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Cbl-b: Its Role of Expression and Regulation in T-lymphocyte Activation and Ageing

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Cbl-b: Its Role of Expression and Regulation in T-lymphocyte Activation and Ageing

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Dedication

献给亲爱的许爸许妈

你们的爱，是我前行的动力

你们的信任，给我飞翔的翅膀

因为你们，我的梦想成真

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The aging process is strongly associated with decreased activity in the immune system. Dysregulation of T-lymphocyte function, such as reduced proliferation, is one problem faced by most elder people, which prevents them from successfully dealing with exogenous pathogens. Effective regulation of T-lymphocyte activity depends on the proper and prompt transduction of both positive and negative signals within T-lymphocytes and reflects the balance between positive and negative effects. Decline of positive signaling in aging has been studied and reported, while mechanisms concerning up-regulation of negative signaling with age and its role in immune senescence are still unclear.

Cbl-b, an E3 ubiquitin ligase, was studied by our lab since it regulates the ubiquitin process, a protein modification process that has suppressive effects on signaling pathways. We first determined the reaction of Cbl-b to different stimuli in young rat splenic T-lymphocytes, and showed that there is a decrease in Cbl-b protein expression

upon CD28 stimulation and such protein degradation is proteasome-dependent only. We also showed the mechanism of Cbl-b expression regulation involves the intracellular movement of Nedd4 toward Cbl-b and an up-regulation of Nedd4 expression. Then we proved that in old splenic T-lymphocytes, decreased proteasome activity was unable to down-regulate the Cbl-b protein. High levels of Cbl-b in old T-lymphocytes are functional in preventing PI3K activity and are associated with reduced T-lymphocyte proliferation upon regular stimulation.

T-lymphocytes from old Cbl-b knock-out mice show similar proliferative reaction to CD3 stimulation as T-lymphocytes from young wild-type, which establishes the cause-effect relationship between sustained Cbl-b expression and decreased T-lymphocyte proliferation. In summary, these data suggest a unique role of Cbl-b in regulating T-lymphocyte signal transduction and provide critical preliminary data for extending Cbl-b studies into other fields, such as carcinogenesis.

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Chapter 1: Review of Literature

1.1 OVERVIEW

Healthcare of aged people is a social problem met by countries all over the world. One of the biggest challenges needing solved in the aging area is improving immune function to reduce the incidence of age-related diseases in the elderly. Characteristics of immune senescence include decreased proliferation rate in lymphocytes, and increased auto-antibody secretion by B-lymphocytes [1, 2]. Various theories of aging have been put forth to explain the cause of aging from different points of view [3]. Some of these aging theories place emphasis on the immune system, focusing on decline in T cell development, alterations in lymphocyte homeostasis, and impaired intracellular signaling pathways.

The network of intracellular [4] signaling pathways is a complicated system incorporating stimulatory and inhibitory signals by both positive and negative regulation. Numerous studies have pointed out that activities and expression of receptors, modulators, and effectors which transduce and amplify activating signals were down-regulated or mutant with age [5]. At the same time, those proteins that will block the signal transduction or decrease the signal strength may have similar impact on the onset of immune senescence. Phosphorylation is probably one of the most popular posttranslational modifications of protein that help to “turn on” the signaling network. Similarly, the role of ubiquitination, another protein posttranslational modification process, in “turning off” the signaling network could be as important as phosphorylation in the whole signal transduction world. E3 ubiquitin ligases not only control the rate-

limiting step in the ubiquitination procedure, but also determine the substrate specificity. Thus, we want to test the relationship between Cbl-b, an E3 ubiquitin ligase and T cell activation. Also, we would like to see whether there is any functional and expressional change to Cbl-b in aged T-lymphocytes as well as the potential mechanisms. Finally, we want to confirm the role of Cbl-b in aged T-lymphocyte function regulation via an old Cbl-b knock-out model.

The ultimate goal of this study is to prove that changes in the ubiquitin/proteasome system during aging may affect the function of T-lymphocytes. A thorough study on this topic would provide a new mechanism of dysregulation in aged T-lymphocyte function, and subsequently, a better dietary or therapeutic strategy against the immune senescence.

1.2 OBJECTIVES

The objectives of the project are:

1) To characterize changes of Cbl-b expression in young stimulated T-lymphocytes

   Hypothesis: Stimulation of young T-lymphocytes down-regulate Cbl-b activity

2) To determine the age-dependent change of Cbl-b in old stimulated T-lymphocytes

   Hypothesis: Cbl-b can not be down-regulated in old T-lymphocytes, and are associated with low proliferation of old T-lymphocyte

3) To establish a cause-effect relationship between Cbl-b activity and T-lymphocyte proliferation

   Hypothesis: Aged Cbl-b knock-out mice show higher T-lymphocyte proliferative ability

1.3 BACKGROUND

Cellular Aging and Associated Theories

Nowadays, the concept of "cellular aging" is more extended than originally put forward. It includes the features of cessation of cell dividing after certain rounds of doubling [6, 7], accumulation of deteriorative molecules within the cell [8], losing control to maintain cell homeostasis [9] and altered regulation of gene expression [10]. Since many common phenotypes of aging such as osteoporosis [11] and inadequate responses of T cells to viruses [12] are due to the features associated with the cellular aging, the *in vitro* cell aging has been correlated with *in vivo* aging and studies on this topic have raised several theories about aging.

Telomere Theory of Aging

The telomere is a region located at the terminal end of the linear chromosome. It is composed of thousands of repeats of TTAGGG in vertebrates. At the time of cell division, DNA replication can not reach the very end of the chromosome. Instead, each round of DNA replication will result in the loss of 40-100 bps of telomere at the tip of the chromosome. Thus, the higher the cell replicative cycle number, the shorter the telomere length, until it reaches a critical value. After that, cells will stay in an arrested state in which they stop replicating themselves even when outside stimuli are strong enough to trigger their division. The observations that the telomere length in fibroblasts and blood cells from aged donor are statistically shorter [13, 14], patients of Hutchinson-

Gilford progeria and Werner's syndrome, diseases caused by a defective telomere system, show symptoms of accelerated aging [15] and shorter telomeres are associated with lower survival rate in people over 60 years old when facing heart disease and infectious disease [16] all support the idea that the telomere works as an internal cell division counter that monitors the "age" of somatic cells and puts "aged" cells into non-dividing state and eventually determines the overall aging state of the whole body.

Telomere can be extended by a specialized reverse transcriptase, telomerase. However, the expression of TERT (telomerase reverse transcriptase, the core catalytic subunit of telomerase) is suppressed in normal somatic cells and is only detectable in germline cells. The hypothesis that stopping the shortening of the telomere can slow or reverse the aging process was proved by *Wright*, *Lichtsterner* and their team [17]. However, the value of the telomere theory is decreased by other concerns. Some animals, like mice, have a high telomerase activity and can retain the telomere length with aging [18]. Also, there is no correlation between the telomere length and the life span within different stains of mice [19]. Even in human cases, increased telomerase activity on one hand can immortalized the cells, but on other hand may put the cells under the threaten of tumor formation, which probably has a more negative impact on human life span than aging itself [20]. Thus, the function of the telomere and telomerase in human aging may be important, but still not decisive.

Membrane Hypothesis of Aging

The fluidity of the cell membrane is one of the most significant features influencing the function of the cell membrane—a permeable lipid bilayer embedded with

proteins [21, 22]. Cell membranes not only mediate molecular transport in and out of the cell, but also regulate signal transduction from cell surface receptors. Membrane fluidity and permeability are mainly determined by the content of phospholipid and cholesterol. With age, composition of membrane lipids will change in many tissues resulting in an increased ratio of cholesterol/phospholipid, such change has been noted in rat liver and skeletal muscle [23], in rat liver microsomal and mitochondrial fractions [24], and in red blood cells [25]. As a consequence, old cell membranes become more rigid and resting potassium (K^+) permeability decreases. In order to maintain the cell excitability, more K^+ must be brought intracellularly [26]. For example, the K^+ concentration within rat brain neural cells can be 32% higher at the age of 24 months compared to 1 month old rats [27]. High concentrations of intracellular ions will lead to a higher viscosity which directly affects biological enzyme activity including the DNA-dependent RNA polymerase [28]. This is of great importance since slowing of DNA or protein synthesis is another hallmark of cellular aging and prompt DNA and protein formation is critical to cell division.

Formation of the immunological synapse is another cell function affected by decreased membrane fluidity. The function of the immunological synapse is to "provide a mechanism for sustained TCR engagement and signaling [29]". T cell activation is initiated by the interaction of T cells with antigen presenting cells (APCs). Namely, major histocompatibility complex—peptide complexes (MHC-peptide) present at the surface of APCs that are recognized by T cell antigen receptors (TCRs). Shortly after the cell-cell interaction, a series of signaling transduction reactions will be triggered and result in various T cell responses including T cell division and cytokine secretion. It has been noticed that a specialized structure is formed at the T cell-APC interface zone [30].

This special structure, termed immunological synapse, is generated by the polarization of cytoskeleton and is characterized by a TCR cluster at central interaction area and an integrin ring formed at the surrounding zone [31]. In old mice T cells, failure to move LAT and Vav into the immunological synapse is responsible for the age-dependent decline of T cell clonal expansion [32]. Similarly, alteration of the immunological synapse composition with age in memory T cell subsets leads to defects in the T cell response [33]. Since cell membrane fluidity is a major factor that determines the immunological synapse formation [34], the observed changes in the immunological synapse formation and hyporesponsiveness of T cells with age can be explained by the age-associated decline of membrane fluidity. However, experiments trying to reverse the membrane lipid composition changes in older populations by using the plasma membrane cholesterol-depleting agent methyl-β-cyclodexrin (MBCD) did not increase cell proliferation in old groups [35] or only increased to a very limited degree [36]. Thus, further studies regarding the "membrane hypothesis" in enhancing cell function and extending human life span are needed.

Free Radical Theory of Aging

ATP, the energy source of all cell functions, is generated in mitochondria by the phosphorylation of ADP. This reaction is driven by an electrochemical gradient produced by the mitochondria electron transport chain. A side effect of this process is that the electron transport chain will leak electrons to oxygen and form superoxide ($O_2^{\cdot-}$). Then, $O_2^{\cdot-}$ will convert into more toxic free radical-containing reactive oxygen species (ROS) by a series of redox reactions. Besides the normal aerobic metabolism, radiation is another source that will generate ROS. ROS can oxidatively damage all cellular

macromolecules, like protein [37], DNA [38], and lipid [39]. Oxidative damage is already present in cells from young animals [40] and increases with age in various species [41]. The accumulation of oxidative damage contributes to many age-related declines in body functions including learning, memory, hearing, locomotion, reproduction and immune response [42] and are responsible for numerous age-related degenerative diseases like cardiovascular disease, cataracts, brain dysfunction, chronic infection and cancer [43].

It is believed that the accumulation of oxidative damage with age is caused by the combination of accelerated ROS production and a compromised damage repair/removal system, but is not associated with decreased antioxidant defense [44]. Many studies have been done to find a way to decrease the rate of ROS production and in turn the rate of aging. It was found that dietary antioxidant supplementation can increase average life expectancy at birth (ALE-B) by 20-30% in experimental animals, but has little beneficial effect on maximum life span (MLS) [45]. So far, by decreasing O_2 utilization in metabolism, caloric restriction (CR) is the only way to increase both ALE-B and MLS. However, to achieve a significant increase in MLS, the level of CR (decrease by 40%) would be too high to maintain normal life quality [46]. Experiments about the application of CR in primates are currently ongoing [47] and the results will give more valuable information about CR in aging study.

Other Theories of Aging

There are many other theories of aging that have been raised and accepted widely. Some of them are not limited to the cellular level, like the wear-and-tear theory, which

states that the normal function of our body depends on the proper function of every organ in the body in order to deal with almost everything we are facing, either beneficial or harmful. However, just like a machine, the constant use will wear out the organs and slow down their working efficiency and finally result in the aging process [48]. Another widely accepted aging theory, the neuroendocrine theory [49], focuses on hormone secretion, which is all controlled by the hypothalamus. The hypothalamus works in a feedback mechanism; it detects the body hormone levels and regulates the hormone release according to the current hormone level. When we become older, the hypothalamus loses both its sensitivity in hormone level detection and its precision in hormone secretion regulation. The result of such dysregulation is a dramatically decreased level of hormones that are important for body function.

It is crucial to realize that these theories are not exclusive to each other. Some theories are the result of other theories. For example, mitochondria, the energy producing organelle of every cell, faces the damaging attacks from free radicals and lacks the necessary defense mechanism to correct them. Thus, when free radicals accumulate with time, mitochondrial function declines and the free radical theory and the mitochondrial decline theory are linked together. Some theories of aging are interlinked, each representing an aspect of a complicated network. For example, the telomerase theory of aging explains the view of the genetic clock [14]; the error and repairs theory focuses on the repair machinery [50, 51]; and the redundant DNA theory concentrates on the ratio of repeated DNA sequences in the genome [52]. Although focusing on different points of view, one common theory underneath them is the genetic control theory, which believes that our lifespan and aging process is pre-determined in

our DNA sequence when we are born. We can speed or slow the aging rate by working on any one of the aspects listed above.

It is also interesting to notice that some aging theories are specific to the immune system, like the autoimmune theory, which suggests the immune system begins attacking itself due to a decline in distinguishing foreign attackers from self-protein [53], and the thymic-stimulating theory, which states that the shrinkage of the thymus and the decrease in thymic hormone production with age cause the malfunction of the immune system in the elderly [54]. Actually, there are multiple changes found in the immune system that contribute to the aging process and will be illustrated in detail in the next section.

The Aging Immune System

There is a well established association between aging and increased morbidity and mortality due to infectious diseases [12, 55]. That is why studies in immune senescence attract so many gerontologists. Humans and rodents show many age-related immune defects including thymic involution, decreased T-cell response to antigenic stimuli, altered cytokine expression by T cells and reduced formation of high-affinity antibodies [12]. Considering that the immune system is a quite complicated network composed of different cell types with distinct functions, declined immune function accompanied by aging must be the comprehensive result of deficiency occurring in individual immune cells at different stages.

<u>Thymic involution and T cell development</u>

T cell development is a multi-step process that takes place in the bone marrow (BM) and the thymus. Hematopoietic stem cells (HSC), with the phenotype of Lineage^{lo} c-kit^{+} Sca-1^{+} (LSK) Flt3^{-} and originally located in the BM, are the precursors of all hematopoietic cells. During the T cell development process, they will leave the BM for the thymus by circulating in the blood and become the earliest intrathymic T progenitors (ETPs). Within the thymus, ETPs undergo a series of developmental progress, which can be defined by the cell surface expression of CD4 and CD8 as double negative (DN), double positive (DP), and single positive (SP). The DN thymocyte can be further divided into four sub-stages of differentiation by the expression of CD44 and CD25, that is, DN1 (CD44^{+}CD25^{-}), DN2 (CD44^{+}CD25^{+}), DN3 (CD44^{-}CD25^{+}), and DN4 (CD44^{-} CD25^{-}). It is in the DN stage that the precursor cells determine to take either the αβ or γδ T-lineage, more precisely, the pre-TCR-α is expressed in the DN3 stage and the TCR β-chain selection happens in the late DN3 and DN4 stage [56]. During the DP stage, cells also experience positive and negative selection according to whether they can recognize self-MHC and the affinity of such recognition. Positive and negative selection can eliminate about 98% of thymocytes in the thymus and are essential for establishing immunological tolerance.

Age will change the number of HSC within the BM, but the number is increased in long-lived C57BL/6 mice, but decreased in short-lived DBA/2 mice [57]. Moreover, the ability of HSCs to replenish themselves decreases with age [58]. Meanwhile, their ability to generate lymphoid precursors is lower in old animals [59]. Thus, the overall function of HSC for lymphocyte development declines in old mice [1]. The molecular mechanism underlying the HSC aging is accumulation of defects in genomic and mitochondrial DNA caused by ROS [58].

There is no change in the size of the thymus since six months after birth, while the weight of the thymus is lower in aged individuals, reflecting the composition change within the thymus that contains more fat tissue with age [60]. As for the thymic progenitors within the thymus, total number and proportion of DN1 cells that commit to the T lineage is reduced in the thymus in old animals [61], probably due to the defects of inputting BM progenitors. In addition, decreased transcription of RAG1 and RAG2 was observed in aged mice [62]. Since the function of RAG family proteins is to regulate β-selection, the defects in RAG expression will lead to retardation of DN3 thymocytes from going through β-selection and less DP thymocyte production in old thymuses [63]. Currently, no evidence exists to suggest that the differentiation steps in DP and SP thymocytes, including positive and negative selection, are changed with age [64].

T cell proliferation and signaling pathway

Reduced proliferation of T cells after stimulation *in vitro* is one of the hallmarks of aging in humans and rodents [12]. This phenomenon may reflect changes in TCR expression and downstream components in the signaling pathway. Proper T cell stimulation and subsequent proliferation requires two extracellular signals. Perturbing the TCR/CD3 complex generates the first, obligatory, signal. The second signal is generated from co-stimulatory receptors, like CD28, which are critical for determining proper T cell activation each time the cell is exposed to antigen. There is little evidence to suggest decreased density in TCRαβ chains or CD3 complex expression with age [65]. As for the expression of CD28, it is well acknowledged that the percentage of CD28-positive cells is significantly lower in T cells from old compared to young mice,

which may help to explain the reduced T-lymphocyte proliferation in aged individuals [66-68]. However, recent research has shown that not all aged T-lymphocytes are characterized by the lack of CD28 expression [69, 70]. The progressive decline in CD28 expression is more closely linked to age-related diseases as opposed to aging itself [71].

Once the antigenic peptides presented by APCs are bound to the TCR/CD3 complex, a cascade of signaling events will be initiated. Immunological synapses are first formed at the sites where APCs and T cells are contacted, the declined formation of immunological synapses with age has been discussed in the previous section "Membrane Hypothesis of Aging" on page 5. TCR associated protein tyrosine kinases (PTK), like Lck and Fyn are then activated. Activated PTKs can phosphorylate the immunoreceptor tyrosine-based activation motifs (ITAMs) of the CD3ζ chain, which leads to the binding of Zap-70 to phosphorylated ITAMs. Zap-70 is further phosphorylated and activated and plays a key role in stimulating more enzymes and protein effectors, like phospholipase C (PLC), LAT, Vav, and Ras. The function of PLC is to hydrolyse the membrane phosphatidylinositol phosphates (PIP_2) and release inositol triphosphate (IP_3) and diacylglyceral (DAG). IP_3 can regulate the intracellular Ca^{2+} concentration by opening the Ca^{2+} channels on the endoplasmic reticulum (ER), and DAG is the second messenger that activates protein kinase C (PKC). Both the intracellular Ca^{2+} movement and PKC activity are essential to the activation of other protein kinases and finally leads to cell proliferation and IL-2 secretion. Meanwhile, the 3-position hydroxyl group of the inositol ring of PIP_2 is phosphorylated by phosphoinositide 3-kinase (PI3K). The product, $PI_{(3,4,5)}P_3$, helps to activate protein kinase B (PKB or Akt). This "PI3K/Akt" signaling pathway is important in oncogenesis since it can regulate various cellular

processes including cycle progression, cell growth and apoptosis. Amplified expression of the p110α catalytic subunit of PI3K and Akt are found in human cancers [72]. Also, in malignant tumors, the PI3K/Akt pathway remain activated due to the function-loss mutation of PTEN, an antagonist of PI3K [72].

Studies show that enzymatic activity of Fyn is reduced in T cells from both old humans [73] and mice [74]. With advanced age, function of Lck is declined as well, because of less association with TCR and less phosphorylation [75, 76]. These alterations found in the elderly contribute to the age-related impairment of Zap-70 activity, although the expression of Zap-70 and its association with CD3ζ chain is normal with age [77, 78]. As a result of decreased Zap-70 activation, PLC activity and IP_3, DAG generation are impaired [79]. Reduced Ca^{2+} flow in old T cells results in the decreased NF-AT activity whose function is to induce the transcription of genes responsible for cell-cell interaction and is important to T cells' proliferation and differentiation [80]. Low DAG formation leads to less PKC activation and decreased NF-κB pathway function [81] which is critical for inflammation, autoimmune response, cell proliferation, and apoptosis [82]. PKC activity can be inhibited by ceramide. Ceramide accumulates in old cells and contribute to cellular senescence [83]. An age-associated decrease in the PI3K/Akt pathway is observed in pancreatic acinar cells [84], indicating that it may be affected in old T cells too.

It is expected that other signaling pathways controlled by activation of Lck/Fyn will show age-related deficiency also. Studies have found that MAPK activity [85] and JNK activity [86] decrease in old human T cells. The deficiency in MAPK can be partially reversed by adding phorbol ester and calcium ionophore [85]. All the data

together suggest that changes within the early response events after TCR/CD3 engagement are the main reasons for improper T cell signal transduction with age.

Homeostasis and age-associated defect in T cell subsets

According to different definitions, T cells can be classified into different, but sometimes, overlapping subsets. Based on the expression of surface markers, T cells can be divided into $CD4^+$ T cells and $CD8^+$ T cells. According to their function, T cells will be grouped into helper T cells and cytotoxic T cells. All T helper cells express CD4 on the surface and most of the cytotoxic T cells express CD8. There also exists a small portion of cytotoxic T cells with CD4 expression [87]. $CD4^+$ T helper cells can be further subdivided into T helper 1 (T_{H1}) and T helper 2 (T_{H2}) cells according to their cytokine secretion and function. Both the $CD4^+$ and $CD8^+$ T cells can be divided into naïve and memory T cells depending on whether they have been recognized and activated by the antigen. Each T cell subset has its unique function within the T cell response to infection. The composition of T cell subsets, as well as the function of individual subsets, will change with advanced age.

In young animals, homeostasis of T cell subsets is established so that the number of recent thymic immigrants and the number of naïve T cells lost each day is nearly equal. However, with age, the thymic output of naïve T cells decreases while the persistent exposure of individuals to acute and chronic pathogens drive more naïve T cells into memory cells. Thus, the proportion of memory T cells in both $CD4^+$ and $CD8^+$ T cells is elevated [88, 89]. Another age-associated feature of T cell subsets is decreased $CD4^+$ T cells in aged human PBLs (peripheral blood lymphocyte), resulting in

an increased CD4/CD8 ratio with age [90]. CD4+ and CD8+ T cells also express significantly more P-plycoprotein with age [91].

Based on these observations, an "immune subset factor" is calculated based on the level of $CD4^+$, $CD8^+$, CD4 memory (CD4M), CD8M, CD4 naïve, CD4 with P-glycoprotein expression (CD4P), and CD8P. This factor has a higher score when there are higher levels of CD8, CD4M, CD8M, CD4P and at the same time, lower levels of CD4, CD4 naïve population. The score (termed "F1_18" for mice at the age of 18 months) is a good predictor of lifespan or mortality risk [92].

On one side, the change in T cell subset composition with age will further influence the function of the whole T cell population, while on the other side, such compositional changes could also be the result of, or at least reflect, functional alterations in some T cell subsets.

Although $CD4^+$ T helper cells do not directly take part in immune protection, their function is indispensable for both humoral and cellular immunity. The major functional defect of $CD4^+$ T cells found in aging happens in naïve $CD4^+$ T cells. Less CD154 (CD40L) are expressed on aged $CD4^+$ T cells, leading to decreased migratory ability. As a result, the cognate helper function of aged naïve $CD4^+$ T cell declines and humoral immunity becomes defective [93]. This functional defect of naïve $CD4^+$ T cell is related to its post-thymic age, since the newly produced $CD4^+$ T cells from old mice are normal in their function when compared with those generated in young mice [94].

The function of the memory $CD4^+$ T cell also depends on the post-thymic age of the naïve $CD4^+$ T cell when it is activated and differentiates into memory cells. If the memory T cells were generated at a young age, then those T cells would maintain a normal function even when the animals become old. Those LCMV-specific memory $CD4^+$ T cells secrete the same, or ever higher amounts of IFN-γ at 206 days after infection as 45 days after infection [95]. Newly generated memory $CD4^+$ T cells in young and old donors showed no difference in persistence, but they did show functional difference that the aged memory $CD4^+$ T cell population having low proliferation and cytokine production *in vitro* and impaired proliferation and cognate helper activity *in vivo* [96, 97]. So, in the $CD4^+$ T cell subset, it is the naïve cells that are more vulnerable to aging.

In the aging process, $CD8^+$ T cells share some features with $CD4^+$ T cells. For example, the age-related defects, like poor recall response and low production of IFN-γ, are observed only when the naïve $CD8^+$ T cells are old [95, 98]. Aged $CD8^+$ T cells also have their own characteristics. Phenotypically, most (>90%) senescent $CD8^+$ T cells lack the CD28 expression on the surface, which does not happen in old $CD4^+$ T cells [99]. These $CD8^+CD28^-$ T cells are CD57 positive [100]. Since CD28 is essential for a variety of critical T cell functions, this phenomenon imparts profound functional changes associated with this particular subset of $CD8^+$ T cells. Consistent with that, the $CD8^+CD28^-$ cells are resistant to apoptosis under various stimuli [101]. Besides this, it is demonstrated that when under the same stimulatory condition at the fourth round, $CD8^+$ T cells lose the expression of CD28 as well as the ability to upregulate telomerase activity while $CD4^+$ T cells continue to show both CD28 expression and telomerase activity [99]. The $CD8^+CD28^-$ T cells are incapable of proliferating, and thus, show

reduced anti-viral suppressive functions. Other important functional changes associated with $CD8^+CD28^-$ T cells include the decreased binding affinity between T cells and endothelial cells, and the subsequent change in T cells moving around within the body [102]. *In vitro* culture of $CD8^+CD28^-$ T cells secrete more IL-6 and TNF-α, this is consistent with the pro-inflammatory status observed in many aged individuals [103].

Another unique feature associated with old $CD8^+$ T cells is the mono- or oligoclonal expansion (TCE). The expanded subset can occupy >80% of all $CD8^+$ T cells [104, 105]. How TCE are formed is still unclear. Some studies show that they are the result of continuous exposure to antigen [106, 107]. While others think their formation is under the control of cytokines [96, 108]. The existence of $CD8^+$ TCE reduces the diversity of $CD8^+$ T cells and contributes to the immunodeficiency shown in aged $CD8^+$ T cells [105].

Antibody production and B cell

Humoral immunity is an immune defense system mediated by antibody production. During old age, both humans and mice experience declines in humoral immunity, displaying retarded reaction to vaccine/foreign antigen and increased secretion of auto-antibodies [109]. Although the B-cell-derived plasma cell is the only source of antibody production within the body, most of its activation is regulated by the T helper (T_H) cell. Thus, humoral immunity aging can be partially explained by the diminished T_H cell function and increased T-cell-mediated suppression with age. However, intrinsic alterations of B cells must not be neglected from the aging studies.

The overall number of peripheral B cells does not change with age [110]. However, the subpopulations of B cells do change with age, leading to an increased proportion of marginal zone (MZ) B cells and "B1" B cells, but decreased proportions of follicular B cells [111]. Such an alteration in the composition of B cell subsets contributes to changes in the B cell receptor (BCR) repertoire during old age and eventually results in the immunoglobulin class of antibodies switching with age from IgG to IgM [112].

Other studies also showed that changes observed in old peripheral B cells are actually associated with the age-related decline of B lymphopoiesis within the BM. In the BM, B lympopoiesis undergoes sequential differentiation as early B-lineage precursors/common lymphoid progenitors (EBP/CLP), pro-B cells, early pre-B cells, late pre-B cells and immature B cells [113]. Aging affects B cell development at stages as early as EBP/CLPs and pro-B cells, [114] which decrease with age. One reason is that the frequency of $AA4.1^+$ pro-B cells declines with age because the $AA4.1^+$ B cell expresses high levels of RAG protein and can proliferate rapidly upon IL-7 stimulation [114]. Besides this, expression of surrogate light chain λ5 reduces with age in pro-B cells. λ5 protein is a component of pre-B-cell receptors (pre-BCR). As a result, pre-BCR formation is impaired and pro-B to pre-B cell transition is blocked in senescence [115, 116]. In addition, pre-BCR can induce the expression of BCL-XL protein, whose function is anti-apoptosis. Thus, only the pre-B cell with functional pre-BCR can survive, making the number of pre-B cells even more decreased with age [117]. Expression of both the RAG and λ5 protein are controlled ultimately by the E2A gene [118-120], so the decline of E2A expression is critical to B lymphopoiesis deficiency in aging [121].

Innate immunity and macrophage

Innate immunity is the first line of defense that pathogenic organisms will meet once they enter the host body. Macrophages, polymorphonuclear leukocytes, and mast cells are the major defense cells in innate immunity. Microbial pathogens usually express certain conserved metabolic products, called pathogen-associated molecular patterns (PAMPs), on their surface. PAMPs can be recognized by the pattern recognition receptors (PRPs) generated by the innate immunity defense cells and initiate a series of host defense reactions including proinflammatory responses, opsonization, phagocytosis, and apoptosis [122]. The innate immunity system is made of several cell types, and macrophages play an especially important role within those cells. Macrophages are among the first responders to pathogenic invasion and are actively involved in the phagocytosis of invading pathogens. In addition, macrophages can regulate the function of neutrophils, natural killer (NK) cells, dendritic cells, and connect the innate immunity to an adaptive immune reaction [123].

No change is witnessed in macrophage number with age in humans, but is increased in mice with age[123]. "Inflamm-aging" is a phenomenon frequently observed in healthy aged persons, this hyperinflammatory state is associated with higher levels of proinflammatory cytokines, suggesting there is a chronic activation of macrophages with age [124]. In contrast to what was observed *in vivo*, *in vitro* studies show a diminished function of macrophages in aged animals. Macrophages from aged mice produce less pro-inflammatory cytokines like LPS-induced TNF-α, IL-6, IL-1β, and IL-12 [125]. This is because a subset of PRRs that mediate the activation of macrophages, such as the Toll-like receptors (TLRs), have a lower level of expression

[126]. Meanwhile, old macrophages have a reduced capability to migrate, adhere, opsonize, and phagocytize the invading organism [127]. It is still not clear whether this is caused by a deficiency in the phagocytosis- promoting receptors (the mannose receptor, scavenger receptor, MARCO, MER, PSR, CD14, and CD36) and downstream signaling pathways. Other important functions of macrophages, like antibacterial peptide secretion in antibacterial defense and angogenic/fibrogenic growth factor production in wound repair, decline with age too [123]. Also, the macrophages from old human or rodent express less class II MHC and consequently provide suboptimal signals to the T cell to induce adaptive immunity [128]. Thus, the overall ability of the elderly to fight the disease-causing pathogen is reduced.

Proteasome degradation pathway in aging

In addition to declining cell function in the immune system with age, reduced cellular degenerations within the aging body are associated with accumulation of abnormal proteins in the cell. The synthesis/degradation balance of protein is crucial to preserve cell function in cell cycle/differentiation regulation, transcriptional control, DNA repair, antigen processing and apoptosis [129]. One of the main proteolytic systems responsible for protein turnover is the proteasome. So, one of the hypotheses raised to explain the damaged cellular protein build-up, and subsequent decline in cell function, is the breakdown of the proteasome degradation pathway with age [130].

<u>Components and function of ubiquitin-proteasome pathway</u>

For degradation, most of the known protein substrates of the proteasome will first be covalently attached to a polyubiqutin chain. It is this polyubiquitin chain that is recognized by and serves as a substrate signal for the proteasome. Ubiquitin (Ub) is a conserved protein of 76 amino acids, and polyubiquitination is a multi-step process which needs the sequential help of three enzymes: an ATP-dependent ubiquitin-activating enzyme (E1), an ubiquitin-conjugating enzyme (E2), and an ubiquitin-protein ligase (E3). This cascade covalently links the C-terminal glycine residue of ubiquitin to a ε-amino of a lysine residue of the target protein. Additional ubiquitin molecules are conjugated to lysine 48 (Lys48) of the preceding ubiquitin [131]. The specificity of the polyubiquitin chain added to a protein is determined by the E2 and E3 enzymes, which are induced by different types of post-translational modifications of the target protein, such as phosphorylation [132], dephosphorylation [133], alkylation [134], or association with ancillary proteins [135]. Thus, entry of substrate into the ubiquitin-proteasome proteolytic pathway is regulated independently of selectivity by the proteasome.

The proteasome is a complex comprised of several subunits and exists in various molecular forms, including 20S proteasome, 26S proteasome, and PA28-20S proteasome complexes [129, 130]. The 20S proteasome is the catalytic core of all three proteasomes and is present in a latent form in cells, but can be activated by heat treatment or addition of low concentrated detergent [136, 137]. Mostly, 20S proteasome degrades the oxidatively modified protein [138, 139]. Also, two different types of proteasome regulatory complexes that bind to the ends of the 20S proteasome can activate it. The addition of a 19S regulatory complex (PA700) to each end of the 20S proteasome results in the formation of the 26S proteasome [129]. Generally, it is the 26S proteasome that mediates the degradation of a polyubiquitinated protein and the 19S regulator functions to

recognize the polyubiquitin signal [140, 141]. The PA28-20S complex is composed of the 20S catalytic core and an 11S regulator (PA28) [142]. The PA28-20S complex does not participate in the degradation of ubiquitinated substrate, but is important in antigen presentation [142-144].

Before a protein becomes associated with the proteasome and is ultimately degraded in it, the protein substrate needs to first disassociate from the conjugated ubiquitin, which is catalyzed by ubiquitin-C-terminal specific processing proteases (UBPs) or ubiquitin carboxy-terminal hydrolases (UCHs). This is an important step because it can: 1) disassemble the polyubiquitin chain and release free ubiquitin to keep sufficient free ubiquitin supply; 2) "proofread" mistakenly ubiquitinated proteins; 3) trim "abnormally" long polyubiquitin chains so that they will be recognized by the 19S regulatory complex; 4) prevent the ubiquitin-binding sites on 26S proteasome being occupied by pure polyubiquitin chain instead of ubiquitinated substrate; and 5) activate biosynthetic precursors by removing ubiquitin [145, 146].

Impairment of ubiquitin-proteasome pathway in aging

It is believed that the ubiquitin-proteasome degradation pathway is impaired in aging, although the specific step being most affected is still unclear.

The efficiency and accuracy of a polyubiquitin chain being added to the substrate protein is closely related to how quick the protein will be recognized by the 26S proteasome and degraded. No enzymes in the ubiquitin system are proven to have a functional change with age [145]. However, *Heydari* et al found that in hepatocytes

isolated from aged rats, caloric restriction feeding can increase the heat shock induction of ubiquitin mRNA when compared to free access diet [147]. These data indicate that defects in the ubiquitin system associated with age are likely existing in ubiquitin expression which is also approved in aged rat soleus muscle [148]. The impairment of ubiquitin system function was also found in age-related diseases. For example, the ubiquitin system works as a neuro-tissue repair mechanism in normal brain, but in Alzheimer's Disease (AD) patients, the generation of aberrant *tau* and *beta* amyloid proteins is related with the accumulation of free ubiquitin and abnormally phosphorylated *tau* protein as well as ubiquitin-conjugated *beta* amyloid protein [149].

In aging, the observation of ubiquitin-protein conjugates accumulated in different tissues also indicates a decreased proteasome function with age. Decline in both proteasome activity [150] and proteasome subunit expression [10] in aging has been reported. The gene expression of proteasome subunits decreases with age in various cell types, including human fibroblasts [10], human epidermal cells [151], and rat skeletal myocytes [152]. The down-regulated proteasome expression is correlated with accumulation of oxidized proteins [151, 153]. Declined proteasome function with age has been observed in numerous tissues, but not universally in all tissues [130, 150]. Generally, this is due to a significant and consistent age-related decline in PGPH activity in the proteasome [154]. Interestingly, as an enzyme, proteasome itself is the target of oxidative damage and shows an age-related decline in peptidase activity [150, 155]. Also, highly oxidized protein may form intra-molecular cross-linking that resistant to proteolysis in proteasome, this will further impair the proteasome activity with age [156-158]. In T cells from the elderly, decreased proteasome-mediated degradation will affect the T cell function greatly. In aged, TNF-α-treated T cells, degradation of IκBα

is severely compromised, which causes decreased NFκB induction and interleukin-2 (IL-2) receptor expression [159], and consequently increases TNFα induced-apoptosis [160]. Similar age-related decline in the 26S proteasome-associated enzymatic activity is also found in both naïve $CD45RA^+$ and memory $CD45RO^+$ T cell subsets [161]. Another possible explanation for the increased ubiquitin conjugates in the elderly is the impairment of deubiquitinating enzymes. Deletion of *S. cerevisiae UBP14* results in accumulation of free ubiquitin chains and inhibition of proteolysis, presumably because some accumulated chains are too long for ubiquitinated substrates to bind to the proteasome [162]. In AD patients, the deubiquitinating enzymes fail to disassemble the mutant form of ubiquitin, named Ub(+1), from the polyubiquitin chain, and this conquently leads to the neurodegenerative disorder [163].

In conclusion, one or more steps in the ubiquitin-proteasome pathway may be affected during the process of aging. The factors influencing the proper function of ubiquitin-proteasome system include, but are not limit to, the modification of the substrate protein, ubiquitin expression, proteasomal enzyme activity and de-ubiquitination.

Ubiquitination and Signaling Regulation

In the past, most ubiquitination studies have been focused on its association with the proteasome and the proteolytic function in protein turnover. More and more evidence now tells us that ubiquitination also plays an important role in signaling pathways that are proteolysis-independent [164, 165].

Proteins can be either polyubiquitinated or monoubiquitinated. The well-known polyubiquitination (polyUb) that can lead to the degradation of substrate protein in the 26S proteasome occurs through residue Lys48. Another type of polyUb, in which ubiquitins are linked through Lys63, can control the protein activity without the proteasomal degradation. Besides TNF-α, engagement of IL-1β can trigger NF-κB's activity too. Activation of NF-κB requires the separation of NF-κB from its inhibitor IκB by ubiquitinating and sending IκB to 26S proteasome for degradation [166]. Ubiquitination and degradation of IkB is triggered by activated IKK, whose function is to selectively phosphorylate IkB at serine residue 32 and 36 [167]. In IL-1β induced NF-κB pathway, function of IKK is regulated through TRAF6 and TAB [168]. TRAF6 contains RING domain E3 Ub ligase activity and can self-ubiquitinate to form Lys63-linked polyUb chain upon IL-1β stimulation. This then results in the sequential activation of TAB and IKK and leads to NF-κB activation [169]. Addition of proteasome inhibitor MG132 or lactacystin can not inhibit the activity of IKK induced by TRAF6 [169], therefore, in this case, ubiquitination is acting independent of the proteasome. Proteasome-independent ubiquitination also exists in other steps of NF-κB signaling. PolyUb through Lys63 also form on IKKγ, the regulatory subunit of IKK complex and enhance the overall activity of IKK [170-172]. Studies by *Spence* et al also showed that a ribosomal protein, L28, is modified by a Lys63-linked polyUb chain when ribosomes are actively involved in translation. The level of L28 ubiquitination varies with cell cycle stage, where it is high in S phase but low in G_0 and G_1 phase. Since L28 itself is quite stable, no proteolytic action is involved in this case [173].

Monoubiquitination can also regulate the protein function in a reversible, non-proteolytic manner. Sometime, monoubiquitin serves as a sorting signal that determines

newly synthesized protein being delivered to plasma membrane or internalized receptor protein being delivered to lysosome [174]. Interestingly, some proteins can be either monoubiquitinated or polyubiquitinated and lead to different protein function. PCNA (proliferating cell nuclear antigen) is a protein involved in DNA replication and repair [175]. Under normal situation, PCNA is sumoylated. While after exposure to UV or chemicals, PCNA is monoubiquitinated at the same site, which will result in DNA damage-induced mutagenesis [176]. Contrast to the monoubiquitination, Lys63-linked polyubiquitination on PCNA will lead to an error-free DNA repair [175].

Ubiquitination has a lot of features similar to another protein modification system--phosphorylation. First, ubiquitination can be induced by upstream signaling events. Stimulation of epidermal growth factor-receptor (EGFR) can induce the monoubiquitination of Eps15 by Nedd4, Eps15 will then participate in subsequent signal transduction [177]. Second, ubiquitination, especially monoubiquitination can be recognized by proteins containing Ub-binding domains. To date, four types of Ub-binding domains have been found, including the ubiquitin associated (UBA) domain, ubiquitin interacting motif (UIM), ubiquitin E2 variant (UEV) and Cue1-homologous (CUE) [164]. These domains have their own specificity and affinity for ubiquitin modified at different sites. Third, the ubiquitination can be regulated and removed by de-ubiquitinating enzymes. The polyUb chain attached to TRAF6 or IKKγ can be removed by the cyclindromatosis tumor suppressor protein (CYLD) and NF-κB activity is down-regulated [178-180]. Thus, the type of ubiquitination modification, its receptor, and the de-ubiquitinating enzyme form an interactive network that may work as another important regulatory system like phosphorylation.

Aging, T cell function and Cbl-b

The Cbl family was first identified as an oncogenic mouse retrovirus, named v-Cbl, for Casitas B-lineage Lymphoma [181]. Since then, three mammalian homologues (Cbl, Cbl-b and Cbl-3) have been characterized [182-184]. These proteins have highly conserved amino-terminal regions which contain: 1) a tyrosine-kinase-binding (TKB) domain that recognizes phosphorylated tyrosine on activated protein tyrosine kinase (PTK); 2) a RING finger domain that functions as E3 ubiquitin ligase, and induces ubiquitin conjugation to activated PTK [185-187]. Besides these common N-terminal structures within the whole Cbl family, Cbl and Cbl-b share some additional features in their carboxyl-terminal regions. They both contain proline-rich regions that lie in the carboxyl-terminal half of the protein, and are believed to be involved in SH3-domain interaction [185]. Also, they have tyrosines at 709 and 655, which can bind to various signaling intermediates that containing SH2 domains, and are substrates of tyrosine kinases [188]. The phosphorylated Cbl-b is activated and then mediates polyubiquitination and down-regulation of these proteins. Therefore, as E3 ubiquitin ligase, a major function of Cbl family proteins is ubiquitinating activated PTKs and mediating their down-regulation. On the other hand, as an adaptor protein, Cbl is associated with various proteins that are essential to signal transduction, and change their intercellular location and interaction with other proteins, thus providing another way to regulate signaling pathway in addition to its E3 ubiquitin ligase activity. Cbl and Cbl-b are found in a variety of normal tissues and hematopoietic cell lines. cbl mRNA is most abundant in testis and thymus while the highest cbl-b mRNA levels are observed in spleen [182, 189]. The expression of cbl and cbl-b mRNA can be modulated during the differentiation of several hematopoietic cell lines [182, 190]. Induction of proliferation

will not change the expression level of cbl [190] but will down-regulate Cbl-b expression in BALB/c splenic T-lymphocytes [191]. These data suggest that Cbl and Cbl-b may have differential effects on regulation immune system. Both Cbl and Cbl-b are crucial in T cell signaling regulation. Because they have a highly homologous structure, Cbl and Cbl-b have some important overlapping functions, such as promoting TCR down-regulation after ligand engagement [192], suppressing the activation of downstream proteins upon TCR stimulation [193, 194], and inhibiting growth factor induced proliferation [195-197]. More importantly, Cbl and Cbl-b show selectively regulation on T cell system at different aspect. Cbl-deficient mice show hypercellularity of lymphoid organs and altered positive selection in the thymus [198]. Thymocytes from these mice show a marked activation of Zap-70 in response to TCR stimulation and increased intracellular protein phosphorylation and surface receptor expression in $CD4^{+}CD8^{+}$ DP thymocytes [199]. The Cbl-b-deficient mouse shows a similar phenomenon, but in peripheral T cells with hyperactivation of Vav as opposed to thymocytes having increased Zap-70 activation. T cells in these mice show enhanced proliferation and IL-2 production in response to TCR triggering, and uncouple T cell proliferation from the costimulaiton of CD28 signal [200, 201], which indicates a lowered threshold for TCR signaling in Cbl-b-deficient mice. As a result, these mice have an increased susceptibility to the development of autoimmunity. So, in T cell regulation, Cbl controls thymocyte selection and development, while Cbl-b plays its role in peripheral T cell activation [185, 186, 202].

Many signaling pathways in different tissues and cell lines are influenced by Cbl-b, including calcium signaling [203], Lyn-Syk-LAT or Gab2-mediated complementary signaling pathway [204], activation of p21-activated kinase (PAK) [205], insulin-

stimulated glucose transport [206], EGFR induced apoptosis [207], chemotaxis [208], cancer transforming [209], and Ig class switch or germinal center formation [210]. The extent of Cbl-b's participation in signaling pathways in peripheral T cell activation is not well understood. It has been shown that upon TCR stimulation, Cbl-b is phosphorylated by protein tyrosine kinase of the Syk-(Syk/Zap-70) or Src-(Fyn/Lck) family [188]. Phosphorylated Cbl-b has higher affinity for CIN85 (Cbl-interacting protein of 85 kDa) to catalyze the ubiquitination and degradation of receptor tyrosine kinase [211]. In downstream events, Cbl-b can interact with, and direct ubiquitination to, the p85 regulatory subunit of phosphatidylinositol-3-kinase (PI(3)K), which phosphorylates phosphatidyl inositol (PI) to form bioactive lipid product, $PI_{(3,4,5)}P$, that act as second messengers in signaling transduction [212]. The ubiquitination of PI(3)K does not lead to the proteolysis of PI(3)K, but blocks the association of p85 to CD28 and TCRζ [213]. Since the product of PI(3)K can bind and regulate Vav, by inducing ubiquitination of PI(3)K, Cbl-b indirectly regulates the exchange activity of Vav and the organization of receptor clustering and raft aggregation, which is essential for a successful TCR-induced T cell activation [214, 215]. Besides p85, Cbl-b can also bind to Zap-70, Lck, PLCγ-1. Expression of these Cbl-b binding proteins remains unchanged [200] in cbl-b-deficient T cells suggesting that Cbl-b can regulate the function of these proteins in a proteolytic-independent manner similar to Vav regulation. In agreement of these observations that role of Cbl-b in T cell signaling is quite crutial, loss of Cbl-b has profound impact on T cell function. $cbl^{-/-}CD4^{+}CD25^{-}$ T effector cells show defects in TGF-β signaling pathway [216, 217]. Th1 cells without Cbl-b expression is tolerant to apoptosis induced by CD3 ligation [218]. Cbl-b deficiency T cells resist T cell anergy induction by ionomycin [219].

Considering that Cbl-b functions as a key regulator in T cell activation, the mechanism by which Cbl-b itself is regulated within the signaling network attracts more and more attention of current research. Previous work suggests Cbl-b is regulated by CD28-mediated co-stimulation since $CD28^{-/-}$ $cbl\text{-}b^{-/-}$ mice have increased proliferation compared to $CD28^{-/-}$ $cbl\text{-}b^{+/+}$ mice [200, 201]. A recent study in *Zhang*'s lab has also shown that CD28 co-stimulation selectively induces greater ubiquitination and degradation of Cbl-b in wild-type BALB/c T cells than CD3 stimulation alone, and TCR-induced Cbl-b ubiquitination and degradation are significantly reduced in CD28-deficient T cells [191]. It is already known that Cbl-b can be ubiquitinated by either HECT (homologous to the E6-AP carboxyl terminus) E3 enzyme [220] or by itself though its RING finger E3 activity [221]. The subsequent degradation of ubiquitinated Cbl-b depends on proteasome only [220] or both the proteasome and lysosome [221]. However, the expression of Cbl-b in old T cells, its regulation upon stimulation, and its relationship with immunosenescence are still unclear. Answering these questions will be the main target of this proposal.

Chapter 2: CD28 Activation Down-regulate Cbl-b Expression in Young Rat Splenic T-lymphocyte

2.1 ABSTRACT

It is well known that T-lymphocyte proliferation declines *in vitro* with age, and is associated with decreased expression and/or activity of stimulatory intracellular signaling proteins. However, the role of inhibitory intracellular signaling molecules like the ubiquitin ligase Cbl-b in regulating T-lymphocyte function in aging is largely unknown. Therefore, we tested the hypothesis that T-lymphocyte proliferation might influence the expression of Cbl-b or vice versa. We show that young splenic T-lymphocyte reduced Cbl-b expression when stimulated with anti-CD3 and anti-CD28 antibodies, while anti-CD3 antibody alone unable to down-regulate Cbl-b expression. This effect appeared to be due to post-translational binding and modification by another ubiquitin ligase Nedd4. The mechanism for Cbl-b down-regulation may involve the proteasome since blocking proteasomal activity in young T-lymphocytes prevented Cbl-b down regulation. These data provide evidence for a novel relationship of T-lymphocyte function with ubiquitin/proteasome pathway and support the idea of further study in role of Cbl-b in aging-associated reduced T-lymphocyte response.

2.2 INTRODUCTION

As a negative regulator of T cell signaling pathway, Cbl-b's function have been frequently studied to see how they are involved in down-regulating other proteins',

mostly, tyrosine kinases', expression and activity. However, who would control the expression of Cbl-b, what is the entire mechanism, how the regulation on Cbl-b will affect the function of Cbl-b's target in T cell signaling transduction are barely known. Research from different labs gave different stories about regulation of Cbl-b.

Regulation of Cbl-b was first studied in EGF stimulated MDA-MB-468 breast cancer cell line. Stimulation of EGF induces the degradation of both EGFR and Cbl-b. This coordinated degradation requires the association of EGFR with Cbl-b and there is a stoichiometric relationship between these two proteins. Experiment using different inhibitors shows that both proteasome and lysosome are involved in their degradation. The regulation mechanism can not tell the difference between Cbl-b and Cbl, since co-expression of Cbl and EGFR show the same co-degradation result. Detailed study shows that Cbl-b is ubiquitinated and self-degraded. Experiments with mutants of Cbl-b find that TKB and RING finger domain of Cbl-b are indispensable for the proper down-regulation of itself. More specifically, point mutation in Cbl-b's RING finger (C373A) will completely eliminate the EGR-induced down-regulation of the EGRF and Cbl-b [221].

In splenic T cells isolated from WT BALB/c mice, Cbl-b is ubiquitinated and degraded when cells are activated by either plate-bound anti-CD3 or anti-CD3 plus anti-CD28 antibodies. The extent of ubiquitination and degradation are greater when CD28 costimulatory signal is present. The level of Cbl-b ubiquitination also correlates with the level of T cell response. Thus, Cbl-b ubiquitination and degradation induced by CD28 costimulation partly set the threshold of T cell activation [191].

Two domains of Cbl-b, proline-rich region and the phosphotyrosine binding domain, can bind with the WW domains on Nedd4 both *in vivo* and *in vitro* [220]. Nedd4 is an E3 ubiquitin ligase using HECT (homologous to the E6-AP carboxyl terminus) domain as its catalytic motif. Structure of Nedd4 family protein all contain a C2 domain and 2-4 WW domains at N-terminal, and the catalytic HECT domain at C-terminal [222]. C2 domain's function is to localize the Nedd4 to membrane by association with the phospholipid at membrane surface [223]. WW domain is the substrate recognition part of Nedd4 [224]. HECT domain catalyzes the addition of ubiquitin to its substrate, and results in the ubiquitinated target be sent either to 26S proteasome for degradation or to lysosome for recycling or endocytosis [225]. Thus, Nedd4 family proteins are involved in a number of diverse cellular processes, especially the signaling of membrane associated protein.

In transfected 293T human embryonic kidney cells, the association between Cbl-b and Nedd4 has functional importance, since Nedd4 can ubiquitinate Cbl-b and lower Cbl-b protein level by proteasomal degradation and subsequently reverse the Cbl-b-mediated ubiquitination and degradation of EGFR and Src. Other WW domain-containing HECT E3s, like Itch, have the same effect on Cbl-b [220]. However, whether these findings observed in transfected system have an *in vivo* significance in human and mouse primary cell and how the regulation events be connected to cell receptor activation are still unknown.

Opposing to the replicative immune response, when accompanied with different transcriptional partner, sustained Ca^{2+} and calcineurin could induce T cell anergy or tolerance [226], characterized by a low IL-2 secretion and low proliferation,

hyporesponsive state upon restimulation [227]. Long term exposure of mouse D5 (Ar-5) T_H1 cell to ionomycin will induce the anergy state of cells, and increase the mRNA level of Cbl-b and Itch but not Nedd4 [203]. This experiment shows that the altered transcription profile cause by T cell anergy are responsible for the regulation of Cbl-b mRNA expression. The detailed mechanism underlying the Cbl-b regulation and whether this regulative process is special for T cell anergy or can be applied to general situation like normal TCR-mediated cell activation are not clear now.

Another research in T cell anergy found that expression of Egr-2 and Egr-3 are increased in anergy. Egr-2 and Egr-3 are two members of zinc-finger transcription factor Egr (early growth response) family. They can regulate the target gene transcription by binding to its canonical binding site (5'-GCGGGGGCG-3') on target gene promoter [228]. The upregulation of Egr-2 and Egr-3 in anergy has correlation with Cbl-b since the co-upregulation of Cbl-b's expression disappear in Egr-$3^{-/-}$ T cells [229]. Whether Egr-2 and/or Egr-3 bind directly to Cbl-b's promoter or indirectly upregulate Cbl-b is unclear now.

The goal in this chapter is to see under normal stimulation condition, whether Cbl-b's expression will change in primary rat T cells and try to figure out the possible pathway that Cbl-b being regulated.

2.3 MATERIALS AND METHODS

Materials

Anti-Cbl-b antibody (G-1), anti-Cbl antibody (C-15), and anti-β-actin (I-19) were purchased from Santa Cruz (Santa Cruz, CA). Anti-CD3 (G4.18) and anti-CD28 (JJ319) monoclonal antibodies were purchased from BD PharMingen (San Jose, CA). The proteasome inhibitor MG-132 and lactacystin were purchased from Calbiochem (La Jolla, CA). All cell culture media were purchased from Mediatech (Herndon, VA) and all other chemicals were reagent grade or higher and from Sigma-Aldrich (St. Louis, MO).

Animals

Male, 4-6-month (young) Sprague-Dawley rats were purchased from Harlan Sprague Dawley (Indianapolis, IN) and maintained on a standard chow diet. All animal procedures were approved by the University of Texas Animal Use and Care Committee.

T-lymphocyte isolation and culture

Rats were sacrificed and spleens aseptically removed. Single splenic cell suspensions were isolated by homogenization and filtering through a 25mm syringe filter. Lymphocytes were isolated via differential migration following centrifugation in Lymphocyte Separation Medium (Mediatech, Herndon, VA). T-lymphocytes were isolated using negative selection Immulan columns (Biotecx, Houston, TX) per manufacturer's instructions as previously described [230]. T-lymphocytes were counted using the Cell-Dyn 900 Hematology Analyzer (Sequoia-Turner, Mountainview, CA). T-lymphocytes, $2.5x10^7$ cells per petri dish, were stimulated at 37ºC for designated time in pre-warmed complete culture media (RPMI 1640 supplemented with 10% heat

inactivated fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 100 mM 2-mercaptoethanol, 2 mM L-glutamine and 25 mM HEPES buffer) with 10 μg/ml anti-CD3 plus 1 μg/ml anti-CD28 antibodies, 10 μg/ml plate-bound anti-CD3, 1 μg/ml anti-CD28 antibody only, or 5 μg/ml anti-CTLA-4 antibody. In the proteasome inhibition experiments, the MG-132 was dissolved in DMSO (25mM stock solution) and used at a 1:500 dilution (50 μM) in the cell culture media or lactacystin (5 mM stock solution) was used at a 1:1000 dilution (5 μM) and added simultaneously with the antibodies. To inhibit the lysosome activity, NH_4Cl (5 M stock solution) was used at a 1:500 dilution (10 mM) in the cell culture media.

Western immunoblotting

Cellular protein was isolated and western immunoblotting performed as previously described [231]. Briefly, total protein from stimulated or unstimulated T-lymphocytes were pelleted and lysed in 50 μl of lysis buffer containing 50 mM Tris (pH 7.4), 10 mM EDTA, 150 mM NaCl, 0.1% Tween 20, 1 μl/ml β-ME, and 7 μl/ml protease inhibitor mix. Lyastes were centrifuged and the protein containing supernatant was quantitated using the Bio-Rad protein assay (Bio-Rad, Hercules, CA). 30 μg of total protein was separated by SDS-PAGE along with Bio-Rad's Kaleidoscope Prestained Standards. The protein was transferred onto PolyScreen PVDF membranes (NEN Life Sciences, Boston, MA) and the membranes blocked with 15 ml blocking solution containing 25 mM Tris (pH 8.0), 125 mM NaCl, 4% non-fat milk, and 0.1% Tween 20 prior to probing with anti-Cbl-b antibody (1:3000 dilution in 15 ml blocking solution) or anti-Cbl antibody. Immunoreactive bands were detected using an alkaline phosphatase-conjugated goat-anti-rabbit secondary antibody and CDP-Star chemiluminescence

reagent (NEN Life Sciences, Boston, MA). Densitometry was performed using a BioRad Gel Documentation System. The membrane was also probed with an anti-β-actin antibody to control for lane loading variation.

RNA Isolation and RT-PCR

T-lymphocyte RNA was isolated from whole cells by acid guanidinium thiocyanate-phenol-choloroform extraction method. Briefly, $10x10^6$ cells were pipetted repeatedly in 1 ml RNA STAT-60™ (TEL-TEST "B", INC, Friendswood, TX). Cell suspensions were stored for 5 min at room temperature then 200 μl of chloroform was added for each ml of RNA STAT-60™ used. Samples were vortexed vigorously for 15 sec and allowed to stand at room temperature for 2-3 min. Following centrifugation at 12,000 *g* for 15 min at 4ºC, the homogenates were separated into a lower phenol/chloroform phase and the RNA in the upper aqueous phase was transferred to fresh tubes containing 500 μl isopropanol. After standing at room temperature for 5 min, samples were centrifuged at 12,000 *g* for 10 min at 4ºC. The supernatant was removed and the RNA pellet was washed once with 1 ml of 75% ethanol by vortexing and subsequent centrifugation at 7,500 *g* for 5 min at 4ºC. The RNA pellet was dried briefly by air-drying and dissolved in diethylpyrocarbonate (DEPC)-treated water by vigorous pipetting and gentle heating at 55ºC for 10 min. RNA was quantitated spectrophotometrically by UV-1201S UV-VIS spectrophotometer (Shimadzu, Columbia, MD).

For cDNA synthesis, 2 μg of RNA was combined with 1 μg of random primer, 5 μl of 10 μM dNTP mix, 40 u of RNasin plus RNase inhibitor, 5 μl of M-MLV RT 5X

buffer, and 200 u of M-MLV reverse transcriptase (Promega, Madison WI) in a 25 μl total reaction volume using the PerkinElmer GeneAmp PCR system 2400 (PerkinElmer, Wellesley, MA) to reverse transcribe (RT) and amplify template RNA. The RT reaction used was 4ºC x 10 min, 15ºC x 10 min, 22ºC x 10 min, 37ºC x 60 min, and 95ºC x 5min.

mRNA levels of Cbl-b, Nedd4, and GAPDH were analyzed by PCR reaction. 2 μl of RT solution was combined with 20 pM of each primer and 12.5 μl of PCR Master Mix (Promega, Madison WI) in a 25 μl total reaction volume and PCR reaction was performed with 25 cycles of denaturation: 1 min at 94°C; annealing: 1 min at 52°C; and extension: 2 min at 72°C using the PerkinElmer GeneAmp PCR system 2400. Primer sequences are listed in Table 2.1. The PCR products were then separated on a 1% agarose gel, stained with ethidium bromide, and scanned by a CCD camera. The band intensities were then analyzed by BioRad Gel Documentation System.

Table2.1 PCR primer sequences for RT-PCR

Gene amplified		Primer sequence
Cbl-b	Forward primer	5'-GGTCACTGTATACTGAATGG-3'
	Reverse primer	5'-ATTGAGACGTGGCGAGACGG-3'
Nedd4 [232]	Forward primer	5'-CCAGATGCTGCTTGCCATTTGCAG-3'
	Reverse primer	5'-GTAATCCCTGGAGTAGGGCACTGC-3'
GAPDH	Forward primer	5'-CCATGGAGAAGGCTGGGG-3'
	Reverse primer	5'-CAAAGTTGTCATGGATGACC-3'

Immunoprecipitation of Cbl-b

Stimulated or unstimulated T-lymphocytes from 4 rats were pooled together. Pooled T-lymphocytes were pelleted and total protein lysed in 500 µl of lysis buffer containing 50 mM Tris (pH 7.4), 10 mM EDTA, 150 mM NaCl, 0.1% Tween 20, 1 µl/ml β-ME, and 7 µl/ml protease inhibitor mix. Lyastes were centrifuged and the protein containing supernatant were pre-cleared by adding 40 µl of protein A/G plus agarose (Upstate, Charlottesville, VA) and rotated at 4ºC for 1 hr. The mixture was pelleted at 2,000 *g* for 2 min at 4ºC and supernatant was quantitated using the Bio-Rad protein assay (Bio-Rad, Hercules, CA). 500 µg of total protein were incubated with 4 µg anti-Cbl-b antibody (H-454) and rotated overnight at 4ºC. Protein A/G plus agarose (20 µl) was added and the mixture was rotated again at 4ºC for 2 hr. The mixture was pelleted at 2,000 *g* for 2 min at 4ºC and the beads washed 3 times with 500 µl lysis buffer. The bead slurry was suspended in 2X Laemmli sample buffer, boiled for 10 min and the supernatant (immunoprecipitated protein) was collected.

Phosphoprotein staining

Immunoprecipitation of Cbl-b are performed as described before. Half of the sample were separated using standard SDS-PAGE techniques. Gels were fixed in 50% methanol : 10% acetic acid solution two times for 30 min each at room temperature and washed three times in ultrapure water for 10 min each. Gels were stained for phosphorylated proteins using Pro-Q Diamond Phosphoprotein Gel Stain (Invitrogen, Carlsbad, CA) for 90 min at room temperature. Gels were destained to reduce the background signal using 5:75:20 1M sodium acetate (pH 4.0) : water : acetonitrile

solution three times for 30 min each at room temperature. Gels were washed three times in ultrapure water for 10 min each and phosphorylated bands were visualized using a Molecular Dynamics FSI fluorimager (Bio-Rad, Hercules, CA) with excitation at 532 nm and emission at 555 nm, respectively. Proteins from stained gels are not transferred sufficiently to PVDF membranes for probing with anti-Cbl-b antibody, therefore, samples were run in parallel, transferred and immunoblotted for anti-Cbl-b as described above.

Small scale sucrose density gradient centrifugation for protein isolation

Prepare the sucrose gradient solution by adding 950 μl of 40% Sucrose, 950 μl of 31.25% Sucrose, 950 μl of 22.5% Sucrose, 950 μl of 13.75% Sucrose, and 950 μl of 5% Sucrose sequentially in Beckman SW55 Ti rotor tubes. Tubes of sucrose solution layers are kept at 4°C for at least 12 to 16 hr (i.e., overnight) and a linear gradient will form. 100 μl crude cellular extract isolated with RIPA buffer (0.01 M sodium phosphate, pH 7.2, 150 mM NaCl, 2 mM EDTA, 1% Nonidet P-40, 0.1% SDS, 1% sodium deoxycholate, 5 μg/ml aprotinin, 50 mM sodium fluoride, 1 μg/ml leupeptin, and 20 mM sodium vanadate) will be layered on top of the sucrose gradient solution and centrifuge at 4°C for 4 hr at 50,000 rpm (approximately 237,000 X g) in Optima XL-100K Ultracentrifuge (Beckman, Fullerton, CA). After centrifugation, carefully transfer 300 μl of fractions from the top of the gradient to the microcentrifuge tubes with large-bore pipette tips. Fractions will be concentrated by Ultrafree-MC 5,000 NMWL centrifugal filter units (Millipore, Billerica, MA) and assayed by western immunoblotting.

Cell fractionation

2 X 10^7 splenic T-lymphocytes with different treatment were lysed in hypotonic buffer E containing 10 mM Tris (pH 7.4), 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM DTT, and 7 μl/ml protease inhibitor mix for 15 min on ice. Lyastes were centrifuged and supernatant (cytosol fraction) was collected. Pellet yielded was resuspended in hypotonic buffer E containing 1% Nonidet P-40 and was recentrifuged to collect the supernatant (detergent-soluble fraction). The pellet (detergent-insoluble fraction) was resuspended in RIPA buffer (0.01 M sodium phosphate, pH 7.2, 150 mM NaCl, 2 mM EDTA, 1% Nonidet P-40, 0.1% SDS, 1% sodium deoxycholate, 5 μg/ml aprotinin, 50 mM sodium fluoride, 1 μg/ml leupeptin, and 20 mM sodium vanadate) by sonication and cleared by centrifugation. Protein concentration from each centrifugation step (cytoplasm, detergent-soluble, and detergent-insoluble fractions) was determined by the Bio-Rad DC protein assay (Bio-Rad, Hercules, CA) and was analyzed for Cbl-b and Nedd4 expression by western immunoblotting.

20S proteasome activity assay

Proteasome activity is determined by CHEMICON 20S Proteasome Activity Assay Kit (Chemicon, Temecula, CA) via manufacturer's instruction. Briefly, total T cell protein is isolated as described before and protein concentration determined. 1 mg of total protein and 5 nM proteasome substrate (Suc-LLVY-AMC) are incubated in 25 mM HEPES (pH 7.5), 0.5 mM EDTA, 0.05% NP-40, and 0.001%SDS in 96-well fluorometer plate (100 μl total volume) for 1 hr at 37ºC. In parallel, AMC standard curve from 0.04 mM to 12.5 mM and proteasome positive control curve (1:4 to 1:256) are prepared. Fluorescences are recorded in DTX 880 Multimode Detector (Beckman, Fullerton, CA) with 380/460 nm filter.

Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) and statistical difference were determined using Tukey's multiple comparison test with GraphPad Prism software (San Diego, CA). A $p<0.05$ was considered significantly different.

2.4 RESULTS

Cbl-b expression in activated splenic T-lymphocytes

T-lymphocytes were purified from rat spleens by negative selection. The T-lymphocytes were stimulated by soluble anti-CD3 (10 μg/ml) plus anti-CD28 (1 μg/ml) antibodies for 30, 60, or 120 min. The results are shown in Figure 2.1. There is a significant 41% reduction in Cbl-b protein expression at 30 min. Similar decrease was also observed in 60 min antibody treatment and the reduction was maximal at 120 min post stimulation (57% reduction). As Cbl has a very similar structure with Cbl-b and these two proteins have some overlapping functions in immune regulation, the expression level of Cbl protein was also tested at the same time. Cbl protein expression showed a moderate but not significant decrease upon stimulation (16%, 15% and 31% reduction at 30, 60, and 120 min). The two T-lymphocyte activating signals, CD3 and CD28 are both presented to cause the down regulation of Cbl-b. In order to tell whether both signals are required to control the expression of Cbl-b, purified T-lymphocytes were also stimulated with plate-bound anti-CD3 antibody or soluble anti-CD28. Plate-bound anti-CD3 antibody provides a stimulating signal strong enough to cause a similar proliferation

response in T-lymphocyte as those being stimulated by soluble anti-CD3 and anti-CD28 antibodies (data not shown). Results of stimulating young splenic T-lymphocytes with plate-bound anti-CD3 antibody are shown in Figure 2.2. Stimulation via CD3 only did not alter Cbl-b expression in young splenic T-lymphocytes. Stimulating young splenic T-lymphocytes with soluble anti-CD28 antibody alone decreased Cbl-b expression significantly (67%, 59%, and 80% reduction at 30, 60, and 120 min) (Figure 2.3). No effects were seen in Cbl protein expression.

Cbl-b expression in inhibited splenic T-lymphocytes

By competing with CD28 for costimulatory molecules expressed on APCs, CTLA-4 can oppose CD28's function and inhibit T-lymphocytes activation. Since soluble anti-CD28 antibody alone can down-regulate the Cbl-b expression, whether the inhibitory signal from CTLA-4 can control Cbl-b expression was tested. Purified young splenic T-lymphocytes were incubated with 5 μg/ml anti-CTLA-4 antibody for 30, 60, or 120 min. The results are shown in Figure 2.4. Cbl-b expression increases after 30 min exposure to anti-CTLA-4 antibody (133.4% compare to untreated control) and further increased expression of Cbl-b is observed at 60 min and 120 min treatment (126.0% and 183.4% respectively).

Cbl-b gene expression in splenic T-lymphocytes under different treatment

In order to determine if the observed Cbl-b protein expression change under different stimulatory conditions is due to the regulation on transcriptional levle or on translational level, mRNA expression of Cbl-b gene was checked. The T-lymphocytes

were unstimulated or stimulated by soluble anti-CD3 (10 μg/ml) plus anti-CD28 (1 μg/ml) antibodies, plate-bound anti-CD3 (10 μg/ml) antibody, or soluble anti-CD28 (1 μg/ml) antibody for 120 min and RT-PCR was performed. The results are shown in Figure 2.5. Stimulation young splenic T-lymphocytes with different stimulatory signals had no effect on Cbl-b mRNA expression.

Role of proteasome system in the regulation of Cbl-b expression

Cbl-b in wild-type BALB/c T cell can be ubiquitinated and degradated upon CD28 co-stimulation, which suggests the involvement of proteasome system in the regualtion of Cbl-b expression [191]. Proteasome inhibitors were added along with anti-CD3 and anti-CD28 antibodies and Cbl-b expression level tested. Figure 2.6 shows that, when the proteasomal inhibitor MG132 is added simultaneously with anti-CD3 and anti-CD28 antibodies, activation fails to reduce Cbl-b expression at 30, 60 and 120 min in young T-lymphocytes. Since MG132 also inhibits calpain [233], these results were confirmed by using a more specific proteasome inhibitor lactacystin (Figure 2.7). Indeed, stimulation did not reduce Cbl-b protein expression in the presence of Lactacystin at 30, 60 or 120 min poststimulation in young splenic T-lymphocytes. Degradation of Cbl-b can be lysosome-dependent too, since its degradation upon activation of EGFR is sensitive to both proteasome and lysosome inhibitors [221]. To test if Cbl-b was sent to lysosome for degradation too, purified T-lymphocytes were stimulated with anti-CD3 and anti-CD28 antibodies in the presence or absence of NH_4Cl, inhibitor of lysosome and Cbl-b expression tested. As shown in Figure 2.8, NH_4Cl can not inhibit the degradation of Cbl-b stimulated by CD3 and CD28 antibodies.

Proteasome activity in splenic T-lymphocytes with different treatment

Since the proteasome system is required for Cbl-b regulation as shown in previous results, we need to determine the proteasome activity in splenic T-lymphocytes under different treatment. The T-lymphocytes were unstimulated or stimulated by soluble anti-CD3 (10 μg/ml) plus anti-CD28 (1 μg/ml) antibodies, plate-bound anti-CD3 (10 μg/ml) antibody, or soluble anti-CD28 (1 μg/ml) antibody for 120 min and proteasome activities in each group were determined. The results are shown in Figure 2.9. There is no significant change in proteasome activity between each group.

Phosphorylation of Cbl-b under different stimulation condition

TCR stimulation in Jurkat T cells can induce a rapid (<10 min) tyrosine phosphorylation on Cbl-b protein [188]. Also, in previous results, we made the observation that mRNA level of Cbl-b didn't change upon stimulation. All these results suggest that post-transcriptional modification, especially tyrosine phosphorylation may play an important role in regulating Cbl-b's function and other activities. Thus, the tyrosine phosphorylation of Cbl-b upon stimulation was determined. The results are shown in Figure 2.10. In both plate-bound anti-CD3 stimulated and soluble anti-CD28 stimulated T-lymphocytes, Cbl-b started being phosphorylated 5 min after stimulation. The phosphorylation level increased at 30 min and continued till 120 min. No significant difference of Cbl-b tyrosine phosphorylation was seen between plate-bound anti-CD3 stimulated and soluble anti-CD28 stimulated condition.

Association of Nedd4/Itch protein with Cbl-b protein

HECT E3 ubiquitin ligases Nedd4 and Itch mediate the proteasomal degradation of Cbl-b protein in transfected 293T cells [220], which implies that Nedd4 and Itch may also play an important role in the regulation of Cbl-b protein in rat splenic T-lymphocytes. The T-lymphocytes were unstimulated or stimulated by soluble anti-CD3 (10 μg/ml) plus anti-CD28 (1 μg/ml) antibodies, plate-bound anti-CD3 (10 μg/ml) antibody, or soluble anti-CD28 (1 μg/ml) antibody for 120 min. The association of Nedd4 or Itch with Cbl-b was then determined by immunoprecipitation in splenic T-lymphocytes. The results are shown in Figure 2.11. There was a constant association between Nedd4 and Cbl-b even when T-lymphocytes were not stimulated, but the extent of association were increased when T-lymphocytes were stimulated. Also, no association between Itch and Cbl-b was observed under either stimulatory condition or rest condition.

Subcellular localization of Nedd4 with different treatment

In Nedd4-mediated PLC-γ1 degradation, the subcellular localization of Nedd4 was changed [203] to be close to its substrate, suggesting that Nedd4 may use the same mechanism to regulate Cbl-b expression. The subcellular localization of Nedd4 and Cbl-b were tested via small scale sucrose density gradient centrifugation, which is frequently used for separating cell organelles from crude cellular extracts and determining the protein location within the cell. The results are shown in Figure 2.12. It showed that Cbl-b expressing location did not change with different stimulatory signals, most of the Cbl-b proteins were expressed at lane 2 and 3. Subcellular location of Nedd4 was more dispersed at resting condition that Nedd4 protein was distributed

from lane 2 to lane 6 (Figure 2.12a). After different stimulatory treatment, Nedd4 showed different subcellular movement pattern. When anti-CD28 antibody was present, Nedd4 expression showed translocation to lane 2 and 3, which was where Cbl-b protein was located in the T-lymphocytes. Compared to unstimulated T-lymphocytes, 18% more Nedd4 was translocated to lane 2 and 3 if anti-CD3 plus anti-CD28 antibodies were added (Figure 2.12b), and 40% more if only anti-CD28 antibody was added (Figure 2.12d). When T-lymphocytes were stimulated with anti-CD3 antibody only, similar translocation was not observed in Nedd4 protein (Figure 2.12c).

Relative cytoplasm expression of Nedd4 and Cbl-b

Since proteins contained in lane 3 in previous result include both cytoplasm and lipid raft portion (data not shown), the cytoplasm and lipid raft portion of T-lymphocytes were isolated respectively and Cbl-b expression determined. The result showed that in splenic T-lymphocytes Cbl-b can only be found in cytoplasm portion. Thus, the relative expression of Nedd4 vs. Cbl-b in cytoplasm portion was determined. The results are shown in Figure 2.13. Cbl-b showed highest expression when T-lymphocytes were stimulated by plate-bound anti-CD3 only, while under the same stimulation condition, Nedd4 expression was lowest. Cytoplasm expressions of Cbl-b and Nedd4 in T-lymphocytes with different treatment were reversely related.

Nedd4 expression in activated splenic T-lymphocytes

In order to know the change of Nedd4 expression in T-lymphocyte cytoplasm is caused solely by intracellular transportation or the protein or gene expression of Nedd4

change with stimulation too, the Nedd4 protein expression was first determined in T-lymphocytes that were stimulated by soluble anti-CD3 (10 μg/ml) plus anti-CD28 (1 μg/ml) antibodies, plate-bound anti-CD3 (10 μg/ml) antibody, or soluble anti-CD28 (1 μg/ml) antibody for 120 min respectively. As shown in Figure 2.14, Nedd4 protein was increased 60% when soluble anti-CD3 plus anti-CD28 signal were received, and had a dramatic increase of 134% when only soluble anti-CD28 antibody was added. Nedd4 protein showed a slightly but not significant increase (27.5%) when T-lymphocytes were stimulated with plate-bound anti-CD3 antibody only.

Nedd4 mRNA level in activated splenic T-lymphocytes

Change of Nedd4 protein expression can be caused by regulation on translational level or on transcriptional level as well. Thus, the mRNA level of Nedd4 was determined at the same time. The results are shown in Figure 2.15. T-lymphocytes were stimulated by soluble anti-CD3 (10 μg/ml) plus anti-CD28 (1 μg/ml) antibodies, plate-bound anti-CD3 (10 μg/ml) antibody, or soluble anti-CD28 (1 μg/ml) antibody for 120 min respectively. As shown in Figure 2.15, Nedd4 mRNA was increased about 20% when soluble anti-CD3 plus anti-CD28 signal were received, and had a larger increase of 35% when only soluble anti-CD28 antibody was added. Nedd4 mRNA showed no increase (4%) when T-lymphocytes were stimulated with plate-bound anti-CD3 antibody only.

2.5 DISCUSSION

Findings in this chapter demonstrate that rat splenic T-lymphocytes will down-regulate Cbl-b expression following perturbation of the CD28 receptor. The mechanism appears to involve Nedd4, an E3-ubiquitin ligase to degrade the Cbl-b protein in a proteasomal dependent manner.

To stimulate an effective T-lymphocyte response and subsequent physiological reaction, both the Ag-specific signal mediated by TCR and the co-stimulatory signal mediated by CD28 are required [227, 234]. T-lymphocyte proliferation experiments using cbl-b$^{-/-}$ mice found that CD28-costimulatory is no longer required, suggesting that there is a closely relationship between Cbl-b protein expression and CD28 signal which are essential for the full activation of T-lymphocytes [200, 201]. Soluble anti-CD3 plus anti-CD28 antibodies, plate-bound anti-CD3 alone, or soluble anti-CD28 alone were used respectively to stimulate T-lymphocyte. The first two stimulation methods generated similar T-lymphocyte proliferative responses while soluble anti-CD28 alone had no effect (data not shown). Utilizing plate-bound anti-CD3 alone and soluble anti-CD3 plus anti-CD28 culture systems allow for the distinguishing between CD3 and CD28 effects under activation conditions that induce similar proliferation. When young T-lymphocytes were stimulated with anti-CD3 plus anti-CD28 antibody, and not anti-CD3 alone, there was a significant reduction in Cbl-b expression. Interestingly, engagement of the CD28 receptor alone can reduce Cbl-b expression to a greater extent than stimulation with CD3 plus CD28. This data confirm that CD3 is not required to down-regulate Cbl-b protein expression. However, the data do suggest that CD3 signaling pathways may be involved in influencing the extent to which Cbl-b is down-regulated. Further experiments will need to be performed to clarify the interaction between the CD3 and CD28 receptors in regulating Cbl-b expression. Involvement of the Cbl family of

proteins appears to be somewhat specific since we did not see a significant effect on Cbl protein expression in stimulated T-lymphocyte. This is consistent with a previous report showing that Cbl recruitment to the TCR/CD3 complex does not change with age in activated T-lymphocytes [235]. Also, this supports the conclusion based on cbl-deficient mice and cbl-b-deficient mice that in T-lymphocyte regulation, Cbl is essential to the development of thymocyte [198], while Cbl-b is involved in the activation of peripheral T cell activation [200, 201]

Transcription of cbl-b gene is not altered by any of the stimulatory signals, suggesting that regulation of Cbl-b is post-transcriptional. This is different from what was observed in BCR/ABL controlled Cbl-b expression that cbl-b mRNA was down-regulated by stimulation of the B cell receptors on a B cell line [236]. This indicates that Cbl-b expression in splenic rat T-lymphocyte is regulated via a signaling pathway other than what happened in B-lymphocyte.

The tyrosine site at C-terminal of Cbl-b got phosphorylated upon TCR engagement and phosphorylation further activates Cbl-b's function to be involved in the intracellular signaling pathway mediated by TCR signal [188]. However, phosphorylation of Cbl-b can not determine Cbl-b's fate to be down-regulated or not. Since CD3 signal alone or CD28 signal alone induce similar phosphorylation on Cbl-b. It is not clear whether the phosphorylaiton induced by CD3 signal or CD28 signal are on different tyrosine site and further experiment are needed to investigate this possibility.

The mechanism by which CD28 down-regulates Cbl-b protein expression appears to be proteasome dependent in our system since addition of the proteasomal inhibitor

MG132 and lactacystin blocked Cbl-b down-regulation in young T-lymphocytes. Our results showing that reduced Cbl-b expression in young rat T-lymphocytes is in a CD28 dependent manner potentially involving proteasome degradation was recently confirmed by another group in CD28 deficient mice [191]. A recent report showed that CD28 ligation did not down-regulate Cbl-b protein expression [213]. This discrepancy may be due to the fact that the authors were using a human T-lymphocyte cell line (Jurkat) while we used primary rat T-lymphocytes. Further support for Cbl-b being down-regulated in a proteasomal dependent manner was shown in epidermal growth factor (EGF) stimulated breast cancer cells [221]. The authors showed that stimulating the EGF receptor with EGF resulted in the proteasomal dependent degradation of Cbl-b, the EGF receptor itself and several signaling proteins associated with the EGF receptor, while this paper also showed that Cbl-b expression may be controlled by both proteasome and lysosome since both proteasome inhibitor and lysosome inhibitor can partially block the down-regulation of Cbl-b [221]. However, our data showed that lysosome activity is not required in Cbl-b protein down-regulation because lysosome inhibitor NH_4Cl can not prevent the loss of Cbl-b protein expression under CD3 and CD28 activation.

So far, the detailed mechanism of how Cbl-b being down-regulated is still unclear. Since the mRNA level of Cbl-b does not change with activation, it is unlikely that Egr family proteins are involved in the direct regulation of Cbl-b expression. The Egr proteins are characterized as transcription regulatory factors. They recognize and bind to a 9-base-pair fragment on the promoter of target genes and induce the transcription of target genes [237]. Nedd4, but not Itch, is associated with Cbl-b in splenic rat T-lymphocytes. More Nedd4 are available for association since they are moving to the same subcellular location as Cbl-b when CD28 receptors on T-

lymophocytes are stimulated by anti-CD28 antibody. Change of Nedd4 location when T cells are under different stimulatory circumstance was also observed in mouse D5 T_H1 cells, Nedd4 moved from cytoplasm portion to detergent insoluble portion if cells are induced to anergy [203]. We also found that Nedd4 protein level was higher when CD28 were activated no matter whether CD3 activation signal was present or not. Thus, under anti-CD28 antibody treatment, there is a net increase of Nedd4 moving closer to Cbl-b, and the association between Nedd4 and Cbl-b became stronger when CD28 is activated. The increased association is achieved by both the enhanced expression and intracellular translocation of Nedd4 upon CD28 stimulation. As a result, at the cytoplasm portion (where almost all Cbl-b are expressed), the protein level of Nedd4 and Cbl-b are reversely related, that is, whenever more Nedd4 were detected, lower Cbl-b protein level were found at the same time. This phenomenon suggests that Nedd4 associated with Cbl-b plays its role as an E3 ubiquitin ligase, ubiquitinate Cbl-b and send them for proteasomal degradation. This is in accordance with the results showed in 293T human embryonic kidney cell line by Weissman's group that Nedd4 can regulate Cbl-b protein level via its E3 ubiquitin ligase activity [220]. Direct evidence of Nedd4 mediating the down-regulation of Cbl-b in primary rat splenic T-lymphocytes is unavailable now, since gripNA or siRNA procedure to knockout Nedd4 gene showed low transfection efficiency in our cell model.

It need to point out that the mRNA level of Nedd4 also changed with CD28 stimulation, which implies some other transcriptional regulation mechanisms are involved too. The full mechanism about how Nedd4 expression being regulated and how Nedd4 being translocated to Cbl-b are still under study now.

Figure 2.1 Cbl-b expression under CD3 and CD28 activation

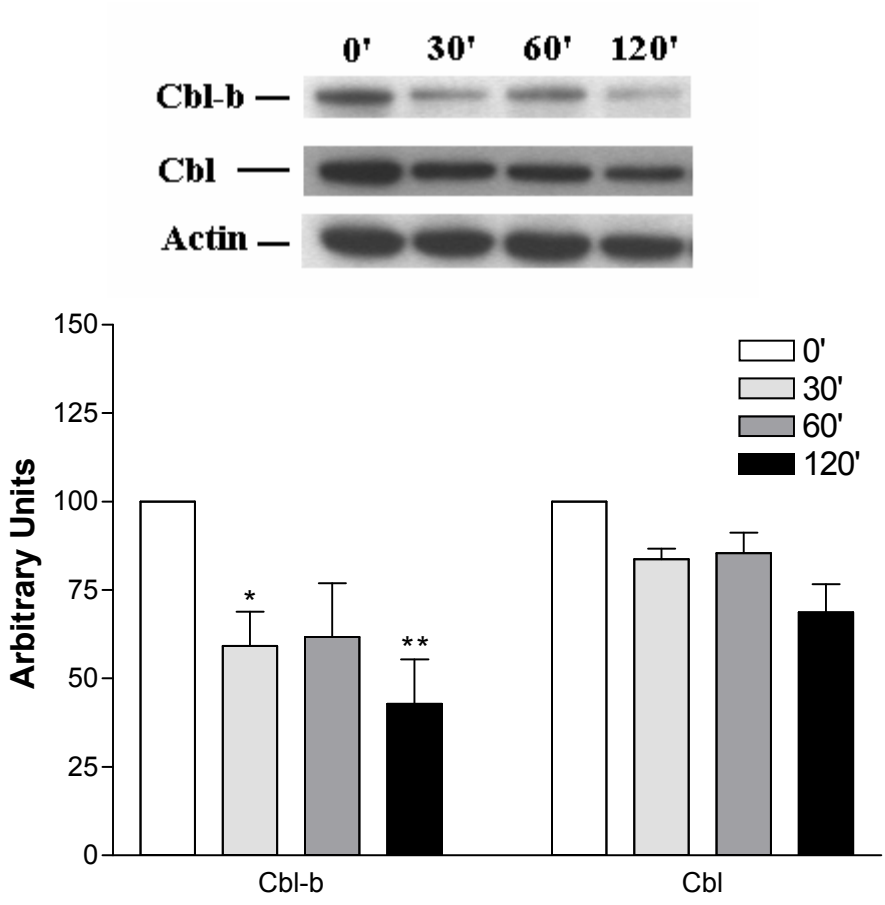

Figure 2.1 Influence of CD3 and CD28 activation on Cbl-b protein expression in young splenic T-lymphocyte. Splenic T-lymphocytes were isolated from young (6 mo) rats. T-lymphocytes were activated with soluble anti-CD3 plus anti-CD28 antibodies for 0, 30, 60, and 120 min. Total cellular proteins were extracted and western immunoblotting performed as described in the Materials and Methods. Values are the mean ± S.E.M. of four individual rats calculated from the densitometric analysis of the autoradiograms. An (*) indicates significantly different ($P<0.05$) and (**) indicates significantly different ($P<0.01$) when comparing stimulated with unstimulated T-lymphocytes.

Figure 2.2 Cbl-b expression under CD3 activation

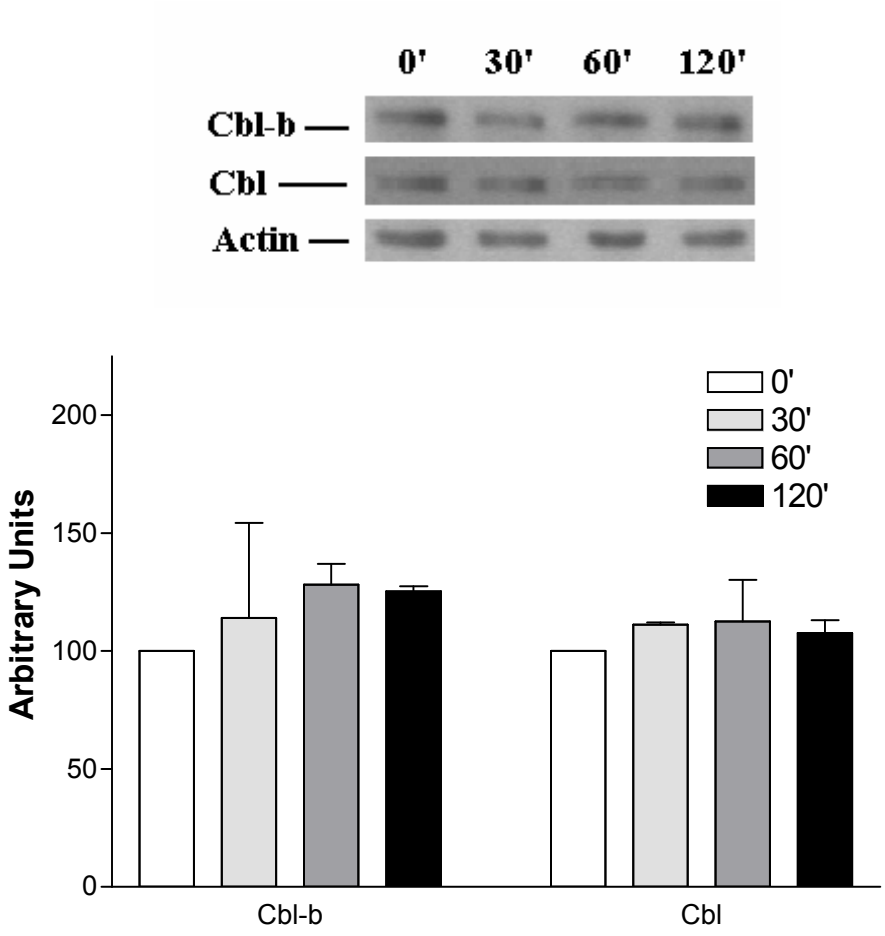

Figure 2.2 Influence of CD3 activation on Cbl-b protein expression in young splenic T-lymphocyte. Splenic T-lymphocytes were isolated from young (6 mo) rats. T-lymphocytes were activated with plate-bound anti-CD3 antibody for 0, 30, 60, and 120 min. Total cellular proteins were extracted and western immunoblotting performed as described in the Materials and Methods. Values are the mean ± S.E.M. of four individual rats calculated from the densitometric analysis of the autoradiograms.

Figure 2.3 Cbl-b expression under CD28 treatment

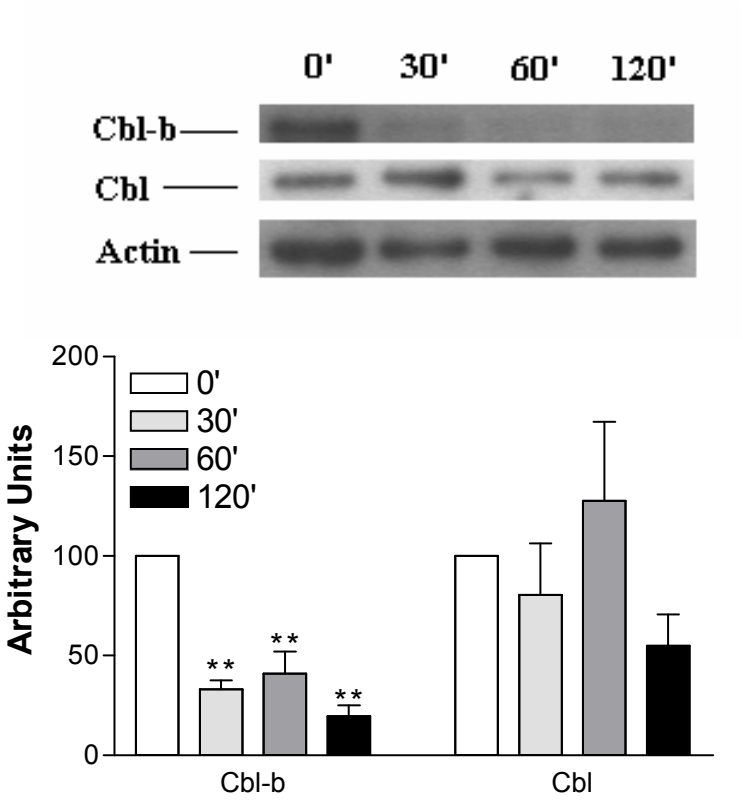

Figure 2.3 Influence of CD28 treatment on Cbl-b protein expression in young splenic T-lymphocyte. Splenic T-lymphocytes were isolated from young (6 mo) rats. T-lymphocytes were activated with anti-CD28 antibody for 0, 30, 60, and 120 min. Total cellular proteins were extracted and western immunoblotting performed as described in the Materials and Methods. Values are the mean ± S.E.M. of four individual rats calculated from the densitometric analysis of the autoradiograms. An (**) indicates significantly different ($P<0.01$) when comparing treated with untreated T-lymphocytes.

Figure 2.4 Cbl-b expression under CTLA-4 treatment

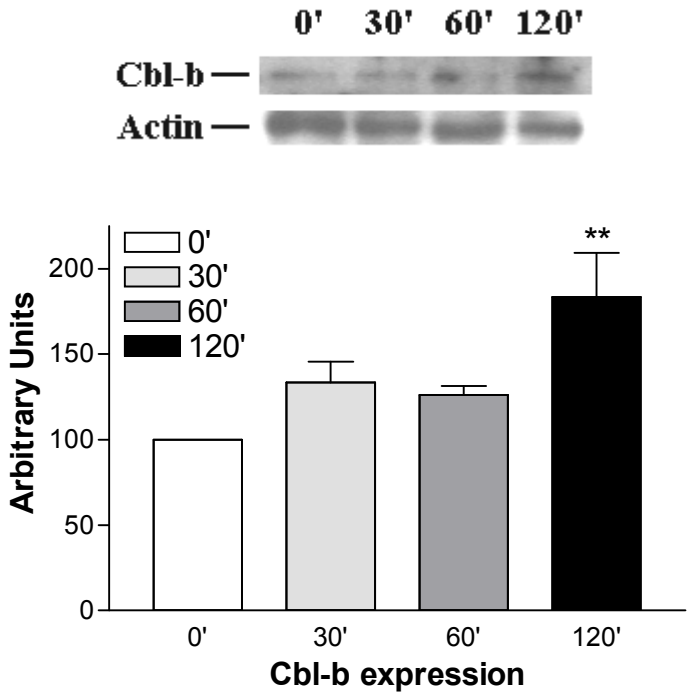

Figure 2.4 Influence of CTLA-4 treatment on Cbl-b protein expression in young splenic T-lymphocyte. Splenic T-lymphocytes were isolated from young (6 mo) rats. T-lymphocytes were activated with anti-CTLA-4 antibody for 0, 30, 60, and 120 min. Total cellular proteins were extracted and western immunoblotting performed as described in the Materials and Methods. Values are the mean ± S.E.M. of four individual rats calculated from the densitometric analysis of the autoradiograms. An (**) indicates significantly different ($P<0.01$) when comparing treated with untreated T-lymphocytes.

Figure 2.5 Cbl-b mRNA expression in activated splenic T-lymphocytes

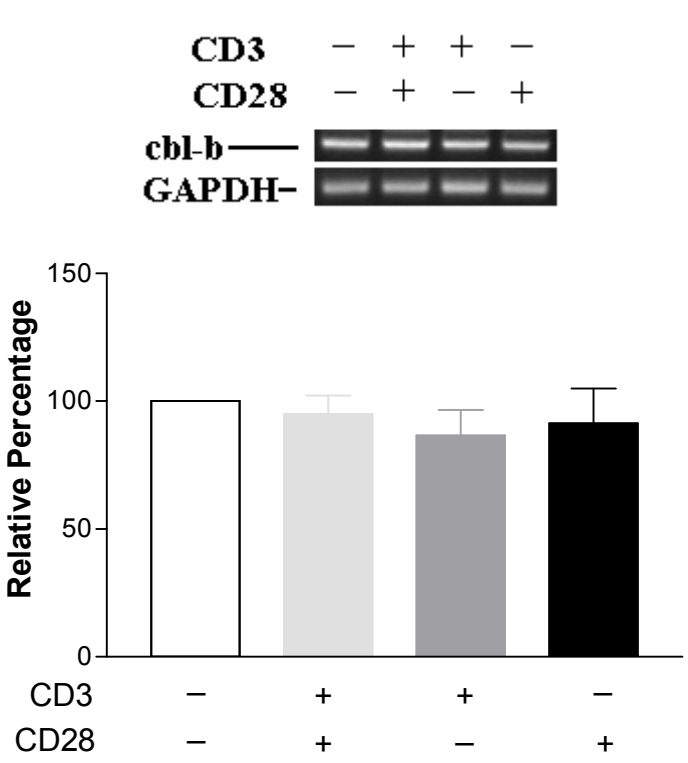

Figure 2.5 Influence of different stimulatory signals on Cbl-b mRNA expression in young splenic T-lymphocyte. Splenic T-lymphocytes were isolated from young (6 mo) rats. T-lymphocytes were activated by soluble anti-CD3 (10 μg/ml) plus anti-CD28 (1 μg/ml) antibodies, plate-bound anti-CD3 (10 μg/ml) antibody, or soluble anti-CD28 (1 μg/ml) antibody for 120 min. Total cellular RNA were extracted and RT-PCR performed as described in the Materials and Methods. Values are the mean ± S.E.M. of four individual rats calculated from the densitometric analysis of the autoradiograms.

Figure 2.6 Cbl-b expression under MG132 treatment

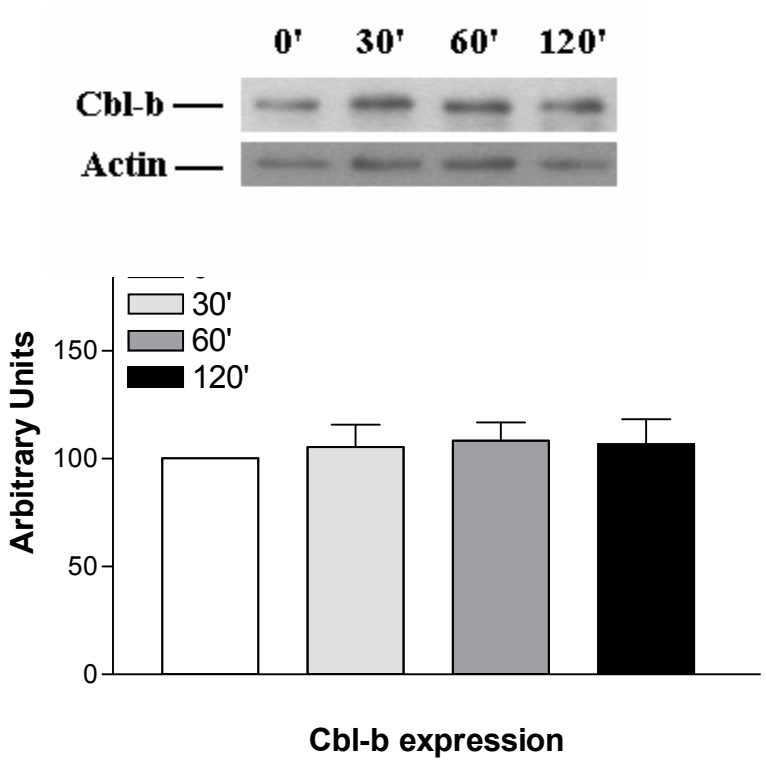

Figure 2.6 Impact of proteasomal inhibition on Cbl-b expression in young splenic T-lymphocytes. Young (6 mo) splenic T-lymphocytes were isolated and stimulated with soluble anti-CD3 plus anti-CD28 as described in Figure 2.1 legend. The proteasomal inhibitor MG132 was added simultaneously with the antibodies. T-lymphocytes were cultured for 0, 30, 60 and 120 min then total cellular proteins were extracted and western immunoblotting performed as described in the Materials and Methods. Values are the mean ± SEM of 4 individual rats calculated from the densitometric analysis of the autoradiograms.

Figure 2.7 Cbl-b expression under Lactacystin treatment

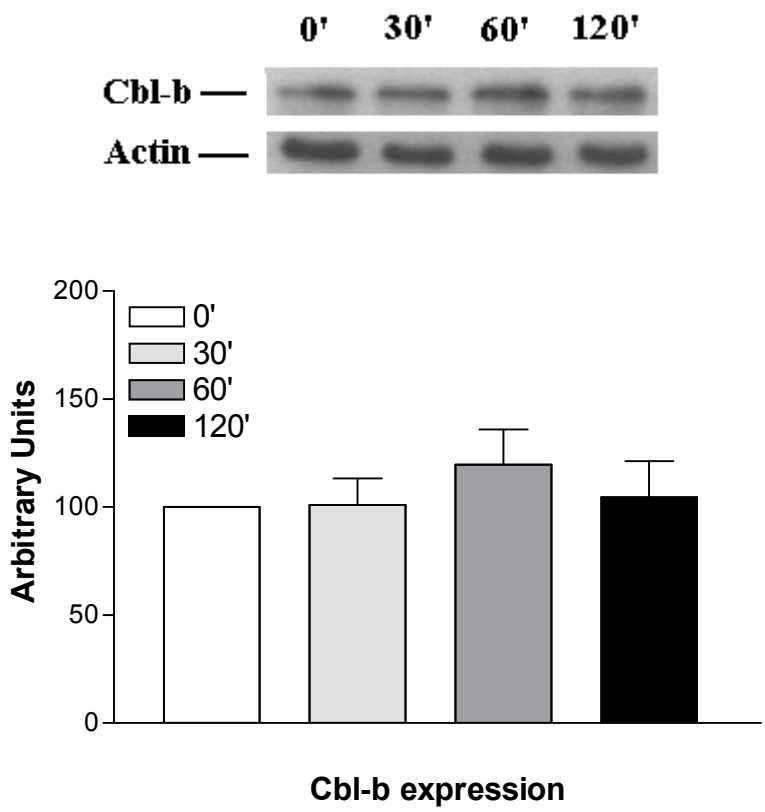

Figure 2.7 Impact of proteasomal inhibition on Cbl-b expression in young splenic T-lymphocytes. Young (6 mo) splenic T-lymphocytes were isolated and stimulated with soluble anti-CD3 plus anti-CD28 as described in Figure 2.1 legend. The proteasomal inhibitor Lactacystin was added simultaneously with the antibodies. T-lymphocytes were cultured for 0, 30, 60 and 120 min then total cellular proteins were extracted and western immunoblotting performed as described in the Materials and Methods. Values are the mean ± SEM of 4 individual rats calculated from the densitometric analysis of the autoradiograms.

Figure 2.8 Cbl-b expression under NH_4Cl treatment

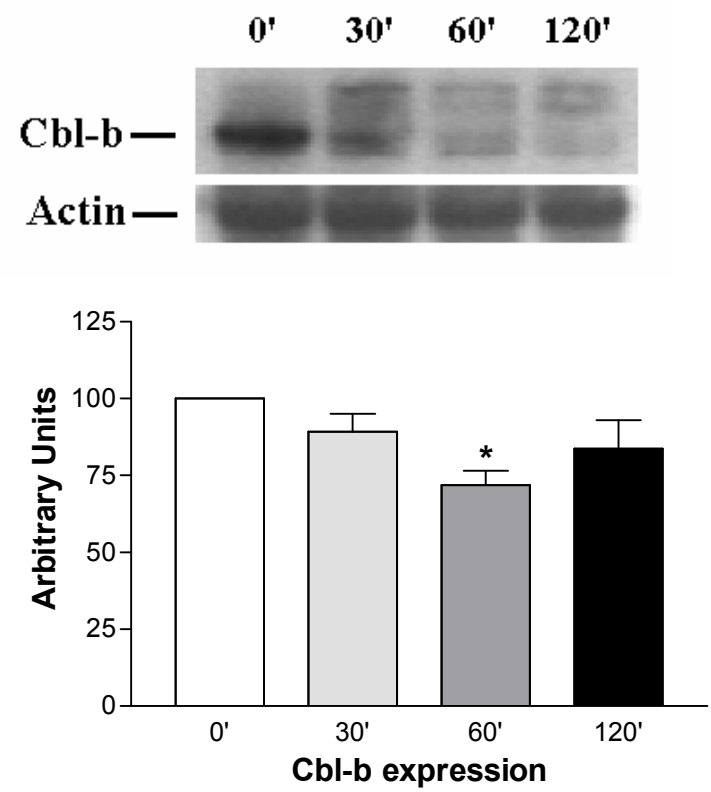

Figure 2.8 Impact of lysosome inhibition on Cbl-b expression in young splenic T-lymphocytes. Young (6 mo) splenic T-lymphocytes were isolated and stimulated with soluble anti-CD3 plus anti-CD28 as described in Figure 2.1 legend. The proteasomal inhibitor NH_4Cl was added simultaneously with the antibodies. T-lymphocytes were cultured for 0, 30, 60 and 120 min then total cellular proteins were extracted and western immunoblotting performed as described in the Materials and Methods. Values are the mean ± SEM of 4 individual rats calculated from the densitometric analysis of the autoradiograms. An (*) indicates significantly different ($P<0.05$) when comparing stimulated with unstimulated T-lymphocytes.

Figure 2.9 Proteasome activity in splenic T-lymphocytes

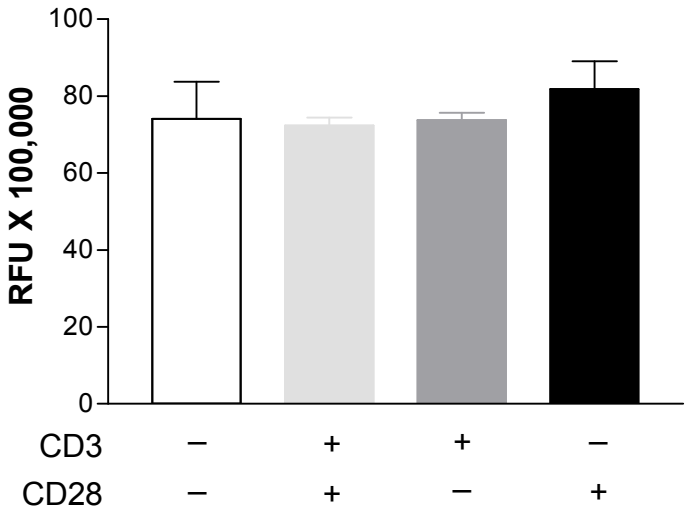

Figure 2.9 Influence of different stimulatory signals on proteasome activity in young splenic T-lymphocyte. Splenic T-lymphocytes were isolated from young (6 mo) rats. T-lymphocytes were stimulated by soluble anti-CD3 (10 μg/ml) plus anti-CD28 (1 μg/ml) antibodies, plate-bound anti-CD3 (10 μg/ml) antibody, or soluble anti-CD28 (1 μg/ml) antibody for 120 min. Total cellular proteins were extracted and proteasome activities determined as described in the Materials and Methods. Values are the mean ± S.E.M. of four individual rats calculated from the densitometric analysis of the autoradiograms.

Figure 2.10 Tyrosine phosphorylation of Cbl-b in splenic T-lymphocytes

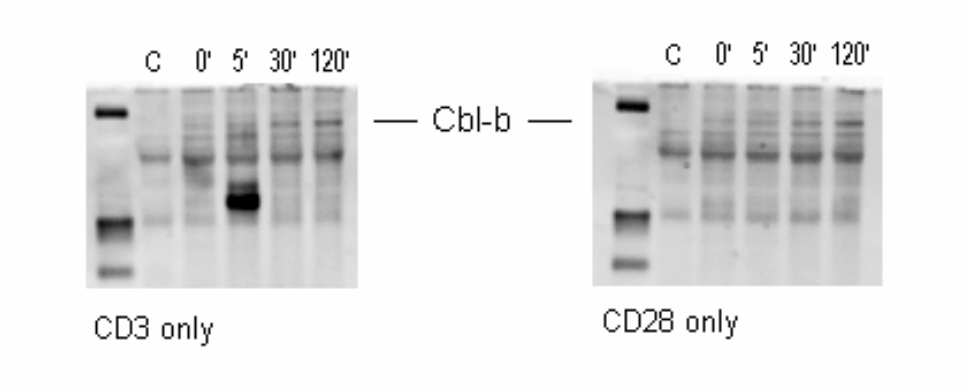

Figure 2.10 Tyrosine phosphorylation of Cbl-b in young splenic T-lymphocyte. Splenic T-lymphocytes were isolated from young (6 mo) rats. T-lymphocytes were stimulated by plate-bound anti-CD3 (10 μg/ml) antibody, or soluble anti-CD28 (1 μg/ml) antibody for 0, 5, 30, and 120 min. Total cellular proteins were extracted and immunoprecipitated by anti-Cbl-b antibody, tyrosine phosphorylation of Cbl-b was determined as described in the Materials and Methods.

Figure 2.11 Association of Nedd4/Itch with Cbl-b

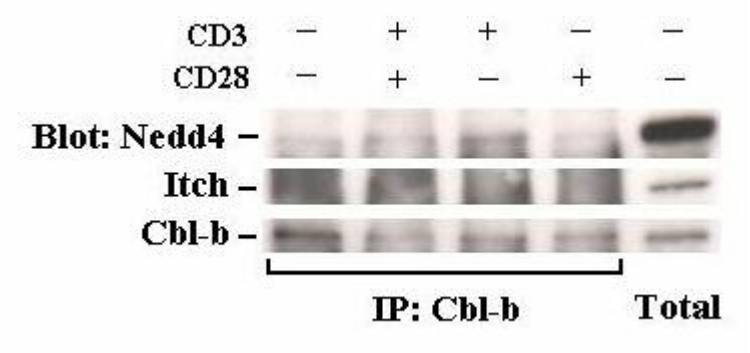

Figure 2.11 Association of Nedd4/Itch with Cbl-b in young splenic T-lymphocyte. Splenic T-lymphocytes were isolated from young (6 mo) rats. T-lymphocytes were stimulated by soluble anti-CD3 (10 μg/ml) plus anti-CD28 (1 μg/ml) antibodies, plate-bound anti-CD3 (10 μg/ml) antibody, or soluble anti-CD28 (1 μg/ml) antibody for 120 min. Total cellular proteins were extracted and immunoprecipitated with anti-Cbl-b antibody. Western immunoblotting was performed as described in the Materials and Methods.

Figure 2.12 Subcellular Protein Location in splenic T-lymphocytes

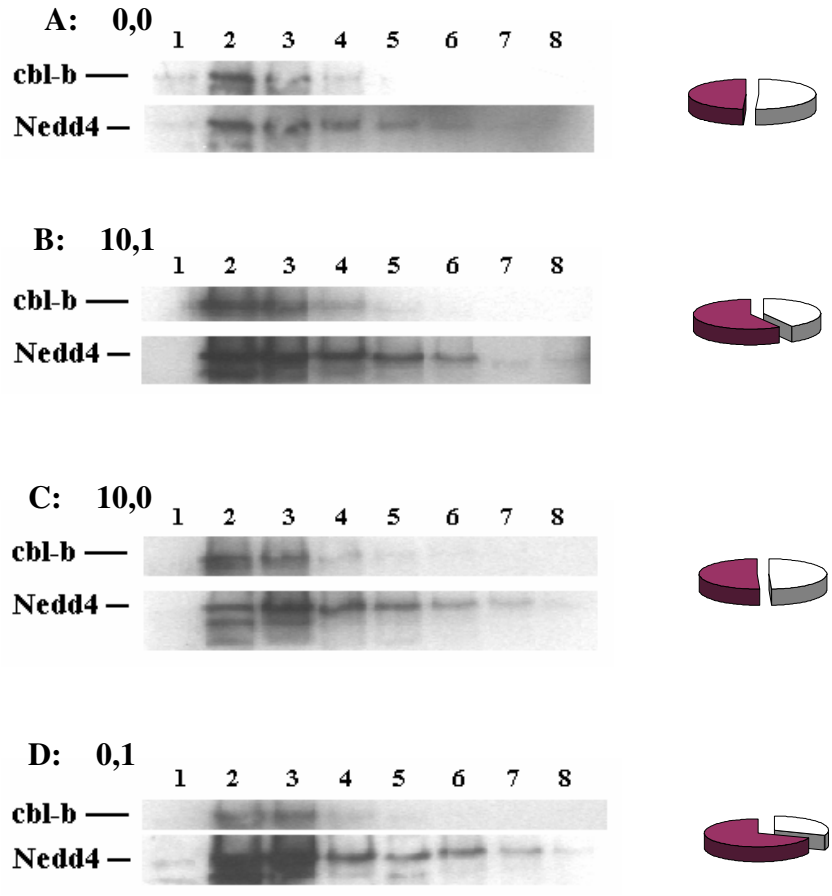

Figure 2.12 Subcellular localization of Nedd4 and Cbl-b in young splenic T-lymphocyte. Splenic T-lymphocytes were isolated from young (6 mo) rats. T-lymphocytes were unstimulated (A) or stimulated by soluble anti-CD3 (10 μg/ml) plus anti-CD28 (1 μg/ml) antibodies (B), plate-bound anti-CD3 (10 μg/ml) antibody (C), or soluble anti-CD28 (1 μg/ml) antibody (D) for 120 min. Total cellular proteins were extracted and small scale sucrose density gradient centrifugation followed by western immunoblotting (left) was performed as described in the Materials and Methods. Pie (right) values show Nedd4 expression in lane 2 and 3 (red) vs. lane 4 to 6 (white) that are calculated from the densitometric analysis of the autoradiograms.

Figure 2.13 Cbl-b and Nedd4 expression in cytoplasm

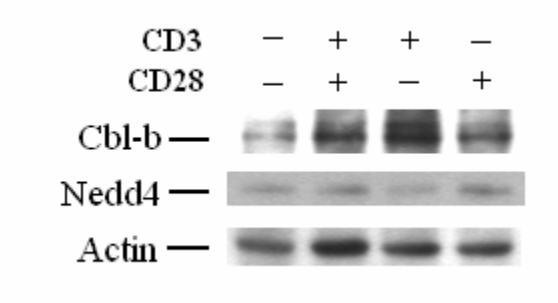

Figure 2.13 Cytoplasm expressions of Cbl-b and Nedd4 in young splenic T-lymphocyte. Splenic T-lymphocytes were isolated from young (6 mo) rats. T-lymphocytes were unstimulated or stimulated by soluble anti-CD3 (10 μg/ml) plus anti-CD28 (1 μg/ml) antibodies, plate-bound anti-CD3 (10 μg/ml) antibody, or soluble anti-CD28 (1 μg/ml) antibody for 120 min. Cytoplasm proteins were extracted and western immunoblotting was performed as described in the Materials and Methods.

Figure 2.14 Nedd4 expression under different stimulatory condition

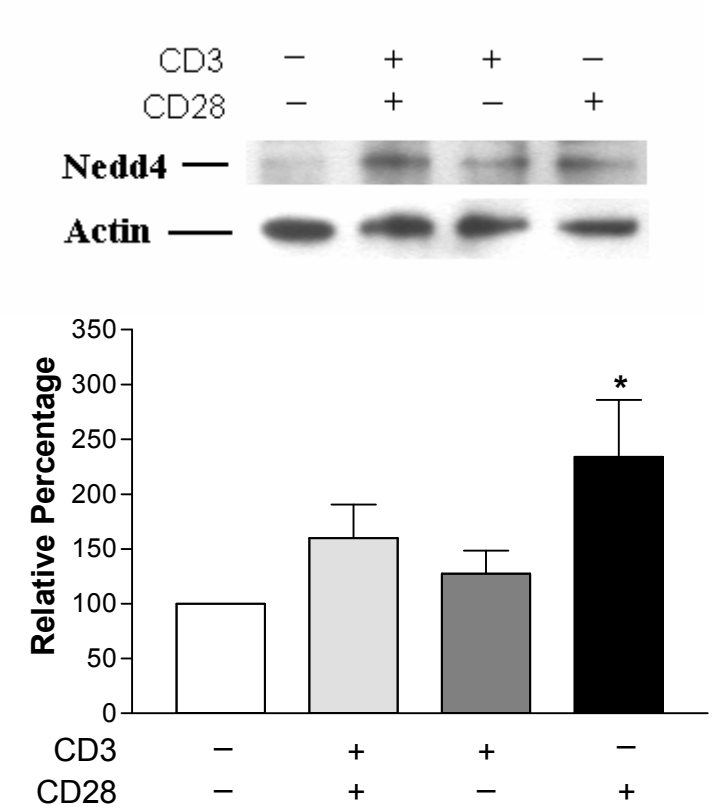

Figure 2.14 Nedd4 expressions in young splenic T-lymphocyte. Splenic T-lymphocytes were isolated from young (6 mo) rats. T-lymphocytes were unstimulated or stimulated by soluble anti-CD3 (10 μg/ml) plus anti-CD28 (1 μg/ml) antibodies, plate-bound anti-CD3 (10 μg/ml) antibody, or soluble anti-CD28 (1 μg/ml) antibody for 120 min. Total cellular proteins were extracted and western immunoblotting performed as described in the Materials and Methods. Values are the mean ± S.E.M. of four individual rats calculated from the densitometric analysis of the autoradiograms. An (*) indicates significantly different ($P<0.05$) when comparing stimulated with unstimulated T-lymphocytes.

Figure 2.15 Nedd4 mRNA level under different stimulatory condition

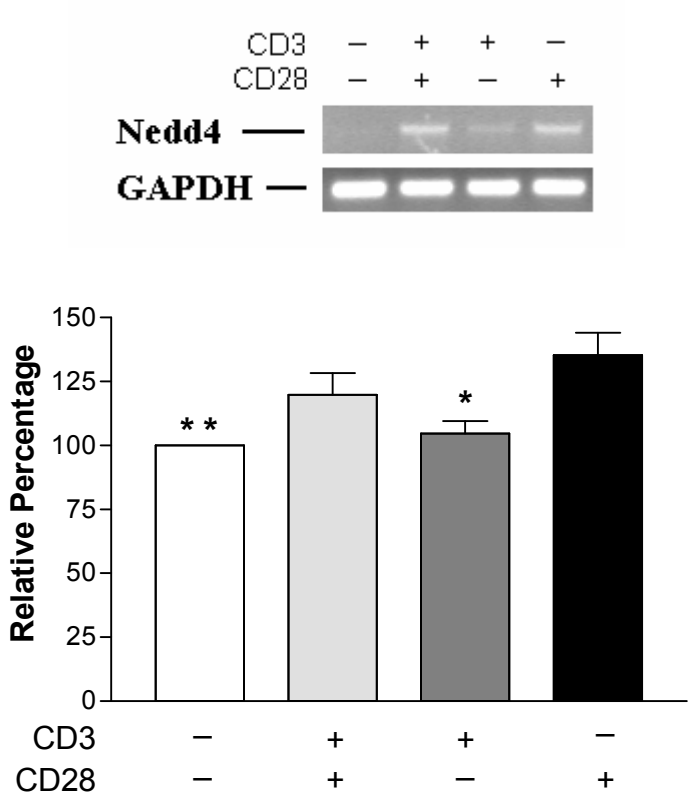

Figure 2.15 Nedd4 mRNA expressions in young splenic T-lymphocyte. Splenic T-lymphocytes were isolated from young (6 mo) rats. T-lymphocytes were unstimulated or stimulated by soluble anti-CD3 (10 μg/ml) plus anti-CD28 (1 μg/ml) antibodies, plate-bound anti-CD3 (10 μg/ml) antibody, or soluble anti-CD28 (1 μg/ml) antibody for 120 min. Total cellular RNA were extracted and RT-PCR performed as described in the Materials and Methods. Values are the mean ± S.E.M. of four individual rats calculated from the densitometric analysis of the autoradiograms. An (*) indicates significantly

different ($P<0.05$) and (**) indicates significantly different ($P<0.01$) when comparing with soluble anti-CD28 stimulated T-lymphocytes.

Chapter 3: Proteasome-dependent Cbl-b Down-regulation does not occur in Aged T-lymphocyte

3.1 ABSTRACT

We have showed that there is a proteasome-dependent down-regulation of Cbl-b with CD28 activation in young splenic T-lymphocytes. Since decreased ubiquitin-proteasome system function is found to be causally related to aging associated malfunction, we studied the actual expression and function of Cbl-b in aged T-lymphocytes. We found in aged T-lymphocyte the CD28-dependent Cbl-b down-regulation did not occur. We also showed that Cbl-b was still functional and its expression was strongly associated with reduced T-lymphocyte proliferation. Such age effect on Cbl-b regulation was not caused by reduced CD28 receptor expression, or increased CTLA-4 expression. Association of Cbl-b and Nedd4 seems to be normal too. Decline in proteasome activity are observed and may be responsible for the dysregulation of Cbl-b in aging. These data indicate Cbl-b level along with proteasome activity are important for T-lymphocyte activation in aging.

3.2 INTRODUCTION

Previous aging research has been focused on decreases in positive signals/regulators/effectors that result in less effective response of aged cells to stimuli. Normally those stimuli can induce optimal reaction in young cells. However, in aging

process, negative signals/regulators/effectors are as important as their positive peers. The up-regulation of negative signal would also lead to decreased cell activity.

CTLA-4 (cytotoxic T-lymphocyte antigen-4) is probably one of the most well-known T cell negative regulators. CTLA-4 is similar to CD28 in their extracellular structure. They all express a conserved amino acid motif, MYPPPY, on their extracellular domain [238]. So CD28 and CTLA-4 bind to the same ligands expressed on APCs--CD80 and CD86. However, the functional outcomes of binding with CD28 or CTLA-4 are opposite. Interaction of CD80/CD86 with CD28 provides costimulatory signal to boost T cell response, while engagement of CTLA-4 with CD80/CD86 will inhibit the T cell activation and proliferation by competing with CD28 for ligands and "turning off" the ongoing cell division [239]. A functional balance between CD28 and CTLA-4 is reached and cell proliferation is tightly regulated. The percentage and relative intensity of both CD28 and CTLA-4 change with age. The difference is CD28 expression decreases while CTLA-4 level increases. Since affinity of CTLA-4-CD80/CD86 ligation is 20-100-fold higher than CD28 ligation [240], up-regulation of CTLA-4 with aging has more profound suppressive impact on T cell function. Thus, T cell senescence is the result of both diminished positive signal and elevated negative signal.

Up-regulation of negative signal in aging that leads to functional loss in immune system is not limited to T cells. In aged mouse macrophage, Toll-like receptor-signaling pathway is down-regulated because the negative regulator of this pathway, IL-1 receptor-associated kinase 3 is increased with age. Directly, such change will cause the dysregulation in cytokine secretion and indirectly, lead to poor production of antibodies

[241]. In old activated B cells, immunoglobulin class switch recombination (CSR) is impaired due to the increase of a negative regulator, Id2. Id2 negatively regulates transcription factor E47, and E47 is required for CSR. Thus, the antibody class diversity in old activated B cells is decreased and the effector function of antibodies to fight the same antigen dramatically reduced [112]. Increased negative signal is also involved in the age-associated thymus involution. TCR β-rearrangement requires the expression of E2A, a transcription regulator. E2A activity is negatively regulated by LMO2, whose expression increases more than 5 times with age [242].

As a negative regulator to early T cell activation events, whether Cbl-b's expression would also be up-regulated with age and whether increased Cbl-b would be able to more effectively inhibit aged T cell signaling pathways are still unknown. Cbl is a close family member of Cbl-b and shares a lot of similarity with Cbl-b in both structure and function. In old mice, P-glycoprotein^{high} CD4 memory cells show low proliferative activity, meanwhile, the proportion of Cbl translocated to immune synapse increased with age, which suggests that Cbl in immune synapse is responsible for the anergy of P-gp^{high} CD4 memory cell subset [33].

Based on these observations, in this chapter, we will test whether Cbl-b's expression will change with age, and if such change is correlated with T cell activity change such as proliferation. Also, the mechanism of how this would happen will also be studied in this chapter.

3.3 MATERIALS AND METHODS

Materials

Anti-Cbl-b antibody (G-1), anti-Cbl antibody (C-15), and anti-β-actin (I-19) were purchased from Santa Cruz (Santa Cruz, CA). Anti-CD3 (G4.18) and anti-CD28 (JJ319) monoclonal antibodies, anti-CD4 (OX-35) FITC labeled and anti-CD8 (OX-8) PE labeled antibodies were purchased from BD PharMingen (San Jose, CA). All cell culture media were purchased from Mediatech (Herndon, VA) and all other chemicals were reagent grade or higher and from Sigma-Aldrich (St. Louis, MO).

Animals

Male, 4-6-month (young) and 18-20-month (old) Sprague-Dawley rats were purchased from Harlan Sprague Dawley (Indianapolis, IN) and maintained on a 12 hour light/dark cycle and standard chow diet. All animal procedures were approved by the University of Texas Animal Use and Care Committee.

T-lymphocyte isolation and culture

Rats were sacrificed and spleens aseptically removed. Single splenic cell suspensions were isolated by homogenization and filtering through a 25mm syringe filter. Lymphocytes were isolated via differential migration following centrifugation in Lymphocyte Separation Medium (Mediatech, Herndon, VA). T-lymphocytes were isolated using negative selection Immulan columns (Biotecx, Houston, TX) per manufacturer's instructions as previously described [230]. T-lymphocytes were counted using the Cell-Dyn 900 Hematology Analyzer (Sequoia-Turner, Mountainview, CA).

T-lymphocytes, $2.5x10^7$ cells per petri dish, were stimulated at 37ºC for designated time in pre-warmed complete culture media (RPMI 1640 supplemented with 10% heat inactivated fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 100 mM 2-mercaptoethanol, 2 mM L-glutamine and 25 mM HEPES buffer) with 10 μg/ml anti-CD3 plus 1 μg/ml anti-CD28 antibodies, 10 μg/ml plate-bound anti-CD3, 1 μg/ml anti-CD28 antibody only, or 5 μg/ml anti-CTLA-4 antibody.

T-lymphocyte proliferation

T-lymphocyte proliferation was determined by MTT cell proliferation assay (ATCC, Manassas, VA). Briefly, $2x10^5$ splenic T-lymphocytes ($2x10^6$ cells/ml) were plated in each well and cultured at 37ºC with complete media in the presence or absence of 10 μg/ml anti-CD3 and 1 μg/ml anti-CD28 for 48 hr. 10 μl MTT reagent was added and continued incubating for 4 hr at 37ºC until purple precipitates were visible. After that, 100 μl detergent reagent was added, plates were then left at room temperature in the dark for 2 hr and absorbance was recorded at 550nm. T-lymphocytes for each treatment were plated out in triplicate and average was used for data calculation.

Western immunoblotting

Cellular protein was isolated and western immunoblotting performed as previously described [231]. Briefly, total protein from stimulated or unstimulated T-lymphocytes were pelleted and lysed in 50 μl of lysis buffer containing 50 mM Tris (pH 7.4), 10 mM EDTA, 150 mM NaCl, 0.1% Tween 20, 1 μl/ml β-ME, and 7 μl/ml protease inhibitor mix. Lyastes were centrifuged and the protein containing supernatant was

quantitated using the Bio-Rad protein assay (Bio-Rad, Hercules, CA). 30 μg of total protein was separated by SDS-PAGE along with Bio-Rad's Kaleidoscope Prestained Standards. The protein was transferred onto PolyScreen PVDF membranes (NEN Life Sciences, Boston, MA) and the membranes blocked with 15 ml blocking solution containing 25 mM Tris (pH 8.0), 125 mM NaCl, 4% non-fat milk, and 0.1% Tween 20 prior to probing with anti-Cbl-b antibody (1:3000 dilution in 15 ml blocking solution), anti-Nedd4 or anti-PI3K p85 antibody. Immunoreactive bands were detected using an alkaline phosphatase-conjugated goat-anti-rabbit secondary antibody and CDP-Star chemiluminescence reagent (NEN Life Sciences, Boston, MA). Densitometry was performed using a BioRad Gel Documentation System. The membrane was also probed with an anti-β-actin antibody to control for lane loading variation.

Flow cytometric analysis

Expression of the CD28 or CTLA-4 receptor on $CD4^+$ and $CD8^+$ T-lymphocytes was determined as we previously described [243, 244]. Splenic T-lymphocytes were stimulated as described before. 1 x 10^6 T-lymphocytes were treated with 200 μl 1X RBC Lysis buffer (eBioscience, San Diego, CA) for 2 minutes to lyse any remaining red blood cells that might interfere with analysis. Lysis was ceased with the addition of 5ml PBS and centrifugation at 250 g for 5 min. Cell pellets were incubated with PE labeled anti-CD28 or anti-CTLA-4 antibody plus FITC labeled anti-CD3, anti-CD4, or anti-CD8 antibody. Excess antibodies were removed by washing with PBS with 0.1% NaN_3 and cells were fixed in 0.5% paraformaldehyde in PBS. CD28 or CTLA-4 receptor expression on T-lymphocyte subsets was analyzed using a BD FACScaliber flow cytometer and CellQuest Pro software (BD Pharmingen, San Diego, CA).

Immunoprecipitation of Cbl-b

Stimulated or unstimulated T-lymphocytes from 4 rats were pooled together. Pooled T-lymphocytes were pelleted and total protein lysed in 500 μl of lysis buffer containing 50 mM Tris (pH 7.4), 10 mM EDTA, 150 mM NaCl, 0.1% Tween 20, 1 μl/ml β-ME, and 7 μl/ml protease inhibitor mix. Lyastes were centrifuged and the protein containing supernatant were pre-cleared by adding 40 μl of protein A/G plus agarose (Upstate, Charlottesville, VA) and rotated at 4ºC for 1 hr. The mixture was pelleted at 2,000 *g* for 2 min at 4ºC and supernatant was quantitated using the Bio-Rad protein assay (Bio-Rad, Hercules, CA). 500 μg of total protein were incubated with 4 μg anti-Cbl-b antibody (H-454) and rotated overnight at 4ºC. Protein A/G plus agarose (20 μl) was added and the mixture was rotated again at 4ºC for 2 hr. The mixture was pelleted at 2,000 *g* for 2 min at 4ºC and the beads washed 3 times with 500 μl lysis buffer. The bead slurry was suspended in 2X Laemmli sample buffer, boiled for 10 min and the supernatant (immunoprecipitated protein) was collected.

20S proteasome activity assay

Proteasome activity is determined by CHEMICON 20S Proteasome Activity Assay Kit (Chemicon, Temecula, CA) via manufacturer's instruction. Briefly, total T cell protein is isolated as described before and protein concentration determined. 1 mg of total protein and 5 nM proteasome substrate (Suc-LLVY-AMC) are incubated in 25 mM HEPES (pH 7.5), 0.5 mM EDTA, 0.05% NP-40, and 0.001%SDS in 96-well fluorometer plate (100 μl total volume) for 1 hr at 37ºC. In parallel, AMC standard

curve from 0.04 mM to 12.5 mM and proteasome positive control curve (1:4 to 1:256) are prepared. Fluorescences are recorded in DTX 880 Multimode Detector (Beckman, Fullerton, CA) with 380/460 nm filter.

Statistical analysis

Data from young and old T-lymphocytes stimulation experiments were analyzed by two-way ANOVA, the significance of the interaction between the age factor and stimulation factor was determined with Minitab software (State College, PA). Other data were analyzed by one-way ANOVA and statistical difference were determined using Tukey's multiple comparison test with GraphPad Prism software (San Diego, CA). A $p<0.05$ was considered significantly different.

3.4 RESULTS

Influence of aging on Cbl-b expression in activated T-lymphocytes

The data in Chapter 2 shows that anti-CD28 stimulation is required to observe a significant reduction in Cbl-b expression in young T-lymphocytes. Therefore, young and old splenic T-lymphocytes were stimulated with soluble anti-CD3 plus anti-CD28 antibodies for 120 min and Cbl-b expression measured (Fig 3.1). Activation significantly ($P<0.01$) reduced Cbl-b expression by approximately 40% in the young T-lymphocytes, but did not change Cbl-b expression significantly in aged splenic T-lymphocytes.

T-lymphocyte proliferation in young and old rat

To confirm that the anti-CD3 plus anti-CD28 stimulation used herein induced T-lymphocyte proliferation and the effective T-lymphocyte proliferation is parallel with reduced Cbl-b expression, the proliferative response in young and old splenic T-lymphocytes was determined. Fig 3.2 shows that anti-CD3 plus anti-CD28 stimulation increased T-lymphocyte proliferation 4.6 times in young splenic T-lymphocytes and 3.5 times in old ones and aging resulted in approximately a 35% reduction in the proliferative response.

Influence of aging on Cbl-b function in stimulated T-lymphocytes

As shown in Fig 3.1 and Fig 3.2, the Cbl-b expression remains unchanged upon anti-CD3 plus anti-CD28 stimulation in old T-lymphocytes and old T-lymphocytes fail to proliferate properly as young T-lymphocytes. One of Cbl-b's functions is to set the activation threshold of T-lymphocytes, suggesting that low proliferation in old T-lymphocytes is probably caused by sustained Cbl-b expression after stimulation. To prove that, function of Cbl-b in stimulated old T-lymphocytes is first determined. Binding of Cbl-b protein with its target, PI3K and the ubiquitination of PI3K (data not shown) are tested. Fig 3.3 shows that in old T-lymphocytes, Cbl-b is still able to bind with p85 subunit of PI3K when stimulated by anti-CD3 and anti-CD28 antibodies.

Influence of aging on Nedd4-Cbl-b association in stimulated T-lymphocytes

In old T-lymphocytes, why stimulation from both CD3 and CD28 fail to down-regulate Cbl-b is still unclear. According to the mechanisms of Cbl-b being regulated in young T-lymphocytes upon stimulation, we are trying to find out the reason causing the dys-regulation of Cbl-b with age.

In transfected 293T cell line, as a HECT E3 ubiquitin ligase, Nedd4 degrades Cbl-b expression via the WW domain [220]. In previous research, we also proved that expression of Nedd4 and its association with Cbl-b was strongly related with the expression change of Cbl-b upon CD28 stimulation, indicating that Nedd4 is responsible for the down-regulation of Cbl-b. Thus, continuous association of Nedd4 with Cbl-b in old rat T-lymphocytes was tested. As shown in Fig 3.4, association of Nedd4 with Cbl-b does not change with age and the extent of such association under CD3 and CD28 activation is comparable in young and old T-lymphocytes with a minor increase with age.

CD28 and CTLA-4 receptor expression in young and old rat

In young rat splenic T-lymphocytes, signals from CD28 or CTLA-4 receptors are required to regulate Cbl-b expression. Meanwhile, short of CD28 costimulatory receptor expression are found in both $CD4^+$ and $CD8^+$ T cell subset with aging [68, 245]. Thus, CD28 levels in $CD3^+$, $CD4^+$, and $CD8^+$ T-lymphocytes were examined. Fig 3.5 shows that CD28 receptor expression was not significantly influenced by age in T-lymphocytes.

Since up-regulation of CTLA-4 expression was also reported in aged human $CD4^+$ T cells [246], the expression of CTLA-4 in $CD4^+$, and $CD8^+$ T-lymphocytes before and

after CD3 plus CD28 stimulation were examined. The results are shown in Fig 3.6. There was no obvious effect of age on CTLA-4 induction in either $CD4^+$ or $CD8^+$ T-lymphocytes.

Proteasome activity in young and old rat

It has been proven that in young splenic T-lymphocytes, Cbl-b is down-regulated in proteasome. However, the aging process is associated with the proteasome inactivation which may in turn weaken the ability of aged T-lymphcytes to down-regulate Cbl-b. Proteasome activities in T-lymphocytes isolated from young and old rats were determined. The results are shown in Figure 3.7. The 20S proteasome activity in old T-lymphocytes is much lower than that in young ones, only 45% of proteasome activity is detected in old T-lymphocytes compared to in the young T-lymphocytes.

Ubiquitination of Cbl-b in young and old rat

In order to make sure the decreased proteasome activity observed in old rat T-lymphocytes is caused by malfunction of proteasome itself or is the result of other failures in ubiquitin system upstream of proteasome, the ubiquitination of Cbl-b in young and old T-lymphocytes before and after stimulation are tested by immunoprecipitation. The results are shown in Figure 3.8. In young rat splenic T-lymphocytes, the relative ubiquitination level of Cbl-b protein slightly decreased after CD3 and CD28 stimulation, while in old T-lymphocytes, ubiquitination of Cbl-b increased upon activation indicating ubiquitinated Cbl-b accumulated in the old T-lymphocyte.

3.5 DISCUSSION

It is well known that aging results in reduced T-lymphocyte proliferation *ex vivo* [247]. To date, attention has focused on understanding the impact of aging on receptor-mediated signal transduction pathways leading to up-regulation of the proliferative response [65, 248]. The data presented here, for the first time, show that aged splenic T-lymphocytes fail to down-regulate Cbl-b expression following perturbation of the CD28 receptor. The mechanism appears to involve the low activity of proteasome in aged T-lymphocytes to degrade the Cbl-b protein in a proteasomal dependent manner.

Soluble anti-CD3 plus anti-CD28 antibody can not effectively induce T-lymphocyte proliferation isolated from old rat spleen. Meanwhile, the same soluble anti-CD3 plus anti-CD28 stimulation condition in old T-lymphocytes fail to reduce Cbl-b expression as what happened in young T-lymphocyte. Related to the fact that T cells with no Cbl-b expression can proliferate much better that T cells with normal Cbl-b expression [200, 201]. Results in this chapter show that, in young peripheral T-lymphocytes, stimulating signals received by T-lymphocytes lead to cell proliferation and IL-2 secretion after Cbl-b protein is first down-regulated, thus, higher Cbl-b expression sets a higher threshold for the activation and proliferation of aged T-lymphocytes. Functional studies of Cbl-b showed that in aged T-lymphocyte, after CD3 and CD28 stimulation, the remaining Cbl-b kept its ability to bind with the p85 subunit of PI3K. No established experiments are available to directly test the function of Cbl-b in stimulated T-lymphocytes. One of the consequences of p85 being mono-ubiquitinated by Cbl-b is PI3K unable to recruit to CD28 receptor [213] and thus its p110 subunit can not be fully activated. Since PI3K activation is important to T cell proliferation and

function [249], data show here strongly supports the idea that Cbl-b is still functional in old T-lymphocyte and sets a higher activation threshold for aged old T-lymphocytes.

The mechanism does not appear to involve the age-dependent changes in the relative proportions of CD28 receptor or CTLA-4 expression in T-lymphocytes. However, this does not rule out the possibility that altered CD28/CTLA-4 expression may take place in other immune organs and/or in other animal models [66]. Age-related deficiency of CD28 expression in T cells and its influence on T cell activity have been studied. However, the results are inconsistent. In human peripheral blood T cells, decline of CD28 expression are found to be more dramatically in $CD8^+$ T cells [67]. The appearance and percentage of $CD8^+CD28^{null}$ T cells in aged individuals are reversely correlated to antibody production in response to vaccine [250, 251], length of telomeres [252], and are considered as a valuable bio-marker of immunocompromision and replicative senescence in human aging. In contrast to that, $CD4^+CD28^{null}$ T cells accumulated in older individuals are functional and long-lived, and probably account for the generation of autoreactive B cells [253, 254]. Changes of CD28 expression with age in mice are different. In C57BL/6 mice, CD28 does not decline in their whole lifespan. There are no age difference in CD28 expression on resting T cells and a significant slower CD28 induction in old stimulated T cells [255]. In DBA/2 mice, percentage of CD28 positive in splenic T cells are slightly increased with age and the increas is statistically significant in $CD8^+$ subset, but the CD28 expression do not change in either subsets in blood peripheral T cells [256]. A unique $CD8^+CD28^{high}$ subset are found in old mice with immune regulation function [257]. All of these data suggest that co-stimulatory function of CD28 signal system in T cell senescence of human are different from its role in rodents T cells aging. In our studies, we showed CD28 signal could

down-regulate Cbl-b expression and enhance T cell activity, but failed to do so in aging. We also showed that CD28 expression in rat splenic T cells did not change with age and was not responsible for the incompetence to stimulate Cbl-b protein degradation. This observation excludes the possibility that human-mouse difference in age-related decline in CD28 expression will exert distinctive regulation mechanism on Cbl-b and indicates human and rodent may share the same regulatory pathway to control Cbl-b expression in aging. Thus, aged rat and mouse will be good models to further study the dysregulation of Cbl-b and immune senescence caused by high Cbl-b level.

The association between Cbl-b and Nedd4, the possible regulator of Cbl-b, remained the same in old activated T-lymphocytes, indicating the CD28-signal-induced intracellular movement of Nedd4 and its association with Cbl-b is intact with age.

It seems that it is the proteasome activity that determines the extent and rate of Cbl-b's down-regulation in old T-lymphocytes. In accordance with the low proteasome activity in old T-lymphocyte, the total ubiquitinated protein is accumulated in aged T-lymphocytes (data not shown) which is also true if only examining the ubiquitination level of Cbl-b protein. This also means age does not have any dramatic influence on the ubiquitination process in T-lymphocytes and indirectly proves that the function of Nedd4 to add ubiquitin tag on Cbl-b for proteasome degradation is unaffected by age too.

Declined proteasome activity with aging have been reported previously [154] with various reasons, including reduced expression of proteasome subunits [258, 259], post-translational modification of proteasome subunits [260], and accumulation of inhibitory damaged proteins such as oxidized [158], HNE-modified [261], or ubiquitinated [262]

proteins. Whether all or part of the factors listed above and which factors lead to dysregulation of proteasome activity in this particular case is still unclear. Thus, detailed experiments on the proteasome system in old rat splenic T-lymphocytes are required to find out the ultimate reason that cause the accumulation of Cbl-b with age and lead to low T-lymphocyte proliferation.

Figure 3.1 Cbl-b expression in young and old splenic T-lymphocytes

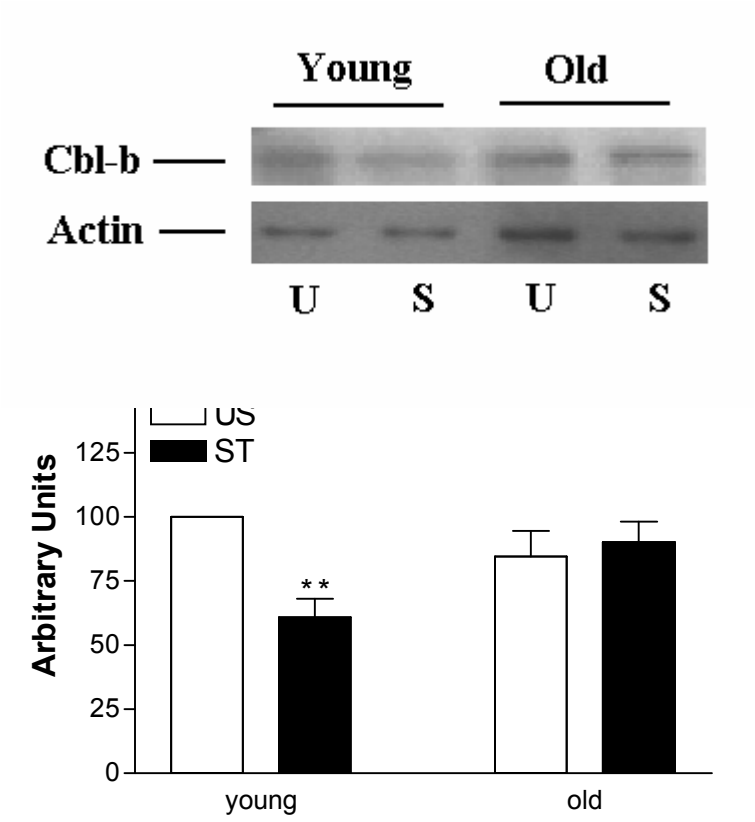

Figure 3.1 Influence of age on Cbl-b protein expression in activated T-lymphocytes. Splenic T-lymphocytes were isolated from young (6 mo) and old (18 mo) rats as described in the Materials and Methods. T-lymphocytes were isolated and stimulated with soluble anti-CD3 plus anti-CD28 for 120 min as described in the Materials and Methods. Total cellular protein extraction and western immunoblotting were performed as described in the Materials and Methods. Values are the mean ± SEM of 9 individual rats calculated from the densitometric analysis of the autoradiograms. An '**' indicates significantly different ($p<0.01$) when the interaction between age and stimulation factors is determined.

Figure 3.2 T cell proliferation in young and old rats

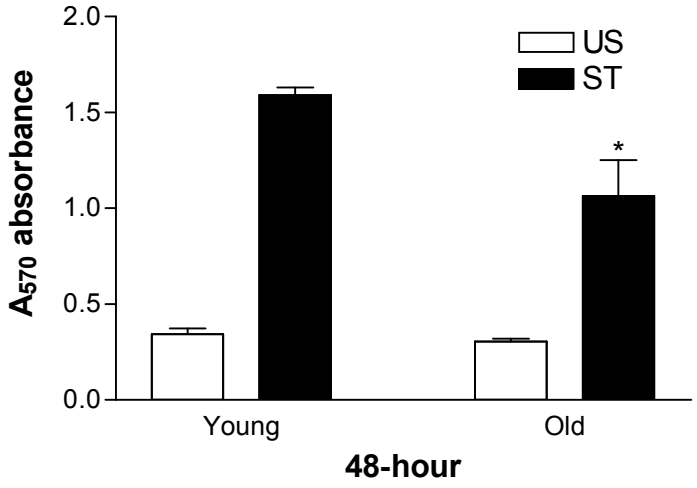

Figure 3.2 Influence of aging on splenic T-lymphocyte proliferation. Splenic T-lymphocytes were isolated from young (6 mo) and old (18 mo) rats as described in the Materials and Methods then activated with soluble anti-CD3 and anti–CD28 antibodies for 48 hours and proliferation determined using the MTT dye uptake assay as described in the Materials and Methods. Data are expressed as the absorbance at 570 nm. Values are the mean ± SEM of 3 individual rats. An '*' indicates significantly different ($p<0.05$) when comparing the young and old anti-CD3 plus anti-CD28 stimulated group.

Figure 3.3 Ubiquitination of PI3K by Cbl-b in splenic T-lymphocytes

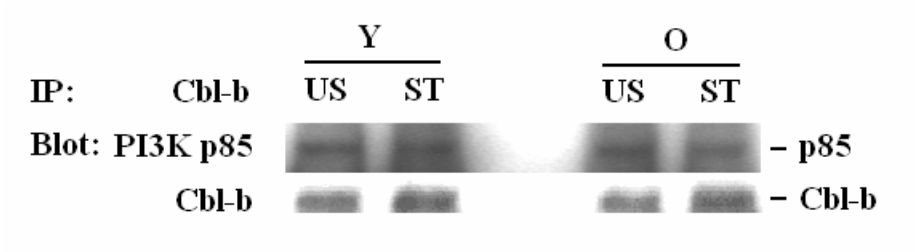

Figure 3.3 Ubiquitination of PI3K by Cbl-b in young and old splenic T-lymphocytes. Splenic T-lymphocytes were isolated from young (6 mo) and old (18 mo) rats. T-lymphocytes were stimulated by soluble anti-CD3 (10 μg/ml) plus anti-CD28 (1 μg/ml) antibodies for 120 min. Total cellular proteins were extracted and immunoprecipitated with anti-Cbl-b antibody. Western immunoblotting was performed as described in the Materials and Methods.

Figure 3.4 Nedd4-Cbl-b associations in young and old stimulated T-lymphocytes

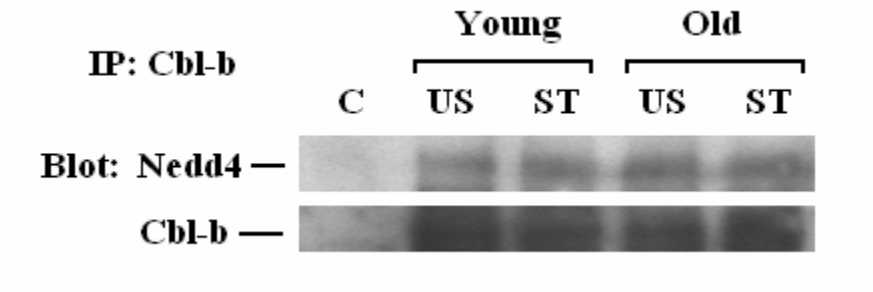

Figure 3.4 Nedd4-Cbl-b associations in young and old stimulated splenic T-lymphocytes. Splenic T-lymphocytes were isolated from young (6 mo) and old (18 mo) rats. T-lymphocytes were stimulated by soluble anti-CD3 (10 μg/ml) plus anti-CD28 (1 μg/ml) antibodies for 120 min. Total cellular proteins were extracted and immunoprecipitated with anti-Cbl-b antibody. Western immunoblotting was performed as described in the Materials and Methods.

Figure 3.5 CD28 expression in young and old splenic T-lymphocytes

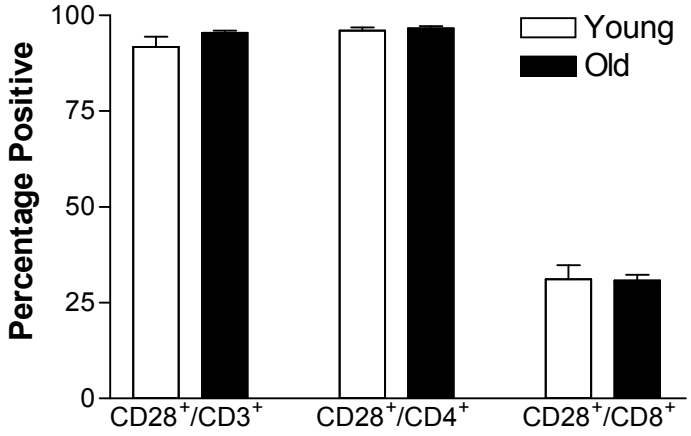

Fig 3.5 Affect of aging on CD28 receptor expression in T-lymphocytes. Splenic T-lymphocytes were isolated from young (6 mo) and old (18 mo) rats as described in the Materials and Methods. CD28 receptor expression was determined by flow cytometry as described in the Materials and Methods. Data are expressed as the percent CD28 receptor positive cells within the $CD3^+$, $CD4^+$, or $CD8^+$ T-lymphocyte population. Values are the mean ± S.E.M. of 5 individual rats.

Figure 3.6 CTLA-4 expression in young and old splenic T-lymphocytes

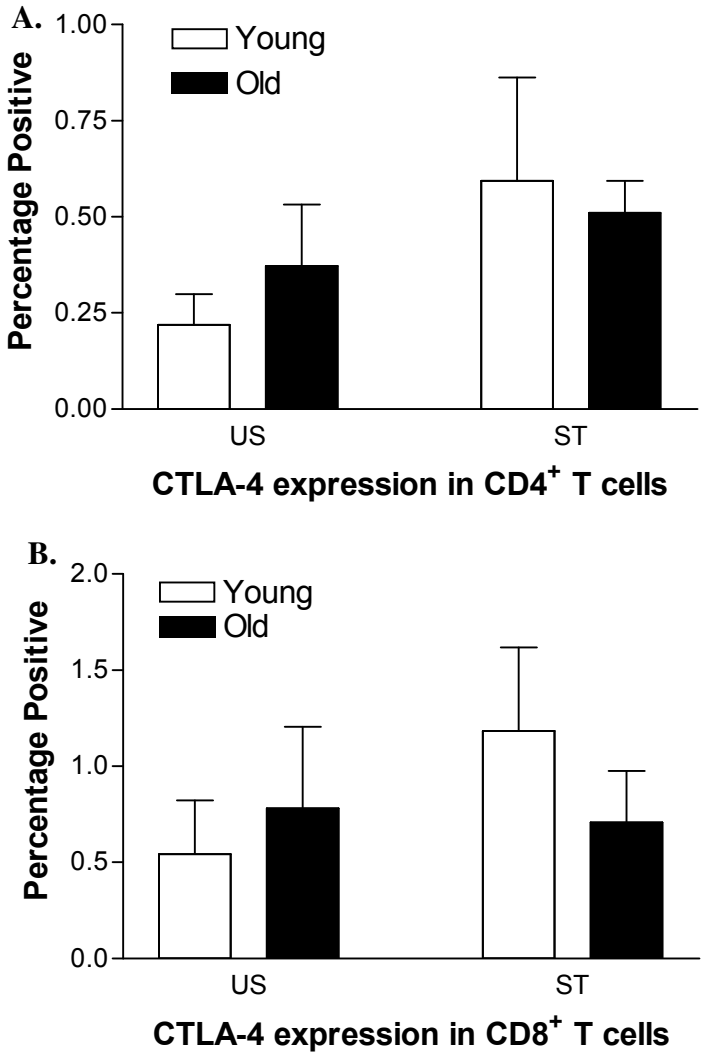

Fig 3.6 Affect of aging on CTLA-4 expression in T-lymphocytes. Splenic T-lymphocytes were isolated from young (6 mo) and old (18 mo) rats and stimulated with soluble anti-CD3 and anti–CD28 antibodies for 2 hours as described in the Materials and Methods. CTLA-4 expression was determined by flow cytometry as described in the Materials and Methods. Data are expressed as the percent CTLA-4 receptor positive

cells within the $CD4^+$, or $CD8^+$ T-lymphocyte population. Values are the mean ± S.E.M. of 4 individual rats.

Figure 3.7 Proteasome activity in young and old splenic T-lymphocytes

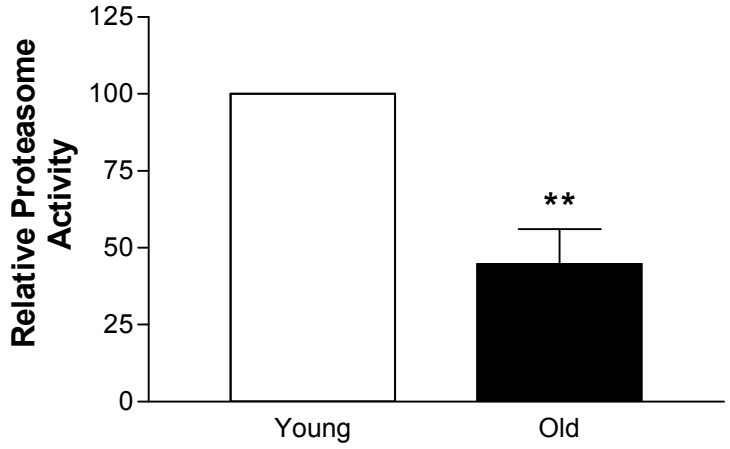

Figure 3.7 Proteasome activity in young and old splenic T-lymphocytes. Splenic T-lymphocytes were isolated from young (6 mo) and old (18 mo) rats. Total cellular proteins were extracted and proteasome activities determined as described in the Materials and Methods. Values are the mean ± S.E.M. of four individual rats calculated from the densitometric analysis of the autoradiograms. An '**' indicates significantly different ($p<0.01$) when comparing the young and old group.

Figure 3.8 Ubiquitination of Cbl-b in young and old splenic T-lymphocytes

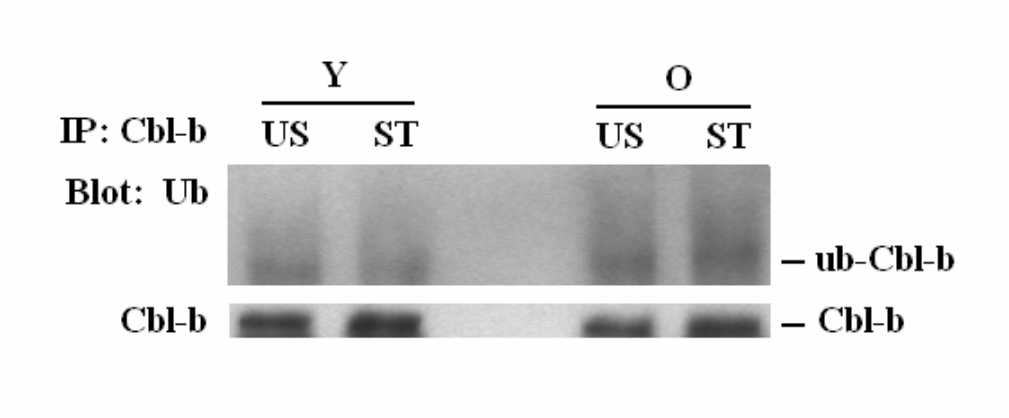

Figure 3.8 Ubiquitination of Cbl-b in young and old splenic T-lymphocytes. Splenic T-lymphocytes were isolated from young (6 mo) and old (18 mo) rats. T-lymphocytes were stimulated by soluble anti-CD3 (10 μg/ml) plus anti-CD28 (1 μg/ml) antibodies for 120 min. Total cellular proteins were extracted and immunoprecipitated with anti-Cbl-b antibody. Western immunoblotting was performed as described in the Materials and Methods.

Chapter 4: Cbl-b Deficiency Partially Reverse T-lymphocytes Function Lost in Aging

4.1 ABSTRACT

In previous studies, we demonstrated that old rat splenice T-lymphocytes fail to down-regulation Cbl-b expression upon CD28 stimulation which is associated with reduced T-lymphocyte proliferation. This suggests that high levels of Cbl-b expression prevent T-lymphocyte activation in aged T-lymphocytes. There is evidence suggesting that deficiency of Cbl-b protein can greatly increase the T-lymphocyte activity. However, whether lack of Cbl-b protein during aging process could reverse the loss of T-lymphocyte proliferation and prevent the immunesenescence. We compared the immune system function between Cbl-b knock-out (KO) mice and wild-type (WT) mice at young and old age. Our results showed that old Cbl-b KO T-lymphocytes demonstrate same proliferation ability as young WT T-lymphocytes. T-lymphocytes from old Cbl-b KO mice also showed less spleen enlargement, decrease in CD4/CD8 ratio, and less amount of suppressive regulatory T-lymphocytes. Taken together, these data indicate that deficiency of Cbl-b in old mice slow down the aging process and down-regulation of Cbl-b might replenish the immune function in the old.

4.2 INTRODUCTION

Dysregulation of the immune system is probably one of the biggest problems that threaten the elder's health. The immune system is a quite complicated network

composed of a variety of cell types. Functional change in one cell type may greatly affect other cell types' function and development. Due to this feature, the work to find out the key component that causes immunosenescence is not easy since in aged immune system, multiple changes have been reported in different immune cell types at different immune system developing stages.

Although there are controversial results concerning the changes happened in aged immune system [12], other studies showed quite consistent transformation in the phenotype of immune system along with advanced age. First, a phenomenon termed "thymic involution" is observed in several species [263-266]. The thymic output of naïve T cell to peripheral T cell pool decreases with age, which is characterized by less weight in thymus, reduced DN1 thymocytes and increased DN3 thymocytes in thymus from aged model. Although the naïve T cell input from thymus decrease with age, the size of entire peripheral T cell pool does not change accordingly. This is because T cell homeostasis plays its role to make up the T cell number loss from naïve T cells by increasing the proliferation of T cells already in the peripheral pool [267] and reducing the activation-induced cell death [268]. As a result, the subpopulation composition within T cell repertoire changed with advanced age. Proportion of $CD8^+$ T cells increase due to the clonal expansion [269]. More memory T cell subsets are observed in both $CD4^+$ and $CD8^+$ aged T cells [270]. Also associated with aging is the higher expression of P-glycoprotein, a membraneous efflux pump, in both T cell subsets [271]. Besides the subpopulation change with age, old T cells become less responsive to extracellular stimulation because the initial steps of T cell activation are defective. T cells express less CD28, the costimulatory molecule of T cell activation and more CTLA-4, the inhibitory molecule of CD28 with age. Meanwhile, CD69—the early T cell

activation marker, CD25—the middle T cell activation marker and HLA-DR—the late T cell activation marker also show age-related expression change [272, 273]. In concord with those T cell changes, upon stimulation T cells from old donor show less proliferative ability and have less IL-2 secretion. But old T cells also express more IFN-γ which reflects the proinflammatory situation experienced by elderly.

Each of those age-related changes that being observed in T cell system carries a piece of information that reflects at which stage of T cell development or in which aspect of T cell function that ageing may have a deleterious effect on T cell. Conversely, if any treatment or gene mutation can reverse one or some of the age-related changes, then the possible target of this treatment or mutation can be easily narrowed to a small field.

The introduction of cbl-b knock-out (KO) mouse by *Gu*'s group [201] and *Penninger*'s group [200] respectively not only show us sufficient evidence that Cbl-b is a negative regulator of T cell signaling pathway, they also provide us a powerful tool to directly study the role of Cbl-b in T cell senescence. The cbl-b KO mice generated by Gu's group [201] are normal in T cell development and immune cell repertoire. While the peripheral T cells from cbl-b KO mouse are hyperactive in their function. cbl-$b^{-/-}$ T cells can proliferate rapidly without costimulation from CD28 and secret high levels of IL-2. Since both the CD28 expression and IL-2 production are what are missing in old T cells, we have the hypothesis that cbl-b KO mice will continue to show a strong proliferative ability and reverse some of the age-related changes observed in T cells. Thus, in this chapter, features that already been proven to change in old wild type immune system will be studied in cbl-b KO mouse to see if the old cbl-b KO mice can

reverse those changes and evaluate the feasibility to use cbl-b KO mice as aging study model.

4.3 MATERIALS AND METHODS

Materials

All antibodies were purchased from BD Pharmingen (La Jolla, CA) unless otherwise noted in the text. All chemicals were reagent grade or better from Sigma-Aldrich (St. Louis, MO).

Animals

C57BL/6 cbl-b knockout mice were obtained from Dr. Hua Gu [201]. Mice were maintained on a 12 hour light/dark cycle and standard chow diet. All animal procedures were approved by the University of Texas Animal Use and Care Committee.

T-lymphocyte isolation

Mice were sacrificed and spleens aseptically removed. Single splenic cell suspensions were isolated by homogenization and filtering through a 25mm syringe filter. Lymphocytes were isolated via differential migration following centrifugation in Lymphocyte Separation Medium (Mediatech, Herndon, VA). T-lymphocytes were isolated using negative selection Immulan columns (Biotecx, Houston, TX) per manufacturer's instructions as previously described [230]. Isolation by negative

selection prevents perturbation of the T-lymphocyte's receptor during the isolation procedure, as occurs with isolation via positive selection. T-lymphocytes were counted using the Cell-Dyn 900 Hematology Analyzer (Sequoia-Turner, Mountainview, CA).

T-lymphocyte proliferation

T-lymphocyte proliferation was determined by MTT cell proliferation assay (ATCC, Manassas, VA). Briefly, $2x10^5$ splenic T-lymphocytes ($2x10^6$ cells/ml) were plated in each well and cultured at 37ºC with complete media in the presence of different concentration of plate-bound anti-CD3 antibody for 48 hr. 10 µl MTT reagent was added and continued incubating for 4 hr at 37ºC until purple precipitates were visible. After that, 100 µl detergent reagent was added, plates were then left at room temperature in the dark for 2 hr and absorbance was recorded at 550nm. T-lymphocytes for each treatment were plated out in triplicate and average was used for data calculation.

T-lymphocyte stimulation

T-lymphocytes, $8x10^6$ cells per petri dish ($4x10^6$ cells/ml) were stimulated at 37ºC for 48 hours in pre-warmed complete culture media (RPMI 1640 supplemented with 10% heat inactivated fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin, 100 mM 2-mercaptoethanol, 2 mM L-glutamine and 25 mM HEPES buffer) with 10µg/ml plate-bound anti-CD3 antibody.

Flow cytometric analysis

Splenic T-lymphocytes were stimulated as described before. 1 x 10^6 T-lymphocytes were treated with 200 µl 1X RBC Lysis buffer (eBioscience, San Diego, CA) for 2 minutes to lyse any remaining red blood cells that might interfere with analysis. Lysis was ceased with the addition of 5ml PBS and centrifugation at 250 g for 5 min. Cell pellets were incubated with PE labeled anti-CD8 plus FITC labeled anti-CD4 antibody. Excess antibodies were removed by washing with PBS with 0.1% NaN_3 and cells were fixed in 0.5% paraformaldehyde in PBS. CD4, CD8 receptor expression on T-lymphocyte subsets was analyzed using a BD FACScaliber flow cytometer and CellQuest Pro software (BD Pharmingen, San Diego, CA).

To detect the proportion of T regulatory cells, cells were first stained with FITC labeled anti-CD4 antibody as described before and washed with PBS with 0.1% NaN_3. Cells were resuspended with 1 ml of freshly prepared Fixation/Permeabilization working solution (eBioscience, San Diego, CA) and incubated for 1 hour at 4ºC. After washing with 2ml of 1x Permeabilization buffer twice, cells were resuspended in 50µl of 1x Permeabilization buffer containing 5ml of PE labeled anti-Foxp3 antibody (eBioscience, San Diego, CA) and incubated for 1 hour at 4ºC. Excess antibodies were removed by washing with 1x Permeabilization buffer (eBioscience, San Diego, CA). Cells were fixed in 0.5% paraformaldehyde in PBS and analyzed as described before.

Statistical analysis

Data were analyzed by one-way ANOVA and statistical difference were determined using Tukey's multiple comparison test with GraphPad Prism software (San Diego, CA). A $p<0.05$ was considered significantly different.

4.4 RESULTS

T-lymphocyte proliferation in wild type and Cbl-b knock-out mice with age

In vitro T-lymphocyte function is usually measured by cell proliferation when stimulated by soluble anti-CD3 antibody plus anti-CD28 antibody. As a negative regulator of T cell activation, lack of Cbl-b expression would greatly improve T cell proliferation even under less optimal stimulatory condition, like without the co-stimulatory signal from CD28 [200, 201]. However, whether the Cbl-b knock-out (KO) mice can keep the high proliferation rate when they are old are still unknown. Thus, we first tested the proliferation ability of wild type (WT) and KO mice at the age of 6-8 month (young) and 18-20 month (old). The results are shown in Figure 4.1. In Figure 4.1A, the proliferation rate of young WT and KO T-lymphocytes are compared when they are stimulated by different concentration of plate-bound anti-CD3 antibody. KO T-lymphocytes showed significantly high proliferation activity at all concentration of anti-CD3 antibody. Proliferation of KO T-lymphocytes is almost 2-fold higher than WT T-lymphocytes when the concentrations of plate-bound anti-CD3 antibody are 2.5 μg/ml (198% higher, $p<0.05$) and 5.0 mg/ml (192% higher, $p<0.001$). When the anti-CD3 concentration increased to 7.5 μg/ml and 10.0 μg/ml, the proliferation of T-lymphocyte from both WT and KO mice increased accordingly, and the proliferation rate in KO mice are 1.53-(7.5 μg/ml) and 1.57-fold(10.0 μg/ml) higher than the rate in WT mice. The proliferation rate of old WT and KO mice T-lymphocytes are shown in Figure 4.1B, the stronger T-lymphocyte proliferative ability were preserved in aged KO mice, but the relative increasing fold in KO T-lymphocyte were decreased with age. At the

concentration of 2.5 μg/ml, the T-lymphocyte proliferation in old KO mice was 38% higher, and was 46% higher at the concentration of 5.0 μg/ml, 43% higher at 7.5 μg/ml, and 50% higher at 10.0 μg/ml. The results of T-lymphocyte proliferation comparison between young and old mice are shown in Figure 4.1C. At low stimulatory condition (e.g. 2.5 μg/ml and 5.0 μg/ml of anti-CD3 antibody), the T-cell proliferation of old KO mice is higher than that of young WT mice (94% higher at 2.5 μg/ml and 34% higher at 5.0 μg/ml). At high stimulation concentration (either 7.5 μg/ml or 10 μg/ml of anti-CD3 antibody), the T cell proliferation ability from old KO mice are similar to young WT mice.

Features of immune system in Cbl-b knock-out mice with aging

Declines of T-lymphocyte function found in aging or other immune-related diseases are associated with certain physiological changes, especially those organs generating new T-lymphocytes and organs storing peripheral T-lymphocytes. Total body weight, thymus weight, spleen weight are measured and the results are shown in Figure 4.2. As shown in Figure 4.2A, the body weight of Cbl-b KO mice are almost the same to WT mice in respective young and old age group. The effects of aging on body weight are alike in both WT and Cbl-b KO mice that the weight increased about 7% with age. The spleen weight of WT and Cbl-b KO mice at young and old age are shown in Figure 4.2B. Spleen weight of young WT and young Cbl-b KO mice are similar too, spleen size increased with age in both WT and Cbl-b KO mice. The spleen size in WT mice almost doubled in WT mice (180% compare to the WT young mice) which is significant, while in Cbl-b KO mice, weight of spleen increase just 24% in aged mice. Figure 4.2C showed thymus weight change in WT and Cbl-b KO mice at young and old

age. Thymus weight decreased with age in both WT and Cbl-b KO mice, but there was no difference in thymus weight between the WT and KO mice in each age group.

CD4/CD8 homeostasis in Cbl-b knock-out mice

Since CD28-dependent down-regulation of Cbl-b in wild-type T-lymphocyte happened only in $CD4^+$ T cell subset, it would be expected that lack of Cbl-b expression in both $CD4^+$ and $CD8^+$ T cells might show different effect on the proliferation of individual subset and result in change of CD4/CD8 homeostasis. So the $CD4^+$ and $CD8^+$ T cells are stained and analyzed by flow cytometry in WT and Cbl-b KO mice at 4-6 month or 18-20 month, before and after 48 hr stimulation by plate-bound anti-CD3 antibody (10 mg/L). The results are shown in Figure 4.3. At young, unstimulated group, the CD4/CD8 ratio in splenic T-lymphocyte shows no difference in WT and KO mice, indicating that composition and activity of immune system at resting condition do not change with Cbl-b deficiency. However, compared to their WT counterparts, CD4/CD8 ratio is 40% lower in young Cbl-b KO T cells when they are stimulated. Similar CD4/CD8 ratio drops were also observed in old Cbl-b KO T-lymphocytes, no matter they were stimulated (45% lower) or not (27% lower).

Regulatory T cell population in Cbl-b knock-out mice

Regulatory T cell (T_{reg}), a subgroup of T cells that express CD4 on cell surface and Foxp3 intracellularly, shows suppressive function on proliferation of other T cell [274, 275]. They play an important role in prevent autoimmune diseases, transplantation tolerance, and cancer immunity [276]. The relative amount of T_{reg} cells

in Cbl-b KO mice comparing to the WT mice are tested. In most previous studies, T_{reg} cells were recognized by surface expression of CD4 and CD25 [277], however, more recent studies show that transcription factor *forkhead box P3 (Foxp3)* is more essential for the generation and activity of T_{reg} cells [278, 279]. $CD4^{+}CD25^{low/neg}Foxp3^{+}$ T cells have suppressive function too [280]. Thus, we use the surface staining of CD4 and intracellular staining of Foxp3 followed by flow cytometry analysis to identify the existence of Treg cells in total splenic T cell pool and the results are shown in Figure 4.4. Similar to what we observed in CD4/CD8 homeostasis data, in young, unstimulated group, WT and Cbl-b KO mice contain same level of T_{reg} cells, T_{reg} cells are 4.1% of total T cells in WT mice, and 4.3% of total T cells in Cbl-b KO mice. But when T cells were stimulated, the T_{reg} composition decreased in both WT and Cbl-b KO mice, and the Cbl-b KO mice have less T_{reg} cells after stimulation (1.78% in KO mice vs. 2.3% in WT mice). The percentage of Treg cells increased with age in both groups (9.2% in WT mice and 6.4% in Cbl-b KO mice) and dropped after stimulation (3.5% in WT mice and 1.7% in KO mice). In both situation, Cbl-b KO mice showed less T_{reg} cells content.

4.5 DISCUSSION

Proliferation of T-lymphocyte is constantly greater in Cbl-b KO mice than in WT mice at all anti-CD3 stimulatory condition if the two groups of mice are at the same age. The hyper-activation of T-lymphocyte because of no Cbl-b expression is observed throughout the life time of Cbl-b KO mice. However, the activity of T-lymphocyte in Cbl-b KO mice decreases with age too, indicating the Cbl-b KO T-lymphocyte is also under the aging regulation. Even though the proliferation rate of old KO T-lymphocyte is significantly lower than young KO T-lymphocyte, it is still as active as young WT T-

lymphocyte. This result suggests that the aging pace in Cbl-b KO mice is slowing down, thus, immune system of aged Cbl-b KO mice would be more functional and be able to fight more effectively against infection, autoimmune diseases and cancers.

Enlarged spleen, also called splenomegaly is a biomarker that associated with aging too. It is commonly observed in old rats [281, 282] and is showed in mice with premature aging diseases [283]. The cause of spleen enlargement can be either disease related, like infection [284], liver problem [285], cancer [286], or life style related, for example, diet induced obesity [287], or lack of exercise [288]. We also observed significantly enlarged spleen in old WT group. In Cbl-b KO mouse, though the spleen also enlarges with age, the extent is much less than its WT counterpart. Since the WT and KO mice are using the same diet and not under exercise training, the difference of spleen enlargement between WT and KO old mice may be a good indication of their healthiness and immune function in aging. Thymus weight are dramatically decreased with age in both WT and Cbl-b KO mice, and there is not much difference in thymus weight when WT and Cbl-b KO mice are compared at the same age. This result is in accordance with previous report that Cbl-b's function is involved in peripheral T cell activation, but not T cell development [289].

Lost of homeostasis in T cell population is another major feature responsible for the dysregulation of T cell function in aging. Change of balance between peripheral CD4 and CD8 T lymphocyte is often observed whenever physiological characters alter due to disease or aging. Here, we found that CD4/CD8 ration is lower in Cbl-b KO mice when splenic T cells are stimulated with anti-CD3 antibody. Skewing and expansion of $CD8^+$ T cell were characterized in B7 transgenic mice in a CD28-dependent

way [290]. Since Cbl-b KO mice uncouple the T cell activation with requirement of CD28 costimulation, the dropped CD4/CD8 ratio strongly suggests it is regulated by CD28 pathway, which is activated in Cbl-b KO mice. The mechanism of CD28/CTLA-4 regulation on $CD4^+$ and $CD8^+$ T cell homeostasis seems to be "CD28 signaling favors $CD8^+$ T cell expansion" [290] and $CD4^+$ T cell is more sensitive to the negative regulation of CTLA-4 engagement [291]. In other words, the activation threshold of $CD8^+$ is lower than $CD4^+$ T cells; this is probably why in WT T-lymphocyte, CD28 co-stimulatory signal only down-regulate Cbl-b expression in $CD4^+$ T cell, thus $CD4^+$ and $CD8^+$ T cells can achieve similar activation level under the same stimulation condition. The detailed pathway is still unclear and need to be elucidated.

Regulatory T cell (T_{reg}), previously characterized as $CD4^+CD25^+$ T cells [277, 292], and now more specifically defined as $CD4^+Foxp3^+$ T cells [293], is a subgroup of T cells with suppressive function. The roles of regulatory T cells include: to suppress autoimmune diseases [277, 294, 295], to inhibit transplantation tolerance [277, 296], and to prevent tumor immunity [297, 298]. The function of T_{reg} cells are achieved by inhibit the proliferation of both $CD4^+CD25^-$ T cells [274] and $CD8^+$ T cells [275]. The correlation of higher T_{reg} cell number with age and suppression of immune response in aging have been approved [299-301].

Increased percentage of $CD4^+Foxp3^+$ T_{reg} cells with age was observed in both WT and Cbl-b KO mice, suggesting that T_{reg} cells are important to the immunesenescence. We also found that at most groups (except the young unstimulated group), Cbl-b KO mice contain less $CD4^+Foxp3^+$ T_{reg} cells than WT mice. Generation and peripheral homeostasis of T_{reg} cells are controlled by the integrated network of CD28/CTLA-4 and

CD80/CD86 expression and interaction [302]. Both the CD28 and CTLA-4 signals contribute to the peripheral maintenance of T_{reg} cells. Absence of CD28 signal reduces the number of T_{reg} cells [302]. Function of CTLA-4 signal on T_{reg} cells' survival is mediated by TGF-β. CTLA-4 increases TGF-β expression [303], and TGF-β can upregulate Foxp3 expression [304-306]. In Cbl-b KO mice, T cells do not need the co-stimulatory signal CD28 to achieve a high level of activation, however, T cells with no Cbl-b expression also can not be regulated by the negative signal from CTLA-4 engagement. Thus, even though Cbl-b KO T cells do not require CD28 signal to maintain the already existing T_{reg} cell number, they are unable to induce Foxp3 expression via CTLA-4/TGF-β pathway. The fact Cbl-b KO mice contains less T_{reg} cells when being stimulated or when being aged suggests that the role of CTLA-4/TGF-β is more dominant in conversion of mature peripheral T cells into peripheral T_{reg} cells.

Figure 4.1 T-lymphocyte proliferation in Cbl-b knock-out mice

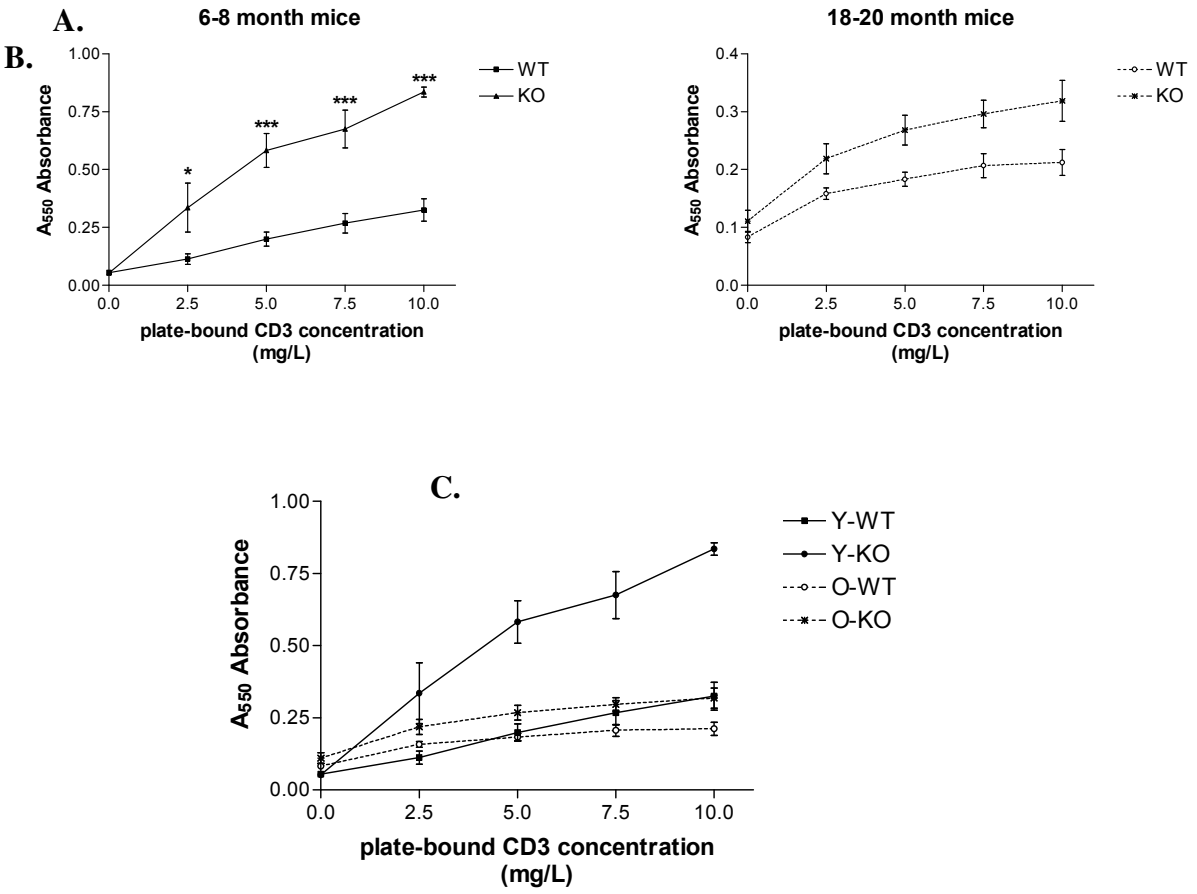

Figure 4.1 Influence of Cbl-b gene and aging on splenic T-lymphocyte proliferation. Splenic T-lymphocytes were isolated from young (6 mo, Y) and old (18 mo, O), wild type (WT) or Cbl-b knock-out (KO) mice as described in the Materials and Methods then activated with different concentration of plate-bound anti-CD3 antibodies for 48 hours and proliferation determined using the MTT dye uptake assay as described in the Materials and Methods. Data are expressed as the absorbance at 550 nm. Values are the mean ± SEM of 4 individual mice. An '*' indicates significantly different ($p<0.05$)

and '***' indicates significantly different ($P<0.001$) when comparing the WT and KO stimulated group.

Figure 4.2 Body, spleen, and thymus weight in Cbl-b knock-out mice

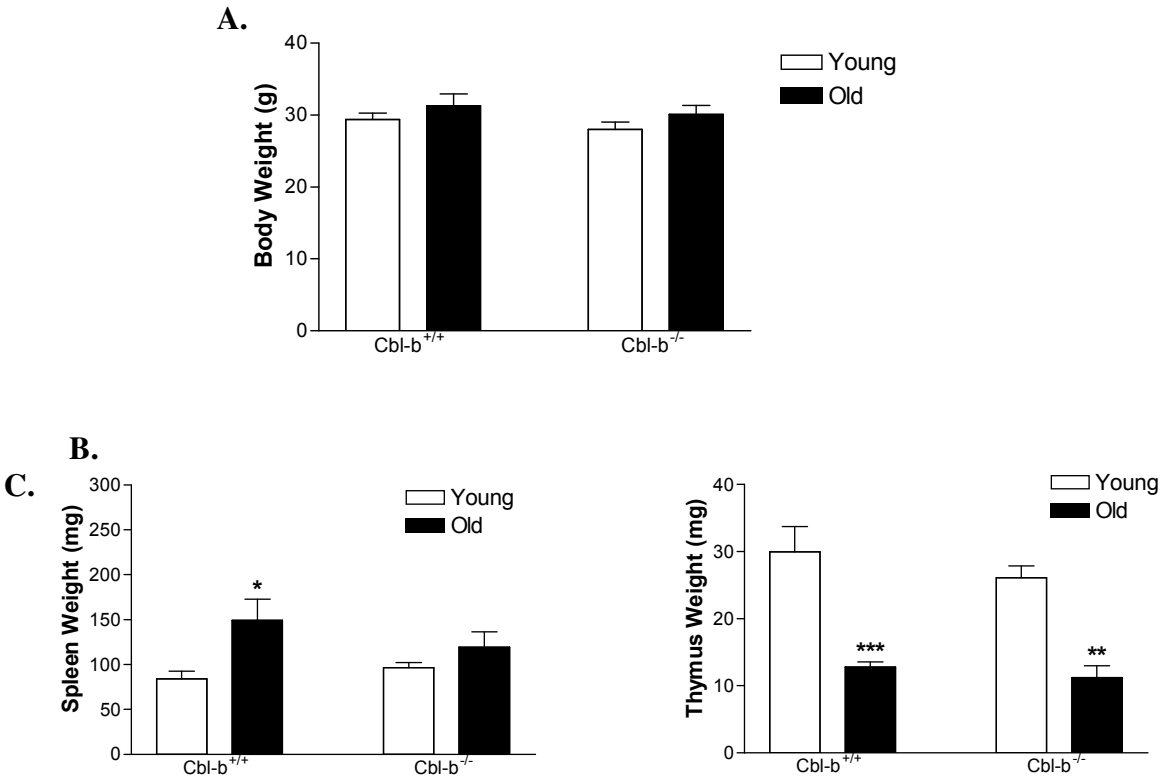

Figure 4.2 Influence of Cbl-b gene and aging on mice body, spleen, and thymus weight. Spleen and thymus were removed from young (6 mo, Y) and old (18 mo, O), wild type (WT) or Cbl-b knock-out (KO) mice and weighed. Values are the mean ± SEM of 8 individual mice. An '*' indicates significantly different ($p<0.05$), '**' indicates significantly different ($p<0.01$) and '***' indicates significantly different ($P<0.001$) when comparing the young and old group.

Figure 4.3 CD4/CD8 ratios in Cbl-b knock-out mice

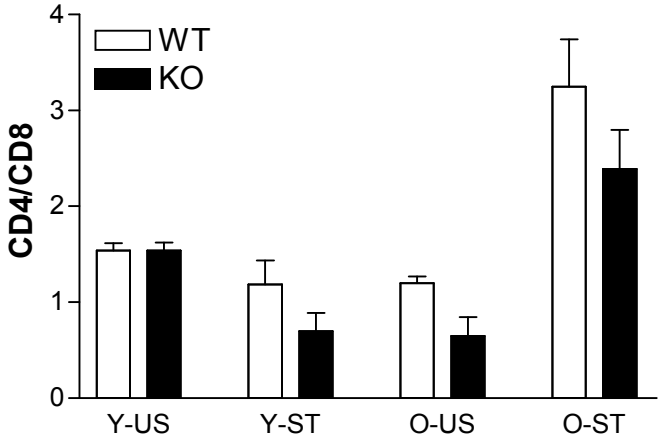

Figure 4.3 Decreased CD4/CD8 ratios in stimulated and aged Cbl-b knock-out mice. Splenic T-lymphocytes were isolated from young (6 mo, Y) and old (18 mo, O), wild type (WT) or Cbl-b knock-out (KO) mice, then activated with 10mg/L of plate-bound anti-CD3 antibodies for 48 hours. Cells were stained and analyzed via flow cytometry as described in the Materials and Methods. Values are the mean ± SEM of 6 individual mice.

Figure 4.4 Regulatory T cells in Cbl-b knock-out mice

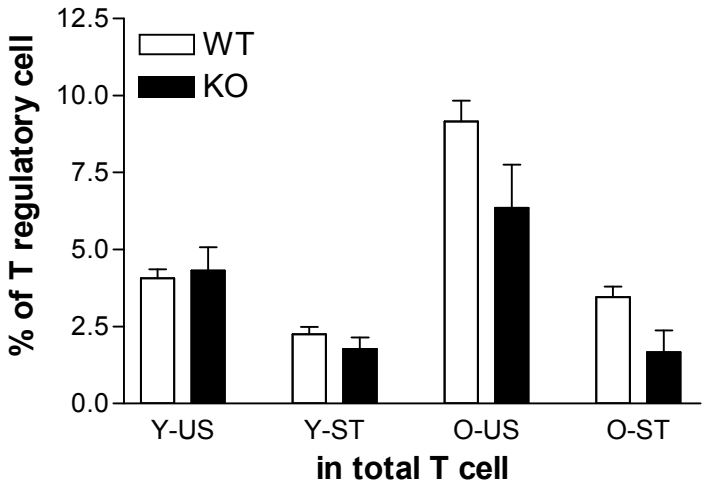

Figure 4.4 Decreased regulatory T cell percent in stimulated and aged Cbl-b knock-out mice. Splenic T-lymphocytes were isolated from young (6 mo, Y) and old (18 mo, O), wild type (WT) or Cbl-b knock-out (KO) mice, then activated with 10mg/L of plate-bound anti-CD3 antibodies for 48 hours. Cells were stained and analyzed via flow cytometry as described in the Materials and Methods. Values are the mean ± SEM of 6 individual mice.

Chapter 5: Conclusions

Prompt and accurate immune response is crucial for persons at all ages to protect themselves from infection caused by microbes and viruses. However, one of the biggest concerns associated with the health care for elderly is the dysfunction of immune system, either inadequate proliferation reaction and cytokine secretion in lymphocytes, or unnecessarily production of auto antibodies. For decades, studies have been focused on alteration of intracellular signaling transduction that leads to abnormal gene transcription. A key regulatory process in signaling pathway is phosphorylation, and one of the important roles of protein phosphorylation is to relay and amplify the stimulatory signals from the original receptor to the ultimate effecter. Changes of protein phosphorylation in aging process had been well studied, while there are still many questions about aging-related alteration in signaling transduction being unaddressed.

Though relatively new to the signaling transduction network, ubiquitin is receiving more and more attention. This is because ubiqutin has the same major features as phosphorylation, thus ubiqutin is considered as another essential protein modification in signaling transduction. Also, ubiquitination usually give negative regulation to involved signaling transductor, which may complement with the positive regulation provided by phosphorylation. So, a better understanding of ubiqutin and its regulation in aging process is urgently required.

This study has been focused on role of Cbl-b, an E3 ubiqutin ligase, in dysregulation of T cell function in aging. First, the expression and change of Cbl-b in

response to stimulatory signals were tested solely in young rat splenic T-lymphocytes, and the mechanism of Cbl-b's reaction to the signals were studied too. We found that protein level of Cbl-b will decrease when T-lymphocytes are stimulated with both anti-CD3 and anti-CD28 antibodies. Thus, all the effecters downstream of Cbl-b will be activated/upregulated since the negative regulatory signal exerted by Cbl-b is removed. The down-regulation of Cbl-b upon stimulation is similar to opening a dam (remove Cbl-b) and let water (stimulatory signal) flow through to keep the whole water system (cell function) manageable (cell proliferation and cytokine secretion). The decrease of Cbl-b disappears if only CD3 receptor being stimulated while the extent of Cbl-b down-regulation increases if only CD28 receptors are activated. The differential reaction of Cbl-b toward CD3 and CD28 stimulation suggests that these two signals play different roles in regulating Cbl-b. Only signals from stimulated CD28 receptor can regulate the protein expression of Cbl-b and control Cbl-b's function quantatively. As for the CD3 signal, since addition of CD3 antibody to CD28 will slow down the pace of Cbl-b being decreased by CD28 signal, CD3 signal also plays an important role in regulating Cbl-b's function, via a mechanism other than expression change.

The detailed mechanism of down-regulation of Cbl-b by CD28 signal was studied in this project. According to the previous research in transfected T cell lines, our research have been focused on another E3 ubiqutin ligase Nedd4, Itch and a transcriptional regulator Egr2/3. Experimental data showed that Nedd4, but not Itch and Egr family proteins are directly responsible for the degradation of Cbl-b. Whenever there is a CD28 stimulatory signal, both the transcription and translation of Nedd4 will increase. The intracellular location of total Nedd4 change with CD28 stimulation too. Nedd4 moves to be associated with Cbl-b, adds ubiquitin to Cbl-b and sends the Ub-

labelled Cbl-b to proteasome for degradation. Although we still lack direct evidence (such as siRNA of Nedd4), while together with the fact that transcription of Cbl-b does not change upon either CD3 or CD28 stimulation, we made the conclusion that the protein level change of Cbl-b is controlled directly by Nedd4 but not Egr family. The mechanism of regulation on Nedd4 mRNA expression is still unknown. Whether Egr family play a role in controlling Nedd4 transcription and thus indirectly regulate Cbl-b protein expression are still under study. The experimental result on this topic will complete our study of detailed mechanism of Cbl-b expression change in young splenic T lymphocytes upon different stimulation.

In old splenic T-lymphocytes, a strong correlation ship was observed between the decreased cell proliferation and anti-down-regulation of Cbl-b protein when cells are stimulated under the same condition as they were stimulated in young splenic T cells. Since in old T cells, Cbl-b maintained its function of binding with and ubiquitinizing PI3K, the sustained high protein level of Cbl-b blocked stimulatory signals being transferred from receptors to downstream effecters, and consequently impeded cell proliferation and cytokine secretion with age. We have tested factors that involved in controlling Cbl-b expression, including CD4/CD8 homeostasis, CD28 and CTLA-4 expression, binding with Nedd4, and ubiquitination of Cbl-b. They all appeared to be normal in aged T lymphocytes. The main reason that causes the inability of down-regulating Cbl-b in aged T cells is insufficient proteasome activity.

Since only an association relationship has been established between the higher Cbl-b expression and lower proliferation rate in old T-lymphocytes, we want to further determine the cause-effect relationship between Cbl-b and cell proliferation in aging.

Cbl-b KO mice showed a much stronger proliferation reaction under CD3 only stimulation. Compare to WT T-lymphocytes, the relative higher proliferative ability of Cbl-b KO T-lymphocytes remained even when they were at old age and the proliferative rate of old Cbl-b KO T-lymphocytes were comparable to young, wild-type T-lymphocytes. Regarding other features of immune system, Cbl-b KO mice also show other advantages, such as less enlargement in spleen, less suppressive T regulatory cells, and less skew in CD4/CD8 homeostasis. The aging process is slowed down but not completely stopped in Cbl-b KO mice.

The study about regulation put on Cbl-b's expression under different stimulatory condition and role of Cbl-b in immunesenescence indicate that Cbl-b play an important role in peripheral T cell function. Accurate manipulation on Cbl-b protein expression will lead to a better understanding and control of many diseases, and consequently improve the overall life quality in aged population.

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