Challenging Development of a Humanized Mouse Model for Evaluating the HTLV-1 Infection and Leukemogenic Process in vivo

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THESE

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Monsieur Thomas SCHULZ, rapporteur
Monsieur Charles RM BANGHAM, examinateur
Monsieur Markus G. MANZ, examinateur
Madame Madeleine DUC DODON, directrice de thèse

Foreword

During my writing of this manuscript, I looked for the first papers ever published on the discovery of HTLV-1 by the group of Robert Gallo 30 years ago. I was astonished by the differences I could notice between the way research is done today and the way it was done not so long ago. Many things were different! The techniques used at that time were so different that I discovered some that were not even taught to me during my learning years! Even the name of proteins was different! The realization that so many techniques that we routinely use in our lab today were not available made a strong impression on me as I realized how much they achieved with technical tools that seems today so limited. This encouraged me to search in a more distant past, the first report on retroviruses and their implication in neoplastic diseases. This led me to contemplate a fascinating story made of many visionary scientists, several scientific revolutions and some Nobel Prize winners, that led us to what is known on retrovirology today. In the first part of my manuscript, I have humbly tried to highlight the key discoveries and concepts of this story before a more traditional introduction on HTLV-1 and its animal models.

Many things have changed since the beginning of this scientific adventure but it appears to me, that all the people involved in it had at least one thing in common that must define what research is: the will to better understand the world.

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Acknowledgments / Remerciements

There it is! More than 3 years have come by and the manuscript is written: it's the dusk of my PhD. The defense is still ahead but I'd already like to warmly thank the members of the Jury who accepted to judge my work. I'd like to thank the "rapporteurs", James Di Franco and Thomas Schulz for their reading of my manuscript. I have very much enjoyed your remarks and it helped me improve it. I'm also grateful to Markus G. Manz and Charles RM. Bangham for their implication in my jury. I am very proud to defend in front of all of you!

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This should end the "acknowledgments" section of this manuscript. And now, Mesdames et Messieurs, la partie "remerciements" !

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Comme je le disais plus haut dans la langue de Shakespeare, ça sent sérieusement la fin de la thèse ! Ecrire un manuscrit de thèse, c'est prendre le temps de regarder tout ce qu'on a fait pendant les dernières années pour le présenter en quelques pages. En condensant ainsi nos résultats et nos réflexions, on laisse forcement des choses de coté. On ne parle, ni des manips (nombreuses) qui ratent, ni des "blonderies" que l'on fait ou dont on est témoin, ni de l'aspect humain qui fait partie intégrante de la vie d'un thésard. On se rend surtout compte que rien n'aurait été possible tout seul. Une thèse est une aventure scientifique extraordinaire. C'est aussi une épopée humaine époustouflante qui implique un grand nombre de personne ! Je vais essayer ici de vous présenter les personnes qui ont fait mon épopée humaine…

Comment ne pas commencer par la personne qui a écrit la trame de l'histoire que je m'apprête à vous livrer : Madeleine Duc Dodon ! Elle est la maman de ce projet de thèse et a eu la folle idée de me confier sa réalisation ! Merci Madeleine pour cette confiance et cette liberté que vous m'avez laissée dans ce travail. Je suis souvent impressionné par vos instincts scientifiques qui vous permettent de vous distinguer dans le milieu de la recherche par votre originalité et votre audace. J'apprécie votre conception « pure », intègre et passionnée de la recherche. J'ai beaucoup appris à vos cotés et je vous remercie pour votre soutien, votre encadrement et votre disponibilité au quotidien. Je vous dois beaucoup !

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Une thèse demande un investissement personnel fort mais il est important de conserver une vie hors du laboratoire. Pour moi, le mardi soir a toujours été le soir du théâtre. Un soir où il faut finir tôt les manips pour pouvoir aller rejoindre Nadine et Sandrine et changer complètement de monde. Ces sessions hebdomadaires ont été autant de bouffées d'air venant ponctuer la routine du laboratoire ! Merci Sévérine pour ma première fois sur scène ! Merci Nadine pour les suivantes et le travail que tu m'as fait faire ! Durant ces années j'ai fait pas mal de belles rencontres pendant ces réunions de la Branche Bâtardes et je tiens à remercier plus particulièrement Ségolène, Cédric, Yann, Alexandre, Julien et Théo pour leur implication dans ce club qui me tient à cœur et pour leur talent ! J'aimerai aussi remercier spécialement Morgane et Etienne pour nos nombreuses heures passer dans l'ombre pour préparer la technique d'un spectacle ! C'est toujours une grande joie de travailler avec vous et je hâte que nous montions "TuBe !"

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Résumé

Développement d'un modèle de souris Rag2$^{-/-}\gamma_c^{-/-}$ humanisée pour l'étude de l'infection et de la leucémogénèse associée à HTLV-1

Le virus HTLV-1 (Human T-cell Leukemia Virus Type 1) est l'agent étiologique de la Leucémie T de l'adulte (ATL) qui est caractérisée par la prolifération de cellules T CD4$^+$ activées. L'absence de modèle animal fiable reproduisant la leucémogénèse associée à l'infection a ralenti la compréhension des étapes précoces du processus leucémogène et le développement de stratégies thérapeutiques efficaces. Récemment l'amélioration des modèles de souris humanisées a permis la reconstitution d'un système immunitaire humain dans des souris. L'injection de cellules souches hématopoïétiques purifiées à partir de sang de cordon humain dans des souris nouveau-nées de la lignée Rag2$^{-/-}\gamma_c^{-/-}$ conduit à la formation *de novo* de cellules dendritiques, B et T humaines. Ces dernières étant la cible de l'infection par HTLV-1, nous avons infecté des souris humanisées avec des cellules productrices de HTLV-1. Cette inoculation conduit à l'infection stable des cellules humaines dans la souris humanisée et la formation de lymphome ou de leucémie à cellules T humaines activées. Cette infection altère le développement des cellules T dans le thymus conduisant à un phénotype plus mature des thymocytes. Ce modèle animal reproduisant l'infection et la pathogénèse associée nous a permis de suivre l'évolution de la clonalité du virus au sein des différents organes lymphoïdes. Basées sur ces observations, des tests préliminaires ont permis d'étudier une nouvelle approche thérapeutique potentiellement applicable en clinique humaine. Ce travail nous a également permis d'affiner le protocole conduisant à l'humanisation des souris afin d'obtenir une meilleure reconstitution humaine dans ce modèle.

Mots clés : HTLV-1, ATL, Leucémie, Lymphome, Souris Humanisées, Thymus, Développement T

Abstract

Human T-cell Leukemia Virus type 1 (HTLV-1) is the etiologic agent of the Adult T-cell Leukemia (ATL), an aggressive lymphoproliferation of activated $CD4^+$ T cells. The lack of a reliable small animal model to reproduce *in vivo* the leukemogenic process associated with HTLV-1 infection has impaired the understanding of the early stages of this process as well as the discovery of effective therapeutic approaches. Recently, improvement in the models of humanized mouse models were achieved allowing the development of a human immune system in mice. Injection of human hematopoietic stem and progenitors cells purified from cord blood into Balb/c $Rag2^{-/-}\gamma_c^{-/-}$ newborns allows the *de novo* production of human dendritic, B and T cells. We infected humanized mice with HTLV-1 producing cell lines resulting in infection of human cells within the mice and the development of lymphomas and leukemias. This infection also results in the alteration of the T-cell development within the thymus pushing the thymocytes toward a more mature phenotype. This small animal model recapitulating *in vivo* the HTLV-1 infection and its associated pathogenesis gave us the opportunity to study the evolution of the clonality of the virus among human cells in different lymphoid organs. Based on these observations, preliminary results on the use of a new therapeutic approach were obtained. We finally tried to adjust the humanization protocol in order to obtain better engraftment in this model.

Key words: HTLV-1, ATL, Leukemia, Lymphoma, Humanized Mice, Thymus, T-cell Development.

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Introduction

I] En route to the discovery of human retroviruses: a retrospective.

The identification and characterization of retroviruses in animals, first in birds, then in rodents and then in monkeys and in humans succeeded all along the 20th century, which was the "Retrovirology century". Retroviruses that belong to the Retroviridae genus are enveloped viruses with two copies of single-stranded, positive-sense RNA genomes. Soon after infection of a target cell, this RNA genome is reverse-transcribed into a complementary DNA strand by a virus-encoded RNA-dependent DNA polymerase, referred to as reverse transcriptase (RT). This DNA product is then integrated into the cellular chromosomal DNA by another virus-encoded protein, the integrase (IN). Once integrated, the DNA provirus, that is therefore replicated along with the host genome, can either stay silent or initiate a productive infection by directing the synthesis of viral proteins and particles, using cellular mechanisms for transcription and translation. New progeny virions are assembled either in the cytoplasm or at the plasma membrane, and are released by budding. In this introduction, I underline the essential information that investigations on the virus-host interactions (mainly replication and transformation) have contributed to the field of molecular and cellular biology. Today, retroviruses represent a large and diverse group of human and animal pathogens that cause a wide variety of diseases including many cancers and various immunological and neurological conditions.

1) The first retroviruses

The retrovirology era began during the first decade of the last century with the demonstration of filterable agents associated with infection anemia of horses in 1904 (Vallée and Carré, 1904) and with erythroid leukemia of chickens in 1908 (Ellermann and Bang, 1908), suggesting the viral etiology of these diseases. Very little was known on the viruses at that time: it was what Beijerinck called a *contagium vivum fluidum*, a

"contagious living fluid", a filterable agent, smaller than a bacteria, not observable in the light microscope, and able to reproduce itself but only in living cells or tissue. Later on, in 1910, Peyton Rous (Figure 1A) proceeded to successive transfers of a sarcoma from a hen to a healthy one (Figure 1B). After several serial transplantations, he injected a cell-free extract from the tumor to a healthy chicken and observed that it was able to induce the formation of a sarcoma similar to the original one (Rous, 1911). For the first time, a viral agent was described as a causative agent for cancer. It was later referred to as the Rous Sarcoma Virus (RSV) (Figure 1). The lack of technical tools available made it impossible to further characterize those newly discovered viruses before several decades (Gross, 1978).

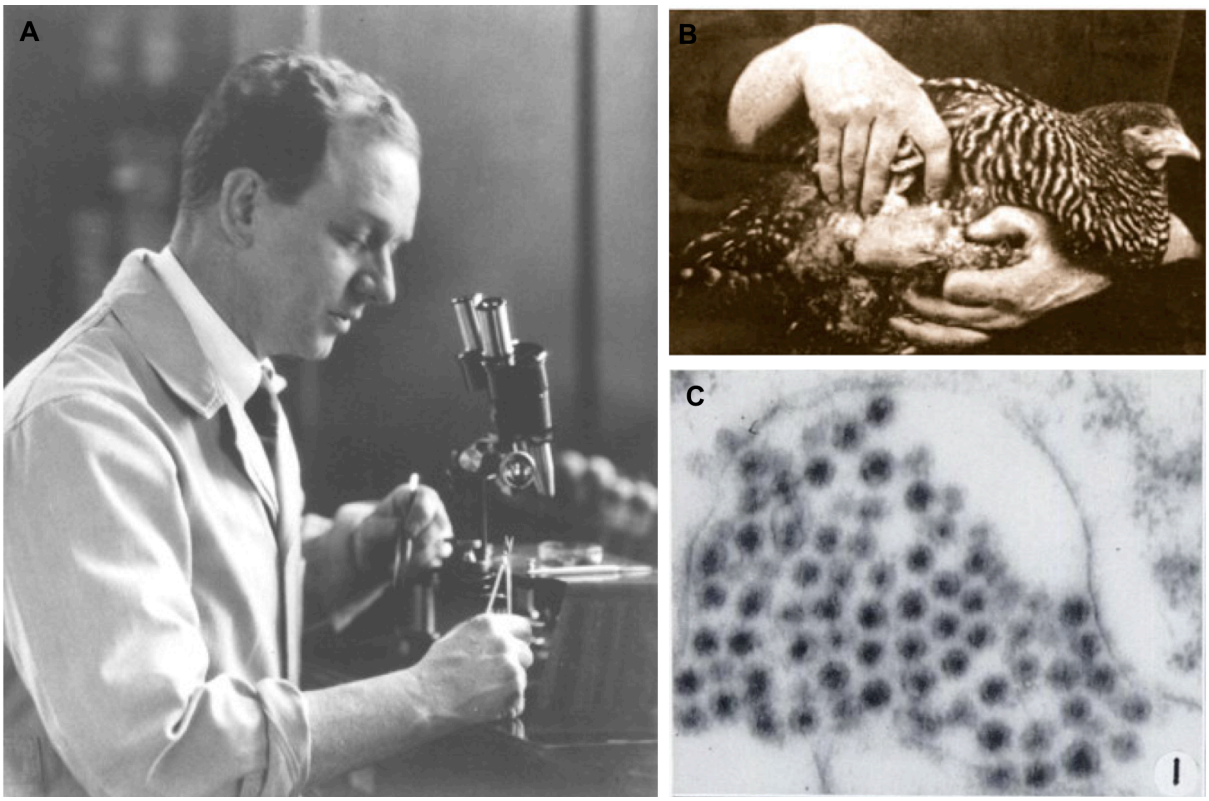

Figure 1 – Discovery and characterization of the Rous Sarcoma Virus

A – Peyton Rous in his lab. **B –** The hen that started everything. In 1909, Payton Rous was brought a hen with a sarcoma. He transferred this sarcoma from this hen to other healthy ones. They developed similar sarcomas that can, in turn, be transplanted to other healthy hens. After several serial transplantations, he injected a cell-free extract to a healthy hen and it developed a sarcoma similar to the original one. The viral origin of this cancer was established. **C –** RSV particles observed in electron microscopy observed in 1955 by Gaylord *et al.* (Gaylord, 1955).

Based on the pioneering work with RSV, many retroviruses were then identified in diverse animal species, most often associated with neoplastic diseases. The first retrovirus in mammals was discovered in 1936, when John Bittner established that mammary carcinomas in mice were found to be caused by a milk-transmitted, filterable agent (Bittner, 1936). In 1957, Ludwik Gross successfully identified a potent murine leukemia virus transmitted in its latent form through the embryos and able to transmit leukemia by inoculation of filtrates in newborns (Gross, 1978). Other leukemia-inducing retroviruses were later described in mice (Moloney Leukemia Virus, Friend Leukemia Virus, Kaplan Leukemia virus, Rich Leukemia Virus, (Gallo, 1991)) and in other species such as cats in 1964 (Feline Leukemia Virus, (Jarrett et al., 1964a; Jarrett et al., 1964b)), cattle (Bovine Leukemia Virus, (Van der Maaten et al., 1972)), and monkeys in 1972 (Gibbon Ape Leukemia Virus, (Theilen et al., 1971)). Retroviruses have been also described in many other species including non vertebrates (Coffin et al., 1997). Interestingly, some retroviruses are not associated with neoplastic diseases, but are rather cytopathogenicinf. For instance, the Feline Immunodeficiency Virus (FIV) described in 1987 by Pedersen (Pedersen et al., 1987) is not responsible for a lymphoproliferative disease, but causes the feline Acquired Immune Deficiency Syndrome (AIDS) characterized by a decrease in the number of lymphocytes.

Progress in the retrovirology field paralleled the development of new tools and techniques. Thus, in 1947, Claude *et al.* were the first to observe RSV particles with an electron microscope (Claude et al., 1947). They observed spherical particles, about 70-80 nm in diameter, but few years were necessary for a more complete description of their morphology (Gaylord, 1955). The analysis of thin sections of cells isolated from Rous sarcoma showed a dense centrally located body called nucleoid surrounded by an external membrane (Figure 1C). At that time, electron microscopy was helpful to

visualize retrovirus-like particles in tissues and cultured cells. These observations led to a first classification of retroviruses into four morphological types, denoted A-, B- C- and D-type particles. Later on, purification of RSV particles by density gradient centrifugation made possible to determine their nucleic acid content and to show that they contained an RNA genome (Crawford and Crawford, 1961). At that time, the replication of other single-stranded RNA viruses, like poliovirus, was known. It involved the synthesis of a RNA-dependent synthesis of partially double-stranded RNA, catalyzed by a polymerase translated from the viral RNA. It was therefore anticipated that RSV would share a similar profile of replication. Surprisingly, such double-stranded RNA was not found in RSV infected cells and more importantly, inhibitors of DNA synthesis were affecting the viral life cycle. These observations led Howard Temin in 1964 to propose the "DNA provirus hypothesis" stating that a DNA intermediate, "the provirus", transcribed from the viral RNA genome and integrated in the genome of infected cells, was necessary for the completion of the life cycle of RSV. This hypothesis was in discordance with the "central dogma of molecular biology" that asserted, at that time, that the genetic information must go one way: from DNA to RNA to protein (Crick, 1958). Consequently, the scientific community did not warmly welcome this DNA provirus hypothesis that remained controversial for several years until it was validated by the discovery of the reverse transcriptase (RT). In 1970, the groups of Temin (Temin and Mizutani, 1970) and of Baltimore (Baltimore, 1970) independently reported the presence of a virus-encoded RNA-directed DNA polymerase, able to reverse-transcribe DNA from an RNA template. These observations that have been published back to back in Nature in 1970, earned them the Nobel prize in Physiology and Medicine, in 1975 (Lindsten, 1992). This discovery was of major importance, because of the positive fallouts in the field of molecular biology. The use of this RT allowed the development of

techniques to study the expression of genes as well as their cloning opening the way to a better understanding of their function. According to the provirus hypothesis, the proviral DNA is integrated in the genome of infected cells. The mechanisms involved in this integration were unraveled only years later, when a virus-encoded integrase (IN) was identified and characterized (Donehower and Varmus, 1984; Grandgenett et al., 1978; Misra et al., 1982; Nissen-Meyer and Nes, 1980). Consequently, the discovery of RT and of IN allowed the identification of a new type of RNA viruses, that of retroviruses. That led Baltimore to propose its famous classification of the viruses based on their "method of transmitting [their] genetic information from one generation to the next and [their] own style of expressing [their] genetic information » (Baltimore, 1971).

Nearly 70 years were necessary for the scientific community to characterize the first retrovirus ever described and understand how RNA genome could be the origin of a DNA provirus and how this provirus could stably alter the host genome by insertion. These researches, and the development of the technical tools needed for them, profoundly modified, on several occasions, the way research was made in Virology and especially in Retrovirology. Through the last century, RSV remained a model of tumor RNA virus and is still actively researched today (Bar et al., 2011; Withers and Beemon, 2010). These discoveries have been essential to unravel the mechanisms through which RSV and other chicken retroviruses can cause leukemias or malignant diseases.

2) Retroviruses, Oncogenes and Proto-Oncogenes: the Oncornaviruses

At the end of the 60s, Weiss described the presence of endogenous retroviruses (ERV) integrated in the genome of uninfected chickens and sharing similar sequences with those of Avian Leukemia Viruses (Weiss, 2006). These observations, first considered as very controversial, have been then confirmed by the discovery of other ERVs in the genome of other animal species such as mice, cats or baboons (Weiss, 2006). It is today

established that such ERVs are present in the genome of all the vertebrates. These retroviruses, most often in a latent form are not producing any viral particles and some of them are defective as an important part of their viral genome is deleted. However, exposing the cells to chemical carcinogens or irradiation was demonstrated to reactivate some of those ERV leading to the production of viral particles (Weiss et al., 1971).

The observation that ERVs are present in a wide range of animal genomes and that they can be reactivated by carcinogens, added to the fact that exogenous retroviruses can cause neoplastic diseases in many animal species, led Huebner and Todaro to propose in 1969 the oncogene hypothesis of cancer (Huebner and Todaro, 1969). They postulated that some of these ERVs contained latent transforming genes (or oncogenes) and that the activation of these transforming ERV could cause cancer. In 1970, it was shown that the RNA genome of a transforming replication-competent strain of RSV was larger than that of a non-transforming replication-competent mutant. They therefore concluded that the additional sequences of the transforming strain were those of an oncogene that has been deleted in the transformation-defective mutant (Duesberg and Vogt, 1970). In 1976, Dominique Stehelin, working in the Howard Bishop-Harold Varmus laboratory prepared a probe specific of this putative transforming gene and demonstrated that this probe recognized homologous sequences in normal various avian DNAs (Stehelin et al., 1976a). The conservation of these sequences, referred to as *src*, in evolution provided evidence that they were of cellular rather than of viral origin. To distinguish these genes from the oncogenes that were postulated of viral origin, the term of proto-oncogene was coined to describe the cellular precursor (*c-src*) of a retroviral oncogene (*v-src*). (Stehelin et al., 1976b). Furthermore, it was shown that *c-src* proto-oncogene had the typical structure of a cellular gene (with coding exons and non-coding introns) that did not have the ability to transform, unless mutated and/or overexpressed.

As a matter of fact, these results opened the way to the identification of several other proto-oncogenes in the genome of normal cells together with the corresponding viral transforming gene in the genome of acute tumorigenic retroviruses. Consequently, these investigations, primarily aimed at the study of the molecular and cellular mechanisms underlying the tumorigenic effects of retroviruses, provided an important contribution in the study of cancer. Indeed, oncogenes were found either mutated or deregulated in many cancers of non-retroviral origin. Considering that the cellular proto-oncogenes are well conserved through the evolution, their normal and well-regulated expression versions were shown to play an essential role in the biology of normal cells. Their study is therefore of primary interest to understand their cellular functions and to characterize the cellular alterations that turned them into oncogenes. Many of these oncogenes were eventually identified as proteins involved in mitogenic signals: growth factors, growth factor receptors, protein kinases, G proteins, and transcription factors… (Coffin et al., 1997) The presence of similar genes in the cellular genome and in the viral genome indicates that these genes of cellular origin have been captured by the virus through a rare recombination event resulting in the capture of a nearby cellular oncogene in the viral genome (Coffin et al., 1997).

Clearly, on the eve of the isolation and characterization of retroviruses in humans, the replication strategy of cancer-inducing retroviruses, also referred to as oncornaviruses, have underlined that reverse transcription and integration confer specific pathogenic mechanisms such as insertional mutagenesis and oncogene transduction. Based on the latency of tumor induction, animal oncornaviruses have been divided into nonacute and acute retroviruses. They differ fundamentally in their mechanisms of tumorigenesis and in their genome structure. Nonacute retroviruses are replication competent and contain a full set of viral replicative and structural genes. They induce neoplasia after a long

incubation period of months to decades, and the tumors are monoclonal or oligoclonal. Tumor development is consecutive to the integration of the proviral DNA at near host proto-oncogenes, a process referred to as insertional mutagenesis that results in the transcription or deregulation of proto-oncogenes. None of them have incorporated sequences of cellular origin, in contrast to acute retroviruses. Whereas nonacute retroviruses are widespread in nature, acute retroviruses are mostly products of laboratory manipulation and have been instrumental in uncovering oncogenes and proto-oncogenes. They cause polyclonal tumor growth within days, are able to transform normal fibroblasts or hematopoietic cells *in vitro* with a one-hit mechanisms. They are derived from nonacute retroviruses through recombination with host proto-oncogenes. During this process known as oncogene transduction, viral oncogenes are mutated and deleted, thus enhancing the oncogenicity of the proto-oncogenes and conferring to these retroviruses a potent tumorigenic ability. Because of the loss of viral replicative and structural genes, the acute retroviruses are replication defective (with the notable exception of RSV).

3) The hunt for the first human retroviruses

In January 1971, during his "state of the union" speech, the American president Richard Nixon announced his project for the "National Cancer Act" that he ratified in December of the same year. This "war on cancer", the second cause of death in the USA at that time, was heavily funded and was aimed at understanding and finding a cure to cancer (NCI, 2011).

Based on the hypothesis proposed by Huebner and Todaro (Huebner and Todaro, 1969), a lot of effort and money were invested in the Virus Cancer Program promoted in 1964 for the search for a human tumor-inducing retrovirus. This program failed to provide clear evidence of tumor retroviruses in humans and was stopped in the late 70s. Indeed,

some reports claiming the isolation of a human retrovirus, were not confirmed, mainly because of experimental flaws masking inter-species contamination of the cell cultures by animal retroviruses (Gallo, 2005; Kontaratos et al., 2010). Contemplating this decade of failures, many stopped researching for human retroviruses, that were even referred to as "Human Rumour Viruses" as many people even doubt their reality (Voisset et al., 2008). This disbelief was also supported by the following scientific arguments: 1) there were little evidence for leukemia viruses in primates; 2) the viruses known to cause cancer among other animal species had been easily found due to a high viremia and if human retroviruses existed they should have the same properties and therefore be equally easy to find; and 3) the human sera was known to lysed all the known animal retroviruses suggesting that humans are protected against such pathogens (Gallo, 2005). In a few years the common belief switches from "a majority of the cancer cases are due to exogenous or endogenous retroviral infections" to "human tumor viruses do not exist". At the light of more recent studies, the reality, as often, lies in between. It is today estimated that one in five cancer cases worldwide is caused by an infectious agent and most of these cases are due to viruses (Parkin, 2006).

One of the major limitations of working on human pathogens is the low availability of human samples. Looking for the potential infectious causes of leukemia in humans requires the use of human hematopoietic cells. Although easiest to collect from humans, several technical limitations did not allow for the isolation of any human retroviruses. In 1975, the Bovine Leukemia Virus (BLV) was discovered and was shown to replicate at a very low level, discarding the notion of high viremia. Therefore, more blood cells might be needed for detecting a putative human retrovirus but the only available human T-cell lines were those derived from patients with Acute Lymphocytic Leukemia (ALL) (Moore and Minowada, 1973; Royston et al., 1974). However, they were relatively rare and did

not display any functional characteristics of normal T-cells. The rare successes in long-term maintenance of primary fresh T cells *in vitro* were by repeated stimulation of T cells with mitomycin-C-treated cells bearing alloantigens (Svedmyr, 1975). However, even under these conditions, cell growth and proliferation were limited to a few days. A breakthrough occurred at the mid'70s, when the Robert Gallo's group worked on conditioned media that represented the supernatant medium of the culture of normal human lymphocytes activated with phytohemagluttinin (PHA) for several days. They demonstrated that the conditioned medium contained a T-cell mitogenic factor that was first called T-Cell Growth Factor (TCGF) and then Interleukin-2 (IL-2) (Morgan et al., 1976; Ruscetti et al., 1977). This factor was later purified (Mier and Gallo, 1980) and better characterized as a 23 kDa protein. After addition of this factor to the medium of normal human activated T cells, they observed that these cells proliferated and could be maintained in culture for several weeks, thus allowing the expansion of normal T cells *in vitro*. This discovery revolutionized many fields including the understanding of the T-cell-mediated immune response itself as, for the first time, it was understood that T cells proliferation requires two steps: an initial activation step, *in vitro* reproduced by PHA stimulation, that would turn the cells into an IL-2 responsive state. That discovery also opened the way to the isolation and identification of the first human retrovirus HTLV-1 in 1980 and of HIV-1 in 1983 (cf. p29).

The following part is devoted to the isolation, the identification and the characterization of HTLV-1. Clinical and epidemiological studies coupled to molecular and cellular investigations have rapidly established this human retrovirus as the etiological agent of adult T-cell leukemia (ATL), have demonstrated specific features in its mechanisms of oncogenesis and have underlined the presence of additional sequences in the viral genome. These hallmarks are shared by BLV, an animal retrovirus that infects cattle. The

mechanisms of pathogenesis of HTLV and BLV that induce long-latency monoclonal T- or B-cell leukemias, differ from those of acute or nonacute retroviruses. Their provirus is not inserted near proto-oncogenes nor contains host proto-oncogenes. They are replication competent and, besides replicative and structural genes, additional open reading frames (ORF) are present coding for regulatory and accessory proteins.

The first classification schemes for retroviruses were based on disease association or morphological features. Later on, the knowledge of the different types of retroviruses was much greater than at the time of the RSV discovery and after the Baltimore classification (Baltimore, 1971), the International Committee on Taxonomy of Viruses has since established and maintained a viral classification (Carstens, 2010). Currently, seven genera are thus recognized and distinguished by the genetic relatedness of the RT protein (see Figure 2).

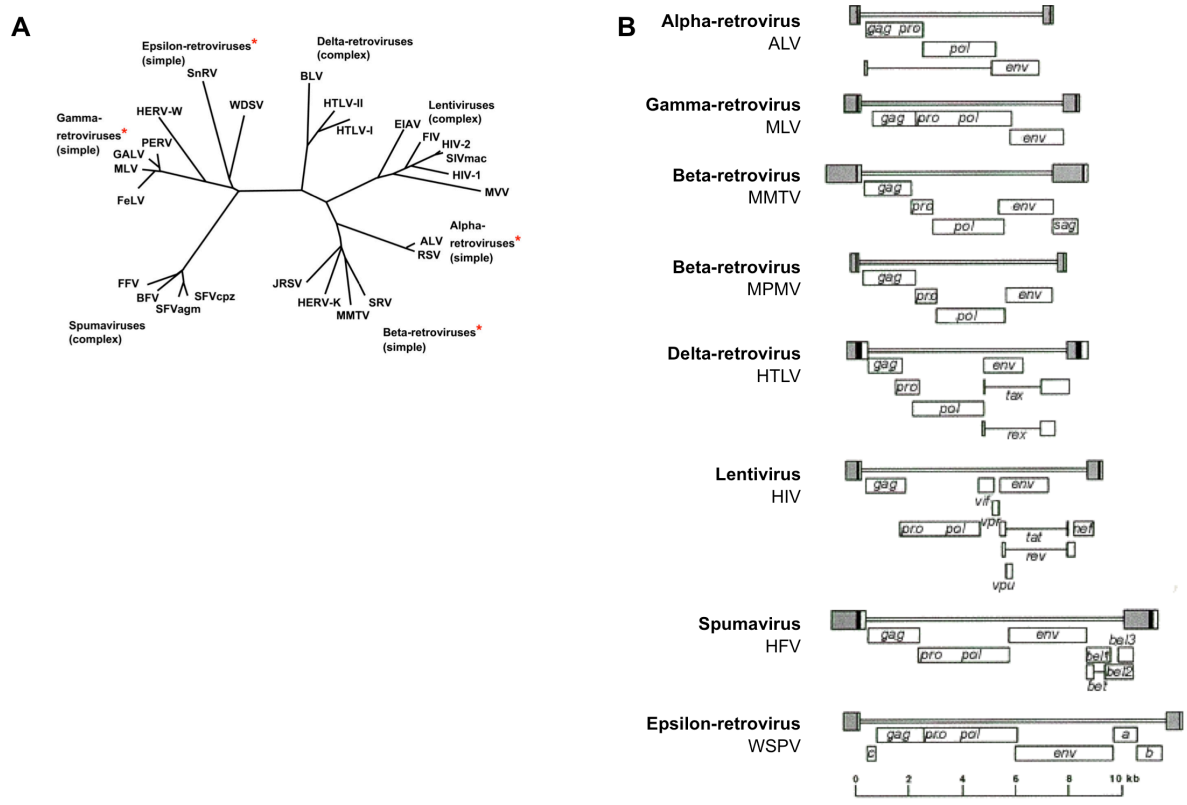

Figure 2 – Retroviruses classification and genomic structure

A – Phylogenetic classification of retroviruses in 7 genera. Red asterisks indicate the genera in which ERV have been described. **B –** comparison of the genomic structure of simple (alpha-, gamma-, beta-, epsilon-retrovirus) and complex retroviruses (delta-retrovirus, lentivirus, spumavirus).

II] The Human T-lymphotropic/leukemia virus type 1 (HTLV-1)

Numerous recent reviews have been devoted to HTLV-1, especially in 2005, 25 years after its discovery. Here, I present a concise overview of the replication and pathogenesis of this human retrovirus.

1) Discovery of HTLV-1, the etiological agent of Adult T-cell Leukemia/Lymphoma (ATL)

After several years of investigation, a group of Japanese clinicians published in Blood in 1977 their description of a new clinical entity (Takatsuki, 2005). An atypical T-cell leukemic disorder was observed among Japanese adults. Most of the patients were born in the southwest of Japan. Further clinical studies have allowed to underline that the pathological features involved were quite distinct from those of other lymphoproliferative malignant diseases. Accordingly, ATL is characterized by: an onset in adulthood, an aggressive clinical course, the presence of leukemic T cells with deeply indented or multilobulated nuclei (Figure 4), frequent infiltrations into skin, liver gastrointestinal tract and lung, hypercalcemia and, as noted above, a striking geographical clustering in Southwestern Japan (Uchiyama et al., 1977). This new clinical entity was reported as Adult T-cell Leukemia (ATL)

Several cases of that new ATL entity were presented at the 16th International Congress of hematology in Kyoto in 1976 and it was stated that “attempts to elucidate leukemogenesis in this disease should be directed towards exploring the genetic background and a possible viral involvement” (Takatsuki, 2005). This statement was premonitory. A few years later HTLV-1 (Human T cell Leukemia Virus type 1), the first human retrovirus, was discovered and identified as the etiological agent of ATL (Poiesz et al., 1980a).

Its discovery was dependent on several conceptual and technical advances. Among them, the development of sensitive RT assays and the use of IL-2 favoring the *in vitro* expansion of human primary activated T cells were of the utmost importance. The *in vitro* culture of peripheral blood cells obtained from patients with cutaneous T-cell lymphoma in presence of IL-2 led to the obtention of an immortalized cell line, HUT-102, that was then able to grow independently of IL-2 (Poiesz et al., 1980b). The presence of retroviral particles, revealed by the detection of an RT activity in the culture supernatants and confirmed by the electron microscopy observation of type C retroviral particles budding from these cells: the first ever human retrovirus associated with a malignancy was thus discovered and designated HTLV-1 (Poiesz et al., 1980a). Serological studies performed with viral antigens prepared from HTLV-1-producing cell lines demonstrated the presence of anti-HTLV-1 antibodies in the sera of several ATL patients and also in non-ATL individuals who were then referred to as asymptomatic carriers. (Hinuma et al., 1981; Kalyanaraman et al., 1982). In 1981, a Japanese team independently isolated a retrovirus associated with ATL. They named it ATLV for Adult T-cell Leukemia Virus (Miyoshi et al., 1981; Yoshida et al., 1982). In 1982, the American and Japanese groups collaboratively publish that it was the same virus and that its name should be HTLV-1 (Popovic et al., 1982). Then in 1983, HTLV-1 was shown to be able to immortalize T cells *in vitro* (Popovic et al., 1983) and the monoclonal integration of HTLV-1 provirus in ATL cells was demonstrated.

The 4 following arguments were made to confirm that HTLV-1 was the etiological agent of ATL and led to a new way of diagnosing ATL patients that include the detection of the virus (Takatsuki, 2005): 1) all patients with ATL have antibodies directed against HTLV-1; 2) areas of high incidence of ATL patients correspond closely with those of high incidence of HTLV-1 carriers; 3) HTLV-1 immortalizes human T cells *in vitro*; 4)

monoclonal integration of HTLV-1 proviral DNA was demonstrated in ATL cells (Takatsuki, 2005).

In 1985, another major lymphocyte-mediated disorder, referred to as HAM/TSP (HTLV-1–associated myelopathy/ tropical spastic paraparesis) was observed among HTLV-1 carriers (cf. p36) (Gessain et al., 1985).

2) Epidemiology of HTLV-1 infection

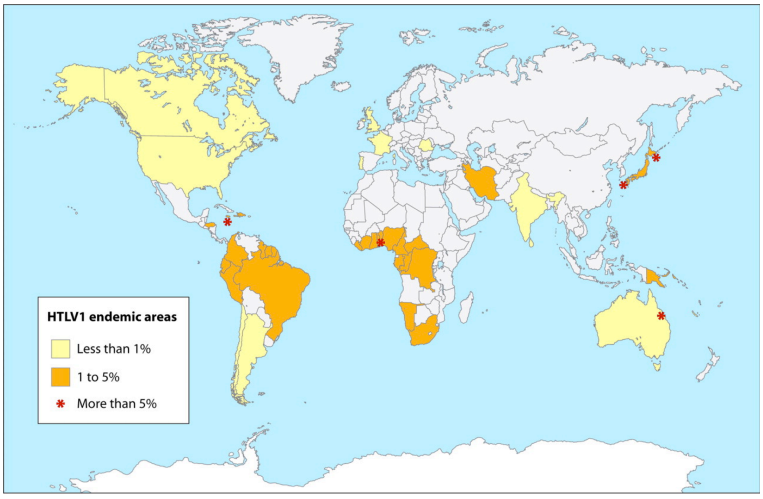

Figure 3 – Geographical repartition of HTLV-1 infected patients

From (Goncalves *et al.*, 2010)

Endemic regions are present in the southwest and in the north of Japan where high prevalence of ATL patients as well as HTLV-1 carriers are found (Yamaguchi and Takatsuki, 1993). In some Japanese areas the HTLV-1 prevalence was as high as 45 % (Ishida and Hinuma, 1986). Other regions in the world such as the Caribbean, sub-saharan Africa countries, Iran, localized area in Melanesia and some south- and central-American countries have endemic foci of infection (Figure 3) (for a review, see (Goncalves et al., 2010; Proietti et al., 2005)). This geographical infection pattern might be associated to infection of particular ethnic groups that might be more susceptible to HTLV-1 infection due to their HLA alleles (Sonoda et al., 2011). Based on epidemiological studies, it has been estimated that approximately 15 to 20 million HTLV-1 carriers exist around the world (de The and Kazanji, 1996). HTLV-1 is mainly

transmitted through contact with bodily fluids containing infected cells, via three routes: from mother to child through breastfeeding; sexual transmission; or parenteral transmission, via blood transfusion or intravenous drug use. Among all HTLV-1-infected persons around the world, the risk of developing an HTLV-1-associated disease is rather low (between 2 to 5%). The factors favoring the development of ATL or HAM/TSP are not completely understood but have been linked to factors regarding host (eg immune response) and virus (eg proviral load). The route of exposure to the virus may influence the clinical outcome of HTLV-1 infection. Individuals subjected to mucosal exposure appear more likely to develop ATL, whereas blood-borne exposure favors the development of HAM/TSP (Hino, 2011).

The initial route of infection is of major importance as it determines which cells are first in contact with the virus and the resulting immune response (Grant et al., 2002). It has been demonstrated in a rat model infected by different routes (oral infection, iv or ip inoculation) that the anti-HTLV-1 immunity is different according to the conditions of infection and that the oral inoculation resulted in a low immune response against HTLV-1 favoring the persistence of infected cells *in vivo* (Kannagi et al., 2000).

3) Adult T-cell leukemia /lymphoma (ATL)

a- ATL is a long-term evolving disease.

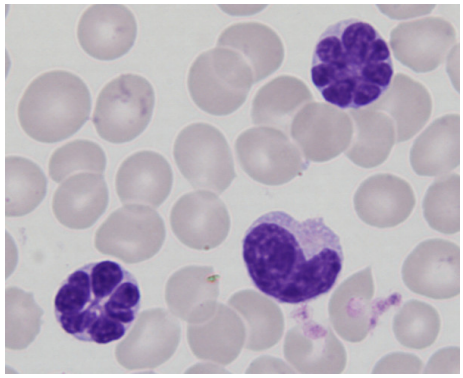

Figure 4 – Flower cells

Presence in ATL patients of multi-lobulated cells. From (Matsuoka and Jeang, 2007)

ATL is an HTLV-1-associated aggressive monoclonal T-cell malignancy that occurs several decades after the primary infection (Cleghorn et al., 1995). This leukemic disease is characterized by the monoclonal proliferation of activated $CD4^+$ T cells. The leukemic cells in the blood of ATL patients, named "flower cells" exhibit a characteristic lobulated nucleus and a cytoplasmic basophilia (Figure 4) (Hollsberg et al., 1992). These patients have been classified into four subtypes, according to the clinical features (Yamaguchi and Takatsuki, 1993):

- Smoldering type: about 5% or more abnormal T lymphocytes in the peripheral blood, normal lymphocyte level (less than 4×10^9/l), no hypercalcemia, lactate dehydrogenase (LDH) with a value up to 1.5-fold the normal upper limit, no lymphadenopathy, no other organ involvement. Skin and pulmonary lesions may be present.

- Chronic type: absolute lymphocytosis (4×10^9/l or more) with T-lymphocytosis (more than 3.5×10^9/l), LDH value up to twice the normal upper limit, no hypercalcaemia. Lymphadenopathy and involvement of liver, spleen, skin, and lung may be present, and 5% or more abnormal T lymphocytes are seen in the peripheral blood.

- Lymphoma type: no lymphocytosis, 1% or less abnormal T-lymphocytes in peripheral blood, and histologically-proven lymphadenopathy with or without extranodal lesions.

- Acute type: remaining ATL patients with leukemic manifestations, but not classified as any of the three other types.

Smoldering and chronic ATL subtypes often progress to acute ATL after a long period of time. The prognosis of patients with acute or lymphoma type is extremely poor, as the median survival time is less than a year. Patients with chronic and smoldering ATL have a longer lifespan but the median survival time is still limited (Shimoyama, 1991) (Yamaguchi and Takatsuki, 1993). This classification is probably artificial and the reality might be less clear-cut with a continuous spectrum of symptoms in constant evolution.

However, the different stages present objective clinical differences, concerning for instance the response to treatments or the quality of the immune response. Taken together, these data demonstrate that the leukemogenic process associated with HTLV-1 infection is complex and multi-step.

The evolution of ATL has been linked to several hallmarks. The number of infected clones during the evolution of ATL decreases and is lower than that observed in asymptomatic carriers (Yoshida et al., 1984). This diminution must result from the selection that occurred for decades before the onset of leukemia. In ATL patients, few clones remains and malignant clones represent a large proportion of the total infected cells (Gillet et al., 2011). Telomerase activity, that is necessary to maintain telomere stability, is demonstrable in a large number of human tumors. Its evaluation provides a convenient tumor marker to appraise the progression of a malignant disease. A comparison of telomerase activity in ATL subtypes and asymptomatic carriers has underlined that telomerase reactivation correlates with development and progression of ATL (Uchida et al., 1999). Serum LDH level also correlated with telomerase activity and its level is assessed to determine the ATL type.

b- ATL cells.

The immunological phenotype of ATL cells is characteristically $CD3^{+/low}$, $CD4^{+}$, $CD8^{-}$ and $CD25^{+}$, a phenotype of activated T-helper cells. The expression of forkhead box P3 (FoxP3), characteristic of $CD4^{+}CD25^{+}$ regulatory T cells (Treg cells), was detected in some ATL patients (Bangham and Toulza, 2011). In addition to CD4 and CD25, ATL cells have been shown to express several membrane antigens that are also expressed on Treg, such as CCR4, CTLA-4 and GITR. However, Treg do not represent the whole population of leukemic cells in ATL patients, excluding that ATL originates from the proliferation of Treg cells. Furthermore, it was also demonstrated that the Treg

population was increased both in ATL patients and in asymptomatic carriers. The Treg population appears to be non-infected and possess regulatory activity, reducing the immune response against HTLV-1. Thus, immunodeficiency favoring the development of opportunistic infections in ATL patients could arise in part from the immunosuppressive action of ATL Treg cells. In the same study, this Treg population was shown to inhibit the proliferation of infected T cells, suggesting a role in the control of the progression of ATL. The presence of this enlarged population might be explained by the Tax-induced production of CCL22, the ligand of CCR4, which favor the survival of Treg cells.

c- Clinical treatment

The acute/lymphomatous subtypes of ATL are associated with a bad prognosis. Different combinations of chemotherapy have been tested and the response is generally low as the most efficient combination, using a highly toxic treatment, leads to an overall survival rate of 23 % at 3 years (Tsukasaki et al., 2007). Allogenic stem cell transplantation has also been tried on patients with the aggressive ATL forms, with an overall survival rate of 36 % at 3 years. This kind of treatment requiring conditioning of the patients using whole body irradiation and treatment has a rather high toxicity. It has been observed that a mild graft vs. host disease is a good prognostic factor as it might be correlated with a graft vs. leukemia effect (Tanosaki et al., 2008; Utsunomiya et al., 2001). After transplantation, the HTLV-1 proviral load dropped to an undetectable level in the patients' PBMC (Tanosaki et al., 2008).

More recently, therapeutic strategies involving monoclonal antibodies directed against markers expressed on ATL cells were carried out on animal models (for reviews see (Matsuoka and Jeang, 2011) and page 55 of this manuscript). A phase I clinical trial has notably been initiated with a monoclonal antibody directed against CCR4, a molecule expressed on Treg cells and often on ATL cells. This phase I clinical trial gave promising

preliminary results. The dosage used appears to be not toxic and partial or complete remissions were observed during this trial on relapsed ATL patients (Yamamoto et al., 2010).

ATL is a form of leukemia but with the specificity of being virus-induced. The use of antiviral therapy has been shown to improve the outcome of ATL patients in comparison with patients only treated with chemotherapy. The positive effect of this antiviral treatment was particularly impressive for chronic or smoldering ATL patients treated with AZT and IFN-α with an overall survival rate of 100% at 5 years (Bazarbachi et al., 2010). However similar treatment administered to acute of lymphomatous patients did not give the same outstanding results.

The development of new original therapeutic strategies and pre-clinical trials of new molecules or new combination of molecules have been hampered by the lack of an animal model to reproduce HTLV-1 infection and pathogenesis *in vivo*. Although much work has been done in that domain, the development of such animal model has been elusive for years slowing down the understanding of the complex mechanisms involved in the HTLV-1-associated leukemogenic process.

4) HAM/TSP, a neurodegenerative disease and other inflammatory diseases

The association of HTLV-1 with HAM/TSP was revealed by the elevated seroprevalence among patients suffering from this neurodegenerative disease (Gessain et al., 1985). Transfusion with HTLV-1–contaminated blood has been associated with a more rapid onset of development of HAM/TSP versus ATL (Osame et al., 1990). HAM/TSP is an inflammatory neurologic disease, most commonly occurring in the lumbar spine in patients typically younger than those affected by ATL. It is characterized by lymphocytic meningitis with demyelination and degeneration of the spinal cord. The first symptoms

of this progressive debilitating pathology usually occur around the age of 40 and there is no remission (Goncalves et al., 2010). As the disease progresses, the composition of the inflammatory component of the spinal cord progresses from $CD4^+$ or $CD8^+$ T cells, B cells, and macrophages to a lesion comprised predominantly of $CD8^+$ T cells. Indeed, cytotoxic T cells (CTLs) directed against HTLV-1 Tax are detectable in the blood and central nervous system of HAM/TSP patients. This infiltration of $CD8^+$ T cells in the central nervous system, leads to inflammatory lesions causing demyelination and axonal degeneration resulting in a slowly progressive spastic paraparesis. Today there is no efficient treatment to cure this chronic disease. Of note, a clinical trial using valporoate, a histone deacetylase inhibitor, to treat HAM/TSP patients has given hopeful results. Indeed, before the treatment, a low level of HTLV-1 expression in infected cells allows their escape from the immune response directed against HTLV-1 and especially from the CTL response. Upon valproate treatment, a transient and massive expression of viral antigens is observed, that exposes the infected cells to the immune response, and to their subsequent elimination (Lezin et al., 2007).

HTLV-1 infected persons can also be subjected to other HTLV-1 associated diseases that exhibit various debilitating degrees. HTLV-1 Associated Uveitis (HAU) is characterized by a granulomatous or nongranulomatous reaction accompanied by vitreous opacities and retinal vasculitis with rare exudative retinochoroidal alterations in one or both eyes. It can affect patients at different ages and was observed in geographical clusters with some minor differences in the clinical signs associated with this disease (Mochizuki et al., 1992). Patients can be treated with topical and systemic corticosteroids associated with lubrication of the eye. (Goncalves et al., 2010).

Dermatological lesions are commonly associated with HTLV-1 infection. It was even part of the initial description of ATL in 1977 (Uchiyama et al., 1977). Several forms of

dermatological lesions are observed such as infective dermatitis, crusted scabies, acquired ichthyosis or lesions similar to mycosis fungoides. These lesions can be due to the immune suppression observed in HTLV-1 carriers, an inflammatory reaction or an infiltration of T cells in the skin (Goncalves et al., 2010). They can be associated with other HTLV-1 associated diseases such as ATL or HAM/TSP or alone in asymptomatic. As seen in the associated skin lesions, a number of opportunistic infections may develop linked to HTLV-1 infections. Stronglyoides stercoralis, Norwegian scabies, molluscum contagiosum and extrapulmonary histoplasmosis have been reported.

Inflammatory rheumatic conditions, including rheumatoid arthritis and Sjögren's syndrome were also associated with HTLV-1 infection. In addition, other autoimmune lesions have been observed such as polymyositis, bronchoalveolar pneumonitis and autoimmune thyroiditis. Muscular lesions (inflammation and destruction) have also been reported (for a review, see (Goncalves et al., 2010)).

5) HTLV-1 and mechanisms of replication

a- The HTLV-1 provirus and the viral life cycle

HTLV-1 is a member of the deltaretrovirus genus, together with Bovine Leukemia Virus (BLV) and the Primate T lymphotropic Viruses (PTLV) including HTLV-1 and its simian counterpart STLV-1 as well as other viruses of the same family (HTLV-2, HTLV-3, HTLV-4, STLV-2, STLV-3). The viral particle has a type C morphology, i.e. an enveloped particle with a condensed, central core (Figure 5) (Poiesz et al., 1980a).

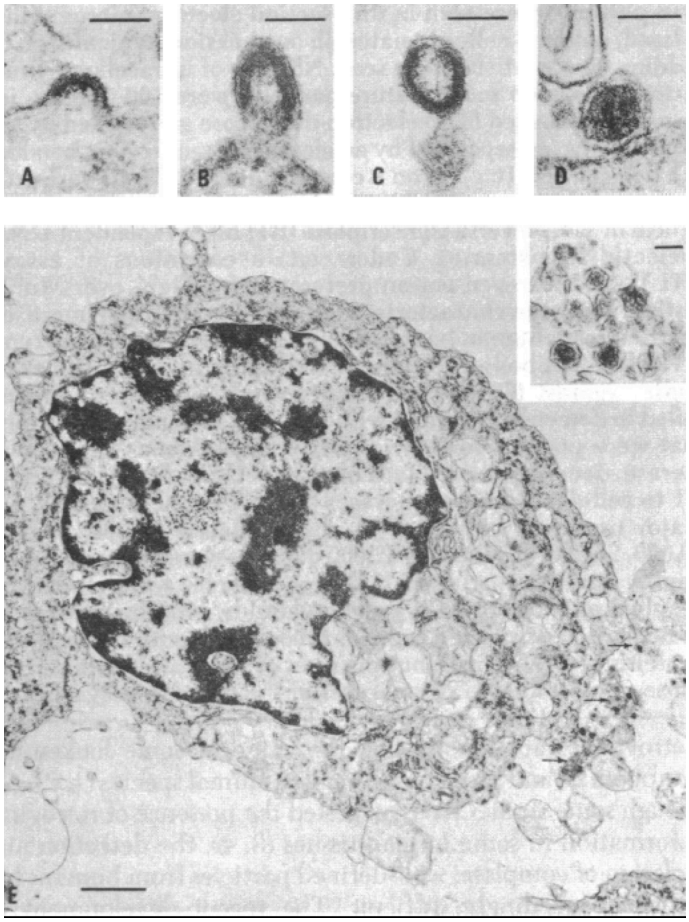

Figure 5 – Detection of HTLV-1 particles by thin-section electron microscopy

This mocrographs were realized on HUT-102 cells and are presented in (Poiesz et al., 1980a) with the following caption. **A –** Early viral bud; **B –** late viral bud with nearly completed nucleoid (specimen was treated with tannic acid prior to chrome osmium); **C -** "immature" extracellular virus particle (top) with incomplete condensation of the nucleoid; **D -** "mature" extracellular virus particle with condensed, centrally located nucleoid surrounded by an outer membrane separated by an electron-lucent area; **E -** HUT 102 cell 72 hr after induction by IdUrd. Many mature particles are found in the extracellular space in clumps associated with cellular debris (Inset). (Bars in A-D and Inset = 100 nm; bar in E 1000 nm.)

It is worth reminding that HTLV-1, unlike animal acute tumorigenic retroviruses, does not use viral capture of a cellular proto-oncogene for pathogenesis. Like other animal replication-competent retroviruses, the HTLV-1 provirus displays structural genes, *gag*, *pol* and *env*, bracketed by long terminal repeats (LTR) containing sequences promoting viral expression. The *pol* ORF encodes the reverse transcriptase, the protease and the integrase. The *gag* ORF provides the viral core proteins, and *env* encodes the viral envelope glycoproteins involved in cell transmission. HTLV-1 is considered as a complex retrovirus as the provirus contains additional sequences, the pX region located between

env and the 3'-LTR. This region encodes regulatory proteins, such as Tax, Rex, p12, p13, p30 and p21 by alternative splicing from 4 ORFs. Tax, encoded by ORF IV, is a 40kDa nuclear protein translated from a doubly spliced mRNA using the same start as *env* (Seiki et al., 1985). Its main function is to trans-activate proviral transcription following its recruitment on the three TRE (Tax Responsive Element) in the 5'-LTR (Felber et al., 1985). Rex, encoded by the ORF III, is responsible for the export of unspliced (*gag/pol*) or singly spliced (*env*) viral RNA to the cytoplasm (Kiyokawa et al., 1985). The other regulatory proteins (p12, p30…) encoded by the pX region are important for infectivity, cell activation and regulation of gene expression (Figure 6).

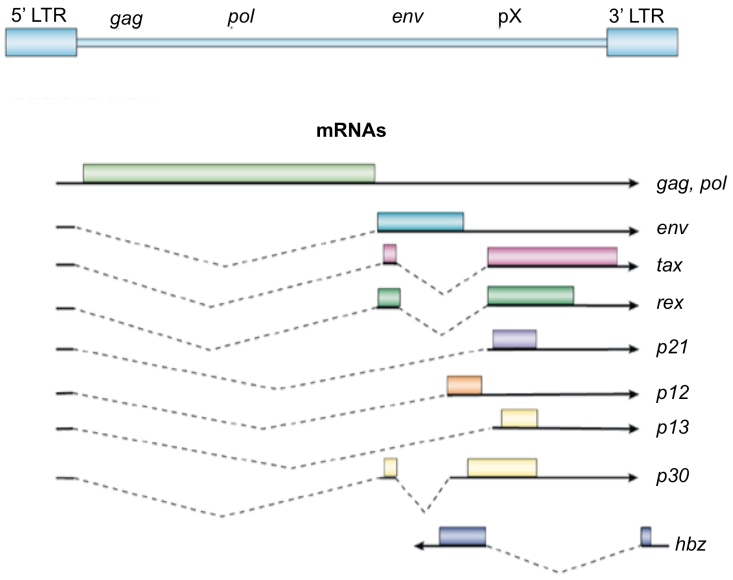

Figure 6 – HTLV-1 genomic organization and transcribed mRNA

In addition to the retroviral gag-pol-env sequences, HTLV-1 is a complex retrovirus with a pX region encoding several accessory and regulatory proteins. Recently a transcript was characterized on the antisens strand of the viral genome, *hbz*. Adapted from (Matsuoka and Jeang, 2007)

Recently, a new non-structural viral protein HBZ (HTLV-1 basic leucine zipper factor) has been identified. It is encoded by an ORF located between the *env* and *tax/rex* genes on the antisense strand of the provirus under the control of a promoter located in the 3'-LTR (Gaudray et al., 2002; Matsuoka and Jeang, 2011). HBZ contains a transcription activation domain at the N-terminus and a leucine zipper at the C-terminus. Two main forms of transcribed RNA, unspliced HBZ (unHBZ) and HBZ spliced (HBZ-SP) have been

detected in several HTLV-1 cell lines as well as in asymptomatic carriers, in ATL and HAM/TSP patients. HBZ protein inhibits Tax-mediated transactivation of proviral transcription through interactions with JUN and ATF/CREB families (Basbous et al., 2003; Gaudray et al., 2002) and is thought to have a crucial role in the leukemogenic process (cf. p43 and 48).

Once the virus has entered into the cell, the RNA genome is reverse-transcribed using the viral reverse transcriptase into the DNA provirus that is then integrated stably into the cell genome by the viral integrase. Once integrated, the provirus is transcribed and allows the formation of new viral particles or the establishment of a chronic infection leading at term to the development of leukemic clones. The analysis of infected cells *in vitro* demonstrated that the viral proteins expression follows a tightly regulated kinetic where the *tax/rex* transcript is the first to be transcribed followed by the structural genes. HBZ expression rises slowly but continuously whereas *tax/rex* decrease after few hours of infection (Rende et al., 2011). This kinetics was also observed in newly infected rabbits (Li et al., 2009) (cf. p60). This pattern is coherent with the fact that Rex is involved in the export of unspliced or singly spliced viral RNA from the nucleus and that Tax is able to transactivate the proviral expression. The initial expression of Tax therefore initiates the expression of the provirus.

b- Tropism of HTLV-1 infection

Even if ATL cells are mostly $CD4^+$ T cells, HTLV-1 infects a wide range of cell types, such as $CD8^+$ T cells, B cells, dendritic cells, monocytes, astrocytes in the central nervous system (CNS) (Grant et al., 2002; Jones et al., 2008; Koyanagi et al., 1993). *In vitro* studies have demonstrated that $CD34^+$ Hematopoietic Progenitor/Hematopoietic Stem Cells (HP/HSC) are sensitive to HTLV-1 infection (Feuer et al., 1996). However, observations made in patients gave contradictory results. The HTLV-1 provirus has been

detected in $CD34^+$ cells of the bone marrow in HAM/TSP patients, but not ATL patients (Grant et al., 2002). An *in vitro* study using fresh human thymocytes has also demonstrated that HTLV-1 was also able to infect immature thymocytes (Maguer-Satta et al., 1995).

This wide distribution of cells sensitive to HTLV-1 infection highlights the broad distribution of its receptors. Today, three cellular molecules are known to be involved in the entry of HTLV-1: Glucose transporter 1 (GLUT1) (Manel et al., 2003), Heparan Sulfate Proteoglycan (HSPG) (Jones et al., 2005) and Neuropilin-1 (Lambert et al., 2009). It is hypothesized that viral particles first entered into contact with HSPG leading to the formation of a complex with Neuropilin-1 followed by GLUT-1 association on the cell surface before membrane fusion and entry into the cell (Ghez et al., 2010).

c- Cell-cell transmission

Viral particles are not detected in the serum of the patients indicating that they are not released from the cell (Fields et al., 2007). Indeed, cell-free virus is largely non-infectious and infection of new cells occurs via a direct cell-to-cell contact. Such a contact between an infected cell and an uninfected one triggers a rapid polarization of the infected cells favoring the infection of new cells (Igakura et al., 2003). This polarization results in the formation of a virological synapse involving cytoskeleton remodeling and adhesion molecules rearrangement at the membrane (Nejmeddine et al., 2009). Through this specialized contact between an infected cell and a naïve cell, direct transfer of viral particles can be achieved without its dissemination in the extracellular medium preventing its detection by the immune system (Majorovits et al., 2008).

Recently, another mode of transfer has been described. Microscopic observations of cell-to-cell contact have demonstrated the presence of a virus-induced biofilm at the surface of the infected cells. In this extracellular structure, made of heparan-sulfate and collagen,

enveloped virus particles are trapped. Upon contact with an uninfected cell, this biofilm can bond to the surface of the naïve cell and allows the entry of the viruses and the infection of the new cell. The importance of this mechanism is underlined by the fact that destruction of this biofilm results in a decrease of 80% of the virus infectivity (Pais-Correia et al., 2010). Of note, the virological synapse and the viral biofilm are two mechanisms that are likely to cooperate to enhance viral infectivity.

6) HTLV-1 and mechanisms of pathogenesis

a- HTLV-1-associated leukemogenesis

i. Functional implications of Tax and HBZ

The viral regulatory Tax protein plays a major role in the early steps of leukemogenic process, not only by controlling proviral transcription, but also by stimulating and repressing the synthesis and function of many regulatory factors involved in a wide variety of normal and pathogenic cellular processes (for reviews, see (Matsuoka and Jeang, 2007)). Thus, Tax has been shown to induce the constitutive expression of regulatory factors involved in T-cell activation and proliferation (cyclin D2, Cyclin E, E2F1, CDK2, CDK4, CDK6, p19, p21 (Iwanaga et al., 2001)) and of the tumor suppressor p53 (de la Fuente et al., 2003). Thus, Tax is able to immortalize and transform human primary T cells. Tax has also been shown to target both the fidelity of chromosomal segregation (aneuploidy) and the mismatch repair (clastogeny). Tax has also been shown to interfere with DNA repair pathways (PCNA (Ressler et al., 1997), ß-polymerase (Jeang et al., 1990)). In addition, Tax is able to abrogate the mitotic checkpoint function and lead to miscounted chromosomes in HTLV-1-transformed T cells (Marriott et al., 2002), thus further intensifying the genome instability of these cells. However, these cells are not arrested into their cell-cycle and do not enter into apoptosis, because they are protected by Tax from these responses

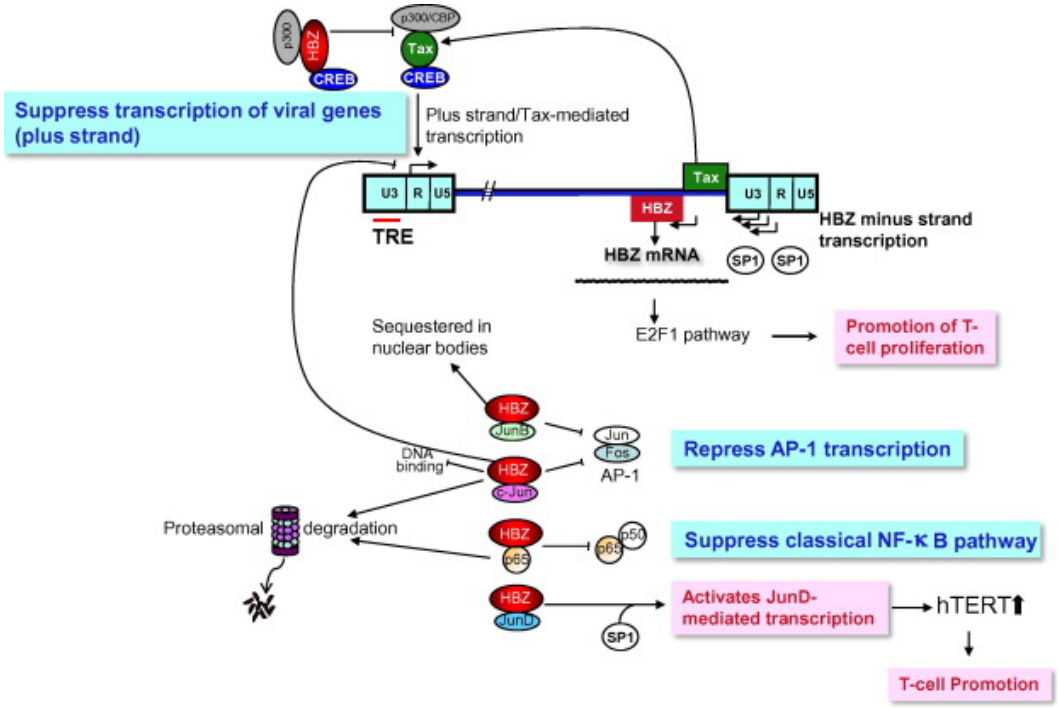

Figure 7 – Functions of HBZ (Matsuoka, 2010).

HBZ is involved in the alteration of cellular pathways (hTERT, AP-1, classical NF-κB pathway, …), playing a critical role in the leukemogenic process.

(bcl-Xl (Tsukahara et al., 1999), bax (Brauweiler et al., 1997)). Clearly, initial expansion of infected T cells is mainly promoted through the pleiotropic effects of Tax (Boxus et al., 2008). In HAM/TSP patients, several cytokines transactivated by Tax such as Tumor Necrosis Factor-α, Monocyte Chemoattractant Protein-1 and Matrix Metallo-Proteinase-9 are over-expressed in the infiltrating mononuclear cells of the patients' spinal cord.

HBZ function has been linked to ATL proliferation. Indeed, reduction of HBZ mRNAs using short hairpin mRNAs slowed down the proliferation of ATL cells (Satou et al., 2006). Conversely, ectopic expression of HBZ in a human T-cell line enhanced cells proliferation. Furthermore, it was found that HBZ RNA, rather than HBZ protein, is upregulating the transcription of E2F1 and many cellular E2F1 target genes (Satou et al., 2006) and that the secondary structure of HBZ RNA is important for its proliferative function. Interestingly, HBZ/JunD heterodimers are stimulating the transcriptional activity of the proximal promoter of the hTERT (human Telomerase Reverse Transcriptase) gene (Kuhlmann et al., 2007). HBZ has also a role in rehulating other AP-

1 factors and is able to inhibit the classical NF-κB pathway (Figure 7). For a review on HBZ roles in HTLV-1 pathogenesis, see (Matsuoka, 2010). These results indicate that HBZ could be associated with the maintenance of the leukemogenic process in collaboration with Tax.

ii. Target cells of the HTLV-1 leukemogenic activity

The observations that HTLV-1 can infect a wide variety of cells types *in vitro* and *in vivo* does not mean that this virus exerts similar effects in all these cells. Identifying the target cell of the leukemogenic activity, i.e. the cell that provides the most favorable environment for the initiation of the leukemic process is of crucial importance. Infection of HP/HSC by animal retroviruses is responsible for the development of leukemias by deregulating pathways controlling hemato-lymphopoiesis . This is for example the case of the Moloney Murine Leukemia Virus (M-MuLV) that induces T-cell lymphoma in mice (Brightman et al., 1990). As noted above, HTLV-1 can infect *in vitro* human $CD34^+$ cells, leading to a latent infection (Feuer et al., 1996). It was supposed that these cells, "hidden" from the innate immune response in the bone marrow represent a reservoir for HTLV-1 infection overtime (Banerjee et al., 2010a). HTLV-1 can also interfere with early progenitors of the T-cell development in the thymus (Maguer-Satta et al., 1995).

Our laboratory has demonstrated that the expression of Tax in a subpopulation of immature thymocytes led to the deregulation of the β-selection checkpoint controlling early T-cell development. Indeed, Tax expression in immature CD4 single positive thymocytes (Figure 10) decreases the expression of pTα, a crucial component of the pre-TCR complex allowing the survival of T cells with correct rearrangement of the β-chain of the TCR. In parallel, Tax induces the expression of the same molecular pathways triggered by the pre-TCR complex resulting in the bypass of the β-selection (Wencker et al., 2007). That event could allow the survival of defective T cells and might represent an

important pre-leukemic step. The hypothesis that ATL cells may derive from infected progenitors is furthermore supported by the deregulation of the miRNA miR223, which is a marker of immature hematopoietic cells, observed in ATL cells (Bellon et al., 2009).

iii. Proviral integration and clonal expansion

In the early stages of HTLV-1 infection, the virus replicates via reverse transcription producing new viral particles that infect new cells. Tax is promoting the survival and the polyclonal/oligoclonal expansion of infected T cells that results from the infection of multiple distinct cells in the host. It is thought that the provirus is randomly integrated in the host genome (Mortreux et al., 2003). However, recent studies using high-throughput sequencing to characterize unique insertion sites in HTLV-1-infected persons have demonstrated that it is not completely true. Initial HTLV-1 integration strongly favors proximity to genes, promoters (as identified by CpG islands), and epigenetic marks associated with the control of gene expression (both activation and repression). Furthermore, it was demonstrated that integration of the provirus near a CpG island or a transcriptionally active region confers a proliferative advantage to the infected clone (Gillet et al., 2011).

The determination of integration sites enables to identify each infected clone and to trace the kinetics of infected cells *in vivo*. Such an analysis has revealed that the proliferation of infected cells become mainly oligoclonal. Clonal expansion becomes the dominant contributor to increasing the number of HTLV-1 infected T cells, rather than the *de novo* infection of cells. The same clones can be detected in infected patients at different times of infection, persist and extend for a long period of time (Cavrois et al., 1996; Etoh et al., 1997; Gillet et al., 2011; Wattel et al., 1995). This clonal expansion is selected against by the host immune response, and most of HTLV-1 infected individuals remain life-long asymptomatic carriers. Thus, CTL killing can control virus replication in

asymptomatic carriers and select for cells that carry latent proviral DNA. However, in 2-5% of them, an HTLV-1 associated disease develops. Continuous proliferation of infected $CD4^+$ T cells that escape from the adverse effects of the immune response favors the emergence of a malignant ATL clone, that emerges after a prolonged asymptomatic period of several decades. Somatic mutations are found more frequently in the T cells from ATL patients compared to those from asymptomatic carriers (Mortreux et al., 2003). These mutations are mostly found in the provirus sequence and in the flanking regions of the insertion site and are thought to occur mostly during the initial phase, when retro-transcription is involved in viral replication rather than during the clonal expansion. The accumulation of somatic punctual mutations might be involved in the onset of leukemias (Mortreux et al., 2001b).

By comparing recently *in vitro* infected cells, and *in vivo* infected cells from patients, it was demonstrated that an *in vivo* selection operates, leading to the emergence of some infected clones (Gillet et al., 2011; Zane et al., 2010). This selection results in the maintenance and/or expansion of clones with a particular phenotype such as the level of Tax expression, the extent of cell-cycling, or the genomic environment of the provirus. All those parameters are probably linked together as the genomic environment impacts the proviral expression that influences the expression of Tax that, in turn, deregulates cell-cycling of the infected cells. Interestingly, Gillet *et al.* have demonstrated that proviral integration in transcriptionally silenced chromatin favors the persistence of the infected cells, whereas its integration near a transcriptional active region give the clone a proliferative advantage (Gillet et al., 2011; Meekings et al., 2008).

Considering that most infected cells have one copy of the provirus, the provirus load therefore indicates the percentage of infected cells among lymphocytes. From several observations, it is commonly admitted that the level of proviral load (PVL) correlates

with the clinical outcome and disease severity; high in ATL patients, intermediate in HAM/TSP and low in asymptomatic carriers (Saito et al., 2009; Usui et al., 2008).

iv. Tax is frequently inactivated in ATL cells, not HBZ

As mentioned above, HTLV-1 infection confers a long lifespan to the infected cells due to the pleiotropic actions of Tax. However, Tax was found to be the dominant antigen recognized by CTLs *in vivo* and its expression result in the reconnaissance of the expressing cell by the immune system (Hanon et al., 2000). Furthermore, loss of Tax expression is frequently observed in ATL cells. Inactivation of Tax is caused by either genetic changes (nonsense mutations, insertions and deletions) of the pX gene or silencing viral transcription by DNA methylation of the provirus or deletion of the proviral 5'LTR (Koiwa et al., 2002; Takeda et al., 2004; Taniguchi et al., 2005). Loss of Tax is therefore protecting ATL cells against a rapid immune clearance by CTL response favoring their survival. Furthermore, some ATL cells that no longer express Tax still continue to show constitutive NF-κB activation. Although the mechanism sustaining NF-κB activation is not fully understood, this finding is consistent with the requirement for Tax in the initiation, but not in the maintenance of the leukemogenic process.

Such a role in the maintenance of that process may be attributed to the HBZ protein. Indeed, the proviral 3' LTR remains intact and unmethylated at all stages of ATL development (Taniguchi et al., 2005). Furthermore, a quantitative analysis of HBZ and Tax mRNA in peripheral blood from HAM/TSP and ATL patients and from healthy asymptomatic carriers indicated that the spliced *hbz* gene was transcribed in all the infected individuals tested. In contrast, *tax* mRNA was not expressed in significant number in the same groups. (Usui et al., 2008). The proviral-adjusted (mRNA loads divided by the proviral load) HBZ and Tax quantification revealed a characteristic imbalanced expression profile of high HBZ and low Tax expression levels in ATL cells

(Uchida et al., 1999). The maintenance of HBZ expression throughout the duration of HTLV-1-infection is of main significance during the late stages of the leukemogenic process, in which HBZ may intervene in the reactivation of telomerase (Kuhlmann et al., 2007).

b- HTLV-1 immune responses, viral latency and clinical outcome.

The host immune response against the virus and infected cells is an important actor that needs to be considered to understand the course of viral infections. This is particularly true for long-term infection. Indeed, the balance between the host immune system and the pathogen may either play a protective role or be involved in pathogenesis. In the case of HTLV-1 infection, humoral and cellular immune responses are observed in asymptomatic carriers as well as in patients. The first antibodies to be produced target the Gag proteins, followed by the Env proteins and later on Tax (Manns et al., 1991). Interestingly the antibody titer correlates with the PVL (Nagai et al., 1998). Moreover, Tax and HBZ-specific T-cell responses either provide an efficient immune surveillance or are of bad prognosis. It further appears that the role of the immune response in controlling the infection is very important as evidenced by the rapid onset of ATL after immuno-suppressive treatment of transplanted patients (Kawano et al., 2006). Another evidence of the influence of this immune response against the virus is the switch from viral replication involving reverse-transcription to clonal proliferation that is concomitant with the onset of the immune response (Mortreux et al., 2003; Mortreux et al., 2001a). Therefore the survival of infected cells is dependent on their escape from the immune system. Furthermore viral and cellular factors are instrumental in reducing viral transcription, thus maintaining a viral latency state. Clearly, the interplay of several parameters (immune and non-immune) should be considered to understand the clinical outcome of HTLV-1 infection.

i. T-cell responses

Although an efficient immune response against HTLV-1 occurs in infected patients, we observed a persistent infection that can last for decades and is never fully eradicated. The equilibrium between host immunity and viral replication is complex and several mechanisms may help the virus in this arms race. The outcome of this equilibrium is of crucial importance as it was demonstrated that patients with a higher PVL are more sensitive to the development of HTLV-1 associated diseases (Nagai et al., 1998). Initially, it was hypothesized that HTLV-1 was transcriptionally silent *in vivo*, allowing infected cells to escape from the immune response (reviewed in (Asquith et al., 2000)). However, the detection of anti-HTLV-1 T-cell responses as well as the use of more sensitive assays have ruled out this hypothesis by showing that viral proteins are expressed *in vivo* (Bangham, 2000, 2003a, b).

Most HTLV-1-infected individuals mount a large and chronically activated $CD8^+$ T-cell response to HTLV-1, with both protective (Asquith and Bangham, 2008) and harmful effects (Jacobson, 2002). Tax, which is one of the first viral proteins to be expressed, has been shown to be the immuno-dominant HTLV-1 antigen for the CTL response (Goon et al., 2004). Thus, a Tax specific $CD8^+$ cytotoxic T-cell response in HTLV-1-infected individuals is particularly effective in viral control by reducing PVL and disease risk. The presence of a specific $CD8^+$ T cell response has been observed in infected patients. Indeed, upon *ex vivo* culture of PBMC obtained from one HTLV-1-infected individual, few $CD4^+$ T cells are expressing Tax *in vitro*, but the percentage of Tax-expressing cells greatly increases, when PBMC are depleted of $CD8^+$ T cells before being cultured, underlining the strict surveillance *in vivo* of Tax-expressing cells by Tax-specific $CD8^+$ T cells (Hanon et al., 2000). The selective pressure exerted by the immune system on Tax-

expressing cells is higher in asymptomatic carriers and to a lesser extent in HAM/TSP patients (Niewiesk and Bangham, 1996).

The observation that the recently discovered antisense protein HBZ was notably found to be always expressed *in vivo* have led to an evaluation of T-cell responses to that viral protein (Saito et al., 2009). Thus, the $CD8^+$ and $CD4^+$ T cell responses to HBZ were recently examined and compared with Tax-specific T-cell responses (Hilburn et al., 2011). To that aim, PBMC from 10 HAM/TSP patients (high proviral load) and from 20 asymptomatic carriers (low proviral load) were stimulated with synthetic HBZ and Tax peptides. Then, enzyme-linked immunosorbent spot (ELISpot) assays were used to detect IL-2- and IFN-γ-secreting T cells. HBZ-specific $CD4^+$ and $CD8^+$ T-cell responses were detected, implying expression of this protein *in vivo* both in asymptomatic carriers and in HAM/TSP patients. As expected, Tax-specific $CD4^+$ and $CD8^+$ T-cell responses (IL-2 and IFN-γ) were also detected with a high frequency compared with HBZ-specific T cells. As expected, the presence of a $CD8^+$ T-cell response to Tax (IL-2 or IFN-γ) was associated with HAM/TSP. Conversely, low viral load and asymptomatic HTLV-1 status were found to be associated with IL-2-secreting $CD8^+$ T cells specific for HBZ. Specifically, the IL-2 production appears to be correlated with a protective response

Next, experiments were performed to identify and quantify the contribution of HLA class I alleles to host protection against HTLV-1 in a cohort of HTLV-1-infected individuals (Macnamara et al., 2010). After using an epitope prediction software, it was found that peptides from HBZ bound to two protective class I alleles significantly more strongly compared to a known detrimental allele. Indeed, HTLV-1 infected individuals whose HLA class I genotype predisposed them to bind HBZ peptides strongly had a significantly lower proviral load, thus implying that strong binding of HBZ peptides is associated with a reduced risk of HAM/TSP and a reduced PVL. Furthermore, an HBZ-specific CTL clone

able to lyse lymphoblastoid cells loaded with HBZ peptide, but not cells from an ATL was used (Suemori et al., 2009). This $CD8^+$ T-cell clone was able to kill naturally infected cells from asymptomatic patients and from HAM/TSP patients. Despite a binding affinity of HLA molecules to HBZ weaker than that of Tax peptides and a frequency lower than that of Tax-specific CD8+ T-cells, the HBZ-specific T-cell response appears to be protective in a majority of patients, the HBZ-specific T-cell response is more protective than the Tax-specific T-cell response, leading to a better control of HTLV-1 infection and viral persistence. These results indicate that the antigenic immunodominance is not linked to a more protective CTL response against the onset of clinical pathologies.

However, it seems that at some point, viral proteins have to be expressed to promote cell-cycling and long-term proliferation of infected cells (Asquith and Bangham, 2008). A model hypothesizes that infected cells could express for a short period of time viral proteins inducing the proliferation mediated by Tax. Some cells expressing Tax would be depleted by the strong anti-Tax CTL response, but most of them could switch back to a low expression profile, hidden from the immune surveillance (Figure 8) (Asquith and Bangham, 2008).

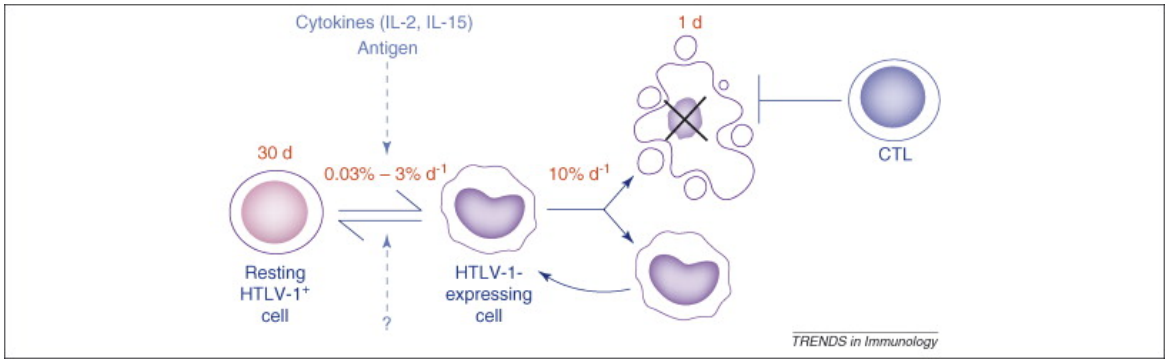

Figure 8 – Alternative model of persistence by Asquith and Bangham (Asquith and Bangham, 2008)

The survival of HTLV-1 infected T cells requires that they escape the adverse effects of the immune response. Several mechanisms that can be involved in this escape have been described.

ii. Induction of viral latency

One way to escape the immune system might be the infection of HP/HSC residing in the immuno-privileged bone marrow. These cells could serve as a viral reservoir for the HTLV-1 infection (Banerjee et al., 2010a). However this mechanism does not explain the persistence of infected clones in HTLV-1-infected patients for several years. A low level of proviral expression provides another way to remain unseen by the immune response. Particularly, as Tax seems to be the immuno-dominant protein, its expression has to be kept under control. Several mechanisms have been described to limit the viral expression. Mutations in the pX region, as well as deletion and total or partial hypermethylation of the 5'-LTR resulting in the inhibition of viral transcription have been reported (Taniguchi et al., 2005).

In addition, the HBZ protein that is expressed independently of other HTLV-1 genes has been demonstrated to play an inhibitory effect on the viral expression and especially on Tax (Gaudray et al., 2002). By limiting Tax expression, one of the major targets of the immune response, this mechanism could prevent the detection of the infected cells. Other similar mechanisms could be involved. The accessory p30 protein is able to retain *tax* and *rex* transcripts in the nucleus limiting the viral expression. An accessory viral protein, p12 can also bind to the heavy chain of MHC-I preventing its membrane exposure and targeting it to degradation. This mechanism could therefore interfere with the viral epitope presentation helping the infected cells to escape the immune response (Johnson et al., 2001). Interestingly, infections using molecular clones mutated for p12, p30 or HBZ have demonstrated an impaired infectivity and persistence potential *in vivo* in a rabbit model (cf. p60) highlighting their importance in the persistence of the infected cells.

iii. HTLV-1 induced immuno-suppression

As evidenced by the presence of anti-Tax and anti-HBZ CTL response, a fierce battle must take place between the infected cells and the immune system. One way to favor the results of a battle for yourself is to weaken your opponent. Interestingly, the infection with HTLV-1 and especially in the patients with ATL is often associated with an immune suppression resulting in a less reactive immune system (Welles et al., 1994) that can lead to associated infections (Gotuzzo et al., 1999). The number of naive T cells is decreased in infected patients (Yasunaga et al., 2001) and ATL cells produce immunosuppressive cytokines (Mori et al., 1996). HTLV-1 infection can also result in the alteration of the normal response of dendritic cells (DC). Indeed, it has been observed that the plasmacytoid DCs (pDC), a major type-1 interferon (IFN) producer, obtained from asymptomatic carriers exhibit an impaired IFN-α producing capacity inversely correlated with the PVL. In addition the absolute number of DC in ATL patients was found to be low (Hishizawa et al., 2004). It was also observed among HTLV-1 carriers that FoxP3$^+$ Treg cells were found in an increased number. This population possesses an immuno-modulatory effect and was demonstrated to suppress the CTL lysis directed against HTLV-1 infected cells (Bangham and Toulza, 2011).

This immuno-suppression may result in the observed co-infection but also weaken the immune response against infected cells. The equilibrium between the immune response and the infected clones is of crucial importance as it controls the development of an HTLV-1 associated diseases.

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III] Animal Models of HTLV-1 infection and pathogenesis: From non-human primates to humanized mice

Viral infections follow a course with discrete stages defined by critical virus-host interactions that determine the clinical outcome. Viruses gain entry to their hosts, multiply at that site and spread through the bloodstream and lymphatics to reach organ systems, where further replication may cause sufficient pathologic damage to result in severe disease or death. More frequently, innate and adaptive immune defenses limit viral replication, spread and adverse pathologic consequences to such an extent that infection is clinically unapparent or manifest as an acute, self-limited illness.

The enduring questions in viral pathogenesis follow from this conceptual framework: 1) in the course of viral infection, where does a virus go? 2) How does it get there? 3) If there is injury to an organ system, what is the mechanism? 4) If infection is persistent, how does the virus elude host defenses? Experimental approaches to answer these questions have evolved from tracing the trail of histo-pathological changes left by a virus, to quantitative assays of infectious virus in tissue culture, to modern methods of measuring viral genomes, mRNAs and proteins. *In vitro* systems are valuable to dissect intracellular and cellular mechanisms involved but fail to provide a good system to approach the complexity of a pathogen. Experimental infection of laboratory animals under controlled conditions provides a wealth of information about the pathogenesis of specific viral agent.

Only an *in vivo* animal model can provide the multi-parameter environment required for the understanding of such a complex system. The complex and prolonged pathogenesis of disease following HTLV-1 infection presents unique challenges to those seeking to develop animal models of HTLV-1–associated lesions or preventive and curative therapeutic interventions. There is not a universal animal model that can be used for

every human pathogen. Instead several species are used and have to be tested to find the appropriate model to study a given infectious agent. We can nevertheless find few parameters that are of particular importance for the development of all animal models: 1) the animal model tested should be sensitive to the infection of the pathogen under scrutiny. Ideally the infected animals should develop similar symptoms as observed in human infection; 2) such a model has to be relatively inexpensive and easy to handle.

The complexity of human infectious diseases cannot be fully understood without taking into account the multiple parameters involved in controlling the relationship between the pathogen, the host and its immune system. Developing an animal model to study HTLV-1 infection and pathogenesis has proven to be a difficult task. Because of the limited tropism of HTLV-1, an alternative to dissect *in vivo* the involved mechanisms could be represented by studying several species naturally infected by close viruses. Thus, *in vivo* modeling may rely on other related deltaretroviruses, such as STLV or BLV, that reproduce similar characteristics of HTLV-1 infection, in non-human primates or in cattle, respectively. However, the specificity of *in vivo* HTLV-1 mechanisms is restricting the use of these non-human hosts. Likewise valuable, but limited observations on essential parameters of HTLV-1 infection and pathogenesis have been obtained in rabbits, rats and mice, either infected, immunodeficient or genetically modified. For these reasons, much emphasis has recently been placed on the so-called “humanized mice”, as evidenced by numerous investigations on the replication and pathogenesis of several human viruses, performed in these animal models.

1) BLV in sheep

Bovine Leukemia Virus (BLV) is a closely related virus of the deltaretrovirus subfamiliy. HTLV-1 and BLV share a high genome homology, the same complex provirus organization, similar transmission routes and can infect and transform cells of the

hematopoietic system (Willems et al., 2000). However, unlike HTLV-1 or STLV-1, BLV induces B-lymphocyte transformation instead of T-lymphocyte, which is a major difference.

BLV naturally infects cows and, in ~5% of the infected animals, induces lymphomas or lymphosarcoma affecting spleen, liver, heart, eye, skin or lung. Although sheep is not a natural reservoir of BLV, it can be experimentally infected by BLV to provide a good animal model. Indeed, in infected sheeps, the frequency of observed pathologies is much higher than in infected cows and has been shown to occur after a shorter latency period. However, several differences can be observed between sheep and cattle. The proviral evolution is notably different as the PVL in sheep gradually rise to reach a leukemic stage whereas in most of infected cattle the PVL reach a plateau where most of the animals stay asymptomatic.

This model of development of a leukemia induced by a HTLV-1- related virus is easy to manipulate as sheep can be infected by intradermal injection of proviral DNA mixed with cationic liposomes (Willems et al., 1992). This technique allows characterization of different mutants to determine the role of the different viral proteins in the infectivity and pathogenicity of the virus (Willems et al., 1995; Willems et al., 1997; Willems et al., 1993) and reviewed in (Willems et al., 2000). This model which reproduces a faithful pathogenesis, addresses the three important steps from the infection to the onset of leukemia: 1) the replication of the virus in the sheep follows a two-step course similar to the one observed in NHP infected with HTLV-1 (Pomier et al., 2008); 2) the immune system response against BLV plays an important role in the development of leukemia (Florins et al., 2009; Van den Broeke et al., 2010); 3) it also brings interesting insights in new therapeutic approaches for HTLV-1 associated diseases (Lezin et al., 2009).

The BLV-infected sheep combine several advantages as it reproduced the apparition of a leukemia induced by the infection by a closely HTLV-1 related virus. However it does not reproduce the whole panel of diseases associated with HTLV-1 and has major differences with its human counterpart as the neoplasia observed is of B cells and not T cells.

2) STLV-1 in Non Human Primates (NHP)

Several field studies have reported that NHP are infected by another virus closely related to HTLV-1, named STLV-1 (Simian T-cell Leukemia Virus Type 1). Such a high homology exists between these two viruses of the same subfamily that antibodies in those animals are recognizing HTLV-1 viral proteins (Homma et al., 1984; Kanki et al., 1985). Macaques of several species (Ishida et al., 1985) as well as baboons (Takemura et al., 2002), chimpanzees (Junglen et al., 2010), mandrills (Souquiere et al., 2009b), gorillas (Masters et al., 2010) and orangutans (Ibuki et al., 1997b) were found to be susceptible to the infection by HTLV-1 simian counterpart.

The replication of STLV-1 in naturally infected NHP appears to be similar to the one observed in HTLV-1 as it involves clonal proliferation (Gabet et al., 2003) and results in the development of malignant lymphomas (Graves et al., 2009; Homma et al., 1984; Kanki et al., 1985).

Although some experiments have been carried out to assess the relation between host and virus (Souquiere et al., 2009a; Souquiere et al., 2009b) and to investigate original therapeutic treatments (Afonso et al., 2010), the work on these infected animals is subject to costs and ethical limitations that prevent their wide usage.

3) HTLV-1 in NHP

NHP are sharing a large part of the human genome. Many diseases affecting humans can be seen in NHP and often with similar symptoms, as it is the case with STLV-1. This

explains why NHP are involved in many studies to test candidate vaccines or drugs and assess their safety and efficiency before clinical trial involving humans (Levenbook, 2011). Several species of NHP are susceptible to HTLV-1 infection performed by injection of HTLV-1-producing cells, such as the MT-2, Ra-1, a rabbit-derived infected cell line or autologous PBMC infected *ex vivo* by coculture with HTLV-1 producing cells (Miyoshi et al., 1984; Nakamura et al., 1986; Yamamoto et al., 1984). Among those species, squirrel monkeys have proven to be a convenient model as, upon infection, they produced antibodies against HTLV-1 at a high titer and stayed infected over a long period of time (Nakamura et al., 1986). The provirus is detected in several lymphoid organs (PBMC, spleen and lymph nodes) as early as 12 days after infection, at which time, *tax/rex* transcripts were detected (Kazanji, 2000). Few weeks after inoculation, the viral protein expression decreases in correlation with the apparition of an immune response against HTLV-1 viral proteins (Mortreux et al., 2001a). Three weeks after infection, antibodies against HTLV-1 can be detected and anti HTLV-1 CTL activity are detected 2 months after infection and thereafter (Kazanji, 2000). Their proximity to humans makes NHP a good model to study potential vaccines and this particularity was used in the case of HTLV-1 with promising results (Ibuki et al., 1997a; Kazanji et al., 2001; Nakamura et al., 1987). Passive immunization obtained by injection of immunoglobulins prepared from asymptomatic carriers was also tested with encouraging results (Akari et al., 1997; Murata et al., 1996).

The replication process of HTLV-1 follows a two-step model. During the first step of infection, the virus replicates through reverse transcription. This step is lasting until the apparition of immune response against HTLV-1. Thereafter clonal expansion of infected cells is seen in the infected animals and is responsible for the maintenance of infected cells (Mortreux et al., 2001a). Other species of NHP can support infection by HTLV-1 and

especially macaques like Cynomolgus monkeys (Murata et al., 1996; Nakamura et al., 1986; Yamamoto et al., 1984). In rhesus macaques, the infection has been associated with development of arthritis, uveitis and polymyositis (Beilke et al., 1996).

NHP are important to study the infection and immunological processes involved in the HTLV-1 control but are not a suitable model to study the pathogenesis associated with HTLV-1 and especially ATL-like disease. Indeed diseases are seldom and obtained after a very long infection time, which, combined to the important costs and ethical limitations associated with NHP, cannot be achieved routinely. The use of a small animal model can answer some of these limitations.

4) HTLV-1 in rabbits

Rabbits were the first animals used for *in vivo* modeling of HTLV-1 (Akagi et al., 1985; Lairmore et al., 1992). Rabbit cells can be infected and transformed *in vitro* by HTLV-1 infection as demonstrated by the generation of the rabbit lymphocyte Ra-1 cell line, derived from co-culture with MT-2 cells. This cell line is infectious and is able to infect naive rabbits (Miyoshi et al., 1985). Inoculation of HTLV-1 infected cells into rabbit results in a persistent infection evidenced by the detection of integrated provirus in the PBMC for at least 90 weeks after inoculation. A sero-conversion occurs as early as 3 weeks after inoculation and antibodies against several viral proteins were detected for several months (Cockerell et al., 1990). Rabbits were mainly used to study the different routes of transmission. It notably demonstrated that the virus could be transmitted through blood transfusion (Kotani et al., 1986), oral route (Uemura et al., 1986) and through sexual intercourse, as the semen of an infected healthy man is able to infect rabbits (Iwahara et al., 1990). This model was also used to ascertain the mother-to-child transmission by milk: infected females are able to infect their progeny after birth (Hirose et al., 1988; Uemura et al., 1987). Breast milk from infected healthy women was

also demonstrated to be infectious for naive rabbits (Iwahara et al., 1990). The rabbit model was also used to study the minimal quantity of infected cells required for an infection (Kataoka et al., 1990) and the difference of infectivity of virus coming from different patients with different diseases (TSP/HAM, ATL, asymptomatic carriers) (Lairmore et al., 1992).

Wild type and mutated molecular clones were used to infect rabbits. It demonstrated the capital roles of accessory proteins such as p12 (Collins et al., 1998), p13 or p30 (Bartoe et al., 2000; Silverman et al., 2004) as well as the necessity of Rex (Ye et al., 2003) in the infectivity and persistence of the virus. More recently, the importance of HBZ has also been demonstrated (Arnold et al., 2006). Interestingly, the mutated molecular clones did not show any difference in the transformation of primary PBMC *in vitro* highlighting the necessity of an animal model to study the viral infection.

As infected rabbits are producing an immune response against HTLV-1, they have been used to characterize the epitopes of infected cells and to test immunization with synthetic peptides in order to produce potential neutralizing antibodies or antibodies eliciting antibody-dependant cellular cytotoxicity (Chen et al., 1991; Lal et al., 1991; Tanaka et al., 1991). After initial failure to produce a protective immune response by injecting heat-inactivated HTLV-1 or a synthetic Env peptide (Takehara et al., 1989), potentially efficient epitopes were found (Conrad et al., 1995; Tanaka et al., 1994). Another way to prevent a new infection through blood transfusion or mother-to-child contamination was the use of passive immunization using immuglobulins coming from healthy infected individuals (Kataoka et al., 1990; Miyoshi et al., 1992; Sawada et al., 1991).

No ATL-like diseases have been obtained in infected rabbits unless a very large amount of HTLV-1 transformed cells was injected resulting in infiltration of these leukemic cells

and death of the rabbit very quickly after inoculation (Ogawa et al., 1989; Seto et al., 1988). Other groups have scarcely reported diseases observed in rabbits, several years after infection, including uveitis (Taguchi et al., 1993), cutaneous lymphoma (Kindt et al., 2000; Simpson et al., 1996) and thymoma (Zhao et al., 2002).

Infected rabbits were used to follow the temporal and spatial events of HTLV-1 infection. The primary target cells of HTLV-1 *in vivo* are recruited among lymphocytes located in both primary lymphoid and gut-associated lymphoid compartments (Haynes et al., 2010b). The kinetics of the expression of the viral genes studied in newly infected rabbits has shown an early transient but strong transcription of *tax/rex* and *gag/pol* genes. These genes are highly expressed in lymphocytes one week post-infection. Their expression level then decreases to reach a plateau. HBZ expression starts later and increases slowly over time. The initial expression of the *tax/rex* transcript is inversely correlated with the observed PVL in the infected rabbits. That might be due to the strength of the immune response developed at that early stage of infection. On the opposite, the *hbz* level positively correlates with the PVL (Li et al., 2009). The study of the very initial steps of the infection is relevant to design potential first-line treatment for infected persons. When animals were treated 10 days before infection with an immunosuppressive drug (Cyclosporine A – CsA), an elevated PVL is observed after infection, highlighting the crucial role of the immune response during the very first steps of infection. Interestingly, when animals are treated 10 days post-infection, it results in a lower PVL (Haynes et al., 2010a).

In summary, infected rabbits that mostly mimic asymptomatic carriers are useful to study the early steps of the infection, the importance of the immune response in the infection and of the viral factors involved in the infectivity and *in vivo* persistence of the virus.

5) HTLV-1 in rats

A few years after the infection of rabbits, rats were successfully inoculated with HTLV-1 producing cell lines (MT-2). This inoculation led to the detection of antibodies directed against HTLV-1 and of the integrated provirus in PBMC. The rats remain chronically infected for more than a year (Suga et al., 1991) even if studies have shown that the infection tends to decrease over time and that not all rats strains are susceptible to the infection (Ibrahim et al., 1994). It appears that HTLV-1 replicates poorly in rats and this might be due to the rat version of CRM1, a cofactor required for the activity of Rex, which has a low ability to help Rex in comparison to its human counterpart (Hakata et al., 2001; Zhang et al., 2006). Using a transgenic rat for human CRM1 allows HTLV-1 to replicate more efficiently (Takayanagi et al., 2007).

The use of the convenient strain allowed important studies of the infectivity of HTLV-1 *in vivo*, particularly concerning the importance of the different routes of infection as well as time of infection. Administration of infected cells by oral, intraveinous (iv) or intraperitoneal (ip) routes all resulted in the infection of adult rats (Kannagi et al., 2000) but with different immune response against HTLV-1. Orally infected rats, thus mimicking the mother-to-child infection through breast-feeding, did not exhibit a humoral immune response against HTLV-1 (Kannagi et al., 2000; Kato et al., 1998). Associated with this immune unresponsiveness, orally infected rats have a higher PVL than iv- or ip-infected rats, probably due to the absence of immune response to control the viral replication (Hasegawa et al., 2003). This is confirmed by the fact that re-immunization of orally infected rats with HTLV-1 leads to the development of anti-HTLV-1 immune response and induces a decrease of their PVL (Komori et al., 2006). Interestingly, this seronegativity has also been described when rats are infected as newborns (Ishiguro et al., 1992), whereas rats infected as adults stay seropositive for an

extended period of time. Taken together these observations could explain the relatively low immune response to HTLV-1 observed in patients infected during their early childhood via breast-feeding.

Several months after infection, a specific rat strain (Wistar-King-Aptekman-Hokudai WKAH) develop a chronic progressive myeloneuropathy with spastic paraparesis of the hind limbs associated with demyelination and mononuclear cells infiltration in the affected regions (Ishiguro et al., 1992; Kushida et al., 1993). However, unlike in HAM/TSP patients, infiltration of T cells is not observed in the rats' lesions (Yoshiki, 1995). It appears that the apoptosis of oligodendrocytes and Schawnn cells is the first event to take place, leading to demyelination, followed by an infiltration of macrophages (Ohya et al., 1997).

Like for the rabbit model, ATL-like diseases are not observed in the natural course of infection, but can be reproduced by inoculating HTLV-1 immortalized cell lines (Ohashi et al., 1999). This model has been used to examine methods of protection against tumor development (Hanabuchi et al., 2001; Kannagi et al., 2000; Nomura et al., 2004).

To study more closely the role of a given viral protein, transgenic rats were designed. Many transgenic strains focused on the role of the Tax protein. Rats expressing the *env* gene together with the pX region under the control of the HTLV-1 LTR develop arthropathies and is a good model for collagen vascular diseases, including arthritis (Yamazaki et al., 1997). T cells of these rats that express a high level of activation markers are hyper-responsive and are involved in the disease onset (Nakamaru et al., 2001). In addition, alterations in the progenitor development of osteoclast and B-cell lineages were observed prior to the apparition of these diseases highlighting the effect of HTLV-1 on the hematopoietic development (Yamazaki et al., 1998).

Rats expressing only the pX region under the control of the promoter of H-2kd MHC-I develop mammary carcinomas (Shikishima et al., 1997) whereas when the Tax expression is restricted to developing thymocytes by the use of the proximal lck promoter, they develop thymomas (Kikuchi et al., 2002). Similar results were obtained in transgenic mice model (cf. p67) (Hasegawa et al., 2006). The cells composing the tumors are from epithelial origin and develop in the medulla of the thymus (Kikuchi et al., 2002). Surprisingly, transplantations of cells from transgenic rats into the bone marrow of wild-type rats indicates that the tumorigenic cells come from the bone marrow and evolved into cells with epithelial characteristics in the thymus (Tsuchikawa et al., 2004). Those models confirm the oncogenic properties associated with the pX region and probably due to the Tax expression.

6) HTLV-1 in mice

Mice can be infected by HTLV-1 as evidenced by the detection of provirus integrated in the murine genome of newborn mice inoculated with MT-2 cells (Fang et al., 1998; Feng et al., 1999; Kushida et al., 1997; Tanaka et al., 2001). The provirus was found integrated in $CD4^+$ T cells as well as in $CD8^+$ T cells, B cells and granulocytes (Feng et al., 1999) and detected in different organs such as thymus, lungs, spleen, lymph nodes and kidneys (Fang et al., 1998). However, provirus integration was described in only 20-30 % of inoculated animals (Fang et al., 1998), no antibodies were found against HTLV-1 (Fang et al., 1998; Kushida et al., 1997) and accordingly no expression of the viral proteins has been detected (Fang et al., 1998). These results were obtained by inoculating newborns. When infected as adults, an antibody response against HTLV-1 can be detected, but the infection and the immune response observed is heterogeneous between different strains, underlining the influence of the genetic background (Nitta et al., 2003). All these

experiments were carried out in immuno-competent mice and no disease developed after the inoculation of infected cells.

Taken together, these results indicate that wild type strains of mice have a limited potential to be considered as an efficient HTLV-1 *in vivo* model. Grafted mice were therefore used to study different aspects of HTLV-1 infection and pathogenesis.

a- Graft of infected cells in immunodeficient mice

Immuno-deficient mice engrafted with leukemic cells were used to determine their proliferative and tumorigenic properties. Inoculation of MT-2 cells into SCID mice leads to the formation of tumors at or near the point of inoculation without invasion of the mouse organs (Ishihara et al., 1992). Inoculation of PBMC from HAM/TSP, ATL or asymptomatic patients resulted in the chronic infection of the mice and the infected clones gained proliferative advantage over uninfected ones. The injection of ATL cells resulted in the apparition of lymphoblastic lymphomas (Feuer et al., 1993). Interestingly, only cells coming from leukemic patients were able to induce the formation of lymphomas and none of the cell lines immortalized *in vitro* demonstrated a tumorigenic potential *in vivo* (Feuer et al., 1993; Imada et al., 1995). These results highlight the fact that *in vitro* immortalization does not reproduce fully the *in vivo* process observed in patients over decades. This was also suggested by the differences observed in the rabbit model in which virus defective for accessory proteins or HBZ were still able to immortalize cells *in vitro* but were impaired for *in vivo* infectiousness and persistence (Bartoe et al., 2000; Silverman et al., 2004). These results were confirmed in more strongly immuno-suppressed mice such as SCID/beige (SCID/bg) or non-obsese diabetic/SCID (NOD/SCID) (Liu et al., 2002; Richard et al., 2001). Interestingly, the tumorigenic process in SCID/bg mice was associated with the

development of humoral hypercalcemia, which is often observed in ATL patients (Richard et al., 2001).

This protocol provides a pertinent *in vivo* model of the late leukemogenic process associated with HTLV-1 and has been used to test therapeutic drugs, such as NF-κB inhibitors (Dewan et al., 2003), proteasome inhibitors, without or with anti-CD25 antibody (Tan and Waldmann, 2002), and various monoclonal antibodies (Zhang et al., 2003a; Zhang et al., 2003b). However, this model does not allow the study of the initial steps of the leukemic process. The relationship between pathogen and the immune system of the host is also unobservable.

b- Transgenic mice

The role of viral factors was also investigated using transgenic mice in which viral genes were inserted into the murine genome under the control of different promoters to study their potential effect on the leukemogenesis.

The first transgenic mice models were developed focusing on Tax, as the wide range of Tax action is thought to be crucial during the first steps of the HTLV-1 infection to promote diseases that develop several decades later. Tax was first inserted under the control of the HTLV-1 LTR (Nerenberg et al., 1987). Its expression was detected in different organs: Tax was highly expressed in skeletal muscles, expressed at a lower level in the thymus and stomach and at an even lower level in most of the other organs (Nerenberg et al., 1987). Mating LTR-Tax mice with transgenic mice expressing the β-galactosidase gene under the control of the HTLV-1 LTR resulted in double-transgenic mice, in which the transactivating effect of Tax on the LTR could be monitored by the expression of the β-gal. It was detected in bone, muscle, cartilage, exocrine glands and mesenchemial tissue (Bieberich et al., 1993). Interestingly the LTR-Tax mice were associated with different pathologies such as: thymic involution and early death at the

age of 3-6 weeks due to secondary infections (pneumonia) (Nerenberg et al., 1987), mesenchemial tumors developing several months after birth especially on the tail, ears, nose, mouth and foots of the mice (Nerenberg et al., 1987), degeneracy of oxidative muscle fibers leading to severe progressive atrophy similar to that observed in human HTLV-1-associated myelopathies (Nerenberg and Wiley, 1989), alteration of bones metabolism with increased bone turn over, osteoclast and osteoblast number and activity, and myelofibrosis (Ruddle et al., 1993), salivary and lacrimal exocrinopathy (that could be compared to Sjögren's syndrome, a disease with an auto-immune origin) (Green et al., 1989), Rheumatoid arthritis-like inflammatory arthropathy (Habu et al., 1999). Interestingly, every tissue in which Tax was found to be active by the expression the LTR-β-gal (Bieberich et al., 1993) can be affected by pathologies, thus emphasizing the potent effect of Tax to deregulate normal cell physiology.

As, in infected patients, the preferential targets for HTLV-1 are lymphocytes, transgenic mice were engineered in which Tax expression controlled by the Granzyme B promoter is therefore restricted to activated $CD4^+$ and $CD8^+$ T cells, NK cells and lymphokine-activated killer cells (Heusel et al., 1991). These mice developed tumors on the ears, legs and tails and develop Large Granular Lymphocytic Leukemia associated with hepato-splenomegaly (Grossman et al., 1995), hypercalcemia and osteolytic bone lesions (Gao et al., 2005), as many symptoms observed in ATL patients. The apparition of these lesions is accelerated when this transgenic strain is mated with an interferon-γ knockout strain (Mitra-Kaushik et al., 2004) opening the way to understand the role of immunity in the development of tumors induced by Tax. A similar approach was used to study the tumor progression in p53 deficient mice model. In this model, the initial kinetics of tumor formation is unaltered but disease progression and death are significantly accelerated suggesting that the functional inactivation of p53 by Tax is not critical for initial tumor

formation, but rather contributes to late tumor progression (Portis et al., 2001). Recently, transgenic mice were developed in which, in addition of Tax under the control of Granzyme B promoter, a luciferase gene was introduced under the control of the HTLV-1 LTR, which is activated in the presence of Tax. This model allows the non-invasive imaging of tumor progression *in vivo*. Using this powerful model, it was observed that microscopic intraepithelial lesions precede the onset of peripheral subcutaneous tumors (Rauch et al., 2009).

In order to control the expression of Tax even more drastically, Tax was inserted under the control of the proximal lck promoter, which restricts its expression to developing thymocytes. After a prolonged latency period, these mice develop diffuse large-cell lymphomas and leukemias with features characteristic of acute ATL. The leukemic cells are of the pre-T double negative ($CD3^-CD4^-CD8^-$) phenotype and express activation markers. Injection of these leukemic cells in naïve SCID mice resulted in their rapid death due to proliferation of cells with multi-lobulated nucleus reminding ATL 'flower cells' (Hasegawa et al., 2006). The development of leukemias and lymphomas was also described in transgenic mice where Tax expression was under the control of the distal lck promoter, which restricts its expression to mature T cells (Ohsugi et al., 2007). In this case, mature $CD4^+$ or $CD8^+$ T cells were found among the leukemic cells. Two years after birth, these transgenic mice develop severe arthropathies (Ohsugi and Kumasaka, 2011). These models were the first to recapitulate *in vivo* ATL-like diseases *de novo*. As such, they were used to obtain "ATL cells" that could be transplanted in naive SCID mice, in order to test promising therapeutic strategies based on a combination of Arsenic and IFN-α (El Hajj et al., 2010).

The evaluation of Tax mutants lacking the capability to activate the NF-κB or the CREB pathways was performed in transgenic mice in which the expression of the mutated Tax

was restricted to the lymphocytes compartment. It was observed that when the capacity of Tax to activate NF-κB was unaltered, transgenic mice developed dermatologic diseases characterized by infiltration of Tax positive T cells into the dermis and epidermis. The presence of Tax was absolutely required as its inhibition by the induction of a tet-off system led to the complete remission of the observed lesions (Kwon et al., 2005).

Altogether, these results underlined the crucial tumorigenic potential of Tax and the importance of its well-regulated expression at a precise stage of the T-cell development to promote T-cell leukemias or lymphomas. Recently, transgenic mice in which the expression of the antisense viral protein HBZ was restricted to $CD4^+$ cells was characterized by an increase of the number of $FoxP3^+$ Treg cells and effector/memory T cells, associated with the development of T-cell lymphomas and systemic inflammatory diseases. Considering that HBZ, unlike Tax, is expressed in all the ATL cases, these results emphasize the critical and underestimated role played HBZ in the pathogenesis associated with HTLV-1 (Satou et al., 2011).

Among the different traditional animal models developed to study HTLV-1 infection or pathogenesis, one of the following limitations are often observed:

- The animal can be infected and reproduce a chronic infection but no associated diseases nor ATL-like diseases are observed,
- The animal develops relevant diseases but they are obtained by transgenic manipulation of the animal genome resulting in an oversimplified model of the HTLV-1 infection and pathogenesis,
- Ethical and cost limitations prevent the wide use of certain animal models.

Although these models allowed to gather important information on different aspects of the viral cycle and pathogenesis of HTLV-1, a faithful animal model able to recapitulate the HTLV-1 infection, the interactions of this human retrovirus with host factors and immune response, and the development of associated pathologies is still lacking.

c- Humanized mice

A review (written in French) on that issue has been accepted for publication by Médecine/Sciences (see p109). Here, a short digest of that review is presented, supplemented with very recent reports.

The possibility to engraft and develop human hematopoietic cells in a mouse requires the absence of rejection of these human cells and therefore a lack of murine immune response. In 1966, the nude mice were the first immuno-compromised mice to be described (Flanagan, 1966). The transplantation of human hematopoietic cells were realized in these mice but failed to engraft (Ganick et al., 1980). The story of humanized mice really began in 1983 with the discovery of the severe combined immunodeficiency mutation $Prkdc^{scid}$ SCID in CB-17 mice (Bosma et al., 1983) and with the pioneering work of McCune and Mosier, who have transferred human peripheral blood mononuclear cells (PBMC or PBL) or implanted with human fetal thymus (thy) and human fetal liver (liv) (McCune et al., 1988; Mosier et al., 1988). These hematolymphoid hu-PBL-SCID and hu-thy/liv-SCID mouse models have opened the way to extensive studies of the development and function of the human immune and hematopoietic systems. These humanized or HIS (Human Immune System) mice also provided valuable tools to document infection of pathogens that mainly replicate in human immune and hematopoietic cells. In particular, Human Immunodeficiency Virus Type 1 (HIV-1) infection of such humanized mice has yielded valuable data ranging from the fields of *in vivo* pathogenesis to drug efficacy and passive immunity. However, in these humanized

mice, HIV-1 infections were often short-term, providing data only on acute infection and no primary adaptive immune response was mounted against HIV-1 (Aldrovandi et al., 1993; Jamieson et al., 1996; Mosier, 1996; Mosier et al., 1991). Indeed, the majority of human cells engrafted were T cells, with few B cells, myeloid cells and natural killer (NK) cells. Furthermore, these humanized mice are "leaky", spontaneously generating murine T and B cells as they age and have high levels of NK activity, both of which prevent efficient/prolonged xenoengraftment (Bosma et al., 1988; Greiner et al., 1998). Such observations have therefore led to the generation of mice with a decreased leakiness and with a diminished level of NK cells. Improvements in the available immuno-deficient mouse strains were reached with the non-obese diabetic (NOD) SCID mouse, which was commonly used because the Prkdc mutation prevents formation of mature T and B lymphocytes while the NOD mutation results in a reduction of NK cell activity (Suwanai et al., 2010). However, a major disadvantage of the NOD strain is a significative incidence of spontaneous thymic lymphomas, which results in a shortened lifespan (Prochazka et al., 1992). Another mouse model was obtained with mice deficient in the recombinase activating genes 1 and 2 (RAG1 and RAG2, respectively). They do not exhibit leaky production of murine lymphocytes (Mombaerts et al., 1992; Shinkai et al., 1992). However, RAG-deficient animals produce normal levels of NK cells, and thus additional mutations are required in order to produce animals better suited for xeno-engraftment studies.

A major technological breakthrough occurred with the creation of $RAG1^{-/-}$, $RAG2^{-/-}$, and NOD-SCID mice that harbor mutations in the common gamma chain receptor (γ_c, also referred to as the IL-2 receptor gamma chain) gene. The γ_c is a component of the IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21 receptors and is the gene involved in X-linked SCID (Sugamura et al., 1996). Consequently, lack of IL-7 signaling further blocks T and B cell

development and lack of IL-15 signaling also prevents maturation and expansion of NK cells (Ohbo et al., 1996; Tassara et al., 1995). Since SCID animals can experience leaky production of T and B cells, the γ_c mutation is useful as a secondary means to block maturation of these cells. Furthermore, the use of human $CD34^+$ HP/HSC have resulted in improved, long-term engraftment of human hematopoietic cell types as well as the ability to generate human immune responses. Combinations of the above mutations so far analyzed for HP/HSC engraftment have included such strains as Balb/c $RAG2^{-/-}\gamma_c^{-/-}$ (BRG), NOD $RAG1^{-/-}\gamma_c^{-/-}$ (NRG), NOD-SCID-$\gamma_c^{-/-}$ (NSG) (Akkina et al., 2011; Brehm et al., 2010a; Ito et al., 2002; Traggiai et al., 2004). It should be noted that the methods used to prepare human HP/HSC and humanized BRG, NRG and NSG mice are relatively straightforward (Baenziger et al., 2008). When engrafted with HP/HSC, these immuno-compromised strains bearing a γ_c mutation have shown to support much higher levels of hemato-lymphoid engraftment than all previous immunodeficient mice, in terms of the production of human immune cells (including T and B cells, monocytes, macrophages, and dendritic cells), of the reconstitution of human cells into various organs, of the duration of engraftment, and of the ability to generate primary human adaptive immune responses (Shultz et al., 2007). Recently, an analysis of the humanization of these three mouse strains has compared engraftment of human HP/HSC derived from umbilical cord blood following intravenous injection into adult mice and intracardiac and intrahepatic injection into newborn mice (Brehm et al., 2010a; Ito et al., 2008). The latter exhibited enhanced engraftment as compared to adult recipients. More interestingly, immunodeficient NOD strains supported enhanced hematopoietic engraftment compared to the Balb/c strain. Indeed, in BRG mice, human B cells develop and survive robustly, but human NK and T cells survive poorly. Thus, the main sign of disturbed human T-cell homeostasis is the low T-cell frequency in peripheral lymphoid

organs. When these mice were complemented either with human IL-7 or human MHC molecules, that both control naïve T-cell homeostasis, they exhibit enhanced human T-cell numbers but only to a limited extent. Likewise, inoculation of BRG mice with human IL-15/IL-15Ra leads to improved but still suboptimal NK-cell accumulation. The different cytokine knocked in these models have been reviewed (van Lent et al., 2010; Willinger et al., 2011)

Interestingly, as the NOD genetic background is known for its defective phagocyte activity, it was inferred that the activity of macrophages and other phagocytic cells plays a role in T and NK cell number regulation. Knowing that the phagocyte activity is inhibited by interactions of CD47 with signal regulatory protein alpha (SIRPα; CD172a), two different experimental approaches have allowed demonstrating that functional CD47/SIRPα interactions are required for optimal human T- and NK-cell homeostasis *in vivo*. The first experimental approach consisted in the introduction of CD47/SIRPα interactions in BRG HIS mice by transducing mouse CD47 into HP/HSC (Legrand et al., 2011). The second experimental approach consisted in the generation of mice that express SIRPα in BRG mice (Legrand et al., 2011; Strowig et al., 2011a). Both procedures resulted in an important and selective improvement of HP/HSC engraftment and of human T- and NK-cell homeostasis. Even if a moderate increase of B cells was observed, total plasma IgG and IgM concentrations significantly increased. Finally, an improved functionality of the human immune system was revealed in these mice, which are comparable to NSG mice. Clearly, CD47/SIRPα interaction appears as a major determinant of escape from phagocyte-mediated cell clearance.

Whatsoever, these current humanized mouse models cannot develop a robust adaptive immune response. Indeed these mice do not express HLA molecules on thymic epithelial cells and human T cells lack the ability to recognize antigen in an HLA-restricted

manner. To achieve that HLA restriction *in vivo*, an immunodeficient NSG-HLA-A2 strain was created, by backcrossing the HLA class I transgene onto the NSG background (Shultz et al., 2010; Strowig et al., 2009). Transplantation of purified human HP/HSC into NSG-HLA-A2 newborns resulted in functional $CD4^+$ and $CD8^+$ T cells expressing cytotoxic molecules and generating cytokines *in vivo*. Furthermore, these humanized NSG-HLA-A2 mice demonstrated functional HLA-restricted CTL in *in vivo* Epstein-Barr virus (EBV) or Dengue virus infection models (Jaiswal et al., 2009; Shultz et al., 2010).

Another humanized mouse model able to mount specific adaptive and innate immune responses is the BLT model (Bone marrow, Liver, Thymus). These mice were obtained after transplantation of autologous human hematopoietic fetal liver $CD34^+$ cells into NOD/SCID mice previously implanted with human fetal thymic and liver tissues (Wege et al., 2008). These mice show long-term systemic repopulation with human T and B cells, monocytes and macrophages and DCs. T cells in these mice generate human MHC class I- and II-restricted adaptive immune responses to EBV infection and are activated by human DCs to mount a potent T-cell immune response to superantigens.

Today humanized NSG mice and BRG mice represent powerful tools for investigation of human biological systems and for translational research, and more particularly for the study of human infectious diseases affecting the hemato-lymphoid system (Table 1).

Pathogen	Associated Disease	Mouse model	Reference
Viruses			
HIV-1	AIDS	BRG NSG NOG NOD/SCID BLT Rag1$^{-/-}\gamma_c^{-/-}$	(Baenziger et al., 2006) (Watanabe et al., 2007a) (Watanabe et al., 2007b) (Brainard et al., 2009) (Akkina et al., 2011)
HTLV	ATL, HAM/TSP	NOD/SCID BRG	(Banerjee et al., 2010b) (Villaudy et al., 2011a)
EBV	Burkitt Lymphoma	BRG NOG NSG NOD/SCID NOD/SCID BLT	(Traggiai et al., 2004) (Yajima et al., 2008) (Strowig et al., 2009) (Islas-Ohlmayer et al., 2004) (Melkus et al., 2006)
KSHV	Kaposi Sarcoma	NOD/SCID	(Wu et al., 2006)
Dengue Virus	Dengue	BRG NOD/SCID	(Kuruvilla et al., 2007) (Bente et al., 2005)
HSV-2	Genital Herpes	BRG	(Kwant-Mitchell et al., 2009)
HCV	Hepatitis C	BRG Fah$^{-/-}$	(Bissig et al., 2010)
HBV	Hepatitis B	BRG Fah$^{-/-}$ BRH uPa$^{-/-}$	(Bissig et al., 2010) (Dandri et al., 2001)
CMV	Cytomegalovirus infection	NSG	(Smith et al., 2010)
Bacteria			
Salmonella typhi	Typhoid fever	BRG	(Traggiai et al., 2004)

Table 1 – Infectious agents studied in humanized mice model

Results

I] Establishing HIS mice as a model to study HTLV-1 infection and pathogenensis

1) Introduction to (Villaudy et al., 2011a)

a- Rationale for the use of HIS mice in the study of infection by HTLV-1

Modeling infectious disease in an *in vivo* model is often required to fully appreciate the multiple parameters involved in the initial stages of infection leading to the development of a latency phase before the onset of a disease. To that goal, several animal models were tested in an attempt to reproduce *in vivo* HTLV-1 infection and pathogenesis (for a review see (Lairmore et al., 2005)). Unfortunately HTLV-1, a human retrovirus therefore requires a reconstituted human system to complete its infection and the associated pathogenesis. Although critical informations were obtained either from infected animals to study infectivity and persistence mechanisms of HTLV-1 or from transgenic animals to study the implication of several viral proteins, no model was satisfying to follow the whole infectious process from primo-infection to the development of a neoplasic disease *in vivo*.

Intensive work in engineering mice strains has recently resulted in new severely immuno-compromised strains able to sustain prolonged human hematopoiesis *in vivo* with higher level of engraftment upon transplantation of $CD34^+$ HP/HSC purified from human cord-blood (cf. p71). Among those strains, BALBc $Rag2^{-/-}\gamma_c^{-/-}$ (BRG) mice have been used to study different human-only infectious agents such as EBV, HIV, Dengue virus (Traggiai et al., 2004). Based on these studies, we decided to infect these BRG mice with HTLV-1.

b- Obtaining T cell development in HIS mice

The first step was to ascertain that we were able to efficiently humanize these immuno-compromised mice by injection of $CD34^+$ cells based on the method developed by

Traggiai *et al.* (Traggiai et al., 2004) (Figure 9C). Briefly, Rag2$^{-/-}\gamma_c^{-/-}$ newborns are sublethally irradiated (Figure 9B) and intrahepatically injected with 200,000 CD34$^+$ HP/HSC purified from human cord blood (Figure 9A). As pictured in Figure 9 and figure 1B in the following paper, humanization levels similar to those published in the literature were obtained in our hands. The thymus was particularly well reconstituted with human cells as early as 3 weeks after humanization (Figure 9D).

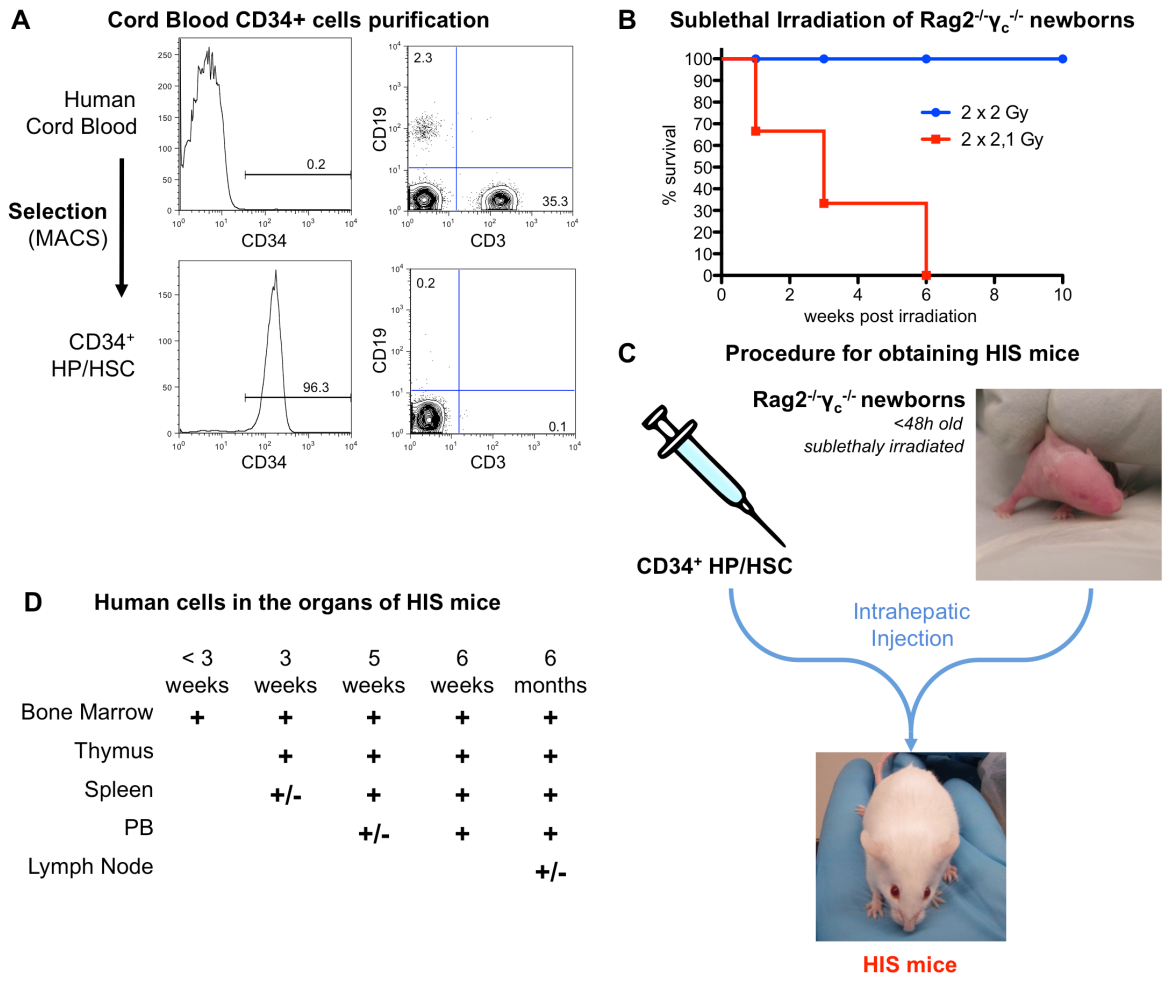

Figure 9 – Generation of HIS mice from human cord blood and Rag2$^{-/-}\gamma_c^{-/-}$ newborns

HIS mice are generated according to the procedure described in (Traggiai et al., 2004). **A –** Representative FACS analysis for CD34, CD3 and CD19. CD34$^+$ cells are obtained by MACS selection from human cord blood (upper panel). After purification, the purity is over 90 % and the B and T cells contamination (lower than 1 % (lower panel). The percentage of CD34$^+$ cells as well as B (CD19$^+$) and T (CD3$^+$) cells are indicated on the plots. **B -** Rag2$^{-/-}\gamma_c^{-/-}$ newborn mice are sublethally irradiated. **C –** Schematic representation of the procedure. Purified CD34$^+$ cells are injected into the liver of sublethally irradiated Rag2$^{-/-}\gamma_c^{-/-}$ newborns. This results in the development of a human adaptive immune system in the HIS mice. **D –** Sequential colonization of the different murine organs by human cells. The humanization can be observed for several months after birth.

HTLV-1 induces T-cells malignancy and its provirus is found integrated mostly in T cells highlighting the need for an efficient T-cell development in the HIS mice. The T-cell development occurs in the thymus and starts by the entry of bone marrow-derived T cell progenitors. The major subsequent steps of the T-cell development can be characterized by the expression of the CD3, CD4 and CD8 antigens. The most immature cells do not express any of these markers and develop into the CD4 immature single positive population, $CD4^{ISP}$ ($CD3^{-}CD4^{+}CD8^{-}$). At this stage, the β-selection, a major checkpoint for the correct rearrangement of the TCR β chain occurs before the evolution to the double positive stage ($CD3^{-/+}CD4^{+}CD8^{+}$). Positive and negative selections allow the survival of mature Single Positive 4 or 8 (SP4 or SP8, $CD3^{+}CD4^{+}CD8^{-}$ or $CD3^{+}CD4^{-}CD8^{+}$ respectively) mature T cells that egress from the thymus to the periphery (Figure 10). The human T-cell development was assessed in the thymus of HIS mice and the results presented in the figure 1A of the following paper exhibit a robust human T-cell development mimicking that observed in human thymus as early as 4 weeks post-transplantation (Blom and Spits, 2006). These data established these mice as an animal model for *in vivo* infection of human T cells by HTLV-1.

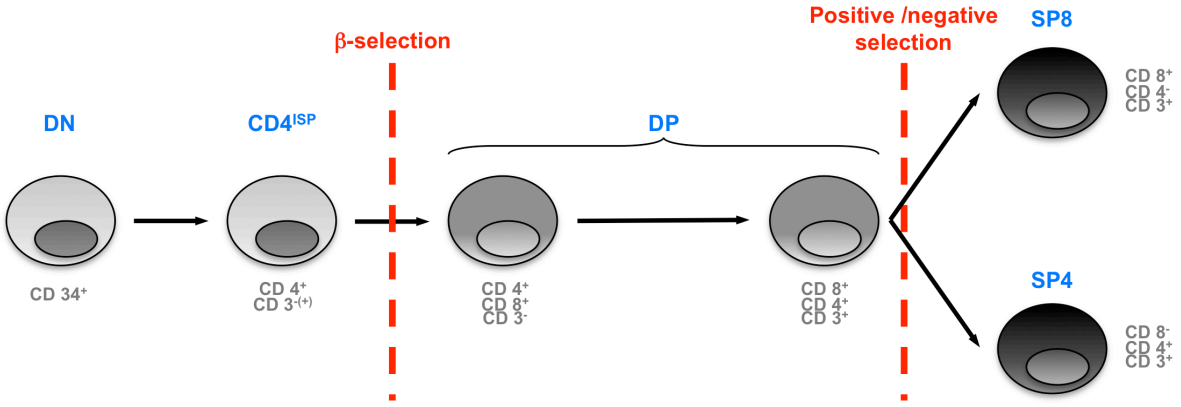

Figure 10 – Human T-cell development

The T-cell development mostly takes place in the thymus. The most immature thymocytes (Double Negative, DN) coming from the bone marrow and expressing CD34 enter the thymus. The CD34 expression is rapidly lost to the sequential expression of CD3, CD4 and/or CD8. $CD4^{ISP}$, CD4 Immature Single Positive Cell. DP, Double Positive. SP8-SP4, mature single positive cells expressing CD8 or CD4 respectively.

c- Efficiently infecting HIS mice

When ready to infect humanized mice with pathogenic agents, several crucial questions arise: **When** is it more relevant to infect the animals? **What** infectious source can be used? **Where** i.e. what is the route of infection more favorable for the infection of the animals?

When? In our search to establish a model to study the infection and pathogenesis of HTLV-1, we are particularly interested in unraveling the very first steps of the infection and assume that the development of an ATL several decades later requires early events occurring in a given target cell at a given stage of its hematopoietic development in a given context. In humans, ATL infection has been linked to the infection of newborn mostly via breast-feeding by an infected mother. Therefore the most relevant time to infect HIS mice is when human hematopoietic development is already initiated, but still at an early stage as it would be observed in human neonates. Furthermore, *in vitro* studies have suggested that infection of hematopoietic progenitor or stem cells resulted in the impairment of their subsequent differentiation ruling out the possibility to infect *ex vivo* the $CD34^+$ cells used to humanized the mice or to co-inoculate $CD34^+$ cells along with the virus. We thus decided to infect the mice at 4 weeks post-humanization. This time was determined to allow a colonization of the thymus with human T progenitor cells and their differentiation in the T-cell development. Experiments of infection between 4 and 10 weeks post-humanization were validated by the observations in the results described later (data not shown) and this protocol was selected for our subsequent experiments.

What? As HTLV-1 infection of new cells occurs mostly through cell-to-cell contact rather than via cell-free particles, we decided to inject infected cells to humanized mice. A quick review of the previous works showed that several HTLV-1-producing cell lines have

been used to infect animals. Among these cells, the MT-2 cell line has been extensively used and we decided to use it in our experiments.. To inhibit their proliferation in the HIS mice, the cells were lethally irradiated without stopping their viral production allowing them to infect human cells. Indeed, these irradiated cells cocultured with human Cord Blood Lymphocytes (CBL) are able to infect the latter (Figure 11). Injecting lethally irradiated HTLV-1 cells into non-humanized Rag2$^{-/-}\gamma_c^{-/-}$ mice demonstrated that the mice were not able to sustain the infection and also that inoculated cells were quickly cleared and were undetectable (figure 2-D in the paper). Based on a bibliographic review (Hasegawa et al., 2003), we initially inoculated 200,000 lethally irradiated infected cells. This protocol produced greatly variable results. We therefore injected 10-fold more MT-2 cells i.e. 2x10^6 cells, resulting in a more reproducible infection and this number of cells was used routinely in our following experiments.

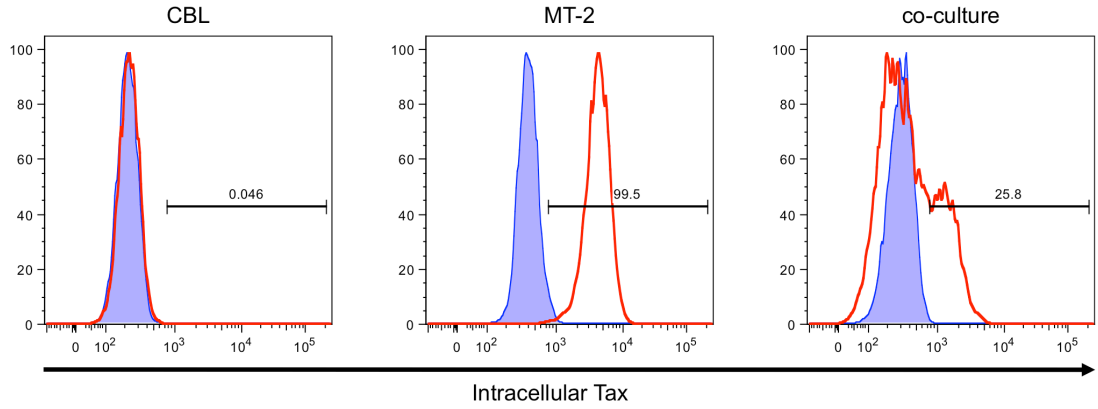

Figure 11 – Irradiated MT-2 cells can infects human CBL *ex vivo*.

MT-2 cells were lethally irradiated and mixed with freshly isolated human CBL. After 3 weeks of co-culture, cells were harvested, stained for intracellular Tax and analyzed by flow-cytometry. Uninfected CBL (CBL) and MT-2 were also analyzed as controls. The blue line represents unstained cells and the red line the Tax-stained cells. The percentage of Tax$^+$ cells among all living cells is indicated for each plot.

Where? Several routes of infection can be used for inoculating infected cells into mice: intravenous (iv), intramuscular (im), or intraperitoneal (ip) injection or oral inoculation of infected cells. Although oral inoculation would have been more relevant to the route of natural infection occurring through breast-feeding, we decided to use ip injection for

practical reasons. ip injection have been successfully used to infect HIS mice with other human virus like HIV (Baenziger et al., 2006) and represent the easiest way to administrate infected cells into the mice. It is not stressful and not harmful for the animal, safe for the PhD student injector and represents a quick, easy, and reproducible procedure.

Once the "when", "what" and "where" questions were answered, the remaining "who" and "how" questions found easy answers, "I" and "with a syringe" to complete the protocol: I will inject ip 2 millions of lethally irradiated MT-2 cells in humanized mice aged between 4 and 10 weeks.

d- Human cells in HIS mice are sensitive to HTLV-1 infection

Using this protocol, we detected the provirus integrated in different organs of the mice (Figure 12 and figure 2D in the paper).

Figure 12 – Detection of the HTLV-1 provirus in different organs of HIS mice.

An HIS mouse was infected 4 weeks after birth and sacrificed at the age of 30 weeks. Genomic DNA was extracted from the different organs and PCR was carried out to detect the gag viral gene.

The provirus is indeed integrated in human genome as evidenced by the result of an ALU-PCR figure 2C in the paper) and was persistent for several months (figure 3A in the paper). The inoculation of non-humanized mice resulted in the absence of provirus detection confirming the low sensitivity of mice cells compared to human cells (figure 1D, lane 1 in the paper). This persistent infection was correlated with an overtime increase of the PVL (figure 3A in the paper). Viral expression was demonstrated both by RT-PCR (figure 5B-D in the paper) and at the protein level (figure 5A in the paper). Furthermore, ATL-like pathologies were observed in infected mice with a high PVL: hepato-splenomegaly, lymphadenopathy, lymphomas or leukemias (figure 2B in the

paper). Interestingly, these symptoms were associated with a profound deregulation of the T-cell development in the infected animals and resulted in the accumulation of infected T cells with a mature phenotype ($CD4^+$ or $CD8^+$)(figure 4 in the paper). We also demonstrated in this *in vivo* model that genes involved in the anti-apoptotic pathway were deregulated similar to what we have previously observed in thymocytes *ex vivo* transduced with the Tax vector (figure 6 in the paper). Using a highly sensitive high throughput sequencing system described elsewhere (Gillet et al., 2011), we were able to quantify the number of unique integration sites (UIS) as well as the respective size of each clone. We analyzed the clonal distribution in the spleen of several animals (figure 3B in the paper) and demonstrated a polyclonal integration pattern. Interestingly, when we analyzed the clonality of different organs in one mouse we observed major differences in the clonal distribution with a high number of UIS in the thymus of this mouse whereas the number of clones in the spleen and the lymph nodes was lower and some clones were quite large in comparison to the others (supporting figure 1 in the paper). More characterization has been realized on the clonality in these HIS mice and are presented in this manuscript (cf. p100).

We concluded that the use of HIS mice represent a valuable model for the infection and the pathogenesis associated with HTLV-1. It could be used to dissect the early stage of the infection and the early events leading to the leukemia.

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HTLV-1 Propels Thymic Human T Cell Development in "Human Immune System" Rag2$^{-/-}$ gamma c$^{-/-}$ Mice

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Abstract

Alteration of early haematopoietic development is thought to be responsible for the onset of immature leukemias and lymphomas. We have previously demonstrated that Tax_{HTLV-1} interferes with ß-selection, an important checkpoint of early thymopoiesis, indicating that human T-cell leukemia virus type 1 (HTLV-1) infection has the potential to perturb thymic human αβ T-cell development. To verify that inference and to clarify the impact of HTLV-1 infection on human T-cell development, we investigated the *in vivo* effects of HTLV-1 infection in a "Human Immune System" (HIS) Rag2$^{-/-}\gamma_c^{-/-}$ mouse model. These mice were infected with HTLV-1, at a time when the three main subpopulations of human thymocytes have been detected. In all but two inoculated mice, the HTLV-1 provirus was found integrated in thymocytes; the proviral load increased with the length of the infection period. In the HTLV-1-infected mice we observed alterations in human T-cell development, the extent of which correlated with the proviral load. Thus, in the thymus of HTLV-1-infected HIS Rag2$^{-/-}\gamma c^{-/-}$ mice, mature single-positive (SP) CD4$^+$ and CD8$^+$ cells were most numerous, at the expense of immature and double-positive (DP) thymocytes. These SP cells also accumulated in the spleen. Human lymphocytes from thymus and spleen were activated, as shown by the expression of CD25: this activation was correlated with the presence of *tax* mRNA and with increased expression of NF-kB dependent genes such as *bfl-1*, an anti-apoptotic gene, in thymocytes. Finally, hepato-splenomegaly, lymphadenopathy and lymphoma/thymoma, in which Tax was detected, were observed in HTLV-1-infected mice, several months after HTLV-1 infection. These results demonstrate the potential of the HIS Rag2$^{-/-}\gamma_c^{-/-}$ animal model to elucidate the initial steps of the leukemogenic process induced by HTLV-1.

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Introduction

HTLV-1 (Human T cell Leukaemia Virus, type 1), the only retrovirus associated with a neoplastic disease in humans, is the etiologic agent of Adult T-cell Leukaemia (ATL), an aggressive clonal lymphoproliferative disorder of mature CD4$^+$ CD25$^+$ T cells [1-3]. It is considered that HTLV-1 infection early in life predisposes to ATL. Indeed, epidemiological studies have stressed that ATL develops after an extremely long (several decades) latency period, often in patients who were infected as neonates through breastfeeding [4]. T-cell leukemias such as T-ALL (T-cell acute lymphoblastic leukaemia) which mainly occur in children and adolescents, are thought to result from malignant thymocytes arising at defined stages of intrathymic T-cell development [5,6]. Thus, HTLV-1 infection within the thymus might be an essential event in the pathogenesis of ATL. *In vitro*, HTLV-1 can productively infect human hematopoietic CD34$^+$ progenitor cells as well as human immature thymocytes [7–9]. It has also been demonstrated that reconstitution of T lymphopoiesis with HTLV-1-infected CD34$^+$ cells in severe combined immunodeficient (SCID) mice engrafted with human thymus and liver tissues resulted in the alteration of thymopoiesis, as shown by abnormalities in the size of thymocyte subpopulations [7]. Recently, Banerjee *et al.* have generated a humanized model, which partially recapitulates HTLV-1-induced T-cell leukemogenesis by inoculating immunodeficient mice with human CD34$^+$ hematopoietic progenitor cells infected *ex vivo* with HTLV-1 [10].

HTLV-1 is a complex retrovirus that relies upon multiple gene products to accomplish replication and disease. Among them, Tax and HBZ are key regulatory proteins. HBZ, encoded by the antisense strand of the HTLV-1 proviral DNA, is permanently expressed and required for efficient viral infectivity and persistence, whereas Tax has been shown to deregulate a number of cellular genes leading to cellular proliferation, to induce genetic instability, thus contributing to malignant

Author Summary

Human T-cell leukemia virus type 1 (HTLV-1) is a retrovirus responsible for the development of Adult T cell Leukemia, an aggressive malignant lymphoproliferation that occurs several decades after viral exposure in some individuals infected when neonates *via* breastfeeding. Here, we used mice that were humanized by transplantation of human hematopoietic stem cells isolated from cord blood. Such an animal model may reproduce key aspects of *in vivo* infection with HTLV-1 and improve the understanding of the early steps of the leukemogenic process. These humanized mice when inoculated with HTLV-1 were indeed productively infected, as shown by the detection and the expression of the provirus in human cells. We observed that T-cell development in the thymus of these mice was profoundly altered, as ascertained by the increased proliferation of activated mature T lymphocytes that accumulated in the thymus as well as in the spleen. These alterations of human thymic T-cell development may be linked to the development of ATL–like pathologies. These results show that this humanized mouse model provides an invaluable tool not only to study the early stages of the infection leading to the development of malignant lymphoproliferations, but also to design new therapeutic approaches.

progression [3]. Studies on Tax-transgenic mice that express *tax* under the control of either the HTLV-1 LTR or alternative promoters that target Tax expression to lymphocytes have demonstrated that Tax can perturb lymphocyte functions. The development of tumors in most of these transgenic mouse models also provided evidence of the leukemogenic potential of Tax. Interestingly, when the transgene expression was placed under the control of the Lck promoter, which restricts Tax expression to developing thymocytes, an ATL-like phenotype developed in the transgenic mice [11]. These observations suggest that the leukemogenic activity of Tax is related to T-cell development in the thymus. We have previously reported that Tax, by silencing E2A transcription factors, down-regulates the expression of the pTα gene thus perturbing β-selection, the early and critical checkpoint of human T-cell development in the thymus [12,13]. These *in vivo* and *in vitro* studies indicated that HTLV-1 infection in the thymus might be a prerequisite for the malignant lymphoproliferation associated with this retroviral infection.

In the present study, we first investigated the *in vivo* effects of HTLV-1 infection on human T-cell development in "Human Immune System" (HIS) mice by transplanting human CD34$^+$ cord blood cells into conditioned newborn BALB/c Rag2$^{-/-}\gamma_c^{-/-}$ recipient mice. The resulting HIS mice were infected with HTLV-1, at a time when the successive stages of the αβT-cell development are evident in the thymus of these mice. In the HTLV-1-infected Rag2$^{-/-}\gamma_c^{-/-}$ mice, we observed efficient viral expression and profound alterations in human thymopoiesis, as shown by the accumulation of activated mature single-positive CD4$^+$ and CD8$^+$ lymphocytes in the thymus and in the spleen. The role of Tax in that process is indicated by an increased expression of NF-κB-dependent genes such as CD25 and Bfl-1 in thymocytes of these mice. In addition, abnormalities frequently observed in ATL such as hepatosplenomegaly, lymphadenopathy and lymphoma were detected in HTLV-1-infected mice, several months after HTLV-1 infection. This HIS Rag2$^{-/-}\gamma_c^{-/-}$ animal model might be of great interest to decipher the initial steps of the leukemogenic process induced by HTLV-1.

Results

HIS Rag2$^{-/-}\gamma_c^{-/-}$ mice were generated by intrahepatic transplantation of human CD34$^+$ cord blood cells into sublethally irradiated immunocompromised newborn BALB/c Rag2$^{-/-}\gamma_c^{-/-}$ mice [14-16]. We first analyzed by flow cytometry the expression of CD4 and CD8 on human CD45$^+$ (hu-CD45) cells in the thymus of HIS Rag2$^{-/-}\gamma_c^{-/-}$ mice transplanted with CD34$^+$ cells. Five weeks after transplantation, immature CD3$^-$ thymocytes, including the double-negative (DN) CD4$^-$CD8$^-$ cells, the CD4$^+$ immature single-positive (CD4ISP) cells and the double-positive (DP) CD4$^+$CD8$^+$cells were found to be predominant, whereas a low percentage of mature CD3$^+$ T-cells containing DP cells and single-positive (SP) CD4$^+$ or CD8$^+$ cells was detected. pTα has been shown to be highly expressed in immature thymocytes and more specially in CD4ISP cells, just before the pre-T-cell Receptor complex formation [17,18]. Eight weeks after transplantation, the population of mature thymocytes predominated over that of immature thymocytes, indicating that human αβT-cell development was completed in the thymus of transplanted HIS Rag2$^{-/-}\gamma_c^{-/-}$ mice (Figure 1A). At that time, approximately 90% of the cells present in the thymus of the HIS Rag2$^{-/-}\gamma_c^{-/-}$ mice expressed hu-CD45$^+$. Circulating human mature T cells were also detected in the blood and secondary lymphoid organs (Figure 1B). Thus, mature T cells represented about 17% of the hu-CD45$^+$ cells in the spleen, 10 weeks after CD34$^+$ transplantation (Figure 1C). These observations are in line with those previously published [16] and confirm that the successive maturational steps of human αβT-cell development were completed in the thymus of these mice within two months following human CD34$^+$ inoculation.

Evaluation of proviral load in HTLV-1-infected HIS Rag2$^{-/-}\gamma_c^{-/-}$ mice

Taking into account the chronology of the different maturational stages of human αβT-cell development in the thymus of HIS Rag2$^{-/-}\gamma_c^{-/-}$ mice, HTLV-1 infection of these mice was carried out between 4 and 8 weeks after CD34$^+$ transplantation. The mice were inoculated intraperitoneally either with 2×10^6 lethally irradiated HTLV-1-producing MT2 cells in Phosphate Buffered Saline (PBS) or with PBS only (mock-infected) (Figure 2A). In parallel, untransplanted BALB/c Rag2$^{-/-}\gamma_c^{-/-}$ mice were inoculated with lethally irradiated MT2 cells.

Autopsy examination between 10 and 35 weeks after HTLV-1 inoculation revealed a significant increase of the size of the thymus, the spleen and lymph nodes of HIS Rag2$^{-/-}\gamma_c^{-/-}$ mice inoculated with MT2 cells, when compared to those of mock-infected animals. Furthermore tumor-like nodules were frequently observed in the spleen and the liver of MT2-inoculated mice (Figure 2B).

To ascertain that the MT2-inoculated HIS Rag2$^{-/-}\gamma_c^{-/-}$ mice were indeed infected, we used ALU-PCR to amplify $gag_{HTLV\text{-}1}$ sequences from human DNA isolated from splenocytes. The results showed that all but two MT2-inoculated HIS Rag2$^{-/-}\gamma_c^{-/-}$ mice were HTLV-1-infected (Figure 2C, lanes 3, 4 and Figure 2D, lane 3). As expected, viral sequences were not observed in mock-infected HIS Rag2$^{-/-}\gamma_c^{-/-}$ mice (Figure 2C lanes 1, 2 and Figure 2D, lane 2). Likewise, they were not detected in MT2-inoculated BALB/c Rag2$^{-/-}\gamma_c^{-/-}$ mice, thus excluding the possibility that the inoculated irradiated MT2 cells were still present in these mice (Figure 2D, lane 1).

The HTLV-1 proviral load (PVL) in HTLV-1-infected HIS Rag2$^{-/-}\gamma_c^{-/-}$ mice was then evaluated by quantitative PCR (qPCR). An increase in the PVL was observed, which correlated with the length of the infection period (Figure 3A). Indeed, a PVL value in

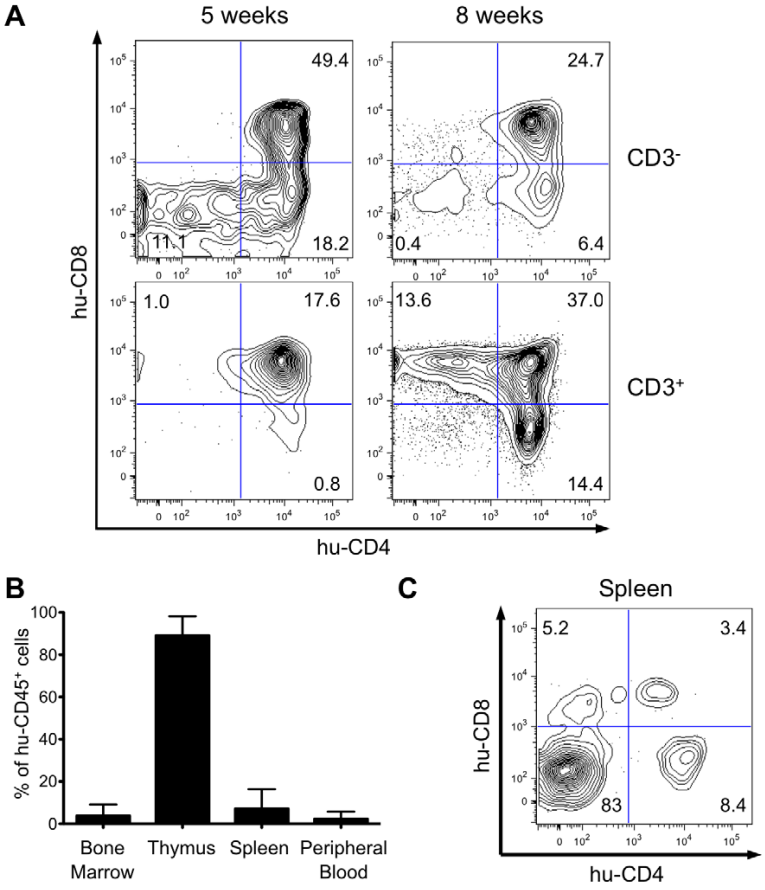

Figure 1. Human hematopoietic cell engraftment and T-cell development in HIS Rag2$^{-/-}\gamma_c^{-/-}$ mice. (**A**) Efficient intrathymic *de novo* development of human T-cells in BALB/c Rag2$^{-/-}\gamma_c^{-/-}$ mice. Animals were sacrificed at 5 and 8 weeks after CD34$^+$ cell transplantation. The flow cytometry dot plots show representative examples of staining for human CD3, CD4 and CD8 markers (values on the dot plots are for the frequency of the corresponding populations). (**B**) Frequency of human CD45$^+$ cells in bone marrow, spleen, thymus and peripheral blood in HIS Rag2$^{-/-}\gamma_c^{-/-}$ mice, at 10 to 30 weeks post transplantation. (**C**) Representative flow cytometry analysis of total splenocytes in transplanted mice sacrificed at 7 weeks after transplantation stained for human CD4 and CD8 lymphoid cell surface markers.
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the range of 10 to 10^3 copies per 10^5 human cells was found in 20 mice that were sacrificed between 5 and 20 weeks after inoculation of MT2 cells. These mice were subsequently referred to as low-PVL mice. Fifteen mice that were sacrificed between 16 and 35 weeks had a PVL between 10^3 to 10^6 copies per 10^5 cells; these were referred to as high-PVL mice. The low PVL values (median 8.9×10^2 copies/10^5 cells) were similar to those in healthy human HTLV-1 carriers, whereas the high PVL values (median 1.1×10^5 copies/10^5 cells) were similar to those in ATL cells [19]. These results show that HTLV-1 infection of human immune cells was established in the HIS Rag2$^{-/-}\gamma_c^{-/-}$ mice and that the PVL correlated with the length of the infection period ($r^2 = 0.9704$).

We then quantified cellular clonality in the spleen of five representative HTLV-1 infected HIS Rag2$^{-/-}\gamma_c^{-/-}$ mice, by measuring the frequency of the different HTLV-1 infected clones present in that organ. The calculation of the oligoclonality index is shown in Figure 3B. Each slice in the pie chart represents a single Unique Insertion Site (UIS) and the size of the slice is proportional to the relative abundance of that UIS. In the mouse with the lowest oligoclonality index (0.45), the slices of the pie chart are of similar size. In contrast, in the two mice with the highest clonality index (0.71 and 0.79), two clones were found to predominate. Although there was no significant correlation between the oligoclonality index and the proviral load, these data indicate that oligoclonal proliferation of infected cells occurred in these mice, as observed in naturally infected patients [20].

The αβT-cell development in HTLV-1 infected HIS Rag2$^{-/-}\gamma_c^{-/-}$ mice is altered in a PVL-dependent manner

To investigate the effect of HTLV-1 infection on αβT cell development in HIS Rag2$^{-/-}\gamma_c^{-/-}$ mice, we proceeded to a comparative FACS analysis of thymopoiesis in infected or in mock-infected mice. In the latter, at either 8 to 20 weeks (early) or 18 to 35 weeks (late) after PBS injection, the relative frequency of the three main human subpopulations (immature, DP and SP) remained mostly unchanged (Figure 4A). In the low-PVL HTLV-1-infected HIS Rag2$^{-/-}\gamma_c^{-/-}$ mice, the respective percentages of these subpopulations were slightly different with a higher frequency of DP cells and a lower frequency of SP cells (Figure 4B). In contrast, in the high-PVL mice, a significant

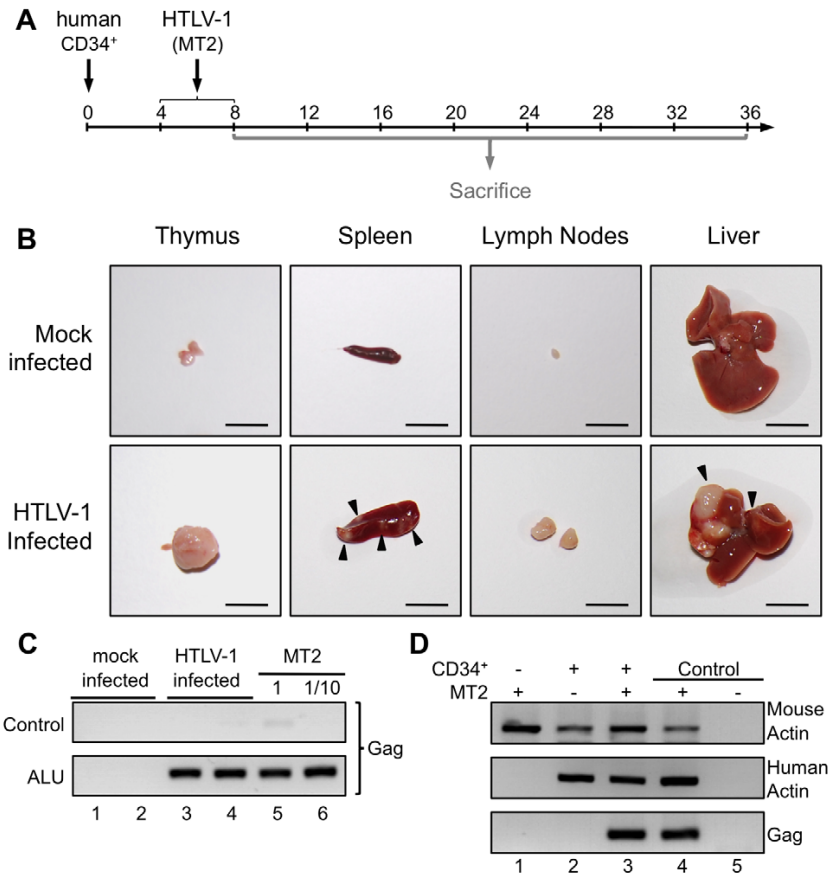

Figure 2. Integration of the HTLV-1 provirus in the genome of human cells in HTLV-1-infected HIS Rag2$^{-/-}\gamma_c^{-/-}$ mice. (**A**) Schematic representation of the experimental infection set-up. Four to eight weeks after CD34$^+$ cell transplantation, the animals were intraperitoneally inoculated with 2×10^6 lethally irradiated MT2 cells in 100 µl PBS or with PBS alone for the mock-infected mice. Animals were sacrificed and analyzed between 8 and 36 weeks after CD34$^+$ cell transplantation. (**B**) Presence of disseminated tumors in HTLV-1 infected HIS Rag2$^{-/-}\gamma_c^{-/-}$ mice, sacrificed between 16 and 35 weeks after infection and referred to as high proviral load (PVL) mice. Infection resulted in thymoma, and splenomegaly and HTLV-1 positive lymphomas in spleen, in mesenteric lymph nodes or in liver (arrows). Are also shown representative photographs of thymus, spleen, lymph node and liver of 34-week-old HIS Rag2$^{-/-}\gamma_c^{-/-}$ control mice. Bars, 10 mm (**C**) ALU-PCR was carried out on the DNA extracted from the thymus of mock-infected (lanes 1 and 2) and infected (lanes 3 and 4) mice. The lower panel (two-rounds of PCR, the first one with ALU primers and the second with gag primers) indicates that the HTLV-1 *gag* gene is integrated in the human genome. The upper panel shows the result of control PCR for *gag* carried out on the initial diluted samples without any ALU-PCR. (**D**) HTLV-1 integration does not occur in the mouse genome: PCR for mouse and human actin and for *gag* were performed with the DNA extracted from the spleen of Rag2$^{-/-}\gamma_c^{-/-}$ mice (not inoculated with human CD34$^+$ cells), infected with HTLV-1 (lane 1), or HIS Rag2$^{-/-}\gamma_c^{-/-}$ mice either mock-infected (lane 2) or infected with HTLV-1 (lane 3).
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depletion of the immature DN subpopulation was observed together with a strong decrease of DP thymocytes and with a large increase of the mature SP cells. Such alterations were evident in mice as young as 18 weeks, *i.e.* 13 weeks after inoculation with MT2 cells. These data show that the main impact of HTLV-1 infection was on the population of the most immature T cells, as indicated by their significant decrease in mice with the highest PVL. In the spleen of infected mice, the population of mature T cells increased with the PVL level to the point where mature T cells were the only human population observed in that organ (Figure 4C).

These observations suggested a positive correlation between the PVL and the alterations of human T cell development observed in HTLV-1-infected HIS Rag2$^{-/-}\gamma_c^{-/-}$mice. To verify this suggestion, we analysed CD4 and CD8 expression on thymocytes of either the less mature CD3$^-$ or the more mature CD3$^+$ subpopulations of four age-matched mice including one mock-infected mouse and three HTLV-1-infected mice, one with a low PVL and two with high PVL (Figure 4D). We observed a significant decrease in the CD3$^-$ subset, which correlated with the increase in PVL. In fact, the immature DN, CD4ISP and DP subpopulations were severely depleted in the mouse with the highest PVL (left panel), whereas the CD3$^+$ SP cells increased to 78.3% in the same mouse (right panel). The thymocyte subpopulations in three mice with a comparable high PVL, but killed at different times after infection (20, 23 and 35 weeks) displayed a similar profile, *i.e.* depletion of CD3$^-$ immature and DP cells and increase of CD3$^+$ mature SP cells (Figure 4E). These results show that HTLV-1 infection led to an increased number of mature T cells in the thymus and in the periphery. We conclude that HTLV-1 infection perturbed the development of αβT lymphocytes in the thymus of HIS Rag2$^{-/-}\gamma_c^{-/-}$ mice, mainly by propelling immature thymocytes

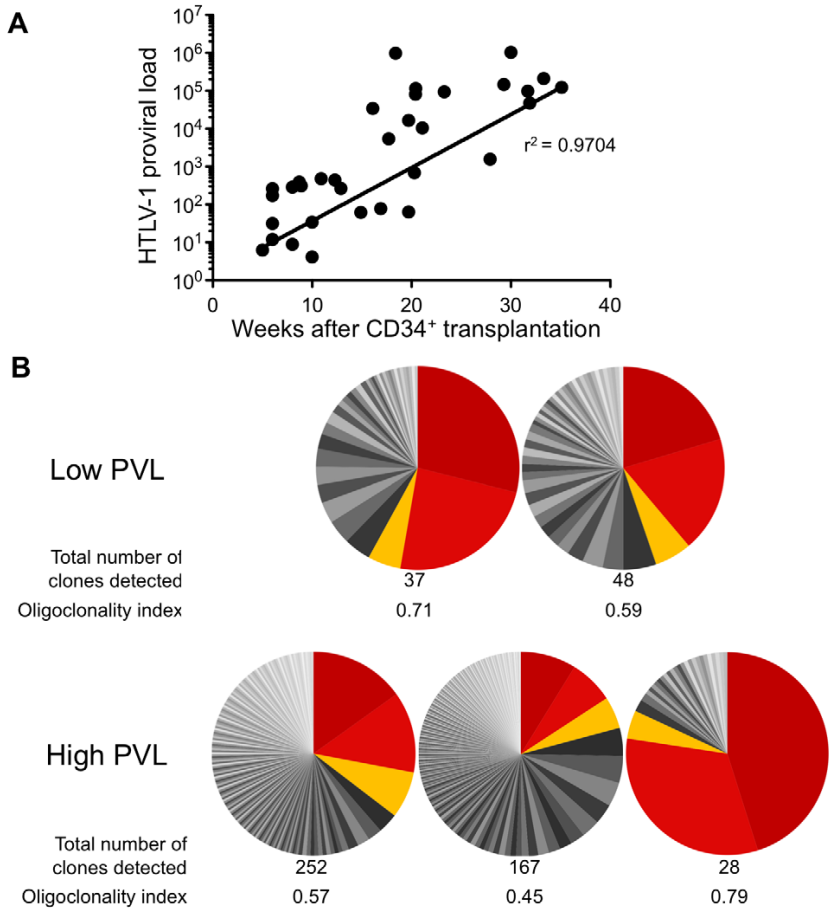

Figure 3. Proviral load and clonality in HTLV-1-infected HIS Rag2$^{-/-}\gamma_c^{-/-}$ mice. (**A**) Temporal evolution of proviral load (PVL) in the thymus. The HTLV-1 PVL (representing the copy number of *tax* per 10^5 human cells) were determined by quantitative real-time PCR at the indicated times after CD34$^+$ cell transplantation. The PVL level correlates with the length of the infection period ($r^2 = 0.9704$). Samples were analyzed at least in duplicate; on all graphs, one dot represents one individual HIS Rag2$^{-/-}\gamma_c^{-/-}$ mouse. (**B**) Clone frequency distribution of HTLV-1 infected cells in the spleen from 5 different mice. The HTLV-1 clonal structure in each genomic DNA sample is depicted by a pie chart. Each slice represents one unique insertion site (clone); the size of the slice is proportional to the relative abundance of that clone. The 3 most abundant clones were colored in red/orange in each spleen sample. The total number of detected clones was given together with the oligoclonality index values calculated as described in [20].

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towards the late stages of maturation. We now propose to investigate whether such an effect is induced by the viral regulatory Tax.

Activation of survival and proliferating pathways in HTLV-1-infected HIS Rag2$^{-/-}\gamma_c^{-/-}$ mice

Of the 35 HIS Rag2$^{-/-}\gamma_c^{-/-}$ mice that were inoculated with HTLV-1-producing MT2 cells, 13 mice displayed pathological features, the severity of which appeared to correlate with proviral load and with the number of activated CD25$^+$ T-cells in the thymus (Table 1). These pathological features were observed only in high-PVL mice that were killed between 16 and 35 weeks (Figure 5A). While most of these mice only displayed enlarged lymph nodes, some of them also developed hepatosplenomegaly, lymphadenopathy, and thymoma (compare panel 2 to panel 1) in which the cells were found to express the Tax protein detected by immunohistochemical analysis (panels 5,6,8,9).

Based on our previous experiments on human immature thymocytes, these *in vivo* observations therefore suggested that Tax induced the alterations in thymopoiesis observed in HIS Rag2$^{-/-}\gamma_c^{-/-}$ mice. *tax* transcription was readily detected in the thymus of high-PVL mice, whereas in the low-PVL mice, it was very low or undetectable (Figure 5B). *hbz* transcription displayed the same pattern (Figure 5C). Finally, the observed increase in Tax transcription correlated with proviral load, unlike HBZ transcripts (Figure 5D).

We previously reported that Tax protein interferes with assembly of the pre-TCR when expressed *in vitro* in human immature thymocytes, such as the CD4ISP thymocytes [12,13]. During normal αβT-cell development, signals from the pre-TCR activate the NF-κB pathways that control the expression of target genes implicated in cell proliferation and survival [17]. We hypothesized that Tax might compensate for the absence of a functional pre-TCR by inducing NF-κB activation in immature thymocytes. We therefore investigated the impact of Tax

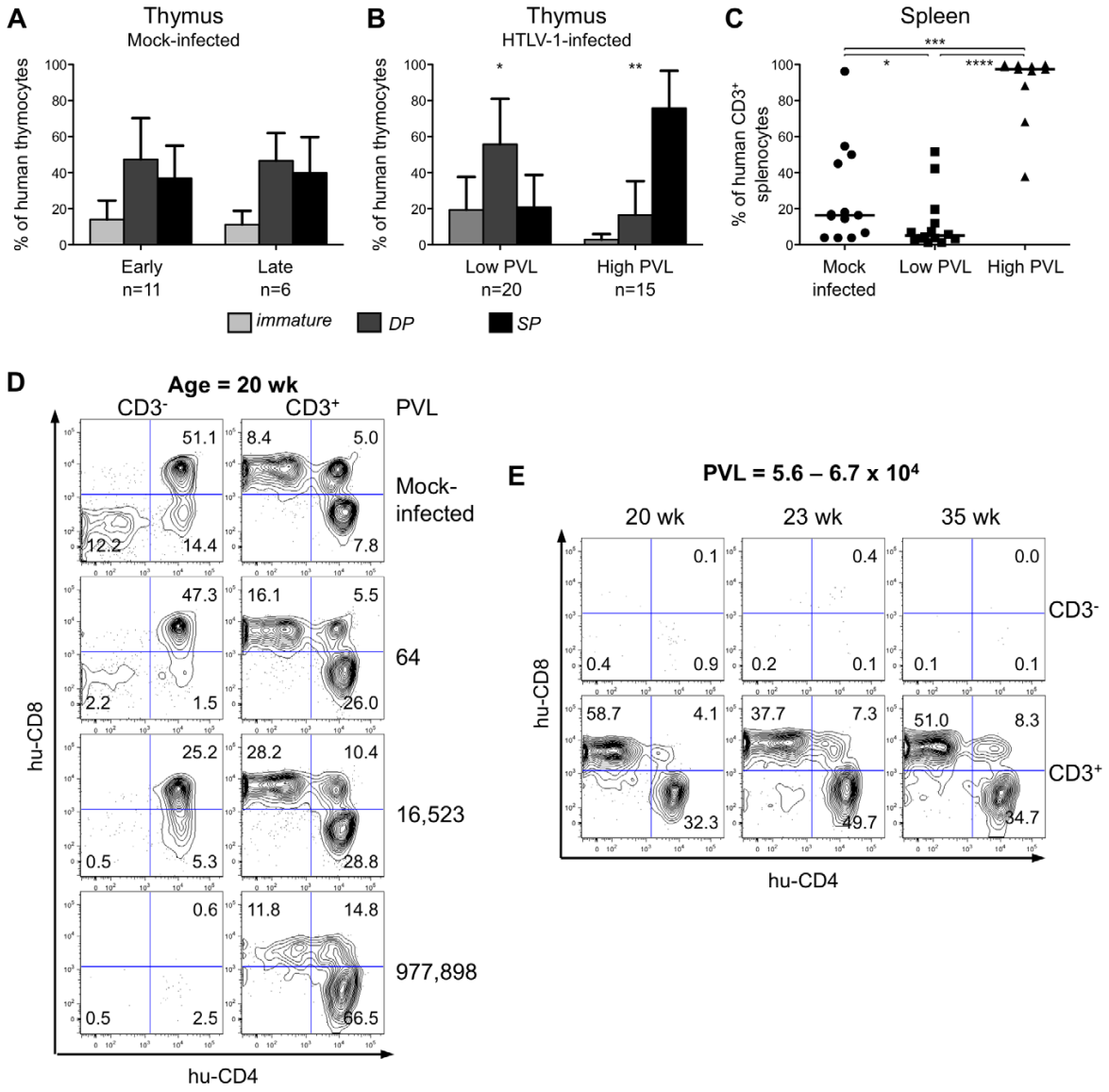

Figure 4. The human T-cell development is altered in HTLV-1-infected HIS Rag2$^{-/-}\gamma_c^{-/-}$ mice. (**A**) Composite data from 11 mock-infected mice sacrificed early after CD34$^+$ cell transplantation (early) and 6 mock-infected mice sacrificed later on (late). Bar graphs represent percent of human CD45$^+$ thymocytes that are CD4$^-$CD8$^-$ and CD4ISP immature cells (light grey), DP cells (dark grey) and SP cells (black). The graphs present the mean and the SD. Statistical differences were calculated using the $\chi 2$ test: *P$<$0.05, **P$<$0.01. (**B**) Composite data from 20 HTLV-1-infected mice with a low PVL and 15 HTLV-1-infected mice with a high PVL showing a decrease in the frequency of the immature thymocytes (light grey) concomitant with an increase of that of the mature SP cells (black) in high PVL mice. (**C**) Expansion of human CD3$^+$ T-cells at the periphery after HTLV-1 infection: FACS analysis of CD3 expression by hCD45$^+$ splenocytes in 35 HIS Rag2$^{-/-}\gamma_c^{-/-}$ either mock- or HTLV-1-infected mice. The median values are indicated by horizontal lines. Statistical differences were calculated using the Mann-Whitney's U test: *P$<$0.05, ***P$<$0.001, ****P$<$0.0001. (**D**) Correlation between HTLV-1 proviral load, the depletion of immature thymocytes and the expansion of mature thymocytes. FACS plots of the immature CD3$^-$ subpopulations containing DN (CD4$^-$CD8$^-$), immature DP and CD4ISP and the mature CD3$^+$ subpopulations, stained for human CD4 and CD8 are shown for representative animals either mock-infected or HTLV-1-infected with different PVL (indicated on the right side) and killed at 20 weeks after CD34$^+$ cell transplantation. (**E**) The alteration of the human thymopoiesis induced by HTLV-1 is independent on the duration of the infection period: FACS analysis of the human CD3$^-$ and CD3$^+$ thymocyte subpopulations in three HTLV-1-infected mice sacrificed at different weeks after CD34$^+$ cell transplantation, but each with a similar high PVL (from 5.6×10^4 to 6.7×10^4 copies/10^5 cells).
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overexpression on the transcription of NF-κB-related genes (RelA/p65, p105/p50, c-Rel) in immature thymocytes. We observed that the amount of the respective transcripts was much greater in TaxGFP-expressing immature thymocytes than in GFP$^+$ control cells (Figure 6A). We also analysed isolated mRNAs to detect transcripts of the anti-apoptotic genes *Bfl-1* and *Bcl-2*,

Table 1. Pathological features in HIS Rag2$^{-/-}\gamma_c^{-/-}$ mice infected with HTLV-1.

Mouse*	Age of Infection	Age of Analysis	Proviral Load °	observations
#209	7 wk	28 wk	1.58×10^3	
#178	5 wk	18 wk	5.39×10^3	Enlarged LN
#365	5 wk	21 wk	1.05×10^4	Enlarged LN
#273	4 wk	20 wk	1.65×10^4	Enlarged LN DP T cells in blood, spleen and Bone marrow
#189	4 wk	16 wk	3.42×10^4	Enlarged LN
#297	7 wk	32 wk	4.75×10^4	
#357	5 wk	23 wk	7.30×10^4	Enlarged LN Tumor in the liver Leukemia
#248	7 wk	20 wk	8.12×10^4	
#240	7 wk	23 wk	9.37×10^4	Enlarged LN Tumor in the liver and the spleen
#307	7 wk	32 wk	9.74×10^4	
#242	7 wk	20 wk	1.15×10^5	Enlarged LN
#279	7 wk	35 wk	1.23×10^5	Enlarged LN Thymoma
#200	8 wk	29 wk	1.46×10^5	
#278	7 wk	33 wk	2.12×10^5	Enlarged LN Tumor in the spleen
#190	4 wk	18 wk	9.78×10^5	Thymoma
#188	4 wk	30 wk	1.04×10^6	Enlarged LN Thymoma

*Mice were inoculated intraperitoneally with irradiated MT2 cells.
° Proviral Load is expressed as number of proviral copies per 10^5 thymocytes.
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because *Bfl-1* is a target of NF-κB transcriptional activity whereas Bcl-2 is independent of NF-κB. Only the expression of *Bfl-1* was significantly increased in Tax-expressing thymocytes (Figure 6B). These results show that Tax expressed in human immature thymocytes activates the transcription of NF-κB factors and that of the Bfl-1 anti-apoptotic gene.

We therefore investigated whether the NF-κB dependent expression of these two genes, *bcl2* and *bfl1*, was increased in HTLV-1-infected HIS Rag2$^{-/-}\gamma_c^{-/-}$ mice. RT-qPCR analysis showed a clear increase in the transcription of *Bfl-1* in these mice, when compared to control animals (Figure 6C); this increase was comparable to that found in Tax-expressing thymocytes (Figure 6B). The increase in *Bfl-1* mRNA correlated with that of *tax* mRNA, as did CD25 mRNA, which is also dependent on NF-κB factors. Indeed, a comparative FACS analysis of mock-infected and HTLV-1 infected HIS Rag2$^{-/-}\gamma_c^{-/-}$ mice indicated that the number of hu-CD45$^+$ CD25$^+$ cells in the thymus and in the spleen cells was significantly higher in high PVL mice than in low PVL or in control mice (Figure 7A, B, C). Lastly, activated T lymphocytes, which were mainly CD4$^+$, were also found in the peripheral blood of an HTLV-1-infected HIS Rag2$^{-/-}\gamma_c^{-/-}$ mouse (Figure 7D). This finding demonstrates that HTLV-1 infection induced expansion of mature T-cells in the periphery. We conclude that Tax activates the transcription of genes involved in cell survival and proliferation in the thymus of HTLV-1–infected HIS Rag2$^{-/-}\gamma_c^{-/-}$ mice and favours the expansion of mature activated T cells. Thus, the presence of T-cell proliferation in HTLV-1 infected HIS Rag2$^{-/-}\gamma_c^{-/-}$ mice indicates that HTLV-1 infection of the thymus might be a critical pre-leukemogenic event.

Discussion

During the last decade, humanized mouse technology has made remarkably rapid progress, allowing the establishment of high levels of human chimerism in various host organ/tissues, particularly the immune system, liver and muscle [14,21–23]. These humanized mice appear ideally suited for direct investigation of human infectious agents [24,25]. In the present study, we have used Rag2$^{-/-}\gamma_c^{-/-}$ mice, which recapitulate the main events in the development of the human lymphoid compartment [26–28]. These mice have already displayed interesting potential for studying infection with lymphotropic viruses, such as HIV (Human Immunodeficiency Virus) and EBV (Epstein-Barr Virus) [29–34].

In the present study, we used HIS Rag2$^{-/-}\gamma_c^{-/-}$ mice inoculated with irradiated HTLV-1-producing cells, at a time when the human lymphoid compartment has been established, *i.e.* within a period of 1 to 2 months after transplanting BALB/c Rag2$^{-/-}\gamma_c^{-/-}$ immunodeficient animals with human CD34$^+$ cord blood cells. This infection protocol was found to be very efficient, as in most of the inoculated HIS Rag2$^{-/-}\gamma_c^{-/-}$ mice the provirus was found integrated in the genome of human cells. These mice were analyzed at regular intervals within a 7-month period after inoculation. We observe a sequential increase of the proviral load, which correlated with the appearance of critical alterations in the distribution of T-cell subsets in the chimeric thymus. Specifically, we observed slight differences in the respective percentages of the three main thymocyte subsets (immature, DP and SP) between HIS Rag2$^{-/-}\gamma_c^{-/-}$ mice with a low proviral load and mock-infected animals. Conversely, in mice with a high proviral load, the SP cells predominated at the expense of the immature and DP populations. These data provide strong evidence that *in vivo* HTLV-1 infection of HIS Rag2$^{-/-}\gamma_c^{-/-}$ mice perturbs human T-cell development in the thymus at the level of immature cells, by propelling development towards the mature stages. In addition, the infected mice had a high proviral load in the same range as that found in ATL patients [19]. These findings demonstrate that HIS Rag2$^{-/-}\gamma_c^{-/-}$ mice infected with HTLV-1 provide a suitable model for viral-induced malignancy. They further suggest that the combination of immature target cells in the thymus and the immunodeficient environment of HIS Rag2$^{-/-}\gamma_c^{-/-}$ mice favours the rapid development of a T-cell malignancy.

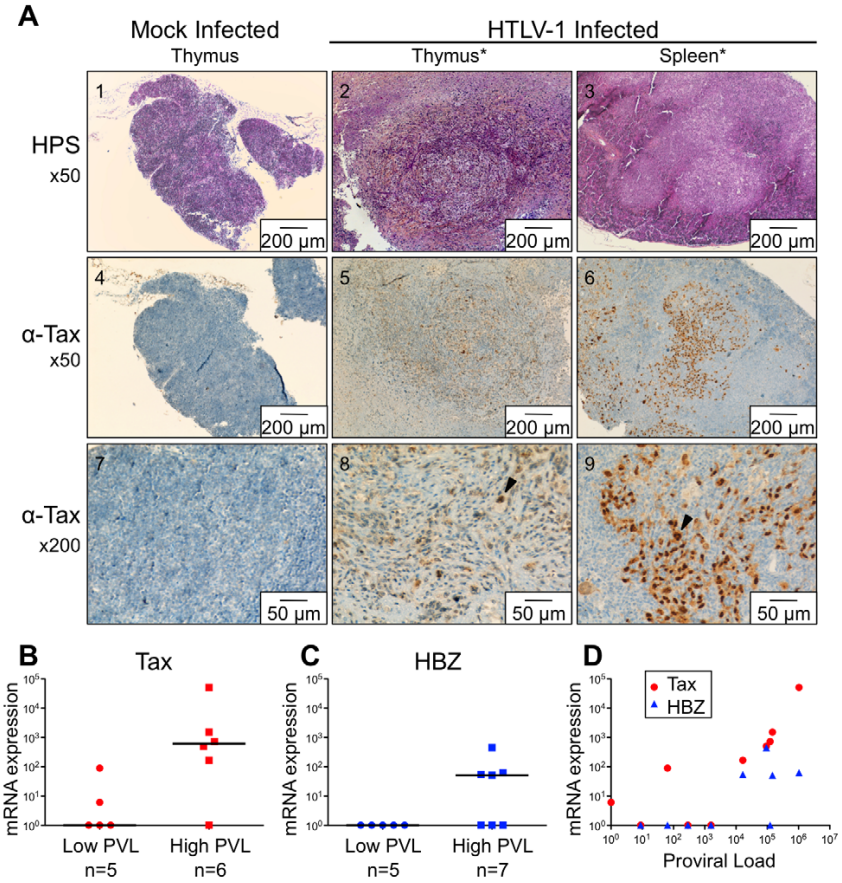

Figure 5. Expression of Tax and HBZ in thymocytes isolated from HTLV-1-infected HIS Rag2$^{-/-}\gamma_c^{-/-}$ mice. (**A**) Immunohistological characterization of representative sections of thymus and spleen from HTLV-1- and mock-infected HIS mice. The thymus of control mice (1) shows a normal architecture, whereas that of HTLV-1-infected mice contains a dense cellular infiltrate made of large lymphoid cells interspersed with giant multinucleated cells (2). A disorganized architecture is also observed in the infected spleen; the white pulp is hyperplastic and is made of large lymphoid aggregates containing large lymphoid cells and multinucleate cells; the red pulp shows extramedullary hematopoiesis with myeloid and erythroid elements (3). Tax immunostaining reveals that the thymus and spleen of infected animals displayed large lymphoma cells with a nuclear localization of Tax (5,6,8,9). Infiltration of lymphomatous cells expressing Tax was not observed in control mice (4,7). (**B-C**) Tax and HBZ mRNA loads in thymocytes isolated from HTLV-1-infected HIS Rag2$^{-/-}\gamma_c^{-/-}$ mice. Total RNAs were extracted from thymocytes of infected mice with either a low or a high PVL, and levels of mRNA coding for Tax and HBZ were measured by RT-qPCR and normalized to b-actin. The zero value of Tax and HBZ gene transcripts was observed in 60 and 100% of mice with low PVL, respectively; the median values are indicated by horizontal lines. (**D**) Dot plot graph of Tax and HBZ mRNA loads as a function of the proviral load.
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This model will also be helpful for studying the involvement of other HTLV-1 genes like *hbz* in viral pathogenesis as well as the role of the proviral integration sites. Thus, an oligoclonal expansion of HTLV-1-infected cells was observed in the periphery of five infected HIS Rag2$^{-/-}\gamma_c^{-/-}$ mice. In contrast, preliminary results obtained from one HTLV-1 infected mouse displaying a thymoma indicate an extraordinary degree of polyclonality of integration sites in the thymus (Figure S1A). Interestingly, the three clones that dominated the population in both spleen and lymph nodes were not dominant in the thymus, and the three most abundant clones observed in the thymus were not detected in the periphery (Figure S1A, B, C).

Our observations are consistent with those reported by Banerjee *et al.* who used a different infection strategy as well as different immunodeficient mice [10]. Indeed, these authors generated HTLV-1-infected humanized nonobese diabetic severe combined immunodeficiency (HU-NOD/SCID) mice by inoculating 4- to-6-week NOD/SCID mice with human fetal liver-derived CD34$^+$ cells infected *ex vivo* with HTLV-1. Less than half of these infected mice developed T-cell lymphomas with a CD4$^+$CD25^{low} or CD4$^+$CD25^{low} phenotype. These mice also showed greater proliferation of infected human CD34$^+$ cells in the bone marrow, leading the authors to propose that HTLV-1 can transform CD34$^+$ cells. However, HTLV-1 proviral sequences were not detected in CD34$^+$ bone marrow cells from ATL patients [35]. Banerjee *et al.* also reported that HTLV-1 infection skewed T-cell development in the thymus of these HTLV-1-infected HU-NOD/SCID mice, as well as in the thymus of HTLV-1-infected HU-NSG (NOD/SCID IL-2γ chain^{null}) mice. These data support our present observations which suggest that alterations of T-cell development play a prominent role in the initiation of HTLV-1-induced leukemogenesis.

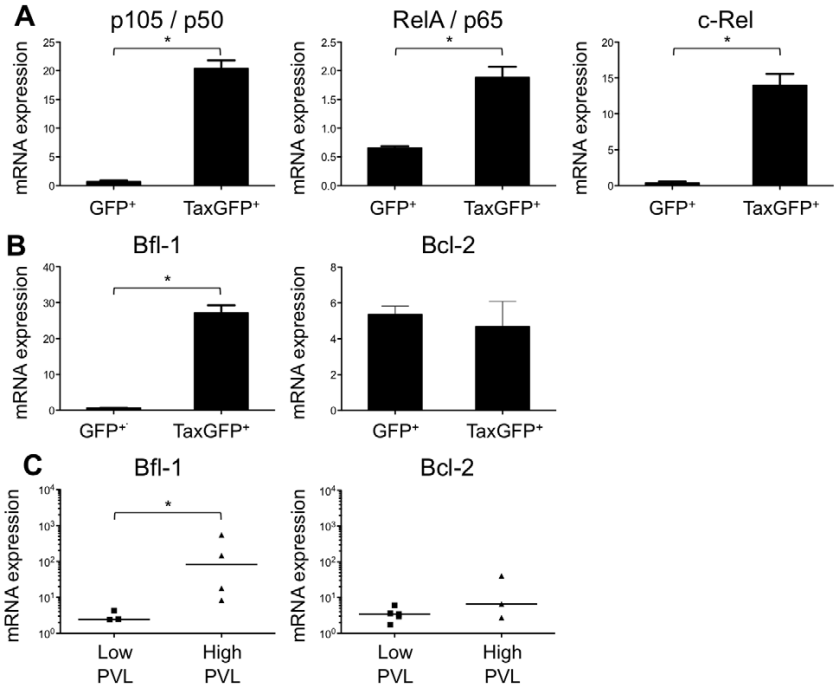

Figure 6. Enhanced transcription of the NF-κB and the antiapoptotic *Bfl1* genes by Tax. (**A-B**) Immature thymocytes isolated as previously described [12] were nucleofected with either pCMV-TaxGFP or pCMV-GFP. Twenty-four hours later, GFP$^+$ cells were sorted, and the total RNAs were isolated and reverse transcribed. The cDNA samples were subjected to qPCR using primers specific for the indicated genes and normalized for the amount of cDNA, using human β-actin as an internal control. Standard deviations are from at least two determinations performed in triplicate. * $P<0.05$ by Mann-Whitney U test. (**C**) *In vivo* correlation between the *Bfl1* gene transcription and the HTLV-1 proviral load: total RNAs were extracted from thymocytes of HTLV-1-infected HIS Rag2$^{-/-}\gamma_c^{-/-}$ mice from each group, and levels of *bfl-1* and *bcl-2* mRNAs normalized to human b-actin were measured by RT-qPCR. Levels of *bfl-1* mRNAs were significantly higher in high PVL mice than in low PVL mice, whereas *bcl-2* transcription was unchanged. The median values are indicated by horizontal lines. * $P<0.05$ using the Mann-Whitney U test.
doi:10.1371/journal.ppat.1002231.g006

In a previous study, we demonstrated that the viral Tax regulatory protein can inactivate the activity of bHLH E2A transcription factors [12]. This inhibitory effect leads to a decrease of the transcription of the pTα gene in immature thymocytes, thus precluding the assembly of the pre-TCR and interfering with the β-selection checkpoint [13]. The pre-TCR plays a crucial role in T-cell development, by allowing the maturation of thymocytes with a functional β chain. Signalling from the pre-TCR in the β-selected cells leads to the activation of NF-κB, which provides survival and proliferative advantages [18,36]. We hypothesize that Tax, which is able to activate the NF-κB pathways, compensates for the absence of the pre-TCR in HTLV-1-infected immature thymocytes. Thus, Tax-activated NF-κB would prevent apoptosis and drive proliferation. We indeed observed that activated transcription of the NF-κB pathways in *ex vivo* Tax-expressing immature thymocytes correlated with increased transcription of *Bfl1*, an anti-apoptotic NF-κB-responsive gene. Likewise, we found increased transcription of Bfl-1 in thymocytes of infected mice. We also observed an expansion of mature SP cells in the thymus and in the spleen of HTLV-1-infected HIS Rag2$^{-/-}\gamma_c^{-/-}$, and an increase in the percentage of CD25$^+$ CD4$^+$ T-cells. Finally, pathological features observed in some of these mice might be related to the disturbances of the αβT-cell development induced by HTLV-1 infection.

We conclude that HTLV-1 infection can override the pre-TCR checkpoint by forcing immature thymocytes to progress towards a more differentiated phenotype (acquisition of CD4 and CD8) in the absence of the pre-TCR. The Tax protein may play a key part in this process. The observed disturbances of thymopoiesis caused by HTLV-1 infection may represent critical events in the initiation of the leukemogenic process associated with this retroviral infection. More specifically, they underline the relationships between dysregulated T-cell development and neoplastic transformation and provide another contribution to the identification of molecular and cellular mechanisms involved in leukaemogenesis [37]. Indeed, the constitutive expression of Tax in immature thymocytes at the level of the β-selection checkpoint alters the developmentally regulated interplay between pre-TCR signalling and E2A activities, resulting in leukemia/lymphoma promotion.

HTLV-1-associated T-cell leukaemia strongly resembles the Notch3-induced T-cell malignancies in several respects [38]. Notch3, a member of the Notch family, is known to play a critical role in T-cell development and its constitutive activation has been related to T-cell leukaemia in both animal models and human diseases. The development of the T-cell leukaemogenic process requires the combined expression of Notch3 and the pre-TCR linked to a sustained down-regulation of E2A activity [37]. It is noteworthy that in the absence of the pre-TCR, Notch3, like Tax, can force immature T-cells through otherwise pre-TCR-dependent developmental stages and favour the progression toward a more differentiated phenotype by acquisition of CD4 and CD8. In contrast with Notch3, Tax is able to activate both the alternative and canonical NF-κB pathways, implying that the appearance of lymphoid tumors in HTLV-1 infected is linked to the exclusive activity of Tax. However, even if Tax down-modulates the

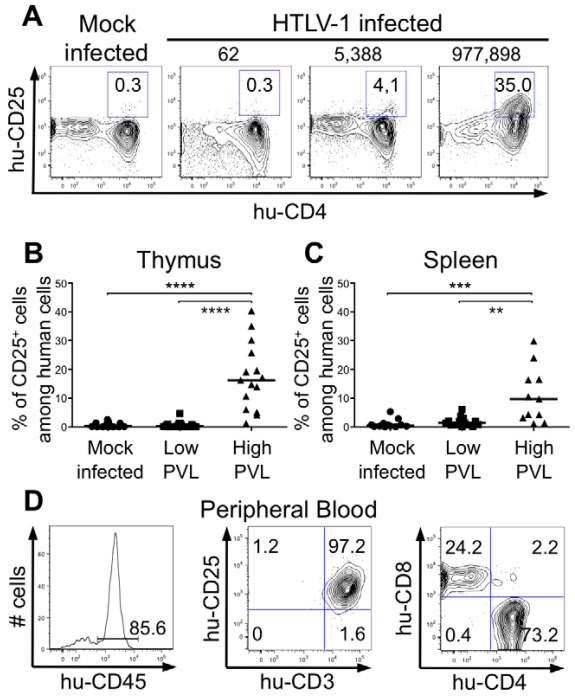

Figure 7. Correlation between expression of CD25 activation marker and HTLV-1 proviral load. (**A**) Representative flow cytometry plots showing the expression of human CD25 and CD4 markers on thymocytes of 17 mock- and 35 HTLV-1-infected HIS Rag2$^{-/-}\gamma_c^{-/-}$ mice with different PVL (indicated on top). (**B**) Frequency of human CD4$^+$ cells expressing CD25 among human thymocytes of 35 HIS Rag2$^{-/-}\gamma_c^{-/-}$ HTLV-1-infected mice; the median values are indicated by horizontal lines. (**C**) Frequency of human CD4$^+$ cells expressing CD25 among human splenocytes of 35 HIS Rag2$^{-/-}\gamma_c^{-/-}$ mice; the median values are indicated by horizontal lines. Statistical differences were calculated using the Mann-Whitney U test: **P<0.01, ***P<0.001, ****P<0.0001. (**D**) Lymphoproliferation in the peripheral blood of one HTLV-1-infected HIS Rag2$^{-/-}\gamma_c^{-/-}$ mouse with a high PVL (7.3×10^4 copies/10^5 cells): this flow cytometry analysis shows that among the 85.6% of huCD45+ cells (left panel), 97.2% are activated T lymphocytes (center panel), the majority with a CD4$^+$ phenotype (right panel).
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transcription of pTα, leukemogenesis may also require continued weak expression of the pre-TCR, which cooperates with pTa to reach a critical level of NF-κB activation. Future experiments should be performed to verify that hypothesis.

In conclusion, our study demonstrates that BALB/c Rag2$^{-/-}\gamma_c^{-/-}$ mice transplanted with human CD34$^+$ cord blood cells provides an *in vivo* model to define the early steps of HTLV-1 infection that might lead to a better understanding of the initiation of the HTLV-1 associated leukemia. Our observations provide evidence that HTLV-1 infection in the thymus propels infected thymocytes toward the mature stages of T-cell development and favours the activation and the proliferation of these cells, thus providing a substrate population for further altered gene expression that would allow the emergence of a malignant clone.

Materials and Methods

Ethics statement

Umbilical cord blood was obtained from healthy full-term newborns with written parental informed consent according to the guidelines of the medical and ethical committees of Hospices Civils de Lyon and of Agence de Biomédecine, Paris, France. Experiments using cord blood were approved by both committees and were performed in full compliance with French law.

Animal experimentation was performed in strict accordance with the French "Comité National de Réflexion Ethique sur l'Expérimentation Animale, n°15" and the ethical guidelines for the care of these mice of the Plateau de Biologie Experimentale de la Souris (PBES, UMS 3444) at Ecole Normale Supérieure de Lyon. The protocol used throughout that study was approved by the Committee on the Ethics of Animal Experiments of the Ecole Normale Supérieure de Lyon (Permit number: 0231). All efforts were made to minimize animal suffering.

Isolation of human CD34$^+$ cells from cord blood samples

After density gradient centrifugation of human cord blood, CD34$^+$ cells were enriched twice using immunomagnetic beads according to the manufacturer instructions (CD34$^+$ MicroBead Kit, Miltenyi Biotec, Bergisch-Gladbach, Germany). Purity (≥95%) was evaluated by FACS analysis using human CD34, CD3 and CD19 antibodies. Cells were either frozen or immediately transplanted when newborn mice were available.

Generation of HIS Rag2$^{-/-}\gamma_c^{-/-}$ mice

Rag2$^{-/-}\gamma_c^{-/-}$ mice on a BALB/c background were bred and maintained under specific pathogen-free conditions in accordance with the French law and the guidelines of PBES. Rag2$^{-/-}\gamma_c^{-/-}$ mice were transplanted with human CD34$^+$ cells, as previously described [16,39]. Briefly, newborn (less than 3-day old) mice were irradiated twice within a 3-hour interval with 2×2 Gy from a ^{137}Cs source (CIS BIO international, IBL 637) at 1.28 Gy/min., a dose that was adjusted to be sub-lethal. After the second irradiation, mice were injected intrahepatically with 2×10^5 human CD34$^+$ cord blood cells in 30 µl PBS.

HTLV-1 infection of HIS Rag2$^{-/-}\gamma_c^{-/-}$ mice

The HTLV-1 T-cell line MT2 [40] was irradiated with 77 Gy from a ^{137}Cs source (CIS BIO international, IBL 637) at 1.28 Gy/min. This dose was adjusted to inhibit cell proliferation but not HTLV-1 production. HIS Rag2$^{-/-}\gamma_c^{-/-}$ mice aged from 4 to 8 weeks were intraperitoneally injected with 2×10^6 lethally irradiated MT2 cells in 100 µl PBS. Mock-infected mice were injected with PBS alone. Mice were sacrificed at different time-points after infection. Infection was performed in a Biosafety Level 3 laboratory in accordance with the PBES guidelines.

Histopathological analysis

Anesthetized mice were killed and tissue specimens (thymus, spleen, bone marrow and lymph nodes) were fixed directly in neutral buffered formaldehyde, embedded in paraffin, sectioned and stained with H&E solution. An indirect immunoperoxidase technique was applied to 4 µm-thick formalin-fixed, paraffin-embedded tissue sections with a rabbit polyclonal anti-Tax antibody. An automated immunostainer was used (Ventana, Tucson, AZ, USA). Antigen unmasking and immunodetection were performed according to the manufacturer's instructions (Ventana). The avidin-biotin technique was used for development.

Flow cytometric analysis

Peripheral blood cells were collected from the retro-orbital venous sinus under Ketamine-Xylazyne anesthesia. When needed, mice were sacrificed after anesthesia and thymus, spleen, bone marrow and lymph nodes were collected, gently minced in PBS to

obtain a single-cell suspension. For FACS analysis, monoclonal antibodies, conjugated with either FITC, PE, PE-Cy7, APC or V450 against the following human antigens were used: CD4 (RPA-T4), CD8 (RPA-T8), CD19 (HIB19), CD34 (581/CD34), CD45 (HI30), (all BD Biosciences, San Diego, CA), CD3 (UCHT1) and CD25 (BC96) (eBioscience, San Diego, USA). Flow cytometric analysis was performed using a FACScanto II and Diva for acquisition (Becton Dickinson Immunocytometry Systems, Mountain View, CA) and FlowJo (Treestar, Ashland, OR) for data analysis.

DNA and RNA isolation

Genomic DNA was extracted from the single cell suspension using the Nucleospin Blood kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions, and resuspended in 60 µl of the supplied elution buffer. For mouse tissues, RNA was extracted from the single cell suspension using the Nucleospin RNA XS kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions, and resuspended in 10 µl of water. For human thymus, total RNA was extracted from $1–2\times10^5$ thymocytes using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Two chloroform extractions were performed on the organic phase to avoid phenol contamination. Before reverse transcription, RNAs were first treated with 10 U of RNase-free DNase I (Qiagen) for 20 min at 27°C and then for 15 min at 60°C.

PCR, RT-PCR and quantitative real-time PCR (qPCR)

The RNA samples were reverse transcribed at 42°C for 1 hour in a total volume of 20 µl reaction buffer containing 100 U of SuperScriptTII Rnase H- reverse transcriptase (RT; Invitrogen). A reaction without RT was performed in parallel to serve as control for the absence of DNA contamination. PCR was performed using the Phusion enzyme (Finnzyme, Espoo, Finland) in a Piko thermocycler (Finnzyme, Espoo, Finland). For ALU-PCR, the first round was carried out using an ALU-specific primer and a *gag* specific primer (see sequence below). The amplification product was then diluted in water (1/50) and used as templates for a *gag*-specific PCR. The control PCR for *gag* was carried out by diluting the initial template (1/1250) instead of applying first ALU-PCR.

Quantitative PCR (qPCR) was performed using the FastStart Universal SYBR Green Master (Roche, Mannheim, Germany) on a LightCycler 2.0 system (Roche Applied Science, Illinois, USA) or on a StepOnePlus system (Applied Biosystem, CA, USA).

Species-specific primers were used to amplified human β-actin, 5′–TGAGCTGCGTGTGGCTCC–3′ and 5′–GGCATGGGGGAGGGCATACC–3′, human p105/50 5′–CTGGAAGCACGAATGACAGA–3′ and 5′–CCTTCTGCTTGCAAATAGGC–3′, RelA/p65 5′–CCTGGAGCCAGGCTATCAGTC–3′ and 5′–CACTGTCACCTGGAAGCAGA–3′, c-Rel 5′-GAACGATTGGGAAGCAAAAG-3′ and 5′-GGCACAGTTTCTGGAAAAGC-3′, human Bfl-1 5′-GGCATCATTAACTGGGGAAG-3′ and 5′-TCCAGCCAGATTTAGGTTCAA-3′, human Bcl2 5′-TGTGGATGACTGAGTACCTGAACC-3′ and 5′-GTTTGGGGCAGGCATGTTGAC-3′, mouse actin 5′–TGGAATCCTGTGGCATCCATGAAAC–3′ and 5′–TAAAACGCAGCTCAGTAACAGTCCG–3′, HTLV-1 gag 5′-CCCTCCAGTTACGATTTCCA-3′ and 5′–GGCTTGGGTTTGGATGAGTA–3′, tax 5′–GTTGTATGAGTGATTGGCGGGGTAA–3′ and 5′–TGTTTGGAGACTGTGTACAAGGCG–3′, hbz 5′-GGCAGAACGCGACTCAACCG-3′ and 5′-GCCGATCACGATGCGTTTCCC-3′; in this later case, PCR amplifications were conducted on oligo(dT)-primed cDNAs with a forward primer spanning the HBZ-SI mRNA exon 1 and a reverse primer located in the exon 2; these primers do not overlap with the splice sites, avoiding false positive. Amplification of human β-actin was done for each experimental sample to normalize results in RT-PCR experiments.

Evaluation of the proviral load

The proviral load was expressed as number of copies of provirus per 100,000 human cells. It was evaluated by qPCR on genomic DNA for the number of copies of tax and the number of copies of human β-actin. To establish the calibration curve we assumed that one ng of DNA extracted from human PBMC contains 333 copies of β-actin. TARL-2 is a rat HTLV-1 infected cell line which has a single copy of HTLV-1 proviral DNA per cell and we therefore assume that one ng of DNA contains 167 copies of the *tax* gene [41].

Cell transfection and plasmids

Human immature thymocytes were obtained as previously described [12], using the EasySep CD3-positive selection cocktail to deplete CD3$^+$ cells followed by negative selection using the human CD4$^+$ T-cell enrichment cocktail (StemCell Technologies Inc, Vancouver, BC, Canada) according to the manufacturer's instructions. The Tax expression plasmid, pCMV-TaxGFP (generous gift from R. Mahieux), contains the *tax* coding sequence in frame with the N-terminal extremity from the *gfp* sequence under the control of the cytomegalovirus promoter. pMax-GFP (Amaxa) was used as a GFP-control expressing vector. Immature thymocytes (2.10^6 cells), cultured overnight in α-MEM complete medium containing 20 ng/ml IL-7 (R&D, Abingdon, UK) and 10 ng/ml SCF (Peprotech, Rocky hill, NJ), were transfected either with 4 µg of pCMV-TaxGFP or pCMV-GFP by nucleofection (Human CD34 cell nucleofector kit, Amaxa, Köln, Germany) according to the manufacturer's instructions. Twenty-four hours later, GFP$^+$ cells were sorted from both cultures using an ARIA sorter (BD-Beckinson).

Selective amplification and quantification of proviral insertion sites

DNA was extracted from tissue samples as described in the section "DNA and RNA isolation" and the selective amplification and quantification of the proviral insertion sites was done as previously described [20]. The DNA was sheared by sonication. A linker containing a tag was ligated, and nested PCR was performed between the end of the HTLV-1 LTR and the linker. Nested PCR products were pooled to construct the sequencing library. A paired-end read (read 1 and read 2) plus a tag index read were acquired on an Illumina Genome Analyzer II. Read 1 and read 2 were mapped against the human genome (build hg18) and the proviral insertion site and the shear site were respectively deduced. The relative abundance (in percent of proviral load) of a given unique insertion site (UIS) was calculated from the number of amplicons of different length (*i.e.* different shear sites). For more details, see [20].

Statistical analysis

Statistical analyses were performed using the GraphPad Prism software and the Mann-Whitney U test or the $\chi 2$ test.

Supporting Information

Figure S1 Distribution of abundance of unique insertion sites in different tissues of the same mouse. The HTLV-1 clonal structure in each genomic DNA sample is depicted by a pie chart. Each slice represents one unique insertion

site (UIS); the size of the slice is proportional to the relative abundance of that UIS. (**A**) The 3 most abundant UISs were colored in blue. These UISs were found neither in the spleen nor in the lymph node sample. (**B, C**) The 3 most abundant UISs were colored in red/orange. 11_82937981 means that the provirus of this clonal population is inserted in chromosome 11, coordinate 82937981. These 3 major UISs were detected in the thymus but at a relatively low abundance.
(TIFF)

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Author Contributions

Conceived and designed the experiments: JV MW LG MDD. Performed the experiments: JV MW NG NAG. Analyzed the data: JV MW NAG LG JYS MGM CRMB MDD. Contributed reagents/materials/analysis tools: NG NAG JYS. Wrote the paper: JV JYS LG MGM CRMB MDD.

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Supporting Information

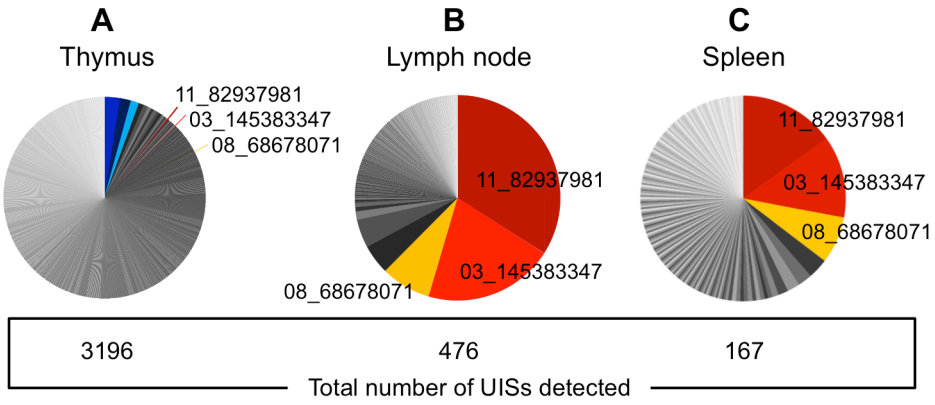

Figure S1 - Distribution of abundance of unique insertion sites in different tissues of the same mouse.

The HTLV-1 clonal structure in each genomic DNA sample is depicted by a pie chart. Each slice represents one unique insertion site (UIS); the size of the slice is proportional to the relative abundance of that UIS. (A) The 3 most abundant UISs were colored in blue. These UISs were found neither in the spleen nor in the lymph node sample. (B, C) The 3 most abundant UISs were colored in red/orange. 11_82937981 means that the provirus of this clonal population is inserted in chromosome 11, coordinate 82937981. These 3 major UISs were detected in the thymus but at a relatively low abundance.

2) Further characterization of the infected HIS mouse model

Along with the results presented in this paper, several complementary experiments were carried out during the publication process. They bring some new interesting insights in the characterization of the HTLV-1 infection of HIS mice and the associated pathogenesis.

a- HTLV-1 infects immature thymocytes in vivo

As it was demonstrated in the paper (figure 2C in the paper), the HTLV1 provirus was integrated in human cells. In the data presented in the paper the provirus detection and PVL quantification were carried out on the total cells extracted from the different organs of infected HIS mice. However we wondered in which cells the HTLV-1 provirus is integrated and we decided to isolate $CD3^+$ cells and $CD3^-$ cells from the thymus of infected HIS mice. A large majority of the cells collected are of human origin as shown in the figure 1B of the paper. The $CD3^+$ thymocytes encompass a mature population with the late double positive thymocytes along with SP $CD4^+$ or $CD8^+$ cells. The $CD3^-$

thymocytes represent immature thymocytes: the early progenitors, the immature CD4 single positive population and the early double positive T cells.

Thymocytes obtained from infected HIS mice were separated on the basis of their CD3 expression using positive selection performed with magnetic beads. The genomic DNA from these 2 populations was extracted and submitted to PVL determination. The results shown in the Figure 13 indicate the presence of the provirus in $CD3^-$ immature thymocytes. Furthermore, the PVL load in the $CD3^+$ population is up to about 40-fold higher than the PVL in the $CD3^-$ population.

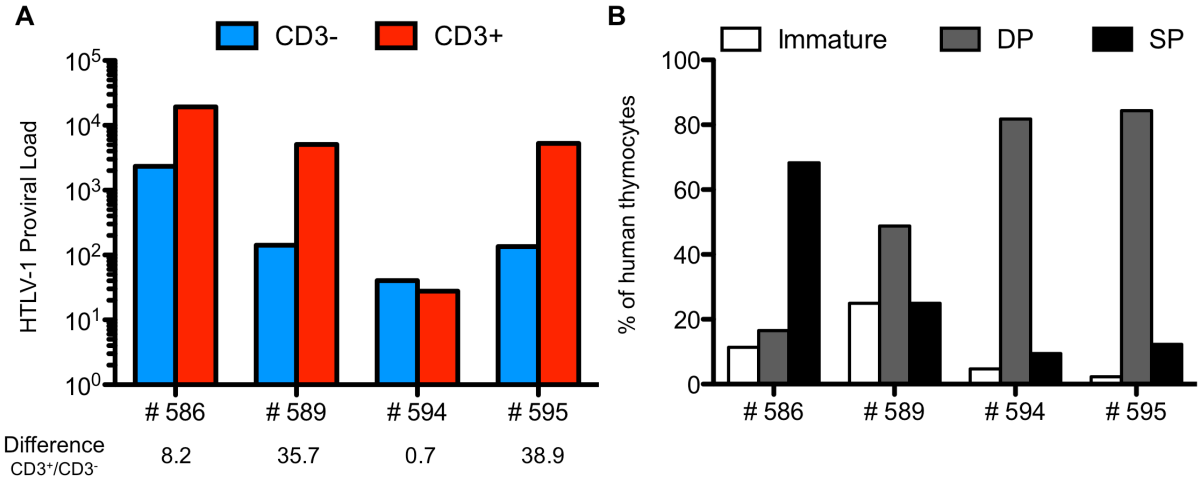

Figure 13 – HTLV-1 provirus is detected in immature thymocytes and higher PVL is found in more mature thymocytes

The cells isolated from the thymus of 4 animals infected at the age of 4 weeks and sacrificed at 18 wk (#594 and #595) or 31 weeks (# 586 and #589) were sorted on their expression of CD3. **A –** PVL was measured on the DNA extracted form the two sorted populations. Except for the mouse # 594, the PVL in the $CD3^+$ populations is higher. PVL is defined as the number of copies of HTLV-1 in 100,000 human cells. ^{B} – FACS analysis of the thymic subpopulations. No correlation with the observed difference in the PVL was evidenced. The Immature group represents the double negative and CD4 immature single positive population (cf. Figure 10). DP, Double Positive population. SP, mature Single Positive population (both $CD4^+$ or $CD8^+$)

The results presented here confirm that immature cells can be infected. Different explanations may be put forward to apprehend the observed differences in the PVL between the immature and mature cells:

- the mature population is globally more susceptible to the infection than the immature population resulting in a larger number of infected cells or infected cells with more sites of proviral integration.

- a small subset of immature thymocytes is infected *in vivo*. For instance if thymocytes can be infected only at a certain stage of the T-cell development occurring during the early double positive stage, few cells will be sensitive in the immature population diluting the PVL among the other uninfected cells. This hypothesis is supported by the expression of HTLV-1 receptors on some T-cell development stage (cf. discussion p148)(Swainson et al., 2005).
- immature cells, or at least a given population, could be susceptible to HTLV-1 infection. This infection and the resulting viral expression could give to the infected cells a proliferative and/or differentiation advantage. An immature infected cell could represent an "infected stem cell" producing more cells entering the differentiation than the non-infected cells resulting in a larger proportion of infected mature cells.

Additional experiments are needed to verify if either one, several or none of these hypothesis are valid. This point is further discussed in this manuscript (cf. p148).

b- HTLV-1 infection in HIS mice is associated with an increased number of FoxP3$^+$ cells.

The presence of an increased proportion of FoxP3-expressing cells was observed in infected patients and especially in some ATL subtypes (Bangham and Toulza, 2011). In our HIS mouse model, we observed an increased number of mature T cells in the thymus as well as in peripheral organs (figure 4 in the paper). Among those T populations, an enlarged population of $CD25^+CD4^+$ is found among the mice with the highest PVL (figure 7 in the paper). $CD3^+CD4^+CD25^+$ are markers expressed by Treg cells. We therefore decided to evaluate by flow cytometry the level of FoxP3 expression by intracellular staining of this protein among the cells from the thymus and the spleen. $FoxP3^+$ T cells are detected in the spleen and the thymus of infected HIS mice (Figure 14). In uninfected animals, very few $CD25^+$ cells are fond in the thymus and the spleen (Figure 7B and C in

the paper), therefore almost no FoxP3+ cells are found in uninfected HIS mice. In Infected animals, FoxP3+ cells also expressed CD4 on their surface in coherence with that observed in infected patients (Bangham and Toulza, 2011). This result indicates that the induction of FoxP3+ cells in infected patients is also found in the HTLV-1 infected HIS mice. It further supports the value of this model to accurately mimic the human pathologies. Additional characterization of these cells is needed to understand their origin, their role in the pathology and if it represents a potential target of a therapeutic strategy.

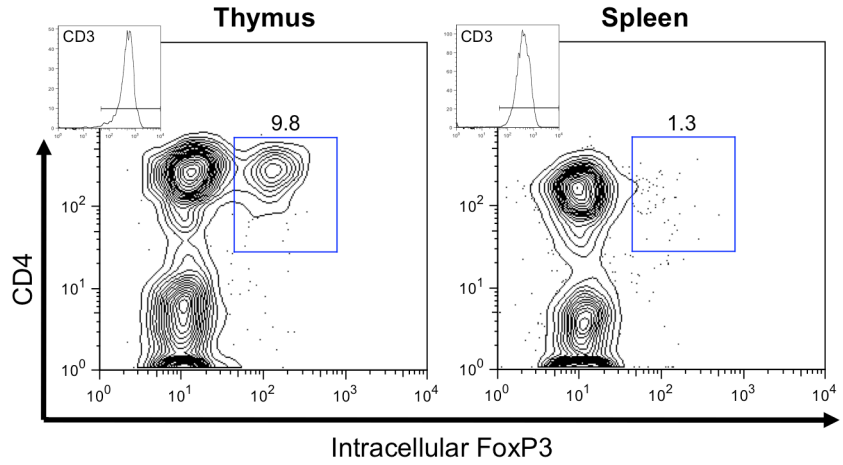

Figure 14 – Detection of FoxP3+ T cells in infected HIS mice.

FACS analysis of the thymus and the spleen from a mouse infected at 4 weeks and sacrificed at 30 weeks. Living cells were gated on human CD45 expression. All of the cells analyzed where CD3+ T cells (insert). The percentage of CD4+FoxP3+ is indicated on each plot.

c- Analysis of the clonality in infected HIS mice

A more in-depth study of the clonality has been carried out on the DNA extracted from the spleen of infected HIS mice. Gillet *et al.* defined an index, the Oligoclonality Index (OCI) to have a measurable objective index of the clonality in a given patient (Gillet et al., 2011). Briefly the value of this index vary from 0 to 1, in which zero represented a highly polyclonal pattern and 1 a strictly monoclonal one. Genomic DNA extracted from infected HIS mice was sent to the lab of Charles RM Bangham in London where Nicolas Gillet and Anat Melamed carried out the clonality experiments. When comparing the OCI

calculated from these samples with the ones from asymptomatic, HAM/TSP or ATL patients, we observed that the OCI of the HTLV-1 infected HIS mice is higher than the one from asymptomatic or HAM/TSP patients and is closer to the one observed in ATL patients (Figure 15A). Gillet et al. described the genomic environment on integration sites and its importance for the persistence and proliferation of each clone. Interestingly when looking at the proximity of several genetic sequences or epigenetic regulatory markers in correlation with the size of the clones, we found similar results between the UIS found in the HIS mouse model and in human patients. First the integration is not random in the host genome and there is a bias for integration in regions near regulatory markers. This is observed in freshly *in vitro* infected cells, in HTLV-1-infected patients as well as in infected HIS mice. The integration near markers of transcriptionally active region seems to favor the proliferation of the clones. Inversely, the integration near negative regulatory markers impairs the proliferation of the clone. These observations indicate that similar mechanisms seem to occur in human patients and in our mouse model and that proviral transcription seems to favor cell proliferation. In human patients, however, it seems that a negative selection occurs: the immune system probably deletes the clone with a high expression level resulting in the difference observed in the proximity of the provirus with transcriptionally active regions between patients and fresh *in vitro* infection. Such a difference is not observed between fresh *in vitro* infection and infected HIS mice. This could be correlated with the absence of a strong selection in our model. Indeed, the immune response observed in the HIS mice is weak and might not be able to control the proliferation of highly Tax-expressing cells. This idea is further supported by the fact that Tax and HBZ expression was readily detected by RT-PCR and immuno-histochemistry in infected HIS mice (figure 5 in the paper) whereas Tax is subjected to a potent CTL response in infected patients

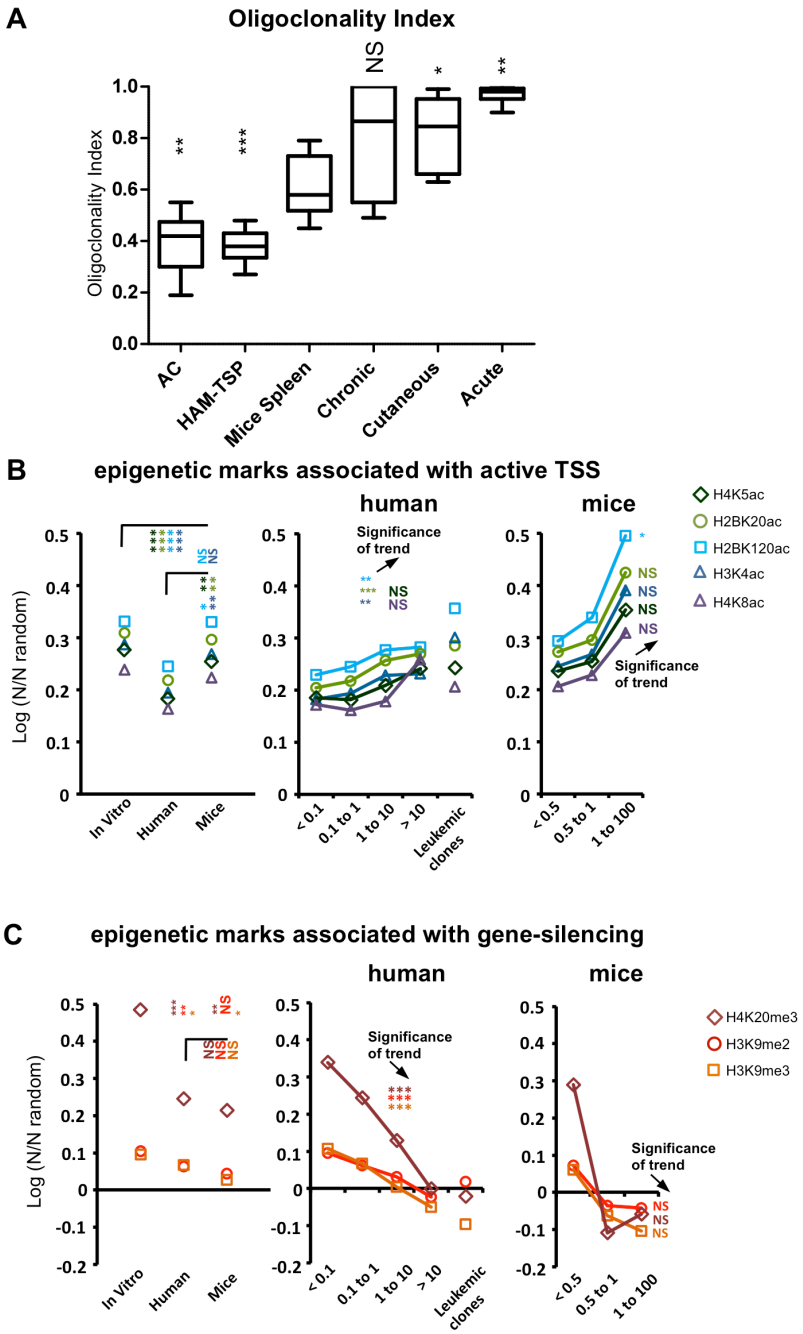

Figure 15 – Genomic environment of integration sites influences the proliferation a clone in HIS mice as in human patients.

A clonality analysis was carried out by Gillet *et al.* on the genomic DNA extracted from infected HIS mice's spleen. *A*- The calculated Oligoclonality Index (OCI) in the spleen of infected HIS mice was compared to the ones obtains in HTLV-1 infected patients (Asymptomatic Carriers, AC; HAM/TSP or ATL – chronic, cutaneous, acute). OCI is an objective measurment of the clonality with 1 being pure monoclonality. Infected HIS mice OCI is higher than AC and HAM-TSP patients. B and C– Relative proximity to epigenetic marks associated with active transcription (B) or gene-silencing (C). A biased proximity to such marks are evaluated in comparison with purely random integration site calculated *in silico*. When Log(N/N random)=0 then the integration is purely random. The first plot compares the proximity to epigenetic marks observed in fresh *in vitro* infection, human patients and infected HIS mice. The second plot dissects the proximity with epigenetic marks for the clone with different size. The size of the clones, in percentage of total PVL, is indicated. The third plot represent the same observation for infected HIS mice.

(Hanon et al., 2000). It therefore appears that the intracellular mechanisms controlling the persistence and the proliferation of the infected clones is reproduced in this HIS mice. The weak immune response seems to be unable to control the proliferation of the clones and might favor the rapid onset of leukemia / lymphoma observed in these animals.

d- Treatment of infected mice with IFN-α, AZT and Arsenic trioxide

Development of therapeutic strategies for treatment of ATL or HAM/TSP patients has been slowed down by the lack of an efficient animal model prone to preclinical trials. ATL patients are known to be poor responders to traditional chemotherapy. Another strategy to address HTLV-1 induced leukemia is to submit ATL patients to an antiviral treatment. Such treatment, involving the use of interferon α (IFN-α) and zidovudine (AZT), has been tested on several patients and has shown promising results as a first-line therapy for ATL patients (Bazarbachi et al., 2010). *In vitro* evidence has demonstrated the beneficial effect of Arsenic Trioxide (As2O3) as, in ATL cell lines, it shuts down the constitutive NF-κB activation and potentiates the anti-apoptotic effects of IFN-α by inducing the proteosomal degradation of Tax (Nasr et al., 2003). Recently a combination of AZT, IFN-α and As2O3 administered to 10 newly diagnosed chronic ATL patients has given successfull results. All the treated patients responded positively with a large majority of them showing complete remission (Kchour et al., 2009). That results in an overall survival rate of 5 years of 100 % of patients affected with chronic ATL (Bazarbachi et al., 2010). Encouraging results have also been reported for acute ATL patients. The molecular and cellular mechanisms involved in this response are not yet understood and should benefit from a reliable *in vivo* model.

Infected HIS mice have been demonstrated to reproduce *in vivo* several stages of HTLV-1 pathogenesis from infection to the development of leukemias/lymphomas. The clonal

expansion seems to be driven by similar mechanisms. These results embolden us to investigate if HTLV-1-infected HIS mice could represent a valid model to test putative therapeutic treatments. To that aim, we injected infected HIS mice with AZT, IFN-α and As2O3 for a week. The treatment was carried out with 20 week-old mice, an age determined as the most efficient to get a high PVL (figure 3A in the paper) and the mice were sacrificed at the end of the treatment to analyze their response in comparison with untreated animals (Figure 16A). The results, presented in Figure 16, illustrate these effects on the PVL, the number of T cells present in the periphery and on the T-cell development in the thymus. After one week of treatment the HTLV-1 PVL measured in the thymus is quite reduced. Due to the low number of mice involved in these preliminary results, not statistical significance can be deduced but the PVL of treated animals seems to be lower than that of untreated animal Figure 16B. This reduction of the PVL is associated with a significant decrease of the spleen size of infected mice (Figure 16C). As this measure is an indicator of the number of cells in the spleen, one can argue that the treatment by itself could induce the observed decrease of the spleen size without affecting infected cells. However the size of the spleen of uninfected and treated animals was not found to differ from that normally observed in our experiments (data not shown). To further characterize the cell population affected by the treatment, FACS analysis was carried out in the spleen, bone marrow and lymph node. The percentages of T cells observed in these organs are presented in Figure 16D. The percentages of activated T cells, evidenced by the CD25 expression, are presented in Figure 16E. The infection is associated with an increased proportion of T cells in the spleen and in the lymph nodes (also described in Figure 4C in the paper) and with the invasion of the bone marrow by T cells. A large number of T cells are activated. Although the number of mice analyzed here is insufficient to reach statistical significance, we nevertheless

observed that after one week of treatment the percentage of T cells is reduced in the spleen and lymph nodes of infected animals. This decrease seems to be even stronger for activated T cells. This observation is coherent with the proposed mechanism of action for this treatment. This combination could target Tax for proteosomal degradation therefore alleviating the constitutive NF-κB activation and potentiating the pro-apototic effect of IFN-α. Such a mechanism could be easily tested in HIS mice. In addition, Tax has been demonstrated to induce the expression of CD25. Therefore the activated T cells showing a high expression of Tax are more sensitive to the treatment explaining their specific loss. The infection is also associated with a deregulation of T-cell development favoring the increase of the mature SP population (figure 4 in the paper and Figure 16). Surprisingly, after just one week of treatment, the T-cell development of 3 out of 5 mice is the same as the one typically observed in mock-infected animals (Figure 16F and G). Another infected and treated mouse shows an intermediate response and the last one has a large majority of mature T cells indicating that it has not been affected by the treatment. Interestingly this mouse also exhibits the highest PVL and the highest percentage of T cells and activated T cells in the bone marrow, the spleen and the lymph node, suggesting that it did not respond to the treatment as the other mice. The effect of the treatment on the T-cell development is also associated with a large decrease in the activated population of thymocytes.

Taken altogether, these results suggest that HTLV-1-infection of HIS mice treated with AZT, IFN-α and As_2O_3 is somehow lessened, as evidenced by the loss of activated T cells as well as the decrease of the PVL. They highlight the fact that infected HIS mice is responding to a therapy that has been shown to be effective in a human clinical trial further validating its use as a preclinical model to evaluate new therapeutic approaches. More experiments are required to ascertain our preliminary results and to elucidate the

mechanism of action of this treatment leading the way to potential future improvement toward a cure of ATL.

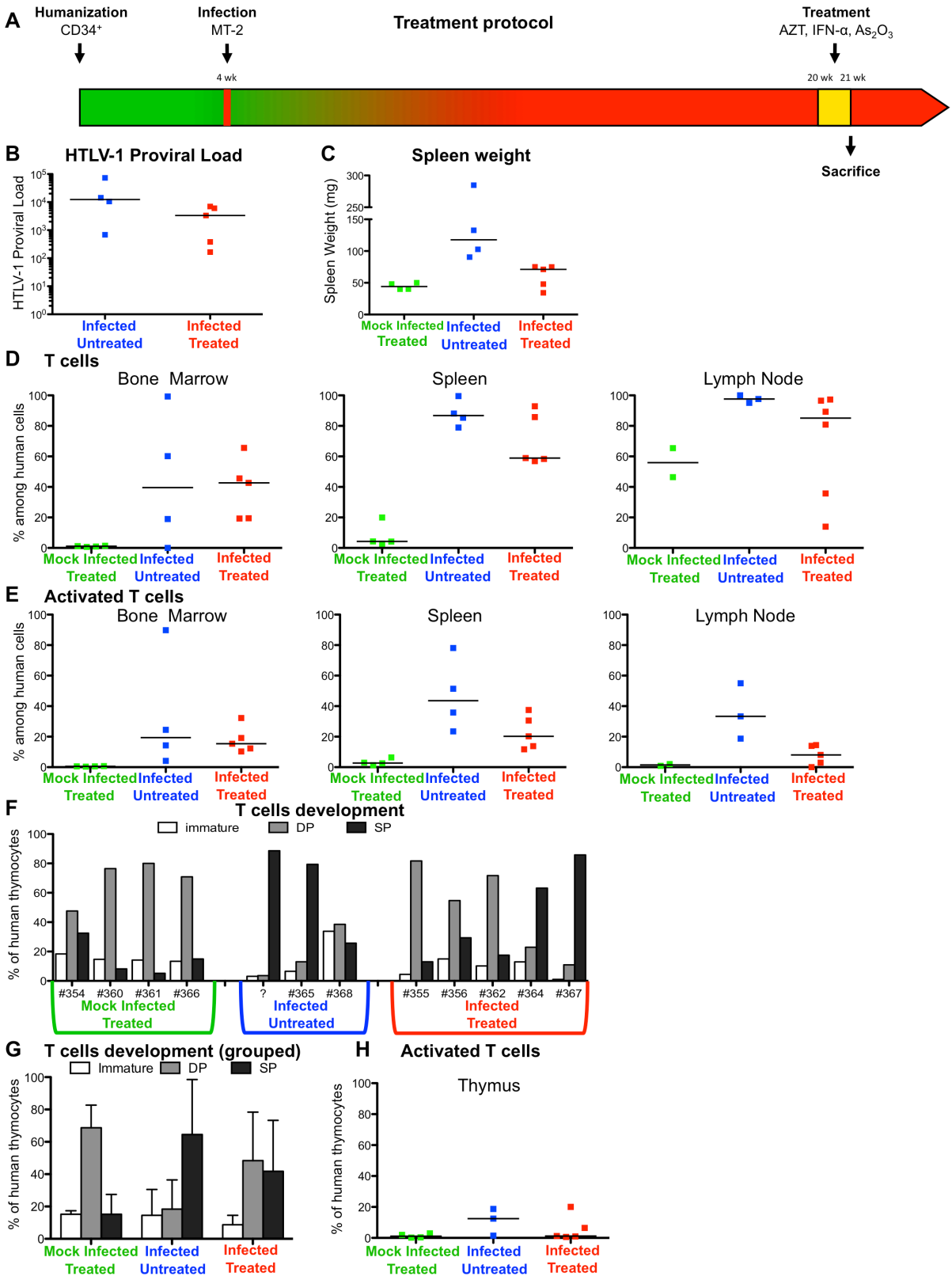

Figure 16 – Treatment of infected HIS mice with AZT, IFN-α and As_2O_3

Three groups of HIS mice were used during this experiment. A first group was mock-infected and treated daily for a week with AZT, IFN-α and As_2O_3 at the age of 20 weeks (Mock Infected Treated, in green). A second group was infected at the age of 4 weeks but injected daily for a week with PBS instead of the treatment (Infected Untreated, in blue). The last group was infected and treated with AZT, IFN-α and As_2O_3 (Infected Treated, in red). **A –** Schematic representation of the procedure used. HIS mice were infected or mock-infected at the age of 4 weeks. They were treated daily for a week with AZT, IFN-α and As_2O_3 or injected with PBS at the age of 20 weeks. Mice were sacrificed after the treatment for analysis. **B –** Effect of the treatment on the HTLV-1 PVL measured in the thymus of the animals. **C –** Effect of the treatment on the weight of the spleen. The weight of the spleen is an easy indicator of the cellularity of the spleen. The significant difference observed between mock-infected animals and infected untreated animals has been previously observed in our experiments. The treatment on mock-infected animals does not alter the weight of the spleen in comparison to what is usually obtained in our lab. **D and E –** Effect of the treatment on the T cells or activated T cell population (D and E respectively) in the bone marrow, the spleen and the Lymph nodes. The percentage of T cells or activated T cells among human cells is plotted for each mice of each group. The bar represents the median for each group. **F and G –** Effect of the treatment on T-cell development in the thymus. The immature, DP and SP population of each mice is plotted (F) and the mean values for each group are plotted in G. The white bars represent the immature population. The light gray bars represent the DP population and the black bars the SP population. The standard deviation is indicated on the grouped plot (G). **H –** Effect of the treatment on activated T cells in the thymus.

II] Review on the humanized mice models (French)

LES SOURIS NE SONT PAS DES HOMMES, ET POURTANT…
Comment les souris humanisées nous renseignent sur les maladies infectieuses humaines

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Titre en anglais: Mice are not Men and yet... how humanized mice inform us about human infectious diseases

Résumé / Aperçu rapide du sujet :

L'étude des pathologies humaines est souvent limitée par l'absence de modèle animal approprié. La souris est le modèle le plus fréquemment utilisé pour l'étude des maladies infectieuses humaines. Cependant, un grand nombre d'agents infectieux spécifiques de l'homme n'infectent pas la souris. Ces vingt dernières années, la greffe de cellules progénitrices ou de tissus humains chez des souris immunodéficiences a permis de générer des souris dites humanisées. Bien que ces modèles demandent encore à être améliorés, ils ont permis de reproduire chez la souris certains aspects des pathologies humaines et laissent ainsi espérer le développement dans un futur proche de thérapies innovantes.

Les propriétés idéales d'un animal servant de modèle d'étude d'un processus pathologique observé chez l'homme sont à la fois une descendance nombreuse, un développement rapide, une taille réduite et un entretien facile. La souris (*Mus musculus*) présente de telles caractéristiques, qu'elle partage avec d'autres espèces animales, comme le ver nématode (*Caenorhabditis elegans*), la mouche du vinaigre (*Drosophila melanogaster*), le poisson-zèbre (*Danio rerio*).

La souris de laboratoire vient de fêter son centième anniversaire : c'est en effet en 1909 que la première souche isogénique de mammifère a été obtenue et identifiée. Il s'agit de la souche de souris DBA (*dilute brown non-agouti*). Cette population d'individus génétiquement identiques représentait alors le groupe témoin essentiel à toute expérimentation biologique. Depuis, la souris est considérée comme le meilleur modèle animal, un « top model » taillé sur mesure pour la recherche. Les premières souris humanisées sont apparues dans les années 80 : ce sont les souris transgéniques dans le génome desquelles a été inséré un gène humain pour en étudier l'expression et la fonction. Ainsi, certaines de ces souris appelées « xénomouse » ont été utilisées pour produire des anticorps humanisés (Bellet et al., 2008). Il faut toutefois admettre que ce qualificatif « humanisées » convient davantage aux souris greffées avec des cellules ou tissus humains. Ce sont ces dernières qui ont retenu notre attention dans la rédaction de cette revue car elles représentent, à ce jour, un outil très performant dans les mains des acteurs de la recherche bio-médicale.

De l'Immuno-déficience à l'Humanisation.

Seules des souris immuno-déficientes se prêtent à une reconstitution d'un système hémato-lymphoïde humain, et sont capables de générer des réponses immunitaires innée et adaptative humaines. La réussite de la prise de greffe des cellules humaines transplantées est conditionnée par l'absence de lymphocytes T et B murins, mais surtout par celle des cellules murines NK. Il est également apparu que l'inoculation de cellules progénitrices hématopoïétiques (CPH) humaines représentait une solution rationnelle pour atteindre un tel objectif.

Les principales souches de souris immuno-déficientes, qui ont été utilisées pour le développement des souris humanisées au cours des récentes décennies, sont présentées dans la Figure 1.

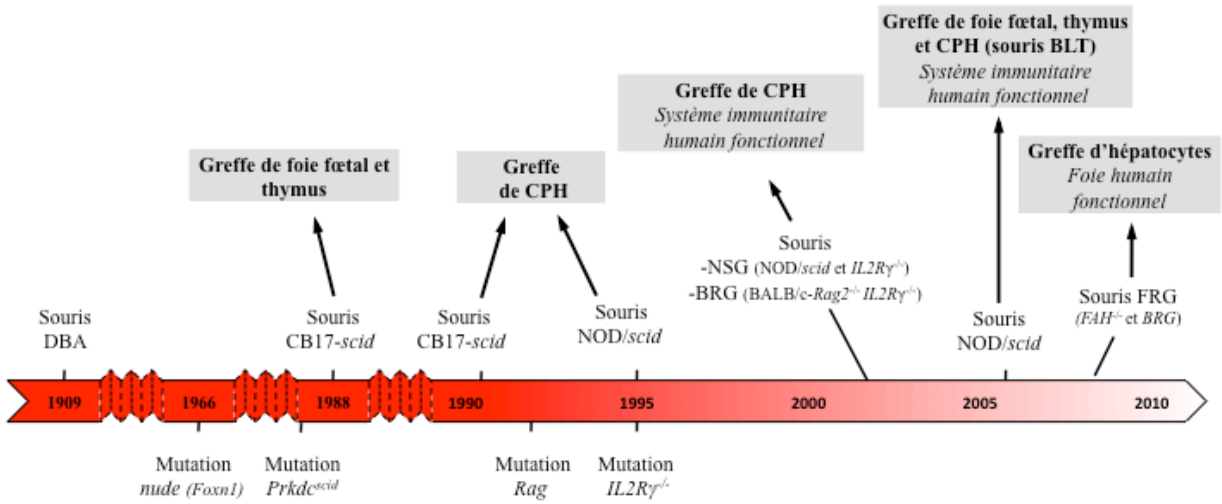

Figure 1. Représentation schématique de la chronologie de la production des différentes souches de souris immuno-déficientes utilisées pour la transplantation de cellules ou tissus humains. BLT : Bone marrow Liver Thymus ; CPH ; cellule progénitrice humaine; DBA :dilute brown non aguti ; FAH : fumaryl acetate hydrolase ; FRG : FAH rag Gamma ; IL2Rγ : nterleukin-2 receptor gamma chain ; NOD : non obese diabetic ; NSG : NOD Scid gamma ; Rag : Recombination-activating gene ; scid : severe combined immuno-deficiency ;

Pour mémoire, citons d'abord les souris *nude* athymiques décrites en 1966 qui ne produisent pas de lymphocytes T. En 1983, les souris CB17-*scid* (severe combined immunodeficiency) ont été décrites (Bosma et al., 1983). Elles présentent une mutation autosomale récessive du gène *prkdc* (protein kinase DNA activated catalytic polypeptide) codant pour une protéine impliquée dans les recombinaisons V(D)J nécessaires aux réarrangements des récepteurs des cellules T et des immunoglobulines (Ig). Cependant, une forte activité des cellules murines NK limitait la prise de greffe de cellules humaines provenant de tissus hématopoïétiques fœtaux (foie, thymus) ou celle de cellules progénitrices hématopoïétiques (CPH) humaines CD34+, empêchant le développement d'un système immunitaire (McCune et al., 1988; Mosier et al., 1988). De même, un tel système n'a pas été observé chez les souris immuno-déficientes NOD (non-obese diabetic)-*scid*, malgré une activité réduite des cellules murines NK (Shultz et al., 1995). Mais la forte incidence de thymomes observés chez ces souris réduit leur durée de vie, écartant la réalisation de longues expériences.

Cependant, les souris NOD-*scid* ont permis après implantation de foie et de thymus de fœtus humain sous la capsule rénale de générer les souris humanisées BLT (pour Bone marrow, Liver, Thymus), implantation suivie d'une irradiation sous-létale et d'une injection intraveineuse de CPH CD34+ isolées du même foie fœtal (Melkus et al., 2006). On observe une repopulation systémique avec présence de cellules hématopoïétiques humaines (lymphocytes T, B, monocytes, cellules dendritiques). La thymopoïèse

humaine a exclusivement lieu dans l'implant thymique, dans lequel l'éducation des cellules T s'effectue dans le contexte d'un CMH humain, comme le confirme la restriction HLA-A1 et HLA-A2 des réponses des cellules T à l'infection par le virus d'Epstein-Barr *in vivo* (Melkus et al., 2006). De plus, ces souris génèrent une forte production d'anticorps (IgG) après immunisation avec des antigènes T-dépendants *in vivo* et la production de cytokines inflammatoires en réponse à la toxine du syndrome du choc toxique (Melkus et al., 2006). Enfin, des cellules B, des cellules T, des monocytes/macrophages, des cellules NK et des cellules dendritiques sont présentes dans l'épithélium intestinal, ainsi que dans les poumons et les muqueuses rectales et vaginales. Cependant, la mise en œuvre de ce protocole étant très laborieuse, le modèle BLT est rarement utilisé.

Plus récemment, ont été générées des souris IL2Rγ$^{-/-}$ présentant une mutation du locus de la chaîne γ du récepteur à l'interleukine 2 (IL2Rγ ou γc pour chaine commune aux récepteurs de l'IL-2, IL-4, IL-9, IL-15 et IL-21). Cette mutation empêche le développement des cellules NK. Ces souris ont été croisées, soit avec les souris NOD/*scid* pour obtenir les souris NOD/*scid*/γc$^{-/-}$ (NSG) (Ishikawa et al., 2005 ; Ito et al., 2008), soit avec les souris BALB/c-Rag2$^{-/-}$, chez lesquelles la mutation Rag2 empêche les recombinaisons V(D)J, pour obtenir les souris BALB/c-Rag2$^{-/-}$γc$^{-/-}$ (BRG) (Traggiai et al., 2004). Chez ces deux souches de souris qui ne développent pas spontanément de tumeurs et sont génétiquement très stables, on note une absence totale de lymphocytes murins T, B, et de cellules NK. Ces souris sont à ce jour les plus utilisées pour étudier les paramètres essentiels à la reconstitution chez ces animaux d'un système hémato-lymphoïde humain et au développement d'un système immunitaire fonctionnel. L'inoculation intrahépatique ou intracardiaque de CPH humaines CD34^{+} préparées à partir de sang de cordon dans des souriceaux âgés de 24 à 48 heures préalablement irradiés représente le protocole idéal (Brehm et al., 2010b). L'efficacité de la greffe de ces CPH autorise la mise en place et le développement d'un système immunitaire humain fonctionnel avec une thymopoïèse humaine active qui a lieu dans le rudiment thymique murin colonisé par des cellules humaines produites *de novo* (Awong et al., 2010; Gimeno et al., 2004 ; Traggiai et al., 2004). A noter que les thymocytes humains sont éduqués principalement contre le complexe majeur d'histocompatibilité (CMH) murin avant leur maturation en cellules T, puis sortent du thymus pour coloniser les organes lymphoïdes secondaires. Deux à 3 mois plus tard, des lymphocytes T et B humains sont détectés dans le sang périphérique, la moelle osseuse, la rate, et les

ganglions lymphatiques (voir tableau). Le nombre de lymphocytes T et B, plus élevé dans les souris NSG souligne que le fond génétique NOD est plus permissif à la greffe xénogénique de cellules humaines. Cette différence est probablement due à une variabilité de l'activité macrophagique, liée notamment à l'expression de différents allèles du gène Sirpα (Takenaka et al., 2007). Comme cette activité est inhibée par les interactions entre CD47 et SIRPα (CD172a), l'expression du CD47 murin dans les CPH humaines ou celle de Sirpα humain dans les souris BRG aboutit à une augmentation significative de la greffe et à une amélioration de l'homéostasie des lymphocytes T et NK (Legrand et al., 2011 ; Strowig et al., 2011b).

Ces modèles autorisent la mise en place d'une réponse immunitaire adaptative. Outre la présence de cellules humaines NK et érythroïdes, celle de cellules myélomonocytaires et dendritiques (DC), dont des DC plasmacytoïdes, souligne l'établissement d'une réponse immunitaire innée (Huntington and Di Santo, 2008 ; Ishikawa et al., 2005 ; Manz and Di Santo, 2009; Traggiai et al., 2004). La production d'anticorps (IgM/IgG) par les lymphocytes B en réponse à une immunisation contre la toxine tétanique est très faible témoignant d'une absence de collaboration T-B dans ces souris chimères, du fait de la non-expression des molécules HLA. Enfin, la détection de cellules B sécrétant des IgA dans la muqueuse intestinale témoigne d'une possible reconstitution de l'immunité mucosale (Ishikawa et al., 2005).

Tissu	**Pourcentage de Cellules hCD45$^+$**			
	Cellules B	Cellules T	Cellules Myéloïdes	Cellules Dendritiques
Moelle osseuse	78-90	3,8-15,1	4,7-11,6	1,6-4,4
Rate	50-85	1,0-39,2	6,4-11,4	1,4-3,3
Thymus	2,1-3,2	95-98	-	-
Sang périphérique	49-76	5,2-27,8	5,6-13,4	-

Composition cellulaire du système hémato-lymphoïde humain observé dans des souris humanisées, 2 à 3 mois après inoculation intra-hépatique de CPH CD34+ humaines, (isolées à partir de sang placentaire) dans des souriceaux NSG ou BRG nouveau-nés, préalablement irradiés avec une dose sous-létale.

De l'Humanisation à l'Infectiologie.

L'émergence, au cours de la dernière décennie, des souris humanisées BLT, NSG et BRG a permis l'étude de divers agents pathogènes humains qu'ils soient d'origine virale, bactérienne ou parasitaire pour lesquels les modèles animaux traditionnels n'apportaient pas de réponse convaincante (pour revues, voir (Legrand et al., 2009 ; Shultz et al., 2007 ; Van Duyne et al., 2009). L'utilisation des souris humanisées permettra de préciser les mécanismes moléculaires et cellulaires de la pathogenèse, de définir de nouvelles stratégies thérapeutiques et de procéder à une évaluation pré-clinique des modalités de prévention vaccinale. Cette recherche translationnelle devrait aboutir à des avancées majeures dans le traitement des maladies infectieuses humaines. Nous analysons quelques exemples illustrant l'apport des souris humanisées à l'étude des virus ciblant les cellules du système immunitaire humain. Il s'agit des virus lymphotropes et monocytotropes (VIH, HTLV-1, virus de la dengue). Récemment, la greffe de cellules du foie a permis d'aborder la pathogenèse des virus hépatotropes (VHB, VHC) (voir Figure 2).

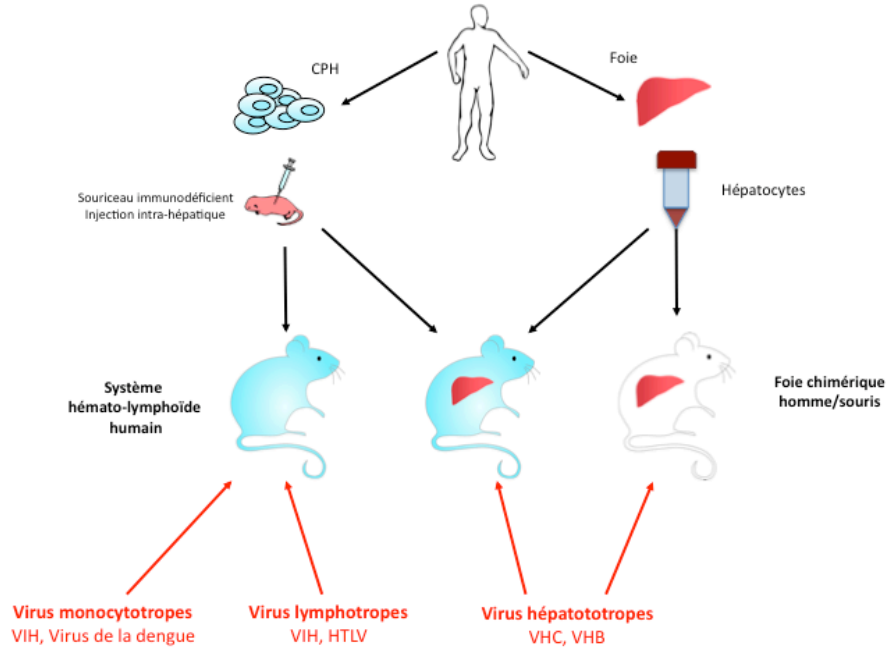

Figure 2. Stratégies mises en œuvre pour établir les modèles de souris humanisées adaptés à l'étude des maladies infectieuses humaines. Voir texte pour détails (de Jong et al., 2010 ; Legrand et al., 2009 ; Van Duyne et al., 2009).

VIH, Virus du syndrome de l'immunodéficience acquise

Un des premiers virus étudié utilisant les souris humanisées est le VIH, lentivirus à la fois lymphotrope et monocytotrope (Baenziger et al., 2006). L'infection des souris

humanisées BRG par ce virus reproduit chez ces souris des aspects spécifiques de la maladie, tels que déplétion de lymphocytes T CD4+, virémie et dissémination du virus dans les organes lymphoïdes (An et al., 2007 ; Berges et al., 2006 ; Gorantla et al., 2007). Cependant, la réponse des cellules B anti-VIH restant très faible dans ce modèle, les études se sont principalement focalisées sur les phases précoces de l'infection, notamment sur la transmission du virus au niveau des muqueuses vaginales et rectales des souris humanisées. Ces muqueuses sont constituées de cellules T, de macrophages et de cellules dendritiques humaines. Lorsque le VIH est directement injecté dans les cavités vaginales ou rectales, l'infection est systémique dès la première semaine (Berges et al., 2006). Ce modèle d'étude est très prometteur pour la validation *in vivo* de nouvelles stratégies thérapeutiques visant à empêcher la transmission du virus au niveau des muqueuses. Dans ce contexte, rappelons qu'un essai de thérapie génique utilisant les shARN (ou ARN en épingle à cheveu) a donné des résultats intéressants (Kumar et al., 2008).

Notons enfin que les souris humanisées permettent l'étude des co-infections. C'est, par exemple, le cas du virus du sarcome de Kaposi qui se réplique dans ces souris en présence ou en absence d'infection par le VIH, et celui de co-infections avec le parasite *Toxoplasma gondii* responsable d'encéphalites (Van Duyne et al., 2009).

HTLV-1, Virus de la leucémie T de l'adulte

Le deltarétrovirus HTLV-1 (Human T-cell leukemia virus type 1) qui infecte 15 à 20 millions d'individus dans le monde est l'agent étiologique de 2 pathologies : la leucémie T de l'adulte ou ATL, et une maladie neurodégénérative invalidante, la paraparésie spastique tropicale ou TSP/HAM (Duc Dodon et al., 2010). Comme pour le VIH, aucun petit modèle traditionnel n'a permis de reproduire l'infection et la pathogenèse *in vivo*. Le modèle des souris *scid* humanisées Thymus/Foie a été d'abord utilisé pour comparer la greffe de cellules CPH préalablement infectées par HTLV-1 à celle de cellules de lignées établies transformées par ce virus (Feuer et al., 1996). Les auteurs ont montré que le virus était retrouvé dans les biopsies de foie et de thymus, mais ne donnait pas lieu à une infection systémique.

Les souris humanisées, ont récemment contribué à l'étude des mécanismes de l'activité leucémogène de ce virus *in vivo* (Banerjee et al., 2010b). Ainsi, quand les CPH CD34+ infectées *ex vivo* par le virus HTLV-1sont transplantées dans des souris NSG, ces

souris développent exclusivement des lymphomes T CD4+ semblables à ceux retrouvés chez les patients ATL et l'intégration du provirus est détectée dans les progéniteurs présents dans la moelle osseuse. Les auteurs concluent que les CPH de la moelle osseuse seraient le réservoir du virus *in vivo* et représentent les cellules cibles de la transformation par HTLV-1 chez l'homme. Nous avons utilisé une approche différente pour étudier les étapes précoces de l'infection par HTLV-1. Des souris BRG humanisées sont infectées par injection intrapéritonéale de cellules irradiées productrices d'HTLV-1, alors que le développement du thymus humain est achevé. L'infection, attestée par une charge provirale élevée dans le thymocytes et les splénocytes humains, perturbe la maturation des thymocytes, provoque une augmentation de la population des lymphocytes T dans les organes périphériques et induit des pathologies caractéristiques de l'ATL. Ces résultats proposent que, chez l'homme, l'infection par HTLV-1 des thymocytes immatures est un événement favorisant le développement de leucémie/lymphomes (Villaudy et al., 2011b).

Virus de la Dengue.

Ce virus, de la famille des Flaviviridae, est le plus souvent à l'origine d'une fièvre bénigne chez les individus infectés (50 à 100 millions par an dans le monde), mais quelquefois d'une fièvre hémorragique, parfois mortelle. Il est transmis à l'homme après piqûre par des moustiques qui jouent à la fois le rôle de vecteurs et de réservoirs. Des souris NOD-*scid* transplantées avec des CPH et infectées par voie sous-cutanée, mimant ainsi la transmission par les moustiques, développent des signes cliniques identiques à ceux observés chez l'homme, à savoir fièvre, érythème et thrombocytopénie (Bente et al., 2005). De même, des souris BRG humanisées après transplantation de CPH, puis infectées par le virus de la dengue, une virémie élevée, accompagnée d'une élévation de température, est observée pendant 3 semaines (Kuruvilla et al., 2007). La détection précoce d'anticorps IgM est suivie de celle d'anticorps IgG, dont certains sont neutralisants. Récemment, des souris NSG, transgéniques pour HLA-A2 ont été utilisées, souris chez lesquelles l'éducation intrathymique des cellules T humaines s'effectue au contact des molécules HLA-A2 exprimées par les cellules de souris. Ces souris humanisées après injection de CPH HLA-A2+ sont infectées par le virus de la dengue (Jaiswal et al., 2009). Des splénocytes humains sont ensuite isolés et stimulés *ex vivo* par des peptides viraux restreints par HLA-A2. La sécrétion d'interféron γ, de TNFα (tumor

necrosis factor) et d'interleukine-2 permet de détecter la présence de lymphocytes T $CD8^+$ dans les souris NSG exprimant HLA-A2, contrairement à des souris NSG non transgéniques pour HLA-A2.

VHB et VHC, Virus des Hépatites

Le hépatocytes humains sont les cellules cibles de plusieurs agents pathogènes, parmi lesquels les virus des hépatites B et C (VHB, de la famille des Hepadnaviridae et VHC, de la famille des Flaviviridae), qui infectent 800 millions d'individus dans le monde. Pour mieux comprendre la pathogenèse de ces infections virales, des souris immuno-déficientes, croisées avec des souris transgéniques pour un gène exprimant une protéine cytolytique pour les hépatocytes murins, ont été produites. Un de ces modèles est le modèle des souris urokinase-type plasminogen activator (uPA)/$Rag2^{-/-}$ (Dandri et al., 2001). Transplantées avec des hépatocytes adultes humains, leur infection par VHB est suivie d'une infection productive. Plus récemment, il a été fait appel à des souris qui n'expriment pas la fumaryle acétate hydrolase (FAH), ce qui conduit à la destruction du foie murin (Bissig et al., 2010; de Jong et al., 2010). Ces souris FAH^-/BRG, transplantées avec des hépatocytes adultes humains, montrent un chimérisme humain/souris élevé au niveau du foie, (corrélé avec la quantité de sérum albumine humaine). Elles sont sensibles à l'infection par VHB et VHC, comme l'indique la détection des antigènes viraux dans le foie chimérique de ces animaux. De plus, les souris infectées par VHC répondent au traitement anti-viral. Récemment, ont été générées des souris BRG transgéniques pour une protéine inductible de fusion (FK506 binding protein-caspase 8), dont l'induction entraîne la dimérisation de la caspase 8, qui ainsi activée, provoque la mort des hépatocytes murins (Washburn et al., 2011). Ces souris sont ensuite greffées à la fois par des CPH et des progéniteurs hépatocytaires isolés du même foie fœtal, puis infectées quatre semaines plus tard par VHC. Elles génèrent alors une réponse immunitaire humaine T-spécifique anti-VHC et développent des hépatites et des fibroses. Ces souris, possédant à la fois des hépatocytes humains et des cellules immunitaires humaines illustrent le potentiel de ce modèle pour étudier l'immunopathogenèse accompagnant l'infection par les virus hépatotropes. Ce modèle sera également utile pour l'étude de *Plasmodium falciparum*, le parasite responsable de la malaria, dont le développement passe par une étape intra-hépatique obligatoire (Morosan et al., 2006).

Le futur des souris humanisées

Actuellement, des protocoles standards relativement faciles à mettre en œuvre pour produire ces souris humanisées sont disponibles (Legrand et al., 2008; van Lent et al., 2010). Une des grandes innovations concernant les souris humanisées est l'augmentation du nombre de tissus humains candidats à la greffe chez la souris, multipliant ainsi le nombre de maladies humaines modélisables. Certains tissus humains cibles spécifiques d'infections virales peuvent être transplantés dans des souris immunodéficientes : c'est le cas de l'épithélium intestinal pour l'étude des virus entériques (Shultz et al., 2007).

Des stratégies sont aujourd'hui entreprises dans ce but. Citons l'ablation des cellules et/ou tissus murins pour créer de « l'espace », soit par irradiation pour la greffe de précurseurs hématopoiétiques, soit par modifications génétiques pour celle d'hépatocytes humains. Par ailleurs, on essaie d'augmenter la spécificité de la réponse immunitaire des souris humanisées par injection ou transgénèse des cytokines humaines et d'éviter la destruction active de la greffe par la souris (Willinger et al., 2011). La contrainte majeure inhérente au modèle des souris humanisées provient de ce que l'éducation des cellules T et B est restreinte du fait de la non-expression des molécules HLA. Ainsi, l'expression transgénique des molécules HLA dans des souris NSG autorise le développement d'une réponse immunitaire HLA restreinte (Jaiswal et al., 2009; Shultz et al., 2010). Il faut espérer que l'effet synergique de chacune de ces modifications permette d'obtenir, dans un avenir très proche, le modèle animal le plus performant pour l'étude des virus, bactéries et autres agents pathogènes humains, ainsi que pour le développement de thérapies innovantes et de vaccins (Manz and Di Santo, 2009).

SUMMARY

Mice are not Men and yet... how humanized mice inform us about human infectious diseases

The study of human pathologies is often limited by the absence of animal models which are robust, cost-effective and reproducing the hallmarks of human infections. While mice have been frequently employed to study human diseases, many of important pathogens display unique human tropism. These last two decades the graft of human progenitor cells or tissues into immunodeficient mice has allowed the elaboration of so

called humanized mice. Humanized mouse technology has made rapid progress, and it is now possible to achieve high levels of human chimerism in various organs and tissues, particularly the immune system and the liver. The present review briefly summarizes the different models of humanized mice available for *in vivo* experiments. With a focus on lymphotropic, monocytotropic and hepatotropic viruses, we here discuss the current status and future prospects of these models for studying the pathogenesis of infectious diseases. Furthermore, they provide a powerful tool for the development of innovative therapies.

CONFLITS D'INTÉRÊTS

Les auteurs déclarent n'avoir aucun conflit d'intérêts concernant les données publiées dans cet article.

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III] HBZ enhances the transcription of hTERT in cooperation with JunD.

In a previous work (Master 1), my project was to characterize the effect of HBZ on the hTERT promoter. hTERT (human telomerase reverse transcriptase) is the catalytic subunit of the telomerase and is responsible of the telomeres replication. Telomeres are specialized structures of non-coding DNA protecting the extremities of the chromosomes. Due to their position and their structure telomeres are shortened at each genome replication and the role of the telomerase is to maintain the telomere length by catalyzing the addition of specific repeats (TTAGGG in vertebrates). The shortening of the telomeres is observed with the aging of the cells and when a minimal length is reached cells enter senescence. In many cancer cells, the telomerase activity is found to be deregulated as a mechanism to maintain the telomeres length through their high division rate. In ATL patients, telomerase activity is also found to be upregulated and especially in the more severe forms of ATL (Uchida et al., 1999). The effect of Tax on the regulation of the hTERT transcription is still controversial and is dependent of the state of the cell (Hara et al., 2008). HBZ is thought to play a major role in the maintenance of the leukemogenic HTLV-1-associated process that might be initiated by Tax (Barbeau and Mesnard, 2011; Duc Dodon et al., 2010). When I started this project, HBZ has been recently discovered, nothing was known on its effect on cellular gene regulation. The fact that HBZ is displaying a bZip domain allowed this viral protein to interact with AP-1 transcription factors, such as JunD which is highly expressed in ATL cells. Thus, we have investigated the role of HBZ/JunD in the regulation of hTERT, known to have AP1 binding sites on its distal promoter. Indeed, we demonstrated that HBZ/JunD up regulates the hTERT transcription. But this action was via its proximal promoter

through an interaction with Sp1 transcription factors. In conclusion, hTERT was the first reported cellular gene to be deregulated by HBZ (Kuhlmann et al., 2007).

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HTLV-I HBZ cooperates with JunD to enhance transcription of the human telomerase reverse transcriptase gene (hTERT)

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Abstract

Background: Activation of telomerase is a critical and late event in tumor progression. Thus, in patients with adult-T cell leukaemia (ATL), an HTLV-I (Human T cell Leukaemia virus type 1)-associated disease, leukemic cells display a high telomerase activity, mainly through transcriptional up-regulation of the human telomerase catalytic subunit (hTERT). The HBZ (HTLV-I bZIP) protein coded by the minus strand of HTLV-I genome and expressed in ATL cells has been shown to increase the transcriptional activity of JunD, an AP-1 protein. The presence of several AP-1 binding sites in the hTERT promoter led us to investigate whether HBZ regulates hTERT gene transcription.

Results: Here, we demonstrate using co-transfection assays that HBZ in association with JunD activates the hTERT promoter. Interestingly, the -378/+1 proximal region, which does not contain any AP-1 site was found to be responsible for this activation. Furthermore, an increase of hTERT transcripts was observed in cells co-expressing HBZ and JunD. Chromatin immunoprecipitation (ChIP) assays revealed that HBZ, and JunD coexist in the same DNA-protein complex at the proximal region of hTERT promoter. Finally, we provide evidence that HBZ/JunD heterodimers interact with Sp1 transcription factors and that activation of hTERT transcription by these heterodimers is mediated through GC-rich binding sites for Sp1 present in the proximal sequences of the hTERT promoter.

Conclusion: These observations establish for the first time that HBZ by intervening in the re-activation of telomerase, may contribute to the development and maintenance of the leukemic process.

Introduction

Adult T-cell leukaemia (ATL) is a T-cell malignancy that develops in about 5% of asymptomatic HTLV-1 (human T-cell leukaemia virus, type 1) carriers after a latent period ranging from 20 to 60 years, indicating a multistage process of transformation of T lymphocytes. ATL cells are gen-

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erally CD4+ T lymphocytes, in which both NF-κB and AP-1 (activator protein-1) transcription factors are constitutively active. Distinct clinical subtypes of ATL include two indolent forms, smoldering and chronic, and extremely aggressive forms, acute and lymphomatous. Chronic ATL often progresses to acute or lymphoma-type ATL and the mean survival time of patients with acute ATL is about one year [1-3]. Interestingly, the close correlation observed between telomerase activity and the clinical stage of the disease indicates that the re-activation of telomerase, by contributing to telomere stabilization, is a key event in development and progression of ATL [4].

A functional basic leucine zipper (bZIP) protein, HBZ (HTLV-1 bZIP factor), that is encoded by a mRNA transcribed from a functional promoter present within the anti-sense strand of the 3' end of the HTLV-1 provirus, was identified, through its expression in several HTLV-1-infected cell lines [5-7]. Moreover, HBZ was found to be the only viral gene product detected in a panel of fresh ATL cell clones [8]. This protein contains an N-terminal transcriptional activation domain, two basic regions corresponding to nuclear localization signals, and a DNA-binding domain upstream of a C-terminal leucine zipper motif [9,10]. Interestingly, HBZ RNA was found to promote T-cell proliferation and to up-regulate the E2F1 transcription factor [8]. Furthermore, the HBZ protein has been shown to interact with other bZIP proteins, in particular with the AP-1 transcription factors, resulting in the modulation of their transcriptional activity [11-13]. Thus, through its interaction with CREB-2 (also called ATF-4), HBZ inhibits Tax-mediated proviral transcription from the HTLV-1 promoter within the viral LTR [10,14-16]. Tax, a viral regulatory protein, encoded by the pX region of HTLV-1, plays a pivotal role in the early steps of the transformation of T lymphocytes infected by HTLV-1, by influencing the transcription of numerous cellular genes, among them NF-κB and AP-1 [17-19].

The hTERT proximal core promoter which contains Sp1 and c-Myc binding sites, is essential for the transcriptional activation of this cellular gene [20-22]. Recently, five putative binding sites for AP-1 have been identified within the distal regulatory sequences of the hTERT promoter [23]. AP-1 is composed of heterodimers of Jun (c-Jun, JunB or JunD) and Fos (c-Fos, Fra1, Fra2, FosB-2) proteins and c-Fos/c-Jun and c-Fos/JunD heterodimers have been shown to decrease hTERT transcription in human cells [23]. Interestingly, HBZ is not able to form stable homodimers and is therefore dependent on heterodimerization with other AP-1 proteins to control gene transcription [11-13]. In the present study, we investigated whether HBZ, in association with c-Jun or JunD, is able to regulate the activity of the hTERT promoter. We demonstrated that HBZ together with JunD synergistically activates hTERT transcription through their recruitment by the Sp1 transcription factors on the Sp1 sites present at the proximal region of the hTERT promoter. These observations provide an original insight by which hTERT transcription is up-regulated by this viral protein.

Results

HBZ regulates the activity of the hTERT promoter

To examine the role of HBZ in regulating the activity of the hTERT promoter, luciferase assays were performed with reporter plasmids containing various lengths of the 5' flanking sequence of the *hTERT* gene fused to the luciferase reporter gene (Fig 1A). The longest reporter pGL3-3300 contains 5 AP-1 binding sites; pGL3-2000 includes two of these sites, whereas the shortest construct pGL3-378 encompassing the proximal region is devoid of any AP-1 binding sequence. Each of these reporter plasmids was co-transfected in HeLa cells along with increasing amounts of an HBZ vector either alone or together with c-Jun or JunD expression plasmids. The expression levels of HBZ and Jun proteins were confirmed by Western blot analysis. Overexpression of HBZ with each of the three constructs did not exert any effect on the hTERT promoter activity (Fig 1B, lanes 2, 3, 4). Overexpression of c-Jun or JunD led to a small, but significant increase of this promoter activity. In presence of c-Jun, a 2-fold increase was observed with pGL3-3300, and a 3-fold increase with pGL3-2000 and pGL3-378. In presence of JunD, a 2-fold-increase was observed only with pGL3-378. Overexpression of HBZ with c-Jun resulted in a reduction of the hTERT promoter activity with the three reporter constructs (compare lanes 6, 7, 8 to lane 5). To note that the increased amounts of HBZ correlated with a decrease of c-Jun detected in cell lysates, as previously shown [12]. Intriguingly, overexpression of HBZ in the presence of JunD led to an increase of the hTERT promoter activity, which also correlated with the transfected amount of HBZ (compare lanes 10,11,12 to lane 9). Taken together, these observations show that HBZ expressed either with c-Jun or JunD is able to repress or enhance the hTERT promoter activity, respectively.

Interestingly, the activation observed in presence of JunD was found to be equally high using reporter pGL3-378 and pGL3-2000 constructs. As no AP-1 binding site was present in pGL3-378, this observation suggests that HBZ exerts an indirect control on the hTERT core promoter. Indeed, our data propose that the up-regulation of hTERT promoter activity is mediated by HBZ in cooperation with JunD and indicate that the proximal region of the promoter contains the responsive sequences necessary and sufficient to increase this activity. To confirm the effect of the *HBZ* gene in T cells, we co-transfected Jurkat T cells with the pGL3-378 reporter construct with either JunD or both JunD and HBZ (Fig 1C). It was observed that HBZ,

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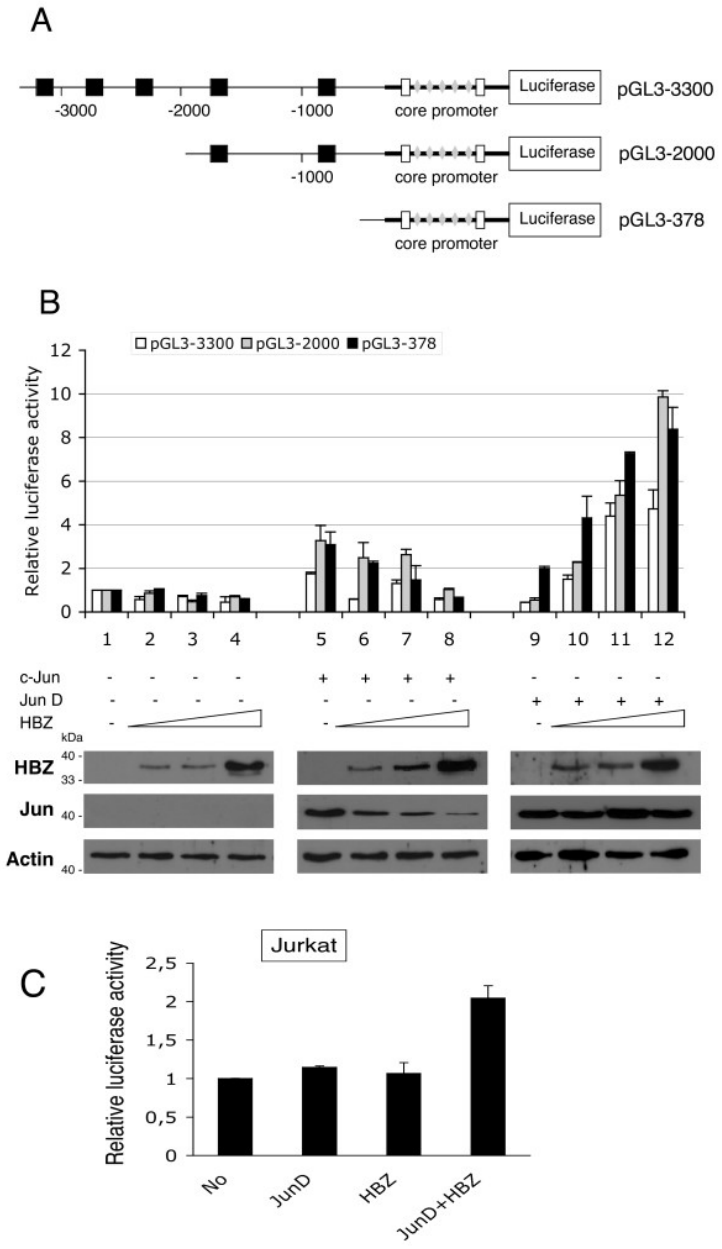

Figure 1

Transient-expression assays to examine the role of HBZ and of AP-1 in the hTERT promoter. (A) Schematic diagram of the luciferase reporter plasmids containing various lengths of the hTERT promoter. The black squares indicate AP-1 responsive sites. The sequence of the proximal core promoter is located between -181 to +80. (B) Effect of HBZ and AP-1 on luciferase reporter constructs. HeLa cells were cotransfected with various lengths of the hTERT promoter plasmids (0.1 μg) and with HBZ- (0.2 to 0.8 μg), and/or c-Jun- (0.2 μg), and/or JunD (0.2 μg)-expression vectors. Luciferase activity was normalized to tk-luc activity and presented relative to cells transfected with the reporter plasmid alone. The values are those obtained in triplicate, from three different experiments. Error bars indicate standard deviations. Shown in the lower panel, a western blot analysis of HBZ and Jun protein levels in whole cell lysates of HeLa samples transfected with pGL3-378. The membrane was probed successively with a polyclonal anti-HBZ antibody, and a mouse anti-flag antibody. Actin was used as a loading control. (C) Transactivation of the hTERT promoter by HBZ and JunD in Jurkat cells. Cells were cotransfected with pGL3-378 reporter plasmid (4 μg), in combination with the indicated HBZ (2 μg) and/or JunD (2 μg)-expression vectors. Luciferase activity was normalized and presented as indicated in B. The values are those obtained in triplicate, from one representative experiment.

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in presence of JunD, increases the hTERT promoter activity, but to an extent lower than that observed in HeLa cells.

Both the N-terminal and leucine-zipper regions of HBZ are required to increase the hTERT promoter activity

To identify the domain(s) of the HBZ protein required for transactivation of the hTERT promoter, a structure-function analysis was first performed including vectors expressing three deleted forms of HBZ. The first one, referred to as HBZΔAD, is deleted of the N-terminal 80 amino acids corresponding to the activation domain (AD), and was found to be unable to increase JunD-mediated transactivation of a synthetic collagenase AP-1-Luc construct [13]. The second one, HBZΔbZIP, lacks the 74aa C-terminal domain, which includes the leucine zipper domain, and is therefore unable to form dimers with other AP-1 proteins [11]. The third one, HBZΔADΔZip, lacks both the 80 aa N-terminal domain and the 46aa Zip domain. The wild type (wt) HBZ or each of its deleted forms was transiently co-transfected into HeLa cells along with the pGL3-378 construct, in the presence or absence of the JunD expression vector. Luciferase assays show that the overexpression of wtHBZ in the presence of JunD resulted in a 5.8-fold transactivation of the hTERT core promoter (Fig 2, lane 7). In cells transfected with HBZ mutants deleted either of the activation domain or of the leucine zipper domain, a decrease of this transactivation

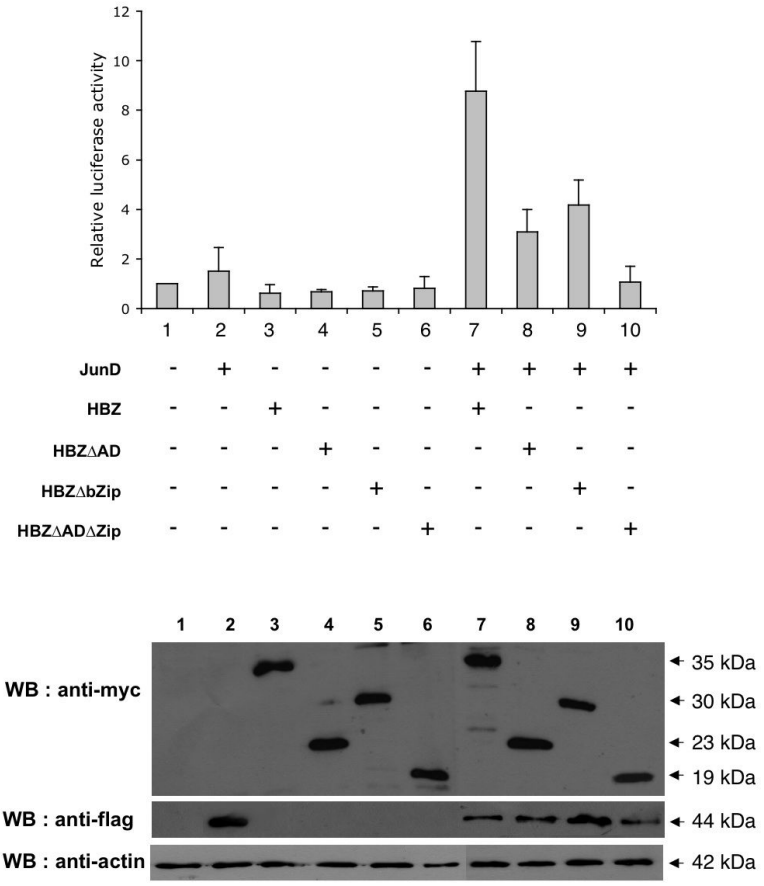

Figure 2

Deletion of the activation domain and leucine-zipper region of HBZ significantly reduces hTERT promoter activity. The luciferase reporter plasmid, pGL3-378 (100 ng) was cotransfected with expression plasmids (200 ng) for JunD, HBZ or HBZΔAD or HBZΔbZIP, or HBZΔADΔZip as indicated. Luciferase activity was normalized to tk-luc activity and is presented relative to that of cells transfected with the reporter plasmid alone. The values are those obtained in duplicate from five experiments. Lower panel: western blot analysis of HBZ and JunD protein levels in whole cell lysates of HeLa samples. The membrane was successively probed with monoclonal anti-c-myc, anti-Flag and anti-actin antibodies.

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by 66% and 53% was observed respectively, suggesting that they still retain enhancing activities (lanes 8 and 9). Finally, the double mutant was found to be unable to increase JunD-mediated transactivation (lane 10). Indeed, these results show that both the N-terminal and the C-terminal domains of HBZ are required to fully transactivate the hTERT promoter in presence of JunD. Thus, the activation functions together with the dimerization properties of HBZ appear to be essential for up-regulating the hTERT promoter activity.

HBZ positively regulates hTERT transcription in presence of JunD

The above results propose for the first time that HBZ, in cooperation with JunD, activates the transcription of the hTERT gene. To examine the effect of HBZ on *hTERT* transcription, HeLa cells were co-transfected either with JunD and HBZ expression vectors or a blank vector. A Western blotting analysis, performed 48 hours later, confirmed the expression of both HBZ and JunD proteins (Fig 3A). The level of hTERT transcription evaluated by RT-PCR analysis showed a slight increase of hTERT transcripts, when HBZ and JunD were overexpressed (Fig 3B). Likewise, a quantitative analysis of hTERT mRNAs using real-time PCR revealed a significant 1.8-fold increase ($P < 0.05$) of hTERT mRNAs in cells co-expressing HBZ and JunD (Fig 3C, lane 6). Such an increase was not observed in cells expressing either JunD alone (lane 2) or HBZ alone (lane 3) or JunD together with one of the mutated forms of HBZ (lanes 7 and 8). These findings demonstrate that HBZ acts synergistically with JunD to increase the transcription of the *hTERT* gene.

To verify that HBZ plays a direct molecular role in the activation of hTERT expression, chromatin immunoprecipitation (ChIP) assays were done to seek evidence of HBZ occupancy at the hTERT promoter. HeLa cells overexpressing HBZ and JunD were crosslinked, sonicated, DNA-protein complexes collected by centrifugation, and ChIP performed (Fig 3D). Both HBZ and JunD were present at the hTERT proximal promoter region. Notably, the specificity of HBZ and JunD ChIP was illustrated by the lack of occupancy at the distal region of hTERT promoter. Collectively, these results confirm that HBZ behaves as a positive regulator of hTERT gene transcription.

Identification of the promoter sequences responsible for the HBZ/JunD-mediated transcriptional upregulation of hTERT

As demonstrated above, the proximal hTERT promoter is responsible for stimulating the transcriptional activity of the *hTERT* gene by HBZ together with JunD. However, the inspection of the nucleotide sequence of this region does not reveal any sequence homologous to the consensus AP-1 binding site (5'-TGAC/GTCA-3'). This region includes the 180 bp core promoter that contains 2 E-boxes and 5 GC boxes, which represent the consensus binding sequences for Myc/Max and Sp1, respectively (Fig 4A). Interestingly, previous studies have revealed that each of the five Sp1 sites cooperatively function as a *cis*-acting element [21]. The presence of a unique Sp1 site in a 32 bp minimal promoter region suggests that Sp1 may recruit basal transcription factors for hTERT. Furthermore, Jun proteins (and among them JunD) are able to interact with Sp1 proteins [24,25]. We therefore hypothesize that a mechanism involving protein-protein interactions between HBZ and JunD is operative for the transactivation of the hTERT promoter, Sp1 being implicated in the recruitment of the HBZ and JunD to the GC boxes. To verify this hypothesis, a construct, pGL2-Sp1-TATA-Luc, containing a minimal promoter with one Sp1 binding site with a sequence (C_4GC_4,) similar to that of the second and fifth Sp1 sites present on the proximal region of the hTERT promoter followed by a TATA box fused to a luciferase reporter gene was co-transfected in HeLa cells together with HBZ- or/and JunD-expression vectors. An identical reporter construct, but lacking the GC box, was used as control. The expression levels of JunD and HBZ proteins were verified by western blotting, 48 h after transfection. In cells cotransfected with the control construct, no significant increase of luciferase activity was observed in presence of either JunD, or HBZ or both (Fig 4B). In HeLa cells transfected with the reporter construct containing the GC-rich Sp1 binding site, the expression of JunD led to a 5-fold increase of the luciferase activity (compared to that of cells transfected with the reporter construct alone), whereas that of HBZ did not exert any effect. The co-expression of JunD and HBZ led to a 15-fold increase of luciferase activity. This observation therefore underlines the intervention of the Sp1 binding sites in the synergistic activation of the hTERT core promoter by HBZ and JunD.

Physical and functional interactions of HBZ, JunD and Sp1 proteins

To confirm the *in vivo* interaction of these three proteins, extracts of HeLa cells co-expressing HBZ, JunD and Sp1 were immuno-precipitated with a rabbit anti-HBZ serum. Proteins in the immuno-precipitate were then analyzed by western blot using a monoclonal anti-Flag antibody or a rabbit anti-Sp1 serum. Under these experimental conditions, JunD was found in the immunoprecipitate only when HBZ is expressed (Fig 5B, lower panel lanes 2 and 3). When the same experiment was performed with extracts from HeLa cells either mock-transfected or transfected with JunD and Sp1, these proteins were not detected in the immunoprecipitate (Fig 5B, lower panel lanes 1 and 4), confirming the specificity of the association between HBZ and JunD. In addition, Sp1 was found to be more abundant in the immunoprecipitate prepared

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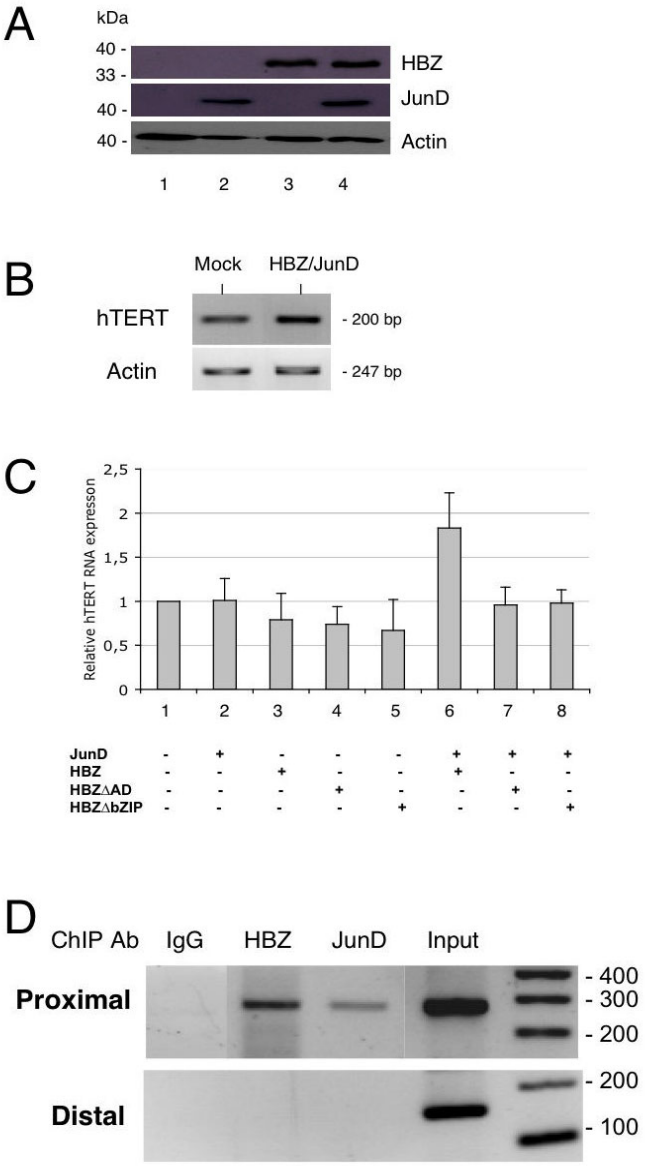

Figure 3
Up-regulation of the hTERT gene transcription in cells overexpressing HBZ and JunD. HeLa cells were cotransfected with expression plasmids for HBZ and JunD and incubated for 48 hours. (A) Western blot analysis of cell lysates using appropriate antibodies; lane 1, mock transfected; lane 2, JunD-transfected; lane 3, HBZ-transfected; lane 4, HBZ/JunD-transfected. (B) RT-PCR analysis of mRNA extracted from transfected cells. The RNAs were isolated and reverse transcribed. PCR was performed using primers specific for hTERT and actin. (C) Real-time PCR quantification of hTERT mRNA expression from cells transfected with indicated plasmids was performed as described in "Materials and methods". The expression level in mock-transfected cells was defined as 1.0. Experimental variations are indicated by error bars. (D) Recruitment of HBZ and JunD to the hTERT proximal promoter by chromatin immunoprecipitation assay (ChIP) in HeLa cells overexpressing HBZ and JunD. PCR results from IP reactions using preimmune rabbit serum (IgG) and antibodies against HBZ and JunD are shown. Each panel shows amplification of 0.4% of the total input chromatin (input). Purified DNA was analyzed by PCR using primers spanning the hTERT proximal promoter (upper panel) or the hTERT distal promoter (lower panel). DNA size standards are indicated. Data are shown for one representative experiment from three independent assays.

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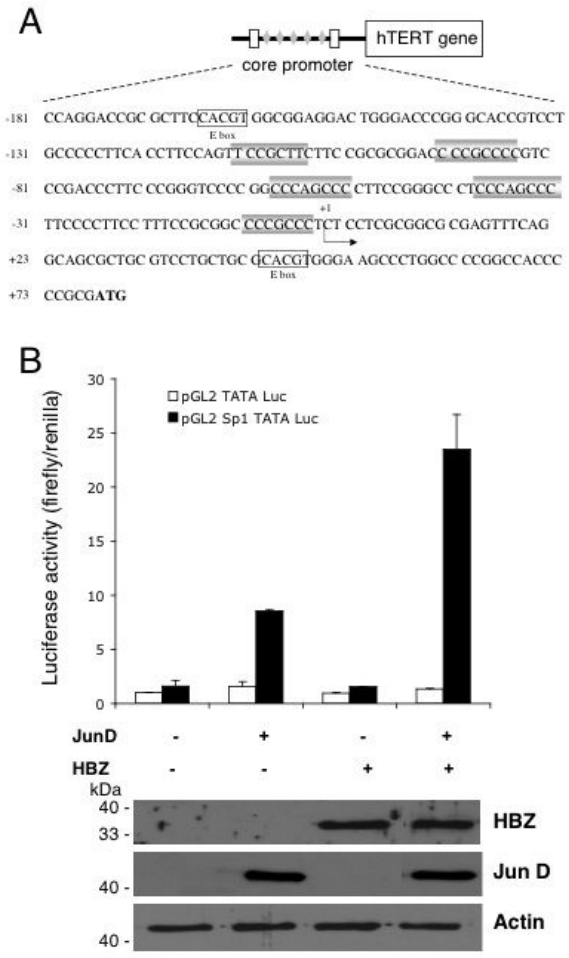

Figure 4

Involvement of Sp1 binding sites in HBZ/JunD-mediated transcriptional activity. (A) Schematic diagram of the hTERT gene promoter corresponding to the sequence of the proximal core promoter (-181 to +80) upstream of the initiating ATG shown in bold. The Sp1-binding sites (shaded box) and E-boxes (white box) are indicated. (B) The luciferase reporter plasmid, pGL2-Sp1-TATA-Luc or the control plasmid, pGL2-TATA-Luc (100 ng) was cotransfected with expression plasmids for HBZ (200 ng) and/or for JunD (200 ng), as indicated. Luciferase activity was normalized to tk-luc activity. Results represent duplicate samples from two different experiments. Lower panel: western blot analysis of HBZ and JunD protein levels in whole cell lysates of HeLa samples transfected with pGL2-Sp1-TATA-Luc plasmid. The membrane was successively probed with a rabbit polyclonal anti-HBZ serum, mouse monoclonal anti-Flag and rabbit anti-actin antibodies.

from HeLa cells overexpressing Sp1, JunD and HBZ (Fig 5B, upper panel, lane 2), underlining the interaction of the three proteins.

To demonstrate that HBZ, JunD and Sp1 co-exist within the same protein complex resident in the proximal promoter, sequential ChIP assays were performed (Fig 5C). In such assays, an initial ChIP was performed with an antibody that recognizes one protein. The precipitated chromatin-DNA complex was washed and eluted, then a second IP was performed with a second antibody. When ChIP was first performed with anti-HBZ, sequential ChIP showed occupancy of Sp1 in the same protein-DNA complex (Fig 5C, lane 6). Alternatively, when ChIP was first performed with anti-JunD, sequential ChIP showed occupancy of Sp1 in the same protein-DNA complex (lane 5). Specificity was again demonstrated, as these complexes were not detected at the distal region of the hTERT promoter

Additional experiments were next performed to establish functional interactions between JunD, HBZ and Sp1. Transient co-transfection experiments in HeLa cells showed that JunD transactivated a synthetic promoter consisting of five tandem high affinity binding sites for the yeast protein Gal4 upstream of a minimal TATA box (5XGal4-Luc) by 11-fold (Fig 6, compare lanes 3 and 2), when it was coexpressed with a Gal4-Sp1B chimeric protein consisting of the DNA binding domain of Gal4 fused to the B domain of Sp1, indicating that JunD and Sp1 are able to interact physically. Overexpression of HBZ in HeLa cells enhanced the JunD-mediated transactivation potential by 2.7-fold (compare lanes 3 and 4). Control experiments showed that the HBZ protein alone in the presence of Gal4-Sp1B had no significant effect on the activity of the 5XGal4 promoter (lane 2). These observations show that cooperative interactions between HBZ, JunD and Sp1 can transactivate promoters containing multiple Sp1-binding sites. Collectively, these results clearly underline that the synergistic transactivation of hTERT promoter by HBZ and JunD is Sp1-dependent.

Effect of Tax on HBZ-mediated activity of the hTERT promoter

It has been previously shown that Tax acts as a transcriptional repressor of the hTERT core promoter through the proximal E-box, by competing with cMyc/Max bHLH proteins for recruitment of the CBP/p300 co-activators [26]. In addition, we have observed a down-regulation of hTERT transcription by Tax in HTLV-1 transformed or in Tax-expressing T lymphocytes [27]. Our present results therefore propose that Tax and HBZ may exert opposite effects on the activity of the core promoter. To verify this possibility, HeLa cells were co-transfected with the reporter pGL3-378 construct together with the Tax-, HBZ- and JunD-expression vectors (Fig 7). As expected, the basal transcriptional activity of the core promoter was repressed by Tax (lane 3). The co-expression of JunD had no effect on the repression exerted by Tax (lane 5). As

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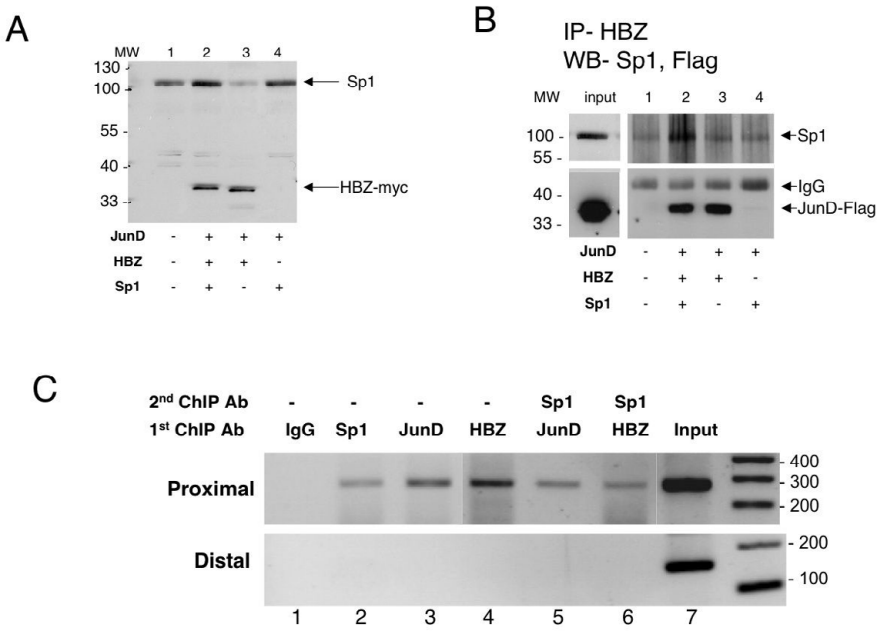

Figure 5

Physical interactions of HBZ, JunD and Sp1 proteins. (A) HBZ and Sp1 expression in transfected HeLa cells by Western blot analysis. (B) *In vivo* interactions detected by Immunoprecipitation and western blot analysis. Five percent (for Sp1) and 0.2% (for JunD) of the whole cell lysate were used as input; lysates (400 μg) were immunoprecipitated as indicated in material and methods with a polyclonal anti-HBZ antibody. (C) HBZ, JunD and Sp1 are tethered to hTERT proximal promoter region. Sequential ChIP assays were performed after an initial IP with either anti-JunD or anti-HBZ. Protein-DNA complexes were detected at the hTERT proximal promoter region, after second IP with anti-Sp1, but not in the distal region. Each panel shows amplification of 0.4% of the total input chromatin (input).

expected, the basal activity was enhanced by 5-fold, when HBZ and JunD were expressed (lane 6); however, only a 2-fold activation was observed when HBZ, JunD and Tax were co-transfected (lane 8). These data provide a clear indication that the HTLV-1 encoded proteins, HBZ and Tax, exert antagonistic effects on the transcription of the *hTERT* gene. They further propose that HBZ is fully active only when Tax is silenced.

Discussion

The novel viral HBZ protein coded in the minus strand of the HTLV-1 provirus has been shown to display a bimodal RNA- and protein-based function (see [16,28] for reviews). Indeed, *HBZ* RNA was found to be implicated in the proliferation of infected cells [8]. The protein, through its interactions with AP-1 proteins acquires the ability to intervene, in the regulation of viral and cellular gene transcription [10,14,15]. Thus, studies performed in HeLa cells with synthetic or natural promoters containing AP-1 consensus sites have indicated that HBZ inhibits the transcriptional activation mediated by c-Jun, while it enhances the activity of JunD [11-13]. Here, we demonstrate that the HBZ protein behaves as a positive or negative regulator of the hTERT promoter depending on the Jun partner. Indeed, HBZ together with JunD activates hTERT transcription, whereas HBZ with c-Jun represses it. We also observe a significant increase of hTERT transcripts in cells expressing HBZ and JunD, in spite of the inhibitory effects exerted by AP-1 proteins on the distal regions of the hTERT promoter [23]. To our knowledge, the present study is the first that describes the effect of HBZ on a cellular gene expressed in tumour cells.

We also report that HBZ and JunD target the proximal region of the promoter in which no AP-1 site is present. Consequently, the activity of HBZ/JunD is independent of the DNA-binding properties of JunD, but instead requires the interaction of these bZIP factors with other nuclear factors. The proximal 180 bp of the hTERT core promoter is important for maintaining basal transcriptional activity of which c-Myc/Max and Sp-1 are the main activators [21-23]. Previous studies clearly demonstrated that c-Jun and related proteins (JunB, JunD and ATF-2) cooperate with Sp1 to transactivate the promoter of the human p21 gene

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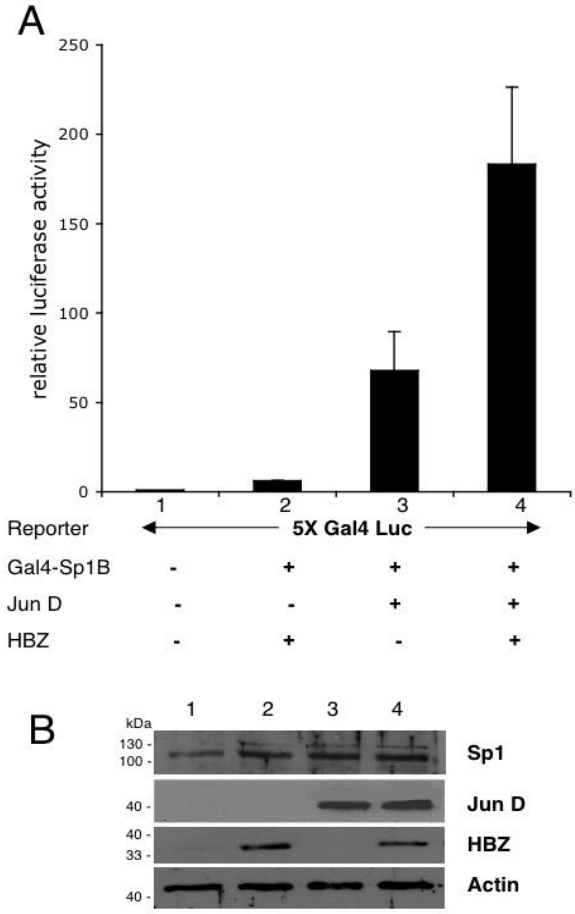

Figure 6

Functional interactions between HBZ, JunD and Sp1. (A) HeLa cells were cotransfected with the reporter construct 5XGal4-Luc (200 ng) consisting of five tandem Gal4-binding sites in the absence (-) or presence (100 ng) of expression vectors for Gal4-Sp1B (containing the B domain of Sp1), and/or HBZ and JunD. Luciferase activity was normalized to tk-luc activity. Results represent duplicate samples from three different experiments. (B) Western blot analysis of Sp1, HBZ and JunD protein levels in whole cell lysates of HeLa samples. The membrane was successively probed with rabbit polyclonal anti-Sp1, anti-HBZ sera, and mouse monoclonal anti-Flag and anti-actin antibodies.

by acting as a superactivator of the Sp1 transcription factors [24,25]. We have therefore hypothesized that these factors together with HBZ and JunD are involved in the activation of the hTERT promoter. We have indeed found that co-expression of JunD and HBZ resulted in a strong synergistic transactivation of a luciferase reporter construct consisting of one Sp1 consensus site upstream of a TATA-box. We have further shown by immunoprecipitation and ChIP assays that the HBZ-JunD heterodimers are tethered to the proximal hTERT promoter via interaction with Sp1. Consequently, we propose that HBZ plays a positive role on hTERT transcription by cooperating with JunD, in an indirect manner through Sp1 transcription factors. Indeed, we have recently demonstrated that HBZ possesses a modulatory domain immediately adjacent to its bZIP domain involved in the stimulation of JunD transcriptional activity [29]. This domain would influence the conformational structure of the AP-1 heterodimers to form a complex with more accessibility to the transcriptional regulators [30,31].

Various viral proteins have also been implicated in Sp1-dependent cellular gene transcription. For instance, the oncoprotein v-Jun downregulates *SPARC* and *collagenase alpha2(I)* transcription through the formation of a DNA-Sp1-v-Jun chromatin-associated complex [32,33]. The E1A tumor suppressor protein of adenovirus upregulates hTERT transcription through Sp1 binding sites, involving recruitment of p300/CBP proteins [34]. Finally, the activity of the proximal promoter of hTERT is upregulated by the interaction of Sp1 with the latency-associated nuclear antigen (LANA), which potentially contributes to the immortalization of Kaposi's Sarcoma-associated herpes virus-infected cells [35]. These data support the hypothesis for Sp1-binding sites in hTERT promoter as the responsive sequences to the Sp1-JunD-HBZ complexes.

Our experimental strategy to apprehend the role of HBZ on hTERT transcriptional regulation was based upon transient transfection assays performed in HeLa cells. These cells, which are widely used in studies on the transcriptional regulation of gene expression, have been shown to display a moderate transcriptional activity of hTERT, when compared to other cancer cell lines and to normal cells. Furthermore, a close correlation has been observed between Myc and Sp1 expression and levels of hTERT transcriptional activity [21]. Last but not least, these cells were found to support the constitutive expression of HBZ, after transduction with a bicistronic retrovirus coding for HBZ and the green fluorescent marker GFP, contrary to T cells lines, such as CEM and Jurkat (data not shown).

Taken into account our present observations together with the relevant published data, a model for the regulation of the hTERT proximal region of the promoter during the multistep process of leukemogenesis is proposed (Fig 8). During the early steps of the transformation of T lymphocytes infected by HTLV-1, Tax is playing a pivotal role, by increasing the transcription of numerous cellular genes, and specially that of NF-κB and AP-1 [28]. In these activated T cells, Tax has been shown to repress the hTERT core promoter activity [26,27,36]. Late in ATL, Tax expression is decreased and/or silenced. Indeed, leukemic cells are selected among cells that carry deletions in the proviral

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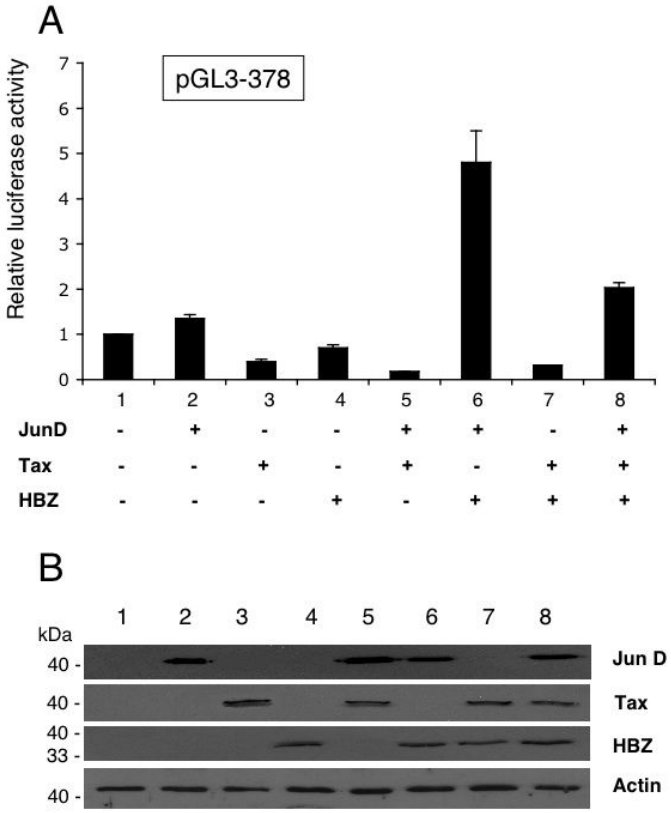

Figure 7
Effect of Tax on HBZ-mediated activation of the hTERT promoter. (A) The luciferase reporter plasmid, pGL3-378 (100 ng) was cotransfected with expression plasmids for HBZ (200 ng), JunD (200 ng) and Tax (10 ng), as indicated. Luciferase activity, normalized to tk-luc activity, is presented relative to that of cells transfected with the reporter plasmid alone. Results represent triplicate samples, from three different experiments. (B) Western blot analysis of HBZ, Tax and JunD protein levels in whole cell lysates of HeLa samples. The membrane was successively probed with a rabbit polyclonal anti-HBZ, anti-Tax sera, mouse monoclonal anti-Flag and rabbit anti-actin antibodies.

DNA and in which the *tax* gene is not expressed [37]. As HBZ is the only viral gene product detected in ATL cells [8], our present observations clearly infer that concomitantly with the loss of Tax, HBZ becomes fully responsible in the increase of hTERT transcription observed during the late stages of leukemogenesis. Now, the telomerase activity in chronic ATL patients was found to be higher than that in HTLV-1 carriers and healthy donors. Furthermore, the reactivation of telomerase in peripheral blood mononuclear cells of ATL patients has been shown to provide a good marker to predict worsening of ATL, especially during the evolution from the chronic to the acute type [4,38]. Finally, the presence of significant shorter telomeres in chronic ATL patients, compared to those of the two other subject groups, is pleading for a telomeric dysfunction. Such an event might favor a genetic instability that would be perpetuated through an increase of hTERT transcription, to which HBZ is participating through its interactions with JunD and Sp1.

Methods

Plasmids

hTERT promoter-luciferase reporter constructs (pGL3-3300, pGL3-2000 and pGL3-378) used in this study have been previously described [23]. The reporter plasmid, pGL2-Sp1-TATA-Luc, contains a single copy of Sp1 binding site fused with a TATA box and the luciferase gene [33]. The pCMV-Tax expression vector was obtained from Dr. W.C. Greene (USA). pcDNA-HBZ-Myc encoding the SI isoform of HBZ and the mutated versions (HBZΔAD, HBZΔbZip and HBZΔADΔZip) were previously described [9,10]. The AP-1 expression vectors pcDNA-c-Jun et

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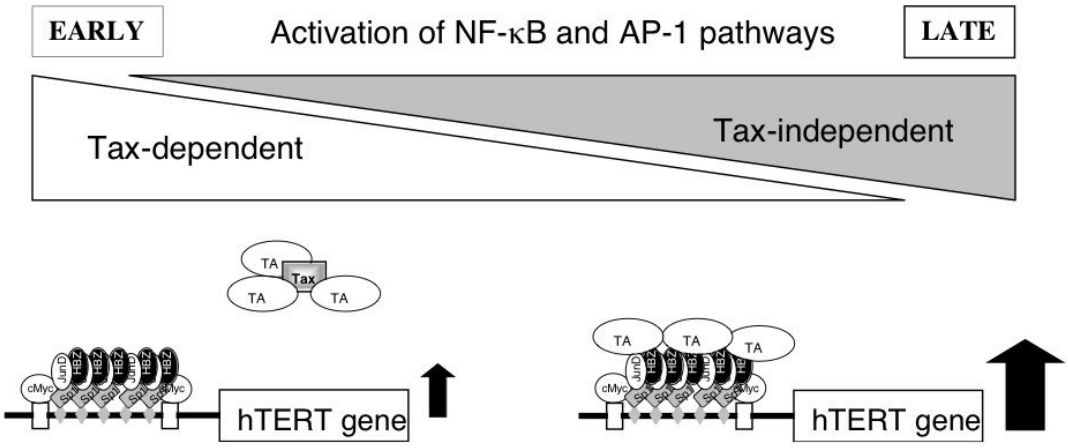

Figure 8
Proposed model for the transcriptional regulation of the hTERT core promoter during the HTLV-1-induced leukemic process (see Discussion). The early steps of the leukemic process during which HTLV-1 infected T-lymphocytes were expressing Tax and HBZ are characterized by the recruitment of transcriptional activators (TA) by Tax. Later, Tax expression is decreased and/or silenced and the TA become available to interact with HBZ/JunD complexes. In both cases, HBZ/JunD complexes are interacting with the Sp1 transcription factor bound to the GC boxes present in the hTERT core promoter.

pCMV-JunD-Flag were obtained from Dr. M. Piechaczyk (Montpellier, France). The Sp1 expression plasmid, pMIC-Sp1, was a generous gift of Dr. J. Marvel (Lyon, France). The Gal4-Sp1B construct containing the Gal4 DNA binding domain portion (aa 1-170) fused to the domain B of Sp1 (aa 263-542) was a generous gift of Dr. D. Kardassis (Heraklion, Greece). The 5XGal4-Luc reporter (pG5Luc) containing five tandem binding sites for the yeast protein Gal4 upstream of a minimal TATA box was purchased from Promega, France.

Cells, transfections, luciferase and Western blot assays

HeLa cells were grown in Dulbecco's modified Eagle's medium (Invitrogen Life technologies, Frederick, MD) supplemented with 10% heat-inactivated fetal calf serum and 100 IU/ml penicillin, 50 μg/ml streptomycin at 37°C in a 5%CO2 atmosphere. They were transfected using the calcium phosphate precipitation method [39]. Jurkat lymphoblastoid T cells were grown in complete RPMI 1640 medium (Invitrogen) and were transfected by electroporation at 250 V and 950 μFd with a Celljject electoporator (Eurogentec). Transfection efficiencies were normalized by cotransfection of a Renilla expression vector (RL-TK, Promega). Assays were performed 30 h after transfection using the dual Luciferase Reporter assay (Promega) and a Berthold luminometer. Western blots were performed using 3 μg of protein lysates, and were revealed by using polyclonal HBZ antibody [5], polyclonal Sp1 antibody (generous gift of J. Marvel, Lyon, France). Mouse monoclonal anti c-myc antibodies were purchased from Roche, anti-Flag and anti-actin (AC-40) antibodies were purchased from Sigma. Secondary HRP-linked antibodies were purchased from Immunotech (France). Blots were developed using an enhanced chemiluminescence detection system (Renaissance, NEN Life Science Products). Bands were visualized by using Hyperfilm (Amersham Pharmacia Biotech).

Immunoprecipitation and in vivo *interaction of HBZ, JunD and Sp1*

HeLa cells (0.7×10^6 cells/10-cm dishes) were transfected with 5.5 μg of each plasmids (pcDNA-HBZ-Myc, pCMV-JunD-Flag and pMIC-Sp1) using the calcium phosphate precipitation method. 48 h post-transfection, cells were lysed in IP buffer (50 mM Tris pH8, 150 mM NaCl, 0.5% Nonidet-P40) supplemented with complete protease inhibitors (Roche Diagnostics). Cell lysates were centrifuged at 12,000 g for 10 min at +4°C. Equal amounts of cell lysates (400 μg) were first incubated with a control serum to preclear the lysate. Precleared lysates were then incubated with anti-HBZ polyclonal antibody overnight at +4°C with rotation and further incubated with protein G Plus/ProteinA Agarose beads (Calbiochem) at +4°C for 30 min with rotation. The immunoprecipitated complexes were washed five times with 0.5 ml of ice-cold IP buffer. The immunoprecipitated pellets were resuspended in 20 μl of 2X-SDS protein sample buffer and then

resolved on 10% SDS-PAGE and detected by Western blot assay using anti-Flag and anti-Sp1 antibodies.

RT-PCR and quantitative real-time PCR (qPCR)

Total RNAs were isolated from cells using Trizol reagent (Invitrogen) according to the manufacturer's instructions. Samples were treated with RNase-free DNase (10 U/μl, Qiagen) for 30 min at 20°C and then for 15 min at 65°C. Five-hundred ng of RNA were reverse transcribed by using oligo(dT)12-18 and Superscript II (InVitrogen). Reverse transcription was performed for 50 min at 42°C. The total cDNA (20 μl) was frozen until PCR was performed. After thawing, 2 μl of cDNA diluted in distilled water were used for each PCR reaction. The real-time quantitative PCR (qPCR) was performed in special lightcycler capillaries (Roche) with a lightcycler Instrument (Roche), by using the Platinium SYBR-Green qPCR SuperMix UDG kit (Invitrogen). The following specific primers were used to detect: hTERT sense, 5'-TGTTTCTGGATTTGCAGGTG-3' and antisense, 5'-GTTCTTGGCTTTCAGGATGG-3', actin sense, 5'-TGAGCTGCGTGTGGCTCC-3' and antisense: 5'-GGCATGGGGGAGGGCATACC-3'. The following program was used: samples were incubated at 50°C for 2 min, followed by 10 min at 95°C, followed by 40 cycles (95°C 10 sec, 61°C 5 sec, 72°C 10 sec). The dissociation curve was measured for each sample. Relative level of hTERT sequence against the reference actin sequence was calculated using the ΔC_t method. A standard calibration curve was performed, using cDNA from HeLa cells. The levels of actin transcripts were used to normalize the amount of cDNA in each sample.

Chromatin immunoprecipitation (ChIP) assay in vivo

ChIP assays were performed essentially by using the Upstate Biotechnology Inc. recommendations with minor modifications. Formaldehyde cross-linked chromatin from 5 × 10^6 cells/antibody was used for each immunoprecipitation. Cross-linking reactions were quenched with 125 mM glycine, cells were lysed, and chromatin was sonicated to obtain an average DNA length of 500 bp. Following centrifugation, the chromatin was diluted 10-fold and pre-cleared with protein A-agarose containing salmon sperm DNA and bovine serum albumin (Upstate Biotechnology). Pre-cleared chromatin (2 ml) was incubated overnight at +4°C with 5 μg of antibody recognizing JunD (sc-74, Santa Cruz Biotechnology) or Sp1 (sc-70, Santa Cruz Biotechnology) or with 10 μl of anti-HBZ or normal rabbit serum, followed by protein A-agarose immunoprecipitation. Eluted protein-DNA cross-links were reversed by heating at 65°C overnight, and 25% of the recovered DNA was used in PCR reaction to amplify the 278-bp region of the hTERT proximal promoter with the Phusion high-fidelity DNA polymerase (Ozyme) enzyme and the forward primer (-190/-171) 5'-CACAGACGCCCAGGACCGCG-3' and the reverse primer (+69/+88) 5'-GCGCGCGGCATCGCGGGGGT-3'. A control PCR (negative control leading to a 150-bp fragment) was also performed from a region devoided of Sp1 or AP1 sites within the distal region of hTERT using forward primer (-2916/-2897) 5'-GGCAGGCACGAGTGATTTTA-3' and reverse primer (-2782/-2763) 5'-CTGAGGCACGAGAATTGCTT-3' to show the specificity of Sp1 sites located on the proximal hTERT in the ChIP assay. DNA samples recovered from chromatin samples before immunoprecipitation, which corresponds to 1% of chromatin samples included in each immunoprecipitation reaction, were also PCR amplified as loading controls. The PCR reactions for hTERT were processed through 32 cycles of 98°C for 10 sec and 71°C for 30 sec, followed by one cycle for 7 min at 72°C. PCR products were separated on 2% agarose gel and visualized with ethidium bromide staining.

For sequential ChIP assays, the initial ChIP was performed with the indicated antibodies, the primary immunocomplex was then eluted by 10 mM dithiothreitol at 37°C for 30 min. The eluate was diluted 50 times with buffer (20 mM Tris-Hcl, pH8.1, 150 mM NaCl, 2 mM EDTA, and 1% Triton X-100) and a second ChIP was then carried out.

Statistical analysis

Data were expressed as mean ± SD, and when required were compared by one-way ANOVA with Dunnett's test, $P < 0.05$, was taken as statistically significant.

Competing interests

The author(s) declare that they have no competing interests.

Authors' contributions

ASK carried out most of the transfection experiments, luciferase assays and western blot analyses and drafted the manuscript. JV has initiated the ChIP assays. MDD has conducted the RT-PCR and IP analyses. MC has participated in the design of the experiments concerning the identification of the Sp1 binding sites. JMM has helped in finalizing the manuscript and has provided important input on the design of the study. LG and MDD have conceived the study, participated in its coordination, helped in drafting and finalizing the manuscript. All authors have read and approved the final manuscript.

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IV] ANNEXE- Modeling Cord Blood Stem Cells Transplantation in HIS mice

1) Cord Blood Stem cells transplantation, a procedure on the rise in human medicine

Allogenic Stem cell transplantation (SCT) is a therapeutic option that has been used with some success to treat ATL patients (Tanosaki et al., 2008), and some blood malignancies or genetic diseases (Pasquini and Wang, 2010). As HP/HSCs come from related or unrelated donor (allogenic), the patients may develop a Graft vs. Host disease (GVHD) which can be prevented with immuno-modulatory treatment. To achieve a better engraftment and prevent GVHD in the transplanted patient, the donor and the receiver have to be HLA-matched.

Human Umbilical Cord Blood is actually an interesting source of HP/HSC. Cord blood transplantations resulted in comparable results with other sources of HP/HSC in the treatment of patients (Brunstein et al., 2010). One of the major limitations of this source is the number of HP/HSC available. The volume of blood collected from an umbilical cord is limited, thus limiting the number of transplantable cells and their use to the SCT of small children due to the fact that the ratio 'number of HP/HSC to the body weight' is determinant. More recently, double-unit CB transplantations in adults has been performed. This procedure, which involves the transplantation of erythrocyte-depleted cord blood units without any purification of HP/HSC has given promising results in adults and its usage is now on the rise.

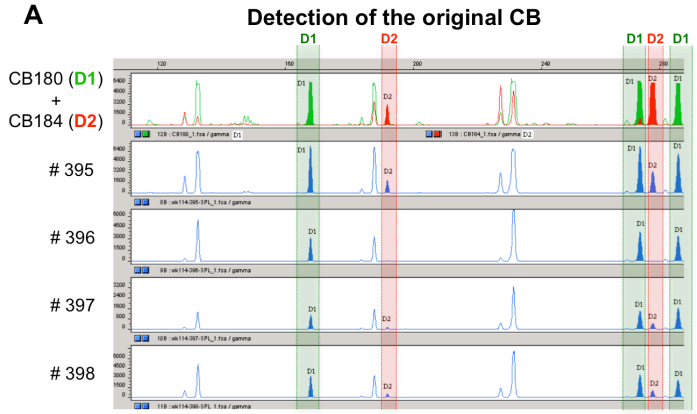
Indeed, the transplantation of two cord blood units results in two engraftment situations in the receiver. In 80 % of the transplantations, only cells from one of the two donors are found to engraft, whereas in the 20 % remaining, the two CB coexist and a chimerism is detected. This latter situation has been associated with a greater risk of relapse (Gutman

et al., 2010). The search for factors governing either the supremacy of one cord blood over the other one or the co-existence of both cord blood is of great interest. Indeed, predicting which CB will dominate is a way to ensure that no chimerism with bad prognosis will be observed in transplanted patients.

In order to bring some understanding in the mechanisms involved in the control of the supremacy of one cord blood we wondered if the HIS mouse system could represent a valuable model to study the fate of a double-unit CB transplantation.

2) Initial observation: No dominance phenomenon but chimerism when using purified CD34$^+$ cells.

A



B

Quantification of each CB

	CB180 (D1)	CB184 (D2)
# 395	79 %	21 %
# 396	96 %	4 %
# 397	86 %	14 %
# 398	85 %	15 %

Figure 17 – Chimerism in HIS mice injected with purified CD34$^+$ cells from two CB

Four littermates were injected with purified CD34$^+$ cells mixed from 2 different CB (CB180 and CB184). The genomic DNA was extracted from the spleen and submitted to genetic profiling. **A –** Result of the genetic profiling. Short Tandem repeats used in the human genetic profiling was amplified and their size was determined by electrophoresis. A particular number of STR (Short Tandem repeats) is specific for every individual and allows the discrimination of the origin of the cells. The first lane represents the analysis of the DNA from the two cord bloods (CB180 = D1 in green and CB184 = D2 in red). This analysis defines informative sizes for each CB (highlighted by green or red boxes). The intensity of signal at a given size allows a quantification of the origin of the cells from one CB or the other (4 lower lanes). **B –** Quantification of the proportion of each CB present in the spleen of 4 mice.

The humanization protocol used in our study of the HTLV-1 infection and pathogenesis involved the inoculation of purified CD34$^+$ cells from several cord bloods in order to have a common batch of CD34$^+$ cells to inject into all the littermates. In collaboration with the Etablissement Français du Sang (EFS), under the supervision of Dr. Dominique Rigal, we tested the chimerism of four littermates injected with CD34$^+$ cells mixed from 2 cord bloods. The results demonstrated the presence of cells coming from several cord

blood (Figure 17). This observation is in accordance with the work of Eldjerou *et al.* (Eldjerou et al., 2010) who demonstrated that injection of $CD34^+$ cells purified from two CB into immuno-compromised mice resulted in the establishment of a chimera. Therefore, it appears that the dominance of one unit over the other observed in human transplantation could be due to the presence of $CD34^-$ cells.

3) Attempts to humanize mice with unpurified cord bloods

In a first set of experiment, $Rag2^{-/-}\gamma_c^{-/-}$ mice were injected either with purified $CD34^+$ cells or with different numbers (10^5 to $5x10^7$) of whole unpurified Mononuclear cells (MNC) from 2 CB. Under the latter condition, a high mortality of mice was observed. In the remaining mice, the engraftment, assayed by the expression of human CD45, was rather low in comparison to what is usually obtained in our lab. Interestingly, the chimerism study demonstrated that only the cells from one CB were found in each mouse. These results seem to confirm the importance of the MNC in the dominance of a CB over the other. The low engraftment could be due to the low number of $CD34^+$ injected in the mice and to prevent this limitation, we refined our protocol.

In a second attempt to assess the importance of the MNC, we injected $CD34^+$ purified cells along with $CD34^-$ MNC. Accordingly, we were able to reduce the number of MNC injected. One of the questions that arose at that time was the nature of the involvement of the MNC in the dominance process. We hypothesized that MNC could either have a feeder effect on the $CD34^+$ cells favoring their engraftment or start an allogenic reaction eliminating the cells of the other CB. In the latter case, the remaining cells would be the survivors of a fierce battle between the two CB. To try to answer this question we injected the MNC from one CB, either under native form, a prerequisite for the establishment of an allogenic reaction or after irradiation allowing their feeder effect. We also injected $CD3^+$-depleted MNC along with the $CD34^+$ cells from 2 CB. Eight weeks

later, the mice were sacrificed and the engraftment level as well as the genetic profiling were determined. The results are presented Figure 18 and Table 2. Interestingly the engraftment seems to be higher in all the cases where MNC were co-injected with $CD34^+$ cells indicating a positive effect of these cells on the engraftment or the proliferation of human cells in HIS mice. Except for the mice injected with the total MNC, the level of B and T cells is in the range of what is expected from our previous results. The most striking effect was from the co-injection of irradiated MNC along with $CD34^+$ cells. It results in a higher engraftment in the bone marrow and the spleen and even more strikingly in the thymus in which the number of human cells is higher than in the other conditions. This increased number of human cells did not affect the T-cell development as indicated Figure 18D. The co-injection of total MNC results in high engraftment with a large majority of T cells in the different organs. This is associated with a very low engraftment in the thymus. This result suggests that the observed cells are the remaining of the inoculated MNC rather than *de novo* produced cells from the engraftment of human cells.

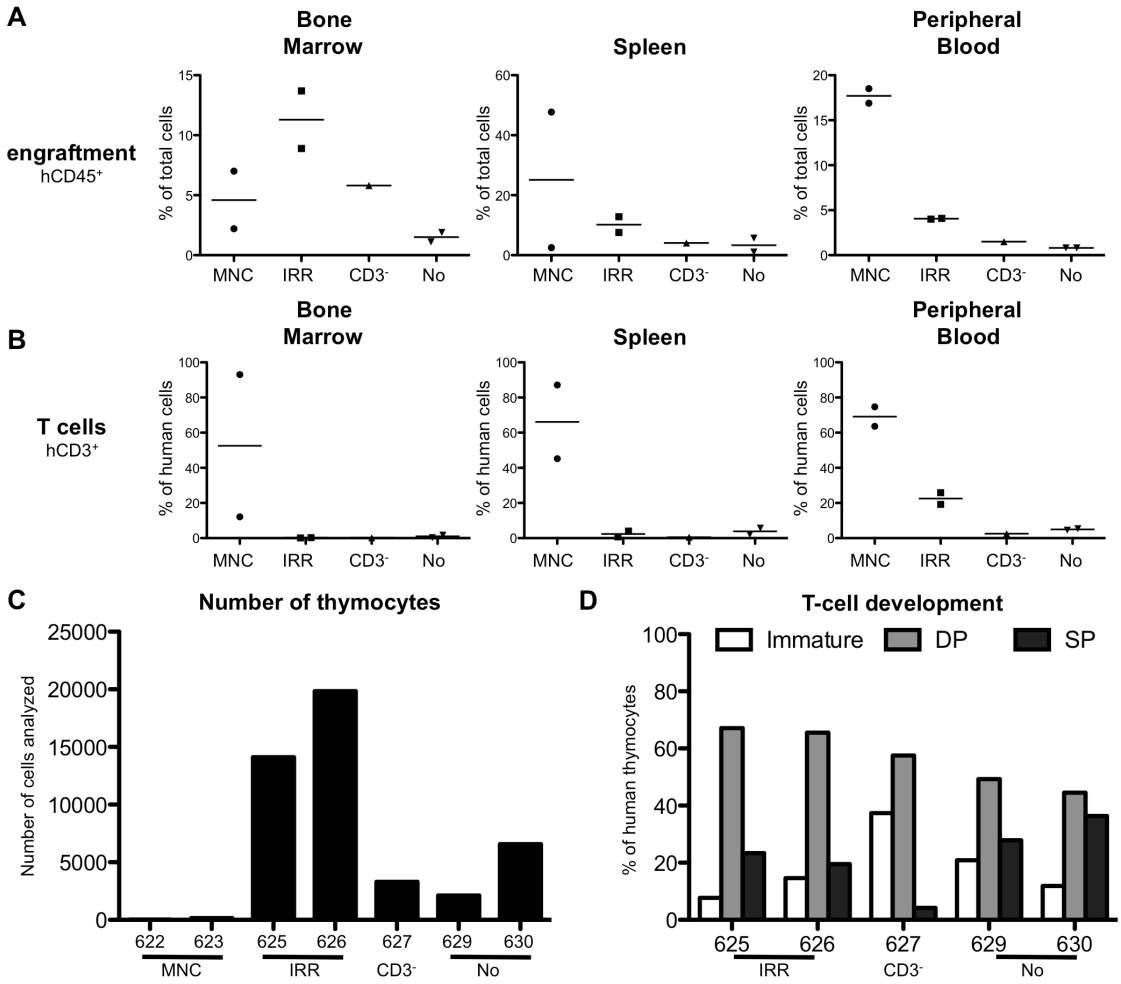


Figure 18 – Effect of the MNC on the engraftment of CD34+ cells in HIS mice

Mice were injected with CD34+ cells purified from 2 cord bloods and co injected with MNC from one CB (MNC), lethally irradiated MNC (IRR), CD3-depleted MNC (CD3-) or nothing (NO). **A –** engraftment, assayed by the percentage of hCD45+ cells in different organs (Bone Marrow, Spleen, Peripheral Blood). Each dot represents a mouse. The bar represents the median. **B –** percentage of T cells in different organs expressed in percentage of CD3+ cells among human cells. **C –**Number of thymocytes analyzed by flow cytometry. This number is proportional to the total number of thymocytes. The number for each mice id indicated. D – T cell development in the thymus of mice colonized by human cells.

mice	CD34+ CB1	CD34+ CB2	MNC	Chimerism analysis BM CB1 – CB2	Spleen CB1 – CB2	Thymus CB1 – CB2	FACS analysis PB	BM	Spleen	Thymus
# 622	5 x 10^4 cells	5 x 10^4 cells	MNC (CB1)	100 – 0	100 – 0	100 – 0	16.9 % - T: 63.6 %	2.2 % - T: 93 %	2.5 % - T: 45.2 %	3.4 %
# 623				100 – 0	100 – 0	100 – 0	18.5 % - T: 74.7 %	7 % - T: 12.1	47.7 % - T: 87.1 %	57.3 %
# 625			MNC Irradiated (CB1)	41 – 59	49 – 51	41 – 59	4 % - 19.2 %	13.7 % - T: 0.1 %	12.8 % - T: 0.7 %	95.4 %
# 626				49 – 51	41 – 59	41 – 59	4.1 % - T: 25.9 %	8.9 % - T: 0.3 %	7.6 % - T: 4 %	98.6 %
# 627			MNC CD3- (CB1)	0 – 100	0 – 100	0 – 100	1.5 % - T : 2.5%	5.8 % - T: 0.2 %	4.1 % - T: 0.5 %	81.4 %
# 628				nd (early death)						
# 629			NO	49 – 51	49 – 51	49 – 51	0.8 % - T: 4.5 %	1.1 % - T: 0.3 %	5.7 % - T: 5.7 %	83.4 %
# 630				49 – 51	nd	49 – 51	0.8 % - T: 5.4 %	1.8 % - T: 1.7 %	1 % - T: 2%	56.2 %

Table 2 – Co-injection of Rag2$^{-/-}\gamma_c^{-/-}$ with purified CD34$^+$ from 2 CB and MNC.

Mice were injected with 50,000 CD34$^+$ cells from the two different CB (CB1 and CB2). Mice were co-injected with with MNC from one CB (MNC), lethally irradiated MNC (IRR), CD3-depleted MNC (CD3$^-$) or nothing (NO). The results from the chimerism analysis is presented as the percentage of cells coming from the CB1 and the one from CB2 (CB1 – CB2). Cells from Peripheral Blood (PB), Bone Marrow (BM), spleen and thymus were analyzed by FACS. The percentage in bold represents the percentage of human cells in the different organs. T, represents the percentage of T cells among human cells. nd means not done. Several mice died at an early age. They are indicated in the table.

The genetic profiling analysis was carried out on cells isolated from different organs and the results are coherent within each mouse. It shows that the mice co-injected with whole MNC resulted in the detection of only the cells coming from the CB from which we injected the MNC. This result is coherent with the hypothesis that the observed cells are MNC surviving in these immuno-suppressed mice. A 8-week survival is conceivable as it was observed that PBMC could be maintained for several months (Mosier et al., 1988). Interestingly, the genetic analysis of mice injected with irradiated MNC indicates a chimerism with the presence of cells coming from both CB. That result seems to rule out the effect of MNC on the dominance via a feeder effect and support that to contribute to the dominance of one CB on the other, the MNC needs to be able to divide. Only one CB cells were detected in the mouse injected with CD3$^+$-depleted MNC. Surprisingly the detected cells are from the CB from which we did not inject MNC. This preliminary result

needs to be confirmed by additional experiments but it raises interesting question on the implication of the $CD3^-$ MNC in the dominance battle. Several trends seem to appears:

The dominance process is not based on a MNC feeder effect and need living MNC to establish a winner CB.

$CD3^+$ might not be indispensable in the dominance process. This fact has yet to be confirmed.

4) Injection of irradiated MNC results in an increase engraftment of the CB in the HIS mouse.

This last striking conclusion emboldens us to address the SCT through a different angle. The main problem of cord blood SCT is the low number of cells available to transplant adults. An easy answer was to mix several CB together to increase to an acceptable level. Other groups have tried to amplify *in vitro* $CD34^+$ cells in order to re-inject them in patients. These studies have not proven very effective up to now and many problems remained to be addressed before this could be a large-scale therapeutic option. Another approach seems possible: could we increase the engraftment capacity of the $CD34^+$ cells so that the number from one CB is sufficient to transplant an adult? This does not seem irrational since in 80 % of the double-unit cord blood transplantation, only one CB remains highlighting that the number of injected cells is sufficient to do so when realized under the right conditions. The observed increase in the engraftment when injecting irradiated MNC tends to indicate that this population can efficiently stimulate the HP/HSC injected in order to increase their engrafting capacity. Experiments to confirm this point are ongoing.

These preliminary data show the interest to use this HIS mouse model to better understand the biology of the double unit CB transplantation and perhaps to test new

approaches that could result in new protocols of transplantation for the sake of patients. More practically for our experiments, the use of irradiated MNC co-injected with $CD34^+$ cells could represent an improvement of our current protocol leading to a better reconstitution of HIS mice favoring our infection experiments.

Discussion and Conclusion

I] Discussion

The work presented in this manuscript describes the development of the first animal model of HTLV-1 *in vivo* infection and pathogenesis based on the use of HIS mice. The development of this model must have been in *l'air du temps* as Banerjee *et al*. published a comparable model last year (Banerjee et al., 2010a). A discussion of the major points being similar and different between our two models has been made in Villaudy *et al.* (Villaudy et al., 2011a). The major difference lies in the protocol of infection we used. Banerjee and colleagues decided to infect CD34+ *ex vivo* and imunodeficient mice by iv injecting those cells.. We tried to be closer to a natural infection by inoculating already humanized mice with lethally irradiated infected cells. We observed similar pathological features in our respective models but our interpretations might differ. Both studies are for the search for the cells at the origin of the leukemic process. Based on their results, they suggest that bone marrow $CD34^+$ cells could represent a reservoir and the initial cells. However the *ex vivo* infection protocol represent a definite bias as those cells are not naturally *in vivo* infected. We infected HIS mice resulting in an *in vivo* infection of human cells, to mimic the conditions favoring the initiation of the leukemogenic process at an early stage of T-cell development. That explains our particular focus on the T-cell development in our approach, a point Banerjee *et al*. did not address in their model. Surprisingly, the analysis of thymocytes of their mock-infected animals shows the presence of an abnormally large population of B cells. Furthermore, the fact that the $CD34^+$ cells are infected before their inoculation prevents the following studies of the PVL evolution.

We demonstrated that human T cells in this HIS mouse model could be efficiently and persistently infected with HTLV-1. This long-term infection correlates with an increase in the PVL and the alteration of the T-cell development in the thymus of these mice with

a significant expansion of mature thymocytes. This leads to the detection of a large proportion of activated T cells in the peripheral organs of infected mice and the onset of lymphomas or leukemias in which activated $CD4^+$ T cells are often detected. Other pathological features, reminiscent of what can be observed in ATL patients, are also observed in leukemic or lymphomatous mice such as hepato-splenomegaly and adenopathy. The clonality analysis demonstrated that infected mice harbored an oligoclonal pattern and the effect of the genomic environment on the control of the persistence and proliferation of a given clone is similar to what is observed in HTLV-1 infected patients (cf. p100). Taken together these results highlight the value of this model to study the HTLV-1 infection and leukemogenic process and could be especially helpful to unravel the firs steps of infection leading to the initiation of the leukemic process such as viral entry and the relative importance of the Tax and HBZ expression. This model also seems to be a good preclinical model to test therapeutic strategies and dissect their mechanisms of action.

However, several issues need to be discussed to ascertain their relevance to the study of HTLV-1 infection and pathogenesis.

One of the first criticisms that can be made is that several aspects differ with regard to the situation observed in patients.

- The percentage of mice developing a pathology after infection is also far above the estimated 5 % seen in humans. From the experiments we have made, it is my belief that almost all of the infected HIS mice will reach a high PVL and subsequently develop some pathological abnormalities if we wait enough time.
- Another possible criticism is the “artificial” infection protocol by ip inoculation of lethally irradiated HTLV-1 infected cells. It mimics none of the natural routes for the entry of the virus in an organism. Such a difference could result in

unanticipated differences in the first steps of infection, exposing specific cell populations that are not naturally infected.

These observed differences might be linked to one of the major limitation of the HIS Rag2$^{-/-}\gamma_c^{-/-}$ mouse model: a reduced immune response. It was recently described that Balb/c Rag2$^{-/-}\gamma_c^{-/-}$ is suboptimal for human engraftment and this is particularly noticeable in the low level of T cells (Brehm et al., 2010a). This lack of T cells could notably explain the absence of others HTLV-1 associated diseases that are often linked to the presence or the infiltration of infected T cells in the periphery. This lack of immune response seems to be confirmed by the observation of the absence of clonal selection in this model (cf. p100). Without the immune force to control the infection and the proliferation of infected clones, HTV-1-expressing cells can freely proliferate explaining both the rapid onset of lymphomatous and leukemic forms and their presence in a large percentage of infected mice.

Facing these limitations, we wonder how to improve this model to be even closer to the reality of the HTLV-1 infection and therefore make more accurate observations of the pathogenic process.

One of the main problems appears to be inherent to the used mouse strain and its limited immune response. Other strains of mice have been developed and are widely used to create humanized mice as well. This is for example the case of the NOD/SCID $\gamma_c^{-/-}$ (NSG) mice that are very similar to the Rag2$^{-/-}\gamma_c^{-/-}$. However, they appear to show a higher engraftment of human cells (Brehm et al., 2010a) due to their allele of the SIRPα gene being able to recognize partially the human CD47 antigen triggering a “Don’t eat me signal” for the murine macrophages, thus resulting in a better survival of human cells (Takizawa and Manz, 2007). A recent improvement of the Balb/c Rag2$^{-/-}\gamma_c^{-/-}$ mouse model has been achieved by creating a Rag2$^{-/-}\gamma_c^{-/-}$ mouse strain with a transgenic human

SIRPα in place of the murine version (Strowig et al., 2011a). This addition resulted in an increased human engraftment to the level observed in the NSG model as well as a significant increase of the immune response in this animal and especially in the T cell development (Strowig et al., 2011a). This recent model is not currently commercially available. Progress has been recently made in the development of mouse strains for the purpose of improving the human engraftment and the immune response observed in these animals. We can notably cite the knock-in of human cytokines as it was observed that the murine version of the cytokines were not always effective on human cells limiting their engraftment, survival or development *in vitro*. Many different cytokines have been and are still investigated (Willinger et al., 2011). Another issue described in these humanized mice models is the selection that are made partially on human and partially on murine MHC (Traggiai et al., 2004). This selection against nature might also alter the strength of the immune response. To bypass this problem, a human HLA class I gene has been recently knocked in a NSG mouse strain resulting in a NSG-HLA-A2 mouse model exhibiting a functional T cell population (Shultz et al., 2010). The use of such improved mouse strains to generate HIS mice in our protocol could bring significant improvement to the model. The development of a stronger and HLA-restricted immune response could be useful to study its role against HTLV-1 infection, as it is known to be crucial for the development of the HTLV-1 associated diseases.

Another diverging parameter is the route of infection we use to infect HIS mice. Intraperitoneal inoculation is an easy procedure but to the best of my knowledge has not been described as a natural route of HTLV-1 infection. The ATL development has been linked to early infection via breast-feeding. It would therefore be interesting to infect HIS mice via the oral route as it has been previously done in other animal models (Lairmore et al., 2005). In order for this inoculation to efficiently infect the human cells

in HIS mice, the infected cells administered to the mice have to meet susceptible cells that could represent the entry point in the organism. HTLV-1 being a human-specific pathogen, we can hypothesize that the presence of human cells somewhere in the digestive tract would be required for an efficient infection. The presence of such cells in our HIS mouse model has not been documented and before proceeding with the oral infection, we are currently testing the presence of human dendritic cells or at least the sensivity of intestine cells to HTLV-1.

MT-2 cells are often used in HTLV-1 infection. They produce large amounts of viral particles and are easy to grow in culture. Alternatively, molecular clones can be used:the genomic sequence of HTLV-1 is incorporated in a plasmid that can be amplified in bacteria as any other expressing plasmid. Their transfection in human cells induces the production of viral particles that can be used to infect in turn naive cells. In addition such a system allows the creation of mutations, deletion, insertion in the genome of the virus in order to study their effect on the infection or the pathogenesis. To that aim, we are currently infecting HIS mice with molecular clones using two different protocols. 1) The molceular clone is transfected into 293T human cells. These cells are then lethally irradiated and injected ip into HIS mice. 2) The molecular clone is mixed with a cationic transfection agent and injected intradermally into HIS mice. This technique has been developed and widely used in the study of BLV in sheep (Willems et al., 1993). The use of molecular clones to infect our HIS mouse model would represent a powerful tool to dissect the role of the different viral proteins and particularly the regulatory proteins such as HBZ, Rex and p30 in an *in vivo* infection and pathogenic process. We would be particularly interested in the study of Tax-mutated molecular clones to address its role in a more integrated way than it has been ever done.

This model also represents a good opportunity of understanding the leukemogenic potential of HTLV-1 in a comparative study with HTLV-2 and HTLV-3 which are closely related to HTLV-1 but have different pathogenesis. HTLV-2 has not been associated with any malignant diseases. The pathogenesis of HTLV-3 is unknown due to the low number of known infected people around the world. Our lab has studied the differences between viral proteins of HTLV-1 and HTLV-2 (Tax1 – Tax2, HBZ – APH2). The infection of HIS mice with HTLV-2 could provide an interesting approach to determine what are the critical steps of leukemogenesis that are created by HTLV-1 and not by HTLV-2 or HTLV-3.

In its actual version and even more if improvements are made to the protocol (use of NSG-HLA-A2 mice, molecular clone, oral inoculation…) this animal model to study HTLV-1 infection and pathogenesis is a powerful tool to study various aspect of the HTLV-1 infection and the development of the associated diseases.

This model represents a unique opportunity to precisely describe the target cells of the infection and the one involved in the initiation of the leukemogenic process. As we have demonstrated here and *in vitro*, HTLV-1 is able to infect immature thymocytes and alter the T-cell development (Wencker et al., 2007). At what precise stage of T-cell development the cell can be infected remained to be elucidated. The use of HTLV-1 env protein fused with fluorescent dye has demonstrated that the viral particles were able to interact strongly with thymocytes at an early double positive stage ($CD3^-CD4^+CD8^+$) (Swainson et al., 2005) that could represent the main target cells. The presence of integrated provirus in specific population could be assessed by sorting the different cell population in the thymus. Similar approaches could be used to determine if $CD34^+$ cells located in the bone marrow are *in vivo* infected. Contradictory results have been found between HAM/TSP patients in whom $CD34^+$ were found infected whereas evidences

tends to demonstrate that they are not in ATL patients (Grant et al., 2002). Of note, the target cell of the virus might not be the one in which its leukemogenic activity can be initiated. As the provirus is stably integrated in the human genome, it is replicated along with the cells and is present throughout all the differentiation steps that the cells go through. Its presence at a (or maybe several) particular development step might be required for the initiation of the leukemogenic process. The study of the early steps of the infection is therefore of crucial importance to understand what is the target cell for the first leukemogenic events. As observed with the clonality analysis, some infected clone expand nicely in this model. To determine at what stage this proliferation takes place, we plan to analyze the presence of the TRECs (T cell Receptor Excision Circle) in peripheral T cells and in the different thymocyte subpopulations. Analysis of TRECs can measure the proliferation rate of thymocytes during the double positive stage (Fry, 2009). (Fry, 2009).

We have observed in the peripheral blood of several-months-old infected animals a large majority of T cells, whereas in age-matched controls, the presence of human cells tends to decrease. This observation could be explained by a continuous proliferation of infected T cells and/or an increase in the survival. That increase in the survival is supported by the observation of the increased expression of anti-apoptotic proteins (figure 6C, (Villaudy et al., 2011a)). It could be easily confirmed in our model by collecting T cells from infected and non-infected HIS mice and measuring their response to apoptotic treatment. Their proliferation rate could also be assessed by *ex vivo* culture.

This model also provides a reliable tool to study the evolution of different cellular or viral factors. The re-activation of the telomerase has been observed in ATL patients and this re-activation could be observed in our model at different times after infection in correlation with the expression of viral proteins such as Tax and HBZ. The study of

HTLV-1 infection with molecular clones mutated for one of these proteins could indicate the relative importance of these two crucial factors in this reactivation. Other cellular proteins could be evaluated in the context of an *in vivo* infection (FoxP3 (Bangham and Toulza, 2011), pTα (Wencker et al., 2007), Fascin (Kress et al., 2011), etc.). A different approach could be imagined to study the implication of cellular factors in the infectious process. Instead of passively following the expression, localization or activation of a given protein, we could knock down its expression in the $CD34^+$ cells to humanize $Rag2^{-/-}\gamma_c^{-/-}$ mice. The resulting HIS mice would allow the determination of the infection and pathogenesis of HTLV-1 in absence of the given protein. This strategy could confirm the importance of the cellular factors essential to the infectious cycle of HTLV-1 like CRM1 (Zhang et al., 2006).

As suggested in this manuscript, this infected HIS mouse model represents a promising preclinical tool to study new therapeutic treatments. Here we tested a combination of AZT, IFNα and As_2O_3 that demonstrated promising results on chronic and smoldering ATL. Other therapies could be evaluated in collaboration with clinicians to interfere at different times after infection. Treatment to block the entry or the replication of the virus could be assessed in order to serve as a post-exposure way to prevent the persistent infection. Other treatment could be designed to specifically treat the acute and lymphomatous most deadly forms of ATL. Recently, a monoclonal antibody directed against CD47 showed promising result in the treatment of Acute Lymphoblastic Leukemia (ALL) (Chao et al., 2011). CD47 is a ubiquitous protein that interacts with SIRPα triggering a “Don’t eat me signal” to the macrophages (Takizawa and Manz, 2007). CD47 was found to be overexpressed on different cancer cells (Chao et al., 2010) and is an inverse prognostic factor (Majeti et al., 2009). The presence of CD47 also correlates with the calreticulin expression at the plasma membrane, which is a strong pro-

phagocytic signal and a bad prognostic factor for various cancers (Chao et al., 2010). The expression of CD47 protects the cancer cells from the phagocytosis that would be activated by the presence of Calreticulin (CRT). The disruption of the CD47-SIRPα "Don't eat me!" signal by the use of monoclonal antibodies resulted in the exposure of the CRL "Eat me!" signal and induces the clearance of leukemic cells in an *in vivo* model (Chao et al., 2010; Gardai et al., 2005). Interestingly, although CD47 is ubiquitously expressed, the use of such a monoclonal antibody has little side effect and the normal cells were not cleared by phagocytosis. This might be due to the absence of CRT at the membrane (Figure 19) (Chao et al., 2010). Nothing is known on the expression of CD47 or CRT at the membrane in the case of HTLV-1 infection and ATL. However, it seems that this mechanism is largely shared by various type of cancer and an unrelated study suggests that CRL expression is increased in ATL cell lines in comparison to uninfected T cell lines (Alefantis et al., 2007). The study of the CD47 and CRT expression at the surface of T cells in infected HIS mice could lead to the use of monoclonal antibody directed against CD47 as a treatment of ATL in this model.

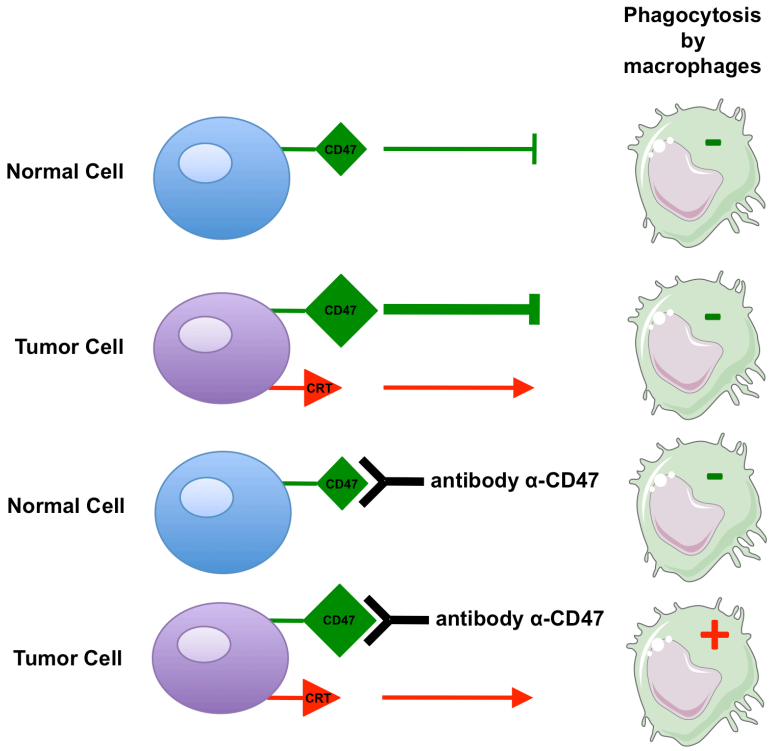


Figure 19 – Integrationof the pro- and anti-phagocytic signals mediated by Calreticulin (CRT) and CD47 respectively.

CD47 is expressed on all the cells. It is over expressed on tumor cells protecting those cells from phagocytosis by the macrophages. Calreticulin is not presence at the membrane of most of the normal cells. Its presence on tumor cells acts as a pro-phagocytic signals but the presence of CD47 protects the cells. The blockage of CD47 signaling using antibodies against CD47, reveals this pro-phagocytic signal leading to its destruction by macrophages whereas normal cells does not express pro-phagocitic signals and are safe. (Chao *et al.*, 2011)

II] Conclusion

Human Immune System (HIS) Rag2$^{-/-}\gamma_c^{-/-}$ mice can be efficiently infected by HTLV-1. The HTLV-1 provirus is integrated into the genome of human cells and persists for several months. During this time a clonal expansion is observed and the cellular mechanisms involved in the regulation of the persistence and proliferation of an infected clone is similar to what is observed in human infected patients. This clonal expansion is associated with an increase of the PVL over time. In the infected HIS mice with the higher PVL, pathological symptoms such as hepato-splenomegaly, adenopathy, thymomas, lymphomas and leukemias can be observed. The tumor cells are expressing the Tax and HBZ viral proteins. This model represents a valuable tool to study the HTLV-1 infection and leukemogenic process as well as the effect of therapeutic treatments.

One of the major limitation of this model is the weakness of the observed immune response unable to control the proliferation of infected clones. The use of alternative immuno-suppressed mice strains could resolve this problem and improve this model. Alternatively, the human cells engraftment might be improved by co-injecting Mononuclear cells from a cord blood. This observation was made in an attempt to develop an animal model to study the mechanisms involved in the dominance of one cord blood over the other in double unit cord blood transplantation.

This model represents the first animal model of *in vivo* HTLV-1 infection leading to the development of ATL-like disease. Its improvement will provide the community with a valuable tool to investigate the early steps of leukemogenesis as well as to unravel what is happening during the long latent period of HTLV-1 infection.

References

The following articles and reviews give a good overview of the different subject treated in this manuscript. They were very useful during the writing of my thesis and can be read by people who wants to have basic, yet complete, idea of the subjected I treated during my PhD.

Concerning the humanized mice model:

- (Traggiai *et al.*, 2004) is the paper describing the obtention of the HIS mouse model used here
- (Legrand *et al.*, 2008) gives many technical and practical information to humanize mice
- (Legrand *et al.*, 2009) gives a nice overview of the use of humanized mice for modeling infectious diseases. (Willinger *et al.*, 2011) highlights the efforts made to improve these models.

Concerning HTLV-1 infection and pathogenesis:

- (Gallo, 2005) is an interesting paper relating the story of the HTLV-1 discovery by Robert Gallo. (Takatsuki, 2005) reports the discovery of ATL and its association to HTLV-1 by a Japanese team of clinicians.
- (Goncalves *et al.*, 2010) is a complete review of HTLV-1 epidemiology as well as the actual knowledge of the HTLV-1 associated pathologies.
- (Matsuoka and Jeang, 2011) presents an overview of the HTLV-1 – associated leukemogenesis.
- (Bangham and Toulza, 2011) is a key review to understand the HTLV-1 relation with the immune system and especially the Treg population.
- (Lairmore *et al.*, 2005) is THE review addressing the animal models used to model HTLV-1 infection and/or pathogenesis

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RÉSUMÉ

Développement d'un modèle de souris Rag2$^{-/-}\gamma_c^{-/-}$ humanisée pour l'étude de l'infection et de la leucémogénèse associée à HTLV-1

Le virus HTLV-1 (Human T-cell Leukemia Virus Type 1) est l'agent étiologique de la Leucémie T de l'adulte (ATL) qui est caractérisée par la prolifération de cellules T CD4$^+$ activées. L'absence de modèle animal fiable reproduisant la leucémogénèse associée à l'infection a ralenti la compréhension des étapes précoces du processus leucémogène et le développement de stratégies thérapeutiques efficaces. Récemment l'amélioration des modèles de souris humanisées a permis la reconstitution d'un système immunitaire humain dans des souris. L'injection de cellules souches hématopoïétiques purifiées à partir de sang de cordon humain dans des souris nouveau-nées de la lignée Rag2$^{-/-}\gamma_c^{-/-}$ conduit à la formation *de novo* de cellules dendritiques, B et T humaines. Ces dernières étant la cible de l'infection par HTLV-1, nous avons infecté des souris humanisées avec des cellules productrices de HTLV-1. Cette inoculation conduit à l'infection stable des cellules humaines dans la souris humanisée et la formation de lymphome ou de leucémie à cellules T humaines activées. Cette infection altère le développement des cellules T dans le thymus conduisant à un phénotype plus mature des thymocytes. Ce modèle animal reproduisant l'infection et la pathogénèse associée nous a permis de suivre l'évolution de la clonalité du virus au sein des différents organes lymphoïdes. Basées sur ces observations, des tests préliminaires ont permis d'étudier une nouvelle approche thérapeutique potentiellement applicable en clinique humaine. Ce travail nous a également permis d'affiner le protocole conduisant à l'humanisation des souris afin d'obtenir une meilleure reconstitution humaine dans ce modèle.

Mots clés : HTLV-1, ATL, Leucémie, Lymphome, Souris Humanisées, Thymus, Développement T

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