Human T-cell leukemia virus type 1 and Foxp3 expression: viral strategy *in vivo*

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Abstract

Human T cell leukemia type 1 (HTLV-1) is the causal agent of adult T cell leukemia (ATL) and inflammatory diseases, including HTLV-1-associated myelopathy / tropical spastic paraparesis (HAM/TSP), uveitis, and infective dermatitis. However, it remains to be elucidated how HTLV-1 induces both neoplastic and inflammatory diseases. A critical component in the regulatory T cell (Treg cell) machinery is the transcription factor Forkhead box P3 (Foxp3), which is expressed in approximately 5% of CD4+T cells of healthy individuals. Foxp3 is expressed in around 80% of ATL cases. Recent studies point to the capacity of Treg cells to convert to other cell types, even to those with an inflammatory phenotype. These characteristics might indicate that Treg cells might be playing a critical role in HTLV-1 infection, either by being targeted by the virus, or by regulating and modulating the immune response. In this review, we will discuss the interplay between Foxp3 expression and HTLV-1, focusing on important viral proteins that might help the virus to trigger the development of such diverse pathologies.

Introduction

Human T cell leukemia virus type 1 (HTLV-1) is a delta retrovirus, and it was the first retrovirus to be associated with a human disease (1). Known as the causative agent of adult T cell leukemia (ATL), a leukemia of $CD4^+$ T cells (2,3), infection with this retrovirus is also associated with several inflammatory diseases, such as HTLV-1-associated myelopathy / tropical spastic paraparesis (HAM/TSP), uveitis, and dermatitis (4-7). Endemic areas of infection with this virus have been described in Southwestern Japan, the Caribbean region, South America and inter-tropical Africa.

HTLV-1 primarily transmits in a cell-to-cell fashion, not by cell-free virions, mainly through three routes: from mother to child by breast-feeding; during sexual intercourse (more common from male to female); and after exposure to contaminated blood products (8-11). It is widely accepted that this virus promotes the proliferation of infected cells after *de novo* infection *in vivo* via the action of viral genes. HTLV-1 preferentially induces the proliferation of $CD4^+$ T cells, especially Forkhead box P3 $(Foxp3)^+$ T cells and effector/memory T cells. In this review, we summarize the close relationship between Foxp3 and the pathogenesis of HTLV-1.

The HTLV-1 provirus

As a retrovirus, HTLV-1 integrates its provirus into the host cellular genome. The provirus is flanked by two long terminal repeat (LTR) sequences and, in between, genes encoding the enzymatic and structural proteins Gag, Pol and Env, common to all retroviruses, are found. In addition, there are several accessory or regulatory genes

encoded in the so-called pX region, located in the 3′ end of the provirus, between the *env* gene and the 3′ LTR (Fig. 1) (12). Among these, Tax protein has been demonstrated to constitute a pivotal component involved in leukemogenesis and inflammation, because of its capacity to highjack several cellular signaling pathways (13,14).

In addition, other viral proteins encoded in the plus strand of the provirus, are expressed under the regulation of Tax, using the 5′ LTR as the promoter. However, several previous reports have demonstrated that the 5′ region of the provirus is especially prone to be affected by genetic and epigenetic modifications (12), leading to an impaired viral gene expression. The 5′ LTR and, the *tax* gene itself, are targets of mutations and deletions (15-17). Furthermore, the methylation of proviral sequences also contributes to down-regulation in the transcription of viral genes (18) (19) (20). Considering that Tax is the main target of cytotoxic T cells (21), these silencing mechanisms are advantageous for HTLV-1, since it is thus able to avoid the host immune response.

In contrast, HTLV-1 basic leucine zipper factor (HBZ), which is encoded in the minus strand of the provirus (22), is constitutively expressed from the 3′ LTR (23) (24). Furthermore, nonsense mutations were frequently found in HTLV-1-encoded genes except for *HBZ* (17). As a result, HBZ has been found to be expressed in all ATL cases and HTLV-1-infected cells, regardless of Tax expression, contributing to their proliferation (23). Therefore, the constitutive expression of HBZ has caught the attention of many laboratories working on HTLV-1 research. HBZ is also known to interact with several transcription factors that may allow the virus to take over the

cellular machinery but, at the same time, to modulate or regulate viral gene expression.

HTLV-1 cellular tropism

It is widely accepted that HTLV-1 infects various kinds of cells (25,26), more efficiently in a cell-to-cell, contact-dependent manner (27). Nevertheless, the CD4^{+} T cell population is the one mainly affected in ATL and HTLV-1-infected individuals. HTLV-1 provirus is mainly detected in effector/ memory T cells and Foxp3^{+} T cells in HTLV-1-infected carriers and HAM/TSP patients (28-30), indicating that these cells are targets of this virus *in vivo*.

Three cellular factors have been identified and proposed to mediate HTLV-1 fusion and entry into the target cells, namely heparan sulfate proteoglycans (HSPG), neuropilin-1 (NRP-1) and glucose transporter 1 (GLUT1) (31-33). The first two are reported to be involved in the fusion step, whereas GLUT1 seems to act in later steps (Fig. 2) (34). These molecules are ubiquitously expressed, and may explain the wide range of target cells. It has been reported that in addition to CD4^{+} T cells, HTLV-1 provirus was also detected in CD8^{+} T cells, monocytes and B cells in patients with HAM/TSP (25).

Interestingly, NRP-1 is constitutively expressed in regulatory T cells (Treg cells) and has been proposed as a surface marker of this population (35). The expression of NRP-1 allows Treg cells to establish longer interactions with dendritic cells (DCs) compared with their naive T cell counterparts (36). Therefore, the presence of NRP-1 on Treg cells might enhance their susceptibility to being infected with HTLV-1.

Foxp3, a transcription factor critical for the development and function of Treg cells, is widely expressed in ATL cases (30,37). These findings have prompted researchers to analyze the potential role of ATL cells in modulating the immune response as Treg cells do. However, the results were not concordant. Some studies attributed regulatory functions to ATL cells, whereas others stated otherwise (38-41).

Foxp3 and HTLV-1 infection

There are different types of characterized Treg cells (Fig. 3). One classification divides Treg cells according to their physical place of development. The so-called "natural" Treg cells (nTreg cells) develop in the thymus, whereas "induced" Treg cells (iTreg cells) differentiate in the periphery after antigenic stimulation (42). Both types express Foxp3, a critical transcription factor that characterizes the Treg population. As described, the receptor proteins for HTLV-1 are expressed on many kinds of cells. However, HTLV-1 provirus is mainly detected in effector/memory T cells and Foxp3$^+$ T cells (29,30). This cell preference of HTLV-1 is attributed to two possibilities. One is that HTLV-1-encoded proteins, in particular HBZ, change the phenotype of infected cells. Another scenario is that effector/memory T cells and Foxp3$^+$ T cells are prone to HTLV-1 infection. It has been well known that regulatory T cells well proliferate *in vivo*, and CC chemokine ligand 22 (CCL22) produced from HTLV-1-infected cells attracts regulatory T cells (43), which might enhance transmission of this virus. Furthermore, expression of Nrp-1 on Treg cells might enhance transmission of HTLV-1 as described above.

Based on the levels of expression of Foxp3 and CD45RA, three distinct populations that differ in their phenotype and function have been described among human CD4$^+$Foxp3$^+$ T cells: CD45RA$^+$Foxp3$^+$ resting Treg cells, CD45RA$^-$Foxp3^{high} activated Treg cells and CD45RA$^-$Foxp3^{low} non-suppressive T cells (44). We have recently reported that HTLV-1 provirus is mainly detected in CD45RA$^-$ cells with high or low Foxp3 expression, namely activated Treg cells and Foxp3^{low} non-suppressive T cells of HTLV-1-infected individuals (30). Furthermore, Foxp3$^+$ ATL cells belong to these subpopulations. Activated Treg cells possess suppressive capacity, whereas the Foxp3^{low} non-suppressive T cells do not. These results could explain the controversial outcomes of previous studies on the suppressive capacity of ATL cells. Consistent with this finding, Foxp3$^+$ ATL cells also fall into the group of activated Treg cells or Foxp3^{low} non-suppressive T cells.

Animal models

How HTLV-1 causes such a range of diseases with such different pathogenic mechanisms still remains unknown. Transgenic models that express viral proteins are useful to study the function of these proteins without triggering an immune response against them. Among HTLV-1-encoded proteins, Tax has been extensively studied, and several reports have demonstrated that it plays a critical role in oncogenesis (45).

Different Tax transgenic mice have been developed differing in the promoter used to control the expression of the protein *in vivo*. Several models were generated that used HTLV-1 LTR-driven expression of genes. One model transgenic for the pX region of

the provirus was an approach that resulted in the induction of inflammatory arthropathy

in transgenic mice (46). These mice expressed not only Tax but also p12, p13, p30 and

HBZ, although the existence of HBZ was not known at that time. Subsequently, another

transgenic mouse strain was generated, to address the role of Tax alone in

leukemogenesis, using the same promoter and the CD4 enhancer/promoter (47). In that

study, Tax expression from LTR and CD4 enhancer/promoter induced both

inflammatory and ankylotic arthropathy, confirming the arthritogenic capacity of Tax.

More recent studies used the *Lck* promoter (which is expressed in T cells) to express

Tax. These mice developed leukemia/lymphoma with an immature T cell phenotype, or

one that resembled ATL, with the difference that the cells in the transgenic mice did not

express CD25, and were either CD4$^+$ or CD8$^+$ (48,49). The authors found that these

mice developed arthritis later in life (50). However, the CD4$^+$ T cells in the spleen were

decreased. These results do not correlate with the usual clinical findings, suggesting that

another factor is required for the maintenance of a malignant or inflammatory

phenotype.

With the discovery of HBZ, encoded in the minus strand of the HTLV-1 provirus, it

has been demonstrated that HBZ could be a qualified candidate to fulfill that role. We

developed an HBZ transgenic (HBZ-Tg) mouse strain that expresses the spliced form of

HBZ in CD4$^+$ cells (23). This mouse model surprisingly resembles the phenotype of

some HTLV-1-infected individuals, developing lymphoma and chronic inflammation

(51). The mice show an increase in the proportion of the effector/memory

sub-population. In particular, CD4$^+$Foxp3$^+$ T cells are increased. In these HBZ-Tg mice,

CD4$^+$ T cells infiltrate various tissues such as skin, lungs and intestines (52). This mouse model is therefore useful, not only to evaluate the role of HBZ in oncogenesis, but also to analyze its role in Treg cells and in the immunological disorders caused by HTLV-1 infection.

HBZ induces Foxp3 gene transcription

CD4$^+$Foxp3$^+$ T cells are increased in HBZ-Tg mice, suggesting that HBZ induces Foxp3 expression *in vivo*. It has been reported that TGF-β induces Foxp3 transcription (53). HBZ enhances the interaction between Smad2/3 and p300, which results in the upregulation of *Foxp3* gene transcription (Fig.4) (54). In HBZ-Tg mice, Foxp3$^+$ T cells are remarkably increased although the level of Foxp3 expression was low compared with that of normal Treg cells.

   Although Foxp3$^+$ T cells are increased in HBZ-Tg mice, these mice frequently show inflammatory lesions in the skin, lung and intestine. This seems to be inconsistent with the increased presence of Foxp3$^+$ T cells. As a mechanism of inflammation in these transgenic mice, Foxp3$^-$CD4$^+$ T cells produce higher amounts of IFN-γ *in vivo* (52). Recent studies have demonstrated that the CD4$^+$Foxp3$^+$ T cell population has the capacity to convert to effector T cells (55). Furthermore, iTreg cells may change their phenotype into cytokine-producing T cells (44,56). We have also provided evidence supporting the hypothesis that, because of epigenetic modifications, HBZ-induced Foxp3 expression is not stable. Specifically, we have found the conserved non-coding sequence 2 (CNS2) region of the *Foxp3* gene to be highly methylated in

CD4$^+$CD25$^+$GITR^{high} T cells of HBZ-Tg mice (Fig. 5). However, it is known that the

non-methylated state of this specific region is required for stable Foxp3 expression (57),

while methylated CNS2 is associated with low and unstable expression of Foxp3 (44).

In HBZ-Tg mice, cells that have lost Foxp3 may convert to IFN-γ-producing cells

leading to inflammation (52). Thus, the labile Foxp3 expression induced by HBZ is a

mechanism that causes inflammation in HTLV-1-infected individuals.

   Why does HBZ change the immunophenotype of T cells expressing it? One possible

scenario is that infected Treg cells can escape the host immune system via the

expression of immunosuppressive molecules. This might be an advantage for infected

cells to survive *in vivo*. However, strong immunosuppression is harmful for the host.

Therefore, HBZ likely interacts with Foxp3, and impairs the suppressive function of

Treg cells.

   Several studies on Tax and HBZ have demonstrated that these two viral proteins have

opposite effects on several signaling pathways (58). With regard to Treg cells, we have

demonstrated that HBZ induces the expression of Foxp3 by enhancing the TGF-β

signaling pathway through its interaction with p300. On the other hand, transfecting the

tax gene into normal CD4$^+$CD25$^+$ T cells led to a decrease in *Foxp3* mRNA levels, and

also to an impaired regulatory function (59). Later, Tax was shown to affect the

expression of Foxp3 through the downregulation of TGF-β receptor II, which rendered

cells unable to induce Foxp3 expression through this pathway (60). It has been reported

that Tax inhibits the TGF-β/SMAD pathway, which likely suppresses the transcription

of the *Foxp3* gene (Fig. 4) (61,62).

Immune dysregulation by HTLV-1

HTLV-1-infected individuals have mildly impaired cell-mediated immunity (63).

Immunodeficiency in HTLV-1 carriers is mostly subclinical. However, ATL patients

show a profound immunodeficiency and are frequently affected by opportunistic

infections caused by bacteria, viruses, fungi and parasites. The immunodeficient state

may be explained by several possible mechanisms, including impaired production of

naive T lymphocytes or deficient innate and adaptive immune responses (29,64). At the

same time, HTLV-1 infection is associated with inflammatory conditions, suggesting a

general disruption in the regulation of the immune response (Fig. 6).

On the one hand, HTLV-1-infected cells secrete CCL22, which attracts non-infected

functionally competent Treg cells expressing CC chemokine receptor 4 (CCR4), in

order to suppress the immune response triggered to clear the infection (43). This

population seems to play an important role, since the abundance of HTLV-1-specific

CTLs was observed to be inversely correlated with the frequency of $CD4^+Foxp3^+$ cells

(65). This phenomenon may contribute to the immune-suppression observed in HTLV-1

infection. The increase of CCL22 was reported to be induced by Tax; thus, how could

we explain immune-suppression in the absence of Tax?

Recently, Sugata *et al*. have demonstrated that HBZ also compromises cell-mediated

immunity and suppresses the production of T_h1 cytokines (66). In that study, the

eradication of herpes simplex virus type 2 (HSV-2) and *Listeria monocytogenes* was

poor in HBZ-Tg mice because of an impaired production of IFN-γ, which is critical for

the clearance of these pathogens. Reporter and chromatin immunoprecipitation assays revealed that HBZ inhibited the recruitment of the transcription factors NFAT (nuclear factor of activated T cells) and AP-1 (activator protein 1) to the promoter of IFN-γ, resulting in insufficient production. Thus, HBZ directly impairs the function of infected T cells.

Conclusion

A particular characteristic of HTLV-1 is its capacity to induce diseases with different pathogenic mechanisms, yet ultimately targeting one cell type of the immune system, the CD4$^+$ T cell population. It is important to take into account that the infection is also characterized by a long latency period, during which many significant events may take place, consistent with the idea of a multi-step process leading to disease. Among the many subtypes of T cells, HTLV-1 provirus has been detected mostly in CD4$^+$CD25$^+$ effector/memory T cells (30), particularly associated with Treg cells. By targeting this population, HTLV-1 is able to disrupt the host immune system, and modulate its activity so that it may succeed in establishing a persistent chronic infection.

HIV-1 is another retrovirus that also targets CD4$^+$ T cells. Infection with HIV-1 leads to immunodeficiency as a result of the depletion of this important T cell population. In contrast, HTLV-1 integrates into the cellular genome to proliferate together with the cell. Free virions are not so effective at spreading the infection. However, it may be possible that this virus follows a unique strategy to persist in its host. Initially, free virions may infect cells that interact with T cells, as was reported for DCs (26), supported by the fact

that the receptors are ubiquitously expressed. For example, NRP-1 has been reported to help in lengthening cell-to-cell interaction between Treg cells and DCs (36). HTLV-1 could take advantage of this approach required for a more efficient viral transmission.

In addition, Foxp3-expressing cells have been demonstrated to migrate more rapidly to sites of inflammation, which, in the case of HTLV-1-infected cells, might facilitate migration and spread of infection. Moreover, adhesion molecules such as LFA-1 (leukocyte function-associated antigen 1) are expressed at higher levels in HBZ-Tg mice and HAM/TSP patients (52). Once infection has occurred, the cellular machinery must be taken over to allow viral replication and survival. For that, HTLV-1 interacts with a set of accessory proteins with multiple functions that change or modulate the phenotype of the infected cell, so that it may spread more efficiently and persist in the host.

A considerable amount of evidence supports the existence of a complex regulatory system in the generation and fate of Foxp3$^+$ Treg cells. It is now widely accepted that Foxp3 alone is not sufficient for a stable lineage specification or function of Treg cells. Epigenetic modifications that involve other important transcription factors, as well as the Foxp3 interactome, have been demonstrated to be critical players too (67,68). Concordant with these concepts, an HBZ-mediated increase in Foxp3 expression does not always lead to functional competence as Treg cells. Further analysis of ATL samples and HTLV-1-infected cells is needed to clear this point.

Nevertheless, HBZ plays a key role in the achievement and maintenance of a persistent infection by HTLV-1 through the targeting of Treg cells, since it seems to

take advantage of the characteristic that Treg cells rely on cell-contact-dependent and -independent mechanisms to exert their regulatory function.

Acknowledgements

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Figure legends

Fig. 1. The HTLV-1 provirus. The structure of the HTLV-1 provirus, integrated in the cellular genome, is shown. The genes encoding structural and enzymatic proteins are flanked by LTR sequences. Between the *env* gene and the 3′ LTR, an additional region, pX, is present, which encodes accessory and regulatory proteins. Viral genes depicted above the provirus are transcribed using the 5′ LTR as their promoter. On the other hand, HBZ, encoded in the minus strand, is expressed under a different regulatory system based on the 3′ LTR. *ORF*, open reading frame.

Fig. 2. Proteins involved in HTLV-1 infection. Virions are thought to first interact with HSPG, subsequently forming complexes with NRP-1 and GLUT-1, leading to fusion and eventual entry of the virus into the host cell. HSPG: Heparan Sulfate Proteoglican; NRP-1: Neuropilin-1; GLUT-1: Glucose transporter 1.

Fig. 3. Types of Treg cells. Natural Treg (nTreg) cells develop in the thymus. Adaptive/induced Treg (iTreg) cells are generated in the periphery from naive T cells. The unstable expression of Foxp3 in this type of Treg cells makes them prone to become exFoxp3 cells, with the ability to cause inflammation in peripheral tissues. Target cells of HTLV-1 infection are shown.

Fig. 4. The *Foxp3* locus, and its regulation by the TGFβ signaling pathway. The TGFβ signaling pathway is known to be important for the induction and maintenance of Foxp3^{+} Treg cells. Sequences in the promoter and CNS1 regions of the *Foxp3* gene locus are known to respond to TGFβ, as well as to TCR stimulation, inducing the expression of the protein. HTLV-1 viral protein Tax inhibits this pathway at different levels. It down-regulates the expression of TGFβRII, constitutively activates JNK and directly interacts with several members of the Smad family of proteins, disrupting their interaction with p300. Overall, the effect of Tax results in the inhibition of the TGFβ signaling pathway, and consequently, down-regulation in the expression of Foxp3. In contrast, we have reported that HBZ enhances the association of Smad3 with p300, which results in the up-regulation of *Foxp3* gene expression. Furthermore, the CNS2 region in HBZ-Tg mice was found to be highly methylated, indicating that the increased expression of Foxp3 is not stable, and prone to be lost.

Fig. 5. CpG island regions in the *Foxp3* gene analyzed in HBZ-Tg mice. CNS2 region was found to be highly methylated, compared to the promoter region in HBZ-Tg mice, suggesting an unstable expression of the *Foxp3* gene induced by HBZ. Percentages of clones displaying methylation of specific CpG motifs in the above-mentioned regions are shown.

Fig. 6. Immunological disorders in HTLV-1 infection. HBZ induces the expression of Foxp3. However, this expression is unstable, and the cells acquire an inflammatory

phenotype, characterized by the increased production of IFN-γ, eventually leading to inflammation. In addition, HBZ inhibits the production of T_h1 cytokines, which is translated into an impaired cell-mediated immunity and, therefore, immunodeficiency. Tax also plays a role in the proliferation of infected cells by acting on Treg cells. This viral protein induces the secretion of CCL22 that attracts non-infected Treg cells that suppress the immune response triggered to clear the infection.
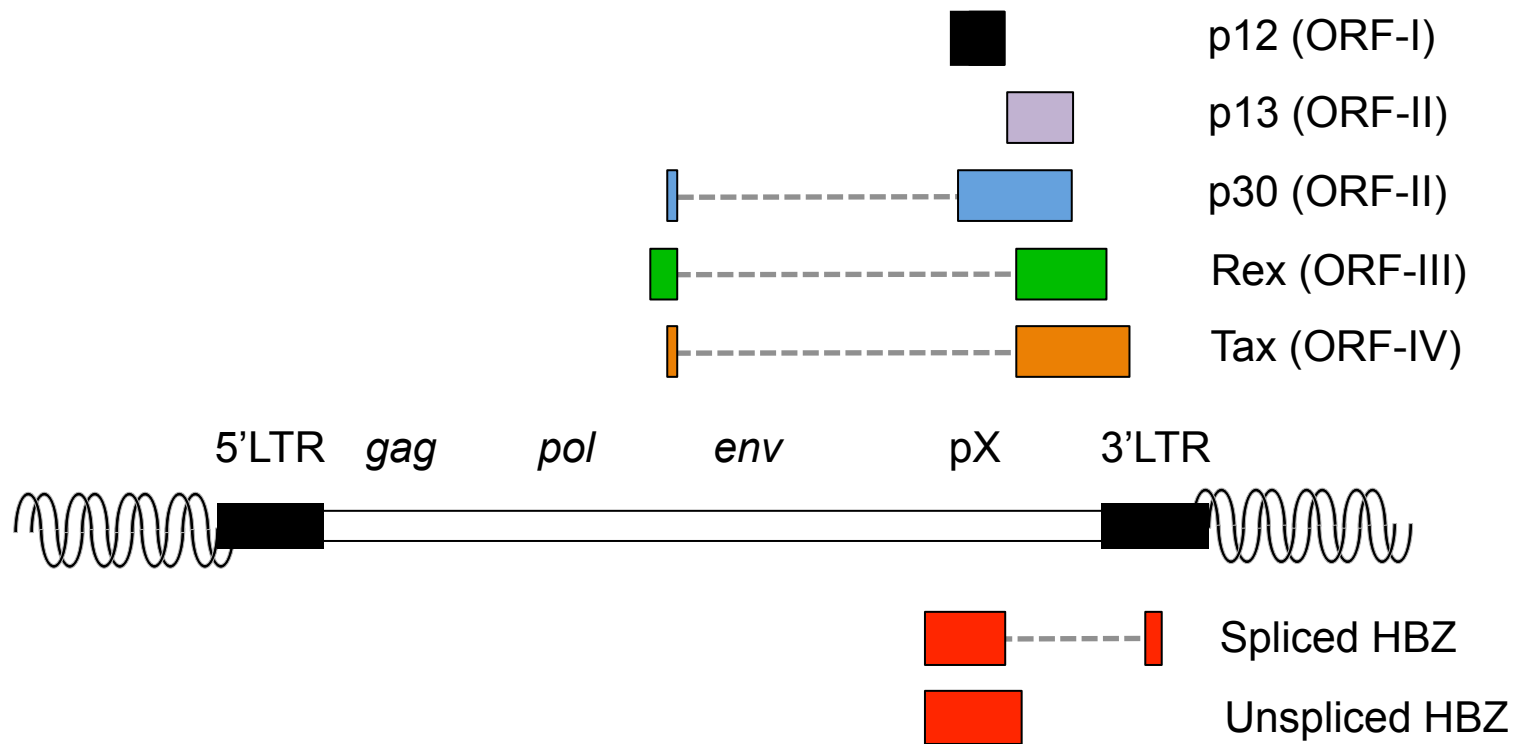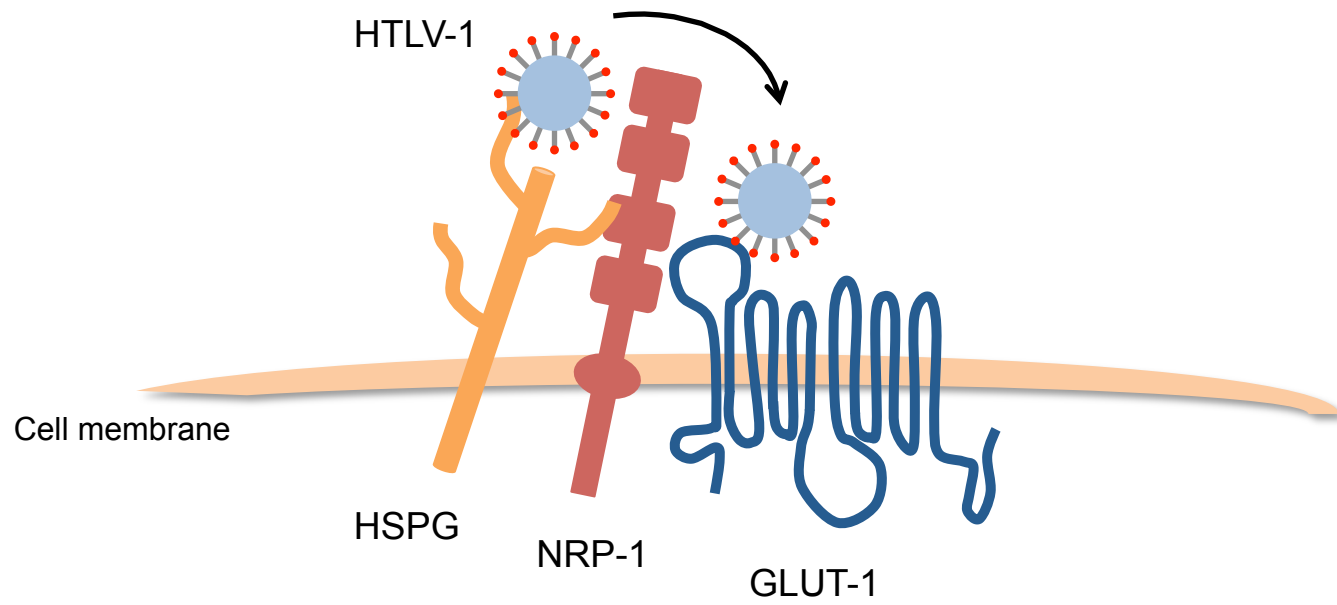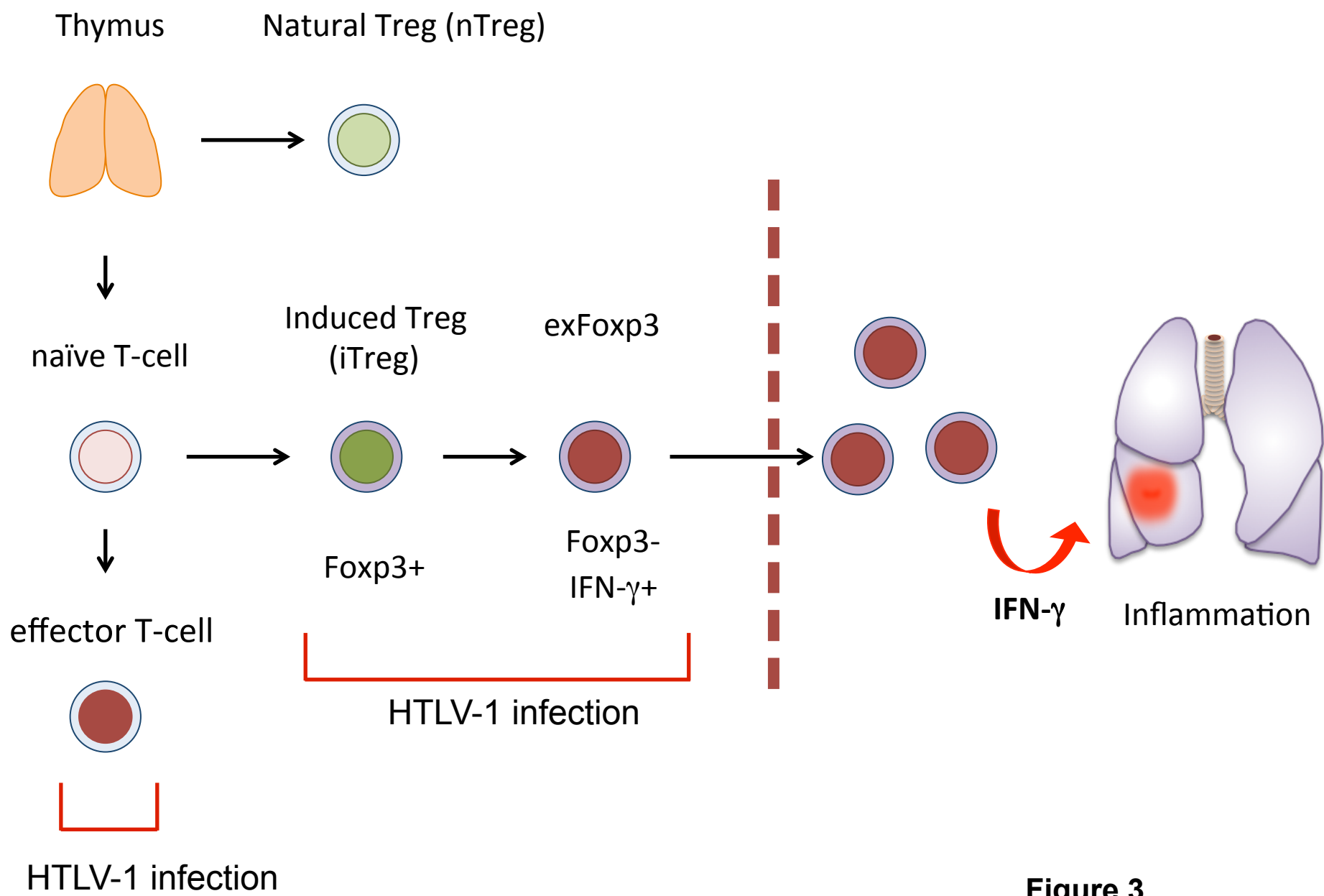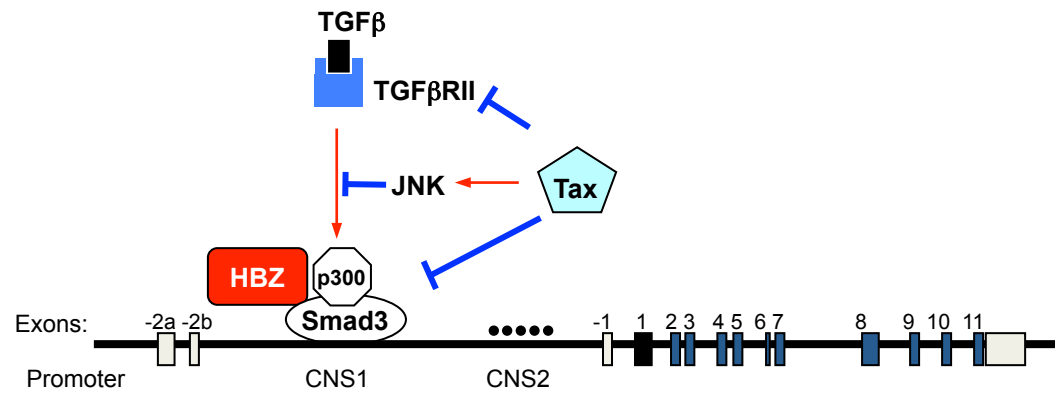
Figure 1

Figure 2

Thymus   Natural Treg (nTreg)

naïve T-cell   Induced Treg (iTreg)   exFoxp3

Foxp3+   Foxp3-
IFN-γ+

effector T-cell

HTLV-1 infection

HTLV-1 infection

IFN-γ   Inflammation

Figure 3

Figure 4

Figure 5

Figure 6