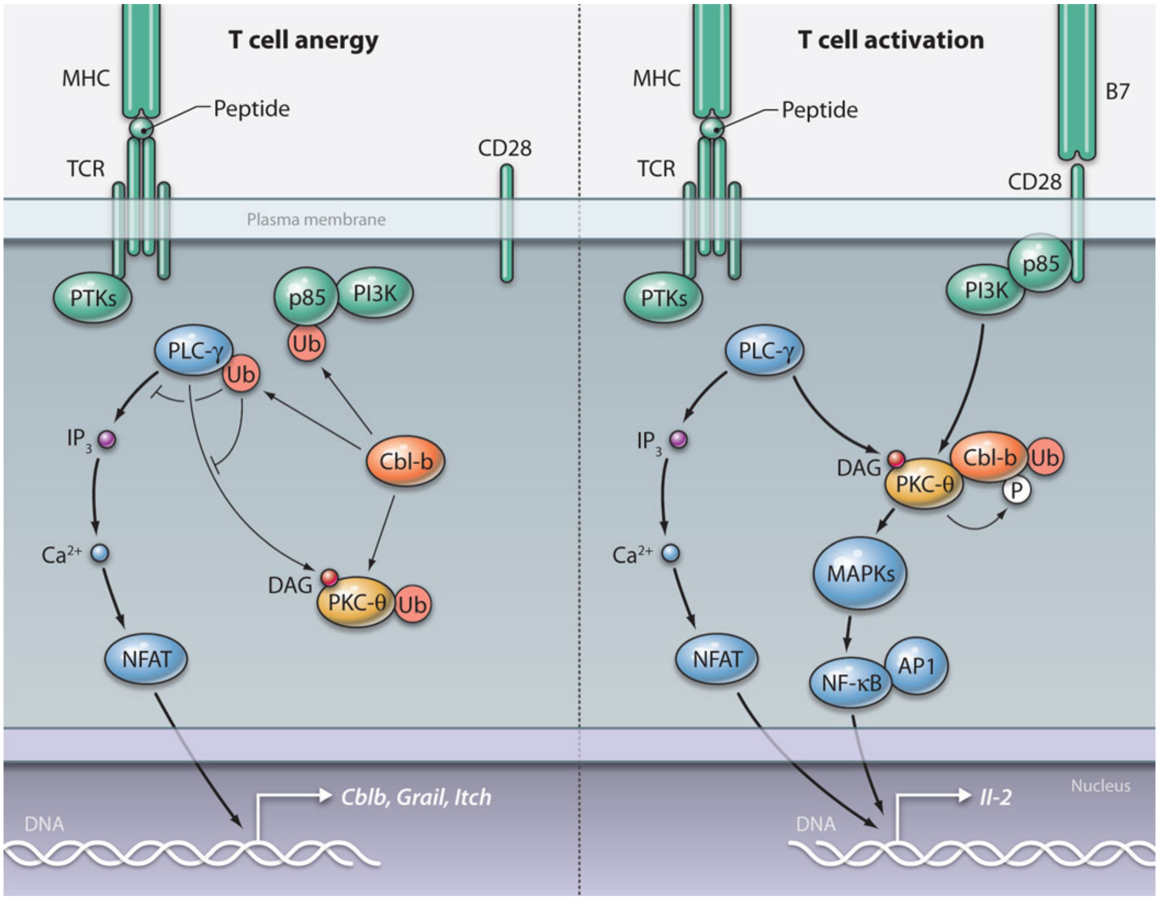
the surface of APCs or target cells (Chen, Flies 2013). During physiological immune responses, the function of costimulatory receptors and ligands is counter-balanced by negative co-stimulatory molecules on T cells, such as CTLA4, and inhibitory ligands on APCs/target cells (Chen, Flies 2013).

CBL proteins in T cells

Role in regulating activation and functions

The CBL-family ubiquitin ligases (E3s) have been established as essential negative regulators of T cell activation and mediate induction of immune anergy/tolerance programs (Loeser, Penninger 2007). Genetic studies using a whole-body CBL-B knockout mouse model demonstrate that CBL-B plays an essential role in coupling T cell activation to the requirement for CD28-mediated co-stimulation through negative regulation of downstream effectors PI3K and Vav1 (Fang, Liu 2001, Chiang, Kole et al. 2000, Krawczyk, Bachmaier et al. 2000). In addition, CBL-B promotes destabilization of the immunological synapse and inhibits T cell activation by negatively regulating integrin activation via its negative regulation of CrkL-C3G interactions (Zhang, Shao et al. 2003). Along with setting the threshold for T cell activation, CBL-B is also a critical regulator of the anergy induction program and becomes transcriptionally up-regulated under T cell anergy-inducing conditions (Jeon, Atfield et al. 2004, Heissmeyer, Macian et al. 2004). This negative regulation of CBL-B on T cell activation is overcome upon co-stimulation through CD28 which leads to the reduction of CBL-B levels (Figure 1.6) (Zhang, Bardos et al. 2002, Schmitz 2009). CBL-B deficiency uncouples T cell activation from the requirement for co-stimulation, leading to hyperactive T cells that display increased proliferation and IL-2 production in response to TCR stimulation alone (Chiang, Kole et al. 2000, Venuprasad 2010). Although CBL-B is expressed in immature

Figure1.6. Regulation of T cell anergy. (Left panel) Stimulation of the TCR in the absence of CD28-mediated costimulation triggers Ca^{2+}-dependent signaling and leads to expression of the *Cblb*, *Itch*, and *Grail* genes. Inhibitory ubiquitination mediated by Cbl-b is shown in red. Ub, ubiquitin. (Right panel) Costimulation of T cells allows for full activation of signaling pathways, including the induction of mitogen-activated protein kinases (MAPKs) and the transcription factors AP1 (activator protein 1) and NF-κB. The discussed mechanisms of PKC-θ–dependent down-regulation of Cbl-b are displayed.


T cell anergy
MHC
Peptide
TCR
CD28
Plasma membrane
PTKs
p85
PI3K
Ub
PLC-γ
Ub
IP3
Cbl-b
Ca2+
DAG
PKC-θ
Ub
NFAT
DNA
Cblb, Grail, Itch
T cell activation
MHC
Peptide
B7
TCR
CD28
PTKs
PI3K
p85
PLC-γ
IP3
DAG
PKC-θ
Cbl-b
Ub
P
Ca2+
MAPKs
NFAT
NF-κB
AP1
Nucleus
DNA
Il-2

thymocytes, CBL-B null mice show no detectable alterations in thymic development (Chiang, Kole et al. 2000, Bachmaier, Krawczyk et al. 2000).

Whole-body CBL-null mice exhibit altered thymocyte development with increased thymocyte numbers and enhanced positive selection of mature $CD4^{+}$ T cells (Naramura, Kole et al. 1998). While $CBL^{-/-}$ thymocytes exhibited hyper-activated Zap70 and MAPK, they also showed reduced activity of PI3K and PLCγ1, and impaired activation-induced TCR down-modulation (Naramura, Kole et al. 1998, Murphy, Schnall et al. 1998). Even though CBL can interact with many of the same substrates as CBL-B, such as PI3K, Vav1 and CrkL, CBL-deficient mice display relatively normal peripheral T cell function to the extent studied (Balagopalan, Barr et al. 2007). Thus, the *in vivo* function of CBL in peripheral T cells remains incompletely characterized.

Double deficient T cells exhibit even higher proliferation compared to $CBL\text{-}B^{-/-}$ T cells when stimulated with anti-CD3 antibody (Naramura, Jang et al. 2002). Double deficient cells also display impaired activation-induced TCR down-modulation, hyperphosphorylated Zap70, and reduced activity of PLCγ and Vav1 similar to CBL deficient thymocytes(Naramura, Jang et al. 2002). Combined deletion of CBL and CBL-B also leads to altered thymic development causing a decrease in the number of total and double positive thymocytes, contraction of the peripheral T cell compartments, and altered ratios of mature $CD4^{+}$ and $CD8^{+}$ T cells (Huang, Kitaura et al. 2006).

Role in autoimmunity

CBL-B null mice exhibit increased susceptibility to spontaneous and peptide induced autoimmunity (Bachmaier, Krawczyk et al. 2000). While $CBL\text{-}B^{-/-}$ mice display increased sensitivity to the development of spontaneous autoimmunity and $CBL^{-/-}$ mice show normal peripheral T cell function, conditional T cell specific CBL deletion in CBL-B null mice leads to a more severe autoimmune disease. Induction of Cre-mediated deletion of a floxed CBL allele by LckCre (deletion at the double negative (DN) stage of thymocyte development) on a CBL-B null background led to severe spontaneous autoimmune organ infiltration, splenomegaly, and auto-antibodies leading to death between 12 and 16 weeks of age (Naramura, Jang et al. 2002). Also, mutations in CBL-B have been linked with increased susceptibility in a number of autoimmune diseases, including type 1 diabetes mellitus (Yokoi, Komeda et al. 2002, Bergholdt, Taxvig et al. 2005), systemic lupus erythematosus (Gomez-Martin, Ibarra-Sanchez et al. 2013), and multiple sclerosis (Sanna, Pitzalis et al. 2010, Corrado, Bergamaschi et al. 2011, Zhou, Wang et al. 2008, Sturner, Borgmeyer et al. 2014).

Role in anti-tumor immunity

CBL-B-null mice exhibit enhanced anti-tumor immunity to spontaneous and transplanted tumor models (Loeser, Penninger 2007, Chiang, Jang et al. 2007). However, the elucidation of mechanisms of enhanced anti-tumor ability in the currently-available CBL-B null mouse models has been challenging since all immune and non-immune cells lack CBL-B expression. Adoptively transferred CBL-B null CD8 T cells or NK cells have been shown to exhibit anti-tumor effects in mouse studies (Lutz-Nicoladoni, Wallner et al. 2012, Hinterleitner, Gruber et al. 2012a, Stromnes, Blattman et al. 2010, Paolino, Choidas et al. 2014). Based on these studies, downregulation of CBL-B in human T cells has been shown to enhance their tumor-killing abilities (Hinterleitner, Gruber et al. 2012a). Recent studies suggest an increased expression of CBL-B within the tumor-associated immune component, consistent with a role in mediating immune tolerance to tumors (Oguro, Ino et al. 2015).

CBL proteins as tumor suppressors

In contrast to a potentially pro-oncogenic role of CBL proteins by promoting immune tolerance associated with tumorigenesis, a potentially opposite role of CBL proteins as tumor suppressors has emerged in the context of leukemogenesis. Mutations clustered in the linker region or RING finger domain of CBL, and rarely CBL-B, which abrogate E3 activity, have been identified in a subset of patients with myelodysplastic syndrome/myeloproliferative neoplasms (MDS/MPN), chronic myelomonocytic leukemia or juvenile myelomonocytic leukemia (Caligiuri, Briesewitz et al. 2007, Grand, Hidalgo-Curtis et al. 2009, Makishima, Cazzolli et al. 2009, Shiba, Kato et al. 2010, Nadeau, An et al. 2012). A majority of these patients exhibit duplication of mutant CBL genes, seen as acquired uniparental disomy (Grand, Hidalgo-Curtis et al. 2009, Niemeyer, Kang et al. 2010, Dunbar, Gondek et al. 2008). In juvenile myelomonyctic leukemia patients, the mutation typically involves the regulatory tyrosine residue in the linker region and many of such patients inherit the mutation from a non-leukemic parent with Noonan Syndrome, followed by somatic duplication of the mutation in hematopoietic stem cells (Niemeyer, Kang et al. 2010). Loss of CBL expression was shown to accelerate BCR-abl induced myeloid leukemogenesis in a mouse model (Sanada, Suzuki et al. 2009). Mice with an inactivating RING finger domain mutation in CBL also exhibited a leukemic disease when the wild type (WT) CBL gene was deleted (Rathinam, Thien et al. 2010). A more rapid leukemic disease was observed upon conditional CBL deletion, using MMTV-Cre, on a CBL-B null background, thus supporting a redundant but essential role of CBL and CBL-B as tumor suppressors in the context of myeloid leukemogenesis (Naramura, Nandwani et al. 2010, An, Nadeau et al. 2015).

In contrast to mutational inactivation of CBL (or CBL-B) as an oncogenic mechanism in leukemia, clustered CBL or CBL-B mutations are not found in other hematological malignancies and the COSMIC database reveals the extreme rarity of such mutations (http://cancer.sanger.ac.uk/cosmic). Whether or not CBL proteins have a role during tumorigenesis of non-myeloid lineages remains unknown; however, recent studies suggest a potentially pro-oncogenic role of CBL as its expression was found to be higher in breast cancer and depletion of CBL/CBL-B reduced tumorigenicity or metastasis of breast cancer cells in the nude mouse model (Zhang, Teng et al. 2015, Kang, Park et al. 2012). These suggestive findings make it vital to design models where tissue-specific and tumor-intrinsic deletion of CBL and/or CBL-B can be induced to assess non-myeloid cell and tumor cell-intrinsic roles of CBL proteins.

Hypothesis

CBL family proteins are critical negative regulators of T cell activation and functions. Previous studies into the role that CBL proteins play in regulating T cells in autoimmunity and antitumor immunity have been inconclusive due to the models utilizing a CBL-B null mouse. CBL-B deficiency leads to altered functions of many different immune cell types. To circumvent this, we have engineered the first CBL-B-flox mouse to allow tissue specific CBL/CBL-B DKO after breeding with the previously available CBL-flox mouse. I hypothesize that DKO in the T cell population will result in an exacerbated autoimmune phenotype and enhanced antitumor immunity.

CHAPTER 2: MATERIALS AND METHODS

Parts of this chapter are derived from the following manuscript:

Benjamin Goetz*, Wei An*, Bhopal Mohapatra*, Neha Zutshi, Fany Iseka, Matthew D. Storck, Jane Meza, Yuri Sheinin, Vimla Band, Hamid Band. A novel *CBL-B*$^{flox/flox}$ mouse model allows tissue-selective fully conditional *CBL/CBL-B* double-knockout: CD4-Cre mediated *CBL/CBL-B* deletion occurs in both T-cells and hematopoietic stem cells. *Oncotarget, 7*(32), 51107-51123.

Mice

In order to generate CBL-B conditional knockout mice, a CBL-B conditional knockout construct was engineered using the "recombineering" technique (Liu, Jenkins et al. 2003). The Clone Finder software (NCBI database) was used to search the NIH's C57BL/6J (B6) mouse BAC library (at the Children's Hospital Oakland Research Institute). We identified clones [RP23-456D16, RP23-122H13, RP24-361F9, RP24-98B21] that contain the mouse CBL-B gene. A series of "recombineering" reactions (Liu, Jenkins et al. 2003) were used to retrieve a 10.5 kb fragment of the BAC DNA containing the first and second exons of CBL-B into a plasmid (with negative selection marker), allowing us to introduce two loxP (Cre recombinase recognition) sites flanking this region. Immediately preceding the second loxP site, an engineered FRT-Neo-FRT selection cassette was inserted which confers G418 resistance to transfected ES cells. FRT sites allow removal of the Neo gene using FLP recombinase thus keeping alterations of the gene locus to a minimum. The correct arrangement and sequence of targeted gene segments at each step have been verified by PCR analysis and sequencing. A NotI linearized targeting vector was submitted to the Mouse Genome Engineering Core Facility at UNMC for electroporation into a C57BL/6-derived ES cell line. Southern blot hybridization with probes located outside the 5' and 3' boundaries of the targeted region was used for screening of 5' and 3' boundaries of the targeted region to identify targeted ES clones after G418 and ganciclovir selection.

C57BL/6 ES clones in which the CBL-B gene was correctly targeted were used to produce chimeric mice using blastocyst injection. Chimeras were mated to B6 mice to test the germline transmission of the targeted CBL-B allele and verified using Southern blot hybridization and PCR analysis of tail-derived genomic DNA.

Next, heterozygous CBL-B targeted mice (CBL-B$^{f\text{-}Neo/+}$) were intercrossed to generate homozygous CBL-B targeted mice (CBL-B$^{f\text{-}Neo/f\text{-}Neo}$). The homozygous CBL-B targeted mice were mated to B6; SJLTg(ACTFLPe)9205Dym/J mice which express the enhanced FLP1 recombinase (FLPe) from the ubiquitously expressed human ACTB (beta actin) promoter to remove the FRT-flanked Neo gene. Heterozygous CBL-B targeted, FLPe transgene-positive mice were crossed to C57BL/6J (wild-type mice) in order to generate heterozygous CBL-B-floxed, FLPe transgene-negative (*CBL-B*$^{fl/+}$) mice. These heterozygous mice were mated to produce homozygous CBL-B floxed mice (CBL-B$^{f/f}$). To examine the functionality of the loxP site in the engineered mice and generate CBL-B null mice, we crossed the CBL-B floxed mice to B6.FVB-TgN (EIIa-Cre) C5379Lmgd, which expresses Cre ubiquitously from the EIIa promoter. Heterozygous *CBL-B*-targeted, EIIa-*Cre* transgene-positive mice were crossed to C57BL/6J (wild-type) mice to generate heterozygous *CBL-B*-deleted, *Cre* transgene-negative (*CBL-B*$^{+/-}$) mice, which were used to produce *CBL-B*$^{-/-}$ mice.

CBL$^{flox/flox}$ (Naramura, Jang et al. 2002), CBL-B$^{flox/flox}$, CD4-Cre [Tg(Cd4-cre)1Cwi/BfluJ] (The Jackson Laboratory), CreERT [B6.129-*Gt(ROSA)26Sor*$^{tm1(cre/ERT2)Tyj}$/J] (The Jackson Laboratory), Cre mT/mGFP reporter [Gt(ROSA)26Sortm4(ACTB-tdTomato,-EGFP)Luo/J] (The Jackson Laboratory), 2D2 TCR Tg [C57BL/6-Tg(Tcra2D2,Tcrb2D2)1Kuch/J] (The Jackson Laboratory), and pmel-1 TCR Tg [B6.Cg-*Thy1*^{a}/Cy Tg(TcraTcrb)8Rest/J] (The Jackson Laboratory) strains were maintained on a C57BL/6 background under specific pathogen-free conditions (Table 2.1) and genotyped using tail DNA PCR with the primers specified in (Table 2.2). All mouse experiments were approved by the UNMC IACUC.

Tissue Preparation and FACS Analysis

Single cell suspensions were made from spleen, thymus, and lymph node by mashing tissue through a 40 µm cell strainer and RBCs were lysed using ACK Lysing

Table 2.1. Genotypes of mice

Strain designation	Genotype
WT	**C57/B6**
Control	**$Cbl^{flox/flox}$; $Cbl\text{-}b^{flox/flox}$**
Cbl/Cbl-b DKO	**$Cbl^{flox/flox}$; $Cbl\text{-}b^{flox/flox}$; $CD4\text{-}Cre^{Tg/0}$**
Cbl/Cbl-b DKO mT/mGFP	**$Cbl^{flox/flox}$; $Cbl\text{-}b^{flox/flox}$; $CD4\text{-}Cre^{Tg/0}$; mT/mG**

Table 2.2. Genotyping primers

Target Allele	Forward 5'-3'	Reverse 5'-3'
Cbl floxed	GTGGTGGCTTGCAATTATAATCCTACCACTTAGG	GTTTGAGATGTCTGGCTGTGTACACGCG
Cbl-b floxed	GGCAGAACCACTGAGACACATTTA	GGCTGCCAAACTGCTACCCAGGAG
CD4-Cre	GCGGTCTGGCAGTAAAAACTATC	GTGAAACAGCATTGCTGTCACTT
Rosa 26 – mT/mGFP	CTCTGCTGCCTCCTGGCTTCT	TCAATGGGCGGGGGTCGTT
CreERT	GCGGTCTGGCAGTAAAAACTATC	GTGAAACAGCATTGCTGTCACTT
Pmel-1 Tg TCR	GGT CCT GTG GCT CCA GTT TAA T	CTG CTT AAC CTG TCC CTC ATG T
	CTG GGC AGT GTT CTG TCT CC	ACC ATG GTC ATC CAA CAC AG
2D2 Tg TCR	CCC GGG CAA GGC TCA GCC ATG CTC CTG	GCG GCC GCA ATT CCC AGA GAC ATC CCT CC

Buffer for 10 min at RT. For cell analysis and sorting, cells were immuno-stained for 20 min at 4^oC in FACS buffer (PBS-1% BSA). The following antibodies were procured from eBioscience: CD4 (RM4-5); CD62L (MEL-14); CD45RB (C363.16A); CD69 (H1.2F3); B220 (RA3-6B2); F4/80 (BM8). The following antibodies were procured from BD Biosciences: CD8 (53-6.7); CD117 (2B8); CD11b (M1/70); anti-CD11c (HL3); Gr-1 (RB6-8C5). CD25 (PC61) and CD44 (IM7) were from Biolegend.

Whole bone marrow cell suspensions were prepared from femurs and tibiae. For stem and progenitor cell analysis and sorting, mature hematopoietic cells (lineage-positive cells) were labeled with antibodies against CD5, B220, CD11b, Gr-1, and 7-4 (mouse lineage depletion kit; Miltenyi Biotechnology) and magnetically depleted using the autoMACS (Miltenyi Biotechnology). Lineage-negative cells were then stained with antibodies followed by cell analysis or sorting. Flow cytometry was performed on a BD LSRII or Aria II at the UNMC Flow Cytometry Research Facility. Data were analyzed using FlowJo software (Tree Star). Cell populations were defined as follows (Seita, Weissman 2010): long-term HSC (LT-HSC): CD34 -FLT3 $^-$Lin$^-$ Sca-1$^+$ c-Kit$^+$; short-term HSC (ST-HSC): CD34$^+$ FLT3$^-$ Lin$^-$ Sca-1$^+$ c-Kit$^+$; MPP: CD34$^+$ FLT3$^+$ Lin$^-$ Sca-1$^+$ c-Kit$^+$; LSK: Lin$^-$ Sca-1$^+$ c-Kit$^+$; CMP: CD16$^-$ CD34$^+$ Lin$^-$ Sca-1$^-$ c-Kit$^+$; GMP: CD16$^+$ CD34$^+$ Lin$^-$ Sca-1$^-$ c-Kit$^+$; MEP: CD16$^-$ CD34$^-$ Lin$^-$ Sca-1$^-$ c-Kit$^+$; CLP: IL-7R$^+$ FLT3$^+$ Lin$^-$ Sca-1^{low} c-Kit^{low}.

Western Blotting

For CBL-B protein expression analysis, splenocytes were lysed using lysis buffer (1 M Tris pH 7.5, 5 M NaCl, 10% Triton, 100 mM VO4, 1 M NaF, 50 mM PMSF). The following antibody for western blotting was procured from a commercial source: rabbit monoclonal antibody (mAb) anti-CBL-B (Clone D3C12, Cell Signaling Technology).

ELISA

Splenocytes were cultured in RPMI supplemented with 10% FBS in triplicates in 96 U-bottom wells ($2x10^6$ cells/well) precoated with 10 µg/ml plate-bound anti-CD3ε (145-2C11) in the presence or absence of 1µg/ml soluble anti-CD28 (37.51) for 48 h at 37^{o}C. Culture supernatants were collected and the IL-2 concentration was measured by Quantikine ELISA kit (R&D systems), according to the manufacturer's instructions.

RNA Isolation and Quantitative Real-Time PCR

RNA extracted from FACS-sorted cells (RNAqueous-Micro kit, Life Technologies) was reverse-transcribed (QuantiTect kit, Qiagen) and subjected to quantitative real-time PCR (QuantiTect SYBR Green Kit, Qiagen) on a BioRad CFX96 thermocycler, following the manufacturer's instructions. Primers are listed in Table 2.3.

Histopathology

Organs were formalin-fixed, dehydrated in 70% EtOH, paraffin-embedded and Hematoxylin and Eosin (H&E) stained. Whole blood Complete Blood Counts (CBCs) were performed on a Scil Vet abc Animal Blood Counter (Scil Animal Care).

Statistics

Quantified results of qPCR and flow cytometry were compared between groups using *Student's t* test, and are presented as mean +/- SD, with p ≤0.05 deemed significant. Statistical analysis and graphical representation of data were performed using GraphPad Prism version 4.0c (GraphPad Software, San Diego, CA). Data shown are mean +/- SD. ns, p≥0.05; *, p≤0.05; **, p≤0.01; ***, p≤ 0.001; ****, p≤0.0001

Table 2.3. Primers used for quantitative real-time PCR

Target	Forward 5'-3'	Reverse 5'-3'
Cbl	AGCTGATGCTGCCGAATTT	TTGCAGGTCAGATCAATAGTGG
Cbl-b	GGAGCTTTTTGCACGGACTA	TGCATCCTGAATAGCATCAA
CD4	GAGAGTCAGCGGAGTTCTC	CTCACAGGTCAAAGTATTGTTG
LCK	CGCATGGTGAGACCTGACAA	TCCGAAGGTAGTCAAACGTGG
CD3	TGCCTCAGAAGCATGATAAGC	GCCCAGAGTGATACAGATGTCAA
GAPDH	CCTGGAGAAACCTGCCAAGTATG	AGAGTGGGAGTTGCTGTTGAAGT

CHAPTER 3: RESULTS

The material covered in the following chapter is the topic of the following manuscript:

Benjamin Goetz*, Wei An*, Bhopal Mohapatra*, Neha Zutshi, Fany Iseka, Matthew D. Storck, Jane Meza, Yuri Sheinin, Vimla Band, Hamid Band. A novel *CBL-B*$^{flox/flox}$ mouse model allows tissue-selective fully conditional *CBL/CBL-B* double-knockout: CD4-Cre mediated *CBL/CBL-B* deletion occurs in both T-cells and hematopoietic stem cells. *Oncotarget, 7*(32), 51107-51123.

Generation of CBL-B floxed mice

Since a regular knockout strategy targeting exons 1 and 2 of CBL-B gene is known to yield mice that completely lack CBL-B protein expression (Chiang, Kole et al. 2000), we chose to target the same exons to generate CBL-B$^{flox/flox}$ mice as described in the Materials and Methods section, using the CBL-B conditional knockout construct shown in Figure 3.1 A. Successfully targeted embryonic stem cell clones and chimeric mice were identified based on the generation of a 10 kb fragment in Southern blots of *Xma1* digested genomic DNA (Figure 3.1B). Successfully-targeted founder mice were crossed with a Flp recombinase strain to excise the Neomycin-resistance cassette. The heterozygous floxed mice were used to generate the CBL-B$^{flox/flox}$ mice whose genotype was confirmed by PCR, with the floxed allele generating a 750 bp fragment while the WT allele generates a 850 bp fragment (Figure 3.1C).

To confirm that the inserted floxed sites were susceptible to Cre cleavage in vivo, we crossed the CBL-B$^{flox/flox}$ mice with an EIIA-Cre transgenic line and assessed the deletion of the floxed CBL-B allele by western blotting of splenocytes, demonstrating a complete loss of CBL-B protein expression similar to that seen with positive control CBL-B (-/-) splenocytes, while CBL-B protein expression in CBL-B$^{flox/flox}$ and WT mouse splenocytes was comparable (Figure 3.2A), excluding any negative impact of the introduced flox sites themselves on CBL-B expression.

To further verify the functional impact of CBL-B deletion in the new conditional CBL-B deletion model, splenocytes isolated from CBL-B$^{flox/flox}$; EIIA-Cre mice were subjected to stimulation using an anti-CD3 antibody with or without an anti-CD28 antibody. It has been established that CBL-B-deficient T cells secrete higher levels of IL-2 upon stimulation with an anti-CD3 antibody alone, or with an anti-CD3 plus anti-CD28 stimulation (Chiang, Kole et al. 2000). Indeed, anti-CD3 or anti-CD3 plus anti-CD28 stimulation induced higher levels of IL-2 production in CBL-B (-/-) T cells from

Figure 3.1. Generation of the CBL-B floxed allele. (A) Strategy for generating the CBL-B floxed targeting vector and CBL-B floxed (targeted) allele. Blue boxes represent exons. The 5′ external probe for Southern Blotting is indicated by the thick black line and 3′ external probe is displayed by thick red lines. The predicted length of Southern fragments is indicated with double arrow lines. (B) Targeted events were identified by Southern analysis of Xma1- digested genomic ES cell DNAs with a 3′ flanking probe. There is a 1.9 KB insertion of the loxP-Frt cassette after proper targeting. B6 ES clones identified after southern blot. (C) Confirmation of the genotype of CBL-B floxed animals generated in our lab using allele-specific PCR primers detecting the floxed CBL-B allele correspond to the insert region containing the loxP site.

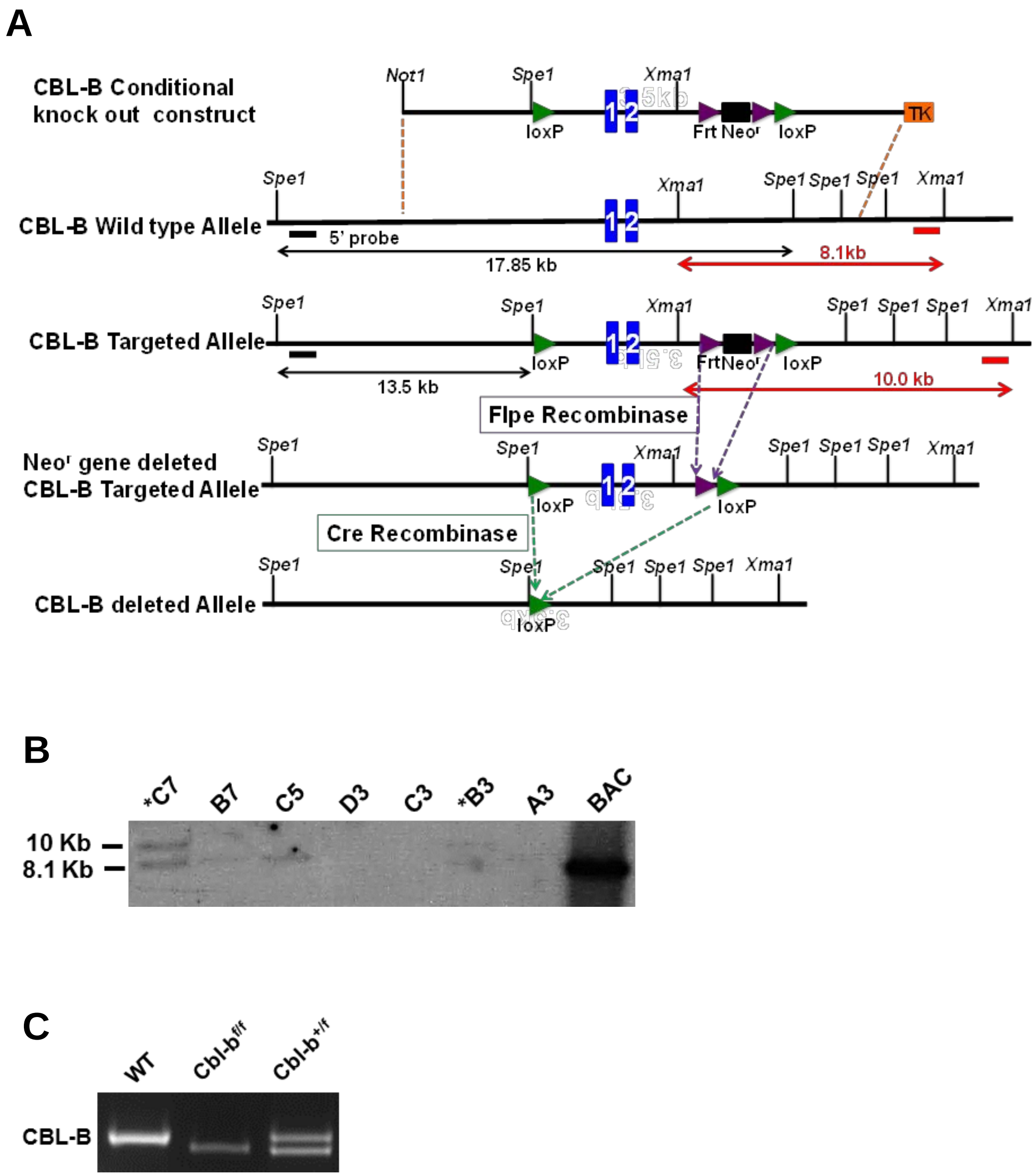
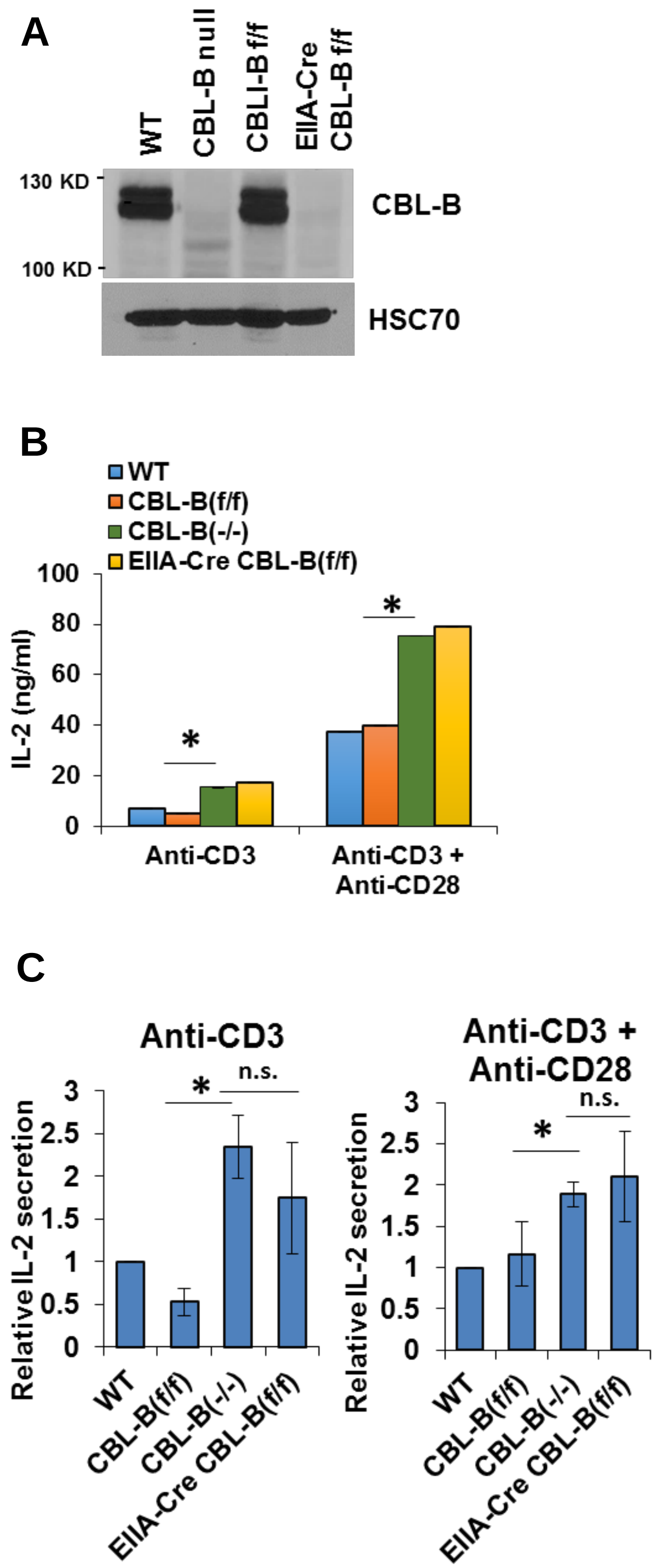

A
CBL-B Conditional knock out construct
Not1
Spe1
Xma1
loxP
Frt Neo^r
loxP
TK
CBL-B Wild type Allele
Spe1
Xma1
Spe1 Spe1 Spe1
Xma1
5' probe
17.85 kb
8.1kb
CBL-B Targeted Allele
Spe1
Spe1
Xma1
loxP
Frt Neo^r
loxP
Spe1 Spe1 Spe1
Xma1
13.5 kb
10.0 kb
Flpe Recombinase
Neo^r gene deleted CBL-B Targeted Allele
Spe1
Spe1
Xma1
loxP
loxP
Spe1 Spe1 Spe1
Xma1
Cre Recombinase
CBL-B deleted Allele
Spe1
Spe1
Spe1 Spe1 Spe1
Xma1
loxP
B
*C7
B7
C5
D3
C3
*B3
A3
BAC
10 Kb
8.1 Kb
C
WT
Cbl-b^f/f
Cbl-b^+/f
CBL-B

Figure 3.2. Characterization of CBL-B floxed mice. (A) Western Blot validating the deletion of CBL-B in EIIA-Cre CBL-B floxed mice. Splenocytes were collected from mice with indicated genotype and total protein lysate were blotted for CBL-B and HSC70. (B–C) IL-2 ELISA. Splenocytes were collected from mice with indicated genotype and plated for 48 hours before medium were collected for IL-2 quantification. (B) is one representative experiment and (C) are pooled data from three experiments and shown as relative level normalized to WT control. Data shown are mean +/− SD. ns, $p \geq 0.05$; *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$; ****$p \leq 0.0001$.



A
WT
CBL-B null
CBLI-B f/f
EIIA-Cre CBL-B f/f
130 KD
100 KD
CBL-B
HSC70
B
WT
CBL-B(f/f)
CBL-B(-/-)
EIIA-Cre CBL-B(f/f)
IL-2 (ng/ml)
100
80
60
40
20
0
*
*
Anti-CD3
Anti-CD3 + Anti-CD28
C
Anti-CD3
Anti-CD3 + Anti-CD28
Relative IL-2 secretion
3
2.5
2
1.5
1
0.5
0
*
n.s.
WT
CBL-B(f/f)
CBL-B(-/-)
EIIA-Cre CBL-B(f/f)

CBL-B$^{flox/flox}$; EIIA-Cre mice similar to the increase in IL2 production seen using T cells from conventional CBL-B (-/-) mice (Figure 3.2B and C). Collectively, these results establish that we have engineered a CBL-B$^{flox/flox}$ model that does not affect CBL-B expression in the absence of Cre-mediated gene deletion and is fully amenable to Cre-mediated gene deletion in vivo, recapitulating the functional impact of whole-body CBL-B deletion on T cells previously reported (Chiang, Kole et al. 2000).

CD4-Cre induced CBL/CBL-B deletion leads to strong hematological phenotype

Previously, CBL/CBL-B double-KO in T cells using Lck-Cre mediated deletion of CBL on a whole-body CBL-B KO was found to produce a spontaneous inflammatory disease that was eventually lethal (within 25 weeks of age), compared to a lack of inflammatory phenotype in the parental CBL-B-null mouse strain (Chiang, Kole et al. 2000). In our effort to examine the impact of T cell specific, concurrent deletion of CBL and CBL-B, we generated CBL$^{flox/flox}$; CBL-B$^{flox/flox}$ mice using the previously generated CBL$^{flox/flox}$ mice (Naramura, Jang et al. 2002) and further crossed these to CD4-Cre transgenic mice to generate CBL$^{flox/flox}$; CBL-B$^{flox/flox}$; CD4-Cre mice for conditional deletion of CBL and CBL-B specifically in T cells (referred to as DKO mice hereafter). We also introduced a dual-reporter of Cre-mediated gene deletion in which ROSA-26 locus-encoded membrane-localized td-Tomato (red) and GFP that are expressed prior to and after successful Cre-mediated deletion of floxed sequence cassettes respectively (Muzumdar, Tasic et al. 2007). This reporter system provided a handy tool to identify cells that have undergone Cre-mediated deletion (GFP+). FACS analysis identified a substantial increase in GFP expression in CD4+ cells of the spleen, and real-time PCR analysis verified the deletion of CBL and CBL-B in these cells (Figure 3.3A, B).

The CBL$^{flox/flox}$; CBL-B$^{flox/flox}$; CD4-Cre+ (DKO) pups were born at expected Mendelian ratios (around 50% when we crossed CBL$^{flox/flox}$; CBL-B$^{flox/flox}$ mice with

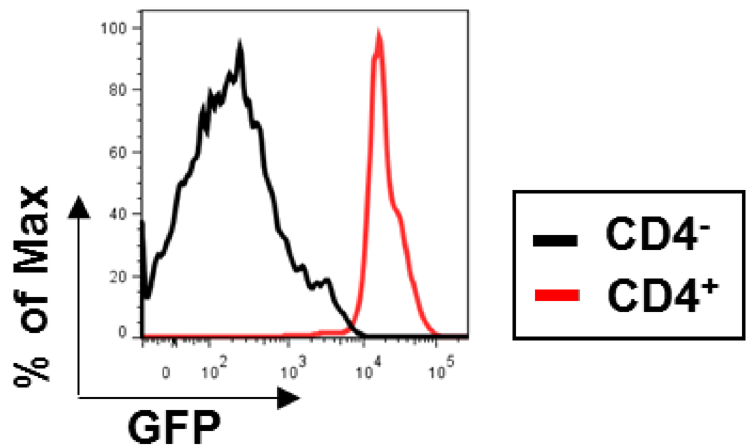
CBL$^{flox/flox}$; CBL-B$^{flox/flox}$; CD4-Cre+ mice). These mice, however, became moribund starting as early as 10 weeks of age and all became moribund and required euthanasia by 25 weeks (Figure 3.4A). Examination of 10-week old female DKO mice revealed the development of lymphomegaly and hepatosplenomegaly in all animals (n=11) (Figure 3.4B). H& E staining of formalin-fixed and paraffin-embedded sections showed a high degree of immune cell infiltration in multiple organs examined (Figure 3.4C). Histological analysis revealed intense immune cell aggregates and features of extramedullary hematopoiesis in the liver, perivascular immune clusters in the kidney, perivascular immune aggregates and signs of acute and chronic inflammation in the lung, as well as perivascular immune clusters in the brain. Pathological changes observed in lymphoid tissues includes enlargement of follicle size and less defined white pulp in the spleen, and increased medullary areas in lymph nodes. The histopathology of the heart and intestine were normal. These data show that CD4-Cre-directed CBL/CBL-B DKO leads to a severe and lethal spontaneous autoimmune/inflammatory phenotype.

CD4-Cre-induced CBL/CBL-B DKO alters T cell development and peripheral T cell activation

Since CBL family proteins are known to function as critical negative regulators of signaling in T cells (Thien, Langdon 2001), we examined the effect of CD4-Cre-induced DKO on T cells within lymphoid tissues. Ten-week old female DKO mice exhibited shrunken thymuses coinciding with a marked reduction in the overall thymocyte numbers compared to control (CBL$^{flox/flox}$; CBL-B$^{flox/flox}$ mice without CD4-Cre) mice (Figure 3.5A). Examination of different thymocyte subpopulations in DKO mice revealed a diminished CD4 and CD8 double-positive (DP) thymocyte population; however, there was an increase in the percentage of CD4/CD8 double-negative (DN) thymocytes and a

Figure 3.3. Confirmation of CBL-B deletion in CD4+ cells. (A) FACS analysis of splenocytes from DKO mice for the expression of GFP in CD4+ or CD4− cells. (B) mRNA expression levels of CBL (left) and CBL-B (right) were analyzed in FACS-sorted CD4+ cells of Control or DKO mice by quantitative real-time PCR.

A


100
80
60
40
20
0
0
10^2
10^3
10^4
10^5
% of Max
GFP
CD4-
CD4+

B

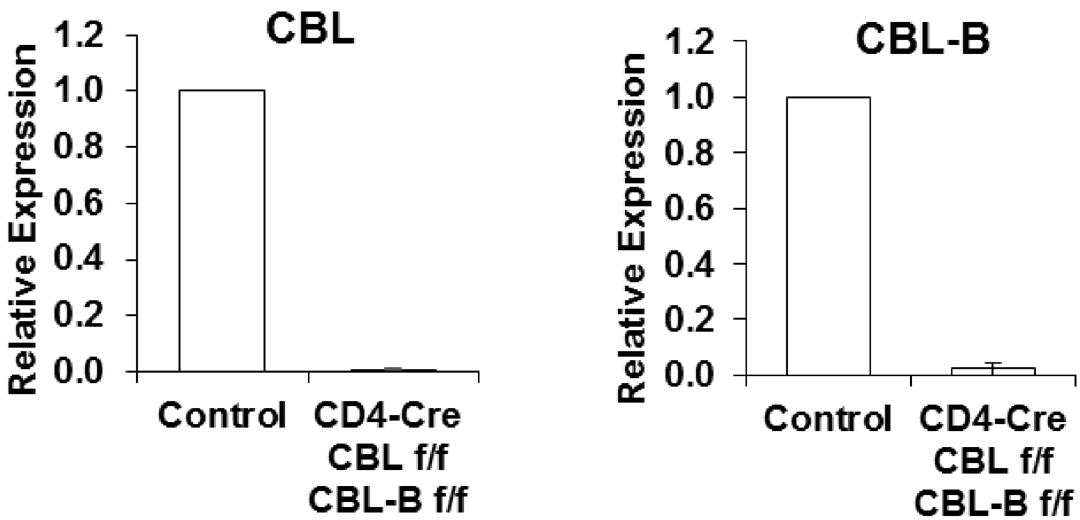
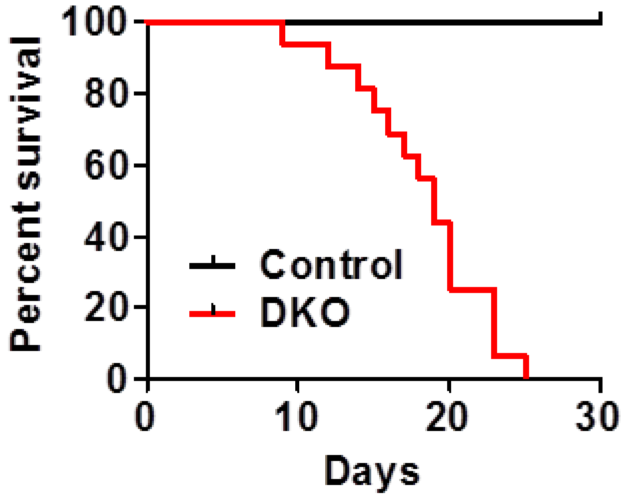
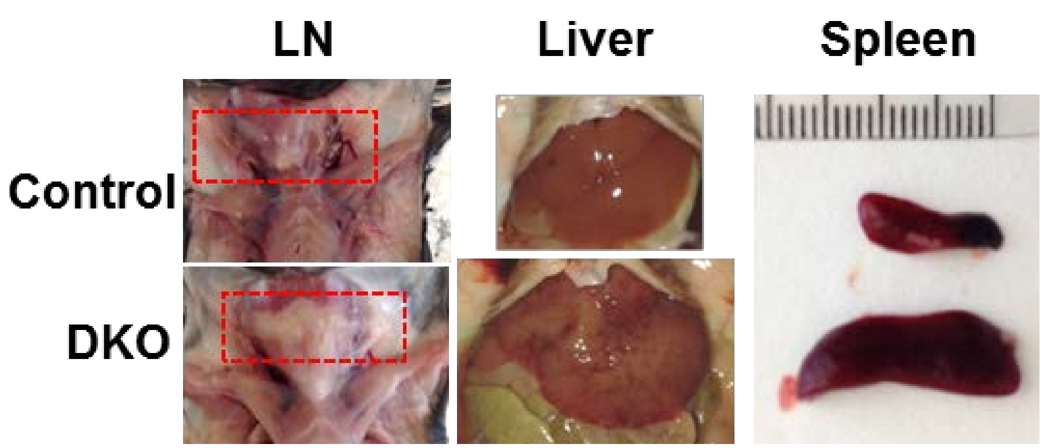

CBL
Relative Expression
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Control
CD4-Cre
CBL f/f
CBL-B f/f
CBL-B
Relative Expression
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Control
CD4-Cre
CBL f/f
CBL-B f/f

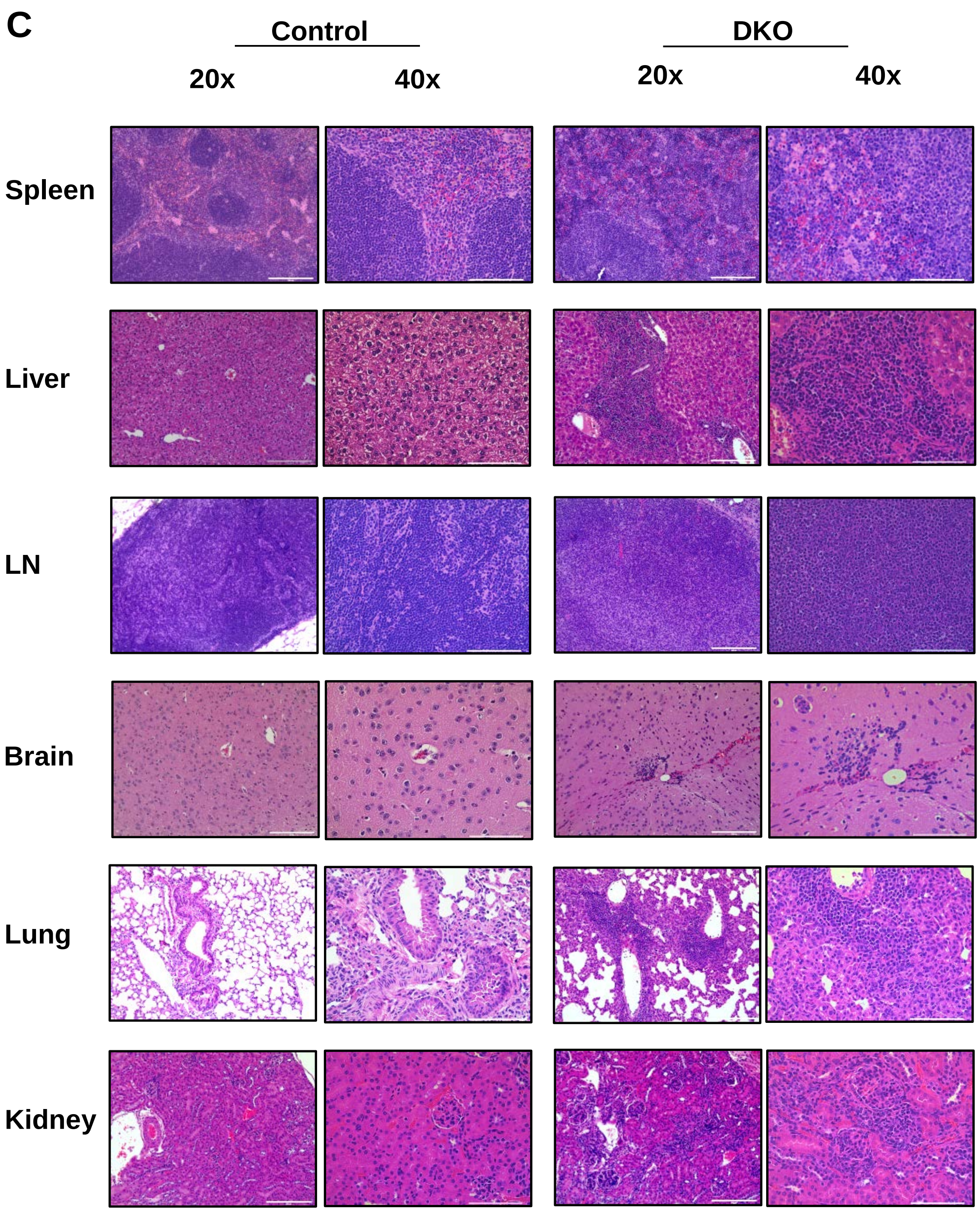
Figure 3.4. CD4-Cre induced CBL/CBL-B deletion leads to strong hematological phenotype. (A) Kaplan-Meyer survival curve of Control and DKO mice; $n = 16$. (B) Representative photos demonstrating lymph node hyperproliferation and hepatosplenomegaly. Red rectangle indicates lymph nodes (C) Representative H & E stained liver, spleen, lymph node (LN), brain, lung and kidney sections. Bar for 20× and 40× images represent 200 μm and 100 μm respectively.

A


Percent survival
100
80
60
40
20
0
0
10
20
30
Days
Control
DKO

B

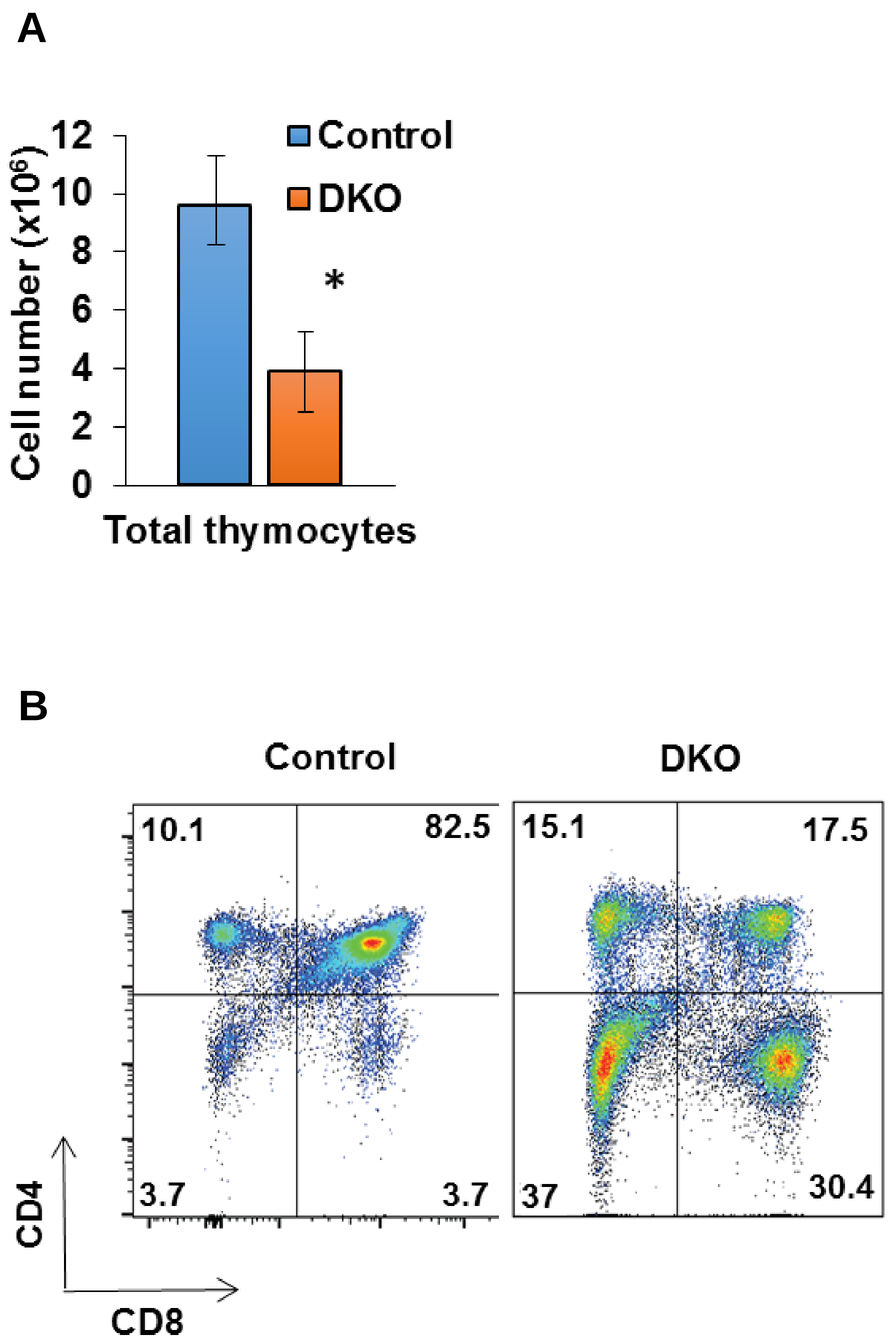

LN
Liver
Spleen
Control
DKO


C
Control
DKO
20x
40x
20x
40x
Spleen
Liver
LN
Brain
Lung
Kidney

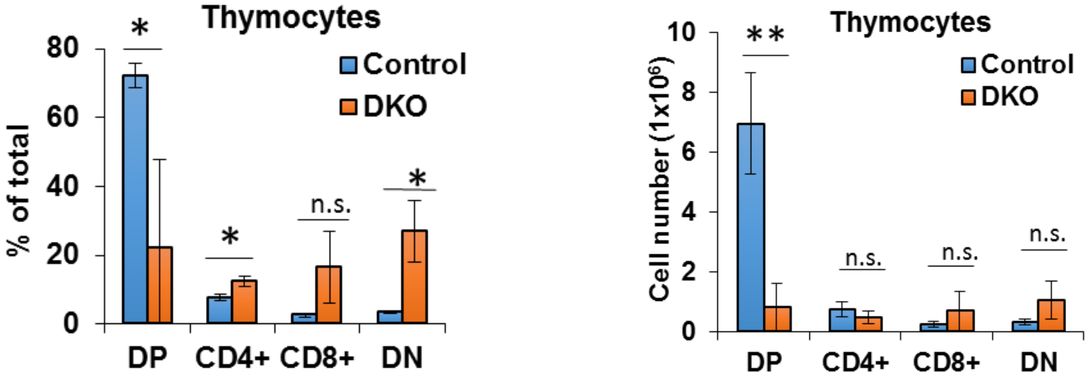
substantial, albeit not statistically significant (with the exception of CD4), skewing of the relative percentages and absolute numbers of single-positive populations compared to control (Figure 3.5B, C). Compared to controls, the percentage of CD4+ thymocytes was modestly higher, however, this was not reflected in the overall CD4+ thymocyte numbers (Figure 3.5B, C). Further dissection of the DN population into DN1-4 subpopulations (based on the markers CD25, CD44, and CD117) revealed an increase in DN4 population in DKO thymuses compared to control (Figure 3.5D). Altogether these data show that concurrent conditional deletion of CBL and CBL-B in T cells using CD4-Cre leads to marked alterations in thymocyte development with a decrease in total double-positive thymocytes, an increase in double-negative thymocytes, and a skewing to CD4+ thymocyte proportions.

CBL/CBL-B deficiency in T cells was previously shown to result in a constitutively activated T cell phenotype (Chiang, Kole et al. 2000, Naramura, Kole et al. 1998, Naramura, Jang et al. 2002), as opposed to hyperactivity only upon extrinsic stimulation of T cells in CBL-B-null mice (Fang, Liu 2001, Chiang, Kole et al. 2000, Krawczyk, Bachmaier et al. 2000). To assess the impact of concurrent deletion of CBL/CBL-B in T cells in CD4-Cre-bearing conditional DKO mice, we evaluated the activation status of peripheral T cells in secondary lymphoid tissues. Compared to control mice, CD4+ and CD8+ T cell populations contributed to a smaller percentage of the total splenic cells in DKO mice despite having overall greater numbers (Figure 3.6A, B). However, both CD4+ and CD8+ splenic T cell populations in DKO mice exhibited lower levels of CD45RB and CD62L, as well as an increase in the ratios of cells positive for activation markers CD25, CD44 and CD69 as compared to control mice (Figure 3.6C, D). Examination of lymph node T cell populations revealed a decrease in the percentage of CD4+ T cells in DKO mice compared to control mice, similar to that seen in the spleen; however, the percentage of the CD8+ T cell population appeared to be

Figure 3.5. CD4-Cre induced CBL/CBL-B deletion leads to altered thymocyte development. (A) Mean values of cell numbers from thymuses of 10-week old Control and DKO mice; $n = 3$. (B) Representative dot plot FACS analysis of anti-CD4 and anti-CD8 stained thymocytes. (C) Flow cytometric analysis of CD4CD8 DP, single-positive, and DN thymocyte populations for percentage of total thymocytes (left) and cell number (right); $n = 3$. (D) Flow analysis of DN thymocyte populations for percentage of total thymocytes (left) and cell number (right); $n = 3$. DN cells are gated (DN1: CD117+ CD44+ CD25−, DN2: CD117+ CD44+ CD25+, DN3: CD117− CD44− CD25+, DN4: CD117− CD44− CD25−). Data shown are mean +/− SD. ns, $p \geq 0.05$; *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$; ****$p \leq 0.0001$.


A
Cell number (x10^6)
12
10
8
6
4
2
0
Control
DKO
*
Total thymocytes
B
Control
DKO
10.1
82.5
3.7
3.7
15.1
17.5
37
30.4
CD4
CD8

C


Thymocytes
% of total
80
60
40
20
0
*
*
n.s.
*
Control
DKO
DP
CD4+
CD8+
DN
Thymocytes
Cell number (1x10^6)
10
8
6
4
2
0
**
n.s.
n.s.
n.s.
Control
DKO
DP
CD4+
CD8+
DN

D

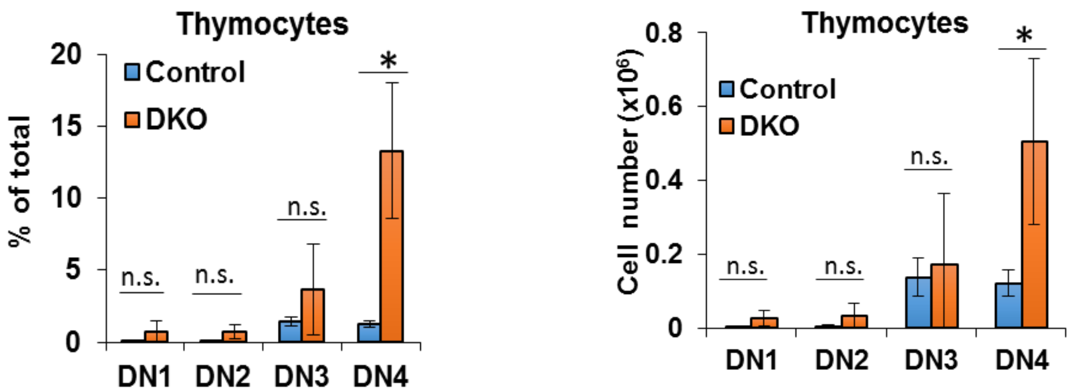
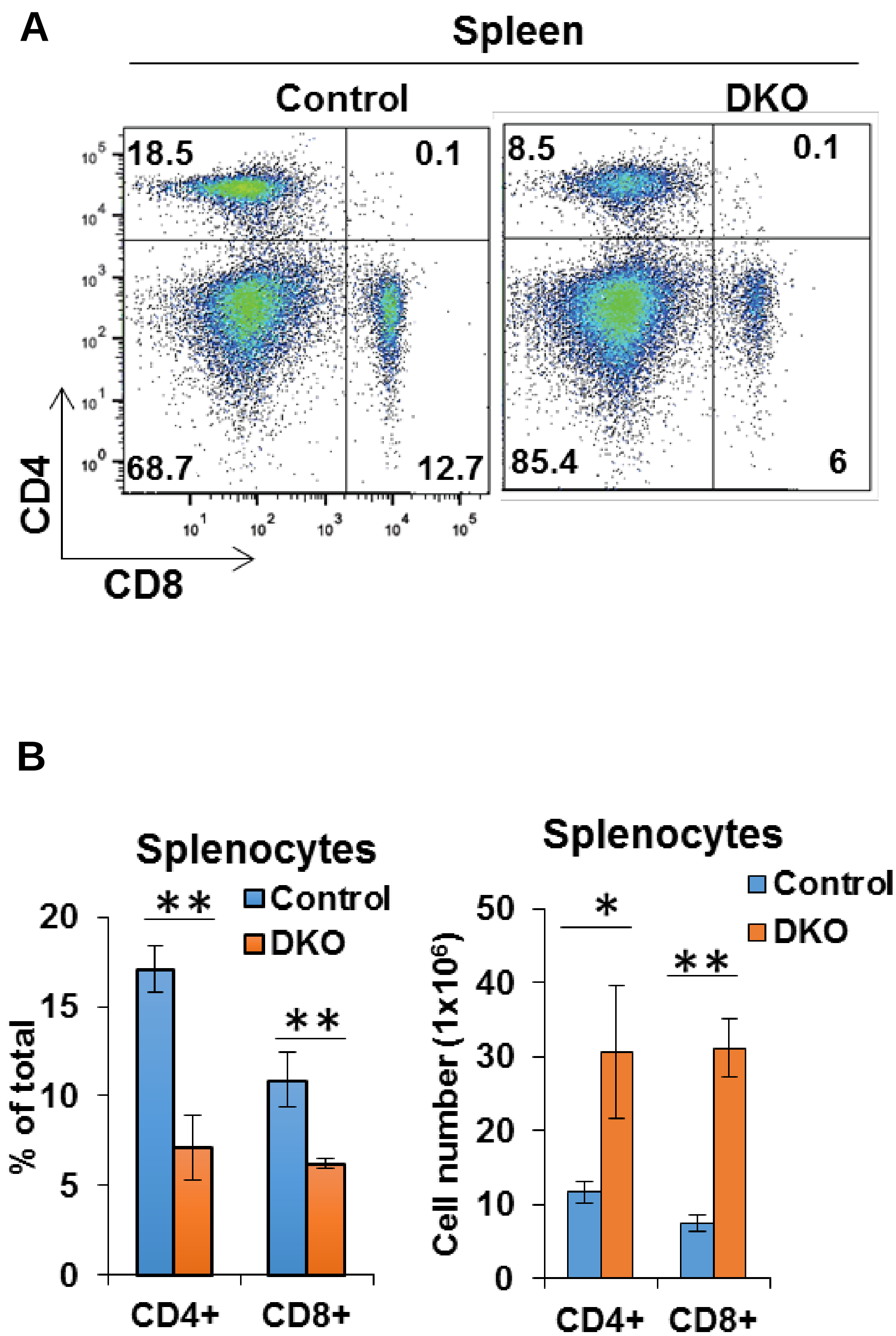
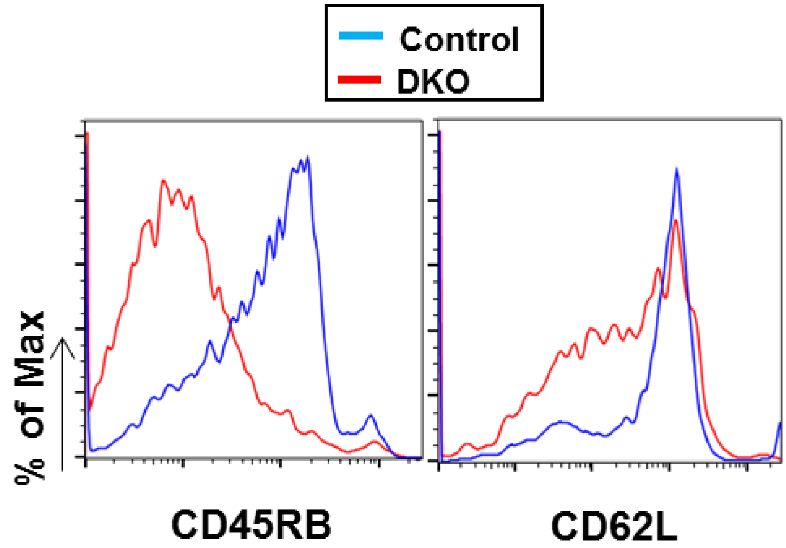

Thymocytes
% of total
20
15
10
5
0
Control
DKO
n.s.
n.s.
n.s.
*
DN1
DN2
DN3
DN4
Thymocytes
Cell number (x10^6)
0.8
0.6
0.4
0.2
0
Control
DKO
n.s.
n.s.
n.s.
*
DN1
DN2
DN3
DN4

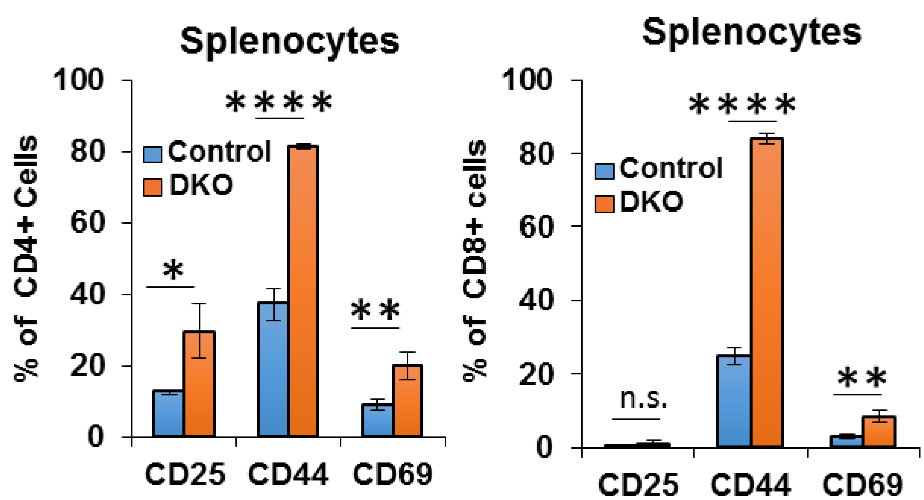
Figure 3.6. CD4-Cre induced CBL/CBL-B deletion leads to altered splenic T cell activation status. (A–B) Representative flow cytometric dot plots (A) and mean values of percentage of total splenic populations stained with anti-CD4 and anti-CD8 (B) in Control and DKO mice; $n = 3$. (C) Representative histograms for flow cytometric analysis of marker expression for CD4+ gated splenic population. (D) Quantification of the percentage of cells positive for markers CD25, CD44, and CD69 in splenic CD4+ (left) and CD8+ (right) gated populations; $n = 3$. Data shown are mean +/− SD. ns, $p \geq 0.05$; *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$; ****$p \leq 0.0001$.


A
Spleen
Control
DKO
18.5
0.1
68.7
12.7
8.5
0.1
85.4
6
CD4
CD8
B
Splenocytes
Control
DKO
% of total
CD4+
CD8+
Splenocytes
Control
DKO
Cell number (1x10^6)
CD4+
CD8+

C


Control
DKO
% of Max
CD45RB
CD62L

D


Splenocytes
% of CD4+Cells
100
80
60
40
20
0
Control
DKO
*
****
**
CD25
CD44
CD69
Splenocytes
% of CD8+ cells
100
80
60
40
20
0
Control
DKO
n.s.
****
**
CD25
CD44
CD69

unchanged between control and DKO mice (Figure 3.7A, B). Also, similar to spleen, CD4+ and CD8+ T cell populations in DKO lymph nodes showed a higher percentage of cells positive for activation markers compared to control mice (Figure 3.7C). Collectively, these findings point to a clear conclusion that CD4-Cre-induced concurrent CBL/CBL-B DKO leads to alleviation of negative regulatory mechanisms of T cell activation resulting in constitutively-activated T cells.

CD4-Cre-directed CBL/CBL-B deletion in Non-T cell lineages

A dramatically activated phenotype of T cells in peripheral lymphoid tissues, coupled with the result that CD4+ T cells represented a smaller percentage of total cells in these tissues, suggested the possibility that CD4-Cre-induced DKO was associated with direct or indirect alterations in non-T cell lineages. To investigate this possibility, we assessed the changes in the percentages and numbers of non-T hematopoietic lineage cells by flow cytometry (Figure 3.8A). Spleen and lymph nodes were collected from control and DKO mice, and B cells and myeloid cells were evaluated. Compared to control mice, DKO spleens showed an expansion of B cells and myeloid cells, evidenced by an increase in the absolute numbers of cells carrying CD11b, CD11c, B220, F4/80 or Gr-1 markers (Figure 3.8B). Similarly, the Gr-1^{+} population was also substantially, albeit not statistically significantly, expanded in DKO lymph nodes (Figure 3.8B). Peripheral blood differential cell counts further demonstrated a dramatic increase in myeloid cell populations in DKO mice compared to Control mice (Figure 3.9A).

To determine whether the alterations of non-T hematopoietic lineage cells was due to cell intrinsic loss of CBL/CBL-B or indirectly due to an effect of extrinsic signals from activated T cells, we carried out flow cytometry-based analysis of GFP reporter gene expression to assess if CD4-Cre-induced gene deletion was confined to T cells as expected, or if non-T cell lineages also showed evidence of gene deletion. As shown in

Figure 3.7. CD4-Cre induced CBL/CBL-B deletion leads to altered T cell phenotype in lymph nodes. (A–B) Representative flow cytometric dot plots (A) and mean values of percentage of total lymph node cells stained with anti-CD4 and anti-CD8 (B) in Control and DKO mice; $n = 3$. (C) Quantification of the percentage of cells positive for markers CD25, CD44, and CD69 in lymph node CD4+ (left) and CD8+ (right) gated populations; $n = 3$. Data shown are mean +/− SD. ns, $p \geq 0.05$; *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$; ****$p \leq 0.0001$.

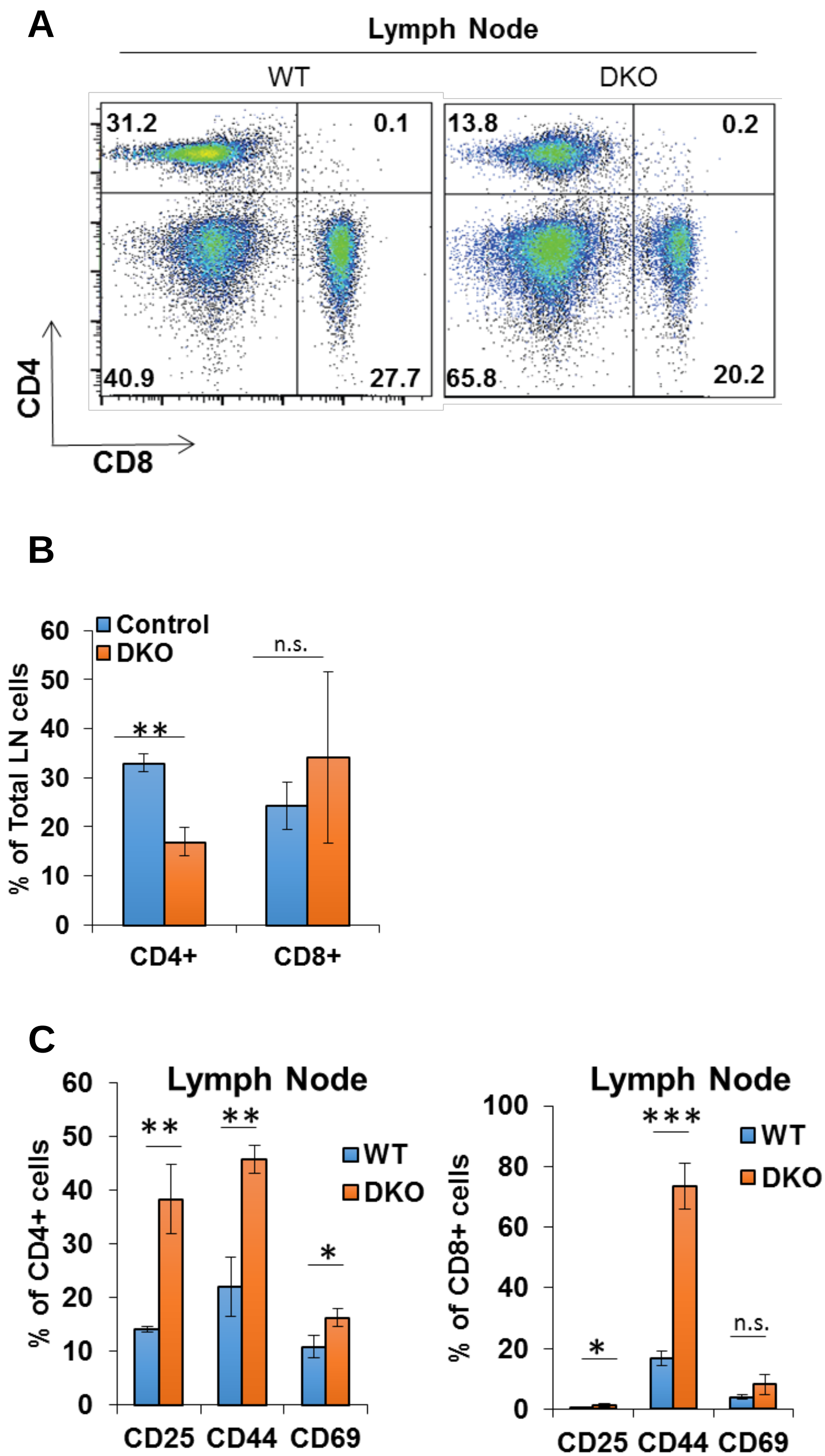
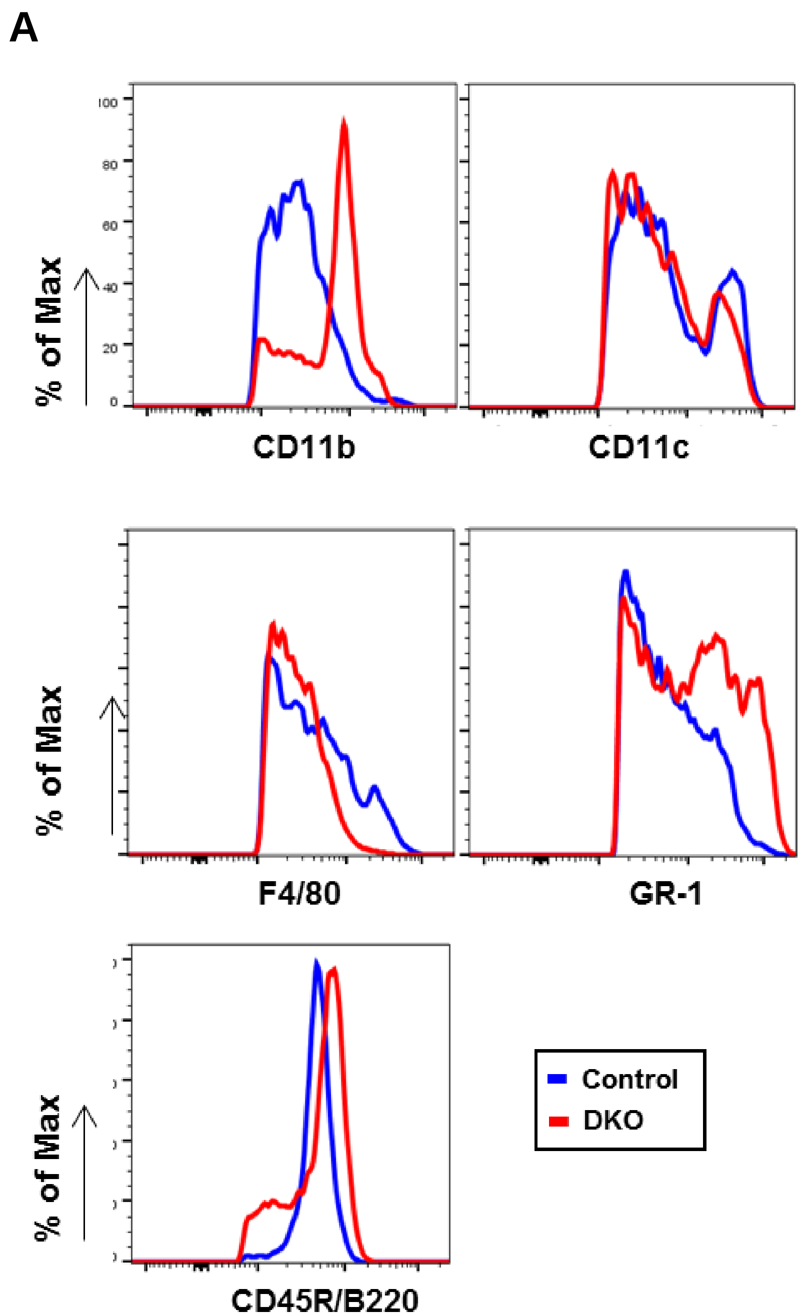

A
Lymph Node
WT
DKO
31.2
0.1
40.9
27.7
13.8
0.2
65.8
20.2
CD4
CD8
B
Control
DKO
% of Total LN cells
**
n.s.
CD4+
CD8+
C
Lymph Node
% of CD4+ cells
**
**
*
WT
DKO
CD25
CD44
CD69
Lymph Node
% of CD8+ cells
*
***
n.s.
WT
DKO
CD25
CD44
CD69

Figure 3.8. Non-T cell lineages were impacted by CD4-Cre. (A) Representative histograms for flow cytometric analysis of non-T cell marker expression in the spleen of Control and DKO mice. (B) Flow cytometric analysis of spleen (left and center) and lymph node (right) from Control and DKO mice for non-T-cell markers; $n = 3$. Data shown are mean +/− SD of at least 3 mice replicates. ns, $p \geq 0.05$; *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$; ****$p \leq 0.0001$.


A
100
80
60
40
20
0
% of Max
CD11b
CD11c
% of Max
F4/80
GR-1
% of Max
CD45R/B220
Control
DKO

B

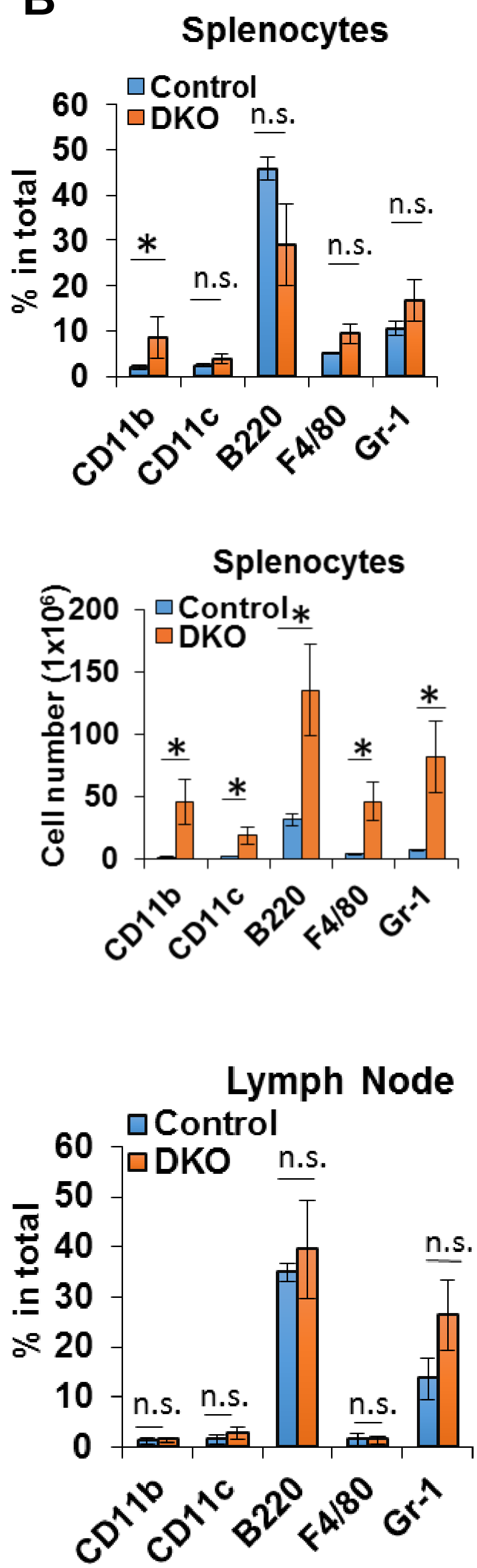
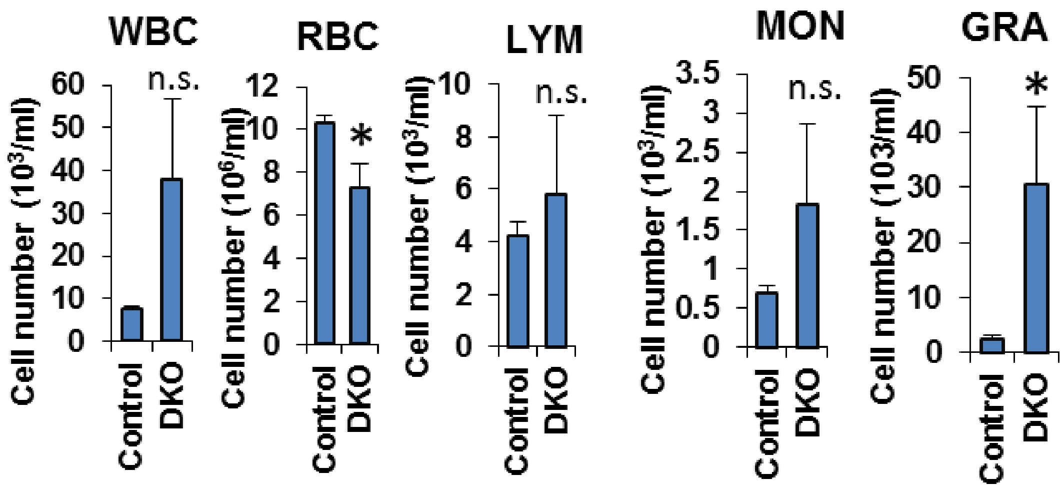

Splenocytes
Control
DKO
% in total
0
10
20
30
40
50
60
*
n.s.
n.s.
n.s.
n.s.
CD11b
CD11c
B220
F4/80
Gr-1
Splenocytes
Control
DKO
Cell number (1x10^6)
0
50
100
150
200
*
*
*
*
*
CD11b
CD11c
B220
F4/80
Gr-1
Lymph Node
Control
DKO
% in total
0
10
20
30
40
50
60
n.s.
n.s.
n.s.
n.s.
n.s.
CD11b
CD11c
B220
F4/80
Gr-1

Figure 3.9. Blood cell counts were affected by CD4-Cre. (A–B) Peripheral blood cell counts (A) and flow cytometric analysis for the expression of GFP (B) of Control and DKO mice; $n = 3$. WBC, white blood cell; LYM, lymphocyte; MON, monocyte; GRA, granulocyte; RBC, red blood cell. Data shown are mean +/− SD of at least 3 mice replicates. ns, $p \geq 0.05$; *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$; ****$p \leq 0.0001$.

A


WBC
RBC
LYM
MON
GRA
Cell number (10³/ml)
Cell number (10⁶/ml)
Cell number (10³/ml)
Cell number (10³/ml)
Cell number (103/ml)
n.s.
*
n.s.
n.s.
*
Control
DKO

B

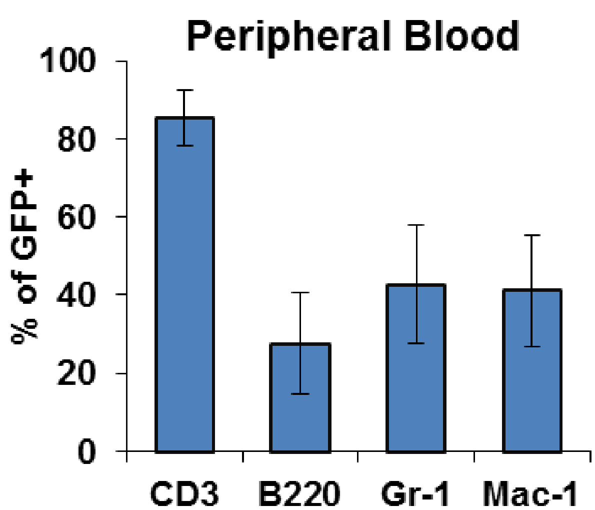
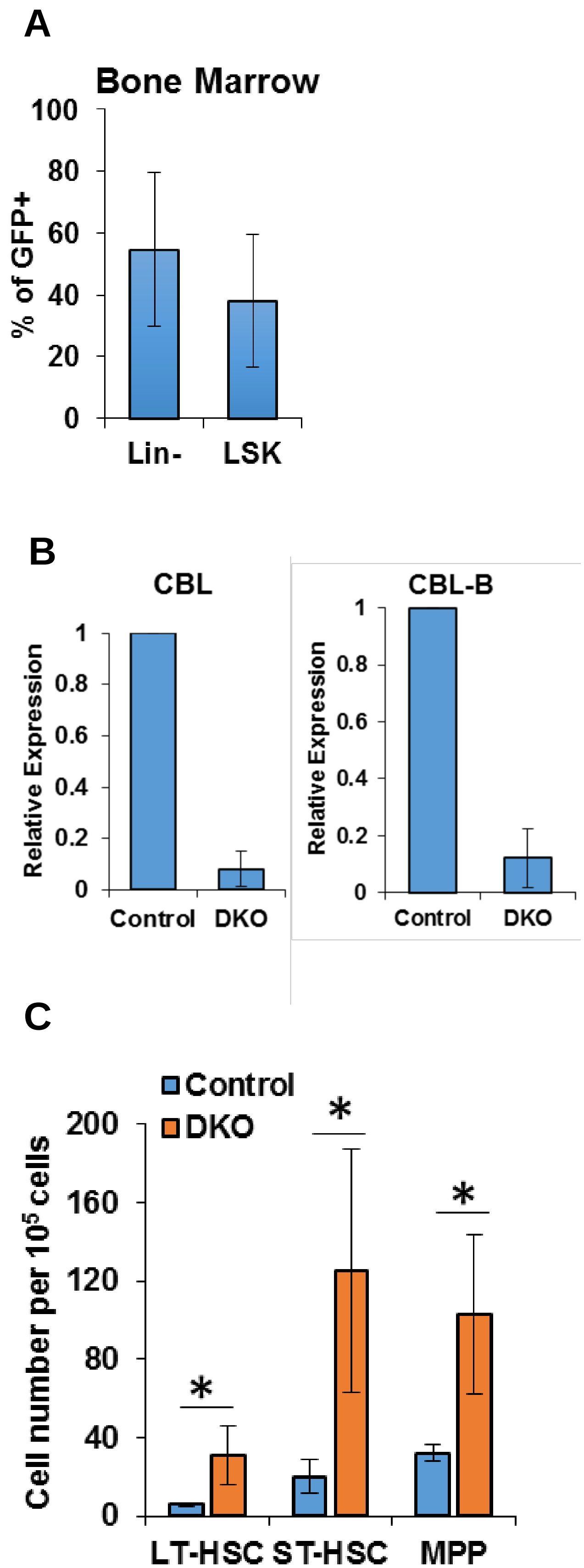

Peripheral Blood
% of GFP+
CD3
B220
Gr-1
Mac-1

Figure 3.9B, GFP was expressed in a majority of T cells as anticipated; however, a subset of both B cells and myeloid populations were GFP+, indicative of unexpected deletion of CBL and CBL-B in non-T lineages.

Our results that CD4-Cre-mediated reporter gene deletion occurred in multiple non-T cell lineages and the myeloid cell proportion was increased in the peripheral blood of mice rendered DKO using CD4-Cre suggests the possibility that CD4-Cre may concurrently direct CBL/CBL-B deletion either in these lineages or in HSCs, since CBL/CBL-B deletion in HSCs using MMTV-Cre leads to a myeloid-skewed expansion (Naramura, Nandwani et al. 2010, An, Nadeau et al. 2015). We therefore assessed whether CD4-Cre-directed GFP reporter gene expression is observed in HSCs from which all hematopoietic lineage cells are derived (Seita, Weissman 2010). To address this, we assessed the GFP expression of lineage marker-negative (Lin^- cells; hematopoietic stem and progenitor cells) and Lin^- $sca\text{-}1^+$ and $c\text{-}Kit^+$ (LSK cells; HSC-enriched population) cells isolated from the bone marrow of DKO mice using flow cytometry. Notably, both Lin^- and LSK populations in DKO mice contained a substantial subset of GFP+ cells (Figure 3.10A) and real-time PCR analysis demonstrated an over 90% reduction in CBL and CBL-B expression in flow cytometry-sorted GFP+ Lin- cells (Figure 3.10B). In addition, CD4-Cre-induced CBL/CBL-B deletion leads to a significant expansion of long-term HSC (LT-HSC), short-term HSC (ST-HSC) and multipotent progenitors (MPP) cell populations compared to those in control mice (Figure 3.10C). This recapitulates the phenotype of mice with MMTV-Cre-induced deletion of CBL and CBL-B in HSCs (Naramura, Nandwani et al. 2010, An, Nadeau et al. 2015). These data lead us to conclude that CD4-Cre can direct gene deletion in a proportion of non-T hematopoietic lineages starting with the HSC stage.

Figure 3.10. Bone marrow populations were impacted by CD4-Cre. (A) Flow cytometric analysis of bone marrow Lin- and LSK cells from DKO mice for GFP expression. (B) mRNA expression levels of CBL (left) and CBL-B (right) were analyzed in FACS-sorted Lin- cells of Control or DKO mice by quantitative real-time PCR. (C) Flow cytometric analysis of bone marrow cells from Control and DKO mice HSCs (LT-HSC, ST-HSC and MPP). Data shown are mean +/− SD of at least 3 mice replicates. ns, $p \geq 0.05$; $^{*}p \leq 0.05$; $^{**}p \leq 0.01$; $^{***}p \leq 0.001$; $^{****}p \leq 0.0001$.

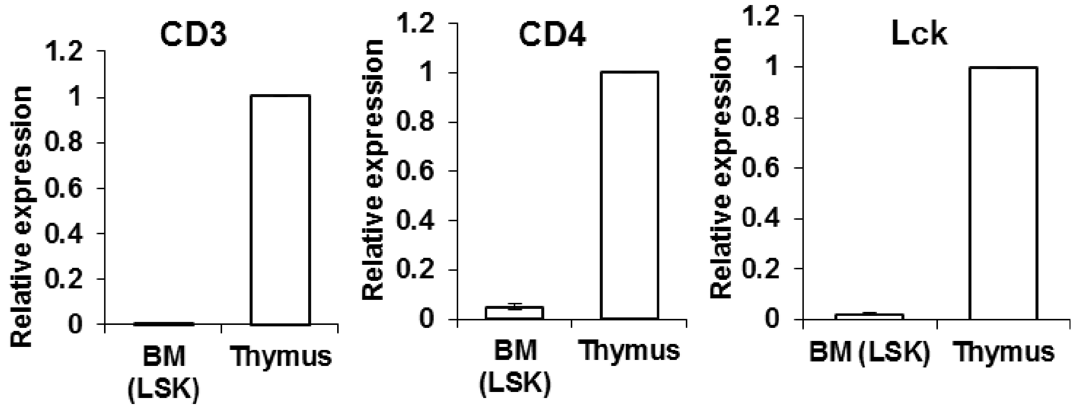

A
Bone Marrow
% of GFP+
100
80
60
40
20
0
Lin-
LSK
B
CBL
Relative Expression
1
0.8
0.6
0.4
0.2
0
Control
DKO
CBL-B
Relative Expression
1
0.8
0.6
0.4
0.2
0
Control
DKO
C
Control
DKO
Cell number per 10^5 cells
200
160
120
80
40
0
*
*
*
LT-HSC
ST-HSC
MPP

Expression of CD4 in HSC

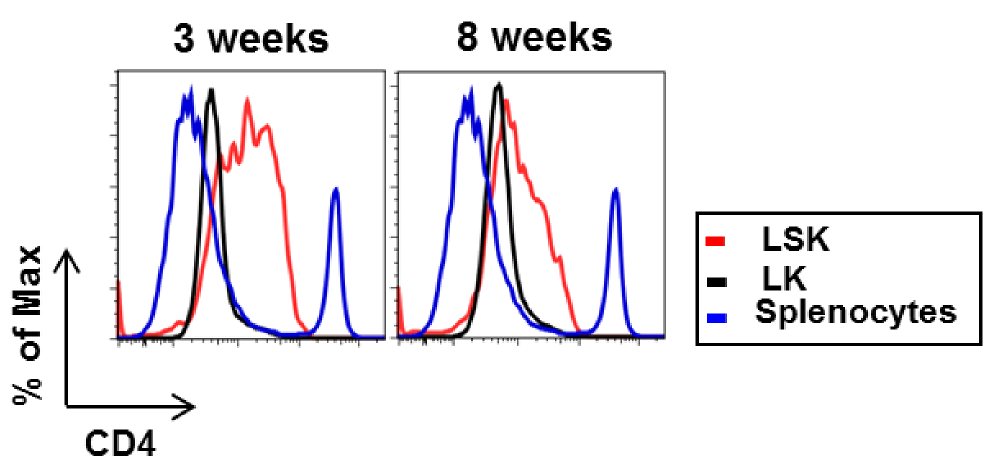
CD4 is considered a T cell marker and CD4 promoter elements have been used extensively in genetic studies to direct T cell specific gene expression or deletion (Yi, Stunz et al. 2013, Hsu, Pajerowski et al. 2011, Johnson, Pao et al. 2013, Buckley, Trampont et al. 2015, Hsu, Shapiro et al. 2014, Jost, Abel et al. 2014, Uddin, Zhang et al. 2014, Maraver, Tadokoro et al. 2007, Zhang, Rosenberg et al. 2005, Mycko, Ferrero et al. 2009). Given our results that CD4-Cre can direct gene deletion in HSCs, and old reports that a small proportion of HSCs express low levels of CD4 that is detectable with antibodies (Wineman, Gilmore et al. 1992, Ishida, Zeng et al. 2002), we next addressed if CD4 was indeed expressed in HSCs using a more sensitive quantitative real-time PCR (qPCR) assay (Figure 3.11A). HSCs (LSK cells) were sorted from WT bone marrow (BM) cells and mRNA was analyzed for CD4 expression by qPCR, with T cell specific CD3ε (Kovacic, Gupta et al. 2010) as a positive control for any T cell contamination. Compared to the undetectable expression of CD3, a low but detectable level of CD4 mRNA expression was observed in HSCs, together with a detectable expression of Lck, a key signal transducer downstream of CD4 (Zhang, Salojin et al. 1998). Furthermore, using WT BM cells from 3-week and 8-week old mice, we performed flow cytometry analysis to evaluate the expression of CD4 on HSCs and hematopoietic progenitors (Figure 3.11B). Notably, compared to splenocytes (used as a positive control), LSK cells express an easily detectable level of CD4 on the cell surface, consistent with previous reports (Wineman, Gilmore et al. 1992, Ishida, Zeng et al. 2002). Collectively, these data suggest that CD4-Cre, by virtue of an authentic expression of CD4 in a subset of HSCs and hematopoietic progenitors, can direct gene deletion in early hematopoietic lineages including HSCs, which can thereby add complexities to phenotypes assigned to T cell-specific gene deletion directed by CD4-Cre.

Figure 3.11. Expression of CD4 in HSCs. (A) BM cells were collected from 3 weeks old WT mice and Lin- cells were flow cytometry sorted followed by mRNA purification. Expression levels of CD3 (left), CD4 (center) and Lck (right) were analyzed by quantitative real-time PCR. Expression of target gene in Lin- cells is normalized to thymus control. Data show mean+/− SD of 3 replicates. (B) BM cells were collected from 3 weeks (left) or 8 weeks (right) mice and labeled with stem cell markers and CD4. Splenocytes were used as positive control for the expression of CD4. The set of data shown is one representative set of three.

A


CD3
CD4
Lck
Relative expression
1.2
1
0.8
0.6
0.4
0.2
0
BM (LSK)
Thymus

B


3 weeks
8 weeks
% of Max
CD4
LSK
LK
Splenocytes

CHAPTER 4: DISCUSSION

Parts of this chapter are derived from the following manuscript:

Benjamin Goetz*, Wei An*, Bhopal Mohapatra*, Neha Zutshi, Fany Iseka, Matthew D. Storck, Jane Meza, Yuri Sheinin, Vimla Band, Hamid Band. A novel *CBL-B*$^{flox/flox}$ mouse model allows tissue-selective fully conditional *CBL/CBL-B* double-knockout: CD4-Cre mediated *CBL/CBL-B* deletion occurs in both T-cells and hematopoietic stem cells. *Oncotarget, 7*(32), 51107-51123.

Discussion

CBL-family ubiquitin ligases are essential negative regulators of T cell activation that impinge on an anergy induction program. Tight regulation of T cell activation and immunological tolerance are essential for effective defense against foreign antigens and immune surveillance against cancer without mounting autoimmunity to self-antigens or producing protracted inflammatory diseases following infections. Previous models have failed to accurately elucidate the role that CBL proteins play in a T cell-specific manner as these models utilized a CBL-B null background (Loeser, Penninger 2007, Bachmaier, Krawczyk et al. 2000), which leads to the altered and/or enhanced function of B cells (Sohn, Gu et al. 2003), macrophages (Hirasaka, Kohno et al. 2007, Abe, Hirasaka et al. 2013), mast cells (Gustin, Thien et al. 2006), neutrophils (Choi, Orlova et al. 2008, Bachmaier, Toya et al. 2007), and NKT cells (Kojo, Elly et al. 2009). Particularly in the context of tumorigenesis, the available CBL-B-null model has not been suitable for in vivo studies to assess the tumor cell-intrinsic roles of CBL proteins since CBL-B-null mice reject tumors due to their activated CD8+ T cells (Loeser, Penninger 2007, Chiang, Jang et al. 2007, Stromnes, Blattman et al. 2010) and NK cells (Paolino, Choidas et al. 2014). Studies described here describe the establishment and characterization of the first inducible model of CBL-B deletion. By crossing this new model to a previously established and sparingly used CBL-flox/flox mouse, we have now established the first fully conditional model of tissue-specific CBL/CBL-B DKO mouse model that will help overcome the current barrier in understanding the redundant roles of these two CBL-family proteins in physiological systems as well as in oncogenesis.

We established the functionality of the new floxed CBL-B alleles engineered in the mouse genome by demonstrating the generation of a whole-body CBL-B null mouse by crossing the CBL-B floxed mice with the EIIA-Cre transgene, which is known to drive Cre expression very early during development, including in germ cells (Lakso, Pichel et al. 1996). Analyses of T cells of these mice recapitulated the hyper-responsiveness to TCR engagement comparable to that seen using T cells of previously generated whole-body KO mice (Chiang, Kole et al. 2000) (Figure 3.6 and 3.7). As a proof of principle of tissue-specific concurrent deletion of CBL and CBL-B in the CBL-flox/flox/CBL-B-flox/flox mice that we generated, we chose to induce a DKO in T cells since prior studies using Lck-Cre-driven deletion of CBL-flox/flox gene on a CBL-null background demonstrated that CBL and CBL-B function redundantly in T cells and the DKO mice exhibit a profound and lethal inflammatory disease (Naramura, Jang et al. 2002). We used a CD4-Cre driver for these studies as this Cre has been extensively used for T cell-specific deletion of conditionally targeted genes (Yi, Stunz et al. 2013, Hsu, Pajerowski et al. 2011, Johnson, Pao et al. 2013, Buckley, Trampont et al. 2015, Hsu, Shapiro et al. 2014, Jost, Abel et al. 2014, Uddin, Zhang et al. 2014, Maraver, Tadokoro et al. 2007, Zhang, Rosenberg et al. 2005, Mycko, Ferrero et al. 2009). CD4-Cre mediated DKO led to severe systemic, autoimmune/inflammatory disease with mice becoming moribund as early as 10 weeks of age accompanied by immune cell infiltration in multiple organs including liver, brain, kidney, and lung (Figure 3.4A, C). Notably, our findings differ from those of the previous T cell DKO studies (Naramura, Jang et al. 2002) in that we also observed immune cell infiltration in brain, kidney, and lung.

CD4-Cre expression is expected to begin at the DP stage during thymic T cell development (Yui, Rothenberg 2014). While this is considerably later than that of Lck-Cre used in the previous studies, which is active as early as DN3 stage of double-negative thymocytes (Huang, Kitaura et al. 2006), T cell development was altered in the CD4-Cre driven DKO mice with a reduction in total thymocyte numbers and skewing of thymocyte populations (Fig 3A-D). These features and the increase in the DN4 thymocyte populations are similar to those seen in mice with Lck-Cre induced CBL deletion on a CBL-B-null background (Huang, Kitaura et al. 2006). An impact on the DN4 populations is somewhat unexpected but consistent with the known role of CBL in negatively regulating the pre-TCR signaling (Panigada, Sturniolo et al. 2002). This feature may be a reflection of CD4-Cre-mediated CBL/CBL-B deletion at earlier stages of hematopoiesis as discussed below. As demonstrated in previous work (Huang, Kitaura et al. 2006), the decrease in the DP thymocyte population can be attributed to alleviation of the negative regulation of TCR signaling, resulting in accentuation of TCR signal strength, which converts a positive into a negative T cell selection.

We also show that CD4-Cre mediated deletion of CBL and CBL-B genes leads to constitutive activation of peripheral T cell populations in the spleen and lymph node, as demonstrated by the changes in the expression levels of activation-related markers (Figure 3.6C, D and Figure 3.7C). These data show that concurrent CBL and CBL-B deletion during T cell development using CD4-Cre largely recapitulates the T cell activation and systemic immune cell infiltration phenotype previously described, providing direct support that the new CBL-B-flox model and its combination with the existing CBL-flox model will allow controlled, tissue-specific deletion of CBL and/or CBL-B in specific cell types. Importantly, the newly validated floxed models will allow, for the first time, a dissection of the specific as well as redundant roles of CBL and CBL-B to fully explore their roles in adult mammalian tissue function and pathology without the inherent developmental issues associated with the whole body CBL KO mice (El Chami, Ikhlef et al. 2005, Molero, Jensen et al. 2004, Rafiq, Kolpakov et al. 2014), immune hyperactivity of CBL-B-null mice (Bachmaier, Krawczyk et al. 2000), and importantly eliminate the issue of embryonic lethality of germline double KO mice. Notably, this model will allow concurrent CBL and CBL-B deletion in non-hematopoietic tissues to understand the role of these proteins in physiology and tumorigenesis, without spontaneous tumor rejection.

While deletion of CBL and CBL-B in T cells (Figure 3.3 A, B) was expected, the alteration of DN thymocyte populations, a more aggressive phenotype of CD4-Cre-mediated DKO mice compared to that of previously described Lck-Cre driven DKO (Naramura, Jang et al. 2002), and a marked decrease in the relative proportions of peripheral CD4+ T cells suggested that CD4-Cre driven CBL/CBL-B DKO may also occur in other hematopoietic lineages, a possibility not considered in previous studies. Indeed, we demonstrated that CBL and CBL-B deletion was present in other hematopoietic lineages (Figure 3.8B). Given our previous studies in which MMTV-driven deletion of CBL in a small percentage of HSCs led to a myeloid-skewed expansion of peripheral blood cell lineages and HSC expansion (Naramura, Nandwani et al. 2010, An, Nadeau et al. 2015), one possible explanation for these discrepancies was that CBL and CBL-B are deleted in a certain proportion of HSCs, which consequently manifests as CBL/CBL-B deletion in other non-intended lineages within the hematopoietic system. This notion is consistent with previous reports, (which have received little attention in the context of the use of CD4-Cre for gene deletion) in which CD4 expression was shown on a small subset of HSCs using antibody-based staining (Wineman, Gilmore et al. 1992, Ishida, Zeng et al. 2002). We provide further support for this idea using real-time PCR and FACS analysis demonstrating the expression of the Cre reporter (GFP) in HSCs as well as differentiated hematopoietic cell populations (Figure 3.9B and 3.10A, B). CD3ε, a marker of T-lineage cells was not detectable in HSCs, reducing the likelihood of the CD4 signals arising from any lingering T cell contamination in our LSK population. Moreover, the Lin- Sca-1- c-Kit+ (LK) population, which represents the more committed myeloid progenitor population (Seita, Weissman 2010), exhibited a lower level of CD4 expression level (Figure 3.11B) compared to LSK cells, suggesting that CD4 expression in immature hematopoietic stem/progenitors is a transient event.

The role of CD4 expression in HSCs is unclear. The likely explanation for why a potentially transient CD4-Cre-directed deletion of CBL and CBL-B in a small percentage of HSCs would manifest more strongly in our studies is that HSCs with loss of CBL and CBL-B acquire a robust proliferative advantage, as has been demonstrated in previous studies (Naramura, Nandwani et al. 2010, An, Nadeau et al. 2015, Rathinam, Thien et al. 2008). The apparent lack of any robust HSC-based phenotype in previous studies (Yi, Stunz et al. 2013, Hsu, Pajerowski et al. 2011, Johnson, Pao et al. 2013, Buckley, Trampont et al. 2015, Hsu, Shapiro et al. 2014, Jost, Abel et al. 2014, Uddin, Zhang et al. 2014, Maraver, Tadokoro et al. 2007, Zhang, Rosenberg et al. 2005, Mycko, Ferrero et al. 2009) could reflect a lack of consideration of such a phenotype due to lack of a proliferative advantage of the targeted gene deletion or a negative impact on proliferation. Regardless of the role of CD4 in HSCs, our results suggest caution in designing CD4-Cre-based deletion strategies and assigning the phenotypes solely to gene deletion in T cells.

Overall, our studies establish a new model of inducible CBL/CBL-B deletion that should allow the immune vs. non-immune cell-intrinsic roles of these key but functionally redundant negative regulators of tyrosine kinase signaling to be determined. Further, for the first time, the new model will allow the role of CBL and CBL-B to be determined in tumorigenesis without the current lack of feasibility of such studies due to tumor rejection from germline deletion of CBL-B using existing models.

CHAPTER 5: FUTURE DIRECTIONS

Determine the role CBL/CBL-B deficiency in CD4+ T cells plays towards inflammatory/autoimmune pathogenesis of multiple sclerosis

Multiple sclerosis (MS) is an autoimmune disease of the central nervous system (CNS) mediated by pathogenic T cells leading to chronic inflammation and demyelination (Calabresi 2004). This study will elucidate the importance of the negative regulatory roles of CBL and CBL-B in controlling the magnitude and nature of antigen-specific T cell immune responses in autoimmunity. More importantly, we hope to identify a specific signature associated with loss of CBL proteins in effector T cells which will help identify future therapies to treat MS patients that have mutations in CBL genes.

Previous studies on CBL-B in MS patients

Mutations in Cbl-b have been linked with increased susceptibility to MS. Genome-wide association studies (GWAS) have linked several CBL-B gene polymorphisms with MS (Sanna, Pitzalis et al. 2010, Corrado, Bergamaschi et al. 2011). Previous studies have shown decreased CBL-B mRNA and protein expression in whole peripheral blood mononuclear cells (PBMCs) of relapsing-remitting MS (RR-MS) patients (Figure 4.1), as well as reduced CBL-B expression and lowered activation threshold of $CD4^+$ T cells from these patients (Zhou, Wang et al. 2008, Sturner, Borgmeyer et al. 2014). One of the risk alleles identified in GWAS was shown to reduce the expression of CBL-B in $CD4^+$ T cells of RR-MS patients. It was further identified that decrease in CBL-B in RR-MS patients with this risk allele occurred in an IFN-β dependent manner (Sturner, Borgmeyer et al. 2014). The mechanisms by which decreased CBL-B expression contributes to MS pathogenesis remain unknown and will be examined in this study.

2D2 mouse model and experimental approach

To address the role that CBL proteins play as barriers to autoimmunity in T cells in MS, we have chosen the 2D2 transgenic mouse model which utilizes a T cell receptor transgene specific for myelin oligodendrocyte glycoprotein (MOG), a protein in the outer surface of the (CNS) myelin sheath. This is a model for experimental autoimmune encephalomyelitis (EAE), which is an experimental model that recapitulates pathological features of human MS (Constantinescu, Farooqi et al. 2011). Four percent of 2D2 mice develop spontaneous EAE within 2.5 to 5 months of age, and EAE can be induced by immunization with MOG protein or peptide using standard protocols (Bettelli, Pagany et al. 2003).

Serial genetic crosses have generated mice homozygous for floxed CBL, CBL-B, or both together with a tamoxifen-inducible CreERT and the 2D2 Tg TCR. I also introduced a dual-reporter (mT/mGFP) of Cre-mediated gene deletion in which the Rosa-26 locus-encoded membrane-localized td-Tomato (red) and GFP are expressed prior to and after successful Cre-mediated deletion of floxed sequence cassettes respectively to allow identification of donor T cells in transplantation experiments. (Figure 4.2)

Adoptive transfer of $CD4^+$ T cells isolated from 2D2 mice into WT C57/Bl6 mice will allow the deletion of CBL and CBL-B specifically in donor cells upon the administration of tamoxifen. Immunization with $MOG_{35\text{-}55}$ peptide could be used to induce EAE if deletion of CBL proteins is not sufficient to elicit disease (Bettelli, Pagany et al. 2003). Clinical EAE scoring will be used to assess disease severity along with percent incidence, mortality, mean day of disease onset, and mean maximum score per experimental group. Histopathological analysis of brains, spinal cords, and optic nerves can be done to look for and quantify inflammatory/demyelinating lesions. I hypothesize that deletion of CBL proteins will lead to earlier disease onset and exacerbated inflammatory/demyelinating disease severity in *in vivo* studies. *Ex vivo* analyses of T cells for proliferation, cytokine release, migration, and gene expression profiles using microarray/RNA sequencing analyses will help to establish the cell-autonomous role of CBL and CBL-B as enforcers of T cell anergy in $CD4^+$ T cells, the impact of the loss of CBL and CBL-B in promoting T cell-dependent autoimmunity, and its cellular and molecular mechanisms.

Figure 4.1. Cbl-b protein levels in peripheral blood lymphocytes isolated from MS patients and control individuals. The bands of Cbl-b protein are located at the molecular weight 109 kDa, while β-actin at 42 kDa.

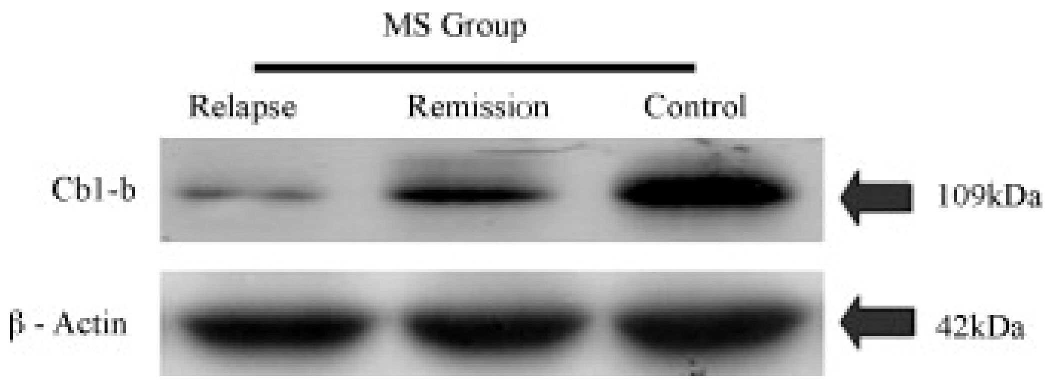
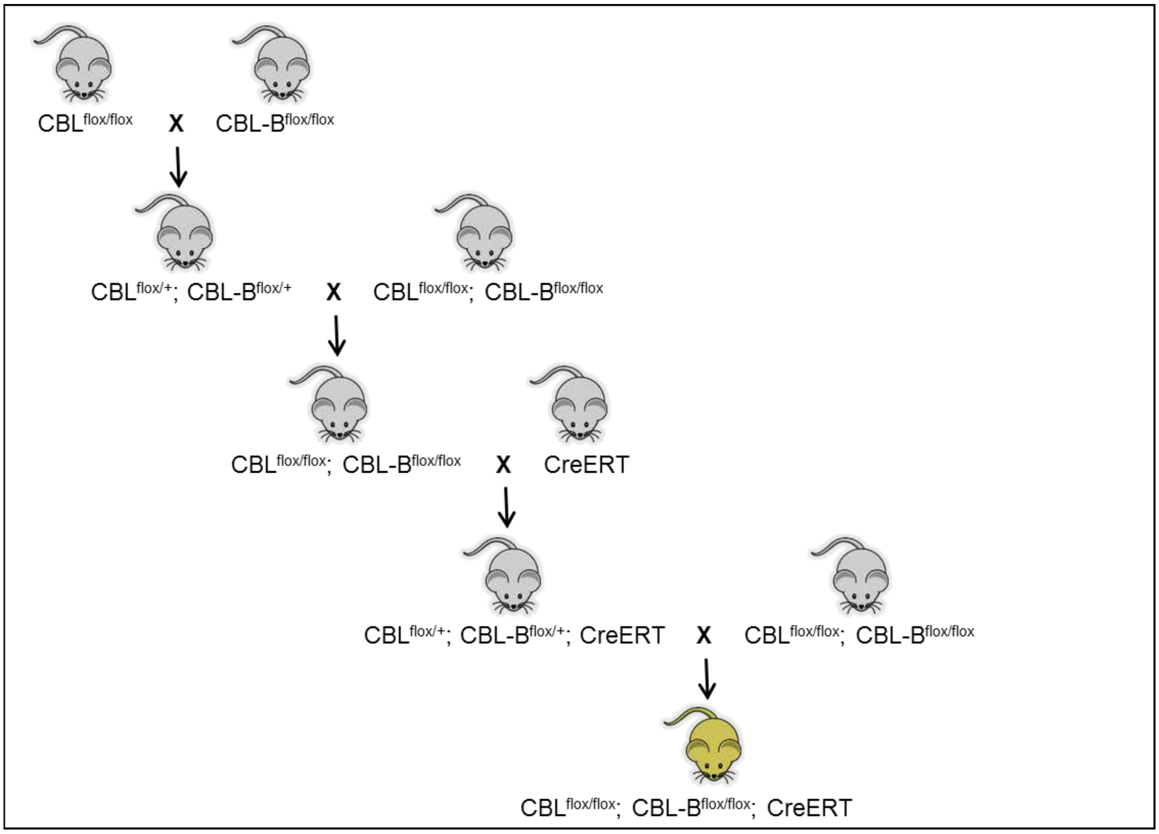
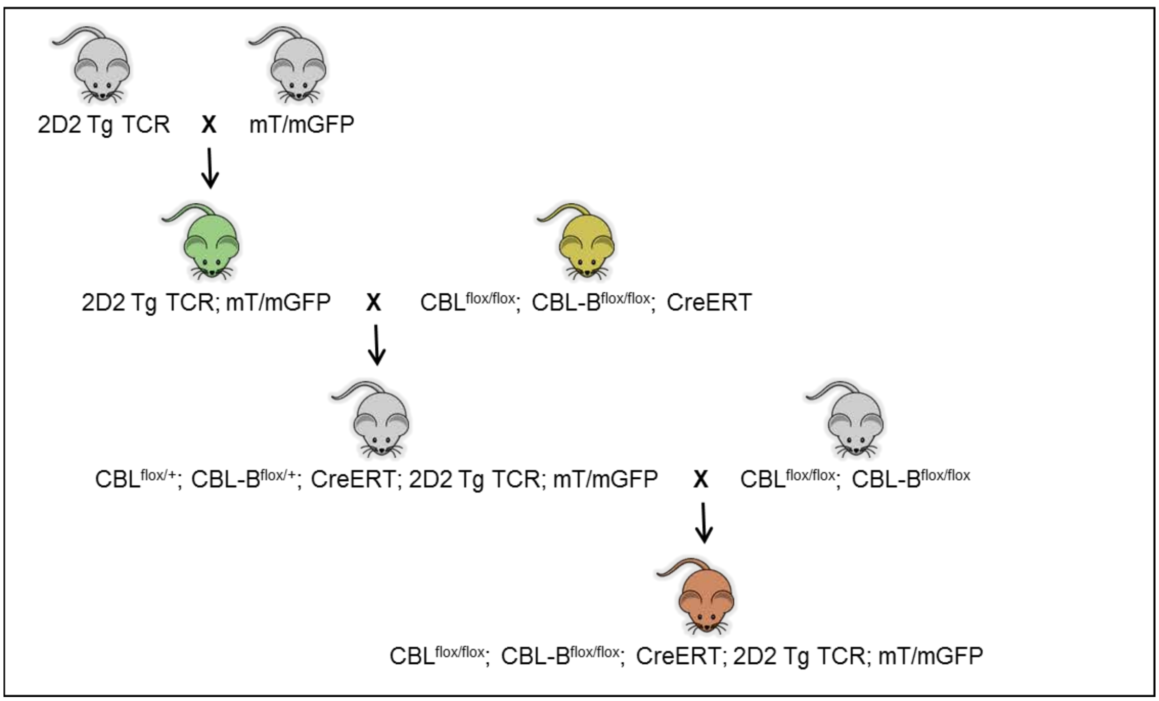

MS Group
Relapse
Remission
Control
Cb1-b
109kDa
β - Actin
42kDa

Figure 4.2. Breeding scheme used to generate CBL/CBL-B DKO EAE mouse model. Mice carrying either the CBL-flox or CBL-B-flox alleles were crossed to generate double floxed mice. These mice were then incorporated with a CreERT Tg. Mice carrying the 2D2 TCR Tg were crossed with mice carrying the Cre mT/mGFP reporter gene, and the resulting mice were crossed with the double floxed CreERT mice.


CBLflox/flox X CBL-Bflox/flox
CBLflox/+; CBL-Bflox/+ X CBLflox/flox; CBL-Bflox/flox
CBLflox/flox; CBL-Bflox/flox X CreERT
CBLflox/+; CBL-Bflox/+; CreERT X CBLflox/flox; CBL-Bflox/flox
CBLflox/flox; CBL-Bflox/flox; CreERT


2D2 Tg TCR X mT/mGFP
2D2 Tg TCR; mT/mGFP X CBLflox/flox; CBL-Bflox/flox; CreERT
CBLflox/+; CBL-Bflox/+; CreERT; 2D2 Tg TCR; mT/mGFP X CBLflox/flox; CBL-Bflox/flox
CBLflox/flox; CBL-Bflox/flox; CreERT; 2D2 Tg TCR; mT/mGFP

Determine the role CBL/CBL-B deficient CD8+ cytotoxic T cells play in anti-tumor immune responses to melanoma

Melanoma is the most serious form of skin cancer which arises from pigment-producing melanocytes, and the incidence of disease has been increasing over the past few years (Bastian 2014, DeSantis, Lin et al. 2014). New immunotherapeutic strategies have been promising in improving the poor prognosis of patients with melanoma (Niezgoda, Niezgoda et al. 2015). Ultimately, this study will allow the dissection of the role CBL proteins play in T cell mediated anti-tumor immune response pathology and provide possible immunotherapeutic strategies for the treatment of patients with melanoma.

Previous studies on CBL-B and anti-tumor immunity

To date, the clinical use of T cell adoptive immunotherapy to treat cancer has been limited due to poor survival and function of transplanted T cells, and many approaches require concurrent administration of cytokines, such as IL-2, which adds complications associated with toxicity. There have been a limited number of studies examining the enhanced anti-tumor ability of CBL protein-deficient T cells. It has been showed previously that adoptive transfer of CBL-$B^{-/-}$ T cells into mice bearing EG7 tumors leads to tumor eradication (Chiang, Jang et al. 2007); however, other studies have shown that transplant alone is not sufficient to eliminate B16-ova or EG7 tumors without the addition of a dendritic cell vaccine (Lutz-Nicoladoni, Wallner et al. 2012). Adoptive CBL-B-null T cell approaches have also shown efficacy in treating leukemia and melanoma in mice (Stromnes, Blattman et al. 2010, Hinterleitner, Gruber et al. 2012b). While these studies raise the prospect of inactivating CBL-B for immunotherapy of tumors clinically, this has not received much consideration.

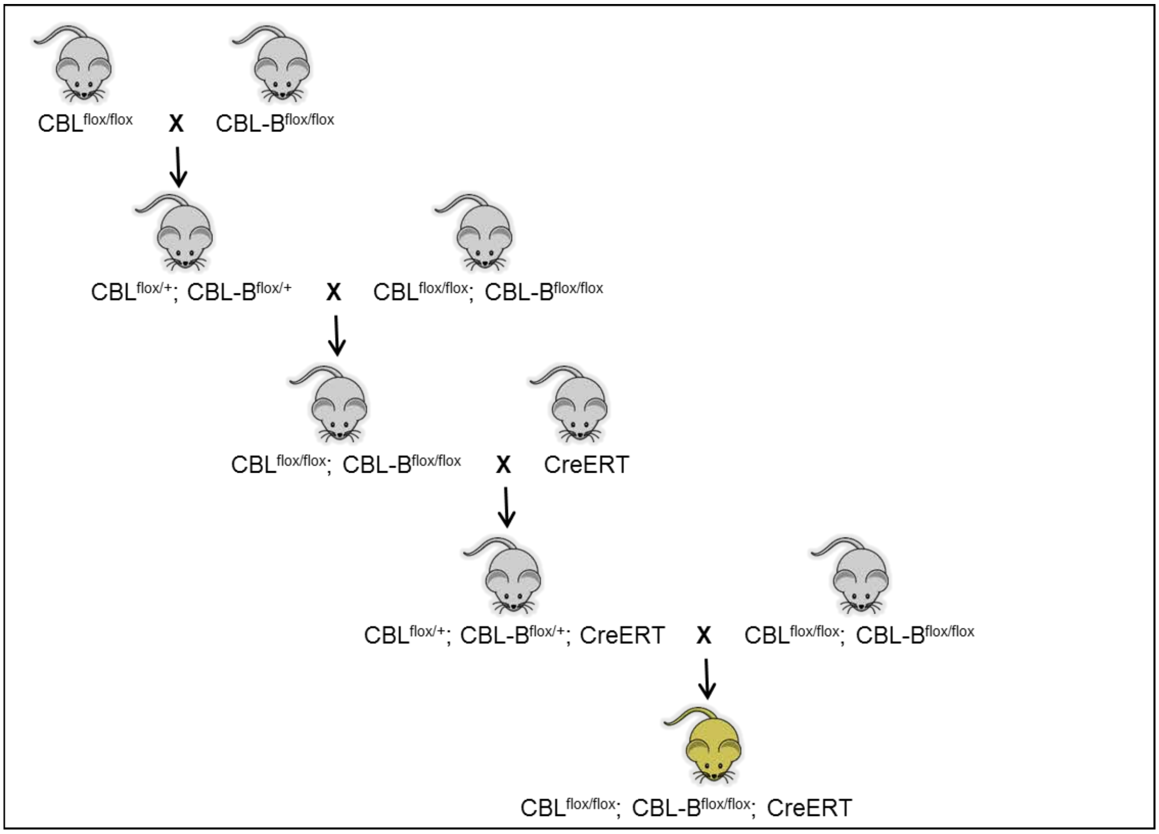
Pmel-1 model and experimental approach

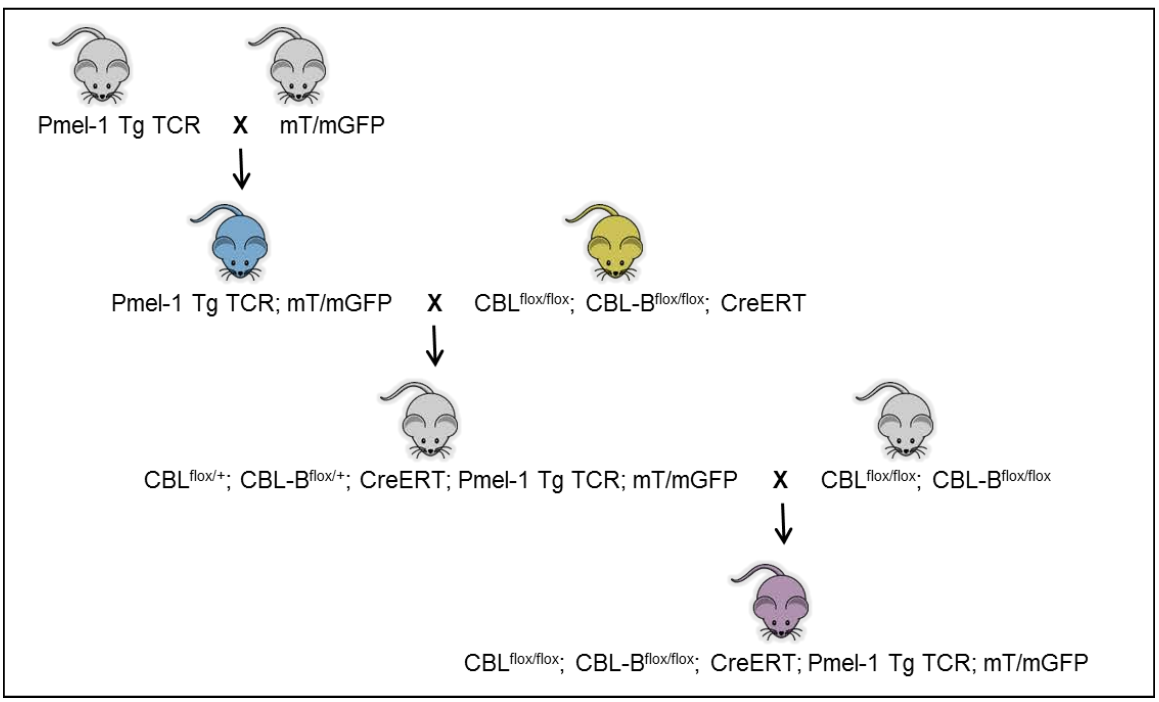
To study the role that CBL proteins play in self/tumor antigen-specific T cell responses in melanoma, we have chosen the pmel-1 transgenic mouse model which utilizes a T cell receptor transgene specific for the mouse homologue (pmel-17) of human SILV (gp100), an enzyme involved in pigment synthesis that is expressed by the majority of malignant melanoma cells including B16 melanoma, as well as by normal melanocytes. Without immunization and concurrent IL-2 therapy, subcutaneously injected B16 tumor cells grow normally in pmel-1 mice and adoptive transfer of pmel-1 splenocytes into tumor-bearing mice alone has no effect on tumor growth (Overwijk, Theoret et al. 2003).

Serial genetic crosses have generated mice homozygous for floxed CBL, CBL-B, or both together with a tamoxifen-inducible CreERT and the pmel-1 Tg TCR. I also introduced a dual-reporter (mT/mGFP) of Cre-mediated gene deletion in which the Rosa-26 locus encoded membrane-localized td-Tomato (red) and GFP are expressed prior to and after successful Cre-mediated deletion of floxed sequence cassettes respectively to allow identification of donor T cells in transplantation experiments (Figure 4.3).

For future adoptive transfer experiments, B16 melanoma cells will be subcutaneously injected into the flank of WT C57BL/6 mice to generate tumors. For mice receiving T cell treatment, $CD8^+$ T cells will be isolated from pmel-1 mice and injected intravenously into the tail vein either 7 or 14 days after tumor cell injection. In addition, some mice will receive peptide vaccination and/or IL-2 therapy. Tamoxifen treatment will allow the deletion of CBL and CBL-B in donor $CD8^+$ pmel-1 T cells. Tumor size will be monitored and analyzed for immune cell infiltrates. We hypothesize that deletion of CBL proteins in $CD8^+$ T cells will lead to a more robust anti-tumor immune response, decreased tumor size, and increased immune cell tumor infiltration. Moreover, we hope to identify a signature associated with loss of CBL proteins in cytotoxic T cells which will help identify future immunotherapeutic approaches for the treatment of patients with melanoma by screening small molecule libraries for compounds that elicit identical gene expression changes.

Figure 4.3. Breeding scheme used to generate CBL/CBL-B DKO B16 melanoma mouse model. Mice carrying either the CBL-flox or CBL-B-flox alleles were crossed to generate double floxed mice. These mice were then incorporated with a CreERT Tg. Mice carrying the pmel-1 TCR Tg were crossed with mice carrying the Cre mT/mGFP reporter gene, and the resulting mice were crossed with the double floxed CreERT mice.


CBL flox/flox
X
CBL-B flox/flox
CBL flox/+; CBL-B flox/+
X
CBL flox/flox; CBL-B flox/flox
CBL flox/flox; CBL-B flox/flox
X
CreERT
CBL flox/+; CBL-B flox/+; CreERT
X
CBL flox/flox; CBL-B flox/flox
CBL flox/flox; CBL-B flox/flox; CreERT


Pmel-1 Tg TCR
X
mT/mGFP
Pmel-1 Tg TCR; mT/mGFP
X
CBL flox/flox; CBL-B flox/flox; CreERT
CBL flox/+; CBL-B flox/+; CreERT; Pmel-1 Tg TCR; mT/mGFP
X
CBL flox/flox; CBL-B flox/flox
CBL flox/flox; CBL-B flox/flox; CreERT; Pmel-1 Tg TCR; mT/mGFP

Conclusions

The information generated by these studies will provide insight into how targeting of CBL proteins may be translated clinically for the treatment of patients with proinflammatory disease or cancer. Targeting of CBL proteins by knocking out protein expression or functionally inactivating the ubiquitin ligase activity will provide therapies in which enhanced T cell activity is desired, such as in the treatment of patients with cancer or infectious diseases. This may be accomplished by *ex vivo* deletion of CBL genes in T cells through targeted genome editing using the technique CRISPR. However, permanent deletion of CBL proteins and constitutively hyperactive T cells may cause additional complications in patients. To avoid complications associated with permanent deletion, an *ex vivo* siRNA approach to temporarily lower CBL protein expression levels will prevent continuous T cell hyperactivity, although limiting the duration of the therapeutic response. Design of small molecules to inactivate CBL protein function may provide a therapeutic strategy that eliminates the need for genetic modification. A caveat to this approach is inactivation of CBL proteins in other cell types in a patient may lead to additional complications such as hyperactivation of other immune cells. Alternatively, enhancement of CBL protein expression in pathogenic T cells of patients with autoimmune/proinflammatory disease may be beneficial in alleviating disease symptoms. Identifying a gene signature associated with loss of CBL proteins will help identify immunotherapeutic approaches for the treatment of patients with proinflammatory disease or cancer by screening small molecule libraries for compounds that can recapitulate or revert the gene expression changes in CBL-deficient hyperactivated T cells.

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