UNIVERSITY OF QUEBEC AT MONTREAL

DEVELOPMENT OF THYMIC REGULATORY T CELLS DURING HUMAN IMMUNODEFICIENCY VIRUS (HIV) INFECTION

THESIS
SUBMITTED
AS PARTIAL REQUIREMENT
FOR MASTER'S IN BIOCHEMISTRY

BY

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JUNE 2019

UNIVERSITÉ DU QUÉBEC À MONTRÉAL

DÉVELOPPEMENT DE LYMPHOCYTES T RÉGULATEURS THYMIQUES PENDANT UNE INFECTION PAR LE VIRUS DE L'IMMUNODÉFICIENCE HUMAINE (VIH)

MÉMOIRE
PRÉSENTÉ
COMME EXIGENCE PARTIELLE
DE LA MAITRISE EN BIOCHIME

PAR

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JUIN 2019

ACKNOWLEDGEMENTS

I extend my gratitude to my supervisor Dr. Mohammad-Ali Jenabian for giving me the opportunity to work in his laboratory, that has helped me build a strong foundation for my research career. His outright criticism and insightful comments have made me learn how to design and conduct a project form scratch. He has taught me to become exceedingly self-reliant and self-confident. I am very thankful to Dr. Tatiana Scorza for agreeing to be my co-supervisor. My discussion sessions with her have always urged me think deeply about scientific problems, have enriched my critical thinking abilities and have rekindled my passion for science. With her humor and wit, she has been my pillar of support during many stressful and challenging situations. I thank Dr. Jonathan Angel and Dr. Gyaandeo Maharajh, for providing us with the precious thymus specimens. I am immensely thankful to the donors and their kind parents for their huge donation to the field of research. I acknowledge the support offered by our collaborators Dr. Cecilia T. Costiniuk, Dr. Petronela Ancuta, Dr. Nicolas Chomont and their teams. I thank Mr. Denis Flipo for helping me with cell sorting. I also appreciate the service offered by the members of IRIC and Genome Quebec for the methylation sequencing. I extend my gratitude to Dr. Omar Farnos, the former research associate in our lab who started working on this project before me and taught me how to work on the thymus specimens. This Master's journey would not have been possible without the physical, moral and intellectual support of my dearest labmates Alexis Yero Diaz, Tao Shi, Syim Salahuddin, Celine Rothan, Pierre Gantner, Zehira Abdelhamid, Elaine Thomson and Oussama Meziane. I thank Dr. Francois Ouellet for helping me cope with many student problems. I thank the Faculty of Sciences, UQAM, for the scholarship awards that provided me with necessary financial support. I thank UQAM for providing me with

this wonderful opportunity to kick-start my research career. I am forever grateful to my family and friends for taking care of me all the time.

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LIST OF ABBREVIATIONS

ADP	Adenosine diphosphate
AIDS	Acquired immunodeficiency syndrome
AIRE	Autoimmune regulator gene
AMP	Adenosine monophosphate
AP-1	Activator protein 1
APC	Antigen presenting cell
ART	Antiretroviral therapy
ATP	Adenosine triphosphate
BMP	Bone-morphogenetic protein
CNS	Conserved non-coding sequences
DC	Dendritic Cell
DN	Double negative
DNMT	DNA methyl-tranferase
DP	Double positive
EAE	Experimental autoimmune encephalomyelitis

ETP	Early T cell Progenitor
GALT	Gut-associated lymphoid tissue
GITR	Glucocorticoid-induced tumour necrosis factor TNF receptor
HAART	Highly active antiretroviral therapy
HCV	Hepatitis C virus
HIV	Human immunodeficiency
HSC	Hematopoietic stem cells
IDO	Indoleamine-2, 3-dioxygenase
IFN-γ	Interferon-γ
IL-10	Interleukin-10
IL-17	Interleukin-17
IL-2	Interleukin-2
IL-21	Interleukin-21
IL-22	Interleukin-22
IL-2R	Interleukin-2 receptor
IL-35	Interleukin-35
IL-6	Interleukin-6

IL-7	Interleukin-7
iTreg	Induced Treg
LAP	Latency-associated peptide
LTR	Long terminal repeat
MHC	Major histocompatibility complex
mTEC	Medullary thymic epithelial cell
Nrp1	Neuropilin-1
NFAT	Nuclear factor of activated T cells
NI	Non-infected
NICD	Notch intracellular domain
PBMC	Peripheral blood mononuclear cells
PD-1	Programmed death-1
PD-L1	Programmed death ligand-1
PFD	Pirfenidone
pTreg	Peripheral Treg
RTE	Recent thymic emigrants
SDF-1	Stromal cell-derived factor-1

SIV	Simian immunodeficiency virus
SMAD	Sma and Mad -Mothers against decapentaplegic
SP	Single positive
STAT-5	Signal transducer and activator of transcription 5
TCR	T cell receptor
TGF-β	Transforming growth factor-β
Th1	T helper type 1
Th17	T helper type 17
TLR-4	Toll-like receptor-4
TNF	Tumour necrosis factor
TREC	T cell Receptor Excision Circles
Tregs	Regulatory T cells
TSS	Transcription start site
TSDR	Treg specific demethylated region
tTreg	Thymic Tregs

RÉSUMÉ

Contexte: Les lymphocytes T régulateurs (Treg) sont des cellules immunosuppressives ayant deux origines développementales: thymiques ou périphérique. FoxP3, le facteur de transcription principal des Tregs, contrôle leur différenciation et leurs fonctions suppressives. L'expression de FoxP3 par les cellules Tregs thymiques est en partie régulée par la cytokine anti-inflammatoire TGF-β, qui contribue également au développement des Tregs dans le sang périphérique et les tissus lymphoïdes. Les personnes vivant avec une infection chronique par le virus de l'immunodéficience humaine (VIH) ont une fréquence plus élevée de Tregs dans le sang périphérique et les tissus lymphoïdes. La fibrose des tissus lymphoïdes induite par le TGF-β chez les individus infectés par le VIH est associée à la progression de la maladie. Malgré la corrélation entre TGF-β et la pathologie associée au VIH, le rôle du TGF-β dans l'induction et le maintien des Tregs dans le thymus pendant l'infection par le VIH demeure incertain.

Hypothèse: Le TGF-β induit l'expression de FoxP3 et contribue ainsi à l'augmentation de la fréquence des Tregs thymiques au cours de l'infection par le VIH.

Objectif: Étudier les modifications sur l'expression de FoxP3 et la génération de Treg thymiques après traitement par le TGF-β lors d'une infection *in vitro* de thymocytes humains par le VIH-1.

Méthodes: Des tissus thymiques humains frais ont été obtenus chez des patients pédiatriques subissant des chirurgies cardiaques correctives. La caractérisation phénotypique des thymocytes a été réalisée *ex vivo* par cytométrie en flux. L'évaluation ex vivo du statut épigénétique de foxp3 a été réalisée à l'aide de MiSeq. L'infection virale par les souches VIH-1 tropiques R5 et X4, ainsi que le traitement par le TGF-β de thymocytes humains ont été réalisés dans un modèle de co-culture *in vitro* avec des lignées cellules OP9-DL1 exprimant le ligand de Notch.

Résultats: La caractérisation phénotypique *ex vivo* des thymocytes a révélé les faits suivants: 1) Quatre-vingt-dix-neuf % des thymocytes CD4+ FoxP3+ expriment Helios, un marqueur controversé des Tregs thymiques. 2) Le facteur de transcription maître des cellules Th17, RORγt, n'ést pas co-exprimé avec FoxP3 dans les cellules T CD4 thymiques, ce qui indique que l'expression dans le thymus est associée à la maturation

des thymocytes plutôt qu'à la différenciation de Th17. 3) Plus de 51% des Treg et des CD4+ FoxP3- non-Tregs dans le thymus expriment CD45RO, indiquant que ces cellules pourraient avoir reçu une forte stimulation du TCR par des auto-antigènes et ont probablement subi une sélection négative. 4) Environ 3% des Treg exprimaient le marqueur inflammatoire CCR6 et 2% les marqueurs de référence intestinaux Integrin-β7 et CCR9, suggérant leur repositionnement dans le thymus. 5) Plus de 87% des Tregs expriment le corécepteur du VIH CXCR4, et les Tregs expriment un niveau plus élevé de CCR5 (18,53%) par rapport aux non-Tregs (1,01%), ce qui suggère que les Tregs sont potentiellement plus susceptibles à l'infection par le VIH. 6) Les thymocytes $CD3^{haut}$ ont la plus haute expression de FoxP3 et une déméthylation de la région CNS1 régulant foxp3. Comme prévu, le traitement des thymocytes par le TGF-β induit l'expression de CD127 et entraîne une augmentation des fréquences de Tregs. L'infection des thymocytes par les souches VIH tropiques R5 et X4 a montré que les thymocytes FoxP3+ sont moins susceptibles à l'infection que les thymocytes FoxP3-. Le traitement par le TGF-β n'a eu aucun effet sur le taux d'infection par le VIH. Lors de l'infection par le VIH et du traitement par le TGF-β, aucune augmentation de l'expression de FoxP3 ou des fréquences de Tregs n'a été observée.

Conclusions : La caractérisation phénotypique *ex vivo* des Tregs thymiques a fourni un aperçu complet des propriétés des Tregs thymiques. L'infection *in vitro* par le VIH et le traitement par le TGF-β de thymocytes humains n'ont pas d'impact sur l'expression de FoxP3 et les fréquences de Tregs.

Mots-clés : Tregs, caractérisation phénotypique, TGF-β, l'infection par le VIH, statut épigénétique de *foxp3*

ABSTRACT

Background: Regulatory T cells (Tregs) are immunosuppressive cells with two developmental origins: thymic or peripheral. FoxP3, the master transcription factor of Tregs, controls their differentiation and suppressive functions. FoxP3 expression by thymic Tregs is partly regulated by the anti-inflammatory cytokine TGF-β, which also contributes to Treg development in the peripheral blood and lymphoid tissues. People living with chronic human immunodeficiency virus (HIV) infection have increased Treg frequencies in peripheral blood and lymphoid tissues. TGF-β-mediated fibrosis of lymphoid tissues in HIV infected individuals is associated with disease progression. However, the role of TGF-β in the induction and maintenance of Tregs within the thymus during HIV infection remains unclear.
Hypothesis: TGF-β induces FoxP3 expression and thus, contributes to increased thymic Treg frequencies during HIV infection.

Objectives: To investigate changes in FoxP3 expression and thymic Treg generation upon TGF-β treatment during *in vitro* HIV infection of human thymocytes.

Methods: Fresh human thymic tissues were obtained from pediatric patients undergoing corrective cardiac surgeries. *Ex vivo* phenotypic characterization of thymocytes was performed by flow cytometry. *Ex vivo* assessment of *foxp3* epigenetic status was performed using MiSeq. HIV infection by both R5- and X4-tropic HIV-1 strains and TGF-β treatment of human thymocytes was performed in an *in vitro* co-culture model with OP9-DL1 cells expressing Notch ligand.

Results: *Ex vivo* phenotypic characterization of thymocytes revealed the following: 1) Ninety nine percent of CD4+ FoxP3+ thymocytes expressed Helios, a debated thymic Treg marker. 2) The master transcription factor of Th17 cells, RORγt, was not co-expressed with FoxP3 in the thymic CD4 T cells, indicating that RORγt expression by thymocytes is rather associated with their overall maturation than Th17 differentiation. 3) More than 51% of Tregs and CD4+ FoxP3- non-Tregs within thymus expressed CD45RO, which indicated that the cells might have received strong TCR stimulation by self-antigens and might be undergoing negative selection. 4) Only about 3% of Tregs expressed the inflammatory marker CCR6 and 2% expressed gut homing markers Integrin-β7 and CCR9, suggesting their re-localization into the thymus. 5) More than 87% of Tregs expressed the HIV co-receptor CXCR4 and Tregs expressed higher CCR5 (18.53%) compared to Non-Tregs (1.01%), suggesting that Tregs are

more susceptible to HIV infection. 6) $CD3^{high}$ thymocytes had highest FoxP3 expression and demethylation of the CNS1 region regulating *foxp3*. As expected, TGF-β treatment of thymocytes induced CD127 expression and resulted in increased Treg frequencies. Infection of thymocytes with R5 and X4-tropic HIV strains showed that FoxP3+ thymocytes were less prone to infection compared to FoxP3- thymocytes. TGF-β treatment had no effect on the rate of HIV infection. Upon HIV infection and TGF-β treatment, an increase in FoxP3 expression or Treg frequencies were not observed.

Conclusions: The *ex vivo* phenotypic characterization provided a detalied overview of thymic Treg properties. The *in vitro* HIV infection and TGF-β treatment of human thymocytes does not have an impact on FoxP3 expression and Treg frequencies.

Key words: Tregs, phenotypic characterization, TGF-β, HIV infection, *foxp3* epigenetic status

CHAPTER I

INTRODUCTION

1.1 T cell development in the thymus

1.1.1 Structure, organization and functions of the thymus

The thymus is a primary lymphoid organ of the immune system, and is morphologically similar in most mammals. It plays a central role in the immune system, as it is the site for maturation of T lymphocytes. The indispensable role of the thymus in establishing the immune system was demonstrated six decades ago: neonatal thymectomy made mice more prone to infections, and these animals developed primitive secondary lymphoid tissues and exhibited very poor immune responses (Miller, 2002). In addition to its immunological role, the thymus also functions as a glandular tissue and produces several hormones including thymosin alpha-1, thymulin and thymopoietin (Hadden, 1998), which regulate both developing thymocytes and mature T cells.

The thymus is located in the thoracic cavity above the heart. It has two lobes, both covered with a capsule of epithelial tissue and held together by connective tissue. Each thymic lobe has an outer cortex and inner medulla. Thymic epithelial cells, macrophages and dendritic cells make the framework of the thymic cortex and medulla. These cells closely interact with developing thymocytes, thereby contributing to their

maturation. Thymic nurse cells have long membrane extensions, which enable efficient interaction with multiple thymocytes, forming multi-cellular complexes (Kindt, 2007). The Hassall's corpuscles are an additional histological identity of the thymus; these are clusters of thymic epithelial cells, mainly involved in the phagocytosis of apoptotic thymocytes (Blau, 1965) or in sustaining their development (Senelar *et al.*, 1976). Hassall's corpuscles also contribute to thymic development of Tregs (Watanabe *et al.*, 2005). Thymic stromal lymphopoietin (TSLP) produced by Hassall's corpuscles stimulate the expression of MHC-II, CD80 and CD86, thereby promoting the interactions between dendritic cells (DCs) and developing CD4 T cells, directing the differentiation of the latter to produce CD4+ CD25+ FoxP3+ Tregs (Watanabe *et al.*, 2005).

Aging causes atrophy and reduces the immune functions of the thymus. In humans, thymic atrophy begins after puberty. Age-associated thymic involution is characterized by significant changes in the thymic microenvironment, decrease in number of thymocytes and increase in the fat content of the tissue. These factors result in a net reduction in the weight of the thymus tissue (Kindt, 2007).

1.1.2 Hematopoiesis and lymphopoiesis

T cells are newly generated throughout the lifetime of an adult to replenish the T cell repertoire in various secondary lymphoid tissues. This is possible because the adult thymus constantly receives thymus-allotted progenitor cells from the bone marrow (Donskoy et Goldschneider, 1992) . Blood cells of all types arise from hematopoietic

stem cells (HSCs) in the bone marrow, in a process termed hematopoiesis. HSCs give rise to two fundamental cell lineages: the myeloid lineage (erythrocytes or red blood cells, megakaryocytes, granulocytes: eosinophils, basophils, neutrophils, and monocytes or macrophages) and the lymphoid lineage (T cell, B cells and NK cells). To develop into a T lymphocyte, a lymphoid progenitor must exit the bone marrow, enter circulation and migrate to thymus (Petrie, 2003). There are several factors influencing the recruitment and migration of HSCs into the thymus. Different sets of markers are used to identify the migrating precursor cells to the fetal and adult thymus. HSCs with migratory potential lack cell adhesion molecules like intergrins α4β1 (VLA-4) or α5β1 (Whetton et Graham, 1999) and express low levels of CXCR4, which is the receptor for stromal cell-derived factor 1 (SDF-1) required to home and be retained in the bone marrow (Whetton et Graham, 1999). These precursors cells exit circulation and settle into the thymus by expressing CD44 (Wu *et al.*, 1993) and L-selectin (CD62L) (Perry *et al.*, 2004). Once in the thymus, these settling precursor cells are called Early T cell progenitors (ETPs), and lack the capacity of self-renewal.

1.1.3 Importance of Notch signaling in the thymus

Thymic development of T cells from ETPs involves complex signaling mechanisms. Two decades ago, severe impairment of thymocyte development was reported in mice lacking Notch-1 signaling (Radtke *et al.*, 1999). Notch is a transmembrane receptor and its ligands are also transmembrane proteins of the Delta/Serrate/LAG-2 (DSL) family. Ligand binding stimulates Notch, following which the Notch intracellular domain (NICD) fragment enters the nucleus and activates gene transcription. T cell progenitors utilize Notch signaling after entering the thymic epithelial microenvironment (Harman *et al.*, 2003). Notch signaling is essential for T cell lineage

commitment, and T cell progenitors in the thymus of Notch-deficient mice differentiated into B cells (Wilson *et al.*, 2001). Notch-1 signalling is also important to prevent ETPs from developing into other cell types like NK-cells, monocytes, or DCs (De Smedt *et al.*, 2005). Experimentally, it was reported that upon interaction with a stromal cell line ectopically expressing Delta-like-1, HSCs develop into CD4+ CD8+ (double positive, DP) and single positive (SP) T cells *in vitro* (Schmitt, T. M. et Zuniga-Pflucker, 2002). Thus, Notch signalling is essential for thymic T cell development.

1.1.4 T cell receptor rearrangement

As for the immunoglobulin receptor on B cells, T cell receptor (TCR) genes also undergo somatic DNA rearrangement in order to generate a diverse repertoire of receptors, assuring recognition of a wide variety of antigens. ETPs entering the thymus do not express TCRs, and have germline TCR genes since they have not yet started the process of TCR gene rearrangement. These cells do not express CD3 or CD4 or CD8, and hence are called triple negative (CD3- CD4- CD8-) cells. The existence of a small proportion of immature $CD4^{low}$ cells, which are CD3-, has been reported, and it has been speculated that these cells later become triple negative cells (Malissen *et al.*, 1999). Triple negative thymocytes do not express either CD4 or CD8 (called double negative, DN cells) are further subdivided into four types based on CD44 and CD25 (IL-2Rα) expression: DN1 (CD44+ CD25−), DN2 (CD44+ CD25+), DN3 (CD44− CD25+) and DN4 (CD44−CD25−) (Godfrey *et al.*, 1993); the numbering corresponds to the stage of maturation. DN2 cells possess a germline TCR gene, while DN4 thymocytes have a rearranged TCR gene (Godfrey *et al.*, 1993).

Three regions in the TCR loci, namely, variable (V), diversity (D) and joining (J) regions are subject to recombination, and therefore, the TCR gene rearrangement is also referred as the VDJ recombination process, which begins in the DN3 stage of thymocyte maturation. T cells can either express an αβ-TCR or a γδ-TCR, depending on which genes (Tcrb, Tcrg, Tcrd) are first successfully recombined. We will be focusing here only on the development of αβ T cells. This somatic DNA rearrangement process, shown in Figure 1.1, ensures the generation a diverse repertoire of T cells, which can recognize a vast variety of antigens.

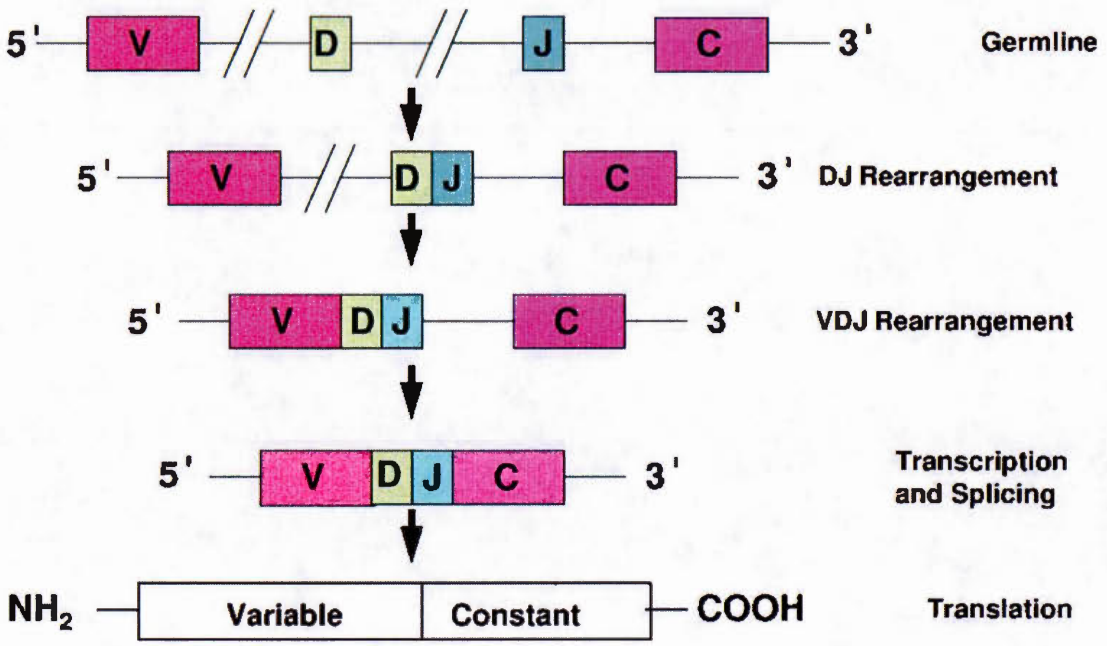

Figure 1.1: Somatic DNA rearrangement (VDJ recombination) process in TCR genes, (Rezuke *et al.*, 1997). The genes encoding the TCRs and immunoglobulin receptors on B cells undergo DNA rearrangement thereby ensuring production of a diversity of receptors, which will enable recognition of a wide variety of antigens.

A crucial step in αβ T cell development is the β-selection checkpoint. Thymocytes at the DN3 stage, which successfully complete TCRβ chain rearrangement, are provided with survival and proliferation signals, which enable them to proliferate and further differentiate. This step also signifies commitment to the αβ T cell phenotype. The rearrangement of Tcrα gene locus follows the β-selection process, resulting in a cell that expresses an αβ-TCR. Signaling through the αβ-TCR causes upregulation of the co-receptors CD4 and CD8 and the developing thymocytes enter the DP stage.

Two important regulatory steps in thymic T cell development are positive and negative selection. Positive selection is the process by which only thymocytes expressing αβ-TCR that can recognize self-antigens presented along with MHC-I/II are selected; thymocytes which have not successfully rearranged their TCR loci are eliminated by apoptosis. DP thymocytes become either CD4SP if they interact with self-peptides presented by MHC-II molecules, or CD8SP if they interact with self-peptides presented by MHC-I molecules. Multitudes of factors influence CD4 or CD8 lineage commitment, some of them being addressed in the following section. The affinity with which TCRs recognize self-peptide-MHC complexes determine whether a T cell is eliminated or selected for survival. Negative selection is the process that discriminates highly self-reactive T cells and prevents autoimmunity by triggering their deletion (Spits, 2002).

1.1.5 Exisiting models for CD4 T cell lineage commitment

The differentiation of DP thymocytes into CD4SP or CD8SP T cells depends on which one of the two co-receptors is downregulated and transcriptionally silenced. Two theoretical models, the instructive and the stochastic models, attempt to explain the CD4-CD8 lineage commitment process in the thymus (Germain, 2002). According to the instructive model, interaction of TCRs with MHC-I generates a downstream signal which is biochemically different from the signals generated upon interaction of TCRs with MHC-II, the former leading to CD8 differentiation and the latter leading to CD4 differentiation. This model is in line with the proposition that TCR affinity for self-peptides is one of the major factors influencing thymic T cell development. Negative selection, an important self-tolerance mechanism that eliminates thymocytes with high-affinity TCRs, is also in line with the instructive model.

The stochastic model proposes that the DP thymocytes randomly express either one of the two receptors, which means that a DP thymocyte may initiate the expression of a co-receptor that is unrelated to the interaction of its TCR with either MHC-I or MHC-II. In such cases, if a mismatch between the MHC specificity of a clonal TCR and its co-receptor occurs, the developing thymocyte will not undergo the maturation process and will become apoptotic. Studies in mice validating both models have been conducted, and it was concluded that a combination of both models provides a complete and precise explanation for thymocyte development (Germain, 2002). A slight modification of the instruction model is the "duration of signal" model of CD4-CD8 lineage commitment, which proposes that the TCR signalling of longer duration results in down regulation of CD8 and favors CD4 differentiation, while TCR signals of shorter duration result in CD8 differentiation and downregulation of CD4 gene

expression (Singer *et al.*, 2008). Signal durations were studied by changing the period for which TCR could engage with the ligand, i.e., by altering the TCR stimulation period during *in vitro* cultures. It was later demonstrated that the TCR signaling in all DP thymocytes is of the same duration and it brings about downregulation of CD8 expression. However, TCR signaling through interaction with MHC-I in CD4+ $CD8^{low}$ thymocytes is of shorter duration than TCR signalling through interaction with MHC-II (Singer, 2002).

The latest, accurate and most widely accepted model of CD4/CD8 lineage differentiation is the kinetic model, which is grounded on crucial experimental evidences, which have defied all of the proposed classical theoretical models. Experimental observations showed that strong TCR signalling in DP thymocytes resulted in downregulation of CD8, leaving CD4 expression unaltered, and the resulting CD4+ $CD8^{low}$ cells remained uncommitted. Thus, the concept that CD4/CD8 lineage commitment is defined by the downregulation of either one of the co-receptors is incorrect. Taking into account all the mentioned factors concerning the duration of TCR signals, the kinetic signaling model proposes that CD4/CD8 lineage commitment occurs not in DP thymocytes but in CD4+ $CD8^{low}$ thymocytes, which represent an intermediate developmental stage after the DP stage. These cells have the potential to differentiate into either CD4SP or CD8SP T cells. If TCR signals persist for a longer duration in CD4+ $CD8^{low}$ thymocytes, they become CD4SP. If the signals last for a shorter duration, cells become CD8SP. The uniqueness of the kinetic signalling model is the explanation provided for CD8 lineage commitment – CD8 differentiation occurs when CD4+ $CD8^{low}$ thymocytes do not receive persistent TCR signal and hence reverse their co receptor expression (that is, they become CD8+ CD4-). More specifically, absence of long duration TCR signals are sensed through γc cytokines like IL-7, crucial for CD8 lineage commitment, which reverse co-receptor expression from CD4+

$CD8^{low}$ to CD4- CD8+ (Singer *et al.*, 2008). The CD4/CD8 lineage choice is also regulated at the transcriptional and epigenetic levels. The gene expression of murine CD4 co-receptor is regulated by two important cis regulatory elements : a transcriptional enhancer and a transcriptional silencer. The transcriptional enhancer is called the cd4 proximal enhancer or E4p is a 300bp sequence located 13kb upstream of the transcription start site (TSS) and has been reported to be active in thymocytes in DN2 or DN3 stages of development (Sawada et Littman, 1991). The transcriptional silencer called S4, is located 1.6kbp downstream of the transcription start site and causes CD4 downregulation in CD8+ T cells (Sawada *et al.*, 1994).

1.2 Regulatory T cells

1.2.1 Phenotype, functions and classification of Tregs

Studies about immune tolerance mechanisms started in the early 1970's (Gershon et Kondo, 1970), but until late in the 1990's, the existence of immunosuppressive cells was much under debate (Green et Webb, 1993). Tregs were first described in 1995 by Sakaguchi *et al.* (Sakaguchi *et al.*, 1995) as immunosuppressive cells, which limit the activity of other types of T cells. The normal physiological role of Tregs is to prevent autoimmunity (Kim, J. M. *et al.*, 2007; Seddon, 2000) and maintain immune homeostasis. The transcription factor FoxP3 is considered the "master regulator" of Tregs since it is required for the expression of several genes responsible for the differentiation and maintenance of the Treg phenotype (CD3+ CD4+ $CD25^{high}$ $CD127^{low}$ $FoxP3^{high}$) (FontenotRasmussen, *et al.*, 2005). From 2000 to 2015, 30,000 research articles referring to Tregs have been published (Zongyi *et al.*, 2016), and the subject continues to be a hotspot in immunological research.

The existence of CD4+ T cells expressing IL-2R (CD25) responsible for immune suppression was first demonstrated in mice (Sakaguchi *et al.*, 1995) and later in humans (Baecher-Allan *et al.*, 2001). Initially, the regulatory phenotype was identified as CD4+ CD25+ T cells. Later, the role of FoxP3 in human Tregs was demonstrated (Yagi *et al.*, 2004), and the low expression of IL-7 receptor (CD127) was shown to be another useful parameter to discriminate Tregs (Liu *et al.*, 2006). Hence, a more complete way to define Tregs phenotypically is CD3+ CD4+ $CD25^{high}$ $CD127^{low}$ $FoxP3^{high}$ T cells (Jenabian *et al.*, 2012). Tregs can be classified based on their origin as follows: 1) natural or thymic Tregs (tTregs) which originate within the thymus; 2) peripheral Tregs (pTregs) which are produced in the peripheral blood, i.e., are generated *in vivo* from CD4+ cells that differentiate into Tregs as they start expressing FoxP3 under inflammatory conditions (Curotto de Lafaille et Lafaille, 2009; Curotto de Lafaille *et al.*, 2004). A third category of immunosuppressive cells are induced Tregs (iTregs), which are generated *in vitro* from CD4+ T cells, which start expressing FoxP3 when stimulated with TGF-β (Chen, W. *et al.*, 2003). However, the expression of FoxP3 alone, either induced by TGF-β or expressed exogenously using transduction methods is insufficient to produce functionally active Tregs exhibiting suppressive capacity (Feuerer *et al.*, 2010; Hill *et al.*, 2007; Yadav *et al.*, 2013). Hence, iTregs are not analogous of pTregs (Yadav *et al.*, 2013) but these names are used interchangeably in articles by different authors. A simplified nomenclature for Tregs has been suggested, which is to use tTregs instead of "natural Tregs"; pTregs to refer to peripherally derived Tregs and iTregs to refer to *in vitro* induced Tregs (Abbas *et al.*, 2013). CD8+ cells expressing FoxP3, hence called CD8 Tregs are also known to originate within the thymus and in the peripheral blood. Although these cells have still not been clearly characterized, they are known to execute suppressive functions on non-Tregs, and secrete immunosuppressive cytokines like IL-10 and TGF- β, similar to CD4+ Tregs (Yu *et al.*, 2018).

1.2.2 Mechanisms of Treg mediated immune suppression

Tregs can execute their suppressive function only after being activated through their TCR (Takahashi *et al.*, 1998; Thornton, A. M. et Shevach, 1998), which is triggered upon binding to a specific antigenic peptide. Sustenance of TCR signal is vital for the maintenance of Treg function (Hoeppli *et al.*, 2016; Levine *et al.*, 2014; Vahl *et al.*, 2014). As CD4+ T cells, Tregs are also antigen-specific, since they contain a functional TCR resulting from somatic rearrangement of gene segments (Corthay, 2009). Based on their antigen specificity, Tregs can be classified as either self-antigen-specific Tregs, which help in preventing autoimmunity and maintain immune homeostasis or foreign-antigen-specific Tregs, such as those recognizing bacterial antigens (McGuirk *et al.*, 2002) or viral antigens (Zhao *et al.*, 2014) and thereby controlling immune response to these pathogens. Several studies have tried to demonstrate the utility of "engineered" Tregs (autologous cells modified *in vitro* according to treatment needs) in immunotherapy approaches for the treatment of cancers (Geldres *et al.*, 2016), autoimmune disorders (Stephens *et al.*, 2009) and in overcoming graft rejections (Brennan *et al.*, 2011).

Tregs exert their immunosuppressive effects on effector T cells through several mechanisms. Figure 1.2 summarizes the different ways in which activated Tregs exert their immunosuppressive functions:

1. Anti-inflammatory cytokines secreted by Tregs such as IL-10 (Vieira *et al.*, 2004), IL-35 (Castellani *et al.*, 2010) and TGF-β (Weiner, 2001) inhibit the proliferation of effector cells.

2. Tregs play a negative role in cancer by repressing anti-tumor immune responses via the granzyme-perforin pathway (Cao *et al.*, 2007). Tregs also control inflammation in

response to viral infection (Loebbermann *et al.*, 2012) using the same pathway. Granzymes are serine proteases while perforins are cell membrane toxins, which are often employed by NK cells and cytotoxic T cells, and are released by exocytosis to trigger apoptosis in target cells (Trapani et Smyth, 2002).

3. Tregs utilize two enzymes CD39 (ectonucleoside triphosphate diphosphorylase-1) and CD73 (ecto-5'-nucleotidase), which break down ATP into ADP, and subsequently, ADP into AMP or adenosine (Borsellino *et al.*, 2007). Adenosine binds to A2A receptors on other immune cells, is converted into cAMP and induces anti-inflammatory effects – it restricts proliferation and cytokine production (Ohta *et al.*, 2009; Ohta et Sitkovsky, 2001; Sitkovsky *et al.*, 2004).

4. Tregs can transport cAMP to effector T cells through gap junctions, thereby inhibiting their proliferation and IL-2 production and preventing HIV replication in the infected T cell (Moreno-Fernandez *et al.*, 2011).

5. DCs are antigen presenting cells that express the co-receptors CD80 and CD86 which interact with CD28/CTLA-4 on T cells (McLellan *et al.*, 1995). Tregs decrease expression of CD80/CD86 on DCs (Wing *et al.*, 2008), via CLTA-4, which leads to downregulation of IDO (indoleamine-2, 3-dioxygenase), an enzyme that converts tryptophan to kynurenine (Hryniewicz *et al.*, 2006).

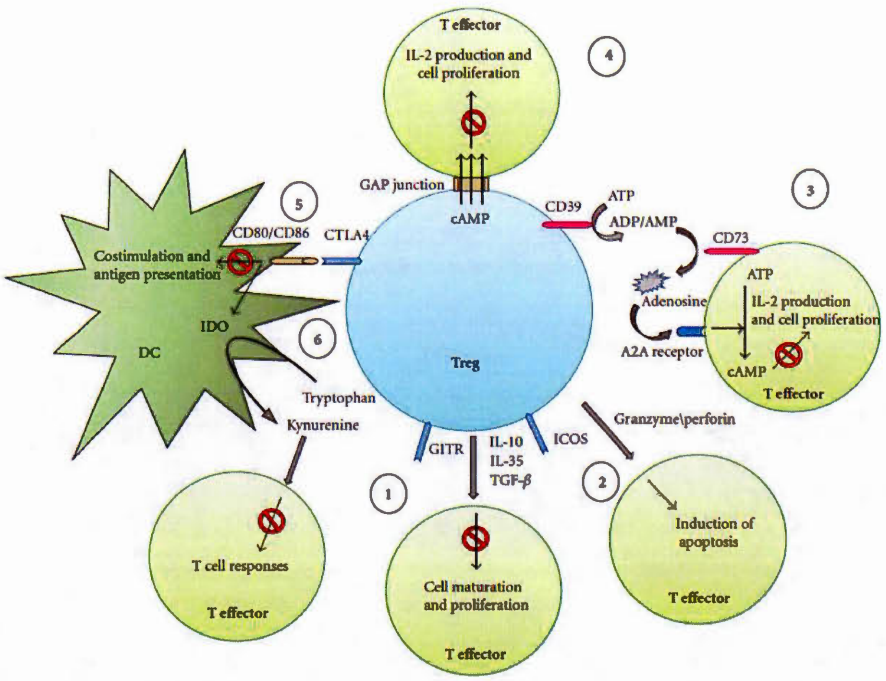

Figure 1.2: Mechanisms of Treg-mediated immune suppression – 1) Cytokine-mediated suppression, 2) Granzyme B-mediated suppression 3) CD39 and CD73-mediated suppression 4) cAMP transport through gap-junctions 5) & 6) DC-mediated suppression (Jenabian *et al.*, 2012).

1.2.3 Models of thymic Treg development

Development of thymic Tregs occurs in two steps: survival of thymocytes bearing TCR with relatively high affinity to self-antigens and cytokine-dependent induction and maintenance of FoxP3 expression (Lio et Hsieh, 2008).

Treg precursors are autoreactive CD4+ CD25+ thymocytes, which have TCRs that bind self-antigens with high affinity (Jordan *et al.*, 2001). Developing thymocytes bearing TCRs with high affinity to self-antigens likely undergo negative selection, the process by which central tolerance is assured in the thymus. Therefore, for Tregs to develop in the thymus, certain autoreactive CD4+ CD25+ thymocytes must escape elimination. However, the exact strength of binding of the TCR with self-peptides exposed on MHC class II molecules that is required for Treg development has not yet been defined, and it is probable that other factors prevent their elimination. As proposed by Hsieh *et al.* (Hsieh *et al.*, 2012), Treg precursor thymocytes are expected to have TCRs that recognize self-antigens with a binding affinity not too high to trigger negative selection, but sufficient to assure survival through positive selection. The latter is a process by which cells with functional TCRs are selected for survival (Jameson *et al.*, 1995). Expression of the cell surface glucocorticoid-induced tumour necrosis factor (TNF) receptor (GITR) is also critical to promote the Treg lineage. Expression of GITR, OX40 and TNF-R2, which belong to the TNF receptor superfamily, corresponds to high TCR signal strength on developing Treg precursors in the thymus (Mahmud *et al.*, 2014). Also, Tregs must respond to cytokines such as TGF-β and IL-2, which promote FoxP3 expression and prevent apoptosis, as discussed in detail later (section 1.2.5.4).

It is presently unclear at what stage developing thymocytes start expressing FoxP3 or whether this process initiates in the thymic cortex or medulla. CD25+ FoxP3+ Treg precursors have been shown to appear in the DP stage (Cabarrocas *et al.*, 2006). DP thymocytes are abundant in the thymic cortex, which is the site for positive selection (Bill et Palmer, 1989). Indeed, Liston *et al.* showed that the thymic cortex alone is sufficient to support initiation of FoxP3 expression - about one fourth of total FoxP3+ cells in thymic cortex are DP. In contrast to what was reported by Liston *et al.* (Liston *et al.*, 2008), Lee *et al.* used more stringent gating strategies to eliminate doublets

(common within the pool of DP thymocytes) and showed that development of thymic Treg from FoxP3+ DP thymocytes is infrequent, close to 1% of total FoxP3+ cells (Lee et Hsieh, 2009). It should also be noted that a small percentage of DN T cells in the thymic cortex, express FoxP3, although not yet having a functional αβ TCR (Tuovinen *et al.*, 2008). It is unknown whether these DN FoxP3+ thymocytes are precursors for functional Tregs.

More recently, it was proven that FoxP3 expression in the human and mouse thymus initiates before the DP stage but requires expression of a functional TCR (Nunes-Cabaco *et al.*, 2010). Interestingly, the thymic cortex hosts thymic cortical epithelial cells expressing MHC- II , which seem essential for generation of CD4+ CD25+ immunoregulatory T cells (Bensinger *et al.*, 2001). All the aforementioned studies indicate that FoxP3 expression starts in the thymic cortex. However, it is to be noted that induction of FoxP3 expression alone is insufficient for the generation of functionally active thymic Tregs.

Several studies have highlighted the importance of the thymic medullary environment for the generation of Tregs. Negative selection occurs in the thymic medulla (Sprent et Kishimoto, 2002), and bypassing apoptosis triggered by negative selection is considered to be one of the major steps in thymic Treg generation. Treg differentiation in the thymus has been shown to be regulated by medullary thymic epithelial cells (mTECs) (Aschenbrenner *et al.*, 2007) and thymic DCs (Watanabe *et al.*, 2005) present in the thymic medulla, that present MHC class II-restricted self-antigens. Furthermore, expression of autoimmune regulator gene (AIRE) in medullary thymic epithelial cells has been reported to be essential for generation of FoxP3+ thymic Tregs

(Aschenbrenner *et al.*, 2007). It has also been shown that migration of CCR7+ developing thymocytes to the thymic medulla is crucial for induction of central immune tolerance (Kurobe *et al.*, 2006). From the above information, it is clear that the expression of FoxP3 in potential Treg precursors depends on various factors including TCR activation, signaling through certain cytokine receptors as well as expression of cell surface molecules and chemokine receptors. In addition, contact with specific types of thymic epithelial cell is crucial (Bettini et Vignali, 2010). In summary, although FoxP3 expression is initiated in developing thymocytes in the thymic cortex, the thymic medulla plays an important and indispensable role in the generation of functional tTregs capable of sustaining immune suppression.

As discussed more elaborately in the upcoming section, tTregs differ from iTregs with respect to the stability of FoxP3 expression (Floess *et al.*, 2007). Several studies indicate that the transcription factor Helios, belonging to the Ikaros family of zinc finger proteins, might be crucial for maintenance of the Treg phenotype, involving stable FoxP3 expression and the ability to inhibit effector responses. For instance, Helios-deficient mice have CD4+ FoxP3+ cells with reduced inhibitory capacity compared to cells from wild type mice (Kim, H. J. *et al.*, 2015), and siRNA mediated knockdown of Helios in CD4+ CD25+ Tregs downregulates FoxP3 expression (Getnet *et al.*, 2010). Helios binds to the IL-2 gene promoter either by itself or together with FoxP3, thereby inducing its transcriptional silencing (Baine *et al.*, 2013). It also plays an important role in maintaining self-tolerance mechanisms in the thymus: self-reactive thymocytes undergoing negative selection co-express Bim and Helios (Baldwin et Hogquist, 2007; Daley *et al.*, 2013). The next section describes the epigenetic regulation of FoxP3 expression, which is crucial in the induction and maintenance of the Treg phenotype.

1.2.4 Epigenetic regulation of *foxp3* expression

Treg development is not only determined by FoxP3 expression but also by the establishment of a Treg-specific CpG methylation pattern, which is also initiated upon TCR stimulation (Ohkura *et al.*, 2012). Unique CpG methylation patterns, called Treg-specific demethylated regions (TSDRs), were found in Treg-specific genes including Foxp3, Ctla-4, Il2ra, Ikzf4 and Tnfrsf18 by genome wide CpG methylation analysis of mice CD4+ T cells versus Tregs (Ohkura *et al.*, 2012). Interestingly, the establishment of this methylation profile seems independent of FoxP3 expression, as demonstrated by Okhura *et al.* in the same study (Ohkura *et al.*, 2012). Tregs are known to contribute to immunosenescence, the process of gradual deterioration of the functioning of the immune system due to age (Jagger *et al.*, 2014). Tregs from aged mice exhibit more demethylation at the foxp3 gene locus compared to younger mice, resulting in higher Treg mediated suppression of effector T cells in older mice (Garg *et al.*, 2014). Lack of TSDR hypomethylation is also the reason behind impaired suppressive functions of iTregs, which in turn is concurrent to unstable FoxP3 expression (Floess *et al.*, 2007). "Stability" and "plasticity" are two close terms, differing subtly from each other as accepted by researchers in the field of Treg biology (Sakaguchi *et al.*, 2013). With respect to Tregs, stability refers to the establishment and maintenance of a molecular profile characteristic of Tregs, i.e., the stable expression of FoxP3 and other Treg-associated genes like CTLA-4 or IL-2R (Sakaguchi *et al.*, 2013). The term plasticity is more often used to describe the functional characteristics of FoxP3+ T cells which might not necessarily have the transcriptional program of Tregs (Sakaguchi *et al.*, 2013). One of the major factors that determine Treg stability is the epigenetic regulation of the *foxp3* gene. In the regulatory region of the *foxp3* gene locus, six elements called the conserved non-coding sequences (CNS), have been identified (Iizuka-Koga *et al.*, 2017). CNS0, enhancer, proximal promoter, CNS1, CNS2, and CNS3 are the six

regions and their positions on the *foxp3* gene locus are shown in Figure 1.3 (Iizuka-Koga *et al.*, 2017). We will now discuss briefly the role of each of these six regions in the order of their location on the *foxp3* gene locus.

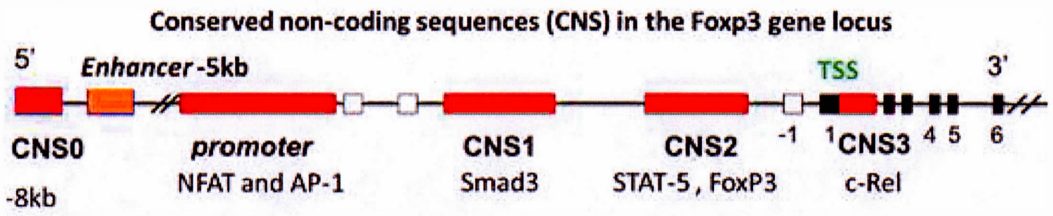

Figure 1.3 Location of CNS elements on the *foxp3* gene locus, adapted from Iizuka-Koga, M. et al. 2017. CNS elements serve as binding sites for specific transcription factors as shown. Methylation status of the CNS elements control the stability of FoxP3 expression on Tregs.

CNS0, also known as a "super-enhancer" element, is located about 8kb upstream of the transcription start site and serves as the binding site for Satb1 (Kitagawa *et al.*, 2017). The major function of Satb1 is to remodel the chromatin architecture to control transcriptional and epigenetic modifications, which in turn facilitates unique tissue-specific gene expression profiles (Cai *et al.*, 2003). Binding of Satb1 to CNS0 triggers changes in chromatin structure, which alters its susceptibility to histone modifications (Iizuka-Koga *et al.*, 2017). Hence, Satb1- CNS0 interaction is a vital first step necessary for the functioning of other CNS elements and the induction of FoxP3 gene (Iizuka-Koga *et al.*, 2017). It is important to note that CNS0 has to be in a demethylated state to be able to support binding of Satb1 (Kitagawa *et al.*, 2017).

The enhancer is a highly conserved sequence located about 5kb upstream of the transcription start site and its demethylation by DNA methyl transferase enzymes (DNMTs) is the key event that initiates FoxP3 expression (Lal *et al.*, 2009). The proximal promoter is located relatively closer to the transcription start site and has binding sites for important transcription factors, such as NFAT and AP-1, STAT-5. Binding of AP- 1 and NFAT to the promoter region is associated with its trans-activation following the activation of TCR. This region is T cell-specific and is responsible for induction of FoxP3 after TCR stimulation (Mantel *et al.*, 2006).

CNS1 serves as the binding site for the transcription factors NFAT, AP-1, retinoic acid receptor and most importantly Smad2/3, the protein complex downstream of TGF- β receptor-mediated signaling. As mentioned previously, TGF-β plays an essential role in pTreg generation. It has been demonstrated that deletion of CNS1 inhibits the differentiation of pTregs highlighting the importance of this region particularly in pTreg generation (Zheng *et al.*, 2010). The role of CNS1 in tTreg generation remains unclear.

CNS2 is also called Treg specific demethylated region (TSDR). As the name suggests, demethylation of this region is specific to Tregs only – activated CD4+ T cells (in mice and in humans) that transiently express FoxP3 protein do not have demethylated CNS2 (Baron *et al.*, 2007). It has binding sites for Stat5, NFAT, Runx1/Cbfb, CREB, and FoxP3 itself. For the stability of FoxP3 expression and hence the maintenance of the Treg phenotype, CNS2 region has to be maintained in a demethylated state. Studies in mice have shown that inheritance of the Treg phenotype is largely associated with stable FoxP3 expression, which is a consequence of demythalted CNS2 (Feng *et al.*,

2014). It has also been shown that tTregs, stably expressing FoxP3, have demethylated CNS2 in contrast to iTregs in which FoxP3 protein expression (induced by TGF-β *in vitro*) is high but unstable with incomplete CNS2 demethylation (Floess *et al.*, 2007). Now arises the question about CNS2 methylation in pTregs (referring to Tregs generated *in vivo*, but not from the thymus). Two studies done in mice have addressed this question – Weiss *et al.* and Yadav *et al.* distinguish pTregs from tTregs using Neuropilin-1 (Nrp1, a receptor initially described in axon guidance, which also has a role in Treg-DC interactions) have demonstrated that both Foxp3+ Nrp1+ (tTreg) and Foxp3+ Nrp1− (pTreg) stably express FoxP3 and have demethylated CNS2 (Weiss, J. M. *et al.*, 2012; Yadav *et al.*, 2012). However, it is controversial if Nrp1 can be used to differentiate tTregs and pTregs. The numbers of Nrp1-expressing Tregs are comparable in genetically modified mice with impaired differentiation of tTregs, in mice with defective pTreg differentiation, and in wild type mice, indicating that high or low expression of Nrp1 is not an appropriate parameter to distinguish between tTregs and pTregs (Yadav *et al.*, 2012). Therefore, it remains unclear if pTregs have stable FoxP3 expression and CNS2 demethylation, since distinguishing tTregs and pTregs remains controversial.

CNS3 includes the binding site for the transcription factor c-Rel, a member of the NF-κB family of proteins. The NF-κB signaling pathway is one of the many signaling cascades activated upon TCR stimulation and c-Rel binding to CNS3 has a direct influence on FoxP3 expression after TCR stimulation (Long *et al.*, 2009). TCR stimulation is a vital step in the process of both pTreg and tTreg generation and hence deletion of CNS3 in mice leads to the deficiency of both tTregs and pTregs (Zheng *et al.*, 2010).

Having discussed the regions in the FoxP3 gene, which are rich in CpG sites, and act as epigenetic switches regulating the gene and contributing to development/maintenance of the Treg phenotype, the mechanisms responsible for controlling their methylation status will now be summarized. DNMTs are a class of enzymes controlling the epigenetic status of *foxp3*. Specifically, the enzymes DNMT1, DNMT3b, MeCP2, and MBD2 are bound to the upstream enhancer of *foxp3*, thereby maintaining it in the methylated state, in iTregs, activated CD4+ T cells, and naïve CD4+ T cells, all of which might unstably express FoxP3 protein and hence do not possess immune suppressive capacity (Lal *et al.*, 2009).Tet enzymes are an additional class of methylcytosine dioxygenases controlling FoxP3 epigenetic status; these enzymes convert 5-methylcytosine (5mC) to 5- hydroxymethylcytosine (5hmC) in CpG islands. Yang *et al.* demonstrated that Tet-1 and Tet-2 enzymes are responsible for the establishment of FoxP3 hypomethylation patterns associated with Treg phenotype and deletion of these enzymes resulted in FoxP3 hypermethylation and defective Treg generation, leading to autoimmune diseases (Yang, R. *et al.*, 2015). Vitamin C also indirectly plays a role in establishing Treg phenotype, since it regulates Tet family of enzymes, which in turn alter FoxP3 methylation patterns (Sasidharan Nair *et al.*, 2016).

1.2.5 Role of TGF-β in Treg development

1.2.5.1 TGF-β: Classification, Isotypes, Structure and Function

Transforming Growth Factor Beta (TGF-β) is a cytokine belonging to the TGF-β superfamily of growth factors, which includes the different forms of TGF-β, bone-

morphogenetic proteins (BMPs), activins and inhibins. TGF-β is involved in the process of cellular differentiation, proliferation, development and embryogenesis, carcinogenesis, fibrosis, wound healing and angiogenesis (Blobe *et al.*, 2000). In mammals, there are three different isoforms of TGF-β (TGF-β1, TGF-β2 and TGF-β3), each encoded by a different gene (Govinden et Bhoola, 2003). The TGF-β1 isoform plays an important role in immune regulation (Li, M. O. *et al.*, 2006) and hence is of our interest.

1.2.5.2 TGF-β: Activation and Signalling

TGF-β was initially identified as a 25kDa protein promoting anchorage-independent growth of mouse fibroblasts (Moses *et al.*, 1981; Roberts *et al.*, 1981). It is produced as a precursor protein (prepro- TGF-β) and exists as a homodimer in its active form. The pre-region of prepro- TGF- β1 contains a signal peptide at the N-terminal end, which undergoes proteolytic cleavage in the Golgi apparatus. The pro-region consists of the latency associated peptide (LAP), which forms a homodimer with mature TGF-β, called the small latent complex, which remains inactive. Once secreted into the extra-cellular matrix, activation of TGF-β is triggered by proteolysis of LAP or by conformational changes in LAP which prevents its binding to TGF-β (Annes *et al.*, 2003). TGF-β mainly signals thorough type I (TGF-β R1) and type II (TGF-β R2) receptors, which belong to the family of serine/threonine kinases. Until now, seven types of TGF-β R1 (Activin-like kinases 1-7) and five types of TGF-β R2 have been reported (Chang, H. *et al.*, 2002). Active TGF-β initially binds to a TGF-β R2, promoting conformational changes of this receptor and recruitment of TGF-β R1 through phosphorylation and subsequent activation (Heldin *et al.*, 1997). The activated

TGF-β R1 phosphorylates Smads (Sma and Mad -Mothers against decapentaplegic), specifically, Smad-2 (Nakao *et al.*, 1997) and Smad-3 which then associate with Smad-4 and translocate into the nucleus (Inman *et al.*, 2002), leading to the activation of target genes in the nucleus (Heldin *et al.*, 1997).

1.2.5.2 Effect of TGF-β on early stages of thymocyte development

TGF-β influences thymocyte development at several stages. Newly arrived ETPs proliferate extensively in response to IL-2 and IL-7. These cells secrete TGF-β1, which provides autocrine regulation of proliferation (Mossalayi *et al.*, 1995). Both TGF- β1 and TGF-β2 restrict proliferation of IL-1,-2, -4, -6, -7 and TNF-α-stimulated thymocytes (Chantry *et al.*, 1989; Ellingsworth *et al.*, 1988; Licona-Limon et Soldevila, 2007; Wahl *et al.*, 1988). Early studies with murine fetal thymic cultures report that TGF- β1 inhibits the differentiation of DN T cells (Plum *et al.*, 1995). In mice, thymic epithelial cells expressing TGF-β1 assist the conversion of CD4-CD8^{low} thymocytes into CD4+ CD8+ thymocytes (Takahama *et al.*, 1994). *In vivo* Cre/lox system-based models of TGF-β RII-deficient mice report increased numbers of proliferating CD8+ T cells, suggesting its importance in thymic T cell development (Leveen *et al.*, 2005).

1.2.5.3 Implications of TGF-β in tTreg development

Studies in mice have shown that tTregs develop from CD4+ CD25+ cells bearing relatively high affinity TCRs, which are however insufficient to cause their elimination

through negative selection (Fontenot *et al.*, 2003). TGF-β1 is anatomically confined to the thymic medulla, and it was initially reported that Treg development most likely occurs in the thymic medulla (Fontenot Dooley, *et al.*, 2005). The role of the thymic cortex and the thymic medulla in Treg development has been discussed in detail (section 1.2.3). The development of tTregs seems to first require the activation of the TCR, followed by cytokine-driven induction and maintenance of FoxP3 expression. The promoter region of the FoxP3 gene contains cis-regulatory elements, called conserved non-coding sequences (CNS), which are crucial for the establishment and sustained maintenance of Treg phenotype (described in detail in section 1.2.4). Particularly, the CNS1 region of mouse and human FoxP3 gene contain a unique Smad-3 binding site (Chakraborty *et al.*, 2017; Li, X. et Zheng, 2015). Smad-3 is one of the primary downstream mediators of the TGF-β signalling pathway and in response to TGF-β1, its binding to CNS1 is crucial for sustained expression of FoxP3 and maintenance of Treg phenotype (Li, X. et Zheng, 2015).

Ouyang *et al.* reported that TGF-β safeguards thymocytes from negative selection i.e., pro-apoptotic proteins are upregulated in TGF-β RII-deficient tTreg cells (Ouyang, W. *et al.*, 2010). Hence, TGF-β promotes Treg generation in the thymus independently of FoxP3 as well. The anti-apoptotic effects of TGF-β in thymocytes was demonstrated in two other earlier studies (Chen, W. *et al.*, 2001; Wahl *et al.*, 2000). However, it is still unclear whether this protective effect is only restricted to tTregs, or may also affect developing thymocytes. Konkel *et al.* reported that apoptosis of developing thymocytes is crucial for tTreg development due to the fact that apoptotic cells and macrophages, which phagocytose apoptotic cells are sources of TGF-β, which in turn induces FoxP3 expression in Treg precursors (Konkel *et al.*, 2014).

Based on these events, a new model for tTreg development has been proposed, described as the "apoptosis-TGF-β-FoxP3 axis" (Figure 1.4) (Chen, W. et Konkel, 2015). This model, proposed by Chen *et al.* summarizes the process of Treg development as follows: a) Developing thymocytes undergo negative selection, which results in high numbers of apoptotic thymocytes and maintenance of elevated TGF-β levels in situ. High concentrations of TGF-β favor the development of tTreg cells from precursors, provided that signals are transmitted through their TCR, and protection against apoptosis is assured. b) These precursors then initiate and maintain FoxP3 expression. As noted by Chen *et al.* several questions still remain unanswered. First, the potential populations of tTreg precursor cells have not been clearly discriminated. Second, the threshold concentrations of TGF-β required for inducing FoxP3 expression in these precursors are unknown. Third, the mechanisms and signaling pathways by which TGF-β stimulates FoxP3 expression in tTregs require investigation.

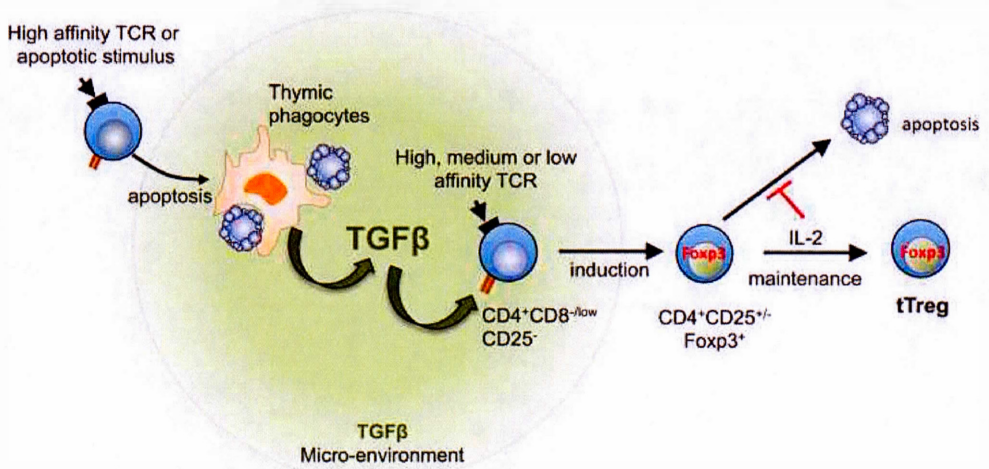

Figure 1.4: Role of TGF-β in tTreg development, shown here is the apoptosis-TGF-β-FoxP3 axis, adapted from Chen, W. and Konkel J.E., 2015.

1.2.6 Development of peripheral Tregs

1.2.6.1 Physiological context of pTreg generation

The existence of extra-thymically generated suppressor cells in mice was described since the early 1990's (Taguchi *et al.*, 1994). Not all the Tregs arise from the thymus since not all Tregs encounter their cognate antigen in the thymus. Tregs which are foreign antigen-specific develop extra-thymically. Differentiation of pTregs occurs more frequently in mucosal surfaces compared to other tissues (Coombes *et al.*, 2007; Sun *et al.*, 2007; Yadav *et al.*, 2013), since they serve as sites for direct host-pathogen interaction, and often there are heightened immune responses, at these sites, which have to be downregulated. Peripherally induced Tregs play a very crucial role in establishing immune privilege (tolerance to the presence of antigens, to prevent inflammatory damage which might have serious consequences), in organs such as the eyes (Sugita, 2009), and the testis (Li, N. *et al.*, 2012). Another peripheral tolerance mechanism mediated by Tregs is the maternal tolerance towards paternal allo-antigens expressed on the fetus, which is crucial for preventing miscarriages (La Rocca *et al.*, 2014).

Development of peripheral antigen-specific Tregs in certain tissues is crucial for preventing immune disorders. For instance, accelerated immune response to gut-resident commensal bacteria could result in the development of inflammatory bowel disease (Elson et Cong, 2012; Jostins, 2012), and one of the mechanisms that helps in tolerance towards gut microbiota is the presence of Tregs (Belkaid et Hand, 2014; Maloy et Powrie, 2011). Interestingly, pTregs in the intestinal mucosa are thought to

be induced by one of the most common intestinal bacterial strains belonging to the genus Clostridium – higher amounts of TGF-β and a higher number of FoxP3 expressing T cells were detected in the colon of mice colonized with Clostridia (of 46 different strains) compared to wildtype mice, suggesting the tole of TGF-β in promoting FoxP3 induction (Atarashi *et al.*, 2011). Polysaccharide A of *Bacillus fragilis*, another species belonging to the intestinal microbiome, also mediates generation of functionally active, IL-10 secreting,FoxP3+ pTregs from CD4+ T cells (Round et Mazmanian, 2010). Yet, another mechanism of pTreg generation occurs through the interaction of CD4+ T cells with specific APCs. For instance, lung resident macrophages, expressing TGF-β and retinoic acid, were shown to induce antigen-specific FoxP3+ Tregs from CD4+ T cells (Soroosh *et al.*, 2013). DCs also play an indispensable role in pTreg generation – depletion of DCs leads to lower Treg numbers (Darrasse-Jeze *et al.*, 2009). Certain classes of DCs, such as migratory DCs, which move from tissues to lymph nodes, carrying self-antigenic peptides from the tissues, are more efficient in generating Tregs compared to tissue resident DCs (Idoyaga *et al.*, 2013). Plasmacytoid DCs, which are specialized DCs that secrete type-I interferons are also known to induce FoxP3+ Tregs in the lungs to maintain immune tolerance (Lombardi *et al.*, 2012).

1.2.6.2 Role of TGF-β in pTreg generation

TGF-β, along with TCR co-stimulation, triggers the expression of FoxP3 in murine naive CD4+ CD25- T cells, and hence, mediates their differentiation to Tregs having immune suppressive functions (Chen, W. *et al.*, 2003). TGF-β is crucial for the induction of FoxP3 in human CD4+ T cells as well (Amarnath, Shoba *et al.*, 2007;

Fantini *et al.*, 2004). The molecular mechanisms underlying induction of FoxP3 mediated by TGF-β have not yet been fully unraveled. Smad-3, which is downstream of TGF-β in the signalling cascade, binds to the enhancer region of FoxP3 gene (Tone *et al.*, 2008). However, phosphorylated Smad2/3 binding to its target is usually considered to be weak and hence, it has been proposed that there must be additional co-factors, which work cooperatively with Smad-2/3 to trigger FoxP3 expression (Chen, W. et Konkel, 2010). TGF-β could also induce FoxP3 through mechanisms not involving Smads, but instead involving other transcription factors like NFAT and AP-1, which can also bind to the FoxP3 promoter region directly (Chen, W. et Konkel, 2010). As for tTreg generation, one of the main steps in pTreg generationis the ligation of TCRs with their specific antigens. FoxP3 expression (and hence pTreg generation) initiates in CD4+ T cells when there is a weak TCR signal, which is insufficient to trigger proliferation (Gottschalk, R. A. *et al.*, 2010; Haxhinasto *et al.*, 2008; Kretschmer *et al.*, 2005; Sauer *et al.*, 2008). However, IL-2 and TGF-β are two indispensable cytokines involved in the process of pTreg induction (Chen, W. et Konkel, 2010; Davidson *et al.*, 2007).

Physiological aging is another interesting aspect to consider in thymic and peripheral Treg development. Thymic involution with aging results in decreased thymic function and decreased production of new, naïve T cells. As such, thymic involution may result in decreased pTreg generation, since these cells arise from naïve CD4+ T cells under inflammatory conditions (Jagger *et al.*, 2014). Recently, Thiault *et al.* reported that relative to non-Tregs, thymic output of tTregs upon aging is less affected (Thiault *et al.*, 2015). These authors suggest that pTregs (characterized by the lack of CD31 expression, which is the marker for recent thymic emigrants), recirculate into the adult human and mice thymuses (Thiault *et al.*, 2015). These recirculating pTregs, which are functionally differentiated, then impair the IL-2 dependent development of Treg

precursor cells in the thymus. Thus, Treg development is possibly regulated by a negative feedback mechanism, as proposed in this study.

1.2.6.3 Common developmental pathway of Th17 and Tregs

Th17 cells are an independent class of CD4+ T cells producing mainly interleukin-17A (IL-17A) and also IL-17F, IL-21 and IL-22 (Korn *et al.*, 2009) and hence Th17 cells are phenotypically characterized as CD4 T cells expressing the transcription factor RORγt and producing IL-17A. In contrast to Tregs, Th17 cells are pro-inflammatory in nature. Th17 cells could be either protective or pathogenic under different biological contexts. For instance, in the context of tuberculosis, IL-17 produced by Th17 cells triggers migration of IFN-γ producing T cells into the lungs, which helps in pathogen elimination, accounting for the protective role played by Th17 cells (Khader *et al.*, 2007). On the other hand, autoimmune disease like rheumatoid arthritis and EAE/multiple sclerosis are associated with IL-17 overexpression, owing to the pathogenic effects of Th17 cells (Korn *et al.*, 2009). Differentiation of Th17 cells from naive T cells was initially described while studying the role of TGF-β in induction of FoxP3 expression in Tregs – in the presence of IL-6, induction of FoxP3+ Tregs by TGF-β was inhibited and instead lead to the generation of Th17 cells (Bettelli *et al.*, 2006). The same group later discovered that IL-21 and TGF- β also induce differentiation of Th17 cells (Korn *et al.*, 2007). All the above mentioned studies were performed in mice. However, it was demonstrated that human Th17 differentiation was mainly controlled by TGF-β and IL-21 (Yang, L. *et al.*, 2008).

pTregs and Th17 cells share common developmental pathways, owing to the fact that they both develop from the same precursor cells. Whether naive CD4+ T cells differentiate into pTregs or Th17 cells depends on the cytokines present in the milieu (Figure 1.5). TGF-β in combination with IL-6 or IL-21, but not just one of them, leads to induction of receptor–type nuclear receptor RORγt, the master transcription factor of Th17 cells, which regulates IL-17 production (Korn *et al.*, 2009). In contrast, higher concentrations of TGF-β alone induces FoxP3 expression and Treg generation as already described in detail. Based on these evidences, it has been proposed that levels of TGF-β in the tissue microenvironment regulates the balance between Tregs and Th17 cells at a molecular level by influencing induction of FoxP3 or RORγt depending on the context (Korn *et al.*, 2009). Under normal homeostatic conditions, TGF-β induces FoxP3+ Tregs, whereas, in the presence of infectious agents, TGF-β synergizes with IL-6, produced mostly by macrophages in response to activation of Toll-like receptors, and leads to the differentiation of RORγt+ Th17 cells (Korn *et al.*, 2009). This also suggests that the phenotype of TGF-β-induced FoxP3+ Tregs is not quite stable. A recent study describes a subset of FoxP3+ Tregs in the context of intestinal inflammation, expressing RORγt (Yang, B. H. *et al.*, 2016). Such RORγt-expressing Tregs displayed greater suppressive capacity compared to their RORγt- counterparts, suggesting a role for the Th17 transcription factor in controlling Treg functions (Yang, B. H. *et al.*, 2016).

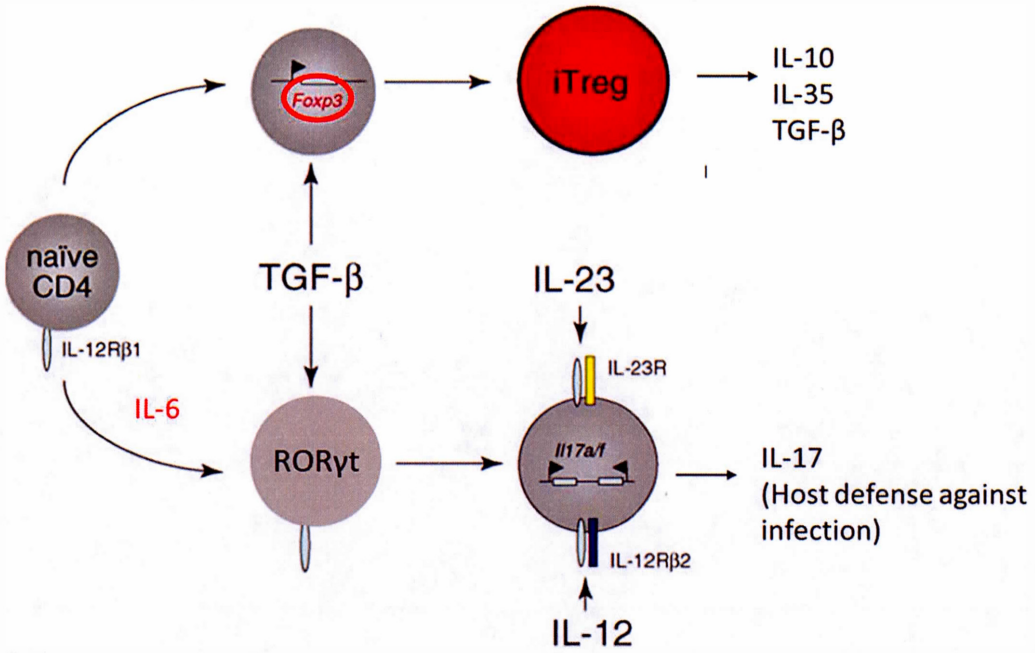

Figure 1.5: Shared developmental pathway of Th17 cells and Tregs in the peripheral blood, adapted from Lee YK et al. 2009. Under homeostatic conditions, TGF-β induces FoxP3+ Tregs, whereas, during inflammation, TGF-β synergizes with IL-6 to induce Th17 cells.

1.3 HIV and AIDS

1.3.1 Viral family and discovery

Human immunodeficiency virus (HIV) belongs to the family of retroviruses and the genus of lentiviruses. It is a single-stranded RNA virus and is the causative agent of acquired immunodeficiency syndrome (AIDS). It was first isolated in 1983 and was

known to specifically infect CD4 T lymphocytes (Barre-Sinoussi *et al.*, 1983). HIV infections in humans are the result of zoonotic transmission from African apes, which are prone to infection by simian immunodeficiency virus (SIV). HIV infections in humans was starting to be recognized in 1981 - even though the causative agent still remained unknown, it was noted that men who have sex with men were prone to acquire many opportunistic infections (Gottlieb *et al.*, 1981).

1.3.2 HIV structure and life cycle

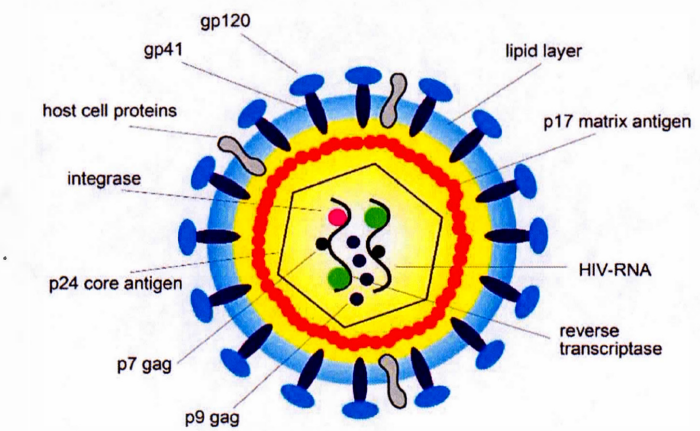

Figure 1.6: Structure of HIV, showing the RNA genome and the glycoprotein envelope, from https://www.hivbook.com/tag/structure-of-hiv-1/. (*HIV book 2011*)

The retrovirus HIV consists of two copies of single stranded RNA encoding nine viral genes, which is enclosed by different layers: nucleocapsid, protein p7, the capsid protein p24 and the matrix protein p17 (Figure 1.6). The viral capsid is covered with a glycoprotein (gp) envelope comprising gp120 and gp41. HIV primarily infects CD4 T cells as the cell surface molecule CD4 serves as the main receptor for HIV. The chemokine receptors CXCR4 and CCR5 serve as co-receptors for HIV, which facilitate the entry of X4-tropic HIV or R5-tropic HIV strains, respectively. Dual tropic viral strains can recognize both co-receptors. The viral replication cycle occurs as follows (Maartens *et al.*, 2014): after binding to the CD4 receptor and co-receptors, the virus fuses with the cell membrane, followed by uncoating of the viral envelope. The viral RNA is then released to the cytoplasm of the host cell and is converted to DNA by the reverse transcriptase enzyme. The viral DNA integrates into the genome of the host cell by the action of integrase enzymes. The integrated viral DNA is then transcribed, and the viral RNA is either translated into viral proteins or used as viral RNA genome for new virion progenitors. Subsequently, the host cell is lysed and infectious viral particles are released into the blood stream of the host. Figure 1.7 describes mechanisms of viral replication and the treatment strategies for controlling HIV replication.

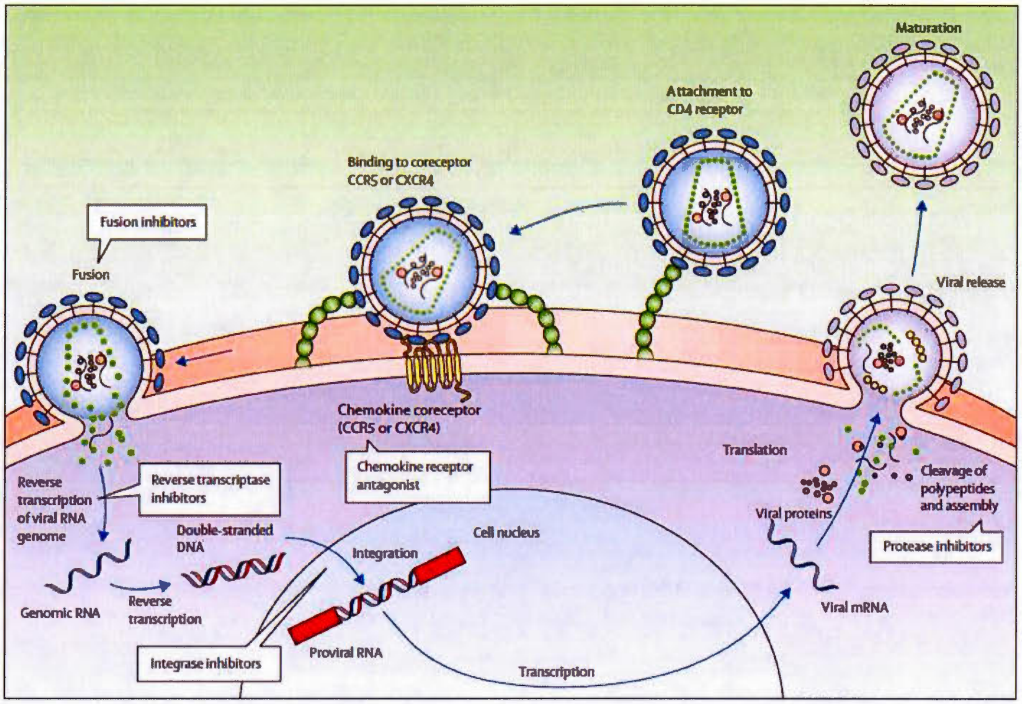

Figure 1.7: HIV life cycle and drugs used in ART, targeting different stages of the viral life cycle (Maartens *et al.*, 2014)

1.3.3 Epidemiology of HIV infection

Since its discovery in the 1980s until today, HIV infection continues to be one of the major public health issues around the world. As of 2016, the estimated number of people living with HIV is 36.7 million comprising 51% of women (Ghosn *et al.*, 2018). Eastern and southern Africa constitute the regions where the disease prevalence is the highest (Ghosn *et al.*, 2018). About one million people died of HIV infections in 2016 and 1.8 million were newly infected (Ghosn *et al.*, 2018). Since HIV infection is

transmitted through sexual intercourse or through sharing of needles with infected users, people prone to greater risk of infection include young women in high prevalence countries and sex workers, drug users, men who have sex with men, and transgender people in countries with low disease prevalence (Ghosn *et al.*, 2018). Figure 1.8 shows the worldwide prevalence of HIV in 2016 as reported by UN-AIDS.

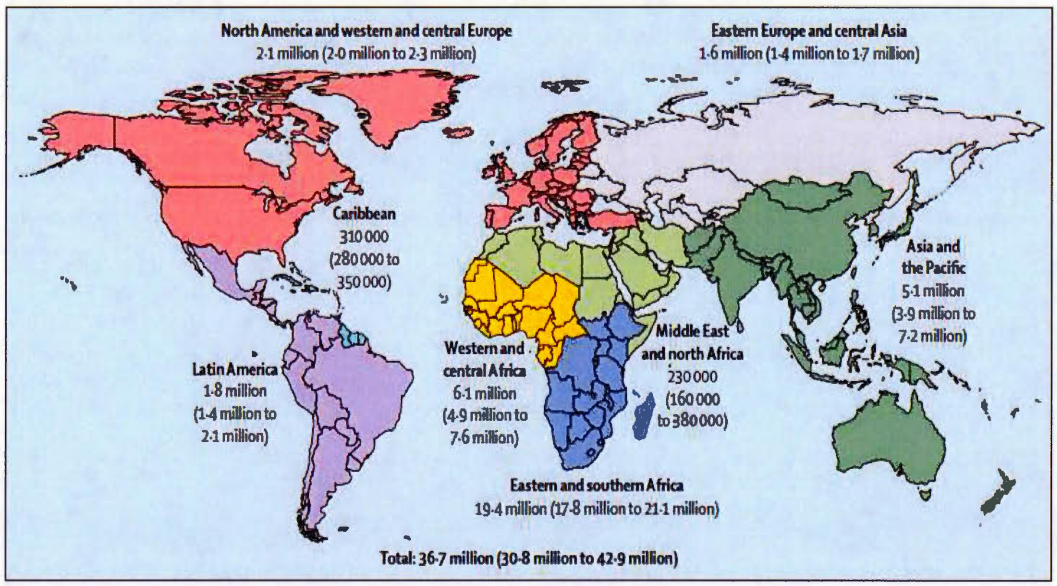

Figure 1.8: HIV-infected people living worldwide, as of 2016 (Ghosn *et al.*, 2018)

One of the major goals in HIV control and prevention is the 90-90-90 target described by UN-AIDS, which aims that by 2020, 90% of all people living with HIV will know their HIV status, 90% of all people with diagnosed HIV infection will receive sustained antiretroviral therapy and 90% of all people receiving antiretroviral therapy will have viral suppression. However, we are still far from achieving the first goal owing to the shortcomings of the existing and most prevalent rapid diagnostic immunological assays, which involve the detection of antibodies to p24 viral antigen. These assays cannot

identify acute phase HIV infection, which occurs before the production of detectable p24 antibodies (Ghosn *et al.*, 2018). HIV-infected people have a higher risk of mortality as they are more prone to a wide range of other diseases. For instance, people living with HIV are at a 50% higher risk of myocardial infarctions than healthy individuals (Freiberg *et al.*, 2013); they are susceptible to co-infections with Hepatitis B (Bodsworth *et al.*, 1991), which shares similar routes of transmission.

1.3.4 Pathophysiology of HIV infection

HIV primarily infects CD4 T cells (Barre-Sinoussi *et al.*, 1983). HIV also infects other cells, like monocytes, macrophages and dendritic cells, which express CD4 and the co-receptors. In a newly infected individual, a founder virus which utilizes the CCR5 co-receptor and not CXCR4 (Keele *et al.*, 2008), is transmitted through the mucosal membranes. This in turn triggers the production of inflammatory cytokines and chemokines (Stacey *et al.*, 2009). HIV-specific adaptive immune responses are then induced, which mainly consists of cytotoxic CD8 T cells, capable of killing HIV-infected CD4 T cells. The CD8 T cell response might also act as driving forces for viral evolution - about 53% of mutations in non-evelope viral antigens were resulting from the selective pressuse of CD8+ T cell responses (Allen *et al.*, 2005), which suggests the role of immune system in shaping viral evolution. Although B cell responses are detected after 1 week of infection, they have no effect on viremia; neutralizing antibodies start appearing in the blood of infected individuals after about 3 months of infection. However, these neutralizing antibodies, being not so diverse, fail to help the host since by this time the virus has already developed resistant mutations to escape immune surveillance (Liao *et al.*, 2013). Depletion of CD4 T cells is a hallmark of HIV infection; there is a disruption of T cell homeostasis in the gut-associated lymphoid

tissue (GALT) leading to immune dysfunction immediately following infection (Mehandru *et al.*, 2007). In addition, Th17 cells, which populate the gut mucosa highly express CCR5, are preferentially infected and hence depleted during the early stages of HIV infection (Bixler et Mattapallil, 2013). The correlation between viral load and CD4 T cell depletion in the blood and GALT in untreated HIV-infected individuals is shown in Figure 1.9.

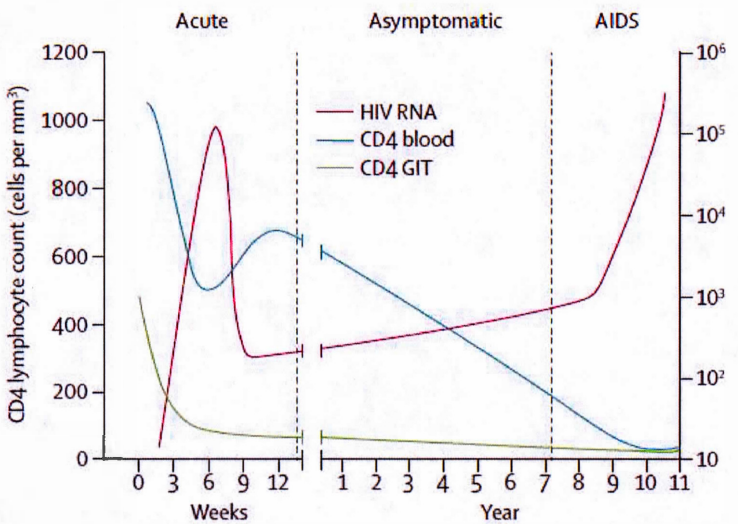

Figure 1.9: CD4 T cell dynamics and HIV viral load during different stages of HIV infection (Maartens *et al.*, 2014)

CD4 T cell depletion in the GALT is accompanied by loss of other important T cell subsets including Th17 cells (Prendergast *et al.*, 2010), which provide defense against microbial organisms in the gut. Since the gut mucosal barrier is disrupted and the protective immune cells present are destroyed, microbial products like LPS enter the blood stream from the gut of affected individual (Brenchley *et al.*, 2006). Microbial translocation activates Toll-like receptor 4 (TLR4) expressed on monocytes and

dendritic cells, resulting in the production of inflammatory cytokines like IL-6 and TNF-α thereby causing systemic immune activation (Brenchley *et al.*, 2006). In addition, HIV infection results in the imbalance of Th17 and Treg cells in the gut mucosa (Prendergast *et al.*, 2010), which is discussed in detail in section 1.4.2.

1.3.5 Antiretroviral therapy (ART) and treatment challenges

Today, more than 25 FDA-approved antiretroviral drugs that block HIV replication at various stages exist (Maartens *et al.*, 2014). Different classes of drugs used in ART 1. Fusion inhibitors that bind viral envelope and block fusion of virus with the host cell; 2. Co-receptor antagonists that block HIV co-receptors on host cells; 3. Reverse transcriptase inhibitors that block conversion of viral RNA into DNA; 4. Integrase inhibitors that prevent integration of viral DNA with the host genome and; 5. Protease inhibitors that prevent production and assembly of new viral particles (Maartens *et al.*, 2014). Some commonly used drugs and their mechanism of action are shown in Figure 1.7.

The regimen in HIV treatment usually consists of a combination of two reverse transcriptase inhibitors, which are nucleoside analogs, along with another reverse transcriptase inhibitor, protease or integrase inhibitor. These combinations try to ensure minimum chances for the development of resistance to antiretroviral drugs (Maartens *et al.*, 2014). ART reduces viral load in the plasma. However, the recovery of CD4 T cell count varies among HIV-infected individuals. As shown in Figure 1.10, despite

recovery of plasma CD4 T cell counts following ART, gut CD4 T cell counts remain low, which poses a persistent problem in achieving HIV cure (Maartens *et al.*, 2014).

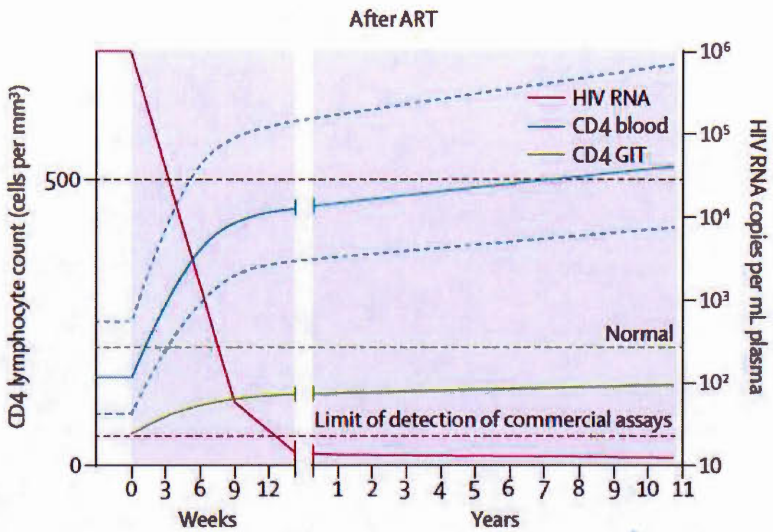

Figure 1.10: CD4 T cell dynamics and HIV viral load after ART, Maartens, G. *et al.* 2014 (Maartens *et al.*, 2014)

So far, only one HIV-infected individual, referred to as the "Berlin patient", has been reported to be cured of HIV. Suffering from acute myeloid leukemia, this individual received two stem cell transplants from a donor with delta-32 mutations in the CCR5 gene, resulting in the truncated non-functional receptor on the cell surface and hence imparted HIV resistance (Alkhatib, 2009). Viral load became undetectable after several years of transplantation, despite discontinuing ART. Very recently in 2019, two other individuals, who were also suffering from leukemia and received similar bone marrow transplants as the Berlin patient were also reported to be cured (Gupta *et al.*, 2019). Clinicians describe "achieving HIV cure" as the prevention of long-term remission by destroying reservoirs housing replication competent viruses (Ghosn *et al.*, 2018).

Hence, viral persistence, as integrated latent viral DNA within a host cell, is one of the major problems preventing the achievement of complete HIV cure. Researchers use the term "viral reservoir" to indicate the presence of latent virus within cells or tissues, hence allowing the persistence of replication competent virus in treated individuals (Churchill *et al.*, 2016). Several cellular and tissue reservoirs have been characterized, and resting central memory CD4 T cells are the major cellular reservoirs of HIV (Churchill *et al.*, 2016). Other cells including naive CD4 T cells, memory stem T cells, transitional memory T cells and macrophages have also been suggested as reservoirs, but it is still not clear if the viral latency in these cells exists during the treatment, as in the case of central memory T cells (Churchill *et al.*, 2016). Reservoirs include tissues that consist of cellular reservoirs (mainly central memory T cells). Lymph nodes, the spleen and the gut mucosa are the major tissue compartments housing central memory T cells carrying latent virus (Churchill *et al.*, 2016). Although the integrated viral genome has been detected in microglia and astrocytes from autopsy samples of brain tissue (Churchill *et al.*, 2006), it is not known whether these cells can continue to harbor the virus during ART and assuming they do so, it is unclear if the virus can be reactivated, leading to remission of the disease after treatment (Churchill *et al.*, 2016). Viral persistence thus continues to be an unresolved issue mainly because of the insufficient understanding of viral reservoirs, and the lack of standardized techniques for its detection and quantification (Ghosn *et al.*, 2018). Many HIV-infected and treated individuals fail to recover a normal CD4 T cell count (Piketty *et al.*, 1998). Such individuals, who have CD4 T cell counts <200cells/μl, are called immunological non-responders (INRs) (Gaardbo *et al.*, 2012). Factors affecting immune reconstitution after treatment include a longer duration of infection prior to treatment initiation (Kaufmann *et al.*, 2005), co-infection with hepatitis C (Greub *et al.*, 2000) and lower CD4 nadir (lowest value till which CD4 count has dropped) (D'Amico *et al.*, 2005).

1.4 HIV and Tregs

1.4.1 Increase in Treg frequencies during HIV infection

Two major experimental parameters must be considered while discussing the available literature concerning the role of Tregs during HIV infection. The first parameter is the phenotype used to define Tregs in different studies, and the second parameter is the stage of HIV infection. The definition of Tregs has been continuously over time and different authors use varying definitions. In addition, the role of these cells during HIV infection can be either beneficial or deleterious depending on the stage of the disease. As mentioned earlier, during HIV infection, there is a depletion of CD4 T cell count, accompanied by immune dysfunction. Owing to the rapid depletion of CD4 T cells, the proportion or frequency of Tregs - not their absolute number - within the T cell pool increases, resulting in higher Treg frequencies compared to non Treg CD4+ T cells (Moreno-Fernandez *et al.*, 2012; Presicce *et al.*, 2011). Several mechanisms account for this increase in Treg frequencies during HIV infection are shown in the Figure 1.11.

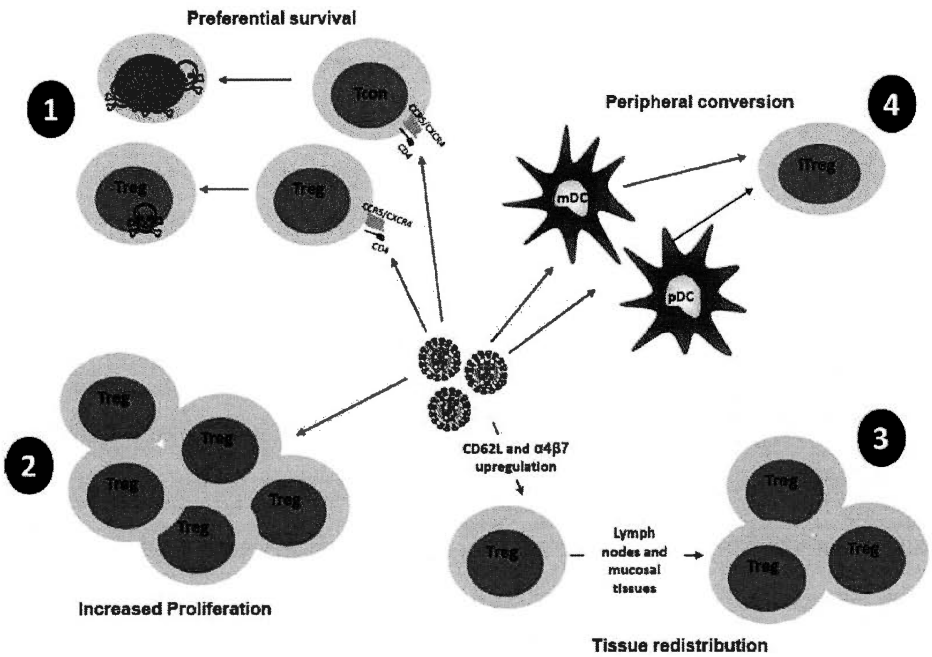

Figure 1.11: Mechanisms accounting for increase in Treg frequencies during HIV infection (Moreno-Fernandez *et al.*, 2012)

1. To understand the reasons for the increased frequencies during HIV infection, it is important to whether these cells are infected by HIV or not. Tregs express CD4, CCR5 and CXCR4 and hence may potentially be susceptible to HIV infection. However, Treg infection by HIV seems lower compared to infection of effector T cells (Moreno-Fernandez *et al.*, 2009). Compared to non-Tregs, Tregs from the colon of SIV-infected macaques are less infected, and are hence protected from SIV-mediated cell death, which accounts for their preferential survival (Allers *et al.*, 2010). FoxP3 expression in Tregs also provides a protective role as it prevents HIV viral transcription by suppressing NFAT binding to the HIV-1 LTR and for HIV gene transcription (Selliah *et al.*, 2008).
2. Presicce *et al.* showed increased Treg proliferation (assessed by the expression of cell cycle markers Ki67 and cyclin B) in HIV-infected untreated individuals,

possibly as a consequence of immune-activation following viral infection and Treg numbers decreased following ART initiation (Presicce *et al.*, 2011). Xing et al. . demonstrated higher turnover (proliferation-apoptosis ratio) of Tregs in untreated vs HAART-treated HIV infected individuals, and showed a direct correlation between high Treg turnover and disease progression resulting from excessive immune activation (Xing *et al.*, 2010). In short, Treg frequencies are higher in untreated compared to HAART-treated HIV infected individuals and higher Treg frequencies correlate with disease progression.

3. Ji *et al.* showed *in vitro* that HIV infected CD4+ CD25+ T cells from healthy donors start expressing CD62L and Integrin α4β7 (gut homing marker), suggesting an increased homing capacity to peripheral and mucosal lymphoid tissues. Hence, the increased migratory potential resulting in redistribution could be a reason for increased Treg frequencies in lymphoid tissues following HIV infection (Ji et Cloyd, 2009). However, other studies have also reported increased Treg frequencies in the peripheral blood of HIV-infected individuals, which does not support the former mechanism of Treg accumulation in tissues (Moreno-Fernandez *et al.*, 2012).
4. The role of TGF-β in mediating fibrosis of lymphoid tissues in HIV-infected individuals and SIV-infected macaques was previously reported (Estes *et al.*, 2007; Zeng *et al.*, 2011). TGF-β is known to induce FoxP3 expression and mediate Treg generation in peripheral blood (Shevach *et al.*, 2008). The effect of TGF-β in the peripheral generation of Tregs during HIV/SIV infection is discussed in detail in section 1.4.5. In addition, HIV-exposed plasmacytoid DCs (Manches *et al.*, 2008) induce Tregs with suppressive capacity from naïve CD4 T cells, through a mechanism involving expression of the tryptophan-catabolizing enzyme IDO, as explained in the section 1.2.2.

1.4.2 Dysregulation of Th17/Treg balance during HIV/SIV infection

One important mechanism that results in higher Treg frequencies during HIV infection is the dysregulation of the Th17 /Treg balance. As described in section 1.2.6.3, Th17 cells and Tregs share common developmental lineages. We also discussed that accelerated depletion of Th17 cells in the gut and peripheral blood is one of the earliest consequences of HIV infection. Indeed, a rapid depletion of Th17 cells that protect against microbes in the gut in microbial translocation and excessive immune activation in the gut mucosa are characteristics of early stages of HIV infection (Prendergast *et al.*, 2010). The fall of Th17/Treg ratio has been correlated with disease progression in SIV (Favre *et al.*, 2009) and HIV infections (Falivene *et al.*, 2015) and this imbalance increases Treg proportion in the CD4 T cell pool. In line with this idea, it was demonstrated that HIV elite controllers (HIV- infected individuals, who are untreated but still maintain undetectable plasma viral load and normal CD4 cell count) have lower Treg numbers, with Th17/Treg ratios similar to healthy individuals (Brandt *et al.*, 2011). Th17/Treg imbalance during early stages of infection can severely affect HIV-specific immune responses. For instance, higher proportion of Th17 cells in the gut correlates with higher CD4 T cell restoration in the gut of HIV- infected individuals undergoing ART (Macal *et al.*, 2008). In addition, ART initiation during early acute HIV infection is known to preserve the gut mucosal Th17 population and reverse the systemic immune activation observed as a consequence of early stages of infection (Schuetz *et al.*, 2014). A higher Th17/Treg ratio during early stages of infection has also been correlated with higher protective HIV-specific CD8+ T cells (Falivene *et al.*, 2015).

1.4.3 Dual role of Tregs during HIV infection

During the early acute phase of HIV infection, there is excessive immune activation, particularly the activation of HIV-specific CD8 T cells, in response to an increased viral load (Goonetilleke *et al.*, 2009). Activation of CD4+ and CD8+ T cells rather than the viral load itself has been more closely associated with higher mortality in HIV-infected individuals (Giorgi *et al.*, 1999). Activation of innate immune cells is an important detrimental parameter affecting the mortality rates of HIV-infected individuals (Hunt *et al.*, 2014). It was shown that CD4+ CD25+ CD127- T cells isolated from untreated HIV-infected individuals (more than 80% being FoxP3+) and CD4+ CD25- CD127+ T cells (about 30% being FoxP+), expressed comparable levels of HIV p24, thereby confirming *in vivo* infection of Tregs (Moreno-Fernandez *et al.*, 2009). It has been demonstrated that *in vitro* HIV infection of Tregs isolated from healthy individuals increases their suppressive capacity (Ji et Cloyd, 2009). Tregs isolated from HIV infected individuals suppress the activity of CD8+ T cells and/or CD4+ CD25- T cells (Kinter, A. *et al.*, 2007; Kinter, A. L. *et al.*, 2007), thereby suggesting the protective role of Tregs against excessive detrimental immune activation during early stages of HIV infection.

In vitro infection experiments have shown that Tregs can control HIV replication in conventional T cells through cyclic AMP-mediated mechanisms (Moreno-Fernandez *et al.*, 2011). As mentioned earlier, HIV elite controllers have Treg frequencies comparable to those of uninfected individuals (Brandt *et al.*, 2011). However, it has been shown that the low Treg levels in elite controllers results in generalized immune activation and immune dysfunction, possibly contributing to enhanced CD4 depletion, and also increasing the risk of non-AIDS associated morbidities (Hunt *et al.*, 2011).

Despite low Treg numbers, the *ex vivo* suppressive capacity of Tregs in elite controllers and untreated infected individuals remained comparable (Angin *et al.*, 2012). In summary, Tregs protect against excessive immune activation during early acute stages of HIV infection.

1.4.4 Deleterious role of Tregs in HIV infection

The accumulation of Tregs in lymphoid tissues in HIV-infected humans and SIV-infected rhesus macaques has been directly correlated with disease progression (Nilsson *et al.*, 2006). TGF-β producing Tregs contribute to collagen deposition and lymphoid tissue fibrosis (particularly in GALT), resulting in disease progression (Estes *et al.*, 2007; Zeng *et al.*, 2011). Tregs suppress the activities of HIV-specific CD4 and CD8 T cells and thereby reduce anti-HIV immune responses (Aandahl *et al.*, 2004). It has been reported that the usage of CTLA-4 antibodies to block Treg suppressive functions in combination with ART, during the chronic phase of infection, in SIV-infected rhesus macaques is associated with increased effector CD4+ and CD8+ T cell function, therefore supporting the importance of controlling Treg function, to improve therapeutic strategies (Hryniewicz *et al.*, 2006). As explained in section 1.2.2, Tregs utilize the ecto-nucleotidase CD39 in combination with CD73 to produce adenosine, which inhibits IL-2 production by conventional T cells. Treg-mediated suppression during *in vitro* HIV infection is mediated by the CD39/adenosine axis (Nikolova *et al.*, 2011). Higher CD39 expression correlates with HIV disease progression, HIV viral load and excessive immune activation (Schulze zur Wiesch *et al.*, 2011).

In the gut mucosa of SIV-infected rhesus macaques, FoxP3-expressing Tregs contained more SIV DNA but lesser SIV RNA compared to CD4 T cells, suggesting that although

there is no active viral transcription in Tregs, they do contain the integrated viral DNA (Allers *et al.*, 2010). In line with the these findings, Tran *et al.* showed that Tregs contribute to viral persistence in prolonged HAART-treated patients, owing to the presence of higher HIV DNA in Tregs compared to non-Tregs (Tran *et al.*, 2008).

One of the major problems in achieving HIV cure is the restoration of normal CD4 T cell count. Since IL-2 is required for CD4 T cell proliferation, combination therapy using IL-2 and ART was expected to produce better immune reconstitution in HIV-infected individuals, and two major clinical trials SILCAAT and ESPRIT were conducted. However, despite substantial increase in CD4 T cell count, IL-2 combined with ART did not provide any clinical advantages to individuals undergoing therapy, compared to ART alone (INSIGHT–ESPRIT Study Group, 2009). In individuals under IL-2 immunotherapy, expansion of naive and memory CD4+ CD25+ FoxP3+ cells was found later, which explained the unexpected failure of this immunotherapy (Weiss, L. *et al.*, 2010). On the contrary, IL-7-treated HIV- infected individuals showed increased circulating CD4+ and CD8+ T cells but no increase in Tregs (Levy *et al.*, 2009; Rosenberg *et al.*, 2006; Sereti *et al.*, 2009).

Immune dysfunction during HIV infection has been correlated with higher expression of programmed death 1 (PD-1) on the surface of HIV-specific CD8 T cells. The interaction of PD-1 (expressed on HIV-specific CD8 T cells), with its ligand PD-L1 leads to inhibition of cell proliferation and cytokine production and the blockade of PD-1 leads to restoration of CD8 T cell functions (Trautmann *et al.*, 2006). Furthermore, T helper type 1 (Th1) cells express PDL-1 and can interact with PD-1 to become Tregs (Amarnath, S. *et al.*, 2011). Hence, blockade of the PD1-PDL1 axis could be an optimal

immunotherapeutic strategy for counteracting the negative effects of Tregs during HIV infection and ART (Jenabian *et al.*, 2012). Another important deleterious role of Tregs during HIV infection is mediated by TGF-β production, resulting in collagen deposition by myofibroblasts leading to lymphoid and mucosal tissue fibrosis (Estes *et al.*, 2007). The next section discusses how TGF-β is involved in the different phases of HIV infection.

1.4.5 Role of TGF-β in HIV infection

Since the early 1990s, the role of TGF-β in HIV infection has been studied (Lotz et Seth, 1993). Overexpression of TGF-β protein and mRNA in the supernatants of PBMCs (cultured for one day) from HIV+ treated and untreated donors compared to uninfected individuals has been demonstrated (Kekow *et al.*, 1990). It was shown that HIV gp160 could induce TGF-β mRNA and protein expression in PBMCs isolated from healthy donors (Hu *et al.*, 1996). HIV Tat (transactivation of transcription) protein induces TGF-β in human PBMCs, which could be one of the mechanisms employed by HIV to cause immune suppression (Reinhold *et al.*, 1999). HIV antigens were also shown to induce TGF-β production by CD8+ T cells, thereby reducing their HIV-specific immune functions and enhancing their immunoregulatory functions (Garba *et al.*, 2002).

Owing to its immunoregulatory effects, increased TGF-β levels in the plasma of HIV-infected individuals has been correlated to disease progression (Wiercinska-Drapalo *et al.*, 2004). One of the contexts in which TGF-β has been extensively studied is renal

fibrosis. This cytokine is known to be the master regulator of fibrosis - it stimulates myofibroblasts to produce collagen, thereby triggering the remodeling of extracellular matrix leading to a fibrotic response (Meng *et al.*, 2016). Consistent with this fact, elevated levels of TGF-β are reported in HIV- associated renal diseases (Bodi *et al.*, 1997). HIV infection enhances HCV replication through TGF-β production, thereby accelerating HCV-associated hepatic fibrosis (Lin *et al.*, 2008). In addition, HIV-infected individuals are at a higher risk of suffering from cardiovascular diseases (Freiberg *et al.*, 2017). TGF-β mediates cardiac fibrosis in HIV-infected, untreated individuals and, in treated individuals, the use of the antiretroviral drug ritonavir has been shown to promote cardiac fibrosis (Ahamed *et al.*, 2016). Using humanized mice models, it was shown that anti-retrovirals like ritonavir induce platelet activation, which leads to excessive amounts of TGF-β, in turn leading to cardiac fibrosis and dysfunction (Laurence *et al.*, 2017). In addition, fibrosis of lymphoid tissues resulting from excessive immune activation in HIV-infected individuals and SIV-infected macaques has been shown to be mediated by TGF-β, as it stimulates collagen deposition by myofibroblast cells (Zeng *et al.*, 2011). The contribution of Tregs in producing TGF-β and enhancing lymphoid tissue fibrosis has been clearly shown in SIV infection models (Estes *et al.*, 2007). Furthermore, anti- fibrotic therapy using Pirfenidone, which is an inhibitor of TGF-β signalling, in combination with ART resulted in higher CD4 T cell counts and better immune reconstitution in SIV-infected macaques (Estes *et al.*, 2015), suggesting the benefits of eliminating negative effects of TGF-β during ART.

TGF-β is also known to affect HIV infection of macrophages. The U1 cell line, of pro-monocytic lineage, can induce virus expression upon addition of phorbol myristate acetate and IL-6. Although TGF-β was shown to inhibit HIV replication in U1 cells and in primary *in vitro* infected human macrophages, it did not have any effect on

ACH-2, an infected T cell line or in infected primary T cell blasts (Poli *et al.*, 1991). TGF-β also increases CXCR4 expression on human monocyte-derived macrophages, thereby increasing their susceptibility to X4 tropic HIV strains (Chen, S. *et al.*, 2005). TGF-β could also play a role in the sexual transmission of HIV, as it has been shown that it is the most abundant cytokine present in the semen and it can upregulate the expression of CD169, the main HIV-1 receptor that captures and transmits the virus to its target cells (De Saint Jean *et al.*, 2014).

As explained in section 1.2.5, TGF-β induces FoxP3 expression in CD4 T cells and thereby plays an essential role in the generation of peripheral Tregs. It was also discussed that HIV infection is associated with increased Treg frequencies and higher levels of TGF-β, both of which correlate with disease progression. Amarnath *et al.*, have demonstrated that HIV infection of TCR-stimulated human CD4+ CD25- T cells isolated from PBMCs, led to the upregulation of FoxP3 and blocking TGF-β using anti-TGF-β1/2/3 antibodies decreased FoxP3 expression, explaining that TGF-β mediates FoxP3 induction from CD4+ CD25- HIV-infected T cells (Amarnath, Shoba *et al.*, 2007).

1.4.6 Thymic fucntion during HIV infection in humans

Following the discovery of HIV in the early 1980s, researchers were interested in examining the role of thymus during HIV infection since it is the site for T lymphocyte maturation. Thymic involution was reported in autopsies of thymuses from chronically HIV-infected individuals (Grody *et al.*, 1985) and in children with AIDS (Joshi *et al.*,

1986). Importantly, SIV infection of rhesus macaques showed that lymphocytes and macrophages within the thymus are infected by SIV within two weeks (Baskin *et al.*, 1991). *In vitro,* it was shown that thymocytes are more susceptible to X4 compared to the R5-tropic HIV strains (Nunes-Cabaço *et al.*, 2015), which correlates with a higher expression of HIV co-receptor CXCR4 compared to CCR5 in human thymocytes. *In vitro* cultures of human thymus tissue explants demonstrated that the different DC subsets within the thymus are infected by HIV; infected thymic DCs could spread HIV within developing thymocytes and also impair T cell development (Schmitt, N. *et al.*, 2006).

Although *in vitro* infection models have shown that human thymocytes are infected by HIV, due to the inaccessibility of thymuses from infected individuals, it is still unclear whether this is also true *in vivo.* As summarized by Ho Tsong Fang *et al.* (Ho Tsong Fang *et al.*, 2008), several studies have attempted to understand the impact of HIV infection on thymus tissue and its contribution to immune reconstitution after ART. Analyses of thymuses obtained from autopsies of infected individuals showed signs of inflammation and thymic dysfunction, explaining that lack of thymic contribution to immune reconstitution (Haynes *et al.*, 1999). A method used to determine thymic activity is the measurement of TRECs (T cell Receptor Excision Circles) which involves detection of the excised DNA byproduct that is generated after TCR rearrangement in the thymus. Using this method, several groups have studied the number of recent thymic emigrants (RTEs) in the peripheral blood of people living with HIV. There was decline in TREC levels in few HIV-infected patients compared to uninfected controls while the effect of ART in recovering this decline was not clear (Zhang *et al.*, 1999). The concentration of TREC in the peripheral T cell pool has been shown to be a measure of HIV disease progression, similar to decreasing CD4 T cell counts (Hatzakis *et al.*, 2000), and infected individuals with lower TRECs in peripheral

blood have higher risk of progression to AIDS (Goedert *et al.*, 2001). It was reported that the use of TRECs to identify RTEs is not optimal since TRECs do not replicate during cell division and hence are diluted by rapid proliferation of cells. Hence, the decline in TRECs in peripheral blood following HIV infection and recovery after ART is a consequence of increased peripheral proliferation of T cells resulting from immune activation and not of changes in thymic output (Hazenberg *et al.*, 2000). In developing thymocytes, TCR rearrangement happens at TCRα, β, and δ loci, resulting in different TRECs (excised DNA segments) and TREC assays described so far measured only the TRECs present after rearrangement of TCRα chain. Since the TCR- β chain rearrangement is a prerequisite for TCRα chain rearrangement, measuring the ratio of TRECs generated from TCRβ as well as TCRα gene rearrangements is more accurate and can be considered as a measure of intra-thymic proliferation (Dion *et al.*, 2004). Using this new assay, which measures intrathymic proliferation and is not affected by peripheral T cell proliferation, Dion *et al.* . showed that HIV infection indeed leads to decreased thymic output (Dion *et al.*, 2004). To study the impact of thymus functions during infection, rhesus macaques either subjected to thymectomy or not, were infected with SIV. Thymectomized animals did not have a higher viral load after SIV infection, nor rapid decline in T cells or faster disease progression compared to infected animals with a thymus (Arron *et al.*, 2005). Hence, the aforementioned study shows that the major cause of T cell depletion is loss in peripheral cells and not due to decrease in thymic output or functions (Arron *et al.*, 2005). However, the role of thymus and the mechanisms of Treg generation in human thymus during HIV infection remains unclear.

CHAPTER II

HYPOTHESIS AND OBJECTIVES

2.1 Rationale

Tregs are immunosuppressive T cells with two developmental origins: within the thymus or in the periphery during inflammation (Sakaguchi, 2003). The transcription factor FoxP3 regulates the differentiation and suppressive functions of Tregs (Bettelli *et al.*, 2005; Hori *et al.*, 2003). TGF-β is an anti-inflammatory cytokine that partly regulates FoxP3 expression by Tregs in the thymus (Chen, W. et Konkel, 2015), and during inflammation in other tissues (Chen, W. *et al.*, 2003). It is well documented that people living with chronic HIV infection have higher Treg frequencies in both peripheral blood (Bi *et al.*, 2009) and secondary lymphoid tissues (Andersson *et al.*, 2005). TGF- β contributes to Treg development in secondary lymphoid tissues, as well as in inflammatory sites, notably in GALT (Konkel et Chen, 2011), and is also the main cause for lymphoid tissue fibrosis and disease progression in HIV-infected individuals (Theron *et al.*, 2017; Zeng *et al.*, 2011). It is plausible that, besides promoting peripheral Treg induction, TGF-β might contribute to the induction and maintenance of Tregs within the thymus during HIV infection.

2.2 Hypothesis

TGF-β induces FoxP3 expression in thymocytes and thus contributes to increased thymic Treg frequencies during HIV infection.

2.3 General Objectives

To investigate changes in FoxP3 expression and thymic Treg differentiation upon TGF-β treatment during *in vitro* HIV-1 infection of human thymocytes.

2.4 Specific Objectives

1. To characterize the *ex vivo* phenotype of human thymocytes and thymic Tregs.
2. To assess *ex vivo*, the epigenetic status of non-coding regulatory sequences of the FoxP3 gene in subsets of thymocytes based on CD3 expression levels.
3. To study the impact of TGF-β treatment on human thymocytes and thymic Treg generation.
4. To evaluate the effect of *in vitro* HIV-1 infection and TGF-β treatment of human thymocytes.

CHAPTER III

MATERIALS AND METHODS

3.1 Human thymus tissues and isolation of thymocytes

Portions of human thymus tissues were obtained from children undergoing emergency cardiac surgery, in collaboration with Dr. Jonathan Angel, Ottawa Hospital and Research Institute (OHRI), Canada and Dr. Gyaandeo Maharajh, Children's Hospital of Eastern Ontario (CHEO), Canada. Age and gender were known only for two specimens; both were females, less than 5 months old. Thymus tissues were cut into small sections using sterile scissors and the pieces were placed on a 70μm cell strainers (ThermoFisher Scientific, Canada) dipped in sterile RPMI (Wisent, Canada) with 10% fetal bovine serum (FBS) (Wisent, Canada) and 1% antibiotic-antimycotic (Wisent, Canada) inside a tissue culture plate. Tissue was macerated using the plunger of a 3mL syringe to facilitate release of cells into the media. Cells were collected and the media was centrifuged for 5 min. The cell pellet obtained was re-suspended in 25mL of RPMI and the cells were carefully added to 20mL of Ficoll (density 1.077 g/mL, Wisent, Canada) kept in a 50mL falcon tube and were centrifuged at 1800 r.p.m. for 25 min with no acceleration and no brakes. After the density gradient separation using Ficoll, lymphocytes were visualized as a ring of cells at the center, were collected and washed twice with 1X PBS (Wisent, Canada) and re-suspended in RPMI + 10% FBS. Cells were counted using a haemocytometer and were either cryo-preserved or used for flow cytometry (Figure 3.1). For cryopreservation in liquid nitrogen, aliquots of 50M thymocytes in cryo-vials were made using FBS and 10% DMSO (Sigma Aldrich, Canada) and kept at -80°C in Mr. Frosty freezer boxes (ThermoFisher) overnight and were transferred to liquid nitrogen the following day.

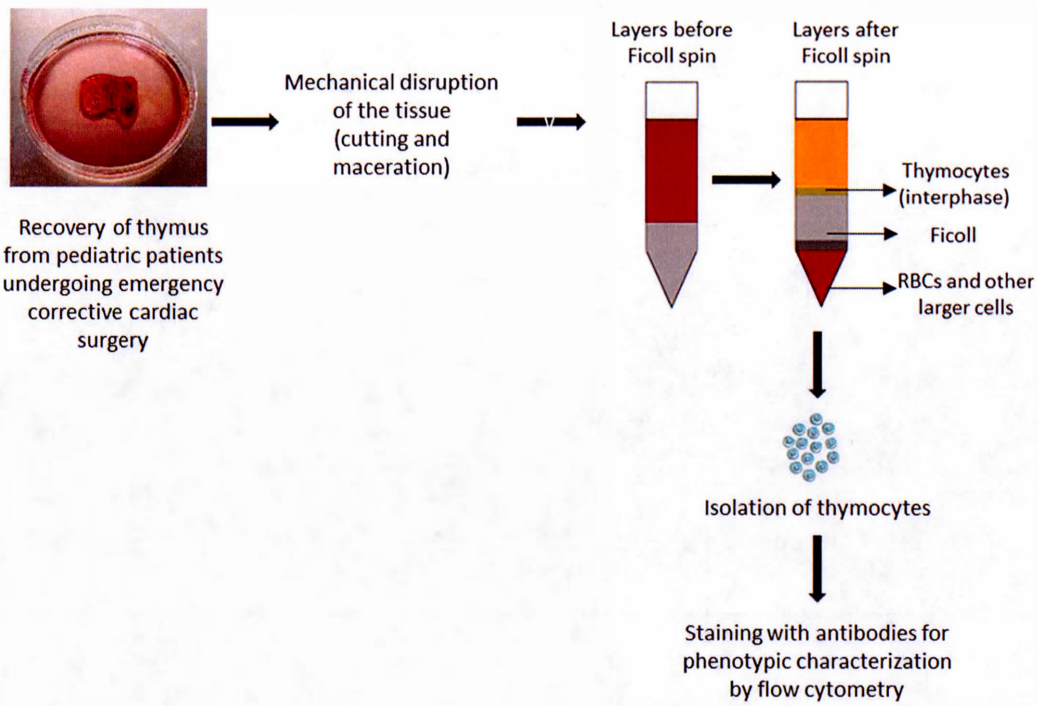

Figure 3.1: Steps involved in the *ex vivo* phenotypic characterization of human thymocytes

3.2 HIV-1 viral stocks

Plasmids of 110NB (macrophage-tropic virus that uses CCR5 co-receptor) and NL4.3 (T cell-tropic virus that uses CXCR4 co-receptor) HIV-1 viral strains were obtained from Dr. Petronela Ancuta, CRCHUM. Stocks of viral strains were generated in the laboratory by transfecting HEK293T cells with the plasmids. Briefly, 293T cells donated by Dr. Nicolas Pilon (UQAM) were seeded in a 10cm tissue culture plate containing DMEM supplemented with penicillin (100 units/mL), streptomycin (100 µg/mL) and 10% heat-inactivated FBS and incubated for 24 hours at 37°C/5% CO_2 until 60-70% of confluence. The following day, transfection complexes were formed by combining an HIV-1 plasmid along with GeneJuice Transfection Reagent (9µg of

plasmid:18μL of GeneJuice) (Novagen, USA) and DMEM. The transfection complexes were allowed to incubate for 30 min at room temperature. The complexes were then added to the plate and incubated at 37°C/5% CO_2 for 4–6 hours. Following a change of media after the 4–6 hours of incubation, the cells were incubated at 37°C/5% CO_2 in complete media for 48 hours. The virus-containing media was then harvested from the plates, centrifuged at 2500 rpm for 20min to remove cell debris, and then passed through a 45-μm-pore-size filter. Viral stocks were stored at -80°C. Viral concentration in the supernatants were measured by p24 ELISA, using homemade kits as described (Gosselin *et al.*, 2010), and were then used for infection of thymocytes.

3.3 *In vitro* assays with primary human thymocytes

In vitro assays were performed either with freshly isolated or thawed thymocytes. Thawed thymocytes were treated with DNase (Stem Cell Technologies, Canada) for 15 min to prevent cell clumping. Dead cells were separated from live cells by staining with LIVE/DEAD™ Fixable Green Dead Cell Stain Kit, for 488 nm excitation (ThermoFisher) for 30 minutes and sorting with BD FACSJazz™ cell sorter. Live cells were collected in FBS and were used for culture.

3.3.1 Co-culture model

Human thymocytes were co-cultured with the mouse stromal cell line OP9-DL1, which expresses delta-like-1 (DL-1), the ligand for Notch. The ratio of thymocyte to OP9-DL1 was 25:1 (Angel, 2012). As explained in section 1.1.3, Notch signaling is essential

for T cell differentiation and lineage commitment in the thymus. A previous study (Holmes et Zúñiga-Pflücker, 2009) reported that *in vitro* differentiation of T cells is achieved by co-culturing hematopoietic or embryonic stem cells with OP9-DL1. This cell line was provided by Dr. Zúñiga-Pflücker, University of Toronto. It maintained in culture in AMEM (Wisent) plus 20% FBS with 1% antibiotic-antimycotic, and passaged every 2 or 3 days using trypsin-EDTA (Wisent).

3.3.2 *In vitro* TGF-β treatment

One million freshly isolated or thawed and FACS-sorted alive thymocytes were re-suspended in McCoy's 5A media (Wisent), with 10% FBS and 1% antibiotic-antimycotic. Treatment with the TGF-β1 inhibitor Pirfenidone (2.6mM) (PFD, Selleck chemicals, Houston, TX) was done for 1 hour and media with PFD was removed prior to addition of TGF-β1. Lyophilized stock of TGF-β protein was diluted in sterile H_2O containing 0.1% BSA and stored in aliquots at -20°C. Thymocytes were treated with 10ng/mL of recombinant human TGF-β (Abcam, Cambridge, UK) and were co-cultured with OP9-DL1 as described in the section 3.3.1.

3.3.3 HIV infection and TGF-β treatment

One million freshly isolated or thawed thymocytes resuspended in RPMI and were treated with polybrene (hexadimethrine bromide, added to increase infection rate, Sigma Aldrich, Canada) at 3μg/10^6 cells for 1 hour, at 37°C in 24 well cell culture

plates (SARSTEDT, Germany). Media with polybrene was removed and laboratory-constructed strains of 110NB (R5) or NL4.3 (X4) HIV-1 viral stocks were added to the cells at a concentration of 50ng of p24/mL per $1x10^6$ cells. The plates were centrifuged at 1200 r.c.f for 3 hours at 25°C to facilitate infection (spinoculation). After 3 hours, cells were washed twice with media and treated with TGF-β1 at 10ng/mL and co-cultured with OP9- DL1, as described in the section 3.3.1. Cells were cultured for 2 days post-infection and flow cytometric staining was performed as shown in Figure 3.2.

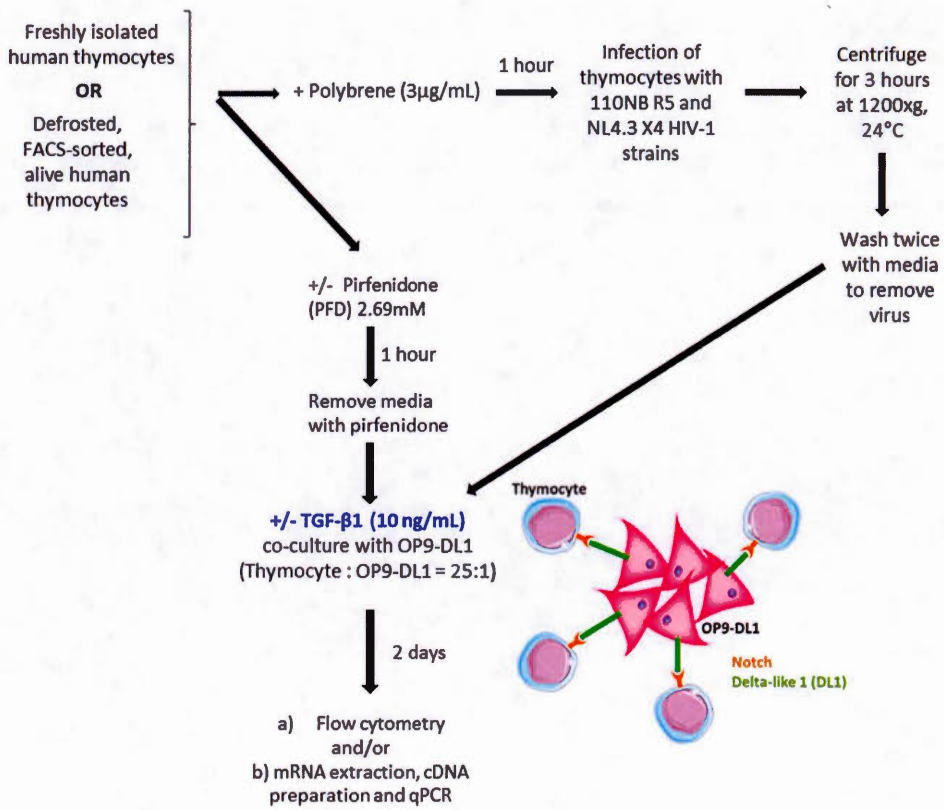

Figure 3.2: Steps involved in the *in vitro* TGF-β treatment and HIV infection of human thymocytes

3.4 Flow cytometry

For *ex vivo* phenotypic characterization and *in vitro* experiments, 1-2 x 10^6 thymocytes (either fresh or thawed) were resupended in 250µL of FACS-staining buffer (1X PBS + 2% FBS). Cells were then incubated with antibody-cocktails for 45-60 min, washed with 3mL of 1X PBS, centrifuged at 1500 rpm for 5 min. The BD transcription buffer kit (BD Pharmigen, Canada) was used to permeabilize cells for intracellular staining. Cells were incubated with intracellular antibodies for 45-60 min and washed according to the protocol described in the BD transcription buffer kit. The antibodies used for staining are listed in Table 3.1 below. Stained samples were acquired through BD LSRFortessa™ X- 20 multicolour flow cytometer and were analyzed with FlowJo version 10.5.3.

Table 3.1: List of antibodies used for flow cytometry

#	Antibody	Flurochrome	Clone	Company	Conc. per test sample (μg)
1	Mouse Anti-human CD3	FITC	HIT3a	BD Pharmingen	0.4
2	Mouse Anti-human CD3	Alexa Fluor 700	UCHT1	BD Pharmingen	0.4
3	Mouse Anti-human CD4	FITC	RPA-T4	BD Pharmingen	1
4	Mouse Anti-human CD4	PEcy5	RPA-T4	BD Pharmingen	1
5	Mouse Anti-human CD4	BV650	L200	BD Pharmingen	1
6	Mouse Anti-human CD8	APC-H7	SK1	BD Pharmingen	1
7	Mouse Anti-human CD25	BV786	M-A251	BD Horizon	1
8	Mouse Anti-human CD25	PE	M-A251	BD Pharmingen	1
9	Mouse Anti-human CD127	PE-Cy7	HIL-7R-M21	BD Pharmingen	1
10	Mouse Anti-human CD39	BV711	TU66	BD Pharmingen	1
11	Mouse Anti-human CD73	BV605	AD2	BD Horizon	1
12	Mouse Anti-human CD45RA	BV650	HI100	BD Horizon	1
13	Mouse Anti-human CD45RO	APC	UCHL1	BD Pharmingen	1
14	Mouse Anti-human CD45RO	PerCP-Cy5.5	UCHL1	BD Pharmingen	1
15	Mouse Anti-human CD183 (CXCR3)	PEcy5	1C6/CXCR3	BD Pharmingen	1
16	Mouse Anti-human CD184 (CXCR4)	BV711	12G5	BD OptiBuild	1

17	Mouse Anti-human CD195 (CCR5)	BV605	2D7/CCR5	BD Horizon	1
18	Mouse Anti-human CD196 (CCR6)	PE	11A9	BD Pharmingen	1
19	Mouse Anti-human CD197 (CCR7)	Alexa Fluor 647	150503	BD Pharmingen	1
20	Mouse Anti-human CD199 (CCR9)	Alexa Fluor 488	112509	BD Pharmingen	1
21	Rat Anti-mouse/human Integrin β7	PE	FIB504	BD Pharmingen	1
22	Mouse Anti-human RORγt	Alexa Fluor 647	Q21-559	BD Pharmingen	1
23	Mouse Anti-human RORγt	BV421	Q21-559	BD Horizon	1
24	Armenian hamster Anti-mouse/human Helios	Pacific blue	22F6	BioLegend	1
25	Armenian hamster Anti-mouse/human Helios	Alexa Fluor 647	22F6	BD Pharmingen	1
26	Mouse Anti-human FoxP3	PE-CF594	236A/E7	BD Horizon	1
27	KC57-RD1 (p24)	PE	Coulter Clone	Beckman Coulter	1
28	LIVE/DEAD™ Fixable Green Dead Cell Stain Kit, for 488 nm excitation	-	-	Thermo Fisher	1
29	LIVE/DEAD™ Fixable Green Dead Cell Stain Kit, for 405 nm excitation	-	-	Thermo Fisher	1

3.5 Methylation sequencing of *foxp3*

3.5.1 Cell sorting

Freshly isolated thymocytes were stained with anti-CD3 and were FACS-sorted using BD FACSJazz™ cell sorter into $CD3^{low}$, $CD3^{int}$, $CD3^{high}$ as shown in the Figure 3.3. One million cells were stored as dry pellets or in 500µL Trizol (Invitrogen), to be used for DNA and RNA extraction respectively.

3.5.2 Genomic DNA extraction and bisulfite treatment

Genomic DNA was extracted from $CD3^{low}$, $CD3^{int}$, $CD3^{high}$ thymocytes using DNeasy Blood & Tissue Kits (Qiagen, Canada). To improve DNA yield, proteinase K (at the concentration specified in the kit) treatment was done overnight at 56°C. The remaining steps of the protocol were the same as described in the kit. Genomic DNA concentrations were measured using nano-drop (ThermoFisher). Isolated genomic DNA was subjected to treatment with sodium bisulfite, according to the protocol described in the EpiTect Bisulfite Kits (Qiagen). Sodium bisulfite deaminates unmethylated cytosine to uracil, which upon PCR amplification is treated as equivalent to thymines as shown in Figure 3.3. The methylated cytosines are not affected by sodium bisulfite treatment. This technique allows to identify methylation at the level of single nucleotides.

3.5.3 Nested PCR and MiSeq

To increase sensitivity and specificity, nested PCR was performed using two sets of primers. The first round of PCR was performed using external primers listed in Table 3.2, with bisulfite treated genomic DNA template from FACS-sorted CD3 subsets of thymocytes. The resulting PCR amplicons were used as the template for the second PCR using internal primers listed in Table 3.3. Platinum™ Taq DNA Polymerase (Invitrogen) was used for both rounds. For the all the six regions in the *foxp3* gene locus, external and internal primers were designed to anneal to bisulfite converted DNA.

Table 3.2: List of external primers used for first round of PCR

Region	Primer Sequence (5' to 3')	Product size (bp)	Annealing Temperature (°C)
CNS0	F:AGTTGTTGTTGTTGGGG R:AAAAATTTTACCTAATCC-CCACATT	583	52
Enhancer	F: CGAAGGAGCGGTTTA-GTACG R: TACTATTCGCCGACGAAA	417	59
Proximal Promoter	F:TTTAAAAACCTTACCTAA-CTAAAATCA R:TTTTATATATTTTTGTTTT AAAAATTGTGG	547	50
CNS1	F:GATGGTTTTATATAGTTA-AATAGGTTAGTTT R:CATATACCCTAAAATACC-CAAATCCA	442	51.6
CNS2	F: TAGGGGTTTGTTATAGTG-GTTAGAT R: TTTCTAAAACACAAA-TTATATTTTCATATCGA	284	52
CNS3	F: TTTGTTTTGATTAGA-GGAGTGTTTA R: AAAACCTCCAATCTCT-AAAACC	553	50.8

Table 3.3: List of internal primers used for second round of PCR

Region	Primer sequence with NEXTRA tags (5' to 3')	Product size (bp)	Annealing Temperature (°C)
CNS0	F: ACACTGACGACATGGTTCTAC-AGTAAAGGGTAGTTGGAAGGTAAAG R: ACGGTAGCAGAGACTTGGTCT-CCCCACATTTTCGCTACCGCCCG	402	52
Enhancer	F:ACACTGACGACATGGTTCTAC-AAATGTGGGGATTAGGTAAAATTT R: TACGGTAGCAGAGACTTGGTCT-AAACCTTAAAACTACCACTAAC	232	59
Proximal Promoter	F: CACTGACGACATGGTTCTAC-AGATATTTTTTATTTTTGTGGTGAG R:TACGGTAGCAGAGACTTGGTCT-CCTCCAATAAAACCCACATCTAAT	401	50
CNS1	F:CACTGACGACATGGTTCTAC-AGTTTTTGTTTTTGGTTTTGTGT-TTAGAA R:TACGGTAGCAGAGACTTGGTCT-CTTAAATTTATTATATATCAAACC-TTATACT	338	51.6
CNS2	F:ACACTGACGACATGGTTCTAC-ATTTGGGTTAAGTTTGTTGTAGG-ATAG	187	52

	R:TACGGTAGCAGAGACTTGGTC-AAATCTACATCTAAACCCTATTA-TCAC		
CNS3	F:ACACTGACGACATGGTTCTAC-ACGATGTTTAATTTTAGGTTTGG-TAAG R: TACGGTAGCAGAGACTTGGTCT-TTCTTAACCCTACAACATCTACAT	387	50.8

The internal PCR primers had a NEXTRA tag (highlighted in grey in Table 3.3) which facilitated library preparation for MiSeq. 20µL PCR reactions were carried out according to the molar concentrations mentioned in the Table 3.4.

Table 3.4: Constituents of PCR reaction

Master-Mix	Volume (µl)
Taq Buffer 10X	2
dNTP (10 mM)	0.5
H_20 Sterile	13.25
Taq Polymerase	0.25 (5U)
Template/H_20	2
F. primer (10 µM)	1
R. primer (10 µM)	1
Total	20

PCR program consisted of the following cycles: initial denaturation, 95°C, 2 min, followed by 40 cycles (for the first PCR) and 25 cycles (for the second PCR) of: 1. second denaturation for 30 sec at 95°C, 2. annealing (at different temperatures for each primer pair) for 40 sec followed by 3. extension step at 72°C for 1 min and a final extension at 72°C for 10 min. PCR amplicons were run on 2% agarose (Bio Basic, Canada) gel at 120V for 30 min and imaged with Gel Doc (BioRad). The PCR amplicons were submitted to IRIC for library preparation, MiSeq and bioinformatic analysis by Bismarck.

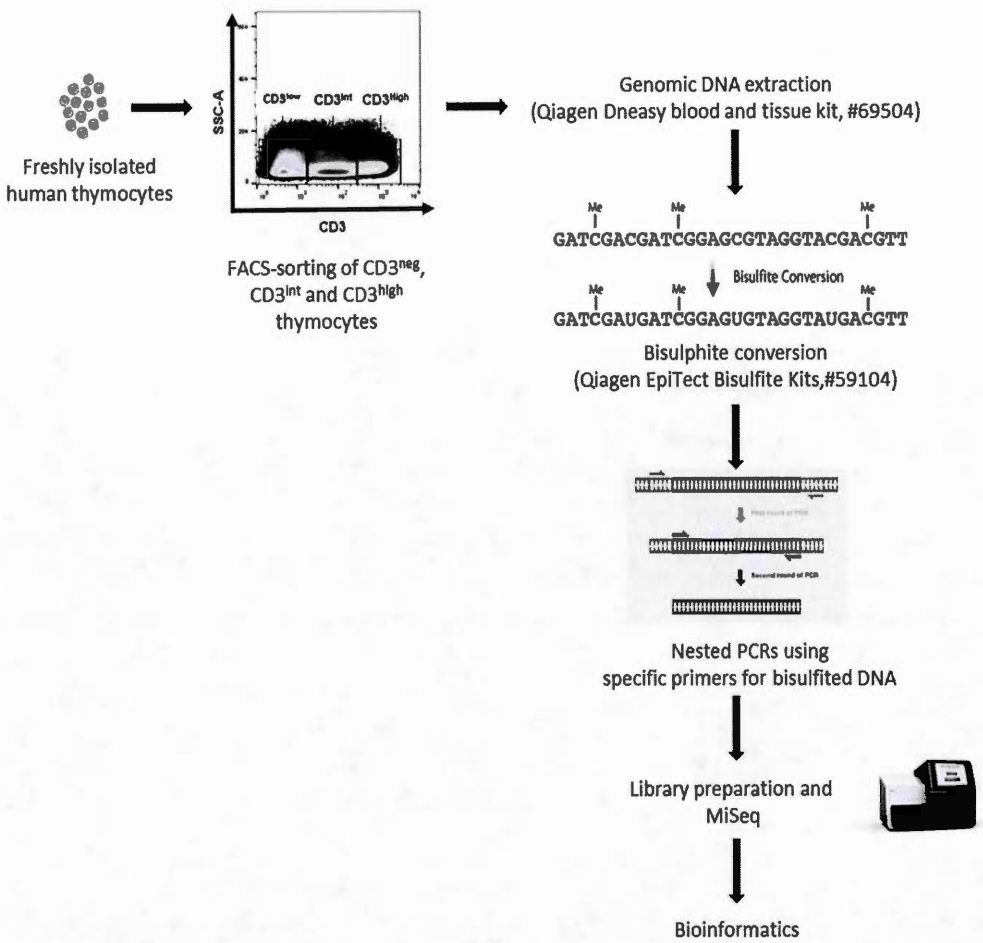

Figure 3.3: Steps involved in the methylation sequencing of *foxp3*

3.6 qPCR

RNA was extracted by the Trizol-chloroform method :1 x 10^6 cells were treated with 0.5mL of Trizol. One mL of choloroform was used per mL of Trizol reagent to achieve phase separation. Acqueous phase was carefully transferred to new tubes after centrifugation at 12000 r.p.m. for 15 min at 4°C and glycoblue, (ThermoFisher) was added as carrier for RNA. One mL of Isopropyl alcohol per mL of Trizol reagent was added to the aqueous phase and incubated for 10-15 min. RNA was pelleted by centrifuging samples at 12000 r.p.m. for 15 min. Pellets were resuspended in RNase free water. RNA concentration was measured using nano-drop. Reverse transcription PCR was performed using MML-V reverse transcriptase (Invitrogen, Canada) and oligo-dT primers (Invitrogen). RNA concentrations were normalized in all samples before cDNA synthesis. To protect RNA from degradation by RNAses, RNaseOUT™ (Invitrogen) recombinant ribonuclease inhibitor was used. qPCR samples were prepared using LightCycler® 480 SYBR Green I Master kit (Roche, Canada) in white, 96 well, LightCycler Plates, (Progene PCR plates, UltiDent, Canada) and were covered with plate seals (Invitrogen). Primers specific to the human cDNA are shown in Table 3.5. Expression levels of the gene of interest was determined relative to expression level of β-actin (housekeeping gene) in each sample. The plates were run in LightCycler® 480 instrument (Roche) with the following program: 95°C for 5min, then 40 amplification cycles of 15 sec at 95°C, 20 sec at 59°C (62°C for TGF- β2) and 20 sec at 72°C; one cycle of 5 sec at 95°C, and 30 sec at 65°C; and 10 sec at 40°C.

Table 3.5: Primers used for qPCR

DNMT1	F: GCAAACCACCATCACATCTCATT R: TCTTTCTCGTCTCCATCTTCGTC
DNMT3a	F: GGGAAGATCATGTACGTCGGG R: TGTCCCTCTTGTCACTAACGC
DNMT3b	F: GACAGCTCTCCAATACTCAGGTT R: AACATTTTCTCTTCTGCTTGCCC
TGF-β1	F: TACCTGAACCCGTGTTGCTC R: AGTGAACCCGTTGATGTCCA
TGF-β2	F: GTGCTCTGTGGGTACCTTGAT R: ATCCCAGGTTCCTGTCTTTATGG
TGF-β3	F: CCGAGTGGCTGTCCTTTGAT R: TCCTCATTGTCCACGCCT
β-Actin	F: CCCTGGAGAAGAGCTACGAG R: CGTACAGGTCTTTGCGGATG

3.7 Biostatistics

Statistical analyses were performed using GraphPad version 6.01. (GraphPad Software, La Jolla, California, USA). Results are presented as median. Wilcoxon matched-pairs signed-rank test was used to compare paired study variables. To compare normally distributed data, paired student's *t* test was used. For comparing the methylation analyses, Chi square test was used. The statistical methods for each data set is also clearly mentioned in figure legends and p values are shown only in cases where the differences were significant.

CHAPTER IV

RESULTS

4.1 *Ex vivo* phenotypic characterization of human thymocytes using flow cytometry

To study the process of T cell development in the human thymus, thymocytes were classified based on their expression level of CD3, which highly correlates with thymocyte maturation (Levelt, Carsetti, *et al.*, 1993; Levelt, Ehrfeld, *et al.*, 1993). Thus, $CD3^{low}$ cells represent the most immature thymocytes and $CD3^{high}$ cells represent the most mature thymocytes (Figure 4.1A and 4.1B). All the analyses shown in Figure 4.1 to 4.5 were performed in these three subsets of thymocytes to define how development and maturation of Tregs occurs in the human thymus. According to the process of thymocyte development (as explained in the introduction section 1.1), the highest percentage of DP CD4+ CD8+ cells was found in the $CD3^{low}$ and $CD3^{int}$ subsets (Figure 4.1C, 4.1D and 4.1E). As expected, higher percentages of SP (CD4+ CD8- and CD8+ CD4-) were found in the $CD3^{high}$ subset (Figure 4.1F).

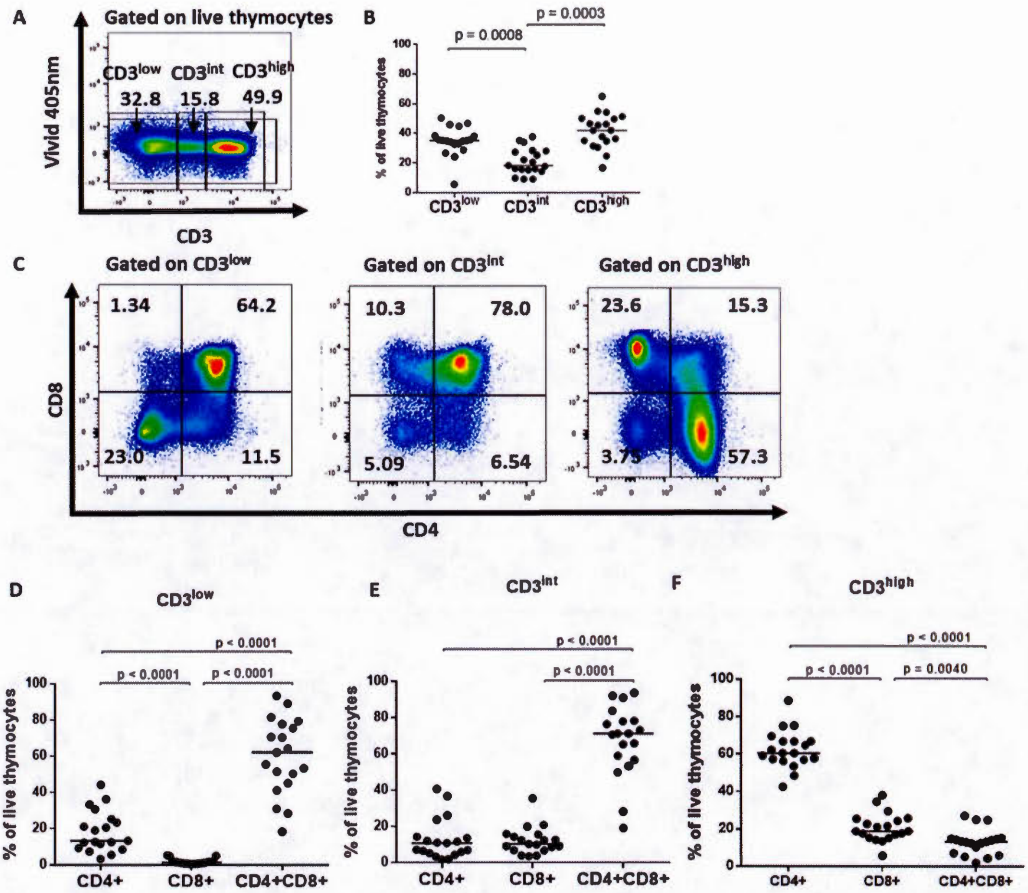

Figure 4.1: Relation between thymocyte maturation and CD3, CD4 and CD8 expression. *Ex vivo* human thymocytes were classified as $CD3^{low}$, $CD3^{int}$ and $CD3^{high}$ as represented by the gating strategy (A) and their frequencies (B). Within each CD3 subset, the populations of SP CD4+ CD8-, CD4- CD8+ and DP (CD4+ CD8+) thymocytes were analysed according to the gating strategy shown in (C). The frequencies of SP and DP thymocytes within $CD3^{low}$ (D), $CD3^{int}$ (E), and $CD3^{high}$ cells (F) are shown. Lines represent median, and the data from 19 thymuses are compared using Wilcoxon matched-pairs signed rank test; p values are shown only in cases where the differences were significant.

Tregs were defined as CD4+ CD127- CD25+ FoxP3+ (Figure 4.2A, 4.2B). CD8+ T cells were also excluded from the analysis. In accordance with the process of CD4 T cell development, the highest percentage of Tregs within CD4+ cells were found within the $CD3^{high}$ subset (Median: $CD3^{high}$ 6.32%, $CD3^{int}$ 4.56%, $CD3^{low}$ 0.21%, Figure 4.2C). Expression of the ecto-nucleotidase CD39 (refer section 1.2.2) within Tregs was also investigated. The frequency of CD39+ Tregs was higher within $CD3^{low}$ (Median: 50%) and $CD3^{int}$ (Median: 48.6%) thymocytes compared to the $CD3^{high}$ subset (Median:17.2%).

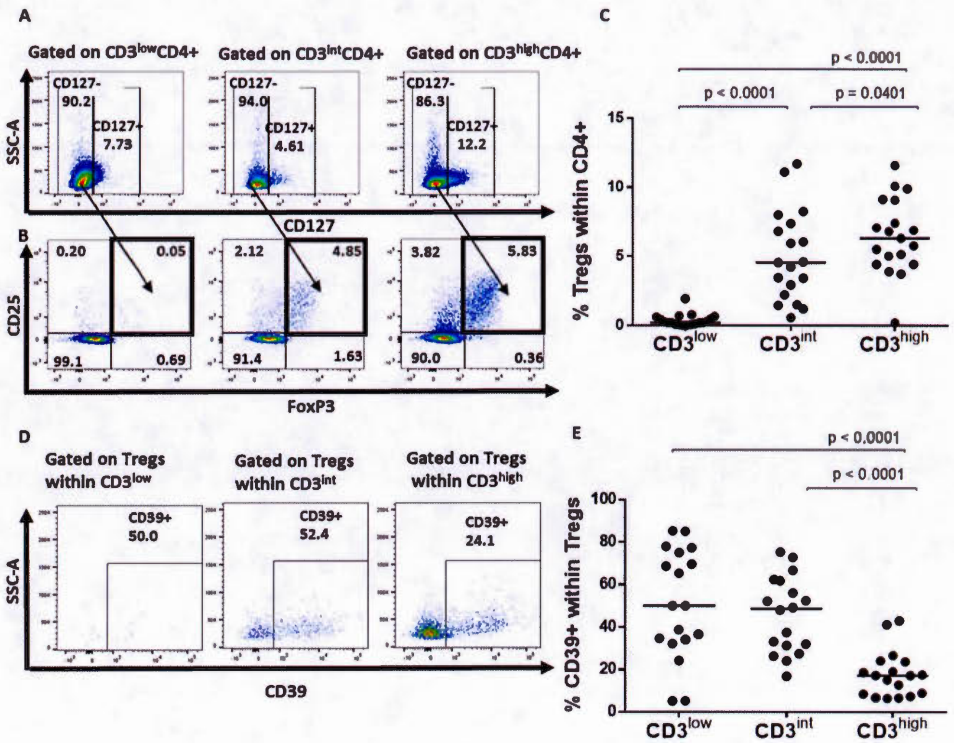

Figure 4.2: Phenotypic characterization of thymic Tregs. A sequential gating strategy was used to define Tregs: for each CD3 subset, CD127- within CD4+ thymocytes (i.e. CD4+ CD8- thymocytes) were gated (A). FoxP3+ CD25+ thymocytes were discriminated within this group as shown in (B). Tregs were identified as CD4+ CD127- CD25+ FoxP3+. The frequencies of tTregs are shown in (C). Expression of ecto-nucleotidase CD39 in human tTregs was analyzed (D). The frequency of CD39+

Tregs is shown in (E). Lines represent median, and the data from 19 thymuses (C), and 18 thymuses (E), were compared using Wilcoxon matched-pairs signed rank test; p values are shown only in cases where the differences were significant.

As mentioned in the introduction section 1.2.6.3, Tregs and Th17 cells from peripheral blood share common developmental precursors. During inflammatory conditions, naïve CD4+ T cells start expressing RORγt (the master transcription factor of Th17 cells) in response to stimulation by the cytokines TGF-β and IL-6. Based on this information, the expression of RORγt was followed in thymocytes within the three different CD3 populations (Figure 4.3A). The percentage of CD4+ RORγt+ thymocytes was higher in $CD3^{low}$ (Median: 34.59%), and $CD3^{int}$ (Median: 25.95%), compared to $CD3^{high}$ thymocytes (Median: 3.88%, Figure 4.3B). Recently, RORγt expressing FoxP3+ Tregs have been described in the context of intestinal inflammation (Yang, B. H. *et al.*, 2016). Despite expressing both the Th17 and Treg master transcription factors, these cells had higher suppressive capacity compared to FoxP3+ RORγt- Tregs and also had the Treg specific demethylation at the FoxP3 gene locus (Yang, B. H. *et al.*, 2016). Hence, it was also checked whether FoxP3 and RORγt are co-expressed within the thymus, and was found that CD4+ thymocytes did not co-express FoxP3 and RORγt (Figure 4.3C).

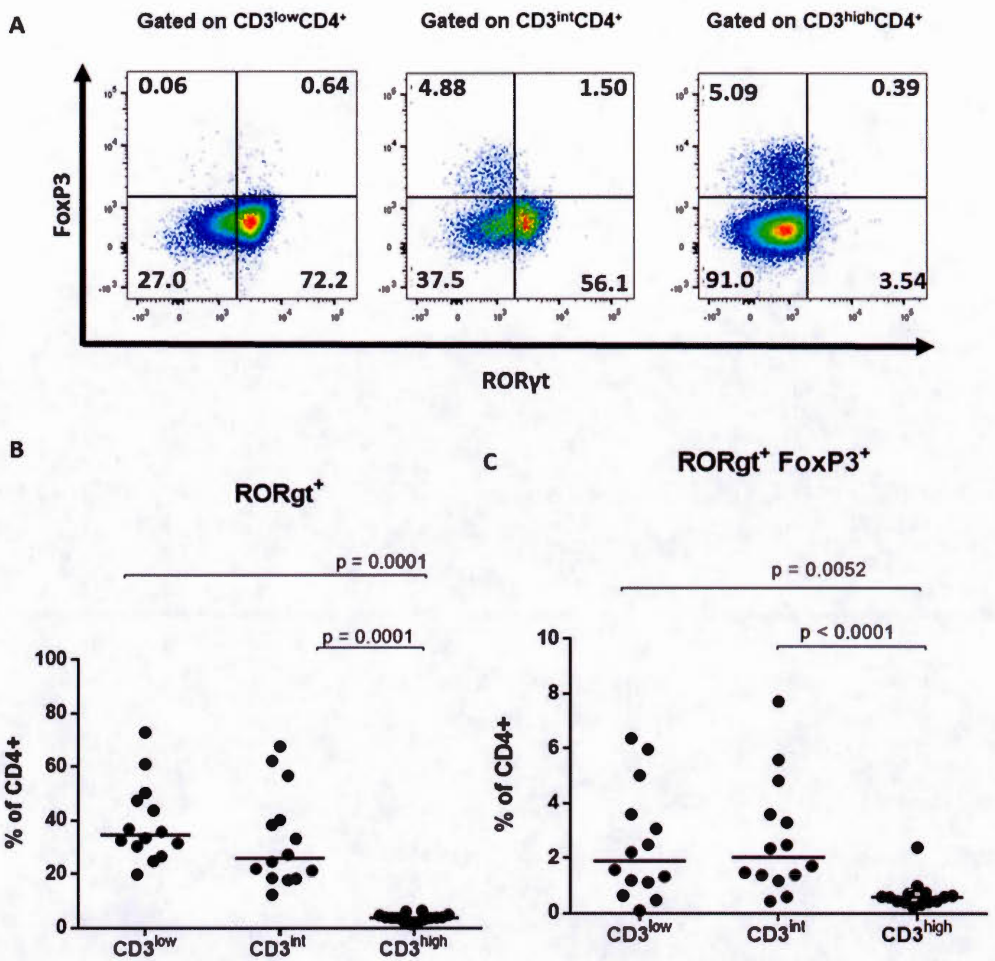

Figure 4.3: Mature thymocytes lose RORγt expression and do not co-express FoxP3 and RORγt. CD4+ thymocytes segregated according to their CD3 expression levels were further analyzed for FoxP3 and RORγt expression (A). The frequencies of CD4+ thymocytes expressing only RORγt (B) or co-expressing FoxP3 and RORγt (C) are shown. Lines represent median and the data from 14 thymuses were compared using Wilcoxon matched-pairs signed rank test; p values are shown only in cases where the differences were significant.

Helios is a transcription factor of the Ikaros family of zinc finger proteins, which is required for sustained inhibitory capacity of Tregs. Indeed, CD4+ FoxP3+ cells from Helios deficient mice had impaired suppressive functions compared to cells from wild type mice (Kim, H. J. *et al.*, 2015), and targeted deletion of Helios in Tregs results in their conversion to effector T cells (Nakagawa *et al.*, 2016). Hence, the expression of Helios was studied in CD4+ FoxP3+ thymocytes within the three CD3 populations. All CD4+ FoxP3+ thymocytes, regardless of their CD3 expression levels were found to be Helios+ (Figure 4.4). Although few Helios- cells were measured, it is to be noted that there is almost no FoxP3 expression by $CD3^{neg}$ population (Figure 4.4C), as shown in the representative image in Figure 4.4A.

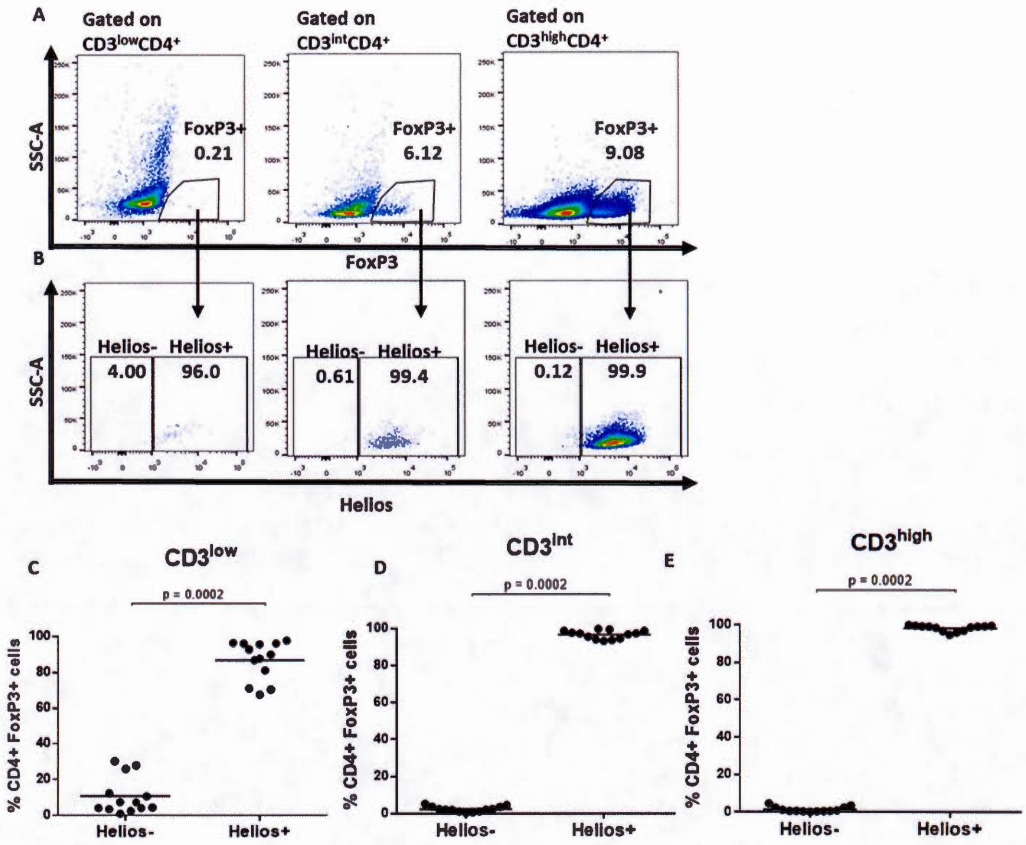

Figure 4.4: CD4+ FoxP3+ thymocytes always express Helios. CD4+ thymocytes segregated according to their CD3 expression levels (low, intermediate or high) were further analyzed for FoxP3 expression (A) and gated as Helios- and Helios+ and their frequencies within $CD3^{low}$ (C), $CD3^{int}$ (D), $CD3^{high}$ (E) are shown. Lines represent median and the data from 13 thymuses were compared using Wilcoxon matched-pairs signed rank test; p values are shown only in cases where the differences were significant.

The protein tyrosine phosphatase CD45RA isoform is a marker for naive T cells while the CD45RO isoform is considered a marker for memory T cells in the peripheral blood

(Akbar *et al.*, 1988; Merkenschlager *et al.*, 1988). CD45RA to CD45RO isoform switching occurs in TCR activated CD4 T cells following antigen recognition, i.e., naive to memory phenotype switching occurs (Johannisson et Festin, 1995). Thymocytes, on the other hand, are only exposed to self-antigens and stimulated by self-antigens through their TCRs. Some of these cells will survive as part of the positive selection process. For these reasons, expression of both CD45RA and CD45RO by $CD3^{high}$ thymocytes was investigated. A higher percentage of CD45RO+ Tregs (Median: 64.42%) was observed compared to CD45RA+ Tregs (Median: 22.95%), and the same was found in non-Tregs as well (CD45RO+ non-Tregs Median: 51.52%, CD45RA+ non-Tregs Median: 20.32%, Figure 4.5). Tregs have been analyzed exclusively within the $CD3^{high}$ subset (and not within $CD3^{int}$ or $CD3^{low}$) in all the upcoming figures, since their numbers are highest within this subset, as shown in Figure 4.2.

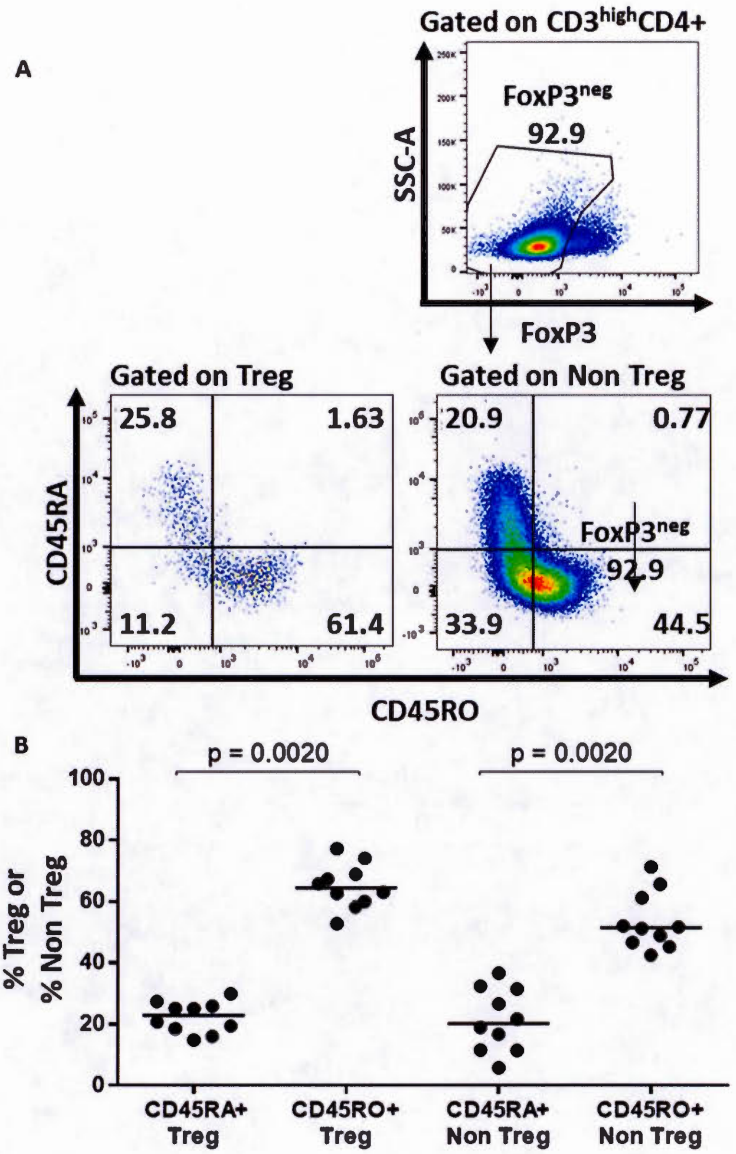

Figure 4.5: Higher expression of CD45RO than CD45RA by thymic Tregs and non-Tregs. Treg ($CD3^{high}$ CD4+ CD127- CD25+ FoxP3+) and non-Tregs (CD4+ FoxP3-) gated on $CD3^{high}$ subset were analyzed for the expression of CD45RA and CD45RO (A). The gating strategy for CD4+ cells excluded CD4+ CD8+ thymocytes from the analysis. The frequencies of Tregs and non-Tregs, either CD45RA+ or CD45RO+ are

shown in (B). Lines represent median and the data from 10 thymuses were compared using paired student t tests; p values are shown only in cases where the differences were significant.

Several studies have suggested the existence of recirculating Tregs in the thymus (Thiault *et al.*, 2015). Cowan *et al.,* reported that recirculating Tregs express CCR6 while *de novo* thymic Tregs express CCR7 (Cowan *et al.*, 2016). In our analyses, compared to non-Tregs, which do not express CCR6 (Median: 0.65%), a small proportion of thymic Tregs (Median: 3.78%) were found to express this receptor, which is required for migration to inflammatory sites. The percentage of CCR7+ (a marker that directs thymocytes from the cortex to the medulla) cells was higher within non-Tregs (Median: 20.17%) compared to Tregs (Median: 7.15%, Figure 4.6).

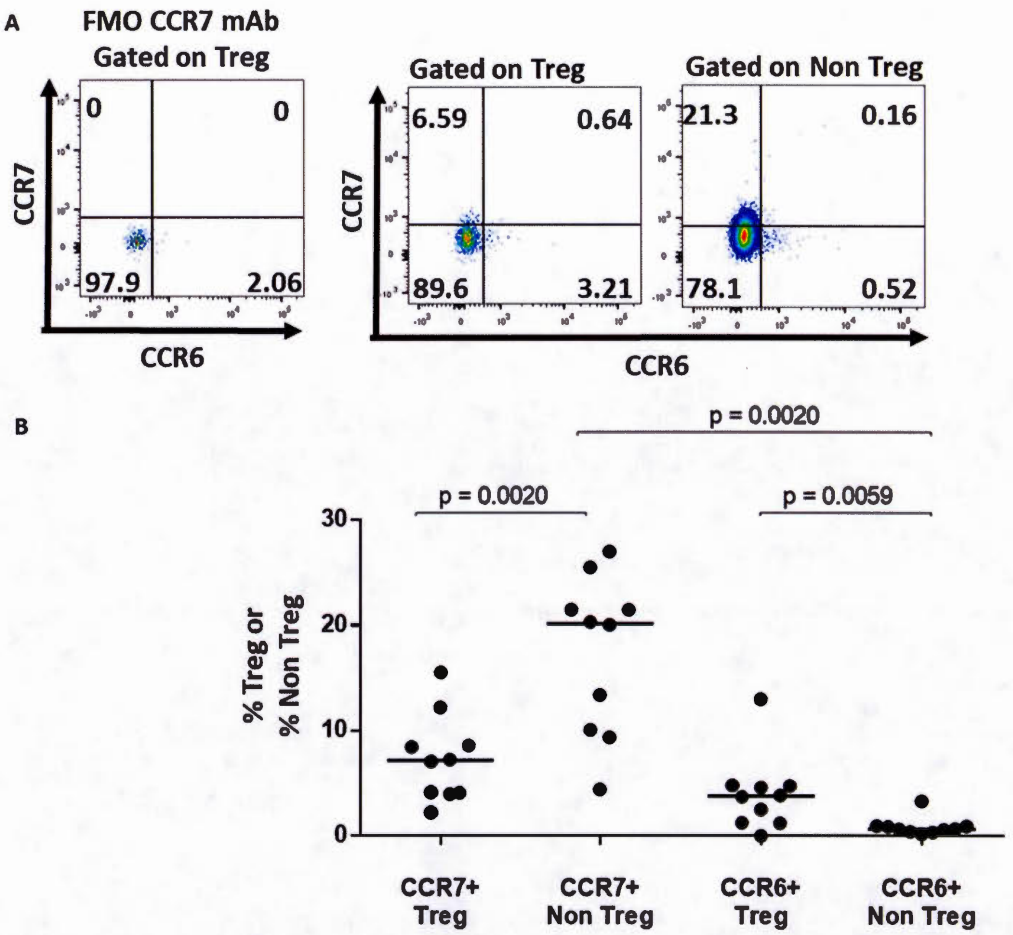

Figure 4.6: Discriminating Tregs of thymic origin from recirculating cells. Tregs and non-Tregs within $CD3^{high}$ subset were analyzed for the expression of CCR7 and CCR6 (A). Frequencies of Tregs and non-Tregs expressing CCR7 or CCR6 are shown in (B). Lines represent median and the data from 10 thymuses were compared using Wilcoxon matched- pairs signed rank test; p values are shown only in cases where the differences were significant. Note: Tregs were identified as $CD3^{high}$ CD4+ CD127- CD25+ FoxP3+ and non-Tregs as $CD3^{high}$ CD4+ FoxP3- cells. The gating strategy for CD4+ cells, excluded CD4+ CD8+ thymocytes.

A small percentage of both Tregs and non-Tregs express the gut homing markers integrin-β7 (Tregs Median: 2.7%, non-Tregs Median: 2.2%) and CCR9 (Tregs Median: 4.5%, non-Tregs Median: 5.%), suggesting their recirculating phenotype as shown in Figure 4.7.

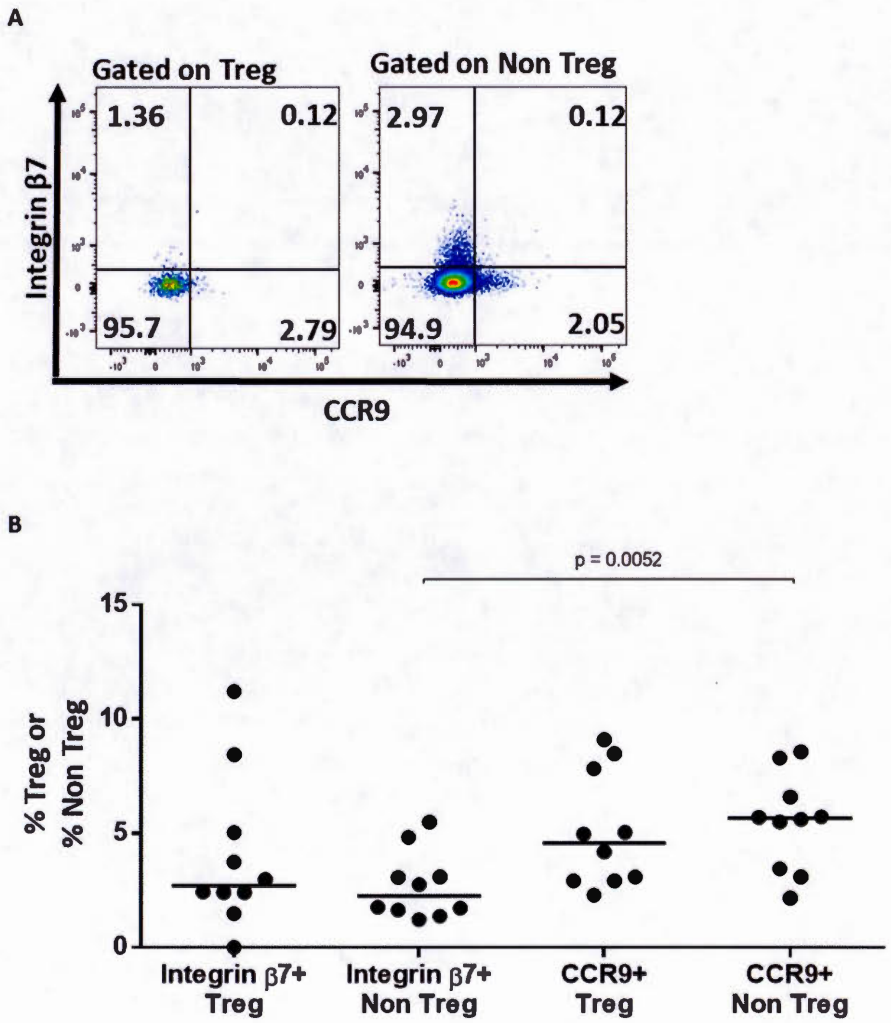

Figure 4.7: Thymic Tregs and non-Tregs express gut homing markers. Tregs and non-Tregs (CD4+ FoxP3-) within $CD3^{high}$ subset were analyzed for the expression of the

gut homing markers Integrin β7 and CCR9 (A). The frequencies Integrin β7+ Tregs and non-Tregs and CCR9+ Tregs and non-Tregs shown in (B). The gating strategy for CD4+ cells, excluded CD4+ CD8+ thymocytes. Lines represent median and the data from 10 thymuses were compared using paired student t tests; p values are shown only in cases where the differences were significant. Note: Tregs were identified as $CD3^{high}$ CD4+ CD127- CD25+ FoxP3+ and non-Tregs as $CD3^{high}$ CD4+ FoxP3- cells.

Next, the expression of HIV co-receptors CXCR4 and CCR5 was evaluated on thymocytes (Figure 4.8). It was found that the majority of Treg and non-Tregs express CXCR4 (Tregs Median: 87.5%, non-Tregs Median: 94.4%). Although few cells expressed CCR5, the percentage of cells expressing CCR5 was higher in Tregs (Median: 18.53%, Figure 4.8) compared to non-Tregs (Median: 1.01%).

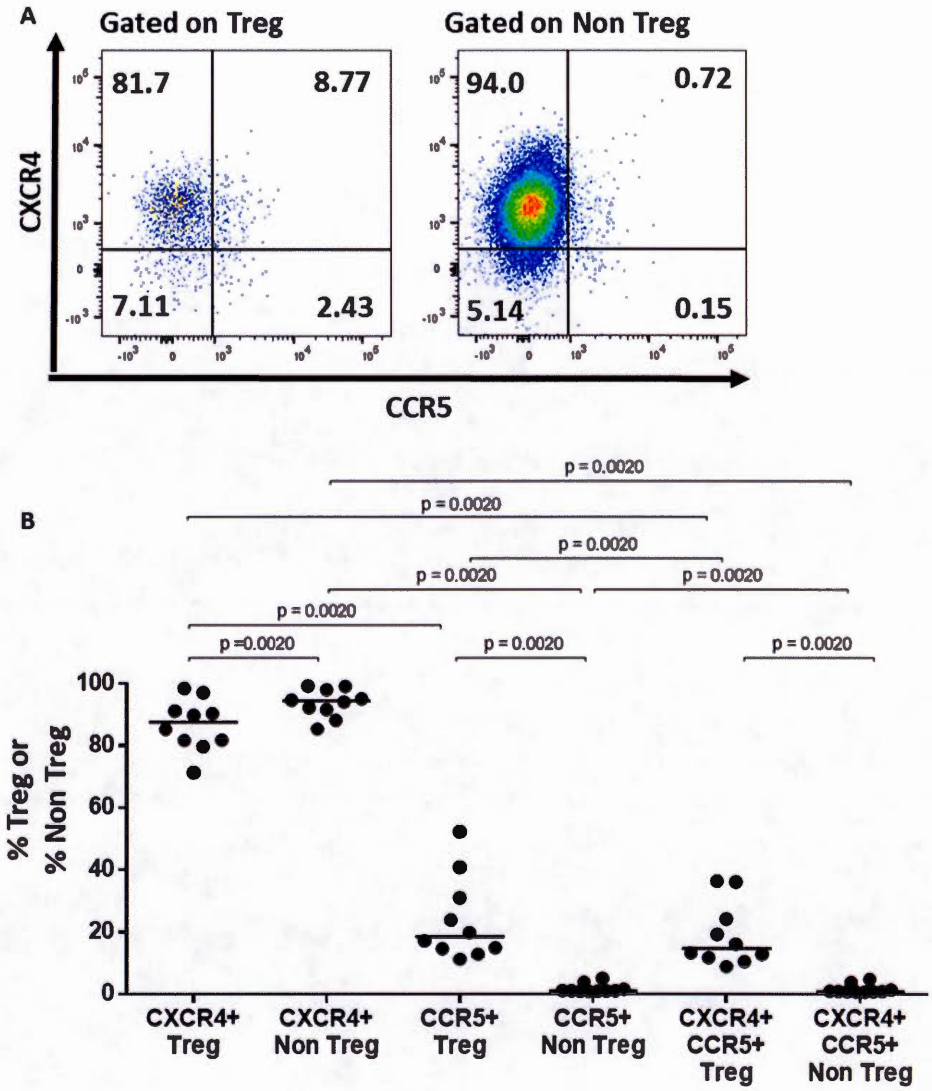

Figure 4.8: Thymic Tregs have higher expression of HIV co-receptor CCR5 compared to non-Tregs. Tregs and non-Tregs (CD4+ FoxP3-) within $CD3^{high}$ subset were analyzed for the expression of the HIV co-receptors CXCR4 and CCR5 (A). The frequencies of cells expressing CXCR4 only, CCR5 only or both CXCR4 and CCR5 are shown in (B). Lines represent median and the data from 10 thymuses were compared using Wilcoxon matched-pairs signed rank test; p values are shown only in

cases where the differences were significant. Note: The gating strategy for CD4+ cells, excluded CD4+ CD8+ thymocytes. Tregs were identified as $CD3^{high}$ CD4+ CD127- CD25+ FoxP3+ and non-Tregs as $CD3^{high}$ CD4+ FoxP3- cells.

CXCR3 is a chemokine receptor expressed at very early stages of CD4 T cell differentiation (Rabin *et al.*, 2003). CXCR3 is required for the migration of T cells (including Tregs) to peripheral sites of inflammation and it is expressed only on memory cells, following antigen-priming and activation (Groom et Luster, 2011). In the peripheral blood, CD45RO expressing CD4 T cells are preferentially infected by HIV (Schnittman *et al.*, 1990). In addition, CXCR3+ memory CD4 T cells in the blood of ART-treated HIV-infected individuals with undetectable viral load, were shown to house replication competent virus (Banga *et al.*, 2018). Therefore, CXCR3 expression on CD45RO+ tTregs could suggest that these cells are recirculating from inflammatory sites, might be more susceptible to infection and might also harbor latent virus following infection. It was noted that CD45RO+ Tregs express the marker CXCR3 (Median: 30.15%), whereas CD45RO+ non-Tregs in the thymus do not express CXCR3 (Median: 3.2%, Figure 4.9), suggesting that CD45RO+ thymic Tregs are potentially recirculating cells, more susceptible to HIV infection compared to CD45RO+ non Tregs.

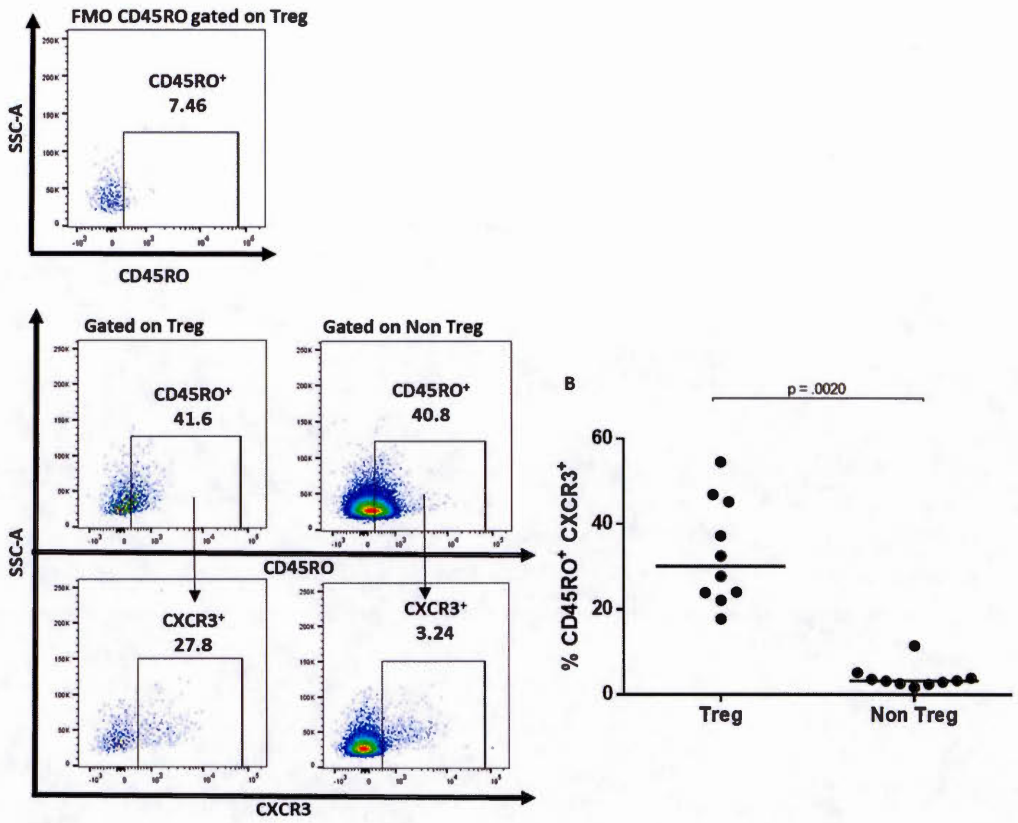

Figure 4.9: Thymic Tregs and not non-Tregs express HIV permissiveness marker CXCR3. CD45RO+ Tregs and non-Tregs (CD4+ FoxP3-) within $CD3^{high}$ subset were analyzed for the expression of the CXCR3, a marker of permissiveness to HIV, as shown in (A). The frequencies CXCR3+ cells within CD45RO+ Tregs and CD45RO+ non-Tregs are shown in (B). Lines represent median and the data from 10 thymuses were compared using paired student *t* tests; p values are shown only in cases where the differences were significant. Note: Tregs were identified as $CD3^{high}$ CD4+ CD127- CD25+ FoxP3+ and non-Tregs as $CD3^{high}$ CD4+ FoxP3- cells The gating strategy for CD4+ cells, excluded CD4+ CD8+ thymocytes.

4.2 Methylation status of foxp3

As shown in Figure 4.2, FoxP3 expression increased with expression of CD3 ($CD3^{high}$ subset had the highest FoxP3 expression). Therefore, the methylation profile of *foxp3* gene was examined in the three different CD3 populations of thymocytes, expecting to see greater demethylation of CpG sites in the $CD3^{high}$ compared to $CD3^{int}$ and $CD3^{low}$, in line with the protein expression patterns. Methylation profile of *foxp3* gene locus was significantly different among the three CD3 subsets in four out of 6 regions described as shown in Figure 4.10. Higher demethylation was observed in the $CD3^{high}$ subset compared to $CD3^{int}$ and $CD3^{low}$, which correlates with higher FoxP3 protein expression and higher Treg frequencies in $CD3^{high}$.

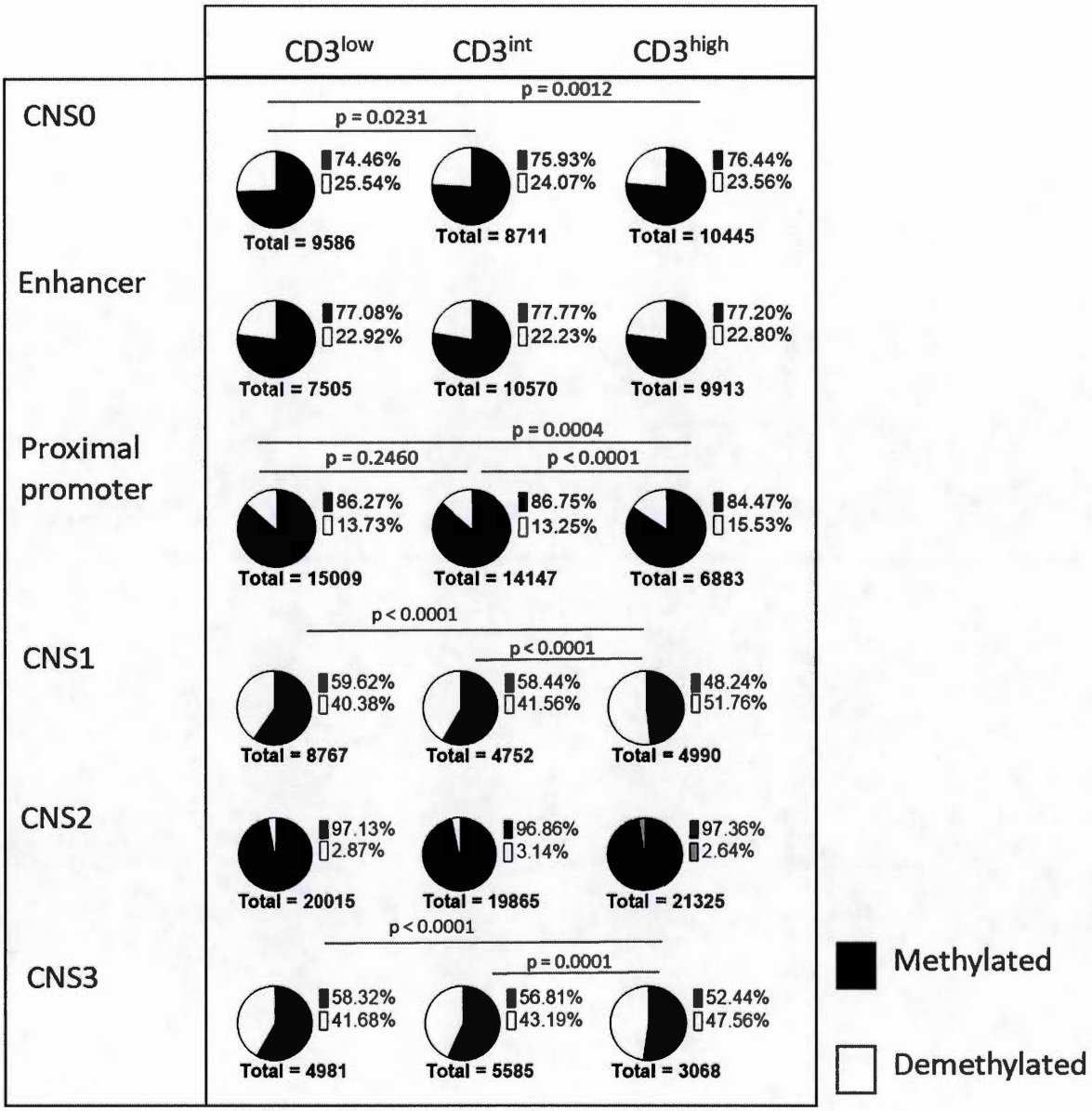

Figure 4.10: Methylation status of CNS elements of *foxp3* in CD3^{low}, CD3^{int}, CD3^{high} thymocytes. Pie charts represent methylation and demethylation percentages. Rows represent respective sequence elements of *foxp3* and columns represent CD3^{low}, CD3^{int}, CD3^{high} thymocytes respectively. Number shown below each pie chart corresponds to total number of PCR amplicons analyzed in each subset of CD3 from two thymus

specimens, by MiSeq followed by Bismark analysis. Data were compared using Chi square test.

To study the regulation of *foxp3* gene methylation, the expression of DNMT enzymes in the three CD3 subsets of thymocytes were assessed by qPCR (Figure 4.11). No differences in the expression of DNA methylation enzymes DNMT1 or DNMT3a were found within the three CD3 subsets of thymocytes. Higher DNMT3b expression was found in in $CD3^{high}$ compared to $CD3^{int}$ and $CD3^{low}$ thymocytes.

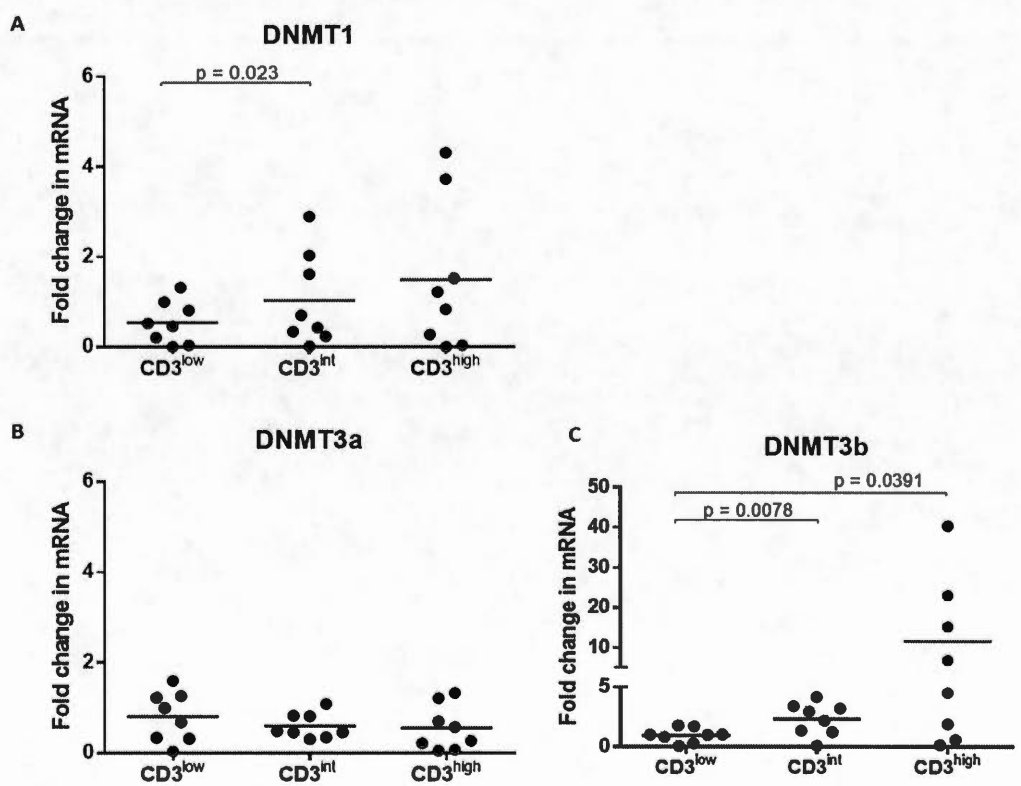

Figure 4.11: Expression of DNA methyl transferases in $CD3^{low}$, $CD3^{int}$, $CD3^{high}$ thymocytes. qPCR results showing mRNA expression of DNMT-1 (A) , DNMT-3a (B) and DNMT-3b (C) from $CD3^{low}$, $CD3^{int}$, $CD3^{high}$ thymocytes (n = 8). $CD3^{low}$ sample chosen at random had fold change = 1. Lines represent median and the data were compared using Wilcoxon matched-pairs signed rank test; p values are shown only in cases where the differences were significant.

4.3 Effect of TGF-β treatment on *in vitro* thymic Treg generation

To understand the role of TGF-β in tTreg generation, human thymocytes were treated with TGF-β, and tTreg frequencies were measured after 2 days. Since TGF-β is known to increase CD127 expression (Ouyang, Weiming *et al.*, 2013), it was used as an internal control for studying the effect of TGF-β. Despite the high inter-individual variability in CD127 expression in untreated samples, TGF-β treatment resulted in a higher percentage of CD127 expressing CD4+ thymocytes (Untreated Median: 65.8%, TGF-β treated Median: 84.10%). Although the differences were not statistically significant, the percentages of CD127+ cells increased in two samples following TGF-β1 treatment, as indicated by the colour code. Addition of Pirfenidone, inhibitor for TGF-β, slightly reversed this effect (Median, 82.15%, Figure 4.12A and B). In addition, the frequency of Tregs increased in all samples upon TGF-β treatment of thymocytes and despite a persisting variability in values, the effect was reversed upon addition of Pirfenidone for two of the four tested samples (Figure 4.12 C and D).

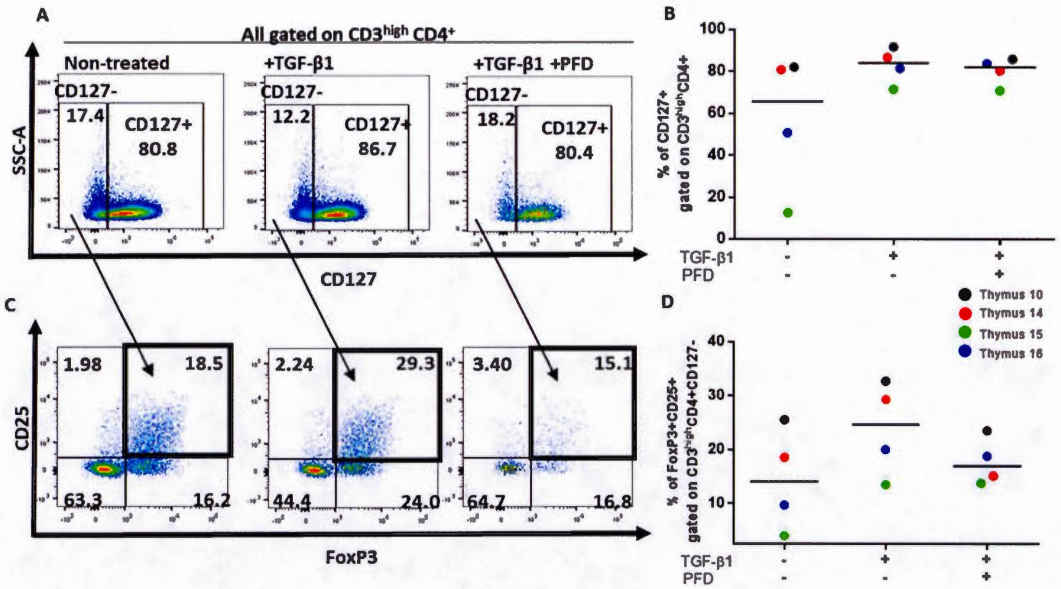

Figure 4.12: Impact of TGF-β treatment on thymic Treg frequencies. Human thymocytes remained untreated or were conditioned with TGF-β1, in the presence or absence of Pirfenidone. TGF-β treatment increases the frequency of CD127+ thymocytes as represented in (A) and (B). The gates for thymic Tregs is shown in (C) and frequencies are shown in (D). Lines represent median and the data from 4 thymuses were compared using Wilcoxon matched-pairs signed rank test. While gating for CD4+ cells, CD4+ CD8+ thymocytes were excluded from the analysis.

4.4 *In vitro* HIV infection of thymocytes

Following the infection of thymocytes with R5 and X4-tropic HIV strains, the susceptibility of FoxP3+ and FoxP3- thymocytes to HIV infection were assessed by

checking for the co-expression of FoxP3 and p24 using flow cytometry. Upon infection of thymocytes with the R5 and X4 tropic HIV strains, $CD3^{high}$ CD8- thymocytes did not co-express FoxP3 and p24, i.e., FoxP3+ p24+ thymocytes were not observed, suggesting that FoxP3+ thymocytes are not infected by either one of these viral strains *in vitro* (Figure 4.13). The percentages of p24+ within $CD3^{high}$ CD8- were analyzed regardless of CD4 expression, since HIV infection causes downregulation of CD4.

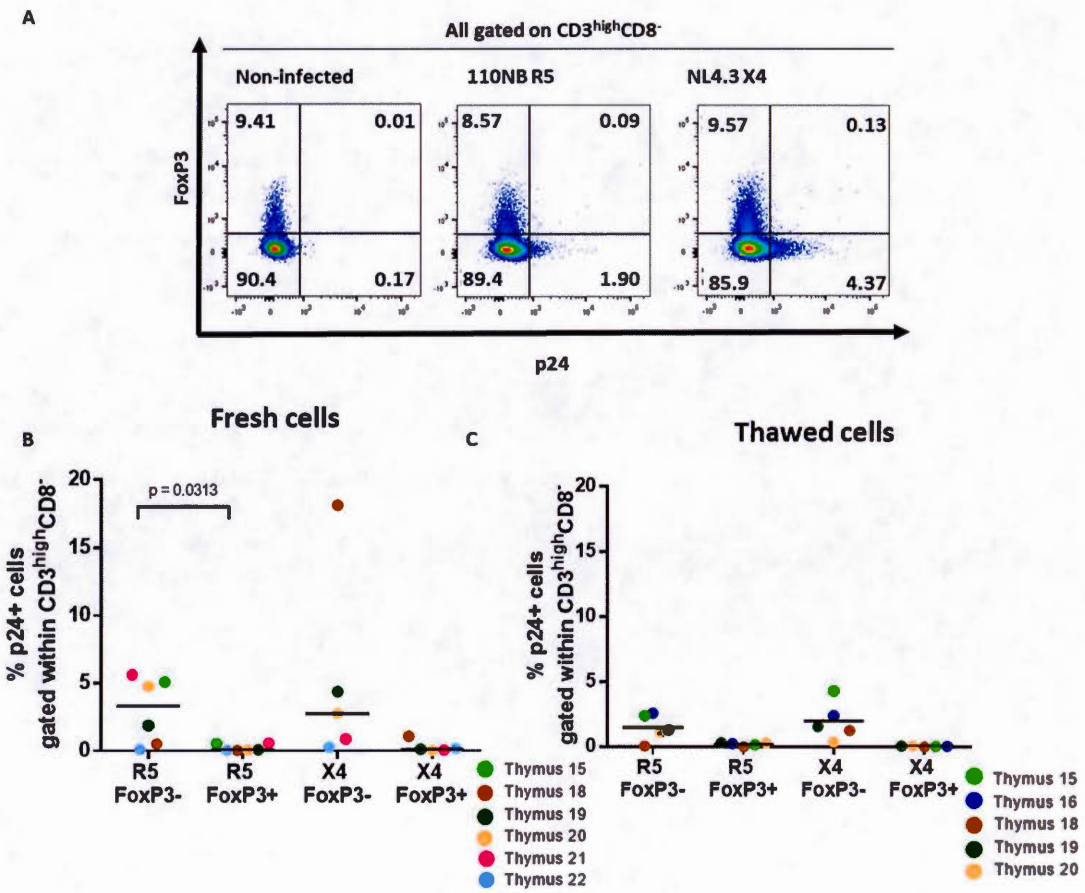

Figure 4.13: FoxP3+ thymocytes are not infected by HIV. Human thymocytes were infected with 110NB R5 and NL4.3 X4 HIV-1 viral strains for 3 hours. The infection rates, achieved with R5 and X4 viral strains, determined by the expression of p24, in FoxP3+ and FoxP3- thymocytes gated on alive, $CD3^{high}$ CD8- are shown in (A). The

frequencies of fresh (B) or thawed (C) p24+ cells are shown. Lines represent median and the data from 6 thymuses were compared using Wilcoxon matched-pairs signed rank test; p values are shown only in cases where the differences were significant.

4.5 Effect of TGF-β on *in vitro* HIV infection of thymocytes

To study the impact of TGF-β treatment on HIV infection rates, p24 expression was compared in TGF-β treated, HIV-infected thymocytes, versus non-treated controls. A non-infected (NI) non-treated control was also used. No difference in the percentage of p24+ $CD3^{high}$ CD8- thymocytes were found upon TGF-β treatment (Figure 4.14). Therefore, TGF-β treatment does not modulate HIV infection of thymocytes *in vitro*.

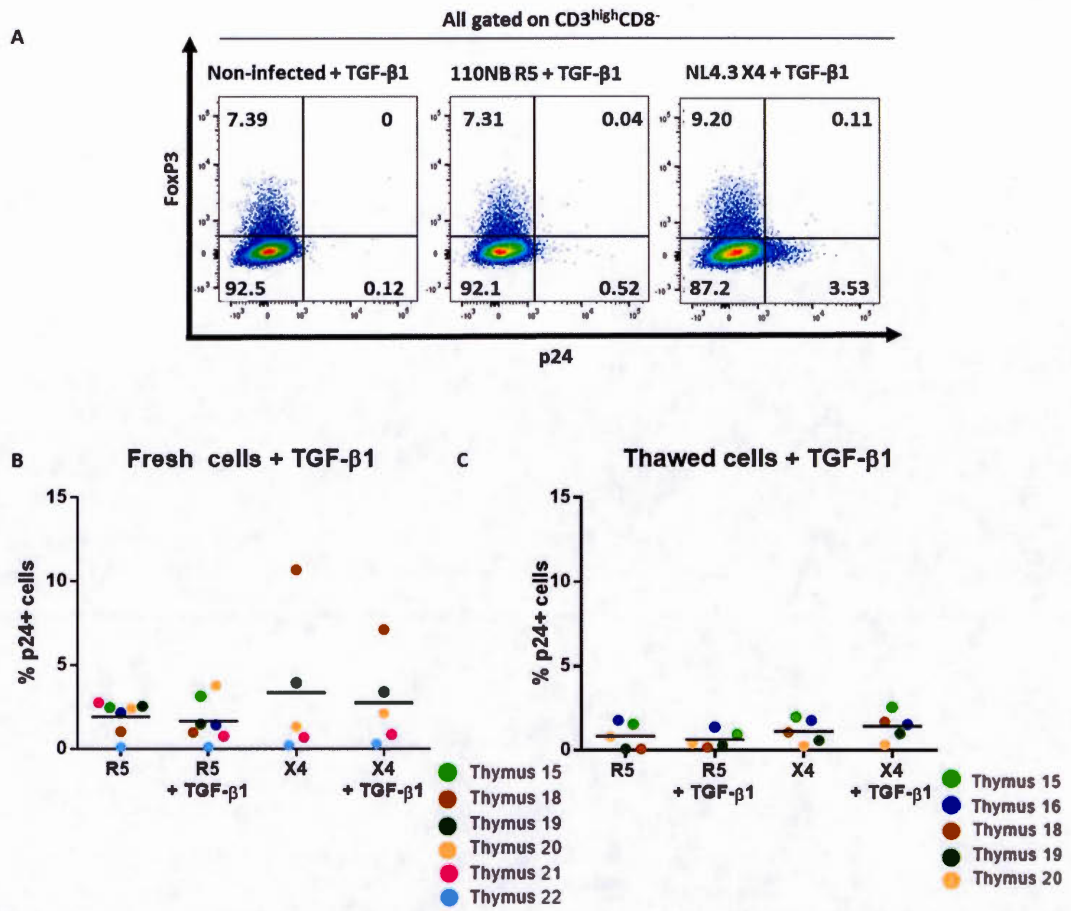

Figure 4.14: HIV infection of thymocytes is not affected by TGF-β treatment. The infection rates achieved with R5 and X4 viral strains in TGF-β treated thymocytes, determined by the expression of p24, is shown in (A). The frequency of fresh (B) or thawed (C) p24+ cells within $CD3^{high}$ CD8- cells of untreated Vs TGFβ-treated thymocytes are shown. Lines represent median and the data from 6 thymuses compared using Wilcoxon matched-pairs signed rank test; p values are shown only in cases where the differences were significant.

To study if TGF-β1 treatment increases HIV infection, FoxP3 expression and Treg frequencies were assed in HIV infected, TGF-β-treated thymocytes (either fresh or thawed). Neither FoxP3 expression by CD4 T cells, nor the frequency of Tregs changed upon HIV infection combined with TGF-β treatment. HIV infection and TGF-β did not affect FoxP3 expression (Figure 4.15A and B), nor thymic Treg frequencies (Figure 4.15C and D).

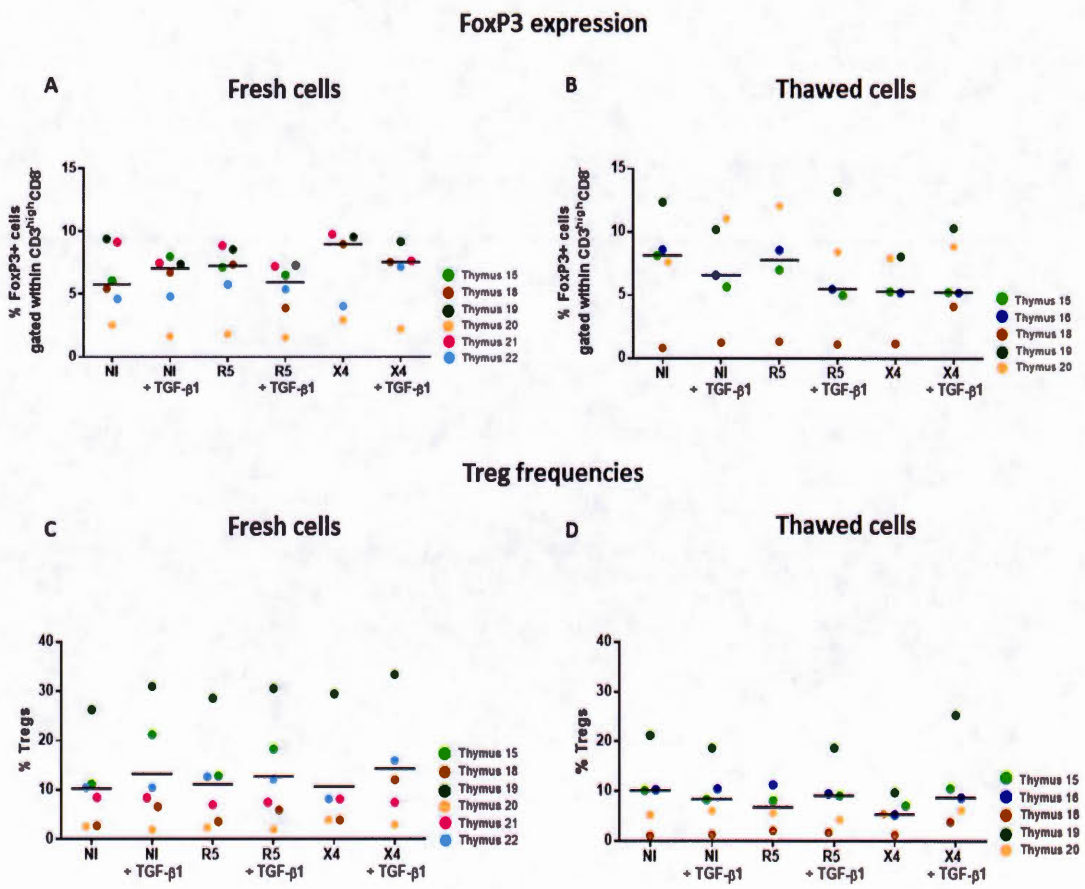

Figure 4.15: FoxP3 expression and thymic Treg generation are not altered by HIV infection and TGF-β treatment. The frequency of FoxP3+ cells within $CD3^{high}$ CD4+ CD8- thymocytes in fresh (A) and thawed (B) samples is shown. The frequency of

thymic Tregs (CD4+ CD127- CD25+ FoxP3+) within $CD3^{high}$ subset in fresh (C) and thawed (D) samples are shown. Lines represent median and the data from 6 thymuses were compared using Wilcoxon matched-pairs signed rank test; p values are shown only in cases where the differences were significant. While gating for CD4+ cells, D4+ CD8+ thymocytes were excluded from the analysis.

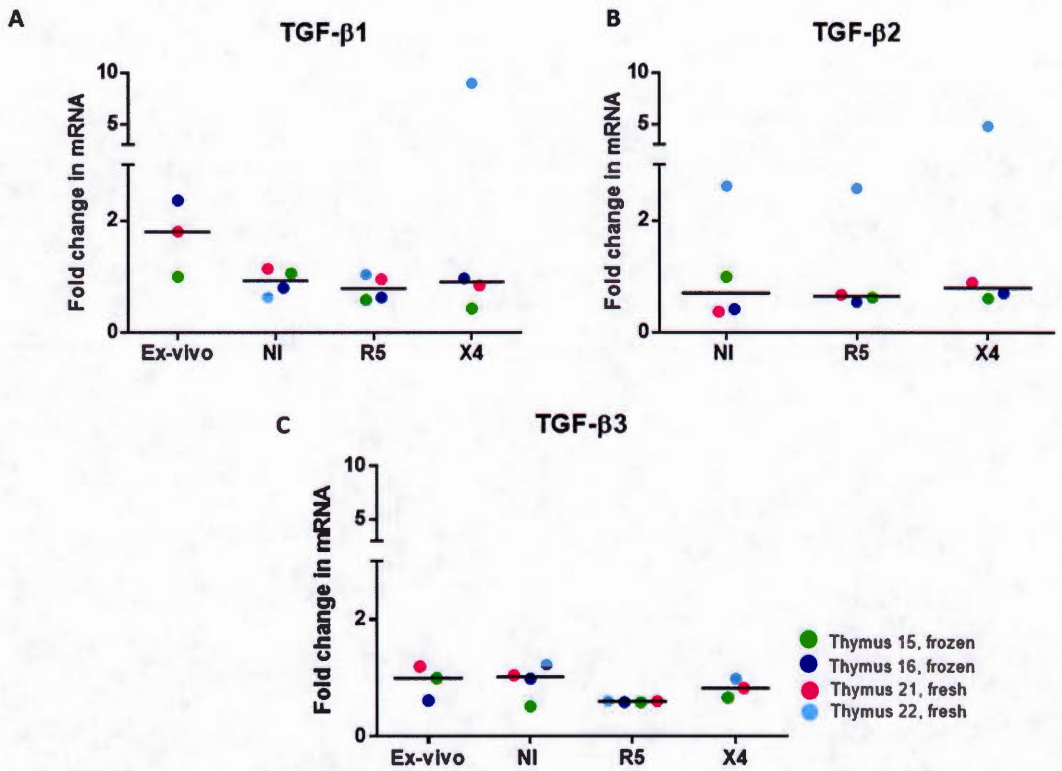

Figure 4.16: HIV infection has no influence on TGF-β production. The fold change in mRNA expression of TGF-β1 (A), TGF-β2 (B) and TGF-β3 (C) in non-infected, R5 and X4 infected thymocytes compared to the *ex vivo* control (or NI control in case of TGF-β2, since there was no detectable *ex vivo* expression) was determined by qPCR. Lines represent median and the data from 4 thymuses were compared using Wilcoxon matched-pairs signed rank test; p values are shown only in cases where the differences were significant. Note: *Ex vivo* refers to cells that were not cultured. NI refers to cells that were cultured but not infected.

Furthermore, no changes in the mRNA expression of TGF-β1/2/3 were noticed in HIV infected samples compared to non-infected control, suggesting that TGF-β is not produced during *in vitro* HIV infection (Figure 4.16). The TGF-β2 isoform, was not detected in the *ex vivo* samples.

CHAPTER V

DISCUSSION

Owing to the difficult accessibility of human thymus tissues due to ethical and logistical limitations, the role of thymic Tregs during HIV infection remains unclear. In this study, *ex vivo* phenotypic characterization of human thymic Tregs was done to investigate markers associated with thymic Treg development, and *in vitro* HIV infection of thymocytes was performed to understand the differentiation of thymic Tregs in the context of HIV infection and in the presence of TGF-β.

For the *ex vivo* characterization, thymocytes were divided into three categories based on their CD3 expression levels. Treg-associated markers were assessed within these groups. Tregs use CD39 in concert with CD73 to produce immunosuppressive adenosine, which inhibits effector T cell proliferation and IL-2 production in effector T cells, and thus helps Tregs to carry out their regulatory functions (Deaglio *et al.*, 2007). Hence, higher expression of CD39 is expected in the thymic $CD3^{high}$ cell population, comprising mature Tregs that should be the most functional cells. However, CD39+ Tregs were more prevalent within the $CD3^{low}$ and $CD3^{int}$ thymocyte populations compared to the $CD3^{high}$ subset. Nunes-Cabaço *et al.* reported that $CD3^{high}$ DP FoxP3+ thymocytes comprise a greater percentage of CD39+ cells compared to SP CD4+ FoxP3+ thymocytes (Nunes-Cabaco *et al.*, 2011). These authors reported that $CD39^{high}$ thymocytes express high levels of FoxP3, CD25 and CTLA-4, thereby displaying characteristics of activated Tregs (Nunes-Cabaco *et al.*, 2011). In addition,

these DP FoxP3+ thymocytes express higher CD127 and BCL-2, which most likely protected from apoptosis due to negative selection, and helped them become CD4SP. Therefore, they concluded that in the human thymus, Treg development is thought to be initiated as early as the double positive stage, as DP cells express most of the Treg markers. Our results also indicate that expression of the Treg-associated marker CD39 occurs in immature thymocytes. However, lesser percentage of CD39+ cells within $CD3^{high}$ thymocyte population was found. It has been reported that CD39 is also upregulated by activated conventional CD4+ and CD8+ T cells (Raczkowski *et al.*, 2018), and activation in the context of the thymus refers to recognition of self-antigens. However, it should be underscored that thymocytes recognizing self-antigens with high affinity are negatively selected and eliminated, to maintain self-tolerance. Therefore, one possible explanation is that the higher percentage of CD39+ Tregs within $CD3^{low}$ and $CD3^{int}$ populations is indicative of encounters with self-antigens and activation . Some of these CD39+ cells may be highly activated by self-antigens and hence eliminated, which would result in lesser CD39+ cells within the $CD3^{high}$ subset. Nunes-Cabaço *et al.* drew their conclusions by comparing DP and SP CD4 T cells expressing FoxP3 within the thymocyte $CD3^{high}$ population, whereas Tregs within CD39 expressing Tregs within $CD3^{high}$, $CD3^{int}$ and $CD3^{low}$ thymocytes are compared in our study . Hence, our expanded analyses extends the reports of Nunes-Cabaço *et al.*. Furthermore, in line with the report of these authors, we also did not observe CD73 expression by human tTregs.

During inflammatory conditions, naive CD4+ T cells in peripheral blood initiate expression of RORγt in response to the cytokines TGF-β and IL-6 and become IL-17-producing Th17 cells. In addition, RORγt-expressing subset of FoxP3+ Tregs are described in the context of intestinal inflammation and such Tregs were reported to have higher suppressive capacity compared to their RORγt-deficient counterparts

(Yang, B. H. *et al.*, 2016). However, in the thymus, RORγt expression seems rather associated with thymocyte maturation, as its expression wanes as thymocytes mature. RORγt is required for maturation of thymocytes in the earlier stages as it inhibits their proliferation, but must be downregulated thereafter since it inhibits IL-2 production and proliferation of SP cells. RORγt is required by DP thymocytes (less mature) since they undergo positive and negative selection, and mitosis must be blocked during these selection events (He *et al.*, 2000).

The transcription factor Helios was shown to be upregulated in CD4+ CD25+ regulatory T cells, binding to the FoxP3 promoter and siRNA-mediated knockdown of Helios in these cells led to FoxP3 downregulation, thereby emphasizing its role in regulating FoxP3 (Getnet *et al.*, 2010). Thorton *et al.* demonstrated that 100% of mouse thymic CD4+ FoxP3+ T cells express Helios, while *in vitro* TGF-β-induced FoxP3+ cells (murine and human) as well as *in vivo* induced antigen-specific FoxP3+ cells do not; hence these authors proposed that Helios is a marker to delineate tTregs (Thornton, Angela M. *et al.*, 2010). However, in a second independent study, Helios+ and Helios- cells were shown to be present within the CD4+ CD45RA+ FoxP3+ naive Tregs isolated from human peripheral blood (Himmel *et al.*, 2013). In addition, Helios has also been demonstrated to be a marker of T cell activation, irrespective of the T cell subset (Akimova *et al.*, 2011) and FoxP3+ Tregs generated extra-thymically, both *in vivo* and *in vitro*, were shown to express Helios, thereby rejecting its use as a marker for tTregs (Gottschalk, Rachel A. *et al.*, 2012). An important role for Helios is its contribution to T cell tolerance, as this transcriptional factor is known to bind to the IL-2 gene promoter on its own or in combination with FoxP3, which leads to IL-2 transcriptional silencing (Baine *et al.*, 2013). Helios has also been associated with self-tolerance mechanisms in the thymus; it is co-expressed with Bim (Daley *et al.*, 2013), a pro-apoptotic protein marker upregulated by self- reactive thymocytes that are

destined to undergo negative selection in the thymus (Baldwin et Hogquist, 2007). As mentioned in the section 1.2.3, the current theoretical models of thymic Treg development suggest that Tregs, which bear TCR responding to self-antigens, escape the negative selection process in the thymus. Indeed, our analysis shows that all thymic CD4+ FoxP3+ cells express Helios, thereby suggesting that despite being self-reactive, these cells survive negative selection. Supporting the role of Helios in the maintenance of self-tolerance, both thymic and peripheral T cells bearing transgenic TCR specific to a self-antigen, regardless of FoxP3 expression, upregulate Helios and become anergic in a mouse model of autoimmune gastritis (Ross *et al.*, 2014). Altogether, these facts suggest that Helios expression in the thymus is more likely to be associated with the maintenance of tolerance in general.

CD45RO and CD45RA are two different isoforms of the CD45 cell surface protein tyrosine phosphatases (PTPs) (Thomas, 1989). As for PTPs, CD45 isoforms are responsible for the regulation of signal transduction mechanisms controlling cell division or apoptosis. Chang *et al.*. demonstrated that murine DN thymocytes do not express CD45RA nor CD45RO mRNAs, and that transcription of CD45RO isoform is maximal at the single positive stage (Chang, H. L. *et al.*, 1991). Subsequently, Fujii *et al.* studied CD45RA and CD45RO protein expression in human thymocytes in detail, reporting that the majority of triple negative (least mature thymocytes) were CD45RA+, while CD45RO+ cells constituted about 80% of DP and SP (CD4 and CD8) thymocytes (Fujii *et al.*, 1992). The latter is consistent with our findings. CD45RA is a marker for naïve T cells and CD45RO is a marker for memory T cells, which are in the peripheral blood. In addition, CD45RA to CD45RO isoform switching occurs in TCR activated CD4 T cells following antigen recognition (Johannisson et Festin, 1995). It is important to note that the terminology "naïve" or "memory" in the thymus is inappropriate, since thymocytes are activated only against self-antigens, to become positively and

negatively selected. Fujii *et al.*. explain that, as in peripheral blood, isoform switching in DP or SP thymocytes also occurs as a result of TCR binding. Higher CD45RO expression by DP and SP thymocytes is concurrent to TCR activation, which in the thymus would indicate higher self-reactivity. In this case, apoptosis and negative selection could be triggered in the thymus. Thus, DP and SP thymocytes expressing CD45RO will likely be eliminated by negative selection, due to high self-reactivity. On the other hand, if developing thymocytes switch back to CD45RA expression, they are egressed out of the thymus (Fujii *et al.*, 1992).

It is well documented that CD45RO+ CD4 T cells in peripheral blood and lymph nodes are preferentially infected by HIV compared to CD45RA+ CD4 T cells, and are destroyed at the earlier stages of infection (Schnittman *et al.*, 1990). In addition, CD45RO+ cells are known to harbor 10 times more HIV DNA compared to CD45RA+ CD4 T cells (Schnittman *et al.*, 1990). Although the complete and exact signaling mechanisms accounting for the differences in susceptibility to HIV infection are unknown, it has been reported that the CD45RO+ cells differentially regulate their downstream tyrosine kinases (Helbert *et al.*, 1997) and as a result prevent endocytic pathways for CD4, facilitating syncytium (fused cells following infection) formation, resulting in higher infection (Chowdhury *et al.*, 1992). It has also been suggested that HIV-infected CD45RA+ CD4 T cells will initiate CD45RO expression, as a result of immune- activation due to the infection (Helbert *et al.*, 1997).We were therefore interested in studying the susceptibility of thymic CD45RO+ Tregs and non-Tregs to HIV infection. CXCR3 is a chemokine receptor for CXCL-9, 10, 11, and serves as a co-receptor for HIV. CXCR3+ is induced during early stages of naïve CD4 T cell differentiation even before CCR5 (Rabin *et al.*, 2003). CXCR3+ CCR6+ memory cells in the peripheral blood are also highly permissive to HIV infection (Gosselin *et al.*, 2010). However, the susceptibility of CD45RO+ tTregs to infection is not known and

role of CXCR3 expression in CD45RO+ thymocytes (which are possibly recirculating cells or cells undergoing negative selection), is unclear. We detected that CD45RO+ tTregs express the chemokine receptor CXCR3 while CD45RO+ non-Tregs do not, suggesting that tTregs are potentially more permissive to infection compared to non-Tregs. However, it is to be noted that thymic CD45RO+ Tregs, unlike their peripheral counterparts, are probably pre-apoptotic and may undergo cell death prior to infection.

CCR7 is the receptor for the chemokines CCL19 and CCL21, which are highly expressed by medullary thymic epithelial cells (Rivino *et al.*, 2004). Studies in mice show that CCR7 expression in developing thymocytes facilitates their migration from cortex to medulla to complete their development (Kwan et Killeen, 2004; Ueno *et al.*, 2002). Surprisingly, increased Treg numbers are found in mice deficient in ccr7 compared to wild-type mice, which complicates understanding of the role of ccr7 in T cell and specifically Treg development. In this context, it has been speculated that CCR7 most likely plays a role in tTregs egress and its deletion resulted in intra-thymic accumulation (Cowan *et al.*, 2014). Later, Cowan *et al.* demonstrated a heterogeneity in CCR7 expression within mouse tTregs. Indeed, CCR7- CCR6+ recirculating cells within the tTreg and their precursor populations (CD25+ FoxP3- or CD25- FoxP3+) exist (Cowan *et al.*, 2016). It was proposed that CCR7 is required by de novo Tregs in the medulla to compete with recirculating Tregs for the medullary niches. In this context, deletion of CCR7 would enable recirculating cells to outcompete intra-thymically generated cells, thereby resulting in increased Treg numbers (Cowan *et al.*, 2016). As reported by Cowan *et al.* 2016, CCR6, the receptor for CCL12, is expressed by recirculating cells in the mouse thymus, and is also expressed by Th17 cells and Tregs, enabling them to migrate to inflammatory sites (Yamazaki *et al.*, 2008). The presence of recirculating Tregs which are distinguished from intra-thymically generated cells by the expression of CD31or PECAM-1, has been shown to inhibit *de*

novo tTreg generation in mice and humans (Thiault *et al.*, 2015). Thirty to fifty percent of Tregs in the mouse thymus and 20-25% of Tregs and 9-12% of Tconv cells in the human thymus were found to be CD31-, suggesting their recirculating nature (Thiault *et al.*, 2015). However, the differential expression of CCR7 and CCR6 by thymus-derived or recirculating cells respectively, in the human thymus have not been reported. We observed that the higher percentage of CCR7+ cells was found in non-Tregs (Median: 20.1%) compared to Tregs (Median: 7.1%). A small percentage (Median: 3.78%) of CCR6+ tTregs was found, whereas non-Tregs did not express CCR6 at all. Two possible explanations could account for these differences in percentages of CCR7/CCR6-expressing Tregs in humans and mice. First, our definition of Tregs as CD4+ CD127- CD25+ FoxP3+ is more specific, compared to the phenotypes defined in other studies, which have considered Tregs as CD25+ FoxP3+ CD4 T cells. Second, out of all the recirculating Tregs in the human thymus, it is likely that only a small subset express CCR6. Third, the study by Cowan *et al.*. was performed on adult mice while our study population involved infants (about 4 month-old, although the age for all donors were not known). This could result in important differences in the expression of inflammatory markers, like CCR6, since these infants would probably have not encountered any foreign antigens at all, and would have much less developed immune systems compared to adult mice.

Knowing that recirculating cells exist within the human thymus, we explored the expression of the gut homing markers Integrin-β7 and CCR9, relevant in the context of HIV infection. HIV gp120 binds to α4β7 receptors expressed on CD4 T cells (Cicala *et al.*, 2011). In HIV-infected individuals, CCR9+β7hi CD4+ T cells have impaired gut-homing capacity and are more present in the peripheral blood compared to the gut, enhancing thereby mucosal immune disruption and contributing to disease progression (Mavigner *et al.*, 2012). We observed that Integrin-β7 and CCR9 are expressed by a

small percentage of Tregs and non-Tregs in the human thymus, which suggest their susceptibility to HIV infection as well as their recirculating nature. We observed higher expression of CCR5 on thymic Tregs compared to non-Tregs which suggests that tTregs are potentially more susceptible to HIV infection with R5 tropic HIV strains.

Methylation sequencing of *foxp3* gene locus was performed on bisulfited converted genomic DNA extracted from the three CD3 subsets of thymocytes to check if their methylation status reflected the FoxP3 protein expression pattern as shown in Figure 4.2. Although significant differences in methylation were observed in four out of the six specified regions, there were no significant differences in the enhancer and CNS2 regions, which is described as the TSDR. It was surprising to find that the enhancer region was not more demethylated in the $CD3^{high}$ subset. Since FoxP3-expressing cells represent around 6% of $CD3^{high}$ thymocytes, it is likely that the demethylation seen in FoxP3+ cells is being masked by the huge majority of FoxP3- thymocytes present within the $CD3^{high}$ population. This shortcoming could potentially be overcome by sorting Tregs. However, since FoxP3 staining is performed intracellular and requires permeabilization and fixation of cells, which might potentially interfere with subsequent steps involved in the assessment of epigenetic status. However, the fact that there is significant difference in methylation between $CD3^{high}$ versus $CD3^{low}$ suggests that the methylation status of *foxp3* gene starts undergoing changes right from early stages of human thymocyte development. In addition, expression of DNMT enzymes was assessed on thymocytes sorted based on CD3-expression, and not on enriched tTregs, which is also a limitation of this study. It is to be noted that DNMT enzymes could regulate multiple genes in addition to *foxp3,* or might regulate *foxp3* through indirect mechanisms. Owing to the limitations of the sorting strategy, a correlation between FoxP3 protein expression and methylation status might not be useful.

TGF-β regulates FoxP3 expression in the thymus (Chen, W. et Konkel, 2015), induces Treg development in secondary lymphoid tissues during inflammation (Chen, W. *et al.*, 2003) and mediates lymphoid tissue fibrosis is HIV-infected individuals (Theron *et al.*, 2017; Zeng *et al.*, 2011). Hence, we postulated that TGF-β might contribute to the induction and maintenance of Tregs within the thymus during HIV infection. When human thymocytes were treated with recombinant human TGF-β1, we observed an increase in the percentage of CD127+ CD4 T cells, which confirmed the TGF-β administration, since TGF-β signalling promotes CD127 expression (Ouyang, Weiming *et al.*, 2013). All TGF-β-treated samples showed increase in Treg frequencies compared to untreated ones, although not statistically significant, likely due to the small sample size.

Our *in vitro* data rather indicated that Tregs are not infected by HIV. The transcription factor FoxP3 is known to inhibit HIV viral transcription by suppressing NFAT (Selliah *et al.*, 2008). The transcription factor NFAT binds the HIV-1 LTR and thereby contribute to HIV gene transcription by binding to the HIV-1 LTR (Cron *et al.*, 2000). Since FoxP3 inhibits NFAT activity, it contributes to reduced viral transcription in Tregs (Selliah *et al.*, 2008). It was also demonstrated that CD4+ CD25+ CD127- cells (of which 80% express FoxP3) and CD4+ CD25- CD127+ (of which 30% express FoxP3) isolated from peripheral blood of untreated HIV-infected individuals have comparable levels of HIV p24 (Moreno-Fernandez *et al.*, 2009). Although this confirms *in vivo* HIV infection of Tregs, the same study showed that *in vitro* HIV infection of Tregs appeared to be lower compared to infection of effector T cells (Moreno-Fernandez *et al.*, 2009). In addition, Tregs coming from the colon of SIV-infected macaques are less infected, and are hence protected from SIV-mediated cell

death, accounting for their preferential survival (Allers *et al.*, 2010). Although previous studies showed higher rate of thymocyte infection with X4 virus compared to R5 virus (Nunes-Cabaço *et al.*, 2015), we only detected infected cells in two out of six samples (thymus #18 and #19). This might be due to our use of laboratory constructed viral strains and not clinically isolated viral strains.

Our results indicate that HIV infection alone does not increase FoxP3 expression, nor does the combination of HIV infection and TGF-β treatment. Furthermore, we did not observe any increase in TGF-β mRNA levels in HIV-infected thymocytes. However, we measured low infection rates both in fresh and thawed thymocytes. One possible explanation for achieving low infection rates could be the use of laboratory constructed viral strains and not clinical isolates as reported (Angel, 2012), which might be more virulent. A major negative effect of TGF-β in HIV-infected individuals is its contribution to lymphoid tissue fibrosis, by enhancing the activity of fibroblast cells, which results in excessive collagen deposition (Zeng *et al.*, 2011). However, in HIV-infected individuals, the direct effect of TGF-β on viral replication has not been yet studied. Our results indicate that TGF-β does not modulate HIV infection. It is to be underscored that *in vitro* infections are not necessarily representative of what happens in vivo. APCs in the thymus are major sources of TGF-β during *in vivo* infection. In the peripheral blood and tissues of HIV-infected individuals, platelets, Tregs and CD8 T cells secrete TGF-β (section 1.4.5). We do not observe TGF-β production most likely because Tregs remain unaffected by infection or probably because the Tregs present are not functionally active.

In summary, our results provide a comprehensive description of the phenotypic markers expressed by human thymic Tregs. Our study remains the first to demonstrate the use of high throughput methylation sequencing, MiSeq, for the assessment of foxp3 epigenetic status on human thymocyte samples. Although our initial hypothesis about the impact of TGF-β on thymic Treg differentiation during HIV infection was not confirmed, the *in vitro* assays validate the potential use of this model for further studies on human thymocytes in the context of HIV infection, as proposed earlier (Angel, 2012).

CHAPTER VI

CONCLUSIONS

Although Treg frequencies are reported to be higher in chronic HIV-infected individuals and TGF-β is known to mediate lymphoid tissue fibrosis and disease progression in such individuals, the role of TGF-β in inducing thymic Tregs during HIV infection remains unclear. This study aimed at determining the contribution of TGF-β in the induction of FoxP3 expression and Treg generation during *in vitro* HIV infection of human thymocytes.

The *ex vivo* phenotypic characterization of thymic Tregs provided a complete overview of the characteristics of these cells. The expression of certain markers by Tregs imparts a different meaning in the context of the thymus, in comparison to the peripheral blood. For instance, although RORγt is the master transcription factor of Th17 cells in the peripheral blood, in the thymus, it is rather associated with thymocyte maturation. The ex vivo assessment of the epigenetic status of non-coding regulatory sequences of the foxp3 gene in subsets of thymocytes based on CD3 expression levels showed that the establishment of methylation status of foxp3 begins at early stages of thymocyte development. The use of high throughput methylation sequencing (MiSeq) for human thymocytes has been performed for the first time and encourages the use of this technique for further methylation studies in human specimens.

The *in vitro* TGF-β treatment of human thymocytes resulted in increased expression of CD127 and increased Treg frequencies. HIV infection of human thymocytes with R5 and X4 tropic viral strains showed that FoxP3+ CD4 T cells are not infected by HIV. TGF-β treatment had no impact on the rate of HIV infection and did not affect FoxP3 expression or Treg generation during HIV infection. Although the initial study hypothesis was not confirmed, the study validates the usefulness of *in vitro* co-culture model of human thymocytes and OP9-DL1. It has taken us one-step further in understanding human thymic Treg development in the context of HIV infection.

As a next step, it would be interesting to check the epigenetic status of *foxp3* in e *ex vivo* sorted-tTregs, which will provide insights on human tTreg development under homeostatic conditions. It would also be interesting to infect thymocytes with clinical virus isolates , which may increase the infection rates. Eventually, sorting of infected (p24+) tTregs and their epigenetic analysis may allow characterizing the effects of HIV infection on tTreg development.

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