MOLECULAR PATHOGENESIS OF THE RETROVIRAL ONcopROTEIN TAX
IN T LYMPHOcyTE TRANSFORMATION

A Dissertation in
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by
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Abstract

Human T cell leukemia virus type 1 (HTLV-1) is the etiological factor that causes adult T cell leukemia and lymphoma (ATL). This virus infects over 20 million people worldwide with about 1 million of HTLV-1-infected patients developing ATL. ATL is a highly aggressive non-Hodgkin's lymphoma and currently there is no effective therapy for this type of leukemia and lymphoma. The viral genome encodes three main structural gene products including Gag, reverse transcriptase (Pol/RT) and envelope protein (Env), together with several viral regulatory proteins such as Tax (Tax-1). Tax is a known viral oncogenic protein that plays a central role in transforming CD4+ T lymphocytes by deregulating oncogenic signaling pathways. Expression of Tax is critical for promoting survival of HTLV-1-infected T cells, which is required for initiation of oncogenesis. Notably, Tax activates IκB kinase (IKK) complex in the cytoplasm, resulting in persistent activation of NF-κB, a transcriptional factor that plays key roles in the pathogenesis of human cancer. This action increases NF-κB responsive gene expressions that are crucial for T cell survival and cell cycle progression. Tax is predominantly distributed in the nucleus with a perinuclear Golgi distribution pattern, shuttling between these two subcellular compartments in HTLV-1-transformed T cells.

My thesis project is to study the molecular mechanism of the retroviral oncoprotein Tax in promoting T lymphocyte survival and proliferation. The central hypothesis of my thesis project is that “modulating IκB kinase activity by Tax is critical for HTLV-1-mediated oncogenesis”. I proposed to study three specific aims. **Aim1** was to investigate the oncogenic activation of the Tax protein in human primary CD4+ T cells in a prospective approach. **Aim2** was to evaluate the
mechanism and function of Tax-mediated autophagy during T cell immortalization and transformation. **Aim3** was to explore the possibility of utilizing Tax as a molecular tool to generate T cell subtype cell lines and to develop T-LGL leukemia model.

My previous work demonstrated that Tax is a viral lipid raft protein that can hijack the IKK complex into its lipid rafts for persistent activation. It is known that T cell receptor engagement results in activation of IKK and PI3KC1, and induces autophagy that is important for T cell survival. Similarly, Tax activates both IKK and PI3KC1. Although these two kinases have been reported to exhibit opposing activities in regulating autophagy, I observed that HTLV-1-infected T cells exhibit constitutively high level of autophagy. These experimental results strongly suggest that autophagy is beneficial for HTLV-1-mediated oncogenesis. HTLV-1 and HTLV-2 are closely related, sharing strikingly similar structure. HTLV-2 infection is prevalent among intravenous drug users and Tax-2 is highly homologous to Tax-1. However, the link between HTLV-2 infection and leukemia has not been established. My proposed research is to understand the survival mechanism of HTLV-1-transformed T cells. Therefore, the proposed study is highly significant not only for a better understanding of HTLV-1 pathogenesis but also for developing molecular therapeutics targeting the oncoprotein Tax.

We found that Tax is a determining factor for dysregulation of autophagy in HTLV-1-transformed T cells and Tax-immortalized CD4 memory T lymphocytes. Tax facilitated the autophagic process by activating IKK complex, which subsequently recruited an autophagy molecular complex containing Beclin1 and Bif-1 to the lipid
raft microdomains. Tax engaged a crosstalk between the IKK complex and the autophagic molecular complex by directly interacting with both, thereby promoting assembly of LC3+ autophagosomes. Moreover, expression of lipid raft-targeted Bif-1 or Beclin1 was sufficient to induce formation of LC3+ autophagosomes, suggesting that Tax recruitment of autophagic molecules to lipid rafts is a dominant strategy to deregulate autophagy in the context of HTLV-1 transformation of T cells. Furthermore, depletion of Beclin1 resulted in impaired growth of HTLV-1-transformed T cells, indicating a critical role of Tax-deregulated autophagy in promoting survival and malignant transformation of virally infected T cells.

The general approach in my thesis project was to investigate the oncogenic activity of Tax in a prospective way, using HTLV-1-transformed T cell lines as controls. Human primary T lymphocytes were isolated and transduced with lentivirus carrying Tax-GFP expression cassette. The transduced cells exhibited green fluorescence after in vitro culture for one month. Although all transduced cells passed typical senescence period (about three weeks at normal culture conditions), two Tax-1-GFP-transduced and four Tax-2-GFP-transduced cell lines were established for long-term growth in culture (more than one year). These cell lines, therefore, represent useful tool cell models for investigating the role of Tax in promoting T cell survival. This approach allows analysis of oncogenic events during the process of T cell immortalization and transformation. Lentivirus transduction in human primary T cells was highly efficient, with about 40-60% of cells able to be transduced. 4 out of 12 Tax-2-GFP transduced cells were capable of maintaining long-term growth in culture. These immortalized cells were consistent with CD4+ memory T lymphocytes demonstrating a CD3/TCRαβ/CD4/CD25/CD45RO/CD69 immunophenotype. In
comparison to normal CD4 T cells, Tax-2-GFP-immortalized cells exhibited constitutive activation of PI3K/Akt, IKK/NF-κB, MAPK and STAT3. The level of Mcl-1, a pro-survival Bcl-2 family member, was increased. Disruption of these oncogenic pathways caused apoptotic cell death of the Tax-2-established T cell lines. I observed that in Tax-2-immortalized T cells, a high level of autophagy occurred which was critical for supporting T cell survival. Further analysis showed that the IKK complex played a key role in Tax-2-induced autophagy.

Some leukemia types that are difficult to be cultured in vitro include CLL, T-LGLL and TPLL. For CLL, a JVM-3 cell line was developed by EBV infection of primary CLL cells. Establishment of T-LGLL cancer model remains to be a challenging task. The Tax protein immortalizes predominantly CD4 T cells and is not able to immortalize normal CD8 T cells. We developed a strategy that can prolong in vitro growth of primary T-LGL leukemia cells. Primary CD8+ populations from T-LGL leukemia patients were stably transduced with the retroviral tax gene derived from HTLV-2. Expression of Tax-2 overrode replication senescence and promoted clonal expansion of the leukemic CD8+ T cells. These cells exhibited features characteristic of T-LGL leukemia, such as resistance to FasL-mediated apoptosis, sensitivity to inhibitors for sphingolipid kinase and IKK as well as expression of cytotoxic gene products including granzyme B, perforin and IFNγ. Collectively, these results indicate that the immortalized CD8+ cells from T-LGL leukemia patients are T-LGL leukemia-like cells, and this leukemia model can duplicate the main phenotypes of the clinical isolates of T-LGL leukemia. The establishment of the T-LGL leukemia model is useful for investigating molecular pathogenesis of the disease and for developing novel therapeutics.
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Abbreviations List

aa: amino acid

AP: Alkaline Phosphatase

AP-1: Activator Protein 1

APC: Antigen-Presenting Cell

ATL: Adult T-cell Leukemia

ATLL: Adult T-cell Leukemia/ Lymphoma

BAF: B-cell Activating Factor

BER: Base Excision Repair

bp: base pairs

CBP: CREB Binding Protein

CD4, CD8: T-cell mature phenotype

cDNA: complementary DNA

CLL: chronic lymphocytic leukemia

CMV: Cytomegalovirus

CNS: Central Nerve System

CRE: Cyclic-AMP Response Element

CSF: CerebroSpinal Fluid

CTL: Cytotoxic T Lymphocyte

CREB: Cyclic AMP Response Element Binding protein

DSBR: Double-Strand Break Repair

DMEM: Dulbecco’s Modified Essential Medium

dsDNA: double stranded DNA

EBV: Epstein-Barr Virus
EGFR: Epidermal Growth Factor Receptor
ELISA: Enzyme-Linked Immunosorbent Assay
EMSA: Electrophoretic Mobility gel Shift Assay
Env: envelope
ER: Endoplasmic Reticulum
FACS: Fluorescence-Activated Cell Sorting
FBS: Fetal Bovine Serum
GAG: Group-Associated Gene
GFP: Green Fluorescent Protein
GLUT1: Glucose Transporter 1
HAM/TSP: HTLV-1-associated myelopathy/tropical spastic paraparesis
HBV: hepatitis B virus
HBZ: HTLV-1 bZIP factor
HCC: Hepatocellular Carcinoma
HCV: hepatitis C virus
hDlg: human homolog of Disk large
hEF: human Elongation Factor
HRP: Horseradish Peroxidase
hScrib: human homolog of Scrib
HSM: Hepatosplenomegaly
hTERT: human Telomerase Reverse Transcriptase
HTLV: Human T-cell Leukemia Virus
IKK: IκB kinase
IL-2: Interleukin-2
IL-15: Interleukin-15
LC3: Microtubule-associated protein 1A/1B-Light Chain 3
LMP1: Latent infection Membrane Protein 1
LT-β: LymphoToxin B
LTR: Long Terminal Repeat
MAGI-3: Membrane-Associated Guanylate kinase Inverted-3
MAPK: Mitogen-Activated Protein Kinase
MβCD: Methyl-β-CycloDextrin
MDC1: Mediator of DNA damage Checkpoint protein 1
MMR: MisMatch Repair
mTOR: mammalian Target Of Rapamycin
NB: Nuclear Body
NER: Nucleotide Excision Repair
NES: Nuclear Export Signal
NF-κB: Nuclear Factor kappa-light-chain-enhancer of activated B cells
NIK: NF-κB-Inducing Kinase
NK: Natural Killer
NLD: Nuclear Localization Determinant
NLS: Nuclear Import Signal
NPC: Nasopharyngeal Carcinoma
nt: nucleotide
OI: Opportunistic Infection
ORF: Open Reading Frame
PBM: PDZ-binding domain motif
PBMC: Peripheral Blood Mononuclear Cell
PCNA: Proliferating Cell Nuclear Antigen
**PCR**: Polymerase Chain Reaction

**PHA**: Phytohaemagglutinin

**PTM**: Post-Translational Modification

**qPCR**: quantitative Polymerase Chain Reaction

**Rex**: Regulator of viral protein expression

**RIP**: Receptor Interacting Protein

**RT**: Reverse Transcriptase

**RT-PCR**: Reverse Transcription-Polymerase Chain Reaction

**SDS-PAGE**: Sodium Dodecyl Sulphate Polyacrilamide Gel Electrophoresis

**STAT3**: Signal Transducer and Activator of Transcription 3

**STLV**: Simian T cell Leukemia Virus

**SUMO**: Small Ubiquitin-like Modifier

**TAB2**: TAK1-Binding protein 2

**TAK1**: Transforming growth factor-β–Activated Kinase 1

**Tax**: Transcriptional activator of pX region

**TCR**: T Cell Receptor

**T-LGL**: T-Large Granular Lymphocyte

**TNFR1**: Tumor Necrosis Factor Receptor 1

**TPLL**: T cell prolymphocytic leukemia

**TRADD**: Tumor necrosis factor Receptor type 1-Associated DEATH Domain protein

**TRAF**: Tumor necrosis factor Receptor-Associated Factor

**TSC1/2**: Tuberous Sclerosis Complex 1/2

**TSS**: Tax Speckled Structure

**WB**: Western Blot

**WHV**: Woodchuck hepatitis virus
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Chapter 1: Literature Review

1.1 Overview

The discovery of the first human retrovirus, human T cell leukemia virus type 1 (HTLV-1), changed the consensus view in the scientific community doubting the possible existence of human retroviruses [1-3]. HTLV-1 was discovered in 1979 in a T cell line isolated from a cutaneous T cell lymphoma patient [1]. Subsequently, four reports in 1981 described additional isolates of HTLV-1 [2, 4-6]. Following these reports and epidemiological studies, HTLV-1 was proven to be the etiological agent for induction of adult T cell leukemia and lymphoma (ATL), a distinct form of CD4+ T cell malignancies first recognized in Japan [3, 7]. In addition to causing ATL, HTLV-1 is also associated with inflammatory diseases such as HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP), arthritis and alveolitis, polymyositis, uveitis and infective dermatitis and some types of skin lesions [8-15]. Two years following HTLV-1 discovery, a virus closely related to HTLV-1, Human T-lymphotropic virus type 2 (HTLV-2), was isolated in patients who had both typical hairy cell leukemia, a B cell type of leukemia, together with a CD8+ T cell lymphoproliferative disorder [16]. HTLV-2 sequence was found in T cells, but not in hairy cell leukemia cells, and subsequent epidemiological study did not support a causal link between HTLV-2 infection and hairy cell leukemia. In recent years, additional HTLV-1-related viruses, including HTLV-3 and HTLV-4, have been identified [17, 18]. Currently no human disease is linked to the infections by these two retroviruses.

HTLV-1 is the most studied human retrovirus because it is known to cause a very aggressive form of leukemia and lymphoma, ATL [10, 19]. HTLV-1/ATL appears to be very useful cancer model as it can be studied in both retrospective and
prospective approaches. Analysis of clinical specimens from ATL patients provided a
great deal of insights into oncogenic activation and potential roles of viral oncogenes
in the differential stages of the development of ATL. HTLV-1 easily infects and
transforms primary human CD4+ T cells in culture, and the transformed cells can be
further tested in animal models [20]. Transgenic mouse model demonstrated the
oncogenic potential of the HTLV-1 viral genome-encoded viral oncogenic proteins,
Tax and HBZ [21, 22]. Humanized mouse model was also established to imitate the
disease course of HTLV-1 infection in humans [23, 24]. With these research
advances, it is generally agreed that Tax and HBZ are two essential viral
oncoproteins with distinct roles in the different stages of leukemogenesis. Tax
executes its oncogenic role in the early stage of oncogenesis while HBZ is
necessary for maintenance of malignancy of ATL in the patients [25-27].

Tax is the most studied viral protein from HTLV-1 because of its indispensible
role in viral pathogenesis. Although the antisense gene product, HBZ, is an
emerging candidate for its role in HTLV-1 leukemogenesis, the fact that HBZ is not
able to immortalize and transform human T lymphocytes indicates that HBZ is less
important than Tax at the early stage of HTLV-1 oncogenesis [28, 29]. In contrast,
Tax appears central in mediating T cell oncogenesis at the early stage of ATL [26,
30]. My graduate work found that Tax connects IkB kinase complex to autophagy
pathways and executes its functions in activating IkB kinases and in deregulating
autophagy in lipid rafts for T cell survival and proliferation. These new experimental
findings provide insights into not only the molecular basis of HTLV-1-mediated T cell
transformation but also the potential development of new therapeutics. Directly
targeting Tax and IkB kinase, inhibiting autophagy pathways and modulating
intracellular cholesterol contents to disrupt lipid raft microdomains may becoming
new options for therapeutic development aimed at controlling this aggressive form of leukemia and lymphoma.

To summarize, HTLV-1, a well-known oncogenic human retrovirus, provides an excellent cancer model for biomedical scientists to understand the nature of disease pathogenesis in both retrospective and prospective ways. HTLV-2, a closely related virus to HTLV-1, is a less pathogenic strain based on current knowledge, which could serves as an important subject for comparative study in understanding the pathogenesis of HTLV-1-associated ATL.

1.2 Human T cell leukemia virus type 1, 2, 3 and 4 (HTLV-1, -2, -3 and -4)

1.2.1 HTLV-1

Human T cell leukemia virus type 1 (HTLV-1) is the etiological factor that causes adult T cell leukemia and lymphoma and is also associated with inflammatory diseases [31]. HTLV-1 is classified as a retrovirus in the genus delta-retrovirus of the subfamily [32]. Virions of HTLV-1 consist of spherical to pleiomorphic enveloped particles and are about 73 nm in diameter [33]. HTLV-1 can be transmitted from person to person in the following ways: perinatal transmission by blood contamination or breast milk feeding, sexual transmission, exposure to contaminated blood products or sharing contaminated needles [34, 35]. The transmission of HTLV-1 is believed predominantly through cell-cell interaction via viral synapse [36]. Evidence showsthat cell-free virions produced by HTLV-infected cells are largely non-infectious [37].

The HTLV-1 genome is composed of two identical copies of a linear single-stranded, positive sense RNA (diploid) about 8507 nt in size with a 5'-cap, 3'-polyadenylation tail and a transcriptional stop [38], as shown (Figure 1.1). As a
retrovirus, HTLV-1 genome contains three characteristic open reading frames (ORFs): gag (structural and core proteins), pol (reverse transcriptase) and env (envelope glycoproteins) whose genes are flanked by the long terminal repeat (LTR). The LTRs are about 600 nt long at both the 5’- and 3’-ends that contain the U3, R and U5 regions, serving as promoters, enhancers and regulators of viral gene expression at pro-viral level. Gag is translated as a polypeptide precursor followed by cleavage into the 19 kDa matrix protein (p19), 24 kDa capsid protein (p24) and 15 kDa nucleocapsid protein (p15). Env is translated as a poly-protein precursor followed by cleavage into the 46 kDa surface glycoprotein (gp46) and 21 kDa transmembrane protein (p21).

In addition to the essential viral genes, HTLV-1 encodes accessory and regulatory genes at a unique region ORF, named pX, which is located between env and the 3’-LTR. The positive strand of the pX region encodes Tax/p40 (ORF IV), Rex/p21 (ORF III), p12 (ORF I), p13 (ORF II) and p30 (ORF II). The negative strand encodes HTLV-1 basic leucine zipper factor (HBZ) protein. Tax transactivates transcriptional initiation through three Tax-responsive elements (TRE1) in the U3 region of the LTR. Tax is important for HTLV-1-induced immortalization and transformation. Rex is essential for regulating viral gene expression post-transcriptionally by facilitating the cytoplasmic accumulation of the incompletely spliced viral mRNA transcripts [39, 40]. Rex binds viral mRNAs containing a cis-acting sequence termed Rex response element (RxRE) at the R region of the viral LTR [41-43]. The p12 protein is a hydrophobic protein that localizes to the endoplasmic reticulum and the Golgi [44-46]. The p12 protein is further cleaved to generate p8 [47]. The p13 protein is an 87-amino acid protein that associates with the inner mitochondrial membrane. Under certain conditions, p12 is transported into
the nucleus. The p30 protein is a negative post-transcriptional regulator of viral gene expression [43, 46]. Unlike Tax and Rex proteins, p12, p8, p30, and p13 are not necessary for viral replication or for immortalization of human primary T cells in vitro [48]. The experimental finding from rabbit model of HTLV-1 infection suggested that p12, p13 and p30 are required for HTLV-1 infectivity [49]. HBZ gene is the only viral gene that is constitutively transcribed and remains intact in ATL cells, suggesting a functional role in malignant transformation of host cells [28]. HBZ suppresses Tax-mediated transcription from 5’-LTR [50, 51]. HBZ also suppresses the classic NF-κB pathway and activator proteins (AP-1) pathway [52]. 30% of HBZ-transgenic mice developed T cell lymphoma and most of HBZ transgenic (Tg) mice developed spontaneous inflammatory lesions [53]. Increasing evidences showed that the interaction between Tax and HBZ are important for the HTLV-1 oncogenesis [25, 50, 51, 54].

HTLV-1 has preferential tropism for CD4 T cells in healthy carriers, HAM/TSP and ATL patients. Other cell types in peripheral blood of infected patients, including CD8+ lymphocytes, monocytes and B-lymphocytes, are found to harbor HTLV-1 [55-57]. In addition, macrophages, dendritic cells, megakaryocytes as well as glial cells (astrocytes and microglial cells) are also cell targets for HTLV-1 in vivo [55, 58-60]. Similarly, HTLV-1 is detected in non-T cells and non-lymphoid cells in vitro. For example, HTLV-1 isolates can be transmitted to primary human endothelial cells [61, 62], monocytes and microglial cells [63], as well as basal mammary epithelial cells [64].

HTLV-1 transmission requires cell-cell contact [65, 66]. Previous studies showed that HTLV-1-infected cells polarize their microtubules and viral components upon contacts with target cells, forming viral synapses before HTLV-1 viruses spread
from cell to cell [36, 67-69]. Both in vitro and in vivo data showed that glucose transporter 1 (GLUT1) serves as a cellular receptor for HTLV-1 entry. GLUT1 is a twelve transmembrane multi-spanning molecule that plays roles in both binding and post-binding steps of HTLV Env-mediated infection [70, 71]. GLUT1 appears to be concentrated in virological synapses that are formed between HTLV-1-infected cells and target cells [36].

The HTLV-1 life cycle begins with the interaction of the surface unit of the virion envelope glycoproteins (SU), gp21/46, with its receptor GLUT1 on the target cell membrane. Then the virion fuses with the recipient cell membrane and transports the capsid and viral genome inside. Reverse transcriptase is released and directs the synthesis of a DNA copy of viral RNA genome. The resulting double stranded DNA enters the nucleus, integrates into the host genome in forming the provirus. Viral RNAs including copies of the viral genome are synthesized by cellular machinery, followed by the synthesis of viral proteins. During the productive phase of viral replication, assembled progeny viruses bud out from the host cell membrane. Proteolytic processing of Gag-Pro-Pol polyprotein is required for producing a mature infectious viral particle. During primary infection, HTLV-1 has a period of active replication. The subsequent proliferation occurs mainly through clonal expansion of infected cells or by viral synapses [72-74]. Thus, HTLV-1 has a low level of intra-individual genetic variation, unlike HIV.

1.2.2 HTLV-2

Human T cell leukemia virus type 2 (HTLV-2), the second human retrovirus ever discovered, was reported in 1982 after the discovery of HTLV-1 [16]. HTLV-2 was isolated from MoT T lymphocytes, a T cell line established from spleen of a
patient (Mo) with hairy cell leukemia [16]. HTLV-2 is also classified as a retrovirus in the genus delta-retrovirus of the subfamily Orthoretrovirinae. This virus is closely related to HTLV-1, sharing about 70% genomic sequence homology with HTLV-1. Similar to HTLV-1, HTLV-2 is a positive single-stranded RNA retrovirus with the genome length about 8.9 Kb. HTLV-2 viral genome has a primer-binding site (PBS) to a tRNAPro, ORFs for gag, pol, env and pX, flanked by 5’- and 3’-LTRs [38]. HTLV-2 Rex regulates the expression of the viral structural and enzymatic gene products [75, 76]. HTLV-2 also encodes a viral transactivator Tax, which is highly homologous to HTLV-1 Tax. However, both Tax proteins exhibit distinct structural and cellular features.

Emerging evidence indicates that the rate of HTLV-2 infection is increasing. It is estimated that there are about 197,000 cases of HTLV-2 infection in the United States alone and several millions worldwide [77]. HTLV-2 is found predominantly in American Indian populations in North, Central and South America, especially in Brazil where some tribes show 30% prevalence among general population [78-82]. HTLV-2 is also prevalent in African pygmy tribes [83, 84], and is found sporadically in other regions of Africa [85-88]. HTLV-2 is detected among intravenous drug abusers in the United States and Europe [89, 90], probably because HTLV-2 has the same pathways of transmission as HTLV-1, including exposure to infected blood products or intravenous drug use, mother-to-child and sexual transmissions [91-96]. Unlike HTLV-1, in vivo studies showed that HTLV-2 has tropism for CD8+ lymphocytes [97]. However, both CD4+ and CD8+ lymphocytes from HTLV-2-infected patients showed spontaneous in vitro proliferation in the absence of exogenous stimulation [98, 99], suggesting that HTLV-2-infected cells may secrete soluble factors that are capable of promoting growth of both CD4 and CD8 T cells.
The mechanism of HTLV-2 tropism has not been well determined. Recent study suggests that similar to HTLV-1 entry, GLUT-1 serves as the receptor on target cells for HTLV-2 [71]. However, the fact that HTLV-2 preferentially infects CD8+ T cells suggests that there exists a CD8+ T cell-specific co-receptor for assisting HTLV-2 entry [100]. This putative receptor has yet to be determined.

HTLV-2 was first detected in CD8 T cells from a patient with hairy cell leukemia, a rare type of leukemia that affects B cells [16]. Further studies indicated that HTLV-2 infection is not causally linked to hairy cell leukemia. HTLV-2 appears to be associated with increased risk of neurological disorders such as HAM/TSP-like, subacute myelopathy, multiple system atrophy (MSA) and ataxia [101-103]. AIDS patients co-infected by HTLV-2 are three times more likely to develop peripheral neuropathy and have increased bacterial infections than the patients with HIV-1 infection alone [104]. HTLV-2 is also associated with increased incidence of respiratory disorders such as pneumonia, bronchitis, tuberculosis and inflammatory conditions, implicating a possible role of HTLV-2 in inhibiting immunologic responses and in inducing autoimmune reactions [77]. The current consensus view is that HTLV-2 is less pathogenic than HTLV-1 and that there is no causal link between HTLV-2 infection and a specific human disease. Comparative studies on HTLV-1 and HTLV-2 have been proposed in order to understand the pathogenesis of HTLV-1-associated diseases.

1.2.3 HTLV-3 and HTLV-4

The initial name for human immunodeficiency virus (HIV), the virus that causes acquired immune deficiency syndrome (AIDS), was HTLV-3. However, this term is no longer in use because HIV apparently belongs to a distinct subfamily of human
retrovirus, lentivirus [105]. The new HTLV-3 of delta-retroviral subfamily was identified in a 62-year old Bakola Pygmy from central Africa in 2005 [17]. HTLV-3 is closely related to simian T cell leukemia virus (STLV)-3, a monkey virus that is divergent from HTLV-1 (60% nucleotide similarity) or from HTLV-2 (62% nucleotides similarity) by sequence comparison. HTLV indeterminate western blot (WB) serological patterns were found in a patient, and HTLV-3 was subsequently isolated in this patient. Subsequent studies showed that HTLV-3 genome is shorter than STLV-3 sequence due to a 366nt deletion in the pX region [106]. HTLV-3 viral genome encodes gag, pol, env and tax/rex genes. Tax3 and Tax-1 share a number of similarities that are related to the transforming activity of Tax-1, which suggests that HTLV-3 might be pathogenic in vivo [107, 108]. Recent study showed that the surface glycoprotein of HTLV-3 binds to both activated CD4+ and CD8+ T cells [109]. Unlike HTLV-1 or HTLV-2, HTLV-3 binds naïve CD4+ T cells, and these cells do not express detectable levels of GLUT-1, the receptor for mediating HTLV-1 and HTLV-1 entry.

HTLV-4 was recently identified from a hunter in the same geographic location as HTLV-3 [110]. Sera from the patient reacted with an indeterminate pattern against HTLV-1 and HTLV-2. Phylogenetic analysis on short fragments of pol and tax genes showed that HTLV-4 belongs to a phylogenetic lineage that is distinct from all known HTLVs or STLVs [18]. The full-length of HTLV-4 viral genome sequence is 8791bp in size, sharing 62%-71% nucleotide identity with HTLV-1, HTLV-2 or HTLV-3 [107]. The data on HTLV-4 is limited, and HTLV-4 genomic clone and HTLV-4-infected cell line are currently not available. The viral transmission, tropism in vivo and pathogenesis of HTLV-4 remain unknown. The continuous detection of unknown human retroviruses opens a new chapter in our understanding of the pathogenesis
of human retroviruses. Although the newly identified HTLV-3 and HTLV-4 have not been linked to any human disease [18], they could serve as crucial molecular tools for determining how these viruses evolved and how a non- or less pathogenic retrovirus acquires disease-causing capacity.

1.2.4 TLGLV, a putative retrovirus in large T granular lymphocytic leukemia

There is some evidence to suggest that patients with the T cell form of LGL leukemia might be infected with a retrovirus related to the HTLV family. A few LGL leukemia patients appear to have been infected with HTLV-2. Serum antibodies that react to synthetic peptide specific for HTLV-2, but not for HTLV-1, in serum were detected in this patient using enzyme-linked immunosorbent assay (ELISA), followed by Western blot (WB) and radioimmuno-precipitation assays that confirmed the positivity. HTLV-2 specific gag, pol, env, and pX gene sequences were also detected by PCR in the LGL patient. Sequence analysis indicated that the pol and pX sequences from this patient differ from HTLV-2 isolates. In other studies, 30% of T-LGL leukemia patients showed serum antibodies that react against BA21, a 34 amino acid peptide derived from HTLV-1 envelope protein p21 [111]. Because large sampling analysis showed that prototypical HTLV-1/-2 infection is rare among LGL leukemia patients [111], the findings on HTLV-1/-2 serum reactivity in LGL leukemia patients suggest that TLGLV might be a new retrovirus that is closely related to HTLV-1/-2. It is also possible that this TLGLV is a variant of clinical HTLV-1/-2 isolates. Molecular cloning of the putative TLGLV viral genome is necessary to confirm the existence of this new virus.
1.3 HTLV-1 and adult T cell leukemia/lymphoma (ATL)

The existence of ATL was first recognized in 1973 at Kyoto University Hospital in Kyoto. Such patients were found to have an unusual leukemia of T cell derivation. Lymphocytic leukemia usually develops from malignant transformation of B-lymphocytes with the T cell type of leukemia being much less common. Subsequent studies found that this type of leukemia was prevalent among adults in Kyushu, southwestern Japan [112, 113]. The association of HTLV-1 and ATL was made from clinical observations of Japanese patient samples [7, 114]. The discovery of HTLV-1 and ATL opened a new era of virology, oncology, epidemiology, molecular biology and other biomedical fields. It is now well established that HTLV-1 is the etiological factor causing ATL, based on extensive experimental findings and epidemiological studies [7, 114-117].

Four clinical variants of ATL have been identified [117]. The most common one is acute ATL with elevated white blood cell counts and abnormal lymphoid cells as well as hypercalcemia, bone lesions and hepatosplenomegaly (HSM). Acute ATL is a highly aggressive form of leukemia/lymphoma, and patient survival is less than one year. The lymphomatous ATL mainly displays lymphadenopathy, and peripheral blood involvement is not typically found in this variant. The chronic ATL is accompanied with frequent skin lesions and slight nuclear abnormality. The smoldering ATL is also associated with frequent lesions in skin or lung with relatively mild disease course. Morphologically, ATL cells are described as flower cells due to a lobated nucleus.

HTLV-1 infects 20 million people worldwide and five percent of the infected patients develop ATL. The HTLV-1 endemic areas include southwestern Japan (Kyushu and Okinawa), the Caribbean islands, Central and South America, Central
Africa, Middle East with sporadic cases in western countries [118-121]. HTLV-1 carriers are also found in isolated populations in Ainus in Hokkaido and Aborigines in Australia. ATL occurs 20-30 years following HTLV-1 infection. The risk factors for developing ATL include low anti-Tax activity, large numbers of abnormal lymphoid cells in peripheral blood, increased white blood cell counts and high level of soluble interleukin-2 receptor (sIL-2R) [122-124]. Proviral load is higher in ATL patients than that in asymptomatic HTLV-1 carriers. The immunophenotype of ATL cells is CD3+CD4+/CD5+/CD7-/CD25+ in most clinical cases [125, 126].

Studies based on ATL showed that HTLV-1 viral genome does not contain a human cellular-derived oncogene, which might explain why it takes a long time to establish malignancy after primary infection [127]. Instead, HTLV-1 has a unique sequence in the pX region that encodes the Tax protein that exhibits transforming activity. Tax is expressed in HTLV-1-infected cells and functions as a transactivator promoting viral gene expression. In addition, Tax is able to activate or repress expression of a large number of cellular genes, leading to immortalization and transformation of the infected T cells. Furthermore, Tax induces chromosomal instability by repressing DNA repair system [128]. The evidence that more than fifty percent of ATL cases lost Tax expression suggested that Tax is important in the early stage of ATL development but is dispensable for maintenance of ATL malignancy [129]. The antisense gene-encoded protein form, HBZ, inhibits the transcriptional activity of Tax whereas its RNA form promotes proliferation of HTLV-infected T lymphocytes [28, 29]. It is known that some viruses produce RNA products that functionally resemble microRNAs. However, HBZ RNA is structurally different from microRNA. How this viral RNA acquires oncogenic activity remains unclear and is currently under active investigation by several groups.
It is noted that ATL cells have not been shown to exhibit specific chromosomal translocations. Somatic alterations of genomic DNA are likely to occur in ATL due to attenuated cell cycle checkpoints and DNA repair system. The clonal expansion of ATL cells causes accumulation of various mutations and genomic abnormalities, eventually leading to malignant transformation of T cells and development of ATL [130, 131].

1.4 HTLV-1 and neurological diseases

HTLV-1 infection is associated with myelopathy/tropical spastic paraparesis (HAM/TSP). HAM is known as chronic progressive myelopathy, an inflammatory neurodegenerative disease caused by HTLV-1 infection of spinal cord, resulting in the motor and sensory disturbance. TSP is commonly found in tropical regions such as Caribbean where the disease was first identified. TSP was described as a chronic and progressive clinical syndrome for decades before the cause was known in 1985, when HTLV-1 infection was found in patients’ blood samples [9]. HAM was linked to HTLV-1 infection in Japan shortly after the virus was identified [132]. TSP and HAM were combined later since they are actually the same disease [133, 134]. Approximately 2% to 3% of HTLV-1-infected patients develop this chronic inflammatory disease involving central nerve system and other organs, suggesting that the virus-host interaction has a role in the pathogenesis of this inflammatory disease [135].

HAM/TSP is a chronic progressive inflammatory disease, causing atrophy of the thoracic spinal cord with loss of myelin. It is clinically characterized by demonstrating spastic paraparesis, hyper-replexia and Babinski signs of the lower limbs [134, 136]. The onset could be years to decades after primary infection. In rare
occasion, the incubation time could be as short as 6 months after receiving HTLV-1 contaminated blood transfusion [137, 138]. HAM/TSP usually exhibits neurological features such as progressive weakness in lower limbs, difficulty in walking, sensory disturbance, bowel/bladder dysfunctions [139]. In some cases, patients may have uveitis, arthritis, pulmonary lymphocytic alveolitis, polymyositis, keratoconjunctivitis sicca and infectious dermatitis.

In the early stage of the disease, the infiltrating cells include CD8+, CD4+ T lymphocytes and B-lymphocytes [8, 140]. In the late stage of the disease, patients are usually wheelchair bound. Pathological analysis shows that perivascular lymphocytic cuffing and parenchymal mononuclear cell infiltration resulting in nerve fiber impairment and loss of sensory-motor ability in the patients [141]. In chronic patients, the cells present in the damaged area of spinal cord are exclusively CD8+ lymphocytes [139, 142]. HTLV-1 infection was suggested to be responsible for the development of the disease by activating highly specific immune response against HTLV-1 antigen. Compared to asymptomatic HTLV-1-infected individuals, HAM/TSP patients have elevated pro-viral load and increased titers of anti-HTLV-1 antibodies in serum and cerebrospinal fluid (CSF) as well as high levels of pro-inflammatory cytokines and chemokines [143-145]. The HTLV-1-specific, CD8+ cytotoxic T cells (CTLs) are found in peripheral blood lymphocytes (PBLs) and CSF of HAM/TSP patients [146, 147].

HTLV-1 crossing the blood-brain barrier is mediated through infected lymphocytes [148]. Viral antigens such as Tax, which is highly immunogenic, trigger CTL response. The activated CTLs target central nerve system (CNS) cells such as neurons, oligodendrocytes, astrocytes, microglia and endothelial cells, causing damage to these cells. It is proposed that the combination of high levels of pro-
inflammatory cytokines and elevated activity of CTLs contributes to bystander damage on CNS [149].

1.5 The retroviral Tax oncoproteins from HTLV-1 (Tax-1) and -2 (Tax-2)

1.5.1 Protein structure of Tax-1 and Tax-2

HTLV-1 and HTLV-2 share similar genomic structure but differ significantly in their pathogenic properties [29, 101]. Both viruses encode viral transactivators, Tax-1 and Tax-2, which display 75% nucleotide sequence homology and 85% amino acid sequence similarity [150]. Tax-1 is also called p40Tax because it is a 40 kDa nuclear phospho-protein that consists of 353 amino acid residues. The Tax-1 protein consists of several important motifs including a CREB (cAMP response element-binding protein)-binding region on the N-terminus, a zinc interacting domain, a domain for interacting with the transcriptional co-activator CBP (CREB binding protein)/p300 and a domain for DNA contact [151-154]. CBP/p300 are the nuclear partners of ATF/CREB that contains bZip (basic region-leucine zipper) domain [155-157]. The nuclear localization signal (NLS) resides within the first 60 aa of Tax-1 [158]. The C-terminus of Tax-1 contains domains for transcriptional activation and for CBP and p/CAF binding [159]. The PDZ-binding domain motif (PBM) is present on the C-terminus of Tax-1, but is absent in Tax-2. This motif is important for the interaction of Tax-1 with several cellular factors such as scaffolding proteins human homolog of disk large (hDlg), human homolog of Scrib (hScrib), membrane-associated guanylate kinase inverted 3 (MAGI-3), pro-interleukin-16 (pro-IL-16) and Erbb2 interacting protein Erbin [160-169]. The central region of Tax-1 contains two leucine zipper-like sequences that are important for DNA binding and protein dimerization [170, 171]. Tax-2 has four subtypes relating to four serotypes of HTLV-
2. Both Tax-2A and Tax-2B are well characterized compared to the other two Tax-2 subtypes [158]. Tax-2A is a 331-amino acid protein while Tax-2B has an additional extension of 25aa at the C-terminus [172, 173]. Tax-2 also contains CREB-binding site, a zinc-finger domain and binding regions for cell progression proteins, proteasomal subunits, transcriptional factors and cell signal regulators. Tax-2 has a nuclear localization determinant (NLD) within its first 42 aa that is different from Tax-1 [174]. Tax-2 contains an additional localization domain at the amino acids 89-113, which is associated with its subcellular localization [175]. The C-terminal region of Tax-2 has a similar ATF/CREB activating domain as that from Tax-1 [176].

1.5.2 Cellular localization of Tax proteins

Tax-1 is localized predominantly in the nucleus and shuttles between the nucleus and the cytoplasm [177]. However, subcellular fractionation and immunofluorescence imaging experiments demonstrated cytoplasmic localization of Tax-1 in transiently transfected cells [178]. The non-canonical nuclear import signal (NLS) of Tax-1 allows it to enter the nucleus and to prevalently localize in sub-nuclear domain, which is also known as Tax speckled structure (TSS) [177, 179]. The fluorescent and electron microscopy data showed that the TSSs are prominent nuclear bodies (NBs) [180, 181]. The NBs are spheres of 2 to 4 µm in diameter in the inter-chromatin space close to transcription sites. Tax-1 NBs contain over 20 proteins that are involved in different cellular functions including transcription, pre-mRNA splicing, DNA damage response, protein modification and nucleo-cytoplasmic transport [180-188]. For transcription activation, two subunits of NF-κB, p50 and p65RelA, are identified in the Tax-1 NBs [180]. The inter-chromatin space is filled with a dense population of nuclear speckles and bodies that are implicated in
mediating key cellular processes [189-191]. The structure and components of the Tax-1 NBs suggest that they are involved in the transcriptional regulation of both the HTLV-1 proviral and cellular genes. Tax-1 NBs sequester DNA repair effectors such as mediator of DNA damage checkpoint protein 1 (MDC1) and prevent it from recruitment to DNA repair foci [185]. These functions correlate with cellular transformation by HTLV-1. The cytoplasmic distribution of Tax-1 may have various patterns, including Golgi-associated lipid raft microdomains, TAB2-containing cytoplasmic foci and centrosome [182, 188, 192, 193].

Lipid raft microdomains are sphingolipid- and cholesterol-rich plasma membranes that function as a signaling transduction platform [194]. Lipid rafts contain various signaling and transport proteins such as IκB kinases (IKKs) in activated cells. Tax-1 hijacks IKK subunits to the Golgi-associated lipid raft microdomains to induce persistent activation of NF-κB [192]. Tax-1 induces TAK1 (Transforming growth factor-β–activated kinase 1) activation via TAB2 (TAK1-binding protein 2) adaptor [195]. Tax-1 is found to interact with the centrosomal protein to induce over-duplication of centrosome, also called microtubule-organizing center [196]. NLS, together with a ‘Rev-like’ nuclear export signal (NES), are likely to be involved in the shuttling of Tax-1 [178]. The intracellular trafficking of Tax-1 is important for NF-κB activation. Although Tax-2 contains a functional NLS domain, all Tax-2 subtypes are mainly localized in the cytoplasm in HTLV-2-infected cells, probably due to the presence of a motif covering aa 90-100) [175, 197]. NES sequence of Tax-2 is dispensable for the Tax-2 localization [197]. Tax-2B was found to be present within the nuclear bodies [198]. Like Tax-1, Tax-3 displays a nuclear localization, similar to that of Tax-1 in transiently transfected cells [108].
1.5.3 Tax post-translational modifications

Tax-1 is modified by several posttranslational modifications (PTMs) including phosphorylation, ubiquitination, sumoylation and acetylation. Six of Tax-1 residues, including T48, T184, T215, S300, S301 and S336, have been identified as phosphorylation sites. Phosphorylation of serine at position 300 and 301 were identified from Tax-1 mutants F2 (S300L and S301A) and F9 (S300D and S301) [199, 200]. F9 mutant is predominantly localized in the cytoplasm and fails to activate NF-κB pathway or ATF/CREB. Phosphorylation of Tax-1 is a prerequisite for further acetylation, ubiquitination and sumoylation [201]. T48 mutant is unable to activate NF-κB pathway and T215 mutant results in Tax-1 inhibition [200].

Ubiquitination is one of the most important universal PTMs, which regulates a variety of cellular processes, including mitosis, signal transduction, endocytosis and apoptosis [202, 203]. During ubiquitination process, carboxylic acid from the di-glycine motif of ubiquitin protein forms a covalent bond to the ε-amino group of the lysine residue in the modified protein [202-204]. Tax-1 sequence has ten lysine residues that are important for cellular function, localization and protein-protein interaction [201, 205-207]. Five of them, including K189, K197, K263, K280 and K284 that are located in the central domain of Tax-1, are major targets for poly-ubiquitination. Whether ubiquitination targets Tax-1 to the proteasomal degradation or whether it mediates Tax activation is still not clear [208]. Tax-1 polyubiquitination is important for Tax-1-induced activation of NF-κB [173]. A previous study showed that K63 ubiquitination could stabilize Tax-1 and this form of Tax-1 is found in the cytoplasm, which is associated with the Golgi apparatus [209]. However, K48-ubiquitinated Tax-1 is predominantly localized in the nucleus and is targeted for proteasomal degradation. Studies showed that the C-terminal PBM domain of Tax-1
is involved in this degradation process [186, 209]. Tax-2B sequence contains fourteen lysine residues and is ubiquitinated, similar to Tax-1 [198].

Sumoylation is a reversible PTM involved in many distinct cellular processes, including chromatin structure, DNA repair, transcription, cell cycle and cellular trafficking [210]. The major molecule in sumoylation process is SUMO (small ubiquitin-like modifier) protein that is similar to ubiquitin. SUMO proteins are 10 kDa polypeptides that form covalent bonds with lysine residues on target proteins to modify their function. K280 and K284 on Tax-1 sequence are also the targets for polysumoylation. Unlike ubiquitinated Tax-1 at K280 and K284, polysumoylated Tax-1 is only detected in Tax-1 NBs, indicating that ubiquitination and sumoylation are mutually exclusive [182]. Sumoylation is essential for Tax-1 NB formation and NF-κB activation. The experimental findings using sumoylation deficient mutants support the idea that ubiquitinated and sumoylated Tax-1 cooperate to activate the NF-κB pathway [182, 211]. Acetylation introduces an acetyl group into Tax-1 at K346 by the transcriptional co-factor p300 in Tax-1 NBs [201]. Sumoylation of Tax-1 is necessary for the assembly of Tax-1 NBs and activation of NF-κB pathway [212]. Acetylation is also observed in Tax-2, though the detailed mechanism is still unknown [201].

1.5.4 The transforming activity of Tax-1

Previous studies showed that Tax is able to transform rodent fibroblasts such as Rat-1 and NIH3T3. This was demonstrated by the ability of Tax-1 to promote colony formation in semi-soft agar in Tax-1 expressing Rat-1 or NIH3T3 cells [26]. These Tax-1 expressing cells were able to form tumor when transplanted into animals. The transforming activity of Tax is dependent on its ability to activate NF-κB, as the Tax mutant that is defective in activating NF-κB fails to transform rodent
fibroblasts [213]. Interestingly, human fibroblast cells are resistant to Tax-mediated transformation, and the fusion of human fibroblast cells with rodent fibroblasts prevents Tax-mediated transformation on rodent cells. This finding suggests that there may be an inhibitory cellular factor that is able to block Tax transformation of human cells. Rodent cells are more sensitive to carcinogen- or oncogene-mediated transformation than human cells. Unlike human fibroblasts that are not immortalized, Rat-1 and NIH3T3 cells are immortalized cells, and introduction of another oncogene expectedly leads to cellular transformation according to “two hits” theory. Indeed, human fibroblast cells are relatively resistant to oncogene-induced transformation, and it was reported that at least four oncogenes are necessary for transformation of human fibroblasts [214].

In Tax transgenic mice model, Tax-1 was shown to induce ATL-like leukemia in mice [21]. These data are supportive for the transforming activity of Tax-1, but not absolutely informative for Tax’s role in human T lymphocytes. For example, SV40 large T antigen transgenic mice develop a variety of cancers, but SV40 has not been reported to cause human cancer [215]. Tax’s transforming activity on human lymphocytes was first demonstrated using HTLV-1 infectious molecular clone in which the tax gene was disrupted. The wild type molecular clone easily transformed human primary T cells, while the tax mutant molecular clone failed to do so, strongly supporting the hypothesis that Tax is the viral transforming protein [213, 216-219]. However, this experiment does not rule out the potential participation of other viral genes within the HTLV-1 genome backbone. In fact, when Tax is expressed alone, it rarely immortalizes or transforms human primary T cells [220]. This is not surprising because any other known viral or cellular oncogene, when acted alone, rarely immortalizes human T cells. In my graduate study, a Tax-immortalized CD4+ cell line
was established from one of twelve healthy donors, with moderate growth rate in
culture. The low rate of Tax's transforming activity on human primary CD4+ T cells is
correlated with a long incubation period of HTLV-1-mediated leukemogenesis,
suggesting that genetic and environmental factors also play important roles in
disease pathogenesis. My graduate work demonstrated that Tax-2 is able to
immortalize human CD4+ T cells more efficiently than Tax-1. This phenomenon will
be discussed in Chapter 5.

1.6 Effects of Tax in signal transduction
1.6.1 Tax and NF-κB signaling
1.6.1.1 NF-κB pathways

NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells, is a
family of transcriptional factors that control a large number of normal and abnormal
cellular and organismal processes, such as immune and inflammatory responses,
developmental processes, cell growth, apoptosis and oncogenesis [221-223]. NF-κB
pathway is frequently deregulated at various pathogenic conditions. It is well
established that Tax-1 deregulates NF-κB pathway, and this process is critical for
immortalization and transformation of HTLV-1-infected T cells [224]. The
transcriptional factors of NF-κB family share a 300 aa N-terminal Rel homology
domain [225]. NF-κB family proteins consist of five DNA-binding proteins, RelA
(p65), RelB, c-Rel, NF-κB1 (p50) and NF-κB2 (p52) [226-228]. RelA, RelB and c-Rel
share a C-terminal transactivation domain. NF-κB1 and NF-κB2 proteins are
synthesized as large precursors, p105 and p100, respectively. The activity of NF-κB
is regulated by inhibitory IκB proteins. The IκB family contains NF-κB precursors
p100, p105, classical IκB proteins IκBα and IκBε, and non-classical IκB proteins IκBβ, IκBδ and IκBNS [227].

In quiescent cells, the NF-κB proteins are sequestered by IκB in the cytoplasm, forming an inactive heterodimeric protein complex, NF-κB-IκB. The activation of NF-κB is modulated by the upstream regulatory IκB kinase (IKK) complex, which consists of two catalytic kinase subunits, IKKα and IKKβ, and one regulatory subunit, IKKγ (or NEMO). Upon extracellular stimulation, IκB kinases are activated, which subsequently phosphorylate two serine residues at the N-terminus of IκB. The phosphorylated IκB proteins are ubiquitinated, leading to their degradation in proteasome. NF-κB dimers are freed from IκB in the cytoplasm, translocating into the nucleus where they bind to the κB cis-element on the enhancer region of a responsive cellular or viral gene to activate target gene expression. Under physiological conditions, activation of NF-κB is transient, primarily due to an autoloop feedback regulation in which NF-κB turns on expression of the inhibitory IκB protein [229].

NF-κB signaling is regulated in two major pathways: canonical (classical) pathway and non-canonical (alternative) pathway. The canonical pathway is triggered by microbial products or pro-inflammatory cytokines, and is often involved in the regulation of inflammation and apoptosis [230]. For example, the binding of an extracellular ligand to relevant cell surface receptor such as tumor necrosis factor receptor 1 (TNFR1) or a Toll-like receptor leads to the recruitment of adaptors such as tumor necrosis factor receptor type 1-associated DEATH domain protein (TRADD), tumor necrosis factor receptor-associated factor (TRAF) or receptor interacting protein (RIP) to the cytoplasmic domain of the receptor. These adaptor proteins assemble a platform to recruit and activate the IKK complex. IKKβ and IKKγ
are the key components of the IKK complex to regulate canonical NF-κB pathway. Activated IKKβ phosphorylates IkB, leading to the nuclear translocation of p65/p50 heterodimers. The non-canonical NF-κB pathway is induced by certain signals such as lymphotoxin B (LT-β), B-cell activating factor (BAF) and CD40 during lymphoid organ development [231-234]. It differs from canonical pathway because the non-canonical pathway involves IKKα-mediated phosphorylation of p100. Extracellular stimulation activates NF-κB-inducing kinase (NIK), which in turn activates the IKK complex that contains two IKKα subunits. The IKK complex mediates the proteolysis of p100 into p52 subunit that forms a transcriptionally active p52/RelB complex.

1.6.1.2 Activation of canonical and non-canonical NF-κB pathway by Tax-1

NF-κB pathway is constitutively activated in HTLV-1-transformed T cells. Tax-1 is known to activate both canonical and non-canonical NF-κB pathways [130, 235-242]. Tax-1 subverts cellular signaling to activate canonical NF-κB pathway. Tax-1 directly binds to the regulatory subunit IKKγ in the cytoplasm, causing conformational change of the catalytic subunits of the IKK complex, IKKα or IKKβ, which subsequently phosphorylates IkBα for its degradation in the proteasome, resulting in the nuclear translocation of the p65/p50 NF-κB heterodimeric complex [243-249]. Co-immunoprecipitation is a common method to pull-down cellular proteins that complex with the Tax protein using Tax-specific antibody for immunoprecipitation. Genetic approach with utilization of small interference RNA-specific for the target protein is best pursued in order to determine the functional relevance of the Tax-target protein interaction. The evidence of the functional interaction between Tax and IKKγ was further strengthened from the finding that Tax fails to activate IKKs in NEMO/IKKγ-deficient cells [250]. A Tax-1 mutant M22
(T130A, L131S) is unable to interact with IKKγ, thus is defective in activating NF-κB pathway [245]. Tax-1 was also reported to interact with IKKβ, and this interaction is likely indirect, probably through the interaction between Tax and IKKγ because both IKKβ and IKKγ are the components of the IKK complex. To support this notion, either the dominant-negative mutant of IKKβ or small interference RNA specific for IKKβ is able to block Tax-mediated activation of NF-κB [192, 251, 252]. These experimental findings indicate that IKKγ is the key target for Tax to activate the IKK complex and that Tax stimulates the activity of IKKβ to activate canonical NF-κB signaling.

Additional experiments showed that IKKα-mediated p65 phosphorylation correlates with Tax-1 expression [253].

In the non-canonical pathway, Tax-1 bypasses NIK, binds IKKγ and recruits IKK complex that contains only IKKα [239, 254-256]. Tax-1 mimics NIK to mediate IKKα binding to p100 and is able to activate non-canonical pathway in the absence of NIK [239, 240]. Tax-1 mediates the processing of p100 to generate the cleaved product, p52, indicating that Tax-1 functions as an adaptor protein that brings IKKα complex and p100 together [257]. Although it is now clear that Tax-1 activates both canonical and non-canonical pathways of NF-κB, it remains to be determined the mechanism of how Tax-1 mediates persistent activation of NF-κB.

1.6.1.3 Tax activation of IκB kinases and lipid raft microdomains

It has been shown that Tax-mediated activation of NF-κB signaling involves the lipid raft recruitment of IκB kinases [192]. Lipid raft microdomains are unique subcellular membrane structures associated with plasma membrane or intracellular membranes in the Golgi, ER or mitochondria [258, 259]. The lipid compositions of
the lipid rafts are cholesterol and sphingolipid, therefore, this specialized membrane structure is quite rigid [260-262]. Cholesterol and sphingolipid are synthesized from the Golgi and are recycled from the Golgi to the plasma membrane or intercellular membranes with a rapid turnover rate [263, 264]. The lipid raft microdomains on the plasma membrane are crucial membrane structures, which is proposed to have an important role in serving as “signal transduction platforms” for receptor tyrosine kinases such as epidermal growth factor receptor (EGFR) and T cell receptor [265-269]. For the intracellular microdomains, it is known that this subcellular structure is critical for recruiting viral structural proteins such as the myristoylated Gag proteins from HTLV-1 or HIV-1 to assemble viral particles [270-272].

In the event of T cell activation, the interaction between the MHC-I/peptide complex from antigen-presenting cells (APCs) and the TCR from T cells that recognize the peptide complex on APCs occurs, which leads to rapid formation of immune synapse on T cells [273]. Lipid rafts are essential components of immune synapse in which TCR and its downstream signaling molecules are rapidly accumulated in this structure [274, 275]. Lck, a lipid raft-associated tyrosine kinase, is activated in response to TCR engagement and phosphorylates tyrosine residues on CD3 molecules [276, 277]. The phosphorylated CD3 molecules recruit SH2 domain-containing proteins such as Grb2 and SLP76 as well as downstream kinases including ZAP70, Syk, PI3K and PKCθ to the plasma membrane-associated lipid raft microdomains for their activation. Subsequently, the Carma1-Bcl10-MALT1 complex is also recruited into plasma membrane lipid rafts for activation [278-280]. The IKK complex is a downstream protein complex of the Carma1-Bcl10-MALT1 complex, and translocates to the plasma membrane lipid rafts in response to upstream
activation signals to acquire full activity within this structure [281]. Consequently, activated IKK phosphorylates IκB, leading to its proteasomal degradation and subsequent activation of NF-κB. It is well known that the activity of NF-κB is essential for T cell activation and proliferation, and that lipid raft recruitment of the IKK complex to the plasma membrane is crucial for acquiring its catalytic activity [282].

The methodologies for analyzing lipid rafts have been well developed. Lipid raft fractionation employs a technique called density gradient ultracentrifugation in which cellular lysates are placed into sucrose gradients in the densities of 40%, 30% and 5% from bottom to top in an ultracentrifuge tube. After ultracentrifugation, the lipid raft-associated proteins are floating at the layer between 5% and 30% gradients because cholesterol-linked proteins turn to be lighter than the proteins without attached lipids, and are unable to penetrate higher density gradients. Each fraction is collected and examined to determine where the target proteins are located by immunoblot analysis. Some known lipid raft-associated proteins such as Lck and LAT (the linker for activation of T lymphocytes) can be used to assist in identification of the lipid raft fraction [283]. Since cholera toxin B has specific affinity to cholesterol, it can also be used to detect cholesterol-containing lipid raft fraction once this toxin is conjugated with horse radish peroxidase (HRP) [284]. Additional methods can be employed to detect lipid raft fraction. For example, alkaline phosphatase (AP) exhibits a peak activity in the lipid rafts and the cholesterol-containing lipid rafts have a peak absorbance at OD 600 nM [285]. Cholesterol chelate, methyl-β-cyclodextrin (MβCD), effectively destroys lipid rafts, which serves as a useful control [286-288].

How the lipid raft-recruited molecules gain their activities remains a debatable subject. One possible mechanism is enrichment of signaling molecules. Signaling
transduction occurs instantaneously requiring molecules to be available and not requiring synthesis. These molecules are usually distributed widely in the cytosol in resting cells. Recruitment of these molecules to the lipid rafts allows their rapid enrichment, increasing chances of cross-phosphorylation and subsequent activation. Alternatively, the lipid raft-associated proteins may confer conformational change, opening their activation motifs. A method was developed to generate constitutively activated kinases without presence of extracellular stimuli or upstream activators. This can be done by attaching an N-terminal myristoylation signal (Myr) from v-Src or Lck to a given kinase, and the resulting kinases will acquire constitutive activity. For instance, Myr-PI3K and Myr-Akt1 exhibit spontaneous and persistent catalytic activity and are oncogenic [289-291]. Furthermore, an activated kinase library was developed with attachment of the myristoylation signal to a panel of cellular kinases. By screening the activated kinase library, IKKε was identified as one of the cellular oncoproteins for malignant transformation of breast epithelial cells [292, 293].

In Tax-expressing T cells, the cytoplasmic Tax accumulates in the Golgi apparatus, hijacking the IKK complex into this subcellular structure. My previous work demonstrated that HTLV-1 Tax is a viral lipid raft-associated protein that can recruit the IKK complex into the Golgi-associated lipid raft microdomains resulting in their persistent activation in HTLV-1-transformed T cells and in Tax-transfected cells [192]. This conclusion is based on following experimental findings: (a) that IkB kinases are constitutively present in HTLV-1-transformed T cells that express Tax, as IkBα and IKKα are intensely phosphorylated in Tax-expressing T cells, correlating with a hyperactivity of NF-κB, (b) that using density gradient ultracentrifugation, together with biochemical, molecular biology and imaging techniques, the IKK complex and the Tax protein are found to constantly reside in the lipid rafts, (c) that
treatment of HTLV-1-transformed T cells with MβCD results in suppression of the NF-κB activity and (d) that the lipid raft recruitment of the IKK complex is mediated by the physical interaction between Tax and IKKγ.

Tax does not have a consensus lipid raft-targeting signal and it is likely that Tax is accumulated in lipid rafts through an indirect mechanism, for example, through the interaction with a cellular lipid raft-associated protein. This hypothesis is yet to be validated. Other lipid raft-attached viral proteins can also modulate NF-κB activity. For example, the latent infection membrane protein 1 (LMP1) of the Epstein-Barr virus resides in the plasma membrane-associated lipid rafts and mediates engagement with tumor necrosis factor receptor-associated TRAF to activate NF-κB via the NF-κB inducing kinase (NIK)/IKKα-dependent pathway [294, 295]. The Nef protein from human immunodeficiency virus type 1 is a myristoylated protein that associates with the plasma membrane lipid raft and potentiates NF-κB activity [296, 297]. Unlike these viral proteins, Tax appears to be attached to the intracellular lipid rafts in the Golgi, by bypassing upstream signaling molecules of TCR. It is concluded that the persistent presence of IKK in lipid rafts indicates that the IKKs are constitutively activated in HTLV-1-transformed T cells.

1.6.2 Tax and other oncogenic signaling

In addition to the activation of NF-κB signaling, it has been reported that Tax deregulates a variety of oncogenic signaling molecules. Tax is able to activate the transcriptional factors CREB, a cellular factor that is critical for promoting the viral gene transcription from the 5’-LTR from HTLV-1 genome, and AP-1 [30, 298-300]. Both CREB and AP-1 have important roles in oncogenesis [301]. Tax activates Akt by interacting with p85 subunit of PI3KC1, and PI3KC1/Akt signaling is known to
support cell survival [302-307]. Tax was shown to activate Jak/Stat3 signaling molecules [308, 309]. All these signaling molecules that Tax deregulates have been implicated in playing crucial roles in cell survival, proliferation, anti-apoptosis and oncogenesis.

### 1.7 Tax, cell cycle progression and genomic damage

Viral oncoproteins are tightly associated with the progression of cell transformation [310-312]. The cell cycle is governed by interactions between cyclins and cyclin-dependent kinases (CDKs) [313, 314]. The cyclin and CDK levels are controlled by the ratio of post-translational phosphorylation/dephosphorylation modifications. The cell cycle is regulated through the formation and stability of cyclin/CDK complex, the accumulation of CDKs by CDK inhibitors and phosphorylation/ dephosphorylation modifications of CDKs. To break T cell senescence and to promote cell cycle progression, Tax upregulates cell-cycle phase activators including CDK2 and CDK4, cyclin D2, cyclin D3, p21\(^{WAF1}\) and E2F1, resulting in accelerated G1–S progression and DNA hyper-replication [315-320].

In addition to serving as an activator for cellular oncogenic proteins, Tax can also act as a repressor. Tax represses tumor suppressors like p53, retinoblastoma protein (pRB), DLG1 and p16 to increase cell cycle phase transition [162, 310, 319, 321]. Tax inactivates the p53-mediated G1/S checkpoint, forcing cell cycle progression to occur even in the presence of DNA damage [321]. There are two mechanisms proposed to contribute to the functional inactivation of p53 by Tax protein. Tax acts as a competitor of p53 to bind CBP/p300 causing the reduction of p53-mediated gene expression [322, 323]. Another mechanism is that the constitutive activated NF-κB pathway leads to the formation of p65/RelA/p53 inactive
complex [324]. A wide range of chromosomal abnormalities such as deletions, translocations, rearrangements and duplications are found in HTLV-1 infected cells probably due to proviral integration [26, 325-333]. One of the characteristic feature of HTLV-1-transformed T cells is aneuploidy, and Tax induces centromere amplification by interacting key cellular components RANBP1 and TAX-1BP2 [114]. Tax-1 suppresses several DNA repair pathways such as nucleotide excision repair (NER), base excision repair (BER), mismatch repair (MMR), or double-strand break repair (DSBR), and these actions would increase genomic mutations [128, 334-336]. Tax-1 can transactivate proliferating cell nuclear antigen (PCNA) promoter to increase endogenous PCNA protein expression that affects DNA replication and repair [333, 337]. Tax inhibits cellular DNA repair by repressing human DNA polymerase β (pol β) gene expression [128]. Another possible cause of chromosomal abnormality is elevated hTERT expression in HTLV-1-infected cells [338-340]. Tax is needed to induce T cell proliferation by activating growth factor receptors and cellular factors that facilitate T cell growth. For example, Tax upregulates cytokine receptors such as IL-2Rα and IL-15Rα as well as their corresponding cytokines IL-2 and IL-15 [341, 342]. Both IL-2 and IL-15 are essential T cell growth factors. This action provides autocrine loop necessary for chronic expansion of HTLV-1-transformed T cells. The net effect is that Tax breaks T cell senescence, suppresses apoptosis and allows accumulation of oncogenic mutations in HTLV-infected T cells. It is apparent that Tax alone is sufficient to acquire full capacity of oncogenic potential and is the key viral component essential for malignant transformation of CD4+ T lymphocytes.
1.8 Tax and autophagy dysregulation

Autophagy is a cellular process self-eating its own aggregated proteins and aged organelles in order to generate energy source for cells under various stresses such as nutrient deprivation [343, 344]. Therefore, the fundamental function of autophagy is pro-survival. However, deregulated autophagy can either promote cell survival or induce cell death, so called type III programed cell death. Autophagy is induced or deregulated following infections of many oncogenic or non-oncogenic viruses [345, 346]. Deregulated autophagy also plays an important role in tumorigenesis of many types of human cancers that are not caused by oncogenic viruses [346].

Autophagy is an evolutionally conserved, pro-survival cellular machinery among living organisms. In the process of autophagy in response to metabolic stress, double-membraned autophagosomes are formed in sequestering aggregated cellular proteins and organelles. Autophagosomes subsequently fuse with lysosomes for degradation of denatured proteins to generate amino acids that are further metabolized to provide energy for the need of cells under stress [347-351]. A great deal of experimental findings indicate that dysregulation of autophagy plays crucial roles in tumor initiation and progression. Tumor initiation is characterized by defects in apoptosis in pre-malignant cells, allowing these cells to overcome oncogene activation-induced senescence or apoptosis, leading to their aberrant proliferation. When tumor progresses, the growth of cancer cell growth exceeds the rate of formation of new blood vessels or angiogenesis, causing the center of the tumor to be under constant hypoxic and metabolic stress [352]. Such stress signals trigger autophagy-mediated survival machinery in cancer cells [353, 354]. Defective autophagy likely occurs primarily due to disrupted expression of autophagy
mediators and oncogenic activation of PI3KC1/Akt signaling that suppresses autophagic process [355]. Defects in both autophagy and apoptosis cause necrotic cell death in the tumor regions under metabolic stress, leading to inflammatory response and DNA damage that facilitates development of somatic mutations [346]. Consequently, tumor progresses into advanced stage in which metastatic cancer foci develop. Chemotherapy is a main therapeutic strategy to treat cancer patients, while many cancers are resistant to this therapy [356]. Because autophagy is frequently seen in patients undergoing chemotherapy, it is hypothesized that chemotherapeutic agent induces bystander effects such as induction of autophagy, which promotes survival of cancer cells. In support of this hypothesis, a clinical trial combining chemotherapeutic agent and [357] an inhibitor of autophagy, significantly improved therapeutic efficacy. Another function of autophagy is to safeguard normal cells by limiting carcinogen-induced genome damage. Defects in autophagy render cells susceptible to carcinogen- or oncogene-mediated genome instability, allowing accumulation of mutant oncoproteins and facilitating tumor growth [358]. Although the role of autophagy in cancer development remains in hot debate, the consensus view is that autophagy is capable of functioning as a double-edged sword by either promoting or suppressing cancer growth depending on the stages of cancer development, types of human cancer and specific carcinogens/oncogenes.

Viruses can hijack cellular autophagy machinery for their own benefits such as productive replication or oncogenesis [359-361]. Because autophagic process is regulated by a variety of cellular signaling pathways, it is proposed that some viral components have the capacity to interfere with cellular signaling and hence have been implicated in playing a role in dysregulation of autophagy. In the case of HTLV-1, the viral oncoprotein Tax activates a variety of oncogenic signaling including IKK
and PI3KC1 [192, 240, 362, 363]. IKK and PI3KC1 have been reported to exhibit opposing activities in regulating autophagy [364, 365]. IKK is known to mediate autophagy in starvation or rapamycin-induced autophagy, whereas PI3KC1 has an opposite activity [366]. My work in this graduate study demonstrated that a high level of autophagy occurs spontaneously in HTLV-1-transformed T cells.

Unlike solid tumors that are constantly under stress signals such as hypoxia and nutrient deficiency, which are known to induce autophagy, HTLV-1-transformed T cells are grown *in vitro* at optimal culture conditions. Cellular stresses are apparently not the cause of induction of autophagy in the virus-transformed cells. My research work identified the viral oncoprotein Tax to be the determining factor to deregulate autophagy. Tax-mediated autophagy is related to the Tax’s ability to facilitate a crosstalk between the IKK complex and the autophagy molecular complex containing Beclin1-PI3KC3-Bif1. Tax is able to interact with the autophagy molecule complex containing Beclin1, PI3KC3 and Bif1, and to sequester these autophagy molecules into lipid rafts in the IKK complex-dependent manner. Genetic disruption of Beclin1 in HTLV-1-transformed T cells led to an impaired autophagy along with reduced cell viability. These experimental findings suggest that Tax links the IKK complex to autophagy pathways, thereby promoting survival and proliferation of HTLV-1-transformed T cells.
Figure 1.1 Scheme of the HTLV-1 pro-virus genome and HTLV-1 encoding genes. The structural proteins are flanked by LTRs. The pX region ORFs are shown. This drawing is modified after Matsuoka, 2003 [367].
Figure 1.2 Schematic representations of Tax-1, Tax-2A and Tax-2B structural and functional domains. Specific domains involved in transcriptional activation pathways are indicated. NLS (nuclear localization sequence), NES (nuclear export sequence) and LZR (leucine-zipper-like region).
Chapter 2: HTLV-1 Tax deregulates autophagy by recruiting autophagic molecules into lipid raft microdomains

2.1 Abstract

The retroviral oncoprotein Tax from Human T cell leukemia virus type 1 (HTLV-1), an etiological factor that causes adult T cell leukemia and lymphoma, plays a crucial role in initiating T lymphocyte transformation by inducing oncogenic signaling activation. We here report that Tax is a determining factor for dysregulation of autophagy in HTLV-1-transformed T cells and Tax-immortalized CD4 memory T cells. Tax facilitated the autophagic process by activating IκB kinase complex, which subsequently recruited an autophagy molecular complex containing Beclin1 and Bif-1 to the lipid raft microdomains. Tax engaged a crosstalk between the IκB kinase complex and the autophagic molecular complex by directly interacting with both protein complexes, promoting assembly of LC3+ autophagosomes. Moreover, expression of lipid raft-targeted Bif-1 or Beclin1 was sufficient to induce formation of LC3+ autophagosomes, suggesting that Tax recruitment of autophagic molecules to lipid rafts is a dominant strategy to deregulate autophagy in the context of HTLV-1 transformation of T cells. Furthermore, depletion of Beclin1 resulted in an impaired growth of HTLV-1-transformed T cells, indicating a critical role of Tax-deregulated autophagy in promoting survival and malignant transformation of virally infected T cells.
2.2 Introduction

Human T cell leukemia virus type 1 (HTLV-1) is the etiological factor that causes adult T cell leukemia and lymphoma (ATL). The HTLV-1 viral genome-encoded oncoprotein, Tax, plays a pivotal role for promoting viral replication and initiating malignant transformation of CD4+ T lymphocytes. Tax deregulates various oncogenic signaling including IκB kinase (IKK)/NF-κB, STAT3 and PI3KC1/Akt for aberrant proliferation of infected T cells [244, 252, 368-371]. Notably, the constitutive activity of NF-κB is thought to be a prerequisite for induction of ATL. Tax activates NF-κB by stimulating the activity of the IKK complex, the key regulator of NF-κB signaling [206, 243-245]. The IKK complex is composed of two highly homologous catalytic subunits, IKKα and IKKβ, and a regulatory subunit, IKKγ [372-374]. The IKK complex can be activated by divergent upstream kinases that connect it to signals from cell surface receptors such as T cell receptor (TCR) [375-381]. Upon T cell activation, IκB kinases are recruited to the plasma membrane lipid raft microdomains where they become catalytically active. This detergent-resistant membrane structure is enriched with cholesterol and sphingolipids, which are generated in the Golgi and can be recycled from the Golgi to the plasma membrane [382]. These unique membrane structures serve as crucial signal transduction platforms. Distinct from TCR activation, Tax bypasses upstream kinases to target the IKK complex through direct interaction with IKKγ [246], recruiting this kinase complex to the lipid raft microdomains for activation [192].

The IKK complex has been implicated in playing an important role in starvation- or rapamycin-induced autophagy [364], though the underlying mechanism remains to be determined. Autophagy is a catabolic process and is common among living
organisms [383, 384]. In response to metabolic stress, autophagosomes form and sequester aggregated cellular proteins and organelles, which are subsequently degraded through their fusion with lysosomes to generate autolysosomes. This process generates energy for the need of metabolically stressed cells and hence, the primary function of autophagy is pro-survival. Although the role of autophagy in oncogenesis remains controversial, it is known that autophagy contributes to chemotherapy resistance due to its cytoprotective function [385]. Furthermore, autophagy is necessary for certain tumorigenic viruses for their productive replication and induction of oncogenesis [360, 386-388]. Hepatitis C virus (HCV)-induced autophagy promotes initiation of viral infection, and inhibition of autophagy represses HCV replication [389]. Latent membrane protein 1 (LMP1), an oncogene product from Epstein-Barr virus (EBV), induces early or late stage autophagy depending on its expression levels [390]. Inhibition of autophagy in EBV-infected cells suppresses transforming phenotypes resulting from accumulation of LMP1. Further, the oncogenic X protein from Hepatitis B Virus (HBV) sensitizes cells to starvation-induced autophagy by increasing Beclin1 (BECN1) expression [391].

The autophagic process is regulated by a variety of oncogenic signaling pathways [392-394]. Given the evidence that HTLV-1 mediates oncogenic activation, it is conceivable that HTLV-1 may deregulate autophagy for its own benefits in viral oncogenesis. In our previous reports, we identified a novel feature of HTLV-1 Tax in dysregulation of autophagy [192], and examined the role of HTLV-2 Tax-deregulated autophagy in supporting survival of Tax-2-immortalized, human memory CD4+ T cells [395]. In the present study, we investigated the underlying mechanism of HTLV-1 Tax in deregulating autophagy in human T lymphocytes. Our data demonstrated that Tax deregulates autophagy by connecting the IKK complex to autophagy.
pathways in a unique mechanism that involves lipid raft recruitment of IκB kinases and autophagic molecular complexes. We further showed that Tax-deregulated autophagy is critical for survival and proliferation of HTLV-1-transformed T cells.

2.3 Results

**Tax deregulates autophagy in HTLV-1-transformed T cells**

During the process of autophagy, LC3 is lipidated, resulting in a mobility shift from LC3-I to -II. The latter is associated with the autophagosome, which can be visualized by fluorescence imaging. Using these techniques, we evaluated the basal activity of autophagy in HTLV-1-transformed T cell lines including MT-1, MT-2 and HUT102. We observed high levels of LC3-II expression in MT-2 and HUT102, but not in non-HTLV-1-infected T cell line, Jurkat (Figure 2.1A). MT-1 cells expressed very low levels of both LC3-I and LC3-II (Figure 2.1A), indicating that these cells had a low basal activity of autophagy. Accumulation of LC3-II was observed in MT-1 cells and Jurkat T cells that were treated with niclosamide (Figure 2.1B), an inhibitor of mammalian target of rapamycin (mTOR) complex [396], which suggested that the autophagy pathway remained responsive to mTOR inhibition in these cells.

MT-2 and HUT102 cells are known to express Tax. Indeed, both MT-2 and HUT102 cells expressed two forms of Tax, a predominant form of the p68 Env-Tax chimeric protein and a minor form, the wild type p40Tax (Figure 2.1C). MT-1 cells have apparently lost Tax expression (Figure 2.1C). Therefore, we reasoned that Tax might be a causative factor for induction of autophagy in HTLV-1-transformed T cells. To test this possibility, Tax was co-expressed with GFP-LC3 in an autophagy cell model, HeLa cells. The cytoplasmic GFP-LC3 punctate dots reminiscent of autophagosome foci were seen in Tax-expressing cells, whereas the GFP-LC3
fluorescence appeared to distribute evenly in vector-transfected cells (Figure 2.1D). At least a 5-fold increase of autophagic cells was detected in Tax-transfected cells as compared to the vector-transfected cells (Figure 2.1E). Tax also induced formation of p40phox-GFP aggregates, indicating that PI3KC3 was activated by Tax (Fig.1F). Depletion of Tax via lentivirus transduction of Tax shRNAs impaired conversion of LC3-I to LC3-II (Figure 2.1G), and resulted in growth arrest of HTLV-1-transformed MT-2 T cells (Figure 2.1H). The p68Tax knockdown efficiency is shown in Figure 2.1G, and the wild type p40Tax in MT-2 cells was easily depleted by Tax shRNAs because it was expressed at a much lower level than p68Tax (Figure 2.1C). Taken together, these results indicate that Tax is the determining factor for formation of increased LC3+ autophagosomes in HTLV-1-transformed T cells.

**Tax promotes constitutive autophagy in human CD4 memory T cells**

We next determined if Tax plays an essential role in deregulating autophagy in primary human T cells. Two Tax-expressing, human primary T cell lines, PTX4-1 and PL9-1, were established by stable expression of the Tax-GFP fusion protein via lentivirus transduction in human peripheral blood lymphocytes obtained from two healthy donors. These two cell lines exhibited a CD3+/TCRαβ+/CD4+/CD25+/CD45RO+/CD69+ immunophenotype, indicating that they were activated memory CD4+ T lymphocytes (Figure 2.2A). Surface expression of TCRαβ was slightly down-regulated in these cells (Figure 2.2A). Constitutive activation of various oncogenic signaling molecules including NF-κB, STAT3, NF-ATc and AP-1 were detected in Tax-GFP-established T cell lines, similarly to that seen in MT-2 cells (Figure 2.2B and 2.2C). Compared to Jurkat T cells, significant
accumulation of LC3-II was detected in PTX4-1 and PL9-1 cells, confirming a constitutive, high level of autophagic activity occurring in Tax-established primary T cells (Figure 2.2D).

**Tax deregulates autophagy via the IKK complex**

HTLV-1-transformed T cells express constitutively activated PI3KC1/Akt and IKK/NF-κB signaling molecules [243]. Previous studies indicated that these two signaling pathways exhibit opposing activities in regulating autophagy [370]. Although the non-Tax-expressing MT-1 cells exhibited a slightly increased NF-κB activity, much higher levels of NF-κB activity were seen in MT-2 and HUT102 cells [397], which correlated with expression of Tax and high levels of autophagic activity in these cells. These results suggest that Tax could utilize IκB kinase-dependent cellular mechanism that potentially overpowers Akt-mediated inhibition of autophagy, thereby facilitating autophagic process.

To investigate the notion that the activation of IKK is required for the Tax-deregulated autophagy, we generated Tax-GFP and its variant forms, M22-GFP and M47-GFP. M22 is defective in activating IKK while maintaining its ability to activate CREB [398]. In contrast, M47 is unable to activate CREB but maintains the capacity to induce full-scale activation of IKK. We found that Tax and M47 induced NF-κB activity whereas M22 did not (Figure 2.3A). The subcellular distributions of Tax and its mutants also differed. Tax-GFP was distributed in both the nucleus and the cytoplasm with a perinuclear cluster pattern in transfected HT1080 autophagy model cells (Figure 2.3B), and this subcellular distribution pattern was similar to that seen in HTLV-1-transformed T cells [399]. M22-GFP lost the perinuclear cluster pattern and was expressed in the nucleus and the cytoplasm, whereas M47-GFP was expressed
predominantly in the perinuclear clusters (Figure 2.3B). When co-transfected with mKate2-LC3, a far-red, monomeric protein mKate2-tagged LC3, Tax-GFP or M47-GFP induced formation of the cytoplasmic mKate2-LC3 foci, while M22-GFP failed to do so (Figure 2.3C). Consistent with this finding, IKKβ$_{KA}$, a constitutively active form of IKKβ, induced formation of LC3+ foci (Figure 2.3C). Similar to the results from Tax mutants, IKKβ$_{KA}$, but not IKKβ$_{KM}$ (a kinase mutant form of IKKβ), induced formation of LC3+ foci in HT1080 cells (Figure 2.3D). Further, depletion of the catalytic subunits of the IKK complex, IKKα or IKKβ, led to reduced conversion of LC3-I to LC3-II in HTLV-1-transformed MT-2 cells (Figure 2.3E). Together, these results strongly suggest a crucial role of the IKK complex in Tax-mediated autophagy.

**Tax recruits the autophagic molecular complex to lipid rafts through IKK**

Our previous study showed that Tax associated with the lipid raft microdomains, hijacking the IKK complex to lipid rafts for activation. We reasoned that Tax might be able to direct lipid raft translocation of the autophagy molecules for their activation through the IKK complex. To test this possibility, we performed lipid raft fractionation analysis. BECN1 and Bif-1 are two key molecules that are involved in the initiation of vesicular nucleation during formation of autophagosomes [400]. We found that Bif-1, together with IKK and Tax, were constitutively present in the lipid raft fraction in Tax-expressing T cell lines including MT-2, HUT102 and PTX4-1 (Figure 2.4A, 2.4B and 2.4C), while it remained in the soluble fractions in non-Tax-expressing T cells including MT-1, Jurkat T cells and normal peripheral blood lymphocytes (PBLs) (Figure 2.4D, 2.4E and 2.4F). BECN1 was also present in the lipid raft fraction in HUT102 cells (Figure 2.4A). These results support the idea that Tax is capable of recruiting the autophagic molecules into lipid rafts.
We next verified the role of Tax in directing the lipid raft translocation of BECN1 and Bif-1. In Tax-GFP-transfected cells, the autophagic molecules including BECN1 and Bif-1 were detected in the lipid raft fraction, whereas these molecules remained in the soluble fractions in GFP- or M22-GFP-transfected cells (Figure 2.5A). To validate the involvement of the IKK complex in Tax recruitment of BECN1 and Bif-1, we generated IKKα- or IKKβ-depleted cells (Figure 2.5B), followed by transfection of Tax into these genetically modified cells. We observed that depletion of either IKKα or IKKβ impaired lipid raft translocation of BECN1 and Bif-1 by Tax (Figure 2.5C and 2.5D).

To investigate further the role of IKK in autophagy induction, we developed lipid raft-targeted IKKβ (Myr-IKKβ) and its kinase mutant form, Myr-IKKβKM, as depicted in Figure 2.5E. Myr-IKKβ is catalytically active, whereas Myr-IKKβKM is not, as demonstrated by their abilities in activating NF-κB (data not shown). Myr-IKKβ, but not Myr-IKKβKM, induced lipid raft translocation of BECN1 and Bif-1 (Figure 2.5F). Expression of Myr-IKKβ, but not Myr-IKKβKM, induced formation of LC3+ autophagosomes in transfected HeLa cells (Figure 2.5G). These data, therefore, validate an important role of the IKK complex in Tax-mediated recruitment of the autophagy molecules to lipid rafts for induction of autophagy.

**The cytoplasmic, lipid raft-associated Tax induces formation of autophagosome**

Tax is a biphasic protein, shuttling between the nucleus and the cytoplasm to mediate distinct cytoplasmic and nuclear functions. To exclude the involvement of the nuclear Tax protein, we constructed a lipid raft-targeted Tax, Myr-Tax, as depicted in Figure 2.6A. We found that this modified Tax protein was exclusively
associated with lipid rafts (Figure 2.6A) and was able to increase NF-κB activity at least 5-fold over the vector control, though it was less potent than the wild type p40Tax (Figure 2.6B). The less potent activity of Myr-Tax in activating NF-κB might be caused by different protein processing from p40Tax. Unlike p40Tax, Myr-Tax failed to activate HTLV-1 LTR (Figure 2.6C), though both p40Tax and Myr-Tax were expressed at comparable levels (Figure 2.6D). Tax-mKate2 co-localized with GFP-LC3 to form the cytoplasmic LC3+ foci, while Myr-Tax-mKate2 did not apparently co-localize with GFP-LC3 but was able to induce dramatic cytoplasmic LC3+ puncta (Figure 2.6E), thereby supporting the notion that the cytoplasmic Tax deregulates autophagy via activation of the IKK complex.

**Lipid raft targeting of BECN1 or Bif-1 is sufficient to increase LC3+ autophagosomes**

In Tax-expressing T cells, Bif-1 was constitutively present in lipid rafts (Figure 2.4), while BECN1 appeared to be transiently associated with lipid rafts as evidenced in Tax-transiently transfected cells. To test the idea whether the lipid raft-associated Bif-1 or BECN1 is capable of promoting autophagy, we constructed Myr-Bif-1 and Myr-BECN1 as depicted in Figure 2.7A. Both the wild type Bif-1 and BECN1 were mainly localized in the soluble fractions (Figure 2.5A), and as expected, both Myr-Bif-1 and Myr-BECN1 accumulated in the lipid raft fractions (Figure 2.7A). Surprisingly, expression of Myr-Bif-1 or Myr-BECN1 alone significantly increased LC3+ autophagosomes (Figure 2.7B and 2.7C).

**Tax interacts with the autophagy molecular complex containing BECN1 and PI3KC3**
To understand the mechanistic nature of Tax-mediated autophagy, we examined a possible physical interaction between Tax and autphagic molecular complex. In co-transfected 293 cells, Tax was strongly co-precipitated with BECN1 and PI3KC3, but not UVRAG (Figure 2.8A). The Tax-BECN1 interaction was readily detected in Tax-immortalized T cells (Figure 2.8B). Tax apparently interacted with the domain situated on the amino acid sequence of BECN1 between aa250-aa300, as BECN1ΔN250 was still co-precipitated with Tax whereas BECN1ΔN300 was not (Figure 2.8C). The strength of physical interaction between Tax and BECN1 was comparable to that between Tax and IKKγ (Figure 2.8C). We further showed that depletion of Beclin1 with lentivirus transduction of specific shRNAs led to significant reduction of cell viability of MT-2 cells (Figure 2.8D and 2.8E). Together, these results validatea crucial role of Tax in dysregulation of autophagy with Tax-mediated autophagy functioning as pro-survival machinery in HTLV-1-infected T cells.

2.4 Discussion

In the present study, we demonstrated that increased autophagic activity occurs spontaneously in HTLV-1-transformed T cells. The viral oncoprotein Tax is the determining factor for dysregulation of autophagy in HTLV-1-transformed T cells and in Tax-immortalized CD4 memory T cells. Disruption of autophagic pathways results in growth retardation of HTLV-1-transformed T cells, thereby implicating a critical role of autophagy in promoting T cell survival and malignant transformation.

Although PI3KC1/Akt and IKK, which are both activated in HTLV-1-transformed T cells, have been reported to exhibit opposing activities in regulating autophagy [366], the overall outcome is in favor of autophagosome induction in HTLV-1-transformed T cells expressing the viral oncoprotein Tax. Similarly, TCR
engagement results in activation of IKK and PI3KC1/Akt, and promotes autophagic process, causing T cell expansion [401, 402]. Our findings strongly suggest that autophagy is beneficial for the retrovirus-mediated oncogenesis by providing crucial survival machinery to HTLV-1-transformed T cells. Dysregulation of autophagy can occur during the process of tumorigenesis in a variety of human cancers in addition to virus-mediated oncogenesis [403-405]. Tumor growth typically exceeds the rate of angiogenesis during the early stages of malignancy, causing the center of tumor to experience constant hypoxic and metabolic stress. These stress signals trigger autophagy-mediated survival machinery in cancer cells [346]. In addition, induction of autophagy by chemotherapeutic agent may contribute to the resistance of cancer cells to therapy [406, 407]. Indeed, cancer chemotherapy in conjunction with an inhibitor of autophagy results in an improved therapeutic efficacy, supporting a pro-survival role of autophagy for cancer cells. During the progression of cancer, activation of PI3KC1/Akt and loss of autophagy mediators cause defective autophagy [408, 409]. Defects in both autophagy and apoptosis result in necrotic cell death in metabolically stressed tumor regions, leading to an inflammatory response, DNA damage and consequent tumor progression. In the context of HTLV-1-induced malignant transformation of T cells, Tax is required for initiating T cell transformation since HTLV-1 infectious clone with lack of the tax gene has no transforming activity on T cells [27, 410]. The finding that Tax expression is lost in roughly 50% of ATL cases suggests that Tax is no longer required at the late stage of leukemia. Accumulation of multiple oncogenic events is likely to replace Tax’s functions in advanced disease. Intriguingly, loss of the beclin1 or bif1 gene showed hyper-proliferative and increased incidence of lymphoma and other malignancies in mice [400]. Heterozygous loss of beclin1 is present in some types of human cancer [358].
This may be attributable to the function of autophagy in limiting genome damage. Therefore, it is possible that a much lower autophagic activity in advanced ATL that lacks Tax expression could further enhance genome instability and accumulation of mutant cellular oncoproteins, facilitating cancer progression.

Tax deregulates autophagy by increasing formation of autophagosomes, and some of them can reach the stage of autolysosome. This conclusion is supported by several experimental findings. When co-transfected with the acid-sensitive GFP-LC3, a majority of the Tax protein was found to co-localize with GFP-LC3 in the cytoplasmic puncta (Figure 6E), suggesting that Tax directly participated in the assembly of autophagosomes. However, Tax only partially co-localized with the cytoplasmic mKate2-LC3 red puncta (Figure 3C), which represents both autophagosomes and autolysosomes since mKate2 is acid-stable. Furthermore, Tax induced aggregation of p40phox-GFP, the substrate of PI3KC3, indicating that Tax increases autophagic influx. A recent report showed that Tax increases autophagosomes by blocking fusion of autophagosomes with lysosomes. This process is IκB kinase-dependent [411]. Although the underlying mechanism of this action is presently unclear, it was suggested that the increased autophagosomes are beneficial for supporting HTLV-1 replication by preventing the Tax protein from degradation in lysosome [411]. Our study showed that Tax physically interacted with the autophagy molecular complex of Beclin1-PI3KC3 and participated in the assembly of the LC3+ autophagosomes. This process was dependent on the activity of the IKK complex. IκB kinases have been reported to play crucial roles in starvation and rapamycin-mediated autophagy, leading to the completion of the autophagic process. Recent reports further demonstrated a crosstalk between Beclin1 and the
components of NF-κB signaling pathway, and autophagy induction may be necessary for activation of IkB kinases [412].

Our study demonstrated that Tax-deregulated autophagy is involved in the lipid raft recruitment of the autophagic molecular complex containing LC3, Beclin1 and Bif-1 and that this action was also dependent on the activity of IkB kinases. Similarly, the viral LMP1 protein from EBV associates with the lipid raft microdomains to activate NF-κB, and it also induces autophagy [413]. However it is currently not clear if the lipid raft microdomain is involved in the LMP1-mediated autophagic process.

Although the autophagy molecule LC3B is found to complex with Fas in lipid rafts to activate extrinsic apoptosis in cigarette smoke-induced emphysema [414], the role of lipid raft-associated autophagy mediators for induction of autophagy is presently not known. In the context of Tax-mediated oncogenesis, the following scenarios may occur. Tax may utilize lipid rafts as a signaling platform to recruit both IkB kinases and autophagy molecules into this structure for activating both NF-κB and autophagy pathways. Therefore, lipid raft associated autophagy molecules such as Beclin1 and Bif-1 are activated to facilitate the processes of autophagy and Tax-mediated oncogenesis. Future studies will be important to investigate further the role of lipid raft-associated Beclin1 and Bif-1 in HTLV-1 associated diseases. For example, we will focus on organization and function of the Beclin1 network in HTLV-1 associated myelopathy/tropical spastic paraparesis (HAM/TSP), the cross-regulation between Beclin1 and several cofactors (Atg14L, UVRAG, Bif-1, Rubicon, Ambra1, HMGB1, nPIST, VMP1, SLAM, IP3R, PINK and survivin).

2.5 Materials and methods

Cell lines, antibodies and chemicals
MT-2 cell line was obtained from Dr. Douglas Richman (AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, National Institutes of Health). MT-1 was kindly provided by Drs. Atsushi Koito and Takeo Ohsugi (Center for AIDS Research and Institute of Resource Development and Analysis, Kumamoto University, Japan). HT1080 and Jurkat cell lines were from ATCC. Human T cell lines including MT-1, MT-2 and Jurkat were cultured in RMPI 1640 medium supplemented with 10% fetal bovine serum plus antibiotics at 37°C, 5% CO2.

Antibodies for IKKα, IKKβ and IKKγ were purchased from IMGENEX. Antibodies for LAT, ERK1, BECN1, HA, GST and GFP were from Santa Cruz Biotechnology, anti-LC3 from Cell Signaling, and anti-β-actin and -FLAG from Sigma. Monoclonal anti-Tax antibody was obtained from AIDS reagent program. Niclosamide were purchased from Sigma.

**Lentivirus vector, viral production and transduction of primary CD4 T cells**

The full-length tax cDNA from HTLV-1 was fused with enhanced green fluorescence protein (GFP), and the tax-gfp fusion fragment was cloned into the lentivirus vector pLCEF8, in which the human elongation factor 1 alpha promoter drives expression of Tax-GFP. pLCEF8 is a modified vector of pLL3.7 in which human elongation factor 1 promoter replaced U6 promoter. The recombinant lentivirus was generated by cotransfection of plasmids with the packaging plasmids (Invitrogen, Carlsbad, Calif.) into 293T packaging cells, and the viruses in the culture supernatants were harvested 48 h posttransfection and were used to transduce human T cells. Human peripheral blood lymphocytes were isolated from healthy blood donors, and stimulated with PHA (1µg/ml) for 24 hours, followed by adding recombinant IL-2 (100u/ml). The activated lymphocytes were cultured for 5-7 days, and the CD4+ cells
were enriched through sorting with anti-CD4 magnetic beads. The purified CD4 T cells were then transduced with the lentivirus carrying the tax-gfp expression cassette. The transduced cells were cultured continuously in complete media containing 20% fetal bovine serum and 100u/ml of recombinant IL-2 (AIDS Reagent Program). Two Tax-established T cell lines, PTX4-1 and PL9-1, were developed.

**Immunophenotype analysis**

Tax-immortalized T cell lines were stained with allophycocyanin (APC) conjugated antibodies that included anti-CD3, -CD4, -CD25, -TCRαβ, -CD45RO and -CD69 (eBioscience) according to the manufacturer’s instructions. The stained cells were subjected for FACS analysis.

**Electrophoretic mobility gel shift assay (EMSA)**

Nuclear extracts were prepared from T cell lines using NE-PER nuclear and cytoplasmic extraction reagents (Pierce). The oligonucleotide was 5’-end labeled with biotin (Integrated DNA Technologies) and annealed to its complementary strand. The oligonucleotide probes are for STAT5 (5’-AGATTTCCTAGGAATTCAATCC-3’), Oct-1 (5’-TGTCGAAATGCAATCACTAGAA-3’), STAT3 (5’-GATCCTTCTGGGAATTCCTAGATC-3’) and NF-κB (5’-GATCCGGCAGGGAATCTCCCTCCTTC-3’). The binding activities were examined by EMSA using Light Shift Chemiluminescent EMSA Kit (Pierce). In brief, 5 μg of the nuclear extracts were pre-incubated in a 20-μl total volume containing 10 mM Tris (pH 7.5), 50 mM KCl, 1 mM dithiothreitol, 20 mM EDTA (pH 8.0), 5% glycerol, and 1 μg of polydeoxyinosinic-deoxyctydilic acid and 2 μl of biotin-labeled probe (20 fmol) for 20 min at room temperature. The reactions were mixed with 5 μl of 5× loading
buffer and run on a 6% non-denaturing polyacrylamide gel in 0.5× TBE (1× TBE: 89 mM Tris borate, 2 mM EDTA, pH 8.3) for 90 min at 100 V on ice, and transferred to nylon membranes (Amersham Biosciences) at 380 mA for 1 h in 0.5× TBE on ice. The membrane was UV light cross-linked. Biotin-labeled DNA was detected by streptavidin-horseradish peroxidase, and followed by chemiluminescence.

**Lipid raft fractionation assay**

The lipid raft fractionation assay was performed with density gradient ultracentrifugation. Cells (4 × 10^7 for T cells and 1 × 10^7 cells for HEK cells) were lysed in 2 ml of extraction buffer (20 mM Tris-Cl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100 plus protease inhibitor mixture). Lysates were sheared by 20 passages through a 22-gauge needle, incubated for 20 min on ice before mixing with the OptiPrep density gradient medium (Iodixanol solution, final concentration, 40% v/v; AXIS-SHIELD PoC AS, Oslo, Norway), and placed at the bottom of a 12-ml tube. By overlaying 4 ml of 30% and 4 ml of 5% of OptiPrep medium, a discontinuous OptiPrep gradient was formed. Ultracentrifugation was performed at 100,000 × g for 4 h at 4 °C in an SW41 rotor. 1 ml of each fraction from the top to bottom was collected and subjected to Western blot analysis. Depletion of plasma and intracellular membrane cholesterol by MβCD in T cells was performed by pretreatment of the cells (4 × 10^7 cells/each sample) with 10 mM MβCD for 45 min at 37 °C in Hanks' balanced salt solution. Following this step, the cells were subjected to density gradient ultracentrifugation for lipid raft fractionation analysis.

**Plasmids, site-directed mutagenesis, immunoblot, co-immunoprecipitation and GST pulldown assay**
The plasmids for FLAG-BECN1, FLAG-UVRAG, FLAG-PI3KC3, Bif-1-myc and GFP-LC3B were reported previously [400], and the BECN1ΔN250 and ΔN300 were generated using a PCR-based mutagenesis method. The myristoylation signal from human Lck was fused to the N-terminus of the full-length of IKKβ to generate a myristoylated IKKβ, Myr-IKKβ. The Myr-IKKβKM (K44M) kinase mutant was constructed using PCR-based site directed mutagenesis method. Myr-Tax, Myr-BECN1 and Myr-Bif1 were generated by adding the Lck myristoylation signal to the N-termini of their corresponding cDNAs. The Tax shRNAs were constructed in the lentivirus vector. shRNA plasmids specific for IKKα, IKKβ and BECN1 was engineered in the pLL3.7 vector to express RNAi for knocking down the endogenous proteins. The recombinant lentivirus expressing IKKα, IKKβ and BECN1 RNAi was generated by cotransfection of pLL3.7 RNAi plasmid with the packaging plasmids (Invitrogen, Carlsbad, Calif) into 293T packaging cells, and the viruses in the culture supernatants were harvested 48 h posttransfection and were used to transduce target cells. The co-immunoprecipitation and GST pulldown assays were performed. HEK293 cells (10^6 cells/well) of a six-well plate in log-phase growth were transiently transfected with expression plasmids as indicated in the figures by using PolyFect tranfection reagent (Qiagen, Valencia, Calif). At 24 h later, the cells were harvested and lysed in 1 ml of the lysis buffer containing 1% Triton X-100, 20 mM Tris-Cl (pH 8.0), and 150 mM NaCl plus protease inhibitors (Sigma). A total of 50 μl of the total cellular protein extracts was saved for measuring protein expression, and the rest was incubated with 30 μl of glutathione-Sepharose 4B beads (GSB; Amersham, Sunnyvale, Calif) at room temperature for 2 h. After incubation, the beads were washed three times with the lysis buffer described above but without protease
inhibitors, and the precipitates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot analysis.

**Western Blot Analysis**

Cells were collected and lysed in lysis buffer B containing 40 mM Tris-Cl (pH 7.6), 1% Triton X-100, 1% deoxycholate, 150 mM NaCl plus protease and phosphatase inhibitor mixtures at 4 °C for 30 min. Equal amounts of cellular proteins were analyzed by 10-12% SDS-PAGE, followed by immunoblot. Anti-β-actin blot was used for the protein loading control.

**Fluorescence imaging and autophagy assay**

To construct fluorescence protein tagged proteins, mWasabi encoding a monomeric green fluorescent protein [415], or mKate2 encoding a monomeric far red fluorescent protein [416], was amplified from pTEC15 or pTEC20 (kindly provided by Lalita Ramakrishnan, Addgene plasmid 30174 or 30179 respectively) and was fused into the N-terminus of LC3 PCR fragment to generate mKate2-LC3, which was cloned in the mammalian expression vector pEF2. The mKate2 PCR fragment was fused to the C-terminus of Tax to generate Tax-mKate2. Transient co-transfection was performed in HT1080 and HeLa cells using FuGene®HD transfection reagent (Roche). 48 hours post-transfection, the cells were fixed in 4% formaldehyde-PBS and mounted with DAPI. Fluorescent images were taken using an OLYMPUS IX81 deconvolution microscope and analyzed using SlideBook 5.0 software (Intelligent Imaging Innovations). For immunofluorescence staining, cells were fixed in 4% paraformaldehyde-PBS, blocked in 3% horse serum-PBS, stained with the indicated
primary antibodies overnight at 4°C followed by incubation with fluorescent conjugated secondary antibodies and then mounted with DAPI (Invitrogen).

2.6 Acknowledgments

We thank Atsushi Koito and Takeo Ohsugi for MT-1 cell line, and Dan Liu, Li Chen and Di Xiang for technical assistance.
Figure 2.1 Constitutive autophagic activity in HTLV-1-transformed T cells. 

A. LC3 immunoblot analysis of HTLV-1-transformed T cell lines including MT-1, MT-2 and HUT102. Non-HTLV-1-transformed T cell line, Jurkat, is used for control. 

B. MT-1 and Jurkat T cells were treated with niclosamide at increasing doses (0.625, 1.25, 2.5, 5 and 10μM) for 6 hours, and total cell lysates were prepared for anti-LC3 immunoblot. 

C. The expression status of the Tax protein in HTLV-1-transformed T cell lines, as examined by anti-Tax immunoblot. 

D. GFP-LC3 was co-transfected with vector or with Tax in HeLa cells. 48 hours following transfection, the cells were analyzed with fluorescence microscopy. 

E. Percentage of autophagic cells in transfected cells seen in (D). 

F. p40phox-GFP was co-transfected with the control vector or with Tax in HeLa cells. The cytoplasmic aggregates of p40phox-GFP were detected by fluorescence imaging. 

G. Tax was depleted by lentivirus transduction of Tax shRNAs, and the efficiency of Tax knockdown was determined by anti-Tax immunoblot (top panel), and the levels of LC3-I and LC3-II in Tax-depleted cells were shown in the middle panel. β-actin was used for protein loading control. 

H. Trypan blue exclusion assay to examine cell viability of MT-2 cells transduced with a non-specific shRNA (NS), and Tax-specific shRNA1 and shRNA2 via lentivirus transduction.
Figure 2.2 Oncogenic activation and autophagy induction in Tax-GFP-established T cell lines. A. Immunophenotype of Tax-GFP immortalized T cell lines, PTX4-1 and PL9-1, as determined by FACS analysis. B. Immunoblot analysis for Tax-GFP expression in PTX4-1 and PL9-1 cell lines. C. EMSA assay to detect the activities of NF-κB, Stat3, NF-ATc and AP-1 in Tax-established cells. D. Anti-LC3 immunoblot to examine the conversion of LC3-I to LC3-II in Tax-GFP-established T cell lines.
Figure 2.3 Tax-induced autophagy is dependent on its ability to activate IκB kinase. A. NF-κB luciferase reporter assay with transient co-transfection of pNF-κB luciferase plasmid with vector, Tax, M22 or M47 in 293 cells. B. Subcellular localization of Tax-GFP, M22-GFP and M47-GFP in transfected HT1080 cells. C. HT1080 cells were co-transfected with mKate2-LC3, together with Tax-GFP, M22-GFP, M47-GFP, GFP alone (negative control) or GFP-IKKβKA. The transfected cells were analyzed by fluorescence imaging 48 hours following transfection. D. Percentage of autophagic cells in GFP-LC3 co-transfected HT1080 cells with vector, IKKβKA or IKKβKM, a kinase mutant form of IKKβ. E. Anti-LC3, IKKα and IKKβ immunoblots with cellular lysates from HTLV-1-transformed MT-2 T cells transduced with NS- (non-specific shRNA), IKKα- or IKKβ-specific shRNA.
**Figure 2.4** Autophagy molecules Bif1 and Beclin1 are associated with the lipid raft microdomains in Tax-expressing T cells. Lipid raft fractionation assay was applied to examine cellular proteins associated with lipid rafts in HUT102 (A) and MT-2 cells (HTLV-1-transformed T cell lines expressing Tax) (B), PTX4-1 cells (Tax-GFP-established primary human T cell line) (C), MT-1 cells (HTLV-1-transformed T cell line with lack of Tax expression) (D), Jurkat T cells (non-HTLV-transformed T cells) (E) and PBLs (normal peripheral lymphocytes) (F) with various antibodies indicated in the figure. HRP-conjugated cholera toxin B that has specific affinity to lipid rafts to detect GM1 and anti-LAT immunoblot to detect LAT (a lipid raft marker protein) were used as indications of lipid raft fractions.
Figure 2.5 Tax recruits autophagy molecules into lipid rafts. **A.** The presence of Bif-1 and BECN1 in lipid rafts in HEK293 cells-transfected with GFP, Tax-GFP or M22-GFP as examined by lipid raft fractionation analysis, followed by anti-Bif-1 and anti-BECN1 immunoblots. **B.** IKKα and IKKβ knockdown efficiency in HEK293 cells via lentivirus transduction as determined by anti-IKKα and anti-IKKβ immunoblots. **C.** Lipid raft presence of Bif-1 and BECN1 in IKKα-depleted HEK293 cells transfected with Tax. **D.** Lipid raft presence of Bif-1 and BECN1 in IKKβ-depleted HEK293 cells transfected with Tax. **E.** Schematic structure of Myr-IKKβ and its kinase mutant form, Myr-IKKβ_{KM}. **F.** Lipid raft presence of Bif-1 and BECN1 in HEK293 cells transfected with Myr-IKKβ or Myr-IKKβ_{KM}. **G.** The presence of the cytoplasmic LC3+ foci in HeLa cells transfected with Myr-IKKβ or Myr-IKKβ_{KM}. 
Figure 2.6 Lipid raft-associated Tax is sufficient to induce autophagy. A.
Schematic structure of Myr-Tax and its lipid raft presence. HEK293 cells were
transfected with NF-κB-luciferase reporter plasmid (B) or HTLV-1LTR-luciferase
reporter plasmid (C), together with vector (control), Tax-HA, Myr-Tax-HA or FLAG-
IKKβKA. 24 hours following transfection, luciferase activity was examined. D.
Immunoblot analysis of transfected cells seen in B and C. E. HeLa cells were co-
transfected with GFP-LC3, together with mKate2, Tax-mKate2 or Myr-Tax-mKate2.
48 hours following transfection, the transfected cells were analyzed with
fluorescence imaging.
Figure 2.7 Lipid raft-targeted Bif1 or Beclin1 is sufficient to induce autophagy.

A. Schematic structure of myristoylated Bif1 and Beclin1 (Myr-Bif1 and Myr-Beclin1).

B. Percentage of autophagic cells transfected with vector, Myr-Bif1, Myr-Beclin1, Bif1-myc or FLAG-Beclin1.

C. LC3+ foci in the transfected cells from (B) with fluorescence imaging analysis.
Figure 2.8 Tax interacts with the autophagy molecular complex containing Beclin1 and PI3KC3. **A.** Tax-binds to BECN1 and PI3KC3. Tax-GST was co-transfected with the autophagy molecules including FLAG-Beclin1, FLAG-PI3KC3 or FLAG-UVRAG in HEK293 cells. 24 hours following transfection, GST pulldown assay was performed. **B.** Co-immunoprecipitation of Tax-GFP and BECN1 in Tax-GFP-established, primary human CD4 T cell lines. **C.** Tax binds to the domain situated on aa250-300 of BECN1. Tax-HA was co-transfected with GST-tagged BECN1, BECN1ΔN250, BECN1ΔN300 in HEK293 cells, followed with GST pulldown assay. **D.** Depletion of BECN1 by lentivirus transduction of BECN1-specific shRNAs in MT-2 cells. **E.** Trypan blue exclusion assay to examine cell viability of MT-2 cells transduced with NS- or BECN1-specific shRNAs.
Chapter 3: HTLV-2 Tax immortalizes human CD4+ memory T lymphocytes by oncogenic activation and dysregulation of autophagy

3.1 Abstract

Human T cell leukemia virus type 1 and type 2 (HTLV-1 and -2) are two closely related retroviruses with the former causing adult T cell leukemia. HTLV-2 infection is prevalent among intravenous drug users and the viral genome encodes the viral transactivator Tax that is highly homologous to the transforming protein Tax from HTLV-1. However, the link between HTLV-2 infection and leukemia has not been established. In the present study, we evaluated the activity of HTLV-2 Tax in promoting aberrant proliferation of human CD4 T lymphocytes. Tax-2 efficiently immortalized CD4+ memory T lymphocytes with a CD3/TCRαβ/CD4/CD25/CD45RO/CD69 immunophenotype, promoted constitutive activation of PI3 kinase/Akt, IκB kinase/NF-κB, mitogen-activated protein kinase (MAPK) and STAT3, and also increased the level of Mcl-1. Disruption of these oncogenic pathways led to growth retardation and apoptotic cell death of the Tax-2-established T cell lines. We further found that Tax-2 induced autophagy by interacting with the autophagy molecular complex containing Beclin1 and PI3 kinase class III to form LC3+ autophagosome. Tax-2-mediated autophagy promoted survival and proliferation of the immortalized T cells. The present study demonstrates the oncogenic properties of Tax-2 in human T cells and also shows the value of Tax-2 in serving as a molecular tool to generate distinct T cell subtype lines.
3.2 Introduction

Adult T cell leukemia/lymphoma (ATL) is caused by infection of CD4+ T lymphocytes with human T cell leukemia virus type 1 (HTLV-1) [1, 29, 114]. This type of leukemia exhibits an aggressive clinical course without cure. HTLV-1 infection is also etiologically linked to myelopathy/tropical spastic paraparesis (HAM/TSP) [132]. Roughly 5% of HTLV-1 infected patients developed ATL with a long incubation period typically longer than 20 years, suggesting that genetic factors also play a role in the pathogenesis of ATL. In contrast, a closely related retrovirus, termed HTLV-2, is not linked to leukemia induction, though the viral genome was detected in some cases of hairy cell leukemia [417]. Thus, HTLV-2 may lack certain abilities to transform virally infected T cells into malignant ones.

HTLV-1 and -2 share strikingly similar structures of the viral genomes and both viruses encode highly homologous transforming proteins, Tax [169, 418]. One of the oncogenic properties of Tax-1 is the induction of persistent activation of NF-κB in host cells by stimulating both canonical and noncanonical pathways of NF-κB signaling [219, 240, 243, 245, 246, 419-423]. Tax-2 only activates canonical NF-κB signaling [169, 418]. The role of Tax-2 in deregulating other oncogenic signaling pathways is poorly studied. Further, the Tax-1 transgenic mice developed ATL-like leukemia [424]. However the oncogenic activity of Tax-2 in mouse model has not been reported.

Several studies suggested that Tax-2 is less oncogenic than Tax-1. Tax-2 transformed rat fibroblast cells less efficiently than Tax-1 [425]. In addition, the transforming activity of Tax-2 on CTLL-2 cell line, an IL-2-dependent murine T cell line, was weaker than Tax-1 [426]. Dissecting the protein structures of Tax indicated that although there is 75% amino acid sequence homology shared by both viral
proteins, Tax-2 lacks the leucine zipper-like region and the C-terminal PDZ binding motif (PBM) of Tax-1 [169, 418]. When Tax-2 was fused with the PBM motif from Tax-1, such chimeric protein induced NF-κB processing and resulted in an equal transforming efficiency to Tax-1 in CTLL-2 cells [426]. Intriguingly, both viruses immortalized primary human T cells at a comparable efficiency [169, 418]. It is conceivable that the oncogenic potential of the Tax proteins is best determined in human primary T cells, the natural host cells for HTLV-1 or -2 infection, rather than in rodent cells that are highly susceptible to viral or cellular oncogene-mediated transformation. Indeed, devoid of other viral components, Tax-1 appeared to be a weak oncoprotein as Tax-1-mediated immortalization of primary human T cells was a rare event [427]. The ability of Tax-2 in immortalizing human T cells has not been previously reported. In the present study, we evaluated the activity of Tax-2 in immortalizing human primary CD4 T cells and established four Tax-2-immortalized T cell lines with unique properties of activated memory helper T cells. In addition to the oncogenic activations known in HTLV-1-transformed T cells, our study identified autophagy dysregulation by Tax-2 as a novel survival mechanism in Tax-2-immortalized T cells.
3.3 Results

**Tax-2 immortalizes human CD4+ memory T cells**

Activated human primary lymphocytes grow in vitro typically for up to 4 weeks before reaching cell senescence. Extending growth beyond cell senescence is one of the essential steps towards immortalization and ultimately, oncogenic transformation if additional genetic alteration events occur. To investigate the ability of HTLV-2 Tax in immortalizing mature human CD4+ T cells, we isolated CD4+ T cell species from healthy blood donors, which were enriched using anti-CD4 antibody-coated magnetic beads, followed by lentivirus transduction of the *Tax-2-gfp* fusion gene. Roughly 40-60% of primary CD4+ T cells were transduced as evidenced by visualization of green fluorescence. Three weeks following transduction, nearly 100% of the transduced cells emitted green fluorescence signal and exhibited clumpy growth patterns. The growth of the Tax-2-GFP-expressing cells was strictly dependent on exogenous IL-2.

The *Tax-2-gfp*-transduced cells had a long-term growth potential. In fact, continuous growth of these cells in vitro for more than eighteen months was achieved without losing growth potential, indicating that these cells were immortalized. In contrast, GFP-expressing primary CD4 T cells ceased growth in less than four weeks following transduction. Analysis of the cell surface markers of four Tax-2-GFP-expressing CD4+ T cell lines demonstrated a CD3+/CD4+/CD25+/TCRαβ+/ CD69+/CD45RO+ immunophenotype (Figure 3.1), indicating that these cells were activated memory T lymphocytes. Unlike HTLV-1-transformed T cells in which TCRαβ and CD3 were down-regulated [428], Tax-2-immortalized T cells expressed normal levels of these cell surface molecules.
Tax-2-immortalized T cell lines represent unique subsets of helper T cells

In determining expression patterns of selected genes in Tax-2-immortalized T lymphocytes, we performed real-time quantitative PCR analysis. Compared to the normal CD4 T cell populations that were cultured at the identical conditions, we found that Tax-2-immortalized T cells had differential gene expression profiles. TX2-3 cells expressed significantly higher levels of IL-4, TGFβ1 and FAS (p<0.05), and TX2-4 cells showed high levels of IL-5, IL-15, IFNγ and FAS (Figure 3.2A and 3.2B). TX2-2 cells produced an extremely high level of IL-10 (Figure 3.2B). IL-13 and IL-2 were undetected or detected at very low levels in all Tax-2-immortalized T cell lines (Figure 3.2A), unlike HTLV-1-transformed T cells that expressed IL-13 [429, 430]. Consistent with the results from real-time PCR, the intracellular FACS analysis showed that TX2-4 cells expressed a significantly higher level of IFNγ than TX2-1 cells (p<0.05), though a majority of cell populations from these two cell lines produced IFNγ (Figure 3.2C). Because these T cell lines expressed comparable levels of Tax-2-GFP (Figure 3.6C), the increased levels of cytokines in some T cell lines were unlikely caused by Tax-2. Instead, these gene expression profiles implicated Tax-2 in selectively immortalizing unique subsets of helper T cells. All Tax-2-immortalized T cell lines expressed higher levels of Fas than normal CD4 T cells (Figure 3.2B), similarly to HTLV-1-transformed T cells [431]. These Tax-2-immortalized T cells also produced PDGFRA, but did not synthesize a detectable amount of its ligand, PDGF-BB (Figure 3.2B), suggesting that the autocrine loop of the PDGFRA signaling is not the driving force for promoting proliferation of Tax-2-expressing T cells.

Tax-2 deregulates oncogenic signaling molecules
We next examined oncogenic activation in Tax-2-immortalized T cells. The expression patterns of molecules in T cell receptor signaling were first evaluated. As shown in Figure 3.3A, Tax-2-expressing T cell lines expressed proximal signaling molecules of TCR, including Lck, ZAP70 and LAT. In contrast, Lck and ZAP70 were undetected in HTLV-1-transformed MT-2 T cells. Analysis of the common oncogenic pathways showed that similar to MT-2 cells, the Tax-2-immortalized T cell lines, TX2-1 and TX2-2, exhibited hyper-phosphorylation of MEK1, ERK1/2 and Akt1 (Figure 3.3B), implying constitutive activities of these kinases. The activities of the transcription factors including STAT3 and NF-κB were detected in both Tax-2-expressing T cells and MT-2 cells (Figure 3.3C), and the activity of STAT5 was only detected in Tax-2-established, IL-2-dependent T cells (Figure 3.3C). We next investigated additional pro-survival molecules, the Bcl-2 family proteins. A comparable level of Bcl-xL was expressed in normal CD4 T cells, Tax-2- T cells and MT-2 cells (Figure 3.3D). The level of Mcl-1 was increased in all Tax-expressing T cell lines (Figure 3.3D). Interestingly, unlike MT-2 cells that produced a significantly high level of Bcl-2, TX2-1 and TX2-2 cells expressed almost undetectable level of Bcl-2 (Figure 3.3D). The expression of Bax, a pro-apoptotic Bcl-2 family protein, was not altered in Tax-expressing T cells as compared to normal CD4 T cells (Figure 3.3D). As expected, the tumor suppressor protein p53 was stabilized in Tax-expressing cells (Figure 3.3D). Examination of TX2-3 and TX2-4 cells demonstrated constitutive activities of NF-κB, STAT3 and STAT5 (Figure 3.3E), similar to that seen in TX2-1 and TX2-2 cells (Figure 3.3C). The oncogenic activation in Tax-2-immortalized T cell lines was apparently similar to that in HTLV-2-infected MoT cells that exhibited constitutive activities of STAT3 and NF-κB (Figure 3.3F). Further, the
activity of telomerase reverse transcriptase was well maintained in all four Tax-2-immortalized T cell lines after prolonged culture (Figure 3.3G).

To determine the role of the oncogenic pathways in Tax-2-mediated proliferation of primary T cells, we applied various chemical inhibitors to Tax-2-immortalized T cells. As shown in Figure 3.4A and 4B, U0126, the inhibitor of MEK1, and LY294002, the inhibitor of PI3K, impaired phosphorylation of ERK1/2 and Akt1, respectively. Similarly, wortmanin, an inhibitor of PI3K, was shown to effectively inhibit Akt1 phosphorylation in TX2-1 cells (Figure 3.4C). Bortezomib, an FDA-approved proteasome inhibitor, inhibited NF-κB activity in a dose-dependent manner, induced caspase activation as evidenced by finding cleaved forms of caspase-3, -7 and PARP and reduced cell viability of the Tax-2-immortalized T cells (Figure 3.4D, 3.4E and 3.4F). The above chemical inhibitors and BAY11-7082, an inhibitor of IκB kinase, negatively affected viability of Tax-2-immortalized T cells (Figure 3.4G). Together, these results indicate that activation of multiple oncogenic signaling pathways is crucial for Tax-2-mediated survival and proliferation of human primary memory T cells.

**Tax-2 deregulates autophagy to promote survival of the immortalized T cells**

Autophagy is a pro-survival cellular process for cells under various stress signals such as nutrient deprivation, and has been implicated to play a role in supporting T lymphocyte proliferation [432, 433]. To determine whether autophagy plays an important role for the survival and proliferation of Tax-2-immortalized T cells, we analyzed autophagic process in these cells. One of the characteristic features of autophagy is generation of a lipidated form of LC3, LC3-II, which
associates with autophagosome. In Tax-2-expressing T cells, a significantly high level of LC3-II (p<0.05) was detected (Figure 3.5A). To determine whether autophagy provides a survival signal to Tax-2-immortalized T cells, TX2-1 cells were treated with the autophagy inhibitor chloroquine or 3-methyladenin (3-MA). Both chemicals inhibited growth of TX2-1 cells and induced cleavage of caspase-3/-7 (Figure 3.5B and 3.5C). Collectively, these results indicate that constitutive autophagy is necessary for promoting survival and proliferation of Tax-2-immortalized T cells.

PI3K/Akt1 signaling is known to inhibit autophagy by activating mammalian target of rapamycin (mTOR) complex, while the IKK/NF-κB signaling has been linked to starvation- or rapamycin-induced autophagy [434, 435]. Tax-2 was shown to activate both signaling pathways in immortalized T cells (Figure 3.3). To evaluate the role of Tax-2 in induction of autophagy, we co-transfected Tax-2 with GFP-LC3 to examine formation of LC3+ autophagosome foci. In the absence of Tax-2, GFP-LC3 was evenly distributed in the transfected cells (Figure 3.5D). Co-expression of Tax-2 and GFP-LC3 led to formation of GFP-LC3 cytoplasmic foci reminiscent of autophagosomes (Figure 3.5D). Similarly, the constitutively active form of IκB kinase beta subunit, IKKβKA, promoted formation of the LC3+ autophagosomes (Figure 3.5D). Further, co-transfection of Tax-2-mKate2, a far-red fluorescent protein (mKate2) tagged Tax-2, and p40phox-GFP induced aggregation of the p40phox (Figure 3.5E), which indicates that the activity of PI3K class III (PI3KC3), one of the key mediators of autophagy pathway, was stimulated in Tax-2-mediated autophagy.

To determine the role of IKKβ and Beclin1 (BECN1), an essential autophagy molecule, in Tax-2-mediated autophagy, we generated BECN1- and IKKβ-deficient
HT1080 cell lines by lentivirus transduction of specific shRNAs (Figure 3.5F). As shown in Figure 3.5G, strong accumulation of LC3-II was readily detected in Tax-2- or IKKβKA-transfected cells. However, in BECN1-depleted cells, Tax-2 or IKKβKA failed to induce accumulation of LC3-II (Figure 3.5H). Similarly, in IKKβ-depleted cells, reduced numbers of LC3+ foci were seen upon Tax-2 transfection (Figure 3.5I). We further verified these findings in Tax-2-immortalized T cells. Depletion of IKKα/β in TX2-1 cells resulted in a significant reduction of LC3-II (Figure 3.5J) and reduced cell viability (Figure 3.5K). In BECN1-knockdown TX2-1 cells, LC3-II was also reduced compared to the control cells (Figure 3.5L) but such reduction was less than the IKKα/β-depleted cells (Figure 3.5J and 3.5L), suggesting that in addition to BECN1, other autophagy molecules are possibly involved in Tax-2-mediated autophagy. Consistent with this finding, BECN1-knockdown cells showed slight reduction of cell viability compared to control cells. Together, these results suggest that Tax-2 deregulates autophagy by modulating a crosstalk between the IKK complex and the autophagy molecular complex.

**Tax-2 connects the IKK complex to autophagy pathways**

The underlying mechanism of Tax-2 induction of autophagy was investigated to determine whether Tax-2 physically interacts with key components of autophagy pathways. The autophagy molecular complex, which consists of BECN1, UVRAG and PI3KC3, is essential for assembling autophagosomes [436]. We found that Tax-2 interacted with BECN1 and PI3KC3 in co-transfected cells, but did not co-precipitate with UVRAG (Figure 3.6A). In addition, Tax-2 did not interact directly with other autophagy molecules such as LC3 (Figure 3.6B). In Tax-2-immortalized T
cells, the interaction of Tax-2 and BECN1 was also detected by co-immunoprecipitation (Figure 3.6C). Next, we applied fluorescence imaging technique to visualize Tax-2 interaction with autophagy molecules. BECN1 and PI3KC3 were evenly distributed in the cytoplasm, and IKKγ exhibited a cytoplasmic cluster pattern (Figure 3.6D). Tax-2 recruited BECN1, PI3KC3 and IKKγ into the cytoplasmic punctate foci (Figure 3.6E). These results provided structural evidence that Tax-2 connects the IKK complex to the autophagy pathways.

3.4 Discussion

Our study demonstrates that Tax-2 efficiently immortalizes human primary CD4+ memory T cells. Tax-2 appears to promote survival and aberrant proliferation of primary T cells via several mechanisms. Similar to HTLV-1-transformed T cells, Tax-2 constitutively activates oncogenic signaling pathways including IKK/NF-κB, PI3K/Akt1, MAPK/ERK1/2 and STAT3 and induces expression of survival factors such as Mcl-1. In addition, Tax-2 deregulates autophagy by connecting the IKK complex to autophagy pathways. Our data show that the oncogenic activation and dysregulation of autophagy contribute significantly to Tax-2 immortalization of human T cells.

In contrast to the previous report showing that Tax-1 is a weak oncoprotein and immortalizes mature human T cells poorly [427], we found that Tax-2 immortalizes primary T cells at a reasonably good efficiency. All Tax-2-gfp transduced T cells were able to grow for more than three months, and four out of twelve Tax-2-GFP-established T cell lines have maintained growth potential for prolonged time. The differential immortalization abilities mediated by Tax-1 and Tax-2 remain to be
solved. One possibility is a progressive loss of Tax-1 expression in the late passages of the immortalized T cells. Unlike ATL leukemia cells in which Tax expression is typically lost, the growth of Tax-established primary T cells requires constitutive production of the Tax protein in order to promote T cell survival and proliferation. The activity of a heterologous viral promoter, such as cytomegalovirus promoter (CMV) that was previously utilized to establish Tax-1-immortalized primary T cells [427], may be deleterious during prolonged cell growth due to a possible promoter silencing [437]. In Tax-1-immortalized T cell line MT-4, Tax-1 was expressed at a much lower level than that in MT-2 cells in p40tax form [427]. MT-2 cells express abundant amount of p68 Env-Tax fusion protein but produce extremely low level of p40Tax [438]. The lower level of p40Tax in WT4 cells indicates that the Tax-1 expression is barely maintained in most Tax-1-established T cell lines. With use of human elongation factor promoter (hEF) to drive the Tax-2 expression in the present study, the expression of Tax-2 in the immortalized T cell lines is sustainable for over eighteen months in culture. To draw the conclusion that Tax-2 performs better than Tax-1 in immortalizing human T cells, the same technical approach must be employed. Nevertheless, our study validated the idea that immortalization of mature human T cells can be efficiently achieved by Tax-2.

Our study also identifies autophagy as a novel survival mechanism in Tax-2-immortalized T cells. Tax-2 induces autophagy to promote T cell survival and proliferation by targeting the autophagy molecular complex containing BECN1 and PI3KC3. The role of autophagy in oncogenesis remains controversial. On one hand, autophagy molecules function as tumor suppressors as mice that lack expression of these genes are prone to tumor development [403, 439]. On the other hand, vast amounts of evidence have shown that autophagy contributes to chemotherapy
resistance due to its cytoprotective function [346, 354]. Although autophagy is a fundamental process for anti-viral defense, viruses have developed strategies to subvert or use autophagy for their own benefit. Autophagic process is necessary for productive replication of some oncogenic viruses. For instance, autophagy is implicated in initiation of hepatitis C virus (HCV) infection [386]. Chloroquine, a chemical inhibitor of autophagy, or depletion of autophagy molecules represses HCV replication [440, 441]. The oncogenic X protein from Hepatitis B Virus (HBV) sensitizes cells to starvation-induced autophagy by increasing BECN1 expression [391]. Further, latent membrane protein 1 (LMP1), an oncogene product from Epstein-Barr virus (EBV), induces early or late stage autophagy depending on its expression levels [390]. Inhibition of autophagy in EBV-infected cells suppresses transforming phenotypes resulting from accumulation of LMP1. Not surprisingly, HTLV-2 Tax also deregulates cellular autophagic process for promoting aberrant proliferation of its host cells.

The underlying mechanism of Tax-2 induction of autophagy appears to be related to the crosstalk between NF-κB signaling and autophagy pathways. Tax-2 induces hyper-activation of IKK and NF-κB, and both factors are implicated in induction of autophagy [434, 435]. NF-κB p65RelA induces autophagy by transcriptional activation of the beclin 1 gene [435]. However, NF-κB has also been shown to inhibit autophagy in the context of TNFα-induced cell death [442]. The activation of IKKα or IKKβ induced by TNFα has been implicated in the phosphorylation of tuberous sclerosis complex 1/2 (TSC1/2), resulting in activation of mTOR and inhibition of autophagy [443]. Conversely, autophagic process has a negative impact on the activation of IKK and NF-κB. For instance, autophagy
facilitates the degradation of the IKK complex and its upstream activator NF-κB-inducing kinase (NIK), contributing to the inhibition of NF-κB signaling [444]. Further, autophagic process depletes p62 (sequestosome1), an activator of the IKK complex, resulting in a diminished activity of NF-κB [445]. Interestingly, IKK has been implicated to be the key regulator of autophagy induced by starvation or rapamycin stimulation that triggers IKK activation [434]. Constitutively active IKKβ or myristoylated IKKγ induces autophagy, while depletion of IKKα or IKKβ prevents autophagy induction, suggesting that the IKK complex is central in connecting upstream NF-κB signaling to autophagy pathway. Our data clearly demonstrate that Tax-2 recruits the IKK complex, together with the autophagy molecule complex containing BECN1 and PI3KC3, to form LC3+ autophagosome foci. This is executed by the interaction of Tax-2 with the IKK complex, BECN1 and PI3KC3, resulting in an increased activity of PI3KC3 and assembly of autophagosomes. Inhibition of autophagy leads to apoptotic death of Tax-2-immortalized T cells, supporting a critical role of Tax-2-mediated autophagy in T cell immortalization. The present study provides insights into possible therapeutic development of autophagy inhibitors in managing HTLV-2 infection.

3.5 Materials and methods

**Lentivirus vector, viral production and transduction of primary CD4 T cells**

The *tax* gene from HTLV-2 was fused with enhanced GFP, and the *Tax-2-gfp* fusion fragment was cloned into the lentivirus vector pLCEF8s, in which the human elongation factor 1 alpha promoter drives expression of Tax-2-GFP. The procedure for lentiviral production and concentration was described in chapter 2.5. Human peripheral blood lymphocytes were isolated from healthy blood donors, and
stimulated with PHA (1µg/ml) for 24 hours, followed by adding recombinant IL-2 (100u/ml) (AIDS Reagent Program). The activated lymphocytes were cultured for 5-7 days, and the CD4+ cells were enriched with anti-CD4 magnetic beads (Invitrogen). These purified CD4 T cells were then transduced with the lentivirus carrying the Tax-2-gfp expression cassette. The transduced cells were cultured continuously in complete media containing 20% fetal bovine serum and 100u/ml of recombinant IL-2. Lentivirus vector-based shRNAs specific for human Beclin1 were obtained from Open Biosystems.

**Cell lines, antibodies and chemicals**

MT-2 and MoT cell lines were obtained from AIDS Reagent Program, and HT1080 line was from ATCC. Antibodies for pERK1/2, ERK1, pMEK1, MEK1, pAkt1, Akt1 and GST were purchased from Santa Cruz Biotechnology, and anti-Bcl-2, Bcl-xL, Mcl-1, pSTAT3, were from Cell Signaling. U0126, wortmanin, LY294002, BAY11-7082, 3-methyladenin, chloroquine and bortezomib were purchased from Sigma.

**Immunophenotype analysis, cell proliferation assay and human telomerase reverse transcriptase activity assay**

The Immunophenotype of Tax-2-immortalized T cell line was determined with FACS. Cells were stained with allophycocyanin (APC) conjugated antibodies including anti-CD3, -CD4, -CD25, -TCRαβ, -CD45RO and -CD69 (eBioscience) according to the manufacturer’s instruction. The stained cells were subjected to FACS analysis. For IFNγ intracellular staining, TX2-1 and TX2-4 cells were incubated in phosphate-buffered saline containing 10µg/ml Brefeldin A (Sigma) for
4h, and were then stained with APC-conjugated anti-IFNγ antibody after fixation and permeabilization using the intracellular staining kit from eBioscience, followed by FACS analysis. Cell proliferation assay was performed using tetrazolium compound based CellTiter 96® AQueous One Solution Cell Proliferation (MTS) assay (Promega). Telomerase reverse transcriptase activity was measured using TRAPEZE Telomerase Detection Kit (Millipore).

**Electrophoretic mobility gel shift assay (EMSA)**

Nuclear extracts were prepared from various T cell lines using NE-PER nuclear and cytoplasmic extraction reagents (Pierce). The oligonucleotide was 5’-end labeled with biotin (Integrated DNA Technologies) and annealed to its complementary strand. The binding activities were examined by EMSA using Light Shift Chemiluminescent EMSA Kit (Pierce) following the protocol reported previously [192]. The oligonucleotide probes are for STAT5 (5’-AGATTTCTAGGAATTCAATCC-3’), Oct-1 (5’-TGTCGAATGCAAATCACTAGAA-3’), STAT3 (5’-GATCCTTCTGGGAATTCCTAGATC-3’) and NF-κB (5’-GATCCGGCAGGGGAATCTCCCTCTC-3’).

**Real-time quantitative PCR**

Total RNA was isolated using the RNeasy kit (Qiagen) and its concentration was determined using the NanoDrop1000 spectrophotometer (Thermo Scientific). Quality and integrity of total RNA was assessed on 1% formaldehyde-agarose gels. cDNA was synthesized using the Omniscript Reverse Transcriptase Kit (Qiagen) following the manufacturer's recommended protocol. Template samples in triplicate
were subjected to real-time qPCR (Stratagene Mx3005P system) using Power SYBR Green (Applied Biosystems). The primer sequences were shown in Table 3.1.

**Plasmids, immunoblot, co-immunoprecipitation and GST pulldown**

The plasmids for FLAG-BECN1, FLAG-UVRAG, FLAG-PI3KC3, p40phox-GFP and GFP-LC3B were described previously [400]. The co-immunoprecipitation and GST pulldown assays were performed using protocol from the previous chapter.

**Fluorescence imaging and autophagy assay**

To construct fluorescence protein tagged proteins, mWasabi encoding a monomeric green fluorescent protein, or mKate2 encoding a monomeric far red fluorescent protein, was amplified from pTEC15 or pTEC20 (kindly provided by Lalita Ramakrishnan, Addgene plasmid 30174 or 30179 respectively) and was fused into the N-terminus of BECN1 PCR fragment to generate mWasabi-BECN1 or mKate2-BECN1 respectively, which was cloned in the mammalian expression vector pEF2. The mKate2 PCR fragment was fused to the C-terminus of Tax-2 to generate Tax-2-mKate2. Transient co-transfection was performed in HT1080 cells using FuGene HD transfection reagent (Roche), and 48 hours post-transfection, the cells were fixed in 4% formaldehyde-PBS and mounted with DAPI. Fluorescent images were taken using an OLYMPUS IX81 deconvolution microscope and analyzed using SlideBook 5.0 software (Intelligent Imaging Innovations). For immunofluorescence staining, cells were fixed in 4% paraformaldehyde-PBS, blocked in 3% horse serum-PBS, stained with the indicated primary antibodies overnight at 4°C, followed by incubation with fluorescent conjugated secondary antibodies and then mounted with DAPI (Invitrogen).
3.6 Acknowledgments

We thank Qi Sun, Xing-Cong Ren and Susan Nyland at Penn State Hershey Cancer Institute for technical assistance.
Figure 3.1 Immunophenotype of Tax-2-immortalized T cells. Expression of cell surface molecules in four Tax-2-established T cell lines, including TX2-1, TX2-2, TX2-3 and TX2-4, analyzed with FACS using APC-conjugated antibodies indicated in the figures.
Figure 3.2 Selected gene expression profiles of Tax-2-immortalized T cells. (A) and (B). Expression of two sets of selected genes was analyzed using real-time quantitative PCR. Relative expression level of a given gene to GAPDH was plotted. The primer sequences were shown in Table 1. (C) Expression of IFNγ in TX2-1 and TX2-4 cells was analyzed by intracellular staining with anti-IFNγ-APC and FACS.
Figure 3.3 Activation of oncogenic signaling in Tax-2-immortalized T cells. (A). Total protein lysates from activated normal CD4 T cells from two healthy donors, Tax-2-immortalized T cell lines TX2-1 and TX2-2 and HTLV-1-transformed T cell line MT-2 were examined with immunoblot using antibodies reacting to PLCγ1, ZAP70, Lck, LAT, c-Cbl and IKKα. (B). Detection of the phosphorylation status of ERK1/2, MEK1 and Akt1 in normal CD4 cells, TX2-1 and TX2-2 cells that were cultured in the presence of IL-2. MT-2 cells were used for control. (C). Activities of the transcription factors in Tax-2-immortalized T cell lines TX2-1 and TX2-2 with EMSA. Jurkat and MT-2 cells were used for control. (D). Expression patterns of Bcl-2 family proteins and p53 in Tax-2-immortalized T cells using immunoblot analysis. (E). The activities of the transcription factors in Tax-2-immortalized T cell lines TX2-3 and TX2-4 with EMSA. (F). The activities of STAT3 and NF-κB in HTLV-2-infected MoT cell line. Jurkat cell line serves as a negative control and MT-2 cell line for positive control. (G). The telomerase reverse transcriptase assay in Tax-2-immortalized T cell lines. AcPBLs were activated peripheral blood lymphocytes, and PosCtl was the positive control from the assay kit. Heat inactivation was applied to each cell line and was used as control.
Figure 3.4 Inhibition of oncogenic signaling results in growth arrest and apoptotic death of Tax-2-immortalized T cells. The phosphorylation status of ERK1/2 and Akt1 in TX2-1 cells treated with U0126 (A), LY294002 (B) and wortmanin (C) at doses of 0.625, 1.25, 2.5, 5 and 10μM for 2 hours. (D). TX2-1 cells were treated with DMSO or bortezomib at 1.25, 2.5, 5, 10 or 20nM. 16 hours following the treatment, the nuclear extracts from these cells were prepared and were used for EMSA using NF-κB probe. Oct-1 was used for nuclear extracts loading control. (E). TX2-1 cells were treated similarly to (D) with DMSO or bortezomib at 1.25, 2.5, 5, 10, 20, 40, 80nM for 16h. The total protein lysates were examined with immunoblot for detecting cleaved forms of caspase-3, -7 and PARP. β-actin was used for protein loading control. (F). TX2-1 cells were treated with bortezomib at indicated doses for 48h, and the cell viability was examined by MTT assay. (G). TX2-1 cells were treated with DMSO, U0126 (10μM), LY294002 (10μM), wortmanin (10μM) or BAY11-7802 (10μM) for 24, 48 or 72 hours. Cell viability was determined with MTT assay.
**Figure 3.5 Tax-2 deregulates autophagy in promoting T cell survival.** (A). Equal amounts of total protein lysates from four Tax-2-established T cell lines were analyzed with immunoblot using antibodies for LC3 and β-actin. Non-Tax-2-expressing Jurkat T cells were used for control. (B). Cell viability of TX2-1 cells treated with DMSO, 3-methyladenin (3-MA) (5mM) or chloroquine (50μM) at 24, 48 and 72h time points. (C). TX2-1 cells were treated with chloroquine at indicated doses for 24 hours, and the total protein lysates were examined with immunoblot using antibodies detecting cleaved forms of caspase-3 and -7. (D). GFP-LC3B fluorescence patterns in HT1080 cells transfected with vector, Tax-2-HA or the constitutively active form of IKKβ, FLAG-IKKβKA. (E). Co-transfection of p40phox-GFP with mKate2 or with Tax-2-mKate2 in HT1080 cells. (F). Depletion of Beclin1 (BECN1) or IKKβ in HT1080 cells using lentivirus transduction of specific shRNAs. Total protein lysates from the modified Tax-2 immortalization of human CD4 memory T cells 12 cell lines were analyzed with anti-BECN1 or anti-IKKβ immunoblot. β-actin was used as protein loading control. (G). Expression patterns of LC3-I and LC3-II in HT1080 cells transfected with vector, Tax-2-HA or FLAG-IKKβKA. (H). Expression patterns of LC3-I and LC3-II in Beclin1 knockdown (KD) HT1080 cells transfected with vector, Tax-2-HA or FLAG-IKKβKA. (I). Co-transfection of GFP-LC3B with vector or Tax-2-HA in IKKβ-knockdown (KD) HT1080 cells. (J). TX2-1 cells were transduced with lentiviruses expressing non-specific shRNA (NS) or IKKα/β-specific shRNAs, and the transduced cells were selected with puromycin for one week and analyzed with immunoblots for IKKα/β, LC3-I/II and actin. (K). Cell viability assay for TX2-1 cells transduced with NS shRNA and IKKα/β shRNAs. P value <0.05 for the time point at 72h between NS and IKKα/β shRNAs-transduced cells. (L). TX2-1 cells were transduced with lentiviruses expressing non-specific shRNA (NS) or BECN1-
specific shRNA, and the transduced cells were selected with puromycin and analyzed with immunoblots for BECN1, LC3-I/II and actin.
Figure 3.6 Tax-2 interacts with the autophagy molecular complex. (A). GST pulldown assay to analyze the interaction of Tax-2 with the autophagy molecules BECN1, PI3KC3 and UVRAG in transiently co-transfected 293 cells. (B). Reciprocal GST pulldown assay to detect Tax-2-BECN1 interaction. (C). Co-immunoprecipitation of Tax-2-GFP and BECN1 in four Tax-2-immortalized T cell lines. (D). Subcellular localization of mKate2-BECN1, FLAG-PI3KC3 and GFP-IKKγ in HT1080 cells. (E). Dual fluorescence imaging to detect co-localization of Tax-2 with BECN1, PI3KC3 or IKKγ in transfected HT1080 cells.
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Chapter 4: Developing an In-Vitro Model of T cell type of large granular lymphocyte leukemia

4.1 Abstract

We developed a strategy that can prolong in vitro growth of T cell type of large granular lymphocyte (T-LGL) leukemia cells. Primary CD8+ lymphocytes from T-LGL leukemia patients were stably transduced with the retroviral tax gene derived from human T cell leukemia virus type 2. Expression of Tax overrided replication senescence and promoted clonal expansion of the leukemic CD8+ T cells. These cells exhibit features characteristic of T-LGL leukemia, including resistance to FasL-mediated apoptosis, sensitivity to inhibitors for sphingolipid kinase and IκB kinases as well as expression of cytotoxic gene products such as granzyme B, perforin and IFNγ. Collectively, these results indicate that this leukemia cell model can duplicate the main phenotypes of the clinical isolates of T-LGL leukemia. This model should be useful for investigating molecular pathogenesis of the disease and for developing effective therapeutics targeting T-LGL leukemia.
4.2 Introduction

Large granular lymphocyte (LGL) leukemia is a hematological malignancy of either T cells or natural killer (NK) cells [446]. T cell type of large granular lymphocyte (T-LGL) leukemia is the malignancy of CD8+ cytotoxic T cells, which usually occurs in elderly patients. The disease course is frequently associated with autoimmune diseases [447]. Many patients with T-LGL leukemia do not respond to the currently available immune-suppressive therapies [448]. Primary T-LGL leukemia cells display the CD3+/CD8+/CD57+ surface markers, representing activated cytotoxic T lymphocytes. TCR rearrangement pattern indicates their clonal nature [449]. The primary leukemic cells exhibit constitutive activities of STAT3 and NF-κB, and both factors are critical mediators of oncogenic signaling and survival pathways [450, 451]. Activated Stat3 contributes to chemotherapy resistance as repression of STAT3 in LGL leukemic cells down-regulates a survival protein Mcl-1, resulting in an increased sensitivity to apoptosis [450]. Thus, STAT3 activation plays an essential role in promoting aberrant proliferation of the leukemic cells. In addition, NF-κB is constitutively activated in LGL leukemia, also playing a crucial role in promoting survival and proliferation of the leukemia cells. It is of paramount significance to further verify therapeutic agents that target STAT3 and NF-κB for treatment of T-LGL leukemia in a well-defined leukemia model. It is also crucial to identify new survival signaling pathways that could serve as therapeutic target in treating T-LGL leukemia.

Current studies of T-LGL leukemia exclusively relies on fresh specimens collected from patients with T-LGL leukemia. There are no cell lines from these patients. Previous studies failed to establish such cell lines largely because T-LGL leukemia cells are terminally differentiated cytotoxic T lymphocytes and undergo rapid replicative senescence. In the present study, we succeeded in generatingT-
LGL leukemia cell lines by stable expression of the retroviral protein Tax derived from human T cell leukemia virus type 2. Our study demonstrates that these established cell lines faithfully duplicate the phenotype of primary T-LGL leukemia cells from patients. This model should be valuable for studying the pathogenesis of T-LGL leukemia and developing targeted therapeutics.

4.3 Results

To establish long-term culture of T-LGL leukemia cells, we utilized the retroviral Tax derived from HTLV-2, a virus that preferentially immortalizes CD8+ T cells. The Tax-2-gfp fusion gene was generated and constructed in a lentivirus vector in which the human elongation factor promoter drives constitutive expression of Tax-2-GFP. CD8+ T cells from healthy donors or from clinically confirmed T-LGL leukemia patients were enriched through sorting with anti-CD8 magnetic beads, followed by lentiviral transduction (Figure 4.1). Roughly 30%-50% of cells were transduced by lentivirus expressing Tax-2-GFP as evidenced by visualization with fluorescence imaging method. About one month following transduction, nearly 100% of cells exhibited green fluorescence, indicating that non-transduced cells lost growth potential and gradually disappeared during extended culture. The Tax-2-GFP-expressing cells were in clumpy growth pattern as seen in Figure 4.1. Untransduced CD8 T cells from healthy donors or T-LGL leukemia patients typically grow in culture for less than three weeks at normal conditions. The Tax-2-GFP-transduced normal CD8 T cells only grew for about two months before dying. In contrast, the Tax-2-GFP-transduced CD8+ T cells from T-LGL leukemia patients grew in culture for at least four months. One of the established T-LGL leukemia cell lines, named TL-1, was able to grow for over two years without losing growth potential. These findings
suggest that Tax-2 alone is not sufficient to immortalize normal CD8+ T cells, yet it can promote long-term expansion of the leukemia cells that harbor potential oncogenic mutations.

We next examined the immunophenotype of the Tax-2-GFP-established leukemia cells. We showed that TL-1 cells exhibited a CD3+/TCRαβ+/CD8+/CD25+/CD45RO+/CD69+ immunophenotype (Figure 4.2A). CD45RO is the surface marker for memory T cells, while CD69 is a marker for activated T cells. This result indicates that these cells are terminally differentiated, activated memory CD8 T cells, similar to the CD8+ leukemia cells seen from T-LGL leukemia patients. CD25, the IL-2 receptor alpha chain, was also detected in TL-1 cells (Figure 4.2A), which rendered the cells responding to exogenous IL-2. Deprivation of IL-2 led to growth arrest and death of TL-1 cells (Figure 4.2B). Another Tax-2-GFP-established leukemia cell line, TL-2, also exhibited IL-2-dependent growth pattern. However, the growth of TL-2 in culture was very slow, suggesting that TL-2 is not suitable for cancer cell model. Analysis of the TCR rearrangement showed that both TL-1 and TL-2 displayed distinct peak of genotyping, whereas the normal activated CD8 T cells exhibited multiple peaks (Figure 4.3). These data indicated that TL-1 and TL-2 were clonal population of CD8+ leukemia cells.

Primary T-LGL leukemia cells are derived from cytotoxic T cells. To determine whether the Tax-2-established T-LGL cells present similar features, we analyzed cytokine and cytotoxic gene expression profiling on these cells. Similar to normal activated CD8+ T cells, the Tax-2-established leukemia cell lines TL-1 and TL-2 expressed cytotoxic gene products such as granzyme B, IFNγ and FasL (Figure 4.4). Perforin was also expressed in TL-2 cells but not in TL-1 cells (Figure 4.4), suggesting that some of T-LGL leukemia cells execute cytotoxic activity via indirect
mechanism, probably by using IFNγ-mediated cytotoxic killing. Additionally, IL-13, an immune modulatory cytokine, was detected in both normal activated CD8 T cells and Tax-2-established leukemia cells (Figure 4.4). Although the antigen that these CD8+ CTLs recognize was not clear, the expression of certain cytotoxic gene products and typical immunophenotype strongly support the notion that the Tax-2-established CD8 T cells from T-LGL leukemia patients are leukemic CTLs.

We further examined expression of TCR signaling molecules using TL-1 as a model cell line. Jurkat cells are CD4+ T cells with intact TCR signaling response and as expected, the signaling molecules in this pathway, such as Lck, ZAP70, CD3ζ chain, LAT, MEK1, IKKβ and p85 subunit of PI3KC1, were abundantly expressed (Figure 4.5A). MT-2 cells are HTLV-1-transformed CD4+ T cells and displayed down-regulated Lck, ZAP70 and CD3ζ (Figure 4.5A), a pattern commonly found in ATL cells that usually do not respond to TCR engagement. SP cells are HTLV-1-immortalized CD8+ T cells, and their growth is dependent on a low level of exogenous IL-2. SP cells showed similar expression patterns of the TCR signaling molecules to Jurkat cells (Figure 4.5A). An intact expression pattern of TCR signaling molecules was present in TL-1 cells (Figure 4.5A), suggesting that these cells likely have the capacity to respond to antigen in vivo.

LGL leukemia cells are known to express sphingosine kinase, which renders these leukemia cells sensitive to the inhibition of this kinase. We examined several isoforms including S1P1, S1P2, S1P3, S1P4 and S1P5 for their expressions on TL-1 cells. Similar to the NK type of LGL leukemia cell lines, RNK16 and NKL, TL-1 cells expressed all five forms of S1Ps (Figure 4.5B). In addition, Mcl-1, a pro-survival Bcl-2 family member, along with the cytotoxic gene produce granzyme B, were abundantly expressed in TL-2 cells (Figure 4.5B). In Tax-2-established T-LGL
leukemia cell lines TL-1 and TL-2, phosphorylated forms of Akt, Stat3, ERK1/2 and MEK1 were detected, indicating that these kinases were expressed in activated forms (Figure 4.5C), and the DNA-binding activities of NF-κB, STAT3 and AP-1 were also detected in TL-1 cells (Figure 4.5D, 4.5E and 4.5F). Because these oncogenic activations were typically seen in primary T-LGL leukemia cells, our data demonstrate that TL-1 cells share similar oncogenic activation patterns to the clinical isolates. Indeed, chemical inhibitors for ERK1/2, PI3K/Akt and IKK/NF-κB effectively reduced viability of TL-1 cells (Figure 4.5G), indicating that these signaling pathways were crucial for promoting survival and proliferation of the established T-LGL leukemia cells in vitro.

Primary T-LGL leukemia cells are resistant to FasL-mediated apoptosis and sensitive to sphingosine kinase inhibition [452]. To test if TL-1 cells present features similar to clinical isolates, we treated TL-1 cells with CH11, a Fas stimulatory antibody that imitates FasL's action, and FTY720, a structural analog of sphingosine, either alone or in combination. The control cells were activated CD8+ T cells transiently expressing Tax-2-GFP. In the absence of exogenous IL-2, TL-1 cells were resistant to CH11-induced apoptosis and sensitive to FTY720, as compared to the NT8 cells that were treated at the same condition (Figure 4.6A). Addition of exogenous IL-2 in culture restored the sensitivity of TL-1 cells to CH11 (Figure 4.6B). Both TL-1 cells and NT8 cells were equally resistant to FTY720 in the presence of IL-2, and the combination of CH11 and FTY720 restored the sensitivity of these cells to FTY720 (Figure 4.6B).

Our previous study demonstrated that Tax-2 is able to activate autophagy pathway in supporting T cell survival. We examined the autophagic activity in Tax-2-established T-LGL leukemia cells, and found a higher accumulation of LC3-II, a
marker for autophagy, in TL-1 cells regardless of the presence of recombinant IL-2 (Figure 4.7A). Suppression of autophagy pathway with the chemical 3-MA or chloroquine effectively reduced viability of TL-1 cells (Figure 4.7B), implying a critical role of Tax-2-mediated autophagy for the survival and growth of Tax-2-established T-LGL leukemia cells in culture.

4.4 Discussion

In the present study, we established T-LGL leukemia-like cell lines that exhibit characteristic features of primary T-LGL leukemia. The Tax-2-established T-LGL cells are clonal population with mature CD8+ CTL phenotype oncogenic activation and drug-sensitivity profiles that resemble clinical isolates. Thus far, only TL-1 cell line has exhibited optimal growth rate suitable for both in vitro and in vivo experiments to investigate the pathogenesis of T-LGL leukemia. Considering all the previous difficulties in establishing a T-LGL leukemia model, our study succeeded by introducing Tax2 into leukemic LGL. Long-term culture of antigen-specific CTL lines has been reported, which might be achieved by introducing human telomerase reverse transcriptase (hTERT) gene into these cells [453]. hTERT is a catalytic subunit of the enzyme telomerase that adds TTAGGG nucleotide repeat sequences, also known as telomeres, to the ends of chromosomal DNA [454]. However, another group reported that hTERT failed to immortalize primary CD8+ T cells [455], and our group was also unable to utilize hTERT to establish T-LGL leukemia cell line. Like any other cancer cells, T-LGL leukemia cells exhibit constitutive activity of hTERT and therefore other unknown factors likely cause replicative senescence in primary T-LGL leukemia cells. Primary T-LGL leukemia cells are activated CD8+ cells primarily due to their reactive capacity to a presumptive antigen in patients.
However, these activation features may not be maintained because of lack of such antigen in culture, which would contribute to their replicative senescence. Therefore, the primary T-LGL leukemia cells need to be engineered in order to imitate antigen stimulated proliferation. The Tax protein appears to be a favorable choice. HTLV-2 preferentially infects CD8+ T cells and is capable of immortalizing primary CD8+ T cells from healthy donors. However, our results indicated that Tax-2 alone is not able to immortalize normal CD8+ T cells but selectively transforms leukemic CD8+ cells.

There are several factors which may contribute to successful development of T-LGL leukemia model. First, Tax-2 induces expression of CD25, the IL-2Rα, which renders the Tax-2-expressing cells responding to exogenous IL-2, providing an autocrine loop in stimulating T cell growth. Second, Tax-2 constitutively activates IκB kinases, causing a hyperactivity of autophagy that promotes T cell survival. Last, Tax-2 maintains the activated phenotype of primary T-LGL leukemia cells, leading to their sustainable growth. It is noted that autophagy is induced in response to antigen stimulation in activated T cells. Primary T-LGL leukemia cells exhibited transient activity of autophagy because they are antigen simulated comparing to resting CD8 T cells. Following prolonged culture, the autophagy activity is expected to disappear in primary T-LGL leukemia cells because of lack of antigen in culture. Thus, Tax-2 is capable of supplementing autophagy activity in the primary T-LGL leukemia cells, leading to their continuous growth in vitro even in the absence of antigen.

HTLV-2 unlike HTLV-1 has not been associated with human disease. The expression of Tax-2 would not significantly affect disease model of T-LGL leukemia. In addition, Tax-2 activates a variety of oncogenic signaling molecules including Stat3, PI3K/Akt, IKK/NF-κB and MAPK/ERK that overlap with the oncogenic activation present in primary T-LGL leukemia cells. The activated features of T-LGL
cells, therefore, can be maintained in culture for prolonged time. It would be certainly interesting to see if such Tax-2 LGL leukemia cell lines could be used to establish an in vivo mouse model as well. An additional advantage of the Tax-2-GFP-established T-LGL leukemia model is that these cells continuously emit green fluorescence, which would differentiate transplanted cells from endogenous cells in trying to establish an in vivo mouse model. This LGL leukemia model could be further modified by inducible expression of Tax-2-GFP in primary T-LGL leukemia cells.
4.5 Materials and methods

Lentivirus vector, viral production and transduction of primary CD8 T cells

The *tax* gene from HTLV-2 was fused with enhanced GFP, and the *Tax-2-gfp* fusion fragment was cloned into the lentivirus vector pLCEF8 [192], in which the human elongation factor 1 alpha promoter drives expression of Tax-2-GFP. The procedure for lentiviral production and concentration was described previously. Human peripheral blood lymphocytes were isolated from healthy blood donors or from clinically confirmed T-LGL leukemia patients, and stimulated with PHA (1µg/ml) for 24 hours, followed by adding recombinant IL-2 (100u/ml) (AIDS Reagent Program). The activated lymphocytes were cultured for 5-7 days, and the CD8+ cells were enriched with anti-CD8 magnetic beads (Invitrogen). These purified CD8 T cells were then transduced with the lentivirus carrying the *Tax-2-gfp* expression cassette. The transduced cells were cultured continuously in complete media containing 20% fetal bovine serum and 100u/ml of recombinant IL-2.

Cell lines, antibodies and chemicals

MT-2 and SP cell lines were obtained from AIDS Reagent Program, and Jurkat T cell line was from ATCC. Antibodies for pERK1/2, ERK1, pMEK1, MEK1 and pAkt1 were purchased from Santa Cruz Biotechnology, and anti-Mcl-1 and pSTAT3, were from Cell Signaling. U0126, wortmanin, LY294002, BAY11-7082, 3-methyladenin and chloroquine were purchased from Sigma.

Immunophenotype analysis, cell proliferation assay and TCR genotyping

The Immunophenotype of Tax-2-immortalized CD8+ T cell line was determined with FACS. Cells were stained with allophycocyanin (APC) conjugated antibodies.
including anti-CD3, -CD4, -CD25, -TCRαβ, -CD45RO and -CD69 (eBioscience) according to the manufacturer’s instruction. The stained cells were subjected to FACS analysis. Cell proliferation assay was performed using tetrazolium compound based CellTiter 96® AQueous One Solution Cell Proliferation (MTS) assay (Promega). The quantitative PCR was used to examine TCR rearrangement using the method in the previous chapter.

Electrophoretic mobility gel shift assay (EMSA)

Nuclear extracts were prepared from various T cell lines using NE-PER nuclear and cytoplasmic extraction reagents (Pierce). The oligonucleotide was 5’-end labeled with biotin (Integrated DNA Technologies) and annealed to its complementary strand. The binding activities were examined by EMSA using Light Shift Chemiluminescent EMSA Kit (Pierce) following the protocol reported previously.

4.6 Acknowledgments

We thank Katie Broeg, Jun Yang and Susan Nyland at Penn State Hershey Cancer Institute for technical assistance.
Figure 4.1 Strategy to develop T-LGL leukemia model

Human peripheral blood cells
(mitogen activated)

\[ \downarrow \]

Enrichment of CD8+ T cells
(magnetic beads cell sorting)

\[ \downarrow \]

Continuous growth in IL-2 supplemented medium
for more than one year

\[ \downarrow \]

Analysis of Tax-immortalized T cells
Immunophenotypes
Oncogenic signaling
Differential gene expression profiling

Lentivirus transduction of Tax-GFP
Figure 4.2 Immunophenotype of Tax-2-established T-LGL leukemia cells. A.
FACS analysis of surface markers on TL-1 cells. B. Cell viability assay to examine IL-2-dependent growth of TL-1 cells.
Figure 4.3 TCR Vβ genotyping of normal activated CD8+ cells and Tax-2-established TL-1 and TL-2 cell lines. Dilutions, RNA extraction, cDNA synthesis and qRT PCR are performed in triplicates.
Figure 4.4 Cytokine and cytotoxic gene expression profiles on Tax-2-established T-LGL leukemia cell lines.
Figure 4.5 TL-1 cells exhibit constitutive activation of various oncogenic signaling molecules. (A). Expression profiles of TCR signaling molecules were shown in various T cell lines including Jurkat (lymphoblastic leukemia cells), MT-2 (HTLV-1-infected CD4 T cells), SP (HTLV-1-infected CD8+ T cells) and TL-1 (Tax-2-established T-LGL leukemia cells). (B). Expressions of S1Ps, granzyme B and Mcl-1 were shown in TL-1 cells, RNK16 (rat NK leukemia cells) and NKL (human NK-LGL leukemia cells). (C). Immunoblot analysis for phosphorylated Akt, Stat3, ERK1, MEK1 in CD8-1 and CD8-2 (normal CD8+ T cells from two healthy donors), TL-1 and TL-2 (Tax-2-established T-LGL leukemia cells) and SP (HTLV-1-immortalized CD8+ T cells). (D). EMSA for detecting NF-κB activity in Jurkat, MT-2, SP and TL-1 cells. (E). EMSA for Stat3 activity in TL-1 cells and NT8 cells (normal CD8+ cells transiently expressing Tax-2-GFP). (F). EMSA for AP-1 and NF-ATc activities in MT-2, Jurkat, SP and TL-1 cells. (G). Cell viability of TL-1 cells treated with U012 (ERK inhibitor), Ly494002 (PI3K inhibitor), Wortmanin (PI3K inhibitor) or BAY11-7082 (IKK inhibitor) at indicated dose and time points.
Figure 4.6 TL-1 cells are resistant to FasL-mediated apoptosis and sensitive to the inhibitor for sphingosine kinase. (A). Apoptosis induction by CH11 (2µg/ml, FasL mimics) or FTY720 (5µM, sphingosine-1-phosphate analog) for 8h after IL-2 withdraw (16h), as measured by FACS analysis of anexin V-APC stained cells. NT8, normal CD8 T cells transiently expressing Tax-2-GFP, was used as control. (B). Apoptosis induction by CH11 (2µg/ml) or FTY720 (5µM) or the combination of CH11 and FTY720 in cells treated with IL-2 for 24h.
Figure 4.7 Constitutive activity of autophagy in TL-1 cells. (A). Conversion of LC3-I to LC3-II in CD8-2 cells (activated CD8+ T cells from donor 2), TL-1 cells (cultured in the presence of IL-2), TL-1 (IL-2-) (TL-1 cells cultured without IL-2 for 24 hours), Jurkat cells without or with rottlerin (5μM for 6 hours) was examined with anti-LC3 immunoblot. Beta-actin is used for protein loading control. (B). Cell viability of TL-1 cells treated with autophagy inhibitors, 3-MA and chloroquine, at indicated time points.
Chapter 5: Overall dissertation discussions

In this chapter, I intend to discuss some important topics directly or indirectly related to my dissertation, reflecting my understanding towards HTLV pathogenesis.

HTLV infection is becoming an increasing health problem

It is estimated that HTLV-1 infects 20 million people, while HTLV-2 infects several million people worldwide. Although the numbers of HTLV-infected individuals are relatively low compared to the infection rates from other oncogenic viruses such as HBV, HCV, EBV and HPV, HTLV infection may be severely underestimated because large scale screening studies of HTLV antibody reactivity have not been conducted among Asian countries other than Japan and South Korean.

The epidemic patterns of HTLV infection are also changing in correlation with lifestyle change and globalization. Although HTLV-1 was discovered about thirty years ago, detection of HTLV-1 proviral DNA sequence in 1,500 years old mummy indicates that this is an ancient virus [456]. Genomic analysis demonstrates that the virus endemic among ethnic Japanese is linked to the HTLV-1 strain seen amongst people in South America, whose ancestors migrated from Asia to America by crossing Bering Strait tens of thousands years ago [457-459]. In addition, HTLV-1 is also genetically linked to STLV (simian T cell leukemia virus) in primates [460-463]. Similar to the fact that HIV-1 is derived from SIV (simian immunodeficiency virus), HTLV-1 likely evolved from STLV. HTLV-1 is a blood-borne pathogen that can be theoretically transmitted in a way similar to that with human immunodeficiency virus type 1 (HIV-1). HTLV-1 transmission, however, has its own unique patterns due to cultural, economical and geographic factors. In endemic regions such as Japan, HTLV-1 infection shows cluster patterns among village people living in Northern and
Southern parts of Japan where the infection rate can be as high as 39%. The village people in Japan were relatively isolated from outside world in the past; therefore, the main transmission pathway among those people was through perinatal transmission by blood contamination or breast milk feeding. Prescreening of HTLV-1 and replacement of breast milk with cow milk has dramatically reduced the rate of HTLV-1 infection among these people. However, life style changes raise new challenges for HTLV-1 transmission. Migration of workers from endemic villages to urban cities and from South America to the United States, unprotected sexual contact and IV drug use with contaminated needles are factors leading to new cases of HTLV-1 infection. Moreover, a sizable HTLV-1 reservoir likely exists, possibly due to following reasons: (a) that asymptomatic HTLV-1 carriers are largely unidentified and usually untreated, (b) that no effective vaccine and no anti-retroviral regimen for HTLV-1 infection are currently available and (c) that HTLV-1 viral genome is integrated into host chromosome and host immunity is not sufficient enough to clear the virus, therefore, chronic HTLV-1 infection can persist for life time. Furthermore, life style changes also increase the rate of co-infection of HTLV-1 and HIV-1, causing more severe consequences of immunodeficiency, opportunistic infections (OI) and lymphoma [113, 117, 464, 465].

The disease penetrance of HTLV-1 infection is higher than other oncogenic virus-associated diseases. HTLV-1 causes ATL among 5% of infected individuals, while EBV is associated with lymphoma or Nasopharyngeal carcinoma (NPC) in less than 1% of infected patients though more than 90% of adults are infected with this virus. Furthermore, HTLV utilizes GLUT1 receptor for viral entry. Because GLUT1 is ubiquitously expressed, this may explain why HTLV-1 is able to infect several different cell types, unlike HIV-1 that exclusively targets CD4+ cells. For this reason,
HTLV infection may cause currently unrecognized human diseases because of a broader host cell tropism range.

**HTLV-1 and T lymphocyte transformation, a useful cancer model**

In order to understand tumorigenesis, cancer biology research is best conducted by using two-way approaches. The retrospective approach is commonly used for cancers that are not related to oncogenic viral infections. For example, colon cancer specimens are surgically collected and analyzed by a variety of cancer biology methodologies including cancer gene expression profiling, oncogenic activation and somatic mutations. The prospective approach is to start with normal colon epithelial cells by introducing oncogenes found in colon cancer cells in order to replicate the disease course. However, this approach has proven to be extremely difficult. EBV infection can easily immortalize human primary B-lymphocytes, however, these immortalized B cells are rarely oncogenic. Although other oncogenic viruses such as HBV or HCV are able to establish infection *in vitro* or in humanized mouse model, the malignant transformation of hepatocytes by these viruses rarely occurs. Woodchuck hepatitis virus (WHV), a closely related virus to HBV, is capable of causing hepatocellular carcinoma (HCC) in woodchucks 18 months following introduction of WHV and is thus a useful animal model for studying WHV/HCC.

HTLV-1 is advantageous for studying virus-induced transformation of human T lymphocytes. As depicted in Figure 5.1, HTLV-1/ATL can be investigated in both retrospective and prospective approaches. Clinical samples from patients provide valuable resources for determining oncogenic activation, viral replication and viral gene expression and epigenetic alteration. More importantly, HTLV-1 can infect and transform primary human CD4+ T cells and can be studied in immune compromised...
mouse models replicating leukemia/lymphoma. To imitate the disease process, HTLV-1 can be used to infect humanized mice whose native immune system was replaced by human immune system.

Normal lymphocytes isolated from healthy donors are usually at quiescent stage. Stimulation of peripheral blood leukocytes with mitogens such as PHA plus exogenous IL-2 simulates the course of TCR engagement, resulting in rapid proliferation. Activated lymphocytes will stop growing after 2-3 weeks in normal culture conditions by reaching the point of T cell senescence. HTLV-1 infection overrides replicative senescence of T cells, promoting continuous growth of HTLV-1-infected T cells or immortalization of T cells. The growth of HTLV-1-immortalized T cells is still dependent on exogenous IL-2. At the certain point during culture, these cells may become IL-2-independent, and present characteristic features of T cell transformation (lymphoblastic morphology and leukemia/lymphoma formation in immune compromised mice). At every step of aberrant proliferation, immortalization or transformation during HTLV-1 infection of T cells, a variety of cellular oncogenic alterations are expected to occur. Thus, studying these processes will certainly provide ample insights into the pathogenesis of ATL. More specifically, because HTLV-1 is known to encode two viral oncogenic proteins, Tax and HBZ, with differential roles in HTLV-1-mediated oncogenesis, Tax, HBZ or the combination of Tax and HBZ can be expressed in primary T cells to determine their roles in every step of oncogenesis. My graduate research focused on the pathological role of Tax in human primary T cells and identified autophagy as a novel survival pathway, which is deregulated by Tax during the early stage of T cell transformation.

HTLV-1 is pathogenic, and HTLV-2 is non-pathogenic?
HTLV-2 is estimated to infect roughly several million people worldwide. The accurate number of such infection is not known because large scale screening of HTLV-2 among the general population has not been conducted. HTLV-2 is not etiologically linked to any known human disease though this viral infection can be rarely associated with neurological diseases like those seen in HTLV-1-infected patients. However, HTLV-2 can infect and immortalize human primary CD8 T cells though it rarely transforms these cells in vitro. Several hypotheses have been proposed to explain the difference in disease prevalence in HTLV-1 infected individuals compared to those infected with HTLV-2. One postulate is that Tax-2 is less pathogenic than Tax-1. Comparative studies on Tax-1 and Tax-2’s oncogenic properties have been performed using rodent fibroblasts. These studies found that Tax-2 is less potent than Tax-1 in transforming rodent cells. Structurally, Tax-2 lacks the C-terminal motif called PDZ domain found in Tax-1. Some studies have shown that the PDZ domain is critical for Tax-1’s transforming activity. Another hypothesis to explain differences in disease prevalence is that the antisense gene product from HTLV-2, is structurally and functionally different from HBZ seen in HTLV-1. This difference may contribute to a lower transforming activity of HTLV-2. HTLV-1 and HTLV-2 share strikingly similar genome structures, life cycle and sequence homology of the viral genomes. Besides, both viruses encode highly homologous Tax proteins. It remains a big mystery why HTLV-1 is pathogenic and HTLV-2 is not. Therefore, a comparative study on both viruses would be crucial for providing insights into the pathogenesis of HTLV-1 infection.

My graduate work leads to a novel hypothesis regarding the differential pathogenesis of HTLV-1 and HTLV-1 infections, “cell tropism”. Both HTLV-1 and HTLV-2 utilize GLUT1 receptor for viral entry. Unlike HTLV-1 that mainly infects CD4
T cells, HTLV-2 preferentially infects CD8 T cells, suggesting that there exists a CD8 T cell-specific co-receptor for this virus. In my experimental findings, both Tax-1 and Tax-2 fail to immortalize human CD8+ T cells. Tax-2 is able to more efficiently immortalize human CD4+ T cells than Tax-1, when Tax-1 and Tax-2 are introduced into these cells via pseudotyped lentivirus with VSV-G as viral envelope. In addition, Tax-2-immortalized CD4+ T cells grow in vitro at a rate that is similar to lymphoblastic leukemia cells. In contrast, Tax-1-immortalized CD4+ T cells are slow growing and frequently die in normal culture conditions. Thus, it can be hypothesized that it is the cell tropism that limits HTLV-2’s pathogenic role in human.

These findings may have significant implications on disease pathogenesis. Many viruses are considered non-pathogenic, because they do not apparently cause human disease. These non-pathogenic viruses usually do not receive as much attention as known pathogenic viruses. From an evolutionary point of view, it is possible that recombination and cell tropism switch among pathogenic and non-pathogenic viruses might occur, leading to a more lethal hybrid virus and increasing the complexity of disease pathogenesis. In the case of HTLV-2, the infection rate among IV drug abusers is as high as 30%. Since no treatment is available for HTLV-2 infection and there is no host immunity against this virus it is possible that the rate of infection is going to rise due to the presence of increased HTLV-2 reservoir.

Indeed, co-infection of HTLV-2 with HTLV-1 or with HIV-1 has been documented and is increasing, probably because these viruses share similar transmission pathways.

IκB kinase complex as a focal point in Tax-mediated autophagy

My graduate study demonstrates that both Tax proteins from HTLV-1 and HTLV-2 are capable of increasing autophagic activity in HTLV-1-transformed T cells
and in Tax-immortalized T cells. The IKK complex appears to be the cellular target for Tax induction of autophagy as Tax fails to induce autophagy in T cells or non-lymphoid cells with genetically depleted IKKs. In addition, both Tax proteins can interact with the autophagic molecular complex containing Beclin1-PI3KC3-UVRAG. Thus, it appears that both Tax proteins are able to mediate the crosstalk between the IKK complex and the autophagic molecular complex, thereby playing a key role in Tax-induced transformation of CD4+ T cells.

Tax-1 and Tax-2 apparently utilize a similar, yet distinct, mechanism in activation of IKK and induction of autophagy. As depicted in Figure 5.2, Tax-1 activates both canonical and non-canonical pathways of NF-κB signaling. By stimulating the activity of NIK or by bypassing NIK in some studies, Tax-1 facilitates the processing of NF-κB2/p100 through IKKα (1). Tax-1 also activates canonical NF-κB signaling by directly interacting with IKKγ, resulting in activation of IKKβ. This leads to subsequently phosphorylation of IκBα and its degradation in the proteasome, causing nuclear translocation of the p65/p50 NF-κB complex (2). Activation of IKKα and IKKβ by Tax-1 appears to occur in the intracellular lipid raft microdomains (3). Tax-1 connects the IKK complex to the autophagic molecular complex containing Beclin1, PI3KC3 and Bif-1, stimulating the activity of the autophagy complex to promote assembly of autophagosomes. These combined activities, which are executed by Tax-1, facilitate the survival and proliferation of T lymphocytes.

**Tax serves as a molecular tool to establish T cell lines representing distinct T cell subsets and T cell leukemia in vitro models**
It is well known that human cells, particularly human T lymphocytes, are extremely difficult to immortalize and it is also difficult to establish primary T cell lines for studying T cell biology. T cell biology is usually studied in mouse model because human primary T cells are not easily accessible for genetic modifications. Establishment of T cell lines would be extremely useful for understanding the mechanisms of cellular factors involved in the process of T cell development, differentiation and immune regulatory functions.

In my research, Tax-1 was found to immortalize T cells with low efficiency while Tax-2 was highly efficient in immortalizing human CD4+ T cells. Tax-2-immortalized T cells were fast growing. Tax-2-immortalized T cell line, TX2-4, expressed a high level of IFNγ, suggesting that they likely represent the Th1 subtype of T helper cells. Genotyping analysis for another Tax-2-immortalized T cell line, TX2-1, indicated that these cells are clonal. To confirm the clonality of the Tax-2-immortalized T cell lines, it needs to be tested in all Tax-2-established T cell lines. In addition, more detailed studies need to be done to more precisely determine the T cell subsets immortalized by Tax-2. Another interesting finding of my research was that Tax-1 and Tax-2 selectively immortalize distinct subsets of T cells that differ from donor to donor and even within the same donor. In one example, CD4+ T cells enriched from one donor were transduced with either Tax-1-GFP or Tax-2-GFP. The Tax-1-GFP-transduced cells yielded a cell line called PTX4-1 (Chapter 2, Figure 2). However, Tax-2-immortalized CD4+ cells from the same donor produced a cell line, called TLR41, with a unique immunophenotype as shown in Figure 5.3. TLR41 cells also had TCRγδ-/CD14-/CD34-/CD19-immunophenotypes (data not shown). The cell type identity of TLR41 remains to be determined. From the immunophenotypic pattern, TLR41 cells resemble CD4+CD3- accessory cells, which may function as supporting
cells to assist CD4 T cells in helping B cells generate antibody. CD4+CD3- cells in peripheral blood are extremely rare and not much is known about their normal function. The fact that Tax-2 can immortalize such a minor population while not affecting the majority of CD4 T cells strongly suggests that Tax-immortalized cells are clonal species. What cellular factors define the selectivity for Tax-mediated immortalization is currently unknown. This work opens a new avenue to further explore cellular factors involved in HTLV oncogenesis, and also provides very useful tools to study T cell biology and functions of human CD4+/CD3- cells

Tax also serves as a useful molecular tool to develop T cell leukemia cell lines. Cancer models are important for investigating mechanisms of tumorigenesis and testing new drug candidates for therapeutic efficacy. Tumors from patients are not always able to be grown in vitro for unknown reasons. For instance, chronic lymphocytic leukemia (CLL), a hematological malignancy of B-lymphocytes, is difficult to grow in vitro. JVM-3, a currently available CLL model cell line, was developed by infection of EBV. T cell prolymphocytic leukemia (TPLL), an aggressive form of T cell type of leukemia, is also not able to be grown in vitro for extended times. In addition to leukemia/lymphoma, some solid tumors, such as breast cancers from surgical specimens, are not able to be established as cell lines. Similarly, there are no cell lines from T-LGL leukemia, as these leukemic cells do not survive in culture for an extended time. My work demonstrated that by genetically modifying T-LGL leukemia cells by expressing the retroviral Tax-2 protein, the leukemia cells were able to grow for at least two years without losing growth potency. Although the Tax-2-established T-LGL leukemia cell line, TL-1, remains dependent on IL-2 for growth, they are transformed leukemia cells. Future experiments could be aimed at developing in vivo animal models of LGL leukemia
using these cell lines as starting material. For example, TL-1 cells could be further modified by expression of IL-2 cDNA, or mice transplanted with TL-2 cells could be given injections of recombinant IL-2 or an IL-2R-stimulatory antibody to support the growth of the leukemia cells. Development of an T-LGL leukemia mouse model would provide an indispensable tool for investigating disease pathogenesis and for developing novel therapeutics.
Figure 5.1 HTLV-1/ATL disease model: respective and prospective studies

Normal T cells → Normal T cells

mitogen

Normal T cells → Extended growth (2-3 weeks) → Senescence

HTLV-1 → Immortalization → Transformation → Additional oncogenic events

HTLV-1 → Oncogenic processes

30 years

Leukemia/lymphoma
Figure 5.2 A hypothetical model for Tax-1 activation of IκB kinase complex and dysregulation of autophagy
Figure 5.3 Immunophenotype of TLR41, a Tax-2-immortalized CD4+/CD3- cell line
References


83. Gessain, A., et al., Isolation and molecular characterization of a human T-cell lymphotropic virus type II (HTLV-II), subtype B, from a healthy Pygmy living in a


Vita
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Education

<table>
<thead>
<tr>
<th>Degree</th>
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Professional Training and Experiences

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Awards

- Excellent Bachelor’s Degree Thesis, USTC, China, 2007
- Outstanding Student Scholarship (Grade 2), USTC, China, 2004
- Outstanding Student Scholarship (Grade 2), USTC, China, 2003

Publications

- Jiannan Huang, Tong Ren (co-first author), Hui Guan, Yixing Jiang, Hua Cheng. (2009). HTLV-1 Tax is a critical lipid raft modulator that hijacks IkB kinases to the microdomains for persistent activation of NF-kB. *The Journal of Biological Chemistry*, 284(10):6208-17
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Selected Abstracts/Scientific Presentations