ABSTRACT

NOVEL ROLE OF HUMAN T-CELL LEUKEMIA VIRUS TYPE-1 ENCODED PROTEIN

HBZ IN VIRAL TRANSMISSION THROUGH CELL-TO-CELL CONTACT

by

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The complex retrovirus Human T cell leukemia virus type I (HTLV-1) is the etiologic agent of several diseases, including Adult T cell leukemia (ATL), a fatal hematological malignancy that affects mainly $CD4^+$ T-cells. Freshly isolated ATL and HTLV-1 infected cells aggregate spontaneously *in vitro*. We have observed this same phenotype in T-cells exclusively expressing one of the viral proteins called HTLV-1 basic leucine zipper factor (HBZ). The *hbz* gene is uniquely encoded by the complementary strand of the HTLV-1 provirus and, in contrast to other HTLV-1 proteins, is constitutively expressed in ATL and HTLV-1-infected cells.

High levels of aggregation in ATL cells have been correlated to an upregulation of the intercellular adhesion molecule-1 (ICAM-1). This protein is usually activated after T-cell stimulation and plays an important role in forming and stabilizing the immunological synapse. Interestingly, we found that cells expressing HBZ have an increase in ICAM-1 mRNA, which correlates with an increase in ICAM-1 at the cell surface. We confirmed by luciferase assay that HBZ expression stimulates ICAM-1 transcription. We found that blocking antibodies or peptides against ICAM-1 and its

ligand, lymphocyte function-associated antigen 1 (LFA-1), dissociate the aggregates formed by HBZ-expressing cells, suggesting that ICAM-1 overexpression by HBZ mediates T-cell aggregation through interaction with LFA-1.

Increased ICAM-1 expression at the cell surface is crucial for the formation of cell-to-cell contacts and efficient HTLV-1 transmission. To determine whether overexpression of ICAM-1 by HBZ plays a role in viral spread, we performed infection assays using a single-cycle, replication dependent reporter system. Interestingly, we found that HBZ expression significantly enhances HTLV-1 transmission. We confirmed that this effect involves the presence of LFA-1 on target cells. In addition, blocking the ICAM-1/LFA-1 interaction with an ICAM-1 antibody significantly reduced viral transmission. Therefore, ICAM-1 overexpression by HBZ plays a role in mediating viral transmission in our assays. A better understanding of the mechanisms used by HBZ to upregulate ICAM-1, induce cell-to-cell contact, and enhance virus transmission is important for future efforts to limit viral spread and prevent diseases in HTLV-1-infected individuals.

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CHAPTER 1: INTRODUCTION

1.1 HTLV-1 Discovery

In 1977, Takatsuki *et al.* described a highly aggressive CD4$^+$ T-cell malignancy in a small geographically clustered group of patients with lymphoid neoplasms in Japan. This disease was named Adult T-cell Leukemia (ATL) [1]. Subsequently in 1980, Poiesz and Gallo in the United States isolated a type C ultra-morphology retrovirus particle from a cell line derived from an African-American 28 year-old patient diagnosed with cutaneous T-cell lymphoma (CTCL) or mycosis fungoides [2].This patient may have been misdiagnosed because he had widespread systemic involvement, which is typical of ATL. The virus was named human cutaneous T-cell lymphoma virus (HTLV). Simultaneously, Dr. Miyoshi and collaborators in Japan recovered from ATL patient cells a retrovirus, which was classified as a type C retrovirus particle. These cells were co-cultured with cord blood to produce MT-2 [3, 4]. Also at this time, Dr. Y. Hinuma and his colleagues detected and described the entire structure of the HTLV virus from a cell line termed MT-1, which was also established from a patient diagnosed with ATL [5, 6]. Furthermore, Dr. Mitsuaki Yoshida and his colleagues in Japan (1982) identified the etiologic agent of ATL and named the virus Adult T-cell Leukemia Virus (ATLV) [7]. Thereafter, based on homology of the viral genome and viral antigens, it was demonstrated that HTLV and ATLV were the same retrovirus. The terms were unified to HTLV-1 [8]. In both the United States and Japan this new virus was causally linked to ATL. It is noteworthy to mention the discovery of the T-cell mitogenic factor made during the initial HTLV-1 studies by Dr.Gallo's group [9]. The T-cell mitogenic factor is currently known as interleukin-2 (IL-2), which allows the prolonged culture of malignant T-cells [9]

[2] [10]. This technological advance also paved the pathway for the research of one of the most challenging viruses of the century: the human immunodeficiency virus (HIV) [11, 12].

1.2   HTLV-1 Epidemiology

1.2.1   HTLV-1 Geographic Distribution

The Centers for Disease Control and Prevention (CDC) has classified HTLV-1 as a dangerous emerging pathogen [13].   HTLV-1 infects up to 20 million people worldwide, although the exact number of HTLV-1-carriers is probably much higher [14].

Its geographic distribution and prevalence can vary greatly: It is endemic in South-Western regions of Japan, Central Africa, the Caribbean basin, Central and South America, Northern Iran, some pacific islands [15-18], within the aboriginal population of Australia and in the Middle East [17, 19-23]. Sporadic infections occur in Europe and North America, and it is mainly concentrated in at-risk groups (intravenous drug users and their sex partners from endemic regions) and certain ethnic groups (African Americans in the United States). Within endemic regions, the seroprevalance is highly variable, ranging from 0.1 to up to 30%. However, these reports are thought to be underestimated in some highly populated areas such as China, India and East Africa [14] .

1.2.2   HTLV-1 Classification and Related Virus

Based on nucleotide and amino acid sequences, it has been determined that HTLV-1 is a complex retrovirus that belongs to the genus Deltaretrovirus of the Orthoretroviridae subfamily [24], which also includes other different types but closely related human T-cell leukemia viruses: HTLV-2, HTLV-3, and HTLV-4. Other non-human members are the three types of the simian T-cell leukemia viruses: STLV-1,

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STLV-2, and STLV-3, and the bovine leukemia virus (BLV). The genomes of HTLVs show 60-70% sequence identities to each other, but the pathogenicity of HTLV-2, -3, and -4 has not been clearly established [25-27]. HTLV-2 was initially identified in cell lines from patients with atypical hairy cell leukemia (HCL). However, several larger studies showed no association between HTLV-2 and HCL [28], or any other type of malignancy [29-31].

HTLV-3 sequences were discovered from the peripheral blood of a Central African native and were found to strongly resemble that of simian T-cell leukemia virus type-3 (STLV-3), a lymphotropic retrovirus that can infect different African monkeys species [32]. HTLV-4 sequences were identified in 2005 in an individual from a population of Central African bush meat hunters who have contact with non-human primates [33]. However, no disease has been associated with HTLV-3 or HTLV-4 infection. [27, 33-37].

1.2.3   HTLV-1 Transmission and Infection

HTLV-1 establishes a lifelong, persistent infection in humans in spite of a strong cellular immune system response against the virus [38-40]. The majority of HTLV-1 infected patients will remain asymptomatic carriers throughout their lives. However, severe clinical progression to ATL or HAM/TSP can occur in 2.5 - 5% of the infected individuals after months or decades of the initial infection [41, 42].

1.2.4   Risk of Mother to Child Transmission

HTLV-1 vertical transmission occurs mainly during breastfeeding from mother to child [43]. Risk of transmission increases with longer and exclusivity of breastfeeding [44-46], high maternal proviral load [47, 48], antigen expression in peripheral blood mononuclear cells (PBMC) and breast milk [17, 46, 49], and the mother's age [50, 51].

The association between breast milk and neonatal infection has been strongly established through large worldwide epidemiological studies comparing transmission rates between breastfed and formula fed infants [45, 52, 53]. Other routes for mother to child transmission of HTLV-1 are through perinatal contamination or *via* a transplacental mechanism, but they are uncommon [49, 54].

1.2.5   Other Routes for HTLV-1 Transmission

The estimated seroconversion rates of an individual exposed to HTLV-1 infected whole blood products due to the infusion of infected lymphocytes is high [55, 56], whereas the recipients of plasma fraction or plasma derivatives from HTLV-1 infected subjects do not become infected [57, 58]. For this reason, in 1988 the Food and Drug Administration (FDA) recommended to blood donation centers the screening of antibodies to HTLV-1 of all donations of whole blood and blood components in the United States, which has been active since then. Globally, the World health Organization (WHO) recommends the screening of blood donor products as a routine in regions in which HTLV-1 is endemic (Geneva: World Health Organization; 2009), such as Japan, the Caribbean area, foci in South America, the middle East and Africa, and Romania [14].   The most common cause of HTLV-1 transmission in the United States occurs among intravenous drug users sharing needles in the United States, Israel and Middle Eastern countries [59]. In addition, horizontal HTLV-1 transmission by sexual contact also occurs, but is a less efficient mode of infection. Male to female transmission through semen is more common than female to male transmission [60-62].

1.2.6   HTLV-1 Prevention

Prevention strategies for HTLV-1 carrier mothers involve the reduction or restriction of breastfeeding by feeding babies with formula [63]. Another recommended

strategy is to freeze milk before feeding, which can significantly decrease the risk of transmission [64, 65]. The elimination or reduction of breastfeeding can be easily implemented in developed nations. However, there are several challenges to this prevention strategy within endemic regions. Breast milk contains various compounds that promote immune development, and antimicrobial factors such as immunoglobulin (mainly IgA), lysozyme, complement, and leukocytes that inhibit many neonatal conditions, including gastrointestinal and respiratory infections, and bacterial meningitis [66]. Other issues to surmount are the access and affordability of formula, clean water and clean bottles. Hence, the development of a treatment that can inhibit the transmission of the virus at an early stage could be of great importance in many countries.

Elimination of persistently infected cells is the primary goal of current research towards a cure for HTLV-1, especially in asymptomatic carriers, because even when they are not currently afflicted by the disease, they still are able to spread the virus [67]. Based on that premise, the work conducted in this dissertation becomes more relevant, as it could potentially lead to an antiviral therapy able to control the dissemination of the virus.

1.3   HTLV-1 Pathogenesis and Disease Association

Retroviruses had been discovered in animals and reported to cause leukemia, but Human T-cell lymphotropic virus type 1 (HTLV-1) was the first verified human retrovirus to be directly related to development of cancer [68-72]. HTLV-1 was established as the causative agent of ATL, and also found to be associated with several other disorders, including a chronic and slowly progressive neurodegenerative, inflammatory disease, described in French Martinique as Tropical Spastic Paraparesis

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(TSP) [73], and in Japan as HTLV-1 Associated Myelopathy (HAM) [74]. In 1988, the World Health Organization (WHO) determined that these two diseases were the same [75], and since then, it has been called HAM/TSP [7, 72, 73, 76, 77].

HTLV-1 has also been implicated as an etiologic agent for numerous inflammatory diseases: disorders such as polymyositis [78], polyarthritis, uveitis [79, 80], infectious dermatitis [17, 81], thyroiditis [82], T- lymphocyte alveolitis, Sjögren's syndrome [83-85], virulent strongyloidiasis [85-87], and arthritis [88-90]. Interestingly, the presence of infective dermatitis in children may predispose them to the development of HAM/TSP [91]. In addition, the co-infection between HTLV-1 and HIV-1 is often associated with a relatively high CD4$^+$ T-cell count and symptoms resembling HAM/TSP [92]. Overall, the association of HTLV-1 with such vast array of clinical disorders indicates the tremendous public-health impact of this human pathogen. In spite of over three decades of study, the molecular mechanisms leading to ATL or HAM/TSP development have not been fully clarified.

1.3.1   Adult T-cell Leukemia (ATL)

ATL is a highly aggressive CD4$^+$ T-cell malignancy. The majority of infected people are asymptomatic carriers through life [68, 93], yet these individuals are still able to transmit the virus. Infection early in life is associated with the development of ATL, and the estimated lifetime risk for people who are infected with HTLV-1 to develop ATL is 2-5 %, which becomes clinically apparent typically thirty to forty years after the initial exposure to HTLV-1 [41, 49, 94-96] . ATL patients have a poor prognosis, even with existing treatment [97].

1.3.2   ATL Causes

The interactions between the host and HTLV-1 that promote the development

of ATL and the mechanism of progression of CD4$^+$ lymphocytes from viral integration to onset of ATL are not completely defined yet. However, it has been suggested a strong association between development of ATL and the exposure to HTLV-1 early in life through breastfeeding [98-100]. Furthermore, the vast time frame that allows the accumulation of genetic alterations and the surge of the malignant T-cell clones from the expanding polyclonal cell population is also considered an increased risk factor for ATL [72, 101, 102]. In addition, HTLV-1 infection regulates cellular activation and death pathways [103].

1.3.3   Signs and Symptoms of ATL

The clinical features of ATL are similar to non-Hodgkin's lymphoma: malaise, fever, jaundice, drowsiness, and weight loss. These patients also suffer from several abnormalities, including frequent lymphadenopathy or diffuse lymph node infiltration, hepatosplenomegaly, and skin rashes [104] [105] [106]. Furthermore, ATL is distinctive for its high rate of osteolytic tumors (lytic bone lesions), and induction of hypercalcemia with increased osteoclastic bone resorption. Frequently, there is widespread or localized skin involvement (in 40% of cases, there are large nodules, plaques, ulcers, and papular rash on the limbs, trunk, or face) in these patients. In addition, ATL patients frequently suffer from massive cellular infiltration of other organs such as the spleen, liver, gastrointestinal tract, and lungs [105, 107]. In addition, ATL is associated with immunosuppression and opportunistic infections caused by bacteria, fungal infections, parasites (e.g. Strongyloides stercoralis), protozoa and viruses [18, 108, 109], which contribute to poor prognosis.

1.3.4   ATL Diagnostic Criteria

The disease presents in a variety of clinical forms, but the typical criteria to

establish a diagnosis of ATL in patients is by the presence of HTLV-1 antibodies in the

sera from infected patients, by either applying an enzyme linked immunosorbent assay

(ELISA), most commonly in the United States, or particle agglutination (PA) in Japan.

The initial diagnosis must be confirmed by western blot analysis [110, 111],

immunofluorescence (IFA), or polymerase chain reaction (PCR) [14], and by clinical

manifestations (See "Signs and Symptoms of ATL"). Another disease characteristic is

the presence of a neoplastic population of circulating infected T-cells, with an atypical

convoluted and multilobulated nuclei ("flower cells"). These infected cells frequently

accumulate in peripheral blood, and also in lymphoid organs and skin. There are also

increased circulating levels of the IL-2 receptor α-chain (IL-2Rα/CD25), and elevated

serum lactate dehydrogenase (LDH) [94, 112-114]. In addition, based on molecular

approaches, it was determined that infected T-cells from ATL patients contained

integrated HTLV-1 proviral DNA, which was considered as another diagnostic criteria

[115].

1.3.5   ATL Subtypes

Approximately one half of the asymptomatic pre-leukemic patients can

develop lymphocytosis [116] that generally undergoes spontaneous regression,

whereas a few progress to the next phase of malignancy [117]. ATL is clinically

characterized in four different types: 1) Smoldering 2) chronic, 3) acute, and 4)

lymphoma [118-121] (Table1). The different subtypes are classified based on a specific

clinical and laboratory criteria, which includes the involvement of different tissues, in

particular skin, lymphocyte count, the presence of circulating atypical lymphocytes or

"flower cells", and percentage and different blood markers such as increased levels of

serum LDH and calcium [49].

The smoldering and chronic forms of ATL carry a better prognosis, with a four year survival of 62.8% and 26.9%, respectively [105]. They are characterized by skin lesions, cough, an absence of visceral involvement [122, 123], elevated numbers of circulating leukemic cells, marrow involvement, and few ATL cells in the peripheral blood (less than 5%). These two stages are indolent, but after a number of years they can develop into the acute forms [67, 94, 105, 124, 125]. The acute form of ATL is a very aggressive lymphoproliferative disease, with rapid progression and refractory to conventional chemotherapy. Patients with the acute form of ATL constitute approximately 55 to 75% of all ATL cases. It is characterized by fever, malaise, skin lesions (there are large nodules, plaques, ulcers, and papular rash on the limbs, trunk, or face in 40% of cases), lymphadenopathy, leukocytosis, hepatosplenomegaly, [105], lytic bone lesions, spleen or liver involvement, and immunodeficiency. It has the worst prognosis with a median survival at 6-12 months from diagnosis [97] even with intense chemotherapy, and a four-year survival rate of 5.0-6.8% [105, 122, 126]. Peripheral lymphocytosis with polyclonal or oligoclonal proviral integration can precede the acute phase of ATL. In addition, the presence of ascites in an acute ATL patient is associated with a poor prognosis, with a survival rate of only 6 months [127].The lymphoma stage is the second most aggressive, with a four-year survival rate of 5.7 % [105, 128, 129]. It is characterized by lymphadenopathy with atypical lymphocytes involving the superficial and deep lymph node chains.

1.3.6   ATL Treatment

ATL treatment options include watchful waiting until the disease progresses (for the indolent forms of the disease, smoldering and chronic), interferon alpha (IFNα)

Smoldering
- Occurs in 5% of ATL patients
- Skin lesions or lung infiltration of leukemic cells without visceral involvement
- Low number of leukemic cells (1-5% peripheral blood lymphocytes)
- Better prognosis and a four-year survival rate of 62.8%

Chronic
- Occurs in 20% of patients with ATL
- Associated with lymphadenopathy and hepatosplenomegaly without infiltration of CNS or bones
- High leukocyte count.
- LDH level is normal or slightly increased
- Four-year survival rate of 26.9%.

Acute
- Occurs in 55- 75% of ATL patients, with rapid progression of the disease and refractory to conventional treatment
- Characterized by fever, malaise, lung infiltration, skin lesions (large nodules, plaques, ulcers, and papular rash on the limbs, trunk or face in 40% of cases), massive lymphadenopathy, hepatosplenomegaly, lytic bone lesions and immunosupression
- Hypercalcemia and elevated LDH in serum
- Very poor prognosis with a four-year survival rate of 5%

Lymphoma
- Occurs in 20% of patients with ATL
- Similar characteristics as acute ATL but in the absence of peripheral blood (< 1% of leukemic cells) involvement.
- Very poor prognosis with a four year survival of 5.7%

ATL
Subtypes

Table 1. Different pathogenic forms of ATL. Clinical presentation and prognosis of the

different types of ATL. Smoldering, Chronic, Acute, and ATL Lymphoma.

and zidovudine (AZT) therapy [130-133], combined chemotherapy, allogeneic hematopoietic stem cell transplantation (allo-HSCT) [127, 134, 135], and clinical trial testing of new agents.

Currently, ATL treatment decisions are based on the patient ATL subtype, the prognostic factors, and the initial response to therapy (Table 2)[136]. Prognostic factors include age, stage, number of skin lesions, LDH and calcium levels, soluble IL-2 receptor, and suffering from opportunistic infections [136, 137].

Chronic and smoldering ATL.

These patients usually have a better prognosis when compared with acute/lymphoma ATL patients. However, the long-term survival of these patients under watchful waiting until the disease progresses was worse than initially thought [136]. Furthermore, patients who received chemotherapy at this stage had even a poorer prognosis [138]. The causes for progression of the indolent subtypes of ATL to more aggressive forms have not been elucidated. Current treatment options include a therapy of AZT/ IFNα, which must be continuous to avoid relapses, or participation in clinical trials, i.e. adding arsenic to the AZT/IFNα therapy to induce cell cycle arrest and apoptosis in leukemic cells [138].

Acute ATL.

These patients are managed mainly with combination chemotherapy regimens, such as VCAP-AMP-VECP, called the LSG15 regimen, which consists of six cycles of vincristine, cyclophosphamide, doxorubicin, and prednisone (VCAP); doxorubicin, ranimustine, and prednisone (AMP); and vindesine, etoposide, carboplatin, and prednisone (VECP). Other chemotherapy regimens involve four anti-cancer drugs known as CHOP: cyclophosphamide (Cytoxan, Neosar), doxorubicin (Adriamycin,

Rubex), vincristine (Oncovin), and prednisone. Usually all of them are given intravenously, except for prednisone which is given orally. Both chemotherapy regimens are supported with G-CSF intrathecal prophylaxis [139]. Other combination regimens include AZT/ IFNα [140].

Lymphoma ATL.

These patients receive a regimen of chemotherapy (VCAP-AMP-VECP) supported by daily G-CSF [127, 128, 139]. Under this protocol, ATL lymphoma patients have a better response to the treatment (66.7%) when compared with acute ATL (19.6%) or chronic ATL (40%) patients [128, 139]. In lymphoma ATL patients with a poor prognosis or no response to initial therapy, allogeneic HSCT is recommended or inclusion in clinical trials with new agents can be attempted [136].

Clinical trials - New agents.

Other treatment strategies have been reported in the literature, such as the use of anti-CCR4 (mogamulizumab), a humanized monoclonal antibody which displays a robust cytotoxic response against the ATL cells that express the chemokine receptor CCR4[141-144]. ATL therapy has also been approached by inducing growth arrest of ATL cells with phosphatidylinositol 3-kinase (PI3K) Akt/mammalian target of rapamycin (mTOR) signaling inhibitors [145]. Another compound with antitumoral properties under investigation as an ATL treatment is the HDAC inhibitor sodium valproate (VPA) ([146]. Belrose *et al.* suggested that VPA relieved the epigenetic control over certain HTLV-1 immunogenic viral proteins, exposing the ATL cells to the immune system[146].

In general, all these treatments can offer some initial improvement in most ATL patients. Unfortunately, none of them results in long-lasting complete remission, and the mortality rate is still very high. The overall median survival of ATL patients is still only

Table 2. Recommended treatment strategy for different ATL patients. Treatment for smoldering/chronic, acute and lymphoma ATL. HSCT indicates hematopoietic stem cell transplantation.

Subtype	ATL Treatment
Smoldering Chronic	Consider inclusion on prospective clinical trials **Asymptomatic patients:** Watch and wait for progression to acute ATL or lymphoma **Symptomatic patients (skin lesions, opportunistic infections)** AZT/IFNα or watchful waiting until disease progression Treat opportunistic Infections
Acute	Inclusion in prospective clinical trials Management of opportunistic infections Outside clinical trials: Consider prognostic factors and response to chemotherapy: **Good prognostic factors:** Chemotherapy (VCAP-AMP-VECP) or CHOP or AZT/IFNα **Poor prognostic factors:** Chemotherapy followed by conventional allogeneic HSCT **Unfavorable response to initial therapy:** Clinical trial testing with new agents Allogeneic BMT if feasible
Lymphoma	Inclusion in prospective clinical trials Management of opportunistic infections Outside clinical trials: Consider prognostic factors and response to chemotherapy: **Good prognostic factors:** Chemotherapy (VCAP-AMP-VECP) + G-CSF intrathecal prophylaxis followed by observation **Poor prognostic factors:** Chemotherapy followed by allogeneic HSCT **Unfavorable response to initial therapy:** Clinical trial testing with new agents Allogeneic HSCT if feasible
All types	**Progession/Relapse:** Monoclonal antibodies i.e. CCR4 Clinical trials testing new agents

between 6 and 18 months [87, 107, 114, 147, 148]. Research is being carried out in an effort to develop a vaccine against HTLV-1, but so far they are limited to animal models [149, 150].

Currently, promising new agents for ATL treatment are under investigation and in clinical trials, or in preparation for translational research [127, 151-154]. A better understanding of HTLV-1 and ATL disease can lead to advances in treatment and patient outcome.

1.3.7   HTLV-1 Associated Myelopathy/Tropical Spastic Paraparesis (HAM/TSP)

In addition to ATL, HTLV-1 was also reported to be associated with a neurodegenerative disorder called HAM/TSP [74]. The development of HAM/TSP typically occurs in up to 5% of HTLV-1 infected individuals in their fourth decade of life [155]. The onset of HAM/TSP has been reported to be strongly associated with the transfusion of HTLV-1 infected blood products [104] often with a progression from months to years instead of decades after transfusion [156]. This disease is a progressive chronic myelopathy that mainly affects the thoracic spinal cord [157-161]. HTLV-1-infected T-cells infiltrate the spinal cord by crossing through the blood-brain barrier. The pathogenesis of HAM/TSP has not been completely unraveled. However, it has been suggested that a strong cellular immune response and the release of inflammatory cytokines lead to bystander neural damage, demyelination and lymphocytic meningomyelitis [162]. Also, it has been shown that risk of development and progression to HAM/TSP is increased when HTLV-1 proviral copy number is high in the leukocytes of infected individuals [163-166]. Other studies have reported an association between certain HLA types with higher proviral loads and HAM/TSP

development, which would suggest a link between genetics and disease susceptibility [167, 168]. Interestingly, molecular mimicry has also been postulated as a contributing factor to the development of HAM/TSP [169]. The disease is diagnosed by elevated anti-HTLV-1 antibodies titers in the cerebrospinal fluid (CSF), and also high white blood cell (WBC) counts or high protein levels in the CSF, detection of proviral HTLV-1 DNA, and presence of atypical lymphocytes [170]. Among clinical features, HAM/TSP patients frequently are afflicted by urinary incontinence, ataxia and tremor [171, 172], progressive, chronic spastic paraparesis, walking disability due to lower limbs weakness [173], and hyperreflexia [174, 175].

Numerous disorders can resemble HAM/TSP symptoms, including multiple sclerosis (MS), familial spastic paraparesis, neurosyphilis, neurotuberculosis, HIV vacuolar myelopathy, Sjögren's syndrome, among others. It is important to exclude the mentioned conditions to avoid a misdiagnosis through specific laboratory and clinical evaluation [170].

1.3.8   HAM/TSP Treatment

After more than two decades of discovery, there is not yet an efficient treatment for HAM/TSP [176-178], and the options are very limited. Among the standard drugs for HAM/TSP therapy are Rapamycin and Cyclosporine, which are immunosuppressive drugs that can prevent the proliferation of T-cells in HAM patients [179]. In addition, corticosteroids, plasmapheresis, cyclophosphamide, and IFN-α occasionally produce improvement in signs and symptoms associated with the disease [180, 181]. Furthermore, several attempts have been made in order to reduce the proviral load, using antiretroviral therapy or by modulating viral expression by histone deacetylase (HDAC) inhibitors or by modulating lymphocyte proliferation or cytokine

production (by the use of interferon) [182]. Though some of the treatments have shown promise, relapses occur in most HTLV-1 infected individuals. It is clear that numerous viral events still remain to be discovered before we can find an effective treatment for HTLV-1-infection and its associated diseases.

1.4   HTLV-1 Genome Organization

1.4.1   HLTV-1 Genome

The HTLV-1 genome is 9 kb in length (Figure 1) and represents a typical replication competent retrovirus, which contains genome regions encoding for common retroviral proteins, group specific antigen (Gag), protease (Pro), polymerase (Pol), and envelope (Env) [183]. In addition, the genome contains four open reading frames (ORFs) in a unique *pX* region, which encodes regulatory and accessory viral proteins.

Once the RNA viral genome is reverse transcribed to create a double-stranded DNA intermediate, the coding sequence or provirus is flanked by the 755 nucleotide long terminal repeat (LTRs). The LTR contains cis-acting regulatory regions, which are essential for viral integration into the host genome, replication, splicing, viral message transport, and regulation of gene expression [184, 185]. The LTRs are divided into three regions: unique region 3' (U3), repeated region (R) and unique region 5' (U5). The U3 region in 5' LTR contains the viral promoter where transcription of all sense strand-encoded genes is initiated. The other regions of LTR, R and U5, provide the leader sequence for HTLV-1 transcripts. In addition, the 3' LTR at the 3' terminus of the HTLV-1 genome also contains a functional promoter for antisense transcription [186-188], which is completely functionally independent from the 5' promoter [187, 189]. Furthermore, it has been reported that sense transcription from the 5' LTR does not interfere with antisense transcription, and *vice versa*, suggesting that the bidirectional

transcription observed along the HTLV-1 genome does not affect the expression of viral

proteins encoded by these two promoters [190].

The HTLV-1 transcripts can be classified in four major classes : a) plus strand

unspliced mRNA (US), coding for Gag-Pro-Pol, used as genomic RNA; b) plus strand

singly-spliced mRNAs (ss), coding for the envelope glycoproteins (Env) and for the

accessory proteins p21rex, p12 (p8) and p13; c) plus strand doubly-spliced mRNAs (ds),

coding for the regulatory proteins Tax and Rex, and for the regulatory/accessory protein

p30. Both the minus strand mRNAs spliced and unspliced code for HBZ proteins.

The structural Gag protein (48 kDa) is processed (proteolysis) into the mature

structural proteins matrix (MA), capsid (CA), and nucleocapsid (NC) subunits that form

the core of the viral particle. Cleavage of Pol (99 kDa) yields the enzymatic components

of HTLV-1, reverse transcriptase (RT), which reverse transcribes viral RNA into double

stranded circular DNA, and integrase (IN),which integrates viral DNA into the host

genome. Proteins encoded by *gag, pro,* and *pol* are cleaved by the viral protease (Pro),

with the progene located between *gag* and *pol* in a different reading frame. Env is

expressed independently from the Gag-Pro-Pol complex, and it is a precursor to

envelope glycoproteins. It encodes the surface unit (SU) gp46, and transmembrane

subunit (TM) gp21.

1.4.2   HTLV-1 *pX* Region

Another unique aspect of the HTLV-1 genome is its *pX* region, located

between the *env* gene and the 3' LTR. It consists of four partially overlapping, and

alternatively spliced open reading frames ORFs (I to IV) on the sense strand that encode

for nonstructural proteins, including p12 (ORFI), p13(ORFI), and p30(ORFII); the

HTLV-1 Transcriptional Activator of *pX* region (Tax)(ORFIV),and the regulator Rex

(ORFIII) [191-194] [117, 195-199]. There is also one ORF located in the antisense strand of the proviral genome that encodes for the HTLV-1 basic leucine zipper factor (HBZ) [186]. The regulatory proteins p12, p30, p13 are involved in virus infectivity, regulation of viral transcription, and viral pathogenesis [200-204]. Rex is an RNA-binding post-transcriptional regulator which specifically binds to the Rex response element (RRE) located at the 3' region of the viral mRNA to promote the transport of the unspliced and singly spliced viral RNA from the nucleus to the cytoplasm to express structural proteins [205-207]. Among all these regulatory proteins, the most studied are Tax and HBZ, which have crucial roles in viral persistence and pathogenesis [187, 207-211]. Plasma antibodies against both Tax and HBZ proteins can be detected in patients utilizing several assay systems, such as Western blot [212], radioimmunoprecipitation/SDS-PAGE [213], ELISA [214, 215] or by highly sensitive luciferase immunoprecipitation systems (LIPS) [216-218]. However, the expression in plasma of the rest of the HTLV-1 viral proteins (p12, p8, p30) is still unclear in these infected individuals [219-221].

1.4.3   Tax

Tax, a 40KDa pleiotropic phosphoprotein of 353 amino acids (aa), is known as the HTLV-1 viral transactivator. Although it is located predominantly in the nucleus, it shuttles between the nucleus and cytoplasm using a nuclear export signal protein [222-225]. Tax transcriptionally activates the HTLV-1 5' LTR promoter through three repeat sequences called the Tax response element 1's (TxRE-1s), found in the U3 region of the 5' LTR [222, 223, 225, 226] (Figure 1). Each TxRE-1 contains a cyclic adenosine monophosphate cAMP response element (CRE)-like sequence [227-230]. In a complex with Tax, the TxRE-1s members of the activating transcription factor/CRE

binding protein (ATF/CREB) family can bind, and then recruit the multifunctional transcriptional coactivator CREB-binding protein or its homolog p300 (CBP/p300) [231]. Formation of a Tax/CREB/p300/CBP complex at the promoter initiates transcriptional activation [232-234]. In addition to viral gene expression, Tax also regulates the expression of many cellular genes, including cytokine and transcription factors genes, and genes involved in apoptosis and DNA repair. Also, Tax alters DNA damage-induced checkpoints that normally monitor chromosomal integrity, and can repress expression of proteins involved in several overlapping DNA repair pathways, such as DNA β-polymerase, which is utilized for nucleotide excision repair (NER), base excision repair (BER)([208], mismatch repair (MMR), and recombination repair [235]. Furthermore, it has been demonstrated both *in vitro* and *in vivo* that Tax is a critical component in the proliferation and transformation of human primary T-cells. [211, 236]. However, it has been reported that Tax it is not required for maintenance of neoplastic clones that are characteristic of ATL [237]. In fact, most of the ATL cells lose viral gene expression, including expression of Tax [238, 239] by abortive genetic changes, deletion or DNA methylation of the 5' LTR, which prevents TxRE-1 mediated expression of viral genes [240]. Insertions and point mutations (missense or nonsense mostly caused by deamination) are also observed. These alterations have not been characterized in the promoter for HBZ [241], which is the only protein consistently found in infected cells, freshly isolated from ATL patients, as well as HTLV-1-infected cell lines, and peripheral blood mononuclear cells (PMBCs) isolated from asymptomatic HTLV-1 carriers [242, 243]. All these data suggest that HBZ is necessary for maintenance of the infected clone.

1.4.4   HBZ

HBZ, encoded by the antisense strand of the provirus, is one of the HTLV-1 proteins with a crucial role in the pathogenesis of ATL. It regulates viral and cellular factors, contributing to the progression of the disease. It was identified in 2002, when an ORF was found on the antisense strand of the HTLV-1 genome [187]. The HBZ promoter is located in the 3' LTR [186, 187], and it is completely independent of the 5' LTR promoter, thus alterations in the 5' LTR promoter do not affect the expression of HBZ [187-189, 244]. Early studies have reported that the *hbz* gene is constitutively expressed in all HTLV-1-infected cells [242, 245]. HBZ transcription is regulated by the transcription factor Sp1, an important basal transcription factor for many TATA-less promoters [188, 246].HBZ can also activate transcription of its own gene by forming heterodimers with JunD (AP-1 family), which then bind to Sp1 sites at the 3' LTR promoter [247]. HBZ is expressed as three transcripts in both cells from ATL patients and HTLV-1 carriers. These antisense transcripts are alternatively spliced and polyadenylated: the major single spliced variant (sHBZ sp1), a second spliced variant, (sHBZ, sp2), and the unspliced variant (usHBZ) [242, 244, 248]. The usHBZ and sHBZ (sp1 and sp2) protein sequences are identical, with the exception of the first seven amino acids [248, 249]. However, it has been reported that both usHBZ and sHBZ display similar functions. It has also been reported that there are differences in cellular localization between spliced and unspliced. In addition, the half-life of sHBZ protein is longer compared to that of usHBZ [188]. Furthermore, sHBZ is predominantly expressed in infected cells [204, 243, 250]. Hence, the majority of studies have focused on sHBZ (sp1), including studies in the
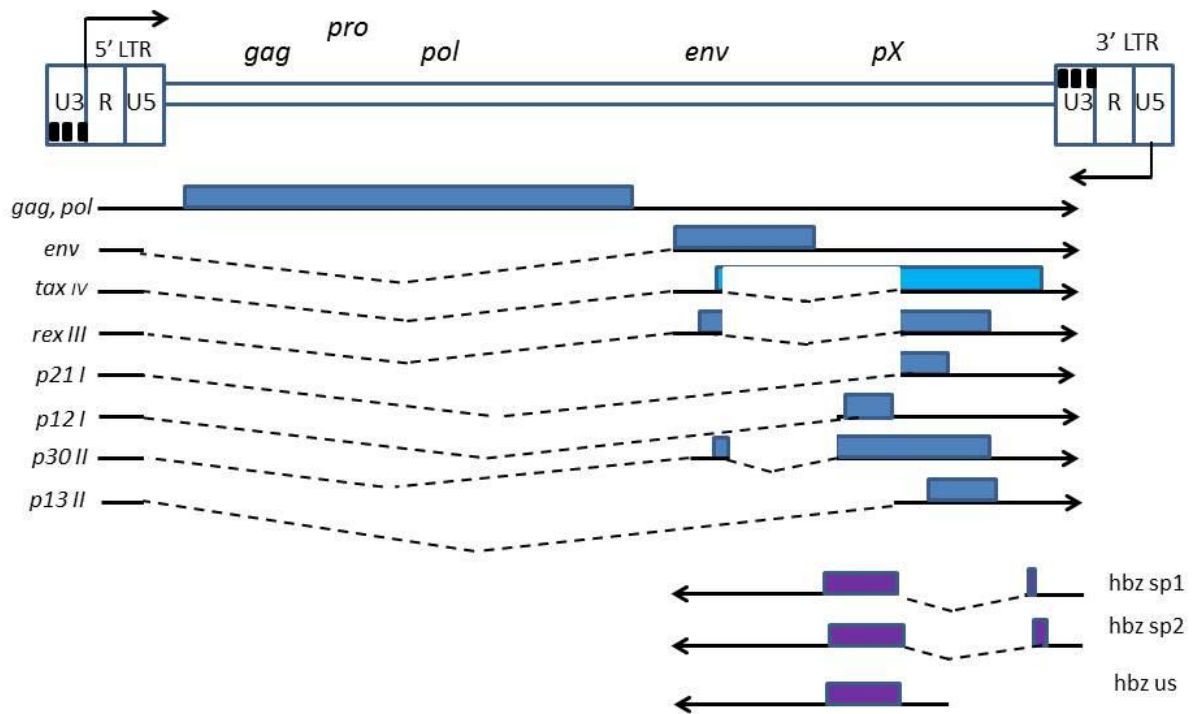
Figure 1. Schematic representation of HTLV-1 genome (~9 Kb), organization, alternate splicing and coding of HTLV-1 mRNAs. The viral genome has 5' and 3' Long Terminal Repeats (LTRs), divided in three regions: U3, R, U5. The LTRs flank the partially overlapping open reading frames (ORFs) of the gag, pro, pol and env genes that code for enzymes and structural proteins of mature viral particles. The region between the env gene and the 3' LTR is called the *pX* region and contains four major partially overlapping ORFs, -I through -IV, coding for regulatory and accessory proteins. The arrows indicate direction of transcription. Dotted lines represent the introns. Locations of the ORFs are indicated by boxes. Adapted from Matsuoka and Jeang, 2007, Nature reviews.

present dissertation.

It has been reported that HTLV-2, as well as HTLV-1, encodes an antisense protein, the antisense protein of HTLV-2 (APH-2) [251]. HTLV-2 has not been causally linked to human pathologies [26]. Nonetheless, HTLV-2 can immortalize and transform T-cells *in vitro* [252], and HTLV-2 Tax is able to immortalize human CD4+ T cells [253]. Thus, there is a growing concern for the increasing endemic infection of HTLV-2 among intravenous drug users in the US and Europe [37, 254] hence the ongoing research is considered of great public health importance. Interestingly, antisense transcripts have been also reported in both HTLV-3 and -4, and the proteins were termed APH-3 and APH-4, respectively [255].   No evidence of human pathology has been reported for these antisense proteins.

1.4.5   Location and Structure of HBZ

Most of the HBZ is detected in the nuclear compartment, where it accumulates in a speckled pattern [256, 257]. Regarding its structure, HBZ contains three main domains (Figure 2): An N-terminal activation domain (AD), a central domain (CD) composed by three basic regions necessary for nuclear localization, and a C-terminal leucine zipper (ZIP) domain [187].

The AD domain of HBZ contains two LXXLL-like motifs located within the N-terminal. Computational analyses predicted that both motifs lie within an amphipathic α-helix, which allow transcription factors to bind KIX domains [258]. In fact, both motifs can directly contact the KIX domain of homologous coactivators p300 and CBP [259]. These homologous coactivators are known to be critical for the activation of HTLV-1 and cellular transcription [259]. Both LXXLL-like motifs are also required for activation of
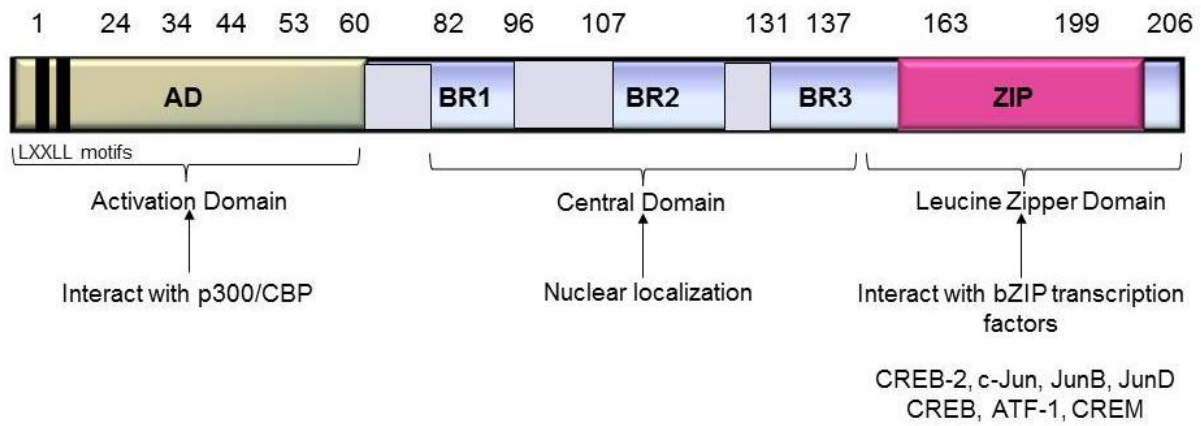
Figure 2. Schematic representation of the structural and functional domains of HBZ. HBZ is composed by an Activation Domain (AD) at the N-terminus, where there are two LXXLL-like motifs, important for the binding of co-factors p300/CBP. The central basic region contains the nuclear localization signals, and the C-terminus is composed of the Leucine Zipper Domain (ZIP). This region interacts with numerous important transcription factors, such as CREB, CREB-2, ATF, CREM, and the AP-1 family (c-Jun, JunB, JunD). Both AD and ZIP activities are necessary for activation of JunD, binding to p65 (NF-kB pathway), and enhance hTERT activity.

cellular genes, such as Dkk1, a protein involved in bone resorption [231]. The three basic regions of the CD domain contain the required positively charged residues, as in many nuclear localization sequences (NLS), necessary to promote nuclear translocation [256].

The ZIP domain located in the C-terminal region of HBZ consists of a basic region followed by heptad repeats of hydrophobic residues forming a leucine zipper. The area containing the heptad repeats of leucine adopts an α-helical conformation, which can form dimers by interacting with a similar α-helix of another polypeptide [256]. Thus it allows the association with different transcription factors that also contain a leucine zipper [187], such as CREB, CREB-2, ATF-1 and c-Jun, which are involved in the transcriptional regulation of HTLV-1 [231].

1.4.6 HBZ: A Selective Regulator of Transcription

So far, there is no evidence that HBZ is capable of binding DNA directly, suggesting that HBZ regulates viral and cellular transcription by interacting with different cellular factors. HBZ can form heterodimers with CREB via its ZIP domain [187, 260, 261] which prevents the recruitment of CREB to the TxRE-1s sites in the HLTV-1 promoter, thus repressing Tax-mediated viral transcription; HBZ also can interact with the homologous transcriptional co-activators CBP/p300 through two LXXLL-like motifs in its N-terminal region, leading to inhibition of the recruitment of CBP/p300 by Tax to the TxRE-1s sites [258, 259]. In addition to ATF/CREB family members, HBZ has been shown to interact and form dimers with several AP-1 transcription factors, JunB, JunD and c-Jun through its ZIP domain [210, 262]. Through colocalization studies it was confirmed that HBZ interacts in vivo with JunB and c-Jun [210].

Generally the interaction with HBZ decreases the DNA-binding activity of these cellular factors, preventing their interaction with Fos [263]. Also, usHBZ has been found to impair the stability of c-Jun by targeting it for proteasomal degradation [264, 265]. In addition, HBZ is able to sequester JunB within nuclear bodies [266]. Furthermore, HBZ suppresses the activation of the canonical NF-κB pathway through inactivation of the transcription factor p65 [204, 267, 268]. However, HBZ does not inhibit the non-canonical NF-κB pathway [267]. In addition, HBZ can upregulate the noncanonical Wnt ligand Wnt5a, which enhances proliferation of ATL cells [269]. Also, HBZ is able to inhibit the interferon regulatory factor-1 (IRF-1) [270, 271], a transcriptional regulator which plays important roles in many cellular processes, such as inflammation, apoptosis, tumor suppression and immune response to viral infections [272]. Interestingly, IRF-1 has been reported to be downregulated in many forms of leukemia [273-275].

In spite of HBZ being considered overall as a transcriptional repressor, in most instances HBZ activates transcription of endogenous cellular genes. For instance, the interaction of HBZ with CBP/p300 activates the expression of cellular genes, such as Dickkopf-1 (DKK1), an inhibitor of the Wnt signaling pathway with an important role in bone resorption [231], suggesting that HBZ may contribute to disease progression. Hypercalcemia is typical of ATL and a significant number of patients experience lytic bone lesions. In addition, HBZ can interact with JunD [262], and activate the human telomerase reverse transcriptase (hTERT) promoter. Constitutively expressed hTERT is a crucial step in development of cancer [276]. These data suggest that the transcriptional stimulation by HBZ may lead to tumor progression in ATL patients. In addition, it has been reported that HBZ can upregulate the expression of a cell survival factor, the brain-derived neurotropic factor (BDNF), which is known to be expressed in HTLV-1

transformed and ATL cells [277]. This interaction induces autocrine and paracrine positive signaling loop in the infected cells that promotes their survival [277] and may be responsible for a poor prognosis. Furthermore, it has been shown that HBZ is able to enhance TGF-β signaling through the promotion of the physical interaction between p300 and Smad3 transcription factors, which induces the activation of the TGF-β target genes [278].

1.4.7   HBZ: Role in Proliferation, Persistence and Cell Survival

Studies have indicated an important role of HBZ in viral pathogenesis. For instance, the majority of ATL cells (60%) do not express Tax protein due to genetic changes within the *tax* gene (nonsense mutation, deletion, and insertion) [237, 279], epigenetic silencing of the HTLV-1 promoter (DNA methylation of the provirus 5' LTR) [240, 280], or deletion of the 5' LTR promoter [281, 282], that results in the loss or suppression of the viral genes encoded on the plus strand of HTLV-1. However, the 3' LTR which contains the *HBZ* promoter is preserved intact [240, 242, 283], and HBZ is expressed constitutively in the HTLV-1 infected cells from both carriers and ATL cells directly from patients [242, 261] suggesting that HBZ has an indispensable functional role in cellular transformation, infectivity, viral persistence *in vivo,* and in the general development of ATL [188, 241, 284].

HBZ has been also involved in the regulation of different cellular mechanism to enhance ATL cell proliferation. HBZ was shown to promote cell proliferation by suppressing the CCAAT/enhancer binding protein alpha (C/EBPα), which is a crucial negative regulator of cell proliferation [285]. Furthermore, it has been reported that HBZ also promotes T-cell proliferation by upregulating expression of E2F1, a critical

transcription factor that regulates cell cycle progression by inducing the G1/S transition [242]. The tumor suppressor, retinoblastoma (Rb), is able to restrain normal levels of E2F1 and thus restrict cell cycle progression [286, 287] by arresting cell growth in the G1 phase [288]. An HBZ-mediated increase in E2F1 might deregulate the Rb-mediated control of E2F1, leading to DNA synthesis. Moreover, it has been reported that HBZ is able to repress the tumor supressor p53, a main regulator of genome stability, hence impairing its ability to activate transcription of genes involved in cell cycle arrest, apoptosis, and the DNA damage response [289]. This effect might also contribute to genome instability and excessive cell proliferation.

1.4.8 HBZ Studies *in vivo*

HBZ function has been investigated *in vivo* using a transgenic (Tg) mouse and a rabbit model of infection. Through the use of Tg mice, it was shown that the presence of CD4$^+$ T-cells expressing HBZ increased the incidence of lymphomas and systemic inflammation after a long asymptomatic period, similarly to what has been observed in HTLV-1 infected patients [290]. In addition, it was demonstrated in a rabbit model that elimination of HBZ expression from an infectious molecular clone of HTLV-1 (deletion mutant) resulted in a decreased proviral load and decreased antibody response to the infection [284]. In addition, the knockdown of HBZ in HTLV-1 positive T-cells correlated with a decrease in cell proliferation *in vitro* and a reduction in tumor growth and organ infiltration when these HBZ-knockdown cells were inoculated into mice [291].

Taken together, these results suggest that HBZ is required for an efficient HTLV-1 infection, proliferation, persistence, and survival of the infected T-cells, thus pointing HBZ as a strong potential target for ATL therapy, especially in the case of

carriers for preventing development of the disease.

1.5 HTLV-1 Viral Structure and Replication Cycle

1.5.1 HTLV-1 Viral Structure

HTLV-1 is an enveloped virus composed of host plasma membrane-derived lipid bilayer with viral glycoproteins gp46, the surface subunit, and gp21, the transmembrane subunit of Env that covers the internal protein core (Figure 3) [292]. The HTLV-1 mature virus particles (virions) possess a central symmetrically placed, high density spherical inner core about 80 to 120 nm in diameter with a C-type particle retroviral morphology that contains two copies of a diploid, positive sense, linear single-stranded RNA (ssRNA).

1.5.2 HTLV-1 Replication Cycle

The first step of the replication cycle of HTLV-1 starts with the binding of the virion to an activated target T-cell. A mature HTLV-1 virion that expresses gp46, which is the surface unit (SU) component of the viral protein envelope (Env), attaches to a multireceptor complex on the cell surface [293], that includes heparan sulfate proteoglycans (HSPGs) [294-297], neuropilin-1 (NRP-1), and the glucose transporter 1 (GLUT-1) [295]. HTLV-1 SU first binds to HSPGs, initiating the attachment of the virus to the uninfected cell [296, 298]. The subsequent binding of the SU to NRP-1 promotes the interaction between HSPGs and NRP-1. Once this interaction becomes stable, it triggers a conformational change in the transmembrane (TM) component of Env (gp21) that allows SU to bind to the GLUT-1 receptor. Once this multireceptor complex is formed, the viral and host cell membrane fusion occurs [297, 299]. After the outer membrane of the viral particles fuses with the host cell membrane, the virus undergoes "uncoating", in
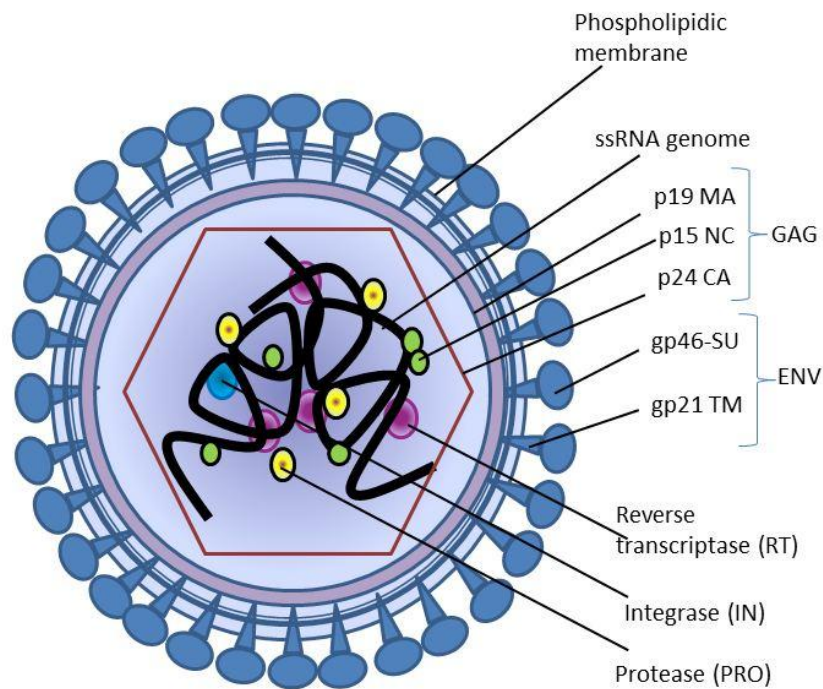
Figure 3. Schematic representation of the HTLV-1 virion structure. The surface glycoprotein (gp46-SU) binds to receptors of host cells. The transmembrane protein (gp21-TM) anchors SU to the virus. The polyproteins encoded by the *gag, pol,* and *env* genes are cleaved by a protease (Pro) into their respective functional components. The nucleocapsid (NC) is a protein found in association with the viral RNA. The matrix protein (MA) is a Gag protein associated with the lipidic area of the envelope. The capsid protein (CA) forms the core internal structure of the virus. The viral Reverse transcriptase (RT) reverse transcribed ssRNA to generate double-stranded DNA (dsDNA) and Integrase (IN) facilitates insertion of the viral genome into the host genome (forming the provirus).

which the viral core is released into the cytoplasm of the host cell, leaving behind the

envelope proteins (Figure 4).The viral genomic ssRNA is reverse transcribed to generate

double-stranded DNA (dsDNA) by the virally-encoded RT [300] using a host derived

t-RNA-dependent DNA polymerase that binds to a unique primer binding site (PBS) in

the retroviral genome. The RT synthesizes a complementary viral DNA strand in the 5' to

3' direction [300, 301], producing an RNA-DNA hybrid. The ribonuclease H (RNAse H)

component of viral RT degrades the RNA strand, and the newly synthesized viral DNA

strand is used as a template by RT, which also has DNA-dependent DNA polymerase

activity, to synthesize a complementary DNA strand. Translocation steps during this

process result in duplication of the U3, R and U5 domains, which generates two direct

repeats, the LTRs. Next, the viral dsDNA associates with cellular and viral proteins to

form a pre-integration complex (PIC) containing CA, RT, IN, and NC proteins (See

Figure 3). This PIC is then transported into the nucleus, and the viral dsDNA is stably

integrated in a random manner into the host DNA by the viral IN, and flanked by the LTR

on both sides. In this way, the provirus is formed, which constitutes the template for

transcription of the viral genome. After integration, the HTLV-1 provirus behaves like

host genomic DNA, requiring the host cellular transcription of RNA polymerase II,

transport machinery and protein synthesis to produce viral RNAs and proteins. After the

viral proteins are translated in the cytoplasm, the packaging of genomic RNA and

assembly into viral particles occurs at the cell membrane. The HTLV-1 genomic RNA

contains a sequence called the Psi element (ψ) for encapsidation. Since ψ is spliced out

in all other viral transcripts, only unspliced genomic RNA (full length) is packaged into the

new virions. The viral particles incorporate two copies of the single stranded RNA
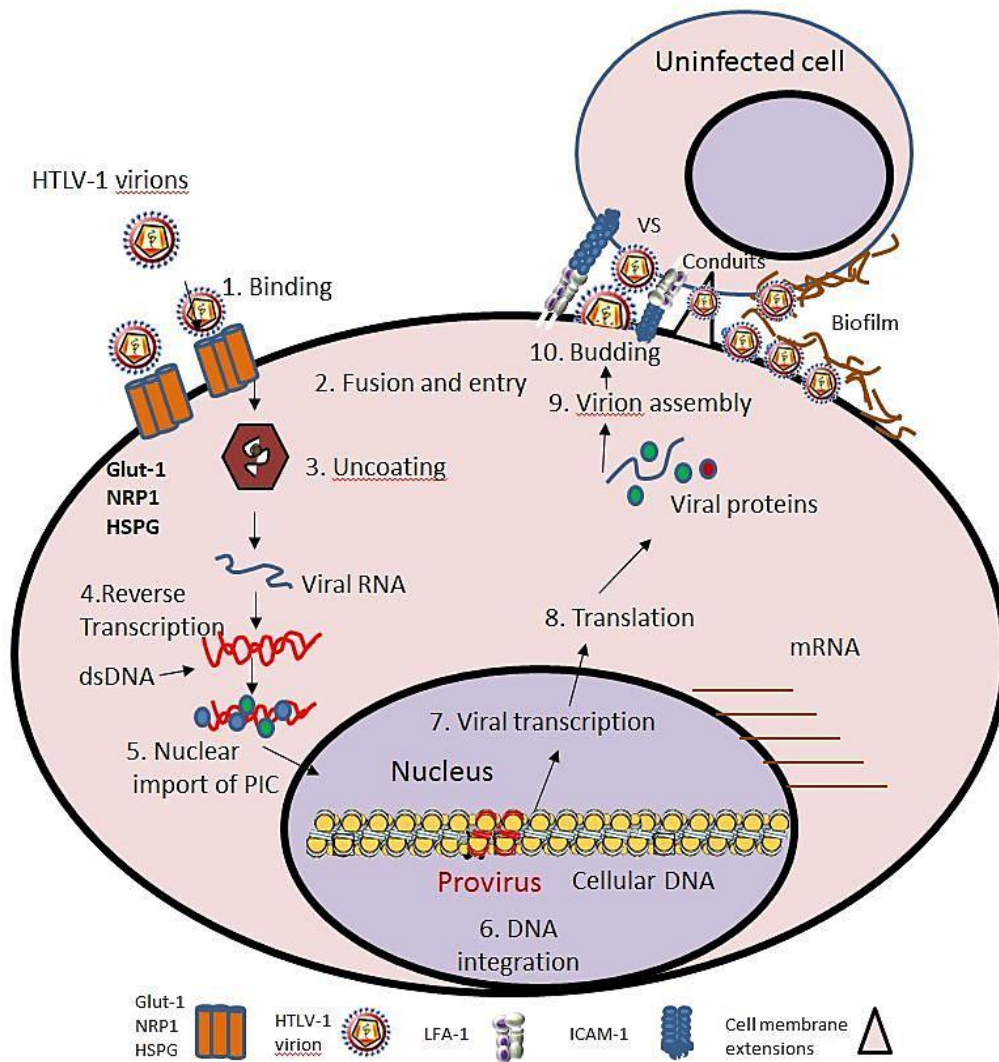
Figure 4. Schematic representation of HTLV-1 replication cycle. After HTLV-1 fusion and entry, the virion is "uncoated" and the viral genomic ssRNA is reverse-transcribed into dsDNA and integrated by IN into the host human genome. The viral mRNA from the provirus is spliced by the cellular splicing machinery and translated into viral proteins. Next, the partially spliced viral mRNAs are translated into precursors polyproteins for Gag, Pro, Pol, and Env. The proteins are used for assembly of the virions to the cytoplasm, which subsequently bud on the cell surface.

genome along with the tRNA, RT, protease and integrase. Thereafter, the virus core is coated with a host cell plasma- membrane lipid bilayer and Env during the budding process at the cell surface. [293, 299-303]. The HTLV-1 replication cycle is completed when virions are released by budding. However, still at this point, the virions are immature; the viral protease cleavage of the structural proteins (Gag, Gag-Pro, and Gag-Pro-Pol precursors) is necessary at, or soon after the budding of the new viral particle for obtaining a fully mature and infectious virion. HTLV-1 free virions infectivity is very low; therefore viral transfer by direct cell contact is a more efficient way of HTLV-1 transmission. Furthermore, it has been demonstrated that through the formation of a "viral synapse" (VS) HTLV-1 can spread efficiently between lymphocytes (T-cell to T-cell) [165].

1.6 HTLV-1 Cell-to-cell Transmission and its Role in Pathogenesis

1.6.1 Overview of HTLV-1 Transmission

There are two HTLV-1 replication patterns: *de novo* infection from infected cells to uninfected cells and clonal expansion of infected host cells [304]. It has been suggested that the effects of the viral proteins Tax and HBZ lead to immortalization and expansion of a polyclonal population of infected T-cells, which under selective pressure from the adaptive immune response develop into an oligoclonal population. It then originates monoclonal T-cells, which are typical of ATL [305, 306] (Figure 5).

1.6.2 Mechanism of Cell-to-Cell Transmission or *de novo* Infection

It has been documented that HTLV-I free particles are weakly infectious [307-310], thus cell-to-cell transmission is widely accepted as the primary mechanism of virus transmission [311]. This is consistent with the observation that HTLV-1 free virus
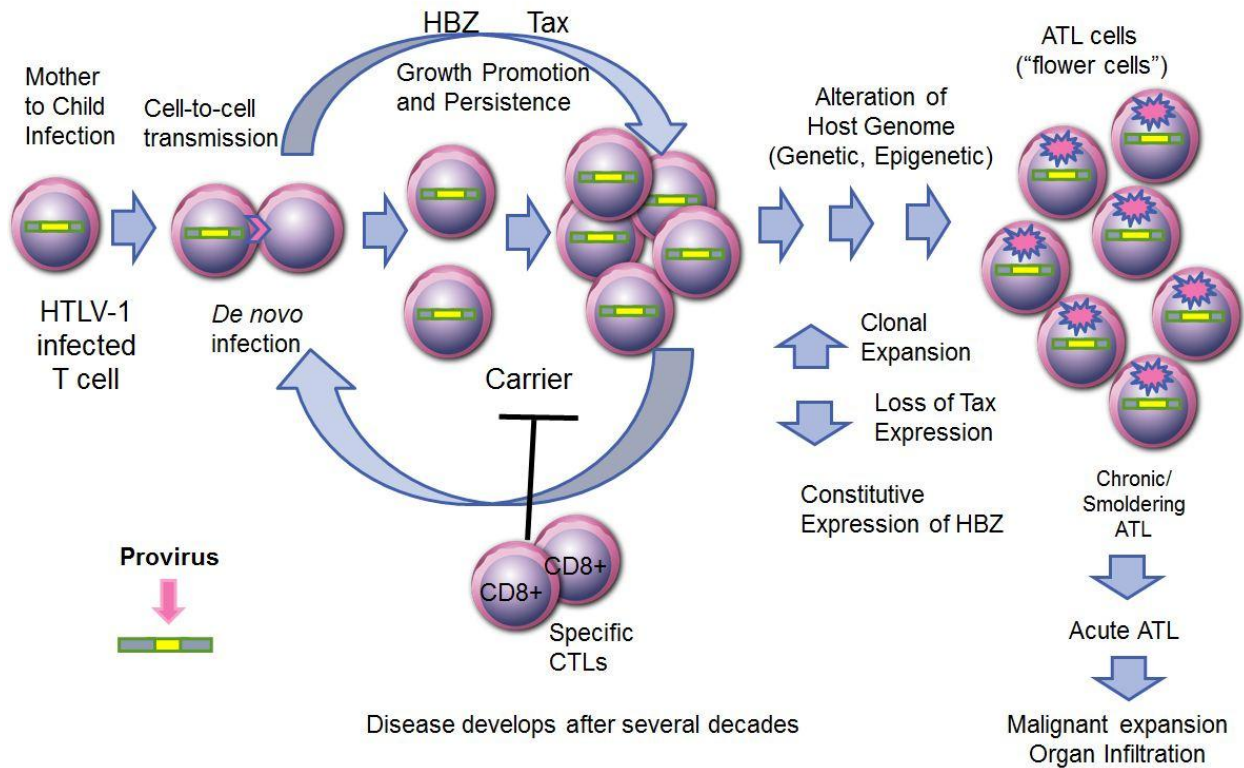
Figure 5. Schematic representation of the onset of ATL. The transmission from mother to child has been defined as the main route to development of ATL later in life. The direct cell-to-cell contact is required for the effective spread of the virus from infected to uninfected cells (*de novo* infection). After infection, HTLV-1 promotes cellular growth and proliferation through its viral proteins, in particular HBZ and Tax. This proliferation is controlled by specific CD8$^+$ cytotoxic T-cells directed mostly against the Tax protein, but the infection is not completely cleared. The virus becomes persistent even in the absence of symptoms (carrier state). Tax expression is inactivated by several mechanisms, such as mutations in the 5' LTR promoter. However, HBZ is expressed constitutively throughout the HTLV-1 infection. After several decades, aneuploid ATL cells ('flower cells') emerge in about 5% of asymptomatic carriers, finally leading to the onset of ATL.

particles are not found in plasma [39], and cell-free blood products are not infectious [55, 309, 312]. Also, a direct cell-to-cell contact constitutes an established mechanism to escape from immune surveillance by HTLV-1-specific neutralizing antibodies, complement, and drug therapy [313].

Studies have suggested that upregulation of the intercellular adhesion molecule-1 (ICAM-1) is required to achieve an efficient transmission of HTLV-1 [314-316]. ICAM-1 was also found to be highly expressed in HTLV-1-positive T-cell lines [317-319] and ATL cells [315, 320, 321]. ICAM-1 (CD54) is an inducible cell surface glycoprotein that belongs to the immunoglobulin supergene family. ICAM-1 expression is constitutively low on most cell types and under non-inflammatory conditions. The full length isoform is composed of five Ig domains, a transmembrane domain, and a short cytoplasmic tail with multiple threonine residues, which interacts with the actin cytoskeleton (Figure 6A). Dimerization of ICAM-1 subunits significantly increases the affinity for its cognate ligand, the membrane-bound integrin receptor Lymphocyte Function-Associated Antigen 1 (LFA-1). LFA-1 is a heterodimeric transmembrane molecule composed of an alpha subunit (αL; CD11a) and a β2 subunit (CD18) (Figure 6B). The interaction between ICAM-1 and LFA-1 plays a crucial role in adhesion mechanisms involved in the immune response, inducing a specific cell-cell adhesion that enables intercellular communication, leukocyte trafficking, immunological synapse formation and stabilization, and numerous inflammatory responses. Interestingly, it has been reported that blocking ICAM-1 on HTLV-1-infected cells abolishes VS formation [322]. These findings indicate that the interaction between LFA-1 on uninfected (target) cells and ICAM-1 on the infected (donor) cells is crucial for HTLV-1 transmission. It also

suggests that ICAM-1 may play an important role in HTLV-1 infection by stabilizing those cell-to-cell interactions [323-326]. The precise mechanism of HTLV-1 cell-to-cell infection is unknown. However, three mutually non-exclusive models based on cell–to-cell transmission have been described: 1)Virus spread through the formation of a virological synapse (VS) [48, 327, 328], or 2) biofilms [329] or 3) through induction of cellular conduits [330].

1.7 Models of HTLV-1 Transmission: Virological Synapse, Biofilm and Conduits

1.7.1 HTLV-1 Virological Synapse Formation.

The VS (Figure 7) is a virus-induced region between the cell-to-cell contact which allows the spread of viral proteins and genomic RNA through a synaptic cleft formed between infected and uninfected cells [327]. The structure was called "virological synapse" because the contact at the surface of the infected T-lymphocyte (donor cell) and the non-infected cell (target cell) resembled an immunological synapse (IS) [327, 331]. The formation of the VS involves a virus-induced polarization of the microtubule-organizing center (MTOC) in the HTLV-1-infected cell towards the uninfected cell, whereas in the case of the IS, the MTOC is polarized in the responding T-cell towards the antigen presenting cell, triggered by the T- cell receptor complex [332]. Adhesion molecules are crucial at the early stage of the VS formation [324], in particular ICAM-1 and its ligand LFA-1 [322]. ICAM-1 is known to cause a significant polarization of the MTOC, which involves cytoskeleton reorganization and reorientation of the MTOC in the HTLV- 1-infected cells [333]. In addition, electron tomography of cell-to-cell contact sites has shown that HTLV-1 particles are released into synaptic clefts between infected
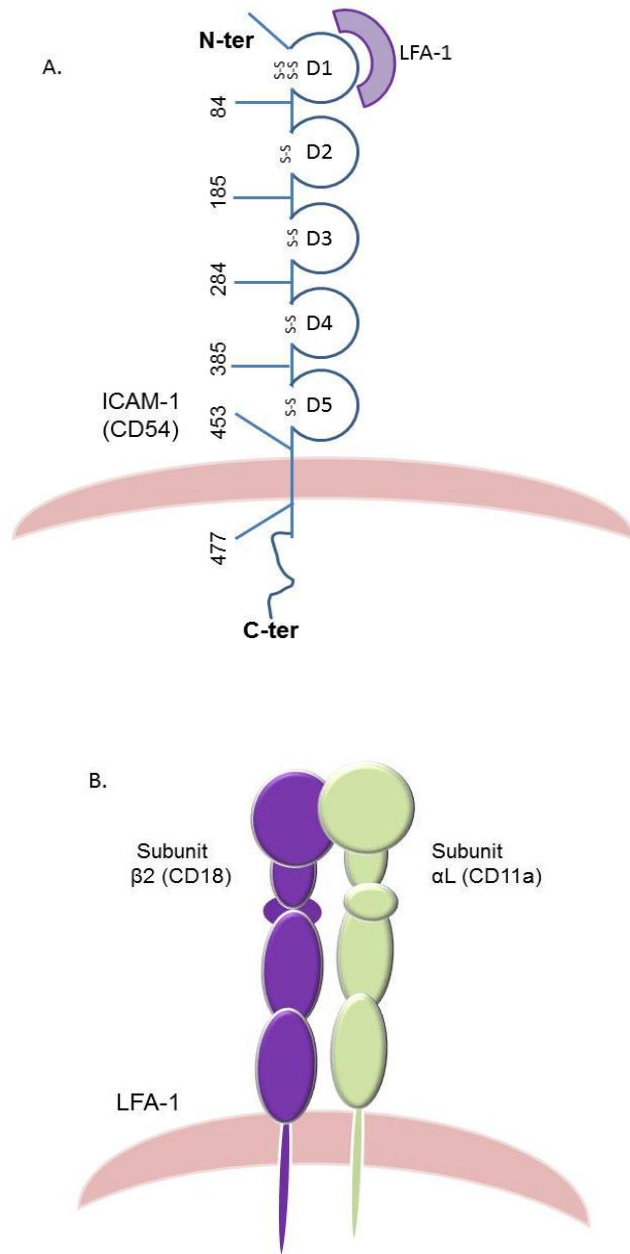
Figure 6. Schematic representation of adhesion molecules involved in virus transmission.
A) ICAM-1 is composed of five Ig domains (denoted as D1-D5), a transmembrane domain, and a short cytoplasmic tail with multiple threonine residues and B) LFA-1 is composed by αL and β2 subunits.

and uninfected primary lymphocytes, suggesting a model involving polarized virus assembly and transmission across the synapse for efficient virus spread [328]. In conclusion, HTLV-1; transmission through the VS during direct cell-to-cell contact is much more efficient than infection by low infectivity cell free-virions [304, 310, 312, 327, 333].

1.7.2   HTLV-1 Biofilm

In addition to the formation of the VS, other studies have reported the presence of extracellular viral clusters on the surface of infected cells in a biofilm-like carbohydrate-rich assembly, composed mostly of secreted extracellular matrix components, such as collagen, agrin, and linker-proteins that may shield viruses at the cell surface from the immune system [329]. This biofilm structure also facilitates a direct-cell-to-cell transmission of HTLV-1. When infected cells contact uninfected cells, the extracellular accumulated virions in the biofilm are rapidly transferred to the target cells, promoting *de-novo* infection. By removing the viral biofilm from the surface of the infected cells, researchers achieved an 80% reduction in infection rates *in vitro* [329].

1.7.3   HTLV-1 Conduits

Another mechanism of virus spread between HTLV-1-infected cells and uninfected cells involves the formation of transient membrane extensions called intercellular conduits [330], through which virions may be transmitted from an infected cell to an uninfected target cells. These conduits may also stabilize cell-cell contacts. Transmission EM studies have shown that, as occurs during the formation of the VS, the HTLV-1 virions concentrate at the contact region between the HTLV-1-infected cell and the target cell, and are transferred to neighboring T-cells through these conduits
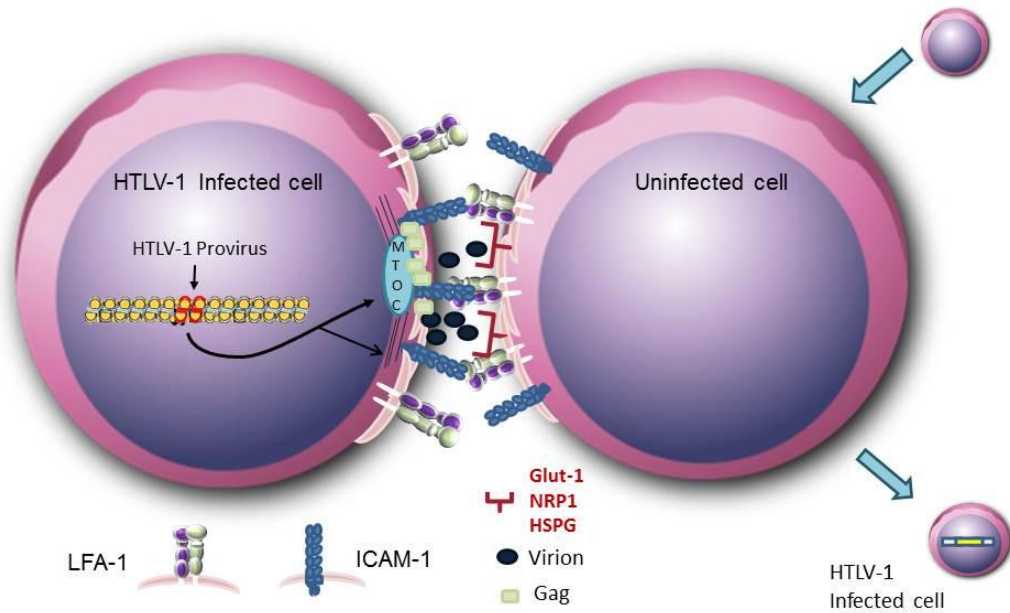
Figure 7. A proposed model for cell-to-cell HTLV-1 transmission. Virus spread from HTLV-1 infected cells to uninfected cells. The infected cells expressing high levels of ICAM-1 are able to make cell contact with the uninfected cell, and through rearrangement of the cytoskeleton, form synaptic clefts between both cell membranes and release mature virions that will attach to the specific receptors on the uninfected cell, and start the viral replication cycle inside the target cell.

[330, 334]. So far, most of the conclusions about HTLV-1 cell-to-cell infection have been derived from fluorescence microscopy, which revealed the accumulation of viral particles at the cell-to-cell contact regions, and from electron microscopy, which showed virions localized between the contacts of infected and uninfected cells [327, 328, 335]. Recently, an efficient method to quantify cell-to-cell infection with single-cycle, replication-dependent reporter vectors has been developed [310]. This system directly measures for viral infection of target cells in the context of cell-to-cell transmission, serving as a valuable complement to image analysis to determine the specific mechanism of cell-to-cell infection (See Chapter 3 Section 3.9).

1.8   HTLV-1 Proteins Involved in Virus Transmission

Regarding the molecular mechanisms of HTLV-1 transmission, research has focused on the participation of the HTLV-1 proteins Tax, p12 and p8 in the induction of cell-to-cell contacts, cytoskeletal reorganization, and virus spread.

1.8.1   Proteins p12 and p8

The HTLV-1 protein p12 is encoded by ORF-I in the *pX* region [336]. The involvement of the nonstructural gene p12 in viral infectivity has been difficult to reveal because the expression of the accessory viral genes *in vitro* is detectable only with reverse transcription PCR [77]. Thus, its role in viral spread is not completely clear. However, the role of p12 in HTLV-1 infection has been assessed using *in vivo* models. In one study using rabbits, a clone of HTLV-1 impaired for production of the p12 mRNA (p12$^{(-)}$) failed to establish infection in the animals [337], suggesting an important role for p12 in viral infection. Furthermore, another study was performed using macaques as the infection model [338]. These animals are susceptible to HTLV-1 and the development of

related diseases [339]. Results showed that   macaques that were challenged with a p12 knock-out, remained infection- free, and following inoculation, only two of the primates became tissue positive for viral DNA after a long period of time [338]. Of note, HBZ was also affected in the p12 mutants utilized in the infection experiments mentioned above [337, 338].

p8 protein originates from the proteolytic cleavage of p12 [336]. p8 is involved in the formation of membrane extensions or filopodium-like protrusions known as cellular conduits, through which HTLV-1 can directly contact and infect new target cells [330]). These data suggest that p8 may induce HTLV-1 transmission by mechanisms that involve cell-to-cell adhesion.

1.8.2   Tax

The Tax protein has also been documented to participate in virus transmission. It has been implicated in the MTOC polarization and formation of the VS in HTLV-1-infected cells [324, 327]. It was also shown that Tax-induced CREB signaling is crucial for the MTOC polarization [324], as is the activity of one or both of the small GTPases: Cdc42 and/or Rac1 [340]. Furthermore, Tax has been implicated in activating transcription of Gem through CREB and CBP. Gem is a member of the small GTP-binding proteins (Ras superfamily) involved in cytoskeleton remodeling and has a crucial role in cell-to-cell viral transmission [333].

Recently, it has been reported that Tax is able to upregulate the host cell factor actin-binding protein Fascin (FSCN-1) in infected cells [341, 342]. Fascin has crucial functions on the cytoskeleton dynamics. It is able to interact with microtubules to modulate cell migration. It also cross-links actin filaments and stabilizes filopodia and cell

protrusions [343]. Using the single-cycle replication dependent HTLV-1 reporter system Gross *et al.,* found that the induction of Fascin by Tax promoted cell-to-cell adhesion and release of virus from the cells [342]. Blocking Fascin on the infected cells diminished the transfer of gag p19 and the release of virus.

CHAPTER 2:   MATERIALS AND METHODS

2.1   Cell culture and Transfection

Jurkat, Jurkat-HBZ and Jurkat-pcDNA clonal T-cell lines, and HTLV-1-uninfected (CEM, Hut-78) and infected T-cell lines (1185, SP, MT-2, C10/MJ, SLB-1, C8166/45, and ATL-2) were cultured in Iscove's modified Dulbecco medium (IMDM) (Sigma, MO, USA) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine (Invitrogen, CA,USA), 100 U/mL penicillin, and 50 µg/mL streptomycin (Invitrogen, CA, USA) Jurkat clonal cell lines were additionally supplemented with 1.5 mg/mL G418 (Geneticin; Invitrogen, USA). HBZ-expressing T-cell clones and control clones were established by transfection of pcDNA-HBZ-SP1-Myc [263] or pcDNA3.1 (Invitrogen) respectively, using lipofectamine LTX reagent, and selection was done with 1.5 mg/ml G418 beginning 48 h post-transfection. The clonal cell lines were produced by expansion of single cells in 96-well plates.

SupT1, a human T-cell lymphoblastic lymphoma cell line [344], and TL-Om1 cells, an ATL-derived cell line [345], were cultured in Roswell Park Memorial Institute medium (RPMI) 1640 (Sigma, MO, USA) supplemented with 10% FBS, 2 mM L-glutamine, 100 U/mL penicillin, and 0.5 mg/mL streptomycin (Invitrogen, CA, USA). Interleukin-2 (IL-2) (50 U/ml, Roche) was added to the culture medium for 1185, and SP cells.

The HeLa cell lines stably expressing wild type (wt)-HBZ, or the empty vector (pcDNA3.1) or different HBZ mutants:HeLa-HBZ-MutAD, MutZIP, and ∆ATG [231]. The HeLa HBZ mutants were generated with point mutations in 1) the N-terminal Activating Domain (MutAD) of HBZ, that abrogates the HBZ interaction with the coactivators p300 and CBP [266]; 2) point mutations in the C-terminal ZIP domain (MutZIP), which inhibits the interaction between HBZ and numerous families of the ATF/CREB and Jun host bZIP

factors [231]; or 3) HBZ with a point mutation in the initiation codon (ΔATG), that suppresses the translation of the HBZ mRNA/transcript [231]). Chinese Hamster Ovary (CHO) cells [346] overexpressing LFA-1 cells (CHO-LFA-1) were suspended in cytomix buffer and electroporated as described elsewhere [347] using the BioRad Gene Pulser System. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin, and 50 µg/ml streptomycin (complete DMEM). The cells were additionally supplemented with 0.5 mg/ml G418.

2.2   Plasmids

To perform single-cycle, replication-dependent vector infection assay, plasmids provided by Dr. Derse's group and Dr. Mazurov were used. The HTLV-1 packaging vector (pCMVHT1M-ΔEnv), HTLV-1-Env and a replication-dependent HTLV-1 reporter vector, pCRU5HT1-inLuc (HTLV-1-inLuc) that contains the two LTRs (the U3 region of the 5'LTR was replaced by a CMV promoter) have previously been described [310]. The plasmids pCMVHT1M-ΔEnv and HTLV-1-Env produce the virus-like particle (VLP). The Env-Stop plasmid, in which a premature stop codon was introduced in the env gene, was provided by Dr. Pique, and it has been described previously [348]. To determine whether HBZ induced the activation of the ICAM-1 promoter, a plasmid consisting of the human ICAM-1 5′-regulatory region (NCBI accession X57151) subcloned into a pGL2 basic vector was provided by Dr. Xue. This expression construct spans the ICAM-1 promoter region -722/+234 as described previously [349]. The β-galactosidase expression vector, provided by Dr. Mesnard, has been described previously [350, 351]. pSG-HBZ-myc, which was constructed by PCR, amplifying HBZ from pcDNA-HBZ-SP1-Myc [263] has

been described previously [231]. To establish the CHO-LFA-1 cells, the plasmids for Human LFA-1 alpha (Addgene plasmid #8630) previously described [352], and Human integrin beta 2 (Addgene plasmid #8640) [353]) were used. Both plasmids were purchased from Addgene, USA.

2.3   Antibodies

The primary antibodies used to detect the indicated antigens, as well as secondary antibodies used for their detection, are listed on Table 3, along with the host species (origin), clone (where applicable), supplier, and application.

2.4   Protein Assay Detection and Western Blot

2.4.1   Whole cell extracts preparation

Cultured cells were centrifuged at 1300 rpm for 3 min at 4°C. Cell pellets were washed once with cold PBS, and resuspended in RIPA lysis buffer (50mM Tris-HCl, pH 8, 100 mM NaCl, 1% Triton X-100, 1 mM MgCl2, and protease and phosphatase inhibitors: 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM benzamidine, 2 ug/mL leupeptin, and 5 ug/ml aprotinin. Cell lysates were vortex and incubated on ice for 20 min, and then were centrifuged at 10,000 rpm for 10 min at 4°C.

2.4.2   Western Blot

Protein concentrations were determined using Bradford protein assay (Bio-Rad laboratories, Inc). Cell lysates amount of proteins were equalized and Sodium Dodecyl Sulphate (SDS) (4x solution)/beta-mercaptoethanol (BME) were added to the lysates, and boiled for 5 min prior to loading to gel. Electrophoresis was performed running the samples on 10% SDS-PAGE polyacrylamide gels in 1X Running Buffer

Primary Antibody	Clone	Host Species	Specificity	Supplier	Application
Alpha L (αL)	38	Mouse	Human	Millipore	Blocking binding of αL integrin
	TS1/22	Mouse	Human	DSHB	Blocking binding of αL integrin
	MHM24	Mouse	Human	DSHB	Western Blot
Alpha M (αM)	M1/70	Rat	Human	Millipore	Blocking binding of αM integrin
Alpha-actin (α-actin)	C4	Mouse	Human	Millipore	Western Blot
Beta 1 (β1)	P4G11	Mouse	Human	DSHB	Blocking binding of β1 integrin
	P5D2	Mouse	Human	DSHB	Western Blot
Beta 2 (β2)	TS1/18	Mouse	Human	Thermo Sc.	Blocking binding of β2 integrin
	P4H9-A11	Mouse	Human	Millipore	Blocking binding of β2 integrin
	H52	Mouse	Human	DSHB	Western Blot
Beta 2 (β2)	MEM-148*	Mouse	Human	Thermo Sc.	Flow Cytometry
Anti-6X His-tag	Polyclonal	Rabbit	Human	Abcam	Western Blot
ICAM-1 (CD54)	P2A4	Mouse	Human	Millipore	Flow Cytometry
	84H10	Mouse	Human	Novus Bio.	Flow Cytometry
	RR1/1	Mouse	Human	Affymetrix Co.	Blocking binding of ICAM-1
LFA-3 (CD58)	TS2/9	Mouse	Human	Thermo Sc.	Blocking binding of LFA-3
Myc tag (Isotype)	4A6	Mouse	Human	Millipore	Blocking binding Isotype Control
Secondary Antibody					
Fluorescein (FITC) conjugate goat anti-Mouse IgG (H+L)	-	Goat	Mouse	SouthernBiotech	Flow Cytometry
Fluorescein (FITC) conjugate goat anti-Rat IgG (H+L)	-	Goat	Mouse	SouthernBiotech	Flow Cytometry

Table 3. List of antibodies used in this study. Antibodies used to detect the indicated antigens, along with the host species (origin), clone (where applicable), supplier, and application. DSHB: Developmental Studies Hybridoma Bank, Iowa, USA.

(190mM glycine, 0.1% (w/v) SDS, 25mM Tris-base), and transferred for blotting to 0.2 μm nitrocellulose blotting membrane (Amersham. GE Healthcare, Germany) in 1X Transfer buffer (190mM glycine, 25mM Tris-base, 20% (v/v) methanol) for 1 hour. Membranes were then blocked with 5% skim milk in 1X PBS-T (PBS, 0.05% Tween 20 and 5% milk) for 1hr at room temperature. Blotting followed with specific primary antibodies at 4°C overnight on a shaker. After blotting, membranes were washed in 1X PBS-T for 10 minutes three times on a shaker, followed by a brief wash in 1X PBS, and incubated with the horseradish peroxidase HRP-conjugated goat anti-mouse or goat anti-rabbit secondary antibody (Promega, CA) at a dilution of 1/5000 -1/10,000 for 1 hour at room temperature on a shaker. The membranes were washed 3 times and incubated with an enhanced chemiluminescence ECL Plus Western Blotting Detection Kit (Pierce™ ECL Plus Western Blotting Substrate, Thermo Fisher Scientific, USA) for 5 min, allow to dry for 15 min and then proteins were exposed using Image Quant software (GE Healthcare Life Sciences or Amersham Biosciences Corp, USA) on a Typhoon fluorescent imager (Amersham Biosciences). The membranes were stripped using Restore Western stripping buffer (Thermo Scientific,) and re-probed with monoclonal anti-α-actin (Millipore, USA) as internal control, and exposed as described above.

2.5   RNA extraction and cDNA preparation

Cultured cells were harvested, centrifuged at 1300 rpm for 3 min at 4°C, and media was discarded. Total RNA from each cell line was extracted adding Trizol reagent (Thermo Fisher Scientific, NJ) to the cell pellet, mixing until obtaining a homogeneous emulsion. Thereafter, 200 μL of chloroform (Fisher Scientific, NJ) was added, mixed vigorously and incubated for 3 min at room temperature. Samples were centrifugated at

12000 g for 15 min at 4°C. Following centrifugation, the top supernatant was collected and mixed with 500 μL isopropanol (Fisher Scientific, NJ) in a new microtube, and incubated for 10 min at room temperature. After, the RNA samples were pelleted via centrifugation and washed once with 75% ethanol (Fisher Scientific, NJ), vortex and centrifuged again at 7500 g, for 5 min at 4°C. Supernantants were removed and the pellets air dried for 15 min. Next, cells were resuspended with a DNAse RNA free enzyme in DNAse recombinant RNA free buffer (Roche, Germany) diluted in Diethylpyrocarbonate (DEPC) water. Samples were incubated for 30 min at room temperature, following incubation for 15 min at 70°C, and stored at -80°C. RNA concentration was determined by using the spectrophotometer ND-1000 (PEQLab) and the software NanoDrop 1000. cDNA was synthetized using the RevertAid RT Kit as described by the manufacturer (Thermo Fisher Scientific), using random hexamers.

2.6   Quantitative Real-time (qRT-PCR)

Subsequent quantitative real-time PCR analysis was performed using SYBR Green Master PCR mix (Applied Biosystems). Standard curves were generated from each experimental PCR plate for all primer pairs on the plate by using serial dilutions of an appropriate experimental sample. PCR efficiencies from all plates and primer pairs ranged from 80% to 120%, with correlation coefficients of 0.98 to 1.0. The reactions were performed in triplicate using the iQ5 real-time PCR detection system (Bio-Rad, California, USA) with the iQ5 optical system software as described in the operator's manual. Primers sequences used for RT-PCR analysis in this study on Table 4.

2.7   Flow Cytometry

Flow cytometry for the expression of cell surface markers ICAM-1 (CD54),

CD11a (αL) and CD18 (β2) was performed using three different Jurkat cell clones expressing HBZ or empty vector controls. A total of 5 x 10^5 cells were collected and centrifuged at 2000 rpm for 3 min at 4^oC, washed once in cold 2 mL of PBS/0.2% BSA (FACS buffer), then resuspended in FACS buffer containing 2 ug of mouse anti-human ICAM-1 monoclonal antibody (clone P2A4, Millipore Corporation), or anti-CD11a mouse Mab(clone 38, Calbiochem ©), or mouse anti-human integrin β2 (Clone P4H9-A11, Millipore Corporation) respectively, and incubated for 1 hour on ice. After that time, cells were washed once, and then incubated in the dark with 0.5 ug of a fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Southern Biotech) as the secondary antibody for 30 min on ice. Also, as a negative control, cells were incubated only with the secondary antibody. Following incubation, cells were washed once, resuspended in 0.5 mL of FACS buffer and flow cytometry was performed within one hour using a BD FACSCan flow cytometer and analyzed using BD CellQuest^{TM} software.

2.8 Infection and Luciferase Assay

Five different cultured Jurkat cells expressing HBZ clones and empty vector control were counted and adjusted to 3 x10^6 cells/mL. These cells, considered as the"donor cells" were centrifuged at 1300 rpm for 3 min at room temperature, and resuspended in 0.6 mL of a RPMI electroporation mix (RPMI base, 10 mM DTT and 0.5M Dextrose), from where 0.3 mL were transferred to an Eppendorf. The respective plasmids (HTLV1-inLuc, pCMV-HT1Env or pCMV-Env-Stop) were added to each Eppendorf, and the samples were electroporated in a Gene Pulser Xcell (Bio-Rad) in 0.4 cm gap width cuvettes. Each cell suspension was subjected to a single exponential decay pulse of 200V/975 μF and ∞ resistance. The electroporated cells were

Gene	Name	Primer	Primer sequence (5' to 3')
CADM1	cell adhesion molecule 1	Forward	CCCAACAGGCAGACCATTTA
		Reverse	TTCCACCGCAGTGTCTTTC
E-Cadherin	cadherin 1, type 1, E-cadherin (CD324)	Forward	GAGAAACAGGATGGCTGAAG
		Reverse	GGATCCTCAACTGCATTCC
ICAM-1	intercellular adhesion molecule 1 (CD54)	Forward	GCTATTCAAACTGCCCTGATG
		Reverse	AGCGTAGGGTAAGGTTCTTG
ICAM-2	intercellular adhesion molecule 2 (CD102)	Forward	AGAGAGGATGGAGGACATCA
		Reverse	CTCACGTGTACCTCGAATACC
ICAM-3	intercellular adhesion molecule 3 (CD50)	Forward	CACCTTCCTCTGTCAGAATGG
		Reverse	TTTCTCAGAGCTGGGACAATC
ICAM-4	intercellular adhesion molecule 4 (CD242)	Forward	CCGGGTTGGGTGTCTTAC
		Reverse	GGCAGCGCAAAGTGTATTT
ICAM-5	intercellular adhesion molecule 5	Forward	CTGCCCAAGTCACCAGAC
		Reverse	CAAGTTCGGCCACCACT
LFA-1 (αL)	lymphocyte function-associated antigen-1	Forward	CACAGGAAGCCTCTATCAGT
		Reverse	GTCTGTTGCCAAGGTCATTC
LFA-3	lymphocyte function-associated antigen-3	Forward	TGTCAGGTAGCCTCACTATC
		Reverse	TAGTGTGGGAGATGGAAGAG
ITGA4	integrin, alpha 4 (CD49e)	Forward	AGAGAGACAATCAGTGGTTGG
		Reverse	TCAGTTCTGTTCGTAAATCAGG
ITGA5	integrin, alpha 5 (CD49e)	Forward	CCTGAGCTGTGACTACTTTG
		Reverse	CGGAGATGAGGGACTGTAA
ITGB1	integrin, beta 1 (CD29)	Forward	CAAAGGAACAGCAGAGAAGC
		Reverse	ATTGAGTAAGACAGGTCCATAAGG
ITGB2	integrin, beta 2 (CD18)	Forward	CATTCTCCTGCTGGTCAT
		Reverse	CTTCACCAAGTGCTCCTAAC
UBE2D2	Ubiquitin Conjugating Enzyme E2 D2	Forward	TGCCTGAGATTGCTCGGATCTACA
		Reverse	ACTTCTGAGTCCATTCCCGAGCTA.

Table 4: List of primer sequences used for quantitative RT-PCR analysis in this study.
Forward primer. Reverse primer. The UBE2D2 gene was used as the housekeeping gene.

immediately transferred to a 6 well-plate containing complete DMEM and cultured at 37°C, 5% CO_2 for 48 hours. Thereafter, a co-cultured assay was performed, using Jurkat cells as the "target cells". The cells were counted and adjusted to 4 x 10^5 cell/mL, and the proportion of effector to target cells was 1:1. The cells were co-cultured at the same conditions as described above.   After 48 hours post-co-culture, cells were collected, washed once, centrifuged at 1300 rpm for 3 min at room temperature, and lysed in 100 uL of a 1X Cell Culture Lysis Buffer 5X (Promega, USA) solution. Samples were vortex and incubated at room temperature for 15 min, centrifuged at 2000 rpm for 3 min, analyzed for protein concentration, and equalized at 60-85 µ with 1X PLB solution. Luciferase activity was measured using 50 µL of Luciferase Assay System (LAS) (Promega, USA) per tube and read in an automated single tube luminometer (Glomax 20/20 Promega Corporation, USA).

2.9   ICAM-1 Promoter Assay

A reporter plasmid consisting of the human ICAM-1 5′-regulatory region (NCBI accession X57151) subcloned into a pGL2 basic vector was kindly provided by Dr. Xue group. This expression construct spans the ICAM-1 promoter region -722/+234 [349]. The sense and antisense nucleotides indicated were used: sense: 5'-TATATAGGTACCTCGGGTGTAGAGAGAGGA-3', antisense, 5'-TATATACTCGAG GTATGCAGGGTCTGGATT-3'. The construct was co-transfected into Jurkat cells with an expression plasmid for HBZ or the empty vector (control).   A standard plasmid expressing ß-galactosidase was also transfected and served as an internal standard. 3 X 10^6 cells were washed twice with serum-free IMDM combined with 5 µg of β-galactosidase-expressing vector, 5 µg of reporter plasmid, and 5 µg of either pSG-HBZ

or psG5, and electroporated in RPMI medium containing 10 mM dextrose and 0.1 mM dithiothreitol (DTT) using a Gene Pulser Xcell (Bio-Rad) in 0.4 cm gap width cuvettes. The cells subjected to a single exponential decay pulse of 200V/975 µF at ∞ resistance. The total amount of DNA in each series of transfection was equal, for a total DNA of 15 µg x electroporation. After electroporation, cells were immediately transferred to a 6 well-plate containing complete IMDM and cultured at $37^{o}C$, 5% CO_2. After 48 hours of incubation, cell extracts equalized for protein content [354] were used for luciferase and β-galactosidase assays.

2.10   Cell Aggregation/Dissociation Assays

2.10.1   Spontaneous Cell Aggregation

Cultured HBZ clones and cells carrying the empty vector were harvested by centrifugation, washed, and resuspended at a concentration of 5×10^5 cells/ml in complete IMDM. The cells were transferred to a 96 well-plate, incubated for 2 hours and allowed to spontaneously formed cell clusters. The cell aggregate formation was subsequently examined under an inverted phase-contrast microscope (Olympus CK2), and photographed at 10X magnification with a microscope camera. Aggregates were defined as containing four or more cells.

2.10.2   Cell Dissociation Assays

Cultured Jurkat HBZ clones, Jurkat cells carrying the empty vector, and SLB-1 ($HTLV^+$ cell line) were harvested by centrifugation, washed, and resuspended at a concentration of 5×10^5 cells/ml in complete IMDM. Blocking monoclonal antibodies directed against the αL and β2 subunits of LFA-1, ICAM-1, αM (CD11b) subunit of Mac-1 (CD11b/CD18), or isotype control (myc tag) (See Table 1) were immediately added to

the cells. A purified short peptide derived from a sequence of the αL subunit of LFA-1 (Sequence: GVDVDQDGETELLIGAPLFYGEQRG, CD11a 441-465 aa) (Genway Biotech, USA) was also used as a blocking agent for ICAM-1 (Tibbetts,S., 2000). Prior to use in the blocking aggregation assay, the peptide was dissolved in Dimethyl sulfoxide (DMSO) and the pH was adjusted between 6.8–7.2 using ammonium hydroxide. Subsequently, at the time of use, it was resuspended in complete IMDM at a concentration of 0.25 mM. DMSO was used as vehicle control. Cells were incubated at $37^{o}C$, $5\%CO_2$ in a 96 well plate at 5×10^5 cells/mL for 6 h, in the presence of the antibodies or the peptide. The cell aggregate dissociation was subsequently examined under an inverted phase-contrast microscope (Olympus CK2), and photographed at 10X magnification with a microscope camera. The images were quantified using ImageJ software (NIH, USA) https://imagej.nih.gov/ij/download.html.

2.11   Static Adhesion Assay

Cultured parental CHO cells and CHO-LFA-1 cells were trypsinized, equalized at 5×10^5 cell/mL and plated over coverslips inserted in 6-well plates. The cells were incubated at 37°C, 5% CO_2 overnight. Jurkat HBZ clones and Jurkat empty vector were stained with 0.1 µg/mL of Hoechst 33342 (a nuclear vital cell stain that emits blue fluorescence when bound to dsDNA) in complete IMDM for 20 min, and washed in un-supplemented IMDM medium. Next, the stained cells were adjusted at a concentration of 5×10^5 cell/mL and co-cultured with the CHO or CHO-LFA-1 cells for 2 hours. After the incubation, the plates with the co-cultured cells were washed to remove non-adherent cells, and the coverslips were photographed with a fluorescent microscope.

2.12   Statistics

The student unpaired t test or two-way analysis of variance (ANOVA) was used for statistical analyses. Differences were considered significant at a P value of <0.05.

CHAPTER 3: RESULTS

3.1   HBZ Expression Stimulates T-Cell Aggregation

Several authors have reported an induction of homotypic cell adhesion (self-aggregation) and cluster formation of ATL cells and HTLV-1^{+} T-cell lines that initiates briefly after the cells are cultured *in vitro* without any stimulation [320, 355, 356]. Thus, the spontaneous formation of large clusters of cells appears to be characteristic of ATL cells and HTLV-1^{+} T-cell lines. This aggregation is likely to be a consequence of alterations in the expression pattern of cell adhesion molecules, which occurs in many types of cancers, including leukemia and lymphoma [357-359]. In the case of ATL, alterations in the cell adhesion molecule pattern may contribute to the observed morbidity at the terminal stage of the disease. Tanaka *et al.,* studied these alterations in the context of ATL and found that there was a differential pattern of specific adhesion molecules that enabled these cells to aggregate and infiltrate specific organs [360]. We observed a similar aggregation phenotype in Jurkat clonal cell lines that stably express HBZ and HTLV-1 infected cell lines that were allowed to spontaneously form aggregates, as shown in Figure 8. HBZ-expressing T-cell clones and control clones were established by transfection of pcDNA-HBZ-SP1-Myc [263] or pcDNA3.1 (Invitrogen) respectively, with lipofectamine LTX reagent [268] and selection with 1.5 mg/ml G418 beginning 48 h post-transfection. The clonal cell lines were produced by expansion of single cells in 96-well plates. For the aggregation assay, concentrations of HBZ clones and cells containing the empty vector were equalized and transferred to a 96 well-plate, incubated for 2 hours, and photographed with a microscope camera. The cultured Jurkat cells containing the empty vector did not form cell aggregates (Figure 8A). In contrast, the HBZ-expressing Jurkat cells spontaneously formed cell clusters (Figure 8B). A similar phenotype can be

observed in HTLV-1 infected T-cell lines, such as SLB-1 (Figure 8D), when compared with non-infected cultured T-cells (Figure 8C). These observations indicate that HBZ induces alterations in the expression of specific adhesion molecules in the clonal cell lines.

3.2   Chelation of Divalent Metal Ions Using EDTA Blocks the Cell Aggregation Induced by HBZ

It has been demonstrated that the transmembrane glycoproteins in the Ig superfamily CAMs (ICAMs) modulate adhesion in a calcium-independent manner [361] [362]. Also it has been reported that Magnesium (Mg^{2+}) is required for the activation of certain integrins [363]. In order to determine the type of adhesion molecules participating in the formation of the homotypic cell aggregation on Jurkat cells stably expressing HBZ, and in a HTLV-1$^+$ cell line (SLB-1), we analyzed the effect of two chelators: ethylene-diamine tetra-acetic acid (EDTA), which chelates divalent cations, such as Mg^{2+} and Mn^{2+}, and Ethylene glycol-bis (beta-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), which binds specifically to Calcium (Ca^{2+}) with high affinity [364]. Briefly, three different HBZ clones, empty vector controls, and SLB-1 cells were incubated with 5 mM of EDTA or EGTA in IMDM complete medium at 37°C, and 5% CO2 (Figure 8E). Microscopy images were taken at different time points (up to 6 hours). We found that EDTA (Figure 8G), but not EGTA (Figure 8H), was able to block the strong homotypic cell aggregation of the Jurkat-HBZ clones, suggesting that EDTA causes a disruption in the interaction between the cell adhesion molecules due to the chelation of Mg^{2+} in the medium. A similar disruption of aggregation was observed in the SLB-1 cells after adding EDTA (Figure 8M) but not with EGTA (Figure 8N). Jurkat cells containing the empty vector

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Figure 8. Spontaneous cell-to-cell adhesion of Jurkat cells expressing HBZ and blocking of self-aggregation by EDTA. Concentrations of Jurkat cells, Jurkat clones expressing HBZ, SLB-1 cells (HTLV-1^{+}), and Jurkat clones cells containing the empty vector were equalized, transferred to a 24 well-plate, and cultured in IMDM complete medium at 37°C, 5% CO_2 for 2 hours. A) Jurkat empty vector clone, B) HBZ clone, C) Jurkat cells and D) SLB-1 cells after 2h of incubation. E) Flow diagram of EDTA or EGTA assay. Cells were equalized and transferred to 24 well-plates, and EDTA or EGTA (5mM) was added. Plates were incubated at 37°C, 5% $CO_{2,}$ and microscopy images were taken at 6 hours. F) Jurkat empty vector clones incubated with G) 5 mM EDTA or H) 5 mM EGTA. I) Jurkat HBZ-expressing cells incubated with J) 5 mM EDTA or K) 5 mM EGTA. L) SLB-1 cells incubated with M) 5 mM EDTA or N) 5 mM EGTA. Images are representative of 3 independent assays.

A. Jurkat-Empty vector

B. Jurkat-HBZ

C. Jurkat

D. SLB-1 (HTLV$^+$)

E.

Equalize Cell Concentration (Culture medium) → Transfer to 24-well plates → Add EDTA or EGTA (5 mM) → Photograph plates at 6h

F. No addition

G. 5 mM EDTA

H. 5 mM EGTA

Jurkat Empty vector

I.

J.

K.

Jurkat-HBZ

L.

M.

N.

SLB-1 (HTLV-1$^+$)
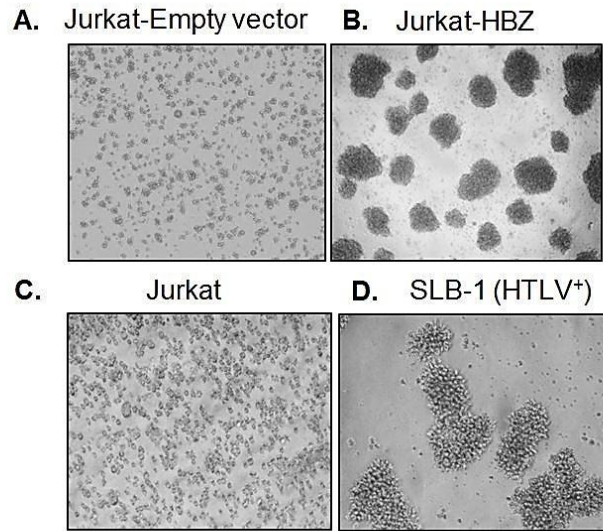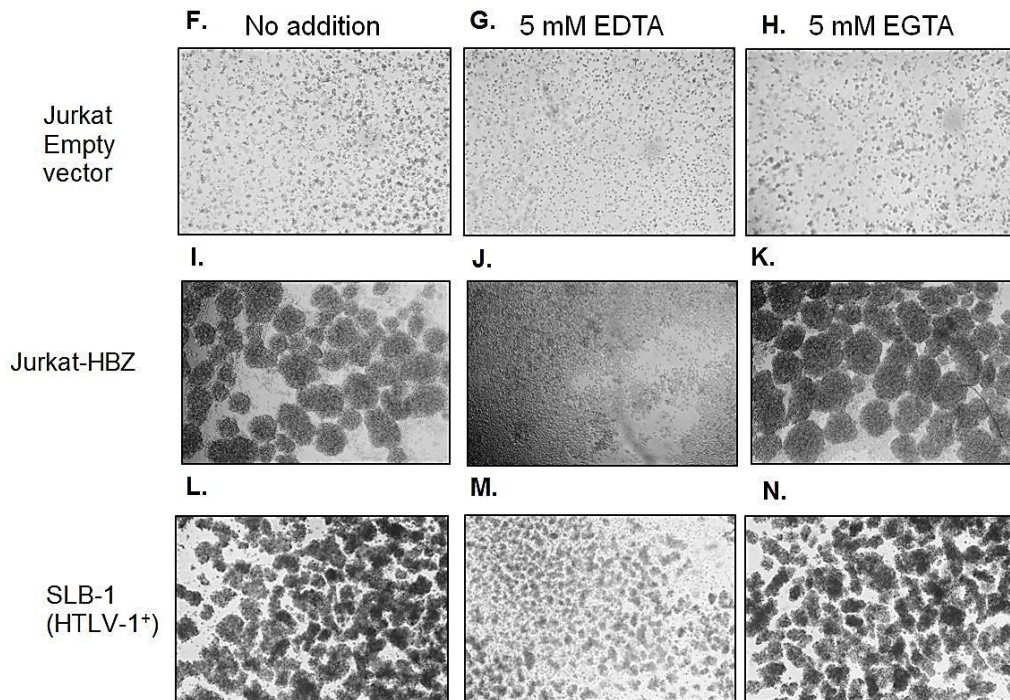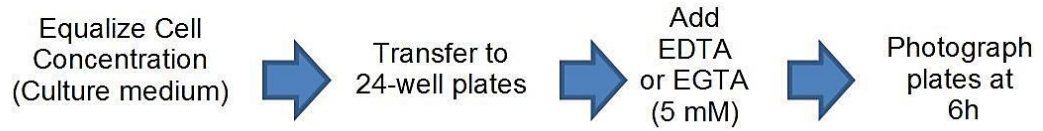
were used as negative controls (Figure 8F to 8H). These data suggest the participation of the same adhesion molecules in the self-aggregation process in HBZ-expressing cells and the HTLV-1$^+$ cell line.

3.3 The Presence of Magnesium in the Cell Culture Promotes T-cell Aggregation

In order to test whether the presence or absence of Mg^{2+} or Ca^{2+} in the medium affects the self-aggregation of the T-cells expressing HBZ, we decided to perform an aggregation assay in which the cells were cultured in a solution depleted of Mg^{2+} and Ca^{2+}. These cations were then added to the culture medium. Jurkat–HBZ clones, SLB-1 cells, and empty vector controls were resuspended in a modified Hepes-Tyrode's solution (which is physiological similar to interstitial fluid) depleted of divalent cations. A concentration of Mg^{2+} similar to the one present in IMDM and also human serum (1mM) was added in triplicate to the respective wells. The cells were incubated at 37°C, 5% CO$_2$. Images were taken with a microscope at 6h) (Figure 9A). We found that after 6 h the presence of Mg^{2+} promoted the cell aggregation of Jurkat HBZ-expressing cells (Figure 9F) and SLB-1 cells (Figure 9I). In contrast, as we observed in the previous EDTA experiment, the absence of Mg^{2+} from the medium prevented the HBZ-expressing cells (Figure 9E) and the SLB-1 (Figure 9H) from forming aggregates. The presence of Ca^{2+} did not induce cell aggregation (Figure 9G and 9J). Jurkat-empty vector were used as negative controls (Figure 9B to 9D).

Taken together, these results suggest that an integrin, which is activated by Mg^{2+}, participates in the cell homotypic aggregation process of HBZ-expressing cells and a
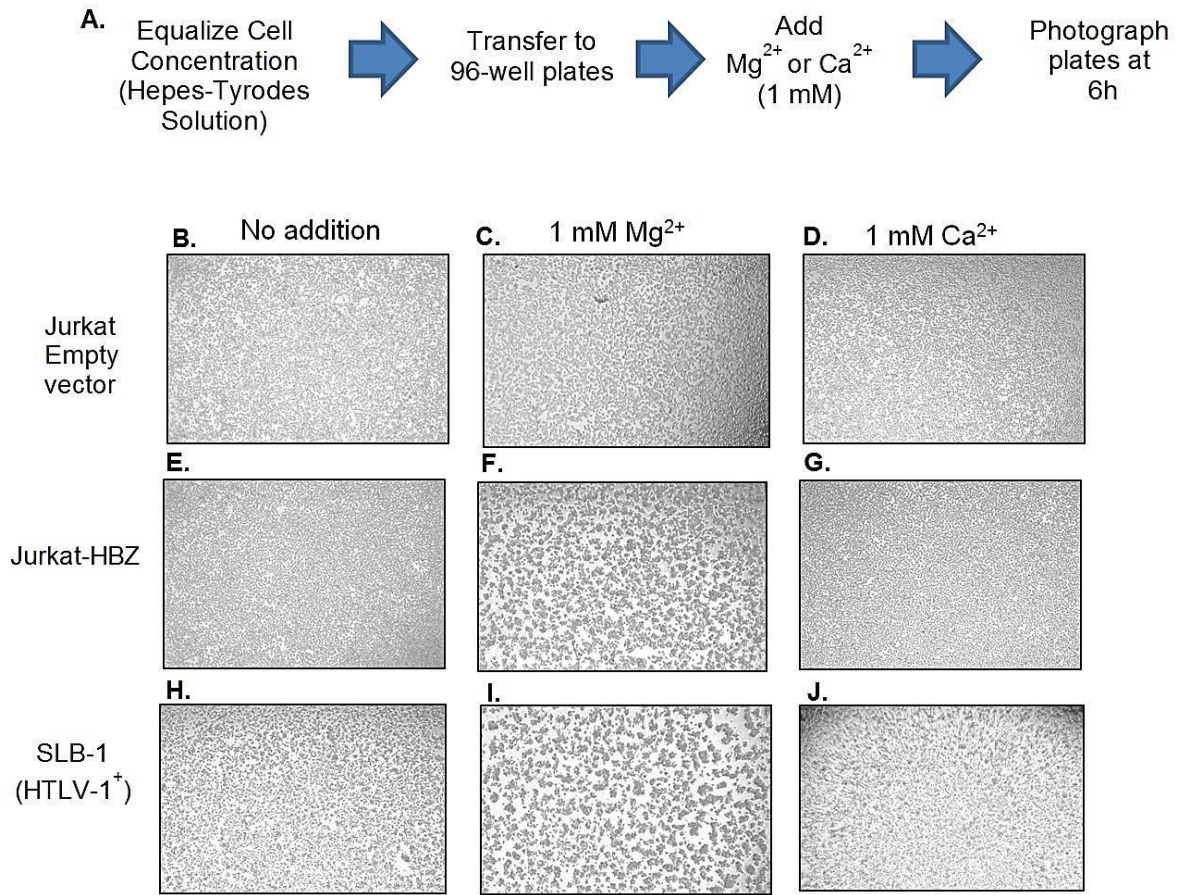
Figure 9. The presence of Mg²⁺ promotes cell self-aggregation. A) Flow diagram of the assay. HBZ-expressing cells, SLB-1 cells (HTLV-1⁺) and empty vector control cells were equalized, resuspended in Hepes-Tyrodes solution, and transferred to a 96 well-plate in triplicate. Mg^{2+} or Ca^{2+} (1 mM) was added in triplicate to the wells, and the plate was incubated at 37°C, 5% CO_2 for 6 hours. Images were taken with a microscope at 6 h. B) Jurkat empty vector cells without Mg^{2+} C) with 1mM Mg^{2+} or D) 1 mM Ca^{2+}. E) Jurkat-HBZ cells without Mg^{2+} F) with 1 mM Mg^{2+} or G) 1 mM Ca^{2+}. H) SLB-1 (HTLV-1⁺) cells without Mg^{2+} I) with 1 mM Mg^{2+} or J) 1 mM Ca^{2+}. Images are representative of 2 independent assays.

HTLV-1 infected cell line, SLB-1. Our next step was to determine what specific adhesion molecules or integrins, which depend on Mg^{2+} for their activation, were involved in the HTLV-1 cell aggregation.

3.4   HBZ Upregulates ICAM-1 Expression

Based on the gene expression data for adhesion molecules from HBZ-expressing cells obtained by microarray in our laboratory (unpublished data), and also analysis on ATL cells and HTLV-1 infected cell lines performed by several groups [320, 365-369], we selected a panel of adhesion molecules and related genes for analysis (Table 5). In order to determine the expression levels of the adhesion molecules genes in Jurkat cells stably expressing HBZ or the empty vector cells (control), we performed quantitative real time-PCR (qRT-PCR). Results were calculated as relative values after normalization to the expression of the housekeeping gene, Ubiquitin-conjugating enzyme E2D 2 (UBE2D2). UBE2D2 is a member of the ubiquitin-conjugating enzyme E2 subfamily that catalizes ubiquitination of cellular proteins prior to degradation [370]. UBE2D2 expression is stable in T-cells [371]. From all the adhesion molecules analyzed, we found a relevant upregulation of ICAM-1 expression in Jurkat HBZ-expressing cells (Figure 10A), while no changes in CADM1, E-Cadherin, ICAM-2,-3,-4,-5, LFA-1, -3, integrin α4, α5, β1 or β2 were observed. We confirmed that HBZ upregulates ICAM-1 expression by transducing another T-cell line, SupT1, with a retrovirus expressing HBZ or GFP as the control (Figure 10B). To determine which domain of HBZ is involved in ICAM-1 upregulation, we used HeLa clonal cell lines stably expressing different forms of HBZ (Figure 10C). The HeLa cell lines used in this experiment consisted of cells stably expressing wild type (wt)-HBZ, or

Gene	Name	Also known as	Fold Change	# Assays **
CADM1	cell adhesion molecule 1	BL2; IGSF4; RA175; TSLC1	0.55	1
E-Cadherin	cadherin 1, type 1, E-cadherin	CDHE; ECAD; LCAM; CD324	1.37	1
ICAM-1	intercellular adhesion molecule 1	CD54; BB2; P3.58	2.50	3
ICAM-2	intercellular adhesion molecule 2	CD102	0.59	3
ICAM-3	intercellular adhesion molecule 3	CD50; CDW50; ICAM-R	0.92	2
ICAM-4	intercellular adhesion molecule 4	LW; CD242	0.42	2
ICAM-5	intercellular adhesion molecule 5	TLN; TLCN	1.07	2
LFA-1 (αL)	lymphocyte function-associated antigen-1	LFA-1, CD11a/CD18	0.71	2
LFA-3	lymphocyte function-associated antigen-3	CD58; ag3; LFA3; LFA-3	1.36	5
ITGA4	integrin, alpha 4	CD49d, α4	0.37	3
ITGA5	integrin, alpha 5	α5, CD49e; VLA-5; VLA5A	1.06	2
ITGB1	integrin, beta 1	β1, CD29	0.79	2
ITGB2	integrin, beta 2	β2, LAD; CD18; LFA-1; MAC-1	0.80	3

Table 5. Upregulated expression of ICAM-1 in HBZ-expressing cells when compared to control. Clones of Jurkat expressing HBZ or carrying the empty vector were analyzed by qRT-PCR. Experiments were performed by triplicate. Results are expressed as the average relative units after normalization with UBE2D2 expression. A biological relevant fold change was set to ≥ 2.0. ** # Assays: Number of Independent Assays.

the empty vector (pcDNA3.1) or different HBZ mutants. HBZ with point mutations in the

N-terminal Activating Domain (MutAD) that abrogates the HBZ interaction with the

coactivators p300 and CBP [266]; HBZ with point mutations in the C-terminal ZIP domain

(MutZIP), which inhibits the interaction between HBZ and numerous families of the

ATF/CREB and Jun host bZIP factors [231]; or HBZ with a point mutation in the initiation

codon (ΔATG), that suppresses the translation of the HBZ mRNA/transcript [231].

Interestingly, among the mutant proteins, MutZIP did not upregulate the ICAM-1

expression (Figure 10B), when compared to the cells expressing wt-HBZ or HBZ MutAD.

It has been reported that the HBZ transcript can exert effects on the cellular machinery

independently from the HBZ protein by promoting cell proliferation [242]. In our study,

HBZ- ΔATG, which does not produce the HBZ protein, did not upregulate the expression

of ICAM-1 in the cells. Our results suggest that HBZ protein is able to upregulate ICAM-1

expression, and also that HBZ regulates the transcription of ICAM-1 through its ZIP

domain. In addition, when we tested the expression of ICAM-1 in a panel of HTLV-1

infected cells (Figure 10D), we observed that the ICAM-1 transcript in these cells was

upregulated in comparison to non-infected cells (CD4+, activated CD4+, Jurkat, CEM,

and Hut-78 cell lines). Interestingly, the SLB-1 and in particular the ATL-2 cell line, which

exhibited the highest ICAM-1 mRNA levels when compared to the other cell lines have

been reported to express high levels of HBZ [277]. Furthermore, the HTLV-1 infected cell

lines (1185), which are immortalized but not yet transformed, also expressed high levels

of ICAM-1, suggesting that increased ICAM-1 expression occurs before the process of

complete cell transformation. In addition, the TL-Om1 cells also exhibited high levels of

ICAM-1 compared to uninfected cell lines. These cells were directly isolated from a

patient with ATL, and only express HBZ due to an inactivation of the 5' LTR promoter

[280], suggesting an essential role for HBZ in ICAM-1 expression.

3.5 HBZ Activates ICAM-1 transcription

To better define the role of HBZ in the homotypic cell aggregation, and to determine whether an autocrine or paracrine signaling loop induced by cytokines might account for this observation, we developed a supernatant transfer assay. HBZ-expressing "donor" cells were cultured at 5% CO_2, 37°C and allow to form aggregates spontaneously (Figure 11A). The conditioned culture media (CM) from the HBZ-expressing cells were collected after 24 hours, cleared by centrifugation, and transferred to thoroughly washed Jurkat empty vector "acceptor" cells. The acceptor cells were incubated for 6 hours at 37°C to determine the effect of the CM from HBZ-expressing cells on cell aggregation, which was monitored every 2 hours. We also cultured the washed HBZ-expressing cells in CM from empty vector cells for 6 hours. In control samples, we did not manipulate the culture media of the Jurkat empty vector cells and HBZ-expressing cells. There were no differences between the aggregates formed by the empty vector cells that were cultured in CM from HBZ-expressing cells (Figure 11B) compared to those formed by the empty vector cells in their own medium (Figure 11C). Furthermore, HBZ-expressing cells that were incubated in the CM from "acceptor" cells were able to aggregate spontaneously (Figure 11D) as the HBZ-expressing cells in their own medium (Figure 11E). These results did not support the autocrine/paracrine loop hypothesis. We decided to perform an analysis at the transcriptional level to determine whether HBZ is able to induce the activation of the ICAM-1 promoter (Figure 12). We performed a luciferase analysis of the ICAM-1 promoter region in order to determine its responsiveness to HBZ. For this purpose, we obtained a plasmid consisting of the

Figure 10. HBZ upregulates ICAM-1 expression. A) Relative ICAM-1 mRNA levels in Jurkat empty vector control (#2, #4, #5) and HBZ clones (F10, G8, H7). Results are expressed as the average of three independent experiments. Values are relative to empty vector #2 (set to 1). *p<0.0005. B) Relative ICAM-1 mRNA levels in SupT1 cells expressing GFP or HBZ. Results are expressed as the average of two experiments. Values are relative to GFP control (set to 1) C) Relative ICAM-1 mRNA levels in HeLa clones expressing wild-type HBZ or HBZ mutants (MutAD, MutZIP, ΔATG). Results are expressed as average of three independent experiment, *p<0.01. Values relative to one of the replicate of the empty vector clones (set to 1). D) Relative ICAM-1 mRNA levels in resting and activated primary CD4+ T-cells and in different T-cell lines: HTLV-1-negative (uninfected) cell lines: Jurkat and CEM (T-cell acute lymphoblastic leukemia); HUT-78 (Sézary syndrome); HTLV-1 infected cells: 1185 and SP (HTLV-1-immortalized); MT-2, C10/MJ, SLB-1, C8166/45 (in vitro HTLV-1-transformed); SP, ATL-2, TL-Om1 (cells derived from ATL patients). Results expressed as the average of two independent experiments. Graphs show real-time PCR data normalized to UBE2D2. Values are relative to resting CD4+ T cells (set to 1). Error bars denote the standard deviation. Increases in ICAM-1 mRNA in infected cells were all significant (p value ranging from p<0.00005 to 0.05).

A. Jurkat clones

B. SupT1 clones

C. HeLa clones

D.
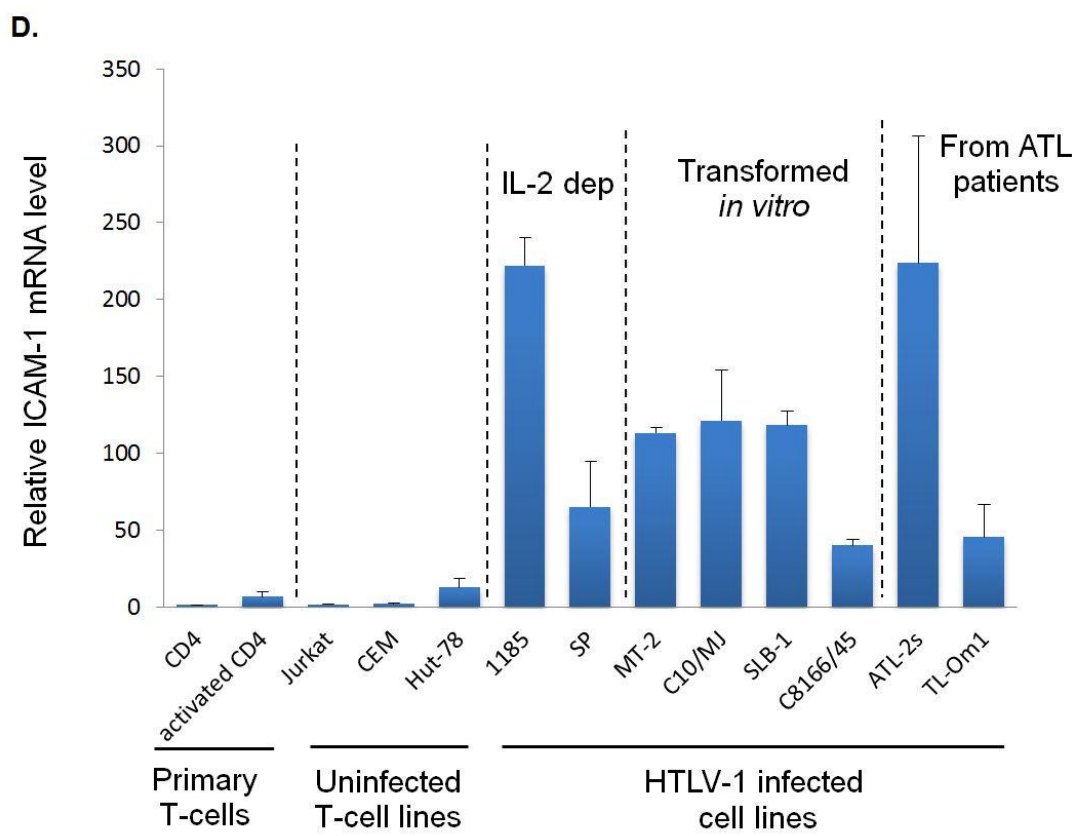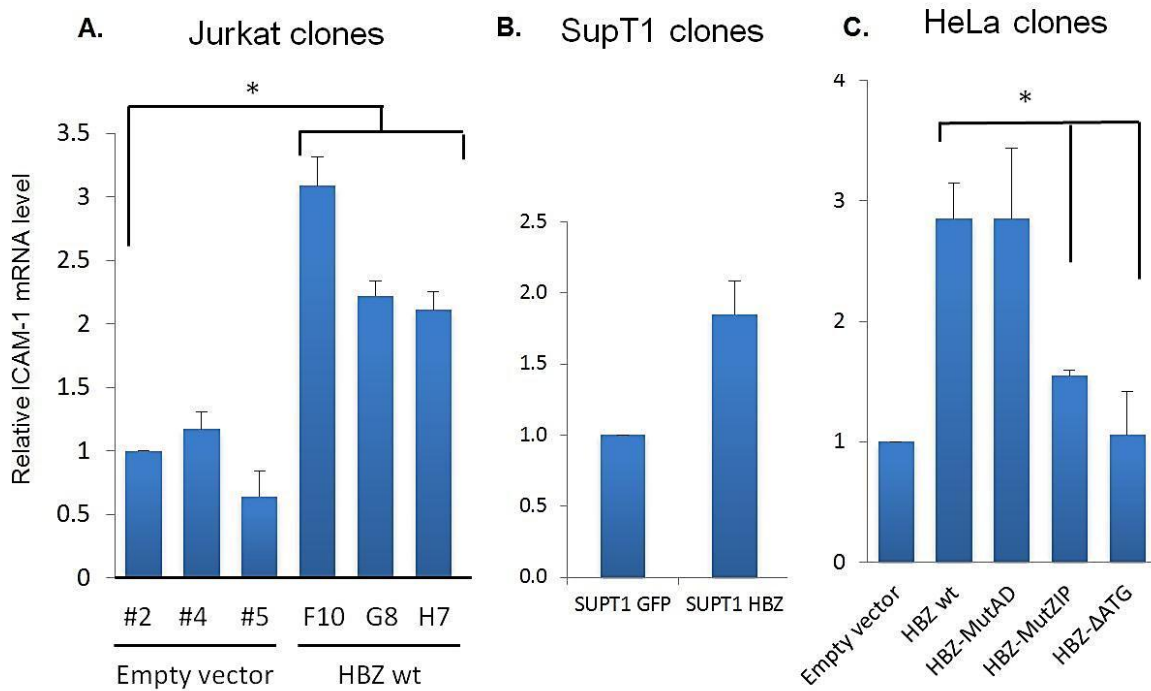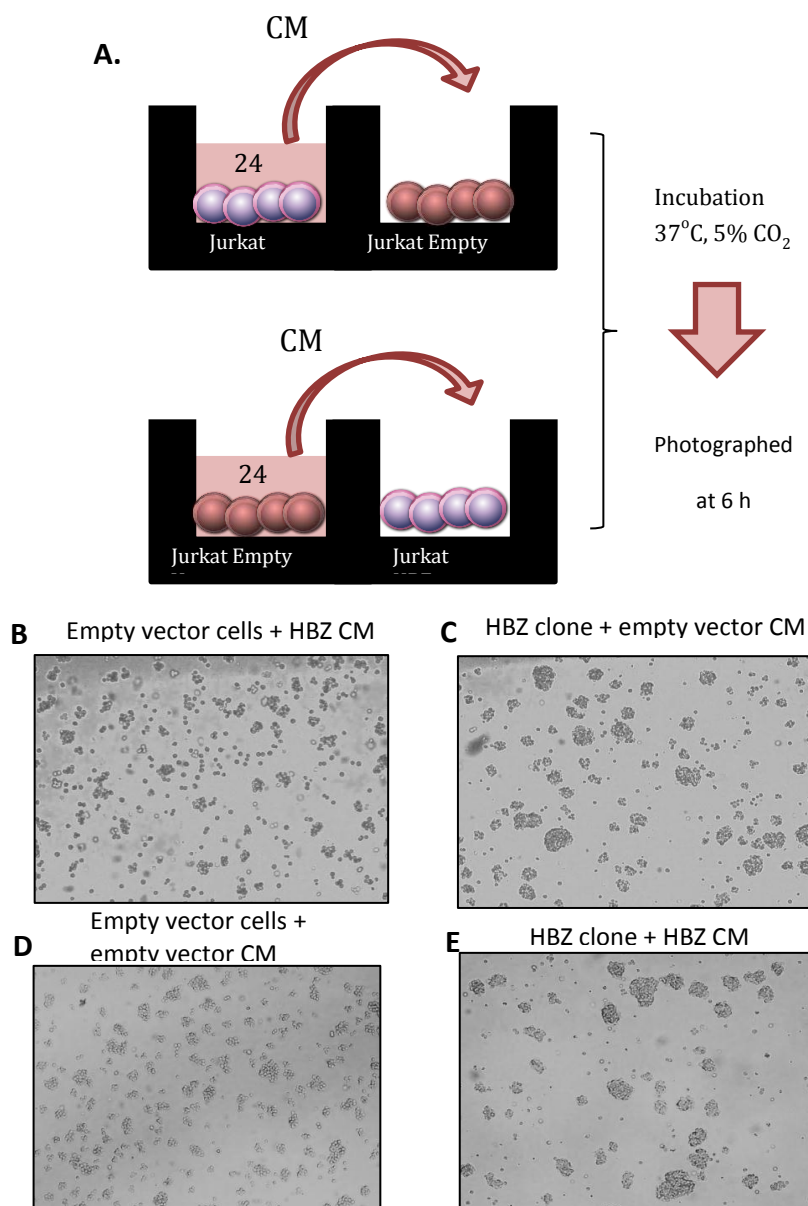
Figure 11. Absence of an autocrine or paracrine signaling loop mechanism for the self-adhesion phenotype in HBZ-expressing Jurkat cells. A) Supernatant Assay Diagram. Jurkat HBZ clones and empty vector cells were cultured at 5% CO_2, 37°C and allow forming aggregates spontaneously. After 24 hours, the conditioned cultured media (CM) from the HBZ clones was transferred to the empty vector cells. As a control, the CM from Jurkat empty vector cells was transferred to Jurkat HBZ clones. The cells were incubated at 5% CO_2, 37°C, and microscopy photograph were taken at 6 h. B) Jurkat empty vector cells with HBZ CM. C) Jurkat HBZ clones with empty vector CM. D) Empty vector cells and D) HBZ clones in their own CM (control). Results are representative of 3 independent assays.

human ICAM-1 5′-regulatory region (NCBI accession X57151) subcloned into a pGL2 basic vector (kindly provided by Dr. Xue). This expression construct spans the ICAM-1 promoter region -722/+234 [349] (Figure 12A). The construct was co-transfected into Jurkat cells with an expression plasmid for HBZ or the empty vector (control). A standard plasmid expressing ß-galactosidase was also transfected and served as an internal standard. Luciferase activity was tested after 48 hours of incubation using a luminometer. As shown in figure 12B, the luciferase activity was increased in the cells co-transfected with the reporter construct and HBZ plasmid, suggesting that HBZ is involved in the activation of the ICAM-1 promoter. The expression of HBZ in the cells was confirmed by Western Blot analysis (Figure 12B, lower panel).

Previously, we have shown that the ZIP domain of HBZ is required to activate the expression of the ICAM-1 gene (Figure 10C). These data suggest that HBZ may bind to a cellular basic leucine zipper (bZIP) factor to activate transcription. Interestingly, it has been demonstrated that JunD is one of the few HBZ-cellular bZIP factor heterodimers that activates transcription instead of repressing it [247, 262, 276, 372, 373]. It has also been reported that the activation of the promoter requires the recruitment and binding of the JunD-HBZ heterodimer to a Sp1 transcription factor that is directly bound to the DNA [247]. Moreover, it has been shown that the complex formed by HBZ, JunD and Sp1 activates transcription from the human telomerase reverse transcriptase (hTERT) promoter [276], the catalytic subunit of the human telomerase. Another study has shown that HBZ also activates its own promoter through the same mechanism [247]. Interestingly, the ICAM-1 promoter contains two Sp1 binding sites (-203 bp and -53 bp) [374, 375]. Based on these data, we proposed a hypothetical model

Figure 12. HBZ activates transcription from the ICAM-1 promoter. A) Schematic representation of the luciferase reporter plasmid ICAM-1 promoter spanning the region -722/+234. B) Jurkat cells were electroporated with the reporter plasmid mentioned above, and also with an empty or a HBZ expression vector. The cells were incubated for 48 hours under standard conditions, and the luciferase activity was performed as described in the Materials and Methods section. A plasmid expressing ß-galactosidase was also transfected and used as an internal standard. The results show the data averaged from four independent experiments*$p<0.05$. A representative western-blot from one experiment is shown. C) The proposed model to explain how HBZ is involved in the activation of the ICAM-1 promoter: HBZ is able to generate an activation complex with JunD, and Sp1 that induces transcription of the ICAM-1 promoter.

A. Reporter plasmid ICAM-1-Luc

NF-kB C/EBP **SP1** NF-kBets STAT **SP1** AP-2

-722 bp   -203 bp   -53 bp   +234 bp

+1

TATA

Luciferase

B.

Relative luciferase activity

10 8 6 4 2 0

*

Empty vector   HBZ

HBZ
Actin

C.

HBZ

Activation Complex

HBZ   JunD   Sp1

RNA polymerase II

+1

GC box

ICAM-1 promoter

70
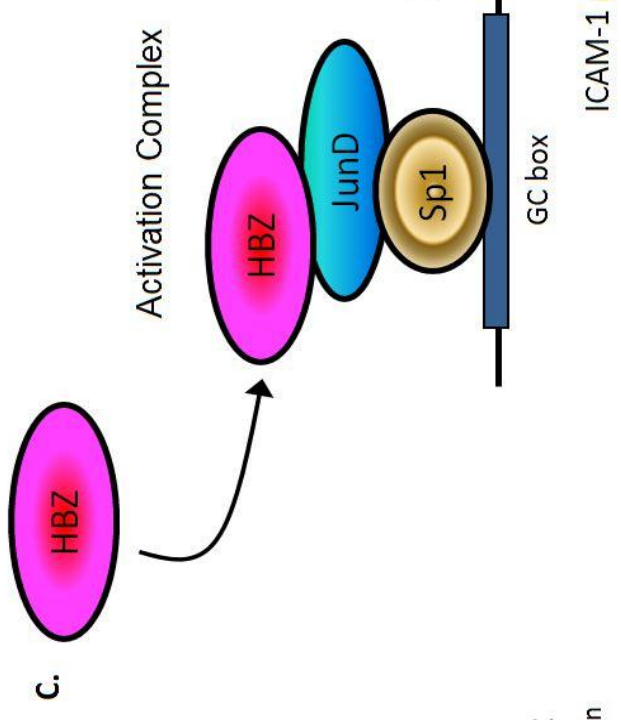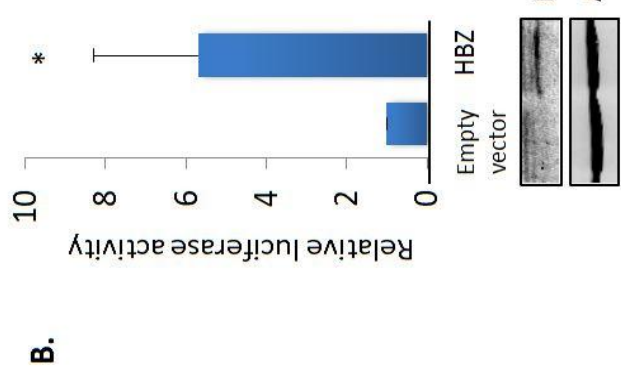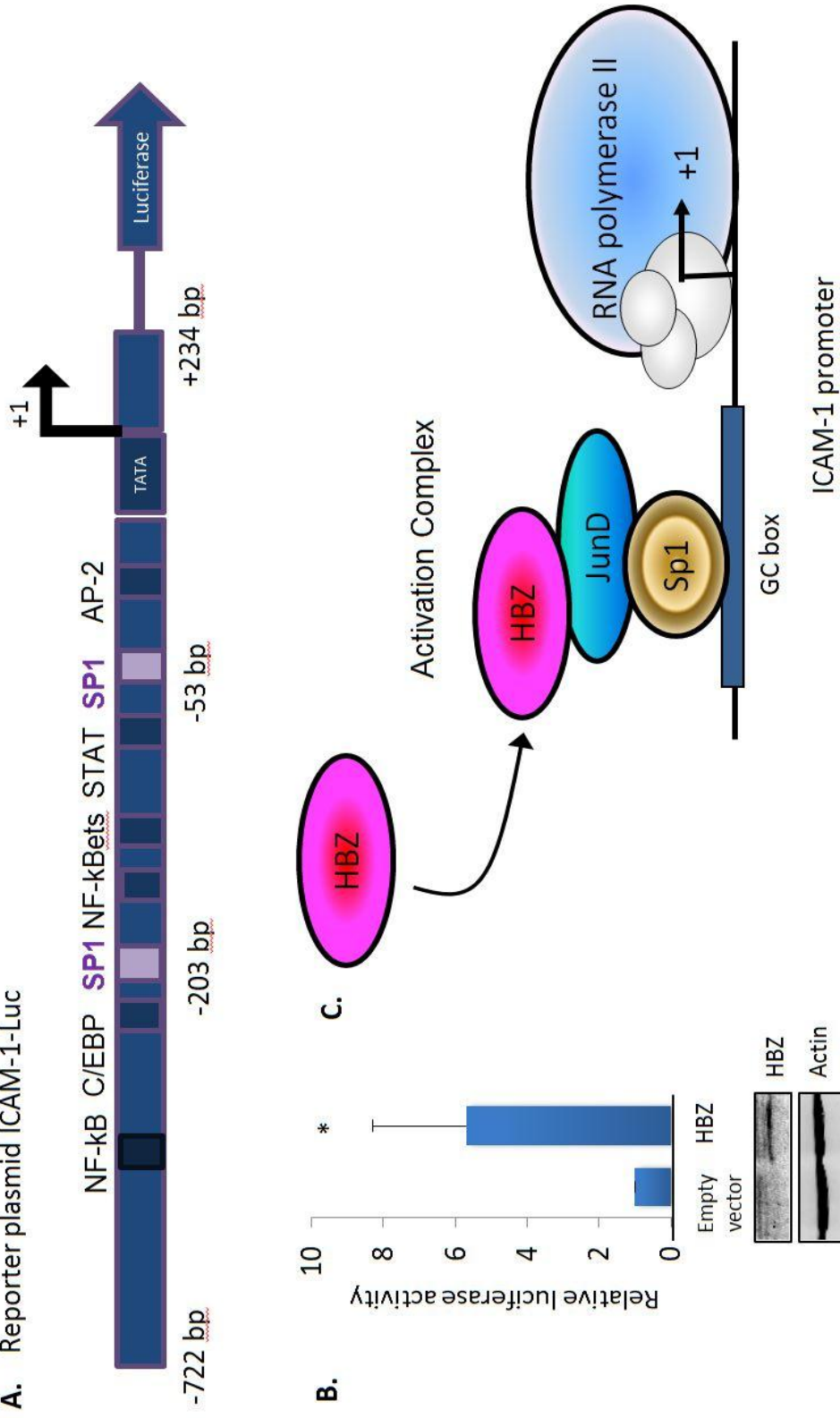
to explain the mechanism of how HBZ activates ICAM-1 transcription. In our model

(Figure 12C), we propose that HBZ is able to activate ICAM-1 transcription by forming an

active HBZ/JunD/Sp1 complex.

3.6   HBZ Increases ICAM-1 Surface Expression

In order to confirm that increased ICAM-1 mRNA correlates with an increase in

ICAM-1 at the cell surface, we examined HBZ-expressing clonal cell lines by flow

cytometry. We also analyzed whether the expression of the ligand of ICAM-1, LFA-1, was

increased on the cell surface. Briefly, the Jurkat HBZ-expressing cells or those containing

the empty vector were incubated with specific monoclonal antibodies against ICAM-1 or

the subunits of LFA-1, β2 (CD18) or αL (CD11a). The secondary antibody alone was used

as a negative control. As shown in Figure 13, we found an increase of ICAM-1 on the

surface of the Jurkat HBZ-expressing clonal cells when compared to the empty vector

cells (Figure 13A). However, the surface amount of the LFA-1 subunit αL (Figure13B)

and β2 (Figure 13C) was similar to that of the empty vector cells. Also, we found no

differences in the level of the activated form of β2 subunit of LFA-1 and empty vector

cells. We confirmed that there were no differences in the β2 and αL protein levels

between HBZ Jurkat cells clones and empty vector clones by western blot (Figure 14).

These results differ from those of Yamamoto-Taguchi *et al.,* who found that

HBZ-transgenic mouse cells exhibited an increased LFA-1 surface expression [321].

However, our data is consistent with those reported by other groups where ICAM-1

surface expression was increased in fresh ATL cells obtained directly from patients, and

the surface levels of LFA-1 in those leukemic cells were on average not different from

those of CD4+ T-cells from healthy donors [314, 320]. Also, a study has shown that

another HTLV-1 protein (p12) is able to induce cell surface clustering of LFA-1 without affecting the level of LFA-1 expression [376]. These data suggest that HBZ does not affect the expression of LFA-1, but may alter LFA-1 functionality.

A way to study LFA-1 function is to analyze its affinity state. LFA-1 can adopt different conformations on the cell surface that affect its affinity for ICAM-1 [377, 378]. Figure 13E shows the LFA-1 closed conformation (bent), which has a low affinity for ICAM-1. It also shows the LFA-1 intermediate conformation, between the bent-closed and extended-open, called the extended closed, conformation that allows LFA-1 to bind to ICAM-1. Finally, figure 13E shows an open conformation with a high affinity for its ligands. These conformations are regulated from both the cell interior (inside-out signaling), and extracellular signaling given by the ligand binding or the divalent cations that trigger intracellular signals (outside-in signaling) [379, 380].

It has been reported that fresh ATL cells exhibit an increase in the activated form (open conformation) of LFA-1 [320]. In order to analyze the open conformation of LFA-1 on HBZ-expressing cells, we performed flow cytometry using MEM-148, which is a specific LFA-1 activation/high affinity reporter monoclonal antibody [381]. MEM-148 binds to the hybrid domain in the β2 subunit of LFA-1 (see figure 13E), and only recognizes the open, fully activated LFA-1 molecule, in which the epitope for MEM-148 becomes exposed [378, 382]. We did not find differences between the levels of β2 activation in HBZ-expressing cells when compared to the empty vector control cells (Figure 13D), suggesting that HBZ does not affect the conformation of LFA-1.

Interestingly, on the extended-closed conformation (Intermediate) which is not recognized by MEM-148, LFA-1 can bind to ICAM-1 [378, 383].

Figure 13. HBZ increases ICAM-1 expression at the cell surface. Cell surface expression of A) ICAM-1 (CD54), B) LFA-1 αL (CD11a), C) LFA-1 β2 (CD18), and D) the activated form of the β2 subunit of LFA-1 in Jurkat empty vector (red) and HBZ (blue) clones analyzed by flow cytometry. Cells labeled with the secondary antibody alone (green) were used as negative control. Results are representative of at least three independent assays. E) Model of LFA-1 conformation states. Closed, Extended-Closed, and Open (High Affinity). The antibodies are depicted according to their respective recognized epitope in the integrin: αL (clone TS1/22), β2 (clone TS1/18), and activated β2 (clone MEM-148).

A. ICAM-1

B. αL (CD11a)

C. ß2 (CD18)

D. ß2 (CD18) activated

E. Closed (Bent) — Extended-Closed (Intermediate) — Open (High Affinity)
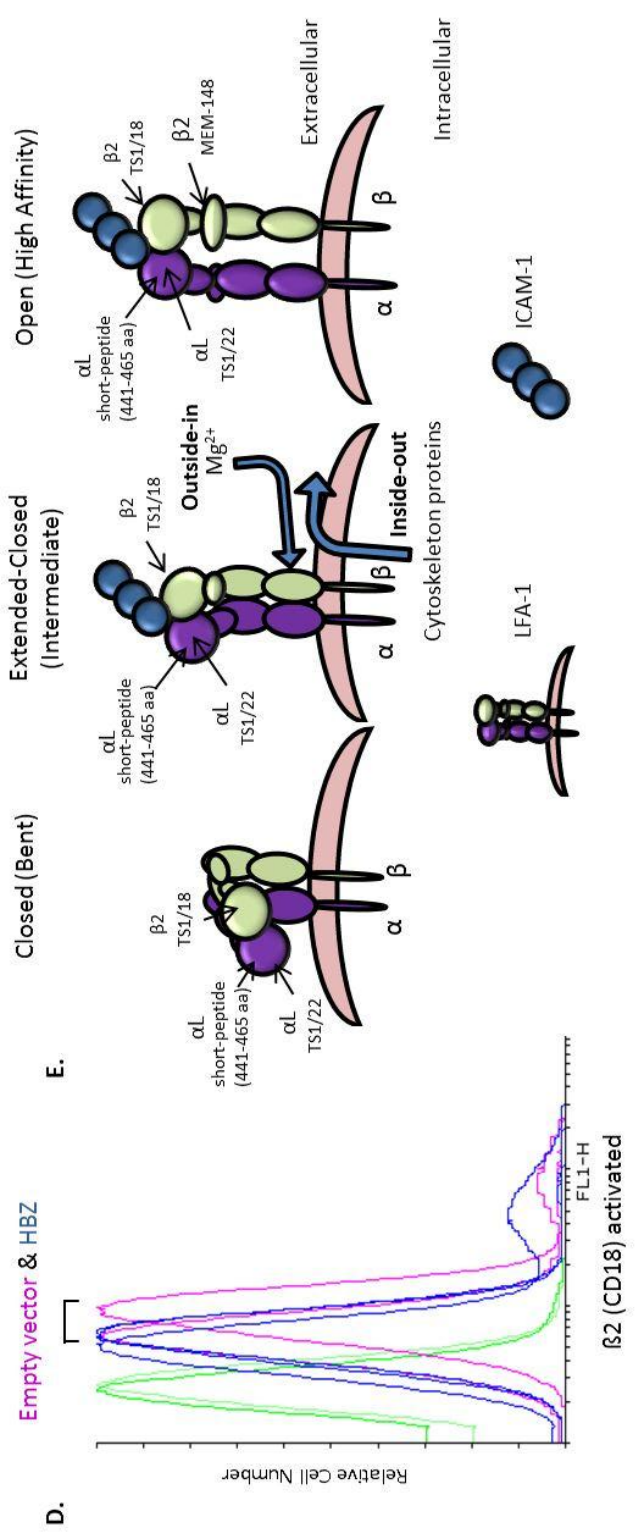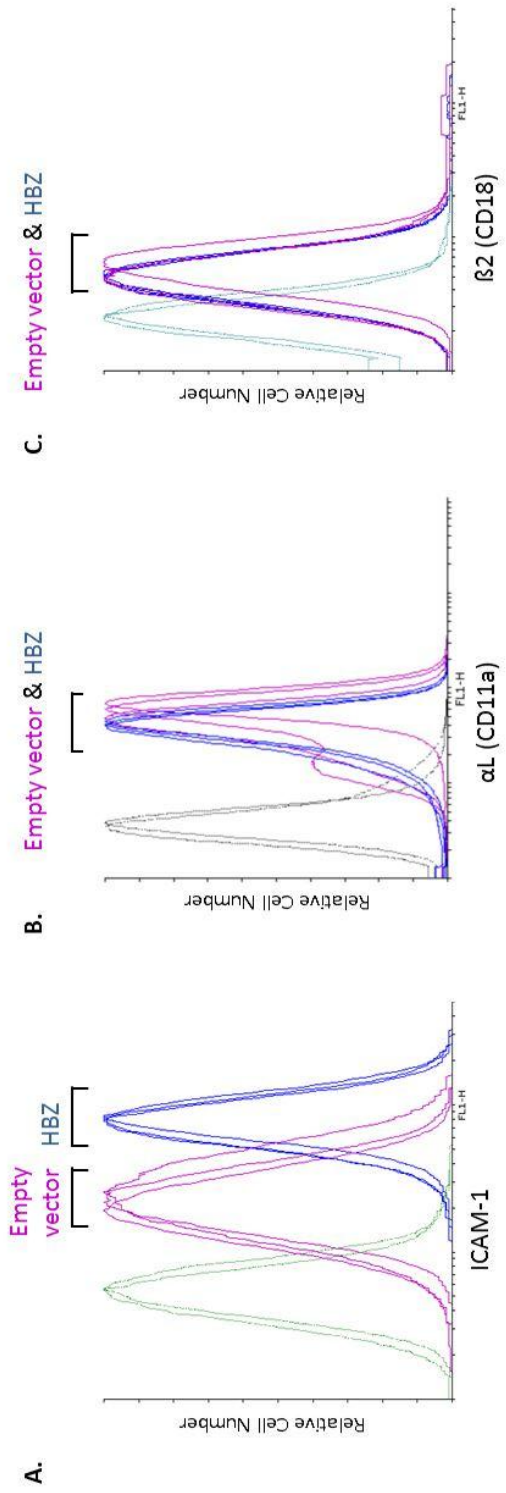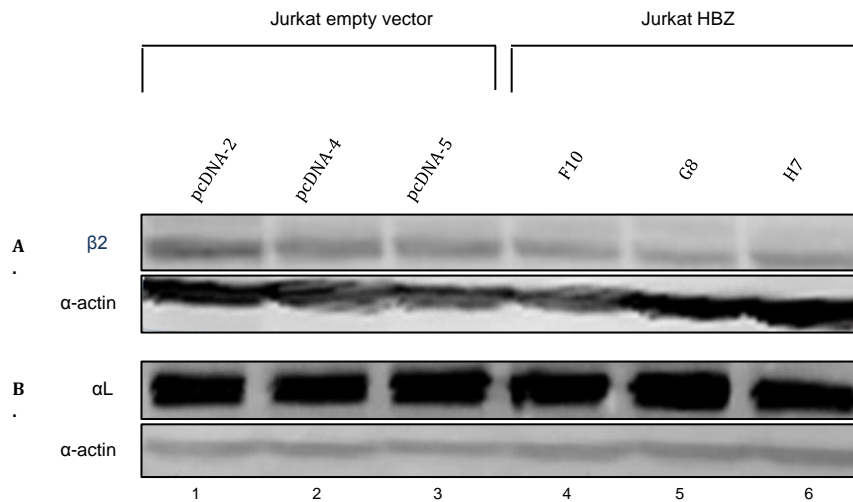
Figure 14. Western blot detection of β2 and αL total protein levels. No differences in A) β2 and B) αL total protein levels from whole cell lysates (50 µg) prepared from empty vector (pcDNA) controls (lanes 1,2,3) and Jurkat HBZ clones (lanes 4,5,6) were observed. The membrane from the upper panel A) was probed with a β2 antibody (1:125). The lower panel B) shows the membrane probed with αL antibody (1:250). α-actin was used as a loading control. A representative western-blot from one experiment is shown.

3.7   HBZ Enhances ICAM-1-Mediated T-Cell-to-Cell Adhesion

To determine whether the interaction between an overexpressed ICAM-1 and its ligand LFA-1 is responsible for the homotypic cell aggregation obser ved in HBZ-expressing cells and also HTLV-1 infected cell lines, we decided to block the ICAM-1/LFA-1 interaction using specific monoclonal antibodies directed against the αL and β2 subunits of LFA-1, ICAM-1, and αM (CD11b) subunit of Mac-1 (CD11b/CD18), which is known to be another specific ligand for ICAM-1 [384]. We also utilized a short peptide (441-465 aa) derived from a sequence of the αL subunit of LFA-1 as a blocking agent for ICAM-1 [385]. We performed several time-course experiments in which we used these reagents to attempt to block aggregation of different clones of Jurkat HBZ-expressing cells, an HTLV-1 infected cell line (SLB-1), and Jurkat cells containing the empty vector. We used an isotype antibody as a negative control. As shown in Figure 15A, after 6 hours of incubation, the antibody against the αL subunit was able to block the cell aggregation in the HBZ clones and the SLB-1 cell line. However, the specific antibody against the αM subunit did not block the cell aggregation (Figure 15B). These results suggest that LFA-1 is directly involved in the homotypic cell aggregation among HBZ-expressing cells and that of SLB-1 cells, but not Mac-1. As expected, the antibodies against ICAM-1 (Figure 15C) and the β2 subunit of LFA-1 (Figure 15D) also decreased the cell-to-cell aggregation. Furthermore, the short peptide against the αL subunit was also able to block the cell aggregation of HBZ-expressing cells and the infected cell line SLB-1 (Figure 15E). Figure 16 shows a graphical comparison of the cell aggregation sizes (Figure 16A to E). Taken together, we demonstrated that the inhibition of the LFA-1: ICAM-1 interaction by using specific antibodies and a short peptide against ICAM-1 and its ligand, LFA-1, can abrogate cell aggregation, suggesting that the homotypic cell

adhesion observed in these cells depends on these specific molecules.

In order to confirm that the enhanced adhesion of the HBZ-expressing cells was caused by the interaction between ICAM-1 and LFA-1, we performed a Static Adhesion assay using CHO (Chinese Hamster Ovary) cells stably expressing LFA-1, and parental CHO cells as the control. CHO cells or CHO-LFA-1 cells were plated over coverslips inserted in 6-well plates and incubated at 37 °C, 5% CO_2 overnight (Figure 17A). HBZ clones were stained with Hoechst 33342 (a nuclear vital cell stain that emits blue fluorescence when bound to dsDNA) for 20 min, washed in unsupplemented IMDM medium. Next, the stained cells were co-cultured with the CHO or CHO-LFA-1 cells for 2 hours. After the incubation, the CHO and CHO-LFA-1 cells were washed, and the coverslips were photographed with a fluorescent microscope. A significant higher number of Jurkat HBZ-clones attached to the CHO-LFA-1 cells when compared to those attached to CHO cells (control) (Figure 17B). As the model shows in Figure 17C, we found that an overexpression of ICAM-1 on the HBZ-clones increases cell adhesion to LFA-1 expressing cells. Cell adhesion results were quantified using ImageJ software (Figure 17D). The presence of LFA-1 on CHO-LFA-1 cells was confirmed by Western blot (Figure 17E).

3.8 HBZ Increases Cell-to-Cell Contact with Uninfected Cells

Cell-free HTLV-1 virus is poorly infectious [386, 387]. Naturally, there is virtually no production of cell-free infectious virions: only 1 out of 10^5 is infectious [309, 312, 388]. In addition, studies of transfusions have demonstrated that the recipients of non-cellular blood products (plasma derivates) from HTLV-1 patients do not become seropositive [58, 389]. These data strongly suggest that HTLV-1 depends highly on cell-to-cell contact for

efficient virus transmission [38, 48, 165, 325].

Studies on T-cells from HTLV-1 infected individuals have demonstrated that these infected cells can spontaneously establish cell-to-cell contacts and form conjugates with uninfected T-cells [322, 327]. It is also known that HTLV-1 can upregulate adhesion molecules, such as ICAM-1 [314, 322, 329]. The interaction between ICAM-1 and LFA-1, which is expressed mostly in T-cells [390], is crucial for virus transmission [322, 340, 391].

We have demonstrated that HBZ-expressing cells are able to increase T-cell contact through the interaction between ICAM-1 and LFA-1. Our next step was to determine the HBZ ability to increase cell-to-cell contact with uninfected T-cells. For this purpose, we performed cell conjugation assays. Briefly, we stained Jurkat HBZ clones with Hoechst 33342, a nuclear vital cell stain that emits blue fluorescence when bound to dsDNA, and co-culture them with Jurkat T-cells lacking the viral protein (uninfected cells) for 2 h, 37°C and 5% CO_2. As it is shown in figure 18, HBZ expressing cells stained with Hoechst 33342 conjugated with cells uninfected (Jurkat target cells). These results suggest that HBZ induces the spontaneous formation of cell conjugates with uninfected cells by regulating the interaction between ICAM-1 and LFA-1 surface adhesion molecules.

3.9 HBZ Enhances the Viral Transmission of Virus-Like-Particles to target cells

Based on our previous results, our next step was to determine whether HBZ was promoting the transmission of the virus though an increased cell-to-cell contact. Cell-to-cell contact and HTLV-1 transmission have been well established by live-cell

Figure 15. HBZ promotes T-cell aggregation by upregulating ICAM-1. A) Different clones of Jurkat empty vector cells (pcDNA2, pcDNA4, pcDNA5), HBZ clones (G8, F10, H7) and a HTLV-1 infected cell line (SLB-1) were incubated with αL (CD11a), or αM (Mac-1), or β2 (CD18) or B) ICAM-1 (clone RR1/1) blocking antibody, or an equal quantity of isotype control antibody at 5% CO_2, 37°C for 6h. The cells were monitored and photographed by microscopy. Monoclonal antibodies were used at a final concentration of 5 μg/mL. Results are representative of three independent assays. C) HBZ clones incubated with 5 μg/mL of LFA-3 (CD58) blocking antibody, or an equal quantity of isotype control. D) Diagram of the possible combinations between the β2 and αL subunit of LFA-1, and their respective ligands. E) SLB-1 cell line incubated with αL blocking antibody for 6 h. F) HBZ clone (H7) and SLB-1 cell line treated with a short αL-derived peptide (250 μM) or equal quantity of DMSO (vehicle) for 6h under same conditions as above.
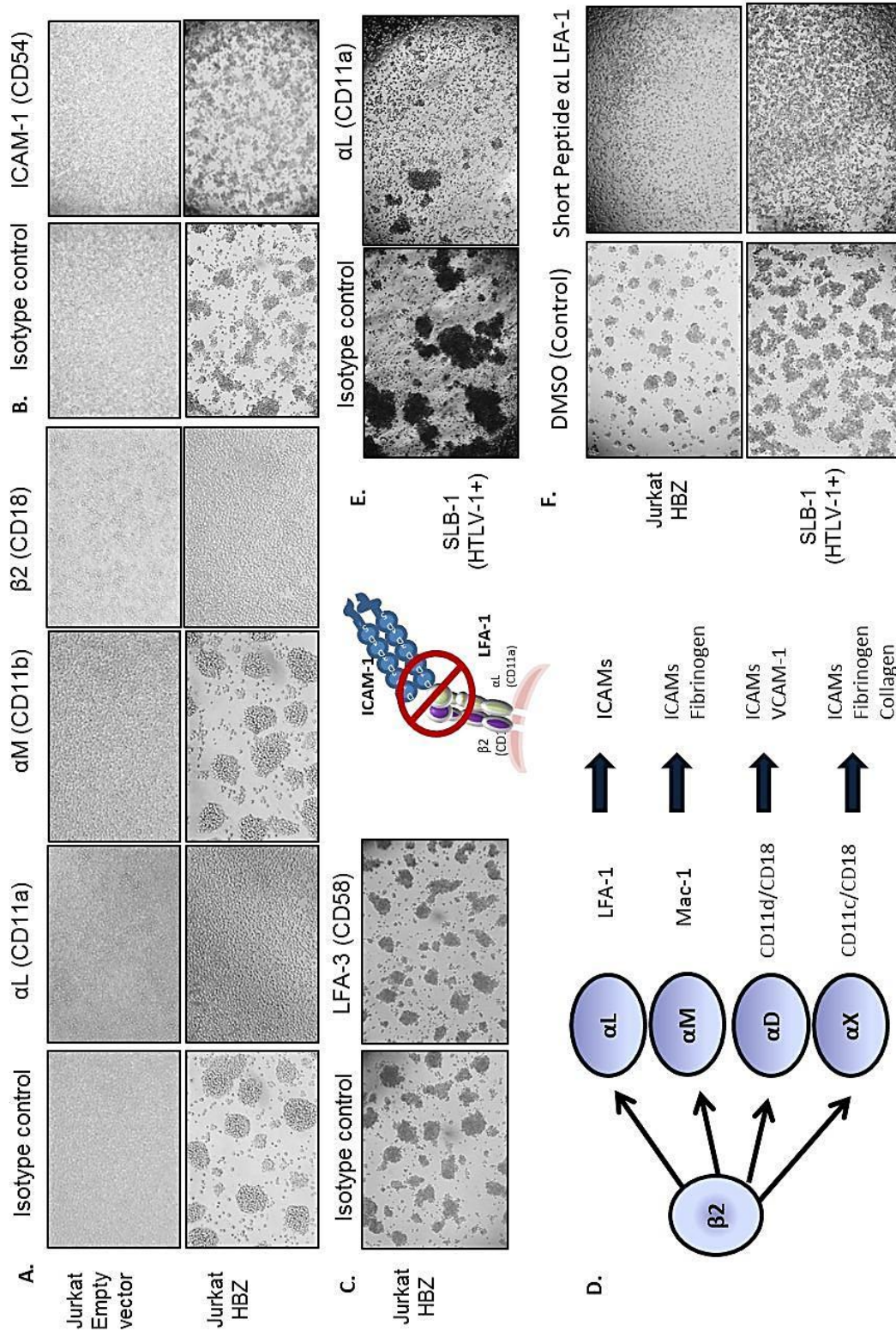
Figure 16. HBZ promotes T-cell aggregation by upregulating ICAM-1, graphical **representation of cell aggregation size.** Results are quantified using ImageJ software. A) Jurkat empty vector cells (pcDNA #2: #4:, #5), HBZ clonal cells (G8, F10, H7) were incubated with αL (CD11a), or αM (Mac-1), or β2 (CD18) or an equal quantity of isotype control antibody. B) HBZ clone H7 incubated with EDTA, EGTA, ICAM-1 (clone RR1/1) or isotype control blocking antibody. C) SLB-1 cell line treated with αL blocking antibody. D) SLB-1 cell line and E) HBZ clone H7 incubated with a short αL-derived peptide (250 μM) or equal quantity of DMSO (vehicle). Monoclonal antibodies were used at a final concentration of 5 μg/mL and EDTA and EGTA were used at 5mM. Results are representative of three independent assays.
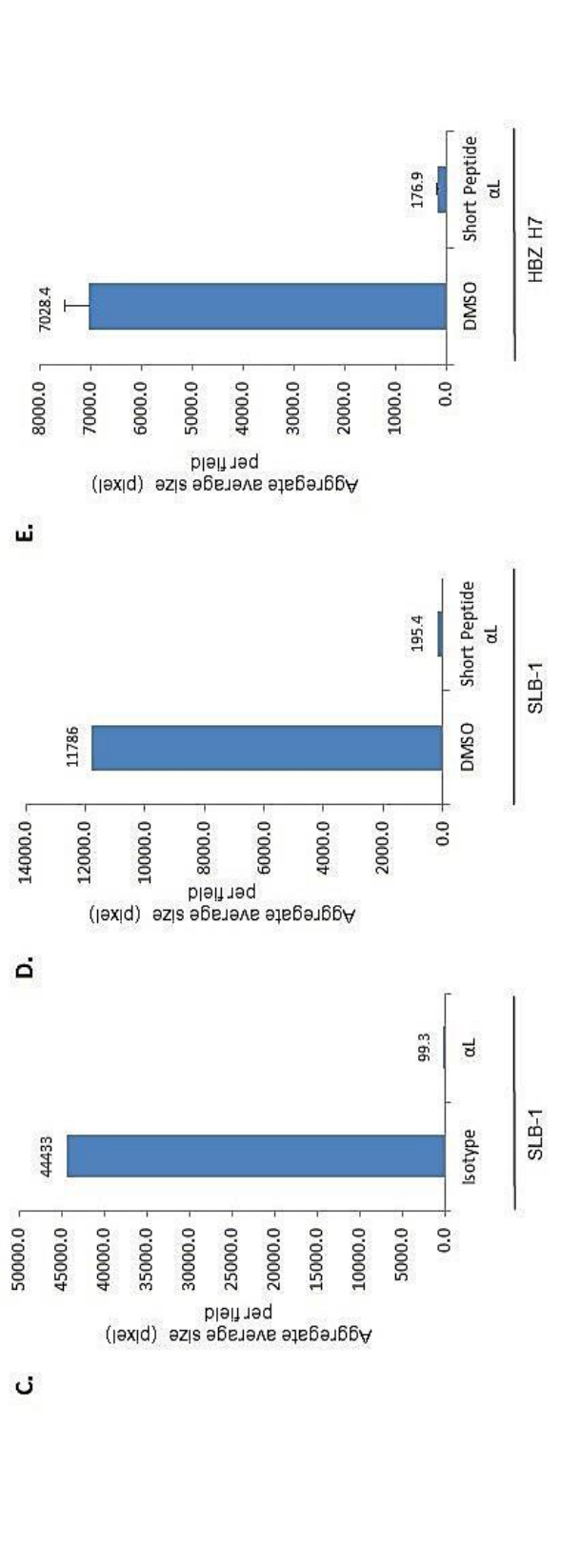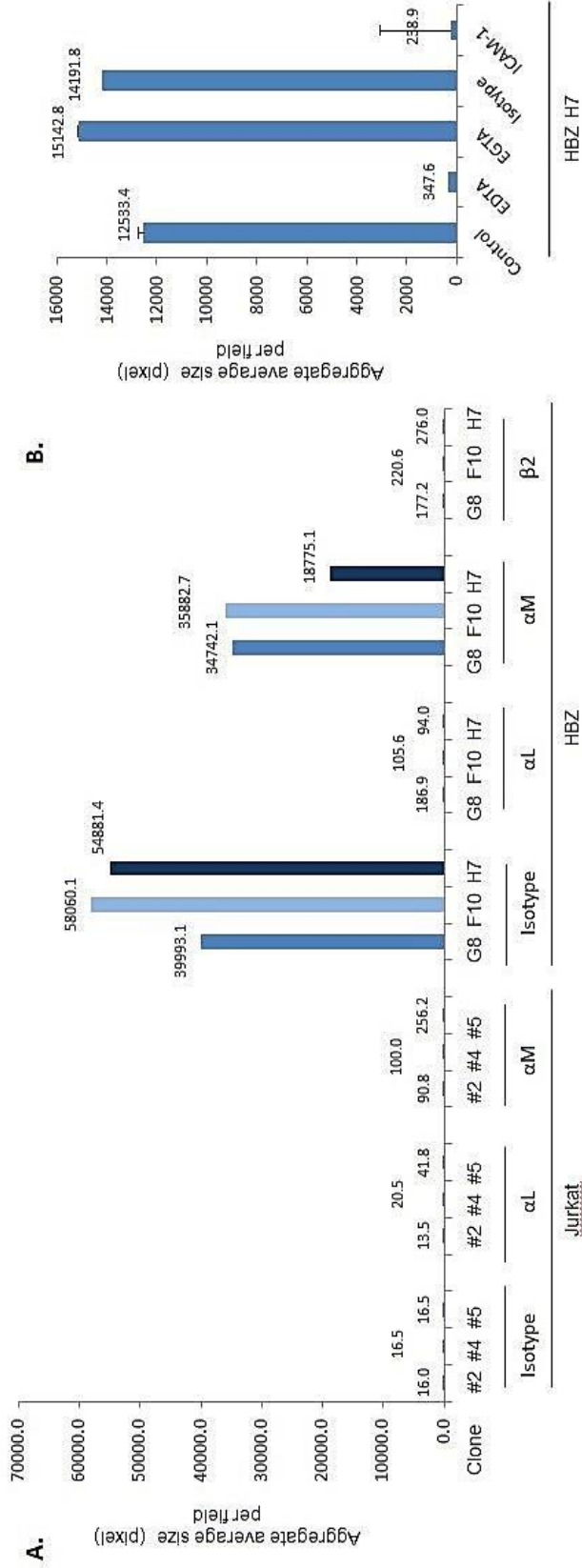
Figure 17. HBZ induces T-cell adhesion by upregulating ICAM-1. A) Diagram of static adhesion assay using CHO (Chinese Hamster Ovary) cells stably expressing LFA-1 and parental CHO cells as the control. CHO cells or CHO-LFA-1 were plated over coverslips, inserted in 6-well plates and incubated at 37 °C, 5% CO_2 overnight. Jurkat HBZ clones were stained with Hoechst 33342 for 20 min, and washed in unsupplemented IMDM medium. The stained cells were then co-cultured with the CHO or CHO-LFA-1 cells for 2 h. After incubation, the CHO and CHO-LFA-1 cells were washed to remove unattached Jurkat-HBZ cells, and images were taken on a fluorescent microscope. B) Jurkat-HBZ cells stained with Hoechst 33342 (live cell stain) were incubated and allow to adhere to parental CHO cells or CHO-LFA-1 cells. Bright field and fluorescent images were taken after 2 h of incubation at 37°C. A neutral-density ND4 filter was used. C) Proposed model to explain how the presence of ICAM-1 in the HBZ clones and LFA-1 in the target cell increases cell adhesion. D) Cell adhesion results were quantified using ImageJ software. Results are expressed in relative % to Jurkat HBZ-clones adhesion to parental CHO cells (control). Data is representative of three independent assays. *$p < 0.05$. E) Western blot showing LFA-1 expression on CHO-LFA-1 cells.

B.

Bright field | Hoechst 33342

CHO + Jurkat-HBZ (Hoechst)

CHO-LFA-1 + Jurkat-HBZ (Hoechst)

A.

CHO-LFA-1 cells

CHO cells (control)

Incubation 37°C, 5% CO₂ O/N
Co-cultured with HBZ Jurkat clones stained with Hoechst 33342

Incubation at 37°C, 5% CO₂ 2h

Wash

Images taken with Fluorescent Microscope

C.

Weak Interaction

Jurkat-HBZ clone

CHO cells

Strong Interaction

Jurkat-HBZ clone

CHO-LFA-1 cells

LFA-1   ICAM-1

D.

*

HBZ

Jurkat + CHO   Jurkat + CHO-LFA-1

E.

CHO
CHO LFA-1

αL (CD11a)   200 150 100

β2 (CD18)   100 75 50

actin   50 37

Figure 18. HBZ increases cell-to-cell contacts. A) Jurkat cells containing the empty vector or HBZ-expressing Jurkat cells were stained for 20 min with Hoechst 33342, a nuclear vital cell stain that emits blue fluorescence when bound to dsDNA, and co-cultured on slides with Jurkat cells (target cells) for 3 h, 37°C and 5% CO_2. B) A proposed model for the cell-to-cell contact between donor and target cells. Cells stained with Hoechst of Results are representative of five independent assays. C) HBZ-expressing Jurkat clones stained with Hoechst 33342 and co-cultured with Jurkat T-cells lacking the viral protein (uninfected cells) for 2 h, 37°C and 5% CO_2. Images were taken using a confocal microscope. Results are representative of at least three independent experiments.

imaging with scanning electron microscopy, confocal microscopy and tomography.

These methodologies have given clearly evidence of the viral particles presence and movement along the surface and inside the target cell, and also the formation of specialized cell-to-cell contact structures, VS [322, 327], biofilm [329], and conduits [330]. Most recently, infection experiments based on single-cycle vectors, allow the specific quantification of the virus transmission and provirus formation after cell-to-cell contact between an infected cell and a target cell [310, 312, 392].

We performed single-cycle, replication-dependent vector infection assays to determine the ability of Jurkat HBZ clones (donor cells) to infect Jurkat cells and other uninfected (target) cells. Jurkat cells containing the empty vector were used as controls. Briefly, we transfected Jurkat cells with single-cycle, luciferase expressing replication-dependent vectors, a packaging plasmid, and an env-expression plasmid, to provide essential structural and enzymatic proteins for viral replication (plasmids kindly provided by Dr. Derse's group and Dr. Mazurov) (Figure 19A). The advantage of this system approach is that the luciferase activity is only present in the target cell, with a very low or null background from the donor cell. The mRNA containing the luciferase gene cannot be translated in the donor cell because of the disruption caused by an inverted intron (sense orientation) in the luciferase gene. The mRNA is spliced and packaged into virions; when infection occurs in the target cell, reverse transcription allows the transcription of the mRNA containing an uninterrupted luciferase gene, and integration in the target cell occurs. A plasmid called pCMV-Env-Stop, which expresses a nonfunctional, truncated version of Env that served as a negative control for infection. 48 hours post-transfection, donor cells (we used different HBZ clones or empty vector controls) were co-cultured with Jurkat (target) cells for an additional 48h in a 1:1 ratio, and

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Figure 19. HBZ Enhances the Viral Transmission of Virus-Like-Particles to T-cells. A) Diagram of Infection Assay. Jurkat-HBZ clones or empty vector cells were transfected with the HTLV-1 replication-dependent reporter system. After 48 h of incubation at 37°C and 5% CO_2, cells were co-cultured with Jurkat cells   or CHO or CHO-LFA-1 (target cells) for 2 h. Cells were washed, donor cells were discarded, and the target cells incubated at 37°C and 5% CO_2 for 24 h. The cells were harvested and luciferase activity was measured with a luminometer. B) Schematic representation of the single-cycle, replication-dependent reporter system used in the infection assay. Three plasmids were transfected into HBZ Jurkat clones: the HTLV-1 packaging vector (pCMVHT1M-ΔEnv), HTLV-1-Env or Env-Stop (negative control), and a replication-dependent HTLV-1 reporter vector (pCRU5HT1-inLuc). The plasmids pCMVHT1M-ΔEnv and HTLV-1-Env produce the virus-like particle (VLP). pCRU5HT1-inLuc (HTLV-1-inLuc) contains the two LTRs (the U3 region of the 5'LTR is replaced by a CMV promoter). The luciferase gene is cloned in the antisense orientation and is controlled by the CMV promoter. mRNA #1 is spliced and packaged into the viral particle. The luciferase protein is not translated from mRNA #2 in donor cells due to the inverted intron that is not spliced from the transcript. The process of infection, reverse transcription and integration in the target cell allows the transcription of mRNA #3 containing an uninterrupted luciferase gene. C) Results from three independent infection assays using Jurkat empty vector or HBZ-expressing cells as donor cells and Jurkat cells as recipient cells, *p<0.0005 (two-way ANOVA test, statistical differences between Env samples).

A)

Electroporation

Empty vector clones
HBZ clones

- Packaging vector (Δenv)
- Env plasmid or Env-STOP plasmid
- HTLV-1 pCRU5HT1-inLuc

Incubation 37°C, 5% CO_2

48h

Cells equalized and Co-culture (1:1) with Jurkat or CHO, or CHO-LFA-1 cells (target)

Jurkat cells | CHO, or CHO-LFA-1 cells

Incubation 37°C, 5% CO_2 48h | Incubation 37°C, 5% CO_2 2h. Effector cells washed. Incubated 37°C, 5% CO_2 24 h

Cell harvested, lysed and Luciferase activity measured by luminometer

B)

1. ΔEnv HTLV-1 packaging vector
2. HTLV-1-Env or Env-Stop
3. pCRU5HT1-inLuc

Effector: Jurkat HBZ or empty vector cell (control)

CMV U5/R <pA i <Luc CMV LTR #1 #2

Virus-like particle (VLP) Virological synapse & Biofilm

CMV U5/R <pA <Luc CMV LTR #3

luminescence

C)

Infection: Jurkat clone → Jurkat

■ Env
■ Env-Stop

Figure 20. HBZ Enhances the Viral Transmission of Virus-Like-Particles to CHO-LFA-1 cells. A) Infection assay using Jurkat -HBZ clones or the empty vector transfected with the HTLV-1 replication-dependent reporter system. Cells were cultured at 37°C and 5% CO_2. After 48 h post-transfection, cells were co-cultured with CHO or CHO-LFA-1 for 2 h, washed and incubated at 37°C and 5% CO_2 for 24 h. Cells were harvested, lysed, and luciferase activity was measured with a luminometer. Results are representative of three independent experiments .B) Infection assays as described above, but with 5 µg/mL of blocking ICAM-1 antibody added to co-cultured cells. Jurkat -HBZ transfected with the replication-dependent reporter system .Jurkat cells were used as target cells. Results are representative of five independent assays. *$p < 0.05$; **$p < 0.005$; ***$p < 0.0005$.

A)   Infection: Jurkat HBZ clone (donor cell) →   CHO or CHO-LFA-1 (target)
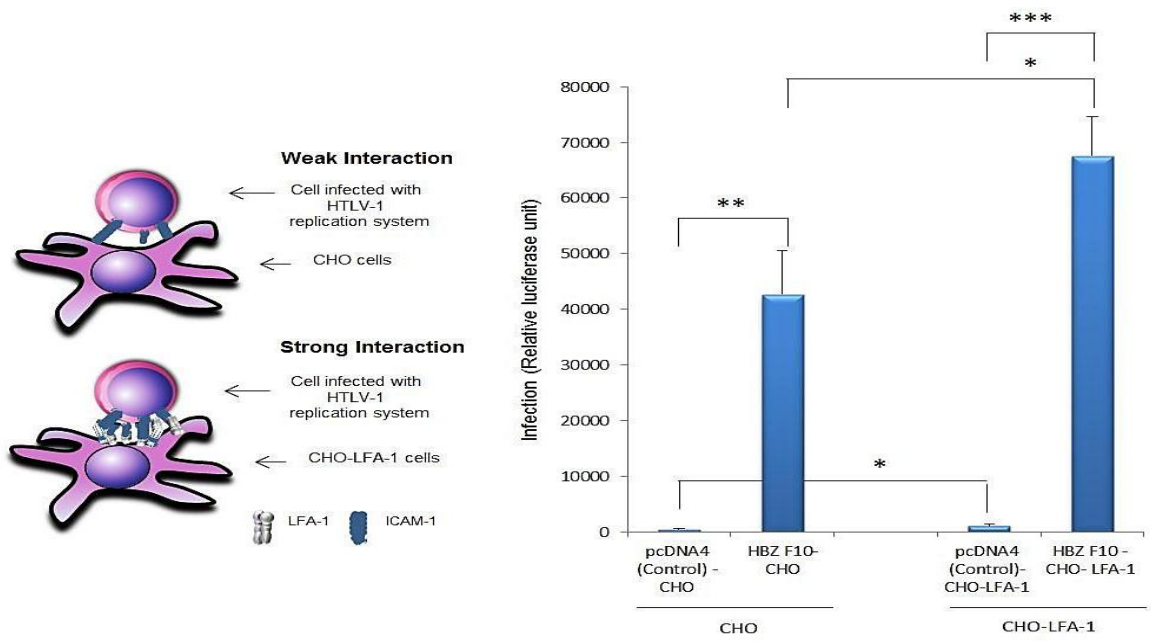
B)   Infection: Jurkat HBZ clone (donor cell) →   Jurkat (target)
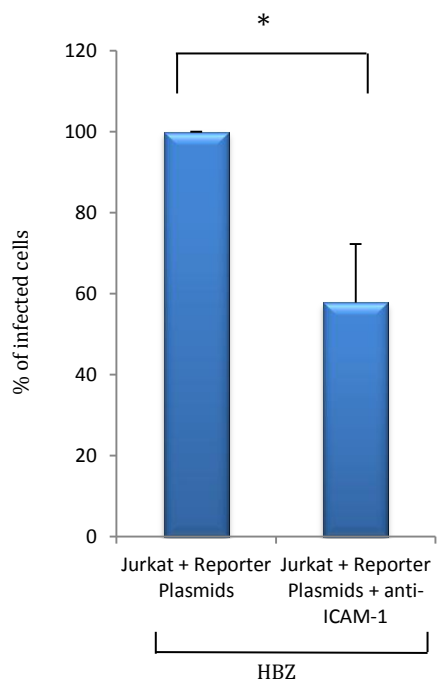
luciferase activity was measured. We observed significant differences in luciferase activity between the Jurkat cells that were co-cultured with the HBZ clones when compared to those that were co-cultured with the empty vector cells (Figure 19C). These results suggest that HBZ increases the transmission of viral components to non-infected cells.

To determine the role of the ICAM-1 upregulation by HBZ in cell infection, we performed an infection assay where HBZ-expressing (donor) cells were co-cultured with target cells that do not express LFA-1 (parental CHO cells), and CHO cells stably expressing LFA-1 (CHO-LFA-1) as the target cells. Also, these adherent cells allow us to demonstrate that the luciferase activity obtained in the previous experiment is not due to HBZ cells infecting each other. We transfected both Jurkat-HBZ clones or empty vector cells with the replication-dependent vectors. After 48 h of incubation at 37°C and 5% CO_2, we co-cultured these cells with CHO or CHO-LFA-1 (target cells). After 2 h, the donor cells were discarded, the target cells washed, and incubated in complete DMEM medium at 37°C and 5% CO_2. The cells were harvested after 24 h and luciferase activity was measured with a luminometer. We detected a higher luciferase activity in the CHO-LFA-1 (target cells) when compared to the CHO control (Figure 20A). These results are consistent with our premise that an overexpression of ICAM-1 in HBZ-expressing cells promotes the adhesion to its ligand, LFA-1 on the target cells, and such interaction enhances viral transmission and provirus formation in the target cell.

To confirm the relevance of ICAM-1 induced by HBZ in virus transmission, we performed the same infection assays as described above, this time adding a blocking antibody against ICAM-1 to the Jurkat HBZ clones transfected with the HTLV-1 replication-dependent reporter system. We found a significant reduction in the

luciferase activity of the Jurkat target cells that were co-cultured with the HBZ clone cells treated with the blocking antibody (Figure 20B).These findings indicate that ICAM-1 induced by HBZ contributes to viral particle transmission. ICAM-1 may play an important role in the infection of target cells by stabilizing cell-to-cell interactions.

CHAPTER 4: DISCUSSION

In vitro homotypic cell adhesion (formation of aggregates) is typical for freshly isolated ATL cells and HTLV-I infected cell lines [320]. Interestingly, we also observed this phenotype in cells exclusively expressing HBZ (Figure 8). High levels of aggregation in ATL cells and HTLV-1 infected cell lines have been correlated to an upregulation of ICAM-1 [320, 355, 356]. ICAM-1 is expressed at low concentrations in the surface of several cell types, including leukocytes and endothelial cells [393, 394]. After stimulation with inflammatory mediators, including tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), and interferon-γ, ICAM-1 expression is upregulated [395, 396]. In this study, we showed an overexpression of ICAM-1 in Jurkat, SupT1 and HeLa cells exclusively expressing HBZ (Figure 10 and 13), suggesting that HBZ alone is sufficient to upregulate ICAM-1. Furthermore, we confirmed that ICAM-1 was also upregulated in ATL cell lines and HTLV-1 infected cells when compared with primary unstimulated and activated human CD4$^+$ T-cells, and uninfected T-cells (Figure 10). Interestingly, the SLB-1, and the ATL-2 cell lines, which exhibited the highest ICAM-1 levels among the cells we tested, both have been reported to express high levels of HBZ [277]. In addition, TL-Om1 cells, an ATL patient-derived cell line [345] that only express HBZ [280], exhibited high levels of ICAM-1 compared to uninfected cell lines, suggesting a regulatory role of HBZ in ICAM-1 expression. Moreover, 1185 cells, which are HTLV-1-immortalized but not yet transformed cells [397], also expressed high levels of ICAM-1.

Thus, our results suggest that HBZ modulates early events just after infection has taken place, and before the process of complete cell transformation occurs. This observation is interesting because HBZ is thought to control exclusively late events occurring during the progression of ATL.

We showed that blocking ICAM-1 using specific antibodies or a short peptide against the ICAM-1 ligand, LFA-1, significantly reduced the formation of aggregates in HBZ clones and SLB-1 cells (Figure 15 and 16), revealing an important role for ICAM-1 in this cell-adhesion process [320, 398]. Of note, we chose to work with SLB-1 cells because it is a well-established HTLV-1 infected T-cell line that expresses high levels of h*bz* mRNA and protein [277].

We performed a Static Adhesion assay using Hoechst 33342-stained Jurkat HBZ clones co-cultured with adherent CHO (Chinese Hamster Ovary) cells stably expressing LFA-1, and parental CHO cells as the control. We found that a significantly higher number of Jurkat HBZ-clones attached to the CHO-LFA-1 cells when compared to those attached to CHO cells (control). We concluded that the interaction between ICAM-1 in the donor cells, and LFA-1 in the target cells, enhances the adhesion properties of the HBZ-expressing cells.

To determine whether HBZ regulates ICAM-1 transcription, we performed a reporter luciferase analysis of the ICAM-1 promoter region, and found that the luciferase activity was significantly increased in the Jurkat HBZ clones when compared to empty vector clones (Figure 12). Interestingly, our results showed that the bZIP domain of HBZ was specifically involved in the ICAM-1 regulation (Figure 10C). The bZIP domain is known to modulate interactions with certain cellular bZIP factors to regulate transcription [187, 210, 259, 260, 262]. Among them, it was shown that the bZIP factor JunD, forms heterodimers with HBZ, which are able to activate transcription from the HBZ promoter, and from the cellular human telomerase reverse transcriptase (hTERT) promoter, the catalytic subunit of the human telomerase [247, 262, 276, 373]. Activation of these promoters requires the recruitment and binding of the JunD-HBZ heterodimer to a Sp1

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transcription factor that is directly bound to the DNA (Gazon *et al.,* 2012). Interestingly, the ICAM-1 promoter contains two Sp1 binding sites (-203 bp and -53 bp) [374, 375]. Figure 21 depicts our hypothetical model to explain the mechanism of how HBZ activates ICAM-1 transcription. HBZ activates ICAM-1 transcription by recruiting and binding JunD to DNA-bound Sp1, forming an active HBZ/JunD/Sp1 complex. Future studies using chromatin immunoprecipitation assays on HBZ-expressing cell lines will allow us to determine the specific host factors involved in ICAM-1 transcription activation induced by HBZ.

Our results also demonstrated that ICAM-1 is increased at the surface of Jurkat-HBZ clones (Figure 13A). These data are consistent with those reported by other groups who showed that ICAM-1 surface expression is increased in HTLV-1-infected cell lines and fresh ATL cells obtained directly from patients [314, 322]. We did not find differences in the cell surface expression of the LFA-1 subunits, αL and β2 (Figure 13B, 13C). These results differ from a study reported by Yamamoto-Taguchi *et al.* that showed an increased LFA-1 surface expression in HBZ-transgenic mouse cells [321]. However, other studies showed that surface levels of LFA-1 in human leukemic cells were, on average, not different from those of CD4$^+$ T-cells from healthy donors [314, 320]. Moreover, studies on LFA-1 levels made directly on HAM/TSP patient's CD4$^+$ cells did not show any changes on LFA-1 surface expression when compared to CD4$^+$ cells from healthy donors [321].

We did not observe differences in the expression of the activated form of β2 in the Jurkat HBZ clones when compared to controls (Figure 13E). It is conceivable that LFA-1 activation occurs in the target cell after the initial contact with its ligand, ICAM-1 on an infected cell. Thus, it would be interesting to determine LFA-1 levels on the target cells

after that initial contact with infected cells or HBZ clones. Also, Yamamoto-Taguchi et al, reported a significant increased expression of CD11a and CD18 expression (LFA-1 subunits) in CD4$^+$ Tax$^+$ cells when compared to CD4$^+$Tax $^-$ cells, both from HAM/TSP patients. Hence, the activation of LFA-1 might depend on a different viral protein, such as Tax. Overall, our results suggest that HBZ can upregulate ICAM-1 expression, but does not affect the expression of LFA-1.

The increase of ICAM-1 expression at the surface of HTLV-1 infected cells has been linked to the formation of close cell-to-cell contacts and HTLV-1 transmission from infected cells to uninfected target cells [48, 322, 324, 327, 335, 340]. In the current study, we demonstrated that HBZ expression is correlated with ICAM-1 upregulation. These data suggested that HBZ might be involved in virus transmission. To determine this possibility, we transfected Jurkat cells with a single-cycle, replication-dependent, reporter vector, a packaging plasmid, and an env-expression plasmid, to provide essential structural and enzymatic proteins for viral replication [310]. A plasmid called pCMV-Env-Stop, which expresses a nonfunctional, truncated version of Env, served as a negative control for infection (Figure 19A). This reporter system can reveal an efficient provirus formation after cell-to-cell contact, and allows the quantification of virus transmission [310, 312, 392]. Another advantage given by this approach is that the luciferase activity produced by the transferred virus particles is only present in the target cell, with very low or null background from the donor cell. Our results revealed a significant increase in luciferase activity in those target cells that were co-cultured with HBZ clones transfected with the vectors of the single-cycle system, when compared to target cells co-cultured with the empty vector cells (Figure 19, 20). These results suggested that HBZ enhances the transmission of infectious viral particles to
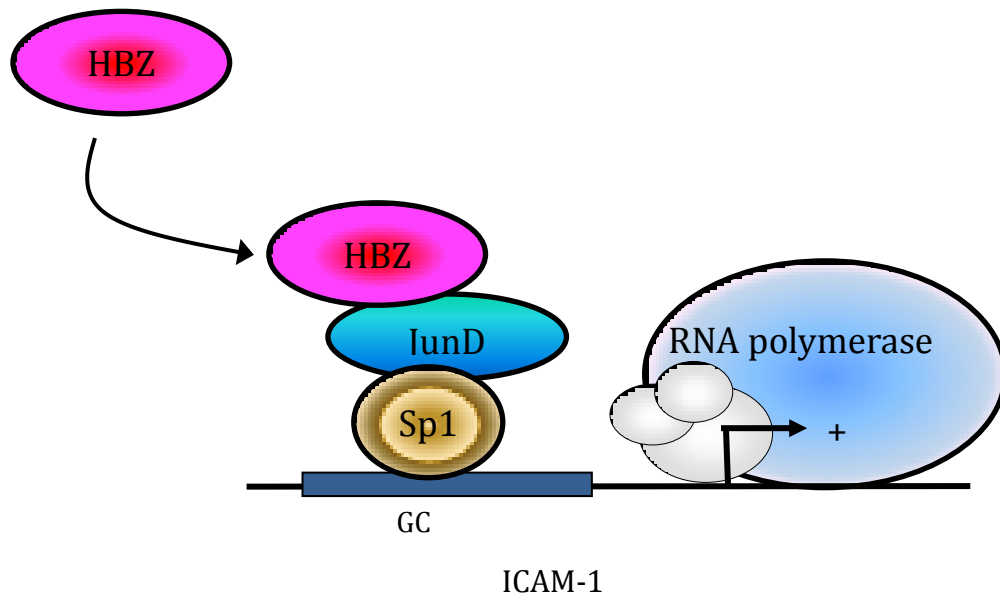
Figure 21. HBZ activates transcription from the ICAM-1 promoter. Proposed model to explain how HBZ is involved in the activation of the ICAM-1 promoter: HBZ is able to generate an activation complex with JunD, and Sp1 that induces transcription of the ICAM-1 promoter.

non-infected cells.

Recently, Dr. Mazurov's group reported a limitation in the single-cycle, replication-dependent, reporter system we utilized in our infection experiments. The intron that interrupts the luc-gene in the reporter vector (HTLV-1 inLuc) in the donor cell, sometimes cannot be efficiently removed once it is transferred to the target cell [392]. Therefore, the sensitivity of the infection assay is reduced, underestimating the quantity of virus transmission [392]. Thus, we can speculate that the number of target cells infected after the co-culture with the HBZ clones in our experiments, is even higher than the results we obtained.

Previously, Liu *et al.,* reported that blocking ICAM-1 on HTLV-1 infected cells reduced viral transmission [399]. Upon the use of ICAM-1 blocking antibody, we found a significant reduction in the virus particle transmission from the HBZ-clones transfected with the three vectors of the single-cycle system, to the target cells, when compared to the same donor-target cell system without ICAM-1 blocking antibody (Fig. 20B). Of note, the infection was reduced, but not completely blocked. These results can be explained by an insufficient amount of antibody added in the experiment, which failed to block completely all the surface molecules of ICAM-1 overexpressed in the HBZ clones. Also, the presence of a soluble form of ICAM-1 may interact with the blocking antibody in the medium and reduce an effective blockage of the surface expression of ICAM-1. In addition, the mechanisms through which HTLV-1 spreads to non-infected cells, might not be exclusively through ICAM-1-dependent cell adhesion. The transfer of stored virions on the extracellular biofilm to a target cell can occur rapidly and initially does not require the formation of the VS. After the donor and target cell have been in contact for a longer time, the formation of more stably structures between the cells, would allow the transfer of

more viral particles to the target cell to ensure an effective infection.

We also performed an infection assay to verify that the direction of the infection was in fact from donor cell to target cell (donor →target cell infection), and discard the possibility that the infection was occurring within the HBZ clones (i.e. HBZ clone F10 cells infecting another F10 clone cell). For that, we co-cultured Jurkat empty vector clones or Jurkat HBZ-clones (transfected with the HTLV-1 single-cycle replication system) with adherent CHO cells stably expressing LFA-1 (CHO-LFA-1), or with parental CHO cells used as control (Figure 20A). We used CHO-LFA-1 cells to determine whether the presence of the ICAM-1 ligand, LFA-1 in the target cells, may enhance virus transmission. After 2 hours, the donor cells were completely discarded, and the luciferase activity of the adherent cells analyzed. We found a significant increase in luciferase activity (infection) in the CHO-LFA-1 target cells that were co-cultured with Jurkat HBZ-clones when compared to parental CHO cells under the same co-culture conditions. These results suggest that HBZ induces infection of target cells, and this infection is enhanced by the presence of LFA-1 on the target cell.

It is known that ICAM-1 enhances cell-to-cell HTLV-1 transmission, and the presence of LFA-1 in the surface of the target cell is required for VS formation and efficient virus spread [322,327]. Based on these premises, we proposed that ICAM-1 induced by HBZ, stabilizes the cell-to-cell contacts required for efficient virus transfer. Hence, HBZ acts as a major contributor in viral transmission. Interestingly, HIV also requires the interaction of ICAM-1 on the infected cell with the LFA-1 on the target cell for efficient virus transmission [400-404] , suggesting a common retrovirus mechanism to infect target cells.

It has been reported that after the interaction of ICAM-1 on the infected cell with

LFA-1 on the target cell, a reorganization of the cytoskeleton occurs in the infected cell [322,324,327]. This reorganization is induced by tight cell-to-cell contacts, and involves the polarization of the microtubule organizing center, MTOC, which is required for the formation of the VS [322,327]. It is known that cell motility is related to the repositioning of the MTOC between the leading edge and the nucleus, in order to create a polarized morphology [405]. Matsuoka's group has reported that HBZ increases the migration of HTLV-1-infected cells by upregulating the expression of the noncanonical Wnt ligand, Wnt5a [269]. Wnt5a promotes cancer invasion and metastasis [406, 407] [408]. In addition, we obtained preliminary evidence that HBZ expression consistently increases cell migration in transmigration assays. Jurkat HBZ clones or empty vector clones were labeled and allowed to transmigrate through a monolayer of HUVEC cells in a Boyden chamber (Figure 22). The invasive cells were trapped in the membrane located under the HUVEC monolayer. These cells were washed, lysed, and the fluorescence intensity was analyzed. We found a significant increased motility in the Jurkat-HBZ clones when compared to the Jurkat empty vector clones. These data suggest that HBZ is able to regulate the molecular machinery for cell motility, supporting the notion that HBZ induces reorganization of the cytoskeleton. Therefore, we speculate that HBZ promotes VS formation by MTOC remodeling and increased ICAM-1 expression, and thus enhances virus transmission. Future studies will determine whether HBZ is able to induce polarization of the MTOC in the infected cell toward the site of cell-to-cell contact. In addition, it would be interesting to explore the possibility that HBZ regulates the expression of cytoskeletal proteins, such as ezrin, an intermediate protein used by ICAM-1 to interact with the actin cytoskeleton [409, 410]. In fact, the interaction of ICAM-1/ezrin/actin concentrates ICAM-1 expression on the uropod of the donor cell,
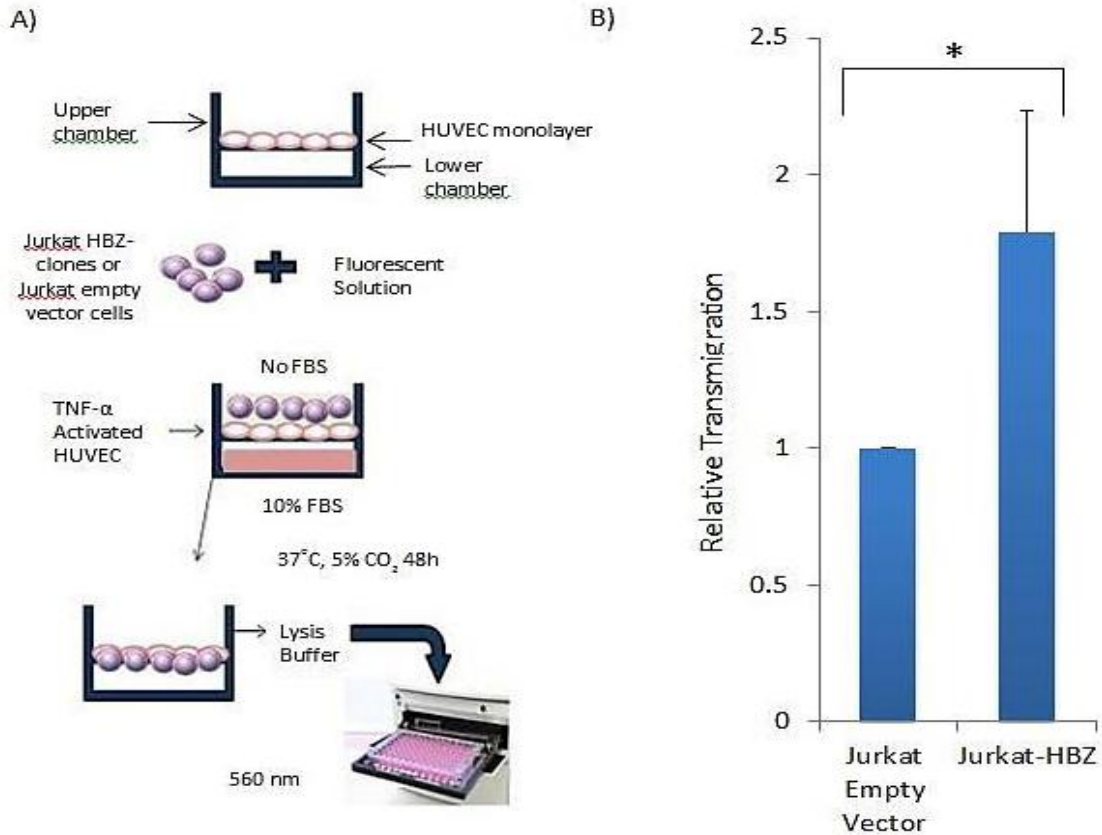
Figure 22. HBZ increases T-cell transmigration. A) Diagram of the transmigration assay. Human umbilical vein endothelial cells, HUVEC, were cultured on a permeable membrane of a Boyden chamber at 37°C and 5% CO_2, and allowed to form a cell-monolayer for 24h. HUVEC cells were activated with 1.2 μg/mL of TNF-α for 4h. Jurkat HBZ (1×10^6 cells/mL) and empty vector clones were labeled with a vital fluorescent solution for 10 min, and 300μL of each set of cells were transferred to the upper chamber in serum free media. Invasive cells passed through the HUVEC monolayer toward the lower chamber that contained medium with 10% FBS. Invasive cells became trapped in the membrane. After 48h, membranes were washed to remove non-migratory cells. Cells trapped in the membrane were lysed and transferred to a 96 well plate and analyzed for fluorescence. B) Relative transmigration of Jurkat HBZ and empty vector clones. Relative transmigration is directly correlated with the fluorescence intensity of the cell lysate associated with the membrane. Results are representative of 3 independent assays performed by triplicate. *$p<0.01$.

which facilitates its interaction with LFA-1 on the target cell [411].

It is intriguing that HTLV-1 appoints several proteins for viral transmission: Tax, p12/p8, and based on our results, HBZ. It is conceivable that these proteins cooperate in the upregulation of ICAM-1 and reorganization of the MTOC, and thus, in the enhancement of HTLV-1 transmission. HTLV-1 clones with impaired production of the p12 mRNA were inoculated in a rabbit model [337]. This clone failed to establish infection in the rabbits [337][412], suggesting an important role for p12 in viral infection. Furthermore, another study showed that macaques that were challenged with cells transfected with a HTLV-1 p12 knock-out clone, remained infection- free. Also, following inoculation, only two of the four primates became tissue positive for viral DNA after a long period of time [338]. Of note, HBZ was also affected in the p12 mutants utilized in the infection experiments mentioned above [337,338]. p8 protein, which originates from the proteolytic cleavage of p12 [336], has been involved in the formation of membrane extensions or filopodium-like protrusions known as cellular conduits. Through these conduits, HTLV-1 can directly contact and infect new target cells [330]. However, these accessory-viral proteins are not detectable in plasma, only with reverse transcription PCR [74], suggesting that there must be other viral proteins involved in HTLV-1 transmission.

After HTLV-1 infection occurs, Tax and HBZ are both simultaneously transcribed and expressed [190]. However, Tax expression is usually low or undetectable [413], and high levels of Tax only occur by punctuated short bursts [414]. HBZ, on the other hand, is stably expressed in the HTLV-1 infected cells. Also, in contrast to HBZ, which has   an extremely low immunogenicity [415, 416], Tax is highly immunogenic, and those cells expressing Tax are rapidly eliminated by cytotoxic T lymphocytes (CTLs) [216, 417].

Tax transactivates the HTLV-1 genome; hence, Tax is required for transcription of the viral proteins regulated by the 5' LTR sense promoter, which are required for virions formation. HBZ is constitutively expressed from the 3' LTR antisense promoter, which is independent from the 5' promoter. Once the virions are formed and Tax is repressed, HBZ can enhance the transfer of the viral particles embedded in the biofilm, and/or enhance the formation of a VS between the infected cells and the uninfected (target) cell (Figure 23). In this way, HTLV-1 ensures an effective viral transmission to target cells. Therefore, we speculate that HBZ has a critical function in HTLV-1 transmission.

Interestingly, there are several lines of evidence in the literature supporting the notion that HBZ plays an important role in viral transmission in animal models. Collins *et al.,* inoculated rabbits with a derivate from a wild type full-length-clone of HTLV-1 (ACH), in which the ORF I message was disrupted by a mutation that affected/abrogated the p12 message (ACH.p12) [337]. The HTLV-1 antibody response was weaker for the ACH.p12-inoculated animals in comparison to animals inoculated with the wild type ACH clone. In addition, it was only possible to isolate the virus from the wild type-inoculated animals [337]. The group attributed this reduced infectivity to the absence of the p12 protein. However, through the introduction of mutations into the ACH clone to generate ACH.p12, HBZ was also altered at several amino acids [338]. Thus, we speculate that a reduced infectivity attributed to p12 was due to HBZ functional alterations.

In addition, Arnold *et al.,* utilized cell lines transfected with HBZ mutants virus to determine their ability to transmit HTLV-1 to a rabbit model. Lymphoblastoid B cells (729) were transfected with one of two HBZ mutant viruses. These HBZ mutants were generated by point mutations that severely truncated HBZ within the first ten amino acids (HTLV-1ΔHBZ) or by a change in the HBZ sequence that eliminates the majority of the

leucine zipper region (HTLV-1HBZΔLZ). Arnold *et al.,* showed that the inoculation of either of the truncated HBZ in rabbits, correlated with a significant loss in infectivity and antibody response against HTLV-1 [284]. The group suggested that viral replication was attenuated in the HTLV-1 HBZ mutant virus-infected animals. (Arnold *et al.,* 2006) but reduction of viral transmission could also be an explanation.

In addition, Valeri *et al.,* inoculated rabbits with 729 B cells electroporated with a HBZ mutant, which was obtained by replacing an arginine at position 11 for a termination codon (TGA). This point mutation did not cause any changes on other viral proteins. In a separate experiment, the group also inoculated the animals with mutants for HTLV-1 proteins p12 and p30. Valeri *et al.* reported that only the absence of HBZ was correlated with a decrease in virus infectivity in the rabbit model when compared to the animals inoculated with the wild-type viruses [338]. In addition, this group inoculated the same 729 B cells with the HBZ KO mutant in Rhesus macaques [338]. Interestingly, they reported that the absence of HBZ (HBZ KO) caused a full reversion to HTLV-1 wild-type in most of the macaques that were inoculated with the mutant [338]. This virus reversion to wild-type genotype suggests high selective pressure to maintain the expression of HBZ. Hence, HBZ is required to maintain an effective infection. Taken together, these results suggest an important role of HBZ in the virus transmission process and point to HBZ as an interesting target for therapeutic intervention.

Figure 23. HTLV-1 viral proteins expression and virus transmission. Diagram shows detectable ("burst") and undetectable levels of Tax, and constitutively expression of HBZ during HTLV-1 infection. After infection occurs and before an immune response takes place, Tax and HBZ are both transcribed and expressed. However, Tax is highly immunogenic; thus, CTLs recognize infected cells expressing Tax and destroy them. Tax expression only occurs at punctuated bursts during the course of the infection. During these bursts of high expression, Tax activates HTLV-1 transcription, and sense viral proteins are expressed, such as p12, p8 and viral structure genes. Also, HTLV-1 virions particles are produced and stored in biofilms. When Tax becomes undetectable to escape from the immune system, HBZ is able to enhance the transmission of the virions adhered in the biofilm of the infected cell to the target cell.

CTL

CTLs eliminates
Tax expressing cells

biofilm

Tax
p12/p8
HBZ

p12/p8 present

Tax high
(burst)

**HBZ level
constant**

virion production/biofilm

virion adhered to biofilm

Target cell

virions

biofilm

HBZ

LFA-1

VS

ICAM-1

Polarized
MTOC

Virus transmission

Tax, p12,p8
undetectable

**HBZ level
constant**

biofilm

virions

virion production/biofilm

virion adhered to biofilm

Tax
p12/p8
HBZ

p12/p8 present

Tax high
(burst)

**HBZ level
constant**

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