UNIVERSIDADE DE LISBOA

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DEPARTAMENTO DE BIOLOGIA VEGETAL

Expression and Regulation of Human Endogenous Retroviruses (HERVs) in developing and mature T Cells

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Trabalho orientado pela Doutora Helena Nunes Cabaço (IMM) e pela
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Mestrado em Biologia Molecular e Genética
Dissertação

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“Agir, eis a inteligência verdadeira. Serei o que quiser. Mas tenho que querer o que for. O êxito está em ter êxito, e não em ter condições de êxito. Condições de palácio tem qualquer terra larga, mas onde estará o palácio se não o fizerem ali?” Livro do Desassossego - Fernando Pessoa

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Abstract

The Human genome comprises almost 8% long terminal repeat (LTR)-retroelements, in which Human Endogenous Retroviruses (HERVs) are included. More than 30 HERV groups have been identified. They share a similar provirus structure with exogenous retroviruses, despite harboring many inactivating mutations. Interestingly, HERVs have been increasingly associated with cancer, autoimmunity and infectious diseases.

Data on HERV expression in T cells is sparse. Here we investigated, for the first time, the expression of several HERV families during human T cell development and differentiation. HERV-K, -W, -P and -R *envelope* (*env*) expression were analyzed in purified T cell subsets from the human thymus, peripheral blood or tonsils using real time RT-PCR. In addition, Env protein expression was studied in thymic and tonsillar tissue using immunohistochemistry.

We observed a similar pattern of HERV *env* transcriptional expression during the development and maturation of thymocytes in the thymus. HERV levels tended to be higher in mature thymocytes than in the early developmental stages, supporting their differential regulation during T cell development. Regarding the peripheral blood compartment, HERVs showed similar transcriptional levels within naïve and memory CD4 and CD8 T cells, with small inter-individual variation. Moreover, HERV expression was modulated during T follicular helper (T_{FH}) differentiation in human tonsils. Importantly, we further confirmed Env protein production in lymphoid tissues, as HERV-K Env protein was expressed in the medullary compartment of the thymus, as well as in the T cell zone of the tonsil.

Our data regarding HERV *env* expression profiles during T cell development and maturation prompted us to test potential pathways of HERV regulation. We demonstrated, using real time RT-PCR, that T cell receptor (TCR) stimulation and Phorbol-12-myristate-13-acetate (PMA)-Ionomycin were able to down-regulate HERV-W, -P and -R transcriptional levels in CD4SP thymocytes. Additionally, a positive correlation was found between HERV *env* expression and the transcriptional levels of the retroviral restriction factor deoxycitidine deaminases apolipoprotein B mRNA-editing enzyme-catalytic polypeptide-like 3G (APOBEC3G) throughout T cell development and differentiation, which was not observed for APOBEC3F.

Overall, our data support a tight regulation of HERV expression during T cell development and differentiation. This appears to be particularly pertinent in the thymus, with possible implications for the process of T cell selection.

Keywords

HERV expression and regulation, human T cell development, human thymus, human T cell differentiation, human tonsils

Resumo

O genoma humano é composto por aproximadamente 8% de retroelementos contendo repetições terminais longas (LTRs), nos quais se incluem os Retrovírus Endógenos Humanos (HERVs). Os HERVs são sequências de ADN que se fixaram no genoma humano após uma primeira infeção da linha germinativa por um retrovírus exógeno, sendo subsequentemente transmitidos verticalmente. A sua diversidade depende das interações com o hospedeiro, no entanto somente alguns HERVs foram capazes de persistir, sofrendo mutações e deleções que resultaram na perda da capacidade de replicação.

Os HERVs dividem-se em mais de 30 famílias e 3 classes, de acordo com a sua homologia a retrovírus exógenos, sendo denominados pela letra que define o ácido ribonucleico de transferência (tRNA) usado para a transcrição reversa do seu genoma. Apesar de a maioria dos HERVs estar defetivo, vários estudos demonstraram a expressão de RNA e de proteína em vários tecidos humanos e linhas celulares, e em raras situações a produção de partículas virais intactas. Pensa-se que eles persistiram no genoma humano por duas razões: exercerem funções biológicas ou devido à incapacidade de o hospedeiro os eliminar. Curiosamente, apenas a função das proteínas do invólucro (Env) do HERV-W e HERV-FRD é reconhecida. Por outro lado, o estudo dos HERVs num contexto de doença tem aumentado, uma vez que eles têm sido associados com a ocorrência de cancro, autoimunidade e doenças infeciosas.

Como não podemos excluir a existência de um HERV infecioso, é importante perceber o que pode regular a sua expressão. Canonicamente, a sua transcrição depende dos seus promotores. No entanto esta pode ser igualmente ativada por fatores de transcrição celulares (i.e., NF-κB) ou proteínas virais. Posto isto, o hospedeiro desenvolveu mecanismos para controlar a sua expressão. O enzima “apolipoprotein B mRNA-editing enzyme catalytic polypeptide-like” 3G (APOBEC3G) e outros membros da família das citidina desaminases foram descritos como fatores de restrição virais em células humanas, atuando sobre a replicação de retrovírus, tais como o HIV e alguns HERVs. Sabe-se igualmente, que a sua transcrição é reprimida por mecanismos de metilação do DNA, modificação das histonas e RNA de interferência, no entanto os estudos em humanos são limitados.

A eficiência do sistema imunitário depende da resposta da imunidade inata e adaptativa para eliminar invasões patogénicas. A imunidade inata representa a primeira linha de defesa, sendo constituída pela barreira epitelial, proteínas solúveis e moléculas bioativas. Subsequentemente, a imunidade adaptativa atua pelo reconhecimento específico de antigénios. Este reconhecimento é feito usando receptores antigénicos expressos à superfície de linfócitos B e T. No entanto, para esta resposta se dar corretamente, as células B e T têm de submeter-se a vários processos de seleção e maturação.

Progenitores de células T expressando CD34+ migram da medula óssea para o timo, onde irá ocorrer o desenvolvimento e maturação das células T. Os timócitos serão distinguidos de acordo com a expressão dos corecetores CD4 e CD8, em: timócitos "triple negative" CD34+ (TN CD34+, CD4-CD8-CD3-), timócitos CD4 "immature single positive" (CD4ISP, CD4+CD8-CD3-), timócitos "double positive" (DP, CD4+CD8+CD3-/+), e timócitos CD4 ou CD8 "single positive" (SP, CD4+/-CD8-/+CD3+). Durante o processo de maturação e seleção, os timócitos sofrem rearranjo do recetor de células T (TCR) β e α. Interações de baixa afinidade entre o TCRαβ de timócitos DP e péptidos do próprio complexados com antigénios no complexo major de histocompatibilidade (MHC) são selecionados positivamente. Por outro lado, a seleção negativa ocorre se esta afinidade for demasiado alta. Timócitos selecionados positivamente diferenciam-se em células T CD4SP ou CD8SP, que após maturação migram para a periferia.

Na periferia, as células T "naïve" circulam através dos órgãos linfóides secundários (p.ex. amígdalas) até sofrerem ativação, processo que envolve o encontro de antigénios apresentados por células dendríticas e o sinal co-estimulator emitido pelas mesmas (CD28). Após ativação, estas células adquirem uma das funções efetoras de células T auxiliar: Th1, Th2, Th17, Th9, T folicular auxiliar ou T reguladora. Em contrapartida, as células T CD8 "naïve" diferenciam-se em citotóxicas (CTLs). Sabe-se que, 5-10% das células T CD4 e CD8 efetoras persistem como células T de memória, caracterizadas por oferecer uma maior e mais eficiente resposta após segunda exposição antigénica.

A expressão dos HERVs em células T tem sido pouco estudada. Neste projeto foi investigado, pela primeira vez, a expressão de várias famílias dos HERVs durante o desenvolvimento e diferenciação de células T. A expressão do gene *env* do HERV-K, -W, -P e -R foram analisadas em subpopulações de células T purificadas do timo humano, sangue periférico e amígdalas, usando Real Time RT-PCR. Paralelamente, foi avaliada a expressão da proteína Env em tecido do timo e amígdalas usando imunohistoquímica (IHQ).

Os resultados revelaram um padrão semelhante de expressão génica dos HERVs durante o desenvolvimento e maturação dos timócitos no timo. Os níveis transcricionais apresentaram uma tendência para serem maiores nas fases mais desenvolvidas (CD4SP e CD8SP) do que nas fases iniciais do desenvolvimento (TN CD34+ e CD4ISP), indicando que possam estar a ser distintamente regulados. Estudos de vários grupos podem explicar este padrão. Conrad e colegas identificaram a expressão no timo humano de superantigénios codificados pelo membro HERV-K18, na fase DP com o intuito de induzir a seleção negativa de timócitos CD4 Vβ7. Adicionalmente, sabe-se que o desenvolvimento de células T no timo é altamente regulado por mecanismos epigenéticos; nas fases iniciais do desenvolvimento o DNA encontra-se hipermetilado, ao passo que ao longo da maturação e seleção o DNA torna-se hipometilado. Os HERVs são reprimidos com a metilação do DNA,

por isso é possível especularmos que estes mecanismos possam explicar o padrão de expressão dos HERVs no desenvolvimento dos timócitos.

Relativamente à periferia, vários estudos analisaram os níveis de HERVs em células mononucleares totais do sangue periférico. Neste projeto analisamos os seus níveis em subpopulações do sangue periférico. Os HERVs revelaram níveis transcricionais similares entre células T CD4 e CD8 "naïve" e de memória, variando pouco entre os indivíduos analisados. Podemos nesse caso especular que, o processo de proliferação homeostático envolvido na manutenção das populações "naïve" e de memória não afeta a expressão dos HERVs.

Adicionalmente, a atividade transcricional dos HERVs parece ser modulada nas amígdalas durante a diferenciação de células T foliculares auxiliares. Apesar do estudo dos HERVs em órgãos linfóides secundários ser inexistente, pensamos que esta modulação se deve à ativação e ação de mecanismos regulatórios que reprimem especificamente a transcrição dos diferentes HERVs durante a diferenciação celular.

É importante referir que os resultados da IHQ confirmaram a expressão da proteína Env do HERV-K no timo (medula) e na amígdala (zona das células T), e apoiam os dados obtidos na análise da expressão génica.

Os dados gerados durante o desenvolvimento das células T no timo, levou-nos a investigar os possíveis mecanismos de regulação da transcrição dos HERVs. Conseguimos demonstrar, usando Real Time RT-PCR, que a estimulação por via de TCR e "PMA-Ionomycin" foi capaz de regular negativamente a expressão do HERV-W, -P e -R em timócitos CD4SP. Adicionalmente, observamos uma correlação positiva entre os níveis transcricionais dos HERVs e do fator de restrição APOBEC3G ao longo do desenvolvimento e diferenciação de células T, mas não com o enzima APOBEC3F. Efetivamente, parece que os mecanismos que controlam os níveis dos HERVs são específicos da célula ou população celular em que eles se expressam.

Em suma, os nossos resultados indicam que os HERVs são estritamente regulados durante o desenvolvimento e diferenciação das células T. Esta regulação aparenta ser mais relevante no timo, com implicações no processo de seleção das células T.

Palavras-chave

Expressão e regulação dos HERVs, desenvolvimento de células T, timo humano, diferenciação de células T, amígdalas humanas

Abbreviations

ERV/HERV - Endogenous Retrovirus/ Human Endogenous Retrovirus

ALV - avian leucosis virus

MLV - murine leukemia virus

MMTV - mouse mammary tumor virus

BaEV - Baboon Endogenous Virus

LTR - long terminal repeat

tRNA - transfer Ribonucleic Acid

DNA - Deoxyribonucleic Acid

Gag - Group specific antigen

MA – Matrix

CA - Capsid

NC – Nucleocapsid;

Pol - Polymerase

PR - Protease

RT - Reverse Transcriptase

IN - Integrase protein

Env - Envelope

SU - Surface

TM - Transmembranar

Rec - Rev-like regulatory

Np9 - env-encoded Nuclear

dUTPase - deoxyuridine triphosphatase enzyme

Myr - Million years

Kbp - kilo base pairs

MSRV - Multiple Sclerosis-associated Retrovirus

ORF - Open Reading Frame

HML - Human MMTV-Like

Real Time RT-PCR - Real Time Reverse Transcriptase Polimerase Chain Reaction

APOBEC3G/3F - Apolipoprotein B mRNA-editing enzyme catalytic polypeptide-like 3G/3F

CDA - cytidine deaminase

Vif - virus infectivity factor

NF-κB - nuclear factor kappa-light-chain-enhancer of activated B cells

HSV - herpes simplex virus

PHA - Phaetoheamoglutinin

PMA - Phorbol-12-myristate-13-acetate

IL - interleukin

TNF -tumor necrosis factor

IFN - interferon

FTOC - fetal thymic organ culture

CD4 or CD8 - cluster of differentiation 4 or 8

TN - triple negative

CD4ISP - CD4 immature single positive

EDP - early double positive

DP - double positive

CD4SP or CD8SP - CD4 or CD8 single positive

V - variable

J - joining

D - diversity

MHC - major histocompatibility complex

cTEC - cortical thymic epithelial cells

TGF - transforming growth factor

CXCR - CXC chemokine receptor

CCR - CC chemokine receptor

SAg - superantigen

RTE - recent thymic emigrants

SLO - secondary lymphoid organs

DC - dendritc cell

CTL - cytotoxic T cell

Th - T helper

T_{FH} - T follicular helper

Gz - Granzyme

CM - central memory

EM - effector memory

GC - germinal centre

Bcl6 - B cell lymphoma 6

PBMC - peripheral blood mononuclear cell

mRNA - messenger RNA

IHC - immunohistochemistry

RPMI - Rosewell Park Memorial Institute 1640 medium

FBS - fetal bovine serum

OCT - optimal cutting temperature compound

PBS - Phosphate Buffered Saline

cDNA - complementary DNA

GAPDH - Glyceraldehyde 3-phosphate dehydrogenase

BSA - Bovine Serum Albumin

DAB - Diaminobenzidine

FV - Friend Virus

mTEC - medullary thymic epithelial cell

1. Introduction

1.1. Human Endogenous Retroviruses

In the late 1960s, Weiss described the existence of an integrated but non-infectious retrovirus in normal chicken embryos, defined later as an endogenous retrovirus (ERV)[1]. ERVs result from the fixation of an exogenous retroviral genome into the germ-line of an ancient host and subsequent vertical transmission. Various insertions appear to be ancient whereas others were acquired by activation or re-infection[1,2]. The endogenous avian leucosis virus (ALV) in birds, the murine leukemia virus (MuLV) and the mouse mammary tumor virus (MMTV) in mice were subsequently identified[1,3].

ERV diversity and distribution depend on interactions with the host, which per se represent key markers to study host-virus evolution[4]. The endogenization process is complex as the virus has to overcome cellular defenses and guarantee its and the progeny's survival[5]. However only some ERVs became fixed and fewer persisted, due to recombination-mediated deletions and accumulated random mutations, which rendered most ERVs replication-defective[6]. After integration in the host's DNA, ERVs can undergo transcription generating viral mRNA, which can either be translated into proteins or be reverse transcribed and re-integrated into the host's nucleus. However they are unable to infect the surrounding cells[7].

1.1.1. Discovery, detection and nomenclature

Malcolm and co-workers were the first to describe a Human Endogenous Retrovirus (HERV)[8], and other HERVs were subsequently identified by different groups[7].

The human genome comprises 8% of long terminal repeat (LTR)-retroelements, in which HERVs are included. It has been difficult to define a common classification system encompassing both endogenous and exogenous retroviruses. HERVs per se are assigned as separate families[9]. A universal nomenclature for transcribed ERV elements has long been required, however the use of several classification methods is hindering its standardization[10]. HERVs are broadly named after the single-letter amino acid code corresponding to the transfer ribonucleic acid (tRNA) primer used for reverse transcription of the HERV genome[11] (Table 1). Usually, HERVs are divided into three general classes based on their homology to exogenous retroviruses: class I gamma- and epsilon-like retroviruses, class II beta-like viruses and class III spuma-like viruses[9,11,12]. So far there are 31-42 families identified[9], some of which are known to be unique to humans (e.g. HERV-K101)[13].

HERV Family	Alternative Name	Primer	Copy No*
Class I			
HERV.Z69907		ND	30
HERV.ADP	ADP-pol	$tRNA^{Thr(?)}$	60
HERV.E		$tRNA^{Glu}$	85
HERV.F		$tRNA^{Phe}$	15
HERV.F (type b)		$tRNA^{Phe}$	30
HERV.FRD		$tRNA^{His}$	15
HERV.H	RTLV-H	$tRNA^{His}$	660
HERV.H49C23		No LTRs	70
HERV.I	RTLV-I	$tRNA^{Ile}$	85
RRHERV.I		$tRNA^{Ile}$	15
ERV-9		$tRNA^{Arg}$	70
HERV.F (type c)		$tRNA^{Phe}$	ND
HERV.P	HuRRS-P	$tRNA^{Pro}$	70
HERV.R	ERV.R	$tRNA^{Arg}$	15
HERV.R (type b)		$tRNA^{Arg}$	15
HERV.T	HERV.S71	$tRNA^{Thr}$	15
HERV.W	MSRV	$tRNA^{Trp}$	115
HERV.XA		$tRNA^{Phe}$	15
Class II			
HERV.K.HML1-4		$tRNA^{Lys}$	170
HERV.K.HML5		$tRNA^{Ile}$	45
HERV.K.HML6		$tRNA^{Lys}$	70
HERV.K.HML9		ND	ND
Class III			
HERV.L		$tRNA^{Leu}$	575
HERV.S		$tRNA^{Ser}$	70
HERV.U2		ND	ND
HERV.U3		ND	ND

*Approximate number of elements encoding domains 1–5 of reverse transcriptase.

Table 1: *HERV general nomenclature overview*. HERV families were grouped into three classes (I, II, III) according to the traditional one letter code represented by the amino acid carried by the cellular tRNA with highest homology to the ERV primer-binding site [Adapted from 4].

1.1.2. Genomic organization and molecular biology

HERVs are eukaryotic multi-copy DNA sequences whose provirus structure is similar to exogenous retroviruses[14]. They most usually encode for three recognized structural proteins: the group specific antigen (Gag), which will be cleaved by the viral protease and encodes for viral structural proteins (matrix (MA), capsid (CA), nucleocapsid (NC)); the polymerase (Pol) includes the protease (PR), the reverse transcriptase (RT) and the integrase (IN); and the envelope (Env) protein, which is subdivided into the surface (SU) and transmembrane (TM) units[3,7]. The provirus is flanked by two identical LTRs, comprising the U3, R and U5 regions. Some HERVs also encode for accessory proteins such as the Rev-like regulatory protein (Rec) and the env-encoded nuclear protein (Np9), as well as the deoxyuridine triphosphatase (dUTPase) enzyme[11]. Moreover, solitary LTRs may be generated as a result of homologous recombination events[14].

1.1.3. HERV Families: similarities and differences

It has been challenging to define the similarities between HERVs derived from different sources[15]. Class I HERVs (HERV-H, -W, -R, -P) are especially difficult to correlate phylogenetically although their elements share a canonical genome organization. Class II HERV-K superfamily differs considerably from the above and within itself in terms of genome structure[4]. Among the existing HERV families we chose to study those best-described: HERV-R, HERV-P, HERV-W and HERV-K, which integrated the human genome between 30 to 63 Myr[15].

HERV-R/ERV3 was first described by Bonner and co-workers[15]; it consists of a 9.9-kilo base pair (kbp) full-length provirus located on chromosome 7[15]. HERV-R has a standard retroviral structure[15] with intact *env* genes and 5' LTR promoter regions[16].

The HERV-P family is localized on chromosome 7q21[17]. Its provirus is flanked by two identical LTRs and is 8.1 kbp long, making up to 20-40 copies per haploid genome[15,17].

HERV-W family shares 82-88% of homology with multiple sclerosis-associated retrovirus (MSRV) particles[18]. Its provirus contains a full coding Env ORF and a functional U3 promoter[19,20]. It is present in the human genome as proviral elements, pseudoelements, truncated elements or solitary LTRs[21].

HERV-K represents the most recently acquired family in humans. As its sequence shares high homology with the mouse mammary tumor virus (MMTV), nine of the existing HERV-K families were named after **H**uman **M**MTV-**L**ike (HERV-K HML 1-9). HERV-K HML-2 represents the most characterized and preserved subgroup of this family. It originated 60 proviral copies with full-length ORFs and 2500 solitary LTRs during evolution through several peaks of proliferation[11,22]. It is subdivided into type I and II proviruses, based on the presence or absence of a 292-bp deletion at the pol-env junction. Additionally, 10% of the HML-2 loci are polymorphic and several of their elements are unfixed[11].

1.1.4. HERV expression profile

Although HERVs are predominantly defective in the human genome, they were found to be expressed in healthy tissues and in both normal and transformed cell lines[5]. Interestingly 7 to 30% of all HERV sequences within the human genome seem to be transcriptionally active. The majority of HERV elements have been identified in somatic cells and reproductive tissues, being preferentially expressed in the placenta, testis and embryonic tissues[7,23]. Moreover, quantitative Real-Time reverse-transcription (RT)-PCR as well as Western-blot analysis revealed a ubiquitous expression of Gag, Pol and Env proteins of several HERV families[24]. Some studies have shown that HERVs are able to produce intact viral particles[25]. The most obvious evidence of HERV expression lies in the immune response triggered against their products in several pathological conditions[26].

1.1.5. Consequence of HERV expression in the host

HERVs' past and present functions remain unclear, as the majority of them are defective and incapable of producing protein, contrary to mice and pig ERVs that are mobile and infectious[7,14]. HERVs persist in the human genome for two possible reasons: to exert biological functions, or due to the hosts' inability to eliminate them during evolution[12]. The results of retrotransposition events may either be neutral or deleterious, with the latter being negatively selected by the host. Positive effects are well described, although not fully understood[27].

1.1.5.1. Beneficial effects

HERVs are thought to confer defense against viral infections as their products bind to exogenous retroviral receptors and prevent their entry in the host[12].

HERVs also appear to have an ancient role in genome evolution. Recombination and transposition events lead to rearrangements, which increase genomes' plasticity[7,12]. Upon insertion they may regulate nearby gene expression through their enhancers and promoters[7,14]. Interestingly the Env proteins of both HERV-W and HERV-FRD are the only ones with recognized functions in humans. These proteins, also named syncytin-1 and syncytin-2, respectively, were co-opted by the host to mediate trophoblast fusion and differentiation[14,28], and ultimately contribute to placenta formation[14,27].

1.1.5.2. Detrimental effects

HERVs have been increasingly associated with cancer, autoimmunity and infectious diseases[27]. It is controversial whether they represent the cause or consequence of a given disease, as HERV expression could be increased as a result of the altered environment[3]. Nonetheless several groups have been able to consistently link some HERV families and a broad range of tumors by studying their expression kinetics in the context of malignancy[29]. Furthermore, distinct data supports a relation between certain HERVs and specific systemic autoimmune disorders[26]. Also, HERV expression levels have been reported to be increased upon HIV-1 infection at both gene and protein level[30].

1.1.6. HERVs infectivity: reality or fiction?

In spite of the lack of evidence of infectious ERVs in humans, it is not clear whether they completely lack pathogenic potential[31]. Moreover, it is reasonable to assume that some HERVs still contain long-term unfixed elements with re-infection capacity, although studies corroborating this in humans are limited[32]. Interestingly, Young Lee and Paul Bieniasz successfully constructed a consensus infectious HERV, using the sequence of several HERV-K HML-2 proviruses[33].

1.1.7. Restriction of Human Endogenous Retroviruses

1.1.7.1. Host Restriction Factors

Cell-intrinsic immunity is essential to protect the host from exogenous and endogenous retroviruses. Restriction factors form part of this line of defense by controlling viral infections. Apolipoprotein B mRNA-editing enzyme catalytic polypeptide-like 3G (APOBEC3G), a member of the cytidine deaminase family, was first identified as a restriction factor, via its capacity to block virus infectivity factor (Vif)-deficient HIV-1 replication[34]. APOBEC3G functions by binding viral nascent DNA and triggering cytosine to uracil deaminations during reverse transcription, thus inducing GG to AG hypermutations. Unlike APOBEC3G, APOBEC3F seems to be partially resistant to HIV-1 Vif in vivo[35]. The impact of APOBEC3 family members on endogenous retroviruses has long been investigated, especially against mobile non-LTR retroelements, and appears to act mainly via cytidine deamination. Although it has been difficult to show in vivo that APOBEC3 members impair HERVs due to their

fixation state, genome-wide analysis showed that some HERV elements carry footprints of APOBEC3G activity[36].

1.1.7.2. Epigenetic silencing

It is known that epigenetic mechanisms in various differentiated cell types and tissues play a major role in controlling ERVs. Although the majority of studies have been conducted using mouse models, these were important in revealing that ERVs' transcription is repressed mainly by DNA methylation, histone modification and RNA interference mechanisms. More interestingly, ERVs are able to maintain their expression in certain cell states (gametogenesis and early development) even though the cellular surveillance mechanisms are highly active. Moreover, the few existing studies suggest that HERVs are not primarily silenced by DNA methylation, suggesting that HERV transcriptome regulation involves the combination of several complex cellular defenses[37].

1.1.8. Activation of Human Endogenous Retroviruses

Although there are more than 50 HERV families, only HERV-K (HML-2) retained complete or near-complete ORFs for all viral proteins[38]. As for any retrovirus, HERV transcription depends on promoter elements incorporated in their flanking LTRs. Additionally, they may also utilize alternative transcriptional start sites, which gives them the ability to be differentially expressed under a given physiological condition[39]. In specific situations they can be activated both by cellular transcription factors and viral proteins. Different expression levels and activation of these transcription elements may direct HERV-K (and other HERVs if prone to be activated) specific expression in a given tissue or physiological state. For instance an impact of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) on HERV-Ks' expression has been reported[40].

Several studies have shown HERV up-regulation upon exogenous retroviral infection[26]. HERV-K[41] and -W[42] are induced by herpes simplex virus (HSV)-1, and HERV-K18 *env* transcription is up-regulated in Epstein-Barr virus infected-B lymphocytes due to transactivation[43]. Moreover, some HERV-K members may also be activated upon HIV-1 infection, by trans-complementation or trans-activation mechanisms.

It has been shown in various experiments that HERV proviruses can be induced by both external and internal signals. Phaetoheamoglutinin (PHA), Phorbol-12-myristate-13-acetate (PMA), some interleukins (IL-1α/β), tumor necrosis factor α (TNF-α) and type I and II interferon (IFN-α and –γ, respectively) were reported to induce HERV transcription and/or release of virus-like particles[26].

1.2. Human T Cell development, activation and differentiation

An efficient immune system relies on both innate and adaptive immune responses to overcome foreign assaults. When the human organism is exposed to a pathogen the innate immune system acts as a first line of defense to protect it from infection. This response is

mediated by physical epithelial barriers, soluble proteins and bioactive small molecules (complement proteins, cytokines and chemokines), membrane bound receptors and cytoplasmic proteins. Subsequently, adaptive immunity acts on specific target antigens, and thus relies on antigen-specific receptors expressed on the surface of adaptive immune cells: T- and B-lymphocytes, to achieve a complete immune response. Before this response, B and T cells have to undergo several selection and maturation steps[44].

1.2.1. αβ-T Cell Development in the Human Thymus

The thymus represents the primary (or central) organ for T cell development and selection, which occurs via a multistep pathway dependent on its complex environment. It is morphologically similar across species, being divided into cortical (more dense) and medullary (less dense) regions separated by a vascular cortico-medullary zone. The thymic medulla also contains structures called Hassall's bodies, which appear to constitute "graveyards" for dead thymocytes[45]. The majority of T cell development studies have been conducted using mouse models. Several in vivo (humanized mouse models) and in vitro (Fetal thymic organ culture - FTOC) assays have been established to help better understand human T cell development[46]. These systems have revealed that there are many differences between humans and mice regarding T cell ontogeny[47].

1.2.1.1. Early Stage T Cell Development

Bone marrow derived lymphoid progenitor cells expressing CD34 migrate to the human thymus through the cortico-medullary junction and undergo T cell commitment and maturation[45,47]. Thymocytes are distinguished based on their expression of the co-receptors CD4 and CD8, being broadly divided in Double Negative (CD4-CD8-, DN), Double Positive (CD4+CD8+, DP) or Single Positive (CD4+CD8-, CD4SP, or CD4-CD8+, CD8SP) cells. The different stages of the human T cell development are detailed in Figure 1.

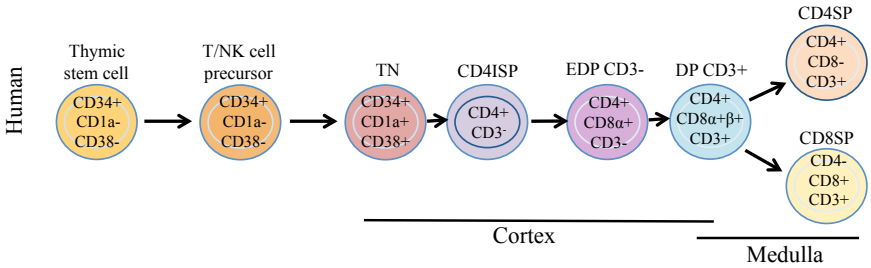

Figure 1: *T cell development in the human thymus.* CD34+CD1a- progenitor cells enter the human thymus and undergo a series of selection and maturation steps to become CD4+ or CD8+ single positive (SP) T cells [Based on 47,48,49].

1.2.1.2. TCR Rearrangements

TCR diversity depends on the random rearrangements of its variable (V), joining (J) and diversity (D) gene segments[47]. In the cortex $CD34^{+}CD1a^{+}$ thymocytes lose the expression of CD34 and start to express CD4 but not yet CD8, becoming CD4 Immature Single Positive

(CD4ISP) cells. These cells can either become αβ T cells or γδ T cells. CD4ISP thymocytes then rearrange the TCR-β gene and, with the aid of a pre-TCR α-chain, undergo β-selection[46]. Developing thymocytes then start expressing CD8, thus becoming double positive (DP) thymocytes, which proliferate and rearrange their TCRα[46,47].

1.2.1.3. Positive and Negative Selection in The Thymus

CD4+CD8+ DP cells test their TCRαβ receptor through a selection process involving the interaction with Major Histocompatibility Complex (MHC) molecules expressed on cortical thymic epithelial cells (cTECs). T cells that interact at low affinity with self-peptides complexed with MHC antigens are positively selected and start expressing the early activation marker CD69. Positively selected DP thymocytes differentiate into either CD4+ or CD8+ single positive (SP) T cells depending on their MHC-II or MHC-I restriction, respectively. SP cells undergo further selection in the medulla, where thymocytes expressing high-affinity receptors for self antigens are negatively selected and die through apoptosis[46,50]. Finally, CD4SP and CD8SP thymocytes expressing the naïve-associated marker CD45RA exit the thymus and migrate to the periphery, thus incorporating the naïve T cell pool[45,47].

1.2.1.4. Important Cytokines and Chemokines

Human T cell development is critically dependent on cytokine production. Several studies have reported the importance of Interleukin 7 (IL-7) for $CD34^+$ thymic T cell progenitor proliferation[50]. Other cytokines have been described as contributing to T cell development both in humans and mice: IL-1, IL-4, IL-6, Interferon γ (IFN-γ), Tumor necrosis factor α (TNF-α) and transforming growth factor β (TGF-β)[51]. Moreover, Notch proteins constitute a family of highly conserved transmembrane receptors whose receptor-ligand interactions have also been shown to play a crucial role in T cell development. The early phases of the development (DN stages) are promoted by Notch-mediated signals and are supported by signals delivered by IL-7 through cTECs[45].

Several chemokines have also been shown to contribute to T cell development. CXC-chemokine receptor 4 (CXCR4), CC-chemokine receptor 7 (CCR7) and CCR9 have been suggested to be involved in the migration of immature thymocytes to the subcapsular region of the thymic cortex[45].

1.2.1.5. HERVs During Human T Cell Development

HERV-K18 has been suggested to provide a continual source of superantigens (SAgs) for negative selection of self-reactive T cells in the human thymus[52]. Nevertheless, the investigation of HERV expression in the human thymus has been limited.

1.2.2. Peripheral T Cell Differentiation

1.2.2.1. Naïve T cells and Peripheral Selection Biases

It has been difficult to distinguish recent thymic emigrants (RTEs) from long-lived peripheral naïve T cells[47]. It has been suggested that the naïve T cell pool in mice is almost entirely maintained by thymic output, whereas in humans it is sustained by a combination of the former and peripheral T cell division[53]. After migration from the thymus, naïve T cells remain in the periphery without proliferating[47,53]. IL-7 appears to be a vital cytokine responsible for naïve T cell homeostasis, via its anti-apoptotic properties. Weak interactions between TCR and self-peptide-MHC complexes also contribute to this process[53].

1.2.2.2. Activation of Naïve T cells

CCR7-expressing naïve T cells circulate through secondary lymphoid organs (SLOs) (e.g. spleen, lymph nodes and tonsils) to sample antigens presented by dendritic cells (DCs). Their TCR-mediated activation is therefore a pre-requisite for their differentiation and subsequent gain of function. T cell activation is triggered by a series of steps: an initial interaction between a TCR and its cognate peptide-MHC complex presented by a DC (signal 1) followed by co-stimulatory signals delivered by the DC, such as those provided through CD28 (signal 2). Upon activation, naïve CD4 T cells acquire one of several possible T helper functions (CD4 helper T cells), whereas their CD8 counterparts differentiate into CD8 cytotoxic T cells (CTLs). CD4 helper T cells release cytokines and chemokines that either activate adjacent cells, or recruit other immune cells to sites of pathogen encounter (e.g. neutrophils). CD8 cytotoxic T cells kill infected cells by several mechanisms, including the release of cytotoxic granules into the cytosol of the infected cell[54].

1.2.2.3. Naïve T Cell Differentiation in Secondary Lymphoid Organs

When they encounter their antigen in SLOs, activated CD45RO+ CD4 T cells proliferate rapidly and differentiate into effector T helper 1 (Th1), Th2, Th17, Th9, T follicular helper (T_{FH}) cells, or regulatory T cells (Tregs), depending on the pathogen encountered and initiating stimulation signal, as illustrated in Figure 2. Each cell type is distinguished in terms of the type of immune response provided and cytokine produced[54,55].

Of note, the majority of effector T cells die by apoptosis after the primary immune response. Around 5-10% of both CD4 and CD8 T cells persist as memory T cells which can be broadly subdivided into: central memory (CM) CD4 or CD8 T cells that express CCR7 and CD45RO and are mainly present in secondary lymphoid tissues; and effector memory (EM) CD4 or CD8 T cells that also express CD45RO but not CCR7[54,56]. On subsequent antigen exposure both memory CD4 and CD8 T cells undergo a rapid and more efficient expansion phase[56,57].

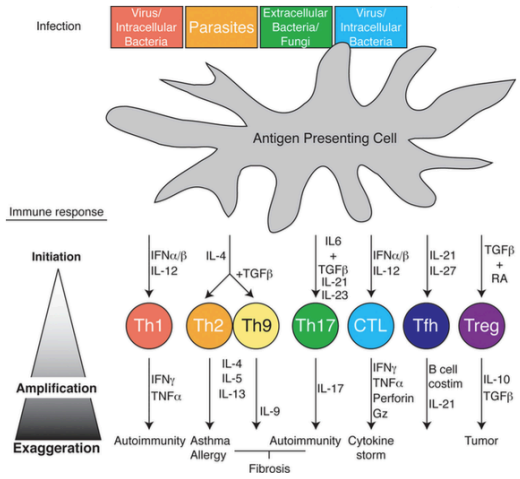

Figure 2: *CD4 T cell differentiation overview*. When a DC presents a given pathogen it releases a panel of characteristic cytokines. Along with TCR engagement various cell population may arise through transcriptional programming: CD4 Th1, Th2, Th9, Th17, T_{FH} and Treg cells. Upon differentiation these cells produce cytokines to orchestrate the immune response against the specific pathogen. If these responses persist for long they may also elicit several immunopathologies. IFN - interferon; TGF-β – transforming factor; Gz – granzyme. [Adapted from 54]

1.2.2.4. T Follicular Helper cell differentiation overview

T_{FH} cells were first described in humans through the study of CD4 T cell subsets in the tonsil. A population enriched in CXCR5 was found in tonsillar germinal centers (GCs)[58].

Naïve CD4 T cells differentiate into T_{FH} in the presence of IL-21 and IL-27 (Figure 2) upon the induction of transcription factor B cell lymphoma 6 (Bcl6)[59]. They then start expressing the chemokine receptor CXCR5, which allows them to migrate to the B cell follicles of SLOs (e.g. tonsils) where they secrete IL-21 to assist in B cell activation, proliferation and differentiation[59].

1.2.2.5. HERV Transcription during T Cell Differentiation

The research into HERV transcription in the periphery has mostly been conducted through the study of their levels in several types of disease, using total peripheral blood mononuclear cells (PBMCs). Some reports investigated whether HERVs can elicit immune responses in multiple sclerosis[60] or during HIV-1 infection[61]. However, their levels in SLO remain poorly studied.

2. Objectives

Although HERVs were long considered as “junk” DNA, there is increasing interest in understanding their role in biological and pathological conditions.

The overall objective of this work was to investigate the expression pattern of HERVs, namely of the most well-described HERV families HERV-K, HERV-W, HERV-P and HERV-R, in human T cells in order to clarify a possible role in human T cell development and/or differentiation.

Our specific aims were:

1. To determine HERV levels during human T cell development by quantifying HERV-K, HERV-W, HERV-P and HERV-R *env* messenger RNA (mRNA) in FACS-sorted thymocyte subsets using Real Time Reverse Transcription Polymerase Chain Reaction (RT-PCR).
2. To analyze whether the HERVs under study maintain their expression when mature T cells exit the thymus, via the assessment of *env* levels in FACS-sorted peripheral naïve and memory CD4 and CD8 T cells using RT-PCR.
3. To investigate HERV *env* levels during human T cell differentiation by measuring their expression in FACS-sorted $CD4^+$ T cell subsets from tonsil tissue using RT-PCR.
4. To demonstrate HERV *env* translation, by assessing HERV Envelope protein levels in the thymus and tonsil using immunohistochemistry (IHC).
5. To study modulation of HERV transcription via the quantification of HERV *env* expression upon exogenous stimuli in stimulated CD4 Single Positive thymocytes using real time RT-PCR.
6. To analyze putative relationships between HERV and APOBEC3G/APOBEC3F expression by comparing their transcriptional levels, determined by real-time RT-PCR, in human T cells.

3. Materials and Methods

3.1. Samples

Thymic specimens were obtained from routine thymectomy performed during pediatric corrective cardiac surgery at the Hospital de Santa Cruz, Carnaxide, Portugal, after parent's written informed consent.

Tonsil specimens were obtained from routine pediatric tonsillectomy at the Hospital de Santa Maria, Lisboa, Portugal, after parent's written informed consent. Blood from the same children was collected.

Adult Blood was collected from volunteer healthy donors. Sample demographics can be assessed in Table 1 (Appendix 1).

3.2. Tissue and Whole Blood Processing

Thymic tissue was thoroughly washed with Rosewell Park Memorial Institute (RPMI) 1640 medium (Gibco Life Technologies) and subsequently mashed using a syringe plunger and a 70-μM filter. Thymocytes were resuspended in 30 ml of RPMI supplemented with 2% fetal bovine serum (FBS, Sigma) (RPMI2), and cell suspensions were underplayed with 10ml Ficoll-Paque PLUS (GE Healthcare). Density gradient separation was performed by centrifuging cells for 15 min at 759 g. The Lymphocyte ring was carefully removed with a sterile Pasteur pipette and transferred to a 20 mL universal tube and washed twice with 20 mL of RPMI2 (5 min centrifugation (Eppendorf 5810) at 441 g). Cells were resuspended in 10 to 20 mL of complete medium (CM) (RPMI1640 supplemented with 10% FBS, 2% Penicillin Streptomycin (100 U/mL; Gibco), 2% Gentamicin (20 μg/mL; Gibco) and 1% L-Glutamin (20 mM; Gibco)) and counted using a hemocytometer (at a 1:10 dilution in trypan blue, a viability dye).

Tonsil specimens were thoroughly washed and sectioned into quadrants, one of which was further divided into two similarly-sized portions. One of these was frozen in an eppendorf containing optimal cutting temperature compound (OCT, VWR) and snap-frozen in liquid nitrogen, whilst the other one was fixed in 4% formaldehyde and then embedded in paraffin. The remaining tissue was mashed and cells were separated using the same procedure as for the thymus. The isolated lymphocytes were washed twice in Phosphate Buffered Saline (Sigma) supplemented with 2% of FBS (PBS2). Cells were resuspended in 10 to 20 mL of PBS2 and counted using a hemocytometer (1:10 dilution in acetic acid, which lyses red blood cells).

Whole peripheral blood was diluted in an equal volume of PBS and PBMCs were again separated using Ficoll-Paque PLUS (20 min centrifugation at 1600 rpm). The lymphocyte ring was removed, and cells washed twice with 1x PBS. Cells were resuspended in PBS2 and counted using a hemocytometer (1:10 dilution in acetic acid).

1 and/or 5 million total thymocytes or mononuclear tonsillar cells were centrifuged at 13200 rpm for 5 minutes (Eppendorf 5415R), supernatant carefully removed and dry pellets were stored at -80 ºC.

3.3. T Cell Sorting

300 million total thymocytes, 34-70 million total adult PBMCs or 200-300 million total mononuclear tonsillar cells were centrifuged for 7 min at 1600 rpm and incubated at 4 ºC in the dark for 30 min with the appropriate antibodies, listed in Table 2 **(**Appendix 1**)**. After washing with PBS2 cells were sorted to a high degree of purity (routinely >98% purity) using a FACSAria (BD Biosciences). The gating strategy used for the abovementioned sortings is detailed in Appendix 2-4.

Total thymocytes were sorted according to human αβ T-cell developmental stages: TN CD34$^+$ (Lineage$^-$CD4$^-$CD8$^-$CD3$^-$CD34$^+$), CD4ISP (CD4$^+$CD8$^-$CD3$^-$), DP (CD4$^+$CD8$^+$CD3$^{-/+}$), CD4SP (CD4$^+$CD8$^-$CD3$^+$) and CD8SP (CD4$^-$CD8$^+$CD3$^+$). Lineage=CD14, CD16, CD19, CD20, CD56, CD123, CD11c, TCRγδ.

Adult peripheral blood T cells were sorted into CD4 (CD3$^+$CD4$^+$CD8$^-$) and CD8 (CD3$^+$CD4$^-$CD8$^+$) T cells, as well as naïve (CCR7$^+$CD45RA$^+$) and effector plus central memory (CCR7$^-$CD45RA$^-$, CCR7$^+$CD45RA$^-$ and CCR7$^-$CD45RA$^+$) T cell subsets[62].

Memory CD4 (CD4$^+$CD45RO$^+$) pediatric mononuclear tonsillar cells were sorted according to the T follicular helper differentiation stages: PD1^{neg}CXCR5^{neg}, PD1^{low}CXCR5^{neg}, PD1^{low}CXCR5$^+$, PD1^{bright}CXCR5^{bright}.

Sorted cells were washed with PBS and dry pellets of 1 and/or 5 million cells were stored at -80 ºC.

3.4. Thymocyte Culture

FACS-sorted CD4SP T cells (200.000-500.000 cells/condition) were stimulated in a 96-well U-bottom tissue culture plate at 37 ºC in the presence of complete medium alone or supplemented with Interleukin-2 (IL-2; 100 U/mL; R&D), IL-4 (100 ng/mL; R&D), IL-7 (50 ng/mL; R&D), Tumor Necrosis Factor-α (TNF-α, 20 ng/mL; eBiosciences), Interferon-α (IFN-α, 50 ng/mL; eBiosciences), IFN-γ (66 ng/mL; BD Biosciences), PHA (5 μg/mL; Sigma-Aldrich), PMA (500 ng/mL; Sigma-Aldrich) + Ionomycin (500 ng/mL; Calbiochem, Merck Biosciences, Nottingham, UK) or soluble anti-CD3 + anti-CD28 mAbs (both 1 mg/mL; eBioscience) for 23h.

After culture, cells were removed from wells, washed in 1x PBS, and supernatant carefully removed. Cell pellets were frozen at -80 ºC.

3.5. RNA Extraction and cDNA Synthesis

Total RNA was extracted using Rneasy mini kit (Qiagen), AllPrep DNA/RNA mini and micro kit (Qiagen) or ZR-Duet DNA/RNA MiniPrep (Zymo Research) according to the manufacturer's guidelines. RNA from all samples was treated with DNase I (Sigma-Aldrich) to remove any genomic DNA contamination. The purified RNA was quantified using NanoDrop™ 1000 Spectrophotometer (Thermo Scientific) and 50 or 200 ng of RNA was used to synthesize complementary DNA (cDNA). The remaining RNA was stored at -80 ºC.

For cDNA synthesis, 2 µL of a mix containing random primers (300 ng/µL; Invitrogen) and dNTPs (Invitrogen) was added to 11 µL of RNA and incubated at 65 ºC for 5 min in a T100^{TM} Thermal cycler (BIO-RAD). After cooling on ice, 7 µL of a mix containing Reverse Transcriptase (RT) buffer (5X; Invitrogen), DTT (0.1 M; Invitrogen), Superscript III reverse transcriptase (Invitrogen) and RNase Out (Invitrogen) were added and the tubes were incubated at 50 ºC for 50 min, and then at 70 ºC for 15 min. The cDNA was stored at -20 ºC.

3.6. Real-Time Reverse Transcriptase Polimerase Chain Reaction (RT-PCR)

Relative HERV-K, -W, -P and -R *env* mRNA levels, as well as APOBEC3G and APOBEC3F mRNA levels, were measured using Real-Time RT-PCR with the appropriate set of primers listed in Table 3 (Appendix 5) and Power SYBR Green PCR Master mix (Applied Biosystems). HERV, APOBEC3G/3F and β-actin primers were used at a concentration of 300 nM and GAPDH at 100 nM. Real time RT-PCR amplification was performed using the 7500 Fast or the ViiA™ 7 Real-Time PCR System (both from Applied Biosystems). In each run, 5 or 2.5 µl of cDNA, respectively, were used as a template for amplification per reaction with the following cycling conditions: 50 ºC for two min, 95 ºC for 10 min, 50 cycles of 95 °C for 10 seconds (s) and 60 °C for 1 min. Melting curve analysis was always included.

All samples were amplified in duplicate. cDNA from total thymocytes was used as positive control and water as negative control. Relative mRNA levels were normalized to GAPDH and β-actin and calculated using the $2^{-\Delta CT}$ or $2^{-\Delta\Delta CT}$ method. All data analysis was conducted using GraphPad Prism 5 software.

3.7. Immunohistochemistry Staining

Paraffin blocks of human thymic and tonsillar tissue were cut into 3µm sections, deparaffinized with xylene for 10 min and rehydrated with alcohol (100%; 96%; 70%, five min each). Endogenous peroxidase blocking was carried out by placing the slides in a mix of methanol+H_2O_2 1.5% v/v, for 15 min. For antigen retrieval, the slides were placed in a water bath at 97ºC for 20 min in 10 mM sodium citrate buffered-0.05% Tween20, pH 6.0 solution. Each tissue section was subsequently treated for 30 min with Bovine Serum Albumin (BSA) 3% for blocking of non-specific staining and washed three times with 1x PBS for 5 min. The sections were stained with anti-HERV-K Env TM antibody (Austral Biologicals, HERM-1811-

5) diluted 1:100 in 1x PBS or anti-MxA (OTTO HALLER, M143) diluted 1:300 in 1x PBS and incubated at room temperature in the dark for 1h. Secondary detection was performed by adding one drop of a Horseradish peroxidase HRP anti-rabbit/mouse kit (Envision, DAKO) for 30 min, washing with 1x PBS and then revealing with Diaminobenzidine (DAB) contained in this kit. Sections were counterstained with hematoxylin for six seconds, dehydrated and diaphanized in running water, alcohols at 70%, 96% and 100% (30 s each) and xylene (10 min). Slides were mounted with Quick-D mounting medium (Klinipath). Bright-field images were acquired with a Leica DM2500 bright-field microscope with either a 20x HC PL FLUOTAR Dry or 40x HCX PL FLUOTAR lens.

4. Results

4.1. Human Endogenous Retroviruses Expression Profile In Human T Cells

Although to date there is no data supporting the existence of a replication-competent HERV, its gene products, when intact, can be expressed at transcriptional and translational levels, interfering with biological and/or contributing to pathophysiological mechanisms. Thus, it is of great interest to improve our knowledge on their role in both contexts.

First we conducted an extensive literature review to identify the appropriate HERV primers that would be subsequently used to in this project. In order to confirm the HERV families we were going to detect we ran the HERV primers' sequences through BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and associated tools. Via this approach, and taking in consideration all the additional information provided by Ahn K., Kim H.-S. and Kim D.-S., the HERV subgroups we chose to study are: HERV-K-21 subgroup located on chromosome 12q14.1, HERV-R (ERV3) locus located on chromosome 7q11.21, HERV-P(b) locus located on chromosome 14q32.12 and HERV-W (ERVWE1) located on chromosome 7q21.2. Although these primers don't amplify all HERV members we chose to use them as each HERV locus harbors an intact *env* Open Reading Frame (ORF).

4.1.1. HERV *env* Expression During Human T Cell Development

Using this approach we focused on studying *env* gene expression during human T cell development. To do so we first validated the primers by Real-Time RT-PCR using a calibration curve comprising serially diluted total thymocyte cDNA. After confirming their high efficiency (close to 100%) we next quantified HERV-K, -W, -P and -R *env* gene expression in the following thymocyte subsets FACS-sorted according to developmental stage: TN, CD4ISP, DP, CD4SP, CD8SP. It is also important to emphasize that each sample RNA was treated with DNase in order to avoid any genomic DNA contamination. Three human pediatric thymuses were collected. The results are presented in Figure 1 and were normalized relative to the endogenous controls, GAPDH and β-actin.

We found that HERV-K, -W and -P were expressed at relatively low levels during the course of T cell development, while HERV-R featured generally higher transcriptional levels. We observed a similar pattern of expression of all HERV families during human T cell development, with slight inter-HERV family and inter-individual variations. It is also worth noting that HERVs tended to be higher in mature thymocytes (CD4SP and CD8SP) than in the early developmental stages, supporting the hypothesis that they might be differentially regulated during the development and maturation of thymocytes in the thymus.

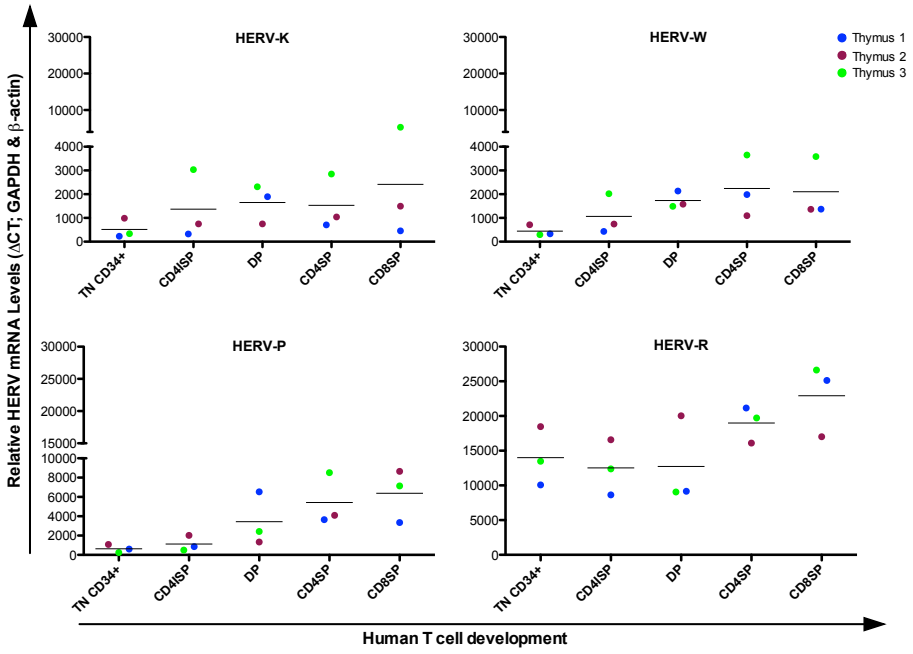

Figure 1: *Real-Time RT-PCR analysis of HERV env mRNA expression during T cell development in the human thymus*. Total thymocytes were FACS-sorted according to CD4, CD8, CD3 and lineage markers expression (Lin= CD14, CD16, CD19, CD20, CD56, CD123, CD11c, TCRγδ). The relative expression levels of HERV-K, -W, -P and -R *env* were normalized to GAPDH and β-actin expression levels. Relative mRNA levels were calculated using the ΔCT method. TN: Triple Negative; ISP**:** Immature Single Positive; DP**:** Double Positive; SP**:** Single Positive. n=3 pediatric thymuses, each color represents a thymus.

4.1.2. HERV *env* Expression Levels in Human peripheral blood T cells

After analyzing the expression of HERVs in the thymus we asked whether HERV-K, -W, -P and -R transcript levels would be maintained when mature T cells exit the thymus to the periphery. To determine this we collected blood from four healthy donors and assessed HERV *env* levels in purified peripheral naïve and memory CD4 or CD8 T cells using real-time RT-PCR.

When we compared the results from Figure 1 and Figure 2 we noticed that peripheral naïve T cell populations showed higher HERV *env* levels than mature thymocytes, with HERV-K featuring highest expression. Moreover, their expression was similar within naïve and memory CD4 and CD8 T cells. It is interesting to note that HERV levels showed small variations between individuals.

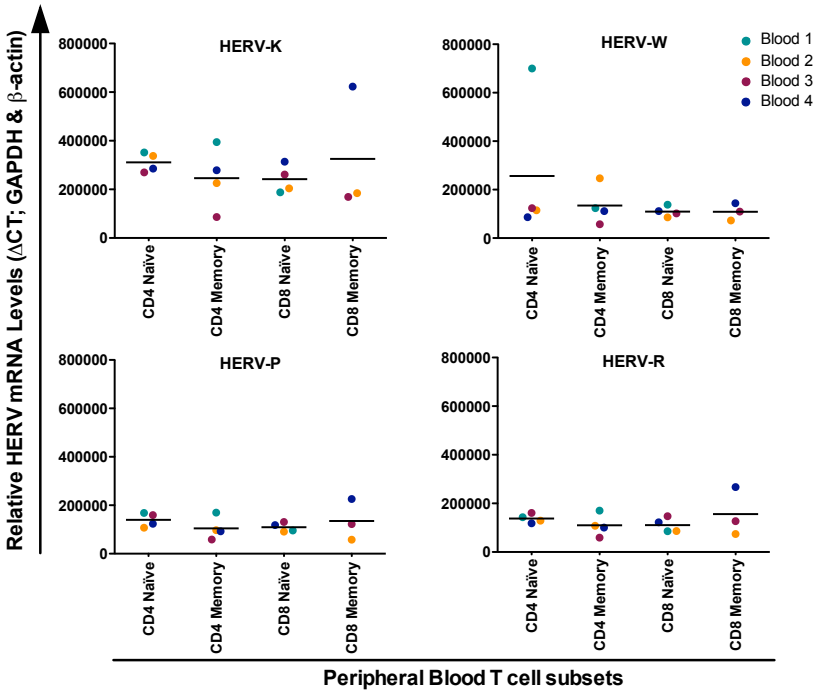

Figure 2: *Real-Time RT-PCR analysis of HERV env mRNA expression in circulating naïve and memory CD4 and CD8 T cells*. PBMCs were FACS-sorted according to Naïve (CD4 naïve: CD4+CCR7+CD45RA+; CD8 naïve: CD8+CCR7+CD45RA+) and Memory (CD4 memory: CD4+CCR7-/+CD45RA-; CD8 memory: CD8+CCR7-/+CD45RA-) subsets. The relative expression levels of HERV-K, -W, -P and -R *env* were normalized relative to GAPDH and β-actin expression levels. Relative mRNA levels were calculated using the ΔCT method. n=4 adult blood donors, each donor represents a donor.

4.1.3. HERV *env* Expression Levels during Human tonsillar T Cell Differentiation

As described in the introduction, circulating naïve T cells can access SLOs, entering the T cell zone to survey antigens presented in the context of MHC complexes expressed by dendritic cells. Once activated they can either exit to the periphery as effector cells or, in the case of appropriately stimulated CD4 T cells, enter into the B cell area of the SLOs, such as tonsils, where their interaction with B cells plays a vital role in the germinal center response.

In order to investigate HERV levels during the stages of CD4 T cell differentiation and activation we quantified HERV-K, -W, -P and -R *env* expression in pediatric tonsillar mononuclear cell populations using Real-Time RT-PCR. We focused our study on memory ($CD45RO^{+}$) CD4 T cells. Within the memory subset we sorted the cells according to T_{FH} expression markers PD-1 and CXCR5: $PD\text{-}1^{neg}X5^{neg}$, $PD\text{-}1^{low}CXCR5^{neg}$, $PD\text{-}1^{low}CXCR5^{+}$ and $PD\text{-}1^{bright}X5^{bright}$ cells. Three samples of tonsil tissue were collected from three children. The HERV *env* levels are shown in Figure 3 and were normalized to GAPDH and β-actin.

Interestingly, HERV transcription was modulated during tonsillar T_{FH} differentiation, showing small inter-individual variations. Moreover, their expression appeared to be regulated irrespective of the HERV family analyzed.

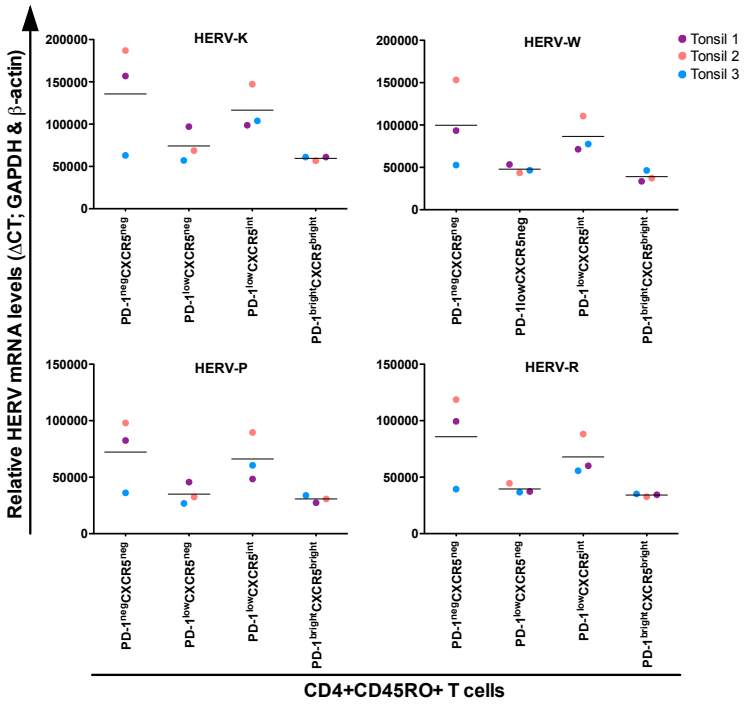

Figure 3: *Real-Time RT-PCR analysis of HERV env mRNA expression in pediatric tonsillar mononuclear CD4$^+$ T cell subsets*. Tonsillar mononuclear cells were FACS-sorted according to T_{FH} differentiation stages: PD-1^{neg}X5^{neg}, PD-1^{low}CXCR5^{neg}, PD-1^{low}CXCR5$^+$ and PD-1^{bright}X5^{bright} cells. The relative expression levels of HERV-K, -W, -P and -R *env* were normalized to the expression levels of GAPDH and β-actin genes. Relative mRNA levels were calculated using the ΔCT method. n=3 tonsil donors, each color represents a donor.

4.1.4. Global analysis of HERV *env* Expression profile in Human T cells

After obtaining the previous results we thought it would be of interest to analyze the overall data as a set, as illustrated in Figure 4.

Interestingly despite the tissue specific differences each HERV shared a similar pattern of expression throughout T cell development and differentiation, with the thymus showing lower HERV transcriptional levels as compared to the other tissues. HERV *env* expression levels appeared to be tissue specific, irrespective of the family analyzed, and appeared to be more regulated in the thymus and tonsil. The conserved pattern and tissue specific expression differences justify further investigations, especially in regard to the mechanisms involved in their transcriptional regulation.

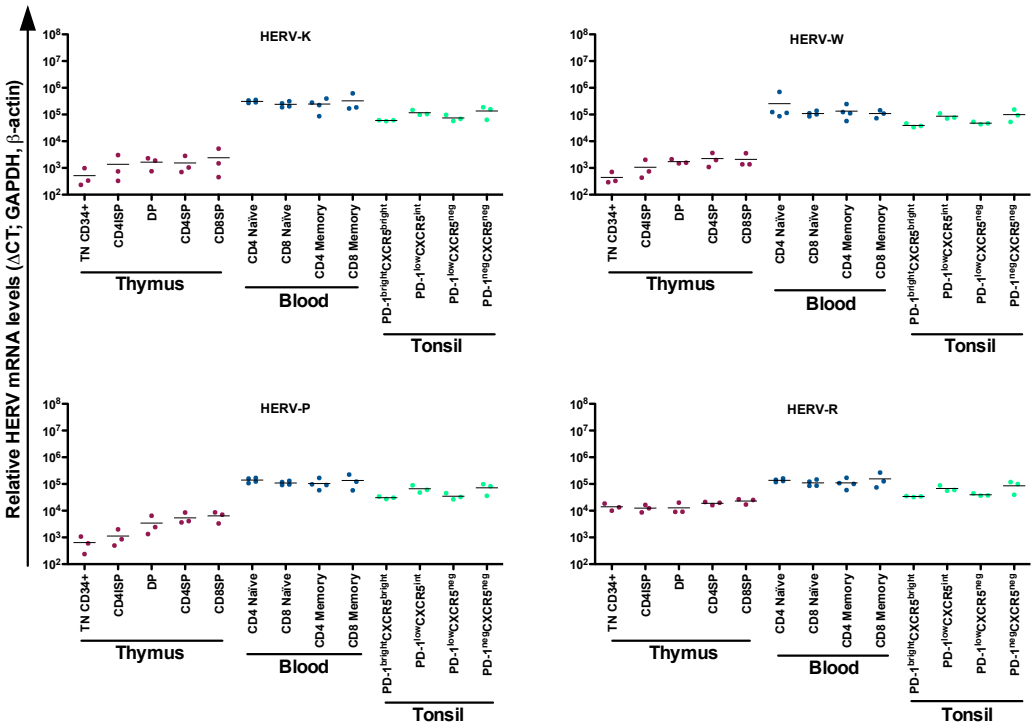

Figure 4: *Overall Real-Time RT-PCR analysis of HERV env mRNA expression throughout T cell development and differentiation*. Each colored circle represents a specific tissue.

4.1.5. HERV Env Protein Expression Levels in the Human Thymus and Tonsil

As we were able to demonstrate expression of the HERV *env* gene at the mRNA level, we next asked whether the transcripts could be translated into proteins. To confirm this we performed immunohistochemistry (IHC) in slide-mounted sections from paraffinized samples of human thymus and tonsil tissue. We analyzed samples from three individual thymus and tonsil biopsies. Each sample was stained with an anti-HERV-K Env antibody that was previously used in other studies[63]. We chose MxA as a positive control because it is known to be constitutively expressed in the thymus[64], and was already optimized in the lab. The addition of the secondary antibody alone served as a negative control for each molecule. IHC staining revealed the expression of HERV-K Env protein in some cells in the thymus (medulla - M; Figure 5 B,C) and tonsil (T cell zone; Figure 5 E,F) as compared to the negative control of each tissue (Figure 5 A,D). In contrast, both the thymic cortex (C) and tonsillar germinal center (GC) regions showed no HERV-K Env staining. In both tissues the protein surrounds the expressing cell, confirming the transmembrane localization of HERV Env protein.

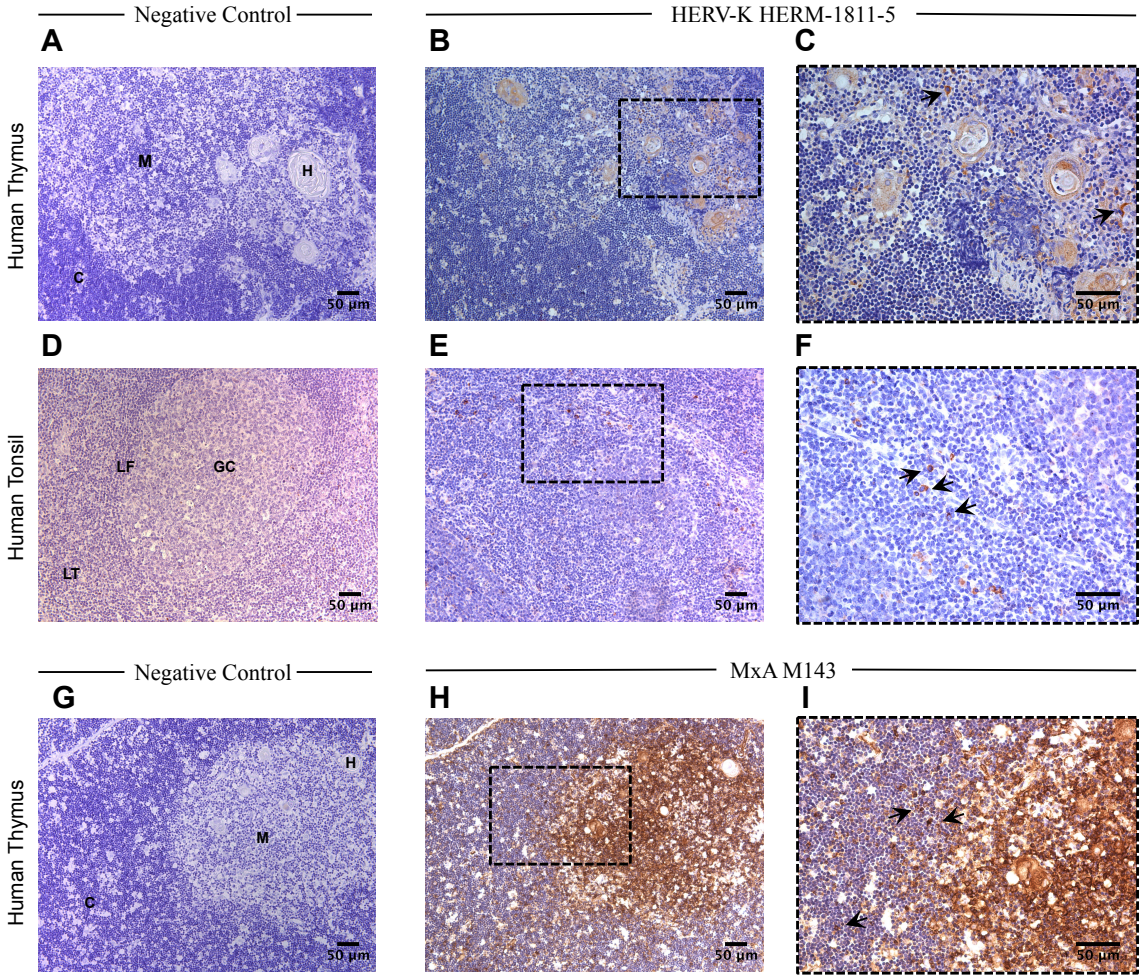

Figure 5: *HERV-K Env protein expression in human thymus (medulla) and tonsil (T cell zone)*. Secondary antibody alone served as a negative control for each gene in the human thymus (A,G) and tonsil (D). Each sample was stained with an anti-HERV-K Env antibody in the thymus (B,C) and tonsil (E,F). Sections were stained with the MxA specific antibody M143 as positive control. Second column: 200X magnification; third column: 400X magnification. Arrows indicate positive cells stained in brown. **C**: Cortex; **M**: Medulla; **H**: Hassall's bodies; **LT**: lymphoid Tissue; **LF**: Lymphoid Follicle; **GC**: Germinal Center. Scale bar = 50 µM.

4.2. Transcriptional Regulation of Human Endogenous Retroviruses

The data we generated regarding HERV *env* expression profiles during T cell development and maturation suggested potential ways in which HERV transcription could be regulated. Thus, we asked whether HERVs were transcriptionally regulated by a given exogenous stimuli (cytokines, mitogens/activators or TCR stimulation) or featured a possible relationship with host restriction factors.

4.2.1. HERV *env* transcription regulation upon cytokine and non-specific stimulation

In order to investigate if HERV *env* levels would be affected upon exogenous stimulation we measured their mRNA expression in cultured thymocyte subsets using Real-Time RT-PCR. A total of three thymuses were collected and thymocytes were FACS-sorted, as previously described. Next, mature CD4SP thymocytes were stimulated for 23 hours with

complete medium alone or in the presence of a panel of cytokines known to impact upon T cells (Interleukin-2 (IL-2), IL-4, IL-7, Tumor Necrose Factor-α (TNF-α), Interferon-α (IFN-α), IFN-γ), non-specific stimulators (Phytohaemagglutinin (PHA), Phorbol-12-myristate-13-acetate (PMA) + Ionomycin) or TCR stimulation (α-CD3+α-CD28). HERV *env* levels were compared to the unstimulated control using the ΔΔCt method[65] (Figure 6).

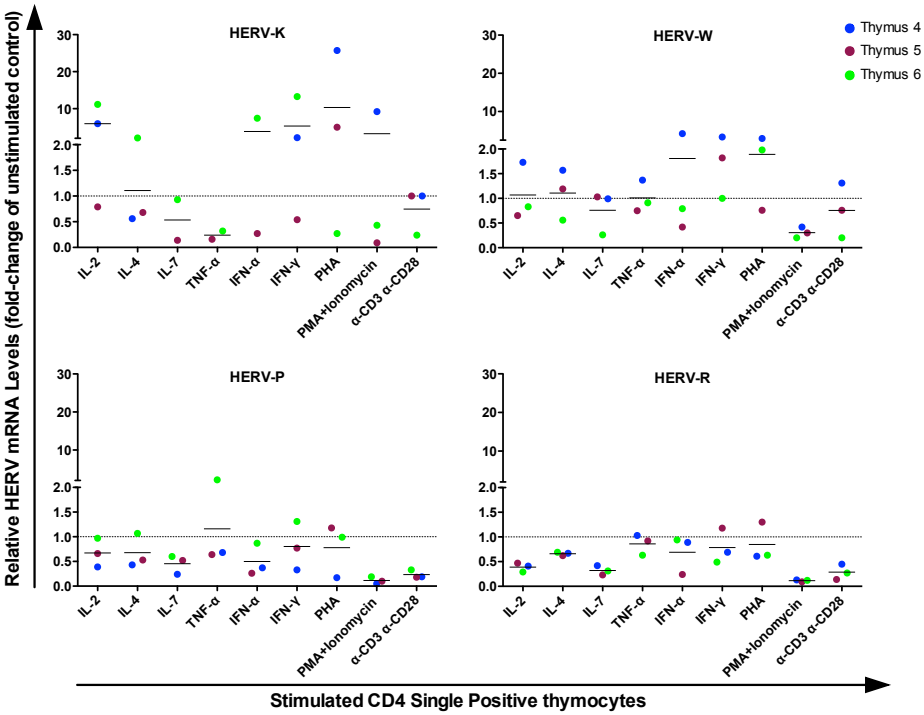

Figure 6: *Real-Time RT-PCR analysis of HERV env mRNA expression in stimulated mature thymocytes*. CD4SP thymocytes were stimulated for 23 hours with medium alone or in the presence of IL-2, IL-4, IL-7, TNF-α, IFN-α, IFN-γ, PHA, PMA + Ionomycin and α-CD3α-CD28. The relative expression levels of HERV-K, -W, -P and -R *env* were calculated as the fold-change relative to the unstimulated control using the ΔΔCT method (delineated with 1). n=3 thymuses, each circle represents a thymus.

A marked inter-individual variation in HERV *env* expression was observed for each stimulation condition. Of note, TCR stimulation as well as PMA-Ionomycin decreased HERV-W, -P and -R transcriptional levels. Moreover, HERV-K and -W showed an increase in some samples upon interferon (α, γ) stimulation. Overall HERVs revealed a robust expression in the human thymus, differentially affected by exogenous stimuli.

4.2.2. Correlations of HERV *env* and host restriction factor expression in Human T cells

As we found no significant differences in HERV transcription upon exogenous stimulation we next asked whether there could be an intracellular defense mechanism capable of regulating HERVs, such as APOBEC3G/3F activity, which has been extensively studied in what concerns the inhibition of retroviruses, non-LTR or LTR retroelements in vitro and ex vivo[33,36].

In order to understand this relationship, we compared the relative HERV *env* and APOBEC3G or APOBEC3F mRNA levels in each previously studied tissue individually (thymus, blood and tonsil), and then in data set as a whole to obtain a general analysis. APOBEC3G or APOBEC3F mRNA levels were measured in parallel with the HERV *env* expression levels and analyzed using the same methodology, with specific primers. Non-parametric spearman correlations were used to evaluate the relationship between parameters, with p values <0.05 considered as significant.

APOBEC3G significantly correlated with the expression of most HERVs in the blood and tonsil, but not in the thymus (Figure 7, Table 1). In contrast, APOBEC3F showed no correlation with the transcriptional levels of any of the HERVs analyzed, except for a positive correlation with HERV-K in the blood (Figure 7, Table 2). Additional functional analysis is required to better understand the interplay between APOBEC3G proteins and HERVs.

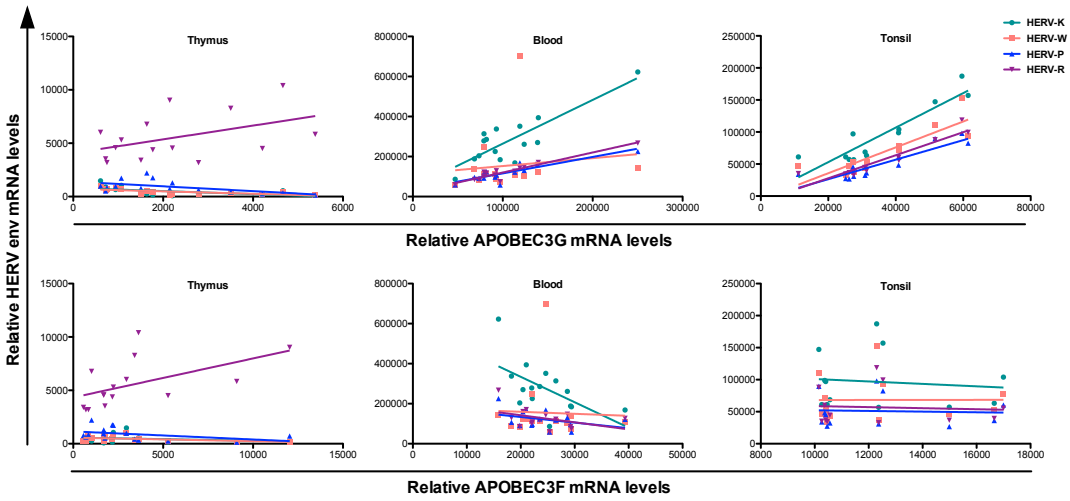

Figure 7: *Relative correlation between the expression of HERV env gene and APOBEC3G or APOBEC3F in the thymus, blood and tonsil.* Relative mRNA levels were calculated using the ΔCT method and were normalized to GAPDH and β-actin genes. Each color and shape represents a different HERV family.

Table 1: Relationship between HERV *env* and APOBEC3G mRNA levels in the thymus, blood and tonsil. Spearman non-parametric test was used to calculate the correlations. p value < 0.05.

APOBEC3G		HERV-K	HERV-W	HERV-P	HERV-R
Thymus	*p*	0,0412	0,1435	0,9195	0,1913
	R^2	-0,5321	-0,3964	0,02857	0,3571
Blood	*p*	0,0365	0,2621	0,0003	< 0,0001
	R^2	0,5429	0,3092	0,8071	0,8786
Tonsil	*p*	0,0008	0,0013	0,0009	0,0002
	R^2	0,8531	0,8322	0,8462	0,9021

Table 2: Relationship between HERV *env* and APOBEC3F mRNA levels in the human thymus, blood and tonsil. Spearman non-parametric test was used to calculate the correlations. p value < 0.05.

APOBEC3F		HERV-K	HERV-W	HERV-P	HERV-R
Thymus	*p*	0,0642	0,2372	0,6664	0,5243
	R^2	-0,4893	-0,3250	-0,1214	0,1786
Blood	*p*	0,0148	0,4159	0,2597	0,1110
	R^2	-0,6143	-0,2270	-0,3107	-0,4286
Tonsil	*p*	0,8004	0,9910	0,6999	0,9910
	R^2	-0,0839	0,0069	-0,1259	0,0069

5. Discussion and Future Perspectives

Although the majority of HERVs have been rendered defective by silencing mutations, some remain transcriptionally active, being detectable at the gene and protein level, as well as releasing viral-like particles under specific physiological conditions. Although several groups were able to show an association between HERV expression and different human pathologies, a direct link is yet to be demonstrated. Moreover, the hypothesis that the persistence of HERVs within the genome could result from an ability to provide functions that benefit the host is still under debate[23]. The best-described example of a biological role for HERV gene products is that of the Envelope protein of specific HERV members. These have been shown to be vital for the syncytiotrophoblast development, as well as for providing immunotolerance in the context of pregnancy[9].

We sought to analyze HERV-K, -W, -P and -R *envelope* expression in human T cells. Our results show, for the first time, that immature populations (TN $CD34^+$ and CD4ISP thymocytes) feature lower levels of HERV *env* expression than their mature counterparts, thus suggesting that HERV transcription is differently regulated during human thymic T cell development.

Given our novel data regarding HERV *env* expression in thymocyte subsets, it is important to understand the physiological relevance of both the presence of HERVs per se in the human thymus, and of the differences observed in their transcription during the course of T cell development. It is reasonable to assume that the presence of HERVs in a given tissue could either result from their exertion of a function beneficial to the host, or that the host was unable to permanently eliminate them from the genome[12]. The situation in the thymus appears to result from a homeostatic balance involving the transcriptional regulation of HERVs, reflected in their pattern of expression. Data from several groups supports this. Conrad and co-workers identified the constitutive expression of a superantigen (SAg) encoded by the HERV-K18 member in the human thymus. SAgs are microbial proteins that bind to MHC class II proteins and the Vβ chain of the TCR, inducing T cell stimulation and expansion[66]. Interestingly, HERV-K18 SAg expression was restricted in time and space, being expressed at the DP stage to induce the negative selection of Vβ7 CD4 thymocytes[67]. In mice, Kassiotis and colleagues showed that a self-peptide, encoded by a mouse endogenous retrovirus, negatively selected Friend Virus (FV)-specific $CD4^+$ T cells and diminished the response to FV infection[68]. Although this evidence relates to specific mice ERV and HERV-K members, it provides us with a possible rationale for the high presence of each HERV in mature thymocytes and emphasizes the importance of unraveling possible roles for HERVs during human T cell development. To better understand this, more studies are required, especially to search for other SAg encoded by the HERVs studied here.

Our data also indicate that HERV expression occurs at low levels in the human thymus, a finding previously reported by other groups but in the context of whole tissue analysis[20,24].

For instance, Hirai and colleagues showed a ubiquitous expression of the HERV-W *env* gene in 20 different human tissues, including the thymus, but at lower levels than reported by others[19]. Moreover in a similar approach Kim and Ahns' group assessed the levels of HERV-P, -K and -R in several tissues and reported that the thymus featured low levels of HERV transcription[69].

As previously mentioned, HERV transcription can be modulated by the methylation status of their LTRs: hypomethylated DNA induces their overexpression, whilst, conversely, hypermethylated DNA represses them[70]. The existing data on this regulatory mechanism focuses on HERV silencing during pre-implantation and germ cell development. However, taking into consideration Lopez-Larreass' work on transcriptional programming by DNA methylation during human T-cell development, it is possible that this mechanism may be involved. Immature thymocyte (TN $CD34^+CD1a^-$) genomes were highly methylated, and from this stage on, de novo methylation levels decreased, with mature SP thymocytes featuring the highest hypomethylation levels[71]. These findings can potentially provide an explanation for our data, in that the hypermethylated state of TN $CD34^+$ thymocytes would result in lowest HERV levels, whereas the highest HERV levels we observe occur in hypomethylated mature thymocytes. To further corroborate this hypothesis, HERV LTR methylation status should be analyzed at each T cell developmental stage.

We noticed a similar expression pattern for all HERVs that varied slightly in terms of magnitude at the inter-HERV family and inter-individual levels. Essentially, the chromosomal location of each HERV defines the extent of its expression in a given tissue[19]. Our results confirm this; HERV-K, -W, -P and -R are integrated in different regions in the genome, and thus are differentially expressed in the thymus. Another reason for this tissue heterogeneity is that each HERV member has a specific transcription regulation mechanism resulting from the interaction of its characteristic LTRs and transcription factors, a process also dependent on their physical localization[40]. The inter-individual diversity is more difficult to explain, however it is known that the integration site may play a major role in determining whether a HERV sequence is positioned in a transcriptionally active genomic locus[72].

As the HERV *env* expression pattern appeared to be modulated in the human thymus, we next decided to investigate HERV levels in the periphery, where mature thymocytes persist as naïve T cells. Some groups confirmed the expression of several HERV families in total human PBMCs. For instance, Sinibaldi-Vallebona recently published data on HERV transcriptional activity in a large study population, in which they demonstrated age-related differential expression of HERV-K and -W[73]. We extend this data by demonstrating the expression of HERV-K, -W, -P and -R *env* mRNA in peripheral naïve and memory CD4 and CD8 T cell subsets. Moreover, our results revealed a significant increase of HERV transcriptional levels in the periphery, as compared to those seen in mature thymocytes. Taking into consideration the existing data on HERV expression analysis in total PBMCs, we

can envisage a situation that could explain our results. As mentioned before, HERV-K18 specific SAg activity negatively selects Vβ7CD4 T cells in the thymus, consequently reducing their frequency in the periphery[67]. Conrad proposed that this negative selection could be an initial checkpoint mediating peripheral tolerance to specific HERV antigens[67]. However, it is problematic to establish a direct link between the high levels of HERVs in the periphery and the selection of a given T cell population.

Our data also revealed that HERVs showed similar transcriptional levels within naïve and memory CD4 and CD8 T cells, with a small inter-individual variation. The details underlying this pattern are not known. However, this may reflect a lack of impact of homeostatic proliferation, that helps maintain peripheral naïve and memory pools, on HERV expression levels. To address the effect of T cell activation on HERV expression an in vitro T cell differentiation system could be used, such as measuring HERV levels in naïve $CD4^+$ T cells prior to, and after TCR-mediated stimulation (αCD3+αCD28+IL-2).

In addition it would be interesting to investigate HERV levels in the context of impaired thymic output (e.g. in primary immunodeficiency disorders, or thymectomized individuals), in which cellular immune responses are compromised[74].

We generated, for the first time, data regarding HERV transcriptional activity upon CD4 T cell differentiation, specifically in T follicular helper cell (T_{FH}) subsets isolated from pediatric tonsils. Notably, HERV transcription levels appeared to be modulated during T_{FH} differentiation, with a tendency to be lowest in the most differentiated cells (T_{FH}). It is well established that CD4 naïve T cells require a complex network of specific cytokine signaling, transcription factor induction together with epigenetic modifications to differentiate into a given subset. Moreover, upon activation the DNA undergoes demethylation[75]. This process, as previously mentioned, would favor HERV expression. However, we believe that other regulatory mechanisms may be activated at the various stages of T cell differentiation, in order to further repress HERV activation, also suggesting that these mechanisms are cell-subset specific.

The ability of HERVs to not only undergo transcription, but also produce active viral proteins is well established. However, the latters functional role remains poorly understood. Mallets et al. confirmed HERV-W Env Glycoprotein expression in human villous cytotrophoblasts via immunohistochemistry (IHC). In this study they were able to demonstrate that the processes of trophoblast cell fusion and differentiation were dependent on the expression of the HERV-W Env protein[28]. In addition Ostrowski and colleagues showed that HERV-K Env protein was induced in $CD4^+$ T cells infected with HIV-1[63]. Here, we were able to confirm HERV-K Env protein expression in the human thymus and tonsil. It is worth mentioning that this antibody recognizes more HERV-K members than the primers used in our gene expression analysis, allowing for a broader analysis. HERV-K Env protein was detected in the thymic medulla, mirroring the higher *env* mRNA levels observed in

mature thymocytes. Interestingly, we couldn't find cells expressing HERV-K Env protein outside the medulla, which is not totally unexpected given the low levels of gene expression we noted during the early stages of T cell development, that mostly occur in the thymic cortex and cortico-medullary region. Moreover, the HERV-K-Env-expressing cells appear to be morphologically distinct. This likely reflects the fact that the thymic medulla is composed of smaller $CD4^+$ and $CD8^+$ thymocytes as well as larger medullary thymic epithelial cells (mTECs) and antigen presenting dendritic cells (mostly DCs)[76]. Overall, our data support a potential role for HERVs in T cell development, particularly in the negative selection process, which occurs in the medulla of the thymus and involves the interplay between mTECs and/or DCs and SP thymocytes.

HERV-K Env protein is also expressed in the tonsil, particularly in the lymphoid tissue-associated T cell zone. In this case however, it is more problematic to discriminate which cells are expressing the protein due to the existing diversity of T cell populations in this area. Nevertheless, it is possible to observe that morphologically distinct cells express the protein. In this regard, further studies are required to specifically identify the HERV-K Env-expressing cells, such as immunofluorescence or flow cytometry. We are currently optimizing both approaches.

It would be of interest to repeat the IHC studies using antibodies specific for each of the other HERV families. Altogether, our IHC results provide evidence for HERV-K Env protein expression in the thymus and tonsil, with these data strongly supporting those obtained via gene expression analysis.

Overall, the data we generated on HERV RNA and Protein expression profiles in human T cells provide interesting insights into the transcriptional and translational regulation of several HERV families. Each tissue analyzed featured distinct HERV *env* levels with the thymus featuring the lowest. We may speculate that, although HERVs appear to be more repressed in the thymus, they may still exert an effect, given their differential expression at different T cell developmental stages.

HERV-products may also have a direct impact on immune cells. Thus, Naito and co-workers showed that a synthetic peptide, isolated from a region of HERV clone 4-1, induced T-cell activation in PBMCs and promoted their production of IL-6 and IL-16[77]. Moreover, Rolland and colleagues showed that the HERV-W ENV-SU protein featured proinflammatory properties, being able to activate monocytes via TLR4 and CD14 pathways, and subsequently trigger the production of proinflammatory cytokines (IL-6)[78]. Furthermore, they also demonstrated that this protein activated DC, enabling them to trigger Th1-like responses[78].

It is also known that under physiological conditions HERVs remain dormant until they are activated. This activation might take place in different tissues at various developmental stages, which means they may be able to respond to a given external stimulus, such as

developmental cues[26]. Our data on HERV *env* expression in the thymus was the most interesting, as it featured a modulated expression pattern shared by all HERVs. Thus, we chose to study modulation of HERV expression in mature thymocytes stimulated with several cytokines, non-specific stimulators, such as mitogens, and TCR-mediated activators. Our findings revealed that TCR stimulation as well as PMA-Ionomycin decreased HERV-W, -P and -R transcriptional levels. Conversely, other studies have shown a general induction of HERV transcripts upon PMA stimulation, in peripheral T cells[79], primary macrophages and monocytes[80]. Using the Jurkat T cell line, Leib-Mösch and colleagues demonstrated that HERV-W, -K, -E and -H were upregulated in these cells, following stimulation with PMA/Ionomycin and CD3/CD28[81]. It is known that PMA activates the transcription factor NF-κB, whilst Ionomycin activates NFAT, via the induction of Calcium ion influx. As both transcription factors were reported to activate HERV transcription[81], we would expect to see an overall upregulation, however this was not the case. We may speculate that the observed differences may be due to tissue-specific effects; e.g. perhaps there are other HERV regulatory mechanisms specific to the thymus. Moreover, the majority of the existing data was generated with cells stimulated for three to four hours, whilst we analyzed CD4 SP thymocytes stimulated for 23 hours. Notably, Gelfand *et al* showed that HERV-H transcription peaked following eight hours of stimulation with PHA, and diminished when stimulated for more than 24 hours[79]. HERV transcriptional levels will be assessed in CD4SP thymocytes stimulated for 4 hours in order to define HERVs' expression kinetics.

Additionally, we observed that HERV-K and -W levels increased in some samples upon interferon (α, γ) stimulation. This is controversial, as some authors have shown an inverse relationship between HERV expression and IFN-inducible genes (type I interferon, IFN-β) up-regulation in the skin of patients with lichen planus[82], whilst others demonstrated that IFN-α induced human endogenous retrovirus HERV-K18 superantigen activity in juvenile rheumatoid arthritis[83].

A marked inter-individual variation in HERV *env* expression for each stimulation condition was observed. This might be due to HERV-specific regulation being impacted by the occurrence of specific polymorphisms. Furthermore, each of the stimuli could potentially act differently on the analyzed HERVs, as they are controlled by variable factors, specific to each individual. It would be of great interest to increase the number of samples in which we assessed HERV levels to confirm this variability.

We have emphasized that epigenetic silencing is one of the main regulators of HERV transcription. Nevertheless, during certain cellular processes or developmental stages transcriptional silencing can be shut down to allow the required expression of specific genes responsible for important cellular functions[70,71]. Thus, in these situations other intrinsic defense mechanisms may be activated to protect the host from foreign assaults. The relationship between HERVs and APOBEC3 proteins is well studied, however, many issues

remain unresolved. It has been very difficult to directly link ongoing HERV regulation to the activity of APOBEC3 proteins, as the majority of HERVs remain inactive, whilst the APOBEC3 proteins act on mobile replication-capable elements[36]. Nevertheless, the HERV elements that are still functional have been cloned in such a way to show, ex vivo, the effect of APOBEC3 proteins on their mobility[84]. Genetic analysis of HERV elements revealed the presence of clear footprints of human APOBEC3G activity[36]. When we analyzed the expression levels of both the HERVs and APOBEC3G or APOBEC3F in the thymus, blood and tonsil, we noted that HERVs appeared to be differentially affected by each restriction factor. To perform in vivo or in vitro functional assays we had to first understand the relationship between their expression profiles. APOBEC3G featured a significant correlation with each HERV analyzed. In contrast, APOBEC3F showed no correlation with the majority of HERV families. Although this data doesn't support a direct relation between LTR retrotransposons and host restriction factors, it provides information to further investigate their inter-relationship. It would be interesting to knock down either HERV or APOBEC3G genes in order to unravel whether HERVs may induce APOBEC3G expression, or whether APOBEC3G expression per se is controlling HERV transcription.

In conclusion, our data support a tight regulation of HERV expression in all T cell populations analyzed. This appears to be particularly pertinent in the thymus, with possible implications for the process of T cell selection.

The data we generated warrants further investigation into HERV expression and regulation. It will be crucial to broaden the knowledge of their tissue-specific activation and regulation as this could reveal unknown, but physiologically relevant functions. Our data will also complement some of the existing paradigms regarding HERV transcription under normal physiological conditions, as well as aid the understanding of their possible pathophysiological role.

6. References

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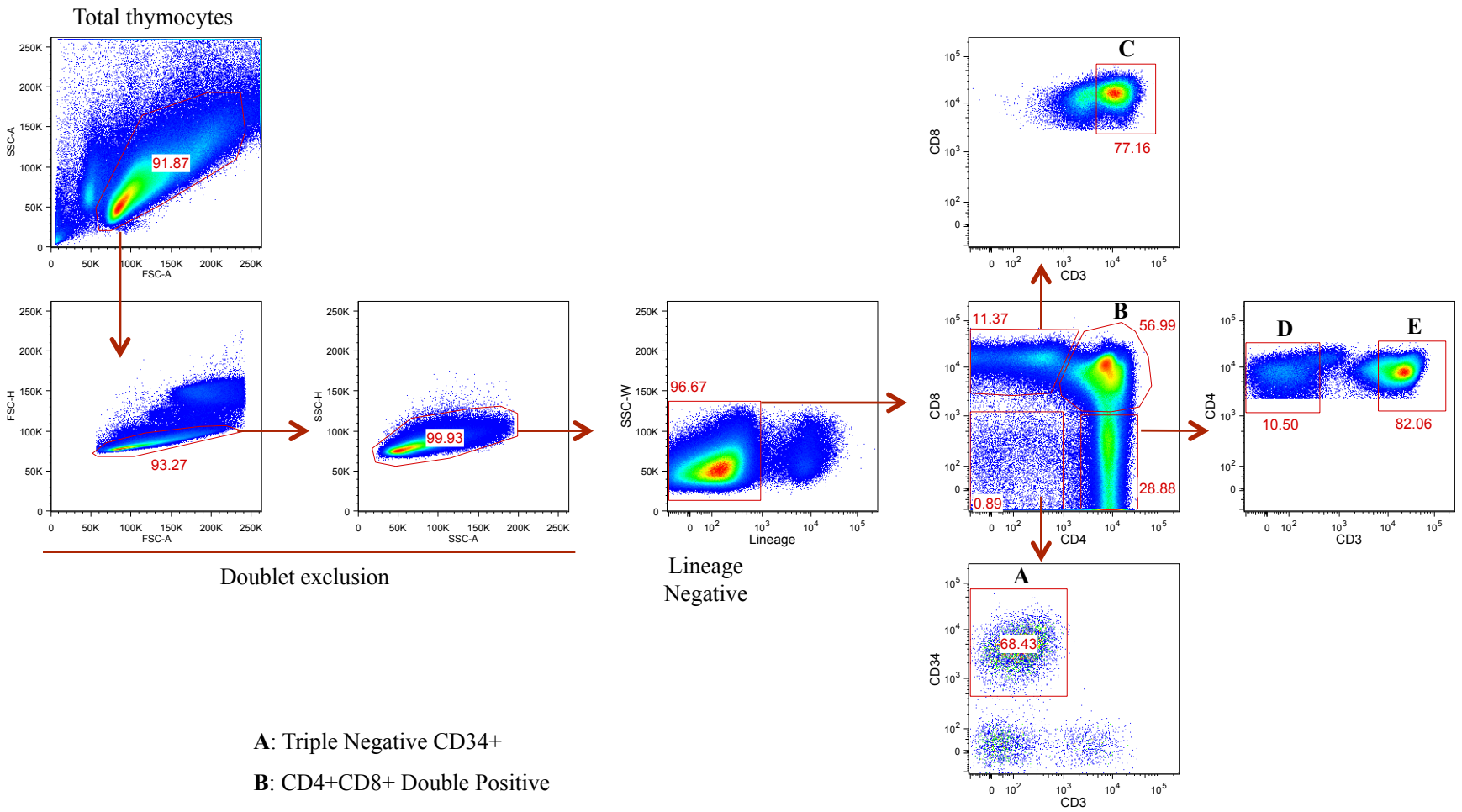
Table 1. List of antibodies used for T cell sorting.

	Sample type		
	Thymus	Blood	Tonsil
Number (n)	6	4	3
Age range	4 months - 5 years	22 - 47 years	5 - 12 years
Gender (Male/Female)	1/5	1/3	2/1

Table 2. List of antibodies used for T cell sorting.

Mouse Antibody	Clone	Fluorochrome	Supplier
CD3	UCHT1	APC	eBiosciences
	OKT3	PerCP-Cy5.5	eBiosciences
CD4	RPA-T4	PE Cy7	eBiosciences
CD8	RPA-T8	PerCP-Cy5.5	eBiosciences
	RPA-T8	PE	eBiosciences
CD34+	4H11	PE	eBiosciences
CD14	61D3	FITC	eBiosciences
CD16	CB16	FITC	eBiosciences
CD19	HIB19	FITC	eBiosciences
CD20	2H7	FITC	eBiosciences
CD56	MEM188	FITC	eBiosciences
CD123	6H6	FITC	eBiosciences
CD11c	3,9	FITC	eBiosciences
TCRγδ	B1.1	FITC	eBiosciences
CD45RA	HI100	APC	eBiosciences
CD45RO	UCHL1	FITC	eBiosciences
CCR7	150503	FITC	R&D
CXCR5	51505	APC	R&D
PD-1	MIH4	PE	eBiosciences
FVD*	NA*	eFluorTM 780	eBiosciences

FVD - Fixable Viability Dye (Live dead staining)
NA - not applicable



A: Triple Negative CD34+

B: CD4+CD8+ Double Positive

C: CD8+ Single Positive

D: CD4+ Immature Single Positive

E: CD4+ Single Positive

Figure 1: Gating strategy for sorting thymocyte subsets according to human T cell developmental stages.

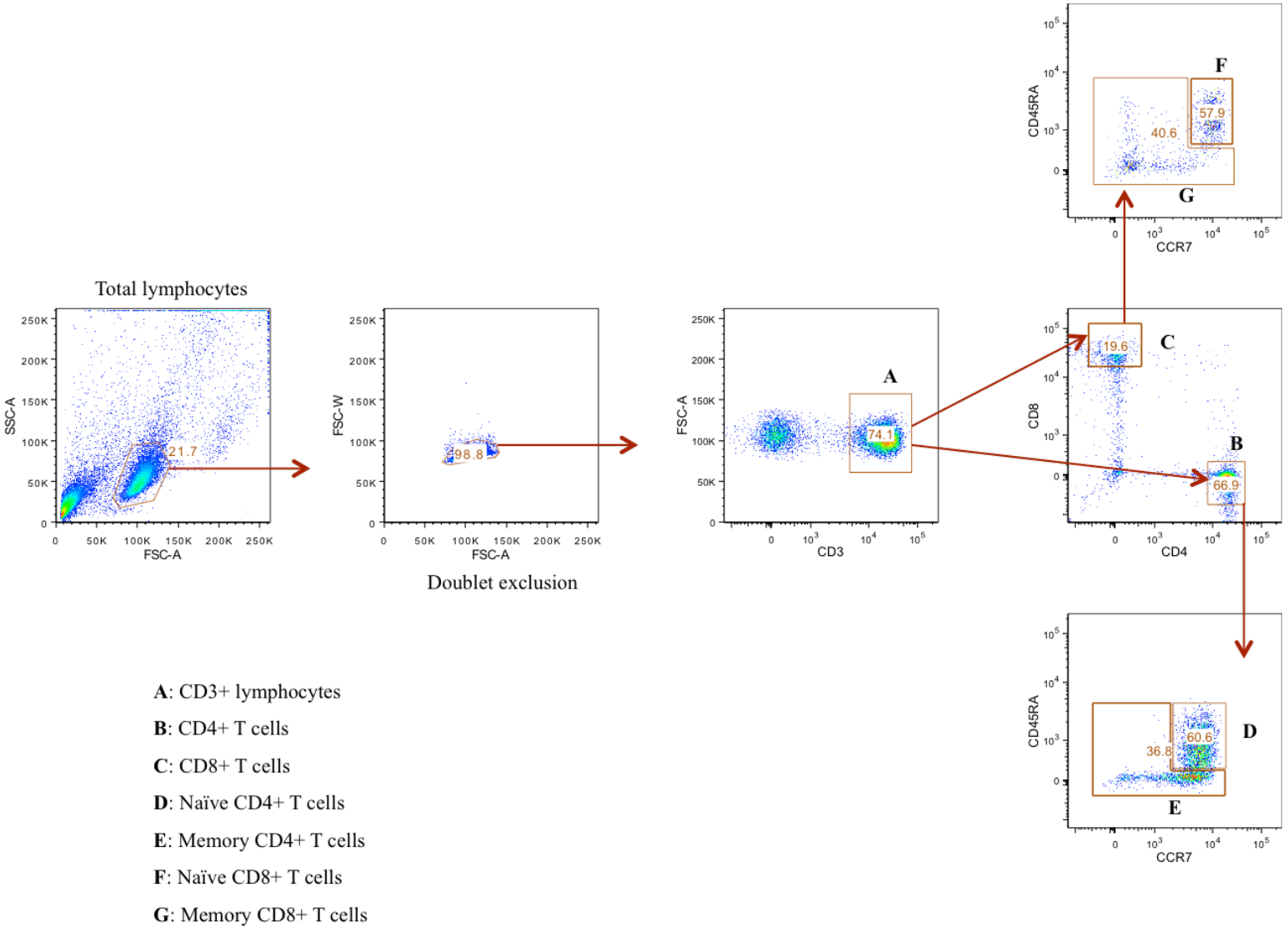
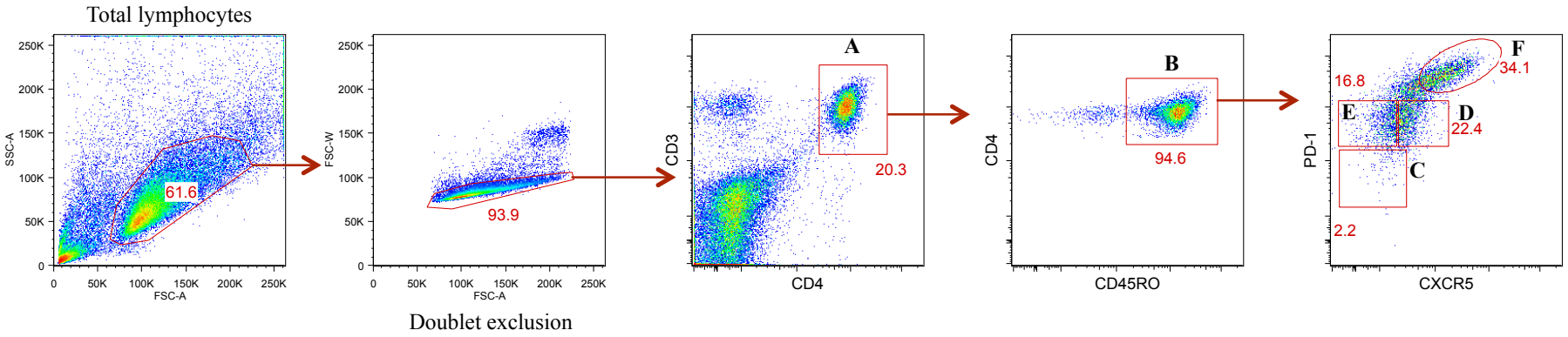


Figure 2: Gating strategy for sorting peripheral blood mononuclear cell subsets according to naïve and memory CD4 and CD8 T cells.



A: CD4+ T cells

B: Memory CD4+ T cells

C: CXCR5- PD-1-

D: CXCR5intermediate PD-1low

E: CXCR5- PD-1low

F: CXCR5bright PD-1bright

Figure 3: Gating strategy for sorting tonsillar mononuclear cell subsets according to memory CD4 T_{FH} differentiation stages.

Table 3: List of primers used for Real Time RT-PCR approach.

Primer Name	**Sequence (5'-3')**	Concentration (nM)
Forward HERV-K *env*	CACAACTAAAGAAGCTGACG	300
Reverse HERV-K *env*	CATAGGCCCAGTTGGTATAG	300
Forward HERV-W *env*	CGTTCCATGTCCCCATTTAG	300
Reverse HERV-W *env*	TCATATCTAAGCCCCGCAAC	300
Forward HERV-P *env*	CAAGATTGGGTCCCCTCAC	300
Reverse HERV-P *env*	CCTATGGGGTCTTTCCCTC	300
Forward HERV-R *env*	CATGGGAAGCAAGGGAACT	300
Reverse HERV-R *env*	CTTTCCCCAGCGAGCAATAC	300
Forward APOBEC3G	CCACATAAACACGGTTTCCTTGAAG	300
Reverse APOBEC3G	CTATGATGATCAAGGAAGATGTCAG	300
Forward APOBEC3F	ATACCGTCTGGCTGTGCTAC	300
Reverse APOBEC3F	ACATTTCTGCGTGGTGCTCA	300
Forward GAPDH	GGTGGTCTCCTCTGACTTCAACA	100
Reverse GAPDH	GTTGCTGTAGCCAAATTCGTTGT	100
Forward β-actin	CTGGCACCCAGCACAATG	300
Reverse β-actin	GCCGATCCACACGGAGTACT	300