Thymic function in HIV-infection

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II Kolte L, Strandberg C, Dreves AM, Ersbøll AK, Jeppesen DL, Ryder LP, Nielsen SD. Thymic involvement in immune recovery during antiretroviral treatment of HIV infection in adults; comparison of CT and sonographic findings. Scand J Infect Dis. 2002;34(9):668-72.

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IV Hansen BR*, Kolte L*, Haugaard SB, Dirksen C, Jensen FK, Ryder LP, Sørensen AL, Flyvbjerg A, Nielsen SD, Andersen O. Improved thymic index, density and output in HIV-infected patients following low-dose growth hormone therapy: a placebo controlled study. AIDS. 2009 Oct 23;23(16):2123-31.

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V Kolte L, Gaardbo JC, Skogstrand K, Ryder LP, Ersbøll AK, Nielsen SD. Increased levels of Regulatory T cells (Tregs) in HIVinfected patients after 5 years of highly active anti-retroviral therapy may be due to increased thymic production of naïve Tregs. Clin Exp Immunol. 2009 Jan;155(1):44-52

VI Kolte L, Gaardbo JC, Karlsson I, Sørensen AL, Ryder LP, Skogstrand K, Ladelund S, Nielsen SD. Dysregulation of CD4+CD25+CD127lowFOXP3+ Regulatory T-cells in HIV-infected pregnant women. Blood. 2011 Feb 10;117(6):1861-8. VII Kolte L, Rosenfeldt V, Vang L, Jeppesen DL, Karlsson I, Ryder LP, Skogstrand K, Nielsen SD. Reduced thymic size but no evidence of impaired thymic function in uninfected children born to HIV-infected mothers. Ped. Infect. Dis. Journal. 2011 Apr;30(4):325-330.

INTRODUCTION

Three decades have passed since the first identification in 1981 of previously healthy patients suffering from severe immune deficiency and dying from opportunistic infections – the syndrome we now know as acquired immunodeficiency syndrome (AIDS), and which in 1983 was recognized to be the result of infection with Human Immunodeficiency Virus (HIV) [4]. Almost 30 years later the HIV epidemic has spread throughout the world with more than 50 million people infected of which 25 million have died [5]. HIV continues to be an enormous global health challenge with immense social and economic consequences.

HIV is a retrovirus belonging to the Lentivirus family. Two isolates of HIV exist, HIV-1 and HIV-2, and there is strong evidence that both HIV-1 and HIV-2 were acquired as zoonotic infections originating from Simian Immunodeficiency Virus (SIV) [6] and transferred to humans from chimpanzees and sooty mangabeys, respectively [7;8]. Globally, HIV-1 dominates, and throughout this thesis HIV refers to HIV-1. HIV causes multiple effects on the immune system with the hallmark of HIV-infection being the progressive depletion of CD4+ T-lymphocytes [9;10]. Viral entry relies on the binding of viral gp120 to the cell surface molecule CD4 and either the co-receptor CCR5 or CXCR4 [11-14] (Figure 1). HIV hereby infects CD4+ T-lymphocytes (CD4+ cells) and monocyte/macrophage populations in peripheral blood and tissue. The natural history of HIV-infection consists of an acute phase lasting 4-6 weeks after infection, during which 30-50 % of patients present with influenza-like symptoms, rash and lymphadenopathy [15;16]. This is followed by a chronic and often clinically asymptomatic phase lasting 2-10 years, and ultimately a symptomatic end stage with progressive immune collapse, AIDS, and finally death [17]. The acute HIV-infection is characterized by high viremia and massive CD4+ cell loss, particularly from gutassociated lymphoid tissue (GALT), and establishment of a reservoir of latently infected CD4+ cells [18;19]. A marked CD4 decline in peripheral blood is observed during acute infection followed by a rebound to subnormal levels. During chronic infection a more gradual loss of CD4+ cells is seen, and as the peripheral CD4 count declines the opportunistic infections and malignancies characteristic of AIDS begin to occur [20;21] (Figure 2). The exact mechanisms of HIV-pathogenesis and progressive CD4 depletion in HIVinfected patients remain a fundamental and controversial issue. After the early phase of the AIDS epidemic where the idea of viral latency ruled, the scientific scene has now been set for a more

dynamic view of HIV-pathogenesis with HIV-associated immune hyperactivation playing a central role in the killing of CD4+ cells [9;22;23]. In this context, a subpopulation of T cells called regulatory T cells (Tregs) with suppressive capacity towards activated T cells has gained much attention [24].



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Figure 1

After binding to receptors and co-receptors HIV fusions with and enters the host cell. HIV is then uncoated, and the virion-associated reverse transcriptase is activated and begins synthesizing viral cDNA. This HIV DNA is integrated into the host cell genome catalyzed by the integrase enzyme. Following transcription new virion proteins (Gag, Env, Gag-Pol) are produced. Subsequent assembly and maturation of virus particles rely on activity of the protease enzyme. These crucial steps of HIV life cycle are targeted with fusion inhibitors, nucleoside and non-nucleoside reversetranscriptase inhibitors (NRTIs and NNRTIs, respectively), integrase inhibitors, and with HIV-protease inhibitors (HIV-PIs)[2].

The first antiretroviral drug was developed in 1986. Ten years later, in 1996, highly active anti-retroviral treatment (HAART) using a combination of anti-retroviral drugs targeting different stages in HIV-lifecycle (Figure 1) was introduced resulting in a markedly reduced morbidity and mortality in HIV-infected patients [25-28]. HAART reduces HIV-RNA load and leads to immune reconstitution with an increase in the CD4 count [29]. However, the degree of immune reconstitution varies among patients and a number of patients with adequate control of viral replication do not succeed in obtaining optimal immunological response with a substantial gain in CD4 count [30-32]. The differences in CD4+ cell recovery between patients are in part believed to be due to differences in the supply of naïve T cells from lymphopoietic sources [33]. The thymus is the site of generation of naïve CD4+ and CD8+ T cells early in life, and data strongly suggest that thymic function is pre-served in adults and contributes to immune reconstitution in HIV-infected patients upon treatment with HAART [1;3]. The role of the thymus in immunological recovery in HIV-infection will be the focus of this thesis.

HYPOTHESES

The purpose of this thesis was to test the following hypotheses:

H1: The thymus remains functional in adults and is important to immunological reconstitution in HIV-infected patients [articles I, II, III, IV, V]

- H2: Alteration/stimulation of thymic function in adults is possible
- i. During treatment with low-dose growth hormone [article IV]
- ii. During pregnancy [article VI]

H3: Natural regulatory T cells (Tregs) are produced in the thymus and may be involved in HIV-pathogenesis (adults, pregnancy, children) [articles V, VI, VII]

H4: HIV-negative infants born to HIV-infected mothers have haematological and immunological abnormalities at birth. The immune function in these children can re-cover and thymic function be normalized. [article VII]

In order to test these hypotheses the following investigations were performed:

S-1: Studies evaluating immune reconstitution by measuring thymic output markers (thymic size, total and naïve CD4+ and CD8+ cells, T-cell receptor excision circles (TRECs) containing CD4+ cells, and immunological repertoire) in adult HIV-infected



Figure 2 HIV time course (http://www.wikimedia.org)

patients treated with HAART [articles I, II, III]

S-2: Randomized, placebo-controlled, double-blind trial evaluating the effect of low-dose, long-term, recombinant human growth hormone (rhGH) on immune reconstitution in adult HIV-infected patients with focus on thymic index, density and output [article IV]

S-3: Study evaluating thymic-derived naturally regulatory T cells (Tregs) in HIV-infected patients during HAART [article V]

S-4: Prospective study of immunological changes in thymic output markers, Tregs, immune activation, and cytokine profiles in HIV-infected women during and after pregnancy [article VI]

S-5: Study of thymic size, thymic output, immune activation, Tregs, and cytokine profiles at 15 months of age in uninfected HIV-exposed children born to HIV-infected mothers [article VII]

CD4+ CELL DYNAMICS

The expression "The first cut is the deepest" has been used to describe the early explosive and massive CD4+ cell loss during acute HIV-infection where especially a large proportion of mucosal memory CD4+ cells (in the range of 60%) is killed [10;34]. This initial strike to the immune system seems to determine the overall course of the following chronic infection and to determine the CD4 set point after acute infection. It is now known that the mechanisms of HIV-pathogenesis both during acute and chronic infection involves not only direct virus-mediated killing of HIVinfected CD4+ cells, but more importantly indirect bystander killing by apoptosis related to the high level of HIV-induced immune activation [35-37]. HIV activates the immune system because of continuous virus production, estimated to be as high as 10¹⁰ viral particles produced per day [38-40]. Activated T cells undergo several rounds of cell division and are hereafter bound to die by apoptosis. Thus, turnover rates of T cells in HIV-infected patients are high, in the range of 2-3 fold increased compared to uninfected individuals [41]. The importance of persistent immune activation in HIV-pathogenesis has been supported by the observation that immune activation is an independent predictor of CD4 decline, a predictor shown in some studies to be even better than the viral load [42-45]. Furthermore, immune activation is associated with disease progression and is the single best predictor of survival in HIV-infected patients [45-47]. Likewise, the role of immune activation as a leading factor in HIV-pathogenesis is underlined by the fact that non-pathogenic SIV-infection in the natural host, as in the African green monkey, results in high viral replication but absence of both immune activation and CD4 decline - while SIV leading to immune deficiency in the chimpanzee, is characterized by both immune activation and CD4 depletion [48;49]. In HIV-infection, the massive CD4 depletion from GALT during acute infection may leave the intestinal mucosa damaged and leaky allowing for microbial products to translocate across it and invade the organism. This so-called microbial translocation can be measured by circulating lipopolysaccharide levels and is considered to play a central role in the induction of immune activation in chronic HIV-infection and to predict the rate of disease progression [18;50-54] Thus, microbial translocation may provide a part of the link between CD4 depletion in GALT and chronic immune activation, two features of chronic HIV-infection. T lymphocytes fall into two categories: Antigen-inexperienced (naïve) and antigen-experienced (memory/effector) T cells. Upon

priming by foreign antigens naïve T cells differentiate into effector T cells that display a different phenotype and cytokine profile. Memory T cells confer long-term immunity and are divided according to function and phenotype into effector memory cells (T_{EM}) that migrate to inflamed sites and display an immediate effector function, and central memory cells (T_{CM}) that home to secondary lymphoid tissues and have the capability to easily differentiate to effector cells in response to antigen stimulation [55]. How immune activation in HIV-infection leads to CD4 depletion remains unclear. The depletion of the memory CD4+ cell pool, especially T_{EM} cells, by repeated activation, increases the outflow of naïve T cells into the memory T cell compartment, and homeostasis becomes increasingly dependent upon renewal of naïve cells.

THYMOPOIESIS

The development of naïve CD4+ and CD8+ T cells take place in the thymus. CD34+ progenitor cells derived from fetal liver and bone marrow migrate to the thymus and undergo intrathymic sequential stages of phenotypic maturation. The process of thymopoiesis begins at the subcapsular zone of the thymus where immature CD1a+ thymocytes differentiate into CD4-CD8- (double negative) cells by interaction with the thymic stroma. As the thymocytes migrate into the thymic cortex, they acquire coexpression of CD4+ and CD8+ (double positive), and the T cell receptor (TCR) is assembled through recombinant rearrangement of $\alpha\beta$ genes resulting in a broad diverse TCR repertoire [1;56] (Figure 3). Subsequently, positive and negative selection events take place. Positive selection refers to the process during which only thymocytes expressing TCRs that recognize self-MHC complexes are selected. The majority of thymocytes does not fulfill this criterion and are killed by apoptosis. During negative selection potentially autoreactive thymocytes, with TCRs recognizing self-MHC complexes with strong avidity, are removed. The selection processes discard about 99% of all thymocytes and lead to the generation of MHC class I-restricted cytotoxic CD8+ T cells and MHC class II-restricted helper CD4+ T cells that exit the thymus as naïve T cells. These newly produced naïve T cells are also referred to as Recent Thymic Emigrants (RTE) [1]. In addition to a dependence upon functional CD34+ progenitor cells, cytokines, growth factors and hormones influence T cell development [57]. The regulation of T cell development is in particular maintained by Interleukin (IL)-7, which is secreted by many different cells, among other stromal cells from the bone marrow and thymus. IL-7 responsiveness is controlled largely by the presence or absence of IL-7 receptor (IL-7R) being present on double-negative thymocytes, absent on double-positive thymocytes, then re-expressed by single-positive thymocytes and remaining present on most mature T cells [58-60]. IL-7 provides proliferative signals to thymocytes and supports TCR rearrangement. In this way, IL-7 is absolutely required for thymopoiesis as illustrated by the fact that absence of IL-7 or IL-7R in humans results in severe combined immunodeficiency (SCID) with complete lack of T cells [61].

The human thymus is colonized with stem cells as early as gestation weeks 7 or 8, and thymic activity is at its highest during fetal life and early childhood. At birth the peripheral repertoire of T cells is already established in such a degree that thymectomy does not cause immediate immune deficiency. The thymus begins to atrophy at the age of one by estimated 1-3% shrinkage in volume per year [33]. Consequently, thymic function in adults has been assumed to be limited and negligible, and the adult thymus



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Figure 3

Thymopoiesis: CD34+CD1A- progenitor cells are believed to have the potential to differentiate into other cell lineages than T cells, such as dendritic cells (DC), monocytes, and natural killer (NK) cells. Those migrating to the thymus undergo distinct stages of development initially by signals of the NOTCH1 pathway. Definitive commitment to the T cell lineage is marked by CD1A expression. Hereafter, CD34+CD1A+ cells begin assembling their T cell receptor (TCR) by recombinant rearrangement of 22 genes. During this process thymocytes differentiate through stages that include CD4-CD8- double negative, immature single positive (ISP) cells, early CD4+CD8+ double positive (DP), CD4+CD8+ DP, and finally CD4+ or CD8+ single positive (SP) cells. For further, see text. [1]

to be dispensable, unless a massive exhaustion of the T cell pool demands for accelerated T cell regeneration, as in the case of chronic HIV-infection and following haematopoietic stem cell transplantation (HSCT). It has been shown, however, that thymectomy during early childhood (removal of the thymus in connection with paediatric heart surgery) causes premature onset of age-associated alterations in the T cell compartment (lower T cell numbers, lower naïve/memory T cell ratio, lower T cell diversity), thus demonstrating that the thymus is important to T cell immunity throughout life even in healthy individuals [62-65]. During HIV-infection thymopoiesis is compromised and HIV induces changes in the thymus resembling accelerated age-associated thymic atrophy. HIV infects and kills the developing CD4+ thymocytes, and inhibits thymocyte maturation by affecting thymic stromal cells [33;56;66-68]. Furthermore, HIV impairs CD34+ progenitor cell function and bone marrow stroma, thus influencing the inflow of stem cells available for thymopoiesis [69-71].

THYMIC OUTPUT MARKERS

As described, T cells exit the thymus as naïve RTE T cells and enter the pool of naïve cells until activated. Thus, thymic productivity can be estimated by quantifying T cells with naïve phenotype. Several surface markers identify naïve T cells including CD45RA+, CD62L+, CD27+ and CCR7+, some of which account for the capability of naïve cells to home to lymph nodes and hereby encounter antigens presented by antigen-presenting cells [72]. When estimating thymic output, it has been a concern that phenotypically naïve cells could expand in the periphery without losing the naïve phenotype and thus not be RTEs. Naïve CD4+ cells can also have a long quiescent lifespan [73-75]. Furthermore, previous studies suggested that CD45RA+ naïve cells could be reverted memory CD45RO+ cells [76;77]. However, when the coexpression of several naïve markers is measured, this contributes to the identification of cells with a true naïve phenotype [72]. Identification of surface markers relies on flow cytometry, a method analyzing single-cell molecules on cell surfaces or intracellular. Multicolour flow cytometry has been a central method applied in all studies included in this thesis.

Advances in molecular methods have allowed for more precise quantification of thymic output with the development of an assay determining the number of T cell receptor excision circles (TRECs) [3]. TRECs are episomal DNA circles that are generated as a by-product during the rearrangement of the variable (V), diversity (D) and joiner (J) genes of the TCR α and β chains. Be-cause TRECs are stable and not duplicated during mitosis, they are diluted out upon cell division and are thus a marker of the cell's proximity to

the thymus. The signal joint (sj) TREC is generated during rearrangement of the TCR α chain, which is a late event in thymopoiesis occurring after the phase of repeated proliferation, and thus only followed by minor dilution compared to the TRECs generated from most other (earlier) TCR gene rearrangements [3;78] (Figure 4). The sj TREC can be detected in peripheral blood mononuclear cells (PBMCs) or in separated CD4+ or CD8+ cells using polymerase chain reaction (PCR). Quantitative PCR is the preferred method since it is sensitive and detects the amplified target in real time alongside with a household gene, such as mannanbinding lectin (MBL) [79]. Comparing the sj TREC with TRECs generated at earlier rearrangement events, such as the delta TREC, contributes with estimates on intrathymic precursor T cell proliferation [80].



Figure 4

T cell receptor excision circles (TRECs) are episomal DNA circles that are generated in the thymus as a by-product during the rear-rangement of the variable (V), diversity (D) and joiner (J) genes of the TCR arrow and arrow chains [3].

Compatible with TREC measurements being a measure of thymic output, TREC frequencies are highest (as well as are naïve cells) in healthy children and are reduced with increasing age, though still detectable in individuals over 60 years of age [3;81-83]. All thymocyte maturation states are found in elderly individuals [84], and so are active TCR rearrangements with constant thymocyte TREC contents [81] suggestive of maintained thymopoiesis. In HIV-infected untreated patients TREC frequencies are reduced compared to age-matched healthy controls [3;85;86]. Lower TREC contents have been associated with faster disease progression [87;88] and both children and adults who are long-term nonprogressors have higher TREC levels than fast-progressors [89-91]. Initiation of antiretroviral therapy leads to increased TREC levels in both children and adults [3;92;93]. However, this recovery may arise from peripheral T cell events as well as reduced thymic function [94]. It should be emphasized and kept in mind when interpreting TREC data, that apart from thymic output, several other factors determine the TREC frequency, such as

peripheral cell division and cell death [78;94-97]. Furthermore, entrapment of RTEs in lymphoid tissue could also in part explain low TREC frequencies in HIV-infection [98]. A mathematical model taking into account these contributing factors has been developed [94]. Results of mathematical modelling, though, have been conflicting [94;99]. The demonstration of impaired intrathymic proliferation, as measured by the ratio between the deltaTREC and the sjTREC, supported the view that reductions in TREC frequencies in HIV-infection are a result of reduced thymopoiesis more than significant peripheral dilution [80]. Thus, even with these above-mentioned limitations TREC measurements are still regarded as gold standard for the evaluation of thymic output [100]. Instead of TREC frequencies (TREC%), total TREC numbers in a population of cells (e.g. per milliliter of blood) should be reported, as these are not to the same extent influenced by T cell proliferation and provide a more reliable measure of thymic output [101;102].

Recently, CD31, also known as PECAM-1, has been suggested as a marker to identify a subset of RTEs within the naive CD4+ cell pool. Naïve CD4+ cells co-expressing CD31+ have significantly higher TREC contents (on average 8 times higher) compared to naïve CD4+ cells lacking CD31 expression. It is believed that CD31 expression is lost when T cells are repetitively stimulated upon recognition of adequate antigen and therefore essentially limited to T cells recently leaving the thymus [103-107] even though some in vivo proliferation without immediate loss of CD31 has been demonstrated in CD31+ naïve cells [108].

Evaluation of TCR repertoire diversity gives a more qualitative measure of thymic output. The complementary determining region 3 (CDR3) β chain of the TCR plays a critical role in antigen recognition and displays enormous diversity with a Gaussian distribution of CDR3 lengths. Analysis of length variation in the CDR3 can be performed by multiplex PCR for the detection of the 23 functional TCR V β families [109]. A diverse immunological repertoire represents a broad spectrum of T cells with the ability of mounting responses to all possible antigens. Depletions within the T cell pool can result in severe disruptions of TCR repertoire and hereby vulnerability to certain pathogens. The diversity of the T cell pool is drastically contracted as a result of high age [83]. Interestingly, even in healthy individuals, such age-related clonal expansions may be driven by the common chronic infection with the herpes virus cytomegalovirus (CMV). Even if asymptomatic, CMV seems to act as a constant stressor to the immune system and accumulation of CMV specific T cells with increasing age leads to shrinkage of the immunological repertoire, and is most likely playing a major role in aging of the immune system also known as immunosenescence [110-112]. A collapse in CD4 T cell diversity is also seen in HIV-infected patients where perturbations in the TCR repertoire give a "holed" oligoclonal appearance representing deletions of T cells of a particular clonal type. The disruptions seen in HIV-infected patients are not or only partly restored during antiretroviral treatment [113-116]. In theory, only the production of new human T cells in the thymus has a chance to increase diversity.

Finally, thymic size has been used as a marker of thymic output. Both size and density of the lymphoid thymic tissue can be estimated on chest computerized tomography (CT) scans [117-119]. In children the thymus occupies the entire pre-sternal space anterior of the heart, whereas in adults it appears as a small triangular mass due to age-related involution. A scoring scale of thymic index from 0 to 5 on CT scans has been described: 0, no visible thymic tissue; 1, minimal thymic tissue, barely recogniz-





В

D





Figure 5

Representative chest CT scan images of patients with thymic indices of 1 (A), 2 (B), 3 (C) and 4 (D) [I].

С

able; 2, minimal, but more obvious, thymic tissue; 3, moderate amount of thymic tissue; 4, moderate but greater amount of thymic tissue; 5, thymic mass large enough to raise concern about thymoma [117] (Figure 5).

THYMOPOIESIS VERSUS PERIPHERAL EXPANSION

Treatment of HIV-infected patients with HAART leads to immunological recovery of CD4+ cells in the blood. The origin of the CD4+ cells that appear in the blood after initiation of treatment has been debated. The initial increase in the CD4 count during the early months after initiation of HAART primarily constitutes of redistributed memory cells sequestered in lymphoid tissues and now released to the blood stream. After this rapid increase, further increase in the CD4 count is slower and believed to be attributed to both peripheral expansion of memory CD4+ cells as well as thymic production of naïve cells [29;120-122]. The contribution from each of these two pathways varies depending on age and remaining thymic function [120]. Reconstitution by active thymopoiesis results in a more diverse TCR with capacity towards a wide range of neoantigens. In contrast, reconstitution by peripheral expansion of existing T cell clones may lead to a skewed, oligoclonal immunological repertoire.

A study was designed to examine the impact of thymic size on immune recovery in HIV-infected patients [I]. The thymus was visualized by CT scans in 25 adult HIV-infected male patients who had received HAART for a period of 6-18 months, were naïve to antiretroviral therapy prior to HAART, and had levels of viremia <500 copies/mL. For comparison, 10 age-matched control subjects were included in the study. Total and naive CD4+ cell counts, as measured by surface markers CD45RA+ and CD62L+, were determined by 3-colour flow cytometry. To determine thymic output, the CD4-TREC% was measured. Qualitative immune recovery was evaluated by determination of CD4+ TCR repertoire in 19 of the 25 HIV-infected patients. The study showed that larger thymic size was associated with higher CD4+ cell counts and higher CD4-TREC%. Furthermore, patients with abundant thymic tissue (as defined by thymic index > 2) seemed to have broader immunologic repertoires, compared with patients with minimal thymic tissue (as defined by thymic index 2). The study concluded that thymopoiesis supposedly is ongoing in the adult thymus and contributes to immune reconstitution in HIV-infected patients receiving HAART leading to a broader immunological repertoire [I]. The results were in line with a study by Smith et al. demonstrating that thymic size in HIV-infected patients correlates to naïve CD4 counts and to the increase in naive CD4+ cells seen during the early phase of treatment with HAART [118]. Subsequent studies have followed demonstrating evidence of thymopoiesis contributing to immune recovery in adult HIV-infected patients receiving HAART resulting in improvements in naive CD4 counts, TREC frequencies and/or TCR repertoire [93;97;123-131], some of which also demonstrate correlations to a greater abundance of thymic tissue. Interestingly, antiretroviral therapy itself may, too, augment thymic output by promoting survival of developing thymocytes [132].

As mentioned, CT scanning is the preferred modality to evaluate thymic size in adults. However, in children below the age of approximately 2 years the thymus is easily visualized by transsternal ultrasound scans through the not yet calcified sternal bone [133-136]. This method is safer and cheaper than CT, but is highly dependent upon performance expertise. A longitudinal prediction model for the evaluation of thymic size by ultrasound in healthy children from birth until 24 months of age has previously been demonstrated [137]. With the advantages of safety and inexpensiveness we tested the applicability of ultrasound in estimating thymic size in adults and predicting the degree of immune reconstitution in HIV-infected patients receiving HAART [II]. We compared thymic size by CT and ultrasound in 25 HIVinfected patients with known thymic output markers. A radiologist and a paediatrician with expertise in thymus ultrasound evaluated thymic size. No association was found between the two scanning methods. Due to the calcified sternal bone in adults, the ultrasound scanning applied a suprasternal, or in a few cases an intercostal, approach. On the ultrasound image the adult thymus has a recognizable homogenous appearance and an echostructure similar to that of the salivary glands, being more hyperechogenic than the paediatric thymus. However, due to the oblique scanning plane it was difficult in some cases to define the limits of the thymus from the surrounding fatty tissue in the anterior mediastinum. This is most likely the explanation why the two measures of thymic size were not correlated. Accordingly, the

study found no association between thymic size measured by ultrasound and thymic output markers such as naïve CD4+ cells, CD4-TREC% and CD4 TCR. Thus, in predicting immune recovery in HIV-infected patients during HAART, CT remains the preferable method for the evaluation of thymic size [II].

Whether or not CD4 counts in HIV-infected patients during HAART return to levels seen in healthy controls is debated. The greatest increase in CD4 count is observed during the first year of HAART where most studies demonstrate CD4+ increases of 100-200 cells/ μ L per year. This is followed by gains of 30-90 cells/ μ L per year afterwards [31;138;139]. Both a tendency to a plateau of the absolute CD4 count and continuously increasing CD4 counts has been reported in patients after until 6 years of HAART [32;138-140]. Data from the Eurosida Study in patients on HAART for more than 5 years demonstrate that plateauing of CD4 counts only occur in patients with a current CD4 count of more than 500 cells/µL [138]. Furthermore, the risk of opportunistic infections in HIV-infected patients in HAART seem to continue to decrease the further the CD4+ cell count is increased above 350 cells/µL suggesting that complete recovery in both numbers and function of CD4 + cells is essential [141].

During the initial phase of HAART, larger thymic size is associated with a higher recovery of total and naive CD4 + cells, higher TREC% and a more diverse immunological repertoire [I]. Furthermore, the CD4 increase obtained during the first approximately 2 years of HAART is associated with thymic size [124;128;142]. Thymic size also predicts CD4 decline in patients during prolonged treatment interruptions, independent of age and nadir CD4 count [143]. To investigate whether the thymus has a prolonged effect on CD4 recovery, total and naive CD4 counts as well as CD4-TREC% were measured prospectively in 25 HIV-infected patients during 5 years of HAART [III]. Patients with larger thymic size had at all time points of follow-up significantly higher CD4 counts than patients with minimal thymic size. The CD4 increase from time of initiation of HAART until 6 months of follow-up differed significantly between the two thymic groups, but did not at later time points. Thymic output remained significantly higher in patients with larger thymic size at follow-up. However, no difference in the increase in thymic output was seen between thymic groups. The conclusion of the study was therefore that the importance of the thymus to the rate of cellular restoration primarily seems to involve the first two years of HAART. The reason for this may be that thymopoiesis is only maximally induced during maximal lymphopenia whereas while CD4 counts reach normal or subnormal levels thymopoiesis is gradually increased and the CD4 counts may at this point be held in check by other T-cell homeostasic mechanisms [III].

IMMUNE-BASED THERAPIES TO ENHANCE IMMUNE RECONSTI-TUTION

As mentioned, reconstitution of CD4+ cells is slow and variable between patients. Approximately one third of HIV-infected patients on HAART experience virological rebound during the first two years following initial virological suppression [28;144], primarily due to non-compliance [145] or to the development of virus resistance [146]. However, even with effective viral suppression a number of patients have abnormally low CD4+ cell gains. These so called immunological non-responders (INR) have been variably defined in the literature, but refer to patients whose CD4 counts remain below a defined threshold (e.g. 200 cells/µL) after a defined period of suppressive treatment (e.g. 1-4 years) [31;124;147]. It might be reasonable also to include pretherapeutic CD4 counts in the definition, and define INR according to their CD4+ cell gains [148]. Because of the lack of consensus on this definition, frequency estimates on INR vary considerably between studies [31;147;149-151]. In a Danish nationwide cohort study of HIV-infected patients with initial low CD4 counts almost 1 out of 5 patients was described as INR defined as CD4 counts below 200 after 3 years of suppressive treatment [147]. The underlying mechanisms of failed immune reconstitution despite viral suppression are not fully understood, but most likely involve thymic impairment and sustained immune activation [148;152-154]. Ongoing low-grade viral replication has also been suggested [148]. Increasing age is associated with INR as is a prolonged period of immune suppression prior to initiation of HAART and low baseline and nadir CD4 counts [147;155]. Immunological non-responders have an increased risk of opportunistic infections and long-term mortality compared to immunological responders [147;149-151;156]. Thus, patients diagnosed with HIV in an advanced state of the disease (HIV late-presenters) are per se in higher risk of being INR [157;158].

To obtain full recovery of the immune system it may, at least in some patients, require more than antiretroviral therapy. Several supplementary immune-based therapies to enhance immune reconstitution are under investigation using cytokines, hormones, and growth factors [148;155]. These strategies also apply to patients suffering from T cell deficiencies of other causes than HIV-infection such as following chemo- or radiotherapy, for instance preceding HSCT.

IL-2

IL-2 is secreted by activated T cells and regulates proliferation and differentiating of T cells [159]. Intermittent subcutaneous recombinant human IL-2 treatment to induce proliferation and raise CD4 counts when used in combination with antiretroviral therapy has been studied in a large set of clinical trials, latest in two large global randomized controlled clinical trials, the ESPRIT (4111 patients) and the SILCAAT trials (1695 patients). Supplementary IL-2 treatment resulted in a substantial and long-lasting increase in CD4 counts compared to HAART alone [160]. Both naïve and memory CD4+ cells were increased by IL-2 treatment, most likely due to peripheral expansion and altered death rate of existing cells [161-163], even though increased thymopoiesis contributing to the expansion of CD4+ cells could not be ruled out [164]. However, contrary to what would have been expected, the clinical implication of this increase in CD4 counts was not beneficial with regard to a reduction in the risk of opportunistic infections or death. In fact, IL-2 treatment was suggested to have overall deleterious effects, supported by the finding that patients receiving supplementary IL-2 treatment had more grade 4 (potentially lifethreatening) events than patients receiving HAART alone. Patients with the greatest IL-2 induced CD4 increases had the highest risk of severe clinical events [160]. Thus, recovery of CD4+ cells is complex and quantity is definitely not all. The CD4+ cells induced by IL-2 treatment may or may not have a role in host defence [165;166]. For instance, no enhanced response to immunization was seen despite dramatic increases in CD4 counts [165]. Most recently, it has been demonstrated that a large part of the CD4+ cells expanded by IL-2 treatment share phenotypic, functional and molecular characteristics with Tregs (will be discussed later), and due to the known function of these cells, this was suspected to be the cause of the unexpected clinical observations following IL-2 treatment [167]. In conclusion, despite the initial high expectations, it has now been concluded that IL-2 has no place as a

supplementary therapeutic agent in the treatment of HIV-infection.

Growth hormone therapy for the stimulation of thymopoiesis

Age-associated reduced thymopoiesis may be caused by impaired thymic microenvironment such as changes in the levels of several hormones and cytokines including sex hormones, growth hormone (GH) and IL-7 [33;168]. Therefore, focus has been on to whether alteration of some of these molecules could stimulate immune reconstitution by enhancing thymopoiesis and thereby broadening the TCR repertoire improving immunity against pathogens.

GH is a neuroendocrine hormone produced in the anterior pituitary mediating many of its endocrine and metabolic effects through insulin-like growth factor-1 (IGF-1). A number of in-vitro and animal studies have appreciated the important role of GH and IGF-1 to the immune system, and especially to mammalian thymopoiesis [169]. GH enhances proliferation of thymic cells and acts through GH receptors found in the thymus and by influencing cytokine production in the thymic microenvironment [170]. Following hypophysectomy the thymus involutes, but this can be partly reversed by GH treatment [171]. Similarly, GH treatment is associated with increases in thymic size and function in animals. In humans, changes in thymic size, structure and function with age are paralleled by the activity of the GH system [33]. Serum GH and IGF-1 levels peak at puberty and thereafter gradually decline with age suggesting a causal relation. Accordingly, HAART treated HIV-infected children who are GH deficient have reduced thymic size and fewer total and naïve CD4+ and CD8+ cells compared to GH non-deficient children [172]. Likewise, withdrawal of GH treatment to adult GH-deficient patients decreases thymic output and intrathymic proliferation [173].

Early studies of GH treatment in HIV-infected patients were conducted to explore the anabolic effect of the hormone in patients with HIV-associated wasting [174;175]. In relation to this, GH treatment has been examined for its anti-lipodystrophic effect in HIV-infected patients suffering from HIV-associated lipodystrophy [176]. Subsequently, the potential thymic-stimulatory effect of GH treatment in HIV-infected patients has been explored [177-182]. Napolitano et al. reported increased thymic density, TREC frequency in PBMCs, and number of total and naive CD4+ and CD8+ cells in an open-label, cross-over study following a supraphysiological dose of 3 mg recombinant human GH (rhGH)/day for 6 months and 1.5 mg rhGH/day for 6 months in 21 HIVinfected patients. However, adverse events were frequent in this high-dose regimen and 9 out of 21 patients (43%) dropped out of the study [178]. In a pilot study of 6 HIV-infected patients receiving low-dose rhGH regimens for 140 weeks as a supplementary treatment to a stable HAART regime, sustained improvements in CD4 counts were demonstrated and patients had few side effects to the treatment [181]. To investigate this further, a randomized, double-blind placebo controlled trial was conducted to study the effect of long-term, low-dose treatment with rhGH on immune reconstitution in HIV-infected patients [IV]. A total of 46 HIVinfected patients were included. Patients were male, aged 21 to 60 years, on a stable HAART regimen for at least 12 months and with no significant co-morbidity. Patients were randomized 3:2 to either rhGH treatment 0.7 mg/day (28 patients) or placebo (18 patients), administrated as daily subcutaneous injections between 1 pm and 3 pm for 40 weeks. Primary endpoints were changes in thymic size and density as well as thymic output measured by TREC contents and total and naïve CD4 counts. We

found that low dose rhGH treatment was associated with significant increases in thymic index, density and area. Furthermore, this increase in visible thymic tissue was compatible with increased thymopoiesis as measured by TREC frequency and content within CD4+ cells. Due to the low physiologic dose regimen in the study, the medicine was well tolerated with few adverse events, and compliance was high. The data from this study strongly support that GH possesses the potential to enhance thymopoiesis and that reversal of age- or HIV-related decreases in thymic function is pharmacologically possible [IV]. In line with this, rhGH treatment restored both CD4-specific cellular and humoral immune responses to commonly employed vaccines when administered to a selected group of HIV-infected patients with defective vaccination responses [183]. In our study, total CD4+ cells increased more in the GH than in the placebo group, though not significantly. However, it is possible that we underestimate the potential benefits of rhGH treatment since the patients studied were already immunologically well recovered and had median baseline CD4 counts above 500 cells/µL. Supportive of this, Smith et al demonstrated significant rhGH-induced CD4 improvements in patients whose baseline CD4 counts were below 350 cells/µL [179]. Thus, patients with lower CD4 counts may benefit more from rhGH treatment possibly because the thymus primarily is involved in immune recovery at maximal lymphopenia []]]].

In conclusion, even if promising, clinical studies on the role of rhGH in immune recovery in HIV-infected patients are until now few and more are definitely warranted to study more detailedly the clinical effect of rhGH on immune recovery. In future studies the GH releasing hormone analogue Tesamorelin may be more attractive than rhGH since it appears to be better tolerated and to stimulate the GH axis in a more physiologic way [184].

IL-7

IL-7 is essential for thymopoiesis. Furthermore, IL-7 is a critical modulator of peripheral T cell homeostasis involved in maintaining the naïve T cell pool by promoting their survival and inducing proliferation without switching naïve phenotype [58;185]. T cell depletion such as CD4 depletion in HIV-infection results in elevated levels of IL-7 [186]. Upon T cell recovery, IL-7 levels fall [127;187]. This inverse correlation between IL-7 and CD4 counts is due to a feedback mechanism, and consequently the potential use of IL-7 in enhancing thymopoiesis and sustaining naïve T cells have been explored. Administration of recombinant human (rh)IL-7 to humans has been promising in preclinical trials with increases in CD4 counts after only short treatment duration. Evidence that IL-7 might accelerate HIV replication initially raised concerns regarding its use. However, used as a supplement to HAART it has, until now, proven safe [60]. Upon IL-7 treatment T cells of both naïve and memory phenotype expand as do TREC containing cells and CD31+ RTE naïve cells with accompanying TCR broadening suggestive of increased thymopoiesis [188-191]. Furthermore, in contrast to rhIL-2, rhIL-7 expands CD4+ cells without selectively expanding Tregs since these are IL-7R (CD127) low (will be discussed later) [190;192].

Several studies examining the potential of rhIL-7 to enhance immune reconstitution are on-going (www.clinicaltrials.gov). In light of the unexpected and unfortunate experiences with IL-2 treatment, it is extremely important that large long-term randomized studies are conducted to investigate whether or not IL-7 induces CD4 increases that translate into increased survival and/or decreased morbidity and without serious side effects.

IMMUNOLOGICAL TOLERANCE AND TREGS

One of the finest tasks of our immune system is immunological tolerance i.e. the ability to distinguish self from non-self, offering protection to own cells and sufficiently fighting foreign incoming antigens. Immunological tolerance has two components, a central and a peripheral one. Central tolerance constitutes of the processes of positive and negative selection that take place in the thymus where only MHC-restricted and adequately responding T cells manage to get through the eye of the needle. However, the system has failures and potentially auto-reactive T cells eventually escape the thymic environment and enter the peripheral blood with the risk of autoimmunity. In the periphery self-tolerance is maintained by regulatory cells of which the best-defined population is the forkhead box P3 (FoxP3+) Tregs, also called natural Tregs [193]. Natural Tregs develop in the thymus along-side and in sync with other T cells by the interaction of the TCR with selfpeptides and through the selection processes [194]. However, natural Tregs can also be peripherally induced by a number of triggers. In contrast, adaptive Tregs (Th3 and Tr1 cells) exclusively develop in the periphery as a consequence of activation of mature T cells [195]. Throughout this thesis Tregs refers to natural Tregs.

There has been intense focus on Tregs since first described in 1995 [196]. Tregs regulate immune responses by suppressing antigen-specific CD4+ and CD8+ T cell responses and controlling inappropriate or exaggerated immune activation induced by pathogens. Furthermore, Tregs down-regulate alloreactive T cells that recognize antigens from tumours and allografts hereby reducing anti-tumour immunity, graft rejection and graft-versushost disease [197]. Finally, Tregs are assumed to play a role in the regulation of chronic viral infections including HIV [197-201]. As mentioned, chronic immune activation plays a central role in HIVpathogenesis; thus, if Tregs down-regulate immune activation they may be beneficial, and their level may be central to the delicate interaction between the host immune system and HIV, and to viral control. In contrast, it has been argued that Tregs may play a harmful role by suppressing HIV-specific effectors and thereby limiting the body's immune response to HIV. In close relation to Tregs are IL-17 secreting Th17 cells that also modulate/regulate the immune system. Th17 cells and Tregs share a reciprocal maturation pathway and seem to function together in opposing ways to determine the inflammatory response to infection. While Tregs inhibit autoimmunity, Th17 cells in contrast play a role in the induction of autoimmune tissue injury [202;203]. During acute SIV-infection in pigtailed macaques the rapid depletion of Th17 cells and disturbed balance between Th17 cells and Tregs is associated with subsequent high chronic immune activation [204]. Likewise, in HIV-infection the loss of balance between Th17 cells/Tregs with depletion of Th17 cells and increases in Tregs may play a part in inducing microbial translocation and chronic immune activation [205;206]. In contrast, if this ratio between Tregs and Th17 cells is maintained, it may favor spontaneous HIV control as in the so called HIV elite controllers, a rare subgroup of HIV-infected individuals (representing approximately 1% of all HIV-infected patients) who demonstrate undetectable viral loads in the absence of therapy [203;207].

The identification of Tregs

The identification of Tregs has been hampered for years by the lack of specific markers. Tregs are CD4+ and share many surface marker characteristics with activated/memory CD4+ cells. Importantly, both cell types express the IL-2 receptor CD25 [196]. Tregs

express CD25 at slightly higher intensity than activated/memory cells, why, to avoid contamination of Tregs with other cells, studies initially defined Tregs as the proportion of CD4+ T cells expressing the highest levels of CD25 (CD25high) [154;208]. However, this most likely underestimates the proportion of Tregs [209]. Furthermore, identifying CD25high is difficult and subjective. When the expression of the transcription factor FoxP3 was appreciated as a central molecular determinant of differentiation and function of Tregs, responsible for the suppressive capability of Tregs, the investigation of Tregs was very much set forward [198;210]. The FoxP3 gene belongs to the forkhead/winged helix family and is located on the X-chromosome. FoxP3 knock-out mice develop fatal autoimmune disease [211]. Likewise, humans with defective FoxP3 function present with IPEX (Immunodysregulation, Polyendocrinopathy and Enteropathy, X-linked), a fatal syndrome characterized by autoimmune diseases in multiple organs, such as type 1 diabetes, thyroiditis, and inflammatory bowel disease [212]. This clearly indicates the important role of FoxP3 to self-tolerance and measuring FoxP3 is now considered gold standard for the quantification of Tregs. It is noteworthy, however, that recent studies have stated that FoxP3 expression not always indicates a regulatory status, and newly activated Tcells can transiently express FoxP3 [213;214]. FoxP3 in human cells can be measured by quantification of FoxP3 mRNA by RT-PCR or determination of intracellular FoxP3 protein expression by flow cytometry after fixation and permeabilization of cells. Recently, cells co-expressing CD25 and lower levels of the α -chain of the IL-7R, CD127low, have been shown to be highly correlated with intracellular FoxP3 expression (identifying over 95% of the FoxP3+ cells in peripheral blood). Why the IL-7R is down regulated in Tregs and maintained in effector/memory T cells has not been clarified but the method provides a simpler identification of Tregs and a means of purifying viable Tregs for functional studies [215-217]. However, the CD25+CD127low phenotype may just be mirroring the elevated number of activated non-regulatory T cells at least in viremic HIV-infected patients [218]. Therefore, choosing a stringent phenotypic method when identifying Tregs combining the markers CD25, CD127 and FoxP3 better distinguish Tregs from activated cells [219;220]. Other markers have also been used to identify Tregs (including Glucocorticoid-induced TNFR family-related receptor (GITR), cytotoxic T-lymphocyteassociated antigen 4 (CTLA-4), CD45RO, HLA-DR, CD38), but all have in common that all Tregs do not constitutively express them [24:201:221-223]. Whatever method used, human Tregs represent a minority of T cells comprising less than 10% of CD4+ cells. The precise mechanism of their suppressive function remains controversial, but most likely is exerted both by cell-to-cell contact and by immunosuppressive cytokines such as IL-10 and TGFbeta [224]. The suppressive function of Tregs can be studied in socalled suppression assays where Tregs suppress polyclonal stimulation (usually with anti-CD3) of T cells [208].

Tregs in HIV-infection

The relation between Tregs and HIV is controversial, and the level and function of Tregs in HIV-infected patients have been eagerly investigated. Persistent antigen exposure as in chronic HIVinfection can induce normal CD4+ T cells to obtain Tregphenotype and -function, thus increasing number of Tregs [225]. In contrast, Tregs are themselves CD4+ and CCR5+, and hereby susceptible to HIV [222]. Therefore, the absolute number of Tregs is expected to decline with CD4 depletion during HIV-progression whereas the percentage of Tregs (%Tregs) more clearly reflects the kinetics of Tregs in HIV-infection. The relative sparing of Tregs in HIV-infection compared to other T cells has been suggested to be a consequence of the ability of FoxP3 to repress retroviral transcription from the HIV promoter [226]. Furthermore, exposure of Tregs to HIV may selectively promote their survival via a CD4-gp120 dependent pathway [227]. In line with this, most studies demonstrate increased %Tregs in blood of HIV-infected patients compared with healthy controls [221;228-233]. However, some studies do show no difference or decreased levels of %Tregs compared to uninfected controls [24;234;235]. The discrepancy in these findings regarding %Tregs may in part be due to the abovementioned different technical approaches in identifying Tregs. Furthermore, not all early studies distinguished clearly between untreated and treated HIV-infected patients. An accumulation of Tregs in lymphoid tissues has been demonstrated in HIV-infection suggesting altered distribution patterns and dynamics of these cells [227;234].

Of notice, the suppressive capacity of Tregs isolated from HIVinfected patients seems unaltered [228;230;231], even if it has been suggested that the function of Tregs in HIV-infected patients with more advanced disease is impaired [228]. Several studies have demonstrated associations between high Treg levels, low CD4 counts, high degrees of immune activation, and/or high viral loads suggesting the expansion of Tregs to be harmful [229;230;232;236;237]. Accordingly, elite suppressors and longterm non-progressors have normal or even low Treg levels compared to non-controllers or HAART-suppressed patients [238-240]. Interestingly, immune activation may still be higher in elite controllers than in controls [239].

In order to examine the influence of HAART on the Treg level in HIV-infected patients we designed a prospective study with measurements at weeks 0, 4, 12, and 24 of treatment in 26 treatment-naïve HIV-infected patients initiating HAART [221]. Tregs were measured by flow cytometry using the (at that time standard) phenotype CD4+CD25high and by mRNA-expression of FoxP3. Both the percentage of CD4+CD25high cells and the expression of FoxP3 were significantly higher in HIV-infected patients compared to controls. Interestingly, during the 24 weeks of HAART where CD4 counts increased and viral loads decreased. neither of the two measures of Tregs changed, indicating that the elevated level of Tregs in HIV-infected patients is independent of both immunological and virological status. The study confirmed similar findings by Lim et al. where 12 treatment-naïve HIVinfected patients were followed for median 36 weeks after initiating HAART and where no significant changes in CD4+CD25+CD127low Tregs were seen [232]. In both prospective studies, immune activation (CD4+ and CD8+ cells expressing CD38+ and HLA-DR+) remained significantly higher in patients compared with controls, and we questioned whether this was the reason why Treg levels were not normalized. We then examined Treg levels in 15 HIV-infected patients during prolonged HAART [V]. CD4+CD25+ CD127low Tregs and FoxP3 mRNA levels were measured after 1 and 5 years of HAART, and so was immune activation. Levels of Tregs were elevated after 1 year of HAART, and they remained elevated despite 5 years of HAART, suppressed viral loads and normalized CD4 counts and immune activation suggesting that the expansion of Tregs in HIV-infection may be irreversible and does not reflect viral load, CD4 depletion or level of immune activation [V].

IMMUNOLOGICAL TOLERANCE AND PREGNANCY

Pregnancy represents a major challenge to the immune system, and how fetal-maternal tolerance develops is intriguing. As the fetus expresses paternal antigens it is to be regarded as a semiallograft; nonetheless it is not attacked by the maternal immune system [241]. Historically, three reasons have been proposed to explain how the fetus evades an immunological reaction from its mother: 1) the fetus is anatomically separated from the mother and to be regarded as an immune privileged site 2) the fetus is unable to present antigens to the mother due to immunological immaturity 3) the mother somehow ignores fetal tissue [242]. It is now known that fetal cells are detectable in the maternal circulation, they are antigenically mature, and the mother does respond to them, yet tolerates them [241], though occasionally suffers from secondary recurrent miscarriage. The association between HY-restricting HLA class II alleles and recurrent miscarriage subsequent to a first-born boy indicates a CD4+ T cell mediated mechanism in these cases [243]. How maternal tolerance to fetal antigens normally develops has not been fully clarified but seems to involve alteration of both central (thymic) tolerance [244-247] and peripheral tolerance mediated by Tregs [248-253]. In most mammals the thymus is reduced in size and changed in structure during pregnancy, and in mice a substantial loss of thymocyte proliferation and reduced thymic output occurs from early pregnancy [246;247;254]. This may promote survival of the fetus by reducing production of new potentially fetus-reactive T cells (in a way similar to deletion of self-reactive T-cells during the process of negative selection). Both thymic size and function return to normal postpartum, at least after cessation of lactation [247].

Regarding peripheral tolerance, studies in mice have shown that Tregs are expanded from early pregnancy by paternal antigens, and absence of Tregs can lead to pregnancy failure [248;252]. Furthermore, mice undergoing abortion have a diminished number of Tregs compared to normal pregnant mice [255]. Likewise, studies in humans have demonstrated an expansion of Tregs during pregnancy [249;251;252], and levels of human decidual Tregs are significantly lower from women undergoing spontaneous compared to induced abortion [250], supporting the important role of Tregs in deter-mining pregnancy success. Inadequate numbers of Tregs have also been linked to infertility [256] and pre-eclampsia [257] and so has an imbalance in the ratio of FoxP3+ Tregs to IL-17-expressing CD4+ cells [258]. In contrast, an increase in Tregs during pregnancy may explain why a number of autoimmune conditions tend to remit during pregnancy [259].

Pregnancy and HIV

It has long been a concern that the already impaired immune system of HIV-infected women might be further challenged by pregnancy [260;261]. Studies conducted before the introduction of HAART showed that pregnancy either slightly increased HIV disease progression (defined as an AIDS-defining event or death) or had no effect [262;263]. In contrast, studies conducted in the HAART-era have demonstrated a protective effect of pregnancy on disease progression [264-266]. Furthermore, there seems to be a survival advantage in women with 2 pregnancies compared with 1 pregnancy [266]. This may be explained by the fact that healthier HIV-infected women are more likely to become pregnant or it may be due to a possible beneficial interaction between pregnancy and HAART. Regarding CD4 counts and HIV viral loads, no negative pregnancy-induced effect has been demonstrated in studies on HIV-infected women conducted during the HAART-era [264;265]. With the improved future prospects for HIV-infected patients and very good results in preventing mother-to-child transmission of HIV, an increasing number of HIV-infected women in the industrialized countries decide to become pregnant and have children. We designed a prospective study to investigate immunological consequences of pregnancy in HIV-infected women receiving HAART [VI]. A total of 20 HIV-infected women and 16 age- and ethnicity matched HIV-negative women were included and blood samples were drawn once during each trimester of pregnancy and once 2-6 months postpartum. All HIVinfected women were treated with HAART during pregnancy, 8 women were naïve to HAART prior to pregnancy and were treated from gestation week 14 according to national Danish guidelines, 12 women were on HAART already prior to pregnancy. The study showed that CD4 counts were significantly lower in HIV-infected women compared to HIV-negative women at all time points. Interestingly, CD4 counts were increased postpartum in both HIV-infected and HIV-negative women. Immune activation (CD4+/CD8+ cells co-expressing CD38+ and HLA-DR+) was significantly reduced in HIV-infected women during pregnancy both in those already on HAART prior to pregnancy and in those beginning treatment from gestation week 14. HIV viral load remained low in HIV-infected women already on HAART prior to pregnancy and was significantly reduced in those women starting HAART at gestation weeks 14. Thus, the study did not indicate that pregnancy adversely affects the virological and immunological course of HIV-infection [VI].

To explore the hypothesis that pregnancy success relies on alteration of tolerance mechanism thymic output and %Tregs were measured in HIV-infected and HIV-negative women during and after pregnancy [VI]. In line with other studies, an expansion of CD4+CD25+CD127lowFoxP3+ Tregs with a peak during the second trimester and a drop during the third trimester was found, but only in HIV-negative women. Interestingly, Tregs in HIV-infected women were not mobilized. Previous studies have reported on an increased risk of spontaneous abortion in HIV-infected women [267], and it cannot be ruled out that lack of mobilization of Tregs play a role in this phenomenon. Interestingly, lack of Treg mobilization in second trimester was accompanied by a decrease in TGF-beta levels in HIV-infected women possibly reflecting the important interaction and mutual regulation between Tregs and TGF-beta levels.

With regard to thymic output significant reductions in naïve CD4 counts and TREC measurements were not found during pregnancy. However, visually there was a trend showing the expected decrease in both CD4-TREC% and total CD4-TRECs during second trimester, but again only in HIV-negative women.

In conclusion, the study demonstrated differences in immunological measures during pregnancy between HIV-infected and HIV-negative women, some of which may have implications for the induction of fetal-maternal tolerance and in part explain the increased risk of abortion in HIV-infected women [VI]. Dysregulation of Tregs may further be associated with reduced immunogenicity of influenza vaccines in HIV-infected pregnant women [268].

ORIGIN OF TREGS

The fact that Tregs, as well as other T cells, are developed in the thymus, complicates the distinction between central and peripheral tolerance, since the thymus hereby, in part, is responsible for both. In the thymus the selection of Tregs resembles the selection of other thymocytes. However, there is a difference in the affinity

of interactions. Tregs have higher reactivity than other T cells to the selecting ligand, and this high self-reactivity of Tregs may guarantee their prompt and efficient activation upon encounter with a diverse range of self-antigen/MHC complexes in the periphery, ensuring dominant control of self-reactive T cells [269]. Even if originating in the thymus, it has been unclear if the thymus still plays a role in maintaining the Treg pool in adults [194;270]. Thymic involution with age is assumed to affect both regulatory and non-regulatory T cells, the latter shown as reduced TREC contents with older age [3;82;83;223;271]. Until recently peripheral Tregs in adults were defined primarily as belonging to the memory T-cell population, and lifelong maintenance of the Treg pool was believed to be dependent upon peripheral proliferation of existing Tregs independent of thymic output [194;223]. However, sub-fractioning Tregs on the basis of the CD45 splice variants CD45RA and CD45RO reveals that in adults a discrete population of Tregs with naive CD4+CD25+CD45RA+ phenotype does exist [223;271;272]. In cord blood and in neonates the majority of Tregs present naïve phenotype [223;271;273]. These naive Tregs may represent RTE Tregs supported by the demonstration of both longer telomeres and higher TREC contents in naive Tregs, similar to telomere length and TREC contents of other naive CD4+ cells [223;274]. In adults naive Tregs decline with age, as do thymic output and other naive T cells [107;223;271;275]. In contrast, memory and total Treg levels are increased with older age, even in HIV-infected patients, suggesting other mechanisms in addition to thymic output to contribute to the lifelong maintenance of these cells [194;276-278]. Naive Tregs have equally potent immunosuppressive properties as do memory Tregs [223;271], but furthermore seem to have unique self-generating capacities and also seem to be more resistant to apoptosis [273]. Their level has been proposed to be critical for the suppressive function of the entire Treg pool [274]. We designed a study to examine levels of naïve Tregs in adult HIVinfected patients with known thymic output in order to investigate if the expansion of Tregs seen in HIV-infection could be due to increased de-novo generation of naive Tregs from the thymus [V]. Naïve Tregs were defined as CD4+CD25+CD45RA+ according to the present literature [223;271;279]. Naïve Tregs, as well as total Tregs, were significantly higher in HIV-infected patients compared to controls. Furthermore, naïve Tregs depended significantly on the TREC%, thus suggesting that higher Treg levels in HIV-infection can be partly explained by increased thymic production of naïve Tregs [V]. Similar associations have been demonstrated between CD4+CD31+CD45RA+ RTE Tregs and TREC% in HIV-infected patients [107]. Treg levels in thymic tissue isolated from adults undergoing cardiac surgery are also increased in HIVinfected compared to HIV-negative individuals, and even if the naïve/memory phenotype was not evaluated in this study, it supports the hypothesis that thymic production of Tregs is increased in HIV-infection [280]. To further investigate the association between naïve Tregs and thymic output, naïve Tregs were measured in HIV-infected and HIV-negative women during pregnancy [VI]. Naïve Tregs were measured by 7-colour flow cytometry and defined as CD3+CD4+ cells co-expressing the markers CD25+, CD127low, FoxP3+, CD45RA+ and CD27+. Thus, compared to our other study [V], an additional naïve marker (CD27+) was now added, hereby narrowing the naïve Treg population. This study confirmed our previous findings and we found that percentages of naïve Tregs depended significantly on the CD4-TREC% in both HIV-infected and HIV-negative women. Interestingly, we further observed changes in naïve Tregs during pregnancy that were paralleling the changes in total %Tregs in HIV-negative

women during pregnancy - that is an expansion during second trimester followed by a decrease thereafter (Kolte, unpublished). This may imply that the expansion of Tregs during pregnancy also relies, at least to some extent, on thymic production of naïve Tregs. No other human studies have investigated this issue so far, however, studies on mice demonstrate similar findings. Even if reduced in size during pregnancy, the thymus is enriched with Tregs in pregnant but not in non-pregnant mice and this is thought to protect the semi-allergenic fetus [247]. In conclusion, the precise mechanisms of Treg expansions in pathological (HIV-infection) and physiological (pregnancy) settings are still unclear. Our studies suggest that an increase in thymic generation of RTE Tregs contribute to Treg increases, but most likely this is accompanied by an increase in peripheral expansion and increased survival of existing Treg cells.

THYMIC FUNCTION IN HIV-EXPOSED UNINFECTED CHILDREN BORN TO HIV-INFECTED MOTHERS

HIV can be transmitted from mother to child (MTC transmission, also referred to as vertical transmission) during pregnancy, at delivery or through breastfeeding. Without any interventions taken, the risk of MTC transmission is 15 - 25 % [281;282]. In resource-rich countries like Denmark, where the studies included in this thesis were performed, appropriate prophylaxis has markedly reduced the risk of MTC transmission to <1% [283;284]. International guidelines include antiretroviral treatment of the pregnant woman during pregnancy (if not already on HAART prior to pregnancy, then initiation of HAART from gestation weeks 14) supplemented with oral treatment of the child with zidovudine for four weeks after birth, and avoidance of breastfeeding [285]. Previous guidelines included intravenous zidovudine at time of delivery, but this is no longer implemented if viral load is undetectable. As a consequence of effective interventions and the increasing success of programmes preventing MTC HIV transmission in developing countries, the number of HIV-exposed, uninfected (HIV-EU) infants in the world is growing. In some African countries it is anticipated that HIV-EU children in not far future will comprise more than 15% of all children being born. Any health problem or immune deficiency that these children may have, even of minor character, may thus be an enormous challenge, especially in developing countries [286]. Despite the fact that HIV-EU children remain uninfected, their immune system at birth has been shown to be impaired. HIV-EU newborns have increased immune activation and reduced total and naïve CD4 counts, and often they have anemia and neutropenia [287-291]. HIV-EU children may have been exposed to maternally derived HIV-proteins diffusing across the placental barrier during pregnancy, influencing the development of the fetal immune system including progenitor cell function and intrathymic lymphocyte maturation. This is supported by the presence of strong HIV-specific CD4 and CD8 responses in HIV-EU newborns [288;292-294]. Furthermore, bone marrow toxicity due to intrauterine and neonatal exposure to HAART probably contributes to lower CD4 counts and other haematological deficiencies in these newborns [295-297]. Both HIV-proteins and some antiretroviral drugs are known to inhibit progenitor cell function [69;70]. Furthermore, thymic abnormalities have been described in foetuses aborted from HIV-positive mothers even in the absence of thymic HIV-infection [298]. We have previously studied immune function in cord blood from 19 HIV-EU newborns and 19 matched controls of HIV-negative mothers [289]. We found decreased CD4 counts and lower thymic output measured as both reduced naïve

CD4 counts and lower CD4-TREC% in HIV-EU newborns compared to control children. Furthermore, HIV-EU newborns had impaired progenitor function evaluated by CFC assay [289]. Thus, both impaired progenitor function and reduced thymic output may be responsible for lower CD4 counts in HIV-EU newborns. The clinical consequence, if any, of these immune abnormalities in HIV-EU newborns is unclear. Morbidity rates seem no higher in HIV-EU children [299], and growth patterns of HIV-EU children in the large European Collaborative Study were comparable to HIVunexposed children [300]. In addition, results from vaccination studies show normal responses both to measles [301], Bacille Calmette-Guérin [302] and rubella [303] vaccination in HIV-EU children. However, three studies have shown that HAARTexposed compared to HAART-non-exposed HIV-EU children have slightly but significantly CD4 counts (although still within normal limits) persisting long after cessation of exposure up to the age of 12 [304], 15 [297] and 24 [295] months, respectively. The clinical relevance of these findings has been discussed especially since no comparison was made to children born to HIV-negative mothers. We designed a study to investigate immunological consequences beyond infancy of in utero exposure to HIV-proteins and antiretroviral drugs [VII]. Thymic size and output, CD4/CD8 lymphocyte subpopulations including Tregs, and immune activation as well as cytokine profiles were evaluated in 20 HIV-EU children at median 15 months of age and compared to HIV-unexposed control children matched for age and ethnicity. Furthermore, the antibody response to Haemophilus Influenzae Type B (Hib) vaccination was evaluated. We found significantly lower thymic size as evaluated by sonography scans in HIV-EU children. However, CD4 counts and thymic output estimated as naïve CD4 counts and TREC measurements did not differ between HIV-EU children and controls. Likewise, levels of immune activation, Tregs and cytokines were comparable between the two groups of children. Furthermore, no difference was seen in Hib vaccination responses indicating that no qualitative immune deficits remain in HIV-EU children at 15 months of age. In HIV-EU children we found no association between thymic size and output, as we demonstrated in adults. An explanation for this difference may be that the thymus in children, large as it is, possesses an excess capacity just as it is known with liver tissue, so that size and function are not associated. In adults, however, thymic size is markedly reduced and the entire remaining tissue may be of functional character. Thymic size in children has been shown to be a general marker of immune function and lower thymic size is associated with childhood mortality in rural areas [305;306]. Thus, it cannot be ruled out that reduced thymic size in HIV-EU children, even not affecting thymic output in childhood, may have consequences later in life. Furthermore, intrauterine exposure to antiretrovirals does seem to significantly reduce haematologic indices [295-297;304], and it is not yet definite if these reductions, although within normal limits, have long-term clinical implications for HIV-EU children. Finally, the data from this Danish study may not translate to the much larger world population of HIV-EU children in developing countries where prophylactic interventions are less available. Therefore, long-term follow-up of uninfected, HIV- and HAART-exposed children into adulthood is indeed needed.

CONCLUSIONS AND PERSPECTIVES

The aim of our investigations was to contribute to the knowledge of the role of the thymus in different aspects of HIV-infection, and in order to explore this we set up four hypotheses (H1 - H4). Assessment of thymic function has been hampered by the inconvenient location of the organ in the anterior mediastinum and lack of appropriate markers. Newer methods, however, including determination of naïve RTE subsets by multicolor flow cytometry, measurements of TREC-containing cells by PCR and definitions of the breath of the immunological repertoire have allowed for further investigation of thymic output in adulthood, and in T cell depleted settings, such as HIV-infection [3;72;104;109]. It is now generally accepted that thymopoiesis is ongoing even at high age. HIV diminishes thymic size and output, but it can recover with HAART [3;93;118;123-131]. We applied several methods for the estimation of thymic output and found that larger thymic size in adult HIV-infected patients on HAART is associated with improved recovery of total and naïve CD4+ cells, increased TREC frequencies, and renewing of the TCR repertoire diversity [I;II]. Upon HAART, and likewise during T cell reconstitution following chemotherapy, the thymus may even "rebound" to greater than normal size when viewed by CT scans, reflecting renewal capacity [118;307]. Thus, mounting evidence points towards a central role of the thymus in immunological recovery in HIV-infection [308], even if the importance of the thymus to the rate of cellular restoration primarily seems to lie within the first two years of HAART [III] (H1).

Insufficient thymic activity may therefore, in part, explain the large variations in CD4+ cell recovery among HIV-infected patients as may a sustained immune activation and, possibly, ongoing lowgrade viral replication [148]. Since a considerable number of patients do not succeed in obtaining optimal immunological responses despite HAART-induced viral suppression, and are hereby in continuous increased risk of morbidity and death [141;150;151;156], strategies to further enhance immune reconstitution by supplementary immune-based therapies have been investigated in a number of studies. Of most promise seemed treatment with IL- that resulted in large increases in CD4 counts, most likely primarily by stimulating peripheral proliferation of CD4+ cells, however, unexpectedly proved non-beneficial with regard to a reduction in the risk of opportunistic infections and death [160]. In our work, we tested the effect of long-term lowdose rhGH in a randomized, double-blind placebo controlled trial for the enhancement of thymopoiesis [IV]. Low-dose rhGH treatment was associated with significant increases in thymic index, density and area and in TREC contents within CD4+ cells. This strongly supports that GH possesses the potential to enhance thymopoiesis and that reversal of HIV-related decreases in thymic function is pharmacologically possible [IV]. IL-7 is another good candidate because it promotes maturation of thymocytes and is a critical modulator of peripheral T cell homeostasis [60]. Even if promising, clinical studies on the role of rhGH and IL-7 in immune recovery in HIV-infected patients are to date few and more are definitely warranted to study in more details the clinical effects and side effects of such therapies. Especially, it is a concern that stimulation of the thymus induces autoimmunity due to an imbalance in the positive and negative selection processes. In contrast, if proven efficient and well tolerated, thymic-stimulatory therapies may in the future have wide implications not only in pathological T cell depleted settings but also to restore the important set of thymic functions that are compromised by ageing. GH or other therapies may possess the potential to correct oligoclonality of the immunological repertoire, which is one of the significant features of immunosenescence leading to susceptibility of elderly people to infectious diseases and declines in immune responses against vaccines [173]. Moreover, increasing knowledge of the processes underlying ageing of the immune system has reevaluated the potential immunological consequences of total

thymectomy, which has been common practice during cardiac surgery in children. Whether or not thymectomized individuals, due to enhanced immune senescence, are at increased risk of developing inflammatory diseases, have diminished immune responses or vaccination responses, and suffer from increased morbidity or mortality, is as yet not clear, but recommendations may likely change to encourage partial resection of the thymus in order to limit premature ageing of the immune system [62-65] (H2). In this regard, our studies on HIV-EU children demonstrating reduced thymic size at 15 months of age may raise concern [VII]. Even if encouraging that HIV-EU children compared to children born to HIV-negative mothers have no qualitative immune deficits at 15 months of age, it cannot at present be ruled out that persistently reduced thymic size may have consequences later in life where age-related thymic atrophy and immunosenescence set in. Further studies on HIV-EU children into adulthood are definitely warranted to enlighten this issue (H4).

Tregs constitute the peripheral strategy of our immune system to tame self-reactive T cells slipping past the defenses in the thymus and to suppress inappropriate or exaggerated immune activation induced by pathogens [309]. Our studies demonstrate increased levels of Tregs in HIV-infected patients despite long-term treatment with HAART, suppressed viral loads, and normalized CD4 counts and immune activation suggesting that the expansion of Tregs in HIV-infection may be irreversible and does not reflect viral load, CD4 depletion or level of immune activation [V]. Our data further suggest that elevated levels of Tregs in HIV-infected adults may in part be due to in-creased thymic production of naive Tregs [V]. Understanding the interplay between HIV and Tregs is complex [310;311], and with recent advances indicating that the balance between Tregs and Th17 cells presumably matters more than levels of Tregs itself in inducing microbial translocation and chronic immune activation, complexity definitely increases [205;206]. Even though it remains blurred whether or not Tregs are directly responsible for HIV-dependent immunodeficiency, reductions in Treg numbers or Treg activity probably will increase HIV-specific T cell responses generating the type of immunity that is seen in elite suppressors and long-term nonprogressors, and at present it cannot be ruled out that this may be a design for future therapies in HIV-infection (H3). During pregnancy, establishing fetal-maternal tolerance is essential to pregnancy success [241], and we found alterations in thymic output and Treg levels compatible with such an establishment in HIV-negative pregnant women. However, HIV-infected women displayed different immunological profiles compared to HIV-negative women, and were not able to mobilize Tregs during pregnancy [VI]. This suggests an immune unbalance during HIVpositive pregnancy possibly interfering with the prevention of fetal rejection and partly accounting for the increased risk of abortion in HIV-infected women [267]. Understanding into more detail the mechanisms that enable maternal Tregs to overcome alloreactivity during pregnancy may have implications not only for a more profound understanding of Treg function but also for development of therapeutic interventions for a variety of human conditions where peripheral tolerance mechanisms fail such as spontaneous abortion, infertility and autoimmune diseases (H2-3).

The greatest need for HIV-infected patients with access to antiretroviral therapy is no longer new antiretroviral drugs, but new therapeutic strategies to ensure successful immune reconstitution and to help decrease the inappropriate immune activation associated with HIV-infection. Biologicals enhancing thymopoiesis hold great promise to complete CD4 restoration and renew the TCR repertoire in those patients who insufficiently respond to HAART. Furthermore, given the probable causal role of an unbalanced ratio between Tregs and Th17 cells in microbial translocation, interventions designed to restore this unbalance in order to decrease microbial translocation and its downstream inflammatory consequences may be future strategies. Finally, strategies to face the non-AIDS complications that remain in HIV-infected patients in the HAART era including cardiovascular disease, cancer, and osteoporosis and osteopenia will become the challenge for the next decade [312;313].

SUMMARY

This thesis is based on seven previously published articles. The work was performed during my employment at The Department of Infectious Diseases, Copenhagen University Hospital, Hvidovre, as a scholarship student from 2000-2001 and as a research assistant in the period 2004-2010.

HIV-infection is characterized by CD4+ cell depletion. The differences between patients in the degree of CD4+ cell recovery upon treatment with highly active antiretroviral therapy (HAART) may in part be due to differences in the supply of naïve CD4+ cells from the thymus. The thymus atrophies with increasing age for which reason the adult thymus was previously assumed to be without function. The aim of these investigations was to examine the role of the thymus in different aspects of HIV-infection: In adult HIV-infected patients, during HIV-positive pregnancy, and in HIV-exposed uninfected (HIV-EU) children born to HIV-infected mothers.

Thymic size and output were determined in 25 adult HIV-infected patients receiving HAART and in 10 controls. Larger thymic size was associated with higher CD4 counts and higher thymic output. Furthermore, patients with abundant thymic tissue seemed to have broader immunological repertoires, compared with patients with minimal thymic tissue. The study supports the mounting evidence of a contribution by the adult thymus to immune reconstitution in HIV-infection. In a follow-up study conducted till 5 years of HAART, the importance of the thymus to the rate of cellular restoration was found to primarily lie within the first two years of HAART.

The effect of recombinant human growth hormone (rhGH) was then investigated in a randomized, double-blind placebo controlled trial in 46 adult HIV-infected patients on HAART. Daily treatment with a low dose of rhGH of 0.7mg for 40 weeks stimulated thymopoiesis as expressed by thymic size, density, and output strongly supporting the assumption that rhGH possesses the potential to stimulate the ageing thymus, holding promise as a future means to complete CD4 restoration and renew the TCR repertoire in patients who respond insufficiently to HAART. Apart from naïve T cells, Regulatory T cells (Tregs) are developed in the thymus. Tregs play a critical role in peripheral tolerance and suppress inappropriate immune activation such as induced by HIV. We studied levels of Tregs in adult HIV-infected patients with known thymic output. Our studies demonstrate increased levels of Tregs in HIV-infected patients despite long-term treatment with HAART, suppressed viral loads, and normalized CD4 counts and immune activation suggesting that Tregs expand irreversibly in HIV-infection independently of viral load, CD4 depletion or level of immune activation. Our data further suggest that elevated levels of Tregs in HIV-infected adults may in part be due to increased thymic production of naive Tregs.

During pregnancy, establishing fetal-maternal tolerance is essential to pregnancy success. In a prospective study on HIV-positive and HIV-negative pregnant women we found alterations in thymic output and Treg levels in HIV-negative pregnant women compatible with such an establishment. HIV-infected women, however, displayed different immunological profiles from HIV-negative women, and this immune unbalance may interfere with the prevention of fetal rejection and may partly explain the increased risk of abortion in HIV-infected women.

We finally examined thymic function in 20 HIV-EU children at 15 months of age. The thymus was reduced in size in HIV-EU children compared with children born to HIV-negative mothers, but no evidence of impaired thymic function, immune regulation, or antibody vaccination response was detected, suggesting that no qualitative immune deficits persist in HIV-EU children beyond infancy.

In conclusion, the thymus is functional in adults, and it contributes to immunological recovery in HIV-infected patients primarily during the first two years of HAART. Treg levels are increased in HIV-infected patients independent of viral load, CD4 cell depletion or level of immune activation, and this may be due to increased thymic production of naïve Tregs. Alteration of thymic function in adults is possible, both by stimulation of thymopoiesis with rhGH therapy and as a result of pregnancy. Finally, immunological abnormalities detected in HIV-EU infants are recovered at 15 months of age, and even if diminished in size, thymic function is normalized at this age.

LIST OF ABBREVIATIONS

AIDS: acquired immunodeficiency syndrome FoxP3: forkhead box P3 GALT: gut-associated lymphoid tissue GH: growth hormone HIV: human immunodeficiency virus HIV-EU: HIV-exposed, uninfected HAART: highly active anti-retroviral treatment IGF-1: insulin-like growth factor-1 IL: interleukin INR: immunological non-responders MHC: major histocompatibility complex MTC: mother to child PBMCs: peripheral blood mononuclear cells PCR: polymerase chain reaction Rh: recombinant human RTE: recent thymic emigrants SIV: simian immunodeficiency virus Sj: signal joint TCR: T cell receptor TREC: T-cell receptor excision circles TREC%: TREC frequency Tregs: regulatory T cells

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