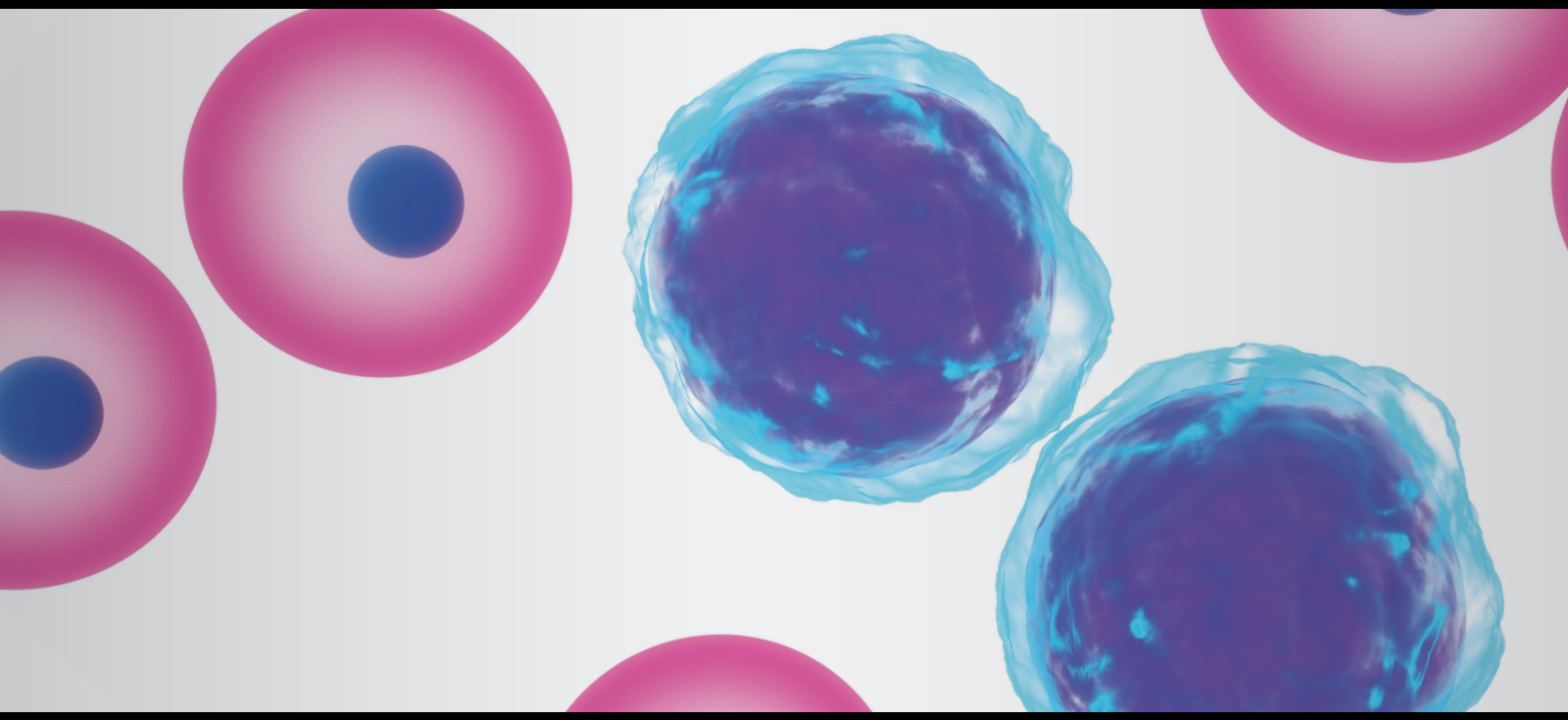
Leukemia Research and Treatment

HTLV-1 Infection and Its Associated Diseases

Guest Editors: Mineki Saito, Pooja Jain, Kunihiro Tsukasaki, and Charles R. M. Bangham



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Editorial Board

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Editorial

HTLV-1 Infection and Its Associated Diseases

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It has been three decades since the discovery of human T-cell leukemia virus type 1 (HTLV-1), which is the first human retrovirus etiologically associated with an aggressive malignancy of CD4+ T cells known as adult T-cell leukemia (ATL) and a disabling chronic inflammatory disease of the central nervous system known as HTLV-associated myelopathy/tropical spastic paraparesis (HAM/TSP). HTLV-1 infection is of particular interest to the field of immunology as well as virology, because HTLV-1 is never eliminated from the host in spite of a vigorous cellular and humoral immune responses against the virus, and it causes no disease in the majority (around 95%) of the infected subjects (asymptomatic carriers: ACs). Although accumulating evidence suggests the importance of complex virus-host interactions and the host immune response in determining the risk and timing of disease, the precise mechanism of disease pathophysiology is incompletely understood and the treatment is still unsatisfactory. The patients with ATL have a poor prognosis and approximately 40 percent of HAM/TSP patients become wheelchair-bound during their clinical course. In this special issue on "HTLV-1 Infection and Its Associated Diseases," we have invited nine papers that address the recent developments in HTLV-1 research in order to elucidate the pathogenetic mechanisms and to identify effective means of treatment and prevention of HTLV-1 associated diseases.

Three papers describe the important advances in understanding the molecular and virological aspects of HTLV-1. One paper explains the significant role of HTLV-1 basic leucine zipper factor (HBZ), a regulatory protein encoded in the minus strand of the HTLV-1 genome, in the viral pathogenesis. Another paper summarizes the comparative biology of HTLV-1 and HTLV-2. The paper by N. Aliya et al. discusses the possible interplay between HTLV-1 infection and miRNA pathways. Two papers describe the clinical trials and treatment for ATL from different groups, to give contrasting views on the current state of knowledge and current approaches to this subject. There is a paper that provides an overview of recent developments in HAM/TSP research, while another pa-per summarizes the current understanding of the host immune response against HTLV-1 infection, especially the potential importance of HTLV-1-specific CTLs in protection against ATL. Two clinical studies for ATL are also included. One of them describes the clinical and pathological characteristics of seven patients with HTLV-1-positive large B-Cell lymphoma, while another one discusses the hypercalcemia that is frequently observed in ATL patients.

The papers in this special issue demonstrate important recent progress in HTLV-1 research and will bring the reader up to date with the current understanding of HTLV-1 infection and its associated diseases.

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Review Article

Clinical Trials of Adult T-Cell Leukaemia/Lymphoma Treatment

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Adult T-cell leukaemia/lymphoma (ATLL) is an aggressive malignancy of mature activated T cells caused by human T-cell lymphotropic virus type I (HTLV-1). Prognosis is severe because of intrinsic chemoresistance and severe immuosuppression. Four different subtypes are described with different outcomes, and treatment strategies vary according to the different clinical courses. Japanese trials show that combinations of chemotherapy can increase the response rates especially in the lymphoma subtype. However, patients have a high rate of relapse and the outcome remains extremely poor. Recently, a worldwide meta-analysis demonstrated that the combination of Zidovudine and Interferon-alpha (IFN) is effective in the leukemic subtypes (smoldering, chronic, and acute) and influences favorably the course of the disease. In order to prevent relapse, clinical trials testing new drugs such as monoclonal antibodies or combinations such as arsenic/IFN are needed. Finally, allogeneic stem cell transplantation is a feasible option but bears a very high rate of complications.

1. ATL Classification and Response Criteria

The classification first described by Shimoyama (1991) used for the initial staging distinguishes four subtypes, which differ regarding their presentation and outcome. This classification has been very useful for comparison between different studies [1].

The complex presentation with both leukemic and lymphomatous components makes response assessment difficult. Recently, an international consensus meeting established new response criteria [2].

Complete response (CR) is defined as the disappearance of all measurable tumor lesions (including normalization of lymph node size) and normalization of absolute lymphocyte (including flower cells less than 5%) count below 4×109/L. Unconfirmed CR is defined as a reduction of 75% of the tumor size and normalization of absolute lymphocyte (including flower cells) count below 4×10^9/L. Partial response (PR) is defined as a reduction of 50% of tumor size and absolute lymphocyte count. Progressive disease is defined as an increase of 50% of the tumor size and/or absolute lymphocyte count. These response criteria require that each criterion is present for at least 4 weeks.

Treatment of ATL is usually dependent on the ATL subtype. Patients with aggressive forms (acute and lymphoma) have a very poor prognosis because of intrinsic chemoresistance, a large tumor burden, hypercalcemia, and/or frequent infectious complications due to profound immune deficiency. Multiple Japanese trials in aggressive ATL clearly demonstrated that although combinations of chemotherapy, in particular those designed for treatment of aggressive non-Hodgkin lymphomas or acute lymphoblastic leukemia, have improved the response rates particularly in ATL lymphoma, they failed to achieve a significant impact on long-term survival. Patients with indolent ATL (chronic or smoldering subtypes) have a better prognosis. However, recent Japanese data showed a poor long-term outcome when patients are managed with a watchful-waiting policy until progression and even worse when patients are treated upfront with chemotherapy [3].

2. Conventional Chemotherapy

The Japan Clinical Oncology Group (JCOG) has conducted six successive prospective clinical trials. All these trials are based on conventional chemotherapy, with various dose

and administration modalities. The first trial JCOG 7801 used VEPA (a CHOP-like regimen that contained vincristine, cyclophosphamide, prednisolone, doxorubicin). The CR rate was only 17% with a median survival time of 5 months. The second trial, JCOG 8101, was a randomized phase III study, which included 54 patients and compared VEPA regimen with VEPA-M (VEPA plus methotrexate) [4]. Although the CR rate was improved in the VEPA-M group (37%), no differences in median survival time (7.5 months) and overall survival (8% at 4 years) were noted.

The third trial, JCOG 8701, was a phase II study with a more aggressive regimen (LSG 4), which combined 3 successive regimens: VEPA-B (VEPA plus bleomycin), M-VEPA (MTX, vindesine, cyclophosphamide, prednisolone, doxorubicin), and VEPP-B (vincristine, etoposide, procarbazine, prednisolone, and bleomycin). The CR rate was improved to 42%. However, median survival rate and overall survival were poor with a median survival time (MST) of 8 months and overall survival rate of 12% at 4 years. These trials enrolled also patients with other subtypes of NHL. MST was 44 months versus 8 months in the ATL group.

Following these initial trials, JCOG designed specific regimens targeting ATL. The JCOG9109 trial (a phase II study conducted between 1991 and 1993) used pentostatin-containing regimen but did not show any improvement (MST 7.4 months and 2 years overall survival rate: 15%) [5].

JCOG 9303 was conducted between 1994 and 1996 and used more intensive multiagent chemotherapy [6]. Treatment was designed as follows: VCAP (Vincristine, cyclophosphamide, doxorubicin, prednisolone), AMP (Doxorubicin, ranimustine, prednisolone), and VCEP (vindesine, etoposide, carboplatin, prednisolone) and include intrathecal injection of methotrexate and aracytine. The use of Granulocyte Colony Stimulating Factor (GCSF) was systematic. Results were encouraging with a CR rate of 35%, an MST of 13 months versus 8 months with historical control CHOP-like regimen. The 2-year OS was 31%. MCNU and carboplatin were used because their activity is not affected by the expression of P-glycoprotein, a product of MDR1, which is frequently expressed by ATLL cells.

In order to confirm these results, a phase III study (JCOG9801) was conducted between 1998 and 2003. This study compared two arms of treatment: VCAP-AMP-VECP versus biweekly CHOP. It included 118 patients (81 acute subtype and 26 lymphoma subtype) [7]. Response rate was higher in the experimental arm (40% versus 25%). Progression-free survival at 1 year was 28% versus 16%, and overall survival was 24% versus 13%. There was a statistically significant difference only in a subgroup analysis (patients younger than 56 years old, poor PS).

3. Allogeneic Stem Cell Transplantation

As most of patients relapse after conventional chemotherapy, allogeneic stem cell transplantation (alloSCT) seems to be an attractive option as consolidation treatment. Most of the reports come from Japan. A number of retrospective studies have confirmed that alloSCT uses either myeloablative conditioning (MAC) or reduced-intensity conditioning (RIC) as a feasible treatment option for ATL patients. The largest retrospective study has been reported recently [8]. This study includes 386 patients allografted between 1995 and 2005. After a median followup of 41 months, 3-year overall survival was 33%. Among patients who received related transplants, donor HTLV-I seropositivity adversely affected disease-associated mortality. Recently, the long-term results of a series of 30 patients who received an RIC was reported. Overall survival rate and progression-free survival rates were 36% (95% IC, 21 to 25%) and 31% (95% IC, 17 to 45%), respectively, [9]. However, the number of ATL patients eligible for alloSCT is very limited because of the low CR rate especially in the acute form, poor performance status, severe immunosuppression, age at disease (median age at onset: 60 years old), and low probability of finding suitable donors in patients from ethnic minorities.

4. Alpha Interferon (Zidovudine) AZT

Even if this treatment association is frequently referred to "antiviral therapy", mechanism of action is not fully understood yet. The combination of Zidovudine (AZT) and alpha interferon (IFN) was first reported in 2 phase II studies [10–12]. High response rate was observed particularly in previously untreated acute ATL. The efficacy of this combination was confirmed in a French trial using AZT/IFN in 19 newly diagnosed ATL patients, and in a UK clinical trial using AZT/IFN in 15 ATL patients [13, 14]. In a recent prospective Phase II study in the USA, 19 ATL patients received infusional chemotherapy (EPOCH regimen) until maximal response, followed by antiviral therapy with daily AZT, lamivudine, and IFN. However, because of disease progression, only 6 patients received antiviral therapy [15].

A worldwide meta-analysis was recently performed on ATL survival since 1995 [16]. In this study, different treatment strategies for ATL has been compared, namely, antiviral therapy alone, chemotherapy alone, and chemotherapy followed by maintenance antiviral therapy in 254 ATL patients treated in the USA, the UK, Martinique, and continental France (116 acute ATL, 18 chronic ATL, 11 smoldering ATL, and 100 ATL lymphoma). Five-year OS rates were 46% for 75 patients who received first-line antiviral therapy, 20% for 77 patients who received first-line chemotherapy, and 12% for 55 patients who received first-line chemotherapy followed by antiviral therapy.

Patients with leukemic forms significantly benefited from first-line antiviral therapy, whereas patients with ATL lymphoma had a better outcome with chemotherapy. In acute ATL, first-line antiviral therapy alone resulted in a significant survival advantage (5-year OS of 28%) as compared with first-line chemotherapy with or without maintenance antiviral therapy (5-year OS of 10%). Achievement of CR with antiviral therapy resulted in 82% 5-year survival. In chronic and smoldering ATL, antiviral therapy resulted in 100% 5-year survival. In ATL lymphoma, first-line antiviral therapy resulted in a significant survival disadvantage (median and 5-year OS of 7 months and 0%, resp.) compared with first-line chemotherapy with or without maintenance antiviral therapy (median and 5-year OS of 16 months and 18%,

resp.). Finally, a multivariate analysis confirmed that first-line antiviral therapy significantly improves overall survival of ATL patients (HR 0.47; 95% CI 0.27–0.83; $P = 0.021$).

5. Arsenic Trioxide (AsO_3)

Arsenic trioxide synergizes with IFN to induce cell cycle arrest and apoptosis in HTLV-I infected and fresh ATL cells through rapid shut-off of the NF-κB pathway and a delayed shut-off of cell cycle-associated genes, secondary to Tax degradation by the proteasome [17–19]. Although it has been demonstrated that arsenic and IFN cooperate to cure murine ATL derived from *Tax* transgenics through selective eradication of leukemia-initiating cell (LIC) activity. This strongly suggests that LIC activity is dependent on continuous Tax oncogene expression. Hence, addition of arsenic to AZT/IFN, through elimination of LIC activity, may result in long-term disease eradication and potential cure [20]. A recent prospective phase II study evaluated the efficacy and safety of the combination of arsenic, IFN, and AZT in 10 newly diagnosed chronic ATL patients. The response rate was 100% including 7 CR, 2 CR but with more than 5% circulating atypical lymphocytes, and 1 partial response. Side effects were moderate and mostly hematologic [21]. We have also recently reported a series of 11 patients with ATL (3 lymphoma type, 3 chronic, and 5 acute) treated with arsenic/IFN after induction chemotherapy [22]. At initiation of AsO_3, 4 patients were in CR, 2 in PR, and 5 in progression. 10 patients received AsO_3 during 3 to 8 weeks. One progressed 3 days after starting AsO_3 and 6 patients died. All were progressive at time of AsO_3 initiation. 5 patients survived: 3-lymphoma type in CR (25, 31, 46 months of followup), 1 acute in CR (9 months followup), and 1 chronic in PR (39 months followup). Tolerance was acceptable with peripheral neuropathy ($n = 4$), hand and foot syndrome ($n = 3$), and drug eruption ($n = 3$, including 2 toxic epidermolysis). While preliminary, these observations nevertheless suggest that in ATL patients arsenic/IFN efficiently targets ATL LIC activity and may be useful as a consolidation therapy for those patients achieving a satisfactory response to induction therapy.

6. Specific Monoclonal Antibodies

ATL cells express CD25 (alpha-chain of IL2 receptor). A first trial reported use of antiCD25 antibody on 19 patients. Authors obtained 6 responses (two CR, four PR) that lasted from 9 weeks to more than 3 years [23].

A second study used CD25 coupled with YTRIUM-90. Seven of 18 patients treated (one with chronic ATL and 6 with acute ATL) obtained a partial remission. The duration of these partial remissions ranged from 1.6 to 22.4 months (mean, 9.2 months). Two patients achieved CR. One died 36 months after initiation of therapy from a secondary AML and the other patient was still in CR at time of publication [24].

A neutralizing monoclonal antibody to the transferrin receptor (mAb A24) has been designed and induces apoptosis of ATLL cell lines and primary ATL cells [25]. Thus far, only preclinical studies have been performed (Hermine et al., personal communication).

7. Anti-CC Chemokine Receptor 4 (CCR4)

ATL cells express the CC chemokine receptor 4 (CCR4). KW-0761 is a defucosylated humanized antibody with enhanced antibody-dependent cellular cytotoxicity (ADCC) that binds CCR4. A phase I study reports 13 patients with CCR4-positive relapsed ATL treated with KW-0761. Overall response rate (ORR) was 31%: 2 CRs and 2 PRs [26]. A pivotal phase II study has been recently presented on the 15th International Conference on Human retrovirology HTLV and related viruses. The primary end point was ORR. Twenty-eight patients with relapsed ATL were enrolled. Among the 26 pts evaluable for efficacy, the ORR was 50% with 8 CRs and 5 PRs with response rates in each affected lesion being 100% (13/13) for peripheral blood, 63% (5/8) for skin, and 25% (3/12) for lymph node disease, respectively. The treatment schedule was one weekly perfusion (1.0 mg/kg) for 8 weeks. Adverse events were mild to moderate.

8. Watch-and-Wait Policy

Patients with smoldering or chronic ATLL subtype have a better prognosis than patients with aggressive forms (acute and lymphoma) and have been considered as indolent forms. Many patients have been managed with a watch-and-wait policy until disease progression or treated with chemotherapy when poor prognostic factors were present. A recent published Japanese study reported 90 patients with indolent form (65 chronic and 25 smoldering) [3]. Forty-four (49%) patients progressed to aggressive form with a median time of transformation of 18.8 months (range 0.3 months to 17.6 years) and 41 died. Median survival time was 4.1 year. No difference between the two subtypes (chronic and smoldering) was observed. The estimated 10-year survival rate was 25, 4% (95% CI, 15.3–36.8%). This study shows that even, in the indolent subtype, prognosis is poor. Moreover, patients who received chemotherapy had a worse prognosis and a shorter life expectancy than patients who were treated was followed with watchful waiting. These results underscore the need for further improvement in the treatment of patients with otherwise indolent forms of ATL.

9. Agents That Have Shown Efficacy on T-Cell Lymphoma outside HTLV-1 Infection

Currently, it is not yet clear whether or not T-cell lymphoproliferation associated with HTLV-1 infection is, with respect to oncogenic mechanisms, different from other T-cell lymphoma and as such whether or not they may benefit from drugs approved or in the development in T-cell lymphoma. We discuss the potential benefit of five agents currently developed in the treatment of T-cell lymphomas: agents with potential cytotoxic effect (pralatrexate and Bendamustine),

T-cell-targeted immunotherapy (Alemtuzumab) and agents interacting with major cellular signaling pathways and/or viral homeostasis (Histone deacetylase inhibitors, Lenalidomide).

9.1. AntiFolate (Pralatrexate). Pralatrexate is a new antifolate that was designed to be efficiently internalized by the reduced folate carrier (RFC). A prospective study has shown its relative efficacy on 111 patients with relapse T-cell lymphoma [27]. Major lymphoma subtypes were peripheral T-cell lymphoma (PTCL) and angio-immunoblastic T-lymphoma (AITL). Only one patient in this study had an ATL. The response rate in 109 evaluable patients was 29% (32 of 109), including 12 complete responses (11%) and 20 partial responses (18%), with a median duration of response of 10.1 months. Median PFS and OS were, respectively, 3.5 and 14.5 months. The U.S. FDA approved Pralatrexate for cutaneous T-cell lymphoma (CTCL) in 2009.

9.2. Histone Deacetylase Inhibitors (Vorinostat, Romidepsin, Panobinostat, and Belinostat). Histone deacetylase inhibitors HDAC inhibitors (HDACI) are a new class of drugs whose activity was initially designed on transcriptional activity by acting on chromatin epigenetic modification, histone deacetylation. However, their antitumor activity seems to occur through others pathways. Indeed, it has been shown that they also increase acetylation of other proteins such as nuclear transcription factors. Whatever the mechanism of action, exposure of cancer cells to HDAC inhibitors results in growth arrest, cellular differentiation, and apoptosis.

Two of these agents (vorinostat and romidepsin) have been approved in the USA for the treatment of relapsed and refractory CTCL. In these studies, they have been used as a single agent. Studies are ongoing to evaluate their efficacy on PTCL.

Vorinostat was evaluated in a phase II study. This study included 74 pts with CTCL who had failed at least two prior systemic therapies [28]. The primary end point was overall response rate (ORR). ORR was 29.7%. Median time to objective response was 56 days (range, 28–171). Median duration of response was not reached but estimated to be more than 185 days (range, 34–441). Major side effects were diarrhea (49%), fatigue (46%), nausea (43%), and anorexia (26%). Eleven patients required dose modification and nine discontinued due to adverse event. On the basis of this study, the U.S. FDA approved Vorinostat for CTCL in October of 2006.

Romidepsin was the second HDAC inhibitor that was approved by the U.S. FDA for CTCL in 2009. Two phase 2 trials were conducted in patients with CTCL with the primary goal of determining response rate and tolerance toxicity profile. The first trial included 71, and 96 patients were treated on a second trial. Response rates were 34% for both studies with median durations of 13.7 and 15.4 months, respectively, [29]. Side effects that were acceptable included nausea, vomiting, fatigue, and transient thrombocytopenia and granulocytopenia. Romidepsin was approved for CTCL after these two studies.

Recently, romidepsin was evaluated on PTCL. A phase 2 study reported forty-seven patients with PTCL of various subtypes including PTCL not otherwise specified (NOS), AITL, ALK-negative anaplastic large cell lymphoma, and enteropathy-associated T-cell lymphoma [30]. All patients received prior systemic therapies. Eighteen (38%) received stem cell transplantation. Overall response rate was 38% (95% confidence interval 24%–53%) with 8 CR and 9 PR. The median duration of overall response was 8.9 months (range, 2–74). Moreover, 6 responses were observed among the 18 patients with prior alloSCT. Side effects were acceptable.

To our knowledge, these drugs have not been yet evaluated in ATL as a single therapy or in combination with other drugs in induction therapy. However, Ramos et al. have reported a clinical trial using IFN-AZT with valproic acid (HDAC inhibitor) during the maintenance treatment phase [31]. The authors hypothesized that HDAC inhibitors could reactivate latent HTLV-1 in ATLL cells harboring intact provirus and help eliminate residual disease. Thirteen patients were enrolled. One showed a serial decrease in clonal ATLL disease followed by PCR. Using fresh cells from this patient treated *ex vivo* with Vorinostat, the authors showed an increase of HTLV-1 expression and an induction of cell death. However, in this study, induction of a putative immune response against virus-infected cells was not addressed.

9.3. Monoclonal Antibody. Alemtuzumab (CAMPATH-1H) is an anti-CD52 antibody that is approved for chronic lymphoid leukemia treatment. It has been showed that it is effective on T-cell prolymphocytic leukemia with high response rate in a prospective study including 39 patients with T-PLL treated with CAMPATH-1H [32]. The overall response rate was 76% with 60% CR and 16% partial remission (PR). These responses were durable with a median disease-free interval of 7 months (range, 4–45 months). In ATL, experience is limited to case report [33]. In addition, a recent study reported efficacy of the association of alemtuzumab and pentostatin in various types of PTCL including one case of ATL, which was in CR [34]. However, association Campath with conventional chemotherapy in PTCL has shown relative efficacy but high rate of infections.

9.4. Lenalidomide. Lenalidomide is a drug currently used for myeloma treatment. Studies have reported its use as single therapy for PTCL treatment. An interim report for a phase 2 clinical trial has been reported [35]. Patients with recurrent and refractory T-cell lymphomas other than mycosis fungoides and untreated patients ineligible for combination chemotherapy were prescribed oral lenalidomide (25 mg daily) on Days 1 to 21 of each 28-day cycle until disease progression. At the time of this interim analysis, 24 patients were enrolled in this study, and 23 were evaluable for response. The overall response rate was 7 (30%) of 23; all were in partial responses. Two patients had stable disease for ≥ 5 cycles. Median PFS was 96 days (range, 8–696 days). Median OS was 241 days (range, 8–696 days). The

most common grade 4 adverse event was thrombocytopenia (33%).

9.5. Bendamustine. Bendamustine is a cytotoxic agent that has been recently approved for the treatment of CLL and indolent lymphoma such as follicular lymphoma. This drug shows structural similarities with alkylating agents or antimetabolites. A phase II study tests his efficacy on relapsed or refractory peripheral T-cell lymphoma. The primary end point is ORR (CR, CRu, PR). Preliminary results have been shown recently for the first 38 patients (G. Damaj et al. 11th International Conference on Malignant Lymphoma, abstract n°126). ORR was 47%: CR + CRu in 11 patients (29%), PR in 7 patients (18%). 20 patients experienced relapse. At the time of analysis, the median duration time for responder patients was 157 days (range, 14–350). The most adverse events (grade 3 and 4) were neutropenia and thrombopenia.

10. Strategy

Suggested treatment strategies according to clinical presentation are described in Figure 1.

10.1. Chronic and Smoldering ATL. Patients with chronic and smoldering ATL have a better prognosis compared to patients with aggressive forms (acute and lymphoma). However, as it has been shown in a recent Japanese study, long-term survival is dismal when these patients are managed with a watchful-waiting policy until disease progression. Moreover, patients who received chemotherapy alone had a poorer outcome indicating that this may be detrimental in these subtypes [3]. So far, no clear prognostic factors have been yet defined in order to predict transformation to an aggressive form and treated patient who are at risk.

Our point of view is that most of patients with chronic and smoldering ATL should be treated. In the recent worldwide meta-analysis, patients with chronic/smoldering ATL who received first-line therapy by AZT-IFN only had an excellent survival (100% OS beyond 5 years). Thus, outside the context of clinical trials, the current standard therapy of chronic and smoldering ATL is combination therapy with AZT and IFN. This requires, however, continuous therapy. Treatment should not be interrupted as relapse always occurs when treatment is stopped. The recommended starting dose is AZT 600 to 900 mg/day (in 3 divided doses) and interferon-alpha (5 to 6 million IU/m2/day). Usually, after one month, AZT dose can be titrated down to 600 mg/day in 2 divided doses and IFN dose can be reduced to 3 to 5 million IU/day or alternatively 1.5 μg/kg of pegylated IFN weekly. Based on preclinical studies, clinical trials are testing the effect of adding arsenic to the AZT/IFN combination as a consolidation therapy with the aim of then stopping therapy and achieving cure by potential elimination of leukaemia-intiating cells [17–20].

10.2. ATL Lymphoma. As has been shown in the recent meta-analysis, the combination AZT-IFN is less effective than first-line chemotherapy in ATL lymphoma [16]. Therefore, chemotherapy should be the preferred option. However, recent unpublished results from the UK suggest that combination of antiviral therapy with CHOP chemotherapy is superior to CHOP alone in patients with ATL lymphoma [36]. Use of chemotherapy is based on the Japanese experience across different trials. The LSG15 protocol is the "standard of care." It is based on multiple drugs. When treated with this LSG15 protocol, ATL lymphoma patients achieved a better CR rate (66.7%) than acute type (19.6%) or chronic type (40.0%). However, relapse occurs rapidly and overall survival rate is low [6]. Therefore, a consolidation therapy is critical. Whenever possible, allogeneic SCT should be considered [8]. For patient failing to achieve remission after chemotherapy or lacking a suitable donor, a consolidation strategy should be discussed. Based on preclinical data, ongoing clinical trials are testing the efficacy of two cycles of arsenic/IFN maintenance as a consolidation procedure following achievement of CR with encouraging preliminary results [22]. Moreover, the addition of AZT/IFN or other novel therapies to chemotherapy may help to achieve remission. HDAC inhibitor might be tested in this indication to induce an immune response against residual tumor cells.

10.3. Acute ATL. Combination chemotherapy regimens have little effect in acute ATL. Even if the most intensive regimen (LSG-15) have increased response rate, MST and OS are low [6, 7]. In the recently published meta-analysis on antiviral therapy for ATL, treatment of acute ATL patients with AZT and IFN showed a higher response rate and significantly prolonged survival. Moreover, patients who achieved CR had a long-term response [16]. Outside the context of clinical trials, the current standard therapy of acute ATL is combination therapy with AZT and IFN. However, it can be difficult to manage patients presenting with bulky tumor or severe hypercalcemia not responding to bisphopshonates, and initial chemotherapy is sometimes required. It would be helpful to predict which patients in the acute form will benefit from this approach. Preliminary results indicate that patients with wild-type functional p53 are more likely to respond to AZT/IFN combination [37]. We, therefore, recommend evaluating p53 by a functional assay in all patients while the treatment is initiated [38]. Long-term disease control requires, however, continuous therapy, since relapse is always noted when treatment is stopped. The recommended dose is the same as with chronic/smoldering form. As in lymphoma subtype, allogeneic HSCT should be considered for young patients with acute ATL and a suitable donor [38]. As in other ATL subtype, based on preclinical data, ongoing clinical trials are testing the efficacy of arsenic/IFN maintenance following achievement of CR.

10.4. Supportive Therapy in ATL. Hypercalcaemia associated with aggressive ATL should be managed with treatment of the disease, hydration, and bisphosphonate therapy. Trimethoprim-sulfamethoxazole, valacyclovir, and antifungal agents are recommended for the prophylaxis of *Pneumocystis jiroveci* pneumonia, herpes simplex virus, and fungal

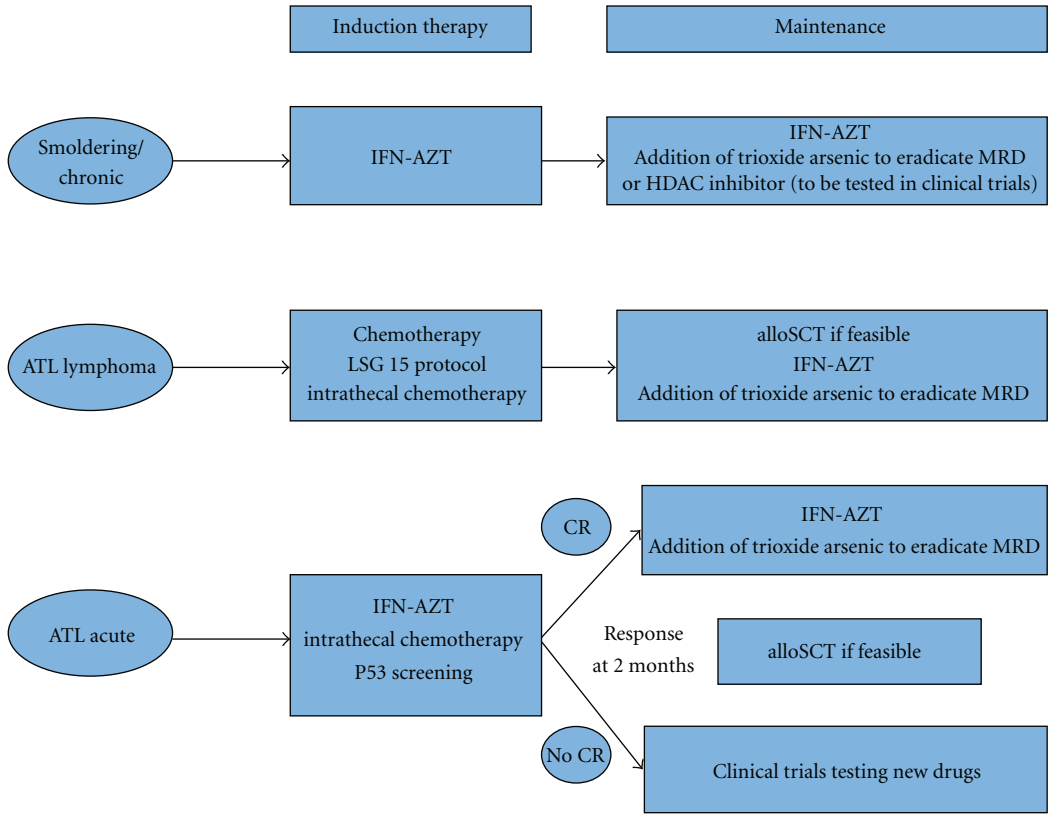

Figure 1: Recommended treatment strategy for patients with acute, lymphoma, or chronic/smoldering ATL (CR: complete remission; MRD: minimal residual disease; AZT: zidovudine; IFN: interferon-alpha; alloSCT: allogeneic stem cell transplantation).

infections, respectively; in the Japanese Trials, prophylaxis with antistrongyloides agents, such as ivermectin or albendazole, should be considered in order to avoid systemic infection in patients with a history of past and/or present exposure to the parasite. Intrathecal prophylaxis should be considered for patients with aggressive ATL even in the absence of clinical symptoms because more than half of relapses at new site after chemotherapy occur in the central nervous system.

11. Conclusion

The combination of AZT and IFN is highly effective in the leukemic subtypes of ATL and should be considered as standard in first-line therapy in that setting. This combination has clearly changed the natural history of the disease through achievement of a significantly improved long-term survival in patients with smoldering and chronic ATL as well as a subset of patients with acute ATL. Prior exposure to chemotherapy increases the rate of complications and of acquiring p53 mutations. We, therefore, recommend that the combination of AZT and IFN is used as a first-line treatment in the leukemic forms and that treatment is initiated with high doses of both agents since reduced doses are often not effective. ATL lymphoma patients benefit from initial induction therapy based on aggressive chemotherapy regimen but constantly relapse and have a poor prognosis. Addition of AZT-IFN in combination with chemotherapy may increase response rate but its long-term effect remains to be determined. We recommend, for those in whom alloSCT is not feasible, that a consolidation treatment with AsO_3 is considered, followed by maintenance therapy with AZT/IFN. This approach should be tested in future clinical trials. Prophylaxis of opportunistic infections and supportive therapy are mandatory. In order to prevent the occurrence of resistance and relapse, clinical trials assessing additional targeted therapies such as arsenic/IFN combination or monoclonal antibodies, particularly the promising anti-CCR4 antibodies, are mandatory after achieving CR. Finally, allogeneic SCT should be considered in suitable patients. HDAC inhibitor may be also an interesting option. Currently, due to the poor outcome of patients with aggressive ATL (acute and lymphoma forms), phase II studies are mandatory in the near future. In chronic form, it is time to set up phase III studies to assess new drugs to avoid relapse for patients treated with AZT-IFN.

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Review Article

Cotranscriptional Chromatin Remodeling by Small RNA Species: An HTLV-1 Perspective

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Cell type specificity of human T cell leukemia virus 1 has been proposed as a possible reason for differential viral outcome in primary target cells versus secondary. Through chromatin remodeling, the HTLV-1 transactivator protein Tax interacts with cellular factors at the chromosomally integrated viral promoter to activate downstream genes and control viral transcription. RNA interference is the host innate defense mechanism mediated by short RNA species (siRNA or miRNA) that regulate gene expression. There exists a close collaborative functioning of cellular transcription factors with miRNA in order to regulate the expression of a number of eukaryotic genes including those involved in suppression of cell growth, induction of apoptosis, as well as repressing viral replication and propagation. In addition, it has been suggested that retroviral latency is influenced by chromatin alterations brought about by miRNA. Since Tax requires the assembly of transcriptional cofactors to carry out viral gene expression, there might be a close association between miRNA influencing chromatin alterations and Tax-mediated LTR activation. Herein we explore the possible interplay between HTLV-1 infection and miRNA pathways resulting in chromatin reorganization as one of the mechanisms determining HTLV-1 cell specificity and viral fate in different cell types.

1. Introduction

In the myriad interactions between viruses and host cells, there is a constant struggle for survival that causes both sides to adopt strategies counteracting each other's effect. More often than not, the error-prone replication of viruses offers them an advantage of selective pressure enabling them to accumulate genetic mutations over time that helps evade host immune defense mechanisms. Most chronic viruses seem to have an edge in this struggle in that they evolve means to manipulate and exploit host molecular pathways to persist in the hostile cellular environment and remain hidden from immune surveillance [1]. In this regard, retroviruses have succeeded in establishing latent infection and developing drug resistance through escape mutants like very few other chronic viruses. One of the strategies utilized by retroviruses is the modulation of chromatin structure and regulation of the rate at which transcription occurs in the target cell. Chromatin remodeling in the context of retroviral infection is being explored as a potent means of long-term persistence.

Many studies have shown that the exercise of chromatin modulation in retroviral infection begins with the proviral integration into the host genome [2]. The site at which this integration occurs is important as it determines the kind of chromatin remodeling that the virus might cause and the rate at which viral proteins are produced. This in turn determines if the viral infection becomes latent or remains active. Persistence, as demonstrated by latent viruses, is thus largely dictated by the nature of virally encoded integrase enzyme. It requires the provirus to integrate into a site that is transcriptionally inactive or less active so that there is minimal viral gene expression. Conversely, a productive infection is a result of integration into transcriptionally active regions on the host genome resulting in a higher rate of viral protein expression [1]. Human T cell leukemia virus 1 (HTLV-1), a deltaretrovirus, behaves preferentially in the former fashion by altering chromatin structure to remain latent and thus aid in its survival and persistence [3]. In addition, methylation along the 5′ long terminal repeat (LTR) region of the virus contributes to regulation of viral persistence [4].

HTLV-1, the first retrovirus to be associated with human malignancies, is the causative agent of adult T cell leukemia (ATL) and HTLV-1 associated myelopathy/tropical spastic paraparesis (HAM/TSP) [5]. The virus has a propensity for infecting $CD4^+$ T cells [6] with $CD8^+$ T cells serving as reservoirs [6]. Other secondary cell types such as $CD8^+$ T cells [7], cells of the monocyte-macrophage lineage, and dendritic cells [8] as well as those belonging to the resident CNS cell population [9] are also known to be infected. One of the factors to be considered during this observation is that some of the cell types refractile to viral transcription also tend to express lower levels of miRNA processing proteins.

A number of independent studies have identified integration sites of HTLV-1 in the human genome [10–13]. Derse et al., in 2007, mapped 541 integration sites of the virus in HeLa cells comparing them to other retroviral integration sites and showed that integration does not correspond merely to transcriptional units and transcriptional start sites. Rather, the apparent nonrandom site integration is monoclonal in nature [14] and predominantly reliant on the structure and/or sequence of viral integrase enzyme [13]. A clear demarcation appears to exist between the integration preferences of HTLV-1 in carrier cells versus leukemic cells. HTLV-1 integrates into nontranscribing heterochromatin alphoid repeats in carrier cells, while in leukemic cells, it preferentially integrates at actively transcribing DNA units [10].

Once integration has occurred, viral replication and successful infection among other factors depend on Tax, the virally encoded transactivator protein largely involved in cellular transformation. A major regulatory function of the retroviral transactivating protein is its ability to interfere with the host cellular miRNA machinery [15, 16]. Altered miRNA expression profiles have been observed between retrovirus-infected and uninfected cells that can also be associated with disease progression and development of cancer [17]. In the context of HTLV-1 infection, a number of recent studies have identified distinct miRNA patterns in infected cells that progress to ATL [18–20]. Although results of the individual studies have disparities between them, all of them identified the tumor suppressor gene TP531NP1 to be commonly repressed in infected cells [21]. A more detailed analysis of miRNA and its differential expression in HTLV-1-infected cells will be discussed in a later section of the paper.

Besides the canonical role of miRNAs as translational repressors of mRNA expression, emerging evidence indicates a significant modulatory role for miRNA at the level of chromatin [22, 23]. The miRNAs accomplish this either through direct methylation along the promoter region of specific genes or more indirectly through the epigenetic modification of histone proteins surrounding the chromatin of the target region [24]. This phenomenon referred to as RNA-induced initiation of transcriptional silencing (RITS) [25, 26] has been implicated in the regulation of a number of human genes [27]. Kim et al. demonstrated that endogenous miRNA recruit argonaute 1 (Ago1), EZH2, a PcG member, and H3K27me3 to the promoter region of the target gene and suppress its expression [28]. In case of HIV-1, another human retrovirus, the proviral promoter is also influenced by RITS in infected cells [29]. Retrovirus-derived miRNAs like those generated by processing of the TAR element of HIV-1 appear to be involved in RNAi-mediated Transcriptional Gene Silencing (RNAi-TGS). Several small ssRNA species cleaved from the TAR region by Dicer have been implicated in dampening cellular as well as viral gene transcription and promoting viral latency [30–32]. Taken together, it is possible that RNAi-TGS keeps viral transcription in check through the recruitment of RITS at the viral promoter [33].

A similar mechanism of gene silencing induced by siRNA is quite likely in HTLV-1-infected cells. From previous studies, it is clear that expression levels of Tax responsive miRNA in infected cells is to a large extent modified through the NFκB pathway [34]. Given that Tax is associated with a number of other cellular transcription factors like CREB, Ap-1, Myc, NFAT, SRF, p53, TGF-β, and so forth, that also regulate siRNA expression levels, its involvement in regulating RITS through these pathways cannot be ruled out. Since Tax is pre-dominantly nuclear, one possible mechanism by which it might influence siRNA-mediated chromatin remodeling could be through sequestering mature siRNA from its target sequence on the chromatin, preventing its regulation. Alternatively, Tax could also interact with the ribonuclease III enzyme Drosha within the nucleus, modulating its function and preventing it from cleaving out the precursor miRNA element from the primary miRNA transcript. Besides the nucleus, Tax occupies a number of subcellular sites in the infected cell. Although mechanistically poorly understood, it is known to shuttle to the cytoplasm from the nucleus, a process that was demonstrated through a heterokaryon fusion assay [35]. Also, there have been studies questioning the nuclear assembly of Tax with its transcriptional activating counterparts—CBP/p300 and RelA, proposing a possible cytoplasmic assembly [35, 36].

In addition, HTLV-1 Rex protein has been demonstrated to interact with Dicer and block it from processing mature miRNA from precursor miRNA and suppressing its RNA silencing activity [37]. The subsequent sections of the paper focus on HTLV-1 infection and the role of Tax protein in disease progression. Further, various small RNA pathways and their capacity to influence chromatin remodeling are described. Finally, a more detailed description is presented on interaction of viruses with miRNA in general and retrovirus and HTLV-1 in specific proposing probable points of intersection that can result in miRNA-mediated chromatin reorganization as depicted in Figure 1.

2. HTLV-1 and Its Transactivator Protein Tax

The virus predominantly gets transmitted through cell-cell contact, prompting the formation of a microtubule-organizing center (MTOC) oriented towards the virological synapse [38]. At the synapse, viral RNA and Gag protein accumulate at the MTOC and get transported into the uninfected cell [39]. Tax plays a role in synapse formation, MTOC orientation, and intracellular adhesion molecule -1 (ICAM-1) engagement with lymphocyte function associated antigen -1 (LFA-1) [40]. Although it was previously believed that cell-free HTLV-1 is largely noninfectious, there is emerging

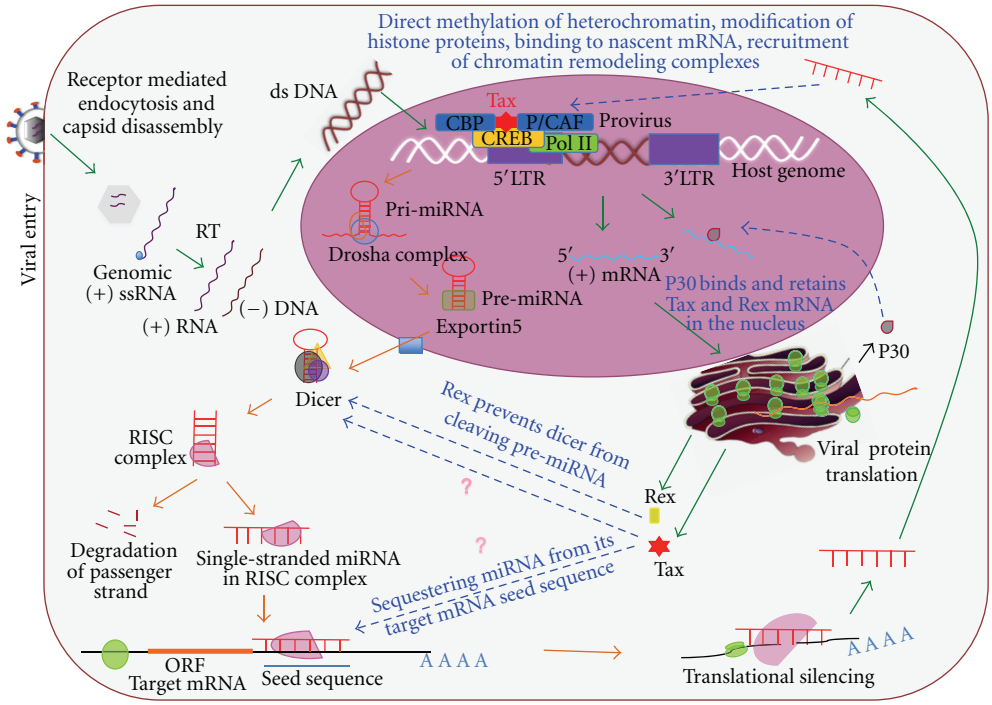

Figure 1: The interaction between HTLV-1 infection pathway and miRNA pathways. HTLV-1 infection begins with viral gp46 and gp21 envelop proteins recognizing and binding receptors on target cell membrane followed by envelop fusion and receptor-mediated endocytosis. Once inside the cell, HTLV-1 loses its capsid and releases the single-stranded (+) ve sense RNA into the cytoplasm that undergoes reverse transcription to (−) ve sense DNA. A second DNA strand is then formed; the two strands form a helix, enter the nucleus, and integrate with the host genome forming the proviral particle. Transcription ensues with cellular pol II, CREB, CBP, p300, P/CAF, and viral Tax assembling at the 5′ LTR of the promoter region. This leads to Tax-dependent transcription of viral mRNA, transport of mRNA into the cytoplasm and its subsequent translation on ribosomes. The continuous green arrows indicate the HTLV-1 infection pathway, while the orange arrows indicate the miRNA biogenesis and mechanism of action pathway. The discontinuous blue arrows indicate points at which viral proteins might interact with the miRNA pathway and also where miRNA might interfere with viral transcription. Chromatin remodeling is one of the interfering mechanisms that can be brought about by siRNA direct binding of siRNA coupled with the RISC complex to DNA, methylation of heterochromatin regions of the chromosome, or modification of histone proteins associated with chromatin.

evidence that HTLV-1 can enter naïve cells through receptor-mediated endocytosis. The human glucose transporter GLUT-1 [41] and surface heparin proteoglycan [42] have been identified as possible receptors for cell-free virus.

HTLV-1 is a relatively complex retrovirus on account of the fact that in addition to the retroviral structural *gag, pol,* and *env* genes flanked by 3′ and 5′ LTR regions, it has a *pX* region located between the 3′ LTR and *env* genes encoding Tax, Rex, and other accessory proteins [43]. Tax is encoded by open reading frame (ORF) IV of the *pX* region and is principally functional in the nucleus. Through DNA array studies, expression profiles of more than 300 of the ~2000 cellular genes assayed were found to be significantly altered under the influence of Tax [44] acting through several pathways as previously mentioned. As an oncoprotein, the most critical function of Tax appears to be cell survival, proliferation, and ultimately transformation of the cell into an ATL state. Oncogenicity is most often associated with genotypic and phenotypic instability of transformed cells. Genomic instability in HTLV-1-induced leukemia is thought to be caused by Tax in two phases:firstly, by inhibition of cellular DNA repair pathways and secondly by the loss of cell cycle checkpoint controls [43, 45, 46].

The 40-kDa Tax protein is essentially involved in HTLV-1 gene expression from three 21-bp Tax responsive elements (TRE) located within the U3 region of the viral promoter [47, 48]. Each TRE is composed of domains A, B, and C, but the central B region is a conserved 8-nucleotide (nt) core sequence (TGACGTCA) that closely mimics a cyclic AMP (cAMP) responsive element (CRE) and is flanked by 5′ and 3′ G/C-rich sequences [49]. Tax activates transcription by recruiting the cellular transcription factors—CRE binding protein (CREB) and serum response factor (SRF or $p67^{SRF}$) to the CRE [50, 51]. Tax interacts with dimeric CREB [52] as a homodimer forming a ternary complex that in turn helps to stabilize the CREB/TRE complex [53]. Once stabilized, Tax then independently recruits the two cellular coactivators-p300/CREB-binding protein (p300/CBP) and p300/CBP-associated factor (P/CAF), both of which bind to two distinct regions in the amino-terminus and carboxyl-terminus of Tax, respectively, and eventually activates transcription by histone acetylation through chromatin remodeling [54–56]. In addition, Tax has shown to reduce histone protein and transcript levels in HTLV-1 infected compared to uninfected T cell lines [57]. The protein also influences transcription of a number of cellular promoters, namely, IL-2, IL-13, IL-15,

IL-2R, c-Fos, GM-CSF, and so forth [58–63]. Modulation of cellular gene expression is through several cellular signaling cascades—four of these cardinal pathways include CREB-ATF [64], NFκB [65], AP-1 [66], and SRF [67]. Regulation of these pathways by Tax has been extensively reviewed elsewhere [68]. One major outcome is the quelling of the tendency of virus-infected cells to undergo apoptosis and senescence [69, 70]. In addition, Tax represses DNA damage control checkpoints and also activates several proliferative factors that facilitate progression of cell cycle into the replicative phase, enhancing cell division [71].

Members of the stimulatory protein (Sp1 and Sp3) family of transcription factors physically interact with the GC regions within TRE-1 repeat III, and purified Sp1 protein competes with purified CREB protein for binding to this site, but in the presence of Tax purified Sp1 can form a protein complex with Tax and CREB [72]. In cells of the monocytic-macrophage lineage (secondary target cell population), factors belonging to the activator protein (AP-1) family of basic region/leucine zipper (bZIP) proteins (Fra-1, Fra-2, JunB, and JunD) are shown to be upregulated [73, 74] and bind to the TRE-1 repeat II site thereby activating basal-and Tax-mediated transactivation of the LTR [75]. However, in the same cell type lineage, another family of bZIP factors, CCAAT/enhancer binding protein (C/EBP), promotes low level of viral gene expression in the absence of Tax (C/EBPβ, C/EBPδ, C/EBPε), while in the presence of Tax it (C/EBPα and C/EBPβ) inhibits high level of viral gene expression [76]. Other transcription factors like members of the CREB family (CREB-2-activating transcription factors—ATF-1 and ATF-2) [77, 78] and the histone deacetylase HDAC1 [77] have also been identified in the LTR complex [79]. The TORC family of transcriptional regulators (viz., TORC1, TORC2, and TORC3) are coactivators of Tax protein and the removal of these factors inhibits Tax activity. The cofactor p300 further enhances for TORC activity [80, 81].

However, majority of these investigations highlighting the importance of the cellular transcription factors (CREB, Sp1, Sp3, AP-1, C/EBP, p300/CBP, and P/CAF) in HTLV-1 Tax-mediated LTR activation [50, 82–85] and the ability of Tax protein to interact with these factors independently [54, 86, 87] have been carried out using transiently transfected viral reporter plasmids or in cell lines that otherwise are not the primary target for HTLV-1 *in vivo*. Studies with HIV-1 have shown that the integrated provirus differs from a transfected viral plasmid both physically [78] and also in the requirement of certain cellular factors especially those belonging to the chromatin-remodeling histone acetyltransferase (HAT) family [88–90] or even in the transcriptional repressor domain [91]. It demonstrates that transient transfection cell systems do not convey the real picture by undermining the crucial role of chromosomal structure in transcriptional regulation. In order to gain a better understanding of viral gene regulation as well as the complex interplay between the integrated provirus, host cellular transcription factors, the viral transcription transactivating protein, and the cellular siRNA machinery during the course of infection and reactivation following latency, it would certainly be more realistic and physiologically relevant if such studies are carried out with stably integrated viral LTR in a clinically relevant cell type that is formatted in the context of cellular chromatin. To this end, we generated HTLV-1 LTR stable integrants with a reporter luciferase gene (HTLV-1 LTR-luc) in the Jurkat cell line, representative of the natural target $CD4^+$ T-cell population, to characterize realistically the intricacies involved in the interplay between the integrated provirus, cellular transcription factors, and the viral transactivating protein Tax (Rahman et al., unpublished data). To investigate the comparative activation/repression of cellular transcription factors between stably integrated and transiently transfected HTLV-1 LTR in at least one native target cell phenotype, both in the absence and presence of Tax, a high-throughput analysis of such factors was performed using protein-DNA array technology. Many substrates and factors associated with the two major chromatin-remodeling complexes, SWI/SNF and HATs, were activated in the stably integrated clones following transfection with Tax. To explore the observed heightened activation of factors necessary for chromatin remodeling complexes, we explored the upstream miRNA regulatory pathway by the microarray approach. A global downregulation in the expression of cellular miRNAs in the HTLV-1 LTR-luc stably integrated $CD4^+$ T-cell clone was observed in the presence of Tax, implying the ability of Tax to modulate the cellular miRNA machinery. When compared to results presented with the transcription factor array, many of the downregulated miRNAs were found to target the mRNA coding for the P/CAF and p300 HAT family members, suggesting a role for Tax in downregulating the expression of cellular miRNAs that are in turn involved in suppressing the expression of transcription factors involved in chromatin remodeling. The results demonstrate that Tax can modulate the cellular miRNA machinery and downregulate the expression of miRNAs identified to be involved in regulating the translation of chromatin-remodeling HAT factors (Rahman et al., unpublished data). Given the rising importance of siRNA-mediated modulation of gene expression in a viral infection context, it would be interesting to explore how HTLV-1 alters the siRNA/miRNA in its primary target cell. Small noncoding RNA species make up the bulk of cellular RNA, and their regulatory potential is increasingly being recognized as significant and proportional to their presence in the cell. Their regulatory potential encompasses chromatin reorganization and the following section of the review focuses on giving a brief description of various noncoding small RNA molecules and their possible involvement in transcriptional modulation through chromatin.

3. Small Interfering RNA and Gene Silencing

Small noncoding RNA species are rapidly being recognized as a significant influence on gene expression and functioning of cells. RNA silencing pathways have been traditionally classified based on the mechanism of action, intracellular location, and the class of RNA molecule involved. There are similarities in the organization of some of the components in these pathways, and an inevitable intersection exists between them in some instances [92]. Three major classes of RNA have been annotated to be involved in modulating cellular

gene expression namely, small interfering RNA (siRNA), micro-RNA (miRNA), and piwi-associated RNA (piRNA). While siRNA and piRNA have an equally potential role in posttranscriptional as well as transcriptional gene repression, miRNA was until recently predominantly descried for its cytoplasmic role of mRNA suppression [93]. From current studies, a transcriptional chromatin-modulating role for miRNA is emerging and gaining importance especially in the context of a viral infection [23].

Small interfering RNAs are ~21 nt single-stranded RNA molecules cleaved out of larger dsRNA that can either be of endogenous or exogenous origin. siRNA identify their target through complete and perfect sequence complementarity and silence target mRNAs through complementary binding [94]. Some organisms have developed mechanisms to amplify their siRNA after target recognition through the expression of an RNA dependent RNA polymerase (RdRP). The RdRP enzyme either by itself produces new single stranded siRNA or amplifies ssRNA into dsRNA that is then cleaved by Dicer to generate mature siRNA [95].

miRNAs are ~19–24 nt short non-coding RNA that modulate ~60% of all human protein coding cellular genes, successfully modifying the outcome of various microbial infections and disease states [96]. Typical miRNA biogenesis is initiated with the RNA pol II mediated transcription of long primary miRNA (pri-miRNA) that contain one or more ~80 nt hairpin (stem-loop) structures. The pri-miRNA is processed by an RNase III enzyme Drosha, which, along with its coeffector DGCR8, recognizes and cleaves ~22 bp down the stem yielding a precursor miRNA (pre-miRNA) approximately 60 nt in length comprising 2-nt 3′ overhangs. The pre-miRNA is transported into the cytoplasm aided by Exportin 5 through the nuclear pore complex. A second RNase III enzyme Dicer in association with Tar RNA binding protein (TRBP) cleaves the terminal loop structure of the pre-miRNA, generating a ~22-bp duplex [97, 98]. One of the strands of the duplex associates with an RNA-induced silencing complex (RISC) functioning as a guide to the target mRNA "seed" sequence, while the other passenger strand gets degraded. The RISC complex is composed of the Argonaute family of proteins (Ago), some of which have endonuclease activity and enzymatically cleave the target mRNA. In addition, Ago proteins guide the complex to the target site and also aid in the degradation of the passenger strand [99]. The following section deals with the involvement of these small non-coding RNA in TGS through reorganization of nuclear chromatin.

4. Chromatin Remodeling

The term refers to the effective shifting of nucleosome core along the length of the DNA molecule [100]. This shift in many cases results in the physical disassembly and reassembly of the nucleosome core and requires the involvement of ATPase containing complexes. The four known ATPase complexes associated with chromatin remodeling are SWI2/SNF2 (mammalian Brm (SNF2α) and Brg1 (SNF2β)), ISWI (imitation switch), Mi-2 (CHD1), and INO80 [101]. During processes such as DNA replication and transcription, the state of chromatin organization and the positioning pattern of associated histone proteins are critical rate determining factors. A large number of histone-modifying enzymes and factors get associated with histones and alter their state to affect winding and unwinding of chromatin DNA as required by the specific cell.

4.1. Chromatin Remodeling by siRNA and miRNA. Heterochromatin reorganization by miRNA is a mechanism that has been generally described to be a result of association of siRNA/miRNA with the RNA-induced initiation of silencing [26] complex, which was first described by Verdel et al. in 2004 [25]. RITS is a multiprotein complex consisting of components that aid binding with RNA and chromatin simultaneously. It has a chromodomain protein, Chp1, that is known to interact with centromeres of chromosomes [102] and an argonaute family protein, Ago1, with endonuclease activity that can bind small RNA. In addition, a recently identified Tas3 protein is present in the RITS complex that is yet to be functionally characterized. Sequences homologous to the *dg* and *dh* region of centromeres were found on siRNA copurified with RITS complexes, indicating plausible complementary base pairing of RITS-associated siRNA with centromeric regions of the chromosome. Also, all three proteins of the complex are essential requirements for H3K9 methylation as well as centromeric chromatin-binding Swi6p protein known to cause heterochromatic gene silencing [102].

It has also been proposed that an alternate mode of interaction could be between RITS-associated siRNA and nascent immature mRNA transcribed from the H3K9 centromeric chromatin region. An interesting outcome associated with this phenomenon is that it serves as a self-amplifying mechanism for siRNA. The inhibition of mRNA from maturing and its subsequent degradation could be the source of new siRNA with a sequence complementary to that of the H3K9 region of the centromere, thus augmenting gene silencing [103]. Although siRNAs with sequences homologous to LTR regions have not been clearly identified, RITS-complex-associated silencing of LTR-associated genes cannot be ruled out. One reason being that LTR-associated gene silencing requires the presence of Ago1 [104] that is generally found in affiliation with other small RNA processing protein complexes namely, RISC and RITS. Also, the relative abundance of siRNA directed against LTR regions might be lower and less obviously discernable compared to *dg* and *dh* regions of centromeres. Since virally encoded proteins directly and indirectly get associated with various stages of the RNAi biogenesis and functioning pathways, it is reasonable to speculate that viral proteins might influence heterochromatin modeling and expression states.

5. Viral miRNAs and Virus-Induced Modulation of Cellular miRNA Pathway

5.1. miRNA of Viral Origin. A number of interactive stages and mechanisms have been described and proposed to elucidate the interplay between viruses and siRNA pathways. Besides cellular miRNAs, viruses also encode their own

species of miRNA that modulate cellular processes favoring efficient viral proliferation and persistence. Herpesviruses were among the first to be shown to express miRNAs [105] which play significant roles in pathogenesis of the virus. For instance, HSV2 expresses miR1 that is a key modulator of ICP34.5 expression and hence progression of neuroinflammatory disease [106].

5.2. Retroviral miRNA. The idea of retroviruses coding for miRNA is in a way counterintuitive in that a cytoplasmic replication will likely yield miRNA precursors that would not be available for processing by the nuclear Drosha complex [107]. Nonetheless, many recent studies revealed retroviral-derived miRNA in the context of HIV-1 infection. Omoto et al. identified several miRNA originating from the nef region that affected transcription from the viral HIV-1 LTR promoter [108]. More recently, it was found that the HIV-1 TAR element is processed by Dicer to yield viral miRNA that could be detected in infected cells and apparently contribute to latency [30]. HTLV-1-encoded miRNA has been relatively less explored, and there is currently no evidence of the virus expressing any miRNA. Li et al., in 2008, performed an extensive computational sequence analysis to identify stem-loop structures resembling those harbored by pri-miRNA and pre-miRNA that could possibly be cleaved to generate microRNAs [109]. Ten such sequences were identified and when aligned with the HTLV-1 genome, it was predicted that two miRNAs could be produced from the plus strand transcript and 4 from the minus strand [110]. Upon further bioinformatics-aided searches of all the sequence reads against the HTLV-1 genome, both sequences mapped to transcribed regions on the plus strand [110]. More extensive studies will be required to investigate if the HTLV-1 genome can act as a source of miRNA.

5.3. Modulation of miRNA Pathway by Viruses. Viruses have been known to exploit cellular miRNAs to induce cell proliferation, suppress apoptosis, influence metabolic pathways, and finally bring about cellular transformation [111]. Increased expression of several oncogenic miRNA in response to retroviral infection has been previously documented [112–114]. There are several instances where viral proteins actively interact with RNA and alter their functioning in infected cells. The nonstructural (NS-1) protein of human influenza A virus has an RNA-binding domain that can associate with a number of RNA species causing RNA silencing suppressor activity [115, 116]. NS1 localizes both in the cytoplasm and the nucleus where it sequesters immature and mature forms of small interfering RNA, this activity is more evident in plants and needs to be elucidated further in mammals [117]. The HCV core protein predominantly localizes in the cytoplasm and interacts with Dicer through its N-terminal, lowering its miRNA processing efficiency. In addition, the HCV envelop protein E2 interacts with argonaute 2 (Ago2) inactivating it. Ago2 is part of the protein complex essential for generation of mature and active miRNA particles [118]. The Ebola virus non-structural protein VP35 consists of a dsRNA-binding domain similar to the NS1 of influenza A. It is generally found in cytoplasmic inclusion bodies and is proposed to sequester siRNA/miRNA preventing them from getting loaded on to RISC complexes, thus preventing them from directing mRNA silencing [117]. Adenoviruses use their dsDNA genome to encode two non-coding RNA molecules apart from viral proteins transcribed by RNA polymerase III. These VA1 RNA transcripts are produced at high levels in the nucleus of infected cells, saturating the Exportin 5 nuclear transport machinery. This in turn prevents precursor miRNA from being exported to the cytoplasm and mature into functional miRNA [119].

5.4. Modulation of miRNA Pathway by Retroviruses. Retroviruses also modulate the host miRNA machinery in a number of ways. Primate Foamy Virus (PFV) primarily encodes a nuclear transactivator of transcription protein—Tas that through a yet unknown mechanism suppresses the effector function of miRNA [120]. HIV-1 Tat protein functions as a transactivator of transcription and is essential for viral replication and is also indicated in suppressing the RNAi pathway. While HTLV-1 Tax protein, which is also a transactivator [47] has not yet been shown to exhibit the similar function. Tat directly interacts with the helicase domain of Dicer and inactivates it, preventing cytoplasmic processing of miRNA [16, 48]. However, it is still not clear how the predominantly nuclear protein Tat interacts with the mostly cytoplasmic Dicer. One explanation could be that small levels of Tat could still remain cytoplasmic and interact with the RNAi pathway. Also, recently small levels of Dicer have been shown to be present in the nucleus where it also participates in processing miRNA and siRNA in association with RNA-dependent RNA polymerase (RdRP) (Figure 2). The Dicer processed TAR-derived miRNA of HIV-1 has been known to suppress RNAi through sequestration of TREB—a Dicer cofactor [121]. A similar possibility can be attributed to the Tax protein of HTLV-1, which is also mostly nuclear but could still interfere with the cytoplasmic phase of miRNA processing.

6. HTLV-1 Infection: Chromatin Remodeling via miRNA

6.1. miRNA Expression in Infected Cells. There have been independent studies linking the differential expression of miRNA profiles in HTLV-1-infected T cells with the progression to ATL. Pichler et al. (2008) performed RT PCR analysis on HTLV-1 transformed cell lines to determine differential expression in $CD4^+$ T cells and Treg cells [19]. Seven miRNAs that had been previously shown to be associated with oncogenic transformation [20], miR-21, miR-24, miR-146a, miR-155, miR-191, miR-214, and miR-223 were considered, for this study as they are specific to Treg cells. Results demonstrated that miR-21, miR-24, miR-146a, and miR-155 were significantly upregulated in the HTLV-1-transformed cell lines, while miR-223 was downregulated. The miRNA profiling of PBMCs from acute ATL samples and HTLV-1 transformed cell lines revealed 6 miRNAs that were upregulated in both the ATL samples and the transformed cell line (i.e., miR-18a, 9, 17-3p, 130b, 20b, and 93), while 9 miRNAs

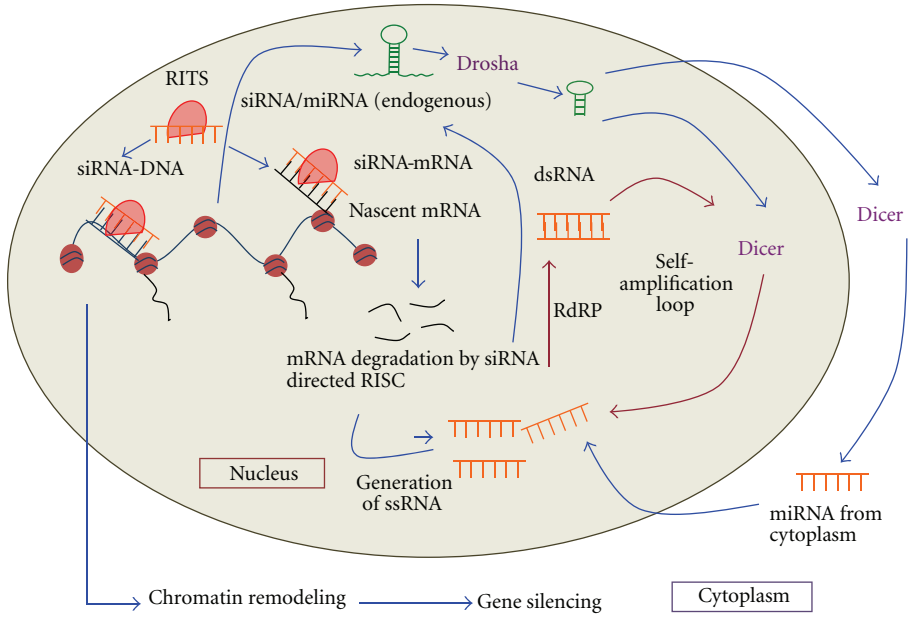

Figure 2: Mechanisms of siRNA-induced transcriptional silencing. miRNA/siRNA is imported into the nucleus from the cytoplasm after processing by Dicer through the nuclear pore complex. Alternatively, Dicer can also localize in the nucleus, allowing siRNA generation and amplification within the nucleus itself. Change in gene expression at the chromatin level directed by siRNA through the RNA-induced initiation of transcriptional silencing complex can be the outcome of a number of interactive mechanisms. Firstly, protein components of the RITS complex possessing both chromatin and RNA binding properties enable siRNA/miRNA to directly bind DNA. siRNA have homologous regions to the dg and dh regions of centromeric heterochromatin indicating a direct interaction between the two components. Secondly, miRNA can methylate promoter regions of genes or epigenetically cause the assembly of histone modifying proteins, changing the transcriptional state of chromatin. Thirdly, an interaction between siRNA and nascent mRNA transcribed from the centromeric region not only causes transcriptional repression of that region but also serves as an siRNA self-amplifying mechanism that uses degraded mRNA as a source of single-stranded RNA. Some of this ssRNA is amplified by RNA-directed RNA polymerase (RdRP) components which generate dsRNA further processed by nuclear Dicer into mature siRNA that augments silencing.

were downregulated (i.e., miR-1, 130a, 199a, 126, 144, 335, 337, 338, and 432) [18]. Further miRNA-profiling studies were performed on ATL cells versus control PBMCs and $CD4^+$ T cells. Several miRNAs, namely, miR-150, miR-155, miR-223, miR-142-3p, and miR142-5p were upregulated and miR-181a, miR-132, miR-125a, and miR-146b were downregulated [20]. A similar miRNA profile was also seen in HTLV-1-infected non-ATL cells. miRNA 27-a controls the F-box protein FBW7/hCDC4-dependent cyclin E degradation by modulating ubiquitylation and turnover of cyclin E during specific stages of the cell cycle progression [122]. Not surprisingly, results from the above studies taken together indicate a highly variant miRNA profile owing to the cell type specificity of the virus and probably also to the differential expression of miRNA-associated cellular proteins.

6.2. Cellular Pathways. Cell longevity in HTLV-1 infection is mediated by Tax through two major cell-signaling pathways. In the first, the protein directly binds PI3K and promotes phosphorylation of Akt, which is a serine/threonine kinase that is involved in maintaining cell proliferation and survival through a series of downstream signaling events involving activating protein −1 (AP-1) [123] that is upregulated in ATL cells [124] as well as a large number of other cancer types. The second pathway involves binding of Tax to Ikkγ in the cytoplasm that triggers phosphorylation of Ikkα and Ikkβ and a complex formation between the 3 Ikk components. The Ikkα-Ikkβ-kkγ complex in turn phosphorylates IκBα and releases it from NFκB. Free NFκB then migrates to the nucleus, activating the transcription of genes associated with the NFκB responsive elements. A number of NFκB target genes are known to enhance cell growth and proliferation and implicated in cancer development.

In spite of the fact that Tax is required for cell transformation and development of ATL, it is interesting to note that more than 50% of ATLs do not show Tax transcripts. In fact cells tend to evolve and accumulate mutations in Tax that silence its activity [39]. It is believed that the presence of Tax is needed for the emergence of ATL, but not for its maintenance. Since the oncoprotein is a major target of T lymphocytes, silencing it is a mechanism to subvert immune detection and clearance. It is quite possible that the maintenance of an active leukemic cell state is mediated through miRNA that are known to be significantly differentially regulated in ATL cells. Expression profile studies suggest that many of these miRNAs are associated with Tax levels in early ATL developmental stages. Chromatin reorganization as previously mentioned is influenced by miRNA and hence the rate of active viral transcription and maintenance of productive/latent viral state could be affected by the miRNA profile of the infected cell. Several of these miRNAs are associated

with prolonging cell proliferation and development of cancer.

6.3. Cell Cycle. Cell cycle progression is tightly regulated by the interaction between cyclins and cyclin-dependent kinases (CDKs). This interaction results in the selective phosphorylation of target proteins involved in regulatory stages of the cell cycle.

Tax-expressing cells seem to show disruptions in cyclin-CDK complex formation. In general, there appears to be a stronger progression of HTLV-1-infected cells through the G1 phase into the S phase [125]. This can be correlated with the upregulation of cyclins D2 and E and the corresponding increase in D2 binding CDK partners, Cdk4/6 and Cdk2 following Tax expression in these cells [126]. Interestingly, Tax directly binds to cyclin D3, D4, and Cdk4 and apparently increases stability of cyclin D/Cdk4 complex formation [127]. Tax has been reported to activate expression of cdk2 and cdk4 in infected cells [128] and downregulate expression of CDK inhibitors like p18 and p19 [129]. The protein also causes repression of cylin A [130], which is required for cells to exit mitosis after one round of chromosome replication [131] thus promoting enhanced DNA replication.

An important phosphorylation target of the cyclin-CDK complexes is the retinoblastoma protein (Rb) that binds transcription factor E2F in a dephosphorylated state. When phosphorylated by cyclin-CDK complexes, it releases E2F that allows transcription of genes required for the progression from the S to G2 phase [14]. Tax disrupts the formation of Rb-E2F complexes by physically degrading dephosphorylated Rb, promoting its phosphorylation and release of E2F, thus pushing the cell into S phase [132–134]. There is a close association between miRNA expression and alteration in the cell cycle progression. Compelling evidence also points at a markedly altered miRNA expression profile in HTLV-1-infected, ATL, and noninfected cells suggesting a probable association between Tax-induced miRNA expression and rate of cell cycle progression.

6.4. DNA Abnormalities. Chromosomal abnormalities associated with Tax-expressing HTLV-1 infection range from deletions, duplications, translocations, and chromosomal rearrangements to aneuploidy [69, 135–140]. Although Tax has not been directly implicated in DNA damage [14, 141], it has been suggested to indirectly promote DNA aberrations through prevention of DNA repair mechanisms that would result in accumulation of mutations [142]. Like most multistage carcinogenic events, virus-induced carcinogenesis is also associated with multiple chromosome defects. A systematic eight-step genetic event sequence has been described by Okamoto et al. (1989) detailing the progression of an HTLV-1-infected cell into a state of cancer [143].

Suppression of antiapoptotic and antitumorigenic factors has been extensively associated with Tax expression in HTLV-1-infected cells. A downregulation of tumor suppressor gene p53 has been observed in 40% of ATL patients [144, 145]. Changes in expression levels of other tumor suppression factors like p15, p16, and Rb cell cycle regulators have also been observed in a number of ATL cases [146–148]. This indicates a strong relationship between suppression of DNA damage repair mechanisms by Tax and transformation of infected cells into leukemia. Some of this suppressive activity could probably be in association with the siRNA pathway that also interacts with chromosomes preventing DNA damage repair.

6.5. Histone Modifications. Eukaryotic histone proteins assemble across the entire length of DNA closely associating with it and packaging it into nucleosomal structures within the nucleus. Histone proteins undergo a variety of post-translational modifications (viz., acetylation, ubiquitylation, methylation, phosphorylation, and SUMOylation) in order to assist in processes such as DNA transcription, replication, and repair.

Tax is known to interact with cAMP response-element-binding protein (CREB) at the enhancer region of viral cAMP response element (CRE) that promotes recruitment of coactivators—CBP (CREB binding protein) and p300 [39]. *In vitro* studies indicate that these coactivators have histone acetylase activity that aids in HTLV-1 transcription [149]. CHIP analysis of the integrated HTLV-1 proviral site in infected SLB1 T cells revealed the recruitment of a number of factors namely, CREB, CREB-2, ATF-1, ATF-2, c-Fos, c-jun, p300, P/CAF, CBP, and Tax at the promoter region. The authors also observed histone H3 and H4 acetylation within the proviral genome of these cells. Interestingly, histone deacetylases (HDACs) were also found to localize at the promoter region and inhibition of the HDACs with specific antagonists-enhanced acetylation states of H4 and an increase in HTLV-1 RNA transcription (see Figure 3).

Lu et al. (2004) showed that the HDAC-1 directly associated with the inactive Tax and not with its transactivated form. Biotinylated chromatin pull down assays revealed that Tax promoted dissociation of HDAC-1 with the promoter region of HTLV-1 [150] suggesting modulation of HDAC recruitment by tax as a means of transcriptional regulation.

Tax promotes its own transactivation by associating with BRIG1 components of the ATP-dependent SWI/SNF chromatin remodeling complex downstream of Pol II [151]. Tax, therefore, appears to modulate the transcriptional state of chromatin by strongly associating with histone modifying proteins that effect alterations between euchromatin and heterochromatin during an HTLV-1 infection both *in vivo* and *in vitro*.

In addition to the oncoprotein, other HTLV-1 associated proteins can also be associated with the miRNA pathway and contribute to chromatin remodeling. Animal studies with mutated forms of accessory genes like p12, p30, Rex, p13, and HTLV-1 basic leucine zipper factor (HBZ) using HTLV-1 infectious clones revealed that these proteins are required to establish *in vivo* latency [152–155]. Rex protein appears to interact with Dicer preventing it from cleaving siRNAfrom shRNA and thus acting as a suppressor of RNA silencing [37]. Although there is a certain degree of sequence similarity between Rex and Tax proteins, it is not very clear if this confers a similar interaction property with host cellular proteins,

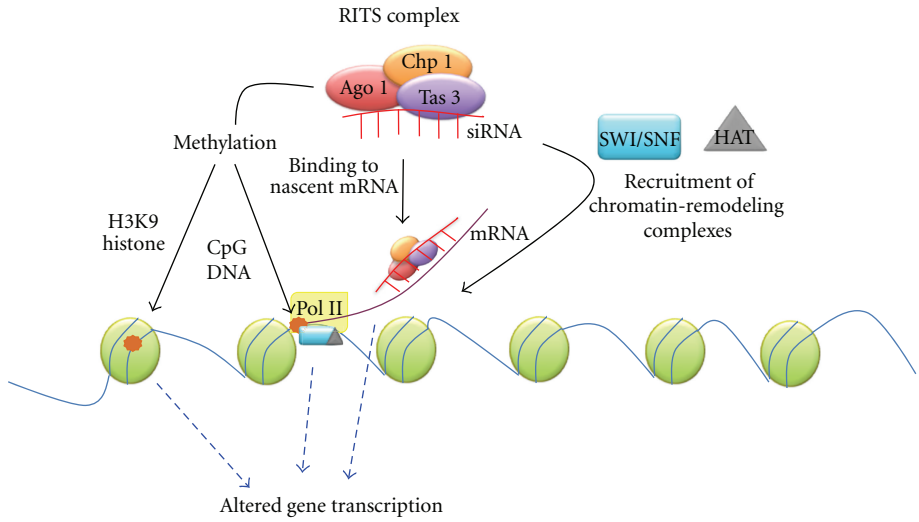

Figure 3: Model for the chromatin remodeling by siRNA/miRNA. siRNA in association with the RNA-induced transcriptional silencing complex can alter the rate of chromosomal transcription in a number of ways. It can directly bind nascent mRNA being transcribed from centromeric heterochromatin preventing its translation. The Ago 1/2 protein of the RITS complex can recruit transcription factors and chromatin-remodeling complexes like SWI/SNF that in turn cause assembly of histone modifying enzymes (viz., HATs, HDACs, etc.). The RITS complex can also recruit methylating enzymes that can either directly methylate CpG regions of centromeric DNA or H3K9 regions on histones associated with the chromosome.

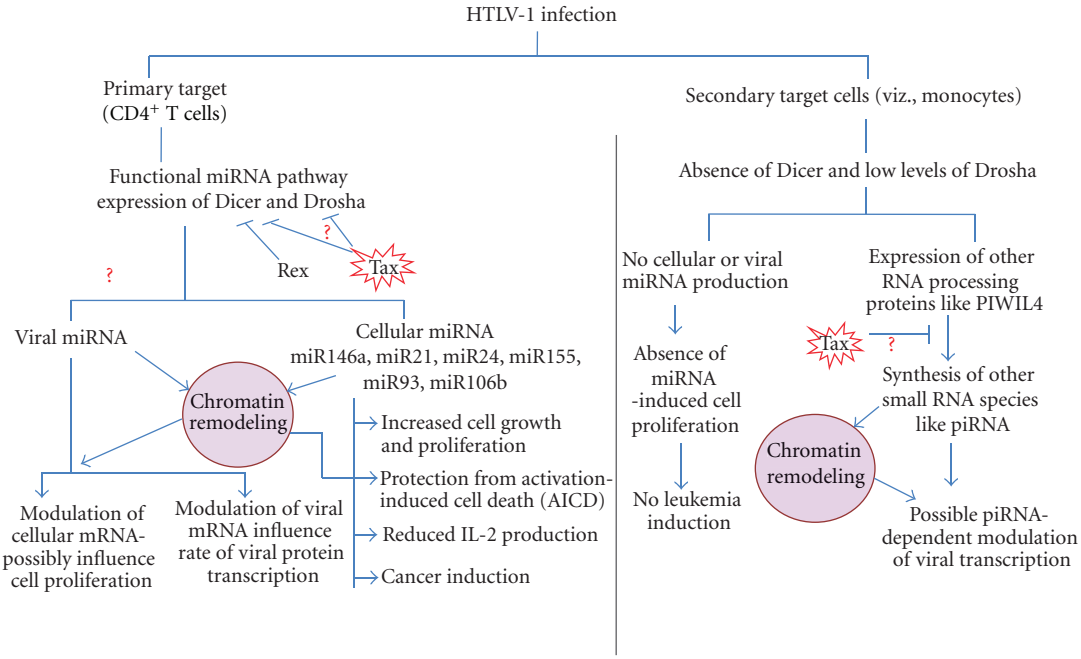

Figure 4: Differential miRNA expression and its impact on chromatin remodeling in primary and secondary target cells during HTLV-1 infection.

like those involved in miRNA processing [156, 157]. The p30 accessory protein can physically bind tax and Rex mRNAs and retain both transcripts in the nucleus, preventing their translation in the cytoplasm. This in turn may influence the rate at which viral transcription occurs and could cause a switch from active to dormant states of viral replication [158]. In addition, p30 also interacts with CREB-binding protein (CBP) and p300, altering rate of transcription of the long terminal repeat region of HTLV-1 as well as CBP and p300-dependent cellular genes [159].

7. Concluding Notes

miRNA-mediated transcriptional gene silencing is rapidly gaining significance as one of the major modes of chromatin remodeling and alteration of the rate of gene expression in

cells. From past and current literature, it is evident that viruses and their proteins actively and effectively utilize the miRNA machinery to alter cellular and viral gene expression, ultimately influencing the progression of disease. Chronic viruses that integrate their genome into that of the hosts' exploit small RNA pathways for establishing latency or increasing the rate of viral protein expression to ultimately transform cells and maximize survival.

Although protein families involved in siRNA-mediated epigenetic silencing pathways are relatively conserved between species and cell types, there exists a noticeable alteration in the level of these proteins. Also, the presence of alternate stages in pathways like RdRP-dependent siRNA amplification in *Saccharomyces cerevisiae* and alternate small RNA species like PIWI-associated RNA being predominantly active in some cell types like germline cells needs to be taken into account. As discussed in the paper, a huge variation exists in miRNA expression profiles of not only different stages of viral infection but also different cell types. The differential progression of viral infection in primary and nonprimary target cells has always been an intriguing aspect of HTLV-1 infection studies. The refractile nature of some cell types in contrast to the ability of others to support viral transcription has been attributed among other factors to the difference in miRNA processing proteins that make certain cell types less supportive of miRNA expression and hence of chromatin manipulation through the siRNA/miRNA-mediated pathways. Figure 4 represents a schematic outline of inherent differences in miRNA expression in primary and secondary target cells of HTLV-1 that could contribute to differing outcomes in both. Deciphering factors that dictate this cell type preference and specificity can go a long way in the advancement of HTLV-1 research and pave the way for developing novel and more effective therapeutic strategies.

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Review Article

Immunopathogenesis of Human T-Cell Leukemia Virus Type-1-Associated Myelopathy/Tropical Spastic Paraparesis: Recent Perspectives

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Human T-cell leukemia virus type-1 (HTLV-1) is a replication-competent human retrovirus associated with two distinct types of disease only in a minority of infected individuals: the malignancy known as adult T-cell leukemia (ATL) and a chronic inflammatory central nervous system disease HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP). HAM/TSP is a chronic progressive myelopathy characterized by spastic paraparesis, sphincter dysfunction, and mild sensory disturbance in the lower extremities. Although the factors that cause these different manifestations of HTLV-1 infection are not fully understood, accumulating evidence from host population genetics, viral genetics, DNA expression microarrays, and assays of lymphocyte function suggests that complex virus-host interactions and the host immune response play an important role in the pathogenesis of HAM/TSP. Especially, the efficiency of an individual's cytotoxic T-cell (CTL) response to HTLV-1 limits the HTLV-1 proviral load and the risk of HAM/TSP. This paper focuses on the recent advances in HAM/TSP research with the aim to identify the precise mechanisms of disease, in order to develop effective treatment and prevention.

1. Introduction

Human T-cell leukemia virus type-1 (HTLV-1) is a human retrovirus etiologically associated with adult T-cell leukemia (ATL) [1–3] and HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) [4, 5]. HAM/TSP is a chronic progressive myelopathy characterized by spastic paraparesis, sphincter dysfunction, and mild sensory disturbance in the lower extremities [6]. Cases of HAM/TSP have been reported throughout the HTLV-1 endemic areas such as Southern Japan, the Caribbean, Central and South America, the Middle East, Melanesia, and equatorial regions of Africa [7]. Sporadic cases have also been described in nonendemic areas such as the United States and Europe, mainly in immigrants from an HTLV-1 endemic area. In contrast to HIV-1 infection, few with HTLV-1 develop disease: approximately 2%-3% of infected persons develop ATL [8] and other 0.25%–3.8% develop HAM/TSP [9–12], while the majority of infected individuals remain lifelong asymptomatic carriers (ACs). However, the ability to evaluate the individual risk of HTLV-1-associated diseases in each AC would make a significant clinical impact, especially in HTLV-1 endemic areas. During the last three decades since the discovery of HTLV-1 as the first pathogenic human retrovirus, advances in HTLV-1 research have helped us to understand the clinical features of HTLV-1 associated diseases, the virological properties of HTLV-1, and the importance of the viral, host, and environmental risk factors as well as the host immune response against HTLV-1 infection. However, the precise mechanism of disease pathophysiology is still incompletely understood, and the treatment is still unsatisfactory, because good small-animal models for studying HTLV-1 infection and its associated diseases were unavailable until recently. In this paper, we summarize the recent developments of HTLV-1 research to try to identify more precisely the pathogenetic mechanisms

of the disease in order to develop effective treatment and prevention.

2. HTLV-1 Infection and Clinical Features of HAM/TSP

2.1. Virological Aspects of HTLV-1. HTLV-1 is classified as a complex retrovirus in the genus *Deltaretrovirus* of the subfamily *Orthoretrovirinae* and infects 10–20 million people worldwide [13–15]. HTLV-1 can be transmitted through sexual contact [16], injection drug use [15], and breastfeeding from mother to child [17, 18]. For over two decades, the investigation of HTLV-1-mediated pathogenesis has been focused on Tax, an HTLV-1 encoded viral oncoprotein, since Tax has been viewed as critical for leukemogenesis because of its pleiotropic effects on both viral and many cellular genes responsible for cell proliferation, genetic instability, dysregulation of the cell cycle, and apoptosis [19]. However, Tax expression is not detected in about 60% of freshly isolated samples from ATL cases [20]. In 2002, another regulatory protein encoded in the minus or antisense strand of the virus genome, named HTLV-1 basic leucine zipper factor (HBZ), was identified [21]. The spliced form of HBZ is expressed in all ATL [22] and HAM/TSP [23] cases, and its expression is strongly correlated with the HTLV-1 proviral load (PVL) in HTLV-1-infected individuals and with disease severity in HAM/TSP patients [23]. Also, HBZ protein promotes proliferation of ATL cells and induces T-cell lymphomas in $CD4^+$ T cells by transgenic expression, indicating the possible involvement of HBZ expression in the development of ATL [22, 24]. Moreover, among the HTLV-1 encoded viral genes, only the HBZ gene sequence remains intact, unaffected by nonsense mutations and deletion [25]. These findings indicate that HBZ expression is indispensable for proliferation and survival of ATL cells and HTLV-1 infected cells, and that Tax expression is not always necessary for the maintenance of ATL [26].

2.2. Clinical and Pathological Features of HAM/TSP. HAM/TSP is a chronic progressive myelopathy characterized by spastic paraparesis, sphincter dysfunction, and mild sensory disturbance in the lower extremities [6]. In addition to neurological symptoms, some HAM/TSP cases also exhibit autoimmune-like disorders, such as uveitis, arthritis, T-lymphocyte alveolitis, polymyositis, and Sjögren syndrome [14]. Among ACs, the lifetime risk of developing HAM/TSP, which is different among different ethnic groups, ranges between 0.25% and 4%. It has been reported that the annual incidence of HAM/TSP is higher among Jamaican subjects than among Japanese subjects (20 versus 3 cases/100,000 population), with a two to three times higher risk for women in both populations [9–12]. The period from initial HTLV-1 infection to the onset of HAM/TSP is assumed to range from months to decades, a shorter time than for ATL onset [11, 31]. HAM/TSP occurs both in vertically infected individuals and in those who become infected later in life (i.e., through sexual contact [almost exclusively from male to female], intravenous drug use, contaminated blood transfusions, etc.). The mean age at onset is 43.8 years, and the frequency of HAM/TSP is higher in women than in men (the male to female ratio of occurrence is 1 : 2.3) [11].

Pathological analysis of HAM/TSP autopsy materials indicates that the disease affects the spinal cord, predominantly at the thoracic level [27, 32, 33]. Loss of myelin and axons in the lateral, anterior, and posterior columns is associated with perivascular and parenchymal lymphocytic infiltration with the presence of foamy macrophages, proliferation of astrocytes, and fibrillary gliosis. In the cases with active-chronic lesions in the spinal cord, perivascular inflammatory infiltration with similar composition of cell subsets was also seen in the brain [28]. The peripheral nerve pathology of HAM/TSP patients with sensory disturbance showed varying degrees of demyelination, remyelination, axonal degeneration, regeneration, and perineurial fibrosis [29, 30]. The presence of atypical lymphocytes (so-called "flower cells") in peripheral blood and cerebrospinal fluid (CSF), a moderate pleocytosis, and raised protein content in CSF are typically found in HAM/TSP patients. Oligoclonal immunoglobulin bands in the CSF, raised concentrations of inflammatory markers such as neopterin, tumor necrosis factor (TNF)-α, interleukin (IL)-6 and interferon (IFN)-γ, and an increased intrathecal antibody (Ab) synthesis specific for HTLV-1 antigens have also been described [34]. Clinical progression of HAM/TSP is associated with an increase in the proviral load in individual patients, and a high ratio of proviral loads in CSF cells/peripheral blood mononuclear cells (PBMCs) is also significantly associated with clinically progressive disease [35]. The clinical and pathological characteristics of HAM/TSP described above are shown in Table 1.

3. Risk Factors for HAM/TSP

3.1. Host Genetic. A previous population association study of 202 cases of HAM/TSP and 243 ACs in Kagoshima prefecture, HTLV-1 endemic Southern Japan, revealed that one of the major risk factors is the HTLV-1 PVL. The median PVL was more than ten times higher in HAM/TSP patients than in ACs, and a high PVL was also associated with an increased risk of progression to disease [36, 37]. A higher PVL in HAM/TSP patients than in ACs was observed in other endemic areas such as the Caribbean [38], South America [39], and the Middle East [40]. It was suggested that genetic factors such as the human leukocyte antigen (HLA) genotype are related to the high PVL in HAM/TSP patients and genetic relatives. In Southern Japan, possession of the HLA-class I genes HLA-A*02 and Cw*08 was associated with a statistically significant reduction in both HTLV-1 PVL and the risk of HAM/TSP, whereas possession of HLA-class I HLA-B*5401 and class II HLA-DRB1*0101 predisposes to HAM/TSP in the same population (Table 2) [37, 41]. Since the function of class I HLA proteins is to present antigenic peptides to CTL, these results imply that individuals with HLA-A*02 or HLA-Cw*08 mount a particularly efficient CTL response against HTLV-1, which may therefore be an important determinant of HTLV-1 PVL and the risk of HAM/TSP. In fact, it has been reported that CTL spontaneously kills autologous HTLV-1-infected

Table 1: Clinical and pathological characteristics of HAM/TSP.

Clinical characteristics		References
Onset	Insidious, slowly progressive	[11]
Major clinical symptoms	Spastic paraparesis Sphincter dysfunction Mild sensory disturbance in the lower extremities	[11]
Complications	Uveitis Arthritis T-lymphocyte alveolitis Polymyositis Sjögren syndrome	[14]
Mean age at onset	43.8 years	[11]
Male-to-female ratio	1 : 2.3 (male : female)	[11]
Laboratory data	Positive anti-HTLV-1 antibody in both serum and CSF Moderate pleocytosis and raised protein content in CSF	[11]
Pathological characteristics		References
Spinal cord	Loss of myelin and axons in the lateral, anterior, and posterior columns-predominantly at the thoracic level Perivascular and parenchymal lymphocytic infiltration with the presence of foamy macrophages, proliferation of astrocytes, and fibrillary gliosis-predominantly at the thoracic level	[27]
Brain	Perivascular and parenchymal lymphocytic infiltration with the presence of foamy macrophages, proliferation of astrocytes, and fibrillary gliosis Perivascular inflammatory infiltration and fibrosis only in the cases with active-chronic lesions in the spinal cord. The composition of cell subsets was similar both in the spinal cord and in the brain	[28]
Peripheral nerve	Varying degrees of demyelination, remyelination, axonal degeneration, regeneration, and perineurial fibrosis	[29, 30]

cells *ex vivo* [42], granzymes and perforin are more highly expressed in individuals with a low PVL [43], and the lytic efficiency of the $CD8^+$ T cell response, that is, the fraction of autologous HTLV-1-expressing cells eliminated per $CD8^+$ T cell per day, was inversely correlated with both PVL and the rate of spontaneous proviral expression [44]. These findings indicate that the CTL against HTLV-1 reduces PVL and risk of HAM/TSP. Moreover, using a combination of computational and experimental approaches, MacNamara et al. recently reported that a CTL response against HBZ restricted by protective HLA alleles such as HLA-A*02 or Cw*08, but not a response to the immunodominant protein Tax, determines the outcome of HTLV-1 infection [45].

Analysis of non-HLA host genetic factors by candidate gene approaches revealed that non-HLA gene polymorphisms also affect the risk of developing HAM/TSP (Table 2). For example, the TNF-α promoter-863 A allele [47] and the longer CA repeat alleles of matrix metalloproteinase (MMP)-9 promoter [48] predisposed to HAM/TSP, whereas IL-10-592 A [49], stromal-derived factor (SDF)-1 +801A, and IL-15 +191 C alleles [47] conferred protection against HAM/TSP. The polymorphisms in the MMP-9 and IL-10 promoters were each associated with differences in the HTLV-1 Tax-mediated transcriptional activity of the respective gene [48, 49]. However, the contributions of these non-HLA genes to the pathogenesis of HAM/TSP are largely unknown, and these data have not yet been reproduced in different populations. Further candidate gene studies together with genome-wide association studies in different ethnic populations in larger sample size may provide evidence for the association of non-HLA genes with HAM/TSP pathogenesis.

3.2. HTLV-1 Genotype and Genomic Integration Site. Although most studies of HTLV-1 genotype have reported no association between variants of HTLV-1 and the risk of HAM/TSP, Furukawa et al. reported the association between HTLV-1 *tax* gene variation and the risk of HAM/TSP [46]. The *tax* subgroup A, which belongs to cosmopolitan subtype A, was more frequently observed in HAM/TSP patients, and this association was independent of the protective effect of the HLA allele HLA-A*02. HLA-A*02 appeared to give protection against only one of the two prevalent sequence variants of HTLV-1, *tax* subgroup B which belongs to cosmopolitan subtype B, but not against *tax* subgroup A in the Japanese population [46]. Interestingly, HLA-A*02 appeared not to give protection against infection with cosmopolitan subtype A in a population in Iran [40]. Moreover, the Iranian HTLV-1 strain has a Rex protein that is 20 amino acids longer than that of the Japanese strain that belongs to cosmopolitan subtype B. Experiments are now underway to compare the functions of these Rex proteins.

Table 2: Host genetic and viral factors associated with the risk of HAM/TSP.

Factor	Condition	Effect	Reference(s)
Viral factors	HTLV-1 *tax* subgroup A	Susceptible	[46]
	Proviral load	Susceptible	[36]
Host factors			
HLA	A*02	Protective	[37, 41]
	Cw*08	Protective	[41]
	B*5401	Susceptible	[41]
	DRB1*0101	Susceptible	[37]
Non-HLA	TNF-α promoter −863 A allele	Susceptible	[47]
	longer CA repeat alleles of MMP-9 promoter	Susceptible	[48]
	IL-10 promoter −592 A allele	Protective	[49]
	SDF-1 promoter +801 A allele	Protective	[47]
	IL-15 +191 C allele	Protective	[47]

Recently, to test whether the genomic integration site determines the abundance and the pathogenic potential of an HTLV-1-positive T-cell clone, Gillet et al. reported the results of high-throughput mapping and quantification of HTLV-1 proviral integration in the host genome [50]. They mapped >91,000 unique insertion sites (UISs) of the provirus from 61 HTLV-1-infected individuals in primary PBMCs and showed that a typical HTLV-1-infected host carries between 500 and 5000 UISs in 10 μg of PBMC genomic DNA. They calculated an oligoclonality index (OCI) to quantify the clonality of HTLV-1-infected cells *in vivo* and found that the OCI did not distinguish between ACs and patients with HAM/TSP and that there was no correlation between OCI and HTLV-1PVL in either ACs or HAM/TSP patients. These results indicate that the higher PVL observed in patients with HAM/TSP was attributable to a larger number of UISs but not, as previously thought, from a difference in clonality. They also obtained evidence that the abundance of established HTLV-1 clones is determined by genomic features of the host DNA flanking the provirus. Namely, HTLV-1 clonal expansion *in vivo* is favored by a proviral integration site near a region of host chromatin undergoing active transcription, or same-sense transcriptional orientation of the provirus. Negative selection of infected clones, probably by CTLs during chronic infection, favors establishment of proviruses integrated in transcriptionally silenced DNA, and this selection is more efficient in ACs than in HAM/TSP, indicating the selection of HTLV-1-infected T-cell clones with low pathogenic potential.

4. Immune Response to HTLV-1

4.1. Innate Immune Response

4.1.1. Natural Killer (NK) Cells . Previous reports indicated that patients with HAM/TSP had both a lower frequency and a lower activity of NK cells (especially the $CD3^+CD16^+$ subset) than ACs although the results were not normalized with respect to PVL [51]. Since an important mechanism of induction of NK cell-mediated killing is recognition by the NK cell of a complex of the nonpolymorphic MHC molecule HLA-E bound to a peptide derived from the signal sequence of some other MHC class I molecules, a synthetic tetramer of HLA-E with the HLA-G signal sequence peptide was used to identify NK cells in HAM/TSP patients [52]. The results showed a significantly lower frequency of HLA-E tetramer-binding cells in HAM/TSP patients than ACs, and as in the earlier studies [51], this reduction in frequency was particularly notable in the $CD3^+$ cells, whereas there was no significant difference in the frequency of HLA-E tetramer-binding $CD3^-$ cells between patients with HAM/TSP and ACs [52]. Recent data also suggest that the frequency of invariant NKT (iNKT) cells in the peripheral blood of HAM/TSP patients is significantly decreased when compared with healthy subjects and/or ACs [53, 54]. These findings indicate that the activity of the NK or NKT cell response was associated with the absence of HAM/TSP. Interestingly, a previous uncontrolled preliminary trial of treatment of HAM/TSP with fermented milk containing viable *Lactobacillus casei* strain Shirota resulted in a significant increase in NK cell activity, with improvements in clinical symptoms [55]. Thus, circulating NK and NKT cells might also play an important role in the disease progression and the pathogenesis of HAM/TSP. Recently, it has been reported that in addition to the previously described $CD8^+$ T-cell spontaneous proliferation [56], $CD56^+$ NK cells also spontaneously proliferated *in vitro*, and spontaneous NK cell proliferation positively correlated with HTLV-1 PVL but not with the presence of HAM/TSP [57]. A hallmark of HTLV-1 infection is the *in vitro* proliferation of PBMCs when cultured in the absence of exogenous antigen or mitogen, referred to as spontaneous lymphocyte proliferation (SLP), and in HAM/TSP patients, the levels of SLP reflect the severity of the disease [58, 59]. Most of the high SLP observed in PBMCs from HAM/TSP patients is likely to be explained by a greater spontaneous expression of the provirus and consequently a greater proliferation of responding $CD8^+$ T cells in culture [56]. The greater proviral expression may be partly attributable to the impaired function and decreased number of NK cells in HAM/TSP patients. Although further

studies are required to clarify the role of NK cells in HTLV-1 infection and HAM/TSP pathogenesis, NK cells might be also an interesting candidate for future immunotherapy.

4.1.2. Interferons. Type I interferon (IFN) is a key innate immune cytokine produced by cells in response to viral infection. The type I IFN response protects cells against invading viruses by inducing the expression of interferon-stimulated genes (ISGs), which execute the antiviral effects of IFN [60]. The ISGs then generate soluble factors including cytokines that activate adaptive immunity or directly inhibit the virus itself [61]. To date, IFN-α is not only one of the effective therapeutic agents for HAM/TSP, but also known as an only therapeutic agent whose efficacy was demonstrated in randomized placebo-controlled trials [62, 63]. However, the therapeutic benefit is small, and IFN-α is not in general use in the treatment of HAM/TSP. The combination of the antiretroviral agent zidovudine (AZT) and IFN-α is also beneficial for overall survival in smoldering and chronic (i.e. indolent) ATL [64] although its efficacy has not yet been confirmed in well-designed prospective studies. It might be interesting to analyse which ISGs are changed in the course of IFN-α treatment and the functional role of ISGs as potential targets for therapy. In PBMCs of HTLV-1-infected individuals, the level of HTLV-1 mRNA is very low, and viral protein is not detectable, but these molecules are rapidly expressed after a short time in culture *in vitro* [42]. However, the mechanisms of this phenomenon are largely unknown. Recently, it has been reported that HTLV-1 expression in HTLV-1-infected T-cells is suppressed by stromal cells, that is epithelial cells and fibroblasts, in culture through type I IFNs [65]. Namely, HTLV-1 Gag protein expression was suppressed when contacted with stromal cells and restored when separated from the stromal cells. Although neutralizing antibodies against human IFN-α/β receptor only partly abrogated this phenomenon, the results indicate that the innate immune system suppresses HTLV-1 expression *in vitro* and *in vivo*, at least through type I IFN.

4.2. Antibody Response to HTLV-1. In 2002, it was reported that antibodies that recognize HTLV-1 Tax protein can cross-react with a heterogenous-nuclear-riboprotein (hnRNP-) A1, suggesting intriguing evidence for antigen mimicry in HTLV-1 infection [66]. However, subsequent analysis using Japanese samples under fully masked conditions indicated that there was no difference in the incidence of anti-hnRNP A1 Abs between HAM/TSP and other neurological diseases [67]. It is unlikely that anti-Tax Ab explains the onset or initial tissue damage of HAM/TSP, as the host protein hnRNP-A1 is not confined to the central nervous system but is widely expressed [68] and is not normally accessible to Ab attack. Anti-Tax Ab might be associated with subsequent inflammation following initial tissue damage and disruption of blood brain barrier, which is probably caused by the antiviral immune responses to HTLV-1 and induces the release of autoantigens.

In HTLV-1 infection, HAM/TSP patients generally have a higher anti-HTLV-1 Ab titer than ACs with a similar HTLV-1 proviral load [69–71]. These anti-HTLV-1 Abs often include IgM in both ACs and patients with HAM/TSP [70, 71]. These findings suggest that there was persistent expression of HTLV-1 proteins *in vivo* and the existence of an augmented humoral immune response to HTLV-1 in HAM/TSP patients. Although Ab responses to the immunodominant epitopes of the HTLV-1 envelope (Env) proteins were similar in all of three clinical groups (HAM/TSP, ATL, and ACs), reactivity to four Tax immunodominant epitopes was higher in HAM/TSP patients (71%–93%) than in ATL patients (4%–31%) or ACs (27%–37%) [72]. Among these anti-HTLV-1 antibodies, anti-EnvAb is particularly important since some anti-Env Abs have neutralizing activity against HTLV-1. Antisera raised against recombinant HTLV-1 Env polypeptides [73, 74], vaccinia virus containing HTLV-1 env gene [75, 76], immunization with neutralizing epitope peptides [77], and passive transfer of human IgG that has neutralizing activity [78, 79] were all shown to neutralize HTLV-1 infectivity. In HTLV-1 infection, the roles of HTLV-1 neutralizing Ab *in vivo* are still largely unknown. It will be interesting to examine whether HTLV-1 neutralizing Ab titres correlate with disease status and PVL in infected individuals. Since the mutation rate of HTLV-1 provirus is significantly lower than HIV-1, passive immunization with human monoclonal Ab may be beneficial and effective method to prevent HTLV-1 infection.

4.3. Cytotoxic T-Lymphocyte (CTL) Response to HTLV-1. Previous reports indicated that the HTLV-1-specific $CD8^+$ CTLs are typically abundant, chronically activated, and mainly targeted to the viral trans activator protein Tax [80]. Also, as already mentioned, the median PVL in PBMCs of HAM/TSP patients was more than ten times higher than that in ACs, and a high PVL was also associated with an increased risk of progression to disease [36, 37]. Furthermore, HLA-A*02 and HLA-Cw*08 genes were independently and significantly associated with a lower PVL and a lower risk of HAM/TSP [37, 41], and $CD8^+$ T cells efficiently kill autologous Tax-expressing lymphocytes in fresh PBMCs in HTLV-1-infected individuals [42]. These data have raised the hypothesis that the class I-restricted $CD8^+$ CTL response plays a critical part in limiting HTLV-1 replication *in vivo* and that genetically determined differences in the efficiency of the CTL response to HTLV-1 account for the risk for developing HAM/TSP. Indeed, as mentioned above (Section 3.1), MacNamara et al. [45] have shown that HLA class 1 alleles which strongly bind oligopeptides from the HBZ protein enable the host to make a more effective immune response against HTLV-1; therefore, such individuals have a lower PVL and are more likely to be asymptomatic. Moreover, another recent report showed the presence of HBZ-specific $CD4^+$ and $CD8^+$ cells *in vivo* in patients with HAM/TSP and in ACs and a significant association between the HBZ-specific $CD8^+$ cell response and asymptomatic HTLV-1 infection [81]. These findings provide strong evidence to support the hypothesis of the crucial role of CTLs and also confirm the importance of HBZ for persistent infection.

Since the frequency of HTLV-1-specific $CD8^+$ T cells was significantly higher in HAM/TSP patients than ACs [82, 83], and these cells have the potential to produce

proinflammatory cytokines [84], there is a debate on the role of HTLV-1-specific-CD8$^+$ T cells, that is, whether these cells contribute to the inflammatory and demyelinating processes of HAM/TSP, or whether the dominant effect of such cells *in vivo* is protective against disease. The analysis of gene expression profiles using microarrays in circulating CD4$^+$ and CD8$^+$ lymphocytes indicated that granzymes and perforin are more highly expressed in individuals with a low PVL [43], suggesting that a strong CTL response is associated with a low PVL and a low risk of HAM/TSP. Indeed, the lytic capacity of HTLV-1-specific CTL in patients with HAM/TSP and ACs, quantified by a CD107a mobilization assay, showed significantly lower CD107a staining in HTLV-1-specific CTL in HAM/TSP than ACs [85]. Recently, it has been reported that the high CTL avidity, which is closely associated with the lytic efficiency of CTL, correlates with low PVL and proviral gene expression [44], indicating that the efficient control of HTLV-1 *in vivo* depends on the quality of CTL, which determines the position of virus-host equilibrium and also the outcome of persistent HTLV-1 infection. However, two caveats must be made here. First, a protective role and a pathogenic role of CTLs are not mutually exclusive. Indeed, there are other examples of viral infections in which the virus-specific CTLs exert both beneficial (antiviral) and detrimental (inflammatory) effects, such as lymphocytic choriomeningitis virus (LCMV) infection in the mouse [86]. Second, it is difficult to separate cause and effect in analyzing the association between T-cell attributes and the efficiency of viral control in a persistent infection at equilibrium.

4.4. CD4$^+$ Helper T-Cell Response to HTLV-1. Antiviral CD4$^+$ T-cell responses are of central importance in driving B-cell and CD8$^+$ T-cell responses *in vivo*. The most common HTLV-1 antigen recognized by CD4$^+$ T-cells is the Env protein [87, 88], in contrast with the immunodominance of Tax in the CD8$^+$ T-cell response [89–91]. At a similar PVL, patients with HAM/TSP had significantly increased frequency of virus-specific CD4$^+$ T cells compared to ACs [88, 92]. The antiviral T-helper (Th)1 phenotype is also dominant among HTLV-1-specific CD4$^+$ T cells in both ACs and patients with HAM/TSP [93], and there is a higher frequency of IFN-γ, TNF-α, and IL-2 production by CD4$^+$ T cells in patients with HAM/TSP compared to AC of a similar PVL [93, 94]. A role for CD4$^+$ T cells in initiating and causing HAM/TSP is also consistent with the immunogenetic observations that the possession of HLA-DRB1*0101, which restricts the immunodominant epitope of HTLV-1 Env gp21, was associated with susceptibility to HAM/TSP in independent HTLV-1-infected populations in Southern Japan [37, 41] and Northeastern Iran [40]. Accordingly, a synthetic tetramer of DRB1*0101 and the immunodominant HTLV-1 Env380-394 peptide was used to analyze Env-specific CD4$^+$ T cells directly *ex vivo* [92]. The results showed that the frequency of tetramer$^+$CD4$^+$ T cells was significantly higher in HAM/TSP patients than ACs with similar PVL. Furthermore, direct ex vivo analysis of tetramer$^+$CD4$^+$ T cells from two unrelated DRB1*0101-positive HAM/TSP patients indicated that certain T-cell receptors (TCRs) Vβs were utilized and antigen-specific amino acid motifs were identified in complementarity determining region (CDR) 3 from both patients. These results suggest that the observed increase in virus-specific CD4$^+$ T cells in HAM/TSP patients, which may contribute to CD4$^+$ T cell-mediated antiviral immune responses and to an increased risk of HAM/TSP, was not simply due to the rapidly growing HTLV-1-infected CD4$^+$ T cells but was the result of *in vivo* selection by specific MHC-peptide complexes, as observed in freshly isolated HLA-A*0201/Tax11-19 tetramer$^+$CD8$^+$ T cells [95] and muscle-infiltrating cells from HAM/TSP patients and HTLV-1-infected polymyositis patients [96].

4.5. Regulatory T Cells (Tregs) in HTLV-1 Infection. Regulatory T cells (Tregs) are important mediators of peripheral immune tolerance and also play an important role in chronic viral infections. In HTLV-1 infection, it has been reported that HTLV-1 preferentially and persistently infects CD4$^+$CD25$^+$ lymphocytes *in vivo* [97], which contain the majority of the Foxp3$^+$ Tregs [98]. In HAM/TSP patients, the frequency of Foxp3$^+$ expression in CD4$^+$CD25$^+$ cells is lower than that in ACs and uninfected healthy controls [97, 99]. This is probably due to the fact that CD25 is transcriptionally induced by HTLV-1 Tax [100], which may result in the reduced proportion of Foxp3$^+$ cells in the CD4$^+$CD25$^+$ population in HTLV-1-infected individuals, especially HAM/TSP patients. It is important to note that the CD4$^+$CD25$^+$ population contains a mixture of Tregs and activated non-Tregs. Therefore, it is inappropriate to use CD25 as a marker of Tregs in HTLV-1 infection: the best current working definition of Treg phenotype is CD4$^+$Foxp3$^+$. Reports from different geographic regions indicate that the percentage of CD4$^+$Foxp3$^+$ cells is higher in the HAM/TSP patients than in ACs [101–103]. It has been reported that the high frequency of CD4$^+$Foxp3$^+$T cells in HTLV-1-infected individuals is maintained by CCL22 produced by HTLV-1-infected PBMCs [104]. The frequency of HTLV-1-negative CD4$^+$Foxp3$^+$ cells was positively correlated with the HTLV-1 proviral load [102, 105], and the CTL activity was negatively correlated with the frequency of HTLV-1-negative CD4$^+$Foxp3$^+$ cells [102], suggesting that CD4$^+$Foxp3$^+$ Tregs may impair the CTL surveillance of HTLV-1. If this is the case, activity of CD4$^+$Foxp3$^+$ cells may also determine the risk of developing HAM/TSP via increasing the HTLV-1 PVL.

4.6. Dendritic Cells (DCs). Dendritic cells are antigen-presenting cells which play a critical role in the regulation of the adaptive immune response. In HTLV-1 infection, it has been shown that the DCs from HAM/TSP patients were infected with HTLV-1 [106], and the development of HAM/TSP is associated with rapid maturation of DCs [107]. As already mentioned, one of the hallmarks of HTLV-1 infection is the spontaneous lymphocyte proliferation (SLP). Interestingly, depletion of DCs from the HAM/TSP patient's PBMCs abolished SLP, whereas supplementing DCs restores proliferation [106]; supplementing B cells or macrophages had no effect. A DC-dependent mechanism of SLP was further supported by data showing that antibodies to MHC

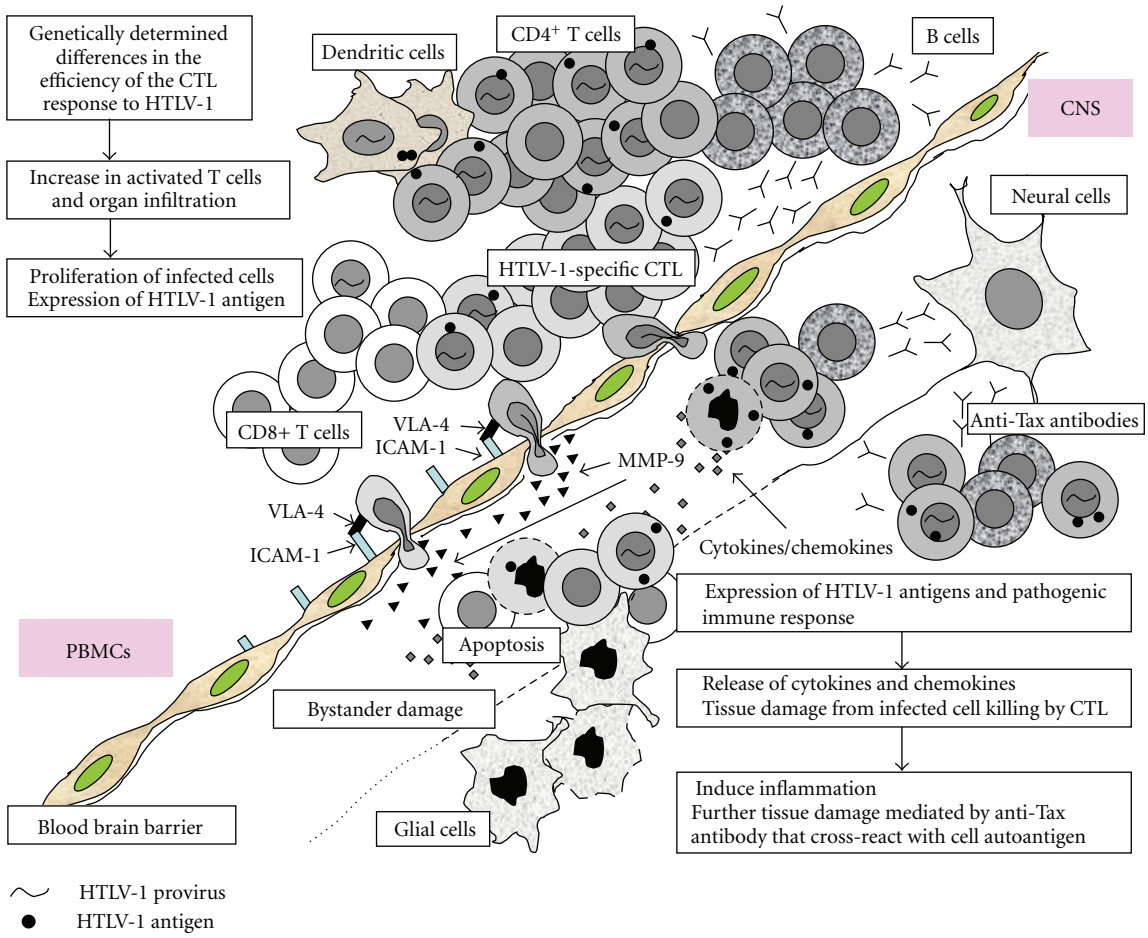

Figure 1: Hypothesis for the pathogenesis of human T-cell leukemia virus type-1 (HTLV-1) -associated myelopathy/tropical spastic paraparesis (HAM/TSP). Accumulating evidence suggests that the virus-host immunologic interactions play a pivotal role in HAM/TSP pathogenesis. Genetically determined less efficient CTL response against HTLV-1 may cause higher proviral load and antigen expression in infected individuals, which lead to activation and expansion of antigen-specific T-cell responses, subsequent induction of large amounts of proinflammatory cytokines and chemokines, and progression of HAM/TSP development. It is also possible that the immunoglobulin G specific to HTLV-1-Tax, which cross-react with heterogeneous nuclear ribonuclear protein-A1 (hnRNP-A1), is associated with subsequent inflammation following initial tissue damage.

class II, CD86, and CD58 can block SLP [108]. Recently, it has been demonstrated that both myeloid and plasmacytoid DCs are susceptible to infection with cell-free HTLV-1, and HTLV-1-infected DCs can rapidly transfer virus to autologous primary $CD4^+$ T cells [109]. In addition, other groups have obtained evidence that HTLV-1 transmission from DCs to T cells was mediated primarily by DC-SIGN [110], and DCs play a major part in generating and maintaining the Tax-specific $CD8^+$ T cells both *in vitro* and *in vivo* [111]. Moreover, using transgenic mouse models that permit conditional transient depletion of $CD11c^+$ DCs, and a chimeric HTLV-1 that carries the envelope gene from Moloney murine leukemia virus, Rahman et al. demonstrated the critical role of DCs in their ability to mount both innate and adaptive immune responses during early cell-free HTLV-1 infection [112, 113]. Since HTLV-1 can impair the differentiation of monocytes into DCs [114], the interaction of DCs with HTLV-1 plays a central part in the persistence and pathogenesis of HTLV-1.

5. Concluding Remarks

As shown in Figure 1, accumulating evidence suggests that the host immune response, especially the CTL response, plays a critical role in determining the risk of HAM/TSP. A less efficient CTL response against HTLV-1 may cause a higher PVL and higher antigen expression in infected individuals, which in turn lead to activation and expansion of antigen-specific T-cell responses, subsequent induction of large amounts of proinflammatory cytokines and chemokines, and progression to HAM/TSP. Since HLA class 1 genotype determines only up to 50% of HAM/TSP risk in infected people [41], it is important to discover other factors that determine the efficiency of the CTL response

to HTLV-1 and the outcome of HTLV-1 infection. Studies of the HTLV-1 receptor and DCs are also critical in the development of vaccine approaches to elicit cellular immune responses to key viral proteins such as Tax and Env to ablate HTLV-1-infected T cells. Newer approaches using genetically engineered and/or humanized mouse models for HTLV-1 infection will help to develop effective treatment and prevention of HAM/TSP in the future.

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Review Article

Clinical Trials and Treatment of ATL

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ATL is a distinct peripheral T-lymphocytic malignancy associated with human T-cell lymphotropic virus type I (HTLV-1). The diversity in clinical features and prognosis of patients with this disease has led to its subtype-classification into four categories, acute, lymphoma, chronic, and smoldering types, defined by organ involvement, and LDH and calcium values. In case of acute, lymphoma, or unfavorable chronic subtypes (aggressive ATL), intensive chemotherapy like the LSG15 regimen (VCAP-AMP-VECP) is usually recommended if outside of clinical trials, based on the results of a phase 3 trial. In case of favorable chronic or smoldering ATL (indolent ATL), watchful waiting until disease progression has been recommended, although the long-term prognosis was inferior to those of, for instance, chronic lymphoid leukemia. Retrospective analysis suggested that the combination of interferon alpha and zidovudine was apparently promising for the treatment of ATL, especially for types with leukemic manifestation. Allogeneic hematopoietic stem cell transplantation (allo-HSCT) is also promising for the treatment of aggressive ATL possibly reflecting graft versus ATL effect. Several new agent trials for ATL are ongoing and in preparation, including a defucosylated humanized anti-CC chemokine receptor 4 monoclonal antibody, IL2-fused with diphtheria toxin, histone deacetylase inhibitors, a purine nucleoside phosphorylase inhibitor, a proteasome inhibitor, and lenalidomide.

1. Introduction

Adult T-cell leukemia-lymphoma (ATL) was first described in 1977 by Uchiyama et al. as a distinct clinico-pathological entity with a suspected viral etiology because of the clustering of the disease in the southwest region of Japan [1]. Subsequently, a novel RNA retrovirus, human T-cell leukemia/lymphotropic virus type I (HTLV-1), was isolated from a cell line established from leukemic cells of an ATL patient, and the finding of a clear association with ATL led to its inclusion among human carcinogenic pathogens [2–5]. In the mid-1980s and 1990s, several inflammatory diseases were reported to be associated with HTLV-1 [6–10]. At the same time, endemic areas for the virus and diseases have been found (reviewed in [11–13]). Diversity in ATL has been recognized and a classification of clinical subtypes of the disease was proposed [14]. This chapter will review the current recognition of ATL focusing on treatment of the disease.

2. Clinical Features and Laboratory Findings of ATL

ATL patients show a variety of clinical manifestations because of various complications of organ involvement by ATL cells, opportunistic infections and/or hypercalcemia [11–14]. These three often contribute to the extremely high mortality of the disease. Lymph node, liver, spleen, and skin lesions are frequently observed. Though less frequently, digestive tract, lungs, central nervous system, bone, and/or other organs may be involved. Large nodules, plaques, ulcers, and erythroderma are common skin lesions [15–17]. Immune suppression is common. Approximately 26% of 854 patients with ATL had active infections at diagnosis in a prior

nationwide study in Japan [14]. The incidence was highest in the chronic and smoldering types (36%) and lower in the acute (27%) and lymphoma types (11%). The infections were bacterial in 43%, fungal in 31%, protozoal in 18%, and viral in 8% of patients. The immunodeficiency at presentation in ATL patients can be exacerbated by cytotoxic chemotherapy. Individuals with indolent ATL might have no manifestation of the disease and are identified only by health checkups and laboratory examinations.

ATL cells are usually detected quite easily in the blood of affected individuals except for the smoldering type with mainly skin manifestations and lymphoma type [14]. These so-called "flower cells" have highly indented or lobulated nuclei with condensed chromatin, small or absent nucleoli, and a agranular and basophilic cytoplasm [18]. The histological analysis of aberrant cutaneous lesions or lymph nodes is essential for the diagnosis of the smoldering type with mainly skin manifestations and lymphoma type of ATL, respectively. Because ATL cells in the skin and lymph node can vary in size from small to large and in form from pleomorphic to anaplastic and Hodgkin-like cell with no specific histological pattern of involvement, differentiating between Sezary syndrome, other peripheral T-cell lymphomas and Hodgkin lymphoma versus ATL can at times be difficult without examinations for HTLV-1 serotype/genotype [13, 19].

Hypercalcemia is the most distinctive laboratory abnormality in ATL as compared to other lymphoid malignancies and is observed in 31% of patients (50% in acute type, 17% in lymphoma type, and 0% in the other two types) at onset [14]. Individuals with hypercalcemia do not usually have osteolytic bone lesions. Parathyroid hormone-related protein or receptor activator of nuclear factor kappa B ligand (RANKL) produced by ATL cells is considered the main factor causing hypercalcemia [20, 21].

Similar to serum LDH, β2-microglobulin, and serum thymidine kinase levels reflecting disease bulk/activity, the level of the soluble form of interleukin (IL)-2 receptor alpha-chain is elevated in the order of acute/lymphoma-type ATL, smoldering/chronic-type ATL, and HTLV-1 carriers as compared with normal individuals, perhaps with better accuracy than the other markers [22–24]. These serum markers are useful for detecting the acute transformation of indolent ATL as well as the early relapse of ATL after achieving responses by therapy.

Prototypical ATL cells have a mature alpha-beta T-cell phenotype, that is, they are terminal deoxynucleotidyl transferase- (TdT-)negative, cluster of differentiation (CD) 1a-negative, T-cell receptor alpha-beta positive, CD2-positive and CD5, CD45RO, and CD29-positive, and frequently do not express CD7 and CD26. A decline in the CD3 level with the appearance of CD25 indicates that the ATL cells are in an activated state. Most ATL cells are CD52-positive but some are negative, and this may correlate with the coexpression of CD30. About 90% of cases are CD4-positive and CD8-negative, and in rare cases either coexpress CD4 and CD8, are negative for both markers, or are only CD8-positive [25]. CC chemokine receptor 4 (CCR4) is expressed in more than 90% of cases and associated with a poor prognosis. Recent studies have suggested that the cells of some ATL may be the equivalent of regulatory T-cells because of the high frequency of expression of CD25/CCR4 and about half of FoxP3 [26–28].

3. Diagnosis of ATL

The diagnosis of typical ATL is not difficult and is based on clinical features, ATL cell morphology, mature helper-T-cell phenotype, and anti-HTLV-1 antibody in most cases [13]. Those rare cases, which might be difficult to diagnose, can be shown to have the monoclonal integration of HTLV-1 proviral DNA in the malignant cells as determined by Southern blotting. However, the monoclonal integration of HTLV-1 is also detected in some HAM/TSP patients and HTLV-1 carriers [29, 30]. After the diagnosis of ATL, subtype classification of the disease is necessary for the selection of appropriate treatment [14, 31].

4. Definition, Prognostic Factors, and Subtype Classification of ATL

ATL is a distinct peripheral T-lymphocytic malignancy associated with a retrovirus designated human T-cell leukemia virus type I or human T-cell lymphotropic virus type I (HTLV-1) [1, 11–14, 31].

Major prognostic indicators for ATL, which have been elucidated in 854 patients with ATL in Japan, the Lymphoma Study Group (LSG) of the Japan Clinical Oncology Group (JCOG) by multivariate analysis, were advanced performance status (PS), high lactic dehydrogenase (LDH) level, age of 40 years or more, more than 3 involved lesions, and hypercalcemia [32]. Also a classification of clinical subtypes into acute, lymphoma, chronic, and smoldering types was proposed based on prognostic factors and clinical features of the disease [14]. The leukemic subtypes include all of the chronic type and most of the acute and smoldering types. The acute type has a rapid course with leukemic manifestation ($\geq$ 2% ATL cells) mostly, with or without lymphocytosis ($> 4 \times 109$/L) including ATL cells and most of the characteristic features of ATL-generalized lymphadenopathy, hepatosplenomegaly, skin involvement, other organ involvement, a high LDH value, and hypercalcemia. The symptoms and signs include abdominal pain, diarrhea, ascites, jaundice, unconsciousness, dyspnea, pleural effusion, cough, sputum, and chest X-ray abnormalities because of organ involvement, hypercalcemia, and/or opportunistic infections. The smoldering type shows an indolent course and 5% or more of leukemic cells in the peripheral blood without lymphocytosis but may include skin/lung involvement. The calcium level is less than the upper limit, and LDH level is less than 1.5 times the upper limit in smoldering ATL. The chronic type, with absolute lymphocytosis (4×109/L) less frequently showing flower cell morphology than the acute type, is frequently and occasionally associated with skin involvement and lymphadenopathy, respectively, and also usually shows a relatively indolent course. The calcium level is less than the upper limit, and the LDH level is less than double the upper limit of the chronic type. The lymphoma type presents with

the manifestations of a nodal-lymphoma without leukemic cells, frequently with high LDH/Ca levels, a rapid course, and symptoms and signs similar to the acute type. In case of ATL, clinical subtype is more important than Ann Arbor stage for predicting prognosis and deciding treatment because of frequent leukemic manifestation defined as stage IV.

Additional factors associated with a poor prognosis include thrombocytopenia, eosinophilia, bone marrow involvement, a high interleukin (IL)-5 serum-level, C-C chemokine receptor 4 (CCR4) expression, lung resistance-related protein (LRP), p53 mutation, and p16 deletion by multivariate analysis [26, 27, 33–37]. Specific for the chronic type of ATL, high LDH, high blood urea nitrogen (BUN), and low albumin levels were identified as factors for a poor prognosis by multivariate analysis [11]. Primary cutaneous tumoral type although generally included among smoldering ATL had a poor prognosis in univariate analysis [15].

5. Clinical Course, Treatment, and Response Criteria of ATL

Treatment decisions should be based on the ATL subtype-classification and the prognostic factors at onset including those related with ATL and comorbidity [31]. As mentioned above, subtype-classification of this disease has been proposed based on the prognosis and clinical manifestations. Without treatment, most patients with acute-/lymphoma/type ATL die of the disease or infections within weeks or months. More than half of patients with smoldering ATL survive for more than 5 years without chemotherapy and transformation to aggressive ATL. Chronic ATL has the most diverse prognosis among the subtypes and could be divided into favorable and unfavorable by clinical parameters (serum albumin, BUN, and LDH levels) after a multivariate analysis [31].

Current treatment options for ATL include watchful waiting until the disease progresses, interferon alpha (IFN) and zidovudine (AZT) therapy, multiagent chemotherapy, allogeneic hematopoietic stem cell transplantation (allo-HSCT), and a new agent [15].

5.1. Watchful Waiting. At present, no standard treatment for ATL exists. Therefore, patients with the smoldering or favorable chronic type, who may survive one or more years without chemotherapy, excluding topical therapy for cutaneous lesions, should be observed and therapy should be delayed until progression of the disease [31]. However, it was recently found that the long-term prognosis of such patients was poorer than expected. In a long-term followup study for 78 patients with indolent ATL (favorable chronic- or smoldering-type) with a policy of watchful waiting until disease progression at a single institution, the median survival time was 5.3 years with no plateau in the survival curve. Twelve patients remained alive for > 10 years, 32 progressed to acute ATL, and 51 died [38]. Recently, the striking benefit of early intervention to indolent ATL by IFN and an antiretroviral agent was reported by a meta-analysis [39]. This modality should be extensively evaluated by larger clinical trials to establish appropriate management practices for indolent ATL.

5.2. Chemotherapy. Since 1978, chemotherapy trials have been consecutively conducted for patients newly diagnosed with ATL by JCOG's Lymphoma Study Group (LSG) (Table 1) [40–45]. Between 1981 and 1983, JCOG conducted a phase III trial (JCOG8101) to evaluate LSG1-VEPA (vincristine, cyclophosphamide, prednisone, and doxorubicin) versus LSG2-VEPA-M (VEPA plus methotrexate (MTX)) for advanced non-Hodgkin lymphoma (NHL), including ATL [40, 41]. The complete response (CR) rate of LSG2-VEPA-M for ATL (37%) was higher than that of LSG1-VEPA (17%; $P = .09$). However, the CR rate was significantly lower for ATL than for B-cell NHL and peripheral T-cell lymphoma (PTCL) other than ATL ($P < .001$). The median survival time of the 54 patients with ATL was 6 months, and the estimated 4-year survival rate was 8%.

In 1987, JCOG initiated a multicenter phase II study (JCOG8701) of a multiagent combination chemotherapy (LSG4) for advanced aggressive NHL (including ATL). LSG4 consisted of three regimens: (1) VEPA-B (VEPA plus bleomycin), (2) M-FEPA (methotrexate, vindesine, cyclophosphamide, prednisone, and doxorubicin), and (3) VEPP-B, (vincristine, etoposide, procarbazine, prednisone, and bleomycin) [42]. The CR rate for ATL patients was improved from 28% (JCOG8101) to 43% (JCOG8701); however, the CR rate was significantly lower in ATL than in B-cell NHL and PTCL ($P < .01$). Patients with ATL still showed a poor prognosis, with a median survival time of 8 months and a 4-year survival rate of 12%.

The disappointing results with conventional chemotherapies have led to a search for new active agents. Multicenter phase I and II studies of pentostatin (2′-deoxycoformycin, an inhibitor of adenosine deaminase) were conducted against ATL in Japan [43]. The phase II study revealed a response rate of 32% (10 of 31) in cases of relapsed or refractory ATL (2CRs and 8PRs).

These encouraging results prompted the investigators to conduct a phase II trial (JCOG9109) with a pentostatin-containing combination (LSG11) as the initial chemotherapy [44]. Patients with aggressive ATL—that is, of the acute, lymphoma, or unfavorable chronic type—were eligible for this study. Unfavorable chronic-type ATL, defined as having at least 1 of 3 unfavorable prognostic factors (low serum albumin level, high LDH level, or high BUN), has an unfavorable prognosis similar to that for acute- and lymphoma-type ATL. A total of 62 untreated patients with aggressive ATL (34 acute, 21 lymphoma, and 7 unfavorable chronic type) were enrolled. A regimen of 1 mg/m^2 vincristine on days 1 and 8, 40 mg/m^2 doxorubicin on day 1, 100 mg/m^2 etoposide on days 1 through 3, 40 mg/m^2 prednisolone (PSL) on days 1 and 2, and 5 mg/m^2 pentostatin on days 8, 15, and 22 was administered every 28 days for 10 cycles. Among the 61 patients evaluable for toxicity, four patients (7%) died of infections, two from septicemia, and two from cytomegalovirus pneumonia. Among the 60 eligible patients, there were 17CRs (28%) and 14 partial responses (PRs) (overall

Table 1: Results of sequential chemotherapeutic-trials of untreated patients with ATL (JCOG-LSG).

	J7801	J8101	J8701	J9109	J9303	JCOG9801	
	LSG1	LSG1/LSG2	LSG4	LSG11	LSG15	mLSG15/mLSG19	
Pts. No.	18	54	43	62	96	57	61
CR (%)	16.7	27.8	41.9	28.3	35.5	40.4	24.6
CR + PR (%)				51.6	80.6	72.0	65.6
MST (months)		7.5	8.0	7.4	13.0	12.7	10.9
2 yr. survival (%)				17.0	31.3		
3 yr. survival (%)				10.0	21.9	23.6	12.7
4 yr. survival (%)		8.0	11.6				

CR: complete remission, PR: partial remission, MST: median survival time.

response rate [ORR] = 52%). The median survival time was 7.4 months, and the estimated 2-year survival rate was 17%. The prognosis in patients with ATL remained poor, even though they were treated with a pentostatin-containing combination chemotherapy.

In 1994, JCOG initiated a phase II trial (JCOG9303) of an eight-drug regimen (LSG15) consisting of vincristine, cyclophosphamide, doxorubicin, prednisone, ranimustine, vindesine, etoposide, and carboplatin for untreated ATL [45]. Dose intensification was attempted with the prophylactic use of granulocyte colony-stimulating factor (G-CSF). In addition, non-cross-resistant agents, such as ranimustine and carboplatin, and intrathecal prophylaxis with MTX and PSL were incorporated. Ninety-six previously untreated patients with aggressive ATL were enrolled: 58 acute, 28 lymphoma, and 10 unfavorable chronic types. Approximately 81% of the 93 eligible patients responded (75/93), with 33 patients obtaining a CR (35%). The overall survival rate of the 93 patients at 2 years was estimated to be 31%, with a median survival time of 13 months. Grade 4 neutropenia and thrombocytopenia were observed in 65% and 53% of the patients, respectively, whereas grade 4 nonhematologic toxicity was observed in only one patient.

Dose intensification of CHOP with prophylactic use of G-CSF was expected to improve survival among patients with aggressive NHL, and our randomized phase II study (JCOG9505) comparing CHOP-14 (LSG19) and dose-escalated CHOP (LSG20) to treat aggressive NHL excluding ATL revealed biweekly CHOP to be more promising [46]. Therefore, we regarded biweekly CHOP as a standard treatment for NHL including aggressive ATL at the time of designing this phase III study.

To confirm whether the LSG15 regimen is a new standard for the treatment of aggressive ATL, JCOG conducted a phase III trial comparing modified (m)-LSG15 with biweekly CHOP (cyclophosphamide, hydroxy-doxorubicin, vincristine [Oncovin], and prednisone), both supported with G-CSF and intrathecal prophylaxis [47].

mLSG19, a modified version of LSG19, consisted of eight cycles of CHOP [CPA 750 mg/m^2, ADM 50 mg/m^2, VCR 1.4 mg/m^2 (maximum 2 mg) on day 1 and PSL 100 mg on days 1 to 5] every 2 weeks [46]. The modification was an intrathecal administration identical to that in mLSG15.

mLSG15 in JCOG9801 was a modified version of LSG15 in JCOG9303, consisting of three regimens: VCAP [VCR 1 mg/m^2 (maximum 2 mg), CPA 350 mg/m^2, ADM 40 mg/m^2, PSL 40 mg/m^2] on day 1, AMP [ADM 30 mg/m^2, MCNU 60 mg/m^2, PSL 40 mg/m^2] on day 8, and VECP [VDS 2.4 mg/m^2 on day 15, ETP 100 mg/m^2 on days 15 to 17, CBDCA 250 mg/m^2 on day15, PSL 40 mg/m^2 on days 15 to 17] on days 15–17, and the next course was to be started on day 29 (Figure 1). The modifications in mLSG15 as compared to LSG15 were as follows: (1) The total number of cycles was reduced from 7 to 6 because of progressive cytopenia, especially thrombocytopenia, after repeating the LSG15 therapy. (2) Cytarabine 40 mg was used with MTX 15 mg and PSL 10 mg for prophylactic intrathecal administration, at the recovery phases of courses 1, 3, and 5 because of the high frequency of central nervous system relapse in the JCOG9303 study. Untreated patients with aggressive ATL were assigned to receive either six courses of mLSG15 every 4 weeks or eight courses of biweekly CHOP. The primary endpoint was overall survival. A total of 118 patients were enrolled. The CR rate was higher in the mLSG15 arm than in the biweekly CHOP arm (40% versus 25%, resp.; $P = .020$). As shown in Table 1, the median survival time and OS rate at 3 years were 12.7 months and 24% in the mLSG15 arm and 10.9 months and 13% in the biweekly CHOP arm [two-sided $P = .169$, and the hazard ratio was 0.75; 95% confidence interval (CI), 0.50 to 1.13]. A Cox regression analysis with performance status (PS 0 versus 1 versus 2–4) as the stratum for baseline hazard functions was performed to evaluate the effect on overall survival of age, B-symptoms, subtypes of ATL, LDH, BUN, bulky mass, and treatment arms. According to this analysis, the hazard ratio and two-sided P value for the treatment arms were 0.62 (95% CI, 0.38 to 1.01) and .056, respectively. The difference between the crude analysis and this result was because of unbalanced prognostic factors, such as PS 0 versus 1, and the presence or absence of bulky lesions between the treatment arms. The progression-free

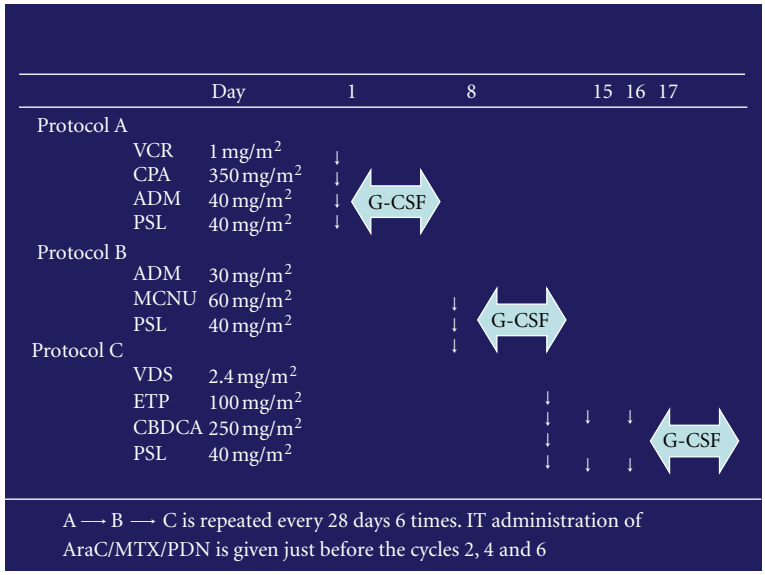

Figure 1: Regimen of VCAP-AMP-VECP in mLSG15. VCAP: vincristine (VCR), cyclophosphamide (CPA), doxorubicin (ADM), prednisone (PSL); AMP: ADM, ranimustine (MCNU), PSL; VECP: vindesine (VDS), etoposide (ETP), carboplatin (CBDCA), and PSL. ∗) MCNU and VDS are nitrosourea and vinca alkaloid, respectively, developed in Japan. A previous study on myeloma described that carmustine (BCNU), another nitrosourea, at 1 mg/kg is equivalent to MCNU at 0.8 to 1.0 mg/kg. VDS at 2.4 mg/m^2 can be substituted for VCR, another vinca alkaloid used in this regimen, at 1 mg/m^2 with possibly less myelosuppression and more peripheral neuropathy which can be managed by dose modification.

survival rate at 1 year was 28% in the mLSG15 arm compared with 16% in the biweekly CHOP arm (two-sided $P = .20$).

In mLSG15 versus mLSG19, rate of grade 4 neutropenia, grade 4 thrombocytopenia, and grade 3/4 infection were 98% versus 83%, 74% versus 17%, and 32% versus 15%, respectively. There were three toxic deaths in the former. Three treatment-related deaths (TRDs), two from sepsis and one from interstitial pneumonitis related to neutropenia, were reported in the mLSG15 arm. Two cases of myelodysplastic syndrome were reported, one each in both arms.

The longer survival at 3 years and higher CR rate with mLSG15 compared with mLSG19 suggest that mLSG15 is a more effective regimen at the expense of higher toxicity, providing the basis for future investigations in the treatment of ATL [47]. The superiority of VCAP-AMP-VECP in mLSG15 to biweekly CHOP in mLSG19 may be explained by the more prolonged, dose dense schedule of therapy in addition to 4 more drugs. In addition, agents such as carboplatin and ranimustine not affected by multidrug-resistance (MDR) related genes, which were frequently expressed in ATL cells at onset, were incorporated [48]. Intrathecal prophylaxis, which was incorporated in both arms of the phase III study, should be considered for patients with aggressive ATL even in the absence of clinical symptoms because a previous analysis revealed that more than half of relapses at new sites after chemotherapy occurred in the CNS [49]. However, the median survival time of 13 months in VCAP-AMP-VECP (LSG15/mLSG15) still compares unfavorably to other hematological malignancies, requiring further effort to improve the outcome.

5.3. Interferon-Alpha and Zidovudine. A small phase II trial in Japan of IFN alpha against relapsed/refractory ATL showed a response rate (all PR) of 33% (8/24), including 5 out of 9 (56%) chronic-type ATL [50]. In 1995, Gill and associates reported that 11 of 19 patients with acute- or lymphoma-type ATL showed major responses (5 CR and 6 PR) to a combination of interferon-alpha (IFN) and zidovudine (AZT) [51]. The efficacy of this combination was also observed by Hermine and associates; major objective responses were obtained in all five patients with ATL (four with acute type and one with smoldering type) [52]. Although these results are encouraging, the OS of previously untreated patients with ATL was relatively short (4.8 months) compared with the survival of those in the chemotherapy trials conducted by the JCOG-LSG (7 to 8 months) [53]. After that, numerous small phase II studies using AZT and IFN have shown responses in ATL patients [54–56]. High doses of both agents are recommended: 6–9 million units of IFN in combination with daily divided AZT doses of 800–1000 mg/day. Therapeutic effect of AZT and IFN is not through a direct cytotoxic effect of these drugs on the leukemic cells [57]. Enduring AZT treatment of ATL cell lines results in inhibition of telomerase which reprograms the cells to p53-dependent senescence [58].

Recently, the results of a "meta-analysis" on the use of IFN and AZT for ATL were reported [39]. A total of 100 patients received interferon-alpha and AZT as initial treatments. The ORR was 66%, with a 43% CR rate. In this worldwide retrospective analysis, the median survival time was 24 months and the 5-year survival rate was 50% for

first-line IFN and AZT, versus 7 months and 20% for 84 patients who received first-line chemotherapy. The median survival time of patients with acute-type ATL treated with first-line IFN/AZT and chemotherapy was 12 and 9 months, respectively. Patients with lymphoma-type ATL did not benefit from this combination. In addition, first-line IFN/AZT therapy in chronic- and smoldering-type ATL resulted in a 100% survival rate at a median followup of 5 years. However, because of the retrospective nature of this meta-analysis based on medical records at each hospital, the decision process to select the therapeutic modality for each patient and the possibility of interference with OS by second-line treatment remains unknown. While the results for IFN/AZT in indolent ATL appear to be promising compared to those with watchful-waiting policy until disease progression, recently reported from Japan [38], the possibility of selection bias cannot be ruled out. A prospective multicenter phase III study evaluating the efficacy of IFN/AZT as compared to watchful-waiting for indolent ATL is to be initiated in Japan.

Recently, a phase II study of the combination of arsenic trioxide, IFN, and AZT for chronic ATL revealed an impressive response rate and moderate toxicity [39]. Although the results appeared promising, the addition of arsenic trioxide to IFN/AZT, which might be sufficient for the treatment of chronic ATL as described above, caused more toxicity and should be evaluated with caution.

5.4. Allogeneic Hematopoietic Stem-Cell Transplantation (Allo-HSCT). Allo-HSCT is now recommended for the treatment of young patients with aggressive ATL [31, 59]. Despite higher treatment-related mortality including graft versus host disease in a retrospective multicenter analysis of myeloablative allo-HSCT, the estimated 3-year OS of 33% is promising, possibly reflecting a graft versus ATL effect [60]. To evaluate the efficacy of allo-HSCT more accurately, especially in view of a comparison with intensive chemotherapy, a prospective multicenter phase II study of LSG15 chemotherapy followed by allo-HSCT is ongoing (JCOG0907).

Feasibility studies of allo-HSCT with reduced intensity conditioning for relatively aged patients with ATL also revealed promising results, and subsequent multicenter trials are being conducted in Japan [61, 62]. The minimal residual disease after allo-HSCT detected as HTLV-1 proviral load was much less than that after chemotherapy or AZT/IFN therapy, suggesting the presence of a graft-versus-ATL effect as well as graft-versus-HTLV-1 activity [61].

It remains unclear which type of allo-HSCT (myeloablative or reduced intensity conditioning) is more suitable for the treatment of ATL. Furthermore, selection criteria with respect to responses to previous treatments, sources of stem cells, and HTLV-1 viral status of the donor remain to be determined. Recently, a patient in whom ATL derived from donor cells developed four months after transplantation of stem cells from a sibling with HTLV-I was reported [63].

However, several other retrospective studies as well as those mentioned above on allo-HSCT showed a promising long-term survival rate of 20 to 40% with an apparent plateau phase despite significant treatment-related mortality.

5.5. Supportive Care. The prevention of opportunistic infections is essential in the management of ATL patients, nearly half of whom develop severe infections during chemotherapy. Some patients with indolent ATL develop infections during watchful waiting.

Sulfamethoxazole/trimethoprim and antifungal agents have been recommended as prophylaxes for Pneumocystis jiroveci pneumonia and fungal infections, respectively, in the JCOG trials [43–45]. While cytomegalovirus infections are not infrequent among ATL patients, ganciclovir is not usually recommended as a prophylaxis [31]. In addition, in patients not receiving chemotherapy or allo-HSCT, antifungal prophylaxis may not be critical. An antistrongyloides agent, such as ivermectin or albendazole, should be considered to avoid systemic infections in patients with a history of exposure to the parasite in the tropics. Treatment with steroids and proton pump inhibitors may precipitate a fulminant strongyloides infestation and warrants testing before these agents are used in endemic areas [31]. Hypercalcemia associated with aggressive ATL can be corrected using chemotherapy in combination with hydration and bisphosphonate even when the performance status of the patient is poor.

5.6. Response Criteria. The complex nature of ATL, often with both leukemic and lymphomatous components, makes response assessment difficult. A modification of the JCOG response criteria was suggested by ATL consensus-meeting reflecting those for CLL and NHL which had been published later [31, 64, 65]. Recently, revised response criteria were proposed for lymphoma. New guidelines were presented incorporating positron emission tomography (PET), especially for the assessment of CR. It is well known and described in the criteria that several kinds of lymphoma including peripheral T-cell lymphomas were variably [18F] fluorodeoxyglucose (FDG) avid [66]. Meanwhile, PET or PET/CT is recommended for evaluations of response when the tumorous lesions are FDG-avid at diagnosis [31].

5.7. New Agents for ATL

5.7.1. Purine Analogs. Several purine analogs have been evaluated for ATL. Among them, pentostatin (deoxycoformycin) has been most extensively evaluated as a single agent and in combination as described above [43, 46].

Other purine analogs clinically studied for ATL are fludarabine and cladribine. Fludarabine is among standard treatments for B-chronic lymphocytic leukemia and other lymphoid malignancies. In a phase I study of fludarabine in Japan, 5 ATL patients and 10 B-CLL patients with refractory or relapsed-disease were enrolled [67]. Six grade 3 nonhematological toxicities were only observed in the ATL patients. PR was achieved only in one of the 5 ATL patients and the duration was short. Cladribine is among standard treatments for hairy cell leukemia and other lymphoid malignancies. A phase II study of cladribine for relapsed/refractory aggressive-ATL in 15 patients revealed only one PR [68].

Forodesine, a purine nucleotide phosphorylase (PNP) inhibitor, is among purine nucleotide analogs. PNP is an

enzyme in the purine salvage pathway that phosphorolysis 2′deoxyguanosine (dGuo). Purine nucleoside phosphorylase (PNP) deficiency in humans results in a severe combined immunodeficiency phenotype and the selective depletion of T cells associated with high plasma deoxyguanosine (dGuo) and high intracellular deoxyguanosine triphosphate levels in those cells with high deoxynucleoside kinase activity such as T cells, leading to cell death. Inhibitors of PNP, such as forodesine, mimic SCID in vitro and in vivo, suggesting a new targeting agent specific for T cell malignancies [69]. A dose escalating phase I study of forodesine is being conducted in Japan for T cell malignancies including ATL.

5.7.2. Histone Deacetylase Inhibitor. Gene expression governed by epigenetic changes is crucial to the pathogenesis of cancer. Histone deacetylases (HDACs) are enzymes involved in the remodeling of chromatin and play a key role in the epigenetic regulation of gene expression. Deacetylase inhibitors (DACis) induce the hyperacetylation of nonhistone proteins as well as nucleosomal histones resulting in the expression of repressed genes involved in growth arrest, terminal differentiation, and/or apoptosis among cancer cells. Several classes of HDACi have been found to have potent anticancer effects in preclinical studies. HDACIs such as vorinostat (suberoylanilide hydroxamic acid: SAHA), romidepsin (depsipeptide), and panobinostat (LBH589) have also shown promise in preclinical and/or clinical studies against T-cell malignancies including ATL [70, 71]. Vorinostat and romidepsin have been approved for cutaneous T-cell lymphoma (CTCL) by the Food and Drug Administration in the USA. LBH589 has a significant anti-ATL effect in vitro and in mice [71]. However, a phase II study for CTCL and indolent ATL in Japan was terminated because of severe infections associated with the shrinkage of skin tumors and formation of ulcers in patients with ATL. Further study is required to evaluate the efficacy of HDACIs for PTCL/CTCL including ATL.

5.7.3. Monoclonal Antibodies and Toxin Fusion Proteins. Monoclonal antibodies (MoAb) and toxin fusion proteins targeting several molecules expressed on the surface of ATL cells and other lymphoid malignant cells, such as CD25, CD2, CD52, and chemokine receptor 4 (CCR4), have shown promise in recent clinical trials.

Because most ATL cells express the alpha-chain of IL-2R (CD25), Waldmann et al. treated patients with ATL using monoclonal antibodies to CD25 [72]. Six (32%) of 19 patients treated with anti-Tac showed objective responses lasting from 9 weeks to longer than 3 years. One impediment to this approach is the quantity of soluble IL-2R shed by the tumor cells into the circulation. Another strategy for targeting IL-2R is conjugation with an immunotoxin (Pseudomonas exotoxin) or radioisotope (yttrium-90). Waldmann et al. developed a stable conjugate of anti-Tac with yttrium-90. Among the 16 patients with ATL who received 5- to 15-mCi doses, 9 (56%) showed objective responses. The response lasted longer than that obtained with unconjugated anti-Tac antibody [73, 74].

LMB-2, composed of the anti-CD25 murine MoAb fused to the truncated form of Pseudomonas toxin, was cytotoxic to CD25-expressing cells including ATL cells in vitro and in mice. Phase I/II trials of this agent showed some effect against hairy cell leukemia, CTCL, and ATL [6]. Six of 35 patients in the phase I study had significant levels of neutralizing antibodies after the first cycle. This drug deserves further clinical trials including in combination with cytotoxic agents.

Denileukin diftitox (DD; DAB(389)-interleukin-2 [IL-2]), an interleukin-2-diphtheria toxin fusion protein targeting IL-2 receptor-expressing malignant T lymphocytes, shows efficacy as a single agent against CTCL and peripheral T-cell lymphoma (PTCL) [75]. Also the combination of this agent with multiagent chemotherapy, CHOP, was promising for PTCL [76]. ATL cells frequently and highly express CD25 as described above, and several ATL cases successfully treated with this agent have been reported [77].

CD52 antigen is present on normal and pathologic B and T cells. In PTCL, however, CD52 expression varies among patients, with an overall expression rate lower than 50% in one study but not in another [78, 79]. ATL cells frequently express CD52 as compared to other PTCLs. The humanized anti-CD52 monoclonal antibody alemtuzumab is active against CLL and PTCL as a single agent. The combination of alemtuzumab with a standard-dose cyclophosphamide/doxorubicin/vincristine/prednisone (CHOP) regimen as a first-line treatment for 24 patients with PTCL showed promising results with CR in 17 (71%) patients; 1 had a partial remission, with an overall median duration of response of 11 months and was associated with mostly manageable infections but including CMV reactivation [80]. Major infections were Jacob-Creutzfeldt virus reactivation, pulmonary invasive aspergillosis, and staphylococcus sepsis.

ATL cells express CD52, the target of alemtuzumab, which was active in a preclinical model of ATL and toxic to p53-deficient cells, and several ATL cases successfully treated with this agent have been reported [81–83].

Siplizumab is a humanized MoAb targeting CD2 and showed efficacy in a murine ATL model. P1 dose-escalating study of this agent in 22 patients with several kinds of T/NK-cell malignancy revealed 6 responses (2 CR in LGL leukemia, 3 PR in ATL, and 1 PR in CTCL). However, 4 patients developed EBV-associated LPD [84]. The broad specificity of this agent may eliminate both CD4- and CD8-positive T cells as well as NK cells without effecting B cells and predispose individuals to the development of EBV lymphoproliferative syndrome.

CC chemokine receptor 4 (CCR4) is expressed on normal T helper type 27 and regulatory T (Treg) cells and on certain types of T-cell neoplasms [20, 21, 35]. KW-0761, a next generation humanized anti-CCR4 mAb, with a defucosylated Fc region, exerts strong antibody-dependent cellular cytotoxicity (ADCC) due to increased binding to the Fcγ receptor on effecter cells [85]. A phase I study of dose escalation with 4 weekly intravenous infusions of KW-0761 in 16 patients with relapsed CCR4-positive T cell malignancy (13 ATL and 3 PTCL) revealed that one patient, at the maximum dose (1.0 mg/kg), developed grade (G) 3 dose-limiting toxic effects, namely, skin rashes and febrile

Table 2: Strategy for the treatment of Adult T-Cell Leukemia-Lymphoma.

Smoldering- or favorable chronic-type ATL
(i) Consider inclusion in prospective clinical trials.
(ii) Symptomatic patients (skin lesions, opportunistic infections, etc.): Consider AZT/IFN or Watch and Wait.
(iii) Asymptomatic patients: Consider Watch and Wait.
Unfavorable chronic- or acute-type ATL
(i) If outside clinical trials, check prognostic factors (including clinical and molecular factors if possible):
(a) Good prognostic factors: consider chemotherapy (VCAP-AMP-VECP evaluated by a phase III trial against biweekly-CHOP) or AZT/IFN (evaluated by a meta-analysis on retrospective studies).
(b) Poor prognostic factors: consider chemotherapy followed by conventional or reduced intensity allo-HSCT (evaluated by retrospective and prospective Japanese analyses, resp.).
(c) Poor response to initial therapy: Consider conventional or reduced intensity allo-HSCT.
Lymphoma-type ATL
(i) If outside clinical trials, consider chemotherapy (VCAP-AMP-VECP).
(ii) Check prognostic factors (including clinical and molecular factors if possible) and response to chemotherapy:
(a) Good prognostic factors and good response to initial therapy: Consider chemotherapy followed by observation.
(b) Poor prognostic factors or poor response to initial therapy: Consider chemotherapy followed by conventional or reduced intensity allo-HSCT.

neutropenia and G4 neutropenia [86]. Other treatment-related G3-4 toxic effects were lymphopenia ($n = 10$), neutropenia ($n = 3$), leukopenia ($n = 2$), herpes zoster ($n = 1$), and acute infusion reaction/cytokine release syndrome ($n = 1$). Neither the frequency nor severity of these effects increased with dose escalation or the plasma concentration of the agent. The maximum tolerated dose was not reached. No patients had detectable levels of anti-KW-0761 antibody. Five patients (31%; 95% CI, 11% to 59%) achieved objective responses: 2 complete (0.1; 1.0 mg/kg) and 3 partial (0.01; 2 at 1.0 mg/kg) responses. Three out of 13 patients with ATL (31%) achieved a response (2 CR and 1 PR). Responses in each lesion were diverse, that is, good in PB (6 CR and 1 PR/7 evaluable cases), intermediate in skin (3 CR and 1 PR/8 evaluable cases), and poor in LN (1 CR and 2 PR/11 evaluable cases). KW-0761 was well tolerated at all the doses tested, demonstrating potential efficacy against relapsed CCR4-positive ATL or PTCL. Recently, results of subsequent phase II studies at the 1.0 mg/kg in relapsed ATL, showing 50% of response rate with acceptable toxicity profiles, reported [87]. A phase II trial of single agent KW-0761 at the 1.0 mg/kg in relapsed PTCL/CTCL and a phase II trial of VCAP-AMP-VECP combined with KW-0761 for untreated aggressive ATL are ongoing.

5.7.4. Other Agents. A proteasome inhibitor, bortezomib (Velcade), and an immunomodulatory agent, lenalidomide (Revlimid), both have potent preclinical and clinical activity in T-cell malignancies including ATL, are now under clinical trials for relapsed ATL in Japan [88–90]. Other potential drugs for ATL include pralatrexate (Folotyn), a new agent with clinical activity in T-cell malignancies including ATL [91–93]. The agent is a novel antifolate with improved membrane transport and polyglutamylation in tumor cells and high affinity for the reduced folate carrier (RFC) highly expressed in malignant cells and has been approved by FDA recently for T-cell lymphoma including ATL.

5.8. Prevention. Two steps should be considered for the prevention of HTLV-1-associated ATL. The first is the prevention of HTLV-1 infections. This has been achieved in some endemic areas in Japan by screening for HTLV-1 among blood donors and asking mothers who are carriers to refrain from breast feeding. For several decades, before initiation of the interventions, the prevalence of HTLV-1 has declined drastically in endemic areas in Japan, probably because of birth cohort effects [94]. The elimination of HTLV-1 in endemic areas is now considered possible due to the natural decrease in the prevalence as well as the intervention of transmission through blood transfusion and breast feeding. The second step is the prevention of ATL among HTLV-1 carriers. This has not been achieved partly because only about 5% of HTLV-1 carriers develop the disease in their life time although several risk factors have been identified by a cohort study of HTLV-1 carriers (Joint Study of Predisposing Factors for ATL Development) [95]. Also, no agent has been found to be effective in preventing the development of ATL among HTLV-1 carriers.

6. Conclusions

Clinical trials have been paramount to the recent advances in ATL treatment, including assessments of chemotherapy, AZT/IFN, and allo-HSCT. Recently, a strategy for ATL treatment, stratified by subtype-classification, prognostic factors, and the response to initial treatment as well as response criteria, was proposed [31]. The recommended treatment algorithm for ATL is shown in Table 2. However, ATL still has a worse prognosis than the other T-cell malignancies [96].There is no plateau with an initial steep slope and subsequent gentle slope without a plateau in the survival curve for aggressive or indolent ATL treated by watchful waiting and with chemotherapy, respectively, although the prognosis is much better in the latter [38]. A prognostic model for

each subgroup should be elucidated to properly identify the candidate for allo-HSCT which can achieve a cure of ATL despite considerable treatment-related mortality. Although several small phase II trials and a recent metaanalysis suggested IFN/AZT therapy to be promising, no confirmative phase III study has been conducted [39]. Furthermore, as described in the other chapters in detail, more than ten promising new agents for PTCL/CTCL including ATL are now in clinical trials or preparation. Future clinical trials on ATL as described above should be incorporated to ensure that the consensus is continually updated to establish evidence-based practical guidelines.

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Review Article

Comparison of the Genetic Organization, Expression Strategies and Oncogenic Potential of HTLV-1 and HTLV-2

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Human T cell leukemia virus types 1 and 2 (HTLV-1 and HTLV-2) are genetically related complex retroviruses that are capable of immortalizing human T-cells *in vitro* and establish life-long persistent infections *in vivo*. In spite of these apparent similarities, HTLV-1 and HTLV-2 exhibit a significantly different pathogenic potential. HTLV-1 is recognized as the causative agent of adult T-cell leukemia/lymphoma (ATLL) and tropical spastic paraparesis/HTLV-1-associated myelopathy (TSP/HAM). In contrast, HTLV-2 has not been causally linked to human malignancy, although it may increase the risk of developing inflammatory neuropathies and infectious diseases. The present paper is focused on the studies aimed at defining the viral genetic determinants of the pathobiology of HTLV-1 and HTLV-2 through a comparison of the expression strategies and functional properties of the different gene products of the two viruses.

1. Introduction

Human T-cell leukemia virus types 1 and 2 (HTLV-1 and HTLV-2) are related deltaretroviruses [1] with similar genetic organization [2–7]. The two viruses share an average 65% homology at the nucleotide level, with higher conservation in the gag, pol, env, and tax/rex genes and lower in the long terminal repeats (LTR), protease, and proximal "X region," a region located at the 3′ end of the genome.

Although both viruses immortalize T cells in culture and establish life-long persistent infections *in vivo*, they exhibit a significantly different pathogenic potential. HTLV-1 is recognized as the causative agent of adult T-cell leukemia/lymphoma (ATLL) and tropical spastic paraparesis/HTLV-1-associated myelopathy (TSP/HAM). In contrast, HTLV-2 has not been causally linked to human malignancy. However, large cohort studies revealed that HTLV-2 infection may be associated with lymphocytosis, increased risk of developing inflammatory neuropathies, infectious diseases, and with increased all-cause mortality [4–6, 8]. Furthermore, coinfection with HTLV-2 plays an important role in the progression of HIV-infected patients to AIDS [9].

In the present paper, we will focus on the discussion of studies aimed at comparing the expression strategies, regulation, and pathogenic properties of HTLV-1 and HTLV-2.

2. The Genetic Organization and Expression Strategy of HTLV-1 and HTLV-2

Like other complex deltaretroviruses, HTLV-1 and HTLV-2 are characterized by the presence of the "X region," in addition to the LTR and the gag, pol, and env genes present in all retroviruses. The coding potential of the HTLV genomes is greatly enhanced by several expression strategies that include ribosomal frame shifting (which generates a Gag-Pro-Pol polyprotein) and alternative splicing (which produce distinct mRNAs coding for Env and the proteins coded by the X region) (Figure 1); in addition, some of the alternatively spliced transcripts are polycistronic [3, 10, 11]. Recent

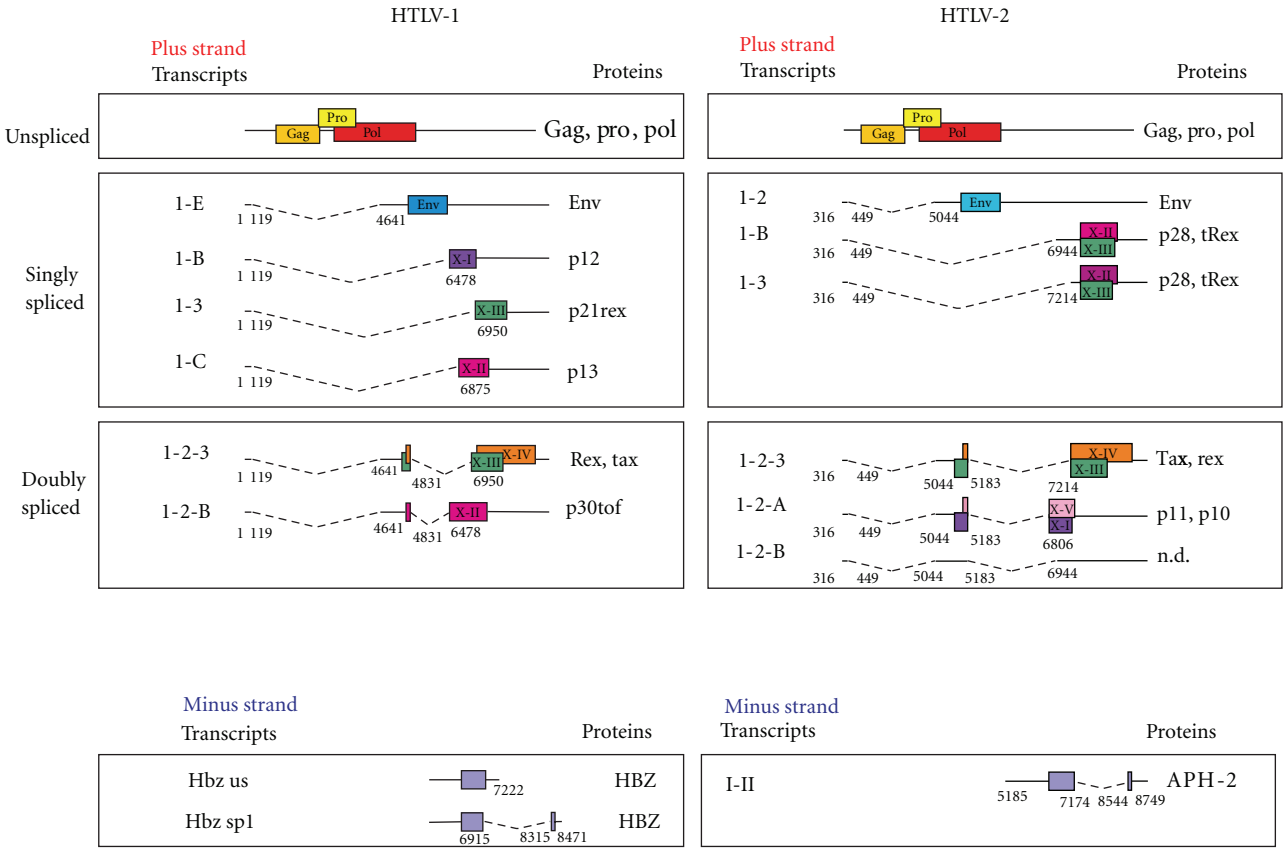

FIGURE 1: Comparison of the organization, alternative splicing, and coding potential of HTLV-1 and HTLV-2 mRNAs. Exon composition and coding potential of HTLV-1 and HTLV-2 alternatively spliced mRNAs. ORFs are indicated by colored boxes. Splice sites are indicated by numbers. n.d.: not determined.

studies showed that HTLV-1 and HTLV-2 also produce complementary-strand mRNAs, transcribed by promoters in the 3′ LTR; these genes were termed HBZ (HTLV-1 bZIP factor) and APH2 (anti-sense protein of HTLV-2), respectively [12, 13].

Transcription from the 5′LTR promoter generates 3 major size classes of mRNAs (Figure 1): (a) full-length (9 kb) genomic mRNA, coding for Gag-Pro-Pol; (b) 4 kb singly-spliced mRNAs, coding for the envelope glycoproteins (Env); (c) mRNAs of approximately 2 kb encoding proteins of the X region. The X regions of HTLV-1 and HTLV-2 encode, respectively, four and five major open reading frames (ORFs), termed x-I through x-V. The x-III and x-IV ORFs code for the essential regulatory proteins Tax and Rex that are produced from a dicistronic doubly spliced mRNA containing exons 1, 2, and 3. Tax and Rex provide, respectively, a positive feedback loop that drives transcription of the viral genome [2] and a posttranscriptional regulatory loop enhancing the nuclear export and expression of a subset of mRNAs [14, 15] (see paragraphs below for details). Other transcripts of the 2 kb class encode the accessory proteins of the X region; these transcripts include singly spliced mRNAs coding for p21rex, p12, and p13 (HTLV-1), tRex and p28 (HTLV-2) and doubly-spliced mRNAs, coding for the accessory proteins p30tof (HTLV-1), p10, and p11 (HTLV-2) [3, 10, 11]. Most of the regulatory and accessory proteins of the two viruses were shown to share structural and functional homologies, while p13 and p8 appear to be unique for HTLV-1 and p11 for HTLV-2 (Table 1).

The discovery of the complex coding potential of deltaretroviruses also raised the question as to whether the different genes are expressed with a particular temporal sequence and whether different patterns of viral gene expression are associated with different disease outcomes. The temporal sequence of HTLV-1 gene expression has recently been investigated using splice site-specific real-time RT-PCR (qRT-PCR) in an *ex vivo* virus reactivation model based on the depletion of CD8+ T cells from unstimulated peripheral blood mononuclear cells (PBMCs) isolated from HTLV-1-infected patients [16]. The results indicated a "two-phase" kinetics with tax/rex expression preceding that of other viral transcripts, a finding that is consistent with the key role of Tax as a master regulator driving overall viral gene expression and suggests an "early-late" switch in HTLV-1 gene expression. Studies in HeLa cells transfected with HTLV-1 molecular clones demonstrated the strict Rex-dependency of this "two-phase" kinetics [16]. Mathematical modelling revealed that the observed "two-phase" kinetics was critically dependent on a delay of Rex function compared to Tax [17], a prediction that was supported by experimental evidence

Table 1: Regulatory and accessory proteins coded by HTLV-1 and HTLV-2. The proteins with recognized functional analogies in the two viruses are indicated in bold. p8 and p13 proteins (indicated in bold italic) are unique to HTLV-1; p11 (indicated in italic) is unique to HTLV-2.

	ORF x-I	ORF x-II	ORF x-III	ORF x-IV	ORF x-V	Minus strand
HTLV-1	p12, ***p8***	**p30, *p13***	**Rex, p21Rex**	**Tax**	—	**HBZ**
HTLV-2	p10	**p28**	**Rex, tRex**	**Tax**	*p11*	**APH-2**

demonstrating a delayed accumulation and longer half-life of Rex compared to Tax [16]. More recently, we also analyzed the expression kinetics of HTLV-2 mRNAs in chronically infected cell lines and in PBMCs obtained from HTLV-2-infected patients. Results indicated a "two-phase" expression kinetics that is reminiscent of that of HTLV-1. Furthermore, this study revealed that HTLV-2 expresses higher levels of mRNAs encoding inhibitors of Tax and Rex, that is, p28 and truncated isoforms of Rex (tRex), respectively (Bender et al., submitted), suggesting that HTLV-2 may be characterized by a more latent expression profile, compared to HTLV-1.

3. Functional Comparison of Tax-1 and Tax-2

HTLV-1 and HTLV-2 Tax (Tax-1 and Tax-2) are required for viral replication, acting as transactivators of proviral transcription from the 5′LTR. Tax may also enhance HBZ transcription through the 3′LTR, although this effect is weaker compared to that on the 5′LTR [18, 19].

In addition to these effects on viral transcription, Tax has a pivotal role in the immortalization and transformation of infected cells by enhancing the expression of cellular genes that control T-cell proliferation and by interacting with proteins that control mitotic checkpoints and inactivate tumor suppressors pathways [20–23]. Tax-1 and Tax-2 present an overall structural and functional homology, although some domains and activities appear to be distinct in the two proteins [24, 25]. Tax-1 is a 353 amino acids (aa), 40 kDa protein, highly conserved in all HTLV-1 serotypes. Tax-2 has been characterized mainly from HTLV-2 subtypes A and B [26]. Tax-2B has 356 aa residues, whereas Tax-2A presents a 25 aa C-terminal truncation. Tax-1 and Tax-2B share an amino acid similarity of 85% and have several common domains including a cyclic AMP response element-binding protein, (CREB-) binding domain, a zinc-finger, domains required for interaction with proteasomal subunits, with transcriptional coactivators and with proteins involved in transcription, cell cycle progression, and in cell signaling regulation [5]. The C-terminal region of both Tax-1 and Tax-2 includes a CREB-activating domain [5, 27]. The central portion of Tax-1 includes two leucine zipper-like regions (LZR), necessary for DNA interaction and for protein dimerization [28, 29], binding domains for proteins involved in chromatin remodeling, cell cycle control, NF-κB activation, and p300 binding. The main structural difference between Tax-1 and Tax-2 is represented by the lack in Tax-2 of a leucine zipper region which is responsible for noncanonical NF-κB activation [30–32] and of the C-terminal motif, which mediates association with protein containing PDZ domains [24] (Figure 2).

Based on the initial studies on the subcellular localization of Tax-1 and Tax-2 indicating that Tax-1 was localized in the nucleus [33] and Tax-2 in the cytoplasm [34], detailed studies have been devoted to the characterization of the nuclear localization signal of both proteins. A nuclear localization signal (NLS) has been mapped to the first 60 aa of Tax-1 [35, 36] and in the first 42 aa of Tax-2 [37]; an additional NLS, located at position 89–113 of Tax-2, is responsible for the divergent cellular localization compared to Tax-1 [34].

Tax-1 localizes mainly in nuclear bodies in which two subunits of NF-κB (p50 and RelA) are present [38]; in the cytoplasm, Tax-1 localizes in organelles associated with secretory pathways [39], in structures associated to the microtubule organizing center (MTOC) [40], and in the cell to cell contact regions termed virological synapses [41]. Interestingly, the posttranslational modification of Tax is tied to its subcellular localization and ability to activate the NF-κB pathway, a key step in HTLV-1-mediated transformation. In particular, ubiquitinated Tax binds and colocalizes IKK subunits at a centrosome-associated signalosome leading to the release of active IKK [42, 43]. Using live-cell imaging, Kfoury et al. also showed that Tax shuttles among nuclear bodies and the centrosome, depending on its ubiquitination and SUMOylation [44]. In contrast, Tax-2 is more abundant in the cytoplasm [34, 37]. Additional functional properties of Tax proteins derive from their effects in the reorganization of TAB2-containing cytoplasmic structures which include RelA and calreticulin [45].

Both Tax-1 and Tax-2 are necessary and sufficient for HTLV-mediated immortalization of primary human T cells [46, 47]. Moreover, Tax-1 induces an ATLL-like T-cell malignancy in transgenic mice [48]. Tax-1 induces IL-2 expression through the transcription factor NFAT in Jurkat cells following stimulation by TPA [49] and upregulates the expression of additional genes encoding cytokines, chemokines, cell surface ligands, and their receptors [24]. Unlike Tax-1, Tax-2 activates IL-2 gene expression through NFAT, even without additional stimulation [24, 50].

Tax-1 alters several cellular signaling pathways by interacting with the cellular transcription factors NF-κB, CREB, serum responsive factor (SRF), and activator protein 1 (AP-1), that control cell proliferation, intracellular protein distribution, cell migration, apoptosis, and genetic stability [51]. More than 100 proteins have been shown to interact with Tax-1 [22]. Comparative studies showed that both Tax-1 and Tax-2 constitutively activate the canonical NF-κB pathway by interacting with RelA and the IκB kinase complex (e.g., IKKα, IKKβ, and NEMO/IKKγ) [22, 52]. Interestingly, Tax-1, but non Tax-2, also activates the noncanonical NF-κB by enhancing p100 processing and nuclear translocation of p52

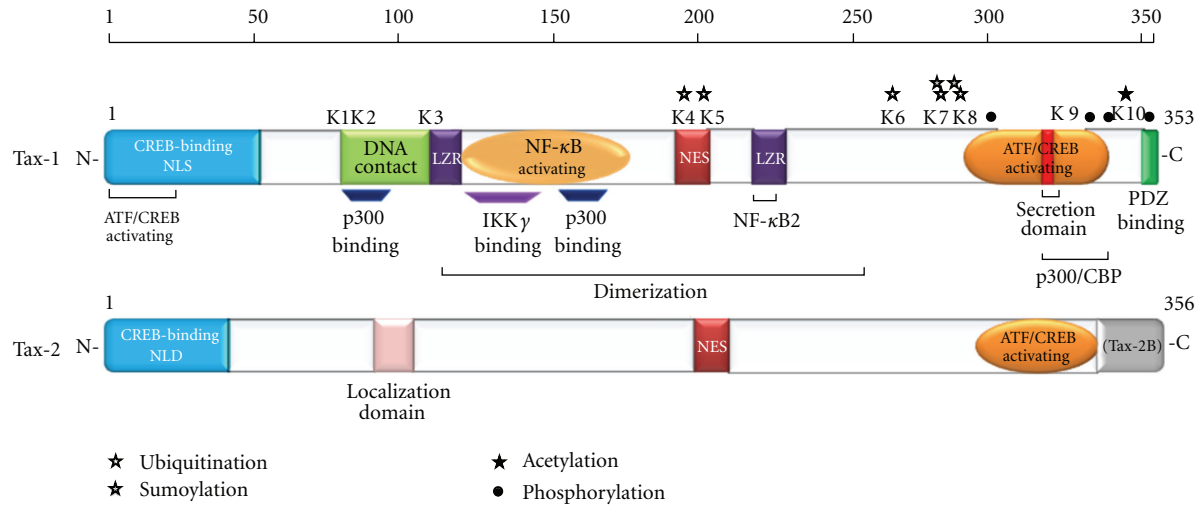

FIGURE 2: Schematic representation of Tax-1 and Tax-2 structural and functional domains. The positions of the amino acids modified by ubiquitination, sumoylation, acetylation, and phosphorylation are indicated.

[31]. Tax-1 and Tax-2 also differ in their mechanism of LTR transactivation, as Tax-1 recruits the CBP, p300, and PCAF coactivators factors, whereas Tax-2 does not require PCAF [53].

Compared to Tax-2, Tax-1 shows an overall higher activity as a transactivator and in transformation capacity as well as inhibition of p53 functions [33, 54, 55]. In contrast to Tax-1, Tax-2 is unable to induce micronuclei [56], does not perturb development and maturation of pluripotent hematopoietic progenitor cells [57], and does not induce G0/G1 cell cycle arrest [58].

4. Rex-1 and Rex-2

Expression of complex retroviruses is controlled at the post-transcriptional level by viral-encoded regulatory proteins that actively transport intron-containing mRNAs in the cytoplasm. In HTLVs, this function is carried out by Rex, that is produced by a doubly spliced dicistronic mRNA that also encodes Tax. HTLV-1 Rex (Rex-1) is a 27 kDa, 189 aa protein; HTLV-2 Rex (Rex-2) is a 170 aa, 26/24 kDa protein that shares 60% homology with Rex-1 at the amino acid level (Figure 3). Both Rex-1 and Rex-2 are phosphoproteins that localize to nucleus, nucleoli, and nucleolar speckles [3, 59–62]. Although mainly detected in the nuclear compartment, Rex actively shuttles between the nucleus and the cytoplasm [60, 63], a property that is intimately linked to its ability to transport incompletely spliced viral RNA form the nucleus to the cytoplasm.

The function of Rex-1 is mediated through binding to a 254-nucleotide stem-loop cis acting RNA element termed the Rex-responsive element (RXRE-1) [64] present in the U3/R region of the LTR. Due to the positions of the transcription start site, major splice donor, and polyadenylation signal/site, the full-length RXRE is located at the 3′ end of all HTLV-1 transcripts, while the 226 nt- Rex-2 responsive elements (RXRE-2), which maps to the R/U5 region, is located at the 5′ end of the unspliced HTLV-2 mRNA (Figure 4) [65–67]. Furthermore, the ability of the RXRE-1 to fold into stem loop structure brings the polyadenylation signal into close proximity to the GU rich polyadenylation site [68, 69] ensuring efficient polyadenylation of viral mRNAs.

Although Rex is not required for cellular immortalization *in vitro*, it is necessary for infectivity and viral persistence *in vivo* [70], since expression of the viral RNAs encoding the structural proteins is Rex dependent. Therefore, the Rex-RXRE interaction was proposed to act as a molecular switch controlling the transition between productive and latent phases of HTLV-1 infection, an hypothesis that is consistent with results of studies of the kinetics of expression of HTLV-1 mRNA (see above) [16].

The domain structure of Rex-1 and Rex-2 is similar, with NLS, RNA binding domains (RBD), multimerization domains, and nuclear export signals (NES) (Figure 3). The N-terminal arginine-rich region (amino acids 1–19) acts as nuclear localization signal (NLS) [61, 71] and as RNA binding domain (RBD) which mediates Rex binding to the RXRE [72]. A leucine-rich sequence located near the middle of the protein (Rex-1 aa 79–99; Rex-2 aa 81–94) functions as activation domain (AD) [73] and contains the nuclear export signal (NES) [63, 74]. The NES interacts with the protein chromosome region maintenance interacting protein 1 (CRM1/exportin 1) and allows export of the Rex-viral mRNA complex from the nucleus to the cytoplasm [75]. CRM1 belongs to the importin-β family, whose members act as RNA transporters between the nuclear and cytoplasmic compartments [76]. Mutations of the four leucine residues within the NES demonstrate that they are critical for nuclear export of mRNA [63, 74]. In addition, a unique C-terminal domain has been described for Rex-2 that is a target for serine phosphorylation and may also contribute to efficient nucleocytoplasmic shuttling [60]. The two regions flanking the NES (aa 57–66 and 106–124 for Rex-1; aa 57–71 and 124–132 for Rex-2) are required for the assembly of

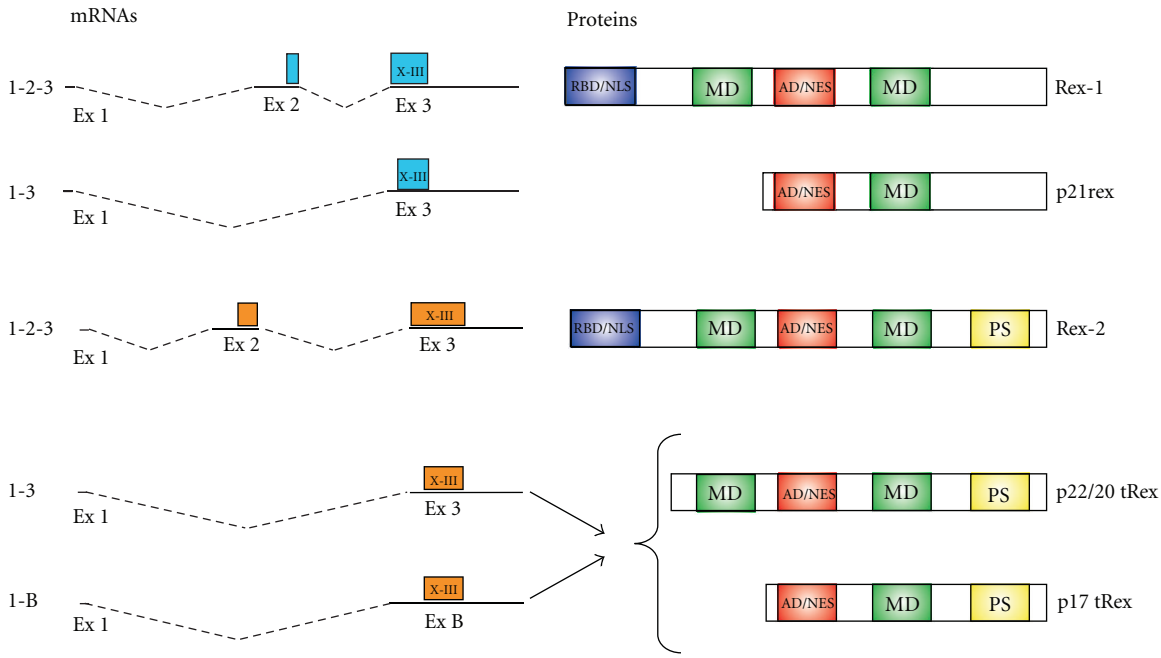

Figure 3: Comparison of the protein isoforms coded by the x-III ORF of HTLV-1 and HTLV-2. Indicated is the exon composition of the mRNAs (left) and the domain structure of the corresponding proteins (right) coded by the x-III ORFs of HTLV-1 and HTLV-2. HTLV-1 produces a single truncated x-III ORF: p21rex; HTLV-2 produces a family of truncated x-III isoforms ranging from 22 to 17 kDa. RBD/NLS: rna binding domain/nuclear localization signal; MD: multimerization domain; AD/NES: activation domain/nuclear export signal; PS: phosphorylation site.

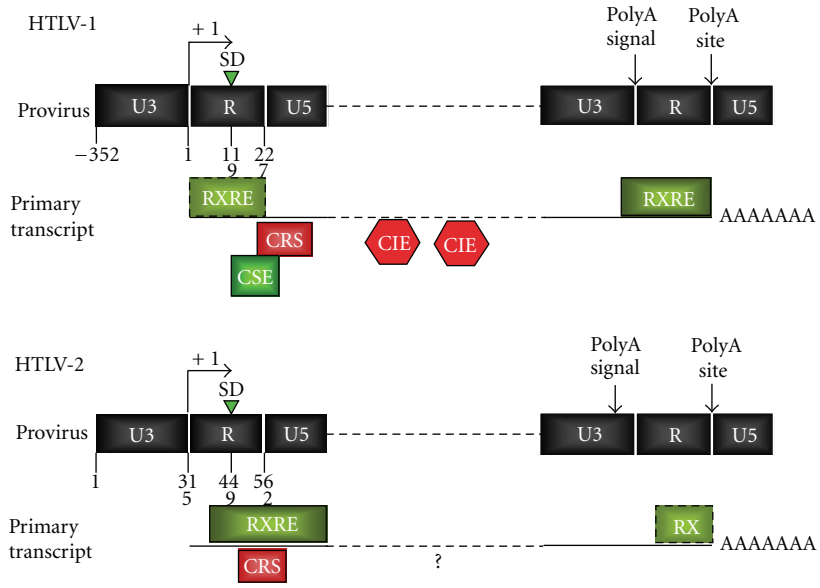

Figure 4: Comparison of the cis acting regulatory elements of HTLV-1 and HTLV-2. Position of the cis acting posttranscriptional regulatory elements of HTLV-1 and HTLV-2 are indicated. RXRE: Rex-responsive element; CRS: cis-acting repressive sequences; CSE: cis-acting stimulatory element; CIE: cis acting inhibitory elements.

Rex into multimeric structures upon binding to the RXRE [75, 77], a process that is critical for the nuclear export of viral mRNA since multimerization-defective mutants of Rex act as dominant-negative mutants [77]. The multimerization of Rex on the viral mRNAs is also enhanced by CRM1 [78, 79] and by the translation-initiation factor eIF-5A [80].

Following Rex-RXRE binding and Rex multimerization, CRM1 is recruited into the complex [75], binds to RanGTP, and translocates the Rex-mRNA complex across the nuclear

pore by interacting with nucleoporins. In the cytoplasm, RanGTP is then converted to RanGDP and is released from the Rex-mRNA complex. Other proteins affecting Rex function include Ran binding protein 3 (RanBP3), a scaffold protein that stabilizes the RanGTP-CRM1-Rex-mRNA complex in the nucleus [78, 81] and SRC-associated in mitosis 68 (Sam 68), which increases the Rex-mRNA binding in a CRM1-independent fashion [82].

In addition, to these effects on viral gene expression, Rex-1 enhances Tax-mediated upregulation of IL-2 and stabilizes the IL-2Rα mRNA [83, 84], thus possibly contributing to transformation of HTLV-1 infected cells. Rex may also enhance the expression of FynB, a src family tyrosine kinase that regulates T-cell receptor stimulation [85, 86] as well as VCAM-1 and LFA-3 [87], two surface molecules important in T-cell adhesion and proliferation. More recently Rex-1 was shown to inhibit the RNA silencing pathway by interacting with Dicer, a member of the RNase III family that converts the double-stranded short hairpin RNA to the single-stranded siRNA (small interfering RNA) [88].

Furthermore, hnRNP A1 (heterogeneous nuclear ribonucleoprotein A1) impairs the posttranscriptional regulation of HTLV-1 gene expression, by interfering with the binding of Rex to the RXRE [89]. Knockdown of hnRNP A1 expression in the chronically infected T-cell lines C91PL using siRNA increased the levels of the unspliced gag/pol mRNA in the nucleus (2-fold) and cytoplasm (3-fold), while a more modest increase of the tax/rex mRNAs was observed in both compartments and the expression and distribution of the env mRNA was not significantly altered [90].

5. Cis Acting Regulatory Elements Important for Rex Function

The expression of the alternatively spliced mRNAs of HTLV-1 and HTLV-2 is controlled by the relative influence of positive and negative sequences present on the primary transcript. Two major types of RNA cis acting elements have been described: (i) the RXRE (described above) which mediates Rex-dependent nuclear export and (ii) cis acting repressive sequences (CRS) that determine poor stability and/or inefficient nucleocytoplasmic export. Although the general layout and function of these elements are similar between the two viruses, several interesting differences can be pointed out (Figure 4) [91–93].

Studies based on heterologous reporter plasmids mapped a CRS in the U5 region of HTLV-1 [93, 94]. Due to the positions of the transcription start site, major splice donor and polyadenylation signal/site, the HTLV-1 CRS is present only at the 5′ end of the unspliced mRNA (Figure 4). Interestingly, an additional CRS at the 3′ end of all transcripts overlaps the RXRE and acts synergistically with the 5′-CRS. Confocal microscopy analysis indicated that both the 5′ and 3′ CRSs act at the posttranscriptional level, most likely as nuclear retention sequences. Deletion of both the 5′ and the 3′ CRS resulted in constitutive Rex-independent nuclear export and expression of mRNAs. A 5′ CRS acting as a nuclear retention sequence was also mapped in the R-U5 region of HTLV-2 [65]. As the 5′ CRS does not bind Rex-1 [66, 95–97], its inhibitory function is likely to be mediated by other viral and/or cellular RNA-binding proteins.

A further layer of complexity is added by a study that analyzed the postsplicing steps of HTLV-1 regulation by testing the expression of individual full length viral mRNAs in their intronless form. Results of this study showed that mRNA 1–3 (encoding p21rex), mRNA 1-2-3 (encoding Tax and Rex), and mRNA 1-2-B (encoding p30/tof) were inefficiently expressed when transcribed in their intronless form. The defective expression of these mRNAs was due to the inhibitory activity of the RXRE and the lack of a 5′ intronic region that counteracted the inhibitory effect of the RXRE. This cis acting stimulatory element (CSE) was mapped to the major splice donor and sequences overlapping with, but functionally distinct from, a previously described transcriptional enhancer [98].

In addition to the CRS, other cis acting inhibitory elements (CIEs) were mapped within the gag-pol and env regions of the HTLV-1 genome [99]. The inhibitory effect of these regions is counteracted by binding of Rex to the RXRE, although it is not clear whether their mechanism of function is mainly at the level of RNA stability or nucleocytoplasmic export. At present, no intragenic CIEs have been described in HTLV-2.

6. Truncated Isoforms of Rex

Both HTLV-1 and HTLV-2 produce truncated isoforms of Rex which lack the N-terminal arginine-rich RBD/NLS and are therefore predicted to be incapable of binding the RXRE and accumulate in the nuclear compartment. HTLV-1 produces a single truncated x-III ORF product termed p21rex, which also lacks the oligomerization domains of full-length Rex (Figure 3) [100, 101]. It was hypothesized that p21rex might act as a repressor of full-length Rex, thus inhibiting the expression of virion-associated proteins and playing a role as a latency-inducing factor in the HTLV-1 life cycle [100].

HTLV-2 produces a family of truncated isoforms ranging from 22 to 17 kDa which differ in the initiation codon usage and phosphorylation status [59]. These truncated x-III ORF products of HTLV-2, that we propose to term tRex, are expressed from two singly spliced mRNAs containing exon 1 linked to either exon 3 or to exon B (Figure 3). Initiation of translation from the first AUG codon located within the x-III ORF gives rise to two major protein isoforms of 22 and 20 kDa and a minor 18-kDa protein differing by posttranslational modification. Translation from the second AUG of the x-III ORF produces a 17-kDa protein [3, 59]. The multimerization and activation domain of Rex2 are also present in the 22–20 and 18 kDa tRex proteins. Cotransfection assays demonstrated that the tRex proteins were able to inhibit the ability of Rex to activate the expression of a Rex-dependent mRNA. Subcellular fractionation studies showed that Rex was preferentially localized in the cytoplasmic or nuclear fraction depending on its phosphorylation status and that coexpression of Rex with tRex changed the phosphorylation pattern of Rex and intracellular localization of tRex [59].

7. Expression and Function of Accessory Genes Coded in the X Region of HTLV-1 and HTLV-2

p30tof and p28 are products of the x-II ORF of HTLV-1 and HTLV-2, respectively. p30tof is coded by a doubly spliced mRNA containing exons 1, 2, and B; p28 is coded by 2 singly spliced mRNAs containing exons 1 and 3 or exons 1 and B (Figure 1). The two proteins are both dispensable for viral propagation *in vitro* but important for viral spread and persistence in animal models [102–104]. p30tof and p28 share some functional analogies as they both sequester the tax/rex mRNA in the nucleus, thus reducing viral expression, an effect that may result in viral latency and blunt immune recognition of infected cells [105, 106]. By interacting with the coactivator CBP/p300 [107, 108], p30tof also affects Tax-mediated transcription of viral and cellular genes at the transcriptional and posttranscriptional levels, including genes involved in T-cell activation and apoptosis [109, 110]. Further studies revealed that p28 function was partly distinct from that of p30tof in that it was devoid of transcriptional modulating activity. Moreover, p30tof interacts with the RNA-binding domain of Rex inhibiting Rex binding to the RxRE [111, 112]. By interacting with PU.1, p30tof inhibits its transcriptional activity, resulting in the downregulation of Toll-like receptor 4 (TLR4) expression from the cell surface [113], suggesting that the protein might also play a role in reducing activation of adaptive immunity in HTLV-1-infected patients.

p13, a short isoform coded by the x-II ORF of HTLV-1 from a singly spliced mRNA containing exons 1 and C (Figure 1), corresponds to the C-terminal 87 amino acids of p30tof [11] and is localized mainly in the mitochondrial inner membrane [114] and in part to the nucleus [115, 116]. p13 increases mitochondrial permeability to K^+ and activates the electron transport chain, resulting in increased mitochondrial reactive oxygen species (ROS) production [117]. These changes in ROS homeostasis affect both cell survival and proliferation depending on the cell's inherent ROS setpoint. While in normal resting T-cells, which have low ROS levels, p13 expression resulted in mitogenic activation, in cancer cells which are characterized by a high ROS setpoint, p13 induced cell death [114, 115, 118–121]. So far, no HTLV-2 ortholog of HTLV-1 p13 has been identified.

HTLV-1 p12 and p8 are coded by the x-I ORF from a singly spliced mRNA containing exons 1 and A or exons 1 and B (Figure 1). p12 localizes in the endoplasmic reticulum (ER) and in the Golgi apparatus, where it interacts with the β and γ_c chains of the interleukin-2 receptor (IL-2R), reducing their surface expression [122]. p12 expression results in activation of STAT-5, which provides a mitogenic signal to T-cells [123]. p12 also decreases surface expression of MHC-I, thus contributing to blunt lysis of HTLV-1-infected cells by CTL [124]. p12 also interacts with calreticulin and calnexin [125] resulting in increased Ca^{2+} release from the ER [126] and activation of the NFAT, a mitogenic pathway in T cells [127–129]. Within the ER, p12 is cleaved into an 8 kDa protein (p8) which traffics to the immunological synapse and favours T-cell anergy. p8 also increases cell-to-cell viral transmission through the formation of intercellular conduits among T cells [130, 131]. In analogy to HTLV-1 p12, HTLV-2 p10 and p11 were shown to bind the MHC heavy chain; however, p10 and p11 did not bind other targets of p12, such as the IL2R β chain or the 16-kDa subunit of the vacuolar H+ ATPase [132]. No functional homologue of p8 has been described in HTLV-2.

8. Functional Comparison of Minus-Strand Genes

Transcription from a promoter in the 3′ LTR generates transcripts from the complementary strand which encode the HBZ (HTLV-1) and APH-2 (HTLV-2) genes [12, 13]. The negative strand of the HTLV-1 genome [133] generates at least 2 different transcripts, one spliced (hbz sp1) and the other unspliced (hbz us) [134–136]. Studies in infected cells indicated that hbz sp1 was more abundant than hbz us [137]. Hbz sp1 has multiple transcriptional initiation sites in the U5 and R regions of the 3′ LTR, whereas transcription of the hbz us mRNA initiates within the tax gene. Both hbz sp1 and hbz us promoters are TATA-less [19]. The transcription of hbz sp1 is dependent on the Sp1 transcription factor and is weakly responsive to Tax [19, 138], a finding that is consistent with the fact that hbz is expressed in HTLV-1-infected cells regardless of the expression levels of Tax and hbz expression exhibits a stronger correlation with provirus load than tax expression. Hbz sp1 is translated into a protein of 206 aa, while hbz us produces a protein of 209 aa differing by 7 aa at the N-terminus [135]. The domain structure of the HBZ protein includes an N-terminal transcriptional activation domain (AD), a central domain (CD), and a C-terminal basic ZIP domain (bZIP) [12]. The HBZ SP1 protein is more abundant and has a longer half-life than the US isoform [19]. HBZ US protein mainly localizes in nuclear bodies while HBZ-SP1 is mainly nucleolar; two regions rich in basic amino acids and a DNA binding domain are associated with nuclear localization of HBZ [139, 140].

HBZ is not necessary for *in vitro* transformation but increases infectivity and viral persistence in *in vivo* animal model [141]; furthermore, HBZ was demonstrated to be oncogenic in a transgenic mouse model [142]. HBZ interacts with a number of transcription factors. Through interactions mediated by its bZIP domain, HBZ inhibits the ability of CREB-2 to bind to the HTLV-1 LTR, resulting in the inhibition of Tax-mediated transcription from the 5′ LTR [12]. HBZ interacts with the KIX domain of CBP/p300 via LXXLL-like motifs in its N-terminal region, leading to suppression of viral transcription by inhibiting the recruitment of CBP/p300 to the 5′ LTR promoter [143]. In addition, by forming a trimeric structure with Smad3 and p300, HBZ enhances TGF-β signalling [144] resulting in upregulation of Foxp3, a marker characteristic of HTLV-1 infected cells as well as of Treg cells [142].

HBZ's bZIP domain also mediates formation of heterodimers with c-Jun, JunB, and JunD [145, 146]. Binding of HBZ to JunB and c-Jun decreases their DNA binding activity by preventing their interaction with Fos, leading to repression of the AP-1 complex [147]. Furthermore, HBZ mediates

proteasomal degradation of c-Jun [147] and sequestration of JunB in nuclear bodies [140]. In contrast, the interaction of HBZ with Jun-D results in the activation of JunD-dependent cellular genes, including hTERT, the catalytic subunit of human telomerase [146, 148]. Through its N-terminal region, HBZ interacts with IRF-1 (interferon regulatory factor 1) reducing its DNA-binding ability and its stability, resulting in a reduction of IRF-1-mediated apoptosis [149]. HBZ inhibits the classical NF-κB pathway by two different mechanisms: by inhibiting the DNA binding of the NF-κB subunit p65 and by increasing the expression of PDLIM2, the E3 ubiquitin ligase of p65, leading to enhanced ubiquitination and degradation of p65 [150]. The downregulation of the NF-κB pathway was proposed to counteract the onset of Tax-induced cellular senescence, which results from hyperactivation of the NF-κB pathway [151]. HBZ expression is associated with proliferation of ATLL cells *in vivo* and *in vitro* [136, 152]. Mutational analyses showed that the hbz mRNA, rather than HBZ protein, has a growth-promoting effect on T cells [136] possibly by up-regulating the transcription of the E2F1 gene and its downstream targets. Interestingly, only hbz sp1, not hbz us, promotes proliferation of T-cells, indicating that the first exon of the hbz sp1 transcript is critical for this activity [19]. Furthermore it has been shown that hbz sp1 mRNA promotes Tax expression by repressing the p30tof, an inhibitor of the tax/rex mRNA; interestingly, this effect is not mediated by the 5′ stem-loop structure of hbz [153]. Moreover, the recent finding that over 90% of the hbz transcripts are localized in the nucleus in the chronically infected cell line C91PL and in HeLa cells transfected with the ACH HTLV-1 molecular clone underscores their importance as noncoding RNAs [16].

Following the discovery of HBZ, an antisense transcript was identified in HTLV-2 [13]. Its product, coded by an ORF located between Tax and Env, was named APH-2. Although the APH-2 mRNA was detected in HTLV-2 infected cell lines and in HTLV-2-infected patients, a quantitative comparison of the APH-2 expression levels with proviral loads has not been reported so far. The APH-2 mRNA is transcribed from the R and U5 regions of the 3′-LTR, spliced, and polyadenylated. APH-2 is a 183-aa protein, localized mainly in the nucleus. Despite the lack of a canonical bZIP domain, APH-2 interacts with CREB (but not with CBP/p300) and represses Tax-2-mediated transcription. In analogy to HTLV-1 hbz, also the APH-2 transcript exhibits a marked nuclear localization in chronically infected cell lines (Bender et al., submitted).

9. Conclusions and Perspectives

HTLV-1 and HTLV-2 are complex retroviruses with similar genetic structures but significantly different pathogenic potential. Many studies were aimed at defining the viral genetic determinants of these viruses by comparing the expression strategies and functional properties of the different gene products of the two viruses. Although these studies added considerable knowledge to the understanding of the HTLV-1 and HTLV-2 gene expression, important questions still remain open. The Tax proteins of the two viruses, although showing many similarities, exhibit some differences, especially regarding their capability to activate the NF-kB pathway and to induce genetic instability. The mechanism of function of HBZ and APH-2 as noncoding RNAs, which was underscored by their predominant nuclear localization [16], also deserves further studies; in particular, it would be interesting to test whether, in analogy tho HBZ, APH-2 may promote T-cell proliferation and exhibit pathogenic properties in transgenic mice. Although most of the accessory proteins appear to be orthologues in the two viruses, p13 and p8 are peculiar of HTLV-1, while p11 of HTLV-2 does not appear to have an HTLV-1 orthologue. Further studies should be aimed at testing whether the functions of these proteins, along with the differences in Tax-1 and Tax-2, might contribute to explain the different pathogenic potential of HTLV-1 and HTLV-2. In both viruses, the Rex-dependence of viral mRNA is determined by cis acting inhibitory sequences that decrease mRNA stability and nuclear export. A thorough analysis of the mechanism of function of these elements, for example, through a definition of their protein binding partners, is likely to shed light into the mechanisms controlling mRNA turnover and nucleocytoplasmic trafficking. The complex and partly redundant splicing pattern of deltaretroviruses also raises the question as to which mechanism(s) determine splice site selection, resulting in distinct patterns of viral gene expression and, possibly, different clinical outcomes of HTLV infection.

Authors' Contributions

F. Rende and I. Cavallari contributed equally.

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Review Article

Pathogenesis of Metastatic Calcification and Acute Pancreatitis in Adult T-Cell Leukemia under Hypercalcemic State

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Human T-cell leukemia virus type-1 (HTLV-1) is the causative agent of adult T-cell leukemia (ATL). Hypercalcemia is common in patients with ATL. These patients rarely develop metastatic calcification and acute pancreatitis. The underlying pathogenesis of this condition is osteoclast hyperactivity with associated overproduction of parathyroid hormone-related protein, which results in hypercalcemia in association with bone demineralization. The discovery of the osteoclast differentiation factor receptor activator of nuclear factor-κB ligand (RANKL), its receptor RANK, and its decoy receptor osteoprotegerin (OPG), enhanced our understanding of the mechanisms of ATL-associated hypercalcemia. Macrophage inflammatory protein-1-α, tumor necrosis factor-α, interleukin-1, and interleukin-6 are important molecules that enhance the migration and differentiation of osteoclasts and the associated enhanced production of RANKL for osteoblast formation. In this paper, we focus on metastatic calcification and acute pancreatitis in ATL, highlighting recent advances in the understanding of the molecular role of the RANKL/RANK/OPG system including its interaction with various cytokines and calciotropic hormones in the regulation of osteoclastogenesis for bone resorption in hypercalcemic ATL patients.

1. Introduction

Adult T-cell leukemia (ATL) was first reported as a new clinical entity in 1977 in Japan [1, 2]. The predominant physical findings are skin involvement, such as erythroderma and nodule formation due to the infiltration of neoplastic cells, lymphadenopathy, and hepatosplenomegaly. The ATL cells are of mature T-helper phenotype and have a characteristic appearance with especially indented or lobulated nuclei. Hypercalcemia is common in patients with ATL, and such patients often show increased numbers of osteoclasts.

A type C retrovirus was isolated from patients with cutaneous T-cell lymphoma by Poiesz and colleagues in 1980 [3]. This virus was later renamed human T-cell leukemia virus type 1 (HTLV-1). In 1981, Hinuma et al. [4] and Yoshida and colleagues [5] reported the isolation of a type C retrovirus named adult T-cell leukemia virus. The two isolates of human leukemia virus, HTLV-1, and adult T-cell leukemia virus, were later confirmed to be the same species of human retrovirus HTLV type I (US isolate) and ATLV (Japanese isolate) [6].

Approximately 16 to 20 million people are infected with HTLV-1 worldwide, and 1 to 5% of the infected individuals develop ATL during their lifetime [7] caused by the transformation of their CD4+ T cells [8]. In Japan, it is estimated that 1.2 million individuals are infected by HTLV-1, and more than 800 new cases of ATL are diagnosed each year [9]. The disease is endemic in southwest Japan, especially Okinawa, Nagasaki, Kagoshima, and Miyazaki, and also in the Caribbean islands, parts of Central Africa, South America, Melanesia, Papua New Guinea, Solomon island, and Australian aborigines [10–12].

HTLV-1 associated myelopathy was recognized in tropical areas independent of that in the Caribbean [13] and Japan [14]. Subsequently, due to its association with HTLV-1, the disease was named HTLV-1 associated myelopathy/tropical

spastic paraparesis (HAM/TSP). HAM/TSP is mainly a chronic inflammation of the white matter of the lower thoracic spinal cord, causing spastic paraparesis in the lower limbs [15]. Clinically, HAM/TSP is characterized by higher production of proinflammatory cytokines, such as interferon-γ and tumor necrosis factor-α (TNF-α), and accumulation of Tax-specific CD8+ T cells in the cerebrospinal fluid [16–20].

Patients with ATL frequently develop hypercalcemia. The authors reported four hypercalcemic ATL autopsy cases with metastatic calcifications [21, 22] including one with acute pancreatitis [21]. The reported incidence of acute pancreatitis in the registered ATL cases in Japan is 4% [23, 24]. Patients with ATL are also reported to be positive for parathyroid hormone-related protein (PTHrP) in ATL cells [25]. Furthermore, marked activation of osteoclasts was noted in the bone marrow of these patients, which could be due to the enhanced production of PTHrP in ATL cells [22].

Mechanical stresses and hormonal changes induce bone remodeling throughout the skeletal system, through osteoclastic bone resorption and osteoblastic bone formation [26]. The osteoclasts are multinucleated cells that originate from the monocytes/macrophages [27, 28]. Experimental evidence suggests that ATL cells stimulate the differentiation of hematopoietic precursors into osteoclasts [29]. The activity of osteoclasts is regulated by various cytokines and calciotropic hormones including macrophage inflammatory protein-1-alpha (MIP-1α), TNF-α, interleukin-1 (IL-1), IL-6, IL-11, macrophage-colony stimulating factors (M-CSF), PTH, PTHrP, 1α,25-dihydroxyvitamin D3 (1α,25$(OH)_2D_3$), and calcitonin [30–32]. Members of the TNF and TNF-receptor (TNFR) superfamily, receptor activator of nuclear factor-κB ligand (RANKL), receptor activator of nuclear factor-κB (RANK), and osteoprotegerin (OPG) also play a key role in the formation and activation of osteoclasts in conjunction with various cytokines and calciotropic hormones [30, 33, 34].

2. Metastatic Calcification

The mechanism of calcification in the viscera is categorized into two groups. Metastatic calcification with hypercalcemia occurs when calcium deposits in previously normal tissue whereas dystrophic calcification occurs in previously damaged tissue. (1) Dystrophic calcification in injured or necrotic tissue in a normal serum calcium level, such as tuberculosis, abscess, and hydatid disease. (2) Metastatic calcification can be divided into malignant and nonmalignant causes. Metastatic calcification in malignancy is reported in parathyroid carcinoma, multiple myeloma, lymphoma, leukemia, hypopharyngeal squamous cell carcinoma, synovial sarcoma, breast carcinoma, and choriocarcinoma. There are many causes of benign visceral metastatic calcification, but chronic renal failure is the most common. Most of the other benign causes are related to calcium balance, such as hypervitaminosis D and hyperparathyroidism [21, 35].

The mechanism of metastatic calcification is not clear. Metastatic calcification deposition can be influenced by release of excess calcium salts from bone, phosphate concentration, alkaline phosphatase activity, and viscera physicochemical conditions under alkalosis. The $Ca_3(PO_4)_2$ and $CaCO_3$ salts precipitate in tissues that have a favorable physicochemical environment under an alkaline pH condition. The liberated $Ca_3(PO_4)_2$ and $CaCO_3$ salts are transported via the blood in soluble form, which increased delivery and precipitation in tissues with alkalosis. Therefore, it is concluded that calcium salts precipitate in an alkaline environment [35, 36].

Hypercalcemia is one of the most difficult complication to treat in patients with ATL and a common direct cause of early death. Hypercalcemia is more severe in patients with ATL than that associated with other hematological malignancies [37]. The high frequency of hypercalcemia is the most striking feature of ATL; about 70% of ATL patients have high serum calcium levels during the clinical course of the disease, particularly during the aggressive stage of ATL [38]. Several pathological studies of ATL patients with hypercalcemia have indicated that high serum calcium levels are due to increased number of osteoclasts and accelerated bone resorption. This disease state is characterized by increased osteoclast activity with demineralization of bones and hypercalcemia. We reported previously that serum calcium levels ranged from 15.4 to 19.4 mg/dL (normal range: 8.4 to 10.4 mg/dL) in ATL patients with metastatic calcification [22]. The possibility of metastatic calcification should be considered in ATL patients associated with hypercalcemia who have abnormal shadow by roentgenogram [35, 39]. Other useful diagnostic procedures are imaging with computed tomography (CT) [35], magnetic resonance (MR) [40], and bone scintigraphy [35].

Metastatic calcification in ATL-hypercalcemia is commonly seen in alveolar septa of the lungs (Figure 1(a)), renal tubules (Figure 1(b)), and myocardium (Figure 1(c)). We reported previously the following rates of metastatic calcification in patients with ATL-hypercalcemia: tubules of kidneys: 100%, pulmonary alveolar septa of lungs: 100%, myocardium: 75%, muscular layer of stomach: 50%, lower portion of the aortic media: 50%, gastric mucosa: 25%, testicular tubules: 25%, and liver: 25% (Figure 1(d)) [22]. Metastatic calcification has also been reported in other organs, including the tongue, pancreas, and spleen [41]. Metastatic calcification of Disse's spaces in the liver of patients with ATL was first reported by Haratake and coworkers in 1985 [42], followed by Senba and colleagues in 1990 [21].

Histopathological examination of osseous tissue sections from ATL patients with hypercalcemia show scattered osteoclasts around the cortex in the vertebrae (Figure 1(e)) [22, 36, 37, 41], although all parathyroid glands were histologically normal [22]. Osteoblast activation is accompanied by osteoclast proliferation.

3. Acute Pancreatitis with Hypercalcemia

ATL associated with hypercalcemia and acute pancreatitis was first described in 1984 by Hosokawa et al. [43], followed later by other reports in 1990s [21, 44, 45]. Hypercalcemia is difficult to treat and can be the cause of death in ATL [46, 47]. The relation between hypercalcemia and acute

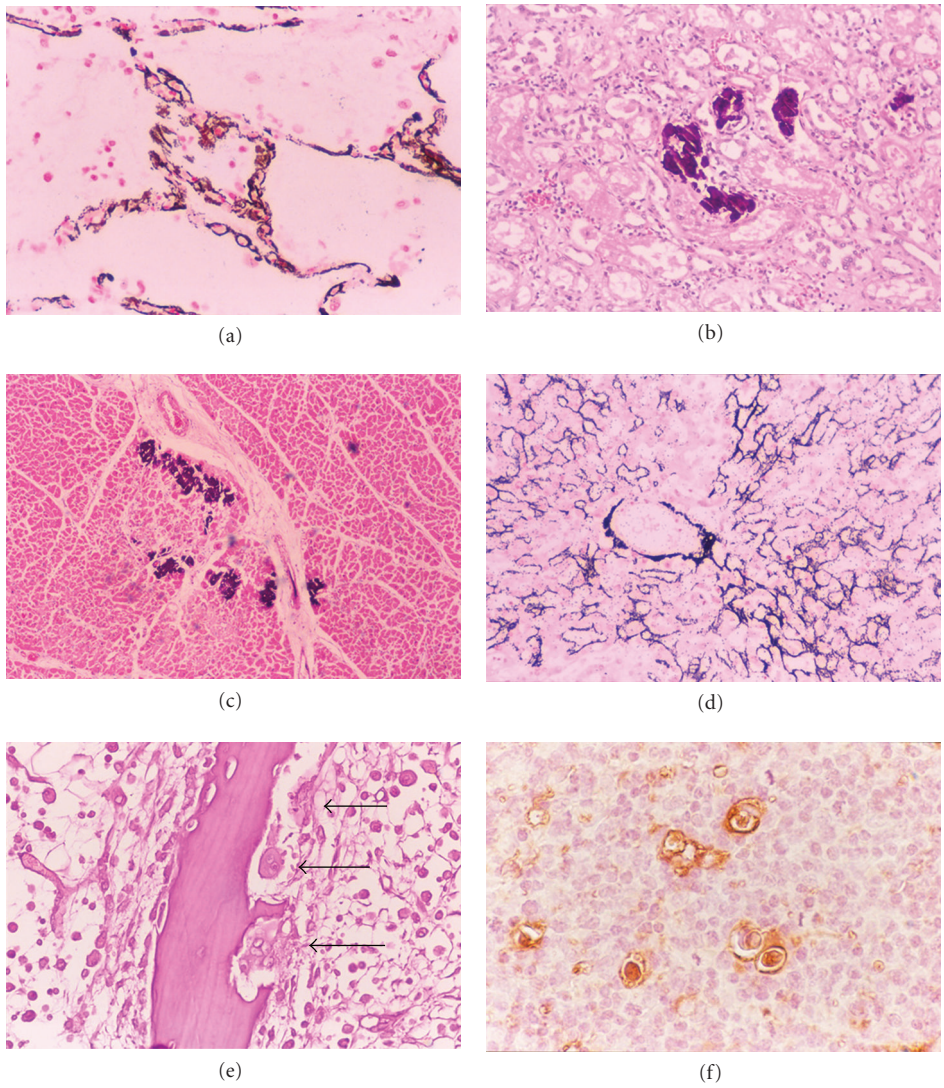

Figure 1: Hypercalcemia in ATL is associated with metastatic calcification. (a)–(d): Metastatic calcification is seen in the pulmonary alveolar septa of the lungs ((a) magnification, ×400), renal tubules of kidneys ((b) magnification, ×200), myocardium ((c) magnification, ×100), and Disses's space, hepatic cell membrane, and central vein wall ((d) magnification, ×200). von Kossa's staining for calcium. (e) Osteoclasts are found in the osseous tissue, and infiltration of numerous leukemic lymphoma cells in the bone marrow of the vertebra. Osteoclasts are multinucleated giant cells. Arrows: typical osteoclasts. Hematoxylin and eosin staining. Magnification, ×400. (f) Immunohistochemistry for PTHrP in leukemic lymphoma cells in ATL. PTHrP-positive cells are stained brown. This case was lymphoma type. The large cells were ATL cells, which were infiltrated in normal lymph nodes. ATL cells produce PTHrP, on the other hand, surrounding normal lymphocytes did not produce PTHrP. Magnification, ×400.

pancreatitis in patients with ATL was suggested based on the observation of pancreatitis in hypercalcemic renal transplant recipients [48]. However, the exact reason linking hypercalcemia and acute pancreatitis in patients with ATL remains to be elucidated. A plausible theory [49] is the following sequence: high serum calcium levels increase calcium levels in pancreatic juice, which result in accelerated calcium-dependent conversion of trypsinogen to trypsin, leading to acute pancreatitis. Another possibility involves the high levels of nephrogenous cyclic adenosine monophosphate [47], which stimulate pancreatic secretion in the extralobular ductal system of the pancreas [50], resulting in acute pancreatitis due to occlusion of the pancreatic duct [23].

4. PTHrP and Hypercalcemia

PTHrP is a polypeptide hormone discovered in 1987 and is structurally similar to PTH [51–53]. The aminoterminal peptides of PTHrP have PTH-like actions in osseous and renal tissues by binding to a common receptor for PTH/PTHrP (PTH-1 receptor), resulting in hypercalcemia [54–56]. PTHrP was originally isolated from specific tumors as the humoral hypercalcemia of malignancy [57], and is overexpressed in many types of neoplasms [58]. Several cytokines, such as IL-1 and transforming growth factor-β (TGF-β), and PTHrP have been implicated in ATL-associated hypercalcemia. Among these factors, PTHrP is considered to

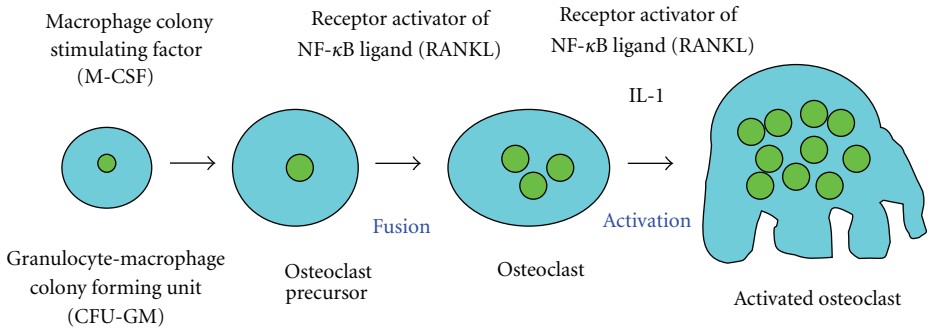

Figure 2: Developmental stages of osteoclast lineage. Osteoclasts are derived from hematopoietic precursor cells, and belong to the monocytes/macrophages lineage. With response to macrophage-colony stimulating factor (M-CSF), hematopoietic stem cells undergo differentiation into the granulocyte macrophage colony forming units (CFU-GM), which are the common precursor cells of granulocytes, macrophages, and osteoclasts. CFU-GM-derived cells differentiate to form mononuclear preosteoclast, which fuse together to subsequently form multinucleated osteoclasts.

stimulate osteoclasts, resulting in increased bone resorption. Moreover, IL-2 increases PTHrP production and secretion in HTLV-1 infected T cells [59, 60]. In addition, PTHrP and IL-6 act synergistically in causing humoral hypercalcemia of malignancy [61, 62]. PTHrP is also overexpressed in ATL cells (Figure 1(f)). The HTLV-1 oncoprotein, Tax is a phosphoprotein localized in the nucleus and acts to transactivate the PTHrP gene in ATL cells and is also involved in the transcription of the PTHrP gene *in vivo* [63, 64]. Furthermore, Tax upregulates PTHrP gene expression *in vitro* and also transactivates the PTHrP promoter [65]. Other studies showed that Tax acts in synergy with Ets-1, AP-1, and AP-2, to increase PTHrP gene transcription [66, 67]. Immunodeficient mice implanted with leukemic cells from patients with ATL exhibited hypercalcemia and overexpressed PTHrP [68]. However, PTHrP cannot directly induce the differentiation of hematopoietic precursor cells to osteoclasts [69]. Furthermore, high serum levels of PTHrP are not always associated with hypercalcemia in patients with ATL, suggesting the involvement of other factors in the development of hypercalcemia [70]. The MET-1/NOD/SCID model demonstrated that RANKL expression correlates with the secretion of PTHrP and IL-6, as well as with hypercalcemia [32]. Therefore, PTHrP is not always the major mediator of hypercalcemia in humoral hypercalcemia of malignancy; rather, the latter involves many other factors.

5. Osteoclast Differentiation and Hypercalcemia

Hypercalcemia is one of the most frequent and serious complications in patients with ATL and is due to marked bone resorption associated with osteoclast accumulation. The osseous tissue is consistently remodeled by the bone forming osteoblasts and the bone resorbing osteoclasts. Osteoclasts are multinucleated giant cells present only in the bone. They are derived from hematopoietic precursor cells, and belong to the monocytes/macrophage lineage. Specifically, they are formed mononuclear preosteoclasts, which fuse to form multinucleated osteoclast. The earliest

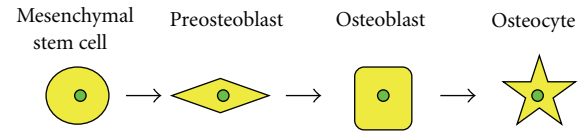

Figure 3: Developmental stages of osteoblast lineage. Osteoblasts are derived from undifferentiated mesenchymal stem cells. The osteoprogenitor cells progress through defined stages from pre-osteoblasts to osteoblasts and finally to osteocytes, which is responsible of mineralization and calcified bone formation.

identifiable osteoclast precursor cells are the granulocyte macrophage colony forming units (CFU-GM), which give rise to granulocytes, monocytes, and osteoclasts. CFU-GM derived cells differentiate to committed osteoclast precursors, which are postmitotic cells, and fuse to form multinucleated osteoclasts (Figure 2) [30, 71]. During differentiation of osteoclasts, precursor cells sequentially express c-Fms (M-CSF receptor) followed by RANK [72]. M-CSF and RANKL produced by osteoblasts appear to play an important role in the proliferation and differentiation of osteoclast progenitors [73]. Osteoblasts are derived from undifferentiated mesenchymal stem cells present in the bone marrow, which further differentiate into osteocytes and are embedded in the calcified bone (Figure 3) [74]. The interaction between RANKL and RANK stimulates osteoclast formation and differentiation by activation of several transcription factors that regulate osteoclastogenesis [75, 76].

Molecular biological research has enhanced our understanding of the mechanism of bone resorption. This process is controlled by a system comprised of three key proteins: the RANK, RANKL, and OPG. These proteins mediate bone remodeling and disorders of mineral metabolism in humoral hypercalcemia of malignancy. RANK, RANKL, and OPG are members of the TNF/TNFR superfamily. Several studies have established a consistent relationship between the RANK/RANKL/OPG pathway and skeletal lesions related to disorders of mineral metabolism [29, 30]. The recognition of the RANK/RANKL/OPG system and its interaction with various cytokines and calciotropic hormones in the regulation

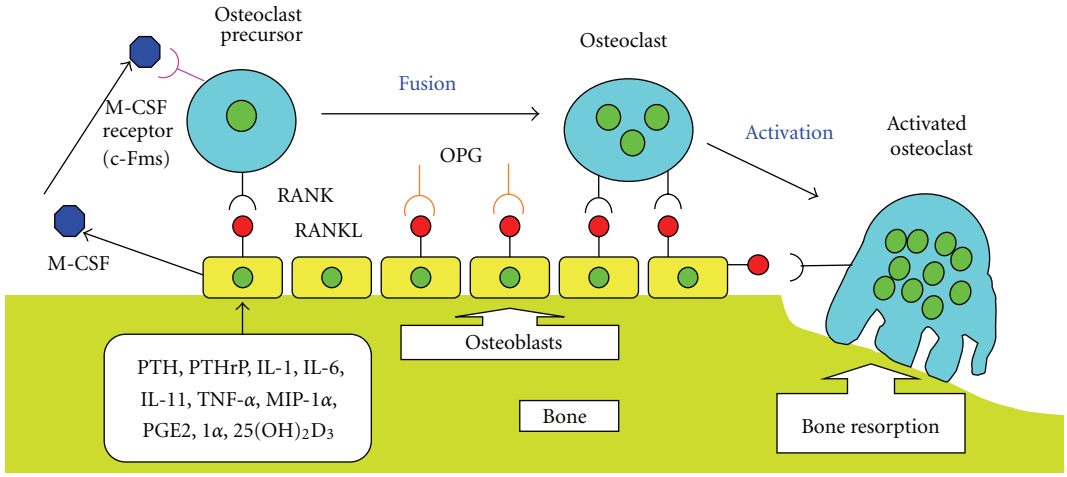

Figure 4: Molecular mechanism of osteoclast differentiation and activation involving the RANKL/RANK/OPG system. Bone remodeling is a balance between formation and resorption through the control of osteoblast and osteoclast activities. Receptor activator of nuclear factor-κB ligand (RANKL), receptor activator of nuclear factor-κB (RANK), and osteoprotegerin (OPG) play important roles in bone remodeling and disorders of mineral metabolism. Bone resorbing factors, such as PTH, PTHrP, IL-1, IL-6, IL-11, and $1\alpha,25(OH)_2D_3$, act on osteoblasts to induce the membrane associated factor called RANKL, which recognizes RANK present on the surface of osteoclast progenitors and osteoclasts. M-CSF is an essential factor for osteoclast proliferation and differentiation, which is produced by osteoblasts in osseous tissue. Reaction of OPG with RANKL inhibits the binding of osteoclast precursors and osteoclasts to RANKL, therefore, OPG acts as a decoy receptor in the RANKL/RANK interaction. Blue structure: M-CSF (macrophage-colony stimulating factor), pink structure: M-CSF receptor (c-Fms), black structure: RANK (receptor activator of nuclear factor-κB), red structure: RANKL (RANK ligand), that is, osteoclast differentiation factor, orange structure: OPG (osteoprotegerin), that is, osteoclastogenesis inhibitory factor.

of osteoclastogenesis have led to further understanding of the mechanism underlying the bone remodeling process in ATL with hypercalcemia.

RANK was discovered by direct sequencing of cDNA from a human bone marrow-derived myeloid dendritic cells [77]. Sequencing of the RANK gene showed it to be a type I transmembrane glycoprotein and also a member of the TNFR family. RKNKL is a TNF-related cytokine expressed by various bone cells including osteoblasts and their immature precursors [78]. Importantly, the target cells of bone resorbing hormones and cytokines are osteoblasts rather than osteoclast progenitors. The expression of RANKL in human and murine osteoblasts is stimulated by various cytokines (IL-1, IL-6, IL-11, MIP-1α, and TNF-α), and calciotropic hormones including PTH, PTHrP, $1\alpha,25(OH)_2D_3$, and prostaglandin E2 (PGE2) [32, 74, 79, 80]. These are also thought to be important in enhancing the migration and differentiation of osteoclast progenitors into mature osteoclasts by stimulating RANKL production by osteoblasts and stromal cells [30]. PTHrP, IL-11, and PGE2 are most important factors in osteoclast differentiation, which results in RANNKL interaction with the surface of immature osteoblasts [30]. Increased production of RANKL by osteoblasts leads to osteoclast differentiation, resulting in increased bone resorption.

The decoy receptor OPG with RANKL is also thought to be a key mechanism in the control of bone turnover (Figure 4). OPG was first identified by sequence homology to the TNFR family [81]. OPG is a soluble a glycoprotein secreted by various mesenchymally derived cells such as osteoblasts and bone marrow stromal cells [82]. Reaction of OPG with RANKL inhibits the binding of osteoclast precursors and osteoclasts to RANKL. Therefore, OPG is produced by osteoblasts and acts as a decoy receptor by binding at high affinity to RANKL, therefore, preventing the interaction with RANK [81, 83, 84]. As a consequence of binding to RANKL, OPG acts as an effective inhibitor of osteoclast proliferation, differentiation, activation, and survival, and therefore, it inhibits bone resorption, resulting in bone protection [83]. In this regard, various metabolic regulators modulate OPG expression and secretion by osteoblasts/stromal cells. These include IL-1, TNF-α, and TGF-β, which increase OPG secretion while various stimulators of bone resorption, such as PTH, PGE2, and $1\alpha,25(OH)_2D_3$ reduce its secretion [78, 79, 85].

6. Molecular Mechanisms of Hypercalcemia in ATL

As discussed above, HTLV-1 is the causative factor of ATL, and patients with ATL often exhibit humoral hypercalcemia of malignancy [86], which is induced by PTHrP and cytokines, such as IL-1, IL-6, TGF-β, and MIP-1α [65, 87–94]. About 70% of ATL patients develop hypercalcemia throughout the clinical course [38]. Overexpression of the RANKL gene correlates with hypercalcemia in ATL. *In vitro* studies have shown that ATL cells obtained from patients with hypercalcemia, which overexpress RANKL gene transcripts, induced the differentiation of human hematopoietic precursor cells into osteoclast in the presence of M-CSF. In contrast, ATL cells from patients with normal serum calcium levels did not induce such differentiation, suggesting

that the expression of the RANKL gene in ATL cells is involved in the induction of differentiation of these cells. These results suggest that ATL cells induce the differentiation of the hematopoietic precursor cells to osteoclast through RANKL expressed on their surface, in cooperation with M-CSF, and ultimately cause hypercalcemia [29]. In ATL patients with metastasis and hypercalcemia, activation of the MIP-1α, TNF-α, IL-1, and IL-6 molecules is induced by Tax-stimulated NF-κB activation [93, 94]. Interestingly, RANKL induces osteoclast formation through the NF-κB signaling pathway, which is critical for osteoclastogenesis. Animals lacking both the p50 and p52 subunits of NF-κB develop severe osteopetrosis [95]. These putative steps in the pathogenesis of disease are supported by evidence derived from tissue culture experiments, xenograft mouse models, and clinical observations in patients [96–100]. Moreover, amino acid sequences homologous to gp46-197 were found in the carboxyl-terminal half of OPG. Administration of the gp46-197 peptide reduced bone mineral density and significantly increased serum calcium levels. The central region of HTLV-1 gp46 acts as an antagonist for OPG and promotes the development of hypercalcemia [101]. HTLV-1 infected cells were found to deregulate the expression of OPG in osteoblast precursors [102]. Ectopic expression of the HTLV-1 basic leucine zipper factor was sufficient to activate Dickkopf-1 transcription in an HTLV-1 infected and uninfected T-cell line [103]. It is possible that HTLV-1 basic leucine zipper factor activates Dickkopf-1 expression at some stage of ATL, thus, indirectly facilitating changes in RANKL and OPG expression, and contributing to the accelerated bone resorption associated with ATL [103].

Conflict of Interests

The authors declare no conflict of interests.

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Review Article

Is There a Role for HTLV-1-Specific CTL in Adult T-Cell Leukemia/Lymphoma?

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ATLL is an aggressive malignancy of T cells that affects about 5% of individuals infected with HTLV-1. The precise mechanism of oncogenesis is not known, but there is evidence that two regulatory viral proteins, Tax and HBZ, are involved. A high set point proviral load is associated with development of ATLL or a chronic inflammatory condition, HAM/TSP. Several lines of evidence, including HLA class 1 association studies and in vitro killing assays, indicate that cytotoxic T lymphocytes are instrumental in determining this proviral load set point. Prior studies have focused chiefly on the CTL response to the immunodominant Tax protein: efficient lysis of Tax-expressing cells inversely correlates with proviral load in nonmalignant infection. However, a recent study showed that strong binding of peptides from HBZ, but not Tax, to HLA class 1 molecules was associated with a low proviral load and a reduced risk of developing HAM/TSP, indicating an important role for HBZ-specific CTL in determining infection outcome. In comparison with nonmalignant infection, HTLV-1-specific CTLs in ATLL patients are reduced in frequency and functionally deficient. Here we discuss the nature of protective CTL responses in nonmalignant HTLV-1 infection and explore the potential of CTLs to protect against ATLL.

1. Introduction

Human T-cell lymphotropic virus-1 (HTLV-1) is a retrovirus which predominantly infects $CD4^+$ T cells, where it is reverse transcribed and integrates into host DNA. The integrated provirus can then disseminate by de novo infection of T cells via the virological synapse, or by inducing clonal expansion of the host cell. Most infected individuals do not experience any symptoms, and HTLV-1-associated disease is rarely observed in individuals with a proviral load of less than 1% of their peripheral blood mononuclear cells (PBMCs) [1]. Approximately 2–6% of individuals HTLV-1 develop adult T-cell leukemia/lymphoma (ATLL), and a slightly lower percentage suffer from inflammatory disorders, including HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP). ATLL is a highly aggressive T-cell malignancy with a poor prognosis, and, even with standard treatment, the median survival time for clinically acute forms of the disease is measured in months [2, 3]. Chemotherapeutic intervention has had limited efficacy despite the development of drugs to specifically target ATLL cells, though some improvement has been reported combining antiviral drugs (zidovudine) and immunomodulators (such as type-1 interferon) [3–5]. Allogeneic hematopoietic stem cell transplantation (HSCT) has also improved survival [6], though may not be a viable treatment option in all cases due to the advanced age of most ATLL patients and the lack of suitable donors [2]. Thus, the ability to harness the host immune system to control ATLL would be an attractive proposition.

Leukemic cells carry at least one copy of the provirus, and express CD4, the IL-2 receptor alpha chain CD25, and typically exhibit a flower-like multilobed nucleus and genetic abnormalities. Downregulation of CD3 and CD7 on leukemic cells has been described [7], and several groups have detected FoxP3 protein in ATLL cells [7–9], although the degree of expression of FoxP3 varies between patients. There is conflicting evidence on whether FoxP3-expressing leukemic cells have regulatory capacity [9, 10]. ATLL is also characterized by uncontrolled expansion of T cells which

share a common T-cell receptor-Vβ chain, implying that the disease is a result of malignant transformation and expansion of a small number of infected cells. High-throughput quantitative sequencing of the site of proviral integration reveals that the bulk of the proviral load in ATLL patients is composed of highly abundant clones with a small number of unique proviral integration sites. This contrasts with asymptomatic carriers (ACs) of the virus and patients with HAM/TSP, whose infected cells consist of a mixture of T-cell clones carrying many unique integration sites with varying abundance in the peripheral blood [11].

$CD8^+$ cytotoxic T lymphocyte (CTL) recognition of viral peptides presented in the context of the human leukocyte antigen (HLA) class 1 (major histocompatibility (MHC) protein class 1) triggers cytokine production by the CTL and lysis of the infected cell. The affinity and stability of the interaction between a given TCR and peptide-MHC complex must exceed a threshold level to allow initial activation of the T cell and to induce clonal expansion to form effector and memory T cells. The nature of a CTL response to its cognate antigen can subsequently be modulated by cell-intrinsic and cell-extrinsic factors, which include the concentration of peptide-MHC complexes on the target cell surface, the length of time which has passed since the CTL has encountered antigen, expression of the peptide-MHC-TCR-stabilizing CD8 co-receptor, inhibition by regulatory T cells, and the cytokine microenvironment. Thus, the efficiency of CTL control is determined by a combination of the host genotype and environmental factors.

HTLV-1 antigens are expressed in vivo and induce a CTL response. In particular, the viral transcription factor Tax is widely recognized by CTLs, and abundant activated Tax-specific CTL can be detected in most infected individuals [12–15]. Tax is expressed early in the life cycle of the virus (ex vivo data) and can induce proliferation of infected cells, but also expression of other viral genes, and contains several well-characterized CTL epitopes. CTL epitopes and CTLs specific for other viral genes have been described; though Tax is immunodominant [12, 16], efficient recognition of other subdominant target antigens appears to be more important in protection: see what follows.

Little is known about the role of HTLV-1-specific CTL in preventing or resolving ATLL. There is anecdotal evidence that immune control of ATLL is achievable: spontaneous regression of ATLL is sometimes observed. One report documents such a patient, in which regression coincided with acquisition of an ability of their PBMCs to kill an ATLL cell line in vitro. Subsequent disease progression was accompanied by a decline in the ability of their PBMC to lyse ATLL cells [17]. Furthermore, there have been several reports of ATLL emergence in $HTLV\text{-}1^+$ individuals after immune suppressive treatment during liver [18] and renal [19] transplantation, indicating the importance of continuous immune surveillance in infected individuals in suppression of ATLL development.

In this paper, we review

(i) what is known about the HTLV-1-specific CTL response in ATLL patients;

(ii) properties of HTLV-1 specific CTLs in patients who have a low proviral load;

(iii) the role of HLA class 1 genes in CTL control of HTLV-1;

(iv) expression of CTL target antigens by ATLL cells.

2. HTLV-1-Specific CTL Response in ATLL Patients

Few data have been published describing the functional properties of CTLs in ATLL, but all reports agree that the ex vivo CTL response to HTLV-1 antigens is weak in these patients [20]. Using a panel of peptide-MHC tetramers Kozako et al. found that, in comparison with asymptomatic individuals, Tax-specific CTLs in ATLL patients were directed at a narrower range of epitopes and present at lower frequencies or were undetectable ex vivo [21]. Env-specific CTLs were also detected but were present at low frequencies and only in asymptomatic carriers. Expression of the programmed death-1 (PD-1) receptor, which delivers inhibitory signals to the CTL, was slightly higher on virus-specific CTLs in ATLL patients than in ACs [22]. A subset of ATLL patients have no detectable CTL when PBMCs are assayed directly ex vivo. After culture, however, functional CTL are sometimes observed [23, 24]: efficient killing of ATLL cells can be demonstrated by $CD8^+$ T cells after being cultured in vitro for a week in the presence of IL-2 and a polyclonal activator or stimulated with HLA-matched infected cells [23, 24].

In peripheral blood of acute ATLL patients, the lymphocyte population consists predominantly of leukemic $CD4^+CD25^+$ cells. Careful analysis revealed that although the ratio of $CD4^+$: $CD8^+$ T cells is grossly elevated, the absolute number of $CD8^+$ T cells detected in peripheral blood is similar to the normal range or reduced by a factor of two [20, 23]. Thus, depletion of the $CD8^+$ T-cell subset does not explain the reduced CTL activity observed. A more likely candidate mechanism to explain this phenomenon is suppression by regulatory T cells. Uninfected $FoxP3^+$ T cells with regulatory activity are significantly expanded in ATLL patients, and lysis of autologous-infected cells by uncultured CTLs was inversely proportional to the frequency of $FoxP3^+$ cells [9]. It is also possible that competition from ATLL cells for resources such as growth factors and cytokines, for example, IL-2, disrupts the antiviral CTL response.

Despite the observed deficiency in the CTL response to HTLV-1 in ATLL, there is evidence for an efficient immune response in a subset of ATLL patients. In three individuals with ATLL, Furukawa et al. observed an amino acid change within the CTL epitope Tax [11–19] which rendered Tax unrecognizable to CTLs specific for the consensus epitope [25]. In addition, premature stop codons within the *tax* gene which abrogate its transactivating activity were observed in another ATLL patient. Familial analysis revealed that the mutation emerged after viral transmission [25], strongly implying that immune selection favoured the variant genes. Loss of Tax expression or activity has the downstream effect of reduced expression of other viral genes such as *gag*, *pol,* and *env* which are transactivated by Tax. Thus, by ablating

Table 1: HBZ and Tax as CTL target antigens: genetics, expression, and antigenicity in ATLL.

	Tax	HBZ
Naturally occurring genetic and epigenetic changes in ATLL		
Point mutations	Detected in Tax 11–19 A*0201 epitope [25]	None described
Deletions	Premature stop codons [26] Deletions of 5′end of genome [25, 27, 28]	None described
Epigenetic silencing	Hypermethylation of 5′LTR [46, 59, 60] and pX region [59]	None described
Expression in ATLL		
In vivo	mRNA and protein low/undetectable [45, 46]	Multiply spliced isoforms of mRNA detected [51, 52]
Ex vivo cultures	Increased mRNA transcription in vitro culture [54] Protein detected in 50% of ATL cases [44]	Increased mRNA transcription after culture in vitro [54] Protein at threshold for detection using currently available antibodies [53]
CTL response in AC and HAM/TSP		
CTL response in vivo	High frequencies Tax-specific CTL detected in 66–94% infected individuals [12, 15, 43]	Low frequencies of HBZ-specific CTL detectable in 25–40% of individuals [42, 43]
Immunogenicity for CTL	Immunodominant, HTLV-1 protein most frequently recognized by CTL [12–14]	Peptides bind weakly to HLA class-1 in general [42] Highly sensitive, cytotoxic CTL which recognise HBZ 26–34 in the context of A*0201 can be generated in vitro [53]
Potential for protection in ATLL	Enhanced ability to present Tax peptides does not confer a significant protective effect [42] but can lyse ATL cells expressing Tax in vitro [23, 24]	Efficient presentation of HBZ peptides significantly associated with low PVL and remaining asymptomatic [42]

a single viral protein, the infected cell may also escape surveillance by CTL specific for other viral proteins. This *tax* mutation was not restricted to ATLL patients, but was also detectable in a minor population of infected cells in ACs and patients with HAM/TSP [26]. There is further evidence of large deletions within the HTLV-1 genome in dominant ATLL clones. In particular, the 5′-long terminal repeat (LTR) region, which encodes the promoter that drives expression of the *tax* gene, is deleted in 28% of ATLL patients, compared with 4% ACs [25, 27, 28]. It is less clear whether these large deletions are due to immune selection or to an inherent genetic instability of the leukemic cells (see Table 1).

3. CTL Control of HTLV-1 Proviral Load

Analysis of HTLV-1-specific CTL responses in individuals who do not develop disease may provide important clues as to the character of a desirable CTL response in ATLL. In cases of nonmalignant infection, proviral load can vary by up to 1000-fold between individuals, though within an individual the load usually remains stable over years of infection. In addition, ACs with a viral load above 1% are significantly more likely to develop inflammatory disease [1, 29]. Thus, we identify a successful immune response as one that results in a low proviral load set point. Many methods have been employed to quantify the frequency of HTLV-1-specific CTLs: primarily identifying cells by functional assays or directly staining reactive TCRs using fluorochrome-labelled peptide-MHC tetramers. However, simply measuring the frequency of HTLV-1-specific CTLs has revealed a positive, negative, or zero correlation with proviral load in different studies [30–32]. This is not an unexpected finding in persistent infection [33], where chronic antigen exposure can drive proliferation of CTLs which are not protective and may even be detrimental in the case of HTLV-1-associated inflammatory disorders. Thus, the absolute frequency of HTLV-1-specific CTLs may not be informative, and alternative methods of quantification of CTL efficiency are desirable.

We have developed a method to quantify the efficiency with which uncultured $CD8^+$ cells suppress Tax expression in autologous infected $CD4^+$ T cells in a perforin-dependent and MHC class-I-restricted manner. $CD8^+$-depleted PBMCs are mixed with autologous $CD8^+$ T cells at a range of effector: target ratios and cocultured overnight, following which the number of Tax-expressing cells is enumerated by flow cytometry, and the rate of elimination of Tax-expressing cells is estimated by using nonlinear regression to fit a mathematical model [34]. Using this assay, we have found that the rate of elimination of Tax-expressing cells is inversely proportional to proviral load, suggesting that a more efficient CTL response has the effect of lowering proviral load [34]. The CTL efficiency parameter measured by this assay could explain up to 50% of the observed

variation among infected individuals in proviral load [34]. Although the precise epitope specificity of CTLs responsible for elimination of Tax-expressing cells is not defined in this assay, we also observed that the rate of infected cell elimination is proportional to the sensitivity with which Tax-specific CTLs detect peptides presented on target cells and that naturally infected cells expressing high levels of Tax protein are killed significantly faster than cells expressing lower levels of Tax [35]. These observations indicate that Tax-specific CTLs can eliminate autologous cells which begin to express the provirus. This is not just a phenomenon observed in cells cultured in vitro: in vivo, $CD4^+CD45^+RO$ cells (which comprise most Tax-expressing cells) proliferate faster in infected individuals than in uninfected individuals and tend to die faster in HTLV-1-infected people [36]. Taken with the observation that, in ACs and patients with HAM/TSP, proviral load is relatively stable over years of infection [11], these data are consistent with the existence of a dynamic equilibrium between proviral expression and CTL surveillance, in which the per-cell efficiency of CTLs is a major determinant of the proviral load set point.

4. HLA Class 1 and the Immune Response to HTLV-1

Some of the most powerful and compelling evidence for the important role of CTLs in controlling HTLV-1 comes from genetic studies. In 1999, Jeffery et al. provided the first definitive evidence that HLA class 1 genes can be protective in HTLV-1 infection [37]. They observed that asymptomatic individuals who possessed HLA-A*02 alleles had a proviral load which was approximately one third that of individuals who lacked HLA-A*02. In addition, the chance of developing HAM/TSP was halved in HLA-A^*02^+ individuals. HLA-A*02 is a common and highly polymorphic allele, present at high frequencies in most populations. HLA-A*0201 binds the Tax 11–19 epitope with extremely high affinity, and up to 10% of circulating CTLs can be specific for this epitope [38]. In ATLL, Yashiki et al. reported that the HLA class 1 alleles HLA-A*26, HLA-B*4002, HLA-B*4006, and HLA-B*4801 were more abundant in individuals who develop ATLL in comparison with AC [39]. They hypothesize that this is due to a reduced ability to bind and present Tax peptides, due to the inability of these alleles to bind anchoring residues in peptides derived from Tax [39].

Although Tax is both expressed and frequently targeted by CTL in HTLV-1 infection, a recent genetic study has shifted the focus of attention to another viral protein: HTLV-1 basic leucine zipper protein (HBZ) [40]. HLA association studies are hampered by the heterogeneity of HLA class 1 alleles in a population. Thus, for rare alleles that may be protective, large numbers of participants are required in a given study for associations to reach statistical significance, and the results may only apply to certain populations. Also, association studies do not reveal the precise mechanism of protection these alleles confer. As an alternative approach, MacNamara et al. conducted a systematic study that tested the capacity of an individual's HLA alleles to bind HTLV-1 peptides and asked whether this metric correlated with the proviral load and the "risk" (i.e., relative prevalence) of HAM/TSP. They used experimentally validated epitope prediction software [41] which predicted the affinity of binding of peptides derived from the HTLV-1 genome to HLA-A and -B alleles in a cohort of 202 ACs and 230 patients with HAM/TSP [42]. They found that the known protective alleles A*0201 and C*0801 bound peptides from HBZ with significantly higher affinity than alleles which were associated with disease progression (B*5401). Further analysis showed that in general ACs had HLA alleles which bound peptides from HBZ significantly more strongly than patients with HAM/TSP, and this was not attributable simply to A*0201, C*0801, and B*5401. The more alleles an individual possessed that strongly bound peptides from HBZ, the lower their proviral load. Interestingly, despite the strong protective effect of effective HBZ presentation, peptides from HBZ bound to class 1 molecules with significantly lower affinity than peptides from Tax. These data were unexpected but robust, and although CTL specific for HBZ could only be detected at low frequencies in 25–40% of infected individuals [42, 43], a CTL clone specific for HBZ could efficiently kill HLA-matched primary HTLV-1-infected $CD4^+CD25^+$ cells, indicating that peptides derived from HBZ are presented by naturally infected cells [42].

5. Expression of CTL Target Antigens by ATLL Cells

Viral antigen expression by ATLL cells in vivo is of course the critical determinant of their susceptibility to lysis by HTLV-1-specific CTL (see Table 1). Although the Tax protein is strongly expressed in cultured cells of ~50% of ATLL cases [44], Tax mRNA is typically undetectable or present at extremely low copy number in peripheral blood [45, 46]. Despite this, ATLL cells which were immediately fixed ex vivo were sufficient to induce a Tax-specific CTL response when used to immunize rats [44]. These data suggest that CTLs are more sensitive than antibody staining in detecting protein expression and lack of detection of mature protein expression does not preclude the presentation of peptides in vivo. A rat model of ATLL in which HTLV-1-transformed cell lines are adoptively transferred into an immunodeficient host has generated data which supports a role of Tax-specific CTL response in prophylaxis against disease development. Immunization with a DNA vaccine encoding full-length Tax protein or with Tax oligopeptides protects against development of a lymphoproliferative disorder in this model [47, 48]. Similarly, adoptive transfer of Tax-specific CTLs was also sufficient to prevent disease [47]. Interestingly, epitopes from Tax are also presented and targeted by newly expanded CTLs in vivo in ATLL patients who have undergone successful HSCT [49, 50].

HBZ is emerging as an alternative viral target antigen in ATLL. Uniquely among HTLV-1 transcripts, several variably spliced isoforms of HBZ mRNA are readily detectable in vivo in all HTLV-1^+ individuals tested [51]. The negative strand of the 3′ genomic region of HTLV-1, from which

HBZ is transcribed, is uniformly conserved in all ATLL cases [28, 52], implying an essential role of HBZ in ATLL persistence, but also making it an attractive target for immunotherapy. The ratio of HBZ mRNA to Tax mRNA is significantly higher in ATLL patients than in patients with HAM/TSP or ACs [51] which may be accounted for by the silencing of Tax by mutation, deletion, or methylation of the Tax-encoding DNA frequently observed in ATLL. Like Tax, however, expression of HBZ protein is usually undetectable in HTLV-1$^+$ PBMCs directly ex vivo. Currently available antibodies reveal extremely low expression of HBZ in cultured, naturally infected cells [53]. Evidence from several groups suggests that HBZ protein is likely to be expressed at lower levels than Tax [53] (Rowan, Bangham, unpublished observations), and the majority of the HBZ mRNA is retained in the nucleus [54], which may inhibit its translation. Suemori et al. performed an elegant study which tested the activity of an in vitro generated CTL clone specific for HBZ 24–36 in the context of A*0201. They showed that sensitive, efficient CTLs specific for HBZ could be generated, at least in A*0201$^+$ individuals, despite the low binding affinity of HBZ peptides for HLA class 1. These cells could lyse HLA-matched HBZ-transfected or peptide-loaded cells, but not freshly isolated ATLL cells in vitro [53]. More testing is needed to ascertain the contribution of HBZ-specific CTLs in controlling proviral load in vivo, and the rat model of leukemogenesis provides one possible system in which to address this question.

6. Discussion

It is clear that the CTL response is crucial in maintaining a low proviral load in nonmalignant cases of HTLV-1 infection. It is also evident that the CTL response to HTLV-1 proteins in ATLL is somewhat lacking, with a reduced frequency of HTLV-1-specific CTLs, a reduced number of viral epitopes targeted, and CTL functional defects. Enhancing weak CTL responses could be a potentially effective, specific method for controlling ATLL: the epitopes are expressed only by infected T cells, and efficient HTLV-1-specific CTLs can provide surveillance for the lifetime of the host; this could be achieved by therapeutic vaccination. In addition, induction or enhancement of HTLV-1-specific CTLs in people at risk of developing ATLL could prevent disease development. Key considerations in designing a vaccine include the choice of antigen, method of delivery, and the need to surmount whatever mechanisms result in a weak HTLV-1-specific CTL response in individuals who develop ATLL. Tax and HBZ are emerging as the key target antigens to be included in a CTL-directed vaccine. Interestingly, both Tax and HBZ have been implicated in the initiation of leukemogenesis, reviewed in [55, 56], and inclusion in a putative vaccine would require functional inactivation of both of these proteins. In fact, several vaccine constructs designed to induce Tax-specific CTLs have already been successfully tested in animal models [57, 58]. HBZ, though less well characterized, plays an indispensable role in maintenance of ATLL cells [52], and the region of the HTLV-1 genome which encodes HBZ appears to be essential as it is preferentially protected from deletion. Since HBZ is constitutively expressed in vivo and ranks as the most protective CTL target antigen in nonmalignant infection, targeting HBZ could be the most important component of a vaccine, particularly for ATLL. It seems logical to target both proteins in order to exploit both the antigenicity of Tax and the potentially highly protective properties of HBZ-specific CTLs. Targeting epitopes from other HTLV-1 proteins may also be beneficial in ATLL; however, no experimental data has been published which supports or indeed refutes this. Finally, it may be advantageous to consider combining therapeutic vaccination with chemotherapeutic ablation of ATLL cells, to increase the effective CTL: target ratio, enabling the CTLs to regain control of the malignancy.

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Review Article

Molecular and Cellular Mechanism of Leukemogenesis of ATL: Emergent Evidence of a Significant Role for HBZ in HTLV-1-Induced Pathogenesis

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Adult T-cell leukemia (ATL) is a leukemia derived from mature $CD4^+$ T cells and induced by human T-cell leukemia virus type 1 (HTLV-1) infection. Previous studies have revealed many possible molecular and cellular mechanisms of HTLV-1-induced leukemogenesis, but it still remains unknown how HTLV-1 transforms peripheral CD4 T cells in infected individuals. Given the fact that only 2–5% of infected individuals develop ATL, HTLV-1 infection alone is not sufficient for the transformation of infected cells. Host genetic and epigenetic abnormalities and host immunological status should be considered in attempting to understand the mechanism of the oncogenesis of ATL. Nonetheless, it is obvious that HTLV-1 infection dramatically increases the risk of leukemia generation from peripheral CD4 T-cells, in which the incidence of leukemia is quite low. Furthermore, the evidence that all ATL cases retain the HTLV-1 provirus, especially the 3′ region, indicates that HTLV-1-encoded genes play a critical role in leukemogenesis. Since increasing evidence indicates that the *HTLV-1 bZIP factor (HBZ)* gene plays a significant role in the pathogenesis of HTLV-1, we will discuss the cellular and molecular mechanism of ATL generation from the virological point of view, particularly focusing on HBZ.

1. Introduction

Human T-cell leukemia virus type 1 (HTLV-1) is a complex retrovirus that infects approximately 10 to 20 million people worldwide [1]. In the late 1970s, adult T-cell leukemia (ATL) was identified as a distinct clinical entity based on its clinical and geographical features, suggesting an association with unknown infectious agents [2]. Thereafter, HTLV-1 was identified in a cell line derived from a patient with cutaneous T-cell leukemia in 1980 [3]. HTLV-1 has been shown to immortalize human T-lymphocytes *in vitro* [4]. In addition, HTLV-1 infection also induces chronic inflammatory diseases, such as HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) [5, 6], HTLV-1-associated uveitis [7], and HTLV-1-associated lung diseases [8]. The entire HTLV-1 sequence was determined [9] and various approaches were used to elucidate the pathogenesis of the virus. However, over 30 years after the discovery of HTLV-I, it is still not fully understood how HTLV-1 transforms mature CD4 T cells. Recent studies have provided emerging evidence of the significance of HBZ in HTLV-1 pathogenesis. In this review, we discuss the present understanding of HTLV-I infection from the virological aspect particularly via focusing on the role of the HBZ gene.

2. The Strategy of Replication in HTLV-1

Once a retrovirus enters into host cells, the RNA genome is reverse-transcribed into a double-stranded DNA form, which is then integrated into the host chromosomal DNA by the viral integrase. The integrated viral DNA expresses viral genes to produce infectious virions. There are two retroviral replication patterns: *de novo* infection from infected cells to

uninfected cells and clonal expansion of infected host cells [10]. Both free viral particle- and cell-to-cell-mediated *de novo* infection require expression of viral structural proteins and assembly of the viral particle. The expression of Tax enhances the transcription of viral structural genes from the plus strand of HTLV-1 in this situation (Figure 1). HTLV-1 has been reported to spread not via free viral particles but via cell-to-cell transmission through the virological synapse [11]. A recent study showed that the biofilm-like extracellular structure of infected cells plays a role in this cell-to-cell transmission of HTLV-1 [12]. This type of viral spread is thought to contribute to initial establishment of a population of infected cells. However the cells expressing Tax could be eliminated by the host CTL (cytotoxic T lymphocyte) response after establishment of host immunity against HTLV-1. After the establishment of anti-HTLV-1 immunity, HTLV-1 replicates predominantly using the second form of replication, clonal expansion of infected cells [13, 14]. In order to escape from the host immunity, HTLV-1 replicates as a provirus by increasing the number of infected host cells. In this phase, survival of HBZ-expressing cells is enhanced by the low immunogenicity of HBZ [15], which could be explained at least in part by the weak binding activity of HBZ peptide to MHC molecules [16].

In the chronic phase of HTLV-1 infection, proviral load becomes stable in most infected individuals; yet there is a broad range of variation of proviral load among infected individuals. Since the variation of HTLV-1 sequence among infected individuals is very limited, host genetic factors including MHC class I molecules are thought to be important determinants of proviral load. Previously Tax has been considered as the most important antigen for the host immune response that controls proviral load [17], but recent evidence concerning lately identified viral protein HBZ has shifted the focus of research. The finding has suggested that individuals who possess MHC alleles which can efficiently bind and present peptides from HBZ have significantly lower proviral load, and are less likely to develop HAM/TSP [16], suggesting that HBZ expression is a critical determinant of viral persistence in chronic phase of HTLV-1 infection. Consistent with this idea, HBZ expression is constitutively detectable whereas Tax expression is frequently suppressed or diminished in ATL cells [18], which could be considered as the most highly expanded clone among many different HTLV-1-infected clones within an infected individual [19]. Even though HBZ might play an important role in viral persistence, *in vivo* persistence of HTLV-1 is decreased by mutation of other accessory genes, such as *p12*, *p13*, and *p30* [20–25], indicating that viral replication and proliferation of infected cells is controlled by these regulatory and accessory genes in harmony.

In summary, previous findings seem to be consistent with the theory that the initial expansion of HTLV-1 infection is due to *de novo* infection, which is driven by Tax, transactivator of HTLV-1 5′LTR. Once the host immune response to HTLV-1 has been established, HTLV-1 propagates in the host mainly by clonal expansion of infected cells via expressing HBZ, a viral antigen with low immunogenicity, as described previously [15].

3. The Host Cell of HTLV-1

HTLV-1 has the potential to infect various cell-types such as T cells, B cells, macrophages, and dendritic cells (DCs) [26], but HTLV-1 can induce clonal expansion and transformation almost exclusively in CD4 T cells [10]. The mechanism underlying why HTLV-1 expands and transforms CD4 T-cell population needs to be uncovered. CD4 T-cells are generally partitioned into two subsets, effector T cells and regulatory T cells. The former plays crucial role in immune response by secreting cytokines that promote and activate immune systems, whereas the latter has been considered to suppress excessive immune responses to maintain the homeostasis of the immune system. Since the differentiation, function, and homeostasis are quite different between these two T-cell subsets, it is of great importance to consider the characteristics of each subset in order to understand how HTLV-1 utilizes and affects these CD4 T-cell subsets.

3.1. HTLV-1 Infection in Effector CD4 T Cells. To exert the function as effector T cells, naive T cells need to encounter their antigens, be activated, and be converted into effector/memory T cells. Previous reports have demonstrated that HTLV-1 infection is more frequently detected in effector/memory CD4 T cells than in naïve CD4 T cells [27, 28]. There is no compelling evidence to explain this tendency. We would propose three possible explanations as follows.

(i) High Susceptibility to De Novo Infection. De novo infection of HTLV-1 is achieved mainly by cell-to-cell infection, which is initiated by LFA-1-ICAM-1 interaction between infected cell and uninfected cells [29]. Since the expression level of LFA-1 and ICAM-1 in effector/memory CD4 T-cells is higher than that in naïve CD4 T cells [30], effector/memory CD4 T cells are likely to be more susceptible to *de novo* cell-to-cell infection than naïve CD4 T cells.

(ii) High Proliferative Capacity. Effector/memory CD4 T cells proliferate faster than naïve CD4 T cells *in vivo*. *In vivo* labeling of lymphocyte using deuterium-labeled glucose has shown that the doubling time of effector/memory CD4 T cells is 28 days, which is much shorter than the doubling time of naïve CD4 T cells, 199 days [31]. Long-term survival, a hallmark of memory CD4 T cell clones, also could contribute to the maintenance of HTLV-1 *in vivo*. Therefore HTLV-1 infection in effector/memory CD4 T-cells is beneficial to clonal expansion of infected cells. Furthermore, HTLV-1-infected effector/memory CD4 T cells are reported to proliferate significantly faster than uninfected cells *in vivo* in HTLV-1 infected individuals [32].

(iii) Enhancement of the Differentiation from Naïve to Effector/Memory CD4 T Cells. Little is known about the impact of HTLV-1 infection on CD4 T-cell differentiation, because few studies have been focused on the effect of viral gene expression on T-cell differentiation to date. We have recently reported that the proportion of effector/memory CD4 T cells was increased in HBZ-transgenic (HBZ-Tg) mice [33],

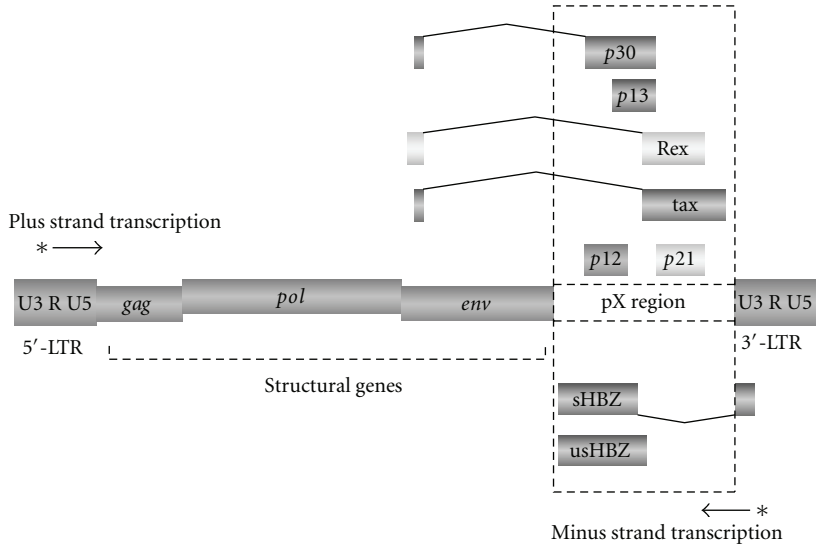

Figure 1: The structure of HTLV-1. HTLV-1 encodes accessory and regulatory genes in the pX region as well as viral structural genes.

suggesting that HBZ expression can drive the differentiation from naïve T cells to effector T cells or enhance cell proliferation more strongly in effector/memory T cells than naïve T cells.

HTLV-1 utilizes this effector CD4 T-cell population as a host cell. It follows that this may induce the dysregulation of helper and effector function, contributing to the viral persistence.

3.2. HTLV-1 Infection of Regulatory CD4 T Cells. $CD4^+CD25^+FoxP3^+$ regulatory T cells (Tregs) have been identified as one of the major immunoregulatory mechanisms which prevent autoimmune disease [34]. Tregs are also involved in the downregulation of specific immune responses during infectious diseases. It has been reported that the frequency of Tregs is elevated in chronic viral infection, such as hepatitis C virus (HCV) [35]. The increased frequency of Tregs may help to prevent immune pathology but on the other hand may facilitate viral persistence. Indeed, the frequency of $CD4^+FoxP3^+Tax^-$ T cells is inversely correlated with HTLV-1 specific CTL response, which could explain the variation of CTL response among infected people [36]. In addition to this general role of Tregs in chronic viral infection, there is a unique role of Tregs in HTLV-1 infection due to direct infection of CD4 T cells by HTLV-1, which include both progenitor Treg cells and Treg cells. The frequency of HTLV-1 infection in $CD4^+FoxP3^+$ cells is higher than other T-cell population [36], which we suggest maybe due to the following.

(i) High Susceptibility to De Novo Infection. Treg cells are known to contact with DCs frequently [37], which could increase the chance of *de novo* infection between DCs and Tregs. DCs are susceptible to HTLV-1 infection, and HTLV-1 infected DCs stimulate proliferation of T cells [38, 39]. A recent study also demonstrated that cell-free HTLV-1 efficiently infects DCs, and the infected DCs promote de novo infection of CD4 T cells [40].

(ii) High Proliferative Capacity. *In vivo* labeling of lymphocytes using deuterium-labeled glucose has shown that the $FoxP3^+$ Tregs were extremely proliferative *in vivo* with a doubling time of 8 days [41]. HTLV-1 infection could further enhance proliferative activity of Tregs via expression of HBZ, which has been shown in Tregs of HBZ-Tg mice [33].

(iii) Enhancement of the Differentiation of Tregs by HTLV-1 Infection. HBZ enhances the generation of $CD4^+FoxP3^+$ T cells in transgenic mice, suggesting that HBZ has enhancing effect on generation and/or expansion of $Foxp3^+$ Treg cells. As a mechanism, HBZ promotes the generation of $FoxP3^+$ Tregs via enhancing the TGF-β signaling pathways [42], which is a crucial pathway for generation of induced Tregs.

(iv) Advantage of Escape from the Host Immune Systems. Tregs have an immune suppressive effect through both cell-contact-dependent and independent mechanisms [34]. Thus HTLV-1-infected Tregs should be more resistant to HTLV-1-specific CTL killing than HTLV-1-infected non-Tregs, resulting in preferential survival of HTLV-1-infected Tregs *in vivo*.

It seems reasonable that ATL cells would be stochastically derived from $FoxP3^+$Tregs because of the high frequency of HTLV-1 infection in $FoxP3^+$ Tregs. But it still remains unclear whether or not ATL is leukemia of $FoxP3^+$ Tregs. Some studies have reported that ATL cells have regulatory function [43, 44], whereas other studies reported no regulatory function in ATL [45, 46]. Given the fact that the detection of FoxP3 expression in ATL cells is variable [47, 48], ATL is likely to be derived from both Treg cells and non-Treg cells. But the situation is more complicated because recent studies have indicated there is a plasticity between

Treg cells and non-Treg cells [49]. Even when ATL cells do not express FoxP3, we cannot exclude the possibility that ATL cells were derived from FoxP3$^+$ Tregs which have lost the FoxP3 expression during the process of leukemogenesis. Conversely, even when ATL cells do express FoxP3, we cannot exclude the possibility that FoxP3 expression is aberrantly induced in non-Tregs by HTLV-1 infection. Furthermore, even when regulatory function of FoxP3$^+$ ATL cells is not evident, there is a possibility that HBZ expression inhibits the function of FoxP3 and impairs the function of the host Tregs [33].

Taken together it is difficult to make a clear conclusion about the relationship between HTLV-1 and the CD4 T-cell subset of host cells at present, but we need to continue the effort to reconcile the complexity to understand the pathogenesis of HTLV-1.

4. The Minus Strand Viral Gene: HBZ

The presence of a transcript from the minus strand of the HTLV-1 provirus has been reported in 1989 [50], but HBZ has not been described until recently. HBZ was identified using the CREB-2 binding protein in the yeast two-hybrid screening system using HTLV-1 infected MT-2 cells [51]. HBZ suppresses Tax-mediated viral gene expression from the 5′LTR by interacting with CREB-2. Therefore, HBZ is a negative regulator for viral gene expression. The following data describing expression of HBZ in primary HTLV-1-infected cells have highlighted the significant role of HBZ in the pathogenesis of HTLV-1 [18, 52–54].

4.1. Minus Strand Transcription of HTLV-1

4.1.1. Transcriptional Pattern of HBZ. There are two major transcripts of HBZ, spliced and unspliced HBZ (Figure 1) [18, 55, 56]. Both HBZ transcripts have been detected in ATL cells [56]. Spliced HBZ mRNA expression is correlated with disease severity of HAM/TSP [53]. The level of HBZ expression is higher than that of tax in uncultured primary cells [52, 53]. A recent study has proposed one possible reason why immunogenicity of HBZ is low. They reported that HBZ-RNA is preferentially retained in the nucleus, which may result in the low translation efficiency of HBZ and contribute to its immune escape [57]. It is of great interest to elucidate the mechanism why HBZ-RNA is retained in the nucleus because that also could also explain how HBZ RNA induces a growth-promoting effect on T cells [18].

4.1.2. Possible Effect of Integration Site on HBZ Transcription. It is possible to evaluate the expression level of HBZ in total PBMC at population level but not at single clone level at present. There are many different clones even within one infected individual [19]. Every infected clone has its own unique integration site; so the difference in the integration site may affect the expression of HBZ; for example, the epigenetic features of HTLV-1 provirus are determined in part by surrounding host genomic features. When HTLV-1 integrated in heterochromatic region of the host genome, the transcriptional machinery has difficulty accessing the 3′LTR promoter region of HBZ (Figure 1) [58], which could inhibit transcription of HBZ. A previous study has indeed shown that integration sites in ATL cells are frequently located within transcriptional units of host genes but rarely located in a heterochromatic region of human genome compared with those in untransformed infected cells [59]. This result suggests that an HTLV-1-infected clone that carries a provirus integrated within a transcriptionally active region is susceptible to ATL generation. A novel powerful method of integration site analysis using a high-throughput technique has been developed recently and provided more detailed and precise information on the integration site of HTLV-1 [19]. In order to elucidate the effect of the integration site on the clonal dominance, further experiments will be required to investigate the underlying molecular mechanism of the transcriptional interaction between HTLV-1 provirus and the surrounding host genome.

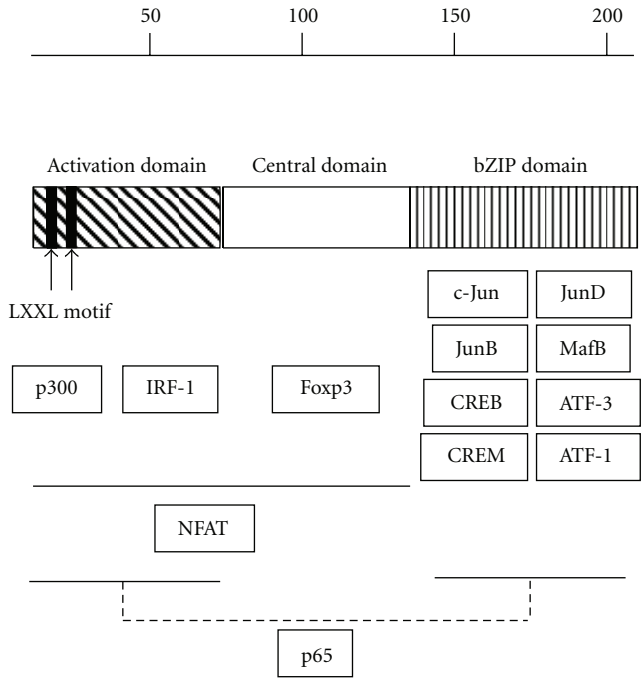

Figure 2: The structure of HBZ and interacting host factors. HBZ could play a crucial role in the HTLV-1 pathogenesis by interacting cellular factor as shown in this figure.

4.2. Molecular Property of HBZ Protein. HBZ has been reported to interact with various host factors via its three distinct coiled-coil domains, including activation domain, central domain, and bZIP domain (Figure 2). Transcriptional coactivator p300 and interferon regulatory factor IRF-1 interact with the activation domain of HBZ [60, 61]. HBZ induces the activation of TGF-β signaling pathway by forming a complex with p300 and Smad proteins [42]. HBZ also activates Dkk1 expression through its KIX domain that interacts with p300 [60, 62]. The central domain of HBZ is responsible for the interaction with Foxp3 [33], which results

in the dysfunction of transcriptional activity of Foxp3. The bZIP domain of HBZ is responsible for the interaction with the host bZIP factors, such as c-Jun, JunB, JunD [63, 64], CREB, CREM, ATF-1 [65], ATF-3 [66], and MafB [67]. These data have demonstrated that HBZ can form complexes with host factors, resulting in the dysregulation of the host cell-signaling pathways. Although it is very difficult to identify how each molecular mechanism affects the fate of infected cells, it could contribute to the phenotype of infected cells in a coordinated manner. It is striking that HBZ interacts with transcription factors that play a critical role in CD4 T cells such as AP-1, NF-κB [68], and FoxP3; therefore these molecular interactions could explain the observed cell-type specificity of transformation induced by HTLV-1.

4.3. Effect of HBZ Expression on Viral Persistence and HTLV-1-Related Pathogenesis. Experiments using a molecular clone of HTLV-1 deleted for HBZ demonstrate that HBZ is dispensable for the HTLV-1-mediated T-cell transformation *in vitro*. However HBZ plays an indispensable role in persistent viral infection *in vivo* [69]. More recently, in the macaque model of HTLV-1 infection, reversion of HBZ knock-out HTLV-1 to wild-type HTLV-1 was observed within weeks from infection, also indicating that HBZ plays a crucial role in the persistent infection of HTLV-1 [24]. Tax is thought to be responsible for the *in vitro* transformation induced by HTLV-1 infection, in which Tax is allowed to express because of the absence of selection pressure by the host immune systems. Also, since Tax has a strong capability to induce genomic instability in infected cells [70–73], Tax could contribute to the accumulation of host genomic abnormalities related to oncogenesis, even at its limited expression level *in vivo.* Nonetheless, Tax-expressing cells would be susceptible to elimination by CTL in an immune competent host. In contrast, the immune selective pressure on HBZ may be significantly lower allowing expression of HBZ mRNA *in vivo* [15, 16], which should contribute to the persistence of HTLV-1. More surprisingly, the phenotype of HBZ-Tg mice has demonstrated that HBZ expression in CD4 T cells *in vivo* could induce the disease phenotype of HTLV-1 infection such as chronic inflammation and T-cell lymphoma [33]. Thus, the transgenic expression of either Tax or HBZ induces both T-cell lymphoma and chronic inflammation [33, 74–76]. It is of great interest how Tax and HBZ synergistically, competitively, or independently contribute to various aspects of the viral pathogenesis. For example, Tax activates the NF-κB signaling pathway, but HBZ represses the canonical pathway of NF-κB [42]. A recent study has shown that HBZ can alleviate cellular senescence induced by Tax-mediated NF-κB hyper-activation [77]. Future experiments should aim to clarify the role of Tax and HBZ in each aspect of HTLV-1-associated pathogenesis.

5. Future Direction of Treatment

Since the identification of ATL as a distinct clinical entity, some progress has been made in preventing and treating the disease. In particular, the identification of a transmission route from the mother to her child through breast milk enables us to reduce *de novo* HTLV-1 infection [78]. However, an effective therapeutic strategy for ATL remains elusive. In particular, there are few available therapies that target HTLV-1 to date. Some reports have shown the efficiency of combination therapy of zidovudine and interferon alpha (AZT/IFN) for treatment of ATL [79], even though little is known about underlying mechanism of AZT/IFN therapy. Recent approaches using allogeneic bone marrow transplantation have significantly improved the prognosis of ATL patients [80, 81], suggesting that enhancement of the immune response to HTLV-1 is a possible strategy for treatment of HTLV-1-associated human diseases [82]. As we discussed in this review, if we could activate viral gene expression, we could remove the infected cells by recruiting HTLV-1-specific CTLs. Several preclinical and clinical studies using valproate, a histone deacetylase (HDAC) inhibitor, have already been performed and shown the efficacy of this therapeutic approach. Histone modification is reported to contribute to the proviral gene expression of HTLV-1 [83–86]. Treatment with valproate enhances the expression of Tax, and the resulting exposure to anti-Tax CTL may explain the observed reduction in HTLV-1 proviral load [87, 88]. The combination of HDAC inhibitor and antiretroviral drugs remarkably reduced the proviral load in STLV-1 naturally infected baboons [89]. In order to find more efficient and specific molecular therapeutic targets, it is necessary for us to clarify the nature of leukemogenesis induced by HTLV-1.

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Clinical Study

Diffuse Large B-Cell Lymphoma in Human T-Lymphotropic Virus Type 1 Carriers

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We describe the clinical and pathological characteristics of seven patients who were human T-lymphotropic virus type 1 (HTLV-1) carriers and had a pathological diagnosis of *de novo* diffuse large B-cell lymphoma. Interestingly, three of our cases showed positive expression of Epstein-Barr-virus, (EBV-) encoded RNA within the tumor cells indicating a possible interaction between these two viruses. Furthermore, our three EBV-positive cases presented with similar clinical characteristics such as early clinical stage and low-risk indices. To the best of our knowledge, this is the first case series describing the characteristics of HTLV-1-positive DLBCL patients. The potential relationship between HTLV-1 and EBV should be further explored.

1. Introduction

Diffuse large B-cell lymphoma (DLBCL) is the most common variant of non-Hodgkin lymphoma (NHL) accounting for approximately 30% of the NHL cases worldwide [1]. Previous reports have associated certain viral infections with the development of DLBCL. For example, HIV-infected individuals have a higher risk of developing DLBCL than the general population. Additionally, the most recent WHO classification has included a provisional entity, EBV-positive DLBCL of the elderly, which seems to be associated with an aggressive clinical course and worse outcome [2]. In general, it is thought that HIV-infected and other immunocompromised individuals are more likely to develop EBV-positive DLBCL.

The human T-lymphotropic virus type 1 (HTLV-1) is a retrovirus regarded as the pathogenic agent for adult T-cell lymphoma/leukemia (ATLL) [3]. HTLV-1 is endemic in Japan, the Melanesian Islands, the Caribbean, South America, the Middle East, and parts of Africa. The prevalence of HTLV-1 in Europe and USA is <1%. However, in Peru, up to 3% of the healthy adult population carries HTLV-1 [4, 5]. Chronic HTLV-1 infection has been associated with immunosuppression and an increased risk of developing other benign and malignant conditions [6].

As the association between HTLV-1 infection and DLBCL has not been previously evaluated, in this study, we aimed to describe the clinical and pathological characteristics of HTLV-1-positive patients with a pathological diagnosis of DLBCL.

2. Materials and Methods

2.1. Case Selection. Cases with a pathological diagnosis of *de novo* DLBCL and concurrent positive serology for HTLV-1 were identified from the medical oncology consultation files at the Edgardo Rebagliati Martins and Jose Alcides Carrion Hospitals, both located in Lima, Peru, from January 2000 to December 2010. Patients with a diagnosis of HIV infection,

Table 1: Clinical characteristics of 7 HTLV-1-positive patients with DLBCL.

Case	Sex	Age	WBC ($\times 10^9$/L)	ALC ($\times 10^9$/L)	Hb (g/dl)	Platelets ($\times 10^9$/L)	Stage	IPI score	Treatment	Response	Overall survival	Outcome
(1)	M	85	5.4	1.6	13.5	145	I	1	CHOP	CR	5 months	Dead
(2)	F	54	6.1	1.5	11.9	230	II	0	CHOP	CR	44 months	Alive
(3)	M	73	5.7	2.5	12.1	293	II	1	R-CHOP	CR	19 months	Alive
(4)	M	47	4.9	1.1	11	240	IV	3	CHOP	PD	8 months	Dead
(5)	F	45	6.2	0.9	10.5	200	IV	3	R-CHOP	CR	24 months	Alive
(6)	F	65	5.4	1.3	11.1	180	III	3	CHOP	CR	48 months	Alive
(7)	F	63	7.2	1.3	10	320	IV	4	R-CHOP	CR	21 months	Alive

ALC: absolute lymphocyte count; Hb: hemoglobin; IPI: international prognostic index; M: male; F: female; CHOP: cyclophosphamide, doxorubicin, vincristine, and prednisone; R-CHOP: rituximab + CHOP; CR: complete response; PD: progressive disease; WBC: white blood cell count.

transformed, primary cutaneous, or primary central nervous system (CNS) DLBCL were excluded. All cases had HTLV-1 detected in serum by ELISA and/or Western blot techniques. Clinical and laboratory information was obtained through medical chart review, after approval of this study by the Institutional Review Board at each center.

2.2. Pathological Evaluation. Routine hematoxylin and eosin-stained sections were prepared from formalin-fixed, paraffin-embedded tissue blocks. Immunohistochemical analysis included a broad panel of antibodies against CD45 (Dako, Carpinteria, Calif; dilution 1 : 400), CD20 (clone L26, Dako; dilution 1 : 100), MUM1/IRF4 (clone MUM1p, Santa Cruz Biotechnology, Santa Cruz, Calif; dilution 1 : 200), bcl-6 (Dako; dilution 1 : 10), and CD10 (Novocastra; dilution 1 : 10). CD10, bcl-6, and MUM1/IRF4 were considered positive if expressed by >30% of the tumor cells. For the detection of EBV, we used a chromogenic *in situ* hybridization (CISH) technique to evaluate the presence of EBV-encoded RNA (EBER; Dako). Cases showing EBER nuclear expression in >10% of the tumor cells were considered positive. The presence of proviral HTLV-1 DNA was evaluated in the tissue blocks using a polymerase chain reaction (PCR) technique as previously described [7].

3. Results

Seven consecutive patients with a pathological diagnosis of *de novo* DLBCL and positive serology for HTLV-1 were identified. One case has been previously reported [8]. Complete clinical and pathological data are shown in Tables 1 and 2, respectively. Clinically, the male-to-female ratio was 3 : 4, with a median age of 63 years (range 45–85 years). Three patients (43%) had early stage, and 4 patients (57%) presented with advanced stages. Low or low-intermediate IPI scores were seen in 3 patients (43%) and high or high-intermediate in 4 cases (57%). Three patients (43%) were treated with R-CHOP and 4 (57%) with CHOP alone. Six patients (86%) obtained a CR after chemotherapy. After 24 months of followup, 2 patients (29%) have died, and the median overall survival (OS) has not been reached. The estimated 2-year OS is 71%. Pathologically, all the cases (100%) had strong expression of CD20 and diffuse large cell morphology. CD10 was positive in 1 out of 5 patients tested (20%), bcl-6 was negative in all cases tested (0/5; 0%), and MUM1/IRF4 was positive in 4 out of 5 cases tested (80%). Three cases (42%) were positive for EBER by CISH. PCR used to detect proviral HTLV-1 DNA in the tumor samples was negative.

Table 2: Pathologic characteristics of 7 HTLV-1-positive patients with DLBCL.

Case	CD20	MUM1	CD10	BCL6	EBER	CD30
(1)	+	+	−	−	+	−
(2)	+	−	+	−	+	ND
(3)	+	+	−	−	+	ND
(4)	+	ND	ND	ND	−	−
(5)	+	+	−	−	−	ND
(6)	+	ND	ND	ND	−	ND
(7)	+	+	−	−	−	ND

EBER: EBV-encoded RNA by chromogenic *in situ* hybridization; ND: not done.

4. Discussion

In this paper, we present a case series of 7 HTLV-1 carriers who have developed *de novo* DLBCL. A most salient point is that three of our cases (42%) demonstrated the presence of EBV genome in the tumor cells. Although this could suggest a high incidence of EBV positivity in HTLV-1 patients with DLBCL, if we consider that the incidence of EBV-positive DLBCL has been reported in the range of 3–15% [9–12], given the small number of cases, it remains speculative. Another important aspect of our study is that these patients were negative to other viruses such as HIV and hepatitis B and C, which could also induce immunosuppression, and have been associated with the development of specific types of lymphoma. Few cases of lymphomas arising in HTLV-1 carriers have been previously published [13–16].

EBV is a recognized oncovirus with B-cell lymphotropism. EBV attaches to CD21 preparing the B-lymphocyte for EBV infection. EBV infection will promote an increased production of IL-6 and EBV-associated mRNAs promoting a blastic transformation. EBV is then inserted into the nucleus

of the B cell where it acquires a circle-shaped configuration. EBV nuclear antigens are the first to be produced after infection, which are essential for immortalization of the cell and upregulation of the expression of other molecules and genes such as latent membrane proteins (LMPs) and C-MYC. LMPs increase expression of bcl-2 and drive the cell into a latent state, which is maintained by the production of EBV-encoded RNA. Hence, EBV-infected B cells enter an apparent resting phase; however, due to their activated phenotype, they are more prone to develop oncogenic changes [17].

HTLV-1, on the other hand, is a retrovirus that infects a wide variety of cells (lymphocytes, monocytes, and fibroblasts) [18]. An important HTLV-1-associated viral protein denominated Tax is a necessary first step in oncogenesis. Tax increases proliferation of virus-infected cells by accelerating all the phases of the cell cycle and renders the affected cells susceptible to a series of genetic and epigenetic changes [19]. The expression of Tax, however, wears out as cells acquire the ability to proliferate independently. Due to its prolonged latency period of decades, HTLV-1-infected cells are more susceptible to acquire malignant phenotypes in a multistep process. Previous studies have indicated a strong association between HTLV-1 infection and the development of ATLL. Interestingly, the frequency of primary malignant neoplasms in HTLV-1 carriers is higher than in HTLV-1-seronegative cases [6], suggesting the oncogenic power of HTLV-1 goes beyond lymphoma and leukemogenesis. Although HTLV-1 has not been associated with the development of B-cell lymphomas, HTLV-1 carriers with B-cell lymphoma tend to have worse prognosis [20].

However, as we suggest in the present paper, there could be a potential lymphomagenetic interaction between EBV and HTLV-1. In a previous paper in patients with ATLL, Ueda et al. indicated that coinfection with HTLV-1 and EBV may induce a more extensive organ involvement through the enhanced expression of adhesion molecules via IL-4 signaling [21]. Similarly, Ogata et al. found a subclinical reactivation of EBV in ATLL patients undergoing chemotherapy [22]. Furthermore, several papers on EBV-associated lymphoproliferative disorders (LPDs) seen in ATLL patients have been reported in the literature. Amano et al. described a case of EBV-associated primary CNS lymphoma arising in a patient with ATLL and was explained by a suppression of the immune system by HTLV-1 [23]. Tanaka et al. described a case of acute type ATLL complicated by the development of EBV-associated LPD which was likely responsible for the patient's demise [24].

Theoretically, HTLV-1 infection can cause immunosuppression *via* T-cell dysfunction and promote reactivation of EBV, which in turn will induce B-cell proliferation and lymphomagenesis. The identification of 3 HTLV-1 carriers with EBV-positive DLBCL in our study may suggest the immunosuppression induced by HTLV-1 could be implicated in the pathophysiology of this rare lymphoma. Additionally, our 3 EBV-positive DLBCL cases had similar clinical characteristics (i.e., early disease, low IPI scores, and achievement of CR with chemotherapy). Hence, we postulate an interesting hypothesis about a potential pathogenetic relationship between HTLV-1 and EBV.

We understand the limitations of small retrospective case series such as ours, in terms of selection bias. However, these cases were identified from nonselected, consecutive patients with a diagnosis of DLBCL who were treated according to their diagnosis and stage with standard therapies. In order to investigate the potential relationship between HTLV-1 and EBV, large prospective cohort or population-based retrospective studies are needed.

5. Conclusion

In this paper, we present 7 cases of DLBCL in HTLV-1 carriers from which 3 were EBV-positive DLBCL. Likely, the interaction between EBV and HTLV-1 could promote T-cell and B-cell dysfunction as well as antiapoptosis and cell proliferation, favoring lymphomagenesis. Further studies are needed to investigate this potential relationship.

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