In compliance with the
Canadian Privacy Legislation
some supporting forms
may have been removed from
this dissertation.

While these forms may be included
in the document page count,
their removal does not represent
any loss of content from the dissertation.

Quantitative and qualitative analysis of human *de novo* T cell production using T cell receptor alpha and beta excision circles

Department of Experimental Medicine
Faculty of Medicine
McGill University
Montréal

August 2002
Final and revised version deposited November 15th 2002

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements of the degree of Philosophiæ Doctor (Ph.D.)

Your file Votre référence
ISBN: 0-612-88558-5
Our file Notre référence
ISBN: 0-612-88558-5

THESIS ABSTRACT

The evaluation of human *de novo* T cell production is critical for a better understanding of T cell homeostasis and the immune reconstitution processes. The presence of a functional thymus post-puberty has been unsuspected due to the paucity of tools. This thesis provides direct evidence for a functional thymus, which can contribute to the diversity of the immune reconstitution in pathological situations where the immune system is severely destroyed. Through peripheral blood PCR-based quantification of TCR α and β rearrangement excision/deletion circles (TREC), by-products of gene rearrangement events, we demonstrated that a diversified *de novo* T cell production occurs throughout life, even though thymic function decreases with age[1]. *De novo* T cell production remains intact following allogenic hematopoietic stem cell transplantation (AHSCT) in the absence of graft-versus-host disease (GVHD) and therefore a reduced thymus function cannot be responsible for the long-lasting reduction in peripheral blood naïve T cells observed in transplanted patients. As naïve T cells from AHSCT patients have reduced levels of IL-7Rα chain (CD127) expression, we propose that their low frequencies reflect an impaired naïve T cell survival rather than thymic dysfunction as signaling through CD127 was previously reported to upregulate Bcl-2 expression[2]. Evidence gathered in this thesis supports the concept that such naïve T cells try to replenish themselves through enhanced levels of proliferation but fail to do so and likely die in the process.

Monitoring of the peripheral α and β TREC ratio, a marker of intrathymic proliferation, demonstrated that HIV infection either induces the cellular depletion or inhibits the cell cycling of differentiating thymocytes[3]. As intrathymic proliferation is important for both the magnitude and diversity of thymic function, the results of this thesis indicate that the replenishment of the naïve T cell peripheral compartment through *de novo* T cell production is both quantitatively and qualitatively limited in HIV-infected individuals leading to the contraction of the peripheral T cell repertoire.

Although peripheral blood quantification of α and β TREC can estimate peripheral blood RTE frequencies, reflective of thymopoiesis levels, it does not constitute a method that

can lead to the characterization of this important T cell subset. To better understand the biology of RTEs, we engineered a transgene with restricted GFP expression in T cell that recently rearranged their TCR. This model would be very useful for the identification of molecules capable of modulating thymic function as well as serving as a source for obtaining a highly purified population of RTEs[4], then allowing the characterization of their gene expression profile.

Taken together, this thesis demonstrates the contribution of the adult thymus to immune reconstitution following AHSCT and during HIV infection.

RÉSUMÉ DE THÈSE

Le thymus est la principale glande qui permet le développement/maturation des thymocytes immatures en lymphocytes T immunocompétents. Jusqu'à tout récemment, la présence d'un thymus fonctionnel chez l'adulte n'avait pas été directement démontrée. La quantification des cercles d'excision α et β, sous-produits générés lors du réarrangement des gènes du récepteur des cellules T (RCT), nous a permis de démontrer que la fonction thymique, bien que décroissante avec l'âge, contribue au renouvellement constant du répertoire des cellules T périphériques en exportant des nouveaux émigrants thymiques (NETs) vers la périphérie[1]. De plus, la fonction thymique n'est pas affectée par la transplantation allogénique de cellules souches hématopoietiques (TACSH) en absence de rejet de greffe (GVHD) et contribue à la reconstitution immunitaire chez des patients transplantés. Nous démontrons aussi que l'immunodéficience observée post-TACSH, qui est caractérisée par une réduction des fréquences des lymphocytes T naïfs dans le sang périphérique, est conséquente à une réduction d'expression de la chaîne α du récepteur de l'interleukine (IL)-7 (CD127) à la surface des lymphocytes T naifs ($CD3^+$ $CD45RA^+$ $CD27^+$) plutôt qu'à un problème homéostatique en périphérie ou encore à une altération de l'activité thymique[2].

Cette thèse documente aussi l'utilisation d'un nouvel outil, le calcul du ratio périphérique des cercles d'excision α/β, comme marqueur de la prolifération intrathymique. Ce ratio s'effondre rapidement chez les adultes infectés par le VIH suggérant ainsi que la production thymique exporte des lymphocytes T qui ont eu une prolifération limitée lors de leur éducation thymique. Nous démontrons très clairement qu'il existe un lien entre la production thymique et la prolifération intrathymique des thymocytes immatures, exposant par le fait même un mécanisme permettant de comprendre la diminution quantitative et qualitative de la production thymique au cours de l'infection chronique du VIH. Conséquemment, nous proposons que le VIH élimine les thymocytes qui prolifèrent (ou inhibe leur prolifération) réduisant par le fait même le bassin de thymocytes doubles positifs et limitant la diversité des lymphocytes T périphériques[3].

Bien que ces techniques permettent d'évaluer le rythme de production ainsi que l'étendue de la diversité de la population lymphocytaire exportée en périphérie, elles rendent impossible l'isolation des NETs. Dans un souci d'approfondir notre compréhension de l'homéostasie des NETs, nous avons construit un transgène théoriquement capable d'induire la production de la protéine fluorescente verte (GFP) uniquement au sein des NETs. La génération d'une telle souris transgénique permettrait le développement de molécules pouvant moduler la production thymique. Ce modèle permettra d'obtenir des populations cellulaires enrichies en NETs et d'étudier leur phénotype et leur profil d'expression génique. La section finale de cette thèse décrit ce modèle ainsi que son importance pour des études visant à comprendre la régulation homéostatique des NETs.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CONTRIBUTION OF AUTHORS

Chapter 2 **Direct evidence of thymic function in adult humans**

Jean-François Poulin, Mohan N. Viswanathan, Jeffrey M. Harris, Krishna V. Komanduri, Eric Wieder, Nancy Ringuette, Morgan Jenkins, Joseph M. McCune and Rafick-Pierre Sékaly

J. Exp. Med., 190 (4), 479-486

J.-F. Poulin and R.-P. Sékaly originally conceived this project. Most of the PCR experiments were performed in J. M. McCune's laboratory by J.-F. Poulin (85%), M. N. Viswanathan (10%) and J. M. Harris (5%). M. Jenkins and J. M. McCune proposed the implantation of the "Reed-Muench" analysis technique for the PCR-based β DC (TREC) quantification. Cells were stained by J.-F. Poulin while E. Wieder and N. Ringuette performed extensive cell sorting. K. V. Komanduri was responsible for the cord blood stimulation experiment. J.-F. Poulin, R.-P. Sékaly, K. V. Komanduri and J. M. McCune wrote the paper and J.-F. Poulin generated the figures.

Chapter 3 **Immunodeficiency following allogenic hematopoietic stem cell transplantation is not due to a thymic function defect but the consequence of impaired naïve T cell survival**

Jean-François Poulin, Myriam Sylvestre, Patrick Champagne, Marie-Lise Dion, Nadia Kettaf, Alain Dumont, Maryse Lainesse, Pierre Fontaine, Claude Perreault, Rafick-Pierre Sékaly and Rémi Cheynier.

Submitted to *Nature Medicine*

The cohort of patients studied in this paper was set up and coordinated by C. Perreault while P. Fontaine stored the study samples. J.-F. Poulin and R. Cheynier developed the real-time, on-line, quantitative PCR assay. M. Sylvestre performed DβJβ TREC quantification (with the help of J.-F. Poulin and R. Cheynier), the phenotypic FACS analysis of PBMCs (with the help of P. Champagne) and spectratyping results (with the help of R. Cheynier). J.-F. Poulin generated the sjTREC data, as well as performed the

CD127 staining and subsequent FACS analysis. All the data of this manuscript were analyzed by J.-F. Poulin and R. Cheynier. The manuscript and the figures were generated by J.-F. Poulin and R. Cheynier in collaboration with R.-P. Sékaly and C. Perreault. A. Dumont performed the cloning of the CD3 amplicon in each of the standard curve plasmid. All other authors contributed only minimally to this paper.

Chapter 4 **Changes in the quality of thymic exportation during early primary human immunodeficiency virus infection**

Marie-Lise Dion, Rebeka Bordi, Jean-François Poulin, Myriam Sylvestre, Rachel Corsini, Nadia Kettaf, Ali Dalloul, Ryan Woods, Julio Montaner, Fred Harris, Patrice Debré, Jean-Pierre Routy, Rafick-Pierre Sékaly and Rémi Cheynier

In preparation

A. Dalloul performed FACS-purification of pediatric thymocyte subsets while R. Bordi and R. Cheynier made the initial observations leading to the design of this project. J.-P. Routy, J. Montaner and R.-P. Sékaly provided all the HIV-infected samples. All the data presented in this manuscript were generated by M.-L. Dion, R. Bordi, M. Sylvestre, N. Ketaff and R. Corsini (with the help of J.-F. Poulin) all under R. Cheynier and R.-P. Sékaly's scientific supervision. J.-F. Poulin had an important intellectual contribution, participated extensively in the data analysis and is currently involved in the writing of the manuscript. All other authors contributed only minimally to this paper.

Chapter 5 **FACS-purification of recent thymic emigrants using a novel transgenic mouse model**

Jean-François Poulin, Martin Bourbonnière, Rafick-Pierre Sékaly and Rémi Cheynier

In preparation

The transgenic GreenMouse project equally originated from J.-F. Poulin, R.-P. Sékaly and R. Cheynier. R. Cheynier designed the cloning strategies and J.-F. Poulin built all DNA

constructions. M. Bourbonnière performed Jurkat E6.1 cell transfection and the subsequent FACS analysis. J.-F. Poulin and R. Cheynier analyzed the data and both contributed to the scientific direction of this project (with the help of Rafick-Pierre Sékaly).

ACKNOWLEDGEMENTS

I would like to acknowledge those who contributed, in their own way, in the realization of this Ph.D. thesis.

I thank my thesis supervisor, Dr. Rafick-Pierre Sékaly, for actively participating in my formation and for providing me with an incommensurable amount of personal and financial resources. I also enjoyed being mentored by him and his senior scientists, one of them being Dr. Rémi Cheynier, who constantly stimulated my scientific curiosity and taught me a lot. Furthermore, I benefited from their network of contacts and had the chance to work and interact with a research group in San Francisco led by Dr. Joseph M. McCune.

Throughout my Ph.D. studies, I also benefited from the constant support of many friends and relatives. Above all, my parents, Denise Beauregard and Jacques Poulin, provided me with the best emotional and financial support that any son would envy. I had the chance to do my graduate studies in "worry-less" spirits. Friends were always interested, curious and supportive of my most recent research developments. I am indebted to my previous roommates who understood when I had to seclude myself at some periods during my Ph.D. studies. I thank my girlfriend for her encouragements and caring during the last year.

I am grateful to the CIHR for their financial support during my Ph.D. studies. I would like to express my gratitude to Helen McGraft for her editorial assistance as well as to my many fellow laboratory colleagues, whom I enjoyed exchanging scientific ideas and working with.

1. Introduction

1.1 The thymic architecture

1.1.1 Embryonic development of the thymus

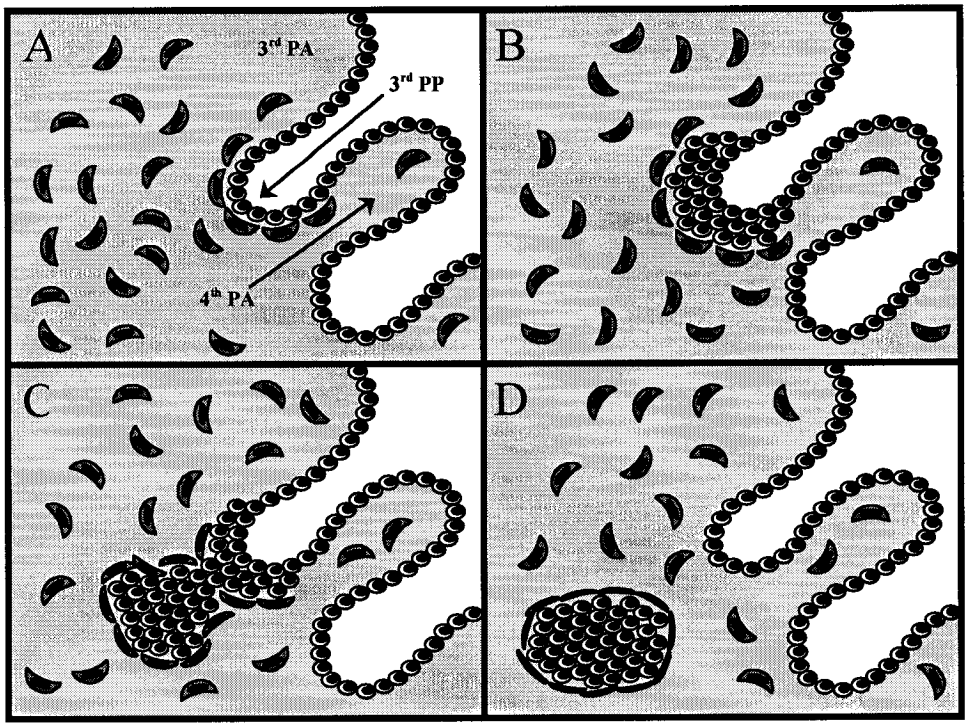
Overview

In the mouse, thymus formation begins early in fetal life with the invagination of the endoderm of the third pharyngeal pouch into the surrounding neural creast (NC)-derived mesenchyme[5-9]. Progressively, this expanding epithelial mass buds and completely detaches from the pharynx to correctly position itself. Further thymus maturation/organization requires colonization by hematopoietic cells. Such cells will finalize thymic architecture development through interactions with thymic epithelial cells (TEC) that will, in return, induce thymopoiesis. A functional thymus is critical for *de novo* T cell production and the establishment of a broad peripheral T cell receptor (TCR) repertoire. This section will describe the events that lead to the formation of the thymic architecture.

Interactions between different embryonic structures lead to thymus rudiment formation

Thymus organogenesis occurs through a series of ordered events. In mice, cells from different embryonic origins congregate during ontogeny to differentiate into the non-hematopoietic structures of the thymus: the endoderm of the 3rd pharyngeal pouch and the surrounding neural crest (NC)-derived mesenchyme from the 3rd and 4th pharyngeal arches[6,10] (Figure 1). It is still unclear whether the ectoderm, which is in close association with the endoderm early in ontogeny, contributes to the initial induction/outgrowth of the 3rd pharyngeal pouch epithelium. NC-derived mesenchymal cells were reported to be essential for the organ morphogenesis[11,12] although they can be substituted by 3T3 fibroblasts in reconstituted organ culture[13]. Delayed growth occurs when mesenchyme from lung or mandibular gland is used suggesting that the interaction of the mesenchymal tissue with the growing epithelial mass appears "permissive" rather than "instructive". In other words, the presence of mesenchyme from an appropriate source at the appropriate

time permits the proliferation and differentiation of already-committed epithelial cells along the normal course of thymus development. The function of NC-derived cells is likely to provide additional signals that allow the growth and differentiation of the epithelial cells from the endoderm/ectoderm, ultimately leading to the formation of the thymic rudiment[14]. Then, the developing thymus detaches from the pharynx and positions itself in the mediastenium.



(Adapted from Manley, N.R., *et al.*, *Semin. Immunol.*, 2000)

Figure 1. *Diagrams depicting the early stages of murine thymic rudiment formation. A Magnified representation of the 3rd pharyngeal pouch endoderm (PP) with the surrounding embryonic tissue, including the neural crest (NC)-derived mesenchymal cells (moon-like cells) from the 3rd and 4th pharyngeal arches (PA).* ***B*** *Initial outgrowth of endodermal cell (round cells) from the 3rd PP penetrates the NC-derived mesenchymal cells.* ***C*** *Around embryonic day 11 (E11), the thymic rudiment buds from the 3rd PP endoderm. The NC-derived mesenchymal cells surrounding the thymic rudiment will condense and gradually form the thymic capsule.* ***D*** *By E12.5, the thymus rudiment has completely detached from the 3rd PP and endodermal cells, through contacts with hematopoietic precursors that will colonize the thymus, begin their differentiation into thymic epithelial cells (TEC).*

Key genes involved in early thymus development

Important transcription factors have been reported to be critical for the early stages of thymus organogenesis. Hoxa3, a member of the Hox transcription factor family, is expressed in the 3rd pouch endoderm as well as in the NC mesenchyme and is implicated in organ positioning in the developing embryo[15]. It exerts its developmental role through regulation of Pax1 and Pax9 genes[6,16]. Mice deficient in Hoxa3 or Pax9 genes result in an athymic phenotype as part of a spectrum of pharyngeal region defects[17-19] whereas loss of Pax1 function leads to a hypoplastic thymus incapable of sustaining thymocyte development[20,21]. Recent studies indicate that Pax1 and Pax9 genes are specifically down regulated in Hoxa3$^{-/-}$ embryos[22]. Taken together, these results illustrate the importance of adequate Hoxa3-dependent regulation of Pax1 and Pax9 for the thymic rudiment formation.

Mutations in the winged-helix nude (Whn) gene Foxn1 present in the *nude* mouse affect the development and/or the maintenance of several organs, including the thymus[23-25]. The *nude* thymic rudiment appears to bud off and migrate normally despite an early block in thymic epithelial cell (TEC) differentiation. Consequently, the thymic rudiment never becomes populated by lymphoid progenitor cells[26]. Therefore, Foxn1 is believed to be required during the latter stage of thymus development (e.g. after the initial induction and outgrowth stage). These results further suggest that migration and differentiation of the thymic rudiment are independently regulated.

The role of hematopoietic-derived cells in thymus organogenesis

The early steps involved in thymus rudiment organogenesis occur in the absence of hematopoietic-derived cells (see above). Further differentiation requires stage-specific interactions between epithelial and hematopoietic cells. At this point, the thymus rudiment is neither vascularized nor compartmentalized and hematopoietic precursors, in the form of lymphoid progenitor cells, need to cross the neural crest-derived capsule and enter the thymic rudiment by a yet unknown mechanism, likely mediated through the secretion of factors by the epithelium[27].

Despite little specific data on the molecular regulation of the interactions between bone marrow-derived cells and epithelial cells during fetal development, it is clear that important "cross-talk" is needed for the adequate development of both thymocytes and the thymic architecture[28,29]. Mouse strains with blocks at distinct stages of thymocyte development show various degrees of epithelial differentiation and histological organization: the earlier the block in thymocyte maturation, the more important its repercussions will be on cortical development/organization. In fact, mice with an early block in thymocyte ontogeny (double negative (DN) 1: $CD44^{+}CD25^{-}$) lack a functional thymic cortex. This defect is reversible during fetal or early post-natal differentiation stages, but not in the adult thymus[30]. These data suggest that the ability to form cortical epithelial cells may be temporally restricted[31]. In contrast, medullary development is less restricted and can be reconstituted even at adult stages[28,29]. Although the molecular nature of this "thymic crosstalk" remains unknown[28,32,33], thymocytes clearly have an important role in both cortical and medullary TEC development.

Encapsulation and vascularization of the thymus

At the end of organogenesis, the thymus is encapsulated by loose connective tissue composed of fibroblasts and extracellular matrix (ECM)[34]. The ECM consists of collagens, glucosaminoglycans, and glycoproteins such as fibronectin and laminin, secreted by epithelial cells and fibroblasts[7,35]. This connective tissue penetrates the cortex, forming septa that often contain blood vessels and consolidate the thymic architecture. Collagen of type I and IV, vimentin and merosin[36] are components of the ECM that were reported to play a role in thymocyte migration, differentiation and proliferation[37].

Thymic vascularization is unusual. Arterioles, which enter the thymus at the corticomedullary junction (CMJ), become capillaries that ascend into the cortex to the subcapsular region where they loop back toward the medulla. As they descend, capillaries join and form larger capillary vessels ultimately fusing into postcapillary venules at the CMJ and in the medulla. Cortical capillaries consist of endothelial cells forming impermeable tight junctions with each other, therefore isolating differentiating

thymocytes present in the cortex from blood-borne proteins by an effective blood-thymus barrier[38].

Although the thymus rudiment consists initially of an aggregate of epithelial cells, hematopoietic cells infiltrating the thymus will soon account for the bulk of thymic mass. In addition to thymocytes, the adult thymus contains a spectrum of hematopoietic-derived cells including macrophages, dendritic cells, B cells, NK cells, eosinophils and basophils. The following section will discuss the anatomical organization of the thymic epithelium and the role of bone marrow-derived cells in thymocyte ontogeny.

1.1.2 Thymic Compartmentalization and Cellular Distribution

Overview

Full thymic development leads to the generation of 4 distinct compartments: the subcapsular region, the cortex, the corticomedullary junction and the medulla. These thymic compartments constitute the thymic epithelial space (TES), where thymopoiesis occurs. Unlike any other lymphoid organ, the thymus contains a dense network of cortical and medullary thymic epithelial cells (TEC) that forms a three-dimensionally oriented network providing the optimal microenvironments required for the generation of immunocompetent T cells from bone marrow-derived precursors[7,33,39-42].In the adult thymus, most of the space between the epithelial "framework" of cells is occupied by developing thymocytes. Other types of thymic bone marrow-derived cells include macrophages, dendritic cells and mature B lymphocytes, believed to play important roles during T cell ontogeny, especially in the induction of tolerance. Previous studies documenting the histological features of the thymus have reported the existence of a perivascular space (PVS), separated from the TES by a basal membrane, containing recirculating peripheral lymphocytes[43]. Given this heterogeneous cellular composition and complex organization, the thymus must be considered as a chimeric organ/gland housing TEC/thymocytes as well as peripheral cells[44]. The following section will describe the organization of the thymic compartments and the localization of several bone

marrow-derived cells within the thymus. An emphasis will be placed on the relevance of this cellular distribution in T cell ontogeny.

The subcapsular region and the thymic cortex

The outermost area of the thymus is referred to as the subcapsular region and contains the most immature population of developing thymocytes (double negative (DN) thymocytes average 1-5 % of total thymocytes) believed to have entered the thymus from post-cappilary venules lining the corticomedullary junction[45] (CMJ) (Figure 2).

Beneath the subcapsular region lies the cortex, which comprise the main body and encompasses almost 80-85% of all lymphoid cells present in the thymus. The thymic cortex is densely packed with double positive (DP) thymocytes ($CD4^+$ $CD8^+$) that will undergo the stringent events of positive/negative selection. Only a small proportion of DP thymocytes (less than 5%) will survive and mature in the medulla to become single positive (SP) thymocytes, waiting to be exported in the periphery.

Thymic nurse cells (TNC) are scattered within the cortex and in the subcapsular area of the thymus (Figure 2). These epithelial structures of unusual size (diameter up to 50 μm), were first documented by Wekerle and Ketelsen in 1980[46,47] and are believed to play a central role in early thymocyte ontogeny given their close association with thymocytes. In fact, TNC have the capacity to "internalize" thymocytes, which are metabolically active, proliferate and are therefore believed to be intact. Furthermore, TNC express high levels of classical MHC class I an II antigens[48,49] and have the ability to bind to other types of cells (macrophages or dendritic cells) and generate TNC complexes. Such complexes contribute to provide an adequate microenvironment required for early thymocyte ontogeny[50]. Moreover, reverse transcription polymerase chain reactions (RT-PCR) have revealed that TNCs contain granulocyte-macrophage colony stimulating factor (GM-CSF) as well interleukin (IL)-5 and interferon (IFN)-γ transcripts[51]. Moreover, TNC complexes can secrete thymic hormones, like thymulin[52], prostaglandin E2[53], thymosin α1 and β that possess immunoregulatory properties as well as have the capabilities to impact on the nervous and endocrine systems[54].

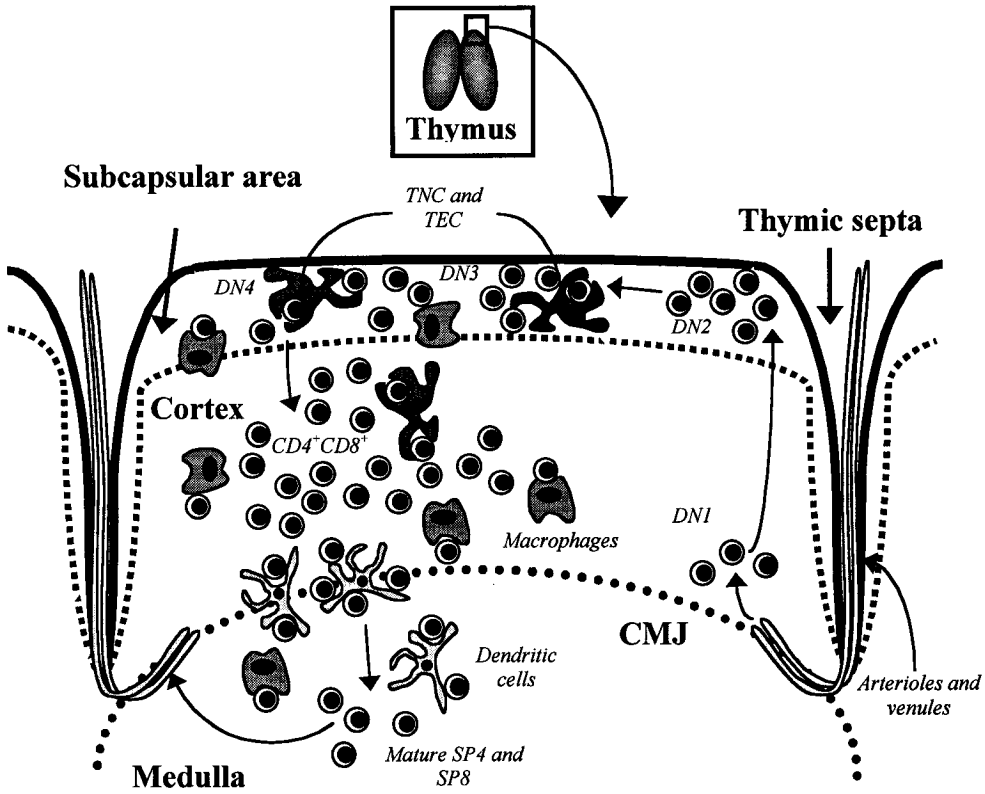


Figure 2. *Magnified representation of a lobule section of the thymus and distribution of its main cellular components. DN1 thymocytes enter the thymus by extravasation from arterioles at the CMJ and migrate outward to reach the subcapsular area. Contacts with TEC and TNC provide the microenvironment required for early T cell ontogeny. Following gene rearrangement (DN2-DN3) and successful β selection (DN4), thymocytes enter the thymic cortex, become DP thymocytes and undergo the stringent T cell selection events mediated by dendritic cells located at the CMJ and in the medulla. Throughout this process, dying thymocytes are phagocytosed by macrophages. Surviving thymocytes commit to either the CD4 or CD8 lineage to become SP4 or SP8 thymocytes, respectively, before exiting the thymus through venules.*

The corticomedullary junction (CMJ) and the medulla

These regions, located underneath the cortex, are enriched in bone marrow-derived cells, especially dendritic cells (DC) and B cells[55,56]. These cells have antigen presenting cell (APC) functions and contribute to the elimination of thymocytes with overt affinity for self MHC[57-60]. Thymocytes surviving this selection process will become single positive (SP) for either CD4 or CD8, express high levels of TCR αβ molecules and migrate to the deeper region of the medulla.

Given that T cell precursors enter the thymus at the CMJ, further thymocyte maturation requires their migration into the subcapsular area, the outermost region of the thymus. Such movement is against the flow of developing thymocytes, migrating in the direction of thymic cortex to medulla. Interactions between chemokine receptors and their corresponding chemokines play a significant role in regulating the migration of developing thymocytes. Stage-specific expression of distinct chemokine receptors could very well be responsible for the migrating patterns followed during thymocyte maturation. This illustrates the importance of the thymic microenvironment during T cell ontogeny and underlines the complexity of cell movement within the thymic 3D architecture.

Thymic macrophages are dispersed throughout the cortex and medulla and also line blood vessels and the connective tissue of the thymic capsule. In the rat thymus, cortical macrophages lack MHC class II expression and have abundant phagolysosomes[61]. This suggests that macrophages contribute to the elimination of the massive number of double positive thymocytes that die as a result of unsuccessful thymic selection. Interestingly, resting DP thymocytes are selectively uptaken by macrophages and completely digested within several hours[62]. Phagocytic macrophages can also be found in the medulla, but are less apparent in the corticomedullary junction (CMJ) where their precursors (immature monocytes) predominate[63]. As thymic post-capillary venules (located at the CMJ) contains high endothelial venules (HEV)[64,65] used by hematopoietic precursors to enter the thymus, it is possible that monocytes colonize the thymus and further differentiate inside the thymic architecture to renew the pool of thymic macrophages.

In contrast to the ubiquitous distribution of macrophages in the thymus, thymic dendritic cells can be found only at the CMJ and in the medulla. Strong expression of both MHC class I and II molecules also characterize thymic dendritic cells. Morphologically, the vast majority of thymic dendritic cells lack phagolysosomes and other cytoplasmic organelles typical of phagocytic cells[61]. Instead, they have elongated and thin cytoplasmic processes relatively free of organelles, that interdigitate with surrounding

human thymocytes expressing high levels of CD1, CD2, CD4 and CD8 but weak levels of CD3[66,67]. Based on these characteristics, dendritic cells are likely to be associated with DP thymocytes that are undergoing thymic selection. As thymic dendritic cells can be generated from hematopoietic progenitors isolated from the bone marrow[68] or from umbilical cord blood[69], it is most probable that thymic dendritic cells originate from a hematopoietic lineage.

Thymic B cells constitute less than 0.5% of total thymocytes in the normal adult thymus and are localized predominantly in the CMJ[70]. Some autoimmune diseases, one of them being myasthenia gravis, lead to a dramatic increase of thymic B cell numbers expressing $CD5^{+}$[56]. Such cells were shown to secrete autoantibodies[71] and be located in the perivascular space of the thymus. The physiological relevance of these processes is yet to be discovered.

The thymic medulla is comprised of SP thymocytes (as well as dendritic and epithelial cells) that progressively undergo the final maturation steps and upregulate a variety of cell surface molecules including CD45RA[72], phenotypically associated with the peripheral naïve T cell compartment. These changes likely "prime" SP thymocytes for further exportation into the recent thymic emigrant (RTE) peripheral pool. After residing in the medulla for a variable period of time, SP thymocytes exit the thymus to enter the periphery while bearing a naïve phenotype.

In summary, thymocytes interact with different types of cells during their differentiation in the thymus. After their entry, thymocytes migrate to the outermost region of the thymus. Association with TNC and TEC provide an adequate microenvironment for the initial events of T cell ontogeny. Thymic selection is mainly mediated through interactions with dendritic cells while macrophages are responsible for phagocytosing unwanted thymocytes. It is important to note that macrophages and dendritic cells are distributed within the thymus in a way that correlates with their biological function during T cell ontogeny.

Cortical and medullary thymic epithelial cells constitute the thymic framework

The greater density of lymphoid cells in the cortex, as compared to the medulla, makes these two compartments easily visible when performing microscopic histochemical analyses of tissue sections. As epithelial cells present in the medulla area (mTEC) are more loosely arranged than thymic cortical epithelial cells (cTEC), it is possible to discriminate between TEC compartments using cytokeratin-specific antibodies (e.g.: more dense regions correspond to the cortex)[73]. Cortical and medullary TEC express distinct cytokeratin species reflective of their differentiation stage[74].

TECs display considerable morphological and histological heterogeneity. Ultrastructural analysis has identified 6 types of thymic epithelial cells[75]. To be brief, type 1 epithelial cells (EC1) line the capsule/septa and surround the perivascular spaces. Types 2, 3 and 4 (starting from the outermost to the deep cortical regions) constitute a graded series of electron-lucent metabolically active (EC2) cells to electron-dense dying cortical cells (EC4). Type 5 epithelial cells (EC5) are rare and likely comprise undifferentiated/unspecialized cells located around the CMJ regions. Type 6 epithelial cells (EC6) form the major medullary population and contribute in the formation of Hassall's corpuscules[29,75-80]. While only a small fraction of TEC were reported to be associated with the vasculature (especially in the cortex where blood vessels are isolated from the TES by EC1 cells), most are interconnected and form a network of elongated cells on which thymocytes "crawl" during ontogeny.

Whether all thymic epithelial cells derive from common progenitors remains to be known, but an understanding of the formation of the medulla is starting to be elucidated[81]. In this elegant study, the authors showed that reaggregated fetal thymic organ cultures (RFTOC), consisting of a mixture of purified Balb/c ($I\text{-}A^b$) and B6 ($I\text{-}A^d$) thymic epithelial cells, have the remarkable ability to self-reorganize into structurally and functionally competent microenvironments capable of sustaining T cell maturation *in vivo* when grafted into MHC class II $^{-/-}$ mice. Furthermore, transplantation of RFTOC in double knockout mice with an early block in T cell ontogeny (MHC class II$^{-/-}$, RAG-2 $^{-/-}$ double knockout mice) still leads to the development of medullary islets. These

experiments also suggest that the sole presence of $CD3^-$ $CD4^-$ $CD8^-$ immature thymocytes, rather than mature CD4 and CD8 single positive (SP) thymocytes, is sufficient to drive medullary islet formation. As these medullary islets are derived from either Balb/c or B6 epithelial cells exclusively, it was proposed that they were generated through expansion/differentiation of a single progenitor. Most mTEC might continuously be regenerated through proliferation/differentiation of stem cells located at the CMJ[82]. Terminally differentiated mTEC take the form of concentric whorls of keratin called Hassall's bodies.

More recently, thymic progenitor cells with the capacity to give rise to both cortical and medullary epithelial lineages were described[83]. Strikingly, these "thymic epithelial stem cells", shown to express the cell surface glycoprotein MTS24, have the ability to generate *in vivo* a full thymic microenvironment capable of attracting hematopoietic precursors and supporting subsequent T cell development following engraftment. It remains to be seen whether these thymic precursor cells can enhance the reconstitution of immune functions during diseases characterized by a thymic hypoplasia. It would be of interest to investigate if a chimeric thymus can shape the peripheral T cell repertoire of an irradiated and thymectomized mice, leading to full acceptance of the grafted organ.

The thymic perivascular space

The perivascular space (PVS) is another anatomical structure of the thymus that has gained interest in recent years because of its localization adjacent to blood vessels, yet still puzzles researchers regarding its function. It is located within the thymic capsule, but separated from the TES by a basal membrane[84-86]. As we age, the PVS grows in size and importance relative to the TES. The perivascular space (PVS) can be identified through immunostainings for laminin and fibronectin, two major components of the extracellular matrix (ECM)[86]. ECM proteins could provide an additional structure capable of influencing intrathymic migration of thymocytes during their maturation process[87].

Thymic function requires an adequate thymus organogenesis as well as a constant supply of bone marrow-derived cells, critical for tolerance induction and elimination of apoptotic

thymocytes. As a result of genetic mutations, aberrant development of either bone marrow-derived cells or TEC occurs leading to a defective thymic activity. The following section will describe immunodeficiency scenarios caused by either a malformation of the thymus or a blockade in T cell differentiation.

1.1.3 Genetic Disorders Leading to Aberrant Thymus Organogenesis and T Cell Deficiencies

Overview

Several developmental disorders lead to malformation of the thymus and surrounding tissues. Inadequate formation of sections of the heart, parathyroid and thyroid glands, are often the consequence of genetic mutations leading to the aberrant differentiation of the pharyngeal pouches as all of these organs/glands are derived from the same embryonic tissue. In general, alterations in the function of early developmental genes lead to more severe anatomical defects compared with tissue-specific gene mutations, which affect fewer lineages and organs/glands. The corresponding anatomical anomalies are classified into an extensive panel of syndromes, one of them being the DiGeorge syndrome (DGS). A brief introduction on the DGS, the severe combined immunodeficiency disease (SCID) and the Bare lymphocyte syndrom (BLS) will be provided in order to illustrate the critical interdependence of a coordinated thymic architecture development and adequate thymocyte differentiation for proper development of the T cell compartment.

The DiGeorge syndrome (DGS)

The DiGeorge syndrome (DGS) is associated with heterozygotic deletions on human chromosome *22q11.21-22q11.23*[88]. This syndrome is characterized by a graded hypoplasia (or even complete absence) of the thymus and parathyroid glands, as well as facial dysmorphogenesis. Aberrant development of these organs/glands (all derived from the pharyngeal pouches) is linked to a failure of neural crest (NC) cells to differentiate appropriately[12,89]. It remains to be shown whether or not key genes involved in NC differentiation are located within the deleted locus.

Various genetic approaches in the mouse have been used to elucidate the underlying molecular basis for the DGS. Hoxa-3 knock-out mice strains show similar phenotypes to that of human patients with DGS[90]. Moreover, the T box transcription factor gene *Tbx1* was also identified as a candidate gene for involvement in the syndrome, both by its chromosomal location and by its specific expression in the mesenchyme and the developing pharyngeal pouches[91-93]. $Tbx1^{-/-}$ mice display a spectrum of defects encompassing most of the common DGS[94,95]. Other candidate genes were proposed (*HIRA*[96-98] and *UFD1L*[99]) but the defect resulting from these genes fall short of encompassing all the spectrum of abnormalities characterizing DGS. Of note is the possibility that DGS abnormalities are a consequence of defects in multiple genes (such as *HIRA*, and *UFD1L*), acting independently or in conjunction with Tbx1 that affects the same developmental pathways. Analysis of mice that lack two or more of these candidate genes may help to address this issue. Although the downstream effectors of Tbx1 remains largely unknown, it is likely that they contribute in ensuring adequate neural crest migration[100].

From the immunological standpoint, DiGeorge patients have a profound T cell deficiency, primarily as a result of very little or no thymic tissue[101-103]. Several therapies have been used to treat such DGS patients ranging from infusion of HLA-identical mature T cells[104,105] to thymus transplantation[106-108]. In fact, a recent report by Markert and colleagues[108] demonstrates that thymus transplantation in complete DGS children can lead to the generation of functional circulating T cells. In this paper, active thymopoiesis was evidenced through peripheral blood quantification of TCR rearrangement excision/deletion circles (TREC), markers for *de novo* T cell production (discussed later in this thesis). As T cells generated through *de novo* mechanisms have a broader TCR repertoire as compared with lymphocytes issued from peripheral expansion, it is likely that thymus transplantation offers great immune reconstitution potential for DGS patients.

The severe combined immunodeficiency diseases (SCID)

Severe combined immunodeficiency diseases are fatal and rare genetic disorders characterized by profound deficiencies in T and B cell functions[109]. The origins of such defects are quite diverse and often involve mutations in key genes implicated in the development of T and B cells. The most common form of SCID (named X-linked SCID) is associated with defects in the shared γ common (γ_c) cytokine chain of IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21 cytokine receptors, which is located on the X chromosome. Several different mutations in γ_c chain have been observed in X-linked SCID. Amino acid substitutions in the extracellular domain impair cytokine binding[110] whereas mutation/truncation of the cytoplasmic tail abrogates signaling. The latter mutation impairs the ability of Jak3, a downstream kinase implicated in cytokine receptor signaling, to bind with the γ_c chain and adequately start the signaling cascade[111,112]. Presumably, any Jak3 mutation interfering with its association with the γ_c chain, its catalytic activity or with the recruitement of downstream substrates/effectors could result in clinical disease. In this regard, it was shown that mice deficient in either Jak3 or the γ_c chain have a similar phenotype[113-116].

Mutations incorporated in a metabolic enzyme, the adenosine deaminase (ADA), also lead to a SCID-like phenotype, characterized by a severe depletion in T, B and natural killer (NK) cells. The ADA deficiency results in the accumulation of its substrates, adenosine and 2'-deoxyadenosine[117]. Such metabolites mediate their cytotoxic effect by either interfering with transmethylation reactions[118], disturbing cell signaling[119-121], interrupting deoxynucleotide synthesis[122,123] and/or activating apoptotic pathways[124]. Thus, ADA deficiency disturbs multiple metabolic pathways important for both intrathymic development and mature T cell function[117].

The inability to rearrange T cell receptor (TCR) or immunoglobulin (Ig) genes causes the complete absence of T and B lymphocytes in the periphery. In infants, such defects, similar to Jak3 and ADA deficiency, result in autosomal-recessive SCID. Similar to the RAG-1[125] and RAG-2[126] mutant mice, infants with mutations in RAG-1 or RAG-2 have very small lymphoid organs containing immature lymphoid cells that all failed to

rearrange the TCR and Ig genes[127]. Unlike the γ_c chain X-linked SCID, Jak3 and ADA deficiencies, infants with RAG mutations do have NK cells in the periphery as these cells do not need to undergo gene rearrangement in order to differentiate adequately.

Thymic hypoplasia is one of the hallmarks of all SCID clinical scenarios presented here. It is important to mention that this phenotype is not due to the incapacity of the thymus to develop but is rather induced by an early blockage in thymocyte ontogeny as a result of mutations in either the γ chain, Jak3, ADA or RAG-1 or RAG-2. A successful attempt has been made to cure X-linked SCID infants (also known as "bubble boys"). Autologous bone marrow transplantation of $CD34^+$ precursors cells, in which a normal γ_c chain gene was retrovirally introduced *ex vivo*, leads to the development of peripheral T lymphocyte subsets, NK cells as well as a normal-sized thymus gland/organ[128]. Furthermore and most importantly, correction of the immunodeficiency eradicated established infections and allowed patients to have a normal life. These impressive results validate once and for all the *in vivo* requirement for a molecular "cross-talk" between both thymocytes and thymic epithelial cells (TEC) and that absence of thymocytes at all differentiation stages induces thymic atrophy.

The bare lymphocyte syndrom (BLS)

The bare lymphocyte syndrom is a rare autosomal recessive disease characterized by the lack of MHC class II expression on all professional antigen-presenting cells (APCs) (e.g. B lymphocytes, macrophages and dendritic cells), which leads to the absence of cellular and humoral immune responses directed against foreign antigens[129-132]. Moreover, cells that are not of bone marrow origins, such as thymic epithelial cells (TECs) are also negative for MHC class II expression. Additional experiments showed that this deficiency concerned the α and β chains of HLA-DR, HLA-DP and HLA-DQ molecules, thus providing evidence of a general block in the expression of MHC class II genes[133-135]. In contrast to most immunodeficiency diseases, BLS patients have normal total T and B lymphocyte numbers but show a reduced proportion of peripheral $CD4^+$ T cells. The remaining $CD4^+$ T cells seem to be functionally normal, based on alloreactivity and responses to mitogens, and do not exhibit major abnormalities in their TCR repertoire.

Therefore, BLS is a disease that originates from a defect in the regulation of MHC class II gene expression.

Several mutations in 4 key transcription factors that control MHC class II expression have been identified in BLS patients (CIITA, RFX5, RFXAP and RFXANK). Whereas the last 3 genes are subunits of RFX, a trimeric complex that binds to all MHC class II promoters[136], CIITA functions as the master control factor for MHC class II expression[134]. It is important to point out that these factors are located on distinct chromosomes than MHC class II genes and that their inactivation leads to defects in *trans* activation of MHC class II expression[134]. Thus, BLS is a syndrom that originate from the inability to express MHC class II genes.

Absence of MHC class II within the thymus likely reduces the number of *de novo* produced $CD4^+$ T cells as well as limits their survival in the periphery (see section 1.3.1). As MHC class I expression is not affected in BLS[129-132], it is anticipated that the thymus would retain its ability to generate $CD8^+$ SP thymocytes and thus, have a normal architecture.

1.1.4 Factors Affecting Thymus Size and Cellularity

Overview

The thymus is the major site for the generation of immunocompetent lymphocytes and is located in the upper part of the mediastinum (behind the sternum). As we age, the thymic epithelial space (TES) decreases in size and is gradually replaced by the perivascular space, mainly consisting of adipose tissue and mature B lymphocytes. This section will evaluate the impact of different hormones and cytokines on thymic size and cellularity. What triggers these histological changes leading to thymic involution is not completely understood and will be explored in this section.

Probable causes leading to thymic involution

As a result of thymic involution, the perivascular space (PVS) becomes a prominent architectural feature during adulthood which has been shown to harbor cells that secrete factors associated with thymic atrophy (IL-6, OMS and LIF). TEC, adipocytes and/or other types of stromal cells could be responsible for this secretion[137]. The histological changes that contribute to thymic involution remain speculative but probable causes are 1) aging of the stem cell population that gives rise to T cells[138,139], 2) a reduced capability to rearrange TCR β gene segments[140], 3) loss of thymic epithelium expressing self-peptide[141,142], and 4) aging of the thymic microenvironment with the loss of cytokines[44]. Each one of these 4 scenarios would induce a reduction in the magnitude of thymic function (either through a decreased number of thymocytes or through an inability of the thymic stroma/epithelium to educate thymocytes).

The impact of hormones and cytokines on the thymus

The first evidence that the endocrine system played a role in the immune system was provided when surgical removal of the anterior pituary gland induced the involution of the rat thymus[143]. The pituary gland is responsible for the secretion of several hormones and neuropeptides like adrenocorticotrophin (ACTH), prolactin (PRL), vasopresin, oxitonin, melatonin, endorphins and encephalins as well as growth hormone (GH)[144]. Aside from their endocrine function, these hormones can modulate several aspects of the T and B cell compartments such as cellular proliferation/activation/immunosuppression, antibody production and cytokine secretion[144-147]. One could postulate that fluctuation in one or several cytokines present within the thymus could participate in the modification of the organ's TEC/PVS ratio as well as its size.

GH has been shown to promote hematopoiesis[148], including cells from the lymphoid[149], myeloid[150], erythroid[150] and megakaryocytic lineages[151]. Despite such strong evidence indicating a role for GH in hematopoiesis, it remains to be demonstrated whether GH-related effects are mediated through modulation of proliferation, activation or survival of cells. Nonetheless, administration of GH was shown to increase thymic volume and density in HIV-infected adults[152]. Moreover, the fact that GH-induced increase in thymic

mass was associated with an increase in naïve T cells, combined with the fact that discontinuation of GH therapy leads to the recurrence of thymic atrophy, strongly suggests that thymopoiesis is enhanced following GH treatment.

The production of GH is pulsatile, mainly occurs at night and is controlled by hypothalamic hormones such as GH-releasing hormones (GHRH), hypothalamic GH releasing inhibiting factor and somatostatin. Although the GH receptor (GHR) can be found on a variety of bone marrow-derived cells (DP and SP8 thymocytes, subsets of B and T peripheral cells, macrophages)[153,154] and could directly mediate its effect on tissues, many of the GH-related effects result from GH-induced secretion of *insulin growth factor* (IGF)-1 which induce TEC proliferation.

Aside from GH, other factors outside the immune system also exert important effects on thymus function. Among these are hormones such as estrogens, glucocorticoids[155] and progesterone[156-161]. Receptors for each of these hormones are expressed on thymic architectural tissues and hematopoietic-derived cells[156,160-164]. Based on the localization of specific receptors, both thymocytes and thymic epithelial cells (TEC) have been implicated as targets of hormones. Fluctuations in androgen levels lead to striking changes in the thymus weight, cellularity and cellular composition. In fact, the thymus gland of male mice is enlarged under conditions of androgen deficiency or in mice with defects in androgen action[165,166]. Similar conclusions can be drawn regarding the role of corticosteroids as thymocytes were shown to be extremely sensitive to corticosteroids.

Furthermore, groups of cytokines whose expression fell during aging (IL-2, IL-9, IL-10, IL-13, IL-14) as well as others whose concentration was increased (LIF, OSM, M-CSF, SCF, IL-6) were identified[137]. Moreover, a third group a cytokines, encompassing G-CSF, IL-7 and IL-15, were demonstrated to remain stably expressed during adulthood[44]. It is thus possible that an age-related modification of thymic cytokine secretion could play a role in the natural process of thymic involution. The age-related accumulation of adipocytes was suggested to induce the changing pattern of thymic cytokine production[137].

Taken together, the size of the thymus can be modulated by several hormones and cytokines that act on both the thymus architectural cells as well as on hematopoietic-derived cells. Age-related changes in the production of these cytokines/hormones are likely to contribute to the involution of the thymus. It is also possible that certain cell types gradually colonize the thymus as we age and, through secretion of distinct cytokine/hormone production profile, regulate the process of thymic atrophy.

1.2 Intrathymic αβ T Cell Development

1.2.1 General αβ T Cell Ontogeny

Overview

From the colonization by T cell precursors of the thymus to the exit of mature cells from the organ, a vast body of interactions promotes the complex process of T cell differentiation. As they mature, thymocytes migrate to specific regions of the thymus and interact with different types of stromal cells. For simplicity and the purpose of clarity, thymologists classified the stages of human thymocyte differentiation according to the expression of several cell surface molecules like CD34, CD1a, CD3, CD4, CD8 and the TCR (αβ or γδ), to name only the most commons. Signals received by thymocytes through contacts with TEC will lead to thymocyte differentiation and survival or death. During these steps, thymocytes undergo phenotypic as well as genomic changes and mature from the $CD4^-$ $CD8^-$ double negative (DN) phenotype, comprising only 1-5% of the total number of thymocytes, to $CD4^+$ $CD8^+$ expressing thymocytes, also known as double positives (DP). Elimination of harmfull and/or autoreactive DP thymocytes within the thymus occurs (a process refered to as positive and negative selection) and surviving thymocytes shut off expression of either the CD4 or CD8 coreceptor[167]. These MHC-restricted, non-self reactive, CD4 or CD8 single positive (SP) thymocytes represent the end product of intrathymic αβ T cell development, and will eventually exit the thymus and migrate to the peripheral lymphoid organs to establish the peripheral T cell repertoire. This section will describe in detail the maturation step characterizing T cell development.

Pluripotency of progenitors and commitment to the T cell lineage

In many ways, the production of functionally mature lymphocytes from immature precursors resembles that of their hematopoietic cousins: the erythroid, myeloid, and megakaryocytic lineages. All of these lineages arise from common, self-renewing hematopoietic stem cells that primarily reside (except for early fetal life) in the bone

marrow, and undergo regulated changes in gene expression that lead to the specialized form and function of the mature cell[168-171].

Through the expression of distinct genes in response to signals from the microenvironment, gradual commitment to one of the hematopoietic lineage occurs and restricts the pluripotency of the differentiating cell. The role of several transcription factors in the early steps of hematopoiesis has been studied. Commitment to the lymphoid and myeloid, but not hematopoietic, lineages depends on transcription factor PU.1[172]. Lymphoid, but not myeloid, commitment further necessitates a functional *Ikaros* gene[173,174]. Strikingly, mutation of the PU.1 gene[175] and a simple null mutation of *Ikaros*[176] were permissive for the differentiation of adult, but not fetal, T cells, further suggesting that the requirements of thymopoiesis change during life. Further supporting this is the fact that the concentrations of several cytokines within the thymus were shown to vary as the human ages[137]. Of note, inactivation of GATA-*3*, a zinc-finger protein, resulted in normal levels of hematopoietic cells, with the sole exception of T cells, which were incapable of progressing beyond the initial stage of thymocyte maturation (DN1)[175]. Therefore, the establishment of thymopoiesis demands bone marrow-derived precursors to commit to the cell lineage, a process regulated through sequential synthesis of distinct transcription factors. As they differentiate, daughter cells acquire new properties in responses to changes in the microenvironment. Key transcription factors mediate this effect through the regulated expression of specific genes that will "instruct" the developing cell to enter a given lineage. Lack of such transcription factors results in the inability to turn on (or turn off) specific genes, further interfering with downstream lineage commitment checkpoints.

The pluripotency of DN thymocytes is evidenced by their ability to give rise to T cells[177,178], natural killer (NK) cells[178,179], dendritic cells[180,181] and even B cells[178,182,183]. The existence of a common progenitor for T and NK cells has been demonstrated at the single-cell level[173]. These precursors are all present at the early $CD44^{+}CD25^{-}$ TN stage (DN1), but only those engendering dendritic cells persist to the next, $CD44^{+}CD25^{+}$ (DN2), stage[181,184]. Thymocytes further differentiating into the DN3 subset can only give

rise to T-cell precursors. This coincides with the first wave of TCR gene rearrangement (encompassing the DN2 and DN3 subsets), irreversibly committing DN thymocytes to αβ or γδ T cell lineage through appropriate gene rearrangements at the TCR β, γ or δ loci (Figures 3 and 4).

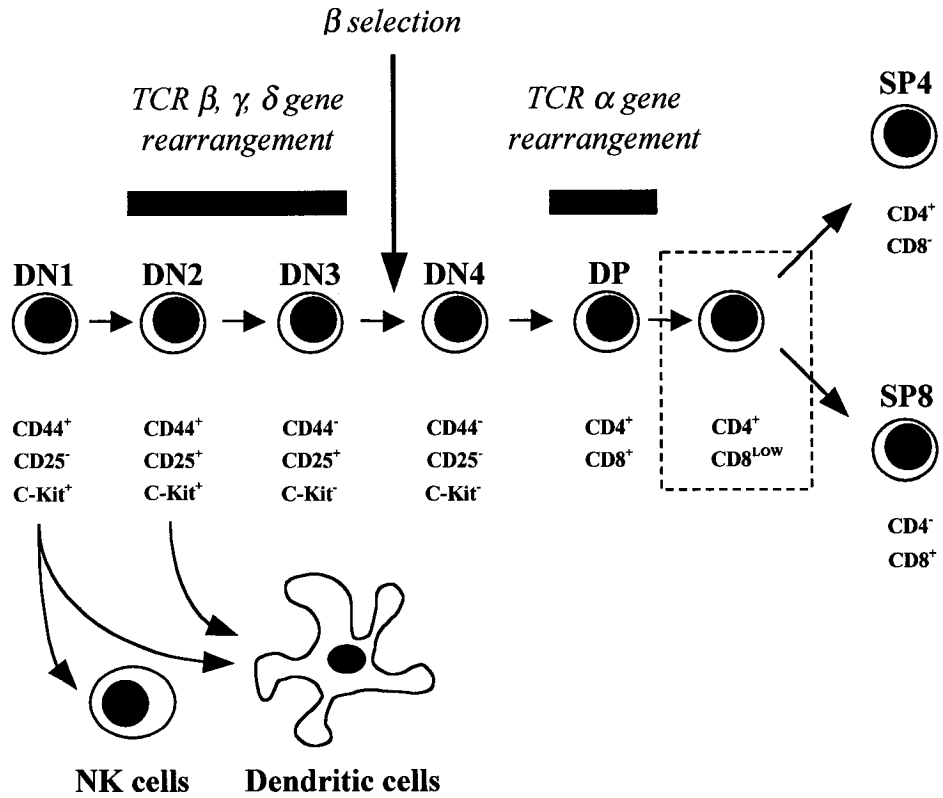


Figure 3. *Murine thymocyte differentiation stages. Immature precursors enter the thymus and begin their differentiation as double negative (CD4⁻ CD8⁻) thymocytes. Initial expansion (at the DN2 stage), TCR gene rearrangement (DN2-DN3 stage) and subsequent β selection (between DN3 and DN4 stage) lead to the generation of the double positive compartment (CD4⁺ CD8⁺) where further elimination of autoreactive thymocytes occurs (T cell selection). Following positive/negative selection, thymocytes become single positive for either CD4 or CD8 before leaving the medulla and enter the periphery. The ability of DN1 and DN2 thymocytes to generate NK and dendritic cells is illustrated at the bottom. Stage-specific expression of CD25, CD44, c-Kit, CD4 and CD8 is also shown. The dashed rectangle represents the newly found thymocyte subset capable of giving rise to both CD4 and CD8 SP thymocytes*[167].

Colonization of the thymus and early thymocyte ontogeny

In the mouse, maturation of αβ thymocytes is characterized by the progression of double negative (DN) precursors expressing neither CD4 nor CD8, to a double positive $CD4^+$ $CD8^+$ stage. DN thymocyte differentiation pathways can be ordered in the following developmental sequence of phenotype: c-$Kit^+CD44^+CD25^-$ (DN1) → c-$Kit^+CD44^+CD25^+$ (DN2) → c-$Kit^-CD44^-CD25^+$ (DN3) → c-$Kit^-CD44^-CD25^-$ (DN4) (Figure 3). Altogether, DN thymocytes represent only a fraction of the normal mouse and human thymocyte population (1-5%) but show tremendous proliferation potential as compared to the more mature DP and SP thymocytes.

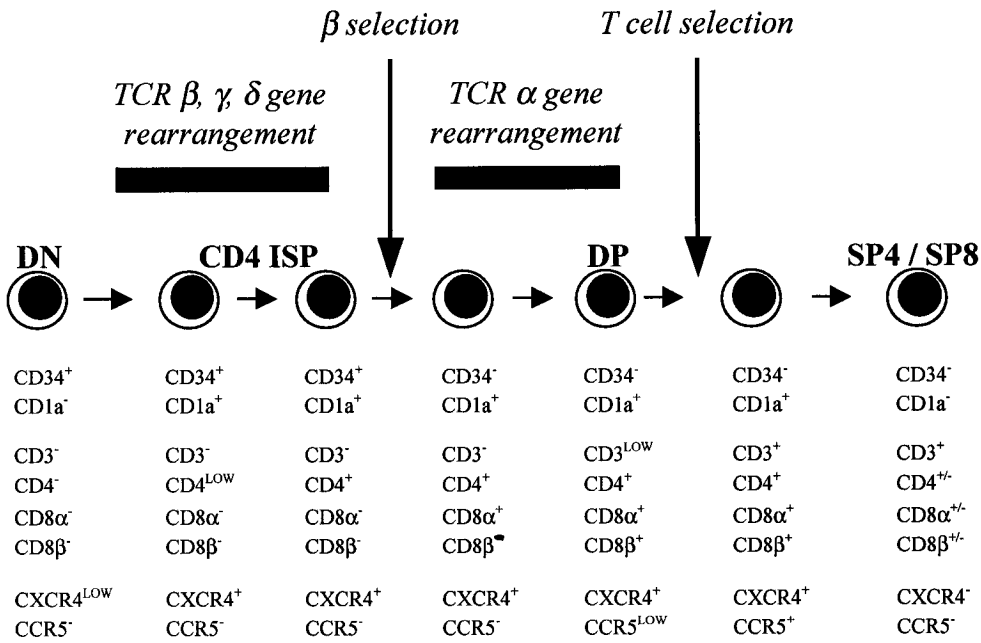


Figure 4. *Human thymocyte differentiation stages. $CD34^+$ T cell precursors colonize the thymus and undergo TCR gene rearrangement. Following β selection, CD4 immature single positive (ISP) thymocytes undergo proliferation before initiating gene rearrangement at the TCR α locus. Sequential upregulation of CD4, CD8α, CD8β and CD3 will lead them to the double positive (DP) stage, where T cell selection occurs. Progressive maturation into the medulla is associated with the appearance of single positive (SP), non-self reactive, thymocytes. Such SP4 and SP8 thymocytes will enter the periphery as RTEs and join the naïve peripheral T cell pool. The HIV coreceptor expression profile (CXCR4 and CCR5) at the surface of developing thymocytes is indicated at the bottom.*

As the initial colonization precedes vascularization, blood-borne precursors must leave the adjacent blood vessels and traverse the mesenchyme and basement membrane surrounding the epithelial rudiment in order to enter the TES and proceed with ontogeny. Currently, little is known of the mechanisms that attract precursors to the thymus or facilitate their migration through the surrounding tissues but chemokines and their receptors are suspected to play a role. In fact, chemotactic factors were reported to be involved in this process more than 20 years ago through innovative functional studies using transfilter migration assays in which the ability of alymphoid fetal thymic lobes to attract stem cells from fetal liver fragments or other lymphoid fetal lobes was assessed[185-188].

Chemokines are basic polypeptides of about 90-100 amino acids, usually containing four cysteine residues linked by intramolecular disulfide bonds[189]. Most chemokines falls into the CXCR or CCR class according to the spacing of two NH_2-terminal cysteines. In the thymus, a vast array of CXCR and CC chemokines are produced (RANTES, SDF-1α, MIP-1α, MIP-1β, TARC, TECK)[190] and thymocytes have been shown to express a wide panel of chemokine receptors including CXCR3, CXCR4, CCR3, CCR4, CCR5, CCR6, CCR7, CCR8 and CCR9 [27,190,191]. Definitive demonstration of the functional relevance of chemokine/chemokine receptors engagement in thymus colonization came from experiments from Wilkinson *et al*[27] who successfully blocked thymic entry of precursor cells by using pertussis toxin, a known inhibitor of G protein-mediated chemokine receptor signaling.

After entry into the thymus at the CMJ, early mouse DN thymocytes (DN1: c-Kit$^+$CD44$^+$CD25$^-$) are required to migrate through the cortex, against the flow of developing DP thymocytes, and localize in the subcapsular region in order to continue differentiation. SDF-1α, the ligand of chemokine receptor CXCR4, is likely to play a role in such early DN thymocyte trafficking as mice CD44$^+$CD25$^-$ thymocytes respond to SDF-1α in chemotaxis assays[192-195]. Furthermore, high levels of SDF-1α were found in the cortex of the adult thymus suggesting that, through the establishment of a "chemokine

gradient", recent immigrants could be guided to the outer areas of the thymus following entry in order to continue maturation. It is also possible that SDF-1α/CXCR4 interactions also promote thymocyte association with stromal elements of the subcapsular region after their arrival[190].

Another chemokine, TECK (thymus-expressed chemokine), a recently described CC chemokine expressed in the thymus and the small intestine, was found to mediate chemotaxis of human G protein-coupled chemokine receptor CCR9[191]. CCR9 expression is restricted to some human thymocytes. NK cells, monocytes, eosinophils, basophils and neutrophils lack expression of this chemokine receptor. Following β selection, thymocytes were shown to vigourously respond to TECK in chemotaxis assays[196,197] as a result of CCR9 upregulation[190]. It is therefore possible that TECK/CCR9 signaling might be required later for proper expansion and/or migration of β-selected thymocytes in order to "guide" them in the cortex and through the CMJ, where DP thymocytes need to go in order to continue the thymic selection events. Chemokines and their receptors control the recruitment of T cell progenitors to the thymus as well as playing a role in their successive intrathymic differentiation and exit to the periphery.

The potential role of other cell adhesion molecules in governing early stages of thymocyte development has also been described. It has been shown that LFA-1/ICAM-1 interactions influence thymocyte maturation and proliferation[198]. Other cell surface molecules, like Thy-1, support adhesion of thymocytes to thymic epithelial cells through heterophilic interactions that can be inhibited by sulfated glycans[199]. The $\alpha_6\beta_4$ and VLA-4 integrins display a developmentally regulated pattern of expression with the highest levels found in thymocytes prior to TCR gene rearrangement, suggesting a role for these molecules in mediating adhesion of early developing thymocytes to TEC/stroma[200]. Additionally, merosin/thymocyte interactions have been suggested to play a role in T cell development[36]. The homophilic interaction between E-cadherins, are implicated as well, and are essential for successful generation of the thymic microenvironment and in cellular interactions occurring in the early phases of T cell development[201].

Notch1 governs the αβ/γδ T cell lineage decision

One important issue in T cell development concerns the lineage relationship between αβ and γδ T cells. What governs the differentiation of the precursor cells to become either αβ or γδ T cells? Notch1, the mammalian counterpart of a cell-surface receptor that regulates well-characterized developmental decisions in flies[202], is thought to influence this checkpoint. Notch1 expression is higher in DN thymocytes as compared to DP and SP thymocytes[203] and Notch1 haploinsufficiency (Notch1$^{+/-}$) results in an increased development of the γδ lineage at the expense of the αβ. Conversely, commitment to the αβ lineage was favored in transgenic mice that express an activated form of Notch1. Therefore, signaling through Notch1 is an important step in the initiation of the αβ T cell developmental pathway. Moreover, mice that are defective in either αβ TCR or γδ TCR do not show an obvious effect on the development of the remaining lineage[204-206] suggesting that precursors commit to one lineage (αβ or γδ) prior to gene rearrangement.

Importance of IL-7 and SCF in intrathymic DN proliferation

As recently reviewed by Prockop *et al*[207], early DN thymocyte ontogeny results in expansion and migration to the subcapsular region, where thymocytes initiate a major developmental transition into the double positive (DP) stage. DN2 and DN4 thymocytes proliferate the most. Such proliferation has been shown to involve signaling through c-Kit and the IL-7 receptor (IL-7R), whose ligands are expressed by MHC class II^{+} TEC[208-210]. Germline mutations in membrane-bound c-Kit protein or its ligand stem cell factor (SCF), affect the development of several cell lineages including melanocytes[211], germ cells[212], hematopoietic cells, and all blood leukocytes [213]. These mutations result in a 10-fold reduction in hematopoietic stem cells (HSC). Furthermore, T cell development is completely abolished in double mutant mice (SCF$^{-/-}$, IL-7R$^{-/-}$) illustrating the critical roles for these cytokines in T cell ontogeny. The precise mechanism by which IL-7 regulates T cell maturation is still not completely clear but may involve multiple effects at different differentiation stages like promotion of SP8 maturation and enhancement of survival/proliferation. It was reported that signaling through the IL-7R induces an up regulation of Bcl-2 expression, leading to enhanced survival of the developing DN thymocytes. Furthermore, the fact that transgenic expression of Bcl-2 restores thymic

cellularity in mice deficient for either IL-7Rα or common γ chain[214], suggests a crucial role for this cytokine in promoting the survival and differentiation of early thymocytes.

Given the small number of precursor cells that enter the human thymus, intrathymic proliferation of DN2 thymocytes increases the pool of thymocytes that will attempt gene rearrangement. Clonal expansion of β-selected thymocytes (mainly at the DN4 stage) contributes to the high DP numbers found intrathymically as well as increases the number of possible different pairing combinations between TCR α and β chains at the surface of developing thymocytes.

Rearrangement at the TCR β locus is a salient feature of T cell development

The mechanism by which TCR germline DNA is rearranged to form functional receptor genes appears to be similar to the mechanism used during Ig-gene rearrangement[215]. To be brief, multiple variable (V), diversity (D) and joining (J) gene segments are fused together during gene rearrangement and generate TCR molecules that will be further selected. The entire human 685 kb TCR β chain locus has been completely sequenced[216] and presents some interesting characteristics conserved in some vertebrate species that have been studied so far (human, mouse, chicken and frog). One interesting feature is the tandem nature of Dβ-Jβ-Cβ regions (Dβ1-Jβ1.1-1.6-Cβ1 and Dβ2-Jβ2.1-2.7-Cβ2) in the TCR β locus. Two nearly identical Cβ coding sequences (Cβ1 and Cβ2) can be found downstream of each Jβ clusters while all the Vβ segments are located upstream of the Dβ, Jβ and Cβ regions. Altogether, a total of 65 Vβ (grouped into 32 families), 2 Dβ, 13 Jβ (grouped in 2 clusters) and 2 Cβ can be found on the locus (Figure 5).

Conserved heptamer and nonamer recognition signal sequences (RSS), containing either 12 bp (one-turn of DNA) or 23 bp (two turns of DNA) spacer sequences, have been identified flanking Vβ, Dβ and Jβ gene segments[217-220]. Like the pre-B cell, the pre-T cell transiently expresses the recombination-activating genes (RAG) 1 and 2 in order to fuse together Vβ, Dβ and Jβ segments. The most conclusive evidence suggesting a role for RAG-1/2 in this rearrangement mechanism came from studies in which mice engineered to lack RAG-1[125] or 2[126] were unable to rearrange either TCR or Ig gene segments

properly. These enzymes, in association with XCCR4, DNA-PK, and Ku proteins, can specifically recognize RSS in a non-random fashion and initiate gene rearrangement through the catalysis of a double strand DNA break[221]. It is likely that RAG's ability to bind to a given RSS as well as the distance between Dβ-Jβ or Vβ-DβJβ segments, influence their rearrangement frequencies. It is important to note that the first wave of RAG-1/2 recombinase activity catalyzes first the Dβ-Jβ followed by Vβ-DβJβ TCR gene rearrangement by the same deletional or inversional mechanism that occurs in the Ig genes.

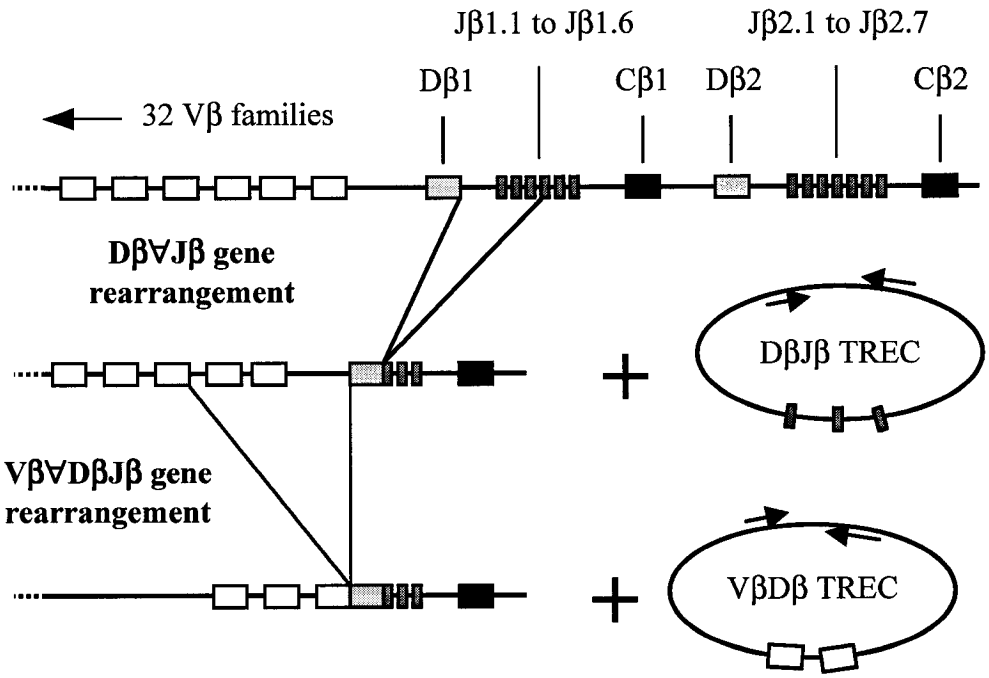


Figure 5. *Human TCR β locus organization and β TREC generation. In total, 65 different Vβ genes (organized into 32 different families), 2 different Dβ genes, 13 different Jβ genes (organized into 2 clusters) and 2 nearly identical Cβ span the 685 kb TCR β locus. Each of these genes are flanked by recombination signal sequences (RSSs) capable of recruiting the RAG machinery that will initiate V(D)J gene rearrangement. The generation of β TREC, by-products of TCR β gene recombination, occurs during the fusion of either Dβ∀Jβ and Vβ∀DβJβ gene segments and is illustrated underneath the full TCR β locus. Arrows indicate primer binding sites for β TREC amplification.*

Fusion between distinct Vβ, Dβ and Jβ segments leads to a vast array of different TCR β chain nucleotide sequences. While this contributes in generating diversified TCR β chain molecules, greater diversification is achieved by the addition of N and P nucleotides which further broaden this pool. In fact, terminal deoxynucleotide transferase (TdT) randomly incorporates deoxyribonucleotides at the junction of segments as they rearrange (N nucleotides). Moreover, the end-loop structure that results from dsDNA break initiation will be further cleaved, leading to the creation of "sticky-ends" that, once filled, will result in the insertion of palindromic sequences (P nucleotides) between rearranged segments. These two events dramatically increase TCR diversity. However, the generation of receptor diversity through recombination of gene segments and addition of N/P nucleotides is not without liabilities. As triplets of nucleotides encode amino acids, the usefulness of any given rearrangement will depend on both the sequence and the number of nucleotides located between the initiation (ATG) and termination codons (TAG, TGA and TAA). Consequently, the majority of such random rearrangements are thought to result in sterile gene configuration, mainly through out-of-frame sequences or premature termination codons. The establishment of a broad repertoire arises from one's ability to generate a vast array of TCR β chains, as well as TCR α chains, that will pair together at the surface of developing thymocytes. Further selection, leading to the elimination of potentially harmful thymocytes, occurs prior to exportation in the periphery.

Past studies indicated that RAG expression is initiated at the $CD44^{+}CD25^{+}$ stage and persists up to the DN3 compartment[222], where rearrangements of the at the TCR β, γ and δ loci can be detected[223]. If a functional γδ receptor is assembled at the surface, thymocytes proceed along the pathway to become mature γδ T cells. If not, only a productive rearrangement at the TCR β locus can rescue developing thymocytes from "death by neglect" upon adequate association with the invariant pre-TCR α chain (pTα).

The role of pTα in β selection of thymocytes

Following the successful generation of an in-frame, coding TCR β chain, thymocytes undergo a series of events that "test" the functionality of the recently rearranged β chain. This period of time is referred to as "β selection" and necessitates the correct assembly of the pre-TCR complex, composed of a rearranged TCR β chain associated with the invariant pTα membrane protein in conjunction with the signaling CD3 chains[224]. Murine thymocytes at the $CD44^{+}CD25^{+}$ stage (DN2) have not been "β-selected" for their TCRβ chain, while those lacking the expression of CD44 and CD25 (DN4) successfully undergo "β selection" and proliferate extensively. Signaling through this complex induces differentiation of β-rearranged DN thymocytes into the $CD4^{+}$ $CD8^{+}$ DP compartment[225-228] as well as strong proliferation[229].

A homologous educational process is taking place in humans. Sequential up regulation of CD5, CD1a and CD4 leads double negative (DN) thymocytes to the "CD4 immature single positive" thymocyte (CD4ISP) stage[223]. These pre-DP thymocytes lack expression of both CD8 chains (α and β) and soon leave the CD34 compartment. Signs of TCRβ rearrangement have been reported in CD4ISP thymocytes. As CD34 down regulation coincides with the expression of pTα, it is believed that $CD34^{-}$ DN thymocytes cells are undergoing β selection (or will in the very near future). CD8 α and β sequential up regulation marks the final stages of pre-DP differentiation.

The existence of ligand(s) capable of triggering the pTα/TCRβ/CD3 complex (and therefore of inducing β selection) has been debated for years and is currently a matter of great interest. As no ligand for the pre-TCR complex has yet been found on the surface of thymic epithelial or stromal cells[230], it is possible that pTα acts as a chaperone that "scans" the 3D structure of the rearranged β chain instead of forming a "pTα/TCRβ" receptor awaiting to be triggered. The fact that thymocytes, engineered to bear a pre-TCR complex containing a truncated TCR β chain (Vβ region missing), can still undergo β selection argues against the need for a ligand. Adequate recognition/pairing would be sufficient to induce a series of conformational changes among some CD3 chains

(ultimately leading to their phosphorylation by Lck/Src kinase) and initiate signaling cascades responsible for the DN-DP transition. Extracellular-regulated kinase (ERK), a downstream effector of the Ras/Raf/MEK/MAPK pathway, has been shown to be implicated in this differentiation step[231].

In short, the pre-TCR mediates β selection of cells expressing in-frame, functional TCR β chains. This process terminates RAG-1/2 expression and contributes in β allelic exclusion (results in only 1 TCR β chain sequence being expressed at the cell surface of developing thymocytes) as well as to induce extensive proliferation of selected thymocytes. This thymocyte proliferation period is referred in this thesis as the "window of proliferation" and will be further explained in chapters 4, 7.4 and 7.5. This leads to a vast number of early DP thymocytes, each one bearing a unique TCR β chain coding sequence as a result of allelic exclusion. As newly generated DP thymocytes stop cycling, they re-express the RAG machinery and initiate rearrangement at the TCR α locus. Proper assembly of TCR αβ heterodimers at the cell surface is a requirement for positive/negative selection to occur.

Rearrangements at the TCR α locus is preceded by excision of most of the TCR δ locus

The genomic organization of the TCR α locus shows some interesting features. In contrast to the TCR β locus organization, the TCR α locus lacks D segments (Figure 6). Furthermore, the TCR δ locus is flanked by Vα and Jα gene segments as well as with specific δ deleting elements (such elements were shown to excise most of the TCR δ locus). Accordingly, any Vα to Jα segment rearrangement will excise the TCR δ locus from the genome. Excision of the TCR δ locus is mediated through the fusion of specific DNA elements (δRec, ΨJα, Jα38 and others) flanking the TCR δ locus, and occurs before Vα-Jα rearrangement. Roughly 2/3 of developing thymocytes excise the TCR δ locus using δRec and ΨJα deleting elements, which are pseudogenes, irreversibly committing thymocytes to the αβ T cell lineage[232]. Although other deleting elements can be found flanking the TCR δ locus, their frequency of utilization is markedly lower. Such

δRec-ΨJα rearrangement leads to the generation of an extrachromosomal circular DNA molecule, referred to as the sjTREC (signal joint TCR rearrangement excision circle) (Figure 6). These by-products of gene rearrangement are the basis of this thesis and will be further described (see chapter 1.2.2).

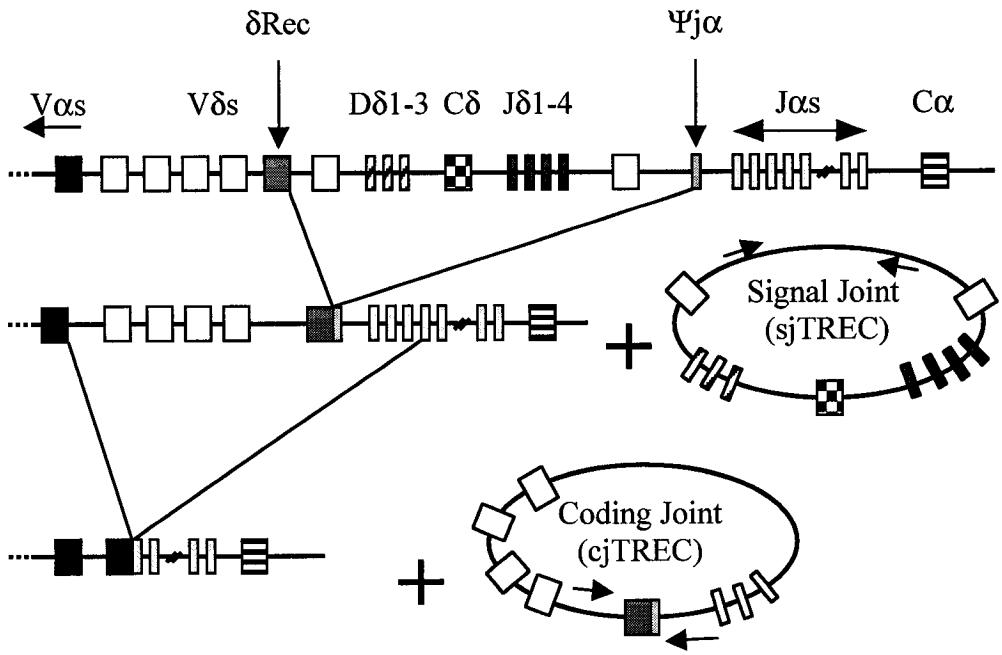


Figure 6. *Human TCR α locus organization and sjTREC generation. Absence of Dα segments and the presence of the TCR δ locus between Vα and Jα segments characterize the human TCR α locus. The TCR α locus spans roughly 1100 kb and encompasses 50-70 Vα genes (grouped in 29 Vα families), more than 50 Jα genes and 1 Cα gene. During T cell differentiation, excision of the TCR δ locus occurs mainly as a result of δRec-ΨJα rearrangement. This leads to the formation of the sjTREC molecule, which is diluted-out following cellular division. Further Vα-Jα rearrangement leads to the generation of a coding TCR α chain as well as to the excision of the fused δRec-ΨJα region from the genomic DNA. Arrows represent primer binding sites that leads to TREC amplification.*

In mice, the first thymocyte subset that shows sign of RAG machinery re-expression is the DN4. By the next differentiation stage (early DP: $CD3^{low}$ DP), substantial rearrangements occur and most early DP possess functional rearrangement at both TCR α and β loci (as evidenced by the expression of the TCRαβ heterodimer on the cell surface), but failed to undergo, or have not yet undergone, positive selection (Figure 3).

During TCR α gene rearrangement, both alleles rearrange simultaneously to relatively coincident loci[233,234], indicating that, at least for the TCR α locus, allelic exclusion neither operates at the recombination nor the gene expression levels. The demonstration that noncycling DP thymocytes express high levels of RAG-1/2 suggests that such cells may be continuously rearranging their TCR genes until a productive Vα-Jα rearrangement is generated within a certain period of time[235].

The expression of RAG proteins is tightly regulated and appears to occur only twice in the life span of a T cell (during TCR β and α chain gene rearrangement events)[222] even though RAG re-expression in peripheral mature T cells has been reported[236].

Temporal relationships between TCR gene rearrangement and programmed cell death

Productive rearrangement at the TCR β locus correlates with the down regulation of Bcl-2 expression. Throughout thymocyte development, an inverse relationship can be seen between TCR α rearrangement and Bcl-2 expression[237]. Productive TCR β rearrangements trigger a series of biochemical events leading to the establishment of a "biological clock" within developing DP thymocytes. As DP thymocytes derived from Bcl-2 transgenic mice possess the capacity to survive extended periods in culture[238] it is likely that DP thymocytes are primed to undergo programmed cell death if they do not assemble a functional receptor with a low affinity/avidity for self-MHC/peptide ligands. In this regard, most murine thymocytes present in the cortex die within a few days and roughly one third of the DP thymocyte population is replaced each day[239]. Upon rescue by positive selection, TCR α gene rearrangement ceases as a result of RAG-1/2 down regulation and high levels of Bcl-2 can also be found. Continuous rearrangement at the TCR α locus maximizes the efficacy of selection (through the generation of a TCR α chain that will efficiently pair with the rearranged TCR β chain) whereas down regulation of Bcl-2 limits the time available to do so. This likely contributes to the maintenance of adequate thymocyte turnover and homeostasis within the thymus.

Generating a self MHC-restricted, self-tolerant repertoire

The events leading to positive/negative selection of DP thymocytes are analogous to "screening" procedures where newly generated DP thymocytes (with low levels of rearranged TCR αβ on the cell surface) await selection by peptide-loaded MHC class I and II molecules[240,241]. Positive/negative selection is a non-proliferative differentiation event that requires expression of TCR αβ molecules at the cell surface of DP thymocytes. Through a developmentally regulated, posttranslational control of TCR α chain molecules[242], surface expression of TCR α β molecules is increased following "productive" engagement with MHC molecules at the surface of cortical TEC[243]. Such engagement is mediated through TCR/MHC/self-peptide interactions.

Positive selection promotes the differentiation step where an immature, short-lived (3-4 days) DP thymocyte escapes from programmed cell death and becomes an end-stage, long-lived, SP thymocyte. Negative selection eliminates DP thymocytes that recognize self-peptide with a high affinity/avidity (potentially autoreactive). This selection process is rigorous, sparing only a small fraction (≈1%)[244] of the DP population and also coincides with CD4/CD8 lineage commitment and migration into the medulla. Thymocytes incapable of recognizing MHC molecules loaded with self-peptides through low affinity/avidity interactions will undergo apoptosis and be eliminated. These steps ensure that only T cells with useful receptors populate the periphery. It is believed that selected thymocytes reside within the thymus for a period (varying from 3 to 14 days) following their selection[245]. Close contacts with medullary TEC complete the final maturation of SP thymocytes and likely prepare them for thymic emigration. It is still unclear by which means mature thymocytes leave the thymus but a recent report suggests that SDF-1α "chemo-repulsion" could contribute to their exportation[246].

The concept of positive selection was developed over several years and has originated from the necessity of explaining the self-MHC restriction of T cell function[247]. Multiple models have been proposed to distinguish the cellular interactions leading to positive selection[248-251]. The most favored one is the affinity/avidity model, where cell fate is determined by cumulative thymocyte/TEC interactions[252].

This model postulates that TCR/MHC interactions are "quantitated" and integrated by thymocytes, leading to their selection or death. Some αβ TCR would have virtually no affinity toward self-peptide/MHC complexes expressed on cortical TEC. Cells bearing such TCR would die owing to a lack of positive selection. At, and above, a certain threshold of low affinity toward self-peptide/MHC complexes, thymocytes would be positively selected. Negative selection would eliminate thymocytes whose TCRs have an affinity at or above a second, higher threshold. The end products of this selection process consist of functional T cells bearing TCR molecules that are in the category of threshold to low affinity for self-peptide/MHC complexes. This model is the oldest explaining the relationship between positive and negative selection, and is among the rare models that have not been disproved so far. In the affinity/avidity model, T cell selection is viewed as a function of quantitative events dictating whether one or both thresholds will be crossed. It also implies that positive selection occurs at a lower threshold (or avidity) than negative selection and does not put constraints on the temporal succession of positive and negative selection. In fact, solid evidence supports the idea than negative selection can occur before, during or after positive selection[253-256].

T cell selection is mediated through interactions between the TCR complex and peptide/MHC molecules at the surface of TECs. Years of research demonstrated that the TCR complex encompass the heterodimeric TCR molecules (αβ or γδ), CD3 chains (γ, δ, ε and ζ) as well as CD4 or CD8 coreceptor molecules. MHC class I and II molecules can interact with CD8 or CD4 molecules respectively and thus, increase the overall affinity/avidity of the TCR complex[257]. Added to this are other interactions between adhesion molecule and their ligand present in the "immunological synapse" (LFA-1 with ICAM-1 or 2[258] and CD2 with LFA-3 or CD59[259]) can contribute to increase the overall affinity/avidity of the TCR complex for the peptide/MHC molecules.

In concordance with this model of T cell selection, direct and indirect measurements of the affinity of TCR/peptide/MHC interactions have been estimated to range from 10^{-5} to 10^{-8} M, on the borderline of detection by affinity-measuring assays[260,261]. Despite the low

affinity of such monomeric complexes, it is possible that *in vivo* oligomerization and interactions with other membrane molecules can strengthen a weak complex[262]. Further validating the affinity/avidity model is the fact that a positively selecting interaction can be transformed into a negatively selecting one through enhanced CD8 expression[263,264]. Moreover, LFA-1/ICAM-1 interactions have been shown to influence negative selection processes[256].

Given that MHC molecules come in several classes, isotypes and alleles within the species as well as the extensive TCR specificities that arise from gene rearrangement, a perfect TCR/MHC fit cannot be expected to spontaneously arise from interactions between any or all of them and some degree of selection must occur[265] (i.e. rescue of useful thymocytes whose TCR can accommodate the individual's MHC molecules and whose coreceptor matches the class of MHC molecules it is restricted by). It is not yet clear how these interactions are translated to intracellular signals that can distinguish between good and useless/harmful thymocytes but it is believed that the extent/duration of phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAM) located on CD3 chains could participate in this selection process[266].

What kinds of peptides mediate positive selection?

The peptide repertoire during thymic T cell development must be considered separately from the peptide repertoire in the context of peripheral T cell activation. While the critical peptides during T cell activation are those foreign peptides expressed transiently in the context of an immune response, the focus during T cell development is on the thousands of different MHC-bound peptides that collectively define self.

The nature of the peptides bound to MHC molecules involved in positive selection has been studied in the FTOC system using an MHC class I mutant in combination with a corresponding transgenic TCR restricted cell[273-276]. From these studies, it was shown that peptides designed to bind MHC molecules must also consist of amino acid residues that have side chains extending toward the TCR. Although the receptors studied seem to have a fairly stringent requirement as far as peptide sequences is concerned, other receptors

may have sufficient interaction with MHC residues leading to positive selection, as long the peptide bound to MHC did not disrupt it. This is in accordance with the results from Schumacher *et al.*[277], who showed that putative "empty" class I molecules in TAP-deficient animals could positively select a small number of $CD8^+$ T cells.

How diverse can thymic selecting peptides be? It is unlikely that the positive selection of every T cell relies on the recognition of a unique self-peptide during thymocyte ontogeny. This is because the extent of diversity of peptide expressed by stromal cells would not be sufficient to select a repertoire of T cells that can respond to peptides derived from a vast universe of microbial/viral pathogens[278,279]. Therefore, it has been argued that recognition of thymic selecting peptides during positive selection is a relatively degenerate event. In fact, single peptide/MHC complexes can induce positive selection of polyclonal $CD4^+$[280,281] and $CD8^+$ T cells[278]. This implies that peptide recognition is a cross-reactive event during positive selection. Specific recognition of self-peptides during positive selection give rise to a repertoire of T cells that, in addition to being MHC-restricted, will also be weakly reactive to self-peptides. Such TCR "tethering" (low-avidity interactions with self-peptides) likely plays a role in the survival of peripheral mature T cells.

Clonal deletion during intrathymic negative selection of thymocytes

In 1987, Kappler *et al*[57] reported that clonal deletion was the dominant form of intrathymic negative selection. Using an antibody directed against the Vβ17 segment of the TCR, they demonstrated that all mature TCR^{HIGH} $V\beta17^+$ were deleted within the thymus. Surprisingly, TCR^{LOW} cells bearing this Vβ segment were not eliminated, further suggesting that the deletion occurred at the DP developmental stage. Not long after, similar phenomenon were reported for Vβ6-[282] and Vβ8.1-[283] bearing T cells (an later for other Vβ segments[284]. It became apparent that the deleting ligands in these experiments were actually the products of endogeneous mouse mammary tumor viruses (MMTV), belonging to a group of microbial products first named "superantigens" by Marrack and Kappler[285].

A series of experiments in TCR transgenic mice systems also supported the clonal deletion theory. In the H-Y TCR transgenic model, it was shown that expression of the male specific antigen eliminates almost all thymocytes bearing the transgenic TCR, including the vast majority of DP cells, leading to a 100-fold reduction in the number of thymocytes[286]. Similar results were obtained with transgenic mice for the ovalbumine (OVA)[287] and the pigeon cytochrome C (PCC)[288] TCRs. Important experiments carried out by Smith *et al* indicated that the main biochemical mechanism for clonal deletion is the apoptotic death induced by TCR stimulation. DNA fragmentation and cell death occur upon TCR ligation by TCR-specific antibodies (that mimick the antigen) or upon the elevation of calcium[289,290].

A vast body of evidence shows that thymic dendritic cells, and to a lesser extent macrophages, are capable of mediating clonal deletion of self-reactive thymocytes[291]. When a deleting element is expressed in the thymic cortex, essentially no deletion occurs in an MMTV system. However, deletion is readily observed if the same deleting element is selectively targeted to the medullary section of the thymus, and more precisely, to macrophages and thymic dendritic cells[292,293]. These findings suggest that the cortical epithelium is not fit to induce negative selection and that medullary hematopoietic components are crucial for this process.

Recently, medullary TECs were identified as being a unique cell type capable of expressing a wide range of tissue-specific antigens, like insulin and albumin. In fact, Derbinski *et al*[294], were able to show through RT-PCR analysis that mTEC expressed a rather random, more than secluded, array of self-antigens. Together with thymic dendritic cells, mTEC participate in the negative selection of thymocytes.

Intrathymic negative selection by nondeletional mechanisms

Although clonal deletion is the best-studied system for removing potentially autoreactive cells from the mature T cell pool, an alternate mechanism of tolerance involves the inactivation of self-reactive T cells. Nondeletional tolerance has been observed in a number of transgenic and superantigen-induced systems. The major characteristic of this

form of tolerance is that cells reactive to a particular antigen are not removed from the mature T cell pool but persist in an inactive state. This is also referred to as clonal anergy, which is defined as an inability of cells to respond to a normal stimulatory encounter with an antigen. This phenomenon appears to be reversible and has been described for both T and B cells. Such cells appear to be functionally silent *in vivo* and are unresponsive (e.g. desensitization/lack of costimulation leading to absence of IL-2 transcription).

On the path to becoming a CD4 or CD8 thymocyte

The events leading to the maturation of self-tolerant DP into SP4 or SP8 thymocytes are of great interest. Maturation of DP is dependant on signals transduced by TCR components and is reflected by changes in expression patterns of CD4 and CD8 coreceptor molecules on the surface of thymocytes. Although most thymocytes do not progress further than the DP stage of development, a fraction of DP will extinguish one coreceptor molecule and ultimately commit to the CD4 or CD8 mature T cell lineage. It was previously thought that DP thymocytes terminate the transcription of one or the other coreceptor molecules, leading to their progression to the SP4 or SP8 pathways. Different mechanisms have been proposed to explain how DP thymocytes can differentiate into SP T cells expressing TCR and coreceptor molecules with matching specificities.

All current models postulate that lineage commitment occurs in DP thymocytes and results in the transcriptional termination of one or the other coreceptor molecules (either through instructional or stochastic mechanisms). However, it is difficult to reconcile these assumptions with the observation that most signaled DP thymocytes initially lose CD8 expression regardless of their ultimate cell fate[295,296].

CD8 coreceptor reversal

Recently, a paper by Brugnera *et al*[167] demonstrated that, prior to becoming SP4 or SP8 thymocytes, DP are pre-programmed to terminate CD8 transcription and become $CD4^{+}CD8^{LOW}$ thymocytes. This intermediate population retains the potential to differentiate into both SP4 and SP8 thymocytes. IL-7 signaling as well as the duration of the TCR signals influence this lineage commitment.

The "kinetic signaling model of lineage differentiation"[167] proposes that the persistence of the MHC/peptide/TCR signal dictates the lineage commitment of $CD4^+CD8^{LOW}$ thymocytes. As a consequence of termination of CD8 transcription, cells with a class I-restricted TCR will be more "loosely attached" to the stroma as compared to cells that have a class II-restricted TCR, leading to short or persisting signaling through the TCR, respectively. The duration of the signal is likely to have strong biological effects on developing thymocytes as it was shown that signaling through the TCR induces a calcium flux in thymocytes. This leads to the activation of calpain, an enzyme capable of proteolyzing the cytosolic domain of the common cytokine γ_c and thereby, abrogating any signal transduction event through the IL-7 receptor. Signaling through this cytokine is crucial for the generation of SP8 thymocytes (through CD8 coreceptor reversal) and is likely to maintain the viability of differentiating thymocytes in the absence of a persisting TCR signal.

This model has the advantage to explain how class I-restricted $CD4^+$ T cells (as well as class II-restricted $CD8^+$ T cells) can be found in the periphery.

How are RTE exported in the periphery?

Several mechanisms have been suggested to explain thymocyte emigration from the thymus. These include down regulation of tethering adhesion molecules, passive exportation into the periphery as a result of local fluid dynamics, and active chemotaxis of mature cells toward chemotactic agents[207,297]. As for thymus colonization, the exportation process is at least mediated through chemokine receptor signaling. This conclusion was drawn from a transgenic mouse model in which the lck promoter drove production of pertussis toxin (inhibitor of $G_{\alpha i}$ protein), resulting in an enlarged thymus incapable of exporting its thymocytes[298]. Further strengthening the point is the fact that similar results were obtained when using cytochalasin D and *Clostridium difficile* toxin[299]. Supporting this idea are other studies involving SDF-1, the ligand of G protein-coupled membrane receptor CXCR4, which was recently identified as one of the molecules involved in exportation of mature thymocytes[246]. They also demonstrated that

only mature thymocytes as well as peripheral T cells, but not immature thymocytes, were susceptible to the chemorepulsion effect of SDF-1. This strongly suggests a role for SDF-1 in thymocyte exportation. Taken together, these reports argue that thymic emigration is mediated through active cell movement involving $G_{\alpha i}$ protein coupled-receptor and not the result of local fluid dynamics operative in the thymus. Therefore, thymocyte emigration is a regulated process.

One of the last physiological events occurring in the thymic medulla is the proliferation of mature pre-emigrant TCR^{HIGH} $CD4^+$ $CD8^-$ and TCR^{HIGH} $CD4^-$ $CD8^+$ thymocytes[245,300,301]. Such proliferation depends on the TCR affinity for self-peptide/MHC ligands and is biologically relevant[302]. In fact, it is a way to increase thymic function as well as to increase the number of cells capable of responding to a given antigenic peptide. The latter is likely to reduce the time necessary to encounter the antigen.

1.2.2 T Cell Receptor α and β Excision Circles: Molecular Markers for Recent Thymic Emigrants

Overview

It is now well known that TCR gene rearrangement (as well as BCR gene rearrangement) is mediated by the recombination-activating genes (RAG) 1 and 2, sharing a common folding topology with member of the bacterial transposase/retroviral integrase family[303,304]. During gene rearrangement, fusion of recombination signal sequences (RSS) flanking V, D and J segments leads to the formation of circular DNA by-products that are excised from the genome. Such extrachromosomal circular DNA excision circles of no known biological function were first isolated and visualized by electron microscopy in the late 1980s at the time when Ig and TCR gene rearrangement processes were starting to become understood[305,306].

TCRα and β gene rearrangement are salient features of thymopoiesis (Figures 5 and 6). Recently, a new method capable of monitoring human *de novo* T cell production has

emerged[1,307]. This method relies on the PCR-based quantification of TCR rearrangement excision/deletion circles (TREC), by-products of T cell receptor (TCR) gene rearrangement events at the α or β locus. This method constitutes a novel way to monitor the peripheral frequency of human RTEs independent of cell-surface marker expression and therefore assess thymic function in a non-invasive way.

The chT1 story

Chicken thymocytes and RTEs specifically express a cell-surface molecule, chT1, first identified by Cooper's group in 1984[308]. Its usefulness in monitoring avian thymic function was shown by the same group early in 1998[309]. As further discussed in this thesis, this paper laid down the first "blue prints" of what is now a well validated concept accounting for the monitoring of human thymic function.

The tissue reactivity to chT1 antigen is restricted to chicken thymocytes undergoing αβ and γδ T cell maturation/differentiation events. Essentially all thymocyte compartments, based on 1 week old chicken CD4 and CD8 expression, showed a good labeling by the chT1 antibody, being more variable in the SP thymocyte populations. More stringent and convincing data regarding tissue expression specificity was obtained when Kong *et al*[309,310] demonstrated that chT1 expression on peripheral T cells positively correlated with the amount of residual chicken thymic lobes following partial thymectomy. Also, immunohistochemical analysis revealed a quick, but not absolute, down regulation of chT1 antigen expression on thymocytes as they proceeded from the thymic cortex to the medulla, suggesting that chT1 expression diminishes as thymocytes maturation events occur. Moreover, detectable levels of chT1 expression were found on peripheral αβ and γδ T cells, albeit at lower level in older chickens, in concordance with the fact that the thymus involutes and its function decreases as the organism ages. Furthermore, by-products of αβ and γδ TCR gene rearrangement, salient features of thymic-dependent *de novo* T cell production, were found at a higher frequency in the peripheral $chT1^+$ population.

Taken together, these data lead to the identification of a chicken RTE-specific marker, chT1, that is rapidly down regulated upon peripheral exportation of mature SP thymocytes but still remains expressed on a small subset of circulating T lymphocytes, likely to be the most recently produced T cells (RTE). Thus, it appears that the avian thymus is constantly supplying new T cells that seed the peripheral compartment. Extensive immunophenotyping of the peripheral chicken $chT1^+$ population has not been done yet. Discovering key molecules expressed at the cell surface of chicken thymocytes could lead to the phenotypic identification of the most recently exported T lymphocytes in humans since no homologue of chT1 has been detected in mammals, although one was found in *Xenopus lavis* (commonly used frog model)[311].

How can thymic function be monitored?

Thymic activity has also been previously studied in the mouse model by intrathymically labeling thymocytes with a fluorescein isothiocyanate injection (FITC) and quantifying the frequency of fluorescent T cells in the periphery[312]. Due to the fact that T cells were shown to recirculate in the thymus following post-selection events[43], these experiments are biased from the start since discrimination between recirculating T lymphocytes present in the thymus and *de novo* produced T cells cannot be made.

Peripheral blood RTE frequencies, reflective of thymic activity, can be quantified using BrdU staining[313]. In fact, RTE can be identified as $BrdU^{LOW}$ cells in peripheral blood as they minimally proliferate. Nonetheless, it is possible that long-lived naïve T cells persist for a long period of time in the blood without dividing and thus contribute to generate false-positive read-out. Eventhough BrdU administration has been approved for use in human[314], it remains to be seen in FACS-purified $BrdU^{LOW}$ cells are enriched in TREC. This would constitute a good validation of the use of BrdU for thymic function evaluation.

In humans, the thymus is relatively difficult to investigate as a consequence of its thoracic localization. Its morphology can be analyzed using computed tomography scans (CT-scans)[315], but its function can only be dubiously estimated through peripheral blood

quantification of RTE. For obvious ethical and practical reasons, invasive experiments aiming at investigating human thymopoiesis and RTE homeostasis in humans are impossible, and even if they were possible, could cause the release of stress hormones which have a depleting action on DP thymocytes and could affect thymic function.

The sjTREC molecule: a marker for the quantification of thymic function

The ability to discriminate peripheral T cells that show evidence of recent TCR gene rearrangement from "old" naïve T cells would be indicative of *de novo* T cell production, most likely occurring in the thymus. Given this, extrachromosomal DNA excision circles appeared to be attractive candidates for quantification of *de novo* produced T cells independent of cell surface molecule expression. Thus, the relative frequency of DNA deletion circles in a T cell population is likely to correlate with the amount of RTEs within that same population, which in turn is reflective of the extent of thymopoiesis. In summary, we hypothesized that thymic function could be monitored through the quantification of "what has recently got out of the thymus". This concept was shown to provide direct evidence of thymic function in adult humans.

In December 1998, Richard Koup's group was the first to successfully demonstrate that TCR rearrangement excision circles (sjTREC), generated during recombination events at the TCR α locus (Figure 6), were present exclusively in CD4 helper and CD8 cytotoxic circulating $CD45RA^+$ T cells (mostly naive T cells), albeit at a lower level in older individuals[307]. Also, as a result of the intrinsic features of gene rearrangement at this locus, two distinct DNA circles are sequentially generated (sjTREC and then cjTREC). The former one is generated when a T cell becomes irreversibly committed to the TCR αβ lineage, deleting most of the TCR δ locus. As previously reported, roughly 2/3 of thymocytes excise the TCR δ locus through δRec→ΨJα recombination event[232].

The cjTREC is excised from the genomic DNA as a result of the fusion of Vα-Jα segments (Figure 6). Even though Douek and colleagues document an age-related decrease in the cjTREC[307], I strongly believe this marker is less stringent for thymic function evaluation. In fact, thymocyte having rearranged a coding TCR α chain will

have excised an sjTREC as well as a cjTREC but because of the primer design[307], a cjTREC PCR product can also be generated from the second, partially rearranged, TCR α locus (δRec-ΨJα-rearranged but no Vα-Jα rearrangement).

These by-products take the form of stable[316], extrachromosomal, circular DNA molecules that do not replicate themselves during mitosis and thus, are "diluted-out" during subsequent cellular proliferation[1,307]. From a theoretical standpoint, a maximum of 2 sjTREC molecules can be generated per cell (given the 2 alleles) and one must consider this biological fact when estimating the peripheral blood frequencies of RTEs. Also, it was reported that the TCR δ locus could be excised through other recombination events that will not generate an sjTREC molecule[232]. Therefore, peripheral blood quantification of sjTREC frequencies leads to an underestimation of the real frequency of RTEs. Nonetheless, it provides a unequivocal way to estimate the blood concentration of RTEs and thus, evaluate the magnitude of thymic function. Quantification of peripheral blood sjTREC frequencies has become a direct indicator of ongoing thymopoiesis[44].

TCR β deletion circles: evaluation of RTE diversity

In 1996, Leroy Hood's group[216] published the full germline sequence of the TCRβ locus located on chromosome 7. Similar to the above-mentioned sjTREC generation process, it is possible to imagine and hypothesize the resulting primary nucleotide sequence of RSS-RSS fusion on the DNA excision circle issued from a distinct TCR Vβ→Dβ or Dβ→Jβ gene rearrangement and synthesize primers allowing for the specific amplification of that deletion circle (Figure 5). In fact each type of gene rearrangement event will lead to the generation of a distinct β TREC, indicative of such recombination. Being much less frequent (averaging 1-30 in 10^5 PBMC, see chapter 3, table 2) than sjTREC molecules, TCR β deletion circles are used to investigate the nature of the gene rearrangement events that occured in the thymus, when T cells were thymocytes. Such DNA deletion circles are enriched in the peripheral $CD4^+$ $CD45RA^+$ $CD62L^+$ T cell compartment but absent in the $CD45RO^+$ $CD62L^-$ T cell subset. Furthermore, various types of Vβ→Dβ and Dβ→Jβ DNA deletion circles can be found within peripheral T cells from healthy adult humans

of varying ages, suggesting that thymopoiesis remains diverse during adulthood, even though its magnitude decreases as we age.

As previously mentioned, these novel tools were used during this thesis to analyze thymic function in clinical situations where a functional thymopoiesis would greatly be beneficial to reconstitute one's immune system, namely following allogeneic hematopoietic stem cell transplantation (AHSCT) and during the various stages of HIV infection.

1.3 T Cell Homeostasis

1.3.1 Homeostatic Regulation of Naïve and Memory T Cells

Overview

A key challenge for the immune system in the periphery is the simultaneous maintenance of T cell diversity and population size. T cells seldom divide, and their inherent longevity combined with the contribution of thymic export of recent thymic emigrants can maintain a steady population level[317]. Recently, it has been shown that RTE are preferentially incorporated into the periphery, causing displacement of resident mature T cells and thereby maintaining the size of the peripheral pool[318]. This ensures that the TCR repertoire is kept diverse, whereby naïve T cells that fail to contact antigens within weeks of export are displaced[318]. Therefore, the immune system can maintain the constant size of the lymphoid compartment concomitantly with the retention of selected antigen-experienced cells.

Peripheral naïve and memory T cells

T lymphocytes are composed of 2 distinct populations that play complementary roles in the immune system[319]. In fact, naïve T cells, responsible for more than 99% of all TCR repertoire diversity, can recognize and respond to a vast array of antigenic peptides[320]. Memory T cells can more rapidly elaborate an immune response following subsequent encounter with the antigen as compared to naïve T cells[321,322].

The memory compartment can be subdivided on the basis of CCR7 expression[323]. $CCR7^+$ T cells will tend to localize in LN and do not demonstrate an effector function (central memory T cells). These cells were shown to persist several years following immunization and their maintenance in the periphery does not require the presence of an antigen[324-326]. In contrast, $CCR7^-$ cells (memory/effector T cells) can migrate to the site of inflammation and die when the antigen is removed or cleared[327-329].

Given the importance of naïve and memory T cells (naïve T cells contribute in broadening the TCR repertoire diversity whereas memory T cells rapidly elicit a strong immune response), the immune system must be able to maintain a cohort of naïve cells, potentially responsive to new antigens encountered during adulthood, without becoming wholly occupied by expanded memory populations. As naïve and memory T cell pools were shown to be independently regulated[330,331], the constant influx of naïve cells is ensured (with the capability of broadening the TCR repertoire) without removing antigen-experienced memory T cells that have previously shown their usefulness in the clearance of foreign antigens.

Regulation of peripheral T cell numbers

The "input" and "output" of cells governs the size of the T lymphocyte compartments at steady state[332]. Input corresponds to the flow of naive cells exported from the thymus, but also to the expanded numbers generated by cell division in response to antigen-specific triggering and nonspecific bystander stimulation. "Output" refers to cell death due to neglect or activation-induced cell death (AICD). Although it is clear that *de novo* T cell production can contribute to increase the input of lymphocytes, modulation of T cell population numbers in an adult organism mainly occurs through regulation of cell death and peripheral expansions.

T cell homeostasis is best understood in terms of competition for survival. Competition may be for soluble factors like cytokines and/or for cell surface ligands displayed on stromal cells. These elements define the basic "peripheral niche", whose availability controls cell numbers. Total T cell numbers, which can vary between individuals or with time, are set by 1) the levels of production of "niche"-specific signals and 2) T cell responses to these factors.

There is evidence that naïve T cells must continuously undergo low affinity TCR triggering to survive in the periphery[317,318,333]. This TCR "tethering" is MHC restricted, contributes in maintaining Bcl-2 expression[333], and does not induce cell division[313] in non-lymphopenic systems. The vast majority of naïve peripheral T lymphocytes appears

to survive as resting T cells, and long-lived naïve T cells, if they do divide, cycle very slowly[334].

Other data also suggests that peripheral T cell survival is not solely dependent on signals mediated by the TCR/CD3 complex, but may also require signals transmitted via cytokine receptors[335]. In this regard, IL-7/IL-7Rα interactions have been identified as important regulators of T cell homeostasis[336] as they impact on cell survival. APC-like cells present in the lymph nodes were shown to produce IL-7 in response to lymphopenia[337]. As IL-7 is a proliferation/survival factor, production of this cytokine will enhance the maintenance of peripheral T cells as well as ensure constant supplies of IL-7 in the thymus (critical for T cell ontogeny). T cells will compete for IL-7 and will restore T cell counts through increased peripheral expansion/maintenance and *de novo* T cell production.

APC-like cells likely constitute a major component of peripheral T cell niches as they can secrete cytokines and express MHC molecules. Other physiological traits, like the ability to enter the lymph nodes (caused by a reduced CCR7 expression) or to be maintained in the periphery might play a role as well in allowing T cells to reach and be in close proximity to these factors. The ability to be receptive to survival signals (impairment of cell survival through a decreased IL-7Rα (CD127) expression) also contributes to the maintenance of cell numbers and thus, is a central factor for peripheral T cell homeostasis and niche maintenance.

1.3.2 Immune Reconstitution following Hematopoietic Stem Cell Transplantation

The principle of hematopoietic stem cell transplantation (HSCT) is to infuse marrow-derived hematopoietic stem cells to reconstitute hematopoiesis and immune functions following high-dose chemotherapy and/or irradiation. HSCT was first pioneered in the 1970s and is now an established treatment for fatal conditions like leukemia, lymphomas, bone marrow failure syndromes as well as immunodeficiency disorders. In many

instances, HSCT constitutes the only form of treatment offering significant prospects for cure.

HSCT is classified as either an autologous or allogeneic procedure depending on whether the patient itself or another individual donates the reconstituting hematopoietic stem cells. Most allogeneic HSCTs (AHSCT) are performed with grafts from HLA-matched sibling donors or HLA-matched unrelated donors. Needless to say, this procedure carries much higher mortality rates than autologous HSCT, mainly as the result of post transplantation complications like graft-versus-host disease (GVHD).

Several requirements need to be fulfilled for the development of GVHD following allogeneic HSCT. First, mature T cells must be present in the graft/infusion. Being HLA-matched, such cells will massively proliferate and participate in the anti-host attack targeting several tissues like the skin, the gastrointestinal (GI) tract, the liver and the thymus[338]. Second, the recipient must express antigenic peptides that are absent in the donor. In fact, these peptides, even though they are self-peptides for the patient, will be recognized as foreign peptide from the standpoint of mature T cells present in the graft/infusion. Such peptides are now referred to as minor histocompatibility antigens and arise from polymorphism observed among HLA-matched humans[339].

T cell restoration following AHSCT occurs through thymic-dependant and thymic-independent pathways . Immediately after transplantation, patients are deficient in all cells derived from the hematopoietic lineage as a result of the myeloablative regimen. Gradually, the T cell count rises, mainly through peripheral expansion of memory T cells[340] present in the graft[341] or that survive the conditioning regimen[342]. A consequence of this repopulation is a biased TCR repertoire[343]. In theory, peripheral expansion of memory T cells constitutes a rapid way to replenish the compartment but results in no additionnal gain in TCR diversity. It was even reported that some Vβ families among memory T cells post transplantation encompass only one clonotype[344]. Therefore, reconstitution of the T cell compartment through expansion restores T cell numbers without reconstituting the diversity of the TCR repertoire.

Contrary to memory cells, naïve T cells take more time to recover. The first peripheral naïve T cells become detectable around 6 months following AHSCT. This involves thymic selection of graft-derived precursors[345-347] and contributes to the broadning of the TCR repertoire. Given the age-related thymic involution, it is assumed that this selection mechanism is more efficient in young AHSCT patients rather than in the elderly[348,349]. Reconstitution of the naïve (CD45RA) compartment has been shown to be thymic-dependant[350]. Furthermore, evidence for *de novo* T cell production has recently been gathered, therefore indicating that the thymus contributes in immune restoration following AHSCT[2,351-353].

1.3.3 The impact of HIV Infection on the Thymus and T Cell Homeostasis

Overview

Human immunodeficiency virus (HIV) is a member of the retroviridæ family and contains 9 genes (*gag*, *pol*, *env*, *vif*, *vpr*, *vpu*, *tat*, *rev* and *nef*) spanning its 9.3 kb RNA genome. HIV infection leads to gradual and profound immunocompromise. It has long been known that the central tenant of this immune dysfunction is the depletion of $CD4^+$ T cells, although the way such destruction occurs *in vivo* is still a matter of debate[354]. Four to six weeks after HIV infection, there is a period of viremia coincident with a significant drop in $CD4^+$ T cell numbers[355]. Following this acute phase, seroconversion occurs; the viral load declines to its "setpoint" and the $CD4^+$ T cell count returns to lower, but quasi-normal levels. During the ensuing period of clinical latency (which can last upward of 10 to 12 years), there is a gradual depletion of $CD4^+$ T lymphocytes in both the peripheral blood and more significantly in the lymphoid tissues[355,356]. Both naive and memory T-helper cells are depleted and the TCR repertoire is perturbed and becomes restricted[357-359]. Added to this, some of the remaining cells that persist in the periphery may be dysfunctional[360]. This has the effect of impairing both the body's ability to coordinate an immune response by decreasing the variety of antigens that can be recognized. With disease progression, existing T-cells become less and less responsive, first to common recall antigens, then to alloantigens and eventually fail to proliferate even when exposed

to mitogens[361,362]. Finally, in late stage disease, the lymph node architecture is destroyed and replaced by fibrosis while the thymus is atrophied. CD4 cells reach critically low numbers and, coincident with this, the viral load again increases and shifts to CXCR4-using viral particules. At this point (< 200 $CD4^+$ T cells per μL of blood), the body is defenseless against a wide range of neoplasms and infectious organisms and eventually succombs (Figure 7).

Highly active antiretroviral therapy (HAART) has been able to slow or even reverse this progressive immune decline[363,364]. Combinations of two reverse transcriptase inhibitors plus one or two protease inhibitors have achieved significant reductions in viral load. Such reductions have been shown to occur equally in both the peripheral blood and the lymphoid tissues[365-368]. Past studies have shown that when the viral load is reduced to between 50 and 500 copies per mL of blood, the suppression is not sustained for more than a few months and inevitably breakthrough of drug-resistant viruses occur and disease progression continues. However, when the viral load is reduced to between 20 and 50 copies per mL of blood, the suppression is maintained for prolonged periods with 50% of such patients maintaining suppression for more than 80 weeks[369,370].

Once HIV suppression is achieved, the $CD4^+$ T cell number quickly begins to rise. The gain is variable and appears to take place in two distinct phases. First there is a rapid increase in the phenotypically-defined memory T cell compartment. Eventually, some 8 to 12 months after initiating HAART, there is an increase in the number of naive CD4 cells coincident with the expansion of the T-cell repertoire[364,371]. Most striking is the significant clinical improvement seen with the increase in total $CD4^+$ T cell numbers. Weight gain, regression of Kaposi sarcoma (KS) lesions[372], and remission of cytomegalovirus (CMV) retinitis[373] have all been noted. While T cells taken from patients with active CMV retinitis have been shown to be anergic to CMV antigens, those taken from patients with quiesent retinal disease following HAART are reactive to CMV antigens[374]. This clinical improvement clearly represents some degree of immune reconstitution. The question of the importance of the thymus to this immune reconstitution observed following HAART is raised.

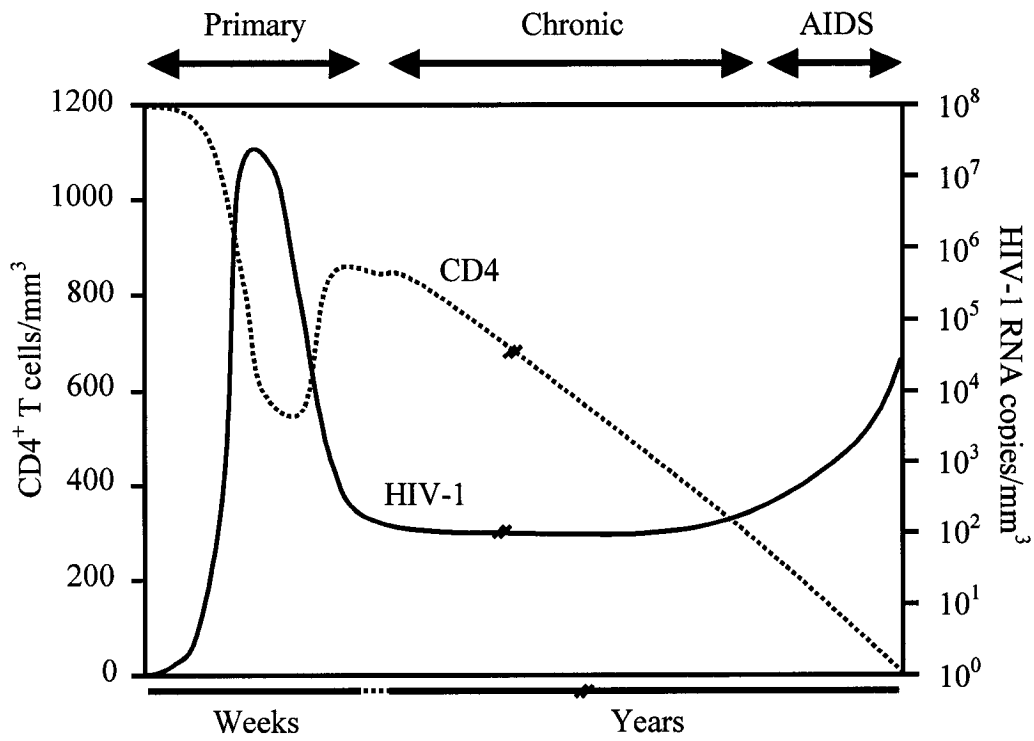


***Figure** 7. Natural course of HIV-infection. Strong viral replication occurs as soon as HIV enters the immune system and the viral load rapidly reaches high blood levels. As a result of this huge viremia, 30-60% of $CD4^+$ T cells are depleted. As the immune response against HIV is gradually elaborated, the viral load becomes under control and reaches a set point, which is indicative of the patient's prognostic. Partial immune restoration of $CD4^+$ T cells occurs and marks the end of primary HIV infection. Chronic HIV infection is asymptomatic but slowly and progressively eliminates peripheral $CD4^+$ T cells to a point where opportunistic infections occur, leading to the onset of the AIDS phase (usually 7-11 years post-infection). The AIDS phase is symptomatic and characterized by the inability of the immune system to contain HIV replication as well as opportunistic infection (Candida albicans, Pneumocysteis Carnii). The viral load increases following a change in coreceptor usage (CCR5 to CXCR4) leading to more depletion of peripheral $CD4^+$ T cells to a point were the immune system can no longer coordinate an immune response against HIV.*

Where do those new T cells come from ?

The nature of the immunological gains as well as the extent and sustainability of the reconstitution are all areas of current uncertainty. While naive ($CD45RA^+$ $CD62L^+$) CD4 cells appear following HAART, the source of these cells has not been determined.

Recently, it was shown that thymopoiesis is still active in adults long after puberty despite the age-related thymic involution[1,141,307,375,376]. Thus, the $CD45RA^+$ $CD62L^+$ T cell rise seen following HAART could represent *de novo* naive $CD4^+$ T cell production. Alternatively, these cells could be generated through proliferation of resting naive cells formed long before HIV infection of the host. This has tremendous impact for the immune reconstitution standpoint as *de novo* T cell production from the thymus will result in a broader TCR reprtoire than would peripheral homeostatic expansion of already existing resting T cells. Furthermore, it is possible that naive cells "trapped" in the lymph nodes are redistributed in the peripheral blood following a reduction in the HIV antigen load. Others have also argued that the rise in $CD45RA^+$ cells does not represent an increase in naive cells but rather a reversion in the phenotype (from $CD45RO^+$ to $CD45RA^+$) of cells following HAART. In this regard, the appearance of the $CD45RA^+$ cells does follow the expansion of the $CD45RO^+$ population. What is naive versus what is memory has become increasingly blurred.

The thymus and HIV

Thymocytes have been shown to be susceptible to HIV infection[377-379]. In fact, CD4 immature single positive (ISP) thymocytes express the CXCR4 and low levels of CCR5 at their surface[380], thereby making them, as well as SP4 and SP8 thymocytes, good target for HIV infection. Moreover, HIV replication has been shown to be dependant on NF-kB[381], which is also expressed following β selection[382]. Taken together, it is very possible that HIV infects the human thymus and induces the depletion of developing thymocytes, leading to the reduction of the magnitude of thymic function.

Spairing developing thymocytes from death could very well contribute to the maintenance of peripheral $CD4^+$ T cell numbers. In fact, it has been shown that HIV viral isolates resistant to protease inhibitor (PI) molecules replicate less efficiently in human fetal thymic organ cultures (FTOCs) as compared to wild-type isolates from the same patients. As PI-resistant viruses replicate less efficiently, it is believed that they induce less destruction within the thymus. Accordingly, it has been demonstrated that patients infected with PI-resistant strains have abundant thymus (according to CT scans) likely as

a result of the preservation of thymocyte supplies[383]. Taken together, these data suggest that depletion of the thymocyte pool directly affect peripheral T cell counts and contributes to the development of AIDS. Furthermore, these results may explain why, despite high viral loads, some patients can maintain high levels of $CD4^+$ T cells (discordant "high CD4/high viral load" HIV-infected patients).

1.4 Project rationale and research objectives of the thesis

The thymus has been recognized as the key site of T cell development for over 4 decades. Most of the first two decades of thymology focused on the identification of the anatomical and macroscopic components of the thymus. Through the use of innovative experiments, thymologists were allowed their first glimpses into the interior of the thymus and witnessed its cellular heterogeneity and complex organization. The advent of multiparametric flow cytometry and monoclonal antibodies directed against membrane molecules further revolutionized immunology and lead to the identification of distinct phenotypic stages that now characterize T cell development stages and the cellular components harbored within the thymic architecture.

Anatomical studies demonstrated the retrosternal localization of the human thymus and documented its age-related involution. In the mid 1980s, Steimann and colleagues[84,85] studied thymus morphology and demonstrated that thymopoiesis began to decrease shortly after birth. Two major components of the thymus, the true thymic epithelial space (TES) where thymopoiesis occurs, and the perivascular space (PVS), were also identified. Furthermore, changes in the proportion of TES and PVS were reported to occur throughout life and lead to thymic atrophy. In fact, as the thymus ages, adipose tissue and mature lympoid cells fill-up the PVS and gradually replace the TES. Given this and despite no direct methods capable of adequately monitoring thymopoiesis levels, it was assumed that the generation of the peripheral T cell repertoire was occurring rather early in life, when the thymus is most active[384,385]. Hence, thymic contribution to peripheral T cell homeostasis in adulthood was considered negligible.

Given the impact of stress on thymopoiesis and the physical inaccessibility of the thymus due to its thoracic localization, non-invasive techniques are prefered when assessing thymic output. Thymic morphology can be visualized by computed tomography (CT) scan[315] but might not be reflective of its activity. Attempts to monitor thymic function were done by evaluating the proportions of peripheral naïve and memory/effector T lymphocytes using CD45RA and CD45RO expression, respectively[341,386]. Given the

existence of memory revertants within phenotypically-defined naïve T cells[387] and the incompletely understood regulation of T cell surface phenotypes, one could easily question the accuracy of reports documenting human thymopoiesis using FACS-analysis of peripheral blood mononucleated cells (PBMCs). Moreover, mature T cells were also shown to recirculate within the thymus leading to a possible overestimation of thymic function levels when performing "pulse-chase" FITC intrathymic labeling experiments[312,388-390]. Thus, the impossibility to phenotypically distinguish recent thymic emigrants (RTEs) from naïve T cells underlines the inaccuracy of FACS-derived methods in the evaluation of thymic function (unlike the avian model, no convincing RTE-specific cell surface molecules has been found in human). The inability to accurately and unequivocally measure human *de novo* T cell production lead to the assumption that thymic function was negligible during adulthood and homeostatic expansion of existing peripheral T cells compensated for the reduced influx of cells from the thymus after puberty.

The research objectives of the work detailed in this thesis were:

1) To determine whether adult humans still exhibit a diversified thymic activity through the development of novel tools capable of uniquevocably monitoring the magnitude of thymic function as well as the diversity of recent thymic emigrants irrespectively of cell surface molecule expression.

2) Having demonstrated that a diversified thymopoiesis still occurs in adult humans, to investigate whether a reduced thymic function is responsible for the observed long-lasting naïve T cell deficiency observed following allogeneic hematopoietic stem cell transplantation (AHSCT).

3) To determine the impact of early primary HIV infection on both the magnitude and diversity of thymic function

2. Direct evidence of thymic function in adult humans

The absence of any direct experimental technique capable of monitoring thymic function as well as the diversity of its exportation contributed to the development of the work presented in this paper. We initially postulated that peripheral blood recent thymic emigrant (RTE) frequencies were representative of thymic function levels and that most peripheral RTEs would encompass by-products of TCR gene rearrangement, namely TCR deletion circles (β DCs or β TREC). Therefore, peripheral blood quantification of these molecular products led to the evaluation of thymic activity and provided, for the first time, information regarding the diversification of *de novo* T cell production.

Direct Evidence for Thymic Function in Adult Humans

Jean-François Poulin[1,2], Mohan N. Viswanathan[3], Jeffrey M. Harris[3], Krishna V. Komanduri[3,4], Eric Wieder[3], Nancy Ringuette[1], Morgan Jenkins[3], Joseph M. McCune[3,6] and Rafick-Pierre Sékaly[1,2,5,7]

[1]Laboratoire d'Immunologie, Institut de Recherches Cliniques de Montréal (IRCM), Montréal, PQ, H2W 1R7, Canada, [2]Department of Medicine, Division of Experimental Medicine, McGill University, Montreal, PQ H3A 1A3, Canada, [3]The Gladstone Institute of Virology and Immunology, University of California, San Francisco, CA, 94141-9100, USA, [4]Division of Hematology and Oncology, University of California, San Francisco, CA 94143, USA, [5]Département de Microbiologie et d'Immunologie, Université de Montréal, Montréal, PQ H3C 3J7, Canada, [6]Departments of Medicine and Microbiology and Immunology, University of California, San Francisco, CA, 94143, USA, [7]Department of Microbiology and Immunology, McGill University, Montreal, PQ H3A 2B4, Canada

Published in The Journal of Experimental Medicine

1999, Vol. 190 (4), pp. 479-486

FOOTNOTES

RTE: recent thymic emigrant, DC: deletion circle, CBMC: cord blood mononuclear cell, APC: allophycocyanin, TC: tri-color, RSS: recombination signal sequence, dNTPs: deoxyribosetriphosphate nucleotides, DCF: deletion circle frequency, RAG: recombination activating enzyme

ABSTRACT

The understanding of human thymic function and evaluation of its contribution to T cell homeostasis are matters of great importance. Here we report the development of a novel assay to quantitate the frequency and diversity of recent thymic emigrants (RTEs) in the peripheral blood of humans. Such cells were defined by the presence of T cell receptor (TCR) rearrangement deletion circles (DCs), episomal by-products of TCR β V(D)J rearrangement. DCs were detected in T cells in the thymus, in cord blood, and in adult peripheral blood. In the peripheral blood of adults aged 22 to 76 years, their frequency was highest in the $CD4^+$ $CD45RA^+$ $CD62L^+$ subpopulation of naive T cells. TCR DCs were also observed in other subpopulations of peripheral blood T cells, including those with the $CD4^+$ $CD45RO^-$ $CD62L^+$ and $CD4^+$ $CD45RO^+$ $CD62L^+$ phenotype. RTEs were observed to have more than one Vβ rearrangement, suggesting that replenishment of the repertoire in the adult is at least oligoclonal. These results demonstrate that the normal adult thymus continues to contribute, even at old ages, a diverse set of new T cells to the peripheral circulation.

INTRODUCTION

It has been assumed that a diverse TCR repertoire is formed during early life, when the thymus is most active, and that T cell homeostasis is maintained without significant thymic input in adults[1,2]. Given the profound effects of stress upon thymopoiesis, intrathymic T cell production in the intact animal is best studied with a minimally invasive assay for RTEs in the peripheral blood. In the chicken, for example, RTEs can be identified by their unique expression of the cell surface marker, chT1[3]. Murine RTEs may be followed kinetically in the peripheral circulation after direct intrathymic labeling, e.g., with fluorescein isothiocyanate[4]. Assays of this type are, however, unavailable for the assessment of human thymic function. Such assessment has relied instead upon autopsy series[5], radiographic observations[6], and/or phenotypic demarcation of circulating human T cells into distinct populations of "naive" or "memory/effector" cells[7]. In aggregate, these studies demonstrate: (a) that there is a correlation between the abundance of circulating $CD4^+$ $CD45RA^+$ $CD62L^+$ human T cells and the presence of thymic tissue[7-9], suggesting that RTEs are included within this T cell subpopulation; (b) that the circulating $CD8^+$ $CD45RA^+$ T cell subpopulation is less clearly associated with human thymic tissue[8]; and (c) that circulating "memory/effector" $CD4^+$ and $CD8^+$ T cell subpopulations bear the phenotypic marker CD45RO instead of CD45RA[10].

Phenotypic measures are imprecise, however, in their ability to distinguish lymphocytes that have recently been made in the thymus or peripheral tissues and those which have reverted from memory status[11,12]. Thus, although it is clear that the human thymus involutes dramatically after puberty[5], the fraction of circulating $CD45RA^+$ T cells remains relatively constant for long periods of time thereafter[13]. These findings suggest that the $CD45RA^+$ $CD62L^+$ T cell subpopulation may contain a higher proportion of RTEs early than later in life and that it harbors heterogeneous cell populations (including revertants of memory/effector cells) throughout.

Recently, Douek, D.C. *et al.*[14] have exploited an intrinsic feature of the TCR rearrangement process to directly demonstrate the presence of continuous thymic output in human adults. This assay relies on the detection of TCR α excision circles (sjTRECs) generated during TCR α gene rearrangement in the thymus. Similar observations have also been made in the avian system whereby *de novo* TCR rearrangement, as measured by excision circle assays, correlated with the expression of chT1 antigen[3]. Moreover, circle-bearing T cells were found in the avian lymph node, spleen and skin[15] suggesting that the thymus may constantly supply new T cells to these peripheral compartments.

In this report, we describe an assay for the detection of recent thymic emigrants within various subpopulations of circulating human T cells. We observe that such cells are most abundant in the $CD45RA^+$ $CD62L^+$ subpopulation, that they are at least oligoclonal in their expression of TCR Vβ regions, and that they are detectable in adults.

MATERIALS AND METHODS

Isolation of thymocytes

Methods for maintenance of SCID-hu mice and harvest of thymocytes from SCID-hu Thy/Liv organs were identical to those previously published[16]. In some cases, SCID-hu Thy/Liv organs were harvested and placed in RPMI 1640 media (Life Technologies) supplemented with 10% fetal calf serum (FCS) (Summit Biotechnology, Fort Collins, Colorado) and transported overnight at 4 °C prior to harvest of thymocytes. Following isolation, thymocytes were resuspended in phosphate buffered saline (PBS) supplemented with 2% FCS and kept on ice prior to staining with monoclonal antibodies for flow cytometric analysis or cell sorting. All procedures and practices were approved by the University of California, San Francisco Committee on Human Research (CHR) or by the University of California, San Francisco Committee on Animal Research.

Isolation of peripheral blood mononuclear cells

Whole blood samples from human subjects were collected by phlebotomy into EDTA collection tubes (Becton Dickinson). Peripheral blood mononuclear cells (PBMC) were isolated from whole blood by density-gradient centrifugation (Life Technologies). PBMC were washed twice with PBS before resuspension in PBS supplemented with 2% FCS prior to staining with monoclonal antibodies for flow cytometry or cell sorting.

Stimulation of cord blood cells in vitro

Human umbilical cord blood cells were obtained (with CHR approval) from healthy delivery specimens and placed in heparinized collection tubes (Becton Dickinson) under sterile conditons. Cord blood mononuclear cells (CBMC) were isolated as described above for whole blood specimens and resuspended at a concentration of 2 x 10^6 cells/ml in RPMI 1640 supplemented with 10% human AB serum (Ultraserum, Gemini Bio-Products). CBMC were then cultured (at 37 °C in 5% CO_2) for 48 hours, 72 hours, 96

hours, or 9 days (time points encompassed in 2 different experiments) and stimulated with 5 μg/mL of phytohemagglutinin (PHA) (Sigma) and 10 U/mL purified interlukin-2 (IL-2) (Boehringer Mannheim). The supplemented medium was changed every 3 days. Cell culture controls did not receive PHA or IL-2 stimulation but were cultured for 72 hours in the same medium. Aliquots of the cell cultures at different time points were analyzed by flow cytometry for the expression of the cell surface markers, CD45RA and CD62L.

Immunophenotypic analysis and cell sorting by flow cytometry

PBMC, thymocytes from SCID-hu mice, or CBMC were stained with fluorescent-conjugated monoclonal antibodies specific for cell surface markers at a concentration of 10^7 cells/ml at 4 °C for 30 minutes. Following staining, cells were washed with PBS supplemented with 2% FCS and sorted either on a FACStar or a FACS Vantage cell sorter (both from Becton Dickinson). The cells were stained with one of the following antibody combinations: 1) anti-CD8-FITC (Becton Dickinson) and anti-CD4-PE (Becton Dickinson); 2) anti-CD45RA-FITC (Immunotech) or anti-CD45RO-FITC (Immunotech), anti-CD62L-PE (Becton Dickinson), and anti-CD4-ECD (Coulter); 3) anti-CD62L-FITC (Pharmingen), anti-CD45RA-PE (Pharmingen), and anti-CD4-TC or anti-CD4-APC (Caltag). Sort purities were checked after each sort and were not less than 97%. For analysis of cord blood CD45RA and CD62L expression, CBMC were stained with anti-CD45RA-FITC (Immunotech) and anti-62L-PE (Becton Dickinson) and analyzed using a FACScan® cytometer and Cell Quest software (both from Becton Dickinson).

Detection of TCRβ rearrangement deletion circles

Total DNA from distinct cell populations was extracted and purified via a standard protocol[17] before spectrophotometric quantitation at 260 nm and 280 nm. The freshly isolated DNA was stored at 4 °C for further processing. Thermal cycling was performed for 30 cycles (1 min at 94 °C, 1 min 30 sec at 65 °C, 1 min 30 sec at 72 °C) for each round

of a semi-nested PCR protocol designed to detect VβDβ-specific deletion circles generated by TCR β recombination. All first and second round primers were generated to fully hybridize with non-coding regions of the TCRβ locus[18] located next to the RSSs (GeneBank accession number: U66059, U66060, and U66061) (see Table 1). Four PCR replicates were done on each total DNA serial dilution to ensure a precise read-out for each experiment. Concentrations of total DNA were adjusted so that a constant volume of 3 μL was added to each 50 mL PCR reaction [200μM dNTPs, 1x PCR buffer (Boehringer Mannheim), 100 ng of each primers and 2 U of Taq polymerase (Boehringher Mannheim)]. From the first PCR amplification, 3 mL were used as template for the second (semi-nested) PCR reaction (same conditions) using the "Circle" primer and the DC-Dβ1 primer.

Quantitative analysis of endpoint dilutions

Second round PCR products were visualized with ethidium bromide on 1.25% agarose gels and digitally photographed. Individual amplifications were scored as positive or negative by two observers. The highest dilution returning a positive amplification was taken as the endpoint for each dilution series. Dilution series with greater than two "skipped" wells (i.e, a failed amplification followed by a successful amplification at higher dilution) were omitted from the analysis. The abundance of deletion circles was estimated by the method of Reed-Muench[19,20]. This method uses information from replicate dilution series to estimate an endpoint (measured in term of ng input DNA) in which 50% of samples were positive for DC (the 50% DC endpoint). The Deletion Circle Frequency (DCF) was arbitrarily defined as the reciprocal of the "50% DC endpoint" (x 100) (see text). Alternatively, the semi-nested PCR data were analyzed by a maximum likelihood estimated method of dilution endpoint with a parametric method[21]. Unlike the Reed-Muench method, this method returns an estimate of goodness of fit of the data to the estimated endpoint. Endpoints estimated by the two methods were highly correlated (R^2=0.93) and the choice of method did not alter the conclusions drawn from the data.

The degree of inter- and intra-assay variation was assessed by performing two independent experiments on two different samples from the same individuals (n=3) and ranged on the order of 2-3 fold (data not shown).

RESULTS AND DISCUSSION

A novel assay to identify recent thymic emigrants

To determine whether the $CD4^+$ $CD45RA^+$ $CD62L^+$ subpopulation of circulating human T cells contains RTEs, we devised an assay to detect physical evidence of recent TCR gene rearrangement. We chose to focus on rearrangements at the β locus because the complete sequence of this locus has been obtained[18], permitting the construction of a panel of Vβ-specific primers to assess the diversity of rearranged TCRs. Moreover, allelic exclusion is more complete at the TCR β locus than at the TCR α locus[22,23]. Rearrangements at this locus are a salient feature of intrathymic T cell production and require expression of the recombination activation genes (RAG-1 and RAG-2) and recognition of conserved heptamer and nonamer recombination signal sequences (RSSs) flanking each V, D, and J gene segment[24-28] (Fig. 1*a*). As the coding segments are brought together, excision-ligation of the heptamer-heptamer signal joint creates an episomal TCR rearrangement deletion circle (DC)[24,29] bearing two identifiers: first, each Vβ-Dβ DC has a precise molecular weight determined by the length of intervening, noncoding DNA; secondly, a unique DNA sequence bridges the signal joint. Using the known nucleotide sequences of the non-coding DNA regions adjacent to Vβ2, Vβ17, Vβ5.1 and to Dβ1[18], primers were designed such that a PCR product would only be amplified if they were facing each other within a closed DC (see Table 1). As shown in Fig. 1*b*, the product amplified for a Vβ2/Dβ1 rearrangement would have a predicted size of 439 bp, with characteristic restriction enzyme sites. In the case of DCs specific for Vβ17/Dβ1 and Vβ5.1/Dβ1 rearrangements, the corresponding molecular weights would be 445 bp and 442 bp, respectively.

The specificity and reliability of this strategy was first assessed in developing human thymocytes, expected to have a high frequency of deletion circles[30]. DNA was extracted from 2 different samples of human $CD4^+$ $CD8^+$ thymocytes (harvested from Thy/Liv

organs of SCID-hu mice)[16]. After amplification using the primers specific for Vβ2/Dβ1 DCs, all were found to generate the expected 439 bp PCR product. As shown in a representative case (Fig. 1*b*), this product carried predicted restriction enzyme recognition sites for SacI, PvuII, and ApaLI, and was not observed with PCR performed on DNA from Jurkat cells (a Vβ8.1 T cell line which should not carry Vβ2/Dβ1 DCs). Nucleotide sequence analysis of the PCR product confirmed its identity to the predicted sequence spanning the signal joint of the Vβ2/Dβ1 DC (not shown).

Quantitative assessment of cells having recently undergone β chain TCR rearrangement

Within a population of cells, the fraction bearing DCs should be proportional to that which has recently undergone TCR rearrangement. To directly compare this fraction between different cell populations, a semi-quantitative assay was developed to measure a dilution endpoint of DC DNA within a given amount of total cell DNA. DNA was diluted in four replicate series and PCR was carried out to determine whether a given well was positive or negative for the DC PCR product. The "50% DC endpoint," measured in terms of nanograms of input DNA, was calculated using either the Reed-Muench method [19,20] or a maximum likelihood estimate[21] (see materials and methods). The 50% DC endpoint represents the median minimal amount of DNA from which a deletion circle may be amplified by nested PCR; the Deletion Circle Frequency (DCF) was arbitrarily defined as the reciprocal of the "50% DC endpoint" (x 100) and is proportional to the number of deletion circles which can be amplified from 100 ng of input DNA. A representative experiment using the assay to quantitate DC is shown in Fig. 2*a*. Four replicate dilution series of DNA from $CD3^+$ $CD8^+$ (single positive, SP) thymocytes were amplified with primers specific for Vβ2/Dβ1 DC, and these yielded a positive PCR signal for deletion circles at final (highest) dilutions of 16, 16, 16, and 3.2 ng input DNA. This corresponds to a 50% DC endpoint of 5.47 ng (as determined by the Reed-Muench method) and a DCF of 18.3 (=100/5.47). Assuming typical recovery of DNA and

amplification sensitivity, this would return minimum estimate of 1 DC in 547 SP8 thymocytes or (since 2-5% of total express a Vβ2/Dβ1 TCR) 11-22 Vβ2/Dβ1 SP8 thymocytes. Similar frequencies of DC were noted in sorted populations of $CD3^+$ $CD4^+$ and $CD4^+$ $CD8^+$ thymocytes, yielding DCFs of 8.4 and 11.7, respectively (Fig. 2*b*).

TCR Vβ deletion circles in circulating peripheral blood T cells

Since prior studies indicated that DCs were detectable within $chT1^+$ RTEs in the chicken[3] and in human children and adults[14,31], the Vβ DC assay was used to determine whether Vβ DCs were present in various populations of human peripheral blood T cells. Reasoning that the frequency of RTEs in the peripheral blood would be highest early in life, T cells in cord blood were examined first. Flow cytometric analysis revealed that >95% of CD4+ T cells in unstimulated cord blood carried the "naive" $CD45RA^+$ $CD62L^+$ phenotype (Fig. 3*a*, panel 1) and all of these cells were "bright" for CD45RA staining. The frequency of DCs within unstimulated cord blood was higher than that observed for single positive thymocytes (with DCFs approximating 43.1 and 41.8 in the two cord blood specimens compared to values of 18.3 and 8.4 for SP8 and SP4 thymocytes, respectively) (Fig. 3*b*). After 9 days of stimulation in vitro with PHA and IL-2, the percentage of CD4+ cord blood T cells with the "naive" ($CD45RA^{BRIGHT}$ $CD62L^+$) phenotype dropped to negligible levels and most cells were instead negative for CD62L and/or dimly positive for CD45RA (Fig. 3*a*, panels 2-4). Within this same time frame, the frequency of DCs dropped from an average of 42.5 DCF to 0.85 DCF, a 50-fold decrease over a 9 day period (Fig. 3*b*). These results indicated that DCs could be detected in circulating T cells and that their detection was correlated with the presence of cells bearing the "naive" $CD45RA^+$ $CD62L^+$ phenotype.

Inverse correlation between frequencies of deletion circles and age

DCs were then quantitated in the peripheral blood of 17 adult individuals, ranging in age from 22 to 76 years. In each, naive $CD4^+$ $CD45RA^+$ $CD62L^+$ and memory/effector $CD4^+$

$CD45RO^+$ $CD62L^-$ cells were quantitated by flow cytometry and sort-purified for determination of DC frequency. Results are shown in Fig. 3*c*. Within the population of circulating $CD4^+$ $CD45RA^+$ $CD62L^+$ T cells, DCs were observed with a frequency that was higher than that found in the $CD4^+$ $CD45RO^+$ $CD62L^-$ population (which had non-detectable levels of DC in these 17 individuals; data not shown). As a function of age, there was a consistent decrease in the frequency of DCs within the $CD4^+$ $CD45RA^+$ $CD62L^+$ subpopulation (Fig. 3*c*, R^2= 0.5026, P=0.0045), even though individuals across this age range had equivalent percentages of $CD45RA^+$ $CD62L^+$ within their $CD4^+$ T cells (Fig. 3*d*, R^2= 0.0233, P=0.5123). These data suggest that RTEs exist within the circulating population of $CD4^+$ $CD45RA^+$ $CD62L^+$ T cells of adults, that their proportion decreases with age, and that the DC assay appears to provide a much more reliable estimate of *de novo* generated T cells than that provided by phenotypic cell surface markers such as CD45RA and CD62L.

Detection of deletion circles in other T cell populations

To determine whether other subpopulations of circulating $CD4^+$ T cells might harbor TCRβ rearrangement DCs, cells were sort purified into subpopulations that were $CD4^+$ $CD45RA^+$ $CD62L^+$, $CD4^+$ $CD45RO^+$ $CD62L^-$, $CD4^+$ $CD45RO^+$ $CD62L^+$, and $CD4^+$ $CD45RO^-$ $CD62L^+$. In eight individuals ranging in age between 22 and 76 years, the highest frequency of DC was found in the $CD45RA^+$ $CD62L^+$ subpopulations and the lowest in the $CD45RO^+$ $CD62L^-$ subpopulation (Table 2). DCs were also found in the $CD45RO^+$ $CD62L^+$ subpopulation in 4 out of 8 individuals tested, albeit at a lower frequency. Finally, DCs were detected in T cells with the phenotype $CD45RO^-$ $CD62L^+$ (data not shown), and $CD45RO^+$ $CD62L^-$, although only one out of 9 individuals showed detectable levels of DCs in the latter compartment. These cells may possibly represent direct progeny of RTEs in the $CD45RA^+$ $CD62L^+$ subpopulation; alternatively, DCs may be present within them as a consequence of extrathymic TCR rearrangements[1,32].

The TCR repertoire in RTEs is at least oligoclonal.

Previous studies have demonstrated the presence but not the degree of TCR diversity of RTEs in adult humans[14,31]. To address this parameter of diversity we generated primers which could amplify DCs issued from three different TCRVβ-Dβ rearrangements (Vβ2/Dβ1, Vβ5.1/Dβ1, and Vβ17/Dβ1). Flow cytometric analyses (data not shown) revealed different percentages of circulating T cells bearing these three Vβs (Vβ2: 8-10%; Vβ5.1: 3-4%; Vβ17: 3-4%). Results illustrated in Table 2 clearly show that DCs detectable in circulating human T cells encompass several (at least two) Vβs and were present not only in the $CD45RA^+$ $CD62L^+$ but also in the $CD45RO^+$ $CD62L^+$ subpopulations of $CD4^+$ Tcells. Interestingly, the relative frequency of DCs from different Vβ regions, as measured by flow cytometry, did not correlate with the proportion of PBLs expressing these TCR Vβ products. For instance, $V\beta2^+$ T cells were always at least two fold more abundant in PBLs from normal individuals compared to $V\beta5.1^+$ or $V\beta17^+$ T cells (data not shown). Yet, analysis of DCF values (shown in Table 2) indicate that, in the two individuals tested (aged 31 and 32 years), Vβ5.1/Dβ1 or Vβ17/Dβ1 DCs were 2- to 5- fold more abundant than Vβ2/Dβ1 DCs. These differences in the relative abundance of Vβ-DCs compared to the expected frequencies of their parental cell populations could reflect both a relative dilutional effect on some Vβ-DCs due to varying degrees of peripheral expansion in Vβ-specific subsets, as well as a relative overestimate of some subpopulations due the detection of DCs from non-productive rearrangements that might be more prevalent in certain Vβ subsets.

In sum, these experiments demonstrate that TCRβ deletion circles can be detected within thymocytes and within circulating human CD4+ T cells with a "naive" ($CD45RA^+$ $CD62L^+$) phenotype. Detection of such circles is specific, reliable, and quantitative; our method also indicates that they are generated upon rearrangement of multiple Vβ coding segments. Finally, DCs in $CD4^+$ $CD45RA^+$ $CD62L^+$ T cells are observed in a pattern

which is consistent with known parameters of intrathymic maturation: their frequency decreases as cord blood T cells are stimulated to divide *in vitro* and in older individuals who have less abundant thymus, as measured in autopsy series or by non-invasive radiography. As such, quantitation of deletion circles within human peripheral blood $CD4^+$ $CD45RA^+$ $CD62L^+$ T cells appears to represent a measure of recent thymic emigrants and, hence, thymic function.

These results serve to directly confirm previous inferences about thymic function. First, the finding of DCs within the $CD4^+$ $CD45RA^+$ $CD62L^+$ population of adult individuals aged 23-76 years underscores the premise that the thymus, though less functional, is nonetheless operative into adulthood (2,5,9,14,32). Secondly, the fact that the frequency of DCs decreases in the $CD4^+$ $CD45RA^+$ $CD62L^+$ population as a function of age demonstrates that this population is heterogeneous[11,12] and that its composition is age-dependent. It may not be useful, in other words, to assume that the presence (or reappearance) of such cells is synonymous with "immune reconstitution"[33-36]. Finally, the finding of DCs within other populations of circulating T cells raises the possibility that extrathymic sources (e.g. gut or liver) may contribute to formation of the circulating TCR repertoire[1,32].

Although further work is required to optimize the quantitative precision of the DC assay and to enhance its applicability for comprehensive studies of human thymic function, it is now applicable to important contemporary questions about thymic function and immune reconstitution in humans. Most immediately, it will be of interest to determine the extent of thymic dysfunction at different stages of HIV infection and following bone marrow reconstitution post myeloablation. It will also be interesting to determine the extent of de novo rearrangement in lymph nodes, which might be induced by chronic viral replication, as recently suggested in a murine model of persistent antigen exposure[37]. This measure of

thymic function may also facilitate the design of studies aimed at augmenting intrathymic T cell production.

Table 1: Primary sequence of primers required for βDCs detection/amplification

Primer name	Nucleotide sequence
DC-Vβ2	5'-gcacacacactcccagatgtctcagtcaggaaagc-3'
DC-Vβ5.1	5'-ttttccccagccctgagttgcagaaagcccc-3'
DC-Vβ17	5'-cgtttcctgccatcatagagtgcagaggagccctgt-3'
DC-Dβ1	5'- gtcatagcttaaaaccctccgagtgacgcacagcc-3
Circle-Vβ2	5'-ggagggcagctgcaggggttcttgc-3'
Circle-Vβ5.1	5'-ccacattgggccagggaggtttgtgc-3'
Circle-Vβ17	5'-gtcggggaagcaggactgggcacatttatgc-3'

Table 2: The TCR repertoire of RTEs is at least oligoclonal

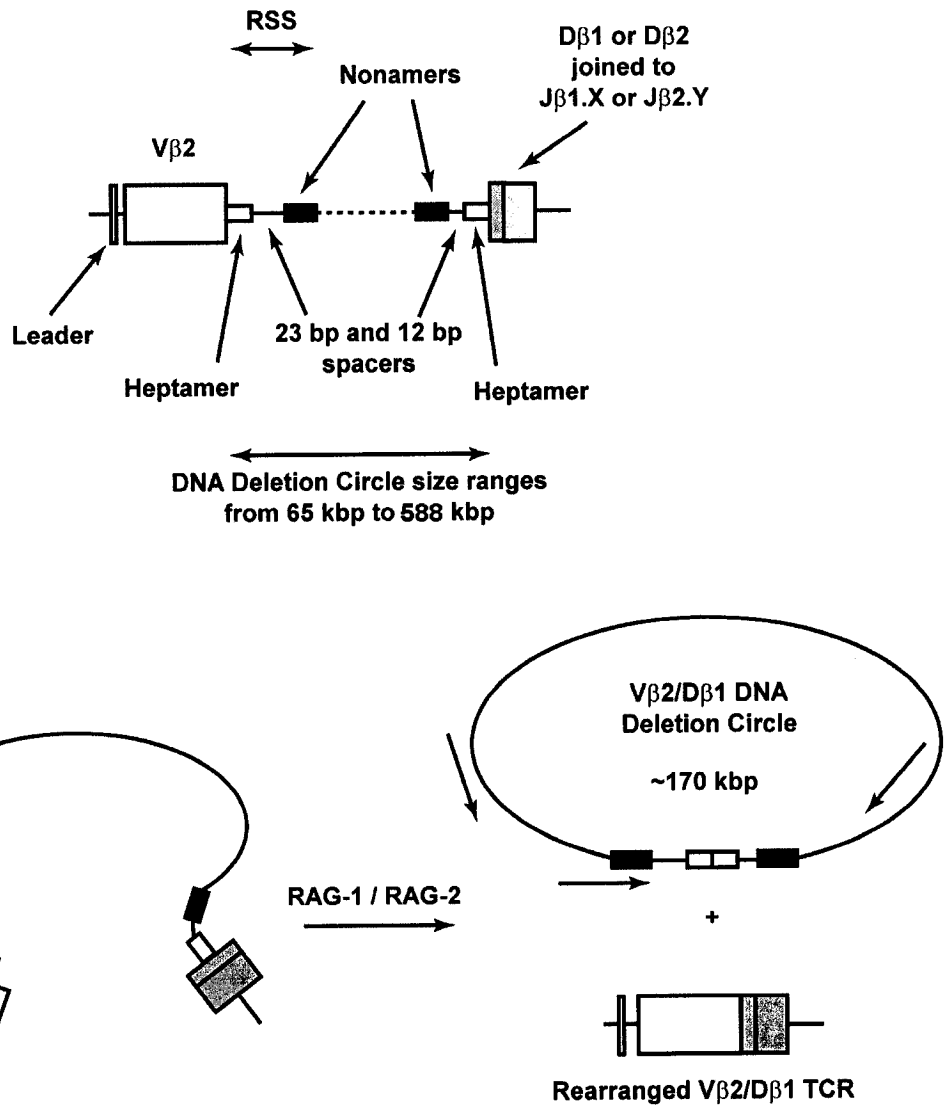
Age	22	23	25	28	31	32	39	76a	76b
%[a]									
$CD4^+CD45RA^+CD62L^+$	32	30	25	36	52	59	35	44	62
$CD4^+CD45RO^+CD62L^+$	18	25	28	42	38	25	18	43	33
$CD4^+CD45RO^+CD62L^-$	39	19	32	13	9	10	23	6	4
% TCR Vb2[b]	N.D.	N.D.	8	N.D.	6	N.D.	N.D.	9	9
DCF (Vβ2/Dβ1 DC)[c]									
$CD4^+CD45RA^+CD62L^+$	1.67	10.12	1.46	3.63	0.50	1.37	0.17	0.50	0.33
$CD4^+CD45RO^+CD62L^+$	0.50	N.D.	0.73	N.D.	0.75	< 0.1	N.D.	< 0.1	0.1
$CD4^+CD45RO^+CD62L^-$	0.22	< 0.07	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	N.D.
DCF (Vβ5.1/Dβ1 DC)[c]									
$CD4^+CD45RA^+CD62L^+$	N.D.	N.D.	N.D.	N.D.	0.29	7.97	N.D.	N.D.	0.71
DCF (Vβ17/Dβ1 DC)[c]									
$CD4^+CD45RA^+CD62L^+$	N.D.	1.72	N.D.	0.85	1.12	N.D.	0.37	N.D.	N.D.

Table 2. Summary of DCF values for multiple TCRVβ-Dβ rearrangements in FACS-sorted subpopulations of $CD4^+$ T cell subpopulations. **a.** Frequency of each sort-purified subpopulation within $CD4^+$ T cells. **b.** Flow cytometry-derived percentages of TCR $V\beta2^+$ T cells found in the $CD4^+$ T cell population. **c.** DCs from different Vβ rearrangements were quantitated in the naive ($CD45RA^+$ $CD62L^+$) and memory/effector ($CD45RO^+$ $CD62L^-$ and $CD45RO^+$ $CD62L^+$) $CD4^+$ T cell subpopulations of healthy adults, as described in Material and Methods. The frequency of TCR $V\beta2^+$ $CD4^+$ T cells (**b**) remains quite constant with increasing age while the corresponding DCF trend (**c**) decreases. N.D.: not done

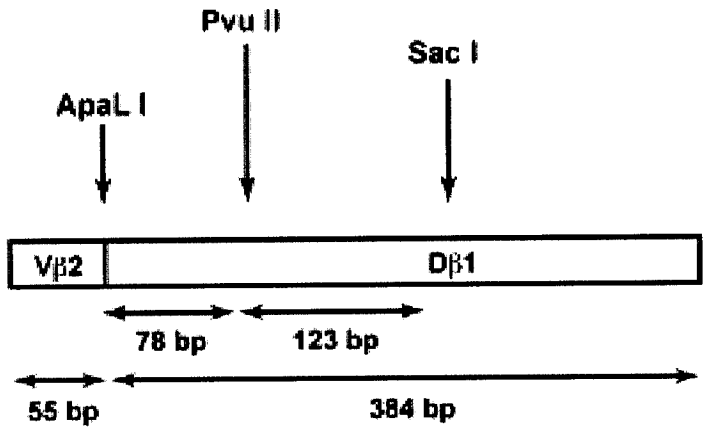
ACKNOWLEDGEMENTS

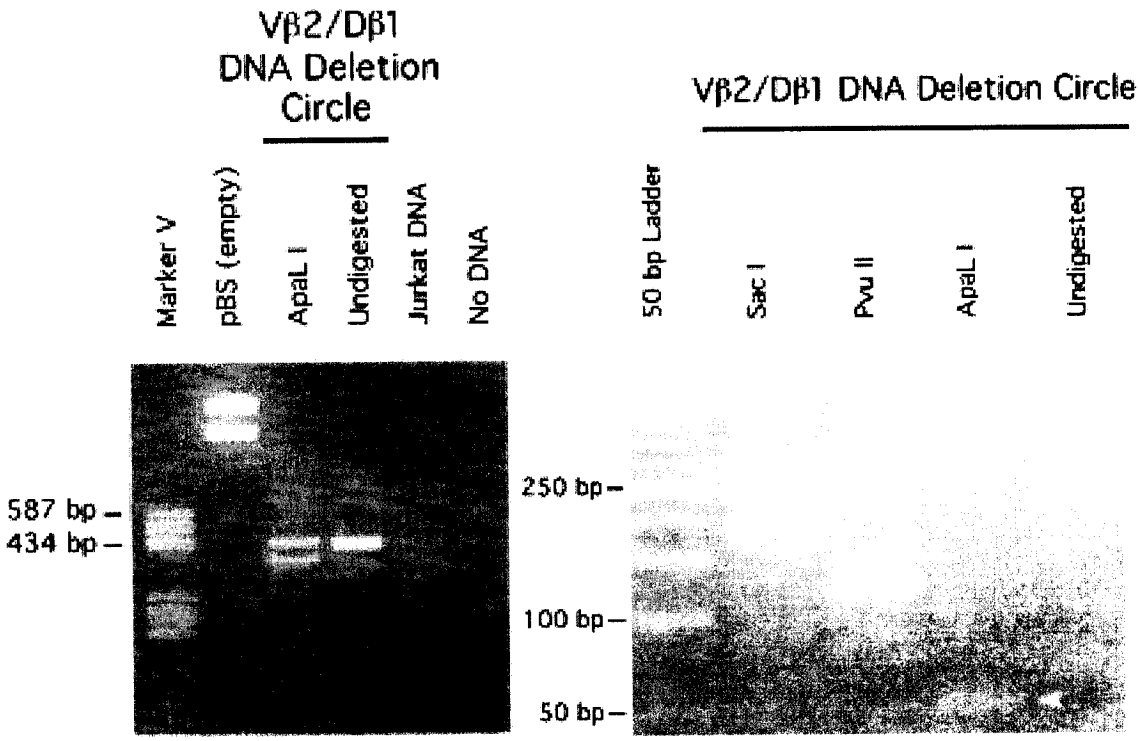
This work was carried out as a partial fulfilment of J.-F.Poulin's Ph.D. thesis at McGill University. The authors would like to thank Drs. Paul MacPherson, François Denis, and François Érard for critical reading of the manuscript and for helpful discussions, Mrs. Nathalie Tessier and Ms. Lisa Gibson for expertise in flow cytometry and Jason Barbour (UCSF) and Dr. Don Brambilla (New England Research Institute) for advice and assistance with statistical analysis. This work was supported by grants from the NIH (RO1 AI235) MRC (MT-14411), and the Réseau FRSQ-SIDA/MI (96007) (to R.-P.Sékaly) and from the NIH (RO1-AI40312 and R01-AI43864) and the Elizabeth Glaser Pediatric AIDS Foundation (to J.M. McCune). J.-F. Poulin holds an MRC student award and R.-P. Sékaly is an MRC scientist. M. Jenkins is the recipient of a KO8 (AI 01425) from the NIH, M.N. Viswanathan is an HHMI Medical Student Research Fellow, K.V. Komanduri is a University of California Universitywide AIDS Research Program Postdoctoral Fellow, and J.M. McCune is an Elizabeth Glaser Scientist supported by the Elizabeth Glaser Pediatric AIDS Foundation.

a

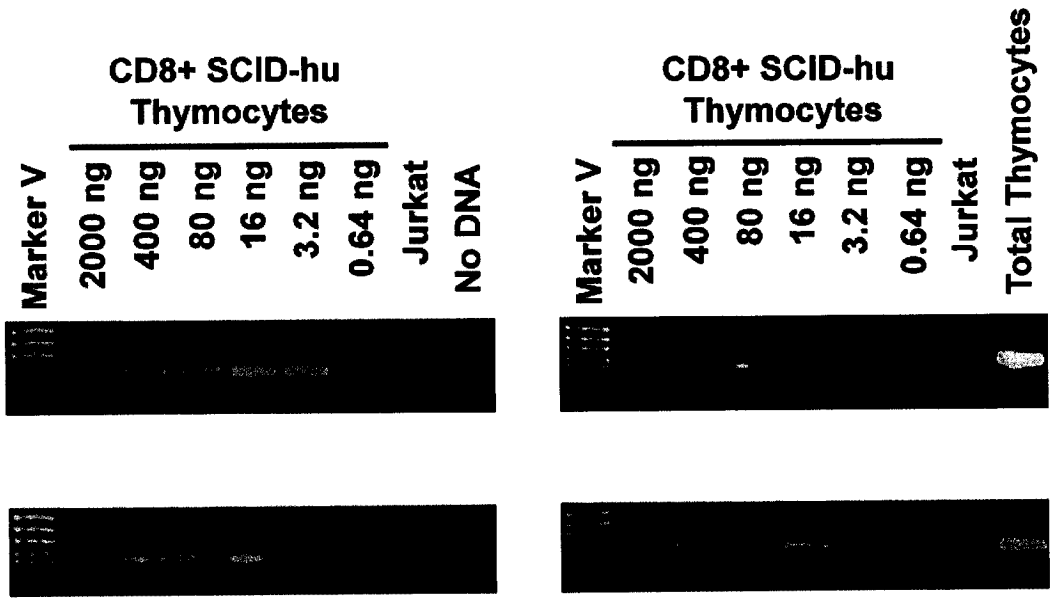

RSS
Nonamers
Dβ1 or Dβ2
joined to
Jβ1.X or Jβ2.Y
Vβ2
Leader
Heptamer
23 bp and 12 bp
spacers
Heptamer
DNA Deletion Circle size ranges
from 65 kbp to 588 kbp
Vβ2/Dβ1 DNA
Deletion Circle
~170 kbp
RAG-1 / RAG-2
+
Rearranged Vβ2/Dβ1 TCR

b

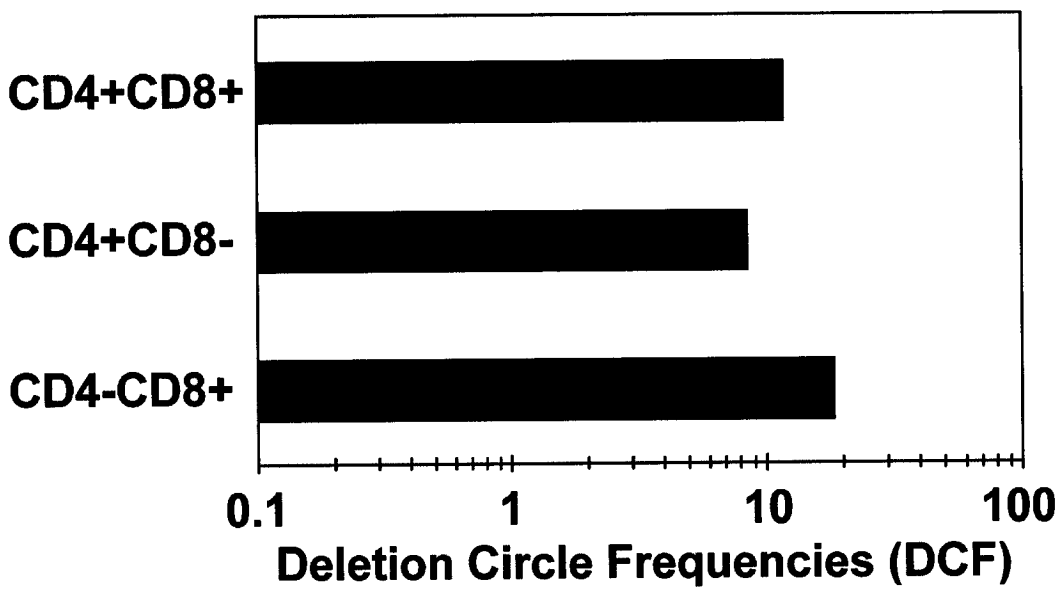

Pvu II
Sac I
ApaL I
Vβ2
Dβ1
78 bp
123 bp
55 bp
384 bp


Vβ2/Dβ1 DNA Deletion Circle
Marker V
pBS (empty)
ApaL I
Undigested
Jurkat DNA
No DNA
587 bp
434 bp
Vβ2/Dβ1 DNA Deletion Circle
50 bp Ladder
Sac I
Pvu II
ApaL I
Undigested
250 bp
100 bp
50 bp

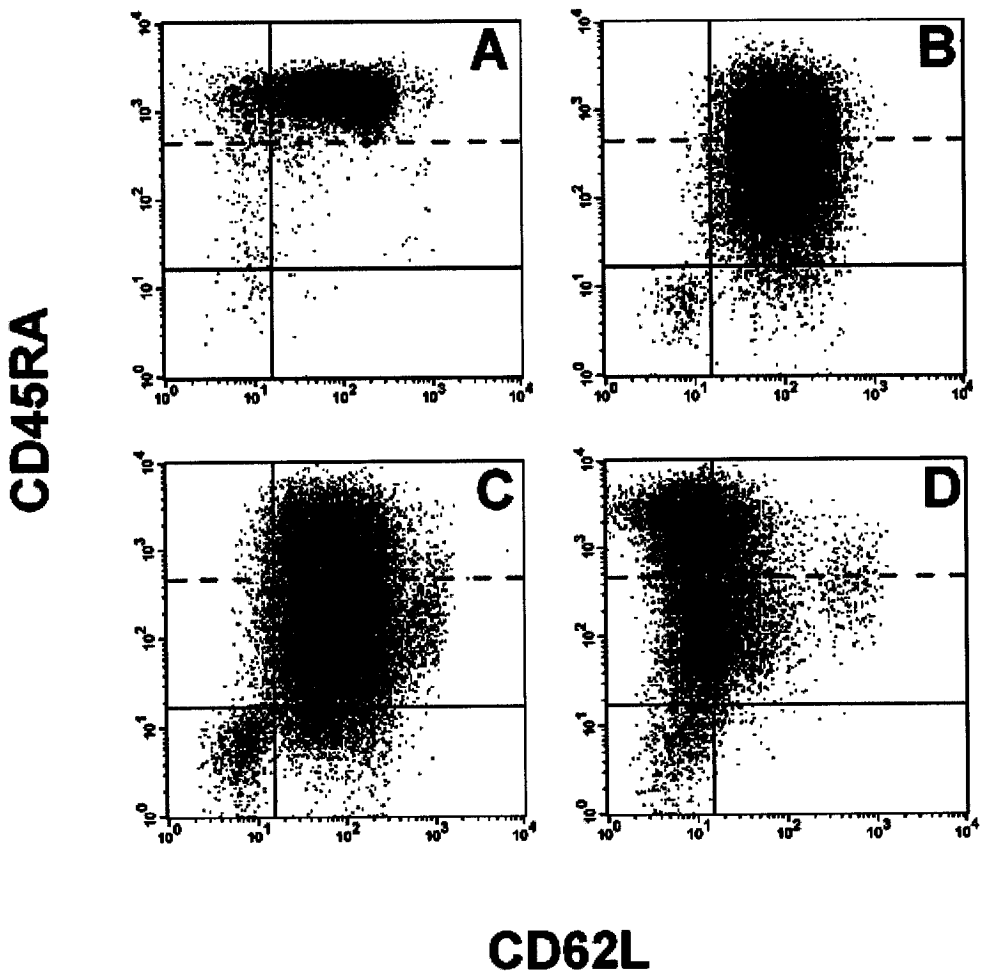
a


CD8+ SCID-hu Thymocytes
Marker V
2000 ng
400 ng
80 ng
16 ng
3.2 ng
0.64 ng
Jurkat
No DNA
CD8+ SCID-hu Thymocytes
Marker V
2000 ng
400 ng
80 ng
16 ng
3.2 ng
0.64 ng
Jurkat
Total Thymocytes

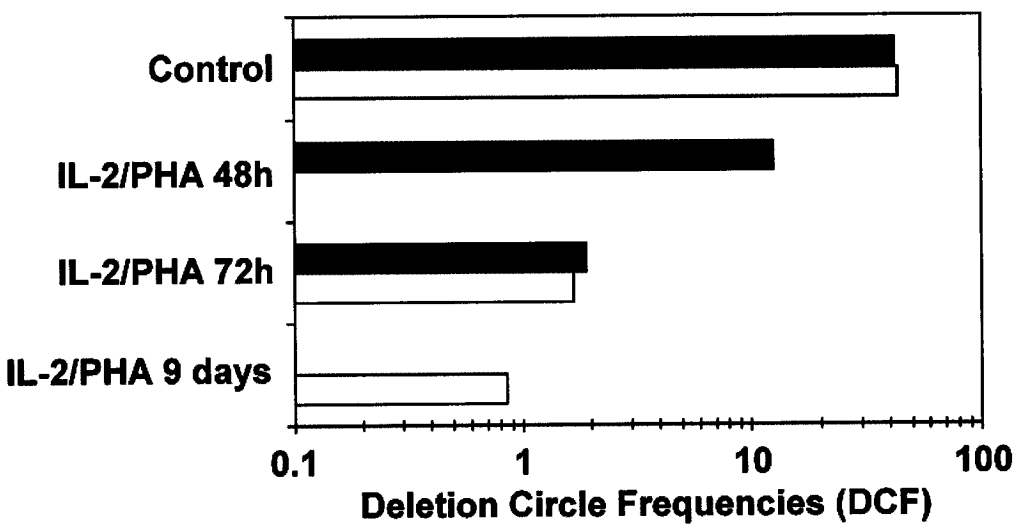
b


CD4+CD8+
CD4+CD8-
CD4-CD8+
0.1
1
10
100
Deletion Circle Frequencies (DCF)

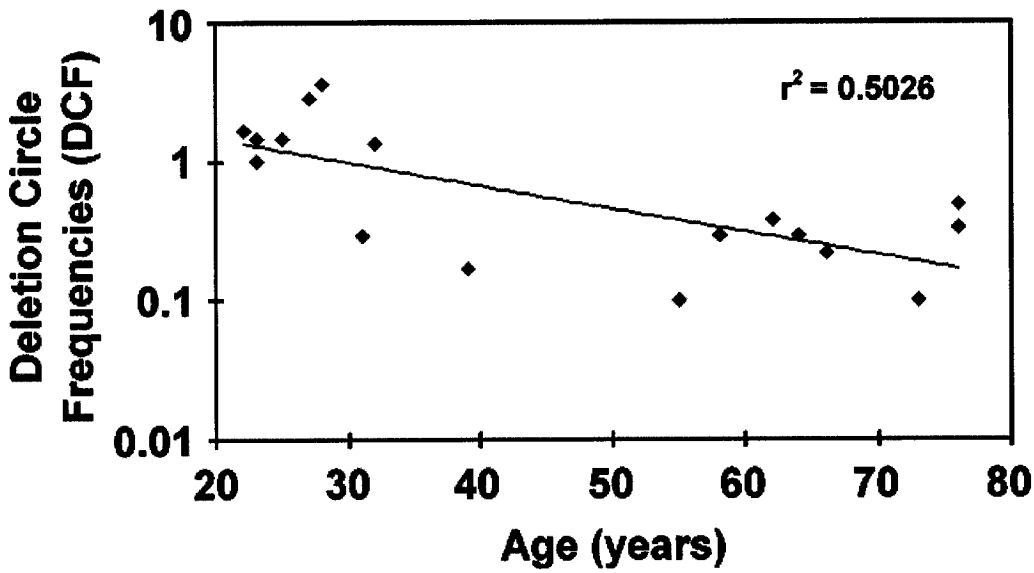
a



b


Control
IL-2/PHA 48h
IL-2/PHA 72h
IL-2/PHA 9 days
0.1
1
10
100
Deletion Circle Frequencies (DCF)

c


Deletion Circle Frequencies (DCF)
10
1
0.1
0.01
$r^2 = 0.5026$
20
30
40
50
60
70
80
Age (years)

d

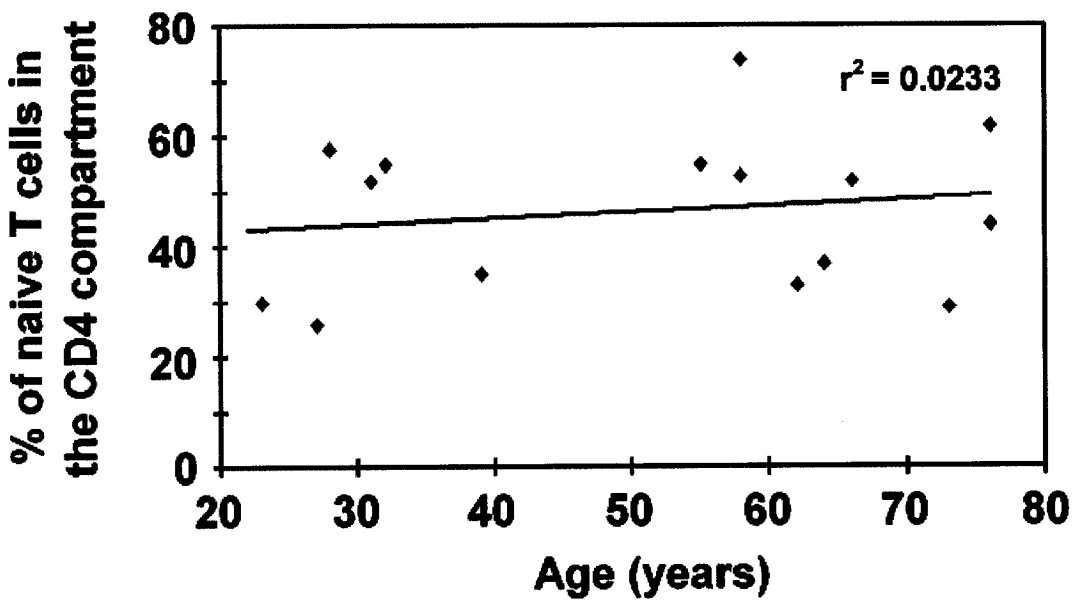

% of naive T cells in the CD4 compartment
80
60
40
20
0
20
30
40
50
60
70
80
Age (years)
r^2 = 0.0233

FIGURE LEGENDS

Figure 1. Formation and detection of TCRβ rearrangement deletion circles. ***a.*** (top) Genomic organization of the region including the Vβ2 and Dβ1 coding segments, flanked by heptamer and nonamer recombination signal sequences (RSSs) and 170 kbp of intervening noncoding DNA. (bottom) Generation of a rearranged Vβ2/Dβ1 coding TCR and a 170 kbp Vβ2/Dβ1 deletion circle after excision-ligation mediated by RAG-1 and RAG-2. The relative location and orientation of the primers used for amplication of the unique signal joint are shown. Note that DCs will have various sizes (from 65 kbp to 588 kbp) depending on the Vβ-Dβ usage. ***b.*** (top) Map of the amplified 439 bp Vβ2/Dβ1 PCR product. (bottom) Representative example of Vβ2/Dβ1 DC products amplified from $CD4^+$ $CD8^+$ human thymocytes or from Jurkat cells. The left gel shows the specificity of the amplification; note the absence of products in both the Jurkat and "no DNA" lane. The PCR product is partially cleavable by ApaLI, likely due to heterogeneity of nucleotide sequence at the circle junction. An ApaLI digestion positive-control was performed at the same time on an empty pBS vector, resulting in complete digestion. The right gel shows restriction analysis of the purified 439 bp Vβ2/Dβ1 DC product, with characteristic cuts by SacI, PvuII, and ApaLI. The white arrow points at the 55 bp fragment released by ApaLI digestion.

Figure 2. Quantification of TCR rearrangement deletion circles. ***a.*** Representative example of endpoint dilution analysis of DC within $CD3^+$ $CD8^+$ human thymocytes. Starting at 2000 ng of input DNA per well, quadruplicate 5-fold serial dilutions were subjected to the nested PCR approach shown in Fig. 1. DNA from Jurkat cells (150 ng) and from total thymocytes (150 ng) served as negative and positive controls, respectively. ***b.*** Relative frequencies of Vβ2/Dβ1 DC in sort-purified populations of $CD4^+$ $CD8^+$, $CD3^+$ $CD4^+$ $CD8^-$, and $CD3^+$ $CD4^-$ $CD8^+$ human thymocytes.

Figure 3. Detection of TCR rearrangement deletion circles in human peripheral blood T cells. ***a.*** Representative flow cytograms of $CD4^+$ human cord blood T cells that were unstimulated (panel 1) or stimulated for varying time intervals (panel 2: 72 hours, panel 3: 96 hours, panel 4: 9 days) with IL-2 (10 U/mL) and PHA (5 ug/mL). $CD4^+$ T cells at each time point were gated and subdivided by staining for CD45RA and CD62L. Based on the staining of cells for CD45RA before stimulation (panel 1), cells were designated as $CD45RA^{BRIGHT}$ or $CD45RA^{DIM}$ (with fluorescence intensities above and below the dotted lines, respectively). ***b.*** Relative frequency of Vβ2/Dβ1 DCs in cord blood T cells that were unstimulated (control) or stimulated for varying time intervals with PHA and IL-2. The black bars show results from one experiment with endpoints at 48 hours and 72 hours; the white bars show results from a second experiment (different cord blood donor) with endpoints at 72 hours and 9 days. ***c.*** Correlation between increasing age and decreasing frequency of Vβ2/Dβ1 DCs in the circulating $CD4^+$ $CD45RA^+$ $CD62L^+$ T cell subpopulation (P=0.0045). Sort-purified $CD4^+$ $CD45RA^+$ $CD62L^+$ human peripheral blood T cells were isolated from individuals of the indicated ages and analyzed for Vβ2/Dβ1 DCs. Such DCs were absent from the $CD4^+$ $CD45RO^+$ $CD62L^-$ subpopulations of each individual (not shown). The point at 55 years old was scored as "undetectable" in the assay (i.e. with a DCF value of 0.1 or less). ***d.*** Percentages of circulating naive ($CD45RA^+$ $CD62L^+$) $CD4^+$ T cells in the peripheral blood as a function of age. No correlation exists between the age and the frequency of such naive $CD4^+$ T cells (P=0.5123).

REFERENCES

1. Mackall, C.L., Hakim, F.T. & Gress, R.E. T-cell regeneration: all repertoires are not created equal. *Immunol Today* **18**, 245-51. (1997).
2. McCune, J.M. Thymic function in HIV-1 disease. *Semin Immunol* **9**, 397-404. (1997).
3. Kong, F., Chen, C.H. & Cooper, M.D. Thymic function can be accurately monitored by the level of recent T cell emigrants in the circulation. *Immunity* **8**, 97-104. (1998).
4. Scollay, R.G., Butcher, E.C. & Weissman, I.L. Thymus cell migration. Quantitative aspects of cellular traffic from the thymus to the periphery in mice. *Eur J Immunol* **10**, 210-8. (1980).
5. Steinmann. Changes in the human thymus during aging, in The Human Thymus - Histopathology and Pathology (Mueller-Hermelink HK, ed.) Springer, New York. 43-48 (1986).
6. Francis, I.R., Glazer, G.M., Bookstein, F.L. & Gross, B.H. The thymus: reexamination of age-related changes in size and shape. *AJR Am J Roentgenol* **145**, 249-54. (1985).
7. Picker, L.J. et al. Control of lymphocyte recirculation in man. I. Differential regulation of the peripheral lymph node homing receptor L-selection on T cells during the virgin to memory cell transition. *J Immunol* **150**, 1105-21. (1993).
8. Heitger, A. et al. Essential role of the thymus to reconstitute naive (CD45RA+) T-helper cells after human allogeneic bone marrow transplantation. *Blood* **90**, 850-7. (1997).
9. McCune, J.M. et al. High prevalence of thymic tissue in adults with human immunodeficiency virus-1 infection. *J Clin Invest* **101**, 2301-8. (1998).

10. Sanders, M.E., Makgoba, M.W. & Shaw, S. Human naive and memory T cells: reinterpretation of helper-inducer and suppressor-inducer subsets. *Immunol Today* **9**, 195-9. (1988).

11. Tough, D.F. & Sprent, J. Turnover of naive- and memory-phenotype T cells. *J Exp Med* **179**, 1127-35. (1994).

12. Bell, E.B. & Sparshott, S.M. Interconversion of CD45R subsets of CD4 T cells in vivo. *Nature* **348**, 163-6. (1990).

13. Erkeller-Yuksel, F.M. et al. Age-related changes in human blood lymphocyte subpopulations. *J Pediatr* **120**, 216-22. (1992).

14. Douek, D.C. et al. Changes in thymic function with age and during the treatment of HIV infection. *Nature* **396**, 690-5. (1998).

15. Kong, F.K., Chen, C.L., Six, A., Hockett, R.D. & Cooper, M.D. T cell receptor gene deletion circles identify recent thymic emigrants in the peripheral T cell pool. *Proc Natl Acad Sci U S A* **96**, 1536-40. (1999).

16. Rabin, L. et al. Use of standardized SCID-hu Thy/Liv mouse model for preclinical efficacy testing of anti-human immunodeficiency virus type 1 compounds. *Antimicrob Agents Chemother* **40**, 755-62. (1996).

17. Ausubel. Preparation of genomic DNA from mammalian tissue, in Current Protocols in Molecular Biology (Wiley Interscience, New York). 2.2.1-2.2.3 (1987).

18. Rowen, L., Koop, B.F. & Hood, L. The complete 685-kilobase DNA sequence of the human beta T cell receptor locus. *Science* **272**, 1755-62. (1996).

19. Reed, L.J. & Muench, H. A simple method of estimating fifty per cent endpoints. *Am. J. Hyg.* **27**, 493-497 (1938).

20. Lenette, E.H. General principles underlying laboratory diagnosis of virus and reckettsial infections, in Diagnostic PRocedures of Virus and Rickettsial Disease (Lenette-Schmidt, ed) New York. (1964).

21. Myers, L.E., McQuay, L.J. & Hollinger, F.B. Dilution assay statistics. *J Clin Microbiol* **32**, 732-9. (1994).

22. Petrie, H.T. et al. Multiple rearrangements in T cell receptor alpha chain genes maximize the production of useful thymocytes. *J Exp Med* **178**, 615-22. (1993).

23. Mason, D. Allelic exclusion of alpha chains in TCRs. *Int Immunol* **6**, 881-5. (1994).

24. Okazaki, K., Davis, D.D. & Sakano, H. T cell receptor beta gene sequences in the circular DNA of thymocyte nuclei: direct evidence for intramolecular DNA deletion in V-D-J joining. *Cell* **49**, 477-85. (1987).

25. Chien, Y.H., Gascoigne, N.R., Kavaler, J., Lee, N.E. & Davis, M.M. Somatic recombination in a murine T-cell receptor gene. *Nature* **309**, 322-6. (1984).

26. Malissen, M. et al. Mouse T cell antigen receptor: structure and organization of constant and joining gene segments encoding the beta polypeptide. *Cell* **37**, 1101-10. (1984).

27. Lewis, S.M. The mechanism of V(D)J joining: lessons from molecular, immunological, and comparative analyses. *Adv Immunol* **56**, 27-150 (1994).

28. Schatz, D.G., Oettinger, M.A. & Baltimore, D. The V(D)J recombination activating gene, RAG-1. *Cell* **59**, 1035-48. (1989).

29. Roth, D.B., Menetski, J.P., Nakajima, P.B., Bosma, M.J. & Gellert, M. V(D)J recombination: broken DNA molecules with covalently sealed (hairpin) coding ends in scid mouse thymocytes. *Cell* **70**, 983-91. (1992).

30. Shortman, K. Cellular aspects of early T-cell development. *Curr Opin Immunol* **4**, 140-6. (1992).

31. Jamieson, B.D. et al. Generation of functional thymocytes in the human adult. *Immunity* **10**, 569-75. (1999).

32. Garcia-Ojeda, M.E., Dejbakhsh-Jones, S., Weissman, I.L. & Strober, S. An alternate pathway for T cell development supported by the bone marrow

microenvironment: recapitulation of thymic maturation. *J Exp Med* **187**, 1813-23. (1998).

33. Autran, B. et al. Positive effects of combined antiretroviral therapy on CD4+ T cell homeostasis and function in advanced HIV disease. *Science* **277**, 112-6. (1997).

34. Fleury, S. et al. Limited CD4+ T-cell renewal in early HIV-1 infection: effect of highly active antiretroviral therapy. *Nat Med* **4**, 794-801. (1998).

35. Pakker, N.G. et al. Biphasic kinetics of peripheral blood T cells after triple combination therapy in HIV-1 infection: a composite of redistribution and proliferation. *Nat Med* **4**, 208-14. (1998).

36. Komanduri, K.V. et al. Restoration of cytomegalovirus-specific CD4+ T-lymphocyte responses after ganciclovir and highly active antiretroviral therapy in individuals infected with HIV-1. *Nat Med* **4**, 953-6. (1998).

37. McMahan, C.J. & Fink, P.J. RAG reexpression and DNA recombination at T cell receptor loci in peripheral CD4+ T cells. *Immunity* **9**, 637-47. (1998).

3. Immunodeficiency following allogenic hematopoietic stem cell transplantation is not due to a thymic functional defect but the consequence of impaired naïve T cell survival

Several hematological disorders are treated by allogeneic hematopoietic stem cell transplantation (AHSCT), which often leads to a prolonged state of immunodeficiency characterized by a low peripheral naïve T cell count and a skewed T cell repertoire. These characteristics, particularly pronounced in patients experiencing chronic graft-versus-host disease (cGVHD), are suspected to be a consequence of impaired thymic function induced by the transplantation and/or autoimmune manifestations following AHSCT. Although it is clear that the GVHD and irradiation/myeloablative therapies can damage the thymic architecture, it remains to be demonstrated whether *de novo* T cell production levels are impaired. The study presented in chapter 3 aims at understanding whether the thymus is implicated in the establishment of the long-lasting immunodeficiency observed following AHSCT. Through FACS analysis and peripheral blood quantification of sjTREC as well as 6 DβJβ TREC frequencies, we demonstrate that thymopoiesis remains intact in GVHD$^-$ patients and that the immune system has a reduced capacity to maintain what has recently been exported from the thymus. Therefore, this work evidences an impaired naïve T cell survival that is most likely responsible for the reduced naïve T cell frequencies observed in non-lymphopenic AHSCT patient (following at least 1 year post transplantation).

Immunodeficiency following Allogeneic Hematopoïetic Stem Cell Transplantation is not due to a Thymic Functional Defect but the Consequence of Impaired Naïve T cell Survival.

Jean-François Poulin*[1,2], Myriam Sylvestre*[1,3], Patrick Champagne[1,2], Marie-Lise Dion[1,4], Nadia Kettaf[1], Alain Dumont[1,2], Maryse Lainesse[1], Pierre Fontaine[5], Claude Perreault[5], Rafick-Pierre Sékaly[1,2,3,4] and Rémi Cheynier[1]

[1]Laboratoire d'Immunologie, Centre de Recherches du CHUM, Hôtel-Dieu, Montréal, Québec, Canada, [2]Department of Medicine, Division of Experimental Medicine, McGill University, Montréal, Québec, Canada, [3]Département de Microbiologie et Immunologie, Faculté de Médecine, Université de Montréal, Québec, Canada, Department of Microbiology and Immunology, McGill University, Montréal, Québec, Canada, [5]Département de Médecine, Université de Montréal and Centre de Recherche Hôpital Maisonneuve-Rosemont, Montréal, Québec, Canada

**both authors equally contributed to this work.*

Submitted to Nature Medicine

ABSTRACT

Allogeneic hematopoietic stem cell transplantation (AHSCT) leads to a prolonged state of immunodeficiency characterized by low levels of peripheral naïve T cells. To determine whether this immunodeficiency results from an impaired thymic function, we assessed both sjTREC and 6 different TCR Dβ-Jβ deletion circles levels, in conjunction with immunophenotype and spectratyping analyses in a cohort of patients sampled from one to ten years following AHSCT. We demonstrate that the biased peripheral T cell receptor repertoire observed in all transplanted patients cannot be attributed to a skewed thymic output, suggesting that it originates from peripheral T cell clonal expansions. However, AHSCT patients incurring chronic graft versus host disease (cGVHD) episodes, clearly demonstrate a quantitative defect in thymic function. Furthermore, we demonstrate, following immune reconstitution, a long lasting reduction of IL-7Rα chain (CD127) expression on naïve T cells that consequently enrich this pool in cells expressing low levels of Bcl-2, which reduces their survival capacity. This defect is partly compensated by increased proliferation and functional thymopoïesis, leading to the establishment of a new homeostatic equilibrium characterized by a reduced level of peripheral naïve T cells and immunosuppression.

INTRODUCTION

Allogeneic hematopoietic stem cell transplantation (AHSCT) is often the only available treatment for several hematological disorders such as severe aplastic anæmia (SAA), acute lymphocytic/myelogenous leukemia (ALL, AML), chronic myelogenous leukemia (CML) and multiple myeloma (MM)[1,2]. The transplantation procedure/conditioning regimen generally leads to a profound state of immunodeficiency that renders patients more susceptible to numerous life-threatening infections. Pioneer studies aimed at better understanding the mechanisms responsible for the regeneration of the T cell compartment demonstrated that although T cells could be generated through both thymus-dependent and thymus-independent pathways[3-8], $CD4^+$ $CD45RA^+$ T cells regeneration following AHSCT could only be achieved in the presence of a functional thymus[9].

PCR-based tools capable of monitoring ongoing human thymopoiesis have recently been developed[10,11]. These aim at quantifying by-products of T cell receptor (TCR) α (δRec-ΨJα) and β loci rearrangement, namely TCR rearrangement excision circles (TREC) or β deletion circles (β DC), which serve as surrogate markers of recent thymic emigrants (RTE). As δRec-ΨJα rearrangement (leading to the generation of sjTREC molecules) occurs in roughly 66% of all developing human TCR αβ thymocytes[12], quantification of sjTREC within peripheral blood mononuclear cells (PBMC) gives a reliable estimate of the extent of ongoing thymopoiesis[10]. Furthermore, quantification of distinct Dβ-Jβ or Vβ-DβJβ DC[11] in peripheral blood illustrates the assortment of the different gene rearrangement events that occurred in the thymus, thereby monitoring thymic output diversity, necessary for the generation of a fully functional naïve T cell repertoire.

Graft-versus-host disease (GVHD) remains the most common complication of AHSCT. Patients are often left with severe lymphoid atrophy and inadequate immune responses to both recall and neo-antigens[13-16]. One of the important hallmarks of this disease is the profound defect in the peripheral T cell repertoire, which was shown to be strongly skewed following AHSCT and particularly during cGVHD episodes[17,18]. Total thymic function has been monitored following allogeneic/syngeneic bone marrow transplantation by sjTREC peripheral blood frequency quantification[19-21]. However, it remains to be

demonstrated whether the perturbation of the peripheral T cell population following AHSCT is a consequence of a biased thymic output or the result of chronic clonal activation in periphery.

In order to evaluate thymic contribution in restoring a diversified naïve T cell compartment, RTE diversity, as defined by the detection of Dβ1Jβ1.1 to Dβ1Jβ1.6 DCs, was evaluated in conjunction with total thymic output and peripheral T cell pool diversity in a cohort of patients following HLA-matched AHSCT. We demonstrate that the skewed peripheral TCR repertoire is not a consequence of a limited or biased thymic output. Both the frequencies and proliferation levels of peripheral blood T cell subsets were assessed by FACS analysis, demonstrating that despite increased proliferation, naïve T cell subpopulation remains limited for several years post transplant, suggesting a permanent defect in peripheral T cell niches. By assessing surface IL-7Rα chain (CD127) expression on naïve T cells, we provide a direct mechanism that can account for the long-lasting reduction of naïve T cell frequencies observed following AHSCT.

PATIENTS AND METHODS

Patient and control subjects

Blood samples were obtained from 38 healthy volunteers and 33 AHSCT recipients. AHSCT recipients had been transplanted 1-10 years before study for the following conditions: SAA (n=1), ALL (n=3), AML (n=10), CML (n=14), myelodysplastic syndrome (n=1), non-Hodgkin lymphoma (n=2) or MM (n=2). The AHSCT myeloablative regimen for patients with hematological malignancies consisted of cyclophosphamide (120 mg/kg) and total body irradiation (12 Gy), or busulfan (16 mg/kg) and cyclophosphamide (200 mg/kg)[22,23]. The patient with SAA received cyclophosphamide 200 mg/kg. Transplanted cells were obtained from the bone marrow or the peripheral blood of an HLA genotypically identical sibling. No specific procedure was carried out to enrich or deplete for a specific cell population. Diagnosis of acute and chronic GVHD was based on standard criteria and treatment consisted of corticosteroids with cyclosporine and/or mycophenolate mofetil and/or tacrolimus[24,25]. Among the patient group, 10 were experiencing cGVHD at sampling time (cGVHD$^+$), while 23 either never suffered from GVHD or were exempt of GVHD clinical symptoms for at least 8 months (GVHD$^-$). No significant age difference was noted between the three groups of individuals (mean age for control group: 46.9 years [23-78], GVHD-: 42.5 years [25-60], cGVHD$^+$: 42.7 years [21-57]; $P \geq 0.17$ for all 3 comparaisons). Clinical protocols were approved by the Human Subjects Protection Committee of the Maisonneuve-Rosemont Hospital. Samples were obtained with the informed consent of the patients.

Quantitative and qualitative analysis of thymic function: sjTREC and DβJβ DC

Primers specific for each of the sjTREC (δRec-ΨJα), the six Dβ1-Jβ1 deletion circles (DβJβ DC) and a fragment of the human CD3γ chain gene were defined on the human germline sequence (Genebank accession numbers AE00061, U66061 and X06026) (Table 1). The amplicons for the DβJβ DCs and the sjTREC, defined by the 5'/3' out primers, amplified from human thymocytes, were individually cloned into Bluescript^{TM} vector (Stratagene) together with the CD3γ amplification product (amplified from human

chromosomal DNA using CD3γ out 3'/5' primer pair). These plasmids, amplified in the same run as the experimental samples, were used to generate standard curves for real time quantitative polymerase chain reaction (PCR)-based assay. Parallel quantification of each deletion circle together with the CD3γ amplicon was performed for each sample using the LightCycler™ technology (Roche Diagnostics). This protocol allows us to precisely measure the input DNA for each quantification thus providing an absolute number of DC per 10^5 cells. Cells (≈$2x10^5$ PBMCs) were lysed in Tween-20 (0.05%), NP-40 (0,05%) and Proteinase K (100 µg/mL) for 30 min at 56 °C and then 15 min at 98 °C. Multiplex PCR amplification was performed for sjTREC and each of the 6 DβJβ DC together with the CD3γ chain in 100µL (10 minutes initial denaturation at 95 °C, 30 seconds at 95 °C, 30 seconds at 60 °C, 2 minutes at 72 °C for 22 cycles) using the "outer" 3'/5' primer pairs (Table 1). The linearity of this first round multiplex assay was demonstrated in triplicate experiments up to 22 cycles when using a maximum of $2x10^5$ PBMCs (data not shown). These PCR conditions were used for all subsequent experiments. Following the first round of amplification, the PCR products were diluted 10-fold prior to on-line, real-time amplification using the LightCycler™ technology. PCR conditions in the LightCycler™ experiments were: 1 minute initial denaturation at 95 °C, 1 second at 95 °C, 10 seconds at 60 °C, 15 seconds at 72 °C for 40 cycles; fluorescence measurements were performed at the end of the elongation steps. For each PCR product, the TREC and CD3γ second round PCR quantifications were performed in separate capillaries and in independent LightCycler™ experiments, but quantified on the same first-round serially diluted standard curve. This highly sensitive nested quantitative PCR assay allows the detection of one copy out of 10^5 cells for each DNA circle. The results are expressed as absolute number of TRECs per 10^5 cells. Since only T cells contribute to the peripheral blood sjTREC content, sjTREC frequencies herein normalized to the relative $CD3^+$ T cells frequency measured by FACS analysis for each sample. Quantification of sjTREC frequencies was performed in duplicate. Inter-experimental variation was always lower than 20% (n=71).

Flow-cytometric analysis

PBMCs cryo-preserved in liquid nitrogen were thawed for staining and analysis. Cell viability, as determined by Trypan blue exclusion assay, was greater than 95%. The following mouse anti-human antibodies were used in different combinations for cell surface staining: anti-CD27 (IgG1, L128) coupled to R-Phycoerythrin (R-PE) (BD Biosciences); anti-CD45RA CyChrome™ (IgG2a, HI100)(BD PharMingen); and anti-CD3 (IgG2a, HIT3a) (BD PharMingen). For CD127 expression analysis, the following combination of antibodies was used: anti-CD3 APC (BD PharMingen), anti-CD45RA Cychrome™ (BD PharMingen), anti-CD27-FITC (BD PharMingen) and anti-CD127 PE (Immunotech). Stainings were performed by incubating cells with the appropriate pool of antibodies for 30 minutes at 4 °C followed by a series of washes with phosphate buffer saline solution (PBS) supplemented with 2% fetal calf serum (FCS). For intracellular Ki-67 analyses, cells were fixed with FACS™ lysing solution (BD Biosciences) after surface marker labeling and then permeabilized with FACS™ Permeabilizing solution™ (BD Biosciences) as per manufacturer's instructions prior to intracellular staining with anti-Ki-67 FITC (IgG1, Ki-67)(DAKO). Isotype-matched controls were used for intracellular staining and specificity was ascertained by performing competition assays of the FITC-labeled antibody with the purified Ki-67-specific antibody. A minimum of $3x10^5$ events in the live cell gate, as defined by forward and side scatter, was accumulated for each sample. Data was acquired on a FACSCalibur™ system and analyzed on a 4-log scale using CellQuest Pro™ software (Becton Dickinson systems).

Spectratyping analysis

A total of six nested PCRs (one for each Jβ1 coding segment) were done for each subject. Briefly, amplification of the CDR3 coding sequences was performed using pan-Vβ primers and any of the Jβ1.1 to Jβ1.6 primers (Table 1). For each sample, PBMCs were lysed in Tween 20 (0,05%), NP-40 (0,05%) and Proteinase K (100 µg/ml) for 30 min at 56 °C followed by 15 min at 98 °C. Nested PCR amplification was performed on cell lysates (approximativelly 10^5 cells) in two successive runs of 30 cycles (10 min initial denaturation at 95 °C, 30 sec at 95 °C, 30 sec at 60 °C and 2 min at 72 °C). Ultimately, nested PCR product was submitted to a run-off PCR (10 cycles) using the corresponding

Jβ1.1 to Jβ1.6 radiolabeled primer. All samples were resolved on a 6% denaturating polyacrylamide gel, revealed using Typhoon™ technology (Molecular Dynamics) and analyzed with ImageQuant™ software (Molecular Dynamics). In order to verify that panVβ primers are able to bind to a majority of Vβs, different PCR products were screened by amplification using Vβ-specific primers. Out of the 24 Vβ families, 17 were present in the different PCR products representing 68% of peripheral T cell repertoire.

Statistical analysis

Statistical analysis (two-tailed Student t test, Pearson's correlation test, *R*, and *P* values) was performed using Microsoft® Excel spread sheets. An *R* value ≥ 0.3, ≤ -0.3, and a *P* value ≤ 0.05 were considered significant. In order to perform two-tailed Student t test for checkerboard representations, we scored a Gaussian distribution (Fig 2*a*) and a detected DβJβ DC family (Fig. 3*b*) with a value of 1. Biased distributions and absence of a DβJβ DC family were attributed the value of 0.

RESULTS

The frequency of peripheral recent thymic emigrant is severely reduced during GVHD in AHSCT.

Quantification of sjTREC was performed by real-time, on-line PCR on PBMCs of patients following AHSCT ($GVHD^-$: n=22 and $cGVHD^+$: n=10) and compared to control individuals (n=37). For each individual, the sjTREC content was normalized to $CD3^+$ T cell counts (Fig 1*a*). In all groups, an inverse association is found between sjTREC frequency and age (Pearson's R=-0.47, -0.41 and -0.93 for control, $GVHD^-$ and $cGVHD^+$, respectively) consistent with previous published reports[10,26]. Peripheral sjTREC frequencies per 10^5 $CD3^+$ T cells average 1255 [range: 3-12521], 897 [15-8738] and 119 [18-328] in controls, GVHD- and cGVHD+ patients, respectively (Fig. 1*b*). While $GVHD^-$ patients exhibit only a 1.4-fold decrease in sjTREC frequencies (P>0.56), a highly significant decrease (10.5-fold, P=0.015) is observed in patients who were experiencing cGVHD, clearly indicating a quantitative impact of cGVHD on thymic function.

Within the $GVHD^-$ group, 15 patients experienced past episodes of cGVHD (more than 8 months before sampling, see patients and methods) while the remaining 8 were exempt of symptoms since AHSCT. No difference in the peripheral sjTREC content was observed between these two groups ($GVHD^-$: 526/10^5 $CD3^+$ T cells, Past GVHD: 1109/10^5 $CD3^+$ T cells, P>0.40), demonstrating the reversibility of the impact of cGVHD on thymopoïesis. The strong reduction of sjTREC frequency observed in peripheral blood during cGVHD episodes indicates that this autoimmune manifestation can lead to a temporary dysfunction of the thymus in sustaining T cell maturation.

Expressed TCRBD1BJ1 peripheral repertoire is biased following AHSCT

The peripheral TCR repertoire, as defined by CDR3 size spectratyping, is characterized by a relative instability following AHSCT, particularly in patients suffering from cGVHD[7,27]. Peripheral TCRBD1BJ1S1 to J1S6 T cell repertoire perturbations were investigated in the aforementioned groups by spectratyping analysis (Fig. 2) using a panel

of primers encompassing the 6 different DβJβ rearrangements. Of note is the fact that, as described in the patients and methods section, we have used a panVβ primer in conjunction with specific Jβ primers to investigate the diversity of T cell repertoire. Representative examples of CDR3 length distributions are shown in Fig. 2*a*. Biased spectratype pattern (i.e. P21, P25 and P38 for Jβ1.3) consists of unevenly distributed CDR3 sizes having unequal amplitude/intensity whereas a Gaussian distribution (i.e. N9 and N13 for Jβ1.3, P18 for Jβ1.2) is similar to a bell-shape curve[28,29]. In the control group (n=31), 94.2% of the analyzed TCRBD1BJ repertoires were polyclonal, as shown by the Gaussian distribution of the CDR3 length diversity. Following AHSCT, biases were observed in 38.9% and 54.3% of the TCRBD1BJ populations of GVHD$^-$ and cGVHD$^+$ patients, respectively (Fig. 2*b*). It is likely that the biases observed in the T cell repertoire diversity represent important perturbations since each expanded clone, represented by individual peaks in the non Gaussian distributions, has overgrown all the sequences amplified by the panVβ primer.

The diversity of TCRBD1BJ populations is significantly different between healthy controls and the two groups of patients in a two-tailed Student t test (controls and GVHD$^-$: P=0.0001; controls and cGVHD$^+$: P=0.0018). Thus, a skewed peripheral T cell repertoire is observed in all patients following AHSCT (except for patients P8 and P35). Of note is the fact that these biases persist for up to 10 years following transplantation (for example, patient P10 still shows a skewed repertoire even after 120 months post transplantation), clearly demonstrating a long-lasting defect in T cell production in cGVHD patients or homeostasis in cGVHD$^+$ and GVHD$^-$ patients.

Thymic output diversity is not impaired following AHSCT

Having demonstrated that peripheral TCR repertoires are skewed following AHSCT, we investigated whether this is a consequence of a biased thymic exportation. Dβ1Jβ1.1 to Dβ2Jβ2.7 DC peripheral blood frequencies reflect the extent of diversity in TCR DβJβ gene segment usage among RTEs. Peripheral blood quantification of 6 DβJβ DCs (Dβ1Jβ1.1 to Dβ1Jβ1.6) was performed for each individual in this study in two independent experiments (table 2) since the same rearrangements are analyzed. Such an analysis can be directly compared to the extent of peripheral T cell repertoire diversity

investigated in the spectratype assay (Fig. 2). Average peripheral blood DβJβDCs frequencies are in accordance with previously published results regarding TCR DβJβ gene segment usage in single positive thymocytes[30], Dβ1Jβ1.3 (range: 0-9) being the less frequent DβJβDC while Dβ1Jβ1.1 (range: 0-17) and Dβ1Jβ1.6 (range: 0-29) DCs are well represented. A biased thymic output would lead to either an over- and/or an under-representation of certain DβJβ DC families in periphery, which in turn would alter the expressed peripheral repertoire. Accordingly, two different analyses of the DβJβ DC results were performed on the three groups of individuals in order to evaluate the relative abundance of DβJβ families in the peripheral blood.

In the first analysis, we investigated whether one or more of the DβJβ DC families were under-represented in peripheral blood of transplanted patients as a consequence of a biased thymic output. For each individual, the number of undetected DβJβ DC families (<1 copy per 10^5 cells) among the 6 tested was evaluated and statistical analysis was performed between the study groups (Fig. 3*a*). The frequency of undetectable DβJβ DC families was not significantly different between the 3 groups (controls: mean=4.22, range: [1-6]; $GVHD^-$: mean=4.18 [0-6] and $cGVHD^+$: mean=4.8 [3-6]; $P \geq 0.15$). In a duplicate experiment, similar means, ranges and statistics were obtained (data not shown). Of note is the fact that the number of detected DβJβ DC families is highly correlated to sjTREC frequencies ($R \geq 0.31$, Fig. 3*b*). Accordingly, this analysis was repeated after normalization for the sjTREC content (number of DβJβ DC divided by sjTREC frequency). Again, no significant difference was observed among groups ($P \geq 0.80$ for all comparisons). Finally, individual analysis of each of the DβJβ DC family did not reveal any significant difference amongst all three groups ($P > 0.05$ for all 18 comparisons; 3 groups and 6 DC families). These statistical analyses demonstrate that under-representation of DβJβ families in thymic output does not lead to the bias observed in the expressed peripheral T cell repertoire (Fig. 2).

The second analysis aimed at evaluating a possible over-representation of DβJβ DC families that could also be the result of a biased thymic function in transplanted patients.

The mean frequency and standard deviation (S.D.) for each DβJβ DC family, normalized to sjTREC content, were calculated for healthy control group. Dβ1Jβ1.1 to Dβ1Jβ1.6 DC frequencies, for the 71 individuals of the study, were compared to these means. A DβJβ DC frequency was considered over-represented when its value was 3 S.D. above the control mean for this particular DβJβ DC. The frequency of over-represented DβJβ DC was 4.05%, 6.82% and 0% in controls, cGVHD- and cGVHD+ respectively. The absence of clear significant differences among the 3 groups in the over-representation of particular DβJβ DC families demonstrates that the skewed peripheral T cell repertoires characterizing AHSCT patients cannot originate from the over-representation of a particular DβJβ rearrangement in thymic output.

Thus, the huge peripheral T cell repertoire biases, characteristic of the AHSCT patients cannot be a consequence of skewed thymic output. It is clear from our results that following AHSCT, when a biased peripheral T cell repertoire is observed, and even during active cGVHD when thymic output is reduced, the thymus continues to produce a diverse population of RTEs.

The naïve T cell population is reduced following AHSCT despite enhanced ongoing proliferation

The observed decreased thymic output during cGVHD could lead to an imbalance in the proportion of naïve and memory T cells in the periphery[9]. To examine this possibility, FACS analysis for CD3, CD45RA and CD27 cell surface expression was performed on all subjects under study (Fig. 4*a*). As compared to healthy controls (n=38), naïve T cell frequency (% of $CD45RA^+CD27^+$ gated on $CD3^+$=32.76%) was significantly reduced in $cGVHD^+$ patients (20.71%, n=10, p=0.02) (Fig. 4*b*). More interestingly, despite the lack of defect in thymic output, naïve T cell frequencies remained reduced in $GVHD^-$ recipients (24.56%, n=21, P=0.03). A positive correlation was observed for both $GVHD^-$ and control groups when comparing sjTREC frequencies to the relative frequency of $CD45RA^+$ $CD27^+$ circulating T cells (Pearson's R=0.42 for control group and 0.43 in the patient group). Reduced naïve T cell frequencies were not associated with time post-AHSCT (R=-0.21) as this defect persists for several years. The mean frequency of $CD3^+$

$CD45RA^+$ $CD27^+$ cells was 25.42% and 17.26% in patients sampled ≥48 months (n=8) and <48 months (n=23) respectively following AHSCT (P=0.06), demonstrating the long-lasting impairment of the naïve T cell compartment.

In previous reports, lymphopenic patients' naïve T cells were shown to undergo homeostatic proliferation in order to replenish the peripheral space[31-33]. Experiments were carried out to define if the observed low levels of naïve T cells (Fig. 4*b*) could be the result of impaired peripheral T cell proliferation rate. Accordingly, Ki-67 expression was quantified in $CD3^+$ $CD45RA^+$ $CD27^+$ T cells (Fig. 4*c* and *d*). Ki-67 is a nuclear antigen expressed throughout all phases of the cell cycle except G_0[34]. Strikingly, naïve T cells from AHSCT recipients free of GVHD clinical symptoms at sampling time demonstrated increased proliferation levels as compared to healthy individuals (controls: 0.37%, $GVHD^-$: 0.59%, P=0.005). Moreover, no significant difference was observed in the frequency of Ki-67-expressing naïve T cells between both groups of transplanted patients ($GVHD^+$: 0.56% P=0.75). In contrast, similar proliferation rates were observed in the memory compartment of all groups (controls: 2.42%, $GVHD^-$: 2.29%, $cGVHD^+$: 1.83%, P≥0.38). The absence of negative correlation (Fig. 4*e*) between sjTREC and naïve T cell proliferation in the three groups (R>0.05) demonstrates that the influence of peripheral proliferation on TREC content, if any, is negligible.

These data, in conjunction with quantitative and qualitative measurements of $GVHD^-$ patients' thymic function, exclude a role for a thymic defect in the observed immunodeficiency of $GVHD^-$ AHSCT patients. Despite enhanced proliferation and normal thymic activity, the peripheral naïve T cell compartment fails to replenish itself, as demonstrated by the long-lasting defect in peripheral naïve T cell concentration.

$GVHD^-$ AHSCT patients demonstrate a reduced IL-7Rα chain (CD127) naïve T cell surface expression.

Naïve T cell survival is regulated through the interaction between interleukin (IL)-7 and its receptor (CD127: IL-7Rα), which modulates Bcl-2 expression levels[31,35,36]. The proportion of $IL\text{-}7R\alpha^+$ naïve T cells was evaluated by FACS analysis in AHSCT $GVHD^-$

patients and compared to healthy individuals in order to investigate whether the observed long-lasting naïve T cell deficiency is attributable to a defect in naïve T cell survival. In healthy adults, most naïve T cells express CD127 (Fig. 5*a*) whereas a wider distribution is observed in AHSCT GVHD$^-$ patients (mean controls: 92.61%, GVHD$^-$: 80.42%, P=0.009). Moreover, evaluation of CD127 mean fluorescence intensity (MFI) within CD127$^+$ naïve T cells is significantly reduced (mean MFI control: 45.44, GVHD$^-$: 38.98, P=0.01) (Fig. 5*b*). Furthermore, most GVHD$^-$ patients show lower levels of CD127-expressing naïve T cells as well as lower CD127 MFI than compared to healthy adults (Fig. 5*c* and *d*). Taken together, both CD127-expressing naïve T cells frequency, and CD127 surface expression level among positive cells, is reduced in AHSCT GVHD$^-$ patients, further indicating a decreased CD127 expression on naïve T cells.

These results highlight for the first time that the long-lasting immunodeficiency associated to HLA-matched AHSCT is a consequence of the incapacity of the immune system to restore naïve T cell population. This defect is neither due to quantitative nor to qualitative impairment of thymic function. However, the observed reduction of IL-7Rαexpression on naïve T cell surface, leading to limited cell survival, provides a possible mechanism explaining the observed immunodeficiency characterizing AHSCT patients.

DISCUSSION

GVHD leads to a skewed peripheral T cell repertoire[38-40]. This disease is known to target the thymus as part of alloreaction that could bias thymocyte ontogeny and/or exportation. In order to investigate whether thymic output is biased during cGVHD, we developed an original approach aimed at precisely quantifying Dβ1Jβ1.1 to Jβ1.6 deletion circles using a panel of primers/probes that covers 46% of the RTE repertoire. Using this tool, we analyzed the repertoire of recent thymic emigrants in a cohort of 38 healthy individuals as well as in a group of 32 ASHCT recipients. Parallel analysis of the TCRBD1BJ1S1 to BJ1S6 spectratypes demonstrated a skewing of peripheral T cell repertoire in AHSCT recipients, as compared to the characteristic polyclonal TCR populations of healthy controls. We unequivocally demonstrated, in two independent experiments, that the human thymus maintains its ability to export a diverse population of RTEs after AHSCT and despite the occurrence of GVHD (Fig. 3*a* and 3*b*). It is important to stress that, although a thymic dysfunction is evidenced during GVHD, it only results in reduced exportation levels. The diversity of thymic output in these patients is comparable to that observed in healthy adults. Furthermore, no biases in RTE diversity were noticed during adulthood despite the age-related involution of the thymus (data not shown). These data are in agreement with previously published findings demonstrating that human thymocytes express a polyclonal TCR repertoire[41]. Our results clearly demonstrate that the peripheral T cell perturbations observed in transplanted patients cannot be the consequence of biases in RTE exportation but likely the result of post-thymic antigen-driven and/or homeostatic expansion. It has been suggested that these clonal expansions might reflect persistent viral infections[42] or immune responses to minor histocompatibility antigens[38,43]. Another possible explanation could be that the observed skewing of the peripheral TCR repertoire is due to the preferential homeostatic expansion of selected T lymphocyte subsets receiving strong TCR signals in the periphery. Indeed, in lymphopenic mice, peripheral naïve T cell homeostatic proliferation is positively modulated by the strength of the interaction between TCR and MHC associated with particular self-peptides[44,45] (e.g.: increased rate of proliferation of T cells bearing high affinity TCR molecules). Nevertheless, the skewing of the peripheral TCRBJ repertoire

illustrates the extent to which post-thymic events can reshape the peripheral T cell repertoire[46].

Peripheral sjTREC frequencies, reflective of thymic output, are severely reduced (>1 log) in patients undergoing cGVHD following AHSCT as compared to cGVHD- patients (Fig. 1). The impact of peripheral T cell proliferation on sjTREC levels[47] in cGVHD patients, if any, is very limited: 1) The proportion of Ki-67$^+$ cells was not significantly increased among CD3$^+$CD45RA$^+$CD27$^+$ T lymphocytes during cGVHD as compared to GVHD$^-$ AHSCT patients (P=0.75) and 2) No inverse correlation was observed between Ki-67 expression in naïve T lymphocytes and sjTREC frequencies (Fig. 4*e*). The decrease in peripheral blood RTE concentration during cGVHD is, hence, most likely the consequence of aberrant thymocyte development/ontogeny[48] induced by autoimmune responses directed at thymocytes or thymic stromal cells and occurring during cGVHD episodes[49]. Of note is the fact that thymic function and naïve T cell peripheral concentrations were comparable between patients who never had GVHD (8/23) and those who did have GVHD episodes but were GVHD-free for at least eight months (15/23) prior the time of study (P=0.40 and 0.54, respectively), clearly indicating that at least the quantitative defect in thymic output is reversible.

AHSCT recipients often exhibit an immunodeficiency of varying severity and duration. This period is characterized by long-lasting (>48 months) reduction of peripheral blood naïve T cell frequencies. In GVHD$^-$ patients, CD45RA$^+$ CD27$^+$ T lymphocyte concentration remained low despite normal sjTREC frequencies, demonstrative of normal thymic function (Fig. 1), and a higher proportion (P=0.005) of CD3$^+$ CD45RA$^+$ CD27$^+$ T cells expressing Ki-67 (Fig. 4*c*). In agreement with this increased proliferation of naïve T cell in GVHD$^-$ patients, a 2-fold decrease in sjTREC frequencies per naïve T cells is observed in this group as compared to healthy control individuals (controls: 2756/10^5; GVHD$^-$: 1427/10^5; P>0.2). These data suggest that despite normal thymic activity and enhanced proliferation levels, the naïve T cell compartment fails to reach normal values, highlighting a defect in the host's ability to maintain T lymphocytes in the naïve T cell compartment. The fact that naïve T cell frequencies remain low for years after

transplantation (lack of positive association with time post-AHSCT) also indicates that a new peripheral naïve T cell frequency "set-point", significantly lower than in controls, has been established, reflective of functional T cell homeostasis mechanisms having reached a new equilibrium.

Naïve T cell homeostasis is a consequence of a delicate balance between cell survival and death. In order to maintain viability, T cells require continual signals from their *in vivo* environment. Cell number regulation is dictated through competition for "sensor cells"-produced survival factors, one of them being IL-7[37]. IL-7 was shown to play a central role in T cell homeostasis[31] by promoting peripheral naive T cells survival[35,50-53]. Signal transduction, induced by IL-7 binding to its receptor complex, involves association with Jak1 and Jak3[54], followed by induction of members of the STAT family, reportedly STAT1, STAT5 and possibly STAT3[55]. As Bcl-2 expression involves signaling through Jak3/STAT[56], a reduced cell surface expression of the IL-7R is likely to impair naïve T cells survival. Although the functionality of this downregulation has not been tested here in this thesis, it is anticipated that it makes naïve cells more prone to die in the periphery.

These findings are thereby consistent with a "steady-state" model in which, naïve T cell homeostasis is regulated through three different parameters: thymic output, peripheral proliferation and cell death. Diminished naïve T cell frequency induces density-dependent proliferation of naïve T cells[47,57]. According to the steady state hypothesis, such proliferation is however compensated by enhanced cell death in a context when thymic function appears to be intact (e.g. in cGVHD$^-$ patients). Therefore, the long-lasting reduction of peripheral naïve T cells frequencies is a consequence of AHSCT recipients' inability to ensure proper naïve T cell survival (Fig. 6).

Human thymopoiesis is an important component of T cell homeostasis. In this study, the impact of AHSCT and cGVHD on the magnitude and the diversity of adult human thymopoiesis were monitored. 1) Thymopoiesis is quantitatively impaired during cGVHD; this effect is reversible upon recovery. 2) Transplanted patients, irrespectively of their GVHD status/history, show a perturbed peripheral TCR DβJβ repertoire that is

not a consequence of a biased thymic function but more likely the result of previous and/or ongoing antigenic stimulation. 3) Patients exempt of cGVHD clinical symptoms at sampling time showed significantly lowered naïve T cell frequencies despite similar sjTREC content and enhanced levels of proliferation. Taken together, these results clearly demonstrate that the thymus remains intact and is still capable of producing a diversified pool of naïve T cells following AHSCT (only in the absence of GVHD). Despite the intact thymic function, naïve T cells IL-7Rα chain expression is reduced in transplanted patients, most likely contributing to the inability to restore adequate naïve T cell frequencies. It remains to be demonstrated whether this defect is a consequence of an AHSCT induced modification of the peripheral environment or intrinsic to thymic educational processes.

Table 1: Oligonucleotides for TREC quantification and spectratyping analysis.

DNA circles quantification		
sjTREC	5'-In:	GCTCTGAAAGGCAGAAAGAGG
	5'-Out:	CTCTCCTATCTCTGCTCTGAA
	3'-In:	ACATTTGCTCCGTGGTCTGTG
	3'-Out:	ACTCACTTTTCCGAGGCTGA
Dβ1	3'-In:	TGTGACCCAGGAGGAAAGAAG
	3'-Out:	CTCATCTGGGCCTGTCCTTGT
Jβ1S1	5'-In:	CCCTCTCTATGCCTTCAATGT
	5'-Out:	GAACCTAGGACCCTGTGGA
Jβ1S2	5'-In:	CAGATCCGTCACAGGGAAAAGT
	5'-Out:	ACAAGGCACCAGACTCACAGTT
Jβ1S3	5'-In:	TGTCCCTGTGAGGGAAGAGTT
	5'-Out:	AAGGGAACACAGAGTACTGGAA
Jβ1S4	5'-In:	TGGACTTGGGGAGGCAGGA
	5'-Out:	GGATCACACGGGGCCTAATT
Jβ1S5	5'-In:	CTCATAAAATGTGGGTCAGTGGA
	5'-Out:	GAAACTGAGAACACAGCCAAGAA
Jβ1S6	5'-In:	TGAATCCAGGCAGAGAAAGG
	5'-Out:	ATCCTCCCTCTTATGTGCATGG
CD3γ	5'-In:	TGGCTGTCCTCATCCTGG
	5'-Out:	ACTGACATGGAACAGGGGAAG
	3'-In:	CTTGGCCTATGCCCTTTTGG
	3'-Out:	CCAGCTCTGAAGTAGGGAACATAT
LightCycler™ Probes		
sj	P1:	AATAAGTTCAGCCCTCCATGTCACACTf
	P2:	(Red!640)TGTTTTCCATCCTGGGGAGTGTTTCAp
Dβ1	P1:	CTGGGAGTTGGGACCGCCAGAGAGGTf
	P2:	(Red!640)TTTGTAAAGGTTTCCCGTAGAGTTGAATCATTGTp
CD3γ	P1:	GGCTGAAGGTTAGGGATACCAATATTCCTGTCTCf
	P2:	(Red!705)CTAGTGATGGGCTCTTCCCTTGAGCCCTTCp
Spectratyping		
Pan TCRBV	5'-In:	CCACGAAACTATGTATTGGTA
	5'-Out:	CCTGCCATGGGCACCAGGCTCCTCTG
TCRBJ1S1	3'-In:	CGGATCTGCAAAAGAACCTGAA
	3'-Out:	CTCTAAAAGGGACACTGTGGA
	3'-RO:	CTGGTGCCTTGTCCAAAGA
TCRBJ1S2	3'-In:	CTGGGATCTCTGTTCTCTTTGA
	3'-Out:	CTGGGAAGCCTGGGTGGATT
	3'-RO:	TCCCCGAACCGAAGGTGTA
TCRBJ1S3	3'-In:	TCCAGCCCCTTTTTGCAAGT
	3'-Out:	CACTTTCACCTAAGCACTAGA
	3'-RO:	GCCAACTTCCCTCTCCAAAA
TCRBJ1S4	3'-In:	CTGGAACTCCGACCTTATGA
	3'-Out:	TATGAGCTTTTCTTGGCTGTGT
	3'-RO:	CAAGACAGAGAGCTGGGTT
TCRBJ1S5	3'-In:	CAATGGCCATACCACCCTGAT
	3'-Out:	TACCCCATGGGGTAAGAGAT
	3'-RO:	CTAGGATGGAGAGTCGAGT
TCRBJ1S6	3'-In:	AATTCCCTTCCCAGCAACTGAT
	3'-Out:	CACAGTGCTCCAGGTAAGAA
	3'-RO:	CTGGTCCCATTCCCAAAGT

Table 2: Representative sjTRECs and DβJβ DCs frequencies in untransplanted adults and AHSCT patients

# ID	Age (years)	Months Post AHSCT	sjTRECs per 10^5 CD3[a]	Dβ1Jβ1.1 to Jβ1.6 DCs/10^5 $CD3^+$ cells[b]					
				1.1	1.2	1.3	1.4	1.5	1.6
Untransplanted Controls									
2	33	N.A.	1547[c]	-	4.35[d]	2.05	-	-	2.92
4	26	N.A.	1631	-	4.44	-	-	-	5.29
7	33	N.A.	3841	-	6.02	-	-	4.79	-
11	42	N.A.	346	2.20	-	0.76	2.58	-	8.58
13	37	N.A.	116	3.64	-	-	4.05	-	2.28
18	65	N.A.	23	-	-	-	-	-	-
24	40	N.A.	596	-	16.43	-	-	6.48	-
$GVHD^-$ AHSCT Patients[e]									
3	39	16	356	-	7.04	-	-	-	-
5	50	32	1348	11.29	-	8.56	-	-	27.67
6	41	108	814	2.90	-	-	-	1.37	-
13	42	48	869	3.85	-	-	-	-	-
20	25	30	3746	7.01	9.69	-	-	2.11	29.81
35	51	36	15	-	-	-	-	6.67	5.76
37	59	36	92	-	-	-	-	6.03	-
$cGVHD^+$ AHSCT Patients[e]									
8	40	44	240	-	2.39	-	-	-	2.45
17	49	20	64	-	5.23	-	-	-	-
18	21	36	328	5.87	5.30	-	-	2.33	-
22	50	19	85	-	3.22	-	-	-	-
25	57	25	77	-	-	-	-	-	-
33	42	12	141	2.19	2.60	-	-	-	-
38	47	48	87	-	-	-	-	0.51	1.07

[a]sjTREC frequencies were normalized according to $CD3^+$ T lymphocyte proportions, as determined by flow cytometry.

[b]DC frequencies were normalized according to $CD3^+$ T lymphocyte proportions, as determined by flow cytometry.

[c]sjTREC content are shown as the mean of 2 independent quantifications.

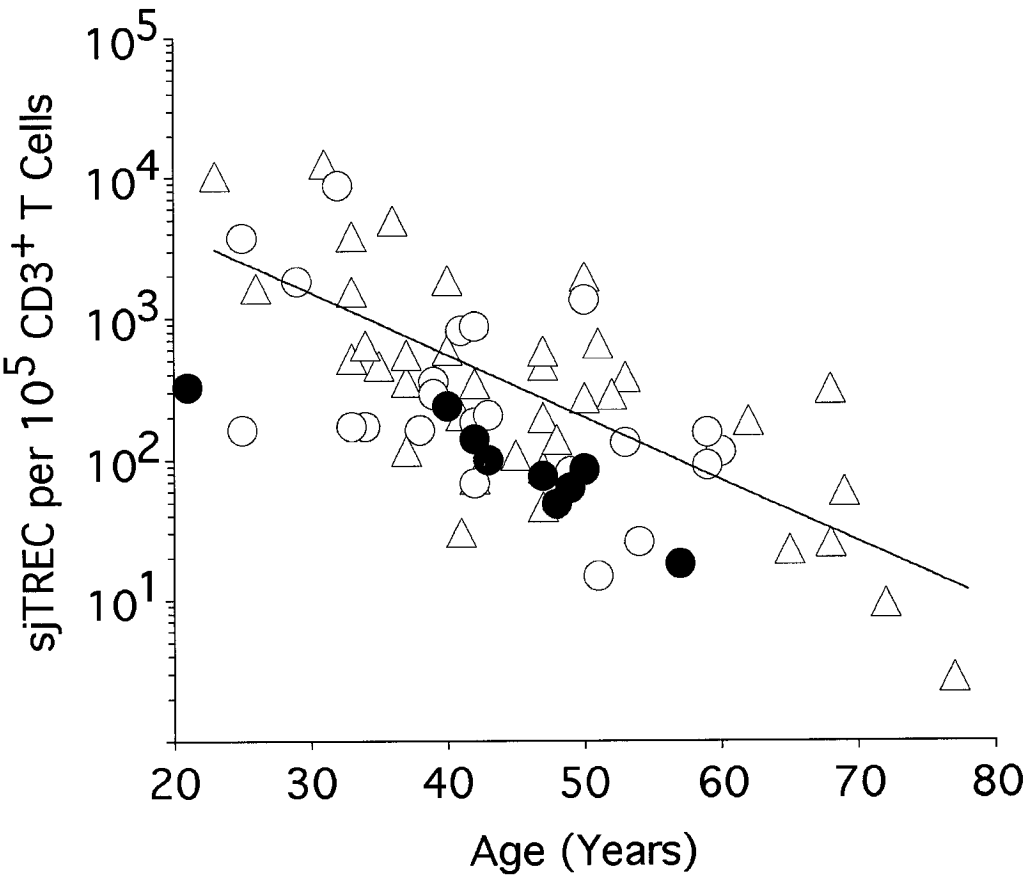
[d]similar results were obtained in a duplicate experiment.

[e]AHSCT transplanted patients were classified according their GVHD history/status. $cGVHD^+$ patients (n=10) were symptomatic at sampling time whereas GVHD- were exempt of symptoms since engraftment (n=8) or for at least 8 months prior sampling (n=14).

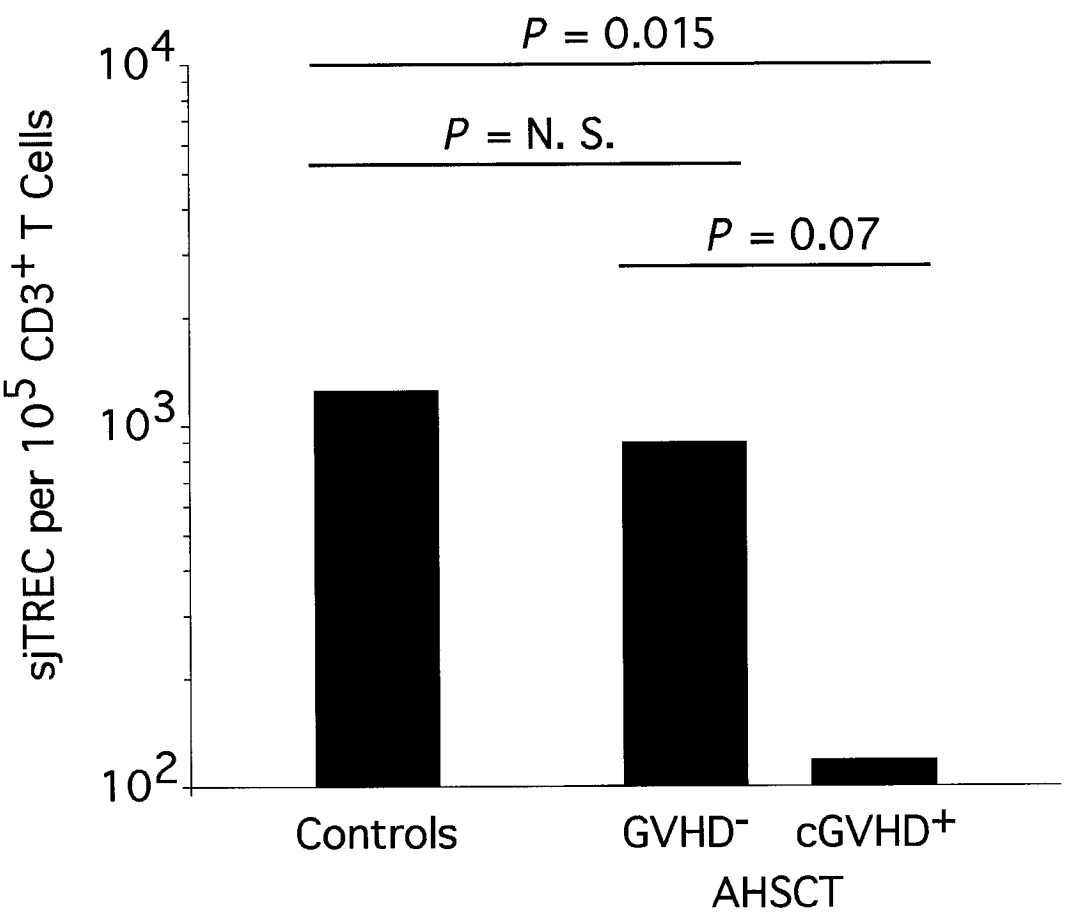
ACKNOWLEDGMENTS

This work was carried out as a partial fulfillment of J.-F. Poulin's thesis at McGill University, Montréal. The authors would like to thank Chantal Baron and Sophie Ouellet for help with flow cytometry and technical assistance. We also thank Zvi Grossman, Martin Meier-Schellersheim, Sophie Gratton and Gaël Dulude for critical reading of this manuscript and insightful thinking. J.-F. Poulin and A. Dumont are doctoral award recipients of the Canadian Institute of Health Research (CIHR). R. Cheynier is an invited/senior visiting scientist from Institut Pasteur (France). This work was supported by grants to C. Perreault from CIHR and to R.-P. Sékaly from NIH, CIHR and CANVAC. R.-P. Sékaly is a CRC chair in human immunology.

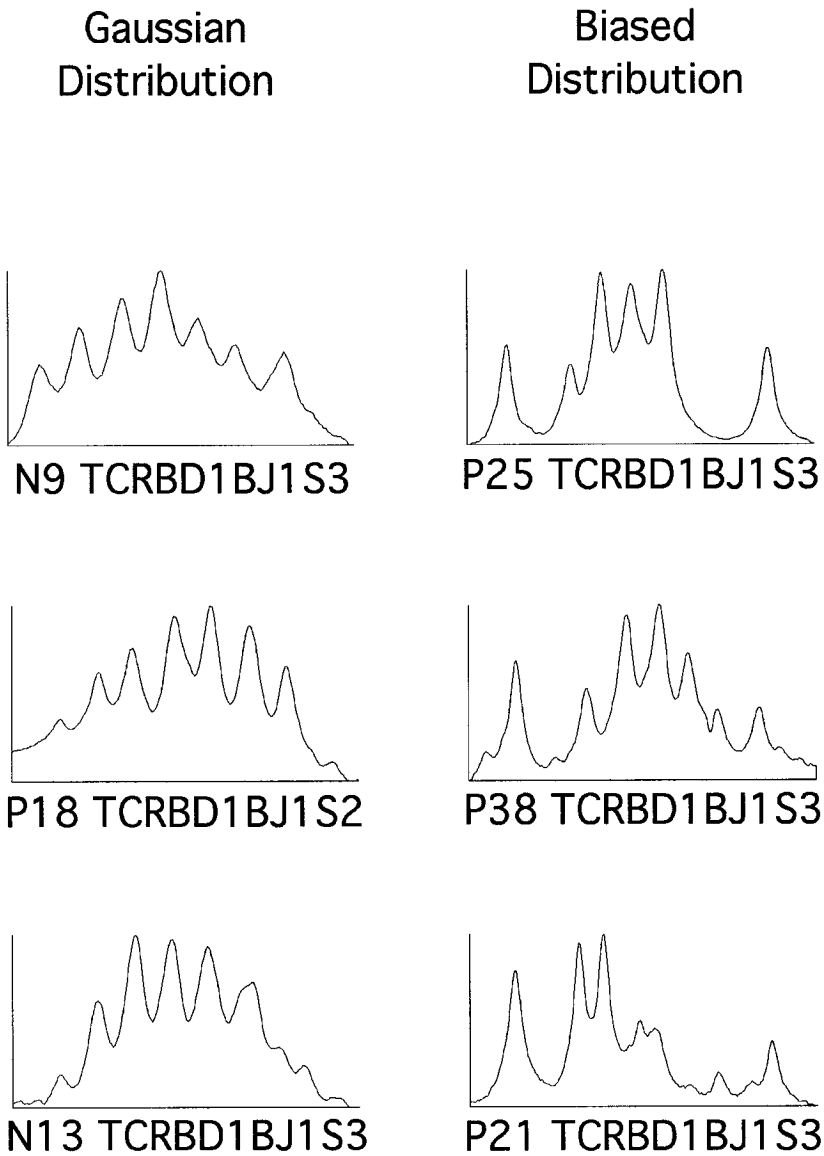
a


sjTREC per 10^5 $CD3^+$ T Cells
10^5
10^4
10^3
10^2
10^1
20
30
40
50
60
70
80
Age (Years)

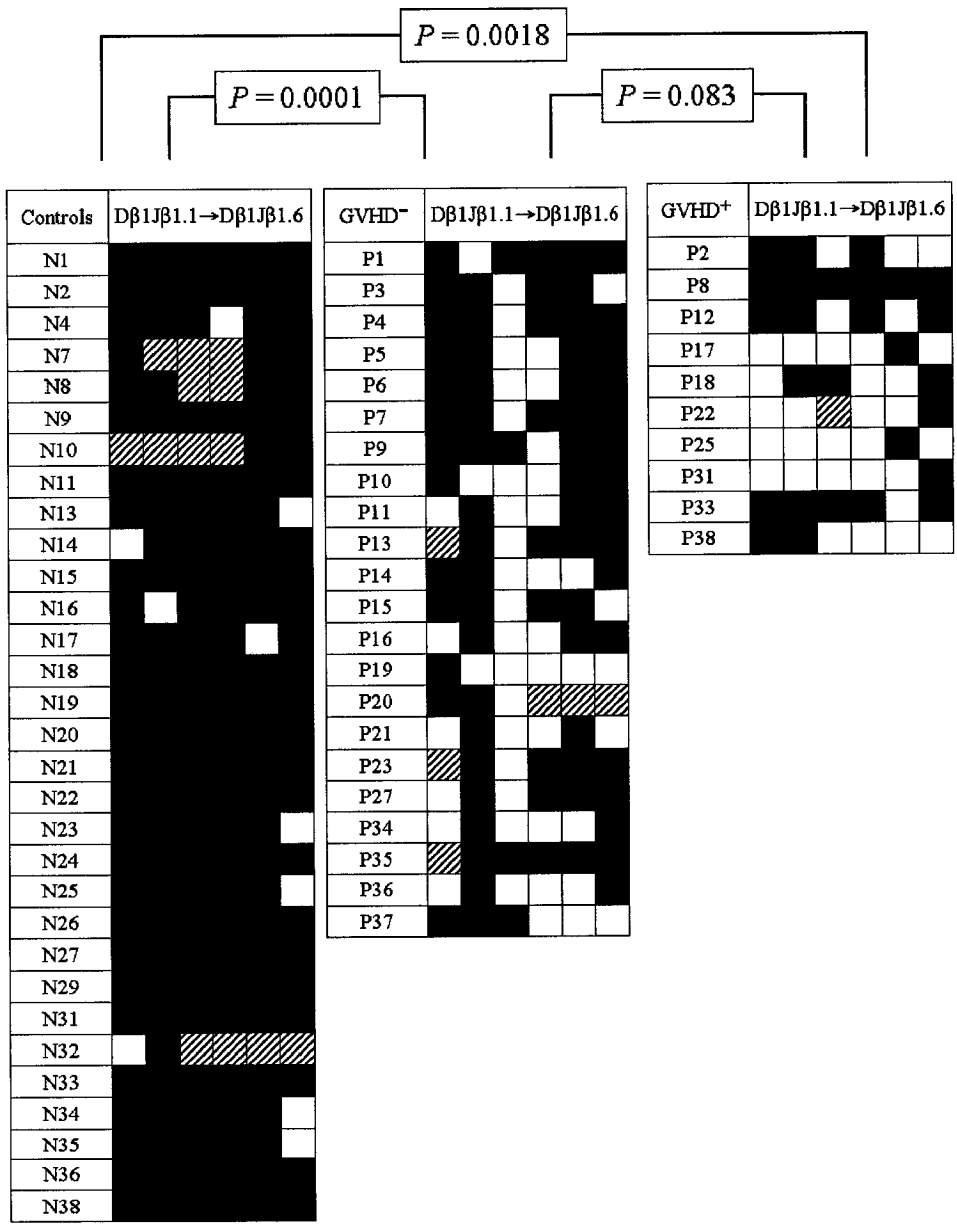
b


P = 0.015
P = N. S.
P = 0.07
10^4
10^3
10^2
sjTREC per 10^5 CD3+ T Cells
Controls
GVHD-
cGVHD+
AHSCT

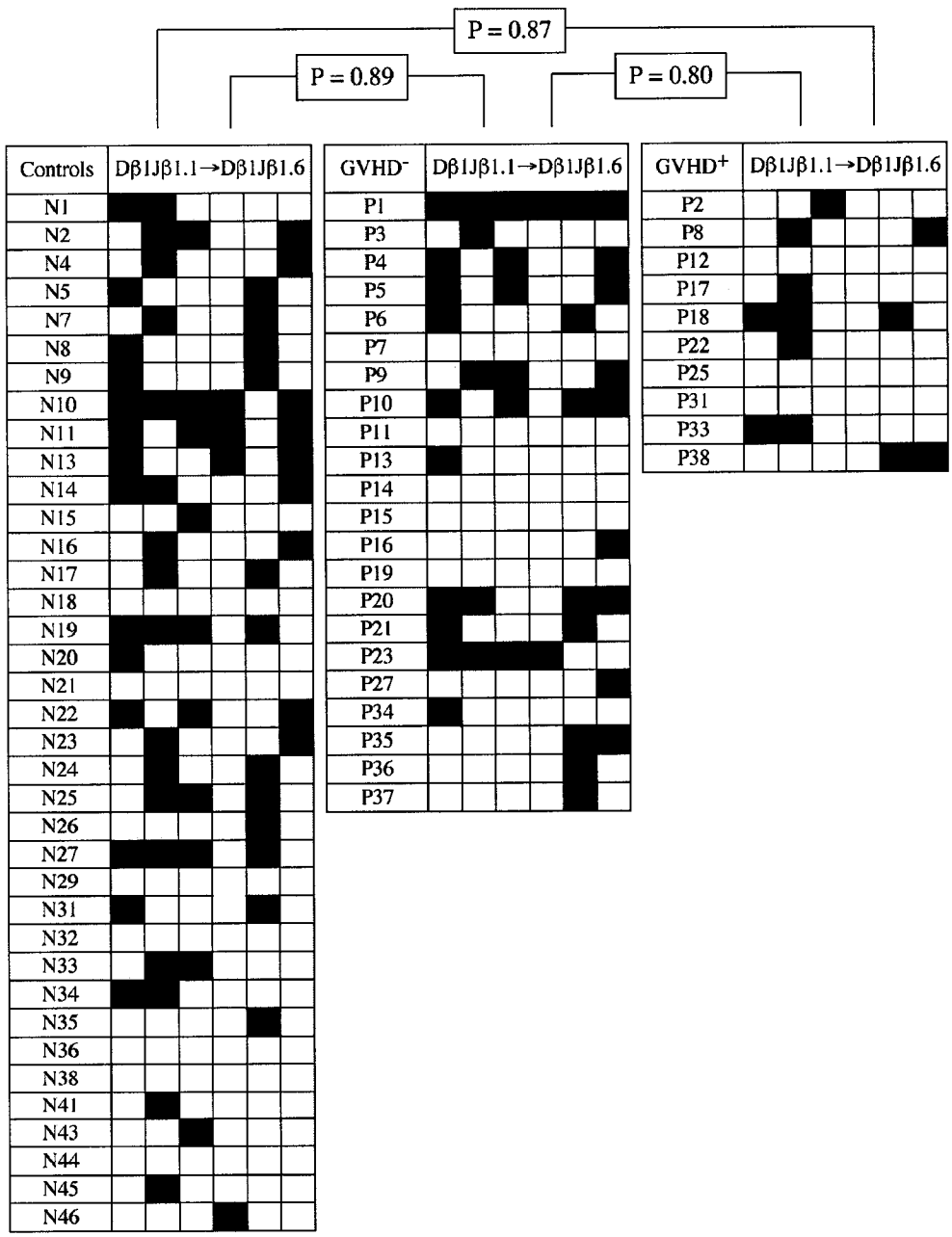
a


Gaussian Distribution
Biased Distribution
N9 TCRBD1BJ1S3
P25 TCRBD1BJ1S3
P18 TCRBD1BJ1S2
P38 TCRBD1BJ1S3
N13 TCRBD1BJ1S3
P21 TCRBD1BJ1S3

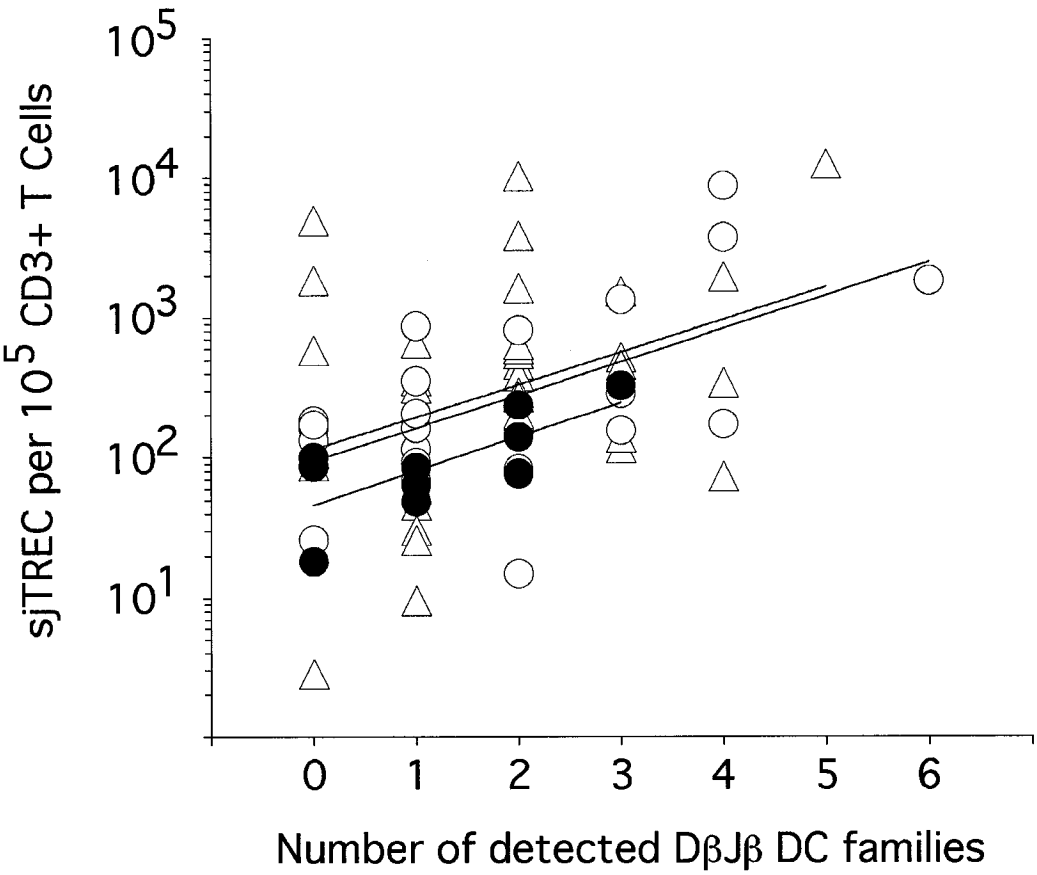
b


P = 0.0018
P = 0.0001
P = 0.083
Controls
Dβ1Jβ1.1→Dβ1Jβ1.6
N1
N2
N4
N7
N8
N9
N10
N11
N13
N14
N15
N16
N17
N18
N19
N20
N21
N22
N23
N24
N25
N26
N27
N29
N31
N32
N33
N34
N35
N36
N38
GVHD⁻
Dβ1Jβ1.1→Dβ1Jβ1.6
P1
P3
P4
P5
P6
P7
P9
P10
P11
P13
P14
P15
P16
P19
P20
P21
P23
P27
P34
P35
P36
P37
GVHD⁺
Dβ1Jβ1.1→Dβ1Jβ1.6
P2
P8
P12
P17
P18
P22
P25
P31
P33
P38

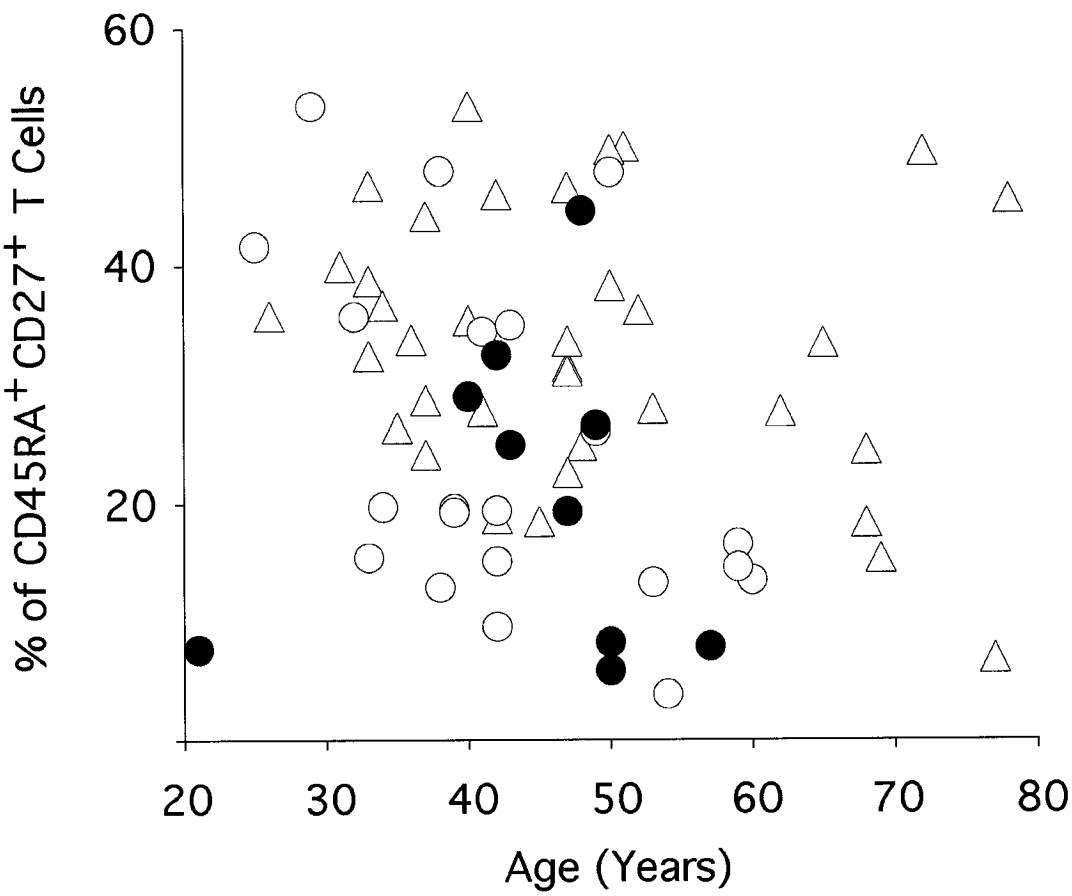
a


P = 0.87
P = 0.89
P = 0.80
Controls
Dβ1Jβ1.1→Dβ1Jβ1.6
N1
N2
N4
N5
N7
N8
N9
N10
N11
N13
N14
N15
N16
N17
N18
N19
N20
N21
N22
N23
N24
N25
N26
N27
N29
N31
N32
N33
N34
N35
N36
N38
N41
N43
N44
N45
N46
GVHD-
Dβ1Jβ1.1→Dβ1Jβ1.6
P1
P3
P4
P5
P6
P7
P9
P10
P11
P13
P14
P15
P16
P19
P20
P21
P23
P27
P34
P35
P36
P37
GVHD+
Dβ1Jβ1.1→Dβ1Jβ1.6
P2
P8
P12
P17
P18
P22
P25
P31
P33
P38

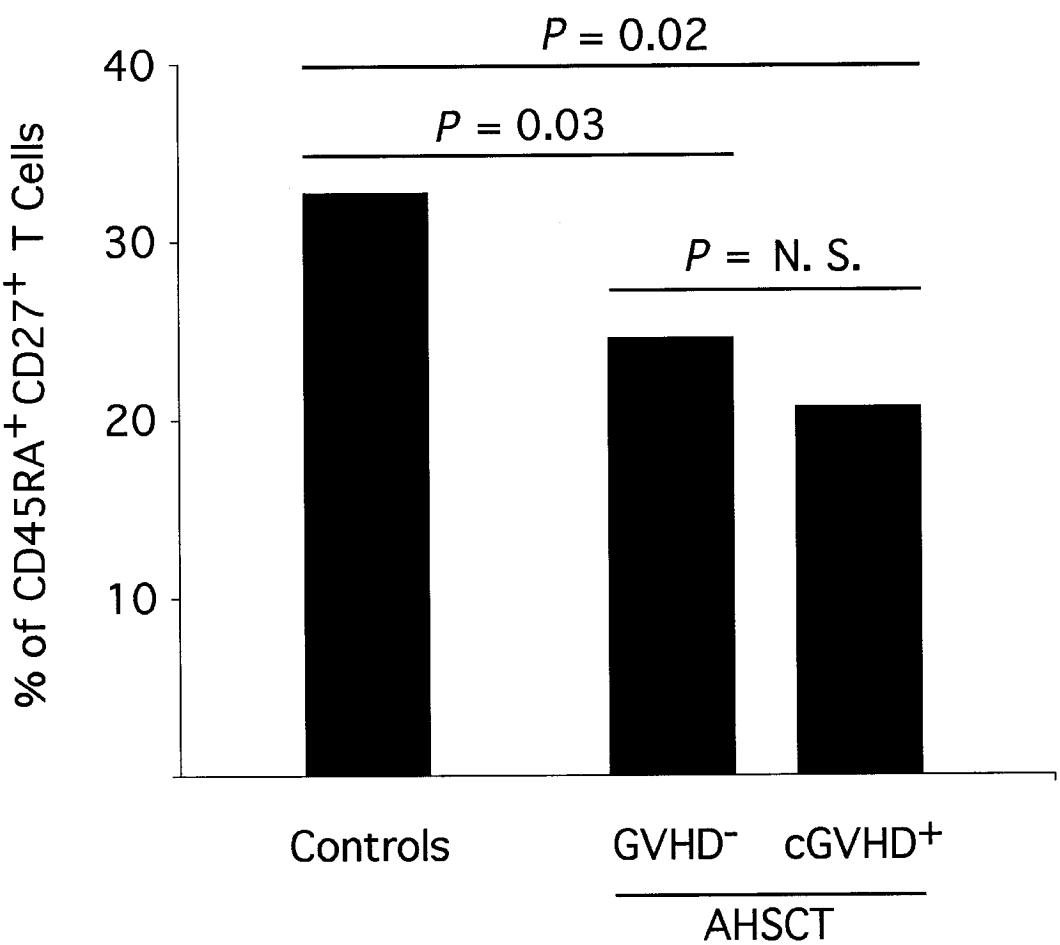
b


sjTREC per 10^5 CD3+ T Cells
10^5
10^4
10^3
10^2
10^1
0
1
2
3
4
5
6
Number of detected DβJβ DC families

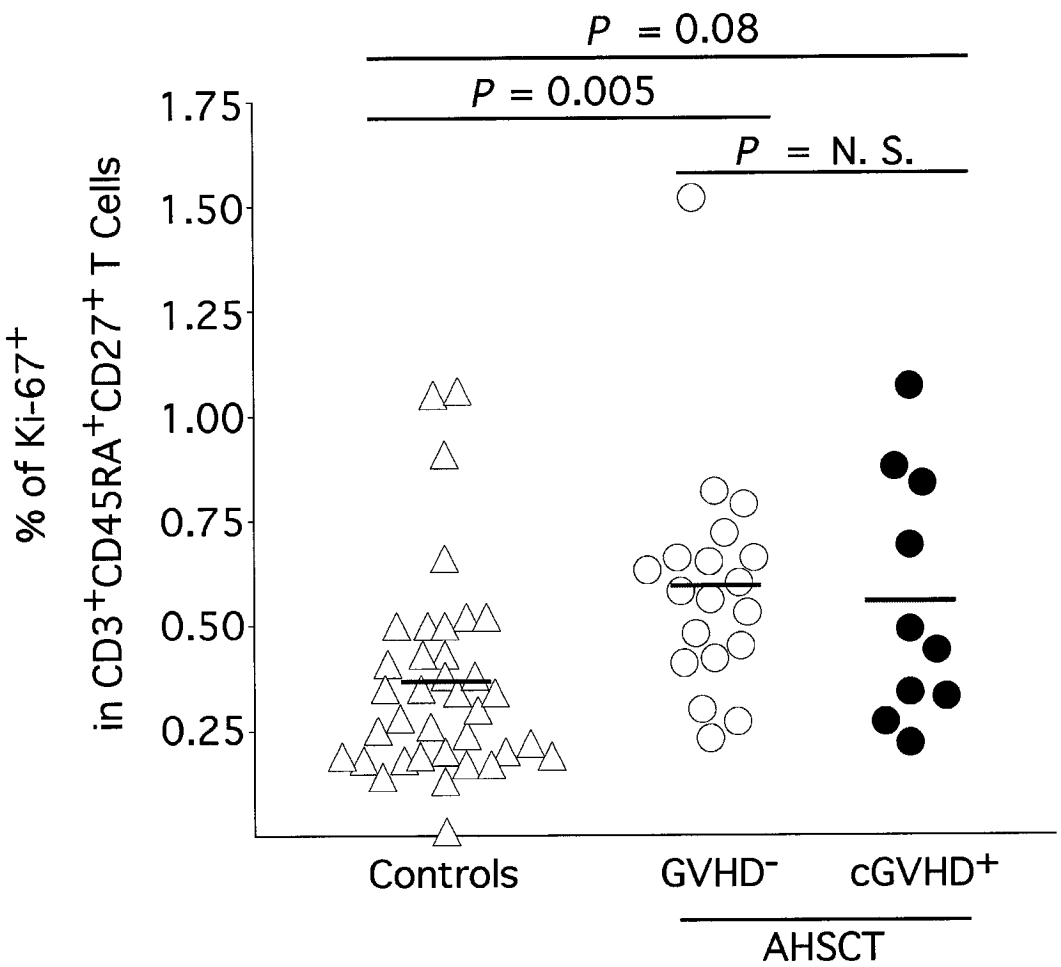
a



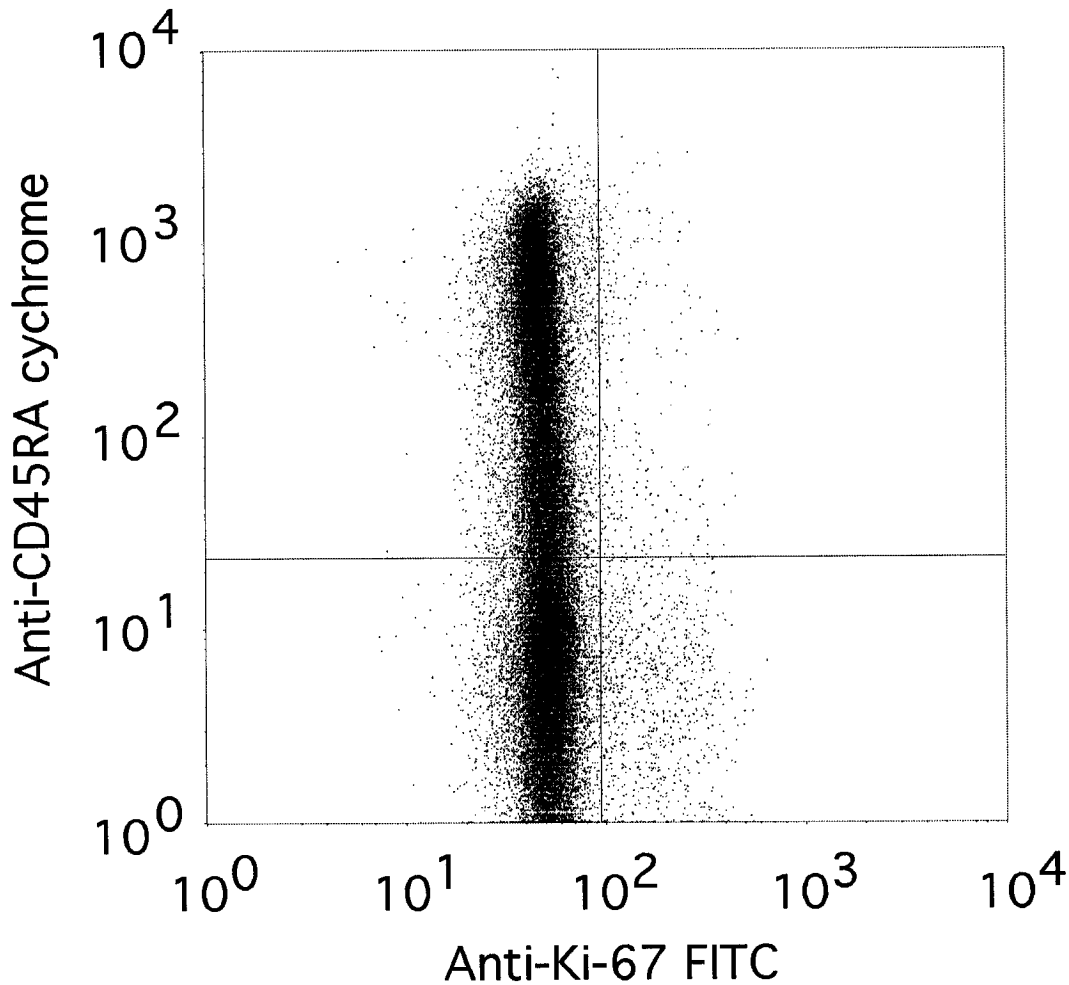
b


P = 0.02
P = 0.03
P = N. S.
40
30
20
10
% of CD45RA+CD27+ T Cells
Controls
GVHD-
cGVHD+
AHSCT

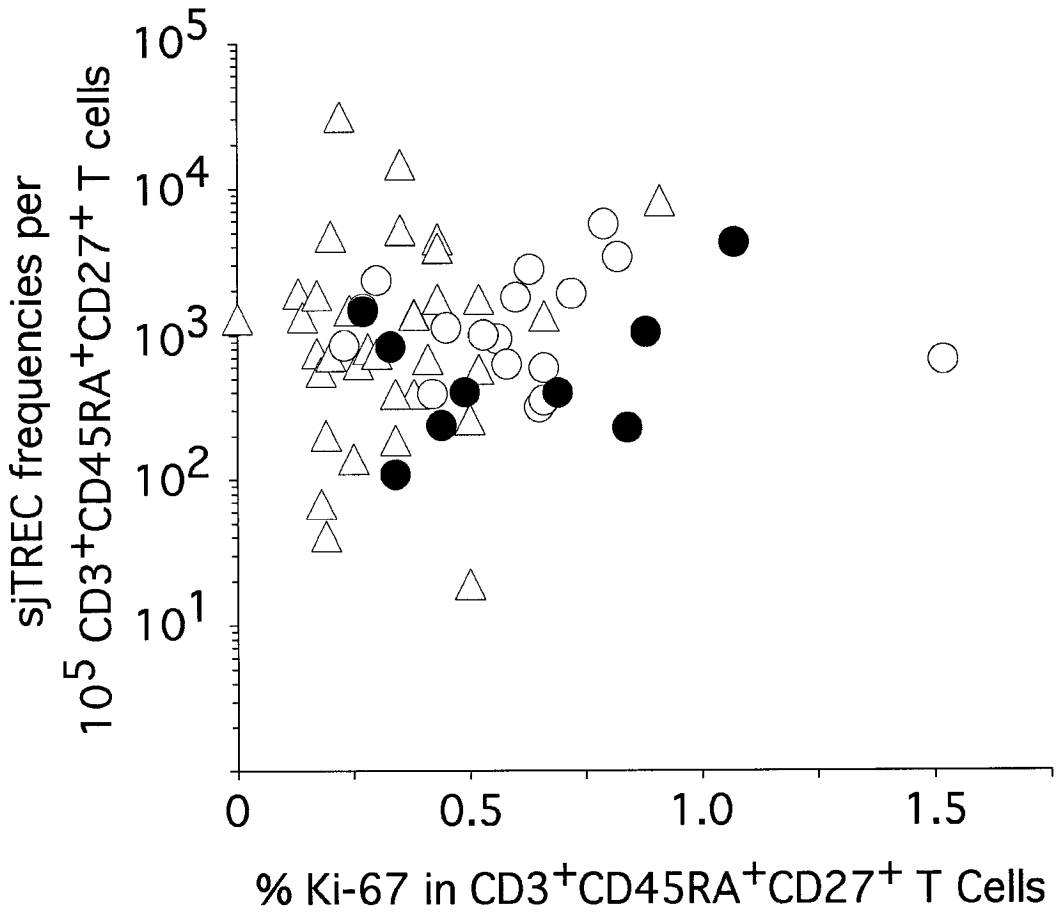
c


P = 0.08
P = 0.005
P = N. S.
% of Ki-67+ in CD3+CD45RA+CD27+ T Cells
1.75
1.50
1.25
1.00
0.75
0.50
0.25
Controls
GVHD-
cGVHD+
AHSCT

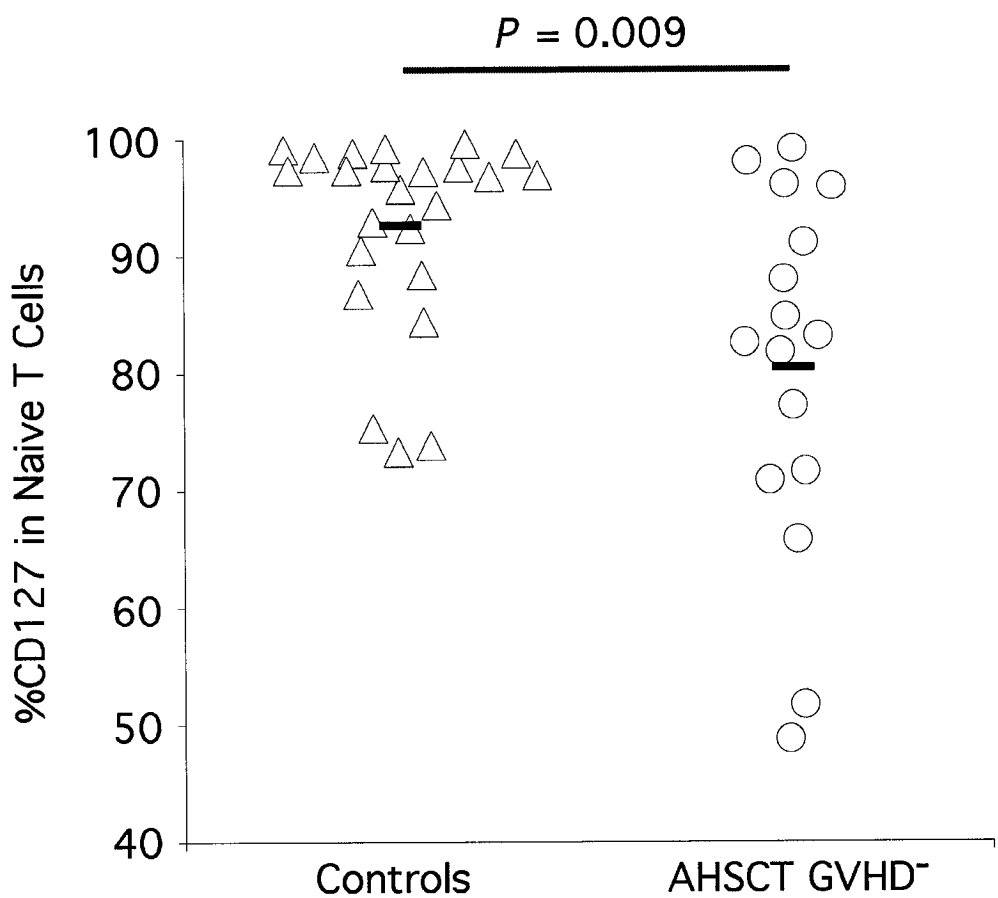
d


10^4
10^3
10^2
10^1
10^0
Anti-CD45RA cychrome
10^0
10^1
10^2
10^3
10^4
Anti-Ki-67 FITC

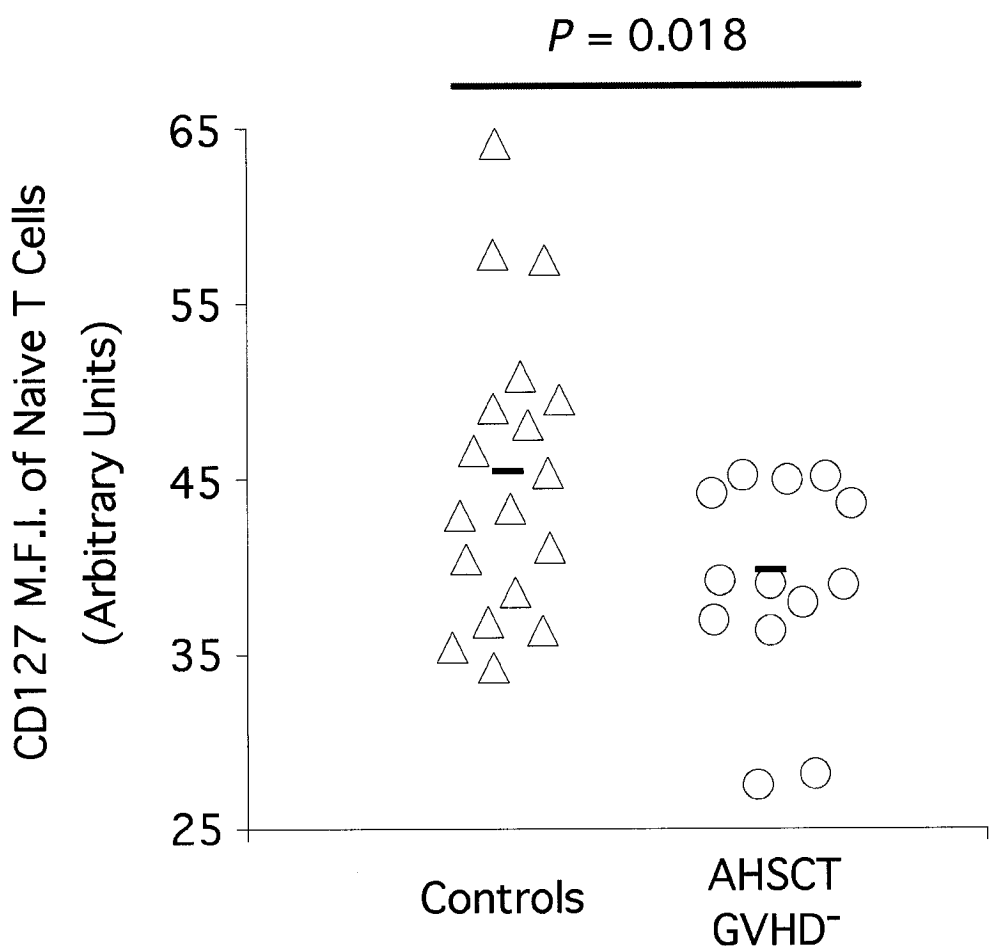
e


sjTREC frequencies per
10^5 CD3+CD45RA+CD27+ T cells
10^5
10^4
10^3
10^2
10^1
0
0.5
1.0
1.5
% Ki-67 in CD3+CD45RA+CD27+ T Cells

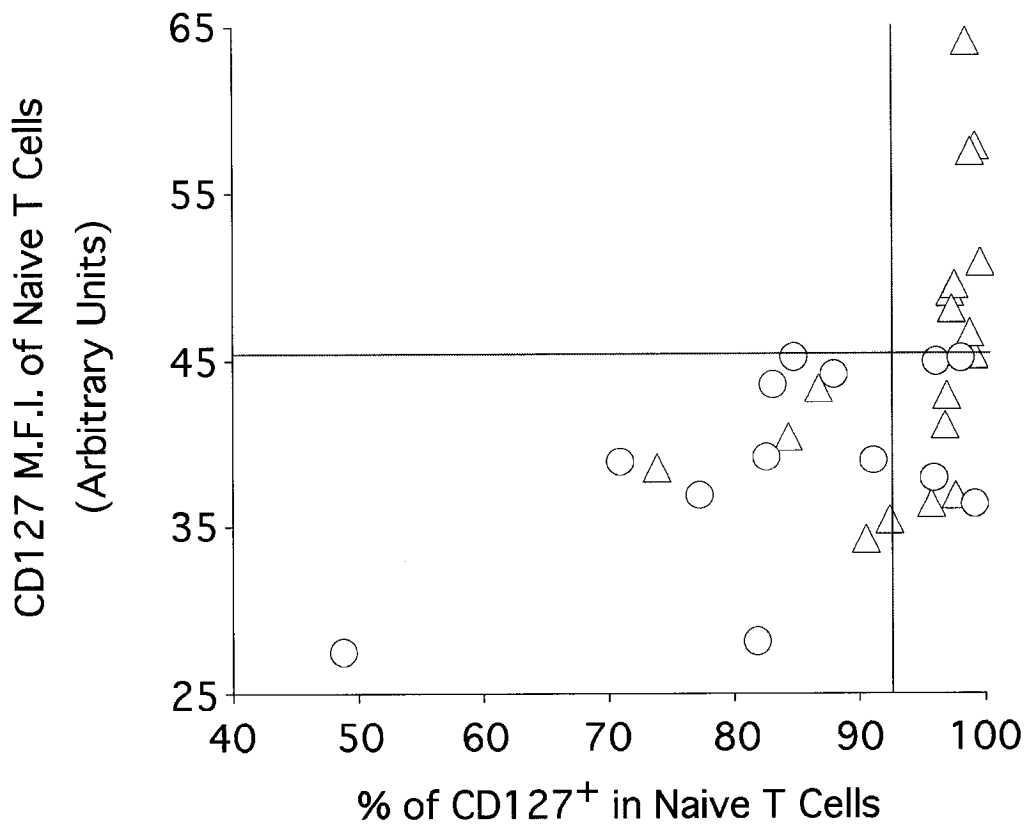
a


P = 0.009
100
90
80
70
60
50
40
%CD127 in Naive T Cells
Controls
AHSCT GVHD-

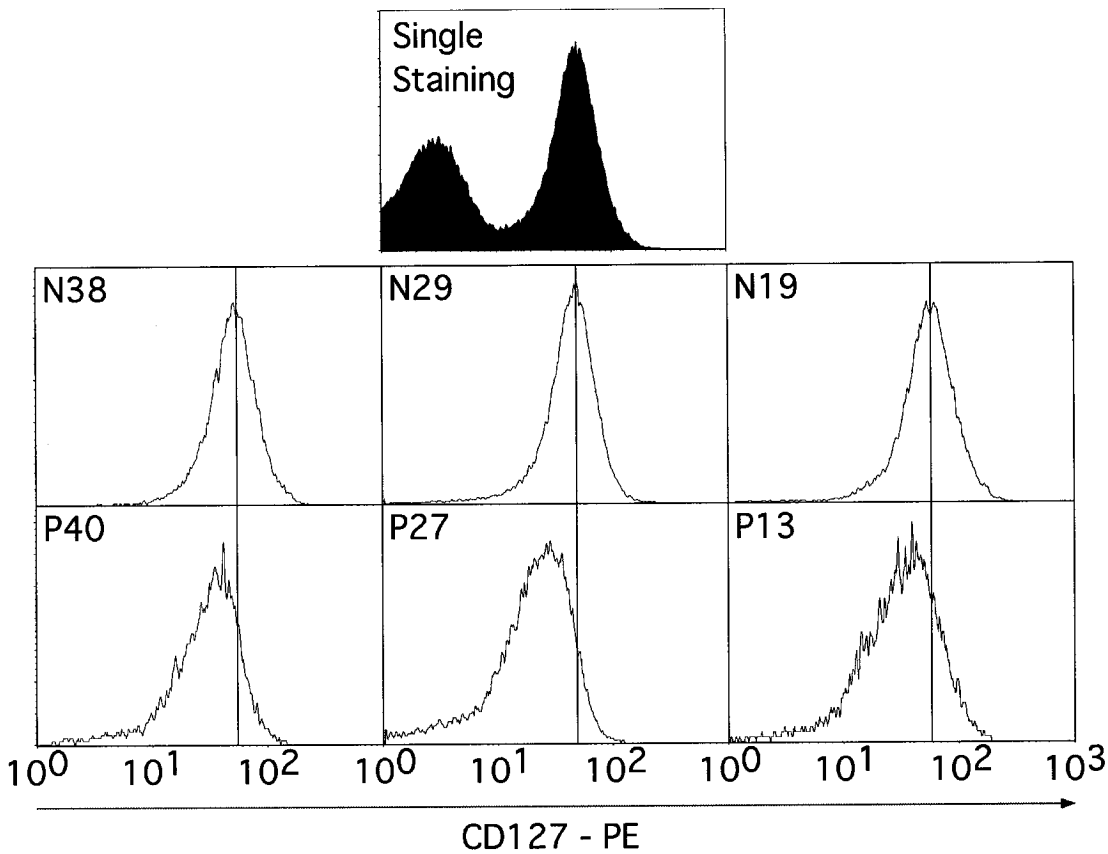
b


P = 0.018
CD127 M.F.I. of Naive T Cells
(Arbitrary Units)
65
55
45
35
25
Controls
AHSCT
GVHD-

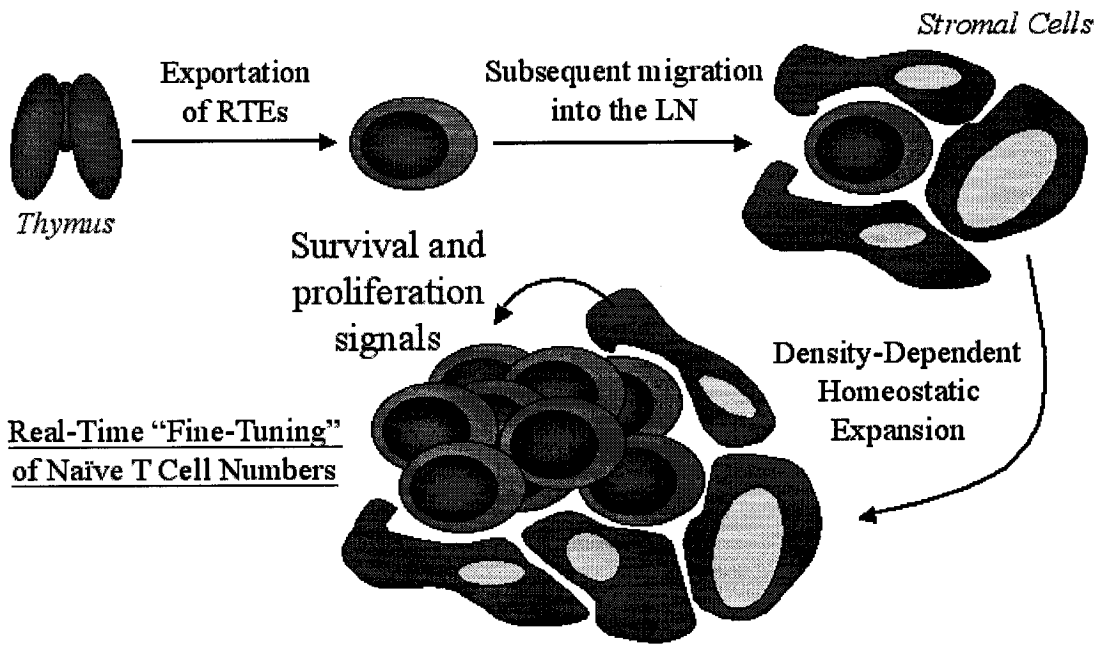
c


CD127 M.F.I. of Naive T Cells
(Arbitrary Units)
65
55
45
35
25
40
50
60
70
80
90
100
% of CD127+ in Naive T Cells

d


Single
Staining
N38
N29
N19
P40
P27
P13
10^0 10^1 10^2 10^0 10^1 10^2 10^0 10^1 10^2 10^3
CD127 - PE

a


Stromal Cells
Exportation of RTEs
Subsequent migration into the LN
Thymus
Survival and proliferation signals
Density-Dependent Homeostatic Expansion
Real-Time "Fine-Tuning" of Naïve T Cell Numbers

b

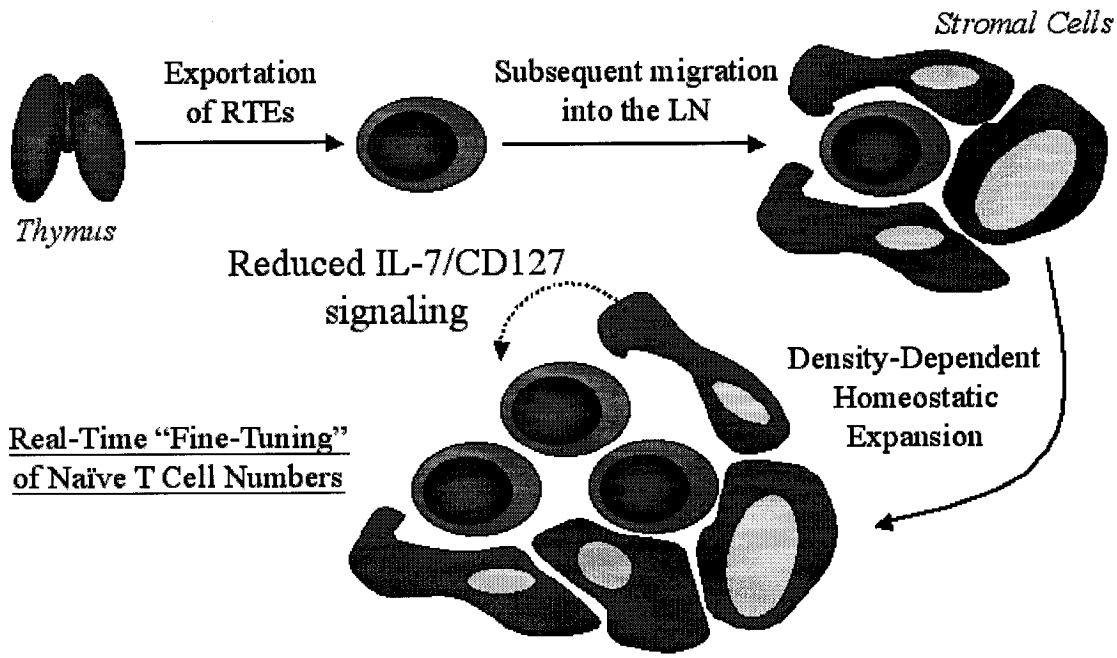

Stromal Cells
Exportation of RTEs
Subsequent migration into the LN
Thymus
Reduced IL-7/CD127 signaling
Density-Dependent Homeostatic Expansion
Real-Time "Fine-Tuning" of Naïve T Cell Numbers

FIGURE LEGENDS

Figure 1. Severe reduction of the peripheral sjTREC frequencies in cGVHD$^+$ patients. ***a.*** Age-related decrease of sjTREC content normalized for CD3$^+$ T cell frequencies. Peripheral blood sjTREC frequencies were obtained using real time quantitative PCR and normalized to CD3$^+$ T cell frequencies as estimated by FACS analysis. Healthy adult controls (n=37) are represented with empty triangles (Δ) while the solid line (—) indicates the negative linear regression trend from control individuals' data ($y = 31581e^{-0,1012x}$). AHSCT patients exempt of cGVHD (n=22) at sampling time are shown as empty circles (○) whereas transplanted patients currently undergoing cGVHD (n=10) are illustrated with filled circles (●). ***b.*** Histogram representation of average peripheral sjTREC frequencies in these cohorts. Statistical significance of the observed differences were calculated using two-tailed Student t test and shown on top. N.S.: not significant.

Figure 2. Peripheral mature TCR Dβ1Jβ1.1 to Jβ1.6 repertoire is biased in AHSCT patients, irrespectively of GVHD status. ***a.*** Representative example of Gaussian (left, *n*=3) and biased (right, *n*=3) DβJβ CDR3 length patterns as obtained by radiolabeled-spectratyping. Each panel represents the CDR3 length distribution for a panVβ-Jβ1.2 or -Jβ1.3 specific amplification. N9 and N13 represent healthy control individuals while AHSCT patients are identified by P18, P21, P25 and P38. ***b.*** Checkerboard representation of peripheral mature TCR Dβ1Jβ1.1 to Jβ1.6 repertoire biases as evaluated by spectratyping analysis of the CDR3 length among untransplanted adults (left), GVHD- (middle) and cGVHD+ (right) AHSCT patients. Biased DβJβ families, as defined by a non-Gaussian CDR3 length distribution, are represented by empty squares whereas a normal distribution is shown with filled squares. Striped squares: not done. In order to perform statistical analysis, a binary code was applied to the data set; "1" and "0" representing "Gaussian" and "biased" distributions respectively. Statistical significance of the observed differences in the diversity of peripheral TCR repertoires between the three experimental groups, calculated using two-tailed Student t test, are shown on top.

Figure 3. Thymic function remains diversified following AHSCT, despite the occurrence of cGVHD. ***a.*** Checkerboard representation of peripheral blood RTE repertoire diversity in controls (left) and AHSCT patients (GVHD-: middle; cGVHD+: right). TCR Dβ1Jβ1.1 to 1.6 deletion circle frequencies were estimated by real time quantitative PCR on 10^5 peripheral blood mononuclear cells. Black squares represent detected (≥1 per $2x10^5$) DβJβ DC families whereas empty squares correspond to frequencies of less than 1 DβJβ DC per $2x10^5$ cells. In order to perform statistical analysis, a binary code was applied to the data set; "1" and "0" representing "detected" and "undetected" DβJβ DC respectively. Statistical significance of the observed differences in the detectability of the TCRDβJβ deletion circle families between the three experimental groups, calculated using two-tailed Student t test, are shown on top. Similar results were obtained in a duplicate experiment (not shown). ***b.*** Positive association between peripheral sjTREC frequencies and the number of detectable Dβ1Jβ1.1 to 1.6 DC families. Empty triangles (Δ) correspond to healthy control adults whereas empty (O) and filled (●) circles represent $GVHD^-$ and $cGVHD^+$ AHSCT patients, respectively. Linear regression curves are shown for the three analyzed populations ($y = 114{,}39e^{0{,}5351x}$, $y = 94{,}266e^{0{,}5464x}$ and $y = 46{,}153e^{0{,}5599x}$ for controls, $cGVHD^-$ and $cGVHD^+$ respectively).

Figure 4. Reduction of naïve T cell frequencies following AHSCT and during cGVHD. ***a.*** FACS analysis of naïve T cell frequencies in all study groups. Naïve T cells, as defined by CD45RA and CD27 expression on $CD3^+$ cells were quantified in PBMCs by flow cytometry and FACScalibur™. A minimum of $3x10^5$ events in the live cell gate, as defined by forward and side scatter, was accumulated for each sample. Empty triangles (Δ) represent healthy adults whereas empty (O) and filled circles (●) correspond to $GVHD^-$ and $cGVHD^+$ AHSCT patients, respectively. Statistical significance of the observed differences in the peripheral blood naïve T cell frequency between the three experimental groups, calculated using two-tailed Student t test, are shown on top. ***b.*** Histogram representation of average naïve T cell frequencies for each study groups. N.S.: not significant. Statistical significance of the observed differences between the three experimental groups, calculated using two-tailed Student t test, are shown on top. ***c.*** Ki-67 expression in $CD3^+$ $CD45RA^+CD27^+$ naïve T cells. Ki67 expressing naïve T cells

were analyzed by flow cytometry as described in the patients and methods section and gated on $CD3^+$ $CD45RA^+$ $CD27^+$ cells, for each of the three study groups. The solid lines represent the average Ki-67^+ frequencies for each group. Statistical significance of the observed differences between the three experimental groups, calculated using two-tailed Student t test, are shown on top. ***d.*** Representative dot plot example for Ki-67 staining of $CD3^+$ $CD45RA^+$ $CD27^+$. ***e.*** Graphic representation of sjTREC content, normalized to 10^5 $CD3^+CD45RA^+CD27^+$ T lymphocytes, as a function of Ki-67 expression among $CD3^+CD45RA^+CD27^+$ T lymphocytes. A minimum of $3x10^5$ events in the live cell gate, as defined by forward and side scatter, was accumulated for each sample. A negative association (i.e. suggestive of an influence of peripheral naïve T cell proliferation on sjTREC frequencies) could not be found in any of the groups.

Figure 5. Reduction of IL-7R (CD127) expression on naïve T cells from AHSCT $GVHD^-$ patients. ***a.*** FACS analysis of IL-$7R\alpha^+$ naïve T cells frequencies of AHSCT patients and control group. $CD127^+$ lymphocytes were quantified in naïve T cells, as defined by CD3, CD45RA and CD27 expression, by flow cytometry and FACScalibur™. A minimum of $1x10^5$ events in the live cell gate, as defined by forward and side scatter, was accumulated for each sample. Empty triangles (Δ) represent healthy adults whereas empty circles (O) correspond to $GVHD^-$ AHSCT patients. Statistical significance between both experimental group, calculated using two-tailed Student t test, is shown on top. Solid bars indicate group averages. ***b.*** Mean fluorescence intensity (MFI) of CD127-expressing naïve T cells of AHSCT patients and control groups. Again, the solid bars indicate averages of the control group (Δ) and AHSCT $GVHD^-$ patients (O). ***c.*** Representation of the MFI of CD127-expressing naïve T cells. The vertical and horizontal solid lines represent the average of IL-$7R^+$ naïve T cells and the MFI of these cells, respectively, for the control group only. ***d.*** Representative CD127 FACS histograms for healthy adults and AHSCT $GVHD^-$ patients. Anti-CD127 single staining was performed on PBMC from a control adult in order to place the positive gate for CD127 (top panel). Representative CD127 FACS histograms (gated on $CD3^+CD45RA^+CD27^+$) are shown for healthy controls N38, N29 and N19 (mid panels) as well as for patients P40, P27 and P13 (bottom panels). The vertical solid lines represent the maxima for each

healthy adult. All histogram pairs (N38/P40, N29/P27 and N19/P13) were acquired on the same day with the same cytometer gains and setup.

Figure 6. Proposed model for the long-lasting naïve T cell deficiency observed in AHSCT patients in the absence of GVHD. ***a.*** The thymus exports T cells in the blood that subsequently migrate to the lymph nodes and provide immune surveillance. Although *de novo* production is essential for the diversification of the TCR repertoire, it is more probable that homeostatic expansion of peripheral naïve T cells is the mechanism of choice for quick regulation of peripheral T cell numbers. The interactions of survival/proliferation factors (produced by APC-like cells located in the lymph nodes) with their corresponding receptor expressed on peripheral naïve T cells regulate the number of cells that can be maintained through in the periphery. Defects in this signaling (through a reduced CD127 expression by naïve T cells) leads to an impaired cell survival (***b***) and the inability to reconstitute naïve T cell numbers.

REFERENCES

1. Roux, E. et al. Recovery of immune reactivity after T-cell-depleted bone marrow transplantation depends on thymic activity. *Blood* **96**, 2299-303. (2000).
2. Gratwohl, A., Passweg, J., Baldomero, H. & Hermans, J. Blood and marrow transplantation activity in Europe 1996. European Group for Blood and Marrow Transplantation (EBMT). *Bone Marrow Transplant* **22**, 227-40. (1998).
3. Mackall, C.L., Hakim, F.T. & Gress, R.E. T-cell regeneration: all repertoires are not created equal. *Immunol Today* **18**, 245-51. (1997).
4. Mackall, C.L., Granger, L., Sheard, M.A., Cepeda, R. & Gress, R.E. T-cell regeneration after bone marrow transplantation: differential CD45 isoform expression on thymic-derived versus thymic-independent progeny. *Blood* **82**, 2585-94. (1993).
5. Mackall, C.L. et al. Age, thymopoiesis, and CD4+ T-lymphocyte regeneration after intensive chemotherapy. *N Engl J Med* **332**, 143-9. (1995).
6. Mackall, C.L. et al. IL-7 increases both thymic-dependent and thymic-independent T-cell regeneration after bone marrow transplantation. *Blood* **97**, 1491-7. (2001).
7. Dumont-Girard, F. et al. Reconstitution of the T-cell compartment after bone marrow transplantation: restoration of the repertoire by thymic emigrants. *Blood* **92**, 4464-71. (1998).
8. Suzuki, K. et al. Gut cryptopatches: direct evidence of extrathymic anatomical sites for intestinal T lymphopoiesis. *Immunity* **13**, 691-702. (2000).
9. Heitger, A. et al. Essential role of the thymus to reconstitute naive (CD45RA+) T-helper cells after human allogeneic bone marrow transplantation. *Blood* **90**, 850-7. (1997).
10. Douek, D.C. et al. Changes in thymic function with age and during the treatment of HIV infection. *Nature* **396**, 690-5. (1998).
11. Poulin, J.F. et al. Direct evidence for thymic function in adult humans. *J Exp Med* **190**, 479-86. (1999).
12. Verschuren, M.C. et al. Preferential rearrangements of the T cell receptor-delta-deleting elements in human T cells. *J Immunol* **158**, 1208-16. (1997).

13. Pignata, C. et al. Defective activation of mitogen-activated protein kinase after allogeneic bone marrow transplantation. *Blood* **88**, 2334-41. (1996).

14. Baker, M.B., Riley, R.L., Podack, E.R. & Levy, R.B. Graft-versus-host-disease-associated lymphoid hypoplasia and B cell dysfunction is dependent upon donor T cell-mediated Fas-ligand function, but not perforin function. *Proc Natl Acad Sci U S A* **94**, 1366-71. (1997).

15. Atkinson, K. Reconstruction of the haemopoietic and immune systems after marrow transplantation. *Bone Marrow Transplant* **5**, 209-26. (1990).

16. Tsoi, M.S. et al. Cellular interactions in marrow-grafted patients. II. Normal monocyte antigen-presenting and defective T-cell-proliferative functions early after grafting and during chronic graft-versus-host disease. *Transplantation* **37**, 556-61. (1984).

17. Dulude, G., Roy, D.C. & Perreault, C. The effect of graft-versus-host disease on T cell production and homeostasis. *J Exp Med* **189**, 1329-42. (1999).

18. Matsutani, T. et al. Restricted usage of T-cell receptor alpha-chain variable region (TCRAV) and T-cell receptor beta-chain variable region (TCRBV) repertoires after human allogeneic haematopoietic transplantation. *Br J Haematol* **109**, 759-69. (2000).

19. Hochberg, E.P. et al. Quantitation of T-cell neogenesis in vivo after allogeneic bone marrow transplantation in adults. *Blood* **98**, 1116-21. (2001).

20. Weinberg, K. et al. Factors affecting thymic function after allogeneic hematopoietic stem cell transplantation. *Blood* **97**, 1458-66. (2001).

21. Storek, J. et al. Immunity of patients surviving 20 to 30 years after allogeneic or syngeneic bone marrow transplantation. *Blood* **98**, 3505-12. (2001).

22. Gyger, M. et al. Quantitative assessment of hematopoietic chimerism after allogeneic bone marrow transplantation has predictive value for the occurrence of irreversible graft failure and graft-vs.-host disease. *Exp Hematol* **26**, 426-34. (1998).

23. Guimond, M. et al. Relapse after bone marrow transplantation: evidence for distinct immunological mechanisms between adult and paediatric populations. *Br J Haematol* **109**, 130-7. (2000).

24. Glucksberg, H. et al. Clinical manifestations of graft-versus-host disease in human recipients of marrow from HL-A-matched sibling donors. *Transplantation* **18**, 295-304. (1974).

25. Vogelsang, G.B. How I treat chronic graft-versus-host disease. *Blood* **97**, 1196-201. (2001).

26. Zhang, L. et al. Measuring recent thymic emigrants in blood of normal and HIV-1-infected individuals before and after effective therapy. *J Exp Med* **190**, 725-32. (1999).

27. Verfuerth, S. et al. Longitudinal monitoring of immune reconstitution by CDR3 size spectratyping after T-cell-depleted allogeneic bone marrow transplant and the effect of donor lymphocyte infusions on T-cell repertoire. *Blood* **95**, 3990-5. (2000).

28. Cheynier, R. et al. HIV and T cell expansion in splenic white pulps is accompanied by infiltration of HIV-specific cytotoxic T lymphocytes. *Cell* **78**, 373-87. (1994).

29. Cochet, M. et al. Molecular detection and in vivo analysis of the specific T cell response to a protein antigen. *Eur J Immunol* **22**, 2639-47. (1992).

30. Jeddi-Tehrani, M. et al. T-cell receptor J beta gene segment usage in immature and mature human thymocytes. *Scand J Immunol* **42**, 449-56. (1995).

31. Schluns, K.S., Kieper, W.C., Jameson, S.C. & Lefrancois, L. Interleukin-7 mediates the homeostasis of naive and memory CD8 T cells in vivo. *Nat Immunol* **1**, 426-32. (2000).

32. Goldrath, A.W., Bogatzki, L.Y. & Bevan, M.J. Naive T cells transiently acquire a memory-like phenotype during homeostasis-driven proliferation. *J Exp Med* **192**, 557-64. (2000).

33. Ge, Q., Hu, H., Eisen, H.N. & Chen, J. Different contributions of thymopoiesis and homeostasis-driven proliferation to the reconstitution of naive and memory T cell compartments. *Proc Natl Acad Sci U S A* **99**, 2989-94. (2002).

34. Gerdes, J. et al. Cell cycle analysis of a cell proliferation-associated human nuclear antigen defined by the monoclonal antibody Ki-67. *J Immunol* **133**, 1710-5. (1984).

35. Vivien, L., Benoist, C. & Mathis, D. T lymphocytes need IL-7 but not IL-4 or IL-6 to survive in vivo. *Int Immunol* **13**, 763-8. (2001).

36. Rathmell, J.C., Farkash, E.A., Gao, W. & Thompson, C.B. IL-7 enhances the survival and maintains the size of naive T cells. *J Immunol* **167**, 6869-76. (2001).

37. Napolitano, L.A. et al. Increased production of IL-7 accompanies HIV-1-mediated T-cell depletion: implications for T-cell homeostasis. *Nat Med* **7**, 73-9. (2001).

38. Brochu, S., Baron, C., Hetu, F., Roy, D.C. & Perreault, C. Oligoclonal expansion of CTLs directed against a restricted number of dominant minor histocompatibility antigens in hemopoietic chimeras. *J Immunol* **155**, 5104-14. (1995).

39. Orsini, E. et al. Changes in T cell receptor repertoire associated with graft-versus-tumor effect and graft-versus-host disease in patients with relapsed multiple myeloma after donor lymphocyte infusion. *Bone Marrow Transplant* **25**, 623-32. (2000).

40. Miura, Y. et al. Characterization of the T-cell repertoire in autologous graft-versus-host disease (GVHD): evidence for the involvement of antigen-driven T-cell response in the development of autologous GVHD. *Blood* **98**, 868-76. (2001).

41. Jamieson, B.D. et al. Generation of functional thymocytes in the human adult. *Immunity* **10**, 569-75. (1999).

42. Ku, C.C., Kappler, J. & Marrack, P. The growth of the very large CD8+ T cell clones in older mice is controlled by cytokines. *J Immunol* **166**, 2186-93. (2001).

43. Friedman, T.M., Gilbert, M., Briggs, C. & Korngold, R. Repertoire analysis of CD8+ T cell responses to minor histocompatibility antigens involved in graft-versus-host disease. *J Immunol* **161**, 41-8. (1998).

44. Surh, C.D. & Sprent, J. Homeostatic T cell proliferation: how far can T cells be activated to self-ligands? *J Exp Med* **192**, F9-F14. (2000).

45. Ge, Q., Rao, V.P., Cho, B.K., Eisen, H.N. & Chen, J. Dependence of lymphopenia-induced T cell proliferation on the abundance of peptide/ MHC epitopes and strength of their interaction with T cell receptors. *Proc Natl Acad Sci USA* **98**, 1728-33. (2001).

46. Correia-Neves, M., Waltzinger, C., Mathis, D. & Benoist, C. The shaping of the T cell repertoire. *Immunity* **14**, 21-32. (2001).

47. Hazenberg, M.D. et al. Increased cell division but not thymic dysfunction rapidly affects the T-cell receptor excision circle content of the naive T cell population in HIV-1 infection. *Nat Med* **6**, 1036-42. (2000).

48. Krenger, W., Rossi, S., Piali, L. & Hollander, G.A. Thymic atrophy in murine acute graft-versus-host disease is effected by impaired cell cycle progression of host pro-T and pre-T cells. *Blood* **96**, 347-54. (2000).

49. van den Brink, M.R., Moore, E., Ferrara, J.L. & Burakoff, S.J. Graft-versus-host-disease-associated thymic damage results in the appearance of T cell clones with anti-host reactivity. *Transplantation* **69**, 446-9. (2000).

50. Vella, A., Teague, T.K., Ihle, J., Kappler, J. & Marrack, P. Interleukin 4 (IL-4) or IL-7 prevents the death of resting T cells: stat6 is probably not required for the effect of IL-4. *J Exp Med* **186**, 325-30. (1997).

51. Hassan, J. & Reen, D.J. IL-7 promotes the survival and maturation but not differentiation of human post-thymic CD4+ T cells. *Eur J Immunol* **28**, 3057-65. (1998).

52. Vella, A.T., Dow, S., Potter, T.A., Kappler, J. & Marrack, P. Cytokine-induced survival of activated T cells in vitro and in vivo. *Proc Natl Acad Sci U S A* **95**, 3810-5. (1998).

53. Boursalian, T.E. & Bottomly, K. Survival of naive CD4 T cells: roles of restricting versus selecting MHC class II and cytokine milieu. *J Immunol* **162**, 3795-801. (1999).

54. Lin, J.X. et al. The role of shared receptor motifs and common Stat proteins in the generation of cytokine pleiotropy and redundancy by IL-2, IL-4, IL-7, IL-13, and IL-15. *Immunity* **2**, 331-9. (1995).

55. van der Plas, D.C. et al. Interleukin-7 signaling in human B cell precursor acute lymphoblastic leukemia cells and murine BAF3 cells involves activation of STAT1 and STAT5 mediated via the interleukin-7 receptor alpha chain. *Leukemia* **10**, 1317-25. (1996).

56. Wen, R. et al. Jak3 selectively regulates Bax and Bcl-2 expression to promote T-cell development. *Mol Cell Biol* **21**, 678-89. (2001).

57. Grossman, Z., Meier-Schellersheim, M., Sousa, A.E., Victorino, R.M.M., Paul, W.E. CD4+ T cell depletion in HIV infection: Are we closer to understanding the cause? *Nat Med* **8**, 319-23 (2002).

4. Changes in the quality of thymic exportation during early primary human immunodeficiency virus infection

Peripheral blood TREC quantification is the method of choice when evaluating the magnitude and diversity of thymic function. Although it is clear that human immunodeficicency infection (HIV) can lead to changes in thymic function levels[375,376,391], the extent at which the diversity of thymic exportation is affected remains to be assessed.

Initial quantification of both α and β TREC frequencies in multiple human thymocyte subsets confirmed the occurrence of several rounds of proliferation between TCR β and α gene rearrangement events. This proliferation is a consequence of thymocyte β-selection and is believed to enhance the number of possible TCR α and β chain pairing combinations within the pool of "pre-selected" DP thymocytes, thereby leading to a vast array of TCR specificities for the thymus to select from. Here we show that α and β TREC peripheral blood frequencies can be transformed into a novel tool, the α/β TREC ratio, reflective of intrathymic proliferation undergone when maturing in the thymus (extensively discussed in chapters 4 and 7.4). Analysis of TREC frequencies as well as calculation of the peripheral blood α/β TREC ratio from chronically infected human immunodeficiency virus (HIV) patients linked the severe reduction of *de novo* T cell production to be the consequence of impaired intrathymic proliferation or selective elimination of proliferating thymocytes. Furthermore, longitudinal analysis of the peripheral blood α/β TREC ratio and sjTREC frequencies at various stages HIV infection demonstrated that thymic function is rapidly impaired (as soon as 2-3 months following infection).

In chapter 4, we put forward the model in which proliferating "β rearranged" thymocytes, expressing CD4 and one of HIV's coreceptor (mostly CXCR4), are preferentially eliminated intrathymically by either viral-, cytokine- and/or cell-mediated effects and therefore, cannot be exported to the periphery. This selective depletion of proliferating thymocytes is likely to reduce the absolute number of early DP thymocytes as well as to limit TCR α and β chain pairing combinations leading to a quantitative and qualitative impairment of human *de novo* T cell production during HIV infection.

Changes in the quality of thymic exportation during early primary human immunodeficiency virus infection

Marie-Lise Dion[1,2], Rebeka Bordi[1], Jean-François Poulin[1,3], Myriam Sylvestre[1,4], Rachel Corsini[1], Nadia Kettaf[1], Ali Dalloul[5], Rachid Boulassel[6], Ryan Woods[7], Fred Harris[7], Julio Montaner[7], Patrice Debré[5], Jean-Pierre Routy[6], Rafick-Pierre Sékaly[1,2,3,4] and Rémi Cheynier[1].

[1]Laboratoire d'Immunologie, Centre de Recherches du CHUM, Hôtel-Dieu, Montréal, Québec, Canada, [2]Department of Microbiology and Immunology, McGill University, Montréal, Québec, Canada, [3]Department of Medicine, Division of Experimental Medicine, McGill University, Montréal, Québec, Canada, [4]Département de Microbiologie et Immunologie, Faculté de Médecine, Université de Montréal, Québec, Canada, [5]Laboratoire d'Immunologie Cellulaire et Tissulaire, URA CRNS 625, Hopital de La Pitié-Salpêtrière, Paris, Fance, [6]McGill University Health Center, Montreal, Quebec, Canada, [7]Canadian HIV trial network, Vancouver, Canada

Manuscript in preparation

ABSTRACT

Human *de novo* T cell production ensures a constant replenishment of the naïve T cell compartment by exporting competent T lymphocytes that can recognize a wide variety of antigens. Although reduced levels of thymic function have been reported during HIV infection, it remains to be proven whether thymopoiesis is qualitatively impaired. In this report, we quantified sjTRECs as well as 10 DβJβ TRECs molecules from peripheral blood mononucleated cells (PBMCs) of HIV-infected and healthy adults and calculated the peripheral blood α/β TREC ratio, a marker for intrathymic proliferation. Here we show that intrathymic proliferation levels decrease during adulthood and are positively associated with the magnitude of thymic function. We also demonstrate that this alteration occurs as soon as 2-3 months following infection and persists throughout the course of the disease. These results are consistent with the selective depletion of proliferating thymocytes and/or inhibition of their proliferation during thymocyte differentiation. As β selection-induced intrathymic proliferation constitutes a way to increase the pool of "pre- T cell selection" double positive (DP) thymocytes as well as to augment the number of different TCR α chain paired with clonally-expanded TCR β chains, we demonstrate that HIV infection impairs both the quantity and the quality of thymic function.

PATIENTS AND METHODS

Thymocyte extraction

Uninfected pediatric thymic tissues were obtained with the informed consent of the child's tutor and according to the guidelines of the bioethic committee of Hôpital de La-Pitié Salpêtrière, Paris, France. Upon reception, thymic tissues were cut into pieces and thymocytes were harvested according to a standard and published protocol[1].

FACS-purification of thymocyte subsets

Thymocytes were immediately processed following several washes with PBS containing 2% FCS. Briefly, ≈80% of all total thymocytes were enriched in triple negative (TN) thymocytes through depletion of CD4 and CD8 expressing cells using magnetic beads (Milteny Biotech). TN thymocytes were then stained using directly-conjugated anti CD34-PE and anti CD1a-FITC cell surface antibodies. Thymocyte subsets were sorted on a FACSvantage (Becton-Dickinson). In a separate staining, the remaining ≈20% of total unsorted thymocytes were FACS-purified using directly-conjugated anti CD3-APC, anti CD4-PE and anti CD8-FITC. Washes between stainings were performed using standard PBS supplemented with 2% FCS. All stainings were performed at 4 °C for 30 minutes and thymocyte purity was always above 80%. TREC quantification was performed immediately on sorted thymocyte subsets (see TREC quantification section).

PBMCs isolation

Peripheral blood mononucleated cells (PBMCs) from healthy adults and HIV-infected individuals were collected in EDTA-containing tubes through phlebotomy and isolated through standard Ficoll density-gradient techniques. PBMC were washed twice with PBS supplemented with 2% FCS and cryopreserved in liquid nitrogen (10% DMSO, 90% FCS) for further processing.

TREC quantification

Frozen PBMCs were thawed into RPMI 1640 medium containing 10% of inactivated fetal calf serum (FCS) and washed several time with PBS supplemented with 2% FCS. Cells were lysed using a Proteinase K-based lysis protocol and peripheral blood TREC frequencies were quantified using a nested PCR-based assay as previously documented[2]. Briefly, multiplex amplification of either the sjTREC or a DβJβ TREC in combination with the CD3 (housekeeping gene) amplicon was performed using a linear 24 cycles first-round PCR protocol. A second round, simplex, real-time, on-line PCR amplification using the LighCycler (Roche Diagnostics) was done following a 10-fold dilution of the first round PCR product. A serially diluted standard curve[2] was processed at the same time but in separate capillaries. Normalization of sjTREC (or DβJβ TREC) frequencies per 100 000 PBMC (using the CD3 value) was done for each samples. The conditions of this PCR assay were shown to be linear when using no more than 10^5 PBMCs and only varied by a 2 fold factor.

Calculation of the α/β TREC ratio

For each patient and uninfected controls, the sjTREC frequencies were divided by the estimated sum of all β TREC frequencies found in all tested families (a/b TREC ratio = sjTREC / {[ΣDβJβ/ Number of DβJβ family tested]x13}. A minimum of 10 DβJβ families (out of 13 possible) was quantified in order to have an accurate α/β TREC ratio.

Statistics

Statistical analysis (two-tailed Student t test, Pearson's correlation test, *R*, and *P* values) was performed using Microsoft® Excel spread sheets. An *R* value ≥0.3, ≤-0.3, and a *P* value ≤0.05 were considered significant.

INTRODUCTION

TCR rearrangement excision circles (TREC) generated during α and β gene rearrangement are powerful tools to use when monitoring the immune reconstitution process as they can assess the magnitude of thymic function[3] as well as the diversity of recent thymic emigrants (RTEs)[2,4]. Through peripheral blood quantification of sjTREC frequencies, a surrogate marker for thymic function, several groups demonstrated that thymopoiesis levels are impaired during HIV-infection[3,5,6]. Given the susceptibility of human thymocytes to HIV infection[7], it is likely that the observed thymic dysfunction is the result of thymocytes depletion. However, peripheral blood sjTREC frequencies can be influenced by naïve T cell homeostatic proliferation, which casts some doubt on the interpretations of sjTREC frequencies[8], especially during acute HIV infection when massive T cell proliferation occurs[9-12]. Furthermore, fluctuations of peripheral sjTREC frequencies can also be induced through modification of normal cellular trafficking patterns (leading to redistribution/sequestration of sjTREC-containing cells) provoked by changes in viral load. Although the *in vivo* impact of cellular proliferation on sjTREC frequencies have not been evidenced, it remains hard to distinguishing a real thymic dysfunction from increased levels of cellular proliferation.

Fusion of Vβ, Dβ and Jβ segments during TCR gene rearrangement leads to generation of TCR β chain molecules. Successful pairing of coding TCR β chains with the invariant pTα chains and CD3 molecules at the cell surface of differentiating thymocytes leads to β selection, which is characterized by rounds of proliferation and induction of CD4 and CD8 expression. This proliferation increases the pool of "pre-selected" double positive (DP) thymocytes that will undergo TCR α gene rearrangement. Furthermore, β selection-induced proliferation leads to different TCR α/β chain pairing combination as each "clonally-expanded" β selected thymocyte will randomly rearrange a TCR α chain and thus, generate multiple TCR specificities with the same TCR β chain clonotype. This report shows that intrathymic proliferation occurring between both gene rearrangement events can be assessed through the calculation of the peripheral blood α/β TREC ratio. Furthermore, this ratio is severely reduced as early as 2-3 months following HIV

infection and remains low throughout the course of the disease. As the peripheral α/β TREC ratio is unaffected by cellular proliferation, this report clearly document a qualitative and quantitative impairment of thymic function in all stages of HIV infection.

RESULTS

Several rounds of proliferation occur between TCR β and α gene rearrangement

Rearrangement of the TCR β locus (leading to the generation of DβJβ TRECs) was confirmed to be initiated in $CD34^{+}CD1a^{-}$ T cell precursors as DβJβ TREC frequencies increases in this subset (Figure 1*a-b*) Assuming no DβJβ TREC degradation, only cellular proliferation induced by successful β selection of thymocytes can lead to the observed decrease in DβJβ TREC frequencies. Following β selection-induced proliferation, double positive (DP) thymocytes continue to mature and initiate gene rearrangement at the TCR α locus, which deletes a large part of the TCR δ locus through δRec-ψJα rearrangement[13]. A maximum of 1 sjTREC molecule per TCR α allele can be generated and such rearrangement mainly occurs in the $CD3^{LOW}CD4^{+}CD8^{+}$ thymocyte subset (Figure 1a-*b*). Therefore, TCR α gene recombination occurs following β selection-induced thymocyte proliferation, and generates a α/β TREC ratio that is representative of the extent of proliferation that occurred between both gene rearrangement events. Single positive (SP) thymocytes (also harboring an α/β TREC ratio) are then exported in the periphery and, in combination with existing peripheral T cells, will contribute to the generation of the peripheral blood α/β TREC ratio. Such ratio is proportional to the magnitude of the intrathymic proliferation between both gene rearrangement events (Figure 1*c*). Although the number of TCR β and α gene rearrangement attempts varies among differentiating thymocytes, it is anticipated that the peripheral α/β TREC ratio fluctuates according to Figure 1*d* as no more than 4 DβJβ TREC and 2 sjTREC can be generated per thymocytes.

Characteristics of the peripheral blood α/β TREC ratio in healthy uninfected adults

Using this tool, we analyzed thymic function in a cohort of uninfected and healthy individuals (n=29). Being proportional to sjTREC frequencies (R=0.777, P=0.0001) (Figure 2*a*), the peripheral blood α/β TREC ratio is age dependant (R=-0.447, P=0.0133) (Figure 2*b*), showing that this parameter is a good surrogate marker for estimating thymic output as well as the quality of thymic exportation. It also demonstrates that the

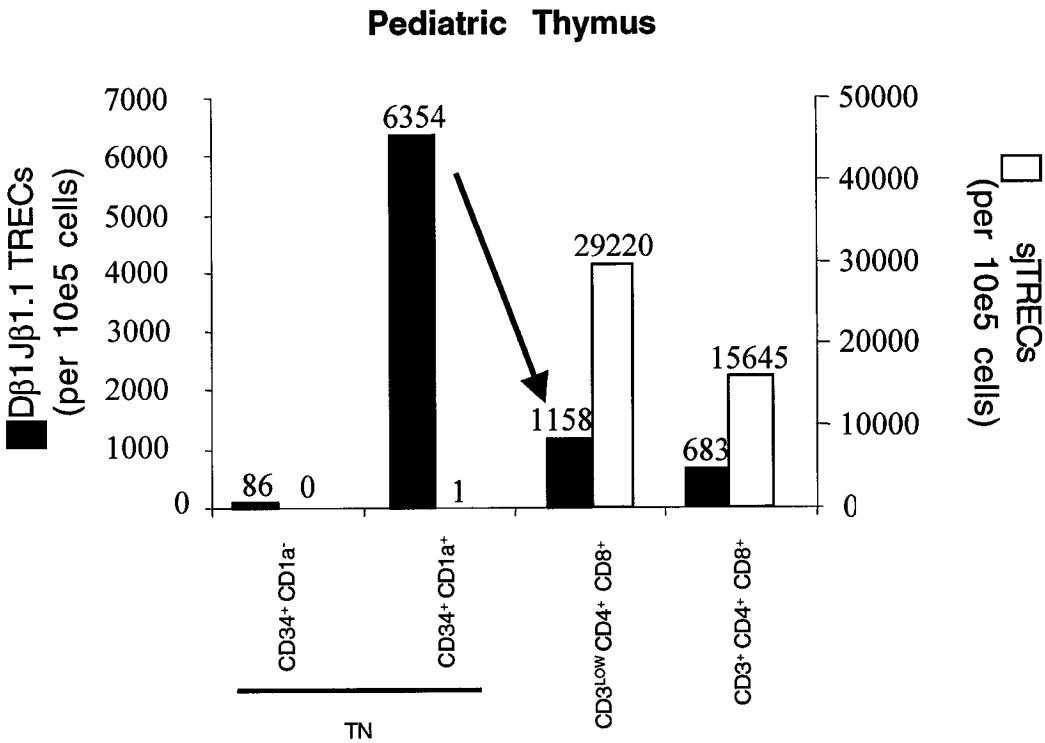
magnitude of thymic function is intimitalely linked to the extent of intrathymic proliferation among developing thymocytes. Moreover, peripheral T cell proliferation, leading to equal dilution of both types of TRECs, does not influence the α/β TREC ratio. Consequently, it can be used to document defects in thymopoiesis irrespective of ongoing proliferation levels or the past replicative history (Figure *2c*).

Severe reduction of intrathymic proliferation levels in early primary HIV-infected patients

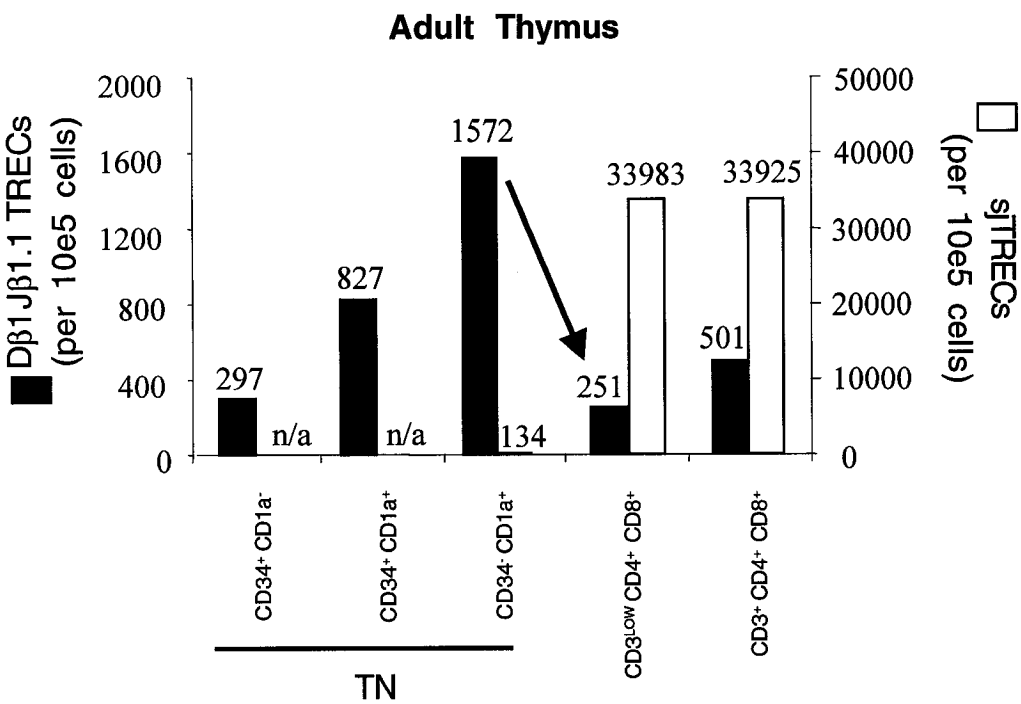
Thymic function was analyzed in a cross-sectional cohort of untreated HIV infected patients (*n*=38) as well as in chronically-infected HIV individuals (*n*=40, 10 patients, 4 time points each) through quantification of peripheral blood sjTREC and DβJβ TREC frequencies, leading to the calculation of the α/β TREC ratio. Patients were sampled less than 98 days (*n*=15, Figure 3*a*), between 109-210 days (*n*=14, Figure 3*b*), between 225-422 days (*n*=9, Figure 3*c*) or more than 422 days (*n*=40, 10 patients, 4 time points, Figure 3*d*) post infection. A drastic reduction of the α/β TREC ratio was observed in each group (α/β TREC ratio = 14.7, 12.2, 13.5 and 13.2 as compared to 55.2 for age matched controls, p=0.004, 0.002, 0.002 and 0.002 respectively) (Figure 3*a-d*). In comparison, the sjTREC levels were barely affected during within the first year post-infection (sjTREC=297, 360, 312 and 247/10^5 cells for all study groups, control individuals sjTREC=668/10^5 cells, p>0.05). As early as 98 days following infection, RTEs present in peripheral blood show a strong reduction of the α/β TREC ratio demonstrating that 1) the RTE population present before HIV infection is rapidly replaced by *de novo* produced T cells harboring a new and lower ratio and 2) striking qualitative modifications of thymic output, rather than quantitative defects, take place early on. The relative stability of sjTREC frequencies may indicate that the lack of intrathymic proliferation is partially compensated by an increased thymic output, possibly as a consequence of increased thymic input[14].

Taken together, these data evidenced a qualitative and quantitative defect of thymic function during all phases of HIV infection.

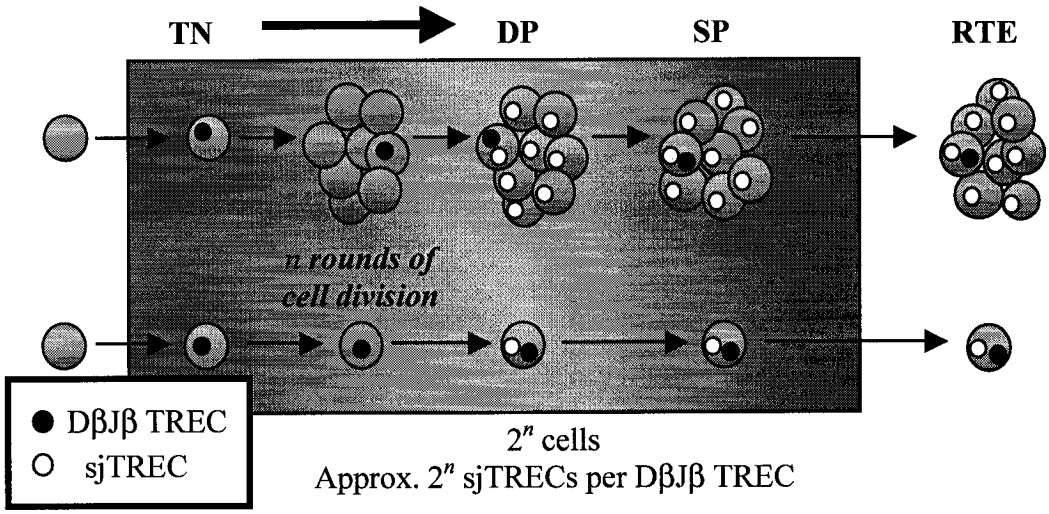
a


Pediatric Thymus
Dβ1Jβ1.1 TRECs (per 10e5 cells)
sjTRECs (per 10e5 cells)
7000
6000
5000
4000
3000
2000
1000
0
50000
40000
30000
20000
10000
0
86
0
6354
1
1158
29220
683
15645
CD34+ CD1a-
CD34+ CD1a+
CD3LOW CD4+ CD8+
CD3+ CD4+ CD8+
TN

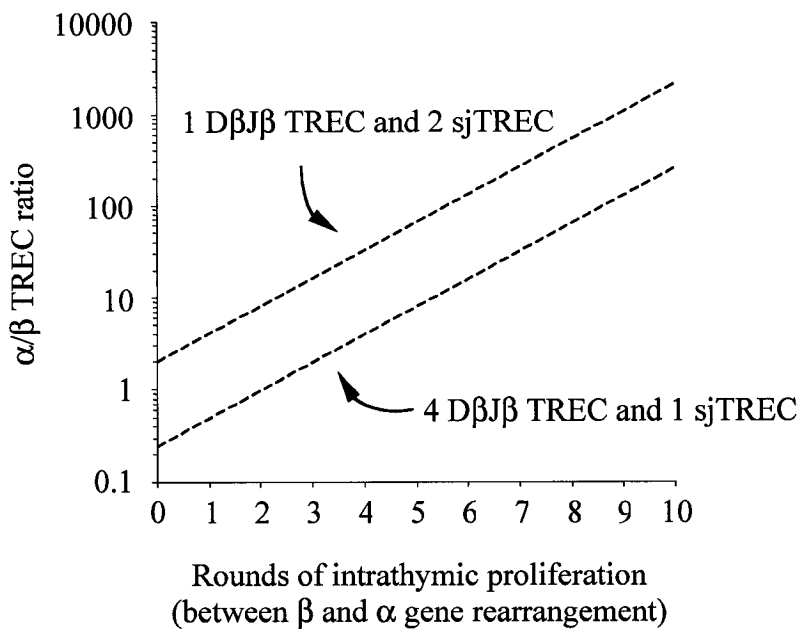
b


Adult Thymus
Dβ1Jβ1.1 TRECs (per 10e5 cells)
sjTRECs (per 10e5 cells)
2000
1600
1200
800
400
0
50000
40000
30000
20000
10000
0
297
n/a
827
n/a
1572
134
251
33983
501
33925
CD34+ CD1a-
CD34+ CD1a+
CD34- CD1a+
CD3LOW CD4+ CD8+
CD3+ CD4+ CD8+
TN

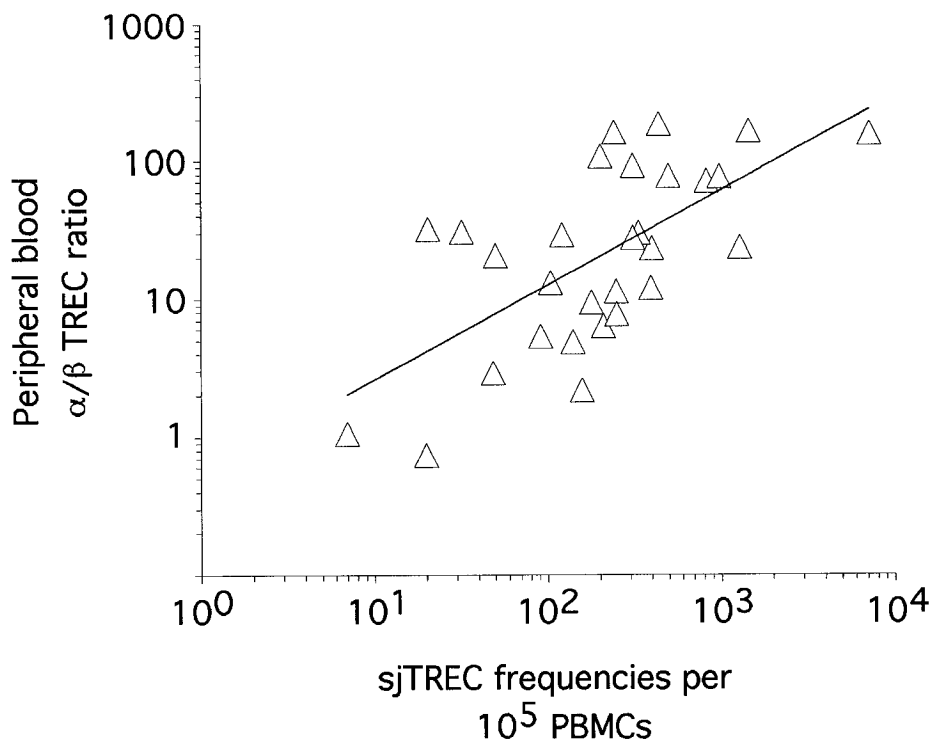
C


TN
DP
SP
RTE
n rounds of cell division
● DβJβ TREC
○ sjTREC
2^n cells
Approx. 2^n sjTRECs per DβJβ TREC

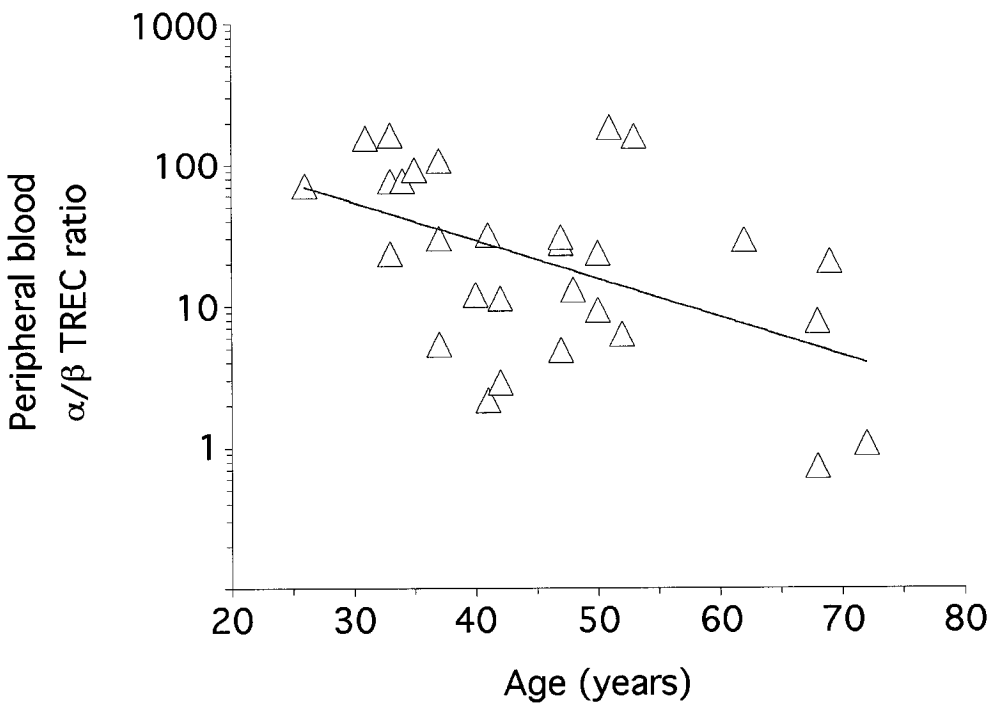
d


10000
1000
100
10
1
0.1
α/β TREC ratio
1 DβJβ TREC and 2 sjTREC
4 DβJβ TREC and 1 sjTREC
0 1 2 3 4 5 6 7 8 9 10
Rounds of intrathymic proliferation
(between β and α gene rearrangement)

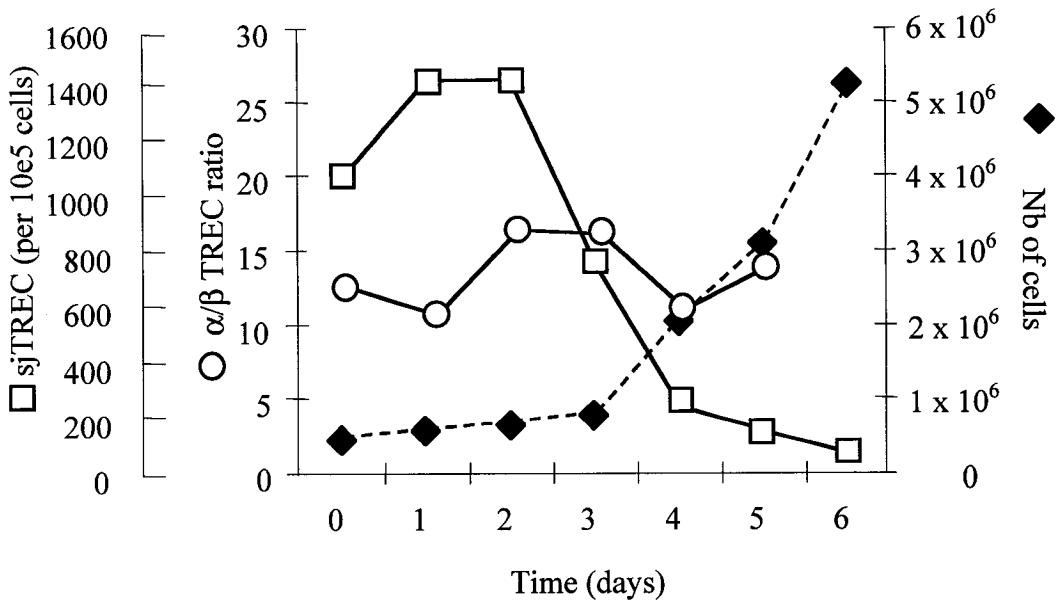
a


1000
100
10
1
Peripheral blood
α/β TREC ratio
10^0
10^1
10^2
10^3
10^4
sjTREC frequencies per
10^5 PBMCs

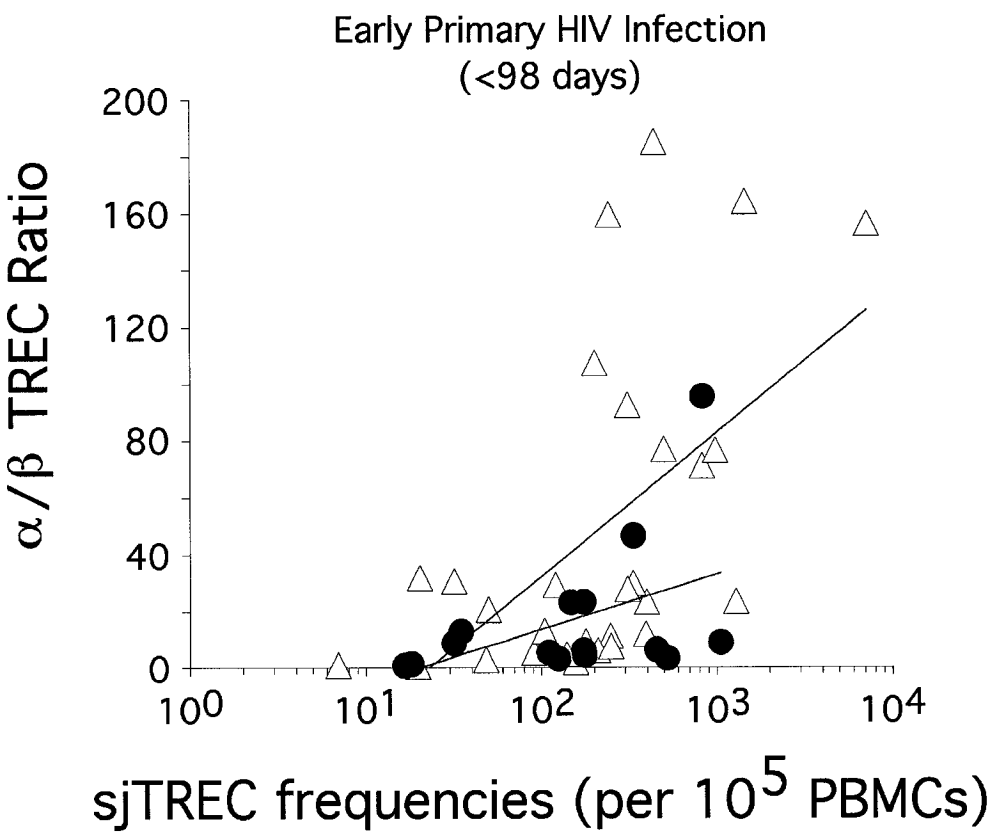
b


Peripheral blood
α/β TREC ratio
1000
100
10
1
20
30
40
50
60
70
80
Age (years)

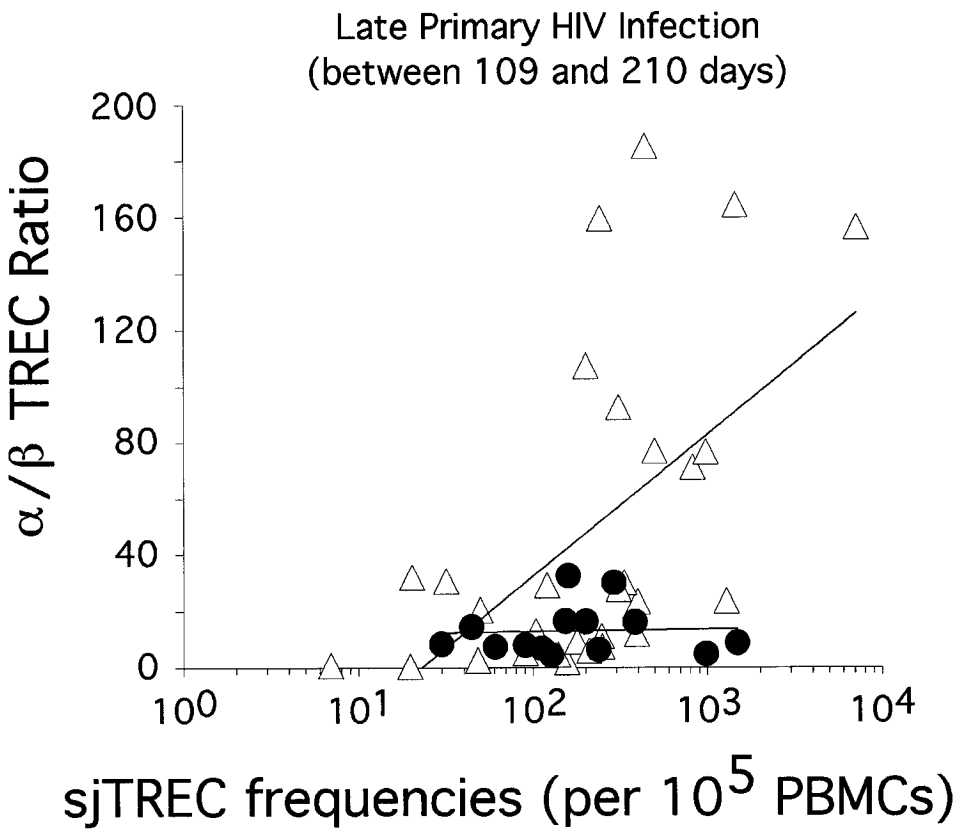
c


□ sjTREC (per 10e5 cells)
1600
1400
1200
1000
800
600
400
200
0
O α/β TREC ratio
30
25
20
15
10
5
0
◆ Nb of cells
6 x 10^6
5 x 10^6
4 x 10^6
3 x 10^6
2 x 10^6
1 x 10^6
0
0 1 2 3 4 5 6
Time (days)

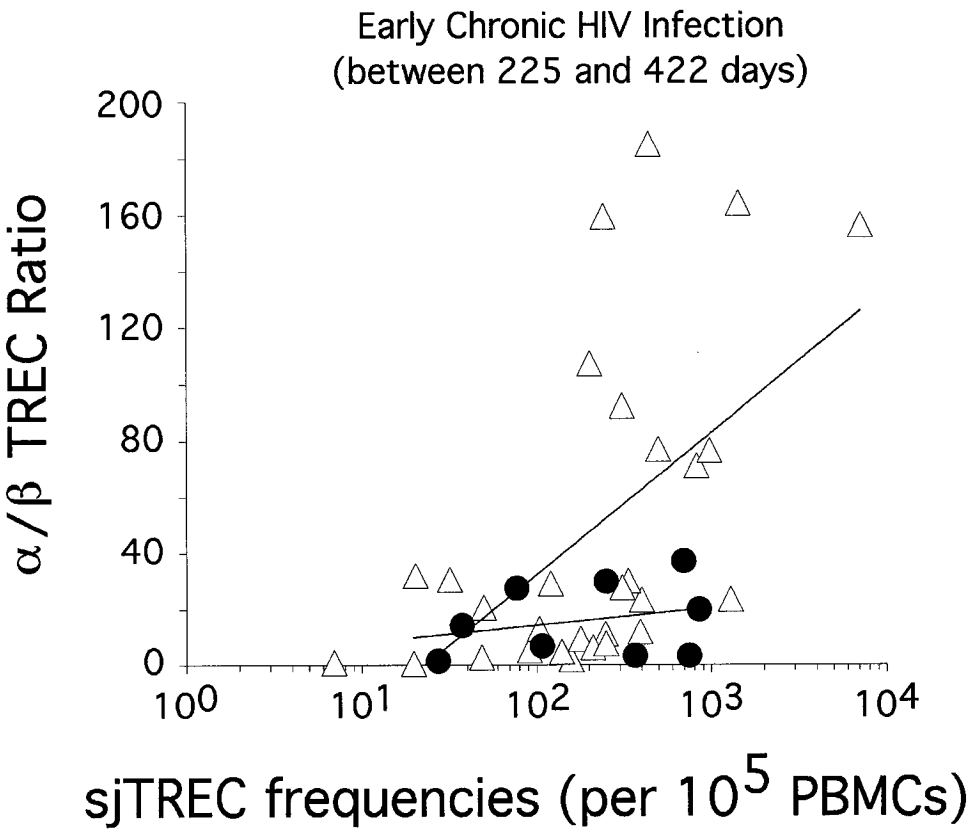
a


Early Primary HIV Infection
(<98 days)
α/β TREC Ratio
200
160
120
80
40
0
10^0
10^1
10^2
10^3
10^4
sjTREC frequencies (per 10^5 PBMCs)

b


Late Primary HIV Infection
(between 109 and 210 days)
200
160
120
80
40
0
α/β TREC Ratio
10^0
10^1
10^2
10^3
10^4
sjTREC frequencies (per 10^5 PBMCs)

c


Early Chronic HIV Infection
(between 225 and 422 days)
200
160
120
80
40
0
α/β TREC Ratio
10^0
10^1
10^2
10^3
10^4
sjTREC frequencies (per 10^5 PBMCs)

d

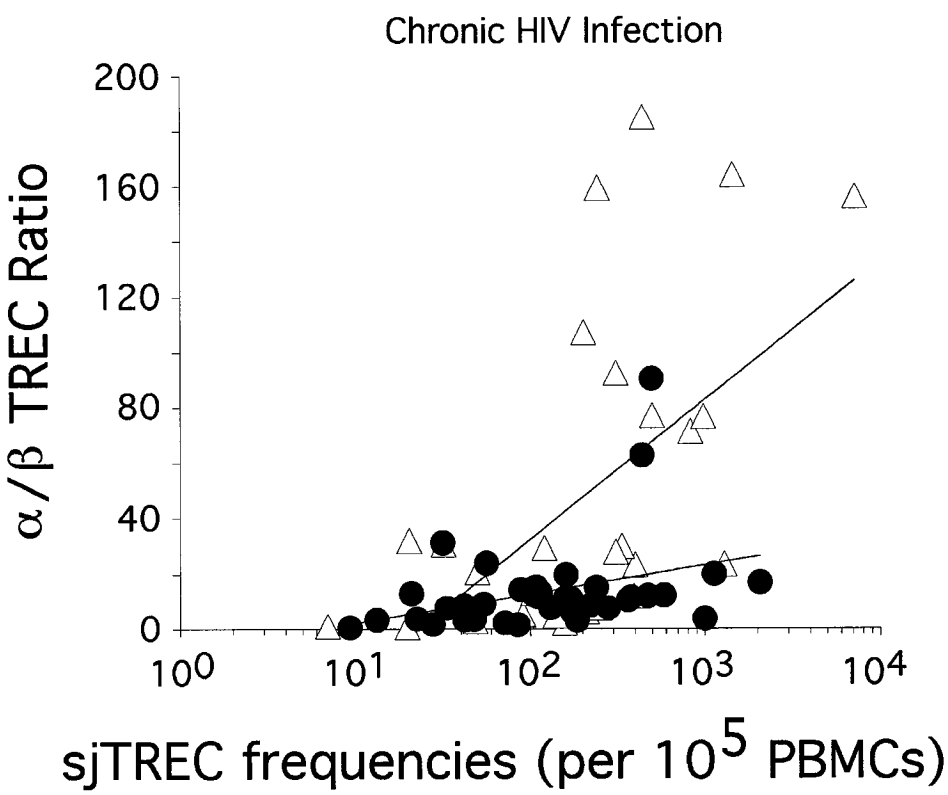

Chronic HIV Infection
200
160
120
80
40
0
α/β TREC Ratio
10^0
10^1
10^2
10^3
10^4
sjTREC frequencies (per 10^5 PBMCs)

FIGURE LEGENDS

Figure 1. Quantification of DβJβ and sjTREC frequencies in multiple thymocyte subsets. Representative histograms illustrating the sjTREC and DβJβ TREC frequencies appearance/dilution patterns from pediatric (***a***) and adult (***b***) thymic tissues. TREC quantification was performed in triple negative (TN) and double positive (DP) thymocytes. Black bars correspond to DβJβ TREC frequencies whereas white bars represent sjTREC frequencies. The number above each histogram bar indicates the calculated frequencies per 10^5 sorted thymocytes. ***c.*** Schematic representation of T cell ontogeny and gene rearrangement events leading to high (above) and low (below) α/β TREC ratio. The extent of proliferation between both gene rearrangement leads to the dilution of DβJβ TREC (black circles) and the α/β TREC ratio is established when sjTREC molecules are generated during TCR α gene rearrangement (white circles). Once established, the α/β TREC ratio remains constant within the thymus as T cell selection/exportation processes are believed to be independent of the presence/absence of a TREC. ***d.*** Maximum and minimum values for the peripheral α/β TREC ratio. Given the genomic organization of each TCR loci, 1 to 4 DβJβ TREC per thymocyte can be generated during TCR β gene rearrangement. Taken in combination with the fact that 1 or 2 sjTREC can be formed during TCR α rearrangement, the peripheral blood ratio is anticipated to range between a maximum of 2 and a minimum of 0.25 for thymocytes that did not proliferate between both gene rearrangement events.

Figure 2. Characteristics of the peripheral blood α/β TREC ratio in healthy individuals. ***a.*** Age-related decrease of the peripheral blood α/β TREC ratio. Quantification of both types of TRECs in uninfected controls leads to the calculation of the peripheral blood ratio, which is representative of intrathymic proliferation. ***b.*** Relationship between sjTREC frequencies and intrathymic proliferation levels. The solid lines correspond to the trendline. ***c.*** Cellular proliferation influences sjTREC frequencies but not the peripheral blood α/β TREC ratio *in vitro* as both types of TRECs are equally diluted. PBMCs from one healthy adult were stimulated by monoclonal CD3 antibodies in

addition with 2 U of recombinant IL-2 for 6 days. Cellular counts (filled diamonds), sjTREC frequencies (empty squares) and the α/β TREC ratio (empty circles) were determined. No changes in the α/β TREC ratio was notice whereas sjTREC frequencies were inversely correlated with cellular proliferation.

Figure 3. Severe reduction of the peripheral α/β TREC ratio in all phases of HIV infection. HIV-infected individuals were classified according to their estimated date of infection based on D-tuned assay, p24 ELISA and Western blotting (***a-d***) and the α/β TREC ratio as well as sjTREC frequencies were determined for each patients and healthy control individuals as mentioned in the patients and methods section. Empty triangles represent healthy controls whereas filled circles correspond to HIV-infected patients. Solid lines represent trendline.

REFERENCES

1. Rabin, L. et al. Use of standardized SCID-hu Thy/Liv mouse model for preclinical efficacy testing of anti-human immunodeficiency virus type 1 compounds. *Antimicrob Agents Chemother* **40**, 755-62. (1996).
2. Poulin, J.-F., Sylvestre, M., Champagne, P., Dion, M.-L., Kettaf, Dumont, A., Lainesse, M., Fontaine, P., Perreault, C., Sékaly, R.-P., Cheynier, R. Immunodeficiency following allogenic hematopoietic stem cell transplantation is not due to a thymic defect but the consequence of impaired naive T cell survival. *Submitted.*
3. Douek, D.C. et al. Changes in thymic function with age and during the treatment of HIV infection. *Nature* **396**, 690-5. (1998).
4. Poulin, J.F., Viswanathan, M. N., Harris, J. M., Komanduri, K. V., Wieder, E., Ringuette, N., Jenkins, M., McCune, J. M., Sekaly, R.-P. Direct evidence for thymic function in adult humans. *J Exp Med* **190**, 479-86. (1999).
5. Hatzakis, A. et al. Effect of recent thymic emigrants on progression of HIV-1 disease. *Lancet* **355**, 599-604. (2000).
6. Zhang, L. et al. Measuring recent thymic emigrants in blood of normal and HIV-1-infected individuals before and after effective therapy. *J Exp Med* **190**, 725-32. (1999).
7. Bonyhadi, M.L. et al. HIV induces thymus depletion in vivo. *Nature* **363**, 728-32. (1993).
8. Hazenberg, M.D. et al. Increased cell division but not thymic dysfunction rapidly affects the T-cell receptor excision circle content of the naive T cell population in HIV-1 infection. *Nat Med* **6**, 1036-42. (2000).
9. Grossman, Z., Meier-Schellersheim, M., Sousa, A.E., Victorino, R.M. & Paul, W.E. CD4+ T-cell depletion in HIV infection: are we closer to understanding the cause? *Nat Med* **8**, 319-23. (2002).

10. Kovacs, J.A. et al. Identification of dynamically distinct subpopulations of T lymphocytes that are differentially affected by HIV. *J Exp Med* **194**, 1731-41. (2001).
11. Mohri, H., Bonhoeffer, S., Monard, S., Perelson, A.S. & Ho, D.D. Rapid turnover of T lymphocytes in SIV-infected rhesus macaques. *Science* **279**, 1223-7. (1998).
12. Mohri, H. et al. Increased turnover of T lymphocytes in HIV-1 infection and its reduction by antiretroviral therapy. *J Exp Med* **194**, 1277-87. (2001).
13. Verschuren, M.C. et al. Preferential rearrangements of the T cell receptor-delta-deleting elements in human T cells. *J Immunol* **158**, 1208-16. (1997).
14. Wykrzykowska, J.J. et al. Early regeneration of thymic progenitors in rhesus macaques infected with simian immunodeficiency virus. *J Exp Med* **187**, 1767-78. (1998).

5. FACS-purification of recent thymic emigrants using a novel transgenic model

This section is currently in progress and aims at validating a concept that would allow the development of a transgenic mouse model leading to the physical isolation of recent thymic emigrants (RTEs). Although it is possible to monitor thymic activity in humans, it remains impossible to purify RTEs from the heterogenous PBMC population. The transgenic model concept presented in chapter 5 would permit the rapid identification of mouse RTEs as a "transgenic TREC", capable of producing the green fluorescent protein (GFP), would be generated during thymocyte development. This concept is currently protected by provisory patent laws and should enable us to obtain a highly purified population of mouse RTE and precisely perform studies aiming at understanding RTE biology as well as mouse T cell homeostasis.

FACS-purification of recent thymic emigrants using a novel transgenic mouse model

Jean-François Poulin[1,2], Martin Bourbonnière[3], Rafick-Pierre Sékaly[1,2,3,4] and Rémi Cheynier[1]

[1]Laboratoire d'Immunologie, Centre de Recherches du CHUM, Hôtel-Dieu, Montréal, Québec, Canada, [2]Department of Medicine, Division of Experimental Medicine, McGill University, Montréal, Québec, Canada, [3]Laboratoire d'Immunologie, Departement de Microbiologie et Immunologie, Université de Montréal, Québec, Canada, [4]Département de Microbiologie et Immunologie, Faculté de Médecine, Université de Montréal, Québec, Canada.

Manuscript in preparation

ABSTRACT

Peripheral blood quantification of TCR α and β rearrangement excision/deletion circles (TREC) is currently the method of choice for monitoring ongoing thymopoiesis as no conclusive human and mouse recent thymic emigrant (RTE)-specific markers were identified. These PCR-based assays require total cell lysate or genomic DNA, thus leading to the impossibility of physically isolating RTEs (e.g. TREC-containing T lymphocytes). The transgenic mouse model presented here aims at inducing green fluorescent protein (GFP) expression in RTEs exclusively. In order to do so, we generated a DNA construct comprising an inverted promoter and GFP sequences flanked by recombination signal sequences (RSS) and TCR α silencer/enhancer elements. It is anticipated that this DNA trangene will be rearranged during T cell differentiation and form a "transgenic TREC" capable of supporting GFP expression. Validation of the proof-of-concept was performed by synthesizing theoretical "post-rearrangement" constructions bearing different promoter (SRα and CD3δ) and enhancer (CD3δ and IgM) sequences and transiently transfecting them in Jurkat E6.1 cell lines. All tested constructions showed strong levels of fluorescence and a maximal GFP expression when the SRα-CD3δ combination was used. Given these promising results, "pre-rearrangement" constructions are currently being generated and will be further tested for their ability to recombine *in vitro* and produce fluorescence. Such DNA constructs will then be packaged in retroviruses for *ex vivo* transfection of hematopoietic precursors before re-infusion in an irradiated mouse. A self-propagating transgenic mouse model (GreenMouse) will then be generated and ought to provide new insights on RTE homeostasis as well as helping defining phenotypic and molecular characteristics of RTEs. Furthermore, we believe this model will facilitate the screening of potential drugs capable of modulating thymic function.

MATERIAL AND METHODS

Generation of DNA constructions

All DNA constructions were generated using standard molecular biology techniques. For practical cloning reasons, the Bluescript plasmid (Stratagene) multiple cloning site (MCS: from SacI to KpnI) was replaced with a customized MCS bearing the appropriate enzyme restriction sites needed for the cloning strategy. Isolation/purification of DNA constructs was done using the QIAGEN midi-prep kit (QIAGEN) and all constructions were sequenced prior transfection.

Transfection of Jurkat E6.1 cells and FACS analysis

Jurkat E6.1 cell lines were grown in RPMI 1640 medium supplemented with glutamine and 10% FCS. Fresh medium was added each 3 days. Transient transfection of Jurkat cells was performed using a standard electroporation-based protocol. Briefly, cells were counted and resuspended at 10×10^6/mL. 5×10^6 Jurkat E6.1 cells prior electroporation at 4 °C (260 V and 950 uF) using 50 µg of the DNA plasmids. Cells were allowed to recuperate for 48 hours before acquisition of GFP fluorescence using a FACS Vantage (Becton-Dickinson).

INTRODUCTION

Conventional PCR-based techniques capable of monitoring *de novo* T cell production and its diversity require total cell lysate or extracted genomic DNA[1-3]. Unfortunately, these experimental assays cannot identify RTEs but only evaluate their frequencies within the peripheral blood mononucleated cells (PBMCs). Hence, it is now impossible to isolate a pure population of RTEs from the heterogeneous naïve compartment and determine its genetic expression profile as no RTE-specific cell surface marker (or a combination of markers) has been identified. This limits our understanding of the biology of RTEs as well as hampers the comprehension of their role in T cell homeostasis.

Attempts have been made to identify a phenotype specific of the human RTE compartment. Using the FTOC system, Lee and colleagues[4] were able to demonstrate that RTEs preferentially express CTLA-4, the ligand of CD80/CD86 (B7.1/B7.2). CD103 was also identified as a possible candidate marker[5] but falls short of being express in both the CD4 and CD8 compartments.

RESULTS

TCR rearragement excision circles (TRECs) were repeatedly demonstrated to be good surrogate markers of the RTE compartment. TRECs are mainly present in naïve T cells and absent from memory T cell and B lymphocytes[1,2]. In order to FACS-purify RTEs from the periphery, we have generated a DNA transgene theoretically capable of *in vivo* recombination leading to GFP expression (Figure 1). The combined presence of RSSs and TCR α silencer/enhancer elements is anticipated to recruit the RAG machinery in T cells and lead to the rearrangement of the DNA transgene. This will fuse a promoter sequence in-frame with the GFP gene. In order to ensure that GFP expression would be limited to RTEs, we relocated the endogenous ATG codon of the GFP gene at the end of the promoter sequence and added STOP codons in all 3 open reading frames (ORF) downstream of the TCR α silencer/enhancer elements to minimize residual GFP expression in non-rearranged cell types. In summary, it is anticipated that during T cell ontogeny, excision of most of the transgene from the genome will occur and lead to the generation of a "transgenic TREC" believed to behave similarly to sjTREC molecules and capable of supporting GFP expression.

"Post-rearrangement" constructs support GFP expression *in vitro*

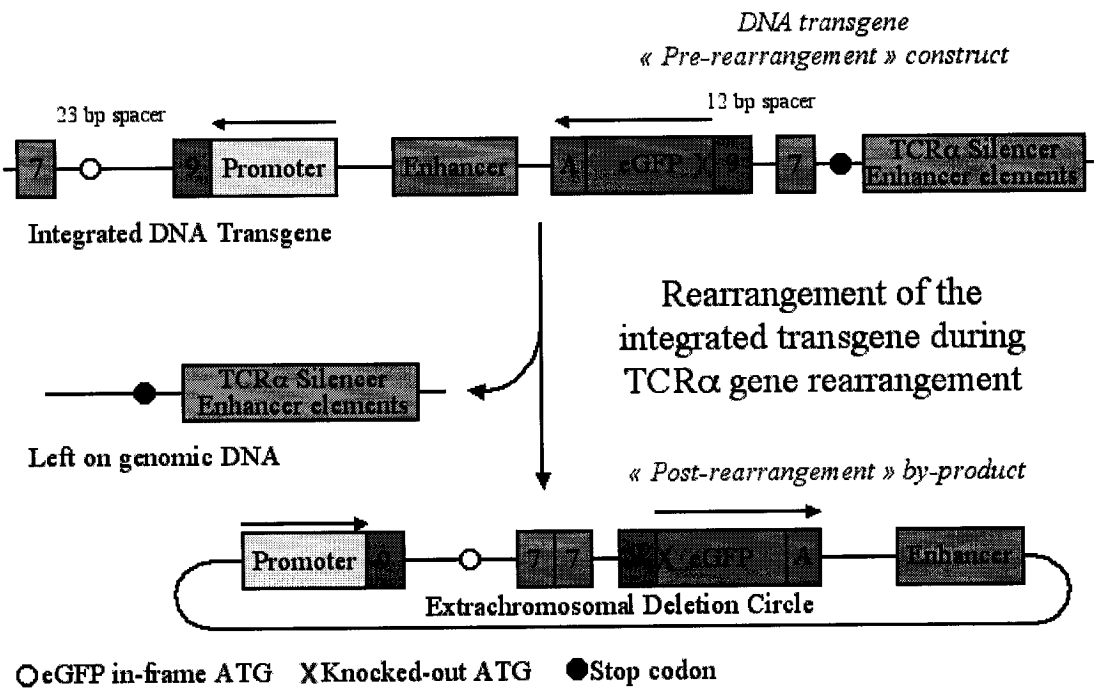
This project was divided in 2 parts. We first validated whether a rearranged transgene could support GFP expression. In order to test this, 4 different "post-rearrangement" constructions, bearing different promoters (CD3δ and SRα) and enhancers (CD3 and IgM) were synthesized (Figure 2). These "post-rearrangement" DNA constructs simulated gene rearrangement of the transgene. As a result of the ATG relocation, a supplementary mRNA transcript sequence, comprising both recombination signal sequence (RSSs), is generated upstream (N-terminal region) of the GFP gene. Furthermore, stem-loop structures could form between the ATG codon and the GFP gene (because of the sequence homology between both heptamers and nonamers), possibly reducing translation efficiency and GFP expression (Figure 3). As illustrated in figure 4*a*, all 4 DNA constructs were capable to induce high levels of fluorescence in Jurkat E6.1 cells, therefore indicating that mRNA molecules can support GFP expression. Furthermore, longitudinal analysis of GFP expression revealed that addition of this

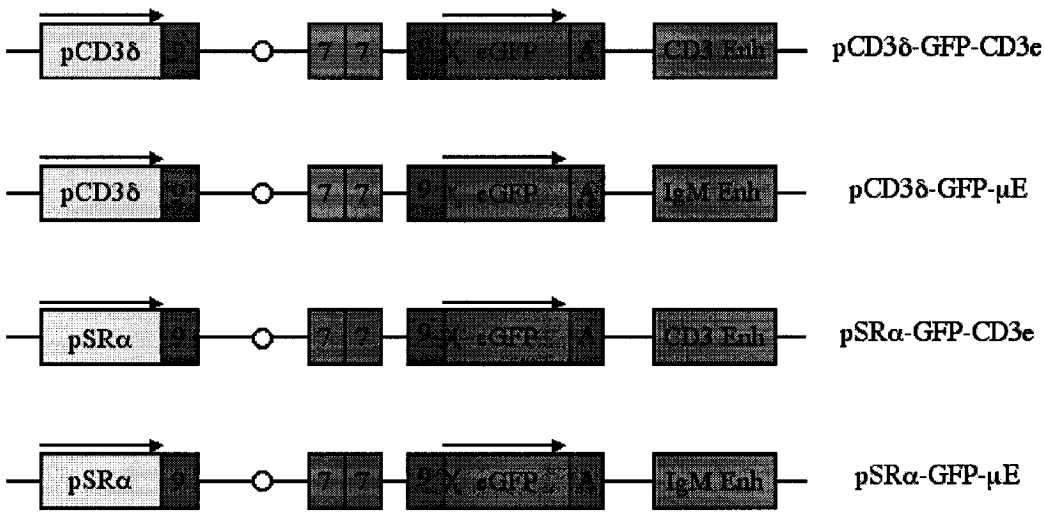
sequence did not alter the stability of the GFP protein, as evidenced by similar decay rate between the positive control vector (peGFP-C1) and all 4 post-rearrangement constructions in the % of GFP^+ Jurkat cells and mean fluorescence intensity (MFI) of positive cells (Figure 4*b* and *c*).

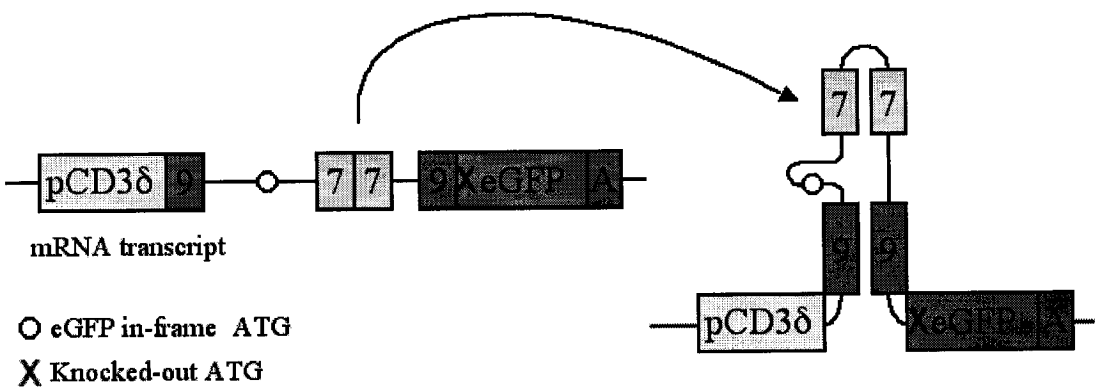
It remains to be proven whether the genetic elements cloned in the "pre-rearrangement" construction (TCR α silencer/enhancer and silencer 2) are capable and sufficient to recruit the RAG machinery and adequately perform rearrangement of the transgene ("pre-rearrangement" construction). In order to determine if rearrangement of the DNA transgene can be achieved *in vitro*, longitudinal FACS-analysis of RAG-1-inducible cell lines transfected with the "pre-rearrangement" construction will be performed. As a result of RAG induction, it is anticipated that rearrangement of the transgene will occur and lead to the gradual appearance of GFP^+ cells.

To this date, all "pre-rearrangement" constructions have been generated and will be transfected in the near future in order to complete the second part of the validation of the proof-of-concept. Following successful induction of "pre-rearrangement" construction recombination, *ex vivo* transfection of mouse T cell precursors with retroviruses carrying the transgene will be used to generate the first generation GreenMouse model. A transgenic version of this model will then follow.

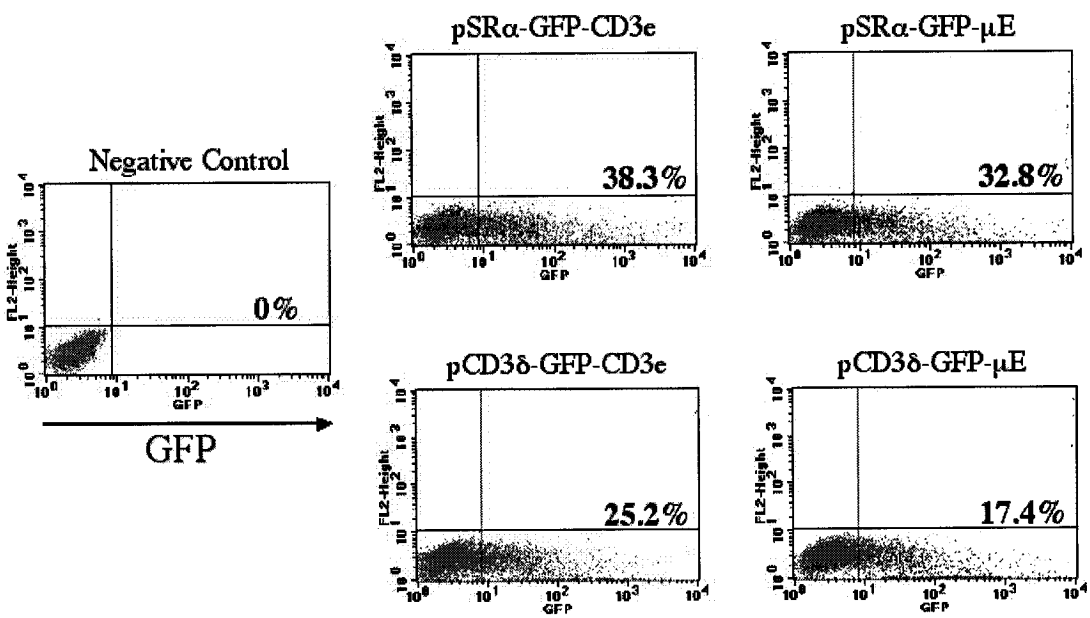
The data presented here pave the way for the generation of a transgenic mouse model from which it will be possible to obtain a pure population of RTEs. Chapter 7.9 summarize the potential of this mouse model and enumerate some experiments that could be performed.


DNA transgene
« Pre-rearrangement » construct
23 bp spacer
12 bp spacer
Promoter
Enhancer
eGFP
TCRα Silencer Enhancer elements
Integrated DNA Transgene
Rearrangement of the integrated transgene during TCRα gene rearrangement
TCRα Silencer Enhancer elements
Left on genomic DNA
« Post-rearrangement » by-product
Promoter
eGFP
Enhancer
Extrachromosomal Deletion Circle
eGFP in-frame ATG
Knocked-out ATG
Stop codon

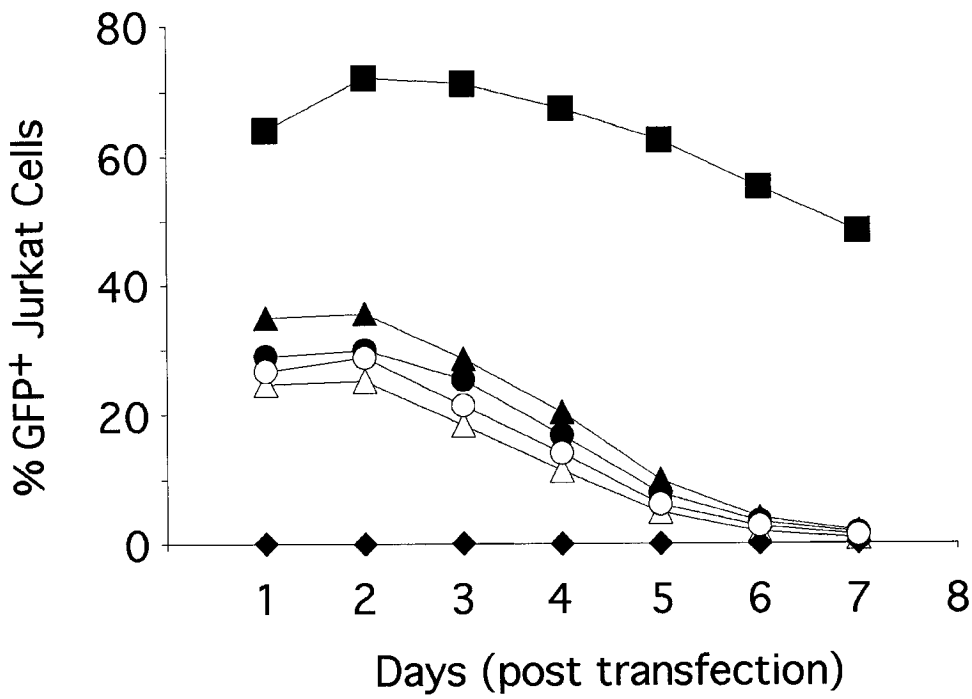

pCD3δ
CD3 Enh
pCD3δ-GFP-CD3e
pCD3δ
IgM Enh
pCD3δ-GFP-μE
pSRα
CD3 Enh
pSRα-GFP-CD3e
pSRα
IgM Enh
pSRα-GFP-μE


pCD3δ
9
7
7
9
eGFP
A
mRNA transcript
O eGFP in-frame ATG
X Knocked-out ATG
7
7
9
9
pCD3δ

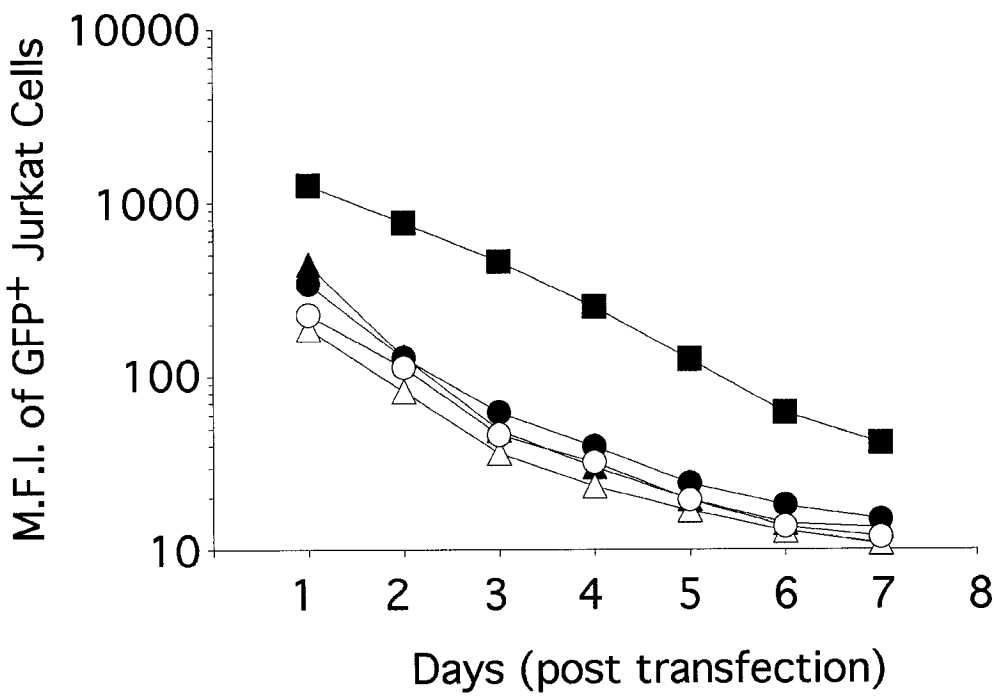
a


Negative Control
0%
GFP
pSRα-GFP-CD3e
38.3%
pSRα-GFP-μE
32.8%
pCD3δ-GFP-CD3e
25.2%
pCD3δ-GFP-μE
17.4%
FL2-Height
GFP

b


80
60
40
20
0
% GFP+ Jurkat Cells
1
2
3
4
5
6
7
8
Days (post transfection)

c


10000
1000
100
10
M.F.I. of GFP+ Jurkat Cells
1
2
3
4
5
6
7
8
Days (post transfection)

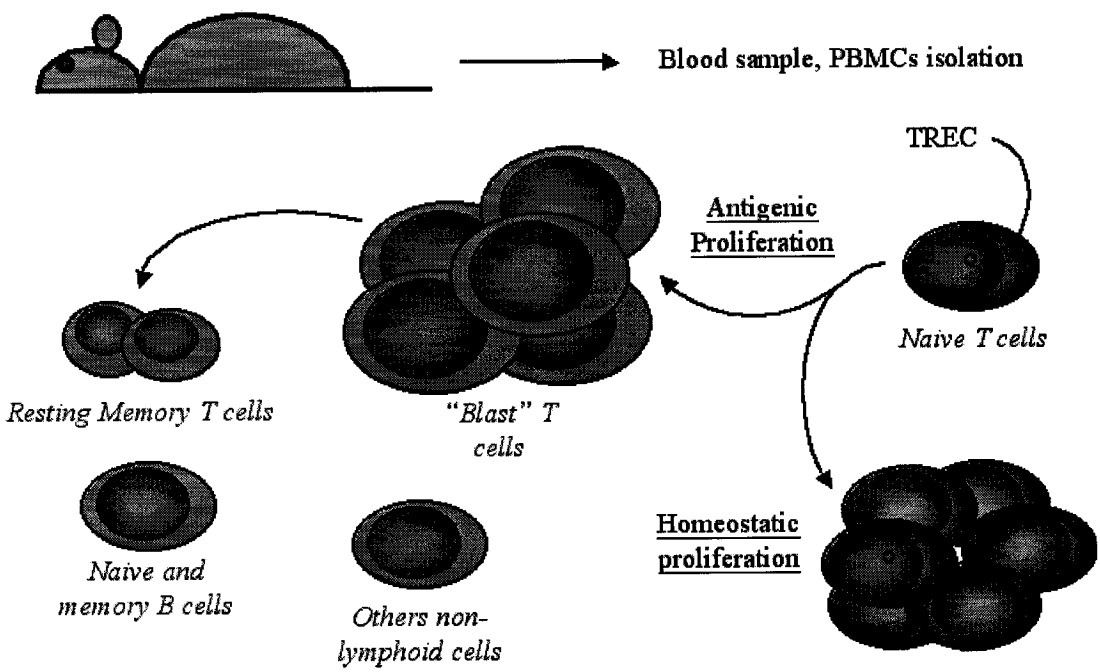

Blood sample, PBMCs isolation
TREC
Antigenic Proliferation
Naive T cells
Resting Memory T cells
"Blast" T cells
Naive and memory B cells
Others non-lymphoid cells
Homeostatic proliferation

FIGURE LEGENDS

Figure 1. Representation of the transgenic DNA capable of supporting GFP expression following *in vivo* recombination. It is anticipated that the DNA transgene will recruit the RAG machinery despite its integration site and become rearranged. Such rearrangement would fuse both RSSs together and bring the promoter sequence in-frame with the GFP gene. Therefore, a "transgenic TREC" capable of supporting GFP expression will be excised from the genome. 9: nonamers, 7: heptamers, triangle: relocated ATG codon, X: knocked-out GFP endogenous ATG, octogone: stop codon, A: polyadenylation signal sequence. The dashed arrows indicate the sequence/gene orientation.

Figure 2. "Post-rearrangement" constructions tested *in vitro* for their abilities to support GFP expression. All 4 constructions were cloned in Bluescript vector and are identical with the exception of their promoter and enhancer sequences.

Figure 3. Plausible secondary structure of the transgene transcript leading to inefficient translation of the GFP gene. Because of the sequence homology of heptamers and nonamers, both RSS could bind to each other and form a stem-loop structure leading to reduced levels of GFP expression. 9: nonamers, 7: heptamers, triangle: relocated ATG codon, X: knocked-out GFP endogenous ATG, octogone: stop codon, A: polyadenylation signal sequence.

Figure 4. "Post-rearrangement" DNA constructions can support GFP expression *in vitro*. ***a.*** FACS analysis of GFP-expressing Jurkat E6.1 cells following transient transfection of 4 different "post-rearrangement" DNA constructs (Figure 2). The x axis represent GFP expression levels and no parameters has been entered for the y axis. Representative FACS dot plot of 2 independent experiments. The % are the mean of both experiments. Time-course experiment where the % of GFP^+ Jurkat (***b***) and the M.F.I. of GFP^+ cells (***c***) were quantified for all 4 constructions. Filled squares correspond to the positive control (peGFP-C1, Stratagene). Filled symbols represent constructions containing the SRα promoter whereas open symbols correspond to constructions containing pCD3δ

promoter. Circles represent constructions containing the IgM enhancer whereas triangles represent DNA construct with the CD3δ enhancer.

Figure 5. The GreenMouse transgenic model. Although each cells from such model will contain the DNA transgene, the presence of theTCR α silencer/enhancer will restrict GFP expression to RTEs. Thymocytes undergoing TCR α gene rearrangement ought to rearrange the DNA transgene and express GFP. Following exportation, RTEs may encounter antigens and participate in the development of an immune response, which involves massive cell division. Consequently, proliferating lymphocytes will quickly "dilute out" the transgenic TREC and lead to decreased levels of GFP expression. Homeostatically-dividing T cell may be able to generate a GFP^{LOW} subset of RTEs. Non-lymphoid cells, B lymphocytes and resting memory T lymphocytes are anticipated to lack GFP expression.

REFERENCES

1. Poulin, J.F. et al. Direct evidence for thymic function in adult humans. *J Exp Med* **190**, 479-86. (1999).
2. Douek, D.C. et al. Changes in thymic function with age and during the treatment of HIV infection. *Nature* **396**, 690-5. (1998).
3. Poulin, J.-F., Sylvestre, M., Champagne, P., Dion, M.-L., Kettaf, Dumont, A., Lainesse, M., Fontaine, P., Perreault, C., Sékaly, R.-P., Cheynier, R. Immunodeficiency following allogenic hematopoietic stem cell transplantation is not due to a thymic defect but the consequence of impaired naive T cell survival. *Submitted.*
4. Lee, C.K. et al. Thymic emigrants isolated by a new method possess unique phenotypic and functional properties. *Blood* **97**, 1360-9. (2001).
5. McFarland, R.D., Douek, D.C., Koup, R.A. & Picker, L.J. Identification of a human recent thymic emigrant phenotype. *Proc Natl Acad Sci U S A* **97**, 4215-20. (2000).

6. Original contribution to scientific knowledge

The results presented in this thesis contribute to scientific knowledge in several aspects.

The work presented in chapter 2 is an original report that documents thymic function in adult humans through the use of a novel assay developed in collaboration with Dr. J. M. McCune. This work elegantly shows that the thymus continues to export T cells that contribute to the diversification of the peripheral T cell repertoire during adulthood. In fact, the assay presented in chapter 2 can monitor the extent of diversity of RTEs through quantification of distinct by-products (β DC or β TREC) specific for the different TCR β gene rearrangement combinations. This work contributes to expose the previously unsuspected importance of the adult thymus in the constant replenishment of the immune system.

The results presented in chapter 3 unequivocably demonstrate that thymopoiesis remains intact in AHSCT patients free of GVHD at sampling time. Critically, we show that the long-lasting reduction in peripheral blood frequencies cannot be the consequence of a decreased thymic activity but the result of an impaired naïve T cell survival. Evidences gathered in this chapter suggest that naïve T cell undergo homeostatic proliferation but die in the process of doing so as a result of a diminished CD127 expression. A peripheral niche defect is evidenced, as the immune system cannot adequately maintains peripheral naïve T cells in the periphery.

The findings presented in chapter 4 illustrate for the first time the impact of HIV on the qualitative nature of thymopoiesis. In fact, we demonstrate that the RTE compartment of HIV-infected individuals essentially encompasses T cells that did not undergo proliferation while being educated in the thymus. This is compatible with a model where HIV selectively depletes proliferating thymocytes or selectively inhibits their intrathymic proliferation. To this date, this work constitutes the first to demonstrate an alteration in the qualitative nature of thymic exportation as a result of HIV infection. We also provide evidences suggesting that the level of intrathymic proliferation is directly linked to the magnitude of thymic exportation. Given the importance of thymocyte/epithelial cells "crosstalks" as well as the age-dependant decrease of intrathymic proliferation levels,

reversal of thymic involution can be envisaged through therapeutical interventions aiming at maintaining high levels of intrathymic proliferation in the adult. This would lead to a greater TCR diversity generation as well as increased exportation levels.

The work presented in chapter 5 could lead to a promising transgenic mouse model that will allow FACS-purification of recent thymic emigrants (RTEs) and thus, pave the way for the identification of a mouse RTE-specific cell surface marker. As will be discusses in greater detail in chapter 7.9, this model will make possible studies that aims at characterizing the genetic expression profile of mouse RTEs and thus, better understand their biology and their role in the immune system.

Taken together, evidences gathered in this thesis support the notion that the human thymus is active in adulthood and contributes to T cell homeostasis by exporting new T cell bearing diverse specificities, even after AHSCT.

7. Discussion

7.1 The importance of human thymic function

"Resurrection" of the human thymus?

Until recently, the thymus had been thought of as an organ/gland that serves no significant function in humans following puberty, mainly because of its age-related involution (gradual replacement of TES by PVS). Further supporting this concept is the fact that adult thymectomy is not associated with a decreased immunity and is often damaged (and subsequently removed) during heart surgery. Moreover, thymectomy constitutes a good treatment for myasthenia gravis (MG), an autoimmune disease characterized by an intrathymic accumulation of autoreactive B cells secreting autoantibodies against the acetylcholin receptor. Despite some arguments suggesting that the thymus plays a negligible role during adulthood, this thesis provides direct evidence supporting the hypothesis that thymopoiesis remains functional in adulthood and participates in the maintenance of a diversified pool of naïve T cells.

Influx of RTE ensures the maintenance of a broad and functional TCR repertoire

Although adult thymectomy is not lethal, efficient thymopoiesis is crucial for the development of an adequate immune system prior to birth as well as in clinical scenarios where immune reconstitution would be beneficial, such as following allogeneic hematopoietic stem cell transplantation (AHSCT), cancer chemotherapy or during highly active antiretroviral therapy (HAART) of human immunodeficiency virus (HIV) infection. Thymic function may well be as important in non-pathological situations, such as contributing to the renewal of a diversified pool of naïve T cells necessary for optimal immune responses against foreign antigens and potentially pathogenic organisms. The thymus is the main organ in which T lymphocytes can develop but other extrathymic compartments, capable of *de novo* lymphopoiesis, also exist (gut-associated lymphoid tissue[392], lymph nodes[393]). The physiological/biological relevance of these potential sites is unknown at this date but they could act as "backup" organs in case thymic function fails for whatsoever reason.

Given the impact of cellular proliferation on telomere length, maintaining adequate levels of thymopoiesis would constantly regenerate the proliferative capacity of the immune system as *de novo* lymphocytes have longer telomeres than resident T cells. It is clear that the age-related thymic involution is accompanied by a reduced thymic output. Increased peripheral homeostatic proliferation levels compensate for the decreased exportation levels in order to maintain the number of T cells in the peripheral pool within precise limits. This biological homeostatic response "fine-tunes" total T cell counts as well as ensures maximal occupancy of peripheral T cell niches. Therefore, falling thymic output would be matched by increased resident T cell proliferation and with age, these proliferating cells will reach their replicative limit, defined by their telomere length. Neo antigen-driven cell division would also exacerbate this phenomenon, as it would gradually empty the naïve T cell compartment. In the absence of thymic function, these mechanisms will ultimately contribute to the accumulation of cells unable to replicate, producing a decline in immune function and a susceptibility to infection, or certain cancers. As memory T cells were shown to have shortened telomeres as compared to naïve T cells[394], it is possible that memory T lymphocytes could indeed reach immunological senescence and contribute to the decreased proliferation potential of the immune response in the elderly[395].

How can we define RTEs?

Since RTEs are, by definition, likely not to have encountered their antigen (because of their short stay in the peripheral circulation), most of them ought to bear naive markers ($CD45RA^+$, $CD62L^+$, $CD27^+$, $CD28^+$, $CCR7^+$, $CD31^+$, $CD11a^{DIM}$)[323,396,397] and preferentially reside in the lymph nodes and the spleen, where potential antigens/APC located. A vast heterogeneity exists within naïve T cells and it is important to note that only a fraction of naive T cells are RTEs. Distinguishing the latter from the heterogeneous peripheral naive T cell population is critical for the understanding of the role and contribution of thymic function to general T cell homeostasis.

Although FACS-purification of RTEs has not been achieved yet, it is possible to estimate their peripheral blood frequencies and establish correlates regarding thymopoiesis levels.

Douek and colleagues[307] were the first to engineer a cell surface marker-independent assay that takes advantage of an intrinsic characteristic of RTEs: the presence of sjTREC molecules (see chapter 1.2.2, figure 6). To this date, a lymphocyte that contains a TREC is the most stringent cellular status defining a RTE. It is important to note that only a fraction of peripheral RTEs (roughly 2/3) encompass sjTREC molecules[232]. Furthermore, some proliferation was shown to occur after T cell selection[300,302] leading to subsequent TREC dilution within SP thymocytes before exportation. Nonetheless, the presence/absence of a sjTREC molecule in a thymocyte/RTE is unlikely to alter its biological properties, as no known function or survival advantage is associated with sjTREC molecules. Therefore, the thymus exports sjTREC-containing thymocytes as well as sjTREC-less thymocytes in the periphery but only the former can be detected using current PCR-based techniques. Homeostatic proliferation following exportation may very well be another factor that contributes to the underestimation of peripheral blood RTE concentration. As sjTREC frequencies were shown to average $\approx 10^3$ molecules / 10^5 PBMC, RTEs represent a minimum of ≈1-2 % of all PBMC (≈2-4 % of T lymphocytes or ≈4-8 % of naïve T cells).

Contribution of RTEs to peripheral T cell homeostasis

Although the human thymus involutes during life and its function decreases as we age, the frequency of T cells bearing naive-associated markers remains relatively constant. It is possible to envisage that the composition of the naïve T cell compartment is age-dependent. In this regard, naïve T lymphocytes can be subdivided in three categories 1) recent thymic emigrants, 2) T cells that proliferate homeostatically and 3) antigen-experienced naïve T lymphocytes (revertants).

Several experiments performed by Tanchot and colleagues[317,331] have identified thymic activity as one of the key players in the regulation of peripheral naïve T cell homeostasis/turnover. In fact, naive T cells were shown to persist for longer period of time in the periphery of mice lacking thymopoietic activity as compared to those with normal thymic function. From a dynamic standpoint, this suggests that thymic exportation reduces the half-life of already existing peripheral naïve T cells. Memory T

lymphocyte homeostasis is not affected by this increased flux in naïve T cells as both compartments are independently regulated. Thymic function causes a displacement of already existing naive T cells and this has the effect of constantly renewing the naïve T cell compartment specificities. It is reasonable to think that naïve T cells, which have not met their corresponding antigen during their stay in the periphery, have yet to prove their usefulness. Thus, it seems logical to replace these cells with others that could play a helpful role and ensure maximal peptide recognition at any time. If a foreign antigen stimulates a naïve T cell, this cell would leave the naïve compartment, participate in the immune response and subsequently placed under the mechanisms controlling memory T cell homeostasis. As memory T cells comprise lymphocytes that previously participated in an immune response, it is important to preserve such specificities for possible future encounters.

Within the naïve T cell compartment, RTEs tend to be excluded from the mechanisms that control naïve T cell homeostasis. In fact, Berzins and colleagues[398] were able to demonstrate that RTEs from mice are excluded from the mechanisms regulating naïve T cell homeostasis for a period of 3-4 weeks following their exportation. This confers to them a selective advantage over residing naïve T cells and likely ensures that *de novo* produced T lymphocytes are not immediately removed from the periphery. Furthermore, RTE are exported with a higher CD4/CD8 ratio (around 3.5)[318]. As the peripheral CD4/CD8 ratio averages 1.5, a post-thymic "correction" of the peripheral CD4/CD8 ratio must occur. This implies that $CD4^+$ RTEs would be more prone to die following exportation whereas a similar proportion of $CD8^+$ RTEs would proliferate. Despite the fact that RTEs are mainly non-dividing cells[318], a minor fraction of them was shown to undergo cellular division and express CD8[300,313]. It is also possible that the CD4/CD8 peripheral blood ratio reflects differences in the homeostatic regulation of CD4 and CD8 expansions/contractions following immune responses.

Eventhough naive T cell half-life has not been precisely calculated in normal humans, it is likely that RTEs are constantly being delivered to the peripheral circulation throughout life and diluted within the naive compartment.

The human thymus: "shrinkable but still functional"

Despite its age-related involution, the thymus retains its ability to generate functional thymocytes bearing polyclonal TCR at their surface[141]. In this paper, the authors demonstrated that the thymus, whatever its age, is capable of generating thymocytes bearing diverse TCR Vβ segments and that they can proliferate following anti-CD3/anti-CD28 antibody stimulation. Although this paper does not provide direct evidence that these diverse thymocytes are exported in the periphery, it nonetheless suggests the thymus retains its ability to produce T cells with a broad specificity during adulthood. Given this, the thymus must be viewed as an organ than shrinks but maintain the same efficacy to generate and educate thymocytes (comparable sjTREC frequencies per mg of thymic tissue). The shrinking (thymic involution) affects the exportation capacity of the thymus and explains why TREC are found at lower frequencies in PBMCs as we age.

These findings are extremely relevant to immune reconstitution studies. Being able to fully identify the elements and factors leading to the involution of the thymus gland would be of great importance, as it appears that the aging process does not hamper the functionality of the thymus. The reduced quantity of thymic tissue found in the vast majority of old individuals, likely account solely for the reduced overall thymic activity.

7.2 β TREC: tools capable of assessing RTE diversity

Complementation of the sjTREC and the TCR β deletion circle assays

Evaluation of one's ability to produce new T cells as well as to generate a broad repertoire are important characteristics in defining the immune reconstitution potential of an individual. Although one of the big advantages of sjTREC quantification is the estimation RTE frequency within the periphery, no statement regarding the TCR diversity of these cells can be made as sjTREC formation occurs before Vα to Jα rearrangement. Consequently, it is impossible to evaluate the diversification of RTEs through sjTREC quantification.

The first part of this study was to develop and validate a novel tool capable of evaluating the peripheral blood frequencies of human recent thymic emigrants (RTE) independent of cell surface markers. We devised a PCR approach aimed at quantifying by-products of TCR α and β gene rearrangement, referred to as TREC. α TRECs (consisting of sjTREC and cjTREC) and β TREC (encompassing Vβ→DβJβ and Dβ→Jβ TREC) are produced when thymocytes undergo α and β gene rearrangement, respectively. Because of the intrinsic nature of T cell ontogeny and TCR gene rearrangement at the α and β loci, the peripheral blood frequencies of sjTREC were shown to be reflective of the overall level of thymic activity whereas Vβ→DβJβ or Dβ→Jβ TREC quantification illustrates the extent of diversity in gene segment rearrangement in newly generated T cells (RTE).

Monitoring RTE TCR β diversity through peripheral β TREC quantification

α and β TREC frequencies were observed in a pattern consistent with known parameters of human thymic function: their frequencies decrease as cells are stimulated to divide *in vitro*[1,307] and are lower in older individuals that have less active thymi, as measured by non-invasive radiography[399] or autopsy series[141]. Also, the detection of several Vβ→DβJβ[1] and Dβ→Jβ[2] TREC families within human peripheral blood mononuclear cells (PBMC) underscores the premise that the thymus is nonetheless capable of producing new and diverse sets of T cells in adulthood.

It is important to note that this quantitative PCR assay monitors RTE diversity only at the level of TCR β gene segment utilization, not at the level of the CDR3 primary nucleotide sequence. For example, all Vβ2-Dβ1-Jβ1.5 gene rearrangement events will generate the same 2 excision/deletion circles (Vβ2Dβ1 and Dβ1Jβ1.5 TREC) but a unique coding sequence is produced each time as a result of N and P nucleotide addition at the segment junction. At this date, it is impossible, with the currently available assays, to evaluate the "real" CDR3 repertoire of RTEs.

Theoretically, a maximum of 4 different DβJβ and 4 VβDβ excision/deletion circles can be found per RTE as developing thymocytes can rearrange the second allele if the first rearrangement is unsuccessful. In fact, each TCR allele has 2 DβJβ clusters and Vβ regions can rearrange to Dβ2Jβ2.X segments during the second rearrangement attempt. This can only happen when the first rearrangement involves Dβ1Jβ1.Y genes and is unsuccessfully rearranged on both alleles (Dβ2 rearrangement excise the Dβ1 locus on the TREC). As the vast majority of peripheral blood T cells only express 1 coding TCR β at their surface (but may have gone through multiple gene rearrangement events), a maximum of 1 VβDβ and 1 DβJβ excision/deletion circle will match the rearranged TCR β coding chain present at the cell surface. Nonetheless, quantification of β TRECs constitutes the best attempt yet performed to evaluate the diversity of newly generated T lymphocytes[1,2]. *A priori*, neither positive/negative T cell selection nor the process regulating thymocyte exportation should favor TREC-less or TREC-containing thymocyte. Therefore, detection of multiple β TREC among peripheral blood T cells from healthy adults strongly suggests that the thymus is able to export diversified RTEs into the periphery. The transgenic GreenMouse model (further discussed in chapter 7.9) will make possible FACS-purification of RTEs and thus, through RT-PCR amplification of the TCR β chains followed by CDR3 sequencing, the ability to fully assess the RTE TCR repertoire.

Following thymic education/exportation, RTE may undergo homeostatic cellular division that could lead to the "dilution" of the β TRECs to a point were they are no longer

detectable (< 1 per 10^5 PBMC). Several reasons can account for the naturally occurring low frequencies of β TRECs in PBMC as compared to the sjTREC molecules: 1) TCRDβJβ gene rearrangement occurs before the onset of δRec-ΨJα rearrangement during thymopoïesis, 2) developing thymocytes are known to undergo several rounds of proliferation between TCR β and α gene recombination events leading to β TREC dilution and 3) no consensus rearrangement event, similar to the δRec-ΨJα rearrangement at the TCR αδ locus, has been shown to occurs at the TCR β locus. In general, DβJβ TRECs average 1 to 30 molecules per 10^5 PBMC of healthy adults and a vast heterogeneity among detected peripheral blood DβJβ families is found within all study groups[2], likely to be the result of previous TCR repertoire expansion/contraction.

7.3 The impact of cellular proliferation on peripheral TREC frequencies

Controversies regarding the interpretation of sjTREC data

A controversial paper published by Hazenberg *et al*[400] followed the initial wave of papers documenting human thymic function through TREC quantification[1,375,376,391]. Through the use of a complex mathematical model, the authors were able to show that the observed decrease in peripheral sjTREC frequencies was a consequence of increased proliferation rather than decreased thymic activity. In fact, as TREC molecules do not replicate themselves, they are diluted-out during mitosis. The "sjTREC content" per volume of blood remains the same but the number of T cells increases, leading to a relative decrease in sjTREC frequencies. With this paper, the authors question the interpretation of sjTREC data, especially when massive proliferation has been shown to occur, namely during primary HIV infection and in the early phase following allogenic hematopoietic stem cell transplantation (AHSCT). Although this concept remains to be debated, recent experimental evidences were shown this to be untrue (Douek, D.C., et al., J. Immunol., 2001)

Increased thymic function or decrease cellular proliferation?

Conceptually, 3 types of peripheral T cell proliferation can affect the sjTREC frequencies: 1) antigen-driven primary immune responses, 2) proliferation of memory T cells in response to a recall antigen and 3) homeostatic proliferation of naïve T cells. The first scenario, elicited by a neo antigen, will minimally contribute to "purge-out" sjTREC-containing cells from the naïve compartment as such stimulation only activates a limited number of naïve T cells. Moreover, these expanded cells will then join the pool of memory/effector T cells, which is phenotypically distinct from the naïve compartment. Therefore, a transient drop in sjTREC frequencies could be observed as a result of massive clonal expansion but only when sjTREC quantification is performed on unsorted cells. If cells are sorted on the basis of naïve markers ($CD45RA^+$, $CD62L^+$, $CD27^+$, $CD28^+$, $CCR7^+$, $CD31^+$, $CD11a^{DIM}$) prior to sjTREC quantification, cellular division due to primary immune responses should not contribute to the dilution effect, as proliferating cells would be excluded from the analysis because of their memory/effector phenotype.

Even though it is true that a population of antigen-experienced cells masquerades within the naïve compartment[327,387] and thus, could contribute to the dilution of sjTREC frequencies, their low peripheral blood frequencies suggest a negligible impact on sjTREC frequencies. Last and foremost, most of the activated/effector T cells will die from AICD during the course of a normal immune response and thus, affect sjTREC frequencies only transiently if at all (i.e.: during the expansion phase, which lasts around 14-21 days). Restoration of "real" sjTREC frequencies should be observed as a result of cell death and *de novo* T cell production.

Secondary immune responses to recall antigen are anticipated to induce similar effects on sjTREC frequencies as primary immune responses. During anamnestic immune responses, only a fraction of memory T cells are stimulated and the naïve T cell compartment remains intact. It is anticipated that sjTREC frequencies (in unsorted PBMCs) are transiently diluted-out as a result of memory T cell expansions. Through the export of RTE and memory T cell contraction, sjTREC levels should be normalized.

On the other hand, homeostatic proliferation could very well contribute to an "apparent" and sustained reduction in sjTREC frequencies as this kind of proliferation is a stochastic events in naïve T cells. Naïve T cells undergo low levels of homeostatic proliferation in order to "fine-tune" total T cell numbers. Such proliferation is driven by the affinity of the TCR/MHC interaction in the periphery[401] and the availability of survival factors like IL-7[336]. One can imagine that if all naïve T cells undergo 1 or 2 round of proliferation, a quick 2-4 folds reduction in sjTREC frequencies will be observed. Therefore, 2 individuals with the same thymic function levels could have different peripheral blood sjTREC frequencies as a result of the changes in homeostatic proliferation levels. It is possible that a vast heterogeneity exists regarding the rate/intensity of homeostatic regulation among different healthy individuals, thus providing an explanation for the wide distribution of sjTREC frequencies (see Figure 3*c* of chapter 2 and Figure 1*a* of chapter 3). Further complicating the picture is the fact that some homeostatically-dividing naïve T cells were shown to retain their naïve phenotype[402] whereas others express memory/effector T cell markers.

In summary, FACS-purification of naïve T cells would limit the impact of cellular proliferation on sjTREC frequencies as memory/effector T cells will be excluded from the analysis. Evaluation of proliferation levels among naïve T cells ($CD45RA^+$ $CD27^+$), using antibodies against the nuclear antigen Ki-67, has to be performed in order to control for the difference in the regulation of T cell homeostasis. The study presented in chapter 3 controls for this proliferation and no inverse correlation between sjTREC levels and the frequencies of cycling naïve T cells was found, undermining a role for cellular division in the "apparent" dilution of sjTREC frequencies (see chapter 3, Figure 4*e*). However, Ki-67 is not an ideal marker for total assessment of cellular proliferation in TREC-related studies as it is only found in cells undergoing cellular division and was reported to remain expressed, albeit at lower levels, following the arrest of mitosis[403]. Moreover, it is important to understand that analyzing proliferation levels at sampling time does not account for past cellular proliferation events, which can dilute out sjTREC frequencies.

The conclusions of Hazenberg and colleagues[400] shook the scientific community and highlighted the importance of assessing the proliferation status of T cells in HIV-infected individuals before drawing conclusions regarding thymic function on the basis of peripheral sjTREC quantification. Nonetheless, recent data by Franco and colleagues[399] demonstrates that the increase in peripheral sjTREC frequencies following HAART is associated with an increased thymic mass as well as increased number of peripheral naïve T cells. This supports the concept that changes in sjTREC frequencies are intimately linked to variations in thymic function. Cellular death could also play a potential role in the modification of peripheral sjTREC frequencies if it preferentially eliminates TREC-less or TREC-containing cells.

The "sjTREC content": independent of cellular proliferation

Representing sjTREC data per unit of volume instead of per number of cells constitutes a way to limit the impact of cellular proliferation on sjTREC interpretation. In fact, *de novo* T cell production will increase the total amount of sjTREC molecules present in the

periphery whether or not cellular proliferation changes. Transforming the sjTREC data per unit of volume (determination of sjTREC content instead of sjTREC frequencies) could provide a better assessment of increases, but not reductions, of thymic function levels. In the light of the experiments performed by Tanchot and colleagues[331], a reduction in thymic activity is likely to limit the flux within the naïve T cell compartment and results in a longer naïve T cell half-life. Given this, one could expect the *in vivo* sjTREC half-life to be the determining factor leading to a reduction of the sjTREC content (aside from homeostatic proliferation). Therefore, conversion of sjTREC frequencies to sjTREC content can provide a better assessment of increases in thymic function. Reductions of thymic activity would be less evident. In fact, reduction of the sjTREC content would be dependant on the *in vivo* half-life of sjTREC molecules rather than decrease in thymic activity (a reduction in thymic function results in a reduced level of thymocyte exportation (sjTREC-containing cells), not a removal of these cells from the periphery).

7.4 Intrathymic proliferation as defined by the α/β TREC ratio

Additional insights on the quality of thymic function

Quantification of both type of TREC not only gives information about the status of thymic function and the diversity of those newly produced T cells but also provides additional data regarding the history of newly produced T cells. It is possible to transform the peripheral α and β TREC values into a new tool, indicative of the extent of intrathymic proliferation RTE underwent between both TCR gene rearrangement events. This new tool allowed us to better understand the behavior of the thymus during HIV infection and indicated that intrathymic proliferation is linked to the magnitude of thymic function.

The peripheral blood α/β TREC ratio is indicative of intrathymic proliferation

Evaluation of the α and β TREC frequencies among several subsets of human developing thymocytes allowed us to obtain kinetic profiles regarding the appearance/dilution of TREC molecules. β TREC frequencies were shown to gradually increase as thymocytes rearrange their TCR β locus[3] (chapter 4, Figure 1*a-b*). After reaching maximal levels in the triple-negative (TN) subset, β TRECs are diluted out as a result of thymocyte clonal expansion following β-selection. While at the $CD3^{-/LOW}CD4^{+}CD8^{+}$ differentiation stage, each clonally-expanded thymocyte rearranges TCR α locus leading to the formation of the sjTREC that, once generated, remains at relatively stable frequencies throughout the thymic selection processes. Both gene rearrangement events mark the boundaries of what we refer to as a "window of proliferation". The average number of replication rounds that thymocytes undergo while in this "window of proliferation" can be determined by calculating the α/β TREC ratio using peripheral α and β TREC frequency data. Assuming that the half-life of both TRECs is similar, fluctuations of the peripheral blood α/β TREC ratio stems from the over/under proliferation of the CD4ISP population or a selective depletion of proliferating/non-proliferating cells.

Intrathymic proliferation affects both the quantity and quality of thymic function

During thymopoïesis, several rounds of cell proliferation occur between the TCR β and the onset of α chain rearrangements. This proliferation results from successful β selection of thymocytes and greatly contributes in the generation of the vast DP thymocyte subset. Recently, data from our lab[3] indicate that each thymocyte undergoes an average of 4-5 rounds of proliferation following β selection. A paper by Kourilski's group[320] group also supports this notion as 25 different TCR a chains were shown to be associated with a given TCR b chain (2^{4-5} equals between 16 and 32).

Such cellular division allows a large portion of TCR diversity to be generated as it enhances the number of possible different TCR α chains that can pair with "clonally-expanded" TCR β chains at the surface of DP thymocytes. As a result of the proliferation following β selection, one could also envisage that each TCR β chain at the surface of a different thymocyte will try to pair with a different TCR α chain. This is likely to ensure maximal rescue of a DP thymocyte TCR β clonotype as well as to generate a diversified pool of thymocytes. Assuming that β selection leads to a 32-fold expansion of thymocytes (5 rounds of replication: 2^5=32), a total of 32 DP thymocytes per each β selected thymocytes will undergo positive/negative selection, each one expressing a different TCR α chain coupled to the same TCR β chain. Data from studies have shown that ≈25 different TCR α chains are associated with a particular TCR β chain clonotype within the peripheral naïve T cell compartment[320].

In contrast, if β selected thymocytes proliferate minimally before rearranging their TCR α genes, the "to-be-selected" pool of DP thymocytes will be greatly diminished and therefore, the magnitude of thymic function will be limited. Despite the fact that a lesser number of different possible TCR α chains are capable to pair with β-selected TCR β chains when the α/β TREC ratio is low, the ensuing TCR diversity is expected to be comparable on a T cell basis but the immune reconstitution will be slower. Therefore, the quantitative and qualitative aspects of thymopoiesis are intimately linked

7.5 Thymopoiesis and T cell turnover during HIV infection

HIV infection of thymocytes

It is clear that HIV has developed the ability to induce the destruction of peripheral $CD4^+$ T cells as well as to shut-off *de novo* T cell production. SIV particules can be found within the macaque thymus as early as few weeks following initial virus entry[404] and can infect thymocytes expressing the CD4 and co-receptor molecules. Due to practical reasons, similar data have not been obtained in human but it is anticipated that a homologue scenario occurs in human.

Accumulating data now strongly suggest that peripheral blood sjTREC content is inversely correlated to HIV plasma viral load. As human thymocytes are susceptible to HIV infection both *in vivo*[377,379] and *in vitro*[378], such cells could become productively infected and die by cell-mediated effects (lysis by HIV-specific CTLs) or through cytopathic viral effects. CD4 immature single-positive (CD4ISP), double-positive (DP) and single-positive CD4 thymocytes (SP4) are candidate target cells for HIV infection as they express the CD4 molecule in conjunction with the CXCR4 and/or CCR5 coreceptors (a fraction of developing thymocytes also express NF-κB, essential for viral replication[381,382]). Death of thymocytes, as a result of HIV infection, leads to a decreased or anihilated thymic function likely through the reduction of the DP thymocyte pool and impacts on the patient's ability to replenish his "holes" in the peripheral TCR repertoire.

Modification of intrathymic proliferation levels by HIV

The reduced peripheral α/β TREC ratio illustrates a change in the qualitative, as well as quantitative, aspects of thymopoiesis. Based on the results presented in chapter 4, we propose that inhibition of thymic activity is mediated through several means: 1) viral production by proliferating thymocytes that leads to the loss of membrane integrity and subsequent cell death, 2) CTLs killing HIV-infected proliferating thymocytes and/or 3) impaired intrathymic proliferation resulting in a reduced quantity of DP thymocytes and thus, limited thymic function.

Given the fact that activated T cells can circulate back to the thymus[43,84,85], it is probable that HIV-specific CTLs are recruited at the site of infection (in the thymus) and are responsible for the elimination of virus-producing cells. Productively infected thymocytes are likely to be "activated" and to proliferate, thereby making them excellent targets for HIV infection. Further enhancing their chances of being infected is the fact that thymocytes proliferate more than others would likely reside in the thymus for longer period of time and thus, would be more susceptible to encounter HIV and die. Therefore, thymocytes contributing to a high α/β TREC ratio would be preferentially eliminated and never reach the periphery.

As thymocytes/TEC "cross-talks" are essential to maintain a normal thymic architecture, the selective depletion of proliferating thymocytes could be a consequence of the observed thymic atrophy observed during chronic HIV infection and could as well contribute to reduce thymic output both quantitatively and qualitatively. It is important to mention that ongoing thymopoiesis should nonetheless remain diverse on a per cell basis but, as a population, less different pairing combinations among TCR αβ chains will be generated. HIV-infected patients having elevated viral loads and thus, low thymic activity, are likely to take more time in order to completely patch "holes" in the peripheral TCR repertoire.

Defects in thymopoiesis occur soon after HIV entry, as the α/β TREC ratio is already low by around 100 days post infection[3]. Similar experiments in the primary infected macaques should be performed in order to document the kinetics of such decreases. The striking and rapid reduction in the α/β TREC ratio indicates that, early in HIV infection, the peripheral RTE compartment has been completely "renewed" by new RTEs harboring a new and lower ratio. This suggests that most of the blood RTE compartment before HIV infection has left the peripheral blood for other anatomical sites like the lymph nodes and the spleen or as died (the persistence of a low α/β TREC ratio during HIV infection also supports the hypothesis that the "pre-infection" RTE compartment is depleted.

Increased sjTREC frequencies: cellular redistribution or *de novo* T cell production?

It is now widely accepted that the number of peripheral blood RTEs during HIV infection is greatly reduced as compared to age-matched, uninfected controls. Although HAART administration increases peripheral blood sjTREC frequencies in a way that is associated with pre-treatment CD4 count levels (lower CD4 T cell counts result in higher sjTREC increases) (data not shown), the origin of these cells has yet to be defined. In fact, it is possible to envisage that restoration of thymic function as well as redistribution/trafficking of sjTREC-bearing T cells previously "sequestered" in peripheral organs (lymph nodes, GALT, spleen) as a consequence of the reduction in the viral load post-HAART, can both theoretically lead to increases in peripheral sjTREC frequencies. How can these hypothesis be distinguished?

Although this is speculation, one could anticipate that "redistributed" lymphocytes might have undergone some cellular divisions while being "sequestered" in the lymph nodes or in the spleen as a result of the massive cytokine secretion. In this regard, naïve T cells were shown to spontaneously proliferate *in vitro* in the presence of IL-2, IL-4 and/or IL-7 without TCR or CD3 stimulation[405]. Given this, "redistributed" cells should have reduced sjTREC frequencies and therefore would minimally contribute, if at all, to the observed increase in peripheral sjTREC frequencies post-HAART. In contrast, if one postulates that "sequestered" naïve T cells undergo very limited proliferation, it is true to say that the "redistribution" of these TREC-containing cells could in part account for the observed increase in sjTREC frequencies following HAART administration.

As the α/β TREC ratio is unaffected by cellular proliferation (although it is possible that massive proliferation could result in extensive dilution of α and β TREC frequencies to a point where the α/β TREC ratio becomes incalculable), it would be of interest to study the early phase of HIV infection (or SIV infection) and document changes in the α/β TREC ratio pre- and post-HAART administration. In fact, HIV infection induces a reduction of sjTREC frequencies as well as a rapid drop in the α/β TREC ratio (data not shown), supportive of cellular "trapping" or migration of most RTE into the lymph nodes as a result of elevated levels of HIV antigens. In the hypothesis that "redistributed" cells

contribute to the increase in peripheral sjTREC frequencies following HAART, one could also anticipate the α/β TREC ratio to quickly rise. Preliminary data from our lab does not support this hypothesis, as long periods of time are needed to recover normal values for the α/β TREC ratio. Another explanation would be that RTE die while in the lymph nodes.

It remains to be evaluated why, despite viral control, does the α/β TREC ratio take so much time to be restored. It is possible that the pharmacokinetics of HAART treatment does not lead to total viral control within the thymus. Residual viral replication could be sufficient to mediate thymocyte depletion and thus lead to the persistence of a low α/β TREC ratio. Restoration of the ratio is likely to take place when the bloodstream has been purged-out of HIV particules, allowing the draining of other organs.

Thymopoiesis as a source for the establishment of the HIV reservoir

One of the salient features of HIV infection is the establishment of a long-lasting, quiescent HIV reservoir within $CD4^+$ T cells[406]. Eradication of HIV must take into account these resting cells that harbor integrated proviral DNA as they can give rise to new viral production following T cell activation. Antigen-stimulation, leading to NF-κB expression, was shown to be the driving force of HIV replication[407,408] and contributes to the perpetual re-seeding of the HIV reservoir by infecting other $CD4^+$ T cells that are downregulating their activation levels at the end of an immune response. These cells are believed to escape recognition by the immune system, as they do not produce sufficient virus for a long enough time in order to "flag" them as virus-producing cells (with a higher peptide density at the cell membrane). Beccause memory T cells are disseminated in the organism and are biologically programmed to persist in the immune system, one can easily envisage that they cells pose a problemg for HIV eradication (they harbor HIV proviral DNA which can be subsequently produced following T cell activation.

The HIV-infected thymus may very well also contribute to the establishment of the HIV reservoir through the exportation of RTEs that have integrated HIV proviral DNA[409]. During thymopoiesis, thymocytes become less transcriptionaly active and leave the

thymus as resting cells (very few division occurs following TCR α rearrangement). Given the susceptibility of thymocytes to HIV infection, it is possible that a fraction of HIV-infected thymocytes escape death (as a result of the gradual downregulation of transcription levels) and leave the thymus to contribute to the establishment of the HIV reservoir. Therefore, such HIV-infected thymocytes would join the periphery and will potentially replicate the virus if they are activated following antigen recognition. As these cells will have broad antigen specificities, it is likely that they will contribute to viral production and reservoir dissemination.

Peripheral repertoire generation during HIV infection

The secretion of cytokines and other mediators, like interferon (IFN)-α, helps the establishment of the adaptive immune response as well as contributes to viral clearance but can also impact on the generation of a functional peripheral RTE repertoire. Preliminary data from our lab tend to show that seric IFN-α levels are increased as a result of primary HIV infection but tend to normalize when the virus is under control[3]. Moreover, IFN-α is known to upregulate MHC class I at the surface of thymocytes and TEC[410]. From the immune reconstitution standpoint, this is particularly interesting as low levels of HIV replication could still exist within the thymus and induce the selection of thymocytes bearing low-affinity TCRs. Although peripheral organs like lymph nodes and the spleen were shown to harbor HIV particules, it remains to be demonstrated whether these anatomical sites secrete similar levels of IFN-α as compared to the thymus. Discrepancy between intrathymic and peripheral seric IFN-α levels would lead to changes in the avidity of MHC class I molecules at the surface of APC and to the inability of peripheral T cells to engage antigen and become stimulated. Supporting this idea are recent studies by Keir and colleagues (personal communication with J.M. McCune) who have been able to show that HIV-infected SCID-hu mice export $CD8^{LOW}$ SP thymocytes, consistent with the theory that low-avidity clones are being exported during HIV infection.

Although not directly proven, HIV infection of the thymus could very well lead to the presentation of HIV peptides by TEC. Such peptides would be in association with MHC

class I and II molecules and would be considered as self-peptides by differentiating thymocytes. Thymocytes capable of recognizing these peptide/MHC complexes with a high affinity/avidity would be depleted and die in the thymus as a result of negative selection. Therefore, it is possible that the residual thymic activity observed in HIV-infected patients comprises RTE that do not have a specificity against HIV.

HIV infection also induces the production of IFN-α, which is believed to play a central role in the control of retroviral replication. Intrathymic injection of IFN-α leads to an increase in MHC class I concentration at the surface of developing thymocytes and TEC[410]. Given the importance of the affinity/avidity of TCR/peptide/MHC interactions for T cell selection, a higher MHC class I density would lead to the selection of thymocytes expressing TCR molecules of lower affinity. Thymic exportation during HIV infection would be biased toward the exportation of RTEs bearing TCR of lower than normal affinity. It is anticipated that these RTE will not contribute in the development of an immune response against foreign pathogens, including HIV, as their affinity/avidity for peptide/MHC class I in the presence of normal levels of IFN-α is unlikely to be sufficient.

Thymic function during HIV infection is dramatically altered. First, elimination of the vast majority of clones capable of contributing to the immune response against HIV are selectively eliminated within the thymus. Second, RTE that could participate in such an immune response were selected in an environment where TCR/peptide/MHC class I affinity/avidity were not representative of the periphery levels. Therefore, *de novo* T cell production generates RTE that are incapable of adequately recognizing peripheral peptide/MHC class I complexes. Given this, HIV gradually depletes peripheral existing T cells as well as limits the patient's ability to replenish its hole in the repertoire.

Regenerative failure or increased T cell turnover?

Mathematical models strongly suggest that HIV leads to an accelerated turnover of $CD4^+$ T cells[411,412] and not a decrease of thymic activity. It proposes that the immune system is able to maintain T cell number relatively constant (through an accelerated generation of T

cells) until the proliferative reserve fails and become exhausted. Although this model is supported by several lines of evidence (rates of change in the $CD4^+$ T cell count and HIV viral load, large number of HIV quasispecies) the amount of HIV-infected T cells present in the circulation is too low to account for the mass destruction of the $CD4^+$ T cell compartment[413,414]. In order to provide an alternative explanation for $CD4^+$ T cell depletion, the "regenerative failure" model was proposed[415] and more recently, evidence supporting such model was gathered[416]. This model implies that the sources of T cells (bone marrow) as well as their maturation site (thymus) become dysfunctional In this regard, defect in bone marrow hematopoiesis[417] and in thymic activity[307] have been observed in HIV-infected individuals. Decreasing the exportation levels of RTEs to the periphery leads to a decrease of input of cells in the periphery and a reduced ability to compensate for cell loss induced by HIV infection. Although both models are opposed, it is likely that they both contribute to the development of AIDS pathogenesis.

7.6 Determination of the minimum average thymic exportation rate during HIV infection

Thymocyte exportation levels

T cell homeostasis allows the immune system to maintain T cell numbers at a relatively constant level throughout life. Through *de novo* production of T cells and peripheral expansion of already existing T cells, the immune system dynamically adjusts the number of T cells. Although evidence of thymic activity was gathered, the exact number of RTEs it pumps-out every day remains unanswered. It was reported that a total of $\approx 70 \times 10^6$ new human T lymphocytes are generated each day through cellular proliferation and *de novo* production[416]. In the mouse, the idea has been advanced that $\approx 10^6$ T lymphocytes are being exported in the periphery each day[313,419]. Humans and mice have different thymic masses and it seems intuitive to think that thymic exportation rates are closely associated to thymic mass (as a bigger thymus encompass a greater number of developing thymocytes leading to an increased exportation capacity).

We believe the peripheral α/β TREC ratio can also be used to quantify the minimum average daily thymic exportation rate. The fact that the α/β TREC ratio is significantly lower in HIV-infected individuals reflects the complete renewal of the peripheral RTE compartment by *de novo* produced T cells harboring a new and lower α/β TREC ratio. Longitudinal analysis of early HIV-infected patients revealed that the α/β TREC ratio rapidly falls following HIV infection, reaching the lowest plateau around 2-3 months post-infection (data not shown). Furthermore, peripheral blood sjTREC frequencies at 2-3 months post infection can be translated into an absolute number of RTEs being present at sampling time. For example, early HIV-infected patients average 100 sjTREC per 10^5 PBMC at that time point, which correspond to 10×10^8 total RTEs (sjTREC X 20 X 5000 X 100), assuming 2×10^6 millions PBMCs per mL of blood, 5000 mL of blood in the body and that blood lymphocytes represent around 1% of the total lymphocyte pool. These 10×10^8 newly produced RTEs have been generated during the first 100 days of HIV infection as they show a reduction in the α/β TREC ratio. Therefore, it is possible to calculate the average daily rhythm at which *de novo* produced T cells are being exported

by dividing total RTE numbers by the length of time between the initial infection and the first time point of the lower α/β TREC ratio plateau. Therefore, a minimum average of 10×10^6 RTEs is produced each day (10×10^8 / 100 days) during early primary HIV infection. One could imagine that thymic function would be increased by several folds in the absence of HIV.

7.7 IL-7 as a key regulator of T cell homeostasis

A feedback loop controlling T cell counts?

The purpose of T cell homeostasis is to maintain, in real-time, adequate number of T lymphocytes in the periphery. Each day, the immune system produces T cells through thymic-dependant and -independent pathways. Consequently, other cells are dying in order to allow the immune system to maintain this state-state equilibrium. Negative feedback loops mechanisms can accomplish this complex regulation. In fact, the existence of this mechanism, through modulation of erytropoietin (EPO) blood concentration, was previously documented for red blood cells[420].

A homologous mechanism has been shown to occur in response to lymphocyte depletion[337]. In this paper, the authors report the existence of sensor cells located in the lymph nodes (likely to be APC-like cells) that have the capacity to increase IL-7 production when lymphocytes become low (< 200 per μL). As mentioned earlier, IL-7 is able to upregulate Bcl-2 expression as well as plays an essential role in naïve T cell homeostasis[336]. Through upregulation of IL-7 production, APC-like cells increase the viability and proliferative potential of existing lymphocytes as well as ensuring that developing DN thymocytes can proliferate intrathymically (and thus, maintain a good thymic function through the maintenance of a vast DP thymocyte pool).

Human IL-7 is a 152 amino acid cytokine produced by stromal cells originally discovered as a growth factor and previously referred to as lymphopoietin-1/pre-B cell growth factor[421]. In addition to being produced by bone marrow stromal cells, IL-7 mRNA has also been detected in the thymus, spleen, kidney as well as in keratinocytes[422,423]. Human IL-7, as well as its murine homologue, has been shown to have pleiotropic effects on a variety of cell types including cells of the B, T, NK and myeloid lineages.

The biological effects of IL-7 are initiated by binding of the cytokine to a cytokine receptor complex consisting of a ligand specific binding component, IL-7Rα (CD127), and a second component, the γ common chain, that is also a constituent of the receptor

complexes associated with IL-2, IL-4, IL-9, IL-15, IL-21 binding and signal transduction[424-426]. Signal transduction following binding of IL-7 to its receptor complex involves association with Jak1 and Jak3[427], followed by subsequent induction of members of the STAT family, reportedly STAT1, STAT5 and possibly STAT3[428]. It was also reported that members of *src*-like kinase[429-431], as well as *insulin receptor substrate 1* (IRS-1)[432] and PI-3-kinase[433] are activated following IL-7/IL-7R interactions.

IL-7 plays an important role in T cell development in the thymus. IL-7 was reported to induce proliferation of immature and mature human and mouse thymocytes as well as to induce V(D)J rearrangement of the TCR β genes[434]. Furthermore, generation of CD8 SP thymocytes also requires on IL-7[167].

7.8 How can we "boost" thymic function?

The key to reversing thymic involution likely resides in finding ways to maintain a constant supply of T cell precursors capable of colonizing the thymus while increasing thymic mass through enhanced TEC proliferation. Evidences presented in this thesis suggests that the magnitude of thymic function and thus, the rapidity at which holes in the repertoire are replenished, is intimately linked with intrathymic proliferation levels[3]. As thymocyte numbers will increase, the demand for survival/proliferation factors required during thymocyte ontogeny will also be greater. In order to permit such intrathymic proliferation to occur, enough thymic space must be generated. Inducing TEC to proliferate ought to lead to an increased secretion of proliferation/survival factors ultimately enhancing thymic function.

It is important to understand that thymocytes and TEC development are intimately interconnected: one cannot fully occur without the other. Clinical scenarios leading to the loss of thymic selecting tissue will impair thymocyte development and lead to decreased levels of thymopoiesis. Alternatively, blocking thymocyte development at distinct and very early stages will abrogate or limit full thymus organogenesis. Given this, the key to the reversal of thymic involution likely resides in the specific *in vivo* induction of epithelium growth as well as ensuring subsequent "filling" of thymic niches (created through this TEC proliferation) by bone marrow-derived cells. One could hypothesize that specific induction of TEC proliferation would increase thymic mass (and reduce the proportion of adipose tissue present in the thymus), allowing enhanced levels of intrathymic thymocyte proliferation and/or colonization by precursor cells. Consequently, these events would lead to greater number of DP thymocytes and ultimately, establish a bigger pool of positively selected thymocytes to be exported to the periphery. Hence, by providing a bigger "house" (the thymus) to thymocytes, more growth/survival factors will be produced and will facilitate thymocyte ontogeny. Reversing the natural process of thymic involution likely requires the understanding of all major development checkpoints and the ability to act on all of them.

How can this be achieved? The induction of TEC proliferation could enhance the TES (and limit thymic involution) and result in an enhanced ability to select thymocytes. Fibroblast growth factor (FGF) was shown to stimulate TEC proliferation[435]. Interestingly, acethylcoline and carbamylcoline (neurotransmitters) enhance proliferation of TEC[436]. Furthermore, GH regulates the production of IGF-1, which induces TEC proliferation[437,438]. Regulation of TEC growth can also be done through control of ECM production (namely fibronectin) by these cells as a positive correlation between TEC adhesion and proliferation has been evidenced[439]. Indeed, low levels of IFN-γ have also been shown to enhance the production of ECM by TEC. In order to proliferate, TEC must adhere to the ECM. Therefore, increases in IFN-γ lead to an enhanced production of ECM by TEC, and thus, sustains proliferation by providing more substrate to grow on.

Sufficient supplies of bone marrow-derived cells must also be available. Given that thymocytes play a critical role in thymic epithelial stroma cell development, it is rational to say that a reduced colonization of the thymus by thymocyte precursor cells ($CD34^+$ in humans) could lead to a reduced thymic function. As $CD34^+$ blood levels do change with age[440], the observed thymic involution leading to a reduced thymic activity, could be the result of a diminished thymic colonization capacity by precursor cells.

Whatever the strategies considered to upregulate thymic function, it is important to make sure that these approaches do not lead to the production of autoreactive T cells. It is possible that a delicate balance exist between the deleting thymocytes and maintaining an adequate thymic function. For example, modulating the intrathymic glucorticoid concentration may influence the stringency at which T cell selection occurs and thus lead to the exportation of T cells that should have dies intrathymically. This would increase the exportation rate of the thymus but also be harmfull as a higher proportion of autoreactive T cells would be produced.

7.9 Relevant questions to answer with the GreenMouse transgenic model.

The quest for the identification of an RTE-specific phenotype

The emergence of novel tools allowing for the *in vivo* assessment of human thymic function has opened new frontiers in the evaluation of the immune reconstitution phenomenon. Nonetheless, it is still impossible at this date to FACS-purify TREC-containing cells as no RTE-specific marker has been associated with a human cell phenotype (other than naïve) highly enriched in TREC. McFarland and colleagues[441] identified CD103, the a_E chain of the $a_E\beta_7$ integrin adhesion molecule, which is only retained at the surface of naïve $CD8^+$ mature lymphocytes which has the expected characteristics of RTE. Although this constitutes a step toward the identification of an RTE-specific phenotype, I believe a good phenotypic thymic function marker should be expressed on both peripheral $CD4^+$ and $CD8^+$ T naïve T cells. Furthermore, FACS-sorting of $CD103^+$ naïve $CD8^+$ T cells only leads to an average 1.4 fold enrichment in sjTREC frequencies as compared to $CD103^-$ naïve $CD8^+$ T cells[441].

Although a unique phenotype for RTE might exist, it could very well be possible that thymocytes are exported and enter the periphery with different sets of molecules at their cell surface. This would complicate the identification of an RTE-specific phenotype. Indeed, naïve T cells need to reach lymph nodes and GALT in order to "scan" peptides presented by APC cells located at different anatomical regions. The expression of distinct adhesion molecules (integrin, selectin) would help naïve T cells (as well as RTE) traffic and reach these sites. It remains to be seen whether RTE are exported in the periphery bearing the identical phenotype or a combination of cell surface phenotypes exists.

The patent-protected GreenMouse transgenic model would allow scientists to find answers to these questions. In fact, the ultimate goal of this model is to specifically induce the rearrangement of a transgene that will lead to a TREC capable of supporting GFP expression (Chapter 5, Figure 1). Such GFP^+ peripheral T lymphocytes could be FACS-purified in conjunction with other fluorescence-coupled antibodies against cell surface molecules in order to define what is expressed on RTE. The analysis of gene

expression in the RTE compartment will allow for the better understanding of their biology and role in the immune system.

Insights on the "real" RTE TCR repertoire (CDR3 nucleotide sequence)

Access to a highly purified population of RTE would permit studies aimed at analyzing the genetic expression profile of RTE, including the TCR α and β CDR3. Contrary to the β TREC, which only assesses the extent of diversity in gene rearrangement events, isolation of GFP^+ cells from the GreenMouse would allow extraction of mRNA molecules, and subsequent conversion to cDNA and processing on DNA microarrays. This could confirm previously found cell surface molecules at the surface of RTE using flow cytometric analysis of GFP^+ cells.

Understanding RTE activation requirements

Current methods allowing for thymic function monitoring involve cell lysis or genomic DNA extraction, both leading to cell death. Therefore, it is impossible to perform functional studies on RTE until FACS-purification of TREC-containing cells is achieved. Such isolation would allow placing these cells in culture without cross-linking any of the surface molecules and testing their activation requirements. As they were recently exported, RTE may need to undergo some maturation steps in the periphery in order to enter the naïve T cell pool. It is also possible that they possess a survival advantage, regulated by Bcl-2 expression, as compared to "old" resting naïve T cell.

The GreenMouse: a source of RTE for adoptive transfer studies

It would be of interest to understand how the immune system, and more specifically the thymus, responds when the periphery is over or under supplied by RTE. The insertion of an IRES-thCD4 (internal ribosomal entry sequence / truncated human CD4) within the transgene allows the simultaneous expression of both the GFP and a portion of the human CD4 molecule. Consequently, we expect TREC-containing cells to be GFP^+thCD4^+. Thus, it will be possible to specifically deplete RTEs *in vivo* using an antibody against the extracellular domain of the thCD4 molecule. As RTEs constitute a minor T cell population, their elimination should not induce lymphopenia. Therefore, study of the

replenishment of the RTE compartment could be performed in a minimally-perturbed model.

Moreover, it will be possible to FACS-purify RTEs from several GreenMouse and inject them into another, non-transgenic, mouse in order to evaluate the impact of overloading the periphery of RTEs on thymic function. Homologous experiment could also be performed in thymectomized recipients.

Is there an extrathymic site capable of *de novo* T cell production?

The ability to distinguish RTEs from naïve T cells makes it possible to follow their movement into the peripheral lymphoid tissue. Therefore, one will be able to assess where RTE go after being produced. It is anticipated that RTE will be enriched within the lymph nodes as these anatomical sites "drain" antigen found in the periphery.

It will be possible to identify other organs, if any, capable of supporting thymopoiesis. A fully thymectomized GreenMouse will be depleted in RTE (using anti-thCD4) and sacrificed several months later in order to screen several organs for the presence GFP^+ cells. As no thymus will be found in these mice, the appearance of GFP has to originate from the extrathymic compartment. Identification of these organs will be easy, as they will encompass GFP^+ cells.

Crossing the GreenMouse with other transgenic mouse models

At this date, no genomic locus has been found to confer an enhanced thymic function. It would be of great interest to cross the GreenMouse with several other mice strains (lck-OM, IL-7 $^{-/-}$, Bcl-2 $^{-/-}$) in order to determine the role of different genes on thymic activity.

8. Perspectives and conclusions

Given the evidences presented in this thesis, there are reasons to believe that the human thymus remains active in adulthood and continues to export T cells that contribute to the diversification of the TCR repertoire. Furthermore, AHSCT alone does not induce thymic dysfunction. This is encouraging given the growing number of transplantation being performed each year in order to attempt to cure several hematological disorders. It is also important to point out the negative, but reversible, effect of GVHD on thymic function.

While an active thymus is essential for full immune reconstitution, the quantity of peripheral niches dictates how much T cells can be maintained. These microenvironments can be viewed as "sensors" that adjust T cell numbers in real-time by modulating homeostatic proliferation levels of peripheral T cells. These cells require survival signals from these peripheral niches in order to persist in the periphery. Disruption of these interactions will result in increased peripheral T cell death, as insufficient amount of "food" will be available. Data presented in this thesis demonstrate that peripheral naïve T cells from AHSCT patients have a reduced IL-7Rα expression, the receptor of a central homeostatic cytokine. This provides a mechanism explaining the inability of these patients to restore normal naïve T cell frequencies and demonstrate that this defect is intrinsic to naive T cells and not related to APCs, as IL-7 plasma concentration were comparable between both study groups.

Altogether, this provides insights on the human thymus and its activity during clinical situations of immune reconstitution, as well as helped to "resurrect" this organ/gland, considered by many to play a negligible role in adulthood. It is important to keep in mind that finding ways to upregulate thymic activity would be of great interest. The identification of molecules capable of modulating thymic activity could accelerate the immune reconstitution process and reduce the morbidity associated with the early phase following AHSCT. Such enterprise can be realized through using the patented and promising GreenMouse model, currently under development.

9. Thesis reference list

1. Poulin, J.F., Viswanathan, M. N., Harris, J. M., Komanduri, K. V., Wieder, E., Ringuette, N., Jenkins, M., McCune, J. M., Sekaly, R.-P. Direct evidence for thymic function in adult humans. *J Exp Med* **190**, 479-86. (1999).
2. Poulin, J.-F., Sylvestre, M., Champagne, P., Dion, M.-L., Kettaf, Dumont, A., Lainesse, M., Fontaine, P., Perreault, C., Sékaly, R.-P., Cheynier, R. Immunodeficiency following allogenic hematopoietic stem cell transplantation is not due to a thymic defect but the consequence of impaired naive T cell survival. *Submitted.*
3. Dion, M.-L., Bordi, R., Poulin, J.-F., Sylvestre, M., Corsini, R., Ketaff, N., Dalloul, A., Woods, R., Montaner, J., Harris, F., Debré, P, Routy, J.-P., Sékaly, R.-P., Cheynier, R. Changes in the quality of thymic exportation during early primary human immunodeficiency virus infection. *In preparation.*
4. Poulin, J.F., Bourbonnière, M., Sékaly, R.-P., Cheynier, R. FACS-purification of recent thymic emigrants using a novel transgenic mouse model. *In preparation.*
5. Bockman, D.E. Development of the thymus. *Microsc Res Tech* **38**, 209-15. (1997).
6. Manley, N.R. Thymus organogenesis and molecular mechanisms of thymic epithelial cell differentiation. *Semin Immunol* **12**, 421-8. (2000).
7. Anderson, G., Moore, N.C., Owen, J.J. & Jenkinson, E.J. Cellular interactions in thymocyte development. *Annu Rev Immunol* **14**, 73-99 (1996).
8. Lobach, D.F. & Haynes, B.F. Ontogeny of the human thymus during fetal development. *J Clin Immunol* **7**, 81-97. (1987).
9. Lobach, D.F., Itoh, T., Singer, K.H. & Haynes, B.F. The thymic microenvironment. Characterization of in vitro differentiation of the IT26R21 rat thymic epithelial cell line. *Differentiation* **34**, 50-9 (1987).
10. Screpanti, I., Modesti, A. & Gulino, A. Heterogeneity of thymic stromal cells and thymocyte differentiation: a cell culture approach. *J Cell Sci* **105**, 601-6. (1993).
11. Kirby, M.L. & Bockman, D.E. Neural crest and normal development: a new perspective. *Anat Rec* **209**, 1-6. (1984).
12. Bockman, D.E. & Kirby, M.L. Dependence of thymus development on derivatives of the neural crest. *Science* **223**, 498-500. (1984).

13. Anderson, G., Jenkinson, E.J., Moore, N.C. & Owen, J.J. MHC class II-positive epithelium and mesenchyme cells are both required for T-cell development in the thymus. *Nature* **362**, 70-3. (1993).
14. Cordier, A.C. & Haumont, S.M. Development of thymus, parathyroids, and ultimobranchial bodies in NMRI and nude mice. *Am J Anat* **157**, 227-63. (1980).
15. Krumlauf, R. Hox genes in vertebrate development. *Cell* **78**, 191-201. (1994).
16. Su, D., Ellis, S., Napier, A., Lee, K. & Manley, N.R. Hoxa3 and pax1 regulate epithelial cell death and proliferation during thymus and parathyroid organogenesis. *Dev Biol* **236**, 316-29. (2001).
17. Chisaka, O. & Capecchi, M.R. Regionally restricted developmental defects resulting from targeted disruption of the mouse homeobox gene hox-1.5. *Nature* **350**, 473-9. (1991).
18. Peters, H., Neubuser, A., Kratochwil, K. & Balling, R. Pax9-deficient mice lack pharyngeal pouch derivatives and teeth and exhibit craniofacial and limb abnormalities. *Genes Dev* **12**, 2735-47. (1998).
19. Manley, N.R. & Capecchi, M.R. The role of Hoxa-3 in mouse thymus and thyroid development. *Development* **121**, 1989-2003. (1995).
20. Dietrich, S. & Gruss, P. undulated phenotypes suggest a role of Pax-1 for the development of vertebral and extravertebral structures. *Dev Biol* **167**, 529-48. (1995).
21. Wallin, J. et al. Pax1 is expressed during development of the thymus epithelium and is required for normal T-cell maturation. *Development* **122**, 23-30. (1996).
22. Manley, N.R. & Capecchi, M.R. Hox group 3 paralogs regulate the development and migration of the thymus, thyroid, and parathyroid glands. *Dev Biol* **195**, 1-15. (1998).
23. Brissette, J.L., Li, J., Kamimura, J., Lee, D. & Dotto, G.P. The product of the mouse nude locus, Whn, regulates the balance between epithelial cell growth and differentiation. *Genes Dev* **10**, 2212-21. (1996).
24. Nehls, M., Pfeifer, D., Schorpp, M., Hedrich, H. & Boehm, T. New member of the winged-helix protein family disrupted in mouse and rat nude mutations. *Nature* **372**, 103-7. (1994).

25. Reth, M. Immunodeficiency. Trapping the nude mouse gene. *Curr Biol* **5**, 18-20. (1995).
26. Blackburn, C.C. et al. The nu gene acts cell-autonomously and is required for differentiation of thymic epithelial progenitors. *Proc Natl Acad Sci U S A* **93**, 5742-6. (1996).
27. Wilkinson, B., Owen, J.J. & Jenkinson, E.J. Factors regulating stem cell recruitment to the fetal thymus. *J Immunol* **162**, 3873-81. (1999).
28. Ritter, M.A. & Boyd, R.L. Development in the thymus: it takes two to tango. *Immunol Today* **14**, 462-9. (1993).
29. Naquet, P., Naspetti, M. & Boyd, R. Development, organization and function of the thymic medulla in normal, immunodeficient or autoimmune mice. *Semin Immunol* **11**, 47-55. (1999).
30. Wang, B., Simpson, S.J., Hollander, G.A. & Terhorst, C. Development and function of T lymphocytes and natural killer cells after bone marrow transplantation of severely immunodeficient mice. *Immunol Rev* **157**, 53-60. (1997).
31. Hollander, G.A. et al. Developmental control point in induction of thymic cortex regulated by a subpopulation of prothymocytes. *Nature* **373**, 350-3. (1995).
32. van Ewijk, W. et al. Thymic microenvironments, 3-D versus 2-D? *Semin Immunol* **11**, 57-64. (1999).
33. van Ewijk, W. T-cell differentiation is influenced by thymic microenvironments. *Annu Rev Immunol* **9**, 591-615 (1991).
34. Van Vliet, E., Melis, M. & Van Ewijk, W. Monoclonal antibodies to stromal cell types of the mouse thymus. *Eur J Immunol* **14**, 524-9. (1984).
35. Savino, W., Vermeulen, M., Dardenne, M. & Lepault, F. The thymic extracellular matrix in genetically engineered mice. *Immunol Today* **19**, 144-5. (1998).
36. Chang, A.C., Wadsworth, S. & Coligan, J.E. Expression of merosin in the thymus and its interaction with thymocytes. *J Immunol* **151**, 1789-801. (1993).
37. Mackay, C.R. & Imhof, B.A. Cell adhesion in the immune system. *Immunol Today* **14**, 99-102. (1993).
38. Raviola, E. & Karnovsky, M.J. Evidence for a blood-thymus barrier using electron-opaque tracers. *J Exp Med* **136**, 466-98. (1972).

39. Thomis, D.C. & Berg, L.J. The role of Jak3 in lymphoid development, activation, and signaling. *Curr Opin Immunol* **9**, 541-7. (1997).
40. Killeen, N., Irving, B.A., Pippig, S. & Zingler, K. Signaling checkpoints during the development of T lymphocytes. *Curr Opin Immunol* **10**, 360-7. (1998).
41. Baird, A.M., Gerstein, R.M. & Berg, L.J. The role of cytokine receptor signaling in lymphocyte development. *Curr Opin Immunol* **11**, 157-66. (1999).
42. Boyd, R.L. et al. The thymic microenvironment. *Immunol Today* **14**, 445-59. (1993).
43. Flores, K.G., Li, J., Sempowski, G.D., Haynes, B.F. & Hale, L.P. Analysis of the human thymic perivascular space during aging. *J Clin Invest* **104**, 1031-9. (1999).
44. Haynes, B.F., Markert, M.L., Sempowski, G.D., Patel, D.D. & Hale, L.P. The role of the thymus in immune reconstitution in aging, bone marrow transplantation, and HIV-1 infection. *Annu Rev Immunol* **18**, 529-60 (2000).
45. Hirokawa, K., Utsuyama, M. & Sado, T. Immunohistological analysis of immigration of thymocyte-precursors into the thymus: evidence for immigration of peripheral T cells into the thymic medulla. *Cell Immunol* **119**, 160-70. (1989).
46. Wekerle, H., Ketelsen, U.P. & Ernst, M. Thymic nurse cells. Lymphoepithelial cell complexes in murine thymuses: morphological and serological characterization. *J Exp Med* **151**, 925-44. (1980).
47. Wekerle, H. & Ketelsen, U.P. Thymic nurse cells--Ia-bearing epithelium involved in T-lymphocyte differentiation? *Nature* **283**, 402-4. (1980).
48. Vakharia, D.D. & Mitchison, N.A. Thymic epithelial cells derived from the cultures of thymic nurse cells. *Immunol Lett* **7**, 261-6 (1984).
49. van Vliet, E., Melis, M. & van Ewijk, W. Immunohistology of thymic nurse cells. *Cell Immunol* **87**, 101-9. (1984).
50. Brelinska, R. & Warchol, J.B. Thymic nurse cells: their functional ultrastructure. *Microsc Res Tech* **38**, 250-66. (1997).
51. Faas, S.J., Rothstein, J.L., Kreider, B.L., Rovera, G. & Knowles, B.B. Phenotypically diverse mouse thymic stromal cell lines which induce proliferation and differentiation of hematopoietic cells. *Eur J Immunol* **23**, 1201-14. (1993).

52. Ezaki, T., Matsuno, K. & Kotani, M. Thymic nurse cells (TNC) in spontaneous thymoma BUF/Mna rats as a model to study their roles in T-cell development. *Immunology* **73**, 151-8. (1991).
53. McCormack, J.E., Wade, T., Morales, H., Kappler, J. & Marrack, P. Analysis of class II MHC structure in thymic nurse cells. *Cell Immunol* **138**, 413-22. (1991).
54. Goya, R.G. & Bolognani, F. Homeostasis, thymic hormones and aging. *Gerontology* **45**, 174-8. (1999).
55. Breel, M., Mebius, R.E. & Kraal, G. Dendritic cells of the mouse recognized by two monoclonal antibodies. *Eur J Immunol* **17**, 1555-9. (1987).
56. Miyama-Inaba, M. et al. Unusual phenotype of B cells in the thymus of normal mice. *J Exp Med* **168**, 811-6. (1988).
57. Kappler, J.W., Roehm, N. & Marrack, P. T cell tolerance by clonal elimination in the thymus. *Cell* **49**, 273-80. (1987).
58. MacDonald, H.R. et al. T-cell lineages, repertoire selection and tolerance induction. *Immunol Rev* **104**, 157-82. (1988).
59. Matzinger, P. & Guerder, S. Does T-cell tolerance require a dedicated antigen-presenting cell? *Nature* **338**, 74-6. (1989).
60. Jenkinson, E.J. & Owen, J.J. T-cell differentiation in thymus organ cultures. *Semin Immunol* **2**, 51-8. (1990).
61. Duijvestijn, A.M. & Hoefsmit, E.C. Ultrastructure of the rat thymus: the micro-environment of T-lymphocyte maturation. *Cell Tissue Res* **218**, 279-92 (1981).
62. Inaba, K. et al. Macrophages phagocytose thymic lymphocytes with productively rearranged T cell receptor alpha and beta genes. *J Exp Med* **168**, 2279-94. (1988).
63. Milicevic, N.M., Milicevic, Z., Colic, M. & Mujovic, S. Ultrastructural study of macrophages in the rat thymus, with special reference to the cortico-medullary zone. *J Anat* **150**, 89-98. (1987).
64. Miodonski, A.J., Bigaj, J. & Plytycz, B. Thymic vascular system of the European common frog, Rana temporaria: a scanning electron-microscopic study of vascular casts. *Cell Tissue Res* **281**, 543-9. (1995).

65. Miodonski, A.J., Bigaj, J. & Plytycz, B. Light and electron-microscopic observations on the thymic capillaries and postcapillary venules in Rana temporaria L. *Folia Histochem Cytobiol* **32**, 91-100 (1994).
66. Pelletier, M. et al. Characterization of human thymic dendritic cells in culture. *Immunology* **58**, 263-70. (1986).
67. Kyewski, B.A., Rouse, R.V. & Kaplan, H.S. Thymocyte rosettes: multicellular complexes of lymphocytes and bone marrow-derived stromal cells in the mouse thymus. *Proc Natl Acad Sci U S A* **79**, 5646-50. (1982).
68. Inaba, K. et al. Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. *J Exp Med* **176**, 1693-702. (1992).
69. Santiago-Schwarz, F., Belilos, E., Diamond, B. & Carsons, S.E. TNF in combination with GM-CSF enhances the differentiation of neonatal cord blood stem cells into dendritic cells and macrophages. *J Leukoc Biol* **52**, 274-81. (1992).
70. Surh, C.D. et al. Two subsets of epithelial cells in the thymic medulla. *J Exp Med* **176**, 495-505. (1992).
71. Kearney, J.F. CD5+ B-cell networks. *Curr Opin Immunol* **5**, 223-6. (1993).
72. Wallace, V.A. et al. CD45RA and CD45RBhigh expression induced by thymic selection events. *J Exp Med* **176**, 1657-63. (1992).
73. Nicolas, J.F. et al. Heterogeneity of thymic epithelial cell (TEC) keratins--immunohistochemical and biochemical evidence for a subset of highly differentiated TEC in the mouse. *J Histochem Cytochem* **33**, 687-94. (1985).
74. Klug, D.B. et al. Interdependence of cortical thymic epithelial cell differentiation and T-lineage commitment. *Proc Natl Acad Sci U S A* **95**, 11822-7. (1998).
75. De Waal, E.J. & Rademakers, L.H. Heterogeneity of epithelial cells in the rat thymus. *Microsc Res Tech* **38**, 227-36. (1997).
76. van de Wijngaert, F.P., Kendall, M.D., Schuurman, H.J., Rademakers, L.H. & Kater, L. Heterogeneity of epithelial cells in the human thymus. An ultrastructural study. *Cell Tissue Res* **237**, 227-37 (1984).
77. Moraux-Moyson, A., Scheiff, J.M. & Haumont, S. Thymus histogenesis in C3H mice. *Thymus* **12**, 89-109 (1988).

78. Nabarra, B. & Andrianarison, I. Ultrastructural studies of thymic reticulum: I. Epithelial [corrected] component. *Thymus* **9**, 95-121 (1987).

79. Kampinga, J. et al. Thymic epithelial antibodies: immunohistological analysis and introduction of nomenclature. *Thymus* **13**, 165-73 (1989).

80. Godfrey, D.I., Izon, D.J., Tucek, C.L., Wilson, T.J. & Boyd, R.L. The phenotypic heterogeneity of mouse thymic stromal cells. *Immunology* **70**, 66-74. (1990).

81. Rodewald, H.R., Paul, S., Haller, C., Bluethmann, H. & Blum, C. Thymus medulla consisting of epithelial islets each derived from a single progenitor. *Nature* **414**, 763-8. (2001).

82. Ropke, C., Van Soest, P., Platenburg, P.P. & Van Ewijk, W. A common stem cell for murine cortical and medullary thymic epithelial cells? *Dev Immunol* **4**, 149-56 (1995).

83. Gill, J., Malin, M., Hollander, G.A. & Boyd, R. Generation of a complete thymic microenvironment by MTS24(+) thymic epithelial cells. *Nat Immunol* **3**, 635-42. (2002).

84. Steinmann, G.G. Changes in the human thymus during aging. *Curr Top Pathol* **75**, 43-88 (1986).

85. Steinmann, G.G., Klaus, B. & Muller-Hermelink, H.K. The involution of the ageing human thymic epithelium is independent of puberty. A morphometric study. *Scand J Immunol* **22**, 563-75. (1985).

86. Bofill, M. et al. Microenvironments in the normal thymus and the thymus in myasthenia gravis. *Am J Pathol* **119**, 462-73. (1985).

87. Savino, W., Villa-Verde, D.M. & Lannes-Vieira, J. Extracellular matrix proteins in intrathymic T-cell migration and differentiation? *Immunol Today* **14**, 158-61. (1993).

88. Moerman, P., Goddeeris, P., Lauwerijns, J. & Van der Hauwaert, L.G. Cardiovascular malformations in DiGeorge syndrome (congenital absence of hypoplasia of the thymus). *Br Heart J* **44**, 452-9. (1980).

89. Couly, G., Lagrue, A. & Griscelli, C. [Di George syndrome, exemplary rhomboencephalic neurocristopathy]. *Rev Stomatol Chir Maxillofac* **84**, 103-8 (1983).

90. Greenberg, F. DiGeorge syndrome: an historical review of clinical and cytogenetic features. *J Med Genet* **30**, 803-6. (1993).
91. Chieffo, C. et al. Isolation and characterization of a gene from the DiGeorge chromosomal region homologous to the mouse Tbx1 gene. *Genomics* **43**, 267-77. (1997).
92. Bollag, R.J. et al. An ancient family of embryonically expressed mouse genes sharing a conserved protein motif with the T locus. *Nat Genet* **7**, 383-9. (1994).
93. Chapman, D.L. et al. Expression of the T-box family genes, Tbx1-Tbx5, during early mouse development. *Dev Dyn* **206**, 379-90. (1996).
94. Lindsay, E.A. et al. Congenital heart disease in mice deficient for the DiGeorge syndrome region. *Nature* **401**, 379-83. (1999).
95. Jerome, L.A. & Papaioannou, V.E. DiGeorge syndrome phenotype in mice mutant for the T-box gene, Tbx1. *Nat Genet* **27**, 286-91. (2001).
96. Pizzuti, A. et al. Isolation and characterization of a novel transcript embedded within HIRA, a gene deleted in DiGeorge syndrome. *Mol Genet Metab* **67**, 227-35. (1999).
97. Farrell, M.J. et al. HIRA, a DiGeorge syndrome candidate gene, is required for cardiac outflow tract septation. *Circ Res* **84**, 127-35. (1999).
98. Magnaghi, P., Roberts, C., Lorain, S., Lipinski, M. & Scambler, P.J. HIRA, a mammalian homologue of Saccharomyces cerevisiae transcriptional co-repressors, interacts with Pax3. *Nat Genet* **20**, 74-7. (1998).
99. Novelli, G., Amati, F. & Dallapiccola, B. UFD1L and CDC45L: a role in DiGeorge syndrome and related phenotypes? *Trends Genet* **15**, 251-4. (1999).
100. Vitelli, F., Morishima, M., Taddei, I., Lindsay, E.A. & Baldini, A. Tbx1 mutation causes multiple cardiovascular defects and disrupts neural crest and cranial nerve migratory pathways. *Hum Mol Genet* **11**, 915-22. (2002).
101. Muller, W., Peter, H.H., Kallfelz, H.C., Franz, A. & Rieger, C.H. The DiGeorge sequence. II. Immunologic findings in partial and complete forms of the disorder. *Eur J Pediatr* **149**, 96-103. (1989).
102. Muller, W. et al. The DiGeorge syndrome. I. Clinical evaluation and course of partial and complete forms of the syndrome. *Eur J Pediatr* **147**, 496-502. (1988).

103. Hong, R. The DiGeorge anomaly (CATCH 22, DiGeorge/velocardiofacial syndrome). *Semin Hematol* **35**, 282-90. (1998).
104. Goldsobel, A.B., Haas, A. & Stiehm, E.R. Bone marrow transplantation in DiGeorge syndrome. *J Pediatr* **111**, 40-4. (1987).
105. Borzy, M.S., Ridgway, D., Noya, F.J. & Shearer, W.T. Successful bone marrow transplantation with split lymphoid chimerism in DiGeorge syndrome. *J Clin Immunol* **9**, 386-92. (1989).
106. August, C.S., Berkel, A.I., Levey, R.H., Rosen, F.S. & Kay, H.E. Establishment of immunological competence in a child with congenital thymic aplasia by a graft of fetal thymus. *Lancet* **1**, 1080-3. (1970).
107. Cleveland, W.W., Fogel, B.J., Brown, W.T. & Kay, H.E. Foetal thymic transplant in a case of Digeorge's syndrome. *Lancet* **2**, 1211-4. (1968).
108. Markert, M.L. et al. Transplantation of thymus tissue in complete DiGeorge syndrome. *N Engl J Med* **341**, 1180-9. (1999).
109. Bortin, M.M. & Rimm, A.A. Severe combined immunodeficiency disease. Characterization of the disease and results of transplantation. *Jama* **238**, 591-600. (1977).
110. Leonard, W.J. The molecular basis of X-linked severe combined immunodeficiency: defective cytokine receptor signaling. *Annu Rev Med* **47**, 229-39 (1996).
111. Macchi, P. et al. Mutations of Jak-3 gene in patients with autosomal severe combined immune deficiency (SCID). *Nature* **377**, 65-8. (1995).
112. Russell, S.M. et al. Mutation of Jak3 in a patient with SCID: essential role of Jak3 in lymphoid development. *Science* **270**, 797-800. (1995).
113. DiSanto, J.P., Muller, W., Guy-Grand, D., Fischer, A. & Rajewsky, K. Lymphoid development in mice with a targeted deletion of the interleukin 2 receptor gamma chain. *Proc Natl Acad Sci U S A* **92**, 377-81. (1995).
114. Thomis, D.C., Gurniak, C.B., Tivol, E., Sharpe, A.H. & Berg, L.J. Defects in B lymphocyte maturation and T lymphocyte activation in mice lacking Jak3. *Science* **270**, 794-7. (1995).

115. Nosaka, T. et al. Defective lymphoid development in mice lacking Jak3. *Science* **270**, 800-2. (1995).

116. Park, S.Y. et al. Developmental defects of lymphoid cells in Jak3 kinase-deficient mice. *Immunity* **3**, 771-82. (1995).

117. Apasov, S.G., Blackburn, M.R., Kellems, R.E., Smith, P.T. & Sitkovsky, M.V. Adenosine deaminase deficiency increases thymic apoptosis and causes defective T cell receptor signaling. *J Clin Invest* **108**, 131-41. (2001).

118. Hershfield, M.S., Kredich, N.M., Ownby, D.R., Ownby, H. & Buckley, R. In vivo inactivation of erythrocyte S-adenosylhomocysteine hydrolase by 2'-deoxyadenosine in adenosine deaminase-deficient patients. *J Clin Invest* **63**, 807-11. (1979).

119. Koshiba, M., Kojima, H., Huang, S., Apasov, S. & Sitkovsky, M.V. Memory of extracellular adenosine A2A purinergic receptor-mediated signaling in murine T cells. *J Biol Chem* **272**, 25881-9. (1997).

120. Apasov, S.G. & Sitkovsky, M.V. The extracellular versus intracellular mechanisms of inhibition of TCR-triggered activation in thymocytes by adenosine under conditions of inhibited adenosine deaminase. *Int Immunol* **11**, 179-89. (1999).

121. Apasov, S., Chen, J.F., Smith, P. & Sitkovsky, M. A(2A) receptor dependent and A(2A) receptor independent effects of extracellular adenosine on murine thymocytes in conditions of adenosine deaminase deficiency. *Blood* **95**, 3859-67. (2000).

122. Ullman, B., Gudas, L.J., Cohen, A. & Martin, D.W., Jr. Deoxyadenosine metabolism and cytotoxicity in cultured mouse T lymphoma cells: a model for immunodeficiency disease. *Cell* **14**, 365-75. (1978).

123. Carson, D.A. et al. Metabolic basis for immune dysfunction in adenosine deaminase deficiency. *Ann N Y Acad Sci* **451**, 34-41 (1985).

124. Liu, X., Kim, C.N., Yang, J., Jemmerson, R. & Wang, X. Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c. *Cell* **86**, 147-57. (1996).

125. Mombaerts, P. et al. RAG-1-deficient mice have no mature B and T lymphocytes. *Cell* **68**, 869-77. (1992).

126. Shinkai, Y. et al. RAG-2-deficient mice lack mature lymphocytes owing to inability to initiate V(D)J rearrangement. *Cell* **68**, 855-67. (1992).
127. Schwarz, K. et al. RAG mutations in human B cell-negative SCID. *Science* **274**, 97-9. (1996).
128. Hacein-Bey-Abina, S. et al. Sustained correction of X-linked severe combined immunodeficiency by ex vivo gene therapy. *N Engl J Med* **346**, 1185-93. (2002).
129. Klein, C., Lisowska-Grospierre, B., LeDeist, F., Fischer, A. & Griscelli, C. Major histocompatibility complex class II deficiency: clinical manifestations, immunologic features, and outcome. *J Pediatr* **123**, 921-8. (1993).
130. Elhasid, R. & Etzioni, A. Major histocompatibility complex class II deficiency: a clinical review. *Blood Rev* **10**, 242-8. (1996).
131. Mach, B., Steimle, V., Martinez-Soria, E. & Reith, W. Regulation of MHC class II genes: lessons from a disease. *Annu Rev Immunol* **14**, 301-31 (1996).
132. Reith, W. & Mach, B. The bare lymphocyte syndrome and the regulation of MHC expression. *Annu Rev Immunol* **19**, 331-73 (2001).
133. Lisowska-Grospierre, B. et al. A defect in the regulation of major histocompatibility complex class II gene expression in human HLA-DR negative lymphocytes from patients with combined immunodeficiency syndrome. *J Clin Invest* **76**, 381-5. (1985).
134. de Preval, C., Lisowska-Grospierre, B., Loche, M., Griscelli, C. & Mach, B. A trans-acting class II regulatory gene unlinked to the MHC controls expression of HLA class II genes. *Nature* **318**, 291-3. (1985).
135. de Preval, C., Hadam, M.R. & Mach, B. Regulation of genes for HLA class II antigens in cell lines from patients with severe combined immunodeficiency. *N Engl J Med* **318**, 1295-300. (1988).
136. Reith, W. et al. Congenital immunodeficiency with a regulatory defect in MHC class II gene expression lacks a specific HLA-DR promoter binding protein, RF-X. *Cell* **53**, 897-906. (1988).
137. Sempowski, G.D. et al. Leukemia inhibitory factor, oncostatin M, IL-6, and stem cell factor mRNA expression in human thymus increases with age and is associated with thymic atrophy. *J Immunol* **164**, 2180-7. (2000).

138. Tyan, M.L. Age-related decrease in mouse T cell progenitors. *J Immunol* **118**, 846-51. (1977).

139. Kadish, J.L. & Basch, R.S. Hematopoietic thymocyte precursors. I. Assay and kinetics of the appearance of progeny. *J Exp Med* **143**, 1082-99. (1976).

140. Aspinall, R. Age-associated thymic atrophy in the mouse is due to a deficiency affecting rearrangement of the TCR during intrathymic T cell development. *J Immunol* **158**, 3037-45. (1997).

141. Jamieson, B.D. et al. Generation of functional thymocytes in the human adult. *Immunity* **10**, 569-75. (1999).

142. Hartwig, M. & Steinmann, G. On a causal mechanism of chronic thymic involution in man. *Mech Ageing Dev* **75**, 151-6. (1994).

143. Smith, O. *Anat Rec* (1930).

144. Morales-Montor, J. *Mod. Asp. Immunobiol.* (2002).

145. De Mello-Coelho, V., Savino, W., Postel-Vinay, M.C. & Dardenne, M. Role of prolactin and growth hormone on thymus physiology. *Dev Immunol* **6**, 317-23 (1998).

146. de Mello-Coelho, V. et al. Growth hormone and its receptor are expressed in human thymic cells. *Endocrinology* **139**, 3837-42. (1998).

147. Robert, F.R. et al. The recognition of hypothalamo-neurohypophysial functions by developing T cells. *Dev Immunol* **2**, 131-40 (1992).

148. Tian, Z.G. et al. Recombinant human growth hormone promotes hematopoietic reconstitution after syngeneic bone marrow transplantation in mice. *Stem Cells* **16**, 193-9 (1998).

149. Chappel, S. Growth hormone in immune reconstitution. *J Acquir Immune Defic Syndr Hum Retrovirol* **20**, 423-31. (1999).

150. Golde, D.W., Bersch, N. & Li, C.H. Growth hormone: species-specific stimulation of erythropoiesis in vitro. *Science* **196**, 1112-3. (1977).

151. Blazar, B.R., Brennan, C.A., Broxmeyer, H.E., Shultz, L.D. & Vallera, D.A. Transgenic mice expressing either bovine growth hormone (bGH) or human GH releasing hormone (hGRH) have increased splenic progenitor cell colony formation and DNA synthesis in vitro and in vivo. *Exp Hematol* **23**, 1397-406. (1995).

152. Napolitano, L.A. et al. Increased thymic mass and circulating naive CD4 T cells in HIV-1-infected adults treated with growth hormone. *Aids* **16**, 1103-11. (2002).
153. Gagnerault, M.C., Postel-Vinay, M.C. & Dardenne, M. Expression of growth hormone receptors in murine lymphoid cells analyzed by flow cytofluorometry. *Endocrinology* **137**, 1719-26. (1996).
154. Thellin, O. et al. Expression of growth hormone receptors by lymphocyte subpopulations in the human tonsil. *Dev Immunol* **6**, 295-304 (1998).
155. Cohen, J.J. Glucocorticoid-induced apoptosis in the thymus. *Semin Immunol* **4**, 363-9. (1992).
156. Tibbetts, T.A., DeMayo, F., Rich, S., Conneely, O.M. & O'Malley, B.W. Progesterone receptors in the thymus are required for thymic involution during pregnancy and for normal fertility. *Proc Natl Acad Sci U S A* **96**, 12021-6. (1999).
157. Rijhsinghani, A.G., Bhatia, S.K., Tygrett, L.T. & Waldschmidt, T.J. Effect of pregnancy on thymic T cell development. *Am J Reprod Immunol* **35**, 523-8. (1996).
158. Rijhsinghani, A.G., Thompson, K., Bhatia, S.K. & Waldschmidt, T.J. Estrogen blocks early T cell development in the thymus. *Am J Reprod Immunol* **36**, 269-77. (1996).
159. Kumar, N., Shan, L.X., Hardy, M.P., Bardin, C.W. & Sundaram, K. Mechanism of androgen-induced thymolysis in rats. *Endocrinology* **136**, 4887-93. (1995).
160. Ferone, D. et al. Somatostatin receptors in the thymus. *Ann Med* **31 Suppl 2**, 28-33. (1999).
161. Ferone, D. et al. In vitro characterization of somatostatin receptors in the human thymus and effects of somatostatin and octreotide on cultured thymic epithelial cells. *Endocrinology* **140**, 373-80. (1999).
162. Kovacs, W.J. & Olsen, N.J. Androgen receptors in human thymocytes. *J Immunol* **139**, 490-3. (1987).
163. Bridges, E.D., Greenstein, B.D., Khamashta, M.A. & Hughes, G.R. Specificity of estrogen receptors in rat thymus. *Int J Immunopharmacol* **15**, 927-32. (1993).
164. Miller, A.H. et al. Glucocorticoid receptors are differentially expressed in the cells and tissues of the immune system. *Cell Immunol* **186**, 45-54. (1998).

165. Olsen, N.J., Watson, M.B., Henderson, G.S. & Kovacs, W.J. Androgen deprivation induces phenotypic and functional changes in the thymus of adult male mice. *Endocrinology* **129**, 2471-6. (1991).

166. Olsen, N.J., Watson, M.B. & Kovacs, W.J. Studies of immunological function in mice with defective androgen action. Distinction between alterations in immune function due to hormonal insensitivity and alterations due to other genetic factors. *Immunology* **73**, 52-7. (1991).

167. Brugnera, E. et al. Coreceptor reversal in the thymus: signaled CD4+8+ thymocytes initially terminate CD8 transcription even when differentiating into CD8+ T cells. *Immunity* **13**, 59-71. (2000).

168. Spangrude, G.J., Heimfeld, S. & Weissman, I.L. Purification and characterization of mouse hematopoietic stem cells. *Science* **241**, 58-62. (1988).

169. Huang, S. & Terstappen, L.W. Formation of haematopoietic microenvironment and haematopoietic stem cells from single human bone marrow stem cells. *Nature* **360**, 745-9. (1992).

170. Terstappen, L.W., Huang, S., Safford, M., Lansdorp, P.M. & Loken, M.R. Sequential generations of hematopoietic colonies derived from single nonlineage-committed CD34+CD38- progenitor cells. *Blood* **77**, 1218-27. (1991).

171. Terstappen, L.W., Huang, S. & Picker, L.J. Flow cytometric assessment of human T-cell differentiation in thymus and bone marrow. *Blood* **79**, 666-77. (1992).

172. Scott, E.W., Simon, M.C., Anastasi, J. & Singh, H. Requirement of transcription factor PU.1 in the development of multiple hematopoietic lineages. *Science* **265**, 1573-7. (1994).

173. Sanchez, M.J., Muench, M.O., Roncarolo, M.G., Lanier, L.L. & Phillips, J.H. Identification of a common T/natural killer cell progenitor in human fetal thymus. *J Exp Med* **180**, 569-76. (1994).

174. Wu, L., Nichogiannopoulou, A., Shortman, K. & Georgopoulos, K. Cell-autonomous defects in dendritic cell populations of Ikaros mutant mice point to a developmental relationship with the lymphoid lineage. *Immunity* **7**, 483-92. (1997).

175. McKercher, S.R. et al. Targeted disruption of the PU.1 gene results in multiple hematopoietic abnormalities. *Embo J* **15**, 5647-58. (1996).

176. Wang, J.H. et al. Selective defects in the development of the fetal and adult lymphoid system in mice with an Ikaros null mutation. *Immunity* **5**, 537-49. (1996).
177. Rodewald, H.R. et al. A population of early fetal thymocytes expressing Fc gamma RII/III contains precursors of T lymphocytes and natural killer cells. *Cell* **69**, 139-50. (1992).
178. Matsuzaki, Y. et al. Characterization of c-kit positive intrathymic stem cells that are restricted to lymphoid differentiation. *J Exp Med* **178**, 1283-92. (1993).
179. Lanier, L.L., Spits, H. & Phillips, J.H. The developmental relationship between NK cells and T cells. *Immunol Today* **13**, 392-5. (1992).
180. Ardavin, C., Wu, L., Li, C.L. & Shortman, K. Thymic dendritic cells and T cells develop simultaneously in the thymus from a common precursor population. *Nature* **362**, 761-3. (1993).
181. Wu, L., Li, C.L. & Shortman, K. Thymic dendritic cell precursors: relationship to the T lymphocyte lineage and phenotype of the dendritic cell progeny. *J Exp Med* **184**, 903-11. (1996).
182. Peault, B., Khazaal, I. & Weissman, I.L. In vitro development of B cells and macrophages from early mouse fetal thymocytes. *Eur J Immunol* **24**, 781-4. (1994).
183. Wu, L., Antica, M., Johnson, G.R., Scollay, R. & Shortman, K. Developmental potential of the earliest precursor cells from the adult mouse thymus. *J Exp Med* **174**, 1617-27. (1991).
184. Moore, T.A. & Zlotnik, A. T-cell lineage commitment and cytokine responses of thymic progenitors. *Blood* **86**, 1850-60. (1995).
185. Jotereau, F.V., Houssaint, E. & Le Douarin, N.M. Lymphoid stem cell homing to the early thymic primordium of the avian embryo. *Eur J Immunol* **10**, 620-7. (1980).
186. Fontaine-Perus, J.C., Calman, F.M., Kaplan, C. & Le Douarin, N.M. Seeding of the 10-day mouse embryo thymic rudiment by lymphocyte precursors in vitro. *J Immunol* **126**, 2310-6. (1981).
187. Pyke, K.W. & Bach, J.F. The in vitro migration of murine fetal liver cells to thymic rudiments. *Eur J Immunol* **9**, 317-23. (1979).

188. Champion, S., Imhof, B.A., Savagner, P. & Thiery, J.P. The embryonic thymus produces chemotactic peptides involved in the homing of hemopoietic precursors. *Cell* **44**, 781-90. (1986).

189. Baggiolini, M., Dewald, B. & Moser, B. Human chemokines: an update. *Annu Rev Immunol* **15**, 675-705 (1997).

190. Norment, A.M., Bogatzki, L.Y., Gantner, B.N. & Bevan, M.J. Murine CCR9, a chemokine receptor for thymus-expressed chemokine that is up-regulated following pre-TCR signaling. *J Immunol* **164**, 639-48. (2000).

191. Zabel, B.A. et al. Human G protein-coupled receptor GPR-9-6/CC chemokine receptor 9 is selectively expressed on intestinal homing T lymphocytes, mucosal lymphocytes, and thymocytes and is required for thymus-expressed chemokine-mediated chemotaxis. *J Exp Med* **190**, 1241-56. (1999).

192. Aiuti, A., Webb, I.J., Bleul, C., Springer, T. & Gutierrez-Ramos, J.C. The chemokine SDF-1 is a chemoattractant for human CD34+ hematopoietic progenitor cells and provides a new mechanism to explain the mobilization of CD34+ progenitors to peripheral blood. *J Exp Med* **185**, 111-20. (1997).

193. Kitchen, S.G. & Zack, J.A. CXCR4 expression during lymphopoiesis: implications for human immunodeficiency virus type 1 infection of the thymus. *J Virol* **71**, 6928-34. (1997).

194. Moepps, B., Frodl, R., Rodewald, H.R., Baggiolini, M. & Gierschik, P. Two murine homologues of the human chemokine receptor CXCR4 mediating stromal cell-derived factor 1alpha activation of Gi2 are differentially expressed in vivo. *Eur J Immunol* **27**, 2102-12. (1997).

195. Suzuki, G. et al. Pertussis toxin-sensitive signal controls the trafficking of thymocytes across the corticomedullary junction in the thymus. *J Immunol* **162**, 5981-5. (1999).

196. Uehara, S., Grinberg, A., Farber, J.M. & Love, P.E. A role for CCR9 in T lymphocyte development and migration. *J Immunol* **168**, 2811-9. (2002).

197. Uehara, S., Song, K., Farber, J.M. & Love, P.E. Characterization of CCR9 expression and CCL25/thymus-expressed chemokine responsiveness during T cell

development: CD3(high)CD69+ thymocytes and gammadeltaTCR+ thymocytes preferentially respond to CCL25. *J Immunol* **168**, 134-42. (2002).

198. Fine, J.S. & Kruisbeek, A.M. The role of LFA-1/ICAM-1 interactions during murine T lymphocyte development. *J Immunol* **147**, 2852-9. (1991).
199. Hueber, A.O., Pierres, M. & He, H.T. Sulfated glycans directly interact with mouse Thy-1 and negatively regulate Thy-1-mediated adhesion of thymocytes to thymic epithelial cells. *J Immunol* **148**, 3692-9. (1992).
200. Wadsworth, S., Halvorson, M.J. & Coligan, J.E. Developmentally regulated expression of the beta 4 integrin on immature mouse thymocytes. *J Immunol* **149**, 421-8. (1992).
201. Muller, K.M., Luedecker, C.J., Udey, M.C. & Farr, A.G. Involvement of E-cadherin in thymus organogenesis and thymocyte maturation. *Immunity* **6**, 257-64. (1997).
202. Robey, E. Regulation of T cell fate by Notch. *Annu Rev Immunol* **17**, 283-95 (1999).
203. Hasserjian, R.P., Aster, J.C., Davi, F., Weinberg, D.S. & Sklar, J. Modulated expression of notch1 during thymocyte development. *Blood* **88**, 970-6. (1996).
204. Mombaerts, P. et al. Mutations in T-cell antigen receptor genes alpha and beta block thymocyte development at different stages. *Nature* **360**, 225-31. (1992).
205. Philpott, K.L. et al. Lymphoid development in mice congenitally lacking T cell receptor alpha beta-expressing cells. *Science* **256**, 1448-52. (1992).
206. Itohara, S. et al. T cell receptor delta gene mutant mice: independent generation of alpha beta T cells and programmed rearrangements of gamma delta TCR genes. *Cell* **72**, 337-48. (1993).
207. Prockop, S. & Petrie, H.T. Cell migration and the anatomic control of thymocyte precursor differentiation. *Semin Immunol* **12**, 435-44. (2000).
208. Moore, N.C., Anderson, G., Smith, C.A., Owen, J.J. & Jenkinson, E.J. Analysis of cytokine gene expression in subpopulations of freshly isolated thymocytes and thymic stromal cells using semiquantitative polymerase chain reaction. *Eur J Immunol* **23**, 922-7. (1993).
209. Oosterwegel, M.A., Haks, M.C., Jeffry, U., Murray, R. & Kruisbeek, A.M. Induction of TCR gene rearrangements in uncommitted stem cells by a subset of IL-

7 producing, MHC class-II-expressing thymic stromal cells. *Immunity* **6**, 351-60. (1997).

210. Shortman, K. & Wu, L. Early T lymphocyte progenitors. *Annu Rev Immunol* **14**, 29-47 (1996).
211. Ito, M. et al. Removal of stem cell factor or addition of monoclonal anti-c-KIT antibody induces apoptosis in murine melanocyte precursors. *J Invest Dermatol* **112**, 796-801. (1999).
212. Rossi, P., Sette, C., Dolci, S. & Geremia, R. Role of c-kit in mammalian spermatogenesis. *J Endocrinol Invest* **23**, 609-15. (2000).
213. Nishikawa, S. et al. In utero manipulation of coat color formation by a monoclonal anti-c-kit antibody: two distinct waves of c-kit-dependency during melanocyte development. *Embo J* **10**, 2111-8. (1991).
214. Kondo, M., Akashi, K., Domen, J., Sugamura, K. & Weissman, I.L. Bcl-2 rescues T lymphopoiesis, but not B or NK cell development, in common gamma chain-deficient mice. *Immunity* **7**, 155-62. (1997).
215. Brack, C., Hirama, M., Lenhard-Schuller, R. & Tonegawa, S. A complete immunoglobulin gene is created by somatic recombination. *Cell* **15**, 1-14. (1978).
216. Rowen, L., Koop, B.F. & Hood, L. The complete 685-kilobase DNA sequence of the human beta T cell receptor locus. *Science* **272**, 1755-62. (1996).
217. Chien, Y.H., Gascoigne, N.R., Kavaler, J., Lee, N.E. & Davis, M.M. Somatic recombination in a murine T-cell receptor gene. *Nature* **309**, 322-6. (1984).
218. Kavaler, J., Davis, M.M. & Chien, Y. Localization of a T-cell receptor diversity-region element. *Nature* **310**, 421-3. (1984).
219. Fanning, L., Connor, A., Baetz, K., Ramsden, D. & Wu, G.E. Mouse RSS spacer sequences affect the rate of V(D)J recombination. *Immunogenetics* **44**, 146-50 (1996).
220. Ramsden, D.A., Baetz, K. & Wu, G.E. Conservation of sequence in recombination signal sequence spacers. *Nucleic Acids Res* **22**, 1785-96. (1994).
221. Bassing, C.H., Swat, W. & Alt, F.W. The mechanism and regulation of chromosomal V(D)J recombination. *Cell* **109 Suppl**, S45-55. (2002).

222. Monroe, R.J. et al. RAG2:GFP knockin mice reveal novel aspects of RAG2 expression in primary and peripheral lymphoid tissues. *Immunity* **11**, 201-12. (1999).

223. Res, P. & Spits, H. Developmental stages in the human thymus. *Semin Immunol* **11**, 39-46. (1999).

224. Kruisbeek, A.M. Development of alpha beta T cells. *Curr Opin Immunol* **5**, 227-34. (1993).

225. Dudley, E.C., Petrie, H.T., Shah, L.M., Owen, M.J. & Hayday, A.C. T cell receptor beta chain gene rearrangement and selection during thymocyte development in adult mice. *Immunity* **1**, 83-93. (1994).

226. Levelt, C.N. et al. Regulation of thymocyte development through CD3: functional dissociation between p56lck and CD3 sigma in early thymic selection. *Immunity* **3**, 215-22. (1995).

227. Fehling, H.J. et al. Restoration of thymopoiesis in pT alpha-/- mice by anti-CD3epsilon antibody treatment or with transgenes encoding activated Lck or tailless pT alpha. *Immunity* **6**, 703-14. (1997).

228. Fehling, H.J. & von Boehmer, H. Early alpha beta T cell development in the thymus of normal and genetically altered mice. *Curr Opin Immunol* **9**, 263-75. (1997).

229. Hoffman, E.S. et al. Productive T-cell receptor beta-chain gene rearrangement: coincident regulation of cell cycle and clonality during development in vivo. *Genes Dev* **10**, 948-62. (1996).

230. Wiest, D.L., Berger, M.A. & Carleton, M. Control of early thymocyte development by the pre-T cell receptor complex: A receptor without a ligand? *Semin Immunol* **11**, 251-62. (1999).

231. Crompton, T., Gilmour, K.C. & Owen, M.J. The MAP kinase pathway controls differentiation from double-negative to double-positive thymocyte. *Cell* **86**, 243-51. (1996).

232. Verschuren, M.C. et al. Preferential rearrangements of the T cell receptor-delta-deleting elements in human T cells. *J Immunol* **158**, 1208-16. (1997).

233. Malissen, M. et al. Regulation of TCR alpha and beta gene allelic exclusion during T-cell development. *Immunol Today* **13**, 315-22. (1992).

234. Roth, M.E., Holman, P.O. & Kranz, D.M. Nonrandom use of J alpha gene segments. Influence of V alpha and J alpha gene location. *J Immunol* **147**, 1075-81. (1991).

235. Petrie, H.T. et al. Multiple rearrangements in T cell receptor alpha chain genes maximize the production of useful thymocytes. *J Exp Med* **178**, 615-22. (1993).

236. McMahan, C.J. & Fink, P.J. RAG reexpression and DNA recombination at T cell receptor loci in peripheral CD4+ T cells. *Immunity* **9**, 637-47. (1998).

237. Petrie, H.T., Livak, F., Burtrum, D. & Mazel, S. T cell receptor gene recombination patterns and mechanisms: cell death, rescue, and T cell production. *J Exp Med* **182**, 121-7. (1995).

238. Siegel, R.M. et al. Inhibition of thymocyte apoptosis and negative antigenic selection in bcl-2 transgenic mice. *Proc Natl Acad Sci U S A* **89**, 7003-7. (1992).

239. Egerton, M., Scollay, R. & Shortman, K. Kinetics of mature T-cell development in the thymus. *Proc Natl Acad Sci U S A* **87**, 2579-82. (1990).

240. Kisielow, P. & von Boehmer, H. Development and selection of T cells: facts and puzzles. *Adv Immunol* **58**, 87-209 (1995).

241. Guidos, C.J. Positive selection of CD4+ and CD8+ T cells. *Curr Opin Immunol* **8**, 225-32. (1996).

242. Kearse, K.P. et al. Developmental regulation of alpha beta T cell antigen receptor expression results from differential stability of nascent TCR alpha proteins within the endoplasmic reticulum of immature and mature T cells. *Embo J* **13**, 4504-14. (1994).

243. Shortman, K., Vremec, D. & Egerton, M. The kinetics of T cell antigen receptor expression by subgroups of CD4+8+ thymocytes: delineation of CD4+8+3(2+) thymocytes as post-selection intermediates leading to mature T cells. *J Exp Med* **173**, 323-32. (1991).

244. Nikolic-Zugic, J. Phenotypic and functional stages in the intrathymic development of alpha beta T cells. *Immunol Today* **12**, 65-70. (1991).

245. Scollay, R. & Godfrey, D.I. Thymic emigration: conveyor belts or lucky dips? *Immunol Today* **16**, 268-73; discussion 273-4. (1995).

246. Poznansky, M.C. et al. Thymocyte emigration is mediated by active movement away from stroma-derived factors. *J Clin Invest* **109**, 1101-10. (2002).

247. Zinkernagel, R.M. & Doherty, P.C. The discovery of MHC restriction. *Immunol Today* **18**, 14-7. (1997).

248. Ashton-Rickardt, P.G. & Tonegawa, S. A differential-avidity model for T-cell selection. *Immunol Today* **15**, 362-6. (1994).

249. Janeway, C.A., Jr. Ligands for the T-cell receptor: hard times for avidity models. *Immunol Today* **16**, 223-5. (1995).

250. Jameson, S.C., Hogquist, K.A. & Bevan, M.J. Positive selection of thymocytes. *Annu Rev Immunol* **13**, 93-126 (1995).

251. Sebzda, E. et al. Selection of the T cell repertoire. *Annu Rev Immunol* **17**, 829-74 (1999).

252. Sprent, J. & Webb, S.R. Function and specificity of T cell subsets in the mouse. *Adv Immunol* **41**, 39-133 (1987).

253. Ohashi, P.S., Pircher, H., Burki, K., Zinkernagel, R.M. & Hengartner, H. Distinct sequence of negative or positive selection implied by thymocyte T-cell receptor densities. *Nature* **346**, 861-3. (1990).

254. Spain, L.M. & Berg, L.J. Developmental regulation of thymocyte susceptibility to deletion by "self"-peptide. *J Exp Med* **176**, 213-23. (1992).

255. Carlow, D.A., Teh, S.J. & Teh, H.S. Altered thymocyte development resulting from expressing a deleting ligand on selecting thymic epithelium. *J Immunol* **148**, 2988-95. (1992).

256. Carlow, D.A., van Oers, N.S., Teh, S.J. & Teh, H.S. Deletion of antigen-specific immature thymocytes by dendritic cells requires LFA-1/ICAM interactions. *J Immunol* **148**, 1595-603. (1992).

257. Janeway, C.A., Jr. The co-receptor function of CD4. *Semin Immunol* **3**, 153-60. (1991).

258. Shaw, A.S. & Dustin, M.L. Making the T cell receptor go the distance: a topological view of T cell activation. *Immunity* **6**, 361-9. (1997).

259. Davis, S.J. & van der Merwe, P.A. The structure and ligand interactions of CD2: implications for T-cell function. *Immunol Today* **17**, 177-87. (1996).

260. Matsui, K. et al. Low affinity interaction of peptide-MHC complexes with T cell receptors. *Science* **254**, 1788-91. (1991).

261. Weber, S., Traunecker, A., Oliveri, F., Gerhard, W. & Karjalainen, K. Specific low-affinity recognition of major histocompatibility complex plus peptide by soluble T-cell receptor. *Nature* **356**, 793-6. (1992).

262. Williams, A.F. & Beyers, A.D. T-cell receptors. At grips with interactions. *Nature* **356**, 746-7. (1992).

263. Robey, E.A. et al. The level of CD8 expression can determine the outcome of thymic selection. *Cell* **69**, 1089-96. (1992).

264. Lee, N.A., Loh, D.Y. & Lacy, E. CD8 surface levels alter the fate of alpha/beta T cell receptor-expressing thymocytes in transgenic mice. *J Exp Med* **175**, 1013-25. (1992).

265. von Boehmer, H., Teh, H.S. & Kisielow, P. The thymus selects the useful, neglects the useless and destroys the harmful. *Immunol Today* **10**, 57-61. (1989).

266. Love, P.E. & Shores, E.W. ITAM multiplicity and thymocyte selection: how low can you go? *Immunity* **12**, 591-7. (2000).

267. Vukmanovic, S., Bevan, M.J. & Hogquist, K.A. The specificity of positive selection: MHC and peptides. *Immunol Rev* **135**, 51-66. (1993).

268. Hugo, P., Kappler, J.W. & Marrack, P.C. Positive selection of TcR alpha beta thymocytes: is cortical thymic epithelium an obligatory participant in the presentation of major histocompatibility complex protein? *Immunol Rev* **135**, 133-55. (1993).

269. Bix, M. & Raulet, D. Inefficient positive selection of T cells directed by haematopoietic cells. *Nature* **359**, 330-3. (1992).

270. Pawlowski, T., Elliott, J.D., Loh, D.Y. & Staerz, U.D. Positive selection of T lymphocytes on fibroblasts. *Nature* **364**, 642-5. (1993).

271. Hugo, P., Kappler, J.W., McCormack, J.E. & Marrack, P. Fibroblasts can induce thymocyte positive selection in vivo. *Proc Natl Acad Sci U S A* **90**, 10335-9. (1993).

272. Jenkinson, E.J., Anderson, G. & Owen, J.J. Studies on T cell maturation on defined thymic stromal cell populations in vitro. *J Exp Med* **176**, 845-53. (1992).

273. Hogquist, K.A. et al. T cell receptor antagonist peptides induce positive selection. *Cell* **76**, 17-27. (1994).

274. Ashton-Rickardt, P.G. et al. Evidence for a differential avidity model of T cell selection in the thymus. *Cell* **76**, 651-63. (1994).

275. Sebzda, E. et al. Positive and negative thymocyte selection induced by different concentrations of a single peptide. *Science* **263**, 1615-8. (1994).

276. Hogquist, K.A., Jameson, S.C. & Bevan, M.J. The ligand for positive selection of T lymphocytes in the thymus. *Curr Opin Immunol* **6**, 273-8. (1994).

277. Schumacher, T.N. & Ploegh, H.L. Are MHC-bound peptides a nuisance for positive selection? *Immunity* **1**, 721-3. (1994).

278. Ashton-Rickardt, P.G., Van Kaer, L., Schumacher, T.N., Ploegh, H.L. & Tonegawa, S. Peptide contributes to the specificity of positive selection of CD8+ T cells in the thymus. *Cell* **73**, 1041-9. (1993).

279. Ashton-Rickardt, P.G. The role of peptide in the positive selection of CD8+ T cells in the thymus. *Thymus* **22**, 111-5. (1993).

280. Ignatowicz, L., Kappler, J. & Marrack, P. The repertoire of T cells shaped by a single MHC/peptide ligand. *Cell* **84**, 521-9. (1996).

281. Tourne, S. et al. Selection of a broad repertoire of CD4+ T cells in H-2Ma0/0 mice. *Immunity* **7**, 187-95. (1997).

282. MacDonald, H.R. et al. T-cell receptor V beta use predicts reactivity and tolerance to Mlsa-encoded antigens. *Nature* **332**, 40-5. (1988).

283. Kappler, J.W., Staerz, U., White, J. & Marrack, P.C. Self-tolerance eliminates T cells specific for Mls-modified products of the major histocompatibility complex. *Nature* **332**, 35-40. (1988).

284. Nossal, G.J. Immunologic tolerance: collaboration between antigen and lymphokines. *Science* **245**, 147-53. (1989).

285. Marrack, P. & Kappler, J. The T cell receptor. *Science* **238**, 1073-9. (1987).

286. Kisielow, P., Teh, H.S., Bluthmann, H. & von Boehmer, H. Positive selection of antigen-specific T cells in thymus by restricting MHC molecules. *Nature* **335**, 730-3. (1988).

287. Murphy, K.M., Heimberger, A.B. & Loh, D.Y. Induction by antigen of intrathymic apoptosis of CD4+CD8+TCRlo thymocytes in vivo. *Science* **250**, 1720-3. (1990).

288. Vasquez, N.J., Kaye, J. & Hedrick, S.M. In vivo and in vitro clonal deletion of double-positive thymocytes. *J Exp Med* **175**, 1307-16. (1992).

289. Smith, C.A., Williams, G.T., Kingston, R., Jenkinson, E.J. & Owen, J.J. Antibodies to CD3/T-cell receptor complex induce death by apoptosis in immature T cells in thymic cultures. *Nature* **337**, 181-4. (1989).

290. Shi, Y.F. et al. In vivo administration of monoclonal antibodies to the CD3 T cell receptor complex induces cell death (apoptosis) in immature thymocytes. *J Immunol* **146**, 3340-6. (1991).

291. Sprent, J., Lo, D., Gao, E.K. & Ron, Y. T cell selection in the thymus. *Immunol Rev* **101**, 173-90. (1988).

292. Widera, G. et al. Transgenic mice selectively lacking MHC class II (I-E) antigen expression on B cells: an in vivo approach to investigate Ia gene function. *Cell* **51**, 175-87. (1987).

293. Benoist, C. & Mathis, D. Positive selection of the T cell repertoire: where and when does it occur? *Cell* **58**, 1027-33. (1989).

294. Derbinski, J., Schulte, A., Kyewski, B. & Klein, L. Promiscuous gene expression in medullary thymic epithelial cells mirrors the peripheral self. *Nat Immunol* **2**, 1032-9. (2001).

295. Lundberg, K., Heath, W., Kontgen, F., Carbone, F.R. & Shortman, K. Intermediate steps in positive selection: differentiation of CD4+8int TCRint thymocytes into CD4-8+TCRhi thymocytes. *J Exp Med* **181**, 1643-51. (1995).

296. Suzuki, H., Punt, J.A., Granger, L.G. & Singer, A. Asymmetric signaling requirements for thymocyte commitment to the CD4+ versus CD8+ T cell lineages: a new perspective on thymic commitment and selection. *Immunity* **2**, 413-25. (1995).

297. Norment, A.M. & Bevan, M.J. Role of chemokines in thymocyte development. *Semin Immunol* **12**, 445-55. (2000).

298. Chaffin, K.E. & Perlmutter, R.M. A pertussis toxin-sensitive process controls thymocyte emigration. *Eur J Immunol* **21**, 2565-73. (1991).

299. Lee, C.K. et al. Thymic emigrants isolated by a new method possess unique phenotypic and functional properties. *Blood* **97**, 1360-9. (2001).

300. Penit, C. & Vasseur, F. Expansion of mature thymocyte subsets before emigration to the periphery. *J Immunol* **159**, 4848-56. (1997).

301. Le Campion, A., Vasseur, F. & Penit, C. Regulation and kinetics of premigrant thymocyte expansion. *Eur J Immunol* **30**, 738-46. (2000).

302. Le Campion, A. et al. Quantitative and qualitative adjustment of thymic T cell production by clonal expansion of premigrant thymocytes. *J Immunol* **168**, 1664-71. (2002).

303. Kim, D.R., Dai, Y., Mundy, C.L., Yang, W. & Oettinger, M.A. Mutations of acidic residues in RAG1 define the active site of the V(D)J recombinase. *Genes Dev* **13**, 3070-80. (1999).

304. Landree, M.A., Wibbenmeyer, J.A. & Roth, D.B. Mutational analysis of RAG1 and RAG2 identifies three catalytic amino acids in RAG1 critical for both cleavage steps of V(D)J recombination. *Genes Dev* **13**, 3059-69. (1999).

305. Fujimoto, S. & Yamagishi, H. Isolation of an excision product of T-cell receptor alpha-chain gene rearrangements. *Nature* **327**, 242-3. (1987).

306. Okazaki, K., Davis, D.D. & Sakano, H. T cell receptor beta gene sequences in the circular DNA of thymocyte nuclei: direct evidence for intramolecular DNA deletion in V-D-J joining. *Cell* **49**, 477-85. (1987).

307. Douek, D.C. et al. Changes in thymic function with age and during the treatment of HIV infection. *Nature* **396**, 690-5. (1998).

308. Chen, C.H., Chanh, T.C. & Cooper, M.D. Chicken thymocyte-specific antigen identified by monoclonal antibodies: ontogeny, tissue distribution and biochemical characterization. *Eur J Immunol* **14**, 385-91. (1984).

309. Kong, F., Chen, C.H. & Cooper, M.D. Thymic function can be accurately monitored by the level of recent T cell emigrants in the circulation. *Immunity* **8**, 97-104. (1998).

310. Kong, F.K., Chen, C.L., Six, A., Hockett, R.D. & Cooper, M.D. T cell receptor gene deletion circles identify recent thymic emigrants in the peripheral T cell pool. *Proc Natl Acad Sci U S A* **96**, 1536-40. (1999).

311. Chretien, I. et al. CTX, a Xenopus thymocyte receptor, defines a molecular family conserved throughout vertebrates. *Eur J Immunol* **28**, 4094-104. (1998).

312. Scollay, R.G., Butcher, E.C. & Weissman, I.L. Thymus cell migration. Quantitative aspects of cellular traffic from the thymus to the periphery in mice. *Eur J Immunol* **10**, 210-8. (1980).

313. Tough, D.F. & Sprent, J. Turnover of naive- and memory-phenotype T cells. *J Exp Med* **179**, 1127-35. (1994).

314. Lempicki, R.A. et al. Impact of HIV-1 infection and highly active antiretroviral therapy on the kinetics of CD4+ and CD8+ T cell turnover in HIV-infected patients. *Proc Natl Acad Sci U S A* **97**, 13778-83. (2000).

315. McCune, J.M. et al. High prevalence of thymic tissue in adults with human immunodeficiency virus-1 infection. *J Clin Invest* **101**, 2301-8. (1998).

316. Livak, F. & Schatz, D.G. T-cell receptor alpha locus V(D)J recombination by-products are abundant in thymocytes and mature T cells. *Mol Cell Biol* **16**, 609-18. (1996).

317. Tanchot, C. & Rocha, B. The organization of mature T-cell pools. *Immunol Today* **19**, 575-9. (1998).

318. Berzins, S.P., Boyd, R.L. & Miller, J.F. The role of the thymus and recent thymic migrants in the maintenance of the adult peripheral lymphocyte pool. *J Exp Med* **187**, 1839-48. (1998).

319. Tanchot, C., Rosado, M.M., Agenes, F., Freitas, A.A. & Rocha, B. Lymphocyte homeostasis. *Semin Immunol* **9**, 331-7. (1997).

320. Arstila, T.P. et al. A direct estimate of the human alphabeta T cell receptor diversity. *Science* **286**, 958-61. (1999).

321. Ehl, S., Klenerman, P., Aichele, P., Hengartner, H. & Zinkernagel, R.M. A functional and kinetic comparison of antiviral effector and memory cytotoxic T lymphocyte populations in vivo and in vitro. *Eur J Immunol* **27**, 3404-13. (1997).

322. Cerwenka, A., Morgan, T.M. & Dutton, R.W. Naive, effector, and memory CD8 T cells in protection against pulmonary influenza virus infection: homing properties rather than initial frequencies are crucial. *J Immunol* **163**, 5535-43. (1999).

323. Sallusto, F., Lenig, D., Forster, R., Lipp, M. & Lanzavecchia, A. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. *Nature* **401**, 708-12. (1999).

324. Di Rosa, F. & Matzinger, P. Long-lasting CD8 T cell memory in the absence of CD4 T cells or B cells. *J Exp Med* **183**, 2153-63. (1996).

325. Swain, S.L., Hu, H. & Huston, G. Class II-independent generation of CD4 memory T cells from effectors. *Science* **286**, 1381-3. (1999).

326. Murali-Krishna, K. et al. Persistence of memory CD8 T cells in MHC class I-deficient mice. *Science* **286**, 1377-81. (1999).

327. Bell, E.B., Sparshott, S.M. & Bunce, C. CD4+ T-cell memory, CD45R subsets and the persistence of antigen--a unifying concept. *Immunol Today* **19**, 60-4. (1998).

328. Ciurea, A. et al. Persistence of lymphocytic choriomeningitis virus at very low levels in immune mice. *Proc Natl Acad Sci U S A* **96**, 11964-9. (1999).

329. Ochsenbein, A.F. et al. Control of early viral and bacterial distribution and disease by natural antibodies. *Science* **286**, 2156-9. (1999).

330. Tanchot, C. & Rocha, B. The peripheral T cell repertoire: independent homeostatic regulation of virgin and activated CD8+ T cell pools. *Eur J Immunol* **25**, 2127-36. (1995).

331. Tanchot, C. & Rocha, B. Peripheral selection of T cell repertoires: the role of continuous thymus output. *J Exp Med* **186**, 1099-106. (1997).

332. Grossman, Z., Meier-Schellersheim, M., Sousa, A.E., Victorino, R.M. & Paul, W.E. CD4+ T-cell depletion in HIV infection: are we closer to understanding the cause? *Nat Med* **8**, 319-23. (2002).

333. Kirberg, J., Berns, A. & von Boehmer, H. Peripheral T cell survival requires continual ligation of the T cell receptor to major histocompatibility complex-encoded molecules. *J Exp Med* **186**, 1269-75. (1997).

334. Bruno, L., Kirberg, J. & von Boehmer, H. On the cellular basis of immunological T cell memory. *Immunity* **2**, 37-43. (1995).

335. Freitas, A.A. & Rocha, B. Peripheral T cell survival. *Curr Opin Immunol* **11**, 152-6. (1999).

336. Schluns, K.S., Kieper, W.C., Jameson, S.C. & Lefrancois, L. Interleukin-7 mediates the homeostasis of naive and memory CD8 T cells in vivo. *Nat Immunol* **1**, 426-32. (2000).

337. Napolitano, L.A. et al. Increased production of IL-7 accompanies HIV-1-mediated T-cell depletion: implications for T-cell homeostasis. *Nat Med* **7**, 73-9. (2001).

338. Vogelsang, G.B. How I treat chronic graft-versus-host disease. *Blood* **97**, 1196-201. (2001).

339. Goulmy, E. et al. Mismatches of minor histocompatibility antigens between HLA-identical donors and recipients and the development of graft-versus-host disease after bone marrow transplantation. *N Engl J Med* **334**, 281-5. (1996).

340. Rocha, B., Dautigny, N. & Pereira, P. Peripheral T lymphocytes: expansion potential and homeostatic regulation of pool sizes and CD4/CD8 ratios in vivo. *Eur J Immunol* **19**, 905-11. (1989).

341. Mackall, C.L., Granger, L., Sheard, M.A., Cepeda, R. & Gress, R.E. T-cell regeneration after bone marrow transplantation: differential CD45 isoform expression on thymic-derived versus thymic-independent progeny. *Blood* **82**, 2585-94. (1993).

342. Mackall, C.L. et al. Age, thymopoiesis, and CD4+ T-lymphocyte regeneration after intensive chemotherapy. *N Engl J Med* **332**, 143-9. (1995).

343. Roux, E., Helg, C., Chapuis, B., Jeannet, M. & Roosnek, E. T-cell repertoire complexity after allogeneic bone marrow transplantation. *Hum Immunol* **48**, 135-8. (1996).

344. Dumont-Girard, F. et al. Reconstitution of the T-cell compartment after bone marrow transplantation: restoration of the repertoire by thymic emigrants. *Blood* **92**, 4464-71. (1998).

345. Peault, B., Weissman, I.L., Baum, C., McCune, J.M. & Tsukamoto, A. Lymphoid reconstitution of the human fetal thymus in SCID mice with CD34+ precursor cells. *J Exp Med* **174**, 1283-6. (1991).

346. Vandekerckhove, B.A., Namikawa, R., Bacchetta, R. & Roncarolo, M.G. Human hematopoietic cells and thymic epithelial cells induce tolerance via different mechanisms in the SCID-hu mouse thymus. *J Exp Med* **175**, 1033-43. (1992).

347. Vandekerckhove, B.A. et al. Thymic selection of the human T cell receptor V beta repertoire in SCID-hu mice. *J Exp Med* **176**, 1619-24. (1992).
348. Keever, C.A. et al. Immune reconstitution following bone marrow transplantation: comparison of recipients of T-cell depleted marrow with recipients of conventional marrow grafts. *Blood* **73**, 1340-50. (1989).
349. Storek, J., Witherspoon, R.P. & Storb, R. T cell reconstitution after bone marrow transplantation into adult patients does not resemble T cell development in early life. *Bone Marrow Transplant* **16**, 413-25. (1995).
350. Heitger, A. et al. Essential role of the thymus to reconstitute naive (CD45RA+) T-helper cells after human allogeneic bone marrow transplantation. *Blood* **90**, 850-7. (1997).
351. Weinberg, K. et al. Factors affecting thymic function after allogeneic hematopoietic stem cell transplantation. *Blood* **97**, 1458-66. (2001).
352. Storek, J. et al. Immunity of patients surviving 20 to 30 years after allogeneic or syngeneic bone marrow transplantation. *Blood* **98**, 3505-12. (2001).
353. Talvensaari, K. et al. A broad T-cell repertoire diversity and an efficient thymic function indicate a favorable long-term immune reconstitution after cord blood stem cell transplantation. *Blood* **99**, 1458-64. (2002).
354. McCune, J.M. The dynamics of CD4+ T-cell depletion in HIV disease. *Nature* **410**, 974-9. (2001).
355. Pantaleo, G. et al. HIV infection is active and progressive in lymphoid tissue during the clinically latent stage of disease. *Nature* **362**, 355-8. (1993).
356. Embretson, J. et al. Massive covert infection of helper T lymphocytes and macrophages by HIV during the incubation period of AIDS. *Nature* **362**, 359-62. (1993).
357. Roederer, M., De Rosa, S.C., Watanabe, N. & Herzenberg, L.A. Dynamics of fine T-cell subsets during HIV disease and after thymic ablation by mediastinal irradiation. *Semin Immunol* **9**, 389-96. (1997).
358. Roederer, M. Getting to the HAART of T cell dynamics. *Nat Med* **4**, 145-6. (1998).

359. Gorochov, G. et al. Perturbation of CD4+ and CD8+ T-cell repertoires during progression to AIDS and regulation of the CD4+ repertoire during antiviral therapy. *Nat Med* **4**, 215-21. (1998).

360. Clerici, M. & Shearer, G.M. A TH1-->TH2 switch is a critical step in the etiology of HIV infection. *Immunol Today* **14**, 107-11. (1993).

361. Clerici, M. et al. Detection of three distinct patterns of T helper cell dysfunction in asymptomatic, human immunodeficiency virus-seropositive patients. Independence of CD4+ cell numbers and clinical staging. *J Clin Invest* **84**, 1892-9. (1989).

362. Clerici, M. et al. Role of interleukin-10 in T helper cell dysfunction in asymptomatic individuals infected with the human immunodeficiency virus. *J Clin Invest* **93**, 768-75. (1994).

363. Lederman, M.M. et al. Immunologic responses associated with 12 weeks of combination antiretroviral therapy consisting of zidovudine, lamivudine, and ritonavir: results of AIDS Clinical Trials Group Protocol 315. *J Infect Dis* **178**, 70-9. (1998).

364. Autran, B. et al. Positive effects of combined antiretroviral therapy on CD4+ T cell homeostasis and function in advanced HIV disease. *Science* **277**, 112-6. (1997).

365. Perrin, L. et al. Virus burden in lymph nodes and blood of subjects with primary human immunodeficiency virus type 1 infection on bitherapy. *J Infect Dis* **177**, 1497-501. (1998).

366. Wong, J.K. et al. Reduction of HIV-1 in blood and lymph nodes following potent antiretroviral therapy and the virologic correlates of treatment failure. *Proc Natl Acad Sci U S A* **94**, 12574-9. (1997).

367. Lafeuillade, A., Poggi, C., Profizi, N., Tamalet, C. & Costes, O. Human immunodeficiency virus type 1 kinetics in lymph nodes compared with plasma. *J Infect Dis* **174**, 404-7. (1996).

368. Cohen, O.J. et al. Decreased human immunodeficiency virus type 1 plasma viremia during antiretroviral therapy reflects downregulation of viral replication in lymphoid tissue. *Proc Natl Acad Sci U S A* **92**, 6017-21. (1995).

369. Gunthard, H.F. et al. Human immunodeficiency virus replication and genotypic resistance in blood and lymph nodes after a year of potent antiretroviral therapy. *J Virol* **72**, 2422-8. (1998).

370. Arno, A. et al. Impact on the immune system of undetectable plasma HIV-1 RNA for more than 2 years. *Aids* **12**, 697-704. (1998).

371. Pakker, N.G. et al. Biphasic kinetics of peripheral blood T cells after triple combination therapy in HIV-1 infection: a composite of redistribution and proliferation. *Nat Med* **4**, 208-14. (1998).

372. Aboulafia, D.M. Regression of acquired immunodeficiency syndrome-related pulmonary Kaposi's sarcoma after highly active antiretroviral therapy. *Mayo Clin Proc* **73**, 439-43. (1998).

373. Macdonald, J.C. et al. Lack of reactivation of cytomegalovirus (CMV) retinitis after stopping CMV maintenance therapy in AIDS patients with sustained elevations in CD4 T cells in response to highly active antiretroviral therapy. *J Infect Dis* **177**, 1182-7. (1998).

374. Komanduri, K.V. et al. Restoration of cytomegalovirus-specific CD4+ T-lymphocyte responses after ganciclovir and highly active antiretroviral therapy in individuals infected with HIV-1. *Nat Med* **4**, 953-6. (1998).

375. Zhang, L. et al. Measuring recent thymic emigrants in blood of normal and HIV-1-infected individuals before and after effective therapy. *J Exp Med* **190**, 725-32. (1999).

376. Hatzakis, A. et al. Effect of recent thymic emigrants on progression of HIV-1 disease. *Lancet* **355**, 599-604. (2000).

377. Su, L. et al. HIV-1-induced thymocyte depletion is associated with indirect cytopathogenicity and infection of progenitor cells in vivo. *Immunity* **2**, 25-36. (1995).

378. Stanley, S.K. et al. Human immunodeficiency virus infection of the human thymus and disruption of the thymic microenvironment in the SCID-hu mouse. *J Exp Med* **178**, 1151-63. (1993).

379. Bonyhadi, M.L. et al. HIV induces thymus depletion in vivo. *Nature* **363**, 728-32. (1993).

380. Berkowitz, R.D., Beckerman, K.P., Schall, T.J. & McCune, J.M. CXCR4 and CCR5 expression delineates targets for HIV-1 disruption of T cell differentiation. *J Immunol* **161**, 3702-10. (1998).

381. Chene, L., Nugeyre, M.T., Barre-Sinoussi, F. & Israel, N. High-level replication of human immunodeficiency virus in thymocytes requires NF-kappaB activation through interaction with thymic epithelial cells. *J Virol* **73**, 2064-73. (1999).

382. Voll, R.E. et al. NF-kappa B activation by the pre-T cell receptor serves as a selective survival signal in T lymphocyte development. *Immunity* **13**, 677-89. (2000).

383. Stoddart, C.A. et al. Impaired replication of protease inhibitor-resistant HIV-1 in human thymus. *Nat Med* **7**, 712-8. (2001).

384. Mackall, C.L., Hakim, F.T. & Gress, R.E. T-cell regeneration: all repertoires are not created equal. *Immunol Today* **18**, 245-51. (1997).

385. Haynes, B.F., Denning, S.M., Le, P.T. & Singer, K.H. Human intrathymic T cell differentiation. *Semin Immunol* **2**, 67-77. (1990).

386. Picker, L.J. et al. Control of lymphocyte recirculation in man. I. Differential regulation of the peripheral lymph node homing receptor L-selection on T cells during the virgin to memory cell transition. *J Immunol* **150**, 1105-21. (1993).

387. Bell, E.B. & Sparshott, S.M. Interconversion of CD45R subsets of CD4 T cells in vivo. *Nature* **348**, 163-6. (1990).

388. Butcher, E.C., Scollay, R.G. & Weissman, I.L. Direct fluorescent labeling of cells with fluorescein or rhodamine isothiocyanate. II. Potential application to studies of lymphocyte migration and maturation. *J Immunol Methods* **37**, 109-21 (1980).

389. Butcher, E.C. & Weissman, I.L. Direct fluorescent labeling of cells with fluorescein or rhodamine isothiocyanate. I. Technical aspects. *J Immunol Methods* **37**, 97-108 (1980).

390. Scollay, R., Jacobs, S., Jerabek, L., Butcher, E. & Weissman, I. T cell maturation: thymocyte and thymus migrant subpopulations defined with monoclonal antibodies to MHC region antigens. *J Immunol* **124**, 2845-53. (1980).

391. Douek, D.C. et al. Assessment of thymic output in adults after haematopoietic stem-cell transplantation and prediction of T-cell reconstitution. *Lancet* **355**, 1875-81. (2000).

392. Suzuki, K. et al. Gut cryptopatches: direct evidence of extrathymic anatomical sites for intestinal T lymphopoiesis. *Immunity* **13**, 691-702. (2000).

393. Boileau, C., Houde, M., Dulude, G., Clegg, C.H. & Perreault, C. Regulation of extrathymic T cell development and turnover by oncostatin M. *J Immunol* **164**, 5713-20. (2000).

394. Wolthers, K.C. et al. T cell telomere length in HIV-1 infection: no evidence for increased CD4+ T cell turnover. *Science* **274**, 1543-7. (1996).

395. Pieri, C., Recchioni, R., Moroni, F., Marcheselli, F. & Damjanovich, S. The response of human lymphocytes to phytohemagglutinin is impaired at different levels during aging. *Ann N Y Acad Sci* **673**, 110-9. (1992).

396. De Rosa, S.C., Herzenberg, L.A. & Roederer, M. 11-color, 13-parameter flow cytometry: identification of human naive T cells by phenotype, function, and T-cell receptor diversity. *Nat Med* **7**, 245-8. (2001).

397. Kimmig, S. et al. Two subsets of naive T helper cells with distinct T cell receptor excision circle content in human adult peripheral blood. *J Exp Med* **195**, 789-94. (2002).

398. Berzins, S.P., Godfrey, D.I., Miller, J.F. & Boyd, R.L. A central role for thymic emigrants in peripheral T cell homeostasis. *Proc Natl Acad Sci U S A* **96**, 9787-91. (1999).

399. Franco, J.M. et al. T-cell repopulation and thymic volume in HIV-1-infected adult patients after highly active antiretroviral therapy. *Blood* **99**, 3702-6. (2002).

400. Hazenberg, M.D. et al. Increased cell division but not thymic dysfunction rapidly affects the T-cell receptor excision circle content of the naive T cell population in HIV-1 infection. *Nat Med* **6**, 1036-42. (2000).

401. Ge, Q., Rao, V.P., Cho, B.K., Eisen, H.N. & Chen, J. Dependence of lymphopenia-induced T cell proliferation on the abundance of peptide/ MHC epitopes and strength of their interaction with T cell receptors. *Proc Natl Acad Sci U S A* **98**, 1728-33. (2001).

402. Soares, M.V. et al. IL-7-dependent extrathymic expansion of CD45RA+ T cells enables preservation of a naive repertoire. *J Immunol* **161**, 5909-17. (1998).

403. Lopez, F. et al. Modalities of synthesis of Ki67 antigen during the stimulation of lymphocytes. *Cytometry* **12**, 42-9 (1991).

404. Wykrzykowska, J.J. et al. Early regeneration of thymic progenitors in rhesus macaques infected with simian immunodeficiency virus. *J Exp Med* **187**, 1767-78. (1998).

405. Hassan, J. & Reen, D.J. Human recent thymic emigrants--identification, expansion, and survival characteristics. *J Immunol* **167**, 1970-6. (2001).

406. Finzi, D. et al. Identification of a reservoir for HIV-1 in patients on highly active antiretroviral therapy. *Science* **278**, 1295-300. (1997).

407. Cheynier, R. et al. Antigenic stimulation by BCG vaccine as an in vivo driving force for SIV replication and dissemination. *Nat Med* **4**, 421-7. (1998).

408. Ostrowski, M.A. et al. Effect of immune activation on the dynamics of human immunodeficiency virus replication and on the distribution of viral quasispecies. *J Virol* **72**, 7772-84. (1998).

409. Brooks, D.G., Kitchen, S.G., Kitchen, C.M., Scripture-Adams, D.D. & Zack, J.A. Generation of HIV latency during thymopoiesis. *Nat Med* **7**, 459-64. (2001).

410. Keir, M.E., Stoddart, C.A., Linquist-Stepps, V., Moreno, M.E. & McCune, J.M. IFN-alpha secretion by type 2 predendritic cells up-regulates MHC class I in the HIV-1-infected thymus. *J Immunol* **168**, 325-31. (2002).

411. Ho, D.D. et al. Rapid turnover of plasma virions and CD4 lymphocytes in HIV-1 infection. *Nature* **373**, 123-6. (1995).

412. Wei, X. et al. Viral dynamics in human immunodeficiency virus type 1 infection. *Nature* **373**, 117-22. (1995).

413. Harper, M.E., Marselle, L.M., Gallo, R.C. & Wong-Staal, F. Detection of lymphocytes expressing human T-lymphotropic virus type III in lymph nodes and peripheral blood from infected individuals by in situ hybridization. *Proc Natl Acad Sci U S A* **83**, 772-6. (1986).

414. Chun, T.W. et al. Quantification of latent tissue reservoirs and total body viral load in HIV-1 infection. *Nature* **387**, 183-8. (1997).

415. Hellerstein, M.K. & McCune, J.M. T cell turnover in HIV-1 disease. *Immunity* **7**, 583-9. (1997).

416. Hellerstein, M. et al. Directly measured kinetics of circulating T lymphocytes in normal and HIV-1-infected humans. *Nat Med* **5**, 83-9. (1999).

417. Thiebot, H. et al. Early and persistent bone marrow hematopoiesis defect in simian/human immunodeficiency virus-infected macaques despite efficient reduction of viremia by highly active antiretroviral therapy during primary infection. *J Virol* **75**, 11594-602. (2001).

418. Blancou, P. et al. The infiltration kinetics of simian immunodeficiency virus-specific T cells drawn to sites of high antigenic stimulation determines local in vivo viral escape. *Proc Natl Acad Sci U S A* **98**, 13237-42. (2001).

419. Gabor, M.J., Scollay, R. & Godfrey, D.I. Thymic T cell export is not influenced by the peripheral T cell pool. *Eur J Immunol* **27**, 2986-93. (1997).

420. Cerami, A. Beyond erythropoiesis: novel applications for recombinant human erythropoietin. *Semin Hematol* **38**, 33-9. (2001).

421. Namen, A.E. et al. B cell precursor growth-promoting activity. Purification and characterization of a growth factor active on lymphocyte precursors. *J Exp Med* **167**, 988-1002. (1988).

422. Appasamy, P.M. Interleukin-7: biology and potential clinical applications. *Cancer Invest* **11**, 487-99 (1993).

423. Heufler, C. et al. Interleukin 7 is produced by murine and human keratinocytes. *J Exp Med* **178**, 1109-14. (1993).

424. Noguchi, M. et al. Interleukin-2 receptor gamma chain: a functional component of the interleukin-7 receptor. *Science* **262**, 1877-80. (1993).

425. Kondo, M. et al. Functional participation of the IL-2 receptor gamma chain in IL-7 receptor complexes. *Science* **263**, 1453-4. (1994).

426. Ziegler, S.E. et al. Reconstitution of a functional interleukin (IL)-7 receptor demonstrates that the IL-2 receptor gamma chain is required for IL-7 signal transduction. *Eur J Immunol* **25**, 399-404. (1995).

427. Lai, S.Y., Molden, J. & Goldsmith, M.A. Shared gamma(c) subunit within the human interleukin-7 receptor complex. A molecular basis for the pathogenesis of X-linked severe combined immunodeficiency. *J Clin Invest* **99**, 169-77. (1997).

428. van der Plas, D.C. et al. Interleukin-7 signaling in human B cell precursor acute lymphoblastic leukemia cells and murine BAF3 cells involves activation of STAT1 and STAT5 mediated via the interleukin-7 receptor alpha chain. *Leukemia* **10**, 1317-25. (1996).

429. Venkitaraman, A.R. & Cowling, R.J. Interleukin 7 receptor functions by recruiting the tyrosine kinase p59fyn through a segment of its cytoplasmic tail. *Proc Natl Acad Sci U S A* **89**, 12083-7. (1992).

430. Seckinger, P. & Fougereau, M. Activation of src family kinases in human pre-B cells by IL-7. *J Immunol* **153**, 97-109. (1994).

431. Page, T.H., Lali, F.V. & Foxwell, B.M. Interleukin-7 activates p56lck and p59fyn, two tyrosine kinases associated with the p90 interleukin-7 receptor in primary human T cells. *Eur J Immunol* **25**, 2956-60. (1995).

432. Johnston, J.A. et al. Interleukins 2, 4, 7, and 15 stimulate tyrosine phosphorylation of insulin receptor substrates 1 and 2 in T cells. Potential role of JAK kinases. *J Biol Chem* **270**, 28527-30. (1995).

433. Dadi, H.K. & Roifman, C.M. Activation of phosphatidylinositol-3 kinase by ligation of the interleukin-7 receptor on human thymocytes. *J Clin Invest* **92**, 1559-63. (1993).

434. Muegge, K., Vila, M.P. & Durum, S.K. Interleukin-7: a cofactor for V(D)J rearrangement of the T cell receptor beta gene. *Science* **261**, 93-5. (1993).

435. Galy, A., Jolivet, M., Jolivet-Reynaud, C. & Hadden, J. Fibroblast growth factor (FGF) and an FGF-like molecule in pituitary extracts stimulate thymic epithelial cell proliferation. *Thymus* **15**, 199-211. (1990).

436. Tominaga, K., Kinoshita, Y., Hato, F., Masuda, A. & Matsuyama, M. Effects of cholinergic agonists on the proliferation and protein synthesis in a cultured thymic epithelial cell line. *Cell Mol Biol* **35**, 679-86 (1989).

437. Savino, W., Postel-Vinay, M.C., Smaniotto, S. & Dardenne, M. The thymus gland: a target organ for growth hormone. *Scand J Immunol* **55**, 442-52. (2002).

438. Sabharwal, P. & Varma, S. Growth hormone synthesized and secreted by human thymocytes acts via insulin-like growth factor I as an autocrine and paracrine growth factor. *J Clin Endocrinol Metab* **81**, 2663-9. (1996).

439. Lannes-Vieira, J., van der Meide, P.H. & Savino, W. Extracellular matrix components of the mouse thymic microenvironment. II. In vitro modulation of basement membrane proteins by interferon-gamma: relationship with thymic epithelial cell proliferation. *Cell Immunol* **137**, 329-40. (1991).

440. Bagnara, G.P. et al. Hemopoiesis in healthy old people and centenarians: well-maintained responsiveness of CD34+ cells to hemopoietic growth factors and remodeling of cytokine network. *J Gerontol A Biol Sci Med Sci* **55**, B61-6; discussion B67-70. (2000).

441. McFarland, R.D., Douek, D.C., Koup, R.A. & Picker, L.J. Identification of a human recent thymic emigrant phenotype. *Proc Natl Acad Sci U S A* **97**, 4215-20. (2000).

The Rockefeller
University Press
floor
0021

June 25, 2002

Dr. Jean-Francois Poulin
Laboratoire d'Immunologie
CR-CHUM, Campus Hotel-Dieu

Dear Dr. Poulin:

We will grant you permission for the print reproduction of JEM-vol:190(4),479-486,1999-article as referred to in your letter dated June 19, 2002.

Permission is granted for one time use only. Please write to us each time for permission concerning future editions and translations, as we do not grant blanket permissions.

Since you are the author, a credit line is our only requirement.

Please give suitable acknowledgment to the source in the following manner: Reproduced from **The Journal of Experimental Medicine, year, vol., pp.** by copyright permission of The Rockefeller University Press.

Sincerely yours,

THE JOURNAL OF
EXPERIMENTAL MEDICINE

Lynette N. Henry
Permissions Coordinator

June 25th, 2002

Object: copyright waiver and statement of authorization

To whom it may concern,

As required in section 1.C.6 of the Guidelines for Thesis preparation of the Faculty of Graduate Studies and Research of McGill University, I hereby authorize Jean-François Poulin to include the following article, of which I am co-author, in his thesis to be submitted to McGill University:

Jean-François Poulin, Mohan N. Viswanathan, Jeffrey M. Harris, Krishna V. Komanduri, Eric Wieder, Nancy Ringuette, Morgan Jenkins, Joseph M. McCune and Rafick-Pierre Sékaly (1999). Direct Evidence of thymic function in adults, *J. Exp. Med.*, Volume 190, number 4, pages 479-486.

Mohan N. Viswanathan

June 25th, 2002

Object: copyright waiver and statement of authorization

To whom it may concern,

As required in section 1.C.6 of the Guidelines for Thesis preparation of the Faculty of Graduate Studies and Research of McGill University, I hereby authorize Jean-François Poulin to include the following article, of which I am co-author, in his thesis to be submitted to McGill University:

Jean-François Poulin, Mohan N. Viswanathan, Jeffrey M. Harris, Krishna V. Komanduri, Eric Wieder, Nancy Ringuette, Morgan Jenkins, Joseph M. McCune and Rafick-Pierre Sékaly (1999). Direct Evidence of thymic function in adults, *J. Exp. Med.*, Volume 190, number 4, pages 479-486.

Jeffrey M. Harris

THE UNIVERSITY OF TEXAS
MD ANDERSON
CANCER CENTER

Eric D. Wieder, Ph.D.
Assistant Professor of Medicine
Section of Transplantation Immunology
Department of Blood and Marrow Transplantation

June 25, 2002

Object: copyright waiver and statement of authorization

To Whom It May Concern:

As required in section 1.C.6 of the Guidelines for Thesis preparation of the Faculty of Graduate Studies and Research of McGill University, I hereby authorize Jean-François Poulin to include the following article, of which I am co-author, in his thesis to be submitted to McGill University:

> Jean-François Poulin, Mohan N. Viswanathan, Jeffrey M. Harris, Krishna V. Komanduri, Eric Wieder, Nancy Ringuette, Morgan Jenkins, Joseph M. McCune and Rafick-Pierre Sékaly (1999). Direct Evidence of thymic function in adults, *J. Exp. Med.*, Volume 190, number 4, pages 479-486.

Sincerely,

Eric D. Wieder, Ph.D.

EW:ms

June 25th, 2002

Object: copyright waiver and statement of authorization

To whom it may concern,

As required in section 1.C.6 of the Guidelines for Thesis preparation of the Faculty of Graduate Studies and Research of McGill University, I hereby authorize Jean-François Poulin to include the following article, of which I am co-author, in his thesis to be submitted to McGill University:

Jean-François Poulin, Mohan N. Viswanathan, Jeffrey M. Harris, Krishna V. Komanduri, Eric Wieder, Nancy Ringuette, Morgan Jenkins, Joseph M. McCune and Rafick-Pierre Sékaly (1999). Direct Evidence of thymic function in adults, *J. Exp. Med.*, Volume 190, number 4, pages 479-486.

Nancy Ringuette

June 25th, 2002

Object: copyright waiver and statement of authorization

To whom it may concern,

As required in section 1.C.6 of the Guidelines for Thesis preparation of the Faculty of Graduate Studies and Research of McGill University, I hereby authorize Jean-François Poulin to include the following article, of which I am co-author, in his thesis to be submitted to McGill University:

Jean-François Poulin, Mohan N. Viswanathan, Jeffrey M. Harris, Krishna V. Komanduri, Eric Wieder, Nancy Ringuette, Morgan Jenkins, Joseph M. McCune and Rafick-Pierre Sékaly (1999). Direct Evidence of thymic function in adults, *J. Exp. Med.*, Volume 190, number 4, pages 479-486.

Morgan Jenkins

THE J. DAVID GLADSTONE INSTITUTES

June 25th, 2002

Ph.D.

GLADSTONE
INSTITUTE OF
VIROLOGY AND
IMMUNOLOGY

PO Box 419100
San Francisco
CA 94141-9100
Telephone
415.826.7500
Facsimile
415.826.1817

University of
California
San Francisco

San Francisco
General Hospital

Object: copyright waiver and statement of authorization

To whom it may concern,

As required in section 1.C.6 of the Guidelines for Thesis preparation of the Faculty of Graduate Studies and Research of McGill University, I hereby authorize Jean-François Poulin to include the following article, of which I am co-author, in his thesis to be submitted to McGill University:

Jean-François Poulin, Mohan N. Viswanathan, Jeffrey M. Harris, Krishna V. Komanduri, Eric Wieder, Nancy Ringuette, Morgan Jenkins, Joseph M. McCune and Rafick-Pierre Sékaly (1999). Direct Evidence of thymic function in adults, *J. Exp. Med.*, Volume 190, number 4, pages 479-486.

Joseph M. McCune

June 25th, 2002

Object: copyright waiver and statement of authorization

To whom it may concern,

As required in section 1.C.6 of the Guidelines for Thesis preparation of the Faculty of Graduate Studies and Research of McGill University, I hereby authorize Jean-François Poulin to include the following article, of which I am co-author, in his thesis to be submitted to McGill University:

Jean-François Poulin, Mohan N. Viswanathan, Jeffrey M. Harris, Krishna V. Komanduri, Eric Wieder, Nancy Ringuette, Morgan Jenkins, Joseph M. McCune and Rafick-Pierre Sékaly (1999). Direct Evidence of thymic function in adults, *J. Exp. Med.*, Volume 190, number 4, pages 479-486.

Rafick-Pierre Sékaly