THE ROLE OF RNASE L IN THYMIC HOMEOSTASIS AND HUMORAL RESPONSES

LIN ZHANG

Bachelor of Science in Applied Chemistry
Nanjing University of Finance & Economics, China
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DOCTOR OF PHILOSOPHY IN CLINICAL AND BIOANALYTICAL CHEMISTRY
at the
CLEVELAND STATE UNIVERSITY
MAY 2014
We hereby approve this dissertation

For

__________________________
Lin Zhang
(Student’s Name)

Candidate for the Ph.D. of Clinical and Bioanalytical Chemistry degree

for the Department of

__________________________
Chemistry

And

CLEVELAND STATE UNIVERSITY’S
College of Graduate Studies by

__________________________
Dr. Aimin Zhou

Department & Date

__________________________
Dr. David Andrson

Department & Date

__________________________
Dr. Ge Jin

Department & Date

__________________________
Dr. Xuelong Sun

Department & Date

__________________________
Dr. Xiang Zhou

Department & Date

05-16-2014
Student’s Date of Defense
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THE ROLE OF RNASE L IN THYMIC HOMEOSTASIS
AND HUMORAL RESPONSE

LIN ZHANG

ABSTRACT

RNase L is highly expressed in the spleen, thymus, and multiple immune cells, suggesting that it may play an important role in the immune system against microbes. Previous studies in the lab have shown that deficiency of RNase L results in enlarged thymuses in mice at young age. However, relatively little is known about its influence on the thymic development and adaptive immunogenicity.

The present Ph.D. study focuses on investigating the role of RNase L in the thymic homeostasis and humoral immune responses, thereby gaining new insights into the molecular aspects in thymocyte development, maturation and adaptive immunity.

By using RNase L gene knockout mice with C57BL/6 background, we found that RNase L deficient mice displayed severe homeostatic defect in the thymus, from birth until adolescence consistently. This homeostatic defect in the thymus was reflected by the increased population of BrdU positive cells, the enhanced growth rate and proliferation capacity in response to mitogens, and the elevated expression level of the pro-survival Bcl-2 protein; while the expression of the pro-apoptotic Bax protein was suppressed. Further investigation indicated that wild type thymocytes were prone to be arrested in the G1/S phase of the cell cycle by p27Kip1. In addition, PTPH1, a protein tyrosine phosphatase, and its substrate VCP, a well-known cell cycle regulator, may be the target molecules of RNase L in preventing excessive cell proliferation by
inhibiting cell cycle progression.

To elucidate the effects of RNase L on immune responses, we immunized RNase L $^{+/+}$ and $^{-/-}$ mice with T-dependent (TD) or T-independent (TI) antigens. Interestingly both spleen size and antibody production in TD or TI antigen immunized RNase L $^{-/-}$ mice were severely altered. A combination of GM-CSF, a hematopoietic growth factor facilitating both humoral and cellular mediated immunities, and the antigen in immunization, augmented TD antigen-directed immune responses in RNase L $^{+/+}$ mice compared to that in RNase L $^{-/-}$ mice. PolyI:C, a synthetic dsRNA, exhibited a significant enhancement of the IgM level in TI antigen immunized RNase L $^{+/+}$ mice.

Our findings suggest that RNase L may play an important role in maintaining thymic homeostasis and regulating humoral immune responses.
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CHAPTER I

INTRODUCTION

1.1 Ribonuclease L

RNase L is an interferons (IFNs)-inducible endoribonuclease and a pivotal component of IFN-mediated antiviral pathway that cleaves viral and cellular single stranded RNA, leading to suppression of majority of RNA virus, certain DNA virus, and cell proliferation [1, 2]. RNase L is localized in both nuclei and cytoplasm, and widely expressed in nearly mammalian cell type [3, 4]. The expression level of RNase L can be up-regulated during cell differentiation and in growth arrest cells [5]. Recently, cancer research unveiled that the increased RNase L level was associated with colorectal tumor genesis [6]. In mice, RNase L is highly expressed in the spleen, thymus, liver, lung, and testis. Generally, it peaks at 5 days after birth and then decreases with age, whereas it remains high until adulthood in spleen [7]. The successfully cloning of RNase L in 1993 allowed the detailed elucidation of its remarkable structural, functional and physiological characteristics in diseases [8].
1.1.1 (2-5A)/RNase L system

RNase L is also known as 2-5A dependent RNase (the “L” stands for “latent”), because its enzymatic activity is tightly controlled and requires an allosteric activator, 2', 5'-linked oligoadenylates (2-5A) [9]. Upon binding with 2-5A with high affinity, RNase L is converted from its inactive, monomeric state to a potent dimeric form (Figure 1) [10]. 2-5A produced from ATP by 2-5A synthetases (OAS), a family of interferon-inducible enzymes, is very unique with the formula ppp(A2'p5')nA (n≥2), consisting of a series of 5'-triprophosphorylated oligoadenylates with 2'-5' phosphodiester bonds, in contrast to the 3'-5' linkages found in RNA and DNA (Figure 2). The initial discovery of 2-5A was made by Ian Kerr’s group in 1974 when they investigated cell-free protein synthesis after interferon treatment [11]. Interestingly, Peter Lengyel’s group observed an increased nuclease activity in the extracts of interferon-treated cells after cells were incubated with double stranded RNA molecules (dsRNA) [12]. With the identification of OAS, the enzymes responsible for 2-5A synthesis, the IFN–induced (2-5A)/RNase L system was discovered (Figure 3) [13]. Serving as a RNA degradation pathway, the (2-5A)/RNase L system is triggered by dsRNA, after viral infection, and mediated by two major enzymes: OAS and RNase L. In summary, IFNs induce the production of OAS which is activated by dsRNA to produce 2-5A from ATP [14]. In turn, 2-5A activates pre-existing RNase L, resulting in the cleavage of RNAs within single-stranded regions, preferentially after UpUp and UpAp dinucleotides [15]. The most common RNA targets were identified as viral and cellular RNA. In recent years, mRNA production of several genes regulated by RNase L was identified [16]. This suggests that RNase L, and the 2-5A pathway, could have a wide biological role in cell physiology.
Figure 1 Functional model for the activation of RNase L by 2-5A

(Robert H. Silverman, Biochemistry, 2003)
Figure 2 Structure of the 2-5A: 2’-5’ oligoadenylates tetramer

(Catherine Bisbal, Robert H. Silverman, Biochimie, 2007)
Figure 3 The 2-5A/RNase L antiviral pathway. The viral pathogen associated molecular pattern, dsRNA, activates IFN induced 2-5A synthetase (OAS) which results in the synthesis of 2-5A from ATP. 2-5A binding to inactive monomeric RNase L leads to the formation of activated dimers of RNase L. The resulting degradation of single stranded loop regions in RNA, including rRNA in intact ribosomes, produces a potent antiviral response in the IFN treated and virus infected cell. (Robert H. Silverman, Cytokine & Growth Factor, 2007)
1.1.2 The structural characteristics of RNase L

Human RNase L is a 741-amino-acid protein with a molecular mass of 84 kDa [1]. Structural characterization of RNase L has revealed that this enzyme consists of three major domains: an N-terminal ankyrin repeat domain (ARD), a protein kinase homology domain (PK) and a C-terminal ribonuclease domain (RNASE) [17] (Figure 4). The ARD could be considered as the suppressor part of RNase L. It contains eight complete ankyrin repeats (R1-R8) and one partial ankyrin repeat R9 appearing as a disordered segment in the crystal structure of the ARD. Two walker A motifs (ATP or GTP fixation) are located within R7 and R8 [18]. ARD is a highly conserved protein-protein interaction domain that functions in transcriptional control, cell cycle regulation, and differentiation. RNase L is the only nuclease known to contain ARD, suggesting that RNase L might exert its function via interacting different proteins. One candidate is the ATP-binding cassette (ABC) homology protein, RLI (HP68), that might interact with RNase L directly or indirectly to regulate RNase L activity [19]. The crystal structure of the 2-5A binding domain of RNase L in a complex with 2-5A shows that the bounded 2-5A directly interacts with R2-R4, providing a detailed view of the RNase L binding pocket at the atomic level (Figure 5) [20]. With a cysteine-rich PK domain, RNase L is also predicted to have a kinase activity. Although some of the proposed kinase residues are either incomplete or differ significantly from known protein kinase domain, it is still difficult to completely rule out the possibility that RNase L may have a kinase function in vivo. The PK and RNASE domains at the C-terminus of RNase L [collectively referred to as the kinase-extension-nuclease (KEN)] are related to Ire1, both a kinase and an endoribonuclease that functions in the unfolded protein response (UPR) in organism from yeast to humans [17]. Although RNase L shares some domain architecture with
Ire1, there are functionally some differences between the two proteins. The N-terminal regions are unrelated, but both receive the activation signal. In contrast, in Ire1, activation is stimulated by unfold proteins through its endoplasmic reticulum (ER) luminal domain that titrate off Bip, an ER chaperone, whereas, RNase L activation occurs in the cytoplasm in response to 2-5A produced during viral infection. Both proteins are regulated at the level of dimerization (or oligomerization). In addition, a high-order assembly is shown for Ire1, but RNase L can only form dimmers. Upon activation, Ire1 degrades host mRNAs (mostly for membrane proteins) via a process named “regulated Ire1-dependent decay” (RIDD) which requires the nuclease activity of Ire1, but doesn’t appear to be dependent on its kinase activity. In this respect, Ire1 is similar to RNase L. RIDD was suggested to play a role in attenuating viral protein synthesis during hepatitis C virus (HCV) infections [19]. Thus, it is likely that RNase L may be involved in RIDD during viral infection. Although the kinase function of Ire1 is well established, RNase L has not been detected to have similar kinase activity and does not require phosphorylation for its ribonuclease activity. However, mutation of the lysine 392 in the protein kinase domain leads to a defect in the ability of RNase L to be dimerized, resulting in a greatly reduced activity of RNase L [21]. Several amino acids in the C-terminal domain of RNase L are required for catalysis, including R667 and H672 [22]. In addition, Tyr712 and Phe716 are important for both binding and cleavage of RNA [23]. Within the RNASE domain there is also a UBX-containing protein domain (PUG) similar to that in peptide N-glycanase which removes glycans from misfolded glycoproteins [24]. Some other proteins also have both ARD and PK domains, such as integrin-linked kinase, and death-associated PK, DAP kinase. Although Ire1 have both PK and RNASE domains, RNase L is the only protein identified that has all three
domains (ARD, PK, and RNASE), and the ARD is more highly conserved than the KEN domain generally [25-27].

As mentioned above, 2-5A binds with high affinity to RNase L; however, in the absence of 2-5A, the ARD represses the RNASE domain, while also maintains RNase L as a monomer. Interaction of 2-5A with the repressor region in RNase L relieves inhibition caused by the ARD and converts it from the inactive monomeric state to a potent dimeric endoribonuclease (Figure 6). Presumably, dimeric RNase L is the result of a conformational change that unmask the interaction sites of repressor, thereby exposing protein-protein interaction domains and releasing the RNASE domain (Figure 7) [28]. Indeed, 2-5A binding to RNase L increases dimerization affinity by a factor of $10^5$-$10^6$ [29]. In the dimer, the RNASE domains are no longer repressed by internal interactions and are thus able to cleave RNA [30]. RNase L cleaves after UpNp dinucleotide sequences (primarily UU and UA) in single-stranded RNA, but also at other sequences with lower efficiency [31, 32]. Accordingly, in viral infected cells, viral RNA is preferentially degraded in compared to cellular RNA [33]. It also has been reported that a truncated, recombinant RNase L lacking the ankyrin repeats, showed constitutive endoribonuclease activity, i.e., no longer requiring 2-5A [34]. RNaseL is cleaved into N- and C-terminal polypeptides in extracts of peripheral blood mononuclear cell (PBMC) from chronic fatigue syndrome (CFS) patients; interestingly, G-actin is also degraded, however the underlying mechanisms and effects are currently unknown [35].
Figure 4 Domain structure of RNase L. The main structural and functional domains of RNase L are shown, including ankyrin repeat domain, ARD; protein kinase homology domain, PK; ribonuclease domain, RNASE; kinase-extension-nuclease domain, KEN, and peptide N-glycanase/UBX-containing protein domain, PUG. The region responsible for binding 2-5A is indicated. (Silverman, new insight, 2010)
Figure 5 Crystal structure of an ankyrin repeat domain (ARD) complexed with 2-5A. (A) Structural and functional domains of RNase L. Ankyrin repeats are shown starting with blue at repeat 1 and ending with red at repeat 8. (B) Structure of the predominant trimeric species of 2-5A. (C, D) Surface (top) and ribbon (bottom) representations of the ARD/2-5A complex. Ankyrin repeats (R1 - R8) are shown as in (A). The bound 2-5A molecule is shown as a ball-and-stick model. The view in (D) was obtained by rotating the view in (C) by 90°. (Tnaka, Structural basis for recognition of 2', 5'-linked oligoadenylates by human ribonuclease L, 2004)
Figure 6 Surface view of the RNase L dimer

(H Huang, Dimeric Structure of Pseudokinase RNase L Bound to 2-5A Reveals a Basis for Interferon-Induce Antiviral Activity, 2013).
Figure 7 Schematic of RNase L regulation by 2-5A. Model of 2-5A-induced conformational changes that result in RNase-L dimerization and activation of enzymatic activity. (H Ezelle, Pathologic effects of RNase-L dysregulation in immunity and proliferative control, 2012)
1.1.3 Biologic activities of RNase L

The biologic functions of RNase L have been extensively investigated [28]. As a cellular RNA regulator, RNase L exerts its impacts on a broad range of physiologic activities, both directly and indirectly. Disruption of one or more of these activities may lead to a disturbed physiological state associated with critical diseases. The role of RNase L for antiviral activities, apoptotic activities, cellular proliferation, and the potentially involved mechanisms will be discussed below.

1.1.3.1 Antiviral activities of RNase L

RNase-L is originally characterized as an antiviral effector of IFNs action against infections [10]. Being on the frontline defense against viral infection, IFN response limits virus propagation by inducing the expression of a number of genes to inhibit or block viral replication before the onset of the adaptive immune response. The 2-5A/RNase L pathway plays a central role in the antiviral activity of IFN. In most cases, RNase L exerts its antiviral activities by directly cleaving the viral RNA; however, the increasing role of RNase L in immunomodulatory activities may provide alternative or additive mechanisms towards the enhancement of its antiviral function. RNase L has been reported to be against numerous RNA viruses (Picornaviridae, Reoviridae, Togaviridae, Paramyxoviridae, and Retroviridae) as well as several DNA viruses (Poxviridae, Herpesviridae, and Polyomaviridae) [36]. The antiviral role played by RNase L has been clearly demonstrated by a number of studies involving 2-5A or stabilized 2-5A analogues transfected or OAS and RNase L overexpressing cells, and RNase L -/- mice in response to infections with EMCV, Coxsackievirus B4, West Nile virus and herpes simplex virus 1 [9-12]. In parallel to the anti-viral activity of RNase L, many viruses have adapted ways to inhibit RNase L and/or OAS as an
alternative to evade their antiviral activities.

1.1.3.2 Apoptotic activity of RNase L

RNase L is involved in multiple pathways of apoptosis in an IFN-dependent and independent fashion. In response to viral infections, an infected cell may undergo apoptosis mediated by the IFN induced 2-5A/RNase L pathway, thus rapidly preventing the infected cell to release viral progeny. Many studies have associated the activation of the 2-5A/RNase L system, RNA breakdown, or ribonuclease activation with cell death or tissue regression. For example, activation of RNase L in 2-5A transfected cells results in specific 18S rRNA cleavage and induction of apoptosis, as measured by TUNEL and annexin V binding assays [37]. Overexpression of RNase L by a recombinant vaccinia virus also leads to apoptosis of mammalian cells, which becomes more pronounced by coexpression of a 2-5A synthetase [38]. It has been demonstrated that the Jun N-terminal kinases (JNK)-dependent stress response is responsible for efficient induction of apoptosis initiated by RNase L through a release of cytochrome c from mitochondria and subsequently activation of caspase cascade [39]. It is also found that RNase L participates in mitochondrial mRNA degradation. Interestingly, a number of evidences suggested that RNase L could participate in an apoptotic pathway other than that induced by 2-5A, which is independent to IFN and the 2-5A/RNase L system. Prostate cancer cells DU145 are resistant to apoptosis when the expression of RNase L is down-regulated by siRNA [40]. RNase L null mice showed enlarged thymuses as a result of a reduced level of spontaneous apoptosis. In addition, thymocytes and MEF isolated from RNase L null mice are resistant to apoptosis induced by staurosporine and irradiation [41]. However, how RNase L mediates the IFN-independent apoptosis remains to be fully understood.
1.1.3.3 RNase L regulated cell proliferation

RNase L has also been shown to have an impact on the cell proliferation. Analysis of components in the 2-5A/RNase L system reveals an inverse correlation between 2-5A/RNase L pathway activities and cell proliferation. For example, RNase L and OAS activities were elevated in proliferatively arrested or differentiated cells as well as in antiproliferative agent-stimulated cells, indicating RNase L is involved in the fundamental control of cell proliferation and differentiation [42]. Ectopic expression of OAS or RNase L resulted in cell quiescence, senescence or apoptosis, and the induction of apoptosis and senescence was reduced when RNase L is absent [43]. Cells expressing a dominant negative RNase L were resistant to the antiproliferative activity of IFN-α. In contrast, it has been reported that the over expression of RNase L in murine NIH 3T3 cell increased IFN-α antiproliferative function. Furthermore, introduction of 2-5A into the cells also results in an inhibition of growth rates, suggesting that RNase L may also regulate cell growth [44]. Similar to RNase L associated apoptotic activities, RNase L also can regulate cell proliferation independent of IFN-α. Without IFN-α treatment, RNase L-/- MEF cells grew 1.6-fold faster compared with RNase L+/- MEF cells. The growth rate of bone marrow cells isolated from RNase L-/- mice was 1.73-fold faster than that from RNase L+/-, when granulate macrophage colony stimulating factor (GM-CSF) was present, suggesting that RNase L regulates cell proliferation stimulated by other factor. Nerveless, the role of RNase L in the inhibition of fibrosarcoma growth in nude mice had been demonstrated [45]. Based on these findings, RNase L is considered as a potent tumor suppressor in the mouse model; however, the molecular mechanism is still need to be elucidated.
1.1.4 The involvement of RNase L in the immune system

Besides having a direct impact on cellular antiviral, apoptotic or anti-proliferative activities, RNase L may also play an important role in the immune system. Since tissue distribution analysis has revealed that RNase L is highly expressed in the thymus, spleen and most of immune cells [46]. Recent genetic and biological studies suggest that RNase L may be a potent regulator in modulating the immune response to exogenous pathogens and endogenous malignancies [2]. The impact of RNase L on the regulation of the thymus gland and subclasses population of T cells is observed. It also has been shown that skin allograft is suppressed in mice lacking RNase L, implicating the involvement of RNase L in T cell immunity. In addition, RNase L also exhibits extraordinary immunomodulatory ability in modifying a number of cytokine secretions in immune cells by which it mediates biological activities [46]. As critical factors in the B cell directed humoral immunity, these cytokines strictly control the initiation of immune response, the antibody class switching process and the populations of immunoglobin isotypes, acting as immunological switches against specific antigens. Indeed, a cancer vaccine study had reported that alphavirus-based DNA vaccination against a non-mutated tumor-associate self-antigen (tyrosinase related protein-1, TRP-1) is severely impaired in RNase L deficient mice, indicating the involvement of RNase L in B cell mediated immunity [47]. In clinical trials, RNase L has been recognized for many years as a marker for diagnosing chronic fatigue syndrome (CFS); an illness associated immunological abnormalities with unknown etiology [2]. All these findings highlight the potential role of RNase L in the immune system. A schematic of the 2-5A/RNase L system, is shown in Figure 8 [18]
Figure 8: the 2-5 A/RNase L System
1.1.5 Hypothesis of Study

RNase L is highly expressed in the thymus, spleen and all types of immune cells. However, relatively little work has been done in studying the effect of RNase L on thymic homeostasis and B cell-related humoral immune response. RNase L-null mice exhibit dramatically enlarged thymus glands containing significantly higher numbers of thymic cells at the early stage than that from wild type mice, suggesting that RNase L may play an important role in maintaining the dynamic balance in the thymus [43]. Indeed, it has been observed that the expression levels of several immunologically related cytokines are different between RNase L-null and wild-type mice, suggesting that RNase L may be involved in adaptive immunity through direct regulation of the level of cytokines. As the major organs of the immune system, abnormality of the thymus and spleen can be associated with altered immune responses, leading to immunological diseases. The proposed study in this dissertation focuses on investigating the role of RNase L in the thymic homeostasis and immune responses. The information obtained from this study will provide direct evidence that RNase L is of paramount significance for the adaptive immune system. In addition, a better understanding of the underlying molecular mechanism will be achieved by identifying the RNase L targeting molecules. The outcome of these projects would provide direct evidence that RNase L contributes to the thymic homeostasis through analyzing differential progress changes in the thymic architecture between RNase L +/+ and -/- mice. By identifying the target molecules of RNase L in the thymic development, we will yield new insight into the molecular mechanism by which RNase L regulates the thymic homeostasis as well as its contribution to the immune responses under pathogen infection.
1.2 Project I: RNase L contributes to thymic homeostasis

Thymus is one of the primary lymphatic organs arming the immune system against infection. It is responsible for the provision of T lymphocytes (T cells) to the entire body, and provides a unique microenvironment in which T cell precursors (thymocytes) derived from bone marrow undergo a sequence of complex, but highly dynamic developmental steps to become mature T cells, thereby participating in host defense via T cells mediated adaptive immune response. The conversation between cells proliferation and apoptosis (programmed cell death) in the thymus is critical in maintaining a homeostatic environment for normal T cell development and efficient initiation of immune response. It has been reported that abnormalities of the thymus are associated with severe immunological disorders or syndromes, such as DiGeorge syndrome, myasthenia gravis, thymic tumor and systemic lupus, et al [48].

1.2.1 Thymic Development

The thymus is a dynamic organ where all thymocytes develop, mature, and subsequently leave as mature T cells. Briefly, during T cell development process, thymus constantly loses thymocytes to apoptosis and emigration, while it replaces the cell loss with progenitor populations from the bone marrow, thereby maintaining overall thymus size and cellularity [49]. In the thymus, these thymocytes undergo a complicated and highly regulated process of proliferation and apoptosis. The journey of T cell development in the thymus can be segregated into three stages: expansion, selection, and maturation (Figure 9) [50].

The thymocyte precursors derived from hematopoietic stem cells (HSCs) in bone marrow first migrate to the thymus. These early T cell progenitors comprising
only 1-3% of total thymus cellularity get populated through their high rate of proliferation, and eventually make up $\geq 80\%$ of the total thymus cellularity [51].

After proliferative expansion, they undergo lineage commitment, repertoire selections, and then emigrate from thymus as mature functional T cells to establish the peripheral T cells pool. When undergo differentiation, 95% of the newly divided thymocytes that mutate during rapid division die from apoptosis, and only less than 5% of the T cells generated in the thymus live to exit as mature cells [52]. The loss of cells in apoptosis is replenished with progenitor populations from the bone marrow and they proliferate as a source to govern overall thymic homeostasis.

It has been demonstrated that sustained thymic homeostasis is strictly controlled by the balance between proliferation and apoptosis of thymocytes. It is known that various inductive, hormonal, pro-apoptotic and proliferative signals may also contribute to this balance. A disturbance within these two processes, which can be caused by an abnormal growth rate and ability, may lead to either unwanted tissue atrophy or tissue hyperplasia. For example, thymus atrophy has been observed in adenalectomized rats treated with the synthetic glucocorticoid dexamethasone, a steroid that induces thymocytes apoptosis. In contrast, tumor forms in the thymus, resulting from impaired apoptosis due to the over-expression of anti-apoptotic bcl-2 [53]. However, the mechanisms that mediate and regulate thymic apoptosis and proliferation are not fully understood.
Figure 9 Models of self-reactivity and TReg cell generation. The figure shows the classic Gaussian distribution model as it was first proposed by Maloy and Powrie19. The graph depicts the relationship between the relative avidity to self antigens (x axis) and the selected cell number (y axis). The distribution of positively selected CD4+ single-positive (SP) cells is shown in blue, with the alternative cell fates of regulatory T (TReg) cell selection and negative selection shown in green and red, respectively. (D. Ribatti, Miller’s seminal studies on the role of thymus in immunity, 2006)
1.2.2 Cell cycle maintains homeostatic thymus

As mentioned above, proper thymic development requires a balance between apoptosis and cell proliferation. Generally, cells proliferate via a mitotic process determined by the cell cycle progression. Apoptosis occurs in a widely variety of physiological settings in order to remove harmful damaged or unwanted cells. In addition, cell cycle genes such as p53, retinoblastoma (RB) and p27 have been shown to participate in both the proliferation and apoptosis [54]. Thus, a balance between apoptosis and proliferation must be strictly maintained to sustain thymic homeostasis. Cell-cycle regulator interconnects both cell proliferation and apoptosis together.

The cell cycle consists of two specific and distinct phases: interphase, consisting of G1 (Gap 1), S (synthesis), and G2 (Gap 2), and the mitotic phase; M (mitosis). During interphase, the cell grows (G1), accumulates the energy necessary for duplication, replicates cellular DNA (S), and prepares to divide (G2) [55]. The cellular decision to initiate mitosis or to become quiescent (G0 phase) occurs during G1 phase [56]. At this point, the cell enters M phase, which is divided into two tightly regulated stages: mitosis and cytokinesis. During mitosis, the parent cells’ chromosomes are divided to two identical sets. In cytokinesis, division of the cytoplasm occurs, leading to the formation of two distinct daughter cells [57-59]. Each phase of the cell cycle is tightly regulated, and checkpoints exist to detect potential DNA damage and allow it to be repaired before a cell dividing. If the damage cannot be repaired, a cell becomes targeted for apoptosis [60-62]. Cells can also reversibly stop dividing and temporarily enter a quiescent or senescent state; G0. The first checkpoint is at the end of G1, making the decision if a cell should enter S phase and start to divide or delay division to enter G0. The second checkpoint, at the
end of G2, triggers mitosis if a cell has all the necessary components [63].

The molecules that regulate cell-cycle progression are well defined. The link between cell-cycle and apoptosis has been demonstrated by a line of evidence. Several studies indicate that cell-cycle regulating proteins have an impact on the cell proliferation and apoptosis. For example, p53 has been shown to activate the Bax promoter and induce a high level of the Bax mRNA and protein. Bax is necessary for 50% of p53 induced apoptosis [64]. It also has been demonstrated that a relationship between p27, cdk2 and apoptosis in thymocytes is regulated by p53, Bcl-2 and Bax. It has been believed that cdk2 activation seems to be the key-point associated with a cell cycle and apoptosis [65]. Nevertheless, Bcl-2 inhibits apoptosis of proliferating cells and reduces the rate of cell division [66]. More importantly, haemopoietic stem cells (HSC) from Bcl-2 transgenic mice proliferate more rapidly and extensively than those of wild type [67].
Figure 10 Schematic of the cell cycle (Image provided courtesy of Abcam Inc.)
1.2.3 Protein tyrosine phosphorylation

At the molecular level, protein tyrosine phosphorylation is considered to be a key regulatory mechanism responsible for immune homeostasis. Comparing with other cell types, all cells in the immune system have relative high levels of tyrosine phosphorylation and express more genes encoding protein tyrosine kinases (PTKs) and protein tyrosine phosphatase (PTPs) [68]. The net result from the opposing effects of PTKs and PTPs contributes to the reversible tyrosine phosphorylation, thereby conducting immune cell signals either in a positive or negative manner, which plays a crucial function in cell-fate determination to proliferation, differentiation or death. Recent years, more attention has been given to certain PTPs and their regulatory function in the immune system, particularly in T cells. For example, PTPH1, a member of non-receptor type phosphatases, has emerged as a potent regulator in the T cell homeostasis. It reduces the TCR-induced activation through dephosphorylating the TCR ζ chain in Jurkat T cells [69]. Over-expressing of PTPH1 inhibited the activation of the transcription regulators NF-AT and AP-1, subsequently suppressed growth rate in Jurkat T cells [70]. The mutant forms of PTPH1 were found in colorectal cancers, indicating that PTPH1 is a tumor suppressor [71]. Being a cytoskeletal PTP, PTPH1 is characterized by N-terminal ezrin-, radixin- and moesin homology (ERM) and PDZ domains. The capability of PTPH1 to prevent persistent TCR signaling from inducing aberrant thymocytes proliferation and expansion depends on its membrane associated targets with ERM domain. In addition, the specialized PDZ domain of PTPH1 can be interacted with the cell surface receptor FAS, suggesting its engagement in FAS related apoptosis [72]. Nevertheless, identification of the proposed substrates/interactors of PTPH1 provides further insights into how PTPH1 exerts its inhibitory effect for T cells. Valosin-containing
protein (VCP), a PI3K/Akt survival pathway associated cell cycle regulator, has been identified as a selective substrate of PTPH1 [73]. All of these well-defined aspects suggest a versatile role of PTPH1 in T cell mediated immunity.

Tyrosine phosphorylation is a key mechanism for signaling transduction, controlling a diverse array of cellular responses including growth, proliferation, differentiation, migration, metabolism and survival, and regulating many pathophysiological processes, such as angiogenesis and oncogenesis [74-76]. Proteins are phosphorylated on tyrosine residues by protein tyrosine kinases (PTKs) and dephosphorylated by protein tyrosine phosphatases (PTPs) (see Figure 11). Tyrosine phosphorylation is a reversible, dynamic process controlled by the activity of PTKs and PTPs. The addition of a phosphate (PO₄) molecule to a polar R group of tyrosine residue can turn a hydrophobic portion of a protein into a polar and extremely hydrophilic portion of molecule. In this way it can introduce a conformational change in the structure of the protein via interaction with other hydrophobic and hydrophilic residues in the protein, resulting in activation or deactivation of protein enzymes, which are associated with a variety of biological activities [77]. Tyrosine phosphorylation is closely linked with T-cell development, proliferation, maturation and function [78]. For example, T-cell proliferation mediated by CD26 can be inhibited by tyrphostin, a specific tyrosine kinase inhibitor [79, 80].
Figure 11 The phosphorylation equation

(Cited from the 2007 Herbert Tabor - Journal of Biological Chemistry Lecture)
1.2.4 RNase L as a potential regulator of thymic homeostasis

RNase L, an interferon (IFN) inducible endoribonuclease, is a central player in the 2-5A system against viral infection and cellular proliferation [81]. In mammalian, the dormant RNase L is activated by 2', 5’-oligoadenylates (2-5A) which are synthesized from ATP by oligoadenylate synthethases (OAS). OAS is induced by IFN, and becomes active upon binding to double-stranded viral RNA or cellular RNA with double-stranded structure. After activation by 2-5A, RNase L degrades viral mRNAs and cellular RNAs, thereby disrupts the replication of certain viruses and cell proliferation. In addition to antiviral action, the other functions of RNase L including pro-apoptotic effect, anti-proliferative activity and differentiation regulation have been documented, but relatively little is known about its role in T cell development and functions. Our preliminary study indicated that RNase L is highly expressed in the thymus and its deficiency leads to significant enlargement and hypercellularity of the thymus gland; however the physiological function of RNase L on thymus and the underlying molecular mechanisms are still unclear. In this study, we found that RNase L contributes to homostasis of thymocytes in mouse models. Identification of PTPH1 and its substrate VCP, by means of mass spectrometry based phosphoproteomics, indicated that RNase L may target PTPH1 to mediate a dephosphorylation cascade in the thymus. As a result, RNase L-deficient mice exhibit dysregulated thymic protein tyrosine phosphorylation signaling which interferes with proliferation and survival pathways, therefore, disrupting the established equilibrium of proliferation and apoptosis. The outcome of this study suggests that RNase L is an important modulator in thymic homeostasis by regulating PTPH1 signaling in thymocytes.
1.3 Project II: RNase L mediates systemic humoral immune responses to
T-dependent and T-independent antigens

In contrast to the cell-mediated immune response, the systemic humoral immune response refers to immunologic actions against different types of extracellular and cell surface molecules, including polysaccharides, lipids, and proteins in non-cellular components of blood. As a highly complex and exceedingly specific response, the elaborated systemic humoral immunity relies on the appropriate activation of B cells upon antigenic stimulation. This activation is initiated through B cell receptor (BCR) crosslinking with an antigen, as evidenced by the phosphorylation of immunoreceptor tyrosine activation motifs (ITAMs) and subsequent assembly of the B cell signalosome in the site where BCR microclusters are formed [82-84]. Activation of naïve B cells stimulates their differentiation into antibody-secreting plasma cells. As a consequence, large amounts of antigen-specific antibodies are secreted, and thereby causing the destruction of extracellular microorganisms and preventing the spread of intracellular infections. According to the size, nature and structure of antigens, humoral immune responses can be divided into T cell dependent (TD) and T cell independent (TI) immune responses.

1.3.1 TD Antigens

Many antigens are unable to stimulate naïve B cells into an antibody-producing phase by themselves, therefore, require the cooperation of helper T-cells (T<sub>H</sub>) to induce B cell division and differentiation. By this reason, such antigens are designed as TD antigens (Figure 12). Protein antigens fall into this category. During a TD antigenic challenge, two signals are necessary for the naïve B cell to become activated: Firstly, its surface immunoglobulin must recognize protein
antigens; secondly, some of these antigens must be degraded, internalized, processed, and presented to the T<sub>H</sub> on a class II- MHC (major histocompatibility complex) molecule. Then, the T<sub>H</sub> will bind the naïve B cell using its antigen receptor, secrete cytokines, and finally activate B cells [85]. After initial T-B interaction outside the follicles, some activated B cells migrate back into the follicles of lymphoid organs accompanied by T<sub>H</sub> to form germinal centers. These germinal center B cells, undergo extensive somatic mutation of antibody-gene variable regions and antibody heavy chain isotype switching, produce the bulk of class-switched and high-affinity antibody responses to protein antigens, and give rise to both long-lived plasma cells and memory B cells [86,87]. Long-lived plasma cells migrate to specific niches within the bone marrow [88] and spleen [89], where they keep secreting high-affinity antibodies for prolonged periods [90]. In contrast, memory B cells continuously circulate without secreting antibodies.

2, 4, 6-Trinitrophenyl Keyhole Limpet Hemocyanin (TNP-KLH) is a classical TD antigen. As a hapten, TNP contains antigenic determinants (epitopes); since is not itself antigenic, it requires the aid of a carrier to stimulate a response from the immune system, under the form of antibody production [91]. KLH, a multisubunit and oxygen-carrying metalloprotein, is the most widely employed carrier protein for eliciting a TD immune response.

1.3.2 TI Antigens

Conversely, antigens that are capable of initiating a serological response independently without the T<sub>H</sub>, such as polysaccharides, lipids, and other non-protein are termed TI antigens (Figure 13). Usually, TI antigens possess multivalent and repeated carbohydrate moieties found on the cell walls of bacteria. This structural
characteristic enables TI antigens to activate naïve B cells through extensive cross-linking of multiple B cell receptors on the naïve B cells surface, which is strong enough to stimulate their proliferation and differentiation, without a requirement for Th [92, 93].

TI antigens can be further subdivided into two categories: TI type 1 (TI-1) and TI type 2 (TI-2). TI-2 antigens are polysaccharide antigens which are mostly high-molecular weight polymers with highly repetitive epitopes, such as Ficoll. Because TI-2 is not protein antigens, they cannot be degraded into a small molecule (peptide) and therefore cannot activate Th cells through presenting peptides to the Th cells in association with MHC class II molecules. However, the highly repetitive epitopes (characteristic of TI-2 antigens) are sufficient to induce BCR cross-linking which in turn efficiently induce plasma cell differentiation to produce antibodies [94]. Although the TI-2 response can be evoked without T-cell help, the regulatory T-cells have an influence on the magnitude of the response [95].

TI-1 antigens differ from TI-2 antigens in the senses they can independently induce antibody response without T cell regulatory activity for B cell activation, as TI-2 antigens usually do [96]. The most known TI-1 antigen, is based on bacterial products, such as lipopolysaccharides (LPS), which have mitogenic activity and induces polyspecific antibodies, though a polyclonal B cell activation when used at high concentrations; while, at low concentrations it induces LPS-specific antibodies. TNP-LPS and TNP-Ficoll are used extensively as TI-1 and TI-2 antigens, respectively, to induce TI immune response.
Figure 12 Activation of B cell by T-dependent antigen. Both, T-B interaction and BCR cross linking induce signaling for B cell activation and plasma cell formation.
Figure 13 Activation of B cell by T-independent antigen. Binding of multivalent antigens to B cells is sufficient to induce massive BCR cross linking, which is sufficient to activate B cells and induce plasma cell differentiation.
1.3.3 Systemic Antibodies to TI and TD Antigens

Upon activation by antigens, systemic antibodies are generated by plasma cells with distinct functions, but share the same antigen binding sites, such as the cell surface antibodies (B cell receptor), allowing identification of the antigen. Antibodies (Ab), also known as immunoglobulins (Ig), act as critical part of a systemic humoral immune response by specifically recognizing and binding to particular antigens and aiding in their destruction. Each isotype of Ig displays unique structural and effector property, as shown in the Table I.

As mentioned above, activation of B cells by TI antigens (polysaccharides and lipids) leads to a strong and rapid antibody response, mainly short-lived IgM, in the absence of T<sub>H</sub> cells [97]. Since TI antigens contain polysaccharide-rich capsules, IgM are prone to bind to capsular polysaccharides and target TI antigens, for phagocytosis. In contrast, protein antigens do not induce full B cell responses themselves but depend on T<sub>H</sub> to stimulate the antibody production.

During a TD antigenic challenge, the majority of activated B cells at the edge of lymphoid follicles are recruited back to the follicles of lymphoid organs, in cooperation with T<sub>H</sub>, to form germinal centers (GC) [98]. Interestingly, only TD antigens can induce germinal center response. GC B cells undergo massive rounds of proliferation, extensive isotype switching and somatic mutation of the Ig gene which induces the production of highly specific antibodies with great affinity. Isotyping switching process changes the constant region of immunoglobulin heavy chain, but without affecting the variable region of the heavy chain and light chain. For instance, an antibody isotype could switch from an IgM to an antibody of any of the possible classes IgG1, IgG2a, IgG2b, IgG3, IgA and IgE in mice [99]. Different antibody
isotypes display different functions, and therefore this process broadens the functional capacities of a systemic immune response. For instance, an important defense mechanism against most extracellular bacteria and viruses is to coat these microbes with opsonizing antibodies, which bind to phagocyte Fc receptors, and cause these microbes to be phagocytosed by neutrophils and macrophages. This reaction is best mediated by antibody isotype IgG1 and IgG3 [85]. The isotype switch is critically dependent on the type of cytokine(s) present. Three major cytokines, interferon-γ (IFN-γ), interleukin-4 (IL-4) and transforming growth factor-β (TGF-β) produced by T_H determine which heavy-chain isotype is produced. IFN-γ can stimulate antibody subclasses IgG1 and IgG3 to mediate phagocytosis. In a helminth infection model, a mouse that lacks the IL-4 gene cannot express IgE in response to a TD antigen. It is also well established that TGF-β induced IgA and IgG2b class-switching recombination in murine B cells, and contributed to IgA secretion at mucosal sites, probably because TGF-β promotes switching in these tissues (Figure 14) [85].

Furthermore, TD antigens also stimulate the somatic mutation process in GC with the help from T_H to produce antibodies with higher and higher affinity for the antigen, and give rise to long-lived plasma cells and memory B cells which are ascribed to provide the humoral memory [100]. Thus, effective host defense against a TD antigen is achieved through making different antibody isotypes in response to different types of microbes and affinity maturation, to improve the quality of the systemic humoral immune response.
<table>
<thead>
<tr>
<th>Name</th>
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| IgG  | Monomer   | ➢ Major Ig in serum  
➤ Key player in the humoral immune response  
➤ Activate the complement system  
➤ Phagocytosis of microorganisms  
➤ IgG are further grouped into subclasses: four in human (IgG1, IgG2, IgG3, IgG4) and four in mice (IgG1, IgG2a, IgG2b, IgG3) |
| IgA  | Monoer (serum); Dimer (secretions) | ➢ Second most common serum Ig  
➤ Hydrolyze carbohydrates in bacterial cell walls enabling the immune system to clear the infection  
➤ Two isotypes (IgA1 and IgA2) in human; mice have only one |
| IgM  | Pentamer  | ➢ Third most common serum Ig  
➤ First Ig during an immune response  
➤ Responsible for agglutination and cytolytic reactions |
| IgE  | Monomer   | ➢ Least common serum Ig  
➤ Allergic reaction, parasitic infections, and hypersensitively reactions  
➤ Widely found in the lung, skin and mucous membranes  
➤ Not fundamental in agglutination or complement activation |
| IgD  | Monomer   | ➢ Found on the surface on immature B cells  
➤ Low levels in the serum  
➤ Function is still not known |

Table I Antibody isotype collection: Immunoglobulins (Ig) are divided in isotypes: nine in humans (IgG1, IgG2, IgG3, IgG4, IgM, IgA1, IgA2, IgD, and IgE) and eight in mice (IgG1, IgG2a, IgG2b, IgG3, IgM, IgA, IgD, and IgE) [85].
Figure 14 Immunoglobulin heavy-chain isotype (class) switching [85]
1.3.4 Immunologic Adjuvant

In immunology, adjuvant has been widely applied in combination with specific vaccine antigens to accelerate, prolong, or enhance antigen-specific immune responses [101]. The use of granulocyte-monocyte colony stimulating factor (GM-CSF) as a molecular adjuvant is considered to be a promising approach for enhancing immune response. Behaving T_H characteristics, GM-CSF is known to promote macrophage differentiation and proliferation, activate antigen presenting cells. In addition, it has been well established that GM-CSF facilitates development of the systemic humoral immune response elicited by both TI and TD antigen, and already been demonstrated in vivo in several experimental and clinical settings [102].

Another one widely used adjuvant for amplifying a systemic humoral immune response, is a synthetic double stranded RNA poly (I:C), which is a potent inducer of IL-12 and type I IFNs that can induce activation of innate immunity via endosomally expressed TLR3 and the cytoplasmic receptor MDA-5 [103]. Pioneering studies showed that poly (I:C), through induction of type I IFNs, enhances dendritic cell maturation and B cell activation, leading to induction of potent T_H and humoral immune response, respectively, in mice with TD antigens [104]. Moreover, the co-administration of a vaccine and poly (I: C) proved that poly (I: C) is an effective adjuvant for inducing a potent antibody and multifunctional T_H response to circumsporozoite (CSP) based vaccine non human primates [105]. Collectively, both GM-CSF and poly (I: C) have been used as effective adjuvants for determination of the magnitude of the systemic immune response after stimulation.
1.3.5 RNase L on the systemic humoral immune response

As an IFN-inducible enzyme, RNase L is present at basal levels in most mammalian cells; however, it is highly expressed in the spleen, thymus and the cells of the immune system. In the RNase L−/− spleen lymphocyte population, the presence of Th, Treg, and B cells are lower than that in wild-type, after stimulation of LPS and PolyI: C [106]. In our preliminary study, it was observed that mice deficient of RNase L, developed enlarged and hypercellular thymus glands at the early age. In the project 1 of the thesis, the results demonstrated that the lack of RNase L led to abnormality of thymus, a consequence of impaired thymic homeostasis, since RNase L contributes to highly dynamic thymic environment through controlling proliferation and apoptosis of thymocytes. These findings confirm the role of RNase L in adaptive immunity. On the other hand, RNase L is known as a regulator of several cytokines, such as IFN-γ, TGF-β, IL-1β, IL-6, IL-10, CCL-2, Cox-2 and TNF-α, in immune cells. It modulates the activity and function of macrophages.
CHAPTER II

THE ROLE OF RNASE L IN TYMIC DEVELOPMENT

2.1 Introduction

The thymus, the central lymphatic organ arming the immune system against infection, plays a prominent role in the T cell development through a sequential process, but highly dynamic steps involving cell proliferation and apoptosis [107]. Specifically, the journey of T cell development in the thymus can be segregated into three stages: expansion, selection, and maturation [108]. The thymocyte precursors derived from hematopoietic stem cells (HSCs) in bone marrow first migrate to the thymus. These early T cell progenitors comprising only 1-3% of total thymus cellularity get populated through their high rate of proliferation, and eventually make up ≥ 80% of the total thymus cellularity. After proliferative expansion, they undergo lineage commitment, repertoire selections, and then emigrate from the thymus as mature functional T cells to establish the peripheral T cells pool.
When undergo differentiation, 95% of the newly divided thymocytes that mutate during rapid division die from apoptosis, and only less than 5% of the T cells generated in the thymus live to exit as mature cells [109]. The loss of cells in apoptosis is replenished with progenitor populations from the bone marrow and proliferating by themselves, which govern overall thymic homeostasis. It has been demonstrated that sustained thymic homeostasis is strictly controlled by the balance between proliferation and apoptosis of thymocytes. A disturbance within these two processes, which can be caused by abnormal growth and ability, may lead to either unwanted tissue atrophy or tissue hyperplasia. For example, thymus atrophy has been observed in adenalectomized rats treated with the synthetic glucocorticoid dexamethasone, a steroid that induces thymocytes apoptosis due to an increase in the rate of apoptosis of thymocytes that is not offset by an increase during mitosis. Appositively, tumor forms in the thymus, results from impaired apoptotic effect induced by the over-expression of anti-apoptotic bcl-2 that is not offset by a reduction in cell proliferation [110].

At the molecular level, dynamic protein tyrosine phosphorylation is considered to be a key regulatory mechanism responsible for immune homeostasis. Comparing with other cell types, cells in the immune system have relative high levels of protein tyrosine phosphorylation and express more genes encoding protein tyrosine kinases (PTKs) and protein tyrosine phosphatase (PTPs) [111]. The net result from the opposing effects of PTKs and PTPs contributes to the reversible tyrosine phosphorylation, thereby conducting immune cell signals either in a positive or negative manner, which plays a crucial function in cell-fate determination, leading to proliferate, differentiate or die. Recent years, increasing attention has been given to a few PTPs and their regulatory function in the immune system, particularly in T cells.
PTPH1, a member of non-receptor type of phosphatases, has emerged as a potent regulator in the T cell homeostasis. It reduced the TCR-induced activation through dephosphorylating the TCR ζ chain in Jurkat T cells [112]. Over-expressing of PTPH1 inhibited the activation of the transcription regulators NF-AT and AP-1, subsequently suppressed growth rate in Jurkat T cells [113]. The mutant forms of PTPH1 were found in colorectal cancers, indicating that PTPH1 is a tumor suppressor [114]. Being a cytoskeletal PTP, PTPH1 is characterized by N-terminal ezrin-, radixin- and moesin homology (ERM) domain and PDZ domain. The capability of PTPH1 to prevent persistent TCR signaling from inducing aberrant thymocytes proliferation and expansion depends on its membrane associated targets by ERM domain. In addition, the specialized PDZ domain of PTPH1 can interact with the cell surface receptor FAS, suggesting its engagement in FAS related apoptosis [115]. Nerveless, the proposed substrates/interactors of PTPH1 provides further insights into how PTPH1 exerts its inhibitory effect for T cells. Valosin-containing protein (VCP), a PI3K/Akt survival pathway associated cell cycle regulator, was identified as a selective substrate of PTPH1 [116]. All of these well-defined aspects suggest a versatile role of PTPH1 in T cells mediated immunity. In this study, we discover a novel signaling pathway associated with PTPH1 contributing to thymic dynamic homeostasis, which is mediated by RNase L.

RNase L, an interferon (IFN) inducible endoribonuclease, is a central player in the 2-5A system against viral infection and cellular proliferation [117-119]. In mammalian, RNase L is activated by 2', 5'-oligoadenylates (2-5A) which are synthesized from ATP by an oligoadenylate synthethase (OAS). OAS is induced by IFN, and becomes active upon binding to double-stranded viral RNA or cellular RNA with double-strand structure [120, 121]. After activation by 2-5A, RNase L degrades
viral mRNAs and cellular RNAs, thereby disrupts the replication of certain viruses and cell proliferation. In addition to antiviral action, the other functions of RNase L including pro-apoptotic effect, anti-proliferative activity and differentiation regulation have been documented, but relatively little is known about their role in T cell development and functions. Our preliminary study has shown that RNase L is highly expressed in thymus, whereas its deficiency lead to significant enlargement and hypercellularity of the thymus gland, however the physiological function of RNase L on thymus and the molecular mechanisms underlying these observations are still unclear. In this study, we first found that RNase L contributes to the well-balanced proliferative and pro-apoptotic thymocytes in mouse models. Importantly, the identification of PTPH1 and its substrate VCP by mass spectrometry based on phosphoproteomics indicated that RNase L targets PTPH1 to mediate a dephosphorylation cascade in the thymus. As a result, RNase L deficient mice exhibit dysregulated thymic protein tyrosine phosphorylation signaling, resulting in promoting proliferation and survival pathways, leading to disrupt the established equilibrium of proliferation and apoptosis. Taken together, our findings demonstrate that RNase L is an important modulator in thymic homeostasis through regulating PTPH1 signaling in thymocytes.

2.2 Materials and Methods

2.2.1 Mice, Cell Culture and Biological Reagents

RNase L-deficient mice were generated as described [122]. Primary thymic cells were isolated from wild-type and RNase L-deficient thymus at day 21 after birth and maintained in RPMI-1640 medium (Media Lab of the Central Cell Service, Cleveland Clinic, Cleveland, OH) supplemented with streptomycin (100µg/mL),
penicillin (100 units/mL) and 10% cosmic calf serum (Hyclone, Logan, UT) in a humidified atmosphere of 5% CO2 at 37 °C. Mitogens Con A and LPS purchased from Sigma (St. Louis, MO) were used as stimulators in vitro. MTS reagents were from R & D Systems (Minneapolis, MN). The BrdU detection kit and antibodies to B220-PE, mouse IgG2a-PE and IgG1-PE were from BD Bioscience. Antibodies to Bax, Bcl2, p-TYR, VCP, and PI3K were from Santa Cruz Biotechnology, Inc (Santa Cruz, CA). Antibodies to pan-actin and PTPH1 were purchased from Thermo Fisher. Antibodies to AKT, p-AKT, FLNA, and Cell cycle check points were from Cell Signaling.

2.2.2 Histological Analysis

Thymus glands were removed and were fixed in 10% neutral formalin overnight. Paraffin-embedded tissue was cut into sections 5 mm in thickness, and then stained with hematoxylin and eosin. The characteristics of thymus tissues were analyzed by researchers ‘blinded’ to sample identity.

2.2.3 BrdU Staining

BrdU (1 mg/ml) in PBS was i.p. injected to mice. Mice were sacrificed in 24 hr after BrdU injection. The same segment of the thymuses was fixed in 10% neutral formalin and paraffin embedded. Proliferating cells were detected with a BrdU detection kit (BD Bioscience). Tissues were counterstained with hematoxylin. The number of BrdU-positive cells was quantified by the number of cells in intact, well-orientated thymi.

2.2.4 Cell Viability Assay
Thymic cells were grown in RPMI 1640 with 10% FBS and incubated with ConA and LPS (Sigma, St. Louis, MO) at different concentrations respectively for 24 h. The effects of ConA and LPS on the cell growth were examined by MTS assays.

2.2.5 DNA Cell Cycle Analysis

For cell cycle analysis, thymic cell DNA was labeled with 20 μg/ml propidium iodine (Sigma, USA). The cell cycle was analyzed using FACScalibur flow cytometry with ModiFit software (Flow Cytometry Core Service, Cleveland Clinic, Cleveland, OH).

2.2.6 Flow Cytometric Immunophenotypic Studies

For membrane antigen staining, cells were trypsinized into single cell suspension in a total volume of 100μl PBS/BSA and immediately stained for 15min at room temperature in darkness with fluorescein (PE) labeled B220 antibody (Becton–Dickinson). Mouse IgG2a-PE or IgG1-PE (Becton–Dickinson) was used as isotype control. Stained cells were analyzed by FACScalibur flow cytometry (Flow Cytometry Core Service, Cleveland Clinic, Cleveland, OH).

2.2.7 Co-immunoprecipitation

Thymus extracts were incubated with 1 mg antibody and 2 ml protein A beads (Sigma). After overnight incubation, beads were washed four times with lysis buffer, and proteins were separated by SDS-PAGE and analyzed by immunoblot.

2.2.8 Western blot analysis

The expressions of several proteins in the apoptotic pathway and protein
phosphorylation profiles were examined by western blot analysis. Thymus glands were harvested from wild type and RNase L-deficient at day 21 after birth. Tissue extracts were prepared by lysing tissue with a homogenizer in Triton X-100 buffer. After centrifugation in a microcentrifuge at 4°C for 20 min, the supernatant was removed and stored at –80°C. Tissue extracts were fractionated on SDS-12% or 10% polyacrylamide gels (SDS-PAGE). Proteins separated by SDS-PAGE were electrophoretically transferred to polyvinylidene difluoride membranes (PVDF, Millipore, Billerica, MA) using a semi-dry blotting system. Membrane was blocked for 1 h with TBS-T buffer [20 mM Tris-HCl (pH 7.5), 200 mM NaCl, 0.05% (v/v) Tween 20] supplemented with 5% (w/v) of non-fat dry milk. Membrane was incubated overnight at 4 °C with different primary antibodies. After rinsing with TBS-T, the membrane was incubated for 1 h at room temperature with a corresponding secondary antibody conjugated with HRP (Cell Signaling Technology, Boston, MA). After washing, the blot was developed with an ECL detection kit (GE) by a chemiluminescence method, according to the manufacturer’s specification (Amersham, Piscataway, NJ) and the images will be scanned.

2.2.9 Phosphorylated Protein Enrichment

Approximately 2mg of total tissue extract was diluted to 0.5mg/mL in Lysis/Binding/Wash Buffer. The diluted tissue extract was added to a pre-equilibrated Phosphoprotein Enrichment Column (IMAC, Thermo Fisher) and incubated for 30 minutes on a rocking platform at 4°C. Non-bound protein fractions were collected by centrifugation at 1000 x g for 1 minute. The Phosphoprotein Enrichment Column was washed with Lysis/Binding/Wash Buffer (3 x 5mL). Each wash fraction was collected by centrifugation at 1000 x g for 1 minute. Phosphoproteins were eluted with elution
buffer (5 x1mL) with a 2-to-3 minute incubation between each centrifugation (1000 x g) step. Elution fractions were pooled and placed in a Thermo Scientific Pierce Concentrator 7 ml/9K MWCO (Part No. 89884) and centrifuged at 2500 x g to achieve 100 to 200μL (approx. 30 minutes). Protein concentrations were determined by Coomassie Plus Bradford Assay. Total purification time was 1.5 h.

2.2.10 LC-MS/MS Analysis

To identify the differential p-proteins, LC-MS/MS analysis was employed. The specific p-protein bands on the PVDF membrane after Western blot were excised, cutted into several pieces, and subjected to DMF-assisted trypsin digestion. The digested peptides were analyzed by LC-MS/MS (Department of Chemistry, Cleveland State University). Specifically, the pieces of the membrane containing p-protein were dried in a Speed-Vac and then dissolved in pure DMF (≥ 99.8%). The suspension were diluted with 50mM NH₄HCO₃ buffer (pH8.0) to contain 40% DMF and added with 20 μl of 2 μg trypsin, and incubated overnight at 37 °C. After digestion, the solution was further diluted by adding three-fold volume of deionized water. After centrifugation, the supernatant was collected. The debris were washed in 80 μL of 70% ACN and 1% TFA with sonication for 30 min and then centrifuged. Both supernatants were combined and concentrated in a Speed-Vac and stored at -20 °C until further use. To identify the unknown proteins, HPLC conjugated electrospray ionization (ESI) ion trap mass spectrometry were employed. The samples were separated on an Alltech Vyduc MS C18 column (300 A, 5µm, 100 mm × 300µm) at a flow rate of 5 μl/min using a mobile phase of (A) 0.1% v/v formic acid in water and (B) 0.1% v/v formic acid in acetonitrile. The chromatography system was directly coupled to an ESI Ion-trap mass spectrometer. Raw spectrum data were processed and
MASCOT-compatible mgf files were created using DataAnalysis software (Bruker Daltonics). Peptide searching was performed using MASCOT software (Matrixscience, London, UK). The NCBI nr database was used for protein identification. The identified proteins were further confirmed by Western blot analysis using antibodies to these p-proteins.

2.3 Results

2.3.1 Thymus Enlargement and Hypercelullarity in RNase L-Deficient Mice at Early Age

Since RNase L is highly expressed in the thymus, gross examination of postnatal thymus development in wild-type and RNase L-/- mice at several time points from birth up to 36 weeks of age, was performed. RNase L-/- mice displayed major thymus abnormalities as evidenced by larger thymus sizes at early age in both female and male mice (n=3, p<0.01) from 1 week after birth. The size and weight of thymus peak at around 3 weeks of age, showing about a 2-fold increase of thymus weight and a 1.5-fold increase of thymus weight/body weight ratio in RNase L-/- mice as compared to that in wild-type mice, then decrease progressively with age (Figure 15 and Figure 16). Besides, the cell counts of dissociated thymi also revealed a significantly elevated cell number of thymocytes in RNase L-/- mice in compared with age-matched wild-type mice. At postpartum week 3, the mass of RNase L-/- thymus had, in average, a 2-fold increase as compared with the cell counts in wild-type thymus (n=3, p<0.01, Figure 17). Histological analysis suggested that the microscopic structure of the thymus was largely unaltered, although increased density of cortex zones and relatively smaller medullary areas were observed in RNase L-/-
thymus from birth until adolescence (Figure 18). Interestingly, RNase L-/− mice showed a population of infiltrated eosinophils which might play a pivotal role in T cell development in the thymus and early initiation of allergic Th2 responses.

Figure 15 Abnormal Thymus Weight in RNase L-Deficient Mice. Thymi were isolated from wild-type (+/+), and RNase L-deficient (-/-) littermates at postpartum week 0.5, 1, 2, 3, 4, 8, 12 and 36. After sacrificing mice, both the body and thymuses were weighed. n=3 for each group. Data are shown as means ± standard deviation (SD).
Figure 16 Abnormal Thymus Cell Count in RNase L-Deficient Mice. Thymic cell counts at postpartum weeks 3 of wild-type (+/+) and RNase L-deficient (-/-) mice. n=3 for each group, p<0.01. Experiments were performed in triplicate, and averages of the cell counts are presented. Data are shown as means ± standard deviation (SD).
Figure 17 Enlarged thymi in RNase L-deficient mice. Thymi dissected from 3-week-old male wild-type (+/+) and RNase L-deficient (-/-) mice were compared. n=5 for each group, p<0.01. [144]
Figure 18 Histological analysis of the thymus. H&E-stainings of sections from wild-type (+/+ ) and RNase L-deficient (-/-) thymi at postpartum week 1, 3, and 12 (4x magnification) were performed and the photos were taken at different magnifications. n=3 for each group, p<0.01.
2.3.2 The Thymus of RNase L-Deficient Mice Exhibits Altered Homeostasis

Previous studies have shown RNase L played a broad role of controlling cellular proliferation and apoptosis in an IFN-dependent or independent fashion (ref 2, 3). It is very likely that enlarged and hypercellular thymi in RNase L-/- mice suffer from an instinct proliferative disorder. Therefore, the proliferation status of the thymi from RNase L-/- mice was compared with that in wild-type mice by labeling the proliferative thymocytes with Brdu administrated by intraperitoneal injection. There was a 3-fold increase in number of BrdU-positive proliferating thymocytes in the thymus cortex as well as in the thymus medulla of the RNase L-/- thymus as compared to that in wild-type mice (Figure 19). Thymic cortex contains immature DN and DP thymocytes subjected to positive selection, while the medulla contains more mature thymocytes that undergo negative selection. Our results indicate that the thymocytes of RNase L-/- mice undergo a dysregulated proliferation during both positive and negative selection. In addition to comparison of proliferative capacity in vivo, in vitro proliferative ability of thymocytes was investigated, under both mitogen dependent and independent conditions. Without mitogen stimulation, there was approximatively 2-fold increase of population in RNase L-/- thymocytes when compared to that in wild-type thymocytes; after exposure to mitogens for 72 h, the difference became more profound as RNase L distinguishably suppressed murine thymocytes proliferation induced by ConA and LPS. In the presence of ConA, RNase L-/- thymocytes grew 3.53-fold faster when compared to wild-type thymocytes (most significantly at a concentration of 1.875μg/ml). Similarly, LPS promoted a 3.10-fold increase of proliferation rate in RNase L-/- thymocytes (most significantly 0.5 μg/ml) (Figure 20). These findings demonstrate our hypotheses that RNase L exerts potent
inhibitory effect on thymocytes proliferation.

On the other hand, we considered that another explanation for the growth inhibition imposed by RNase L could be the induction of apoptosis appropriately. To elucidate the pro-apoptotic effect of RNase L on thymocytes, (B220 is not an aapoptotic marker) immune blotting of Bcl-2 family: pro-survival Bcl2 and pro-apoptotic Bax (Figure 21 and Figure 22) provides direct evidence showing impaired apoptosis in RNase L-/thymus. In summary, RNase L probably functions as an important modulator in governing dynamic homeostasis of thymus by proliferation and apoptosis control of thymocytes.
Figure 19 Increased BrdU positive thymocytes in RNase L deficient thymi. Bromodeoxyuridine (BrdU, 1mg/ml) was administrated to age- and sex-matched wild-type (+/+) and RNase L-deficient (-/-) mice by i.p. injection. After BrdU injection for 24 hr, the organs were excised and the tissue sections were stained for BrdU-positive cells (stained brown). Hematoxylin (blue) was used as counter staining to visualize the whole tissue. n=3 for each group. Data are quantified. Error bars present ±SEM, and Student’s t test was used. P<0.01.
Figure 20 Increased thymocytes proliferation in RNase L deficient thymi. Effect of RNase L on murine thymocytes proliferation stimulated by Concanavalin A (ConA) and Lipopolysaccharide (LPS). Thymocytes isolated from 3-week-old and sex-matched wild-type (+/+) and RNase L-deficient (-/-) mice (1 x 10^5) were suspended in RPMI 1640 supplemented with 5% FBS. Cells were exposed to ConA or LPS with different concentrations. Cell proliferation was determined using CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega). A treatment group consisting of six wells and independent experiments were performed twice. The data are expressed in terms of mean ±SD, P<0.01.
Figure 21 FACS analysis of B220 positive thymocytes
Figure 22 Western blot analysis of Bcl-2 family expression in the thymus
2.3.3 RNase L-mediated Thymocytes G1/S Cell-Cycle arrest

To determine the role of RNase L in thymocyte cell-cycle intervention and identify which stages of cell cycle are affected, total thymocytes were isolated from wild–type and RNase L-/- thymi and the percentile of these cells at each phase of a cell cycle were analyzed by flow cytometry. As shown in Figure 23, there was a slight but still significant increase (% increase; p<0.01) in the percentage of total thymocytes in the G0/G1 phase of the cell cycle in thymocytes isolated from wild-type mice. Apparently the number of RNase L-/- thymocytes was higher in the S phase and lower G0/G1 populations. These results indicate that RNase L may mediate the late G1/S arrest of the cell cycle.

In order to elucidate how RNase L contributes to the cell cycle control, western blot analysis for key proteins involved in G1 and S check points, was performed. As shown in Figure 24, the elevation of cyclinD1, cyclinD3, CDK2, CDK6, and p-RB during cell cycle progression was significantly abolished by RNase L. Most importantly, we found that the level of P27, a cell cycle inhibitor, was distinctively associated with RNase L and lack of RNase L remarkably down regulated the protein, suggesting that RNase L mediated cellular growth suppression may be through regulating p27. These data demonstrate our hypothesis that RNase L plays an important role in cell cycle control by up-regulating p27 to inhibit thymocytes growth, leading to thymus homeostasis.
Figure 23 Distribution of thymocytes in the cell cycle. The age- and gender-matched wild-type (+/+) and RNase L-deficient (-/-) mice were euthanized, and thymocytes suspensions were prepared, stained with PI. Cells were subsequently analyzed by using a FACS flow cytometer, and the cell population of the cells at each phase was determined with Dean and Jett cell cycle algorithms. Wild type (+/+, left panel) or RNase L-deficient (-/-, right panel).
Figure 24 RNase L inhibits cell cycle progression by regulating the proteins at the G1/S cell-cycle check points.
2.3.4 RNase L mediates tyrosine-phosphorylation cascades to attenuate cell cycle progression

It is well recognized that protein tyrosine phosphorylation is an important event in the regulation of cell cycle progression [12]. The facts that higher numbers of RNase L-/- thymocytes at the S phase and lower population at G0/G1 implicate a possible role of RNase L in mediating cell cycle progression. To test this hypothesis, we assessed and compared the global phosphotyrosine profiling in RNase L-/- thymus with wild-type thymus in order to gain new insight into candidate factor(s) that participate in the cell-cycle dependent tyrosine phosphorylation. Western blotting analysis probed with a p-Tyrosine (p-Tyr) antibody was performed for the thymus tissue extracts prepared from RNase L-/- and wild-type mice. Strikingly, the tyrosine-phosphorylated proteins profile, particularly a 100 kDa band, was dramatically different in the thymus tissue extracts from RNase L-/- mice compared with that from wild-type mice (Figure 25). It is important to point out that addition of ATP and P3A3, the activator of RNase L, did not affect the phosphotyrosine profiling and the expression level of the 100 kDa protein, suggesting that this tyrosine-phosphorylated protein exists in vivo and they were not phosphorylated by any kinases in the tissue extract. Actually, this observation has been further substantiated by comparison of p-Tyr-protein profiles in the samples with or without incubation at 30°C. The outcome of this study implies that the reduced tyrosine phosphorylation signals in the RNase L +/+ thymic extract may be associated with the activity of certain tyrosine phosphatases in the thymus, resulting in specific dephosphorylation of those tyrosine-phosphorylated cell-cycle regulator candidates, therefore, leading to thymocytes cell-cycle homeostasis.
Figure 25 Determination of Tyrosine-phosphorylated proteins in the thymus. Thymus tissue extracts prepared from RNase L deficient and wild type mice were incubated in the presence or absence of ATP (1μM) and P3A3 (1μM) at 30 °C for 30 min, and then subjected to Western blot analysis using an antibody to p-Tyr (Santa Cruz: sc-7020).
2.3.5 Identification of phosphorylated substrate VCP by phosphoproteomic analysis

To identify the 100 kDa unknown protein, a systematic phosphoproteomic analysis of the tyrosine-phosphorylated protein was performed by using RNase L/- and wild type thymuses. After phosphoprotein enrichment, the concentrated samples were separated by SDS-PAGE, followed by Coomassie blue staining (Figure 26) or Western blot analysis probed with a p-Tyrosine (p-Tyr) antibody (Figure 27). Before enrichment, the 100 kDa tyrosine-phosphorylated protein existed in the presence of a large excess of relatively abundant proteins. After enrichment, the protein of interest was purified and further concentrated in the eluted fraction. The entirely absence of this protein from the wash fraction and its emergence in the eluted fraction demonstrated the efficiency and specificity of our enrichment methodology. The parallel western blot probed with a p-Tyr antibody revealed that partial enriched proteins were phosphorylated on tyrosine residue.

Next, a mass spectrometric based phosphoproteomic strategy was implemented to identify and characterize the tyrosine-phosphorylated 100 kDa protein. By comparing gel-based with membrane-based [16] separation and enrichment strategies, we were able to reliably identify the 100 kDa candidate as VCP which was highly phosphorylated in RNase L/- thymus. Several novel tyrosine-phosphorylated sites were identified as shown in Figure 28. The qualitative protein identification results from two parallel sample preparation experiments were consistent with each other.

To further confirm its identity, tyrosine-phosphorylated VCP in RNase L/- thymus exacts was validated using dual-direction immunoprecipitation and
immunoblot assays. Tyrosine phosphorylated proteins or VCP itself in wild type and RNase L−/− thymus extract were pulled down by utilizing a primary antibody for p-Tyr (Santa Cruz: sc-7020) or VCP (Santa Cruz: sc-20799). Western blot analysis indirectly confirmed those P-Tyr pulled down proteins in RNase L−/− thymus were VCP and those VCP pulled down proteins were highly phosphorylated at tyrosine residue (Figure 29). Measuring relative tyrosine-phosphorylated protein as well total protein, surprisingly it was observed that although the level of total VCP did not change, the tyrosine-phosphorylated VCP was significantly reduced in the presence of RNase L. These results indicated that RNase L regulated VCP, a cell-cycle regulator, in a dephosphorylation-specific manner. Probably, RNase L exerts its regulatory role in thymic homeostasis through the dephosphorylation of VCP.
Figure 26 Phosphorylated protein enrichment followed by CCB staining. SDS-PAGE followed by Coomassie blue staining of phosphorylated proteins from wild-type (+/+), and RNase L-deficient (-/-) thymus extracts. Lane A and B, phosphoprotein + mixture of unphosphorylated proteins; lane C and D, buffer wash; lane E and F, flow through; lane G and H, concentrated flow through fraction.
Figure 27 Phosphorylated protein enrichment followed by western blot. Western blot analysis was performed using an antibody for p-Tyr (Santa Cruz: sc-7020) that detect tyrosine-phosphorylated events in wild-type (+/+) and RNase L-deficient (-/-) thymus extracts. Lane A and B, phosphoprotein + mixture of unphosphorylated proteins; lane C and D, buffer wash; lane E and F, flow through; lane G and H, concentrated flow through fraction.
DMF assisted on membrane digestion and MS/MS

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In gel digestion after phospho-protein enrichment

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**Variable modifications (PTMs): p-STY modification**

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Figure 28 Identification of the 100 kDa tyrosine-phosphorylated protein. Identification of tyrosine-phosphorylated Vcpp97 from RNase L deficient mouse thymus by LC-MS/MS and Mascot Search.
Validation of Phospho-VCP: Western Blot after IP

Antibody Information:
- p-TYR: Santa Cruz Phospho-Tyrosine Antibody #sc-7020
- VCP: Santa Cruz VCP Antibody #sc-20799

Figure 29 Validation of the 100 kDa tyrosine-phosphorylated protein. Validation of tyrosine-phosphorylated Vcpp97 from the RNase L deficient mouse thymus using immunoprecipitation followed by western blot. Left: p-Tyr IP; Right: VCP IP
2.3.6 RNase L mediates PI3K/Akt cell survival pathway through PTPH1

Because of this reduced VCP phosphorylation signal associated with RNase L, it is possible that certain protein tyrosine phosphatases (PTPs) are regulated by RNase L directly or indirectly, which dephosphorylate the cell cycle regulator VCP, ultimately governed the dynamic homeostasis of thymus. Since VCP has shown to be the substrate of protein tyrosine phosphatase PTPH1, it is likely PTPH1 is the targeting molecule of RNase L to suppress the tyrosine phosphorylation cascade in regulating a variety of cellular processes including cell growth, differentiation, and mitotic cycle. To demonstrate it, we compared the expression level of PTPH1 in wild type thymus with RNase L−/− thymus. Clearly, PTPH1 was highly expressed in wild type thymus, whereas its level was significantly reduced in RNase L−/− thymus. The results suggested that the attenuated VCP phosphorylation in RNase L+/+ thymus might be due to an increased level of PTPH1 (Figure 29). Next, the impact of RNase L on PTPH1 was analyzed. Co-immunoprecipitation was performed to examine the interaction between RNase L and PTPH1. After pulling down proteins by using an anti-RNase L antibody, the expression level of PTPH1 was compared. Although PTPH1 was unable to be pulled down by the RNase L antibody, our data indicate that PTPH1 might be the target molecule of RNase L responsible for dephosphorylating VCP.

In addition to tyrosine-phosphorylated VCP, serine and threonine phosphorylation of VCP, from mascot peptides searches, was identified. Previous studies reported that VCP was a selective substrate of the Akt signaling pathway involved the control of cell survival and growth [12]. Interesting, the expression of p-Akt was highly induced in the thymus tissue of RNase L−/− mouse (with equivalent
expression of Akt), as well as PI3K (Figure 30). The hyperactivation of PI3K/Akt pathway in RNase L-/ thymus was probably responsible for the increased thymocytes proliferation and thymus growth. In conclusion, the results showed that due to the abnormality of PTPH1, VCP was highly phosphorylated during the early stage of thymus development in RNase L-/ mice, which in turn promoted the activation of PI3K/Akt pathway important for thymocytes survival and proliferation.

![Western blot analyses of PTPH1 and the PI3K/Akt pathway](image)

Figure 30 Western blot analyses of PTPH1 and the PI3K/Akt pathway
2.4 Discussion

It is well established that RNase L has an essential role in IFN-involved host defense against viral infection. In this study, we investigated the RNase L-involved cellular proliferation, differentiation, and immune activation in an IFN-independent fashion. The outcome of this study reveals a novel role of RNase L, both in anti-proliferative and pro-apoptotic responses of thymocytes, aiming at maintaining dynamic thymic homeostasis. Our results demonstrated that there is a fine balance of molecular signaling governing thymic environment critically involving RNase L; possibly relying on PTPH1, a well-known protein tyrosine phosphatase of T cells activation and growth, and its substrate VCP, a strict phosphorylation-dependent cell cycle regulator. On the other hand, deficiency of RNase L significantly suppresses the expression of PTPH1 in thymocytes, which leads to sustained phosphorylation of VCP, affecting cell cycle progression, disrupting the homeostatic balance of the proliferation and apoptosis. This result is consistent with the observation that the enlarged and hypercellular thymus gland was developed in RNase L-/- mice. Therefore, this study extends the function of RNase L to the adaptive immune system, involving thymocytes proliferation and apoptosis.

In this report, we show evidence of imbalanced thymic homeostasis related to defect of RNase L. RNase L-/- mice exhibited severe thymus abnormality, characterized by time-dependent thymic hypertrophy. The number of total thymocytes was significantly higher in these mice starting from 1 week after birth and the increase was further evident at 3 weeks. In addition, there were much more BrdU incorporated proliferating cells during S phase in RNase L-/- thymus than that in control mice, indicating an intrinsic proliferative disorder in RNase L-/- thymocytes. Nerveless, a
significant elevation of thymocytes proliferation under mitogens stimulation was also observed in RNase L-/- thymocytes. Most importantly, the results of this study demonstrated that the presence of RNase L caused thymocytes G1/S arrest associated with p27KIP upregulation, as supported by both cellular DNA content measurement and cell cycle checkpoint analysis, while absence of RNase L resulted in accelerated proliferation and impaired growth arrest. Most recent studies have already reported that RNase L was capable of modulating certain proteins to exert its regulatory activity in cell cycle phase. For examples, RNase L down modulated HuR, a regulator of cell cycle progression, to retard cellular growth in fibroblasts [116]. Similarly, RNase L mediated tristetraprolin (TTP) expression which orchestrated p21CIP1-WAF1 induced cellular growth arrest in MEFs. Thus, our findings suggested that blockage of p27 might be the cause to disrupt homeostatic thymic environment in RNase L-/- mice, which expanded the cell-cycle regulatory role of RNase L in p27 induced growth arrest at G1/S phase, offering a plausible explanation on how RNase L contributes to thymocytes proliferation in an appropriate manner.

The fact that thymic homeostasis is strictly controlled by the balance between cell proliferation and cell death in order to guarantee proper immune function [123], suggesting that the induction of apoptosis by RNase L in thymus is likely to be another important factor for maintaining dynamic thymic environment. Compared with wild-type, RNase L-/- thymus contains relative less pro-apoptotic thymocytes indicating the increased survival of RNase L-/- thymocytes. The regulation of cell survival is believed to rely on the effect of central anti-apoptotic and pro-apoptotic Bcl2 family proteins. Interestingly, we found an elevated level of anti-apoptotic Bcl2 and reduced level of pro-apoptotic Bak in RNase L-/- thymus. These results provided a mechanism for the increased thymocytes survival in RNase L-/- mice, supporting
the hypothesis that RNase L mediates the proliferation and apoptosis of thymocytes to maintain overall thymic homeostasis. Future efforts are directed toward investigating the thymocyte subsets and their function that are responsible for adaptive immunity.

It has been reported that reversible protein tyrosine phosphorylation serves as an exquisite switch to activate or abolish proliferative and apoptotic pathways [124-126]. Indeed, we demonstrated by using systematic phosphor-proteomic approaches, that RNase L influence the tyrosine phosphorylation of the cell cycle regulator VCP, suggesting that VCP may be a possible target of RNase L to exert the regulatory effect on thymocytes growth. Being a member of the AAA family of ATPase, VCP highly tyrosine-phosphorylated in activated T cells is involved in diverse biological activities such as cell cycle regulation, protein degradation, organelle biogenesis and vesicle-mediated protein transport [127]. The function of VCP for normal cellular function relies on its cell-cycle-dependent phosphorylation on tyrosine residues through a conformational change, which adds phosphoric acid groups on acidic residues repelling the carboxyl terminus further to the core of the protein and exposing the targeting signal sequence previously masked by a starch of acidic residues [128]. Inappropriate tyrosine phosphorylation of VCP would be expected to result in disorder of cell division. Previous studies showed that mutation of tyrosine residue 834 can abolish tyrosine phosphorylation of VCP, leading impaired cell cycle progression and growth retardation [127]; whereas mutation of the same residue allowing phosphorylation on tyrosine residue generated normal cell growth [129]. Thus, the enlarged hypercellular thymus and related disturbance of thymocytes proliferation and apoptosis in RNase L-/- mice may be attributed to the enormous tyrosine phosphorylation of VCP. The regulatory effect of RNase L on thymic homeostasis appears to be associated with downregulating the inhabitable tyrosine
phosphorylation of VCP, possibly via protein tyrosine phosphatase. Interestingly, recent studies reported that rather than tyrosine residues, several serine/threonine residues on VCP can also be selectively phosphorylated by pro-survival serine/threonine kinase Akt [127]. The influence of Akt induced VCP phosphorylation occurs mainly in promoting anti-apoptotic effects and cell survival. In addition to profile VCP tyrosine phosphorylation, those phosphorylation sites on either serine or threonine of VCP were identified by means of mass-spectrometry. Western blot results clearly indicated that constitutive activation of PI3K / Akt signaling was present in the RNase L deficiency-thymus. Although, VCP immunoprecipitations followed by proteomic analysis didn’t prove any direct interaction between RNase L and VCP, however, a group of putative VCP-interacting proteins were identified belonged to PI3K/Akt signaling pathway. As discussed above, cell cycle inhibitor P27, which has been regulated by Akt negatively, was significantly suppressed in RNase L-/- thymus. It is possible that hyperactivation of Akt signaling may be responsible for the decreased P27 presence in the RNase L-/- thymocytes during their failure of cell cycle arrest. The outcome of this study provided novel insights into the mechanisms underlying the facts that (a): the differential phosphorylation level of VCP between wild type and RNase L-/- thymus involves RNase L to targets protein tyrosine phosphatase; (b) dephosphorylation of p-VCP existed on tyrosine residue; (c) RNase L down-regulates PI3K/Akt signaling which dephosphorylates VCP on serine/threonine residue; (d) the phosphorylation of VCP in turn inhibits thymocyte proliferation and promotes pro-apoptotic response through cell cycle arrest, leading to the thymic homeostasis.

Furthermore, it was demonstrated that PTPH1, a potent protein tyrosine phosphatase selectively dephosphorylates the C-terminal tyrosine of VCP, was the
targeting PTP of RNase L. The fact that loss of RNase L dramatically reduced the expression level of PTPH1 in thymus was associated with the elevated level of tyrosine phosphorylated VCP in RNase L-/− thymus implicated that RNase L may interfere with the phosphotyrosine-dependent regulation of VCP via PTPH1 in the cell cycle, resulting in growth inhibition. The results were consistent with a previous discovery that PTPH1 was necessary for growth arrest in NIH3T3 cell lines, also inhibited T cells proliferation through inactivating TCR signaling, which were manifested primarily through dephosphorylation of VCP (\(\text{VCP}\)). Importantly, the inhibitory ability of PTPH1 is believed to rely on its ERM domain which targets F-actin at the plasma membrane. In addition, other transmembrane proteins containing a PDZ binding motif, also interact with PTPH1, such as TACE (tumor-necrosis-factor converting enzyme), a member of the ADAM protein family of integrins and metalloproteinases, leading to shedding and releasing a diverse variety of membrane-anchored cytokines, cell adhesion molecules, receptors, ligands, and enzymes upon stimulation (such as TNF-\(\alpha\)). It is well known that RNase L functions as a key player in IFN function against viral infections and regulates the production of a broad range of inflammatory cytokines such as TNF-\(\alpha\), IL-1\(\beta\), Cox-2, IL-6 and IFN-\(\beta\). It was also found that RNase L is located in cytoskeleton and associated with diverse cytoskeleton proteins although the underlying mechanism of action is unclear. For instance, filamin A, a scaffold protein binding constituents of signal pathways such as plasma membrane receptors, calmodulin, caveolin, protein kinase C, transcription factors, etc., was reported to interact with RNase L [116]. Nevertheless, the structure analysis of RNase L reveals that RNase L contains 9 ankyrin repeats, which are typical protein-protein interaction domains [130]. Hereby, it was hypothesized that RNase L may be either directly interacted with PTPH1 or indirectly
through other binding partners on the membrane, resulting in profound impacts on the cellular level involving cytokines shedding, that triggers an apoptotic cascade in the thymus and cell-cycle-dependent dephosphorylation of VCP to repress thymocytes growth, which in turn contributes to thymic homeostasis.

In summary, based on the findings in this study, we proposed a model in which RNase L is responsible for the dynamic balance of the thymic environment in mice. Mechanistic studies suggested that RNase L may exert its regulatory role in thymic homeostasis through PTPH1 and its substrate VCP. Thus, RNase L may be a promising therapeutic target for immunological disease [131, 132].
CHAPTER III

RNASE L MEDIATES HUMORAL IMMUNE RESPONSE TO T-DEPENDENT AND T-INDEPENDENT ANTIGEN

3.1 Introduction

Ribonuclease L is a pleiotropic interferon (IFN) inducible enzyme in the 2’, 5’-linked oligoadenylates (2-5A) system that was originally characterized as an antiviral effector of IFN [133, 134]. Serving as a RNA degradation pathway, the 2-5A system is triggered by double stranded RNA molecules (dsRNA) after viral infection and regulated by two major enzymes: 2-5A synthetase (OAS) and RNase L [135]. RNase L is the terminal component of the 2-5A system and requires 2-5A for its activity in order to degrade single-stranded RNA (ssRNA) [136], leading to suppression of certain viruses replication [137-139]. In most cases, it has been demonstrated that RNase L directly cleaves viral , as a result, inhibit the replication of RNA viruses, however, the role of RNase L in immunomodulatory activities suggest an alternative or additive mechanism for its antiviral function may exist.
RNase L is expressed constitutively at basal levels in most mammalian cells. Quite interesting, tissue distribution analysis has revealed that RNase L is highly expressed in many immune cells including murine bone marrow cells, B cell through ontogeny, subsets of T cells, natural killer cells, macrophages, granulocytes, neutrophils, platelets and pancreatic β cells, as well as in lymphatic organs such as the thymus and spleen [140]. The expression level of RNase L in immune cells is tightly regulated and the extent is markedly enhanced after stimulation by immune mediators or toxins such as granulocyte-monocyte colony stimulating factor (GM-CSF), a molecular adjuvant for enhancing immune response, and polyinosinic:polycytidylic acid (polyI:C), a harmful inducer of IL-12 and type I IFNs [141]. In clinical trials, RNase L has been recognized for many years as a marker for diagnosing chronic fatigue syndrome (CFS), an illness characterized by debilitating fatigue associated immunological abnormalities with no known etiology [142]. More recently, genetic and biological studies have suggested that RNase L may be a potent regulator in modulating the immune response to exogenous pathogens and altered cells [143-147]. Given that RNase L deficient mice displayed remarkably enlarged thymus glands containing excessive numbers of thymocytes is believed to result from reduced T cell apoptosis, whereas overexpression of RNase L in the cells leads to enhanced apoptotic activities [148]. The impact of RNase L on the subclasses population of T cells was also observed. Physiological studies have revealed that skin allografts rejection was suppressed in RNase L-/- mice, suggesting a crucial role of RNase L in CD4+ (T\textsubscript{H}) mediated immunity [149]. Nevertheless, it also has been showed that T lymphocytes isolated from RNase L deficient mice displayed markedly attenuated cytotoxic activity by 46% lower against retinal endothelial cells, indicating its influence on CD8+ population. These observations implicate that RNase L may contribute to T
cells development and therefore regulate T cell mediated immune response to combat microbes.

In addition to its impact on the development and activation of T cell mediated immunity, RNase L also exhibits extraordinary immunomodulatory ability in modifying a number of cytokine secretions in immune cells. Early microarray research already identified a line of proinflammatory cytokines such as TNF-α, IL-1β, IL-6, cox-2, IFN-α and IFN-γ as targets of RNase L [150]. Subsequent investigations of cytokine production in various immune cells during viral and bacterial infection provided definitive evidences for the immunomodulatory role of RNase L [151]. For example, polyI:C treatment significantly induced an increase of TNF-α and IFN-γ production in RNase L+/+ islets, offering an explanation for the enhanced apoptosis of pancreatic β cells, resulting in diabetes onset. Lack of RNase L remarkably attenuated macrophage function and altered cytokines levels of TNF-α, Cox-2, CCL-2, IL-10 and TGF-β. As critical factors in the B cell-directed humoral immune system, cytokines strictly control the initiation of immune response, antibody class switching process and the populations of immunoglobin isotypes and responsible for determining which isotype is produced against specific antigens. During the antibody isotype switching processing, the IgH constant (C) region μ (Cμ) and C (Cδ) gene coding IgM and IgD are replaced by any of downstream IgH C region exons such as Cγ, Cα or Cε encoding IgG (IgG1, IgG2a, IgG2b, IgG3 in mice), IgA and IgE respectively. Two major cytokines, IFN-γ and TGF-β have been well documented for the event. IFN-γ is capable of stimulating antibody subclasses IgG1 and IgG3 to mediate phagocytosis. On the other hand, it has been established that TGF-β induces IgA and IgG2b class-switching recombination in murine B cells, and contributes to IgA secretion at the mucosal sites. Although the exact mechanism by
which RNase L modulating cytokines secretion remains to be determined, it has been shown that regulation of ARE-binding protein tristetraprolin (TTP) dependent cytokine mRNA stability by RNase L might be involved in cytokine production [152]. Nevertheless, our preliminary study revealed that the population of B220+/IgD double labeled B cells was remarkably down after LPS and poly I: C treatment in the spleen of RNase L deficient mice, implicating that deficiency of RNase L might attenuate the activation of B cells and lead to reduced B cell population in response to harmful molecules. Indeed, a cancer vaccine study had shown that alphavirus-based DNA vaccination against a non-mutated tumor-associate self-antigen (tyrosinase related protein-1, TRP-1) was severely impaired in RNase L deficient mice, indicating the involvement of RNase L in B cell-mediated immunity [153]. Thus, the regulation of proinflammatory cytokines by RNase L might provide an explanation for the diminished antigenicity observed. Taken together, these data suggest that RNase L may mediate certain antibody response in B cells mediated adaptive immune system via regulating the production of cytokines.

In the present study we utilized RNase L deficient mice to elucidate the biological functions of RNase L on systemic humoral immune responses, as well as to extend our understanding of the role of RNase L in B cell activation pathways. Our results unveiled that deficiency of RNase L caused impaired systemic humoral response compared with that in RNase L+/+ mice, which was consistent with the observation that relative smaller spleen and insufficient protein phosphorylation in the spleen tissue extract of RNase L-/- mice after antigen challenges. Furthermore, RNase L influenced adjuvant-directed immunogenicity, which demonstrated RNase L might participate in different regulatory pathways according to the type of antigens. These findings highlight the impact of RNase L on humoral immunity, suggesting the
potential benefit of targeting RNase L in immunological disease treatment.

3.2 Materials and Methods

3.2.1 Animals and Reagents

Wild type and RNase L -/- male mice (8-10 weeks) on the C56BL/6 genetic background (23 to 26 g) were kept under specific pathogen-free conditions. Trinitrophenyl (TNP)-Ficoll (TI type II antigen), TNP–LPS (TI type I antigen) or TNP-KLH (TD protein antigen) were used as stimulators in vivo to initiate immune response. All antigens and adjuvants were obtained from Biosearch Technologies.

3.2.2 Immunization protocol in vivo

For regular immunization, eight-to-ten week-old mice were immunized intraperitoneally (i.p.) with either 100 μg/100μl of TI or TD antigens in complete Freud’s adjuvant (CFA) on day 0 for a primary immunization, and again on day 14 with either 100 μg/100μl of TI or TD antigens dissolved in incomplete Freud’s adjuvant (IFA) for a secondary immunization. Each experimental group contained 8-10 mice. Mice were bled on day 2 before the experiment and day 21 after immunization. Blood samples were allowed to clot for 4 h at room temperature. Sera were placed in new tubes after centrifugation and stored at –20°C for further antibody measurement by ELISA. For booster immunization, mice were challenged with GM-CSF or PolyI: C intraperitoneally (i.p.) 2 weeks after second immunization. The booster injection was carried out daily from day 14 to day 21. Booster reagents were prepared by mixing GM-CSF or PolyI: C stock solution with PBS. Each dose contained 100 μg of GM-CSF or PolyI: C in a total volume of 100μl. Animals were bled before each immunization and 21 d after the last one. Sera were stored at –20°C.
Figure 31 Schematic of immunization

(http://www.abgenex.com/custom-services/monoclonal-antibody, abgenex, Inc)
Figure 32 Regular immunization chart

(http://www.abgenex.com/custom-services/monoclonal-antibody, abgenex, Inc)
Figure 33 Booster immunization chart

(http://www.abgenex.com/custom-services/monoclonal-antibody, abgenex, Inc)

3.2.3 Measurement total serum immunoglobulin level

Total serum IgM, IgG1, IgE, IgG2a, IgG2b, IgG3, and IgA antibody titers were assayed by the Thermo Scientific Pierce Rapid ELISA Mouse mAb Isotyping Kit. ELISA strip-plates were pre-coated in different wells with anti-mouse heavy-chain capture antibodies (anti-IgG1, IgG2a, IgG2b, IgG3, IgA and IgM). The sample and a detection antibody were added together to the wells of the pre-coated microplate. After 60 minutes incubation at room temperature, TMB substrate was added to reveal the antibody isotype, based on which wells in the strip produce color. Results were evaluated qualitatively by measuring the absorbance at 450nm.
Figure 34 Protocol summaries for the ELISA Isotyping Kit. The simple ELISA procedure involves only one probing-incubation step. Sample and detection antibody are added together to the wells of the precoated microplate. After 30 to 60 minutes, TMB substrate is added to reveal the antibody isotype based on which wells in the strip produce color.

Figure 35 Schematic of ELISA Isotyping strip-well plates. Pre-coated strip-well plates for the Pierce Rapid ELISA Isotyping Kit clearly identify monoclonal antibodies. Wells A-H of each eight-well strip (microplate column) was pre-coated with eight different class- or subclass-specific capture antibodies. Sample is tested by adding 50µL of sample to all eight wells of a strip.

3.2.4 Measurement of antigen-specific immunoglobulin level

TNP-specific IgM, IgG1, IgE, IgG2a, IgG2b, IgG3, and IgA antibody titers were assayed by enzyme-linked immunosorbent assay (ELISA). Microtiter plates (96-well) were coated with 100 μl/well of 2μg/ml TNP–BSA. Antigen-specific antibodies were detected with HRP conjugated antibodies to mouse IgM, IgG1, IgG2a, IgG2b and IgG3 (all from PharMingen). After overnight incubation at 4°C, plates were washed with 0.9% (wt./vol) NaCl containing 0.1% (wt/vol) Tween 20 and blocked for 1 h at 37°C in 1%(wt/vol) BSA/ PBS; 100μl appropriately diluted samples were added to the microtiter plate and incubated for 2 h at room temperature. Plates were washed again three times. Horseradish peroxidase (HRP) conjugated goat anti-mouse IgM and IgG subclasses (Nordic Immunological Laboratories, Tilburg, The Netherlands) were diluted at 1:4,000 in the same diluent and incubated with the plates for 1 h at 37°C. The plates were washed three times. The amount of bound peroxidase was visualized by incubation with tetramethylbenzidine (Sigma Chemical Co., St. Louis, MO). After 5 to 15 min, the reaction was stopped with 0.1 M H₂SO₄ and measured at A450 with a microplate reader (Model 3550; Bio-Rad).
Figure 36 Main steps of the Sandwich ELISA

3.2.5 Western Analysis

Tissues were rinsed with cold TBS and lysed in the buffer containing 50 mM Hepes (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10% glycerol, 0.5% Nonidet P-40, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 2.5 mg/ml leupeptin, 0.5 mM NaF, and 0.1 mM sodium vanadate (lysis buffer) and grounded with a French Douncer. After centrifugation, proteins (150 μg) in the tissue extract were resolved by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane or polyvinylidene difluoride membrane. Blots were blocked in TBST (TBS plus 0.05% Tween) containing 5% instant milk and incubated with a primary antibody in PBST. Proteins recognized by the antibody were detected by enhanced chemiluminescence using a horseradish peroxidase-coupled secondary antibody, as specified by the manufacturer (Pierce).

3.2.6 Statistical analysis

The data were expressed as mean ± SD and compared using Student’s t-test.
3.3 Results

3.3.1 RNase L deficiency reduces the weight of spleen to TD and TI antigens

RNase L is highly expressed in the spleen where adaptive immune responses to microbes are initiated. To investigate the potential impact of RNase L on systemic humoral immunity, we first performed necropsies to evaluate spleen responses by examining the phenotypic characteristics of the spleens in RNase L+/+ and RNase L -/- mice with or without antigen challenges. Obviously both TD (TNP-KLH) and TI (TI-1: TNP-LPS; TI-2: TNP-Ficoll) immunization lead to the spleen enlargement. The increment of the spleen size in RNase L+/+ mice was much more significantly than that in RNase L -/- mice. Quite interestingly, TD antigen immunized mice displayed massively enlarged spleen as evidenced by the size and the ratio of spleen weight to the body weight. Particularly the ratio in RNase L+/+ mice was 3-folds higher than that in RNase L -/- mice. In the TI-1 immunized group, the ratio was increased moderately, 77% and 25% respectively, in TI-1 antigen immunized RNase L+/+ and RNase L -/- mice compared to that in control mice. In contrast, the TI-2 immunized group did not produce significant difference in the size of spleens compared to the control groups. The increment of the ratio of spleen/ body weight in TI-2 immunized RNase L+/+ mice was only 35%, while in the RNase L -/- mice was only 9% (Figure 37). Obviously, RNase L is necessary for the efficient induction of spleen response to TD and TI antigens. The deficiency of RNase L severely impaired TD antigen-elicited spleen enlargement although TI induced spleen enlargement was attenuated at a moderately extent in RNase L -/- mice.
Figure 37 Comparison of spleen weight between TD and TI antigens immunized mice. RNase L -/- mice displayed severely impaired spleen enlargement to TD and TI antigen challenge. Body and spleen weight were obtained at endpoints of TD (TNP-KLH), TI-1 (TNP-LPS) and TI-2 (TNP-Ficoll) immunization. Results are shown as the means ± SD from eight mice in each group, p<0.01.
3.3.2 RNase L deficiency results in a decreased IgM level after immunization by TI antigen

According to the data shown in Figure 3.1, in TI-1 (TNP-LPS) and TI-2 (TNP-Ficoll) antigens immunized mice, significant difference was found in the size and weight of spleen between RNase L+/+ and RNase L−/− mice, indicating RNase L might influence TI antigen-directed B cell activation and immune response. Subsequently, the production of IgM in the sera from TI-1 and TI-2 stimulated mice was measured by ELISA at day 0 before immunization and 21 days after immunization. Serum samples collected before immunization were utilized as a negative control. As shown in Figure 3.2, both RNase L+/+ and RNase L−/− mice showed similar amounts of IgM production at day 0 before immunization; however, RNase L−/− mice displayed a significant lower increment of the IgM level after immunization, especially in the TI-1 immunized group. At day 21 after TI-1 stimulation, the increment of the serum IgM levels in RNase L+/+ mice was 1.21 folds, while it was only 1.01 fold in the sera of RNase L−/− mice compared to that in the control mice. To prove the influence of TI-1 antigen on IgM production, we measured the production of TNP-specific IgM. Consistent with the results for total IgM, it also showed that the amount of TNP-specific IgM was 1.57 fold higher in the sera of RNase L+/+ mice immunized by TI-1 as compared with that in the control RNase L+/+ (Figure 3.8). Thus, the data indicate that RNase L is critical for the IgM response to TI antigens. Lack of RNase L results in a suppressed IgM production in serum after TI immunization, which is most pronounced for a TI-1 challenge.
A. Total IgM

![Bar chart showing the average OD (450 nm) for control, TI-1, and TI-2 groups.]

B. TNP-specific IgM

![Bar chart showing the average OD (450 nm) for control and TI-1 groups.]

Figure 38 Level of IgM in the sera of RNase L+/+ and RNase L-/- mice after immunization with TI-1 (TNP-LPS) and TI-2 (TNP-Ficoll) antigens. Mice were immunized on days 0 and day 14 (40 μg/mouse, i.p.) respectively. Blood was taken on day 21. The titers of (A) total IgM and (B) TNP-specific IgM in sera were analyzed by ELISA. The values represent the mean ± SD of a minimum of eight mice at endpoints, p<0.01.
3.3.3 RNase L deficient mice produce markedly less IgG and IgM after immunization by TD antigen

It has been demonstrated that RNase L is able to mediate the production of a large number of cytokines under stimulation, such as IFN-γ and TGF-β, which are critical factors in turn triggering TH participated B cell activation to stimulate TD response. Since our results already showed that RNase L was involved in TI antigen-directed immune response, we were intrigued to determine if RNase L also be able to regulate the differentiation and immunoglobulin production of B cells via TD immune response. Age and gender matched RNase L+/+ and RNase L-/- mice were immunized with TD antigen (TNP-KLH) as described in Methods. The total IgG and TNP-specific IgG were measured in the sera of pre-immune and immunized mice. The amount of total IgG was elevated in the sera of both RNase L+/+ mice RNase L-/- mice, showing 1.3- and 1.1- fold higher respectively (Figure 39 A). However, the increment was more significant when we measured TNP-specific IgG, showing about 6-folds higher in the sera of RNase L+/+ mice, where the increase of IgG production in RNase L-/- mice was only one third of that in RNase L+/+ mice (Figure 39 B). Interestingly, the initial IgG level in RNase L-/- control serum was slightly higher than that in RNase L+/+mice, suggesting the potential role of RNase L in maintaining humoral immune homeostasis. Similarly, the total IgM and TNP-specific measurement displayed enhanced antibody production in response to TD antigen for RNase L+/+ mice, with an increase of 38% and 1.88 folds in total IgM and TNP-specific IgM respectively (Figure 39 C and Figure 39 D). Taken together, we demonstrated that the production of specific IgG and IgM antibodies was dramatically enhanced when RNase L+/+ mice was exposed to TD antigen.
A. Total IgG

![Graph showing Total IgG levels for RL +/- and RL -/- genotypes.]

B. TNP-specific IgG

![Graph showing TNP-specific IgG levels for RL +/- and RL -/- genotypes.]

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Figure 39 Level of IgM in the sera of RNase L+/+ and RNase L-/− mice after immunization with TD antigens (TNP-KLH). Mice were immunized twice on days 0 and day 14 (40 µg/mouse, i.p.) respectively. Blood was taken on day 21. The titers of (A) total IgG; (B) TNP-specific IgG; (C) total IgM; and (D) TNP-specific IgM in the sera were analyzed by ELISA. The values represent the means ± SD of a minimum of eight mice at endpoints, p<0.01.
3.3.4 RNase L deficiency impairs B cell immunoglobulin isotype switching

To further elucidate whether RNase L deficiency affects immunoglobulin isotype switching of B cells, the production of IgG subclasses IgG1, IgG2a, IgG2b, IgG3 and IgA was measured before and after immunization of mice with TD antigen (TNP-KLH). As shown in Figure 40 A, a slightly lower IgG subclasses and IgA production were found in normal RNase L+/+ mice without any stimuli, which was consistent with previous observation during total IgG measurement. On the other hand, the production of all these antibodies was remarkably promoted in the RNase L +/- serum after TD antigen treatment (Figure 40 B). The production of IgG2a and IgG1 was slightly changed in RNase L+/+ mice in comparison with that in RNase L-/- mice. However, the amount of IgG2b, IgG3 and IgA was significantly induced in the RNase L+/+ serum after TD antigen immunization, suggesting that RNase L impacts the antibody isotyping switching process during a TD antigenic challenge. To confirm this result, we performed TNP-specific ELISA assays to measure the production of TD antigen specific IgG subclasses (Figure 40 C). As expected, the production of IgG1, IgG2b and IgG3 was about 1.5-, 1.9- and 2.4-folds higher respectively in the serum of RNase L+/+ mice in compared to that in the serum of RNase L-/- mice after immunization with TD antigen. Collectively, these results indicated that RNase L facilitated the antibody isotype switching from IgM to IgG1, IgG2b, IgG3 and IgA. Although the molecular mechanism remains unclear, it is very likely that the secretion of cytokines such as IFN-γ and TGF-β regulated by RNase L under stimulation condition plays an important role,
A. Total immunoglobulin isotypes

Pre-Immunization

B. Total immunoglobulin isotypes

TD Immunization
Figure 40 Level of IgG1, IgG2a, IgG2b, IgG3 and IgA in the sera of RNase L+/+ and RNase L-/- mice after immunization with TD antigens (TNP-KLH). Mice were immunized on days 0 and day 14 (40 µg/mouse, i.p.) respectively. Blood was taken on day 21. The titers of (A) total immunoglobulin isotypes in pre-immunized sera; (B) total immunoglobulin isotypes in TD antigen immunized sera; and (C) TNP-specific IgG isotypes in sera were analyzed by ELISA. The values represent the means ± SD of a minimum of eight mice at endpoints, p<0.01.
3.3.5 RNase L deficiency attenuates immunogenicity in mice receiving booster immunization

It has been well established that GM-CSF and polyI: C are potent immunologic adjuvants to boost immunogenicity. In addition, we also observed that both of them are able to stimulate the expression of RNase L in mice. To exam the impact of RNase L on the capacity of immunogenicity, we first assessed the production of total IgG, IgM and IgA in RNase L+/+ and RNase L−/− mice with the presence or absence of GM-CSF or poly I: C. Mice immunized with TD or TI antigens at the dose in previous experiments (100 μg/mouse) were used as positive controls whereas PBS-immunized mice were used as negative controls. As shown in Figure 41 A, RNase L+/+ mice receiving TD immunization in the presence of GM-CSF produced significantly higher total IgG, IgM and IgA levels than all control groups. The ratios of the production of immunoglobulin isotypes in RNase L+/+ and RNase L−/− mice provided strong evidence that GM-CSF increased TD directed immunogenicity by 1.6-, 1.2- and 1.4- folds respectively for IgA, IgM and IgG in compared with the single immunization with TD antigen alone (positive control). In comparison with the negative control group, we found the increment of GM-CSF stimulated TD response is more pronounced with 3.0-, 2.0- and 1.7- folds higher respectively for IgA, IgM and IgG. However, there is no remarkable difference was detected in the group receiving polyI: C booster TD immunization.. Quite interestingly, polyI: C seemed to suppress the activated TD immune response and neutralize it to the basal level in RNase L+/+ mice. Furthermore, as measured by ELISA, GM-CSF also induced a significant switching in the levels of TD antigen related IgG heavy-chain isotypes IgG1 (1.4-folds) and IgG2b (2.1-folds) compared to the levels in groups immunized only with TD antigen (Figure 41 B). The production of IgG1 and IgG2b became
more pronounced with 1.84- and 2.5-folds higher respectively than that in the negative control group. In contrast, the production of IgG was suppressed in RNase L+/+ mice in the presence of poly I:C after TD immunization. Taken together, our data demonstrated that RNase L mediated TD antigen associated immunogenicity in the presence of GM-CSF, and promoted the production of IgG2b and IgA. However, the event is barely influenced by polyI: C.

We also investigated the effect of GM-CSF and poly I:C on TI antigens induced immune response in RNase L+/+ and RNase L-/- mice. As shown in Figure 41 C, GM-CSF did not induce remarkable enhancement of total IgM in the TI immunized RNase L+/+ sera. However, polyI: C significantly increased total IgM titers in the TI immunized RNase L+/+ sera, particularly in the TI-1 immunized group. The ratios of IgM production in RNase L+/+ and RNase L-/- mice showed 41% higher and 27% higher in TI-1 and TI-2 immunized sera respectively compared to control groups without any stimuli. Apparently RNase L is unlikely to contribute to promoting the TI induction of IgM by GM-CSF. In contrast, polyI: C enhanced the IgM level in the RNase L+/+ sera, induced by TI antigen. In summary, the results suggest that RNase L participates in TI or TD immunogenicity via two separate mechanisms. RNase L deficiency attenuates TD or TI antigen induced immune response, which become more significant in the presence of GM-CSF or poly I:C, especially for the production of IgA and IgG2b.
A. Immunoglobulin isotypes in TD immunization with or without

GM_CSF or poly I:C
B. IgG heavy-chain isotypes in TD immunization

With or without GM-CSF or poly I:C

![IgG Heavy-Chain Isotypes graph]

- polyI:C Booster+TD
- GM-CSF Booster+TD
- TD Immunization
- Control

Average Ratio of OD, 450 nm (RL+/−: RL−)
C. IgM in TI immunization with or without GM-CSF or poly I:C
Figure 41 Immunoglobulin isotype distributions in RNase L+/+ and RNase L-/− mice after the immunizations in the presence of GM-CSF or polyI: C. Mice were immunized with TI or TD antigens on days 0 and day 14 (40 µg/mouse, i.p.) respectively. On day 15, all mice were challenged with GM-CSF (250 ng/mouse) or polyI: C (150 ng/mouse). Blood was taken on day 21. The titers of immunoglobulin isotypes in the sera were analyzed by ELISA. (A) The ratio of immunoglobulin isotypes production in RNase L+/+ and RNase L-/− mice immunized by TD antigen; (B) the ratio of IgG heavy-chain isotypes production in RNase L+/+ and RNase L-/− mice immunized by TD antigen; and (C) the ratio of IgM production in RNase L+/+ and RNase L-/− mice immunized by TI antigens. The values represent the means ± SD of a minimum of eight mice at endpoints, p<0.01.
3.3.6 RNase L regulates the protein phosphorylation in splenocytes of TD immunized mice

Based on the evidences obtained above, we hypothesized that RNase L might regulate immune response in a way that controls the interaction of antigens and B cells, the phase of B cell activation and their signaling transductions. To investigate how RNase L is involved in the process of B cell activation, we profiled protein phosphorylation cascades by western blot analysis probed with p-Threonine/Serine (p-Thr/Ser) and p-Tyrosine (p-Tyr) antibodies in the spleen tissue extracts prepared from RNase L+/+ and RNase L−/− mice with or without TD/TI-1 immunization. Strikingly, the p-protein profile, particularly the p-Thr, was dramatically different in the spleen tissue extracts from TD immunized RNase L+/+ and RNase L−/− mice when compared with that from control and TI immunized groups. As shown in Figure 42, proteins (54 kDa and 24 kDa) phosphorylated on threonine and tyrosine residues were significantly induced in TD immunized RNase L+/+ splenocytes, however, these phosphorylations were barely observed in TD immunized RNase L−/− splenocytes. In contrast, TI-1 antigen failed to induce additional protein phosphorylation in splenocytes after immunization, suggesting that RNase L involved protein phosphorylation in the spleen is antigen-specific. Overall, our observation was consistent with the previous results, which implied that RNase L might impact TI and TD antigens stimulated B cell activation via two different molecular mechanisms. Obviously, the phosphorylation cascade in RNase L+/+ spleen was only TD antigen dependent. Further identification of these TD antigen stimulated p-protein substrates and their upstream kinases would be able to elucidate the role of RNase L in TD antigen induced B cell activation and its underlying molecular mechanism.
Figure 42 RNase L regulates the protein phosphorylation in the splenocytes of TD immunized mice. Mice were immunized with TI-1 or TD antigens on days 0 and day 14 (40 µg/mouse, i.p.) respectively. Spleen tissues were taken on day 21. The phosphorylated proteins in spleen tissue extracts were profiled by western blot analysis using monoclonal antibodies against mouse phosphorylated-tyrosine (p-Tyr) or phosphorylated-threonine/serine (p-Thr/Ser). Experiments were performed twice.
3.4 Discussion

In the present study, we investigated the role of RNase L in immune responses in RNase L wild type and deficient mice after challenging with a TI or TD antigen challenge with or without in the presence or absence of GM-CSF or poly I:C. Our results revealed that RNase L contributes to adaptive immunity through regulating the production of subclasses of immunoglobulin. This is the first systematic study for the role of RNase L in adaptive immunity although it has been reported that RNase L is associated with the antigengenecity of a replicase-based DNA vaccine in mice [154]. Furthermore, RNase L might participate in adaptive immunogenicity via two separated mechanisms upon the stimulation of different antigen types. The outcome of this study provides strong evidence showing the immunoregulatory role of RNase L and expands our horizon of understanding the biological functions of RNase L other than antiviral infection and anticellular proliferation.

As an exceedingly specific response, the elaborated systemic humoral immunity provides more potent host defense against microbes that greatly relies on the appropriate activation of B cells to secrete antibodies upon antigenic stimulation. TI antigens, such as polysaccharides, lipids and other none protein antigens are able to impact B cell directly to elicit antibody responses without the participation of T_H. They extensively cross-link many antigen receptors on B cells through multivalent arrays of the same epitope, thereby activating B cells to promote proliferation and differentiation. RNase L seems to play a critical role in the TI antigen induced antibody production. The observation of enlarged spleen organ in RNase L +/+ mice after TI antigen immunization implies the enhanced B cells proliferation in response to TI antigen stimuli. In addition, the measurement of IgM level demonstrates that
RNase L is required to initiate TI antibody secretion by B cells, particularly in TI-1 antigen immunization scenario, whereas the increment of IgM was suppressed in RNase L -/- mice. The TI-1 antigens we chose for the immunization experiment was TNP-LPS. As a hapten, TNP contains antigenic determinants (epitopes), but not enough to stimulate immune response, which requires the aid of a carrier LPS to stimulate a TI response [155]. As a bacterial product, LPS has mitogenic activity and induces polyspecific IgM though a polyclonal B cell activation. Obviously, RNase L may be a key player in initiating anti-bacterial response in order to neutralize and eliminate LPS. This result is consistent with previous findings that higher percentages of B220+/IgD B lymphocytes were induced by LPS in RNase L+/+ mice compared to RNase L-/- mice [141]. Besides TI-1 response, we also measured TI-2 antigen induced IgM in RNase L+/+ mice. TI-2 antigens differ from TI-1 antigens as they induce the production of IgM dependent to Treg activity for B cell activation[154]. It has been reported that the expression of TGF-β was regulated by RNase L in immune cells, and TGF-β is believed to contribute to the generation of Treg, perhaps by stimulating expression of the Foxp3 transcription factor. Although we have not determine if RNase L is involved in the development and activation of CD4+CD25+Foxp3+ Tregs, our unpublished result indeed revealed that higher level of CD4+CD25+ Treg in RNase L+/+ upon LPS stimulation, suggesting that the regulation of Treg by RNase L may provide an explanation for the higher level of IgM induced by TI-2 antigen TNP-Ficoll in RNase L+/+ mice.

In addition to direct influence on B cells, the impact of RNase L on B cells could be indirect due to its role in promoting T_H. In the present study, RNase L -/- mice exhibited significantly reduced production of IgG and slightly increased the IgM level in response to TNP-KLH. The serum concentration of IgG1, IgG2b, IgG3 and
IgA was markedly lower in RNase L-/- mice immunized with TNP-KLH. TNP-KHL is the most well-known protein antigen that elicits TD antibody response and is able to induce immunoglobulin isotype switching from IgM and IgD to IgG (IgG1, IgG2a, IgG2b, IgG3) and IgA. The TD antigen-induce immunoglobulin production is highly TH dependent [155]. The B cell response to a TD antigen requires cognate interaction between TH and B cells as well as stimulation by TH derived cytokines [156]. Recently, a number of studies have demonstrated that TH1, TH2 cells and secreted cytokines are not unique in their ability to induce TD associated B cell responses, but both Treg and its cytokines are also capable of supporting B cell clonal expansion and antibody synthesis [157-159]. Specifically, TH1 cytokines (IFN-γ, TNF-α and IL-2) stimulate proliferation of both B and T lymphocytes [160]. TH2 cytokines (IL-4, IL-5 and IL-10) induce the activation and differentiation of B cells, and thus it reinforces the antibody response and plasma cell isotype switching to produce IgG1, while IgG2a, IgG2b and IgG3 isotype switching is promoted by IFN-γ [161]. TGF-β functions mainly as an inhibitor of immune response for the maintenance of self-tolerance. However, it also has been suggested that TGF-β may be immunostimulatory because it facilitates IgG2b and IgA isotype switching [162]. Therefore, the disturbed TD humoral response of RNase L-/- mice might correspond to the reduced levels of these cytokines in RNase L-/- mice. In the previous studies, we have reported that reduced cytokine production in RNase L-/- immune cells, which is more pronounced for IL-10, TNF-α, TGF-β and IFN-γ production. Flow cytometry analysis of T cell subsets also revealed that RNase L promoted the development and differentiation of CD4+ T cells. Thus, it is possible that RNase L mediates the function of TH cells by regulating the expression of cytokines to promote switching subclasses of immunoglobulin. Interestingly, we have also found a slight
higher immunoglobulin level in pre-immunized RNase L-/- mice compared to RNase L+/+, which might be due to the impaired apoptotic prosperities of RNase L-/- B cells, resulting in a delayed humoral immune response.

GM-CSF and poly I:C distinctly promote immune response in RNase L+/+ and -/- mice. Importantly, the elevated antibody response induced by GM-CSF and poly I:C is antigen dependent. GM-CSF has been demonstrated to affect the immune system, including enhancement of the generation of immune and hematological cells and augmentation of antibody response [163]. One of the main functions of GM-CSF in the amplification of immune response is to generate the microenvironment of high concentration of cytokines. In our study, although GM-CSF was not effectively boosted the level of IgM induced by TI, its role in enhancing the production of Ig A and IgG2b was remarkable in RNase L+/+ serum. These T_{H2} and Treg related immunoglobulin profiles indicate that RNase L might exert immunomodulatory function with highly specificity to T_{H2} and Treg. On the other hand, polyI: C failed to promote TD response, but a predominant IgM production was found in TI antigen immunized RNase L+/+ serum when polyI: C was present. PolyI: C is a potent inducer of IL-12 and type I IFN [154]. Apparently, in vivo signals triggered by polyI:C are more correlated with T_{H1} cytokines and efficient for B cell activation and initiation of TI antigen driven response. With the same adjuvant stimulation, B cells generated significant different TI and TD response in RNase L+/+ and -/- mice, suggesting that RNase L might participate in two independent pathways for B cell activation. Based on these observations, we postulated that RNase L deficiency leads to a reduced sensitivity to the signal delivered through BCR, resulting in a requirement for a higher antigen dose to induce B cell activation, whereas RNase L+/+ mice mounted immunoglobulin isotype switching is mainly induced by T_{H2} or
Treg. The fact that altered protein phosphorylation profile of RNase L+/+ splenocytes in response to TD and TI antigens also tested this hypothesis. Spleen is the specialized secondary lymphoid organ where B cells undergo massive rounds of development and differentiation. Thus, RNase L mediated protein phosphorylation may plays an important role in the B cell activation. The two phosphorylated proteins must be highly correlated with the activation process of $T_{H1}$ or $T_{reg}$. In addition, considering that RNase L has 9 typical ankyrin-repeats and a conserved kinase domain in its protein structure. Hereby, it is possible that these two phosphorylated proteins may be phosphorylated either directly by RNase L or indirectly by kinase associated RNase L to regulate B cell activation. These hypotheses are currently under investigation in the laboratory.

In conclusion, our results clearly show that RNase L is involved in both TI and TD antibody production and isotype switching in B cells. Although the immunological mechanism of this effect is unclear, the further identification of two novel phosphorylated substrates and determination of the relationship between upstream kinases and RNase L, will be one focus of our future studies. RNase L is highly expressed in the lung and intestine of mice. Thus, it will be of interest to evaluate RNase L-deficient mice with respect to local mucosal humoral response of other organ systems that express RNase L extensively, such as the pancreas, lung, and intestine. In summary, our findings provide new insight into the contribution of RNase L to systemic humoral immune responses, and suggest a novel physiological role of RNase L in adaptive immunity.
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