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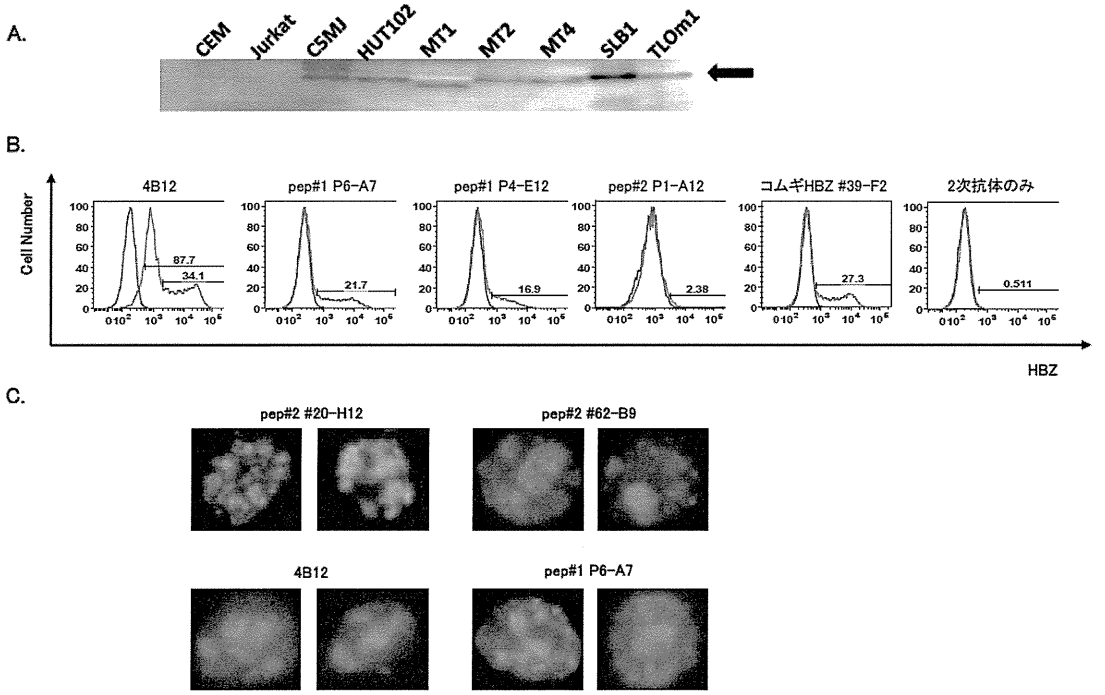
H．知的財産権の出願・登録状況
１．特許取得
該当なし

２．実用新案登録
該当なし

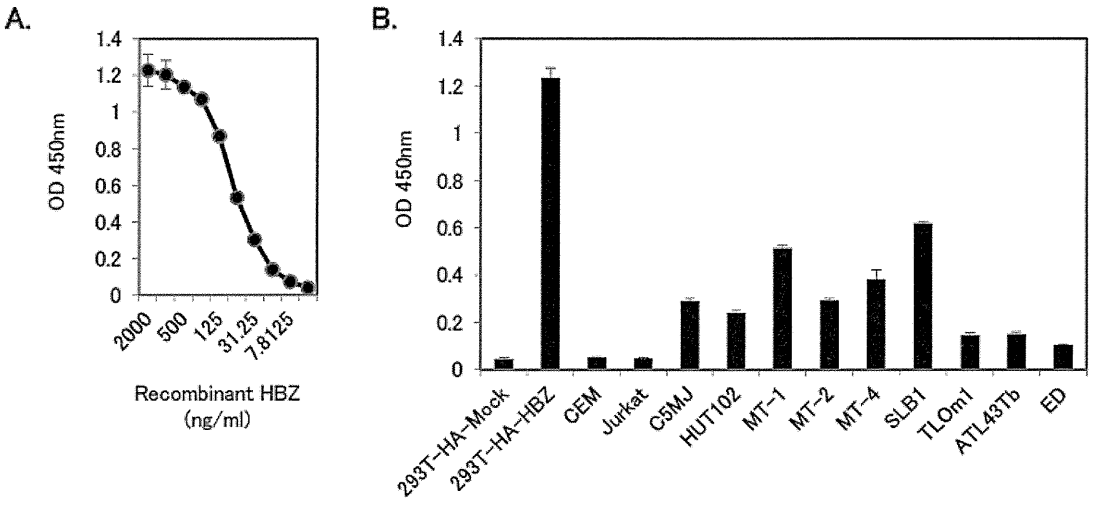
３．その他
特記なし

図１：新規抗 HBZ モノクローナル抗体による細胞内 HBZ 蛋白質の検出



A. ウエスタンブロッティング　B. フローサイトメトリー　C. 蛍光免疫染色（間接法）

図２：サンドイッチ ELISA 系による HBZ 蛋白質の定量的検出



別紙４

研究成果の刊行に関する一覧表

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雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
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Matsuoka M, **Yasunaga J**.	Human T-cell leukemia virus type 1: replication, proliferation and propagation by Tax and HTLV-1 bZIP factor.	Curr Opin Virol.	3 (6)	684-691	2013
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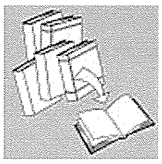
REVIEW



Virological and immunological mechanisms in the pathogenesis of human T-cell leukemia virus type 1

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SUMMARY

Human T-cell leukemia virus type 1 (HTLV-1) was the first retrovirus shown to cause human disease, such as adult T-cell leukemia and HTLV-1 associated myelopathy/tropic spastic paraparesis. HTLV-1 mainly infects CD4 T cells and deregulates their differentiation, function and homeostasis, which should contribute to the pathogenesis of HTLV-1, for example, inducing transformation of infected CD4 T cells and chronic inflammatory diseases. Therefore, not only virological approach but also immunological approach regarding CD4 T cells are required to understand how HTLV-1 causes related human diseases. This review focuses on recent advances in our understanding of the interaction between HTLV-1 and the main host cell, CD4 T cells, which should provide us some clue to the mechanisms of HTLV-1 mediated pathogenesis. Copyright © 2013 John Wiley & Sons, Ltd.

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INTRODUCTION

Human T-cell leukemia virus type 1 (HTLV-1) is a delta-type retrovirus, which infects approximately 10 to 20 million people worldwide [1]. In the late 1970s, ATL was identified as a distinct clinical entity on the basis of 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 [3]. HTLV-1 has a potential to immortalize human T-lymphocytes *in vitro* [4]. In addition to leukemia of infected cells, HTLV-1 infection also induces chronic inflammatory diseases, such as 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 investigations were performed to elucidate the pathogenesis of the virus, resulting in significant impact not only on HTLV-1 research but also on the more general virological and molecular biological field [10–14]. However, many questions still remain to be answered. How does HTLV-1 transform mature CD4 T cells? What is the exact mechanism of HTLV-1-associated inflammatory diseases? How can we treat or prevent HTLV-1-related human diseases? We review recent advances of HTLV-1 research and discuss the present understanding of HTLV-I-related human disease.

THE STRATEGY OF REPLICATION IN HTLV-1

Once HTLV-1 infects host cells, the RNA genome is reverse transcribed into a double-stranded DNA form by reverse transcriptase, and then it is integrated into the host chromosomal DNA by the viral integrase. The integrated viral DNA contains two long terminal repeats (LTRs) at each end of the genome. 5′LTR is a promoter of plus strand viral genes, whereas 3′LTR is a promoter of minus strand viral genes. Similar to other retroviruses, HTLV-1 encodes viral structural genes, including

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Abbreviations used
ATL, adult T-cell leukemia; HAM/TSP, HTLV-1 associated myelopathy/tropic spastic paraparesis; HBZ, HTLV-1 bZIP factor; CREB, cAMP response element-binding protein; Sp-1, specificity protein-1; T_{reg} cells, regulatory T cells; FoxP3, forkhead box P3; LFA-1, lymphocyte functional antigen-1; ICAM-1, intercellular adhesion molecule 1; DC, dendritic cell; AZT/IFN, zidovudine and interferon-α.

gag, *pol*, and *env*, which generate viral particles (Figure 1). Additionally, there is the pX region encoding various regulatory and accessory genes such as *tax*, *rex*, *p30*, *p12*, and *HBZ*. These regulatory and accessory genes control the proviral gene expression and proliferation of infected cells.

HTLV-1 has two major propagation patterns; *de novo* infection from infected cells to uninfected cells and clonal expansion of infected host cells [15]. In *de novo* infection, HTLV-1 rarely spreads via free viral particles but via cell-to-cell transmission through the virological synapse [12] and biofilm-like extracellular viral assemblies [13]. This type of viral spread is primarily critical for the initial spread of infected cells to uninfected cells in an individual and also to transmission from infected individuals to uninfected individuals. The expression of Tax enhances the transcription of viral structural genes from the plus strand of HTLV-1 in this situation. In contrast, clonal expansion of infected cells seems to be optimal fashion to achieve persistent infection [16,17], because clonal expansion of infected cells does not require the production of viral particles but allows the virus to minimize expression of viral antigens, enabling the virus to escape from the host immune surveillance. This idea is supported by the fact that antiretroviral therapy could not reduce the proviral loads in HAM/TSP patients [18]. Furthermore, infection of CD4 T cells, the central player of the host immune system, places HTLV-1 in a unique position by which to evade and manipulate the host immune response.

VIRAL ANTIGEN EXPRESSION AND THE HOST IMMUNE RESPONSE

In principle, HTLV-1 needs to express a viral gene to increase a viral copy number via both *de novo* infection and clonal proliferation of infected cells. On the other hand, viral antigen expression induces the host immune response to HTLV-1, which could reduce the number of infected cells. Accordingly, there should be some kind of equilibrium between the proliferation of infected cells and the host immune response against the virus [19,20]. 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. Because 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 [21,22]. Among various CTLs against viral antigen, the anti-Tax CTL is the most predominant and most likely to contribute to the pathogenesis of chronic inflammatory diseases [23,24]. Therefore, Tax has been

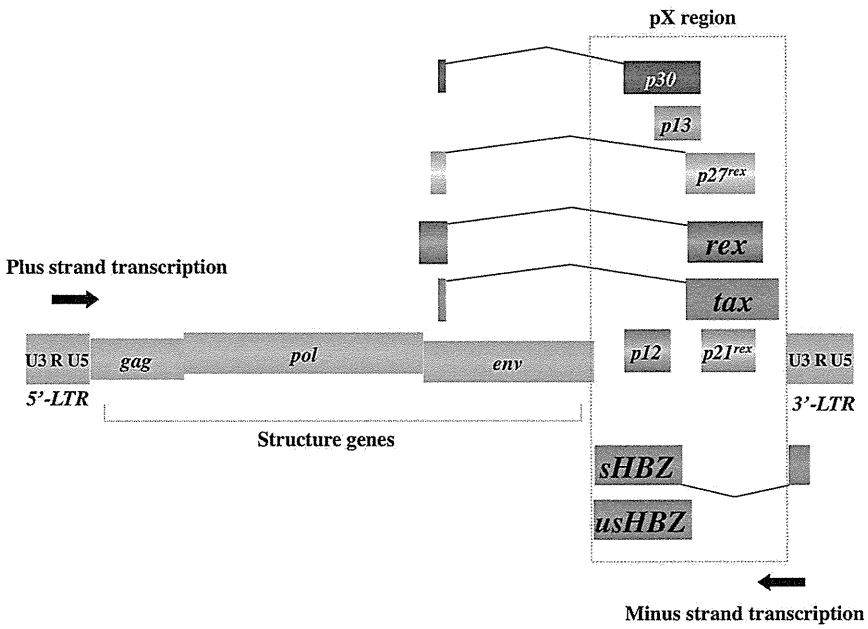

Figure 1. The structure of HTLV-1. HTLV-1 encodes accessory and regulatory genes in the pX region as well as viral structural genes

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considered as the most critical determinant for the host immune response that controls proviral load [25]. However, recent evidence regarding the recently identified viral protein HBZ has proposed an alternative target. HTLV-1-infected individuals with MHC alleles that can efficiently bind and present peptides from HBZ have significantly lower proviral load, and are less likely to develop HAM/TSP [26]. Because HBZ play a crucial role in the clonal expansion of HTLV-1 infected cells, anti-HBZ immune response should be a critical determinant of viral persistence in the chronic phase of HTLV-1 infection regardless of its low immunogenicity.

In summary, the host immune response against HTLV-1 is a critical determinant in the survival of infected cells. Immunodeficient or immunocompromised circumstances, such as *in vitro* cell culture [4], humanized mice [27,28], and immunocompromised host [29], might allow HTLV-1 to express Tax, resulting in the immortalization of infected cells within a relatively short period. That is because Tax has a strong transforming capacity via deregulating the host systems [14,30,31]. In contrast, the immunocompetent host can eliminate tax-expressing cells, so only HTLV-1 infected cells without Tax expression or with very low Tax expression would be able to survive in the host. Just only HTLV-1 infection without Tax expression alone is not sufficient to induce the malignant transformation of infected cells but requires additional genetic and epigenetic host genome abnormality related with oncogenesis to generate ATL cells. Taken together, these findings suggest that the host immune surveillance is a critical determinant to control viral persistence and transformation of infected cells *in vivo*.

TRANSCRIPTIONAL REGULATION OF HTLV-1 PROVIRUS

How does HTLV-1 regulate proviral gene expression to achieve persistent infection *in vivo*? Previous studies have elucidated promoter characteristics of 5′LTR and 3′LTR [32,33]. Repeated 21-nucleotide sequence in the LTR region is critical for the transactivation of 5′LTR [34]. Tax activates 5′LTR via this tax-responsive element collaborating with CREB [35]. Therefore, transcription from 5′LTR has been assumed to be inducible and highly variable. Contrastingly, 3′LTR for the minus strand transcription as a TATA-less promoter seems to have constant activity driven by Sp-1 [33]. Additionally, because the HTLV-1 provirus is integrated in the host genome, transcriptional activity of the provirus is inevitably affected by the genomic environment near the integration site. Recent research by using deep sequence technology has shown that there are thousands different HTLV-1 infected clones in asymptomatically infected individuals. Also, there is a significant difference of clone size among different clones [36]. Given that there is little variation of HTLV-1 provirus, factors other than the HTLV-1 provirus must determine the difference in the clone size. One possible factor is the difference of the integration site. Every infected clone has its own unique integration site, so the particular features of each integration site may affect the activity of 5′LTR and 3′LTR. For example, epigenetic features of the HTLV-1 provirus could be affected by the surrounding host genomic features. When HTLV-1 is integrated in the heterochromatic region of the host genome, it is difficult for the transcriptional machinery to access the 5′LTR and 3′LTR, which should inhibit proviral transcription. A previous study has indeed shown that integration sites in ATL cells are frequently located within the transcriptional unit of the host gene but rarely located in the heterochromatic region of the human genome in comparison with integration sites of untransformed infected cells [37]. This result suggests that an HTLV-1 infected clone that has an integration site within a transcriptionally active region may be predisposed to the ATL generation. A novel powerful method of integration site mapping by using a high-throughput technique should provide more detailed and precise information about the integration sites of HTLV-1 [36]. Further experiments will be required to elucidate the underlying molecular mechanism to describe the topological and transcriptional interaction between the HTLV-1 provirus and the surrounding host genome.

THE HOST CELL OF HTLV-1, CD4 T CELL

Both human retroviruses HIV and HTLV-1 target CD4 T cells as the host cells, but they have opposing effects on the survival of infected CD4 T cells. HIV induces apoptosis of infected CD4 T cells, whereas HTLV-1 induces clonal proliferation/survival of infected CD4 T cells. Regardless of this difference, HIV and HTLV-1 show some similarities in terms of pathogenicity, such as immunodeficiency.

It has been assumed that HTLV-1 causes the expansion of CD4 T cells but changes the quality. In other words, HTLV-1 seems to change the function and/or differentiation of CD4 T cells, which should influence immunological disorder observed in HTLV-1 infection. Therefore, elucidating the mechanism of alteration of CD4 T cells in HTLV-1 infection is a key step to understand how HTLV-1 causes these diseases.

CD4 T cells are generally partitioned into two subsets, effector T cells (T_{eff} cells) and T_{reg} cells. The former plays a 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. Because the differentiation, function and homeostasis of these two T-cell subsets are quite different, we discuss about HTLV-1 infection in T_{eff} cells and T_{reg} cells separately.

HTLV-1 infection in CD4 T_{eff} cells

To exert the effector function as CD4 T_{eff} cells, naive CD4 T cells need to encounter their antigens, be activated, and be converted into CD4 T_{eff} cells. Previous reports have demonstrated that the HTLV-1 provirus is more frequently detected in effector/memory CD4 T cells than naïve CD4 T cells [38,39]. The effector/memory CD4 T cells in these previous analyses include $Foxp3^+$ T_{reg} cells as well. We recently reported that HTLV-1 provirus is frequently detected in $FoxP3^+$ T cells and $FoxP3^-$ effector/memory T cells [40] (Figure 2). We would like to propose three possible explanations of the frequent presence of HTLV-1 in effector/memory CD4 T cells as the following. Firstly, effector/memory T cells are assumed to be highly susceptible to *de novo* infection. *De novo* infection of HTLV-1 is achieved mainly by cell-to-cell transfer, which is initiated by the LFA-1–ICAM-1 interaction between infected cells and uninfected cells [41]. Because the expression level of LFA-1 and ICAM-1 in effector/memory CD4 T cells is higher than those in naïve CD4 T cells [42], effector/memory CD4 T cells are likely to be more susceptible to *de novo* cell-to-cell infection than naïve CD4 T cells. Secondly, effector/memory CD4 T cells proliferate faster than naïve CD4 T cells *in vivo*. It has been shown that the doubling time of effector/memory CD4 T cells is 28 days, which is much shorter

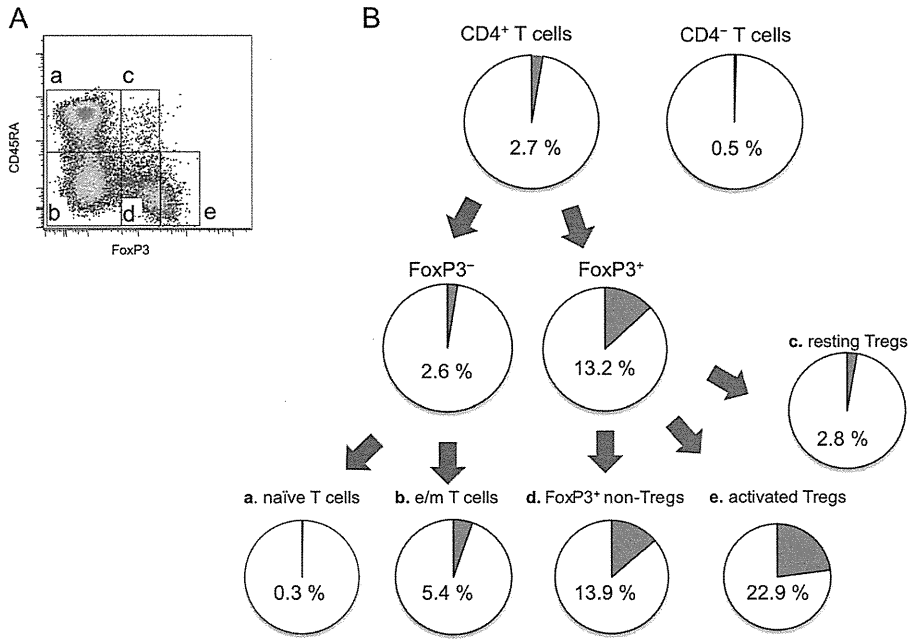

Figure 2. Proportion of HTLV-1-infected cells in each CD4 T-cell subset. (A) Flow cytometric analysis for CD4 T-cell subset. On the basis of the expression of CD45RA and FoxP3, CD4 T cells are divided into five subsets [76]. (a) $CD45RA^+FoxP3^-$, naïve T cells; (b) $CD45RA^-FoxP3^-$, effector/memory T cell; (c) $CD45RA^+FoxP3^+$, resting T_{reg} cells; (d) $CD45RA^-FoxP3^{low}$, non-T_{reg} cells; and (e) $CD45RA^-FoxP3^{high}$, activated T_{reg} cells. **(B)** Based on the expression of Tax after *ex vivo* culture, HTLV-1 infectivity was calculated on the basis of viral antigen Tax positivity and shown as an average value of 23 asymptomatic infected individuals. Methods were described previously [40]

than the doubling time of naïve CD4 T cells, 199 days [43]. Therefore HTLV-1 infected effector/memory CD4 T cells tend to induce clonal expansion, resulting in the predominant presence of HTLV-1 in effector/memory CD4 T cells. Thirdly, HTLV-1 infection can enhance the differentiation from naïve to effector/memory CD4 T cells. We have recently reported that the proportion of effector/memory CD4 T cells was increased in HBZ-transgenic (HBZ-Tg) mice [44]. This result has suggested that HBZ expression in HTLV-1 infected cells can drive the differentiation from naïve CD4 T cells to CD4 T_{eff} cells.

What is the consequence of HTLV-1 infection to T_{eff} cells?

There are several reports describing that some viral proteins affect effector/helper cytokine production. Tax is reported to increase production of proinflammatory cytokine such as IL-2 and IFN-γ [45,46] (Figure 3), which contributes not only to inflammatory diseases related with HTLV-1 but also possibly enhances anti-HTLV-1 immunity via the activating helper T-cell function. In contrast, it has been reported recently that HBZ expression in effector CD4 T cells impairs the production of Th1 cytokines, such as IFN-γ, IL-2, and TNF-α, resulting in cellular

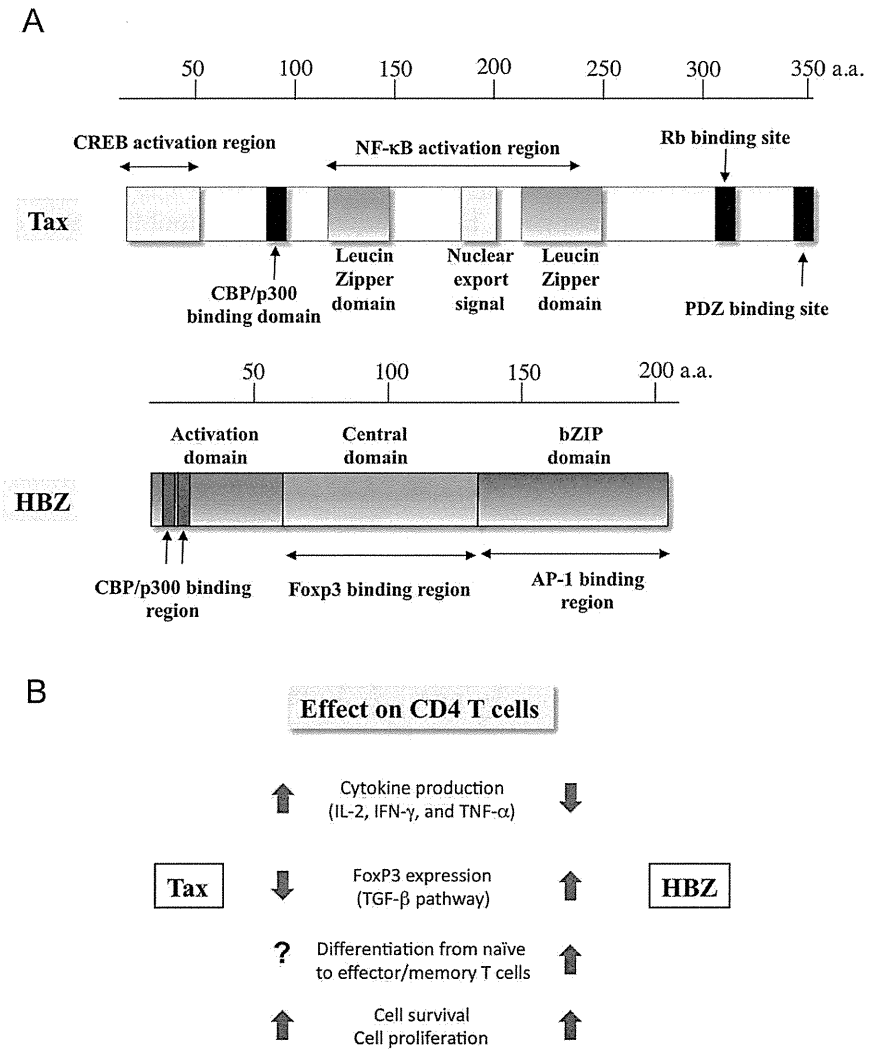

Figure 3. The structure of Tax and HBZ, and their function on CD4 T cells. (A) The structure of Tax and HBZ. **(B)** Functional role of Tax and HBZ on CD4 T cells

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immunodeficiency [47] (Figure 3). These Tax and/or HBZ-mediated deregulation of helper and CD4 T_{eff} cell function should contribute to inflammatory disease or immunodeficiency that are frequently observed in HTLV-1-infected individuals (Figure 4). In terms of persistent infection with the virus, HBZ expression possibly confers an advantage on HTLV-1-infected cells to escape from the host immune surveillance via suppressing helper T-cell function.

HTLV-1 infection in CD4 T_{reg} cells

$CD4^+CD25^+FoxP3^+$ T_{reg} cells have been identified as one of the major immunoregulatory mechanisms which prevent autoimmune disease [48]. T_{reg} cells are also involved in the downregulation of specific immune responses during infectious diseases. It has been reported that the frequency of T_{reg} cells is elevated in chronic viral infection, such as HCV [49]. The increased frequency of T_{reg} cells may help to prevent immune pathology but on the other hand may facilitate viral persistence by suppressing the host immune response against the virus. 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 [50]. In addition, there should be a specific feature for HTLV-1, because HTLV-1 directly infects CD4 T cells including T_{reg} cells, which could affect the differentiation, function, and homeostasis of T_{reg} cells at a cell intrinsic manner. The frequency of HTLV-1 infection in $CD4^+FoxP3^+$ cells is higher than other T-cell subpopulations [40,50] (Figure 2), which might be due to the following two explanations. First, T_{reg} cells are highly susceptible to *de novo* infection from DCs to T_{reg} cells. T_{reg} cells are known to contact with DCs frequently [51], which could increase the chance of *de novo* infection between DCs and T_{reg} cells [52–54]. Secondly, HTLV-1 infection induces the differentiation of T_{reg} cells. HBZ enhances the generation of $CD4^+Foxp3^+$ T cells in transgenic mice, suggesting that HBZ has an enhancing effect on the generation and/or the expansion of $Foxp3^+$ T_{reg} cells [44]. As a mechanism, HBZ promotes the generation of $FoxP3^+$ T_{reg} cells via enhancing the TGF-β signaling pathways [55], which is a crucial pathway for generation of induced T_{reg} cells [56].

What is the advantage for HTLV-1 to infect T_{reg} cells?

T_{reg} cells contain two possible characteristics that can contribute to the survival or the proliferation

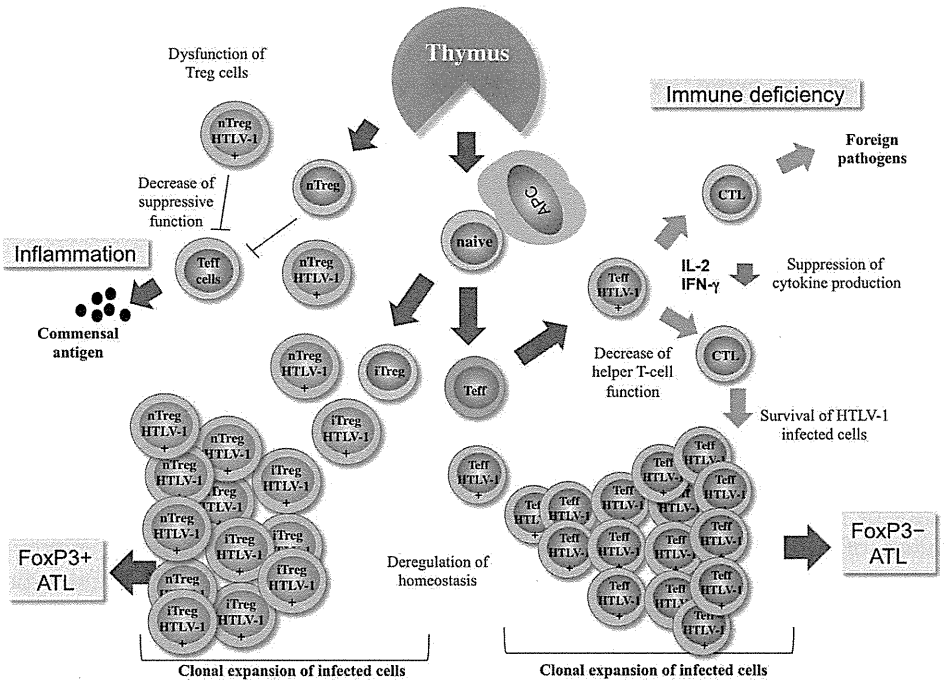

Figure 4. Schematic figure of the relationship between deregulation of CD4 T cells and pathogenesis of HTLV-1

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of infected cells. Firstly, T_{reg} cells have immune suppressive effects through both a cell-contact dependent and independent manner [48]. Thus, HTLV-1-infected T_{reg} cells should be more resistant to HTLV-1-specific CTL killing than HTLV-1-infected non-T_{reg} cells, resulting in the preferential survival of HTLV-1-infected T_{reg} cells *in vivo*. Secondly, the high proliferative capacity of T_{reg} cells could result in the dominant expansion of the HTLV-1-infected cell in T_{reg} cell populations. *In vivo* labeling of lymphocytes by using deuterium-labeled glucose has shown that the FoxP3$^+$ T_{reg} cells were extremely proliferative *in vivo* with a doubling time of 8 days [57]. Therefore, HTLV-1 is likely to utilize the hyper-proliferative characteristic of CD4$^+$FoxP3$^+$ cells to achieve the clonal expansion of infected cells (Figure 2).

In summary, both CD4$^+$ T_{eff} and T_{reg} cells are susceptible to HTLV-1. However naïve T cells in both FoxP3$^+$ and FoxP3$^-$ are rarely positive for HTLV-1. HTLV-1 seems to utilize CD4$^+$ T-cell activation and/or differentiation to achieve infection to the host cells. As a result, the host immune system is disturbed by HTLV-1 infection, inducing human diseases related with HTLV-1. Although most of infected individuals are asymptomatic, when HTLV-1 infects CD4$^+$ T cells that is critical for immune regulation or the defense against for pathogens, the infected host individuals show disease phenotypes such as chronic inflammation or immune deficiency. Further investigations should be performed to make clear how the virus and the host CD4$^+$ T cells are associated with each other.

LEUKEMOGENESIS OF ATL

How are the virus, and the host genetic and epigenetic alterations involved in the transformation of infected cells?

As described, HTLV-1 increases its copy number by proliferation of infected cells instead of viral replication, especially in persistent infection phases. As a consequence of this strategy, HTLV-1 induces the transformation of infected cells in the part of infected individuals [15]. This transformation of infected cells should not be the primary purpose for the virus but just a consequence of the clonal proliferation of infected cells. ATL cells should have some characteristics that are beneficial for clonal expansion. As we mentioned in the previous text, ATL cells constitutively express HBZ, whereas Tax expression is frequently lost by deletion or DNA methylation of the 5'LTR, indicating that HBZ plays an indispensable role in the clonal expansion of infected cells [58]. This notion is supported by several *in vivo* experiments by using an HTLV-1 molecular clone [59,60].

Considering the low frequency of ATL, 2–5% of infected individuals, and long latency, 50–60 years, HTLV-1 alone is not sufficient to develop ATL. Additional genetic and epigenetic alteration of the host genome are required for ATL leukemogenesis. Various genetic changes (mutations, deletion, amplification, and chromosomal translocation) and epigenetic abnormalities have been reported in ATL genomes (Table 1). What triggers such oncogenic event only in the part of infected cells? Are they completely stochastic events? Or are there any regulatory roles to accumulate such genetic alteration only in some particular infected cells? If the circumstance allows infected cells to express Tax, the infected cells tend to accumulate genetic abnormality because of the mutagenic activity of Tax [14,61–63]. HBZ expression is thought to promote proliferation of infected cells [58,64], which can also predispose infected cells to accumulate genetic abnormalities. In fact, HBZ expression in ATL cells is six times higher than asymptomatic HTLV-1 carriers [65]. These two possibilities are not mutually exclusive but could work coordinately. And as discussed in the previous text, the site of proviral integration affects both tax and HBZ expression. It might be necessary to examine the characteristics of the nearby host genome near the integration site to understand the way of virological effects on leukemogenesis in ATL.

Is ATL a leukemia of T_{reg} cells?

It still remains unclear whether or not ATL is a leukemia of FoxP3$^+$ T_{reg} cells. Several previous studies have shown that ATL cells frequently express FoxP3 [66–70]. It is obvious that FoxP3 is a master molecule in T_{reg} cells. Genetic mutation of FoxP3 gene induces fatal inflammatory diseases both in humans and mice [71–73]. Ectopic expression of Foxp3 confers regulatory phenotype on CD4 T cells [74]. However, recent reports have shown that FoxP3 expression can be observed in non-T_{reg} cells [75]. Thus, even when ATL cells express FoxP3, we cannot exclude the possibility that FoxP3 expression is

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Table 1. Genetic and epigenetic abnormality associated with leukemogenesis of HTLV-1

Method	Genes or location	Type of abnormality	Cause	Frequency	Reference
RT-PCR or WB or RNase Protection assay	*Notch1*	Overexpression	Activating mutation	7/12	[83]
	Fas	Downregulation	Mutation	2/47	[84]
	p53	Downregulation	Mutation	2/12	[85]
	p53	Downregulation	—	4/10	[86]
	p16	Downregulation	DNA methylation	5/7	[87]
cDNA microarray	*TSLC1*	Overexpression	Epigenetic mechanism	8/8	[88]
	p21 (CDKN1A)	Downregulation	DNA methylation	5/5	[89]
miRNA microarray	*miR-125a*	Downregulation	—	14/13	[90]
	miR-132	Downregulation	—	14/14	[90]
	miR-93	Upregulation	—	4/4	[91]
	miR-132b	Upregulation	—	4/4	[91]
	miR-31	Downregulation	Epigenetic mechanism	40/40	[92]
Southern Blotting	*p15, p16*	Downregulation	Deletion	9/23	[93]
MCA-RDA[#]	*KLF4, EGR3*	Downregulation	DNA methylation	5/5	[94]
	MEL1S	Upregulation	DNA methylation	4/4	[95]
CGH	*7p22*	Gain	CARMA1*	10–13/66	[96]
	13q21.1-q32.1	Loss	—	10–21/66	[96]
Spectral karyotyping	*10p11*	Chromosomal break	EPC1/ASXL2*	21/61	[97]
	14q11	Chromosomal break	TCF8*	20/61	[98]

[#]Methylated CpG island amplification coupled with representational difference analysis.
*Genes involved.

aberrantly induced in non-T_{reg} cells by some mechanisms such as HTLV-1 infection itself. Therefore, the best way to distinguish T_{reg} from non-T_{reg} cells would be the investigation of regulatory function of ATL cells. Some studies have reported that ATL cells have regulatory function [67,68], whereas other studies reported no regulatory function in ATL [69,70]. In addition, the fact that HBZ expression inhibits the function of FoxP3 and partially impairs the T_{reg} function makes the situation more complicated [44]. Furthermore, it has been reported that there are three distinct T_{reg} subsets in human $FoxP3^+CD4^+$ T cells [76]. We have recently reported that $FoxP3^{low}CD45RA^-CD4^+$ T cells, which do not have suppressive function, are increased and frequently infected with HTLV-1 in HTLV-1-infected asymptomatic carriers with high proviral load [40]. In the same study, some ATL cells showed $FoxP3^{low}$ non-T_{reg} phenotype and others showed $FoxP3^{high}$ T_{reg} phenotype. In summary, it is still in controversy about the origin of ATL cells, but HTLV-1 is frequently detectable in $FoxP3^+CD4^+$ cells in asymptomatic carriers and ATL cells frequently express FoxP3, and at least some ATL show suppressive function. Taken together these findings, we would like to propose the idea that HTLV-1 preferentially infect $FoxP3^+$ T_{reg} cells and the transformation of infected cells is not exclusively but frequently occurs in $FoxP3^+$ T_{reg} cells.

PERSPECTIVE

In the virological aspect, several lines of recent evidences indicate that HBZ plays a significant role in the pathogenesis of HTLV-1 as Tax does [15,58,65,77]. Further investigations will uncover not only a more detailed understanding of the functions of HBZ but also should address several key questions regarding the pathogenesis of HTLV-1. In addition, recent remarkable progress of high through-put sequencing technology enables us to address many questions regarding the role of the host genome in HTLV-1 pathogenesis. What kinds of

polymorphism are associated with the susceptibility to ATL and HAM/TSP? What is the exact content of the multi-step leukemogenesis of ATL? How is the HTLV-1 integration involved in the clonal expansion and the transformation of infected cells? Among several key questions, the most urgent question to be addressed should be how we could prevent and treat ATL and related chronic inflammatory diseases. ATL patients with progressive disease are generally treated with combination chemotherapy with several anticancer drugs, yet the prognosis has not been significantly improved [78]. Long-term survival is rarely achieved with the exception of a small number of patients treated with hematopoietic cell transplantation [79]. However, there are several promising progresses in ATL treatment. Anti-CCR4 antibody is now available for the ATL patients who are refractory to conventional chemotherapies [80]. AZT/IFN treatment is significantly efficacious not in all but in a subset of ATL patients [81]. Viral life cycle is generally inactive in ATL cells, so it is very interesting to know the underlying mechanism of the efficacy of the AZT/IFN therapy. In addition, it has been reported that cancer/testis antigen is expressed in more than 85% of ATL cells, suggesting a potent tumor antigen for the immunotherapy against ATL [82]. Because HTLV-1 has coexisted with humans for 20 000–50 000 years, the virus is extremely well-adjusted to the human system. However, now lots of efforts are in progress to control HTLV-1 infection.

CONFLICT OF INTEREST

The authors have no competing interest.

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Current Opinion in Virology

Human T-cell leukemia virus type 1: replication, proliferation and propagation by Tax and HTLV-1 bZIP factor

Masao Matsuoka and Jun-ichirou Yasunaga

Human T-cell leukemia virus type 1 (HTLV-1) spreads primarily by cell-to-cell transmission. Therefore, HTLV-1 promotes the proliferation of infected cells to facilitate transmission. In HTLV-1 infected individuals, the provirus is present mainly in effector/memory T cells and Foxp3+ T cells. Recent study suggests that this immunophenotype is acquired by infected cells through the function of HTLV-1 bZIP factor (HBZ). Tax, which is encoded by the plus strand, is crucial for viral replication and *de novo* infection, while HBZ, encoded by the minus strand, is important for proliferation of infected cells. Importantly, HBZ and Tax have opposing functions in most transcription pathways. HBZ and Tax cooperate in elaborate ways to permit viral replication, proliferation of infected cells and propagation of the virus.

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Introduction

Transmission of human T-cell leukemia virus type 1 (HTLV-1) is confined to three routes; mother-to-infant, sexual parenteral transmission, and blood transfusion/needle sharing [1]. A striking feature of this virus is that HTLV-1 is transmitted primarily in a cell-to-cell fashion, and infectivity of free virions is very poor. By contrast, another human retrovirus, human immunodeficiency virus (HIV), transmits by both cell-free and cell-to-cell contact. The transmission of HTLV-1 requires living infected cells in breast milk, semen and blood products. To facilitate its transmission, this virus increases the number of infected cells *in vivo* by stimulating their proliferation.

HTLV-1 was discovered in 1980 as the first human retrovirus [2,3]. Thereafter, this virus was found to be linked with a human disease, adult T-cell leukemia (ATL) [4]. Subsequently it was found that this virus also causes another disease, HTLV-1 associated myelopathy/tropical spastic paraparesis (HAM/TSP), as well as HTLV-1 uveitis, infective dermatitis, and myopathy [1]. These diseases are thought to be associated with the fact that infected host immune cells proliferate *in vivo*. In this review, we summarize recent findings on the replication of HTLV-1, the proliferation of infected cells, and HTLV-1 propagation — matters which are closely related for this virus.

Virus entry and cell-to-cell transmission

Unlike HIV, HTLV-1 can infect a variety of cells; its receptor is thought to be a commonly expressed molecule [5]. It has been reported that HTLV-1 envelope protein interacts with three cellular molecules, heparan sulfate proteoglycan (HSPG) [6], neuropilin-1 [7], and a glucose transporter, GLUT1 [8], for entry into cells. Conformational changes of the complex consisting of the HTLV-1 virion and these molecules are thought to occur sequentially during the entry step. First, the HTLV-1 envelope attaches to HSPG, and it then forms complexes with neuropilin-1, which results in stabilization of the complex. Thereafter, GLUT1 is associated with the complex, and finally triggers the fusion process necessary to viral entry [9•].

In vitro experiments showed that free virions had poor infectivity, while co-culture of uninfected cells with HTLV-1 infected cells easily established HTLV-1 infected cells [10]. It has been reported that cell-mediated infection of HTLV-1 is 10,000 times more efficient than cell-free infection, while cell-to-cell infection by HIV-1 is only twice as efficient as cell-free infection [11]. Three models for the mechanism of cell-to-cell infection by HTLV-1 have been proposed: (1) virological synapse [12] and (2) biofilm [13••], and (3) cellular conduits [14]. HTLV-1 infected cells form a virological synapse with uninfected cells; ICAM-1 and LFA-1 are implicated in this synapse formation. Tax is also implicated, specifically in microtubule reorientation [15]. Indeed, Tax enhances cell-to-cell infection [11]. On the other hand, there is evidence to support the biofilm model as well. HTLV-1-infected T cells retain viral particles with virally-induced extracellular matrix components, including collagen, agrin, tetherin and galectin-3 [13••]. By cell contact, these viral assemblies adhere to other cells, resulting in infection with HTLV-1.

An increased number of infected cell augments the chances of transmission. Indeed, for mother-to-infant

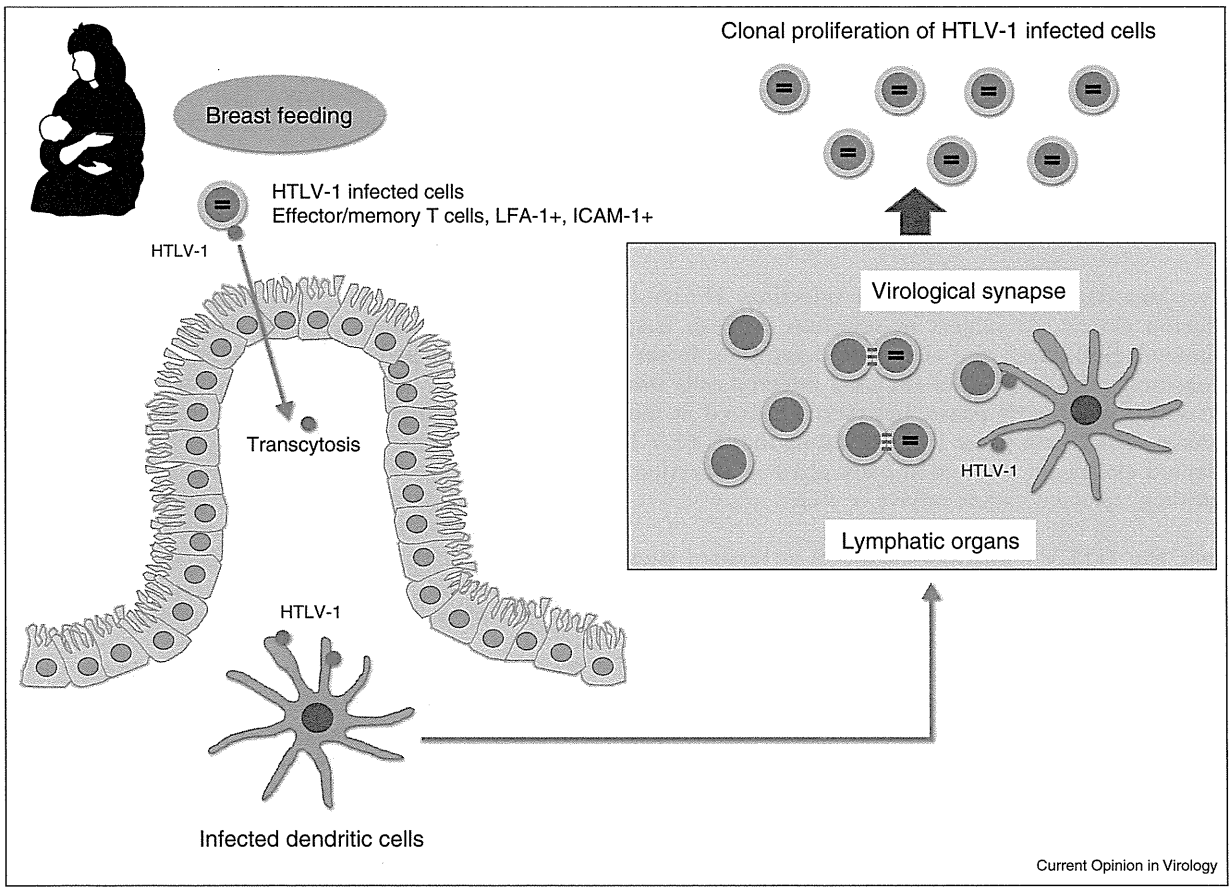
transmission, it has been reported that infants have higher chances of getting infected from mothers with higher proviral loads [16].

Transmission of HTLV-1

HTLV-1 can be transmitted by breast-feeding, sexual contact and blood transfusion. This transmission requires living infected cells, since this virus transmits mainly by cell-to-cell contact (Figure 1). Therefore HTLV-1 infected cells are hypothesized to have attributes that promote their entry into breast milk and semen. It has been reported that breast milk contains T-cells, most of which are effector/memory T cells expressing LFA1 and ICAM-1 [17], and HTLV-1 provirus has been detected in such effector/memory T cells [18]. These findings suggest that HTLV-1 may confer a phenotype to infected cells that facilitates their entry into breast milk. What component of HTLV-1 confers this effector/memory phenotype to HTLV-1 infected cells? Transgenic expression of HBZ in CD4+ T cells increased the number of effector/memory T cells and regulatory T cells, while transgenic mice expressing Tax had no change in the phenotype of CD4+ T cells [19••]. This clearly demonstrates that the immunophenotypes of ATL cells and HTLV-1 infected cells are conferred by HBZ, not by Tax. This conferred phenotype, which involves high levels of expression of adhesion molecules, enables HTLV-1 infected cells to enter into breast milk and semen (Figure 1).

Next, the virus must override epithelial barriers. How does HTLV-1 cross the alimentary tract? Recently, it has been shown that free infectious HTLV-1 virions could cross the epithelial barrier via a transcytosis mechanism [20•]. HTLV-1 virions could then infect human dendritic cells (DCs) that exist in the epithelial barrier [21]. Infected DCs likely migrate to draining lymph nodes and then form virological synapses with T cells (Figure 1). It is difficult to infect T cells by free virus *in vitro*.

Figure 1



Transmission and *de novo* infection with HTLV-1. HTLV-1 is transmitted via breast feeding, sexual intercourse, and blood transfusion. For any of these routes, living infected cells are essential. HTLV-1 infected cells have the immunophenotypes of effector/memory T cells or regulatory T cells. These cells tend to enter breast milk. HTLV-1 enters into the alimentary tract by transcytosis, and infects dendritic cells. Infected DCs transmit virus to uninfected T cells via virological synapses. Then infected T cells expand clonally *in vivo*.

However, free virus can infect DCs, and infected DCs can propagate HTLV-1 infection, suggesting that DCs are the spreader of this virus *in vivo* [21]. Expression of adhesion and co-stimulatory molecules is crucial for immunological synapses between T cells and DCs [22]. Thus, the immunophenotypes (effector/memory T cells, regulatory T cells, and enhanced expression of adhesion molecules) conferred by HBZ are crucial for the further spread of this virus *in vivo*. Thus, using HBZ, HTLV-1 induces infected cells to acquire certain immunophenotypes that facilitate its entry into the body and its subsequent spread within the body.

Clonal proliferation of HTLV-1 infected cells

After infection, HTLV-1 spreads by cell-to-cell infection and DC mediated infection. This *de novo* infection of cells is thought to form a pool of infected cells at an early phase of infection. In an experiment using immunodeficient mice with human lymphocytes, administration of reverse transcriptase inhibitors, tenofovir disoproxil fumarate (TDF) or azidothymidine (AZT) beginning after one week of infection could neither block nor decrease proviral load of HTLV-1, while TDF or AZT could block infection when they were injected at the same time of infection [23]. These results suggest that a pool of HTLV-1 infected clones is generated at very early phase of infection, and after that time, clonal proliferation of infected cells is predominant. This notion is also supported by clinical findings that reverse transcriptase inhibitors or integrase inhibitors did not alter proviral load in HTLV-1 infected individuals [24,25].

After this early stage of *de novo* infection, HTLV-1 infected clones are subject to selection by both host immunological attack and viral gene expression. In seroconvertors, the clonality of HTLV-1 infected cells was not stable at an early phase, but then stabilized at the chronic carrier state phase [26], indicating that HTLV-1 infected clones are selected at early phase of infection, and then, selected clones survive *in vivo*.

Since the HTLV-1 provirus integrates at random sites within the host genome, the clonality of HTLV-1 infected cells can be analyzed by studying these integration sites. Inverse PCR has been used to identify the integration sites and determine the clonality of infected cells [27,28]. Recently, high-throughput sequencing has been shown to be capable of detailed analysis of clonality [29••]. It is well known that HAM/TSP patients possess higher proviral loads compared with asymptomatic carriers. Analysis of clonality using high-throughput sequencing revealed that the abundance of each clone did not differ, but the number of different clones increased in HAM/TSP patients compared with asymptomatic carriers [29••]. By contrast, the abundance of certain clones increased in patients coinfected with HTLV-1 and strongyloides, and in infective dermatitis patients with HTLV-1 infection (IDH patients) [30•]. It is noteworthy that ATL develops relatively frequently in IDH patients and HTLV-1 carriers coinfected with strongyloides, while the occurrence of ATL is not so frequent in HAM/TSP patients [31]. Thus the enhanced abundance of clones and increased cell division might promote the development of ATL.

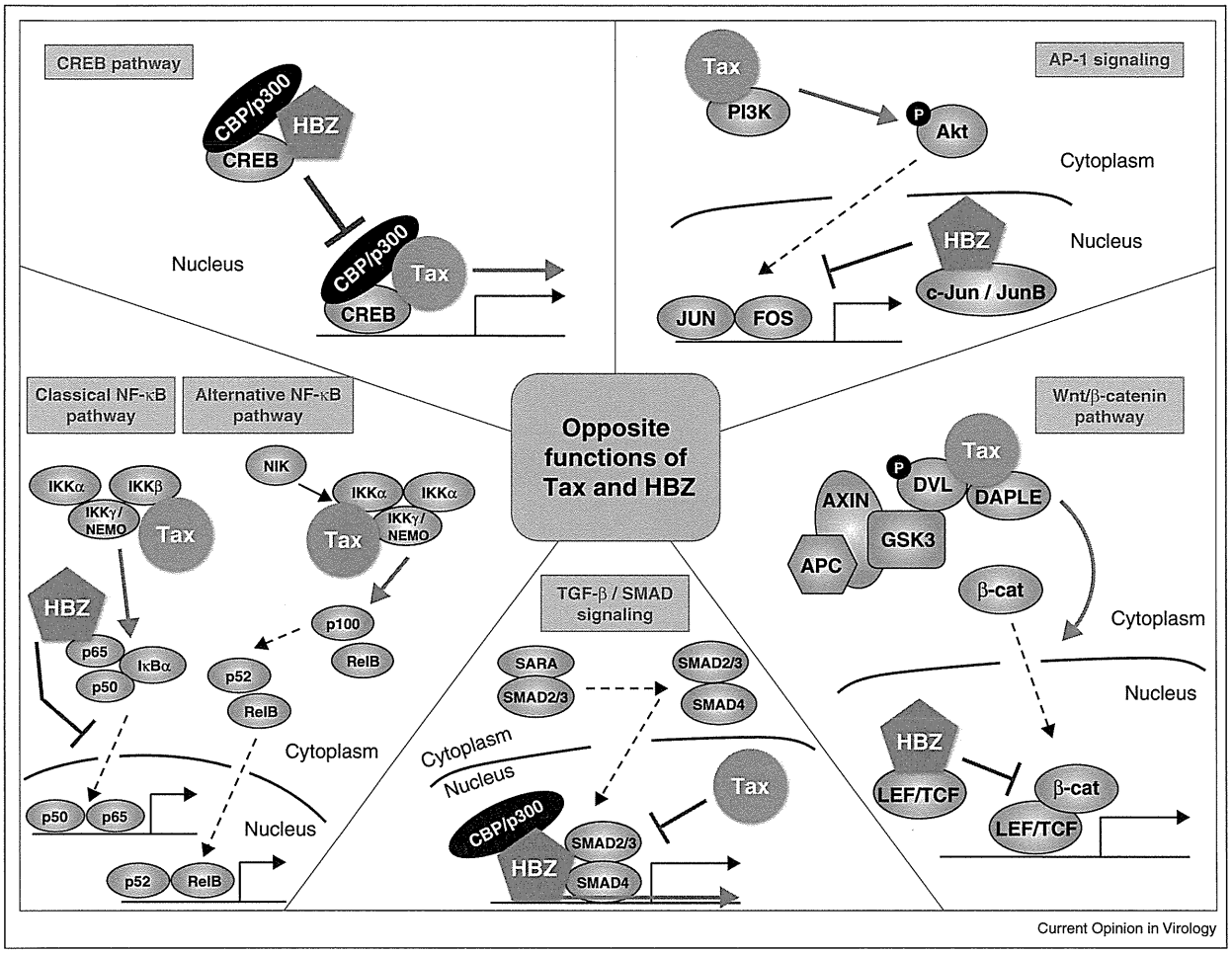
What drives cell division of HTLV-1 infected cells? HBZ is ubiquitously expressed in ATL cells and HTLV-1 infected cells *in vivo*, and promotes their proliferation [32]. In addition, Tax enhances mitogenic antigen-receptor signals [33,34]. The details of the mechanisms by which HBZ and Tax stimulate cell proliferation are complex and fascinating. In fact, HBZ and Tax have opposite effects on most signaling pathways [35] (Figure 2). For example, Tax activates the AP-1, NFAT, and CREB pathways while HBZ suppresses them [36,37]. Conversely, Tax inhibits TGF-β/Smad pathway whereas HBZ activates it [38]. Tax activates both the canonical and non-canonical NF-κB pathways [39]. HBZ inhibits only the canonical NF-κB pathway by interacting with p65. Expression of Tax promotes cell proliferation and simultaneously induces cellular senescence by induction of p21 and p27. HBZ prevents Tax induced cellular senescence by inhibiting p65 [40]. Thus, the elaborate interactions of various signaling pathways with Tax and HBZ control the proliferation of HTLV-1 infected cells. In addition to this relationship between HBZ and Tax, it has been reported that HBZ mRNA has growth-promoting activity [32], indicating another complex connection of HBZ as RNA and protein.

Furthermore, we have reported that HBZ suppresses the canonical Wnt pathway by inhibiting DNA binding by TCF-1/LEF-1 transcription factors, while Tax activates canonical Wnt signaling [41]. By contrast, HBZ enhances the transcription of Wnt 5a, which is a ligand for the non-canonical Wnt pathway. The canonical Wnt pathway is predominant during the development of T cells in the thymus, while non-canonical Wnt signaling is activated in peripheral T cells. These findings suggest that HBZ modulates the intra-cellular environment of peripheral T cells, which are natural target of this virus.

Control of transcription of viral genes

The HTLV-1 provirus encodes the regulatory genes (*tax* and *rex*) and the accessory genes (*p12*, *p13*, *p30*, and *HBZ*) in pX region; these genes regulate viral replication and the proliferation of infected cells [1]. For their transcription, the LTRs at each end of the provirus are used as promoters: the 5′LTR and 3′LTR control the transcription of the viral genes encoded in the plus and minus strands of the provirus, respectively (Figure 3). Since the plus strand of the provirus encodes all structural proteins and the viral genomic RNA, 5′LTR-mediated transcription is required for viral replication and transmission. Tax

Figure 2

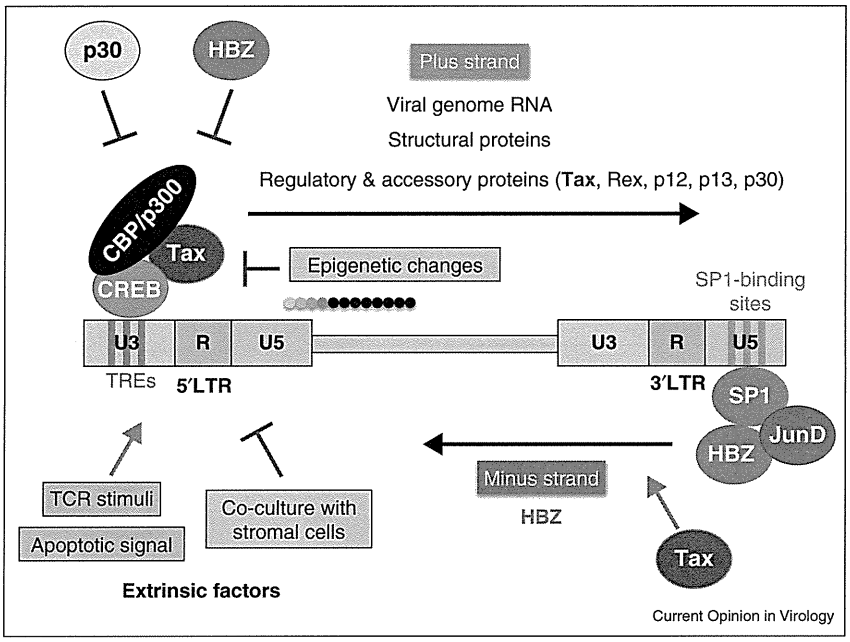


Opposite functions of Tax and HBZ. Tax and HBZ have opposite functions in many signaling pathways. Tax activates the CREB pathway by recruiting CREB and CBP/p300 to the promoters of target genes, whereas HBZ also interacts with the same proteins, suppressing Tax-mediated transcription. Tax activates both the classical and the alternative NF-κB pathways, and HBZ selectively suppresses classical signaling by targeting p65. Tax activates PI3K and induces the transcription of AP-1 target genes. HBZ negatively regulates this pathway by its inhibitory interactions with c-Jun and JunB through their bZIP domains. Tax forms a complex with DAPLE and DVL, and activates the canonical Wnt pathway. HBZ interacts with LEF-1/TCF-1 at point further downstream in this pathway and suppresses the transcription of the target genes. Tax has a negative effect on the TGF-β/SMAD pathway; however, HBZ activates it by interacting with SMAD2/3 and recruiting CBP/p300 to the promoters of the target genes.

is a potent activator of viral transcription through the 5′LTR. Tax does not bind to DNA, but activates the transcription of target genes by recruiting various transcription factors and modifying the epigenetic status of promoter regions [42]. The association between Tax and CREB is crucial for viral gene transcription. There are three 21-bp repeat elements, called Tax-responsive elements (TREs), located in 5′LTR, and the Tax-CREB complex recruits several histone acetyltransferaeses including CREB biding protein (CBP), p300, and p300/CBP-associated factor (PCAF) to the LTR, resulting in induction of viral expression. In addition to Tax, some cellular signaling machinery can enhance the activity of the 5′LTR. It has been shown that immune stimulation via T-cell receptor signaling activates the 5′LTR [34,43]. Another study showed that apoptotic signals induced viral transcription [44]. 5′LTR activation by these signals might be advantageous to efficient viral transmission and to viral 'escape' from a dying host cell.

Importantly, viral replication is actually suppressed *in vivo* [45], while viral antigens including Tax are quickly expressed in infected cells after they are transferred to *ex vivo* culture [46]. Host immune surveillance eliminates infected cells by targeting viral antigens. Among viral proteins, Tax is a major target of cytotoxic T-cells (CTLs)

Figure 3



Transcriptional control of HTLV-1 provirus. The 5′LTR is a promoter and enhancer of the plus strand transcripts that encode the viral genomic RNA, the structural proteins (Gag, Pol, and Env), and the regulatory/accessory proteins (Tax, Rex, p12, p13, and p30). Transcription via the 5′LTR is induced by recruiting the Tax-CREB-CBP/p300 complex to TREs in U3 region of 5′LTR, whereas the other viral factors (HBZ and p30) and epigenetic modifications on the 5′LTR suppress it. Some extrinsic factors are also associated with the activity of 5′LTR. By contrast, the 3′LTR is constitutively activated, and recruitment of SP-1 to its binding elements in U5 of the 3′LTR is important for 3′LTR activity. HBZ is encoded in the minus strand, and the HBZ-JunD complex enhances the transcriptional function of SP-1 on the 3′LTR.

[47]. It is well known that removal of CD8+ T-cells from PBMC allows infected cells to express Tax in the *ex vivo* cell culture [45], suggesting the presence of immune pressure against Tax *in vivo*. In addition, it was shown that, even in immunodeficient animal models, viral transcription from 5′LTR was suppressed, indicating that other mechanisms are involved in the silencing [48]. HTLV-1 can suppress its replication by its own proteins; p30 and HBZ are known to counteract Tax by competing for the binding to CREB, resulting in suppression of HTLV-1 replication [49]. p30 also inhibits the nuclear export of tax/rex mRNA [50]. Epigenetic changes, such as DNA methylation and histone modifications, are also involved in the silencing of HTLV-1. HTLV-1 differs from HIV in this respect. The LTR of HIV contains few CpG sites, while there are DNase hyper-sensitive regions, which explains the resistance of the HIV LTR to silencing [51,52]. On the other hand, the HTLV-1 LTR has many CpG sites, suggesting that HTLV-1 is susceptible to gene silencing mediated by DNA methylation. CpG methylation in the HTLV-1 provirus is observed in HTLV-1 carriers, and methylation tends to increase and to spread toward the 5′LTR during disease progression [53]. Indeed, Tax expression is frequently missing in ATL cells by epigenetic silencing of the 5′LTR as well as by genetic destruction of the 5′LTR or the *tax* gene [54,55]. Destruction of Tax expression enables ATL cells to escape from Tax-specific CTLs. Recently, it was reported that a histone deacetylase inhibitor, valproate (VPA), enhanced the expression of Tax and Gag in cultured HTLV-1-infected cells from asymptomatic carriers and HAM/TSP patients, suggesting that viral expression is suppressed by epigenetic mechanisms even in the carrier state [56•].

The 3′LTR functions as a promoter of the minus strand of the provirus [57]. It has been shown that the 3′LTR is conserved in all cases and CpGs are hypomethylated, suggesting that transcription through the 3′LTR is required for infected cells [53,58]. The *HBZ* gene is encoded in the minus strand, and alternative splicing makes the splice variants, the spliced and unspliced isoforms [59,60]. The spliced *HBZ* gene is transcribed from the 3′LTR, and the SP1 binding elements in 3′LTR are important for its transcription [57]. SP1 is a transcription factor ubiquitously expressed in a variety of cells, a fact which corresponds to the finding that HBZ is constitutively expressed in all ATL cases and HTLV-1 infected individuals [61]. It was also reported that SP1 forms a complex with HBZ and JunD and enhances the promoter