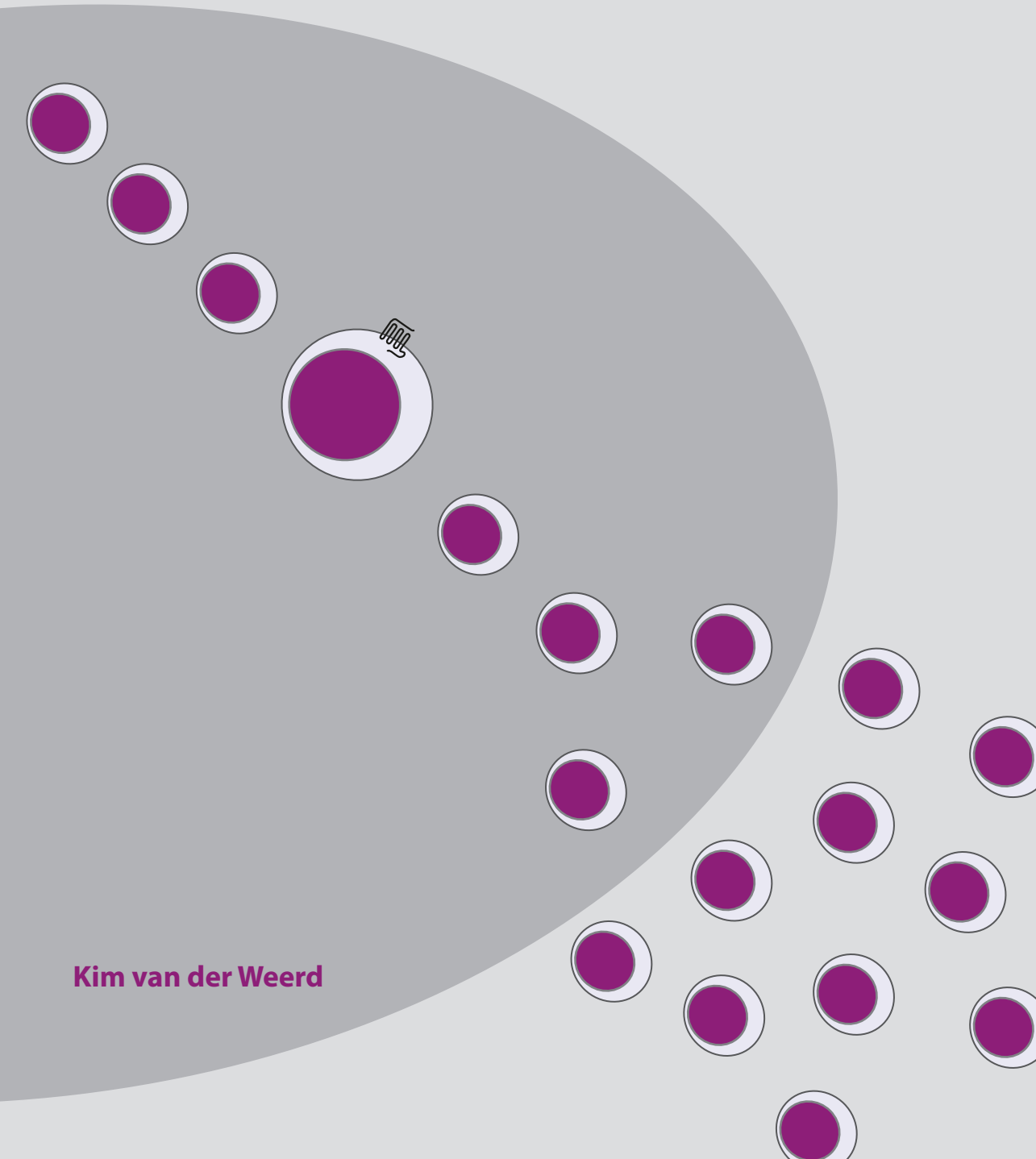
Endocrine regulation of T-cell development and peripheral T-cell maturation



Kim van der Weerd

Endocrine Regulation of T-cell Development and Peripheral T-cell Maturation

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ISBN: 978-94-91811-01-2

Endocrine Regulation of T-cell Development and Peripheral T-cell Maturation

Endocriene regulatie van T-cel ontwikkeling en perifere T-cel maturatie

Proefschrift

ter verkrijging van de graad van doctor aan de
Erasmus Universiteit Rotterdam

op gezag van de
rector magnificus
Prof.dr. H.G. Schmidt

en volgens besluit van het College voor Promoties.

De openbare verdediging zal plaatsvinden op
donderdag 27 juni 2013 om 11.30 uur

door

Kim van der Weerd

geboren te Almelo

PROMOTIECOMMISSIE

Promotoren:	Prof.dr. P.M. van Hagen Prof.dr. F.J.T. Staal
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Copromotor:	Dr. W.A. Dik

The printing of this thesis was financially supported by: Erasmus Medical Center Rotterdam, Erasmus University Rotterdam, J.E. Jurriaanse Stichting, Merck Sharp & Dohme B.V., Ipsen Farmaceutica B.V.

Illustrations: Sandra de Bruin-Versteeg, K. van der Weerd
Printing: Ridderprint B.V., Ridderkerk
Cover: K. van der Weerd, Ridderprint B.V., Ridderkerk
Lay-out: K. van der Weerd

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Chapter I

General introduction

T-CELL DIFFERENTIATION AND DEVELOPMENT

The thymus

The thymus is a lymphoid organ located in the upper anterior thorax behind the sternum. Functionally, the thymus is involved in the formation and maintenance of the peripheral T-cell compartment by supporting the differentiation process of bone-marrow derived T-cell progenitors into mature naive T cells.[1-3]

The origin of the word thymus is uncertain. It might be called after the leaf of the plant *Thymus vulgaris* due to the macroscopic resemblance between the organ and the leaf or it might be derived from the Greek word θυμος which means warty excrescence but also soul or spirit.[4, 5] Although the thymus was already described by Galen of Pergamum (130-200 AD),[5] its function remained obscure for centuries. The thymus has long been considered as an endocrine organ or even an evolutionary remnant with no function.[5] The immunological function of the thymus was first described in the early 1960's by Miller.[6]

The thymus embryonically originates from the third pharyngeal pouch endoderm and surrounding neural crest-derived mesenchymal cells from the third and fourth pharyngeal arches, which together form the thymic primordium.[7-10] The fetal thymic epithelial microenvironment develops from epithelial cell precursors of endodermal origin, and is supported by growth and differentiation signals from surrounding mesenchymal cells.[7-10] Hematopoietic cells colonize the fetal thymus before the thymic microenvironment is fully matured, as early as E11-12 in mice and week 7-8 in humans.[7] The resulting lymphostromal interactions further support maturation and differentiation of the fetal thymic epithelial microenvironment.[7]

The fully developed thymus consists of two lobes enclosed in a fibrous capsule which divides the lobes into lobules.[1] The thymus consists of several morphologically different regions which, from outside to inside, are defined as the subcapsular zone, the cortex, the cortico-medullary junction and the medulla.[1] The thymic epithelial cells (TECs) present in these regions slightly differ from each other, thereby creating highly specialized microenvironments facilitating distinct stages of T-cell development.[8, 11]

T-cell development in the thymus

T-cell development in the thymus is a highly complex process during which bone-marrow derived precursors develop into mature T cells. This process generates a functional peripheral T-cell compartment with a large T-cell receptor (TCR) diversity that is required to fight off foreign antigens.

The maturation of thymus-seeding progenitor cells into fully mature T cells comprises several sequential stages that can be distinguished by phenotypical (surface molecules) and genotypical (TCR gene rearrangements) characteristics (figure 1).[12, 13] Four main T-cell developmental stages can be distinguished based on CD4 and CD8 surface expression patterns in humans and mice (figure 1). T-cell precursors entering the thymus lack CD4 and CD8 surface expression and are therefore called double negative (DN). In the mouse, DN thymocytes can be further

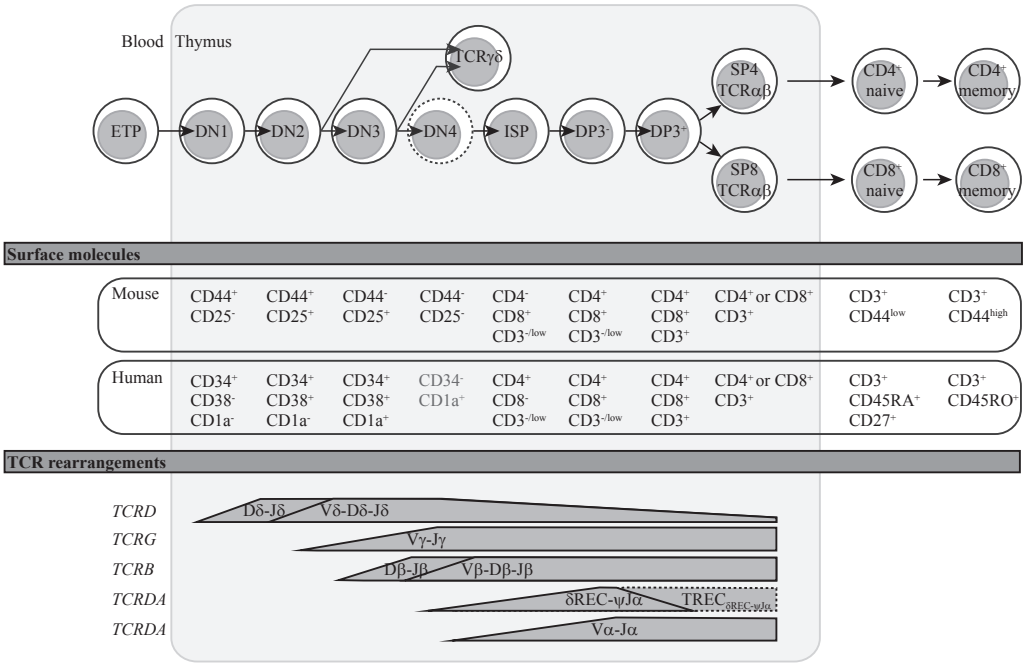

Figure 1. Schematic overview of consecutive T-cell developmental stages. Progenitor cells that enter the thymus develop into mature T cells via consecutive developmental stages, followed by further maturation and differentiation in the peripheral blood upon encounter of antigens. The different developmental stages, in both man and mice, can be recognized by surface markers and by TCR rearrangements; Adapted from Dik et al, van Dongen et al.[21, 26] ETP: early thymic progenitor, DN: double negative, ISP: immature single positive, DP: double positive, SP: single positive, TCR: T-cell receptor.

subdivided into $CD25^-CD44^+$ (DN1), $CD25^+CD44^+$ (DN2), $CD25^+CD44^-$ (DN3) and $CD25^-CD44^-$ (DN4) thymocytes. The human thymus DN counterparts are $CD34^+CD38^-CD1a^-$ (DN1), $CD34^+CD38^+CD1a^-$ (DN2) and $CD34^+CD38^+CD1a^+$ (DN3) thymocytes. So far, DN4 are hard to identify in the human thymus but most likely are $CD34^-CD1a^+$. DN cells subsequently develop into immature single positive (ISP) cells expressing CD4 in humans or CD8 in mice, followed by further maturation into double positive (DP) cells expressing both CD4 and CD8 and finally single positive (SP) mature T cells expressing either CD4 or CD8. Key processes of T-cell development are seeding of the thymus, lineage restriction and specification, lineage commitment, (pre)TCR formation, positive and negative selection and thymic emigration. These processes are described in more detail in the following paragraphs.

Thymic seeding and lineage commitment

Similar to all other hematopoietic cells, T cells originate from pluripotent hematopoietic stem cells (HSCs) which reside in the bone marrow. Seeding of the thymus with progenitor cells

(early thymic progenitors, ETPs) from the bone marrow is therefore necessary to sustain T-cell development in the thymus. ETPs enter the thymus at the cortico-medullary junction (CMJ) via postcapillary venules (figure 2).[11] Only very few ETPs enter the thymus, probably less than ten per day.[14, 15] Massive expansion of these early progenitors is therefore required to generate a sufficient pool of immature thymocytes and takes place during the earliest stages of T-cell development. It is estimated that DN1-2 thymocytes undergo ~10 cell divisions which results in an approximately 1000-fold expansion of this cell population.[15] In these early stages of development, the $CD34^{+}CD1a^{-}$ fraction in the human thymus has been shown to contain multi-lineage differentiation capacity into NK cells, B cells, myeloid cells and even erythrocytes. These multi-lineage differentiation capabilities are lost at the $CD34^{+}CD1a^{+}$ stage of development where thymocytes become T-cell lineage comitted.[14, 16]

DN thymocytes migrate through the cortex towards the outer subcapsular zone, which is associated with the initiation of TCRγδ formation and maturation or TCRαβ formation and phenotypical transition from DN to ISP and DP thymocytes (figure 2).[17]

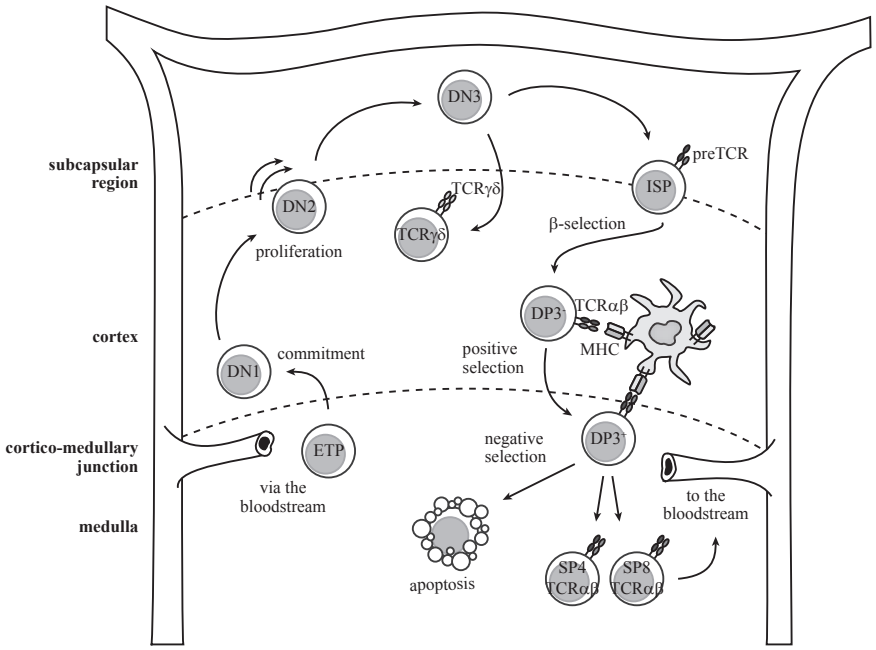

Figure 2. Schematic overview of T-cell development in the thymus. The thymic microenvironment can be divided into different compartments each having their own unique microenvironment supporting different stages of T-cell development. ETPs enter the thymus at the CMJ via postcapillary venules. These ETPs then migrate through the distinctive microenvironments of the thymus and develop into DN, ISP, DP and SP thymocytes. ETP: early thymic progenitor, DN: double negative, ISP: immature single positive, DP: double positive, SP: single positive, TCR: T-cell receptor, CMJ: cortico-medullary junction.

TCR formation

TCR formation takes place during the DN2-3, ISP and DP stages of T-cell development. Four different TCR chains can be formed, a TCRδ, TCRγ, TCRβ and a TCRα chain. A functional TCR consists of two disulfide-linked TCR chains. Most T cells express a TCR consisting of a TCRα and a TCRβ chain and are referred to as TCRαβ^{+} T cells. A small proportion of T cells express a TCR that consists of a TCRγ and a TCRδ chain and are called TCRγδ^{+} T cells. All TCR chains consist of a constant domain which is important for signaling, and a variable domain which is important for MHC binding and antigen recognition.
The ability of the immune system to eliminate a wide spectrum of pathogens depends on the presence of a highly diverse TCR repertoire, which requires a large heterogeneity of the variable domains. This heterogeneity is not encoded in the germ-line DNA, but generated during a process called V(D)J recombination.

V(D)J recombination
The *TCRD*, *TCRG*, *TCRB* and *TCRA* gene loci that are required for TCRδ, TCRγ, TCRβ and TCRα formation respectively, are build up by variable (V), diversity (D) and joining (J) genes. D segments occur only for the *TCRD* and *TCRB* loci. Unique exons encoding the variable domain of a TCR chain are assembled through recombination of one V, (D) and J gene (figure 3). Joining of the different genes occurs in a tightly regulated order with D to J rearrangements preceding V to DJ rearrangements in the loci containing V, D and J genes.[18-21]
All V, D and J genes are flanked by specific sequences called recombination signal sequences (RSS). An RSS consists of relatively highly conserved heptamer and nonamer sequences that are separated by a spacer with a length of either 12 or 23 nucleotides.[20] RSSs are recognized by a complex of recombination activating gene (RAG) 1 and 2 enzymes that bind the RSS sequences and subsequently generate a double strand DNA break between the RSS and the rearranging gene.[20] After cleavage, hairpins are formed at the sides of the rearranging genes (coding ends) and blunt ends on the side of the RSS (signal end). The two rearranging gene segments are than ligated again via the non-homologous end joining pathway to form the so-called ‘coding joint’ (CJ), the genomic DNA sequence encoding (part of) the variable part of the TCR. The two RSSs on the excised gene sequence are also ligated and as a result form a circular DNA structure, the TCR excision circle (TREC). The coupled RSSs together form the so-called ‘signal joint’ (SJ).
When functional recombination occurred on one allele, V(D)J recombination on the other allele is stopped, a phenomenon called allelic exclusion. This process, however, appears less strict for the *TCRG* and *TCRA* loci.[22]
V(D)J recombination of TCR loci, together with heterodimerization of TCR chains (TCRαβ or TCRγδ) can be expected to generate a TCR repertoire of over 10^6 different TCR molecules with a unique variable domain (combinational diversity) in humans. Diversity of the variable domain is further enhanced during the ligation process involved in coding joint formation as random insertions and deletions of nucleotides at the junction are introduced (junctional diversity). When it is assumed that V(D)J recombination is a random process, a TCR repertoire consisting of 10^{11} different TCR molecules could be expected.[23, 24] However, as gene segment usage is not completely random and due to negative selection (see below), the actual TCR repertoire will comprise less than 10^{11} different TCR molecules.[25]

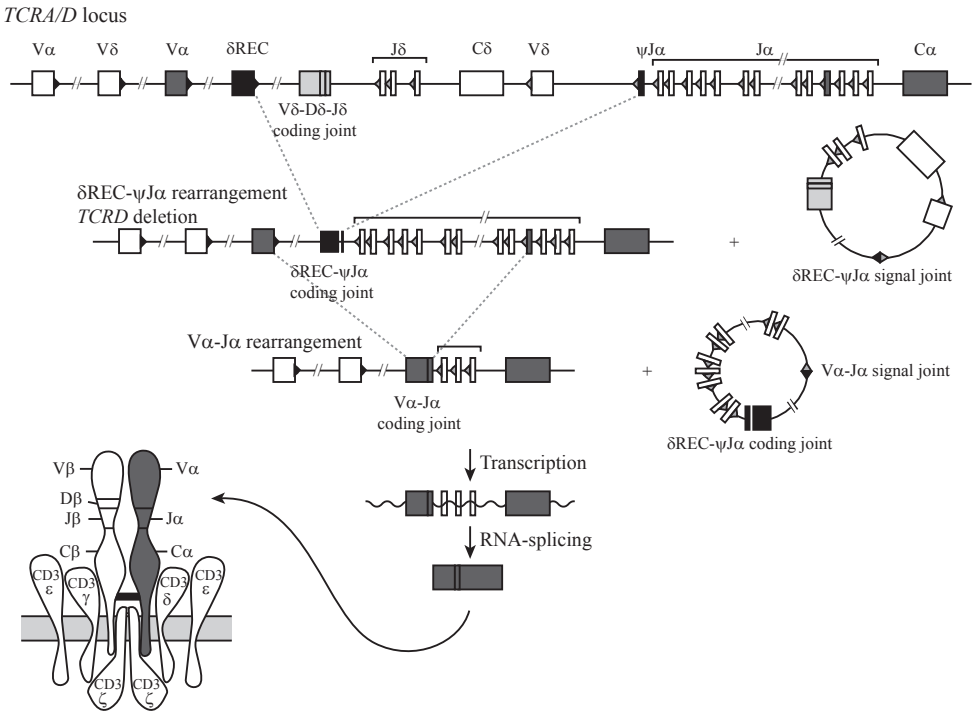

Figure 3. Schematic overview of TCR gene rearrangement and TREC formation. Before *TCRA* rearrangements occur within the TCRαβ lineage, the rearranged *TCRD* gene is deleted, preferably by the δREC-ψJα rearrangement. This rearrangement results in a $CJ_{\delta REC\text{-}\psi J\alpha}$ present in the genomic DNA, and a $SJ_{\delta REC\text{-}\psi J\alpha}$ on the corresponding TREC. The deletion of the *TCRD* gene is immediately followed by *TCRA* (Vα-Jα) rearrangements, during which the $CJ_{\delta REC\text{-}\psi J\alpha}$ will also be excised and become a signal joint on the corresponding TREC; Adapted from Verschuren et al, Breit et al.[59, 60] The rearranged *TCRA* gene is than transcribed and spliced into mRNA and translated into a TCRα protein. This protein together with a TCRβ protein and a CD3 complex, consisting of CD3γ, CD3δ, CD3ε and CD3ζ proteins, will form a mature TCRαβ on the surface of a T cell. CJ: coding joint, SJ: signal joint, TCR: T-cell receptor.

TCR gene rearrangements occur in a well-defined order and at defined stages of T-cell development. During the DN stages of development, rearrangements take first place in the *TCRD* locus, followed by the *TCRG* and the *TCRB* loci (figure 1).[21, 26] Cells that productively rearrange the *TCRD* and *TCRG* loci will express a functional TCRγδ on the cell membrane, enabling these cells to develop into mature TCRγδ$^+$ T cells. Productive rearrangement of the *TCRB* locus results in the expression of a preTCR signaling complex consisting of the TCRβ chain, the invariant preTα chain and CD3 molecules on the cell surface (figure 2).[27] PreTCR signaling induces TCRβ allelic exclusion, suppression of apoptosis, clonal expansion through proliferation and further maturation into the ISP and DP stages of development and finally initiation of *TCRA* locus rearrangements.[26, 28] These preTCR mediated events are collectively referred to as β-selection. When a *TCRA* locus is then also rearranged successfully, a TCRαβ will be expressed on the cell surface (figure 2).

Positive and negative selection of the formed TCRs

During the DP stages, thymocytes migrate via the subcapsular zone and the cortex to the medulla (figure 2).[17] During this migratory process, TCRαβ molecules interact with MHC molecules with the goal to select cells with the appropriate avidity for self-peptide/MHC complexes (positive selection). In addition, cells with too high avidity for the self-peptide/MHC complexes and thus potentially autoreactive, and cells with too low avidity for the self-peptide/MHC complexes and thus non-functional will die by apoptosis (negative selection).[29] These selection processes eliminate the majority of newly formed DP thymocytes (~98%; figure 2). Additionally, TCR-MHC interactions determine CD4 SP (MHC-II) and CD8 SP (MHC-I) lineage commitment.[8]
Altogether, the processes of TCR formation and selection generate a self-tolerant T-cell compartment capable of recognizing a large variety of foreign antigens.

Thymic emigration

The mechanisms by which thymic emigration is regulated are so far not completely understood.[30] Thymic emigration is thought to take place at the CMJ via lymphatic and blood vessels[30] and is mediated by several chemokines. For instance, sphingosine 1-phosphate (S1P) is present in high levels in blood and lymph compared to the thymus and lymph nodes creating a chemotactic gradient towards blood. Binding of S1P to the sphingosine 1-phosphate type 1 receptor ($S1P_1R$) present on mature thymocytes might mediate thymic emigration.[30, 31] In addition, CXCL12, produced by thymic stromal cells binds the CXCR4 receptor on mature thymocytes and acts as a chemo-repellent signal.[31] Additionally, CCL19 is thought to bind to the CCR7 receptor on thymocytes thereby contributing to the recruitment of mature thymocytes out of the thymus into the periphery.[31] T cells that recently entered the peripheral T-cell compartment are called recent thymic emigrants (RTEs).[32]

Peripheral T-cell compartment

The peripheral T-cell compartment refers to the total pool of T cells that continuously recirculate through primary lymphoid organs (thymus, bone marrow), secondary lymphoid organs (spleen, lymph nodes, tonsils, mucosa-associated lymphoid tissue; MALT), and non-lymphoid organs (blood, lungs and liver).[33] Generally, only 2% of the T cells are present in the peripheral blood, while most T cells reside in the spleen and lymph nodes.[33]

Mature T cells that have not yet encountered antigen are called naive T cells, which express CD45RA in humans and are $CD44^{low}$ in mice (figure 1). The naive T-cell pool is maintained by homeostatic survival that depends on TCR and self-peptide/MHC-complex interactions and the availability of the cytokine interleukin-7 (IL-7).[34]
Naive T cells are activated upon encounter of foreign antigens presented by antigen presenting cells (APCs) in a lymph node. Adequate activation of a T cell requires interaction between the TCR and a foreign-peptide/MHC-complex, co-stimulatory signals such as interactions of CD28 on T cells and CD80/CD86 on APCs, and the availability of cytokines such as IL-2, IL-12 and IL-15.[34] When co-stimulatory signals are not provided, T cells become anergic in order to prevent

responses to self- antigen.[34]
Antigen-activated T cells expand clonally and differentiate into T helper (Th) or cytotoxic effector T cells (CTLs). $CD4^+$ T cells differentiate into Th subpopulations, of which several different types have been described: Th1, Th2, Th9, Th17, Th22 and Tfh (T follicular helper cells).[35] Differentiation into one of the Th subpopulations is highly dependent on the cytokine milieu present during T-cell activation.[36] For instance, IL-12 enhances the expression of the transcription factor T-bet, which commits cells to the Th1 lineage.[35] Th1 T cells are characterized by the production of interferon-γ (IFN-γ) and pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α), and are predominantly involved in cell-mediated immunity against intracellular pathogens.[36] In contrast, IL-4 enhances the expression of the transcription factor GATA-3 which commits cells to the Th2 lineage.[35] Th2 T cells produce IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13,[36] and enhance humoral immunity by activating B-cell proliferation and antibody production. Th2 T cells play an important role in immunity against extracellular pathogens and parasites.[36] More recently, the Th17 lineage has been described. Commitment of this lineage is enhanced by the transcription factor retinoic acid-related orphan receptor-γt (RORγt) and requires the presence of IL-6 (or other pro-inflammatory cytokines such as IL-1 or TNF-α) and transforming growth factor-β (TGF-β).[35] Th17 cells produce IL-17 and IL-22 and are thought to be involved in immunity against extracellular pathogens particularly at mucosal surfaces.[36]
$CD8^+$ T cells differentiate into CTLs that are involved in the destruction of virus-infected cells and tumor cells.[37] Recognition of a target cell results in contact-mediated cytotoxicity, a process in which cytolytic molecules, such as perforin and granzyme B, released by CTLs are involved in inducing apoptosis of the target cell.[37] Additionally, binding of Fas-ligand (Fas-L) present on CTLs to Fas molecules present on target cells induces the formation of a death-inducing signaling complex, which also initiates apoptosis pathways in the target cell. Furthermore, activated $CD8^+$ T cells secrete pro-inflammatory cytokines such as IFN-γ, TNF-α and IL-2.[37]
Effector T cells are short-lived cells, and ~90% will die during resolution of inflammation, thereby restoring the resting state of the immune system. Only ~5-10% of the effector cells survive the first inflammatory state and become long-lived memory cells that can rapidly respond to re-infection with the same pathogen.[34] These memory T-cell populations depend on IL-7 and IL-15 signals for survival and proliferation.[34]
The memory T-cell compartment consists mainly of central and effector memory cells[38] which both express CD45RO in humans and are $CD44^{high}$ in mice (figure 1). Effector memory T cells (T_{em}) express homing receptors that facilitate migration through non-lymphoid tissues. T_{em} are capable of producing effector cytokines within hours after TCR stimulation, thereby providing protective memory.[38] Central memory T cells (T_{cm}) express the lymph node homing receptors CCR7 and CD62L (L-selectin) and recirculate through lymph nodes and the spleen. T_{cm} largely lack effector functions but readily proliferate and differentiate upon antigenic re-encounter, providing reactive memory.[38]

Additionally, several subpopulations of regulatory T cells (Tregs) with immunosuppressive function exist to regulate immune responses.[39] Natural Tregs (nTregs) are $CD4^+$ T cells generated as a separate subpopulation in the thymus.[39] In humans and mice these cells express CD25 and the transcription factor FoxP3.[39] Naive T cells can also acquire FoxP3 expression in the presence of TGF-β, IL-2 and retinoic acid, these cells are called induced Tregs (iTregs).[39] Next

to FoxP3$^+$ Tregs, several other types of Tregs, such as Tr1 and Tr3, can be induced from naive CD4$^+$ T cells.[39] Tr1 T cells secrete IL-10 and TGF-β, while Tr3 T cells mainly secrete TGF-β.[39] Also, several CD8$^+$, CD4$^-$CD8$^-$ and TCRγδ$^+$ T-cell subpopulations have been reported to exhibit immunosuppressive activity.[39]

MEASUREMENT OF THYMIC FUNCTION

The intricate process of T-cell development in the thymus is essential in the formation and maintenance of the peripheral T-cell compartment with a diverse TCR repertoire.[2, 3] As described above, V(D)J recombination is an essential step during T-cell development that determines the diversity of the TCR repertoire and is thus critical in the establishment of a peripheral T-cell compartment that is well equipped to fight off foreign antigens.[40, 41] After thymic emigration, extra-thymic homeostatic peripheral T-cell proliferation results in further expansion of the naive T-cell compartment, without further expansion of the pre-existing repertoire.[41]
Thymic function is maximal early in life during which T-cell homeostasis is mainly sustained by thymic output.[42] With ageing, however, the thymus involutes, eventually resulting in strongly diminished thymic output.[43, 44] To compensate for this, extra-thymic homeostatic T-cell proliferation is enhanced to maintain peripheral naive T-cell numbers at desired levels during ageing.[2] Nevertheless, ageing will eventually result in a less diverse TCR repertoire, as repertoire formation largely depends on thymic output of *de novo* generated naive T cells.[45] It is, however, largely unknown to what extent thymic output and homeostatic T-cell proliferation contribute to the maintenance of the peripheral T-cell compartment and how this is affected by ageing. Importantly, two recent studies, demonstrated that large differences exist between mouse and human T-cell maintenance during ageing.[46, 47] In mice, naive T-cell numbers are largely maintained by thymic output up to very old age, while, in human elderly the majority of naive T cells is formed by peripheral proliferation.[46, 47] These recent studies underscore the importance of detailed knowledge about the contribution of thymic output and peripheral T-cell proliferation to T-cell pool maintenance. Moreover, they provide important evidence that insights from mice studies cannot simply be extrapolated to the human situation.[46, 47]

Despite the importance of thymic output for a functional T-cell compartment with a large TCR repertoire, direct quantitative measurements of thymic function are still lacking, especially in humans, since it is difficult to obtain thymic samples of healthy individuals.[48] Several indirect assays to measure thymic function, however, have been developed in the last decades.[49]
Most indirect measurements of thymic function rely on the discrimination of RTEs from mature naive T cells. In mice, intra-thymic labeling of thymocytes with bromodeoxyuridine (BrdU), carboxyfluorescein diacetate succinimidyl ester (CFSE) and fluorescein isothiocyanate (FITC) has been used to identify RTEs in peripheral blood. Long term follow up, however, remains impossible as the signal is rapidly lost due to dilution of the label during cell proliferation.[48, 49]
Moreover, RTEs have been identified using flow cytometric markers in chicken (chT1$^+$)[50] and rat (Thy-1$^+$CD45RC$^-$RT6$^-$).[51] In mice and humans such markers remain largely unknown, although recently, in humans (a combination of) several markers to identify RTEs have been proposed (CD31$^+$; PTK7$^+$; CD8$^+$CD103$^+$).[52-54] The use of CD31 as a marker for RTEs in humans is however

controversial, as it was demonstrated that in adults ~75% of naive $CD4^+CD31^+$ T cells was formed by peripheral T-cell proliferation instead of thymic output.[46, 47]
Besides RTE measurements, estimates of thymic function have also been made indirectly via measurement of thymic volume by computed tomography (CT) scans of the thymus.[55, 56]
Finally, quantification of DNA excision circles formed during TCR gene rearrangements (TRECs) has been suggested as a measure for determining thymic output,[57-60] and is nowadays the most-commonly used measure of thymic function in humans.[48] This last technique will be explained in more detail in the following section.

Excision circle analyses

DNA excision circles are formed during the V(D)J recombination processes of the *TCRD*, *TCRG*, *TCRB* and *TCRA* genes in developing T cells (TRECs) and the immunoglobulin genes in B cells (B-cell receptor excision circles; BRECs; figure 3).[61, 62] These non-genomic circular DNA structures have been shown to be very stable structures,[57, 63] which will not be replicated during consecutive cell divisions.[58-60, 63] Due to these characteristics, excision circles have been used as markers for thymic output and as markers for proliferative history.[64, 65]

Many different TRECs are formed during T-cell development, however, due to low frequencies, most of them are hardly detectable in the peripheral blood T-cell compartment.[64] However, during the first stages of *TCRA* rearrangements the *TCRD* locus, which is positioned within the *TCRA* locus, is deleted.[21, 64] Deletion of the *TCRD* locus is thought to be an important event for $TCR\alpha\beta^+$ T-cell lineage commitment and occurs on both alleles in the majority of $TCR\alpha\beta^+$ T cells, as allelic exclusion occurs infrequently during *TCRA* rearrangements.[21, 66] Although different rearrangements can induce *TCRD* deletion, in most cells (~70%) deletion of the *TCRD* locus occurs via the δREC-ψJα rearrangement.[59] This results in the formation of a TREC that contains the δREC-ψJα signal joint sequence (figure 3).[59] This δREC-ψJα TREC can easily be detected by RQ-PCR with primers specific for the δREC-ψJα signal joint sequence present on the TREC.[59, 64]

The δREC-ψJα TREC is formed in the ISP and DP3 stages of T-cell development in nearly all $TCR\alpha\beta^+$ T cells (figure 3), after the phase of massive thymocyte expansion. Therefore, the frequency of δREC-ψJα TRECs will be relatively high in RTEs, making measurement of δREC-ψJα TRECs a marker for RTEs.[57-59, 64]
However, controversy exists as to whether δREC-ψJα TREC quantification is a good marker of RTEs. Due to the stable nature of excision circles and the longevity of naive T cells, not only recent thymic emigrants but also 'older' thymic emigrants contain δREC-ψJα TRECs.[67] Moreover, δREC-ψJα TREC levels are diluted during peripheral T-cell proliferation.[67] Therefore, quantification of δREC-ψJα TREC levels in peripheral blood T cells more likely reflects a combined measure for thymic output and peripheral blood proliferation, rather than a measure for thymic output and thus thymic function alone.[67]
It is thought that intra-thymic proliferation largely determines thymic output,[68, 69] and thus measurement of intra-thymic proliferation could be an indirect measure for thymic output. An adapted TREC analysis was introduced to measure intra-thymic proliferation. This assay measures the classical δREC-ψJα TREC as well as multiple βTRECs formed during different D to J gene

rearrangements of the *TCRB* locus. Because the classical δREC-ψJα TREC and the βTRECs are equally affected by peripheral events such as proliferation and apoptosis, the δREC-ψJα TREC/βTRECs ratio has been interpreted as a marker for intra-thymic proliferation between *TCRB* and *TCRA* gene rearrangments.[69, 70]

Recently, a quantitative assay to measure B-cell proliferation based on excision circle analysis of the κ-deleting recombination excision circle (KREC) was introduced.[65] The advantage of this system is that this deleting rearrangement renders the *IGK* locus nonfunctional, thus preventing further rearrangements in the *IGK* locus. Therefore the coding joint of this rearrangement remains stably present in the genome,[65] while the excision circle containing the signal joint will dilute out upon proliferation. Consequently, the difference between the levels of coding joint and signal joint present can be used as a robust quantitative measure of *in vivo* proliferative history.[65] Clearly, assays based on this principle would be of great value to study T-cell biology. However, no such assays are available to date to reliably quantify proliferative history of T cells.

REGULATION OF T-CELL DEVELOPMENT

The different processes during T-cell development, such as differentiation, survival and proliferation, are tightly regulated by a variety of extracellular signals, such as cytokines, chemokines, adhesion molecules and growth factors as well as transcription factors. Although many of these stimuli are produced by the developing cells themselves and the microenvironment, also factors of extra-thymic origin might affect primary T-cell development. There is growing evidence that the neuro-endocrine/metabolic system largely affects immune system development and function. In the next paragraphs the interactions between the immune system and the neuro-endocrine and metabolic systems will be further described, with special focus on the effects of thyroid stimulating hormone (TSH) and obesity on T-cell development.

NEURO-ENDOCRINE-IMMUNE INTERACTIONS WITHIN THE T-CELL COMPARTMENT

Neuro-endocrine-immune interactions

During the last century it became clear that complex interactions exist between the neuro-endocrine and the immune system.[71-75] This interactive system is thought to support the induction of coordinated responses to internal and external danger to maintain homeostasis of the body.[71-75] The immune system recognizes non-cognitive stimuli – stimuli that are not recognized by the nervous system – such as bacteria, viruses, tumors and antigens.[76] Cytokines and lymphocyte-derived hormones produced by the activated immune system will subsequently activate the neuro-endocrine system resulting in alterations in energy homeostasis, behaviour (sleep, appetite, malaise) and neuro-endocrine hormone production to support the inflammatory response.[74, 76, 77] However, the neuro-endocrine system also regulates the amplitude and duration of immune activation in order to prevent extensive tissue damage.[72, 78] On the other hand, cognitive stimuli

such as physical or emotional stress recognized by the nervous system will alter immune homeostasis due to alterations in neuro-endocrine hormone production.[71, 76, 79]

Bidirectional communication between the neuro-endocrine and the immune system occurs via an extensive range of shared messenger molecules and receptors.[72] Several autocrine, paracrine and endocrine routes of communication have been described (figure 4):

1) hormones produced by the neuro-endocrine system interact with hormone receptors present on immune cells, and thereby regulate immune function.[74, 79, 80]
2) hormones produced locally within the immune system interact with hormone receptors present on immune cells and with hormone receptors present within the neuro-endocrine system, thereby regulating both immune and endocrine function.[80]
3) thymic hormones (thymulin, thymosin-α, thymopoietin) produced within the thymus interact with hormone receptors present on immune cells and within the endocrine system, thereby regulating both immune and endocrine function.[81, 82]
4) cytokines and growth factors produced by the immune system and by the neuro-endocrine system interact with cytokine receptors present within the neuro-endocrine system, thereby regulating neuro-endocrine function.[72, 80, 83]

Neuro-endocrine regulation within the T-cell compartment

Within the T-cell compartment hormone receptors are present on TECs, thymocytes and mature T cells (table 1). Additionally, TECs, thymocytes and T cells produce a broad range of hormones (table 1). A large number of processes involved in T-cell development in the thymus and T-cell responses in the peripheral T-cell compartment have been described to be regulated by neuro-endocrine-immune interactions. Neuro-endocrine involvement in the function of the thymic microenvironment, T-cell development and peripheral T-cell responses are described in the following paragraphs. The effects of thyroid stimulating hormone (TSH) within the immune system will be discussed in more detail.

Thymic microenvironment
The existence of neuro-endocrine-immune interactions became first evident from the observation that thymic involution found in pituitary-deficient rodents could be restored by administration of prolactin (PRL) or growth hormone (GH).[75] Thymic involution was also found in rodents with stress-induced enlargement of the adrenal glands, while adrenalectomy was associated with thymic hypertrophy.[84] In concordance with these early studies, it was demonstrated that PRL, GH and insulin-like growth factor I (IGF-I) enhance TEC proliferation, while somatostatin and steroid hormones inhibit TEC proliferation *in vitro*.[85] In humans, thymic enlargement has been demonstrated to occur in patients with AIDS after treatment with GH.[86]
The production of extracellular matrix molecules (ECM; such as fibronectin, laminin, type IV collagen) and thymic hormones by TEC, which support T-cell differentiation, have also been demonstrated to be under neuro-endocrine control.[8] Glucocorticoids, triiodothyronine (T3), PRL, GH and IGF-I were found to enhance the production of ECM *in vitro*, which increased

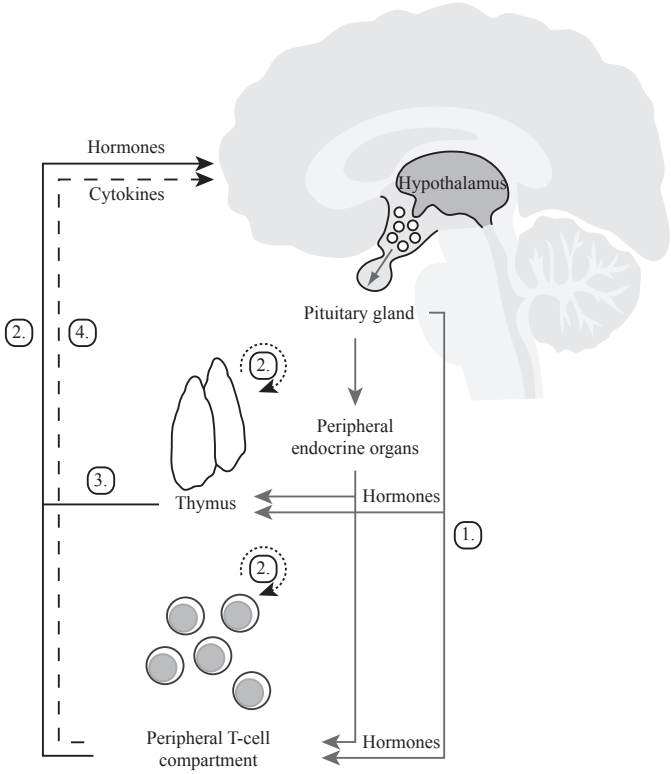

Figure 4. Schematic overview of neuro-endocrine-immune interactions within the T-cell compartment. Scheme showing the different autocrine, paracrine and endocrine routes of communication between the neuro-endocrine system and the T-cell compartment. Hormones produced in the hypothalamus, the pituitary and the peripheral endocrine organs interact with the T-cell compartment (1). Endocrine hormones (2), thymic hormones (3) and cytokines (4) produced within the T-cell compartment interact with the neuro-endocrine system.

adhesion of thymocytes to TEC.[85] Glucocorticoids, GH, PRL, endorphins and T3 were found to enhance thymulin secretion *in vitro*.[85, 87] Moreover, increased thymulin serum levels have been described in patients with hyperthyroidism, hyperprolactinemia and acromegaly, while patients with hypothyroidism and GH deficiency had decreased thymulin serum levels.[85, 88]

T-cell development

Neuro-endocrine circuits influence multiple developmental events during T-cell development, such as thymocyte proliferation, survival and subpopulation composition. In addition, some studies have suggested neuro-endocrine regulation of TCR rearrangements and positive and negative selection.

Thymocyte proliferation is enhanced i*n vitro* by GH, IGF-1, PRL, GH-releasing hormone (GHRH) and luteinizing hormone releasing hormone (LHRH), while this is inhibited by glucocorticoids,

Table 1. Hormone receptors and hormone production in the T-cell compartment

	TEC		Thymocytes		T cells	
	Receptor expression	Hormone production	Receptor expression	Hormone production	Receptor expression	Hormone production
PRL	+[85, 125]		+[73, 85, 125]	+[85]	+[75, 79, 93, 125]	+[75, 93]
GH	+[73, 85]	+[85]	+[73, 79, 85, 125]	+[85]	+[75, 125]	+[75, 208]
ACTH		+[85]			+[79]	+[75, 208, 209]
LH				+[85]		+[208]
FSH						+[208]
TSH					+[93]	+[75, 93]
Vasopressin		+[85]	+[73, 85]	-[85]		
Oxytocin		+[85]	+[73, 85]	-[85]		
LHRH				+[85]		
GHRH			+[85]	+[85]	+[75]	+[75]
CRH				+[85]		
TRH				+[85]		
somatostatin	+[85, 210]	+[85]	+[85, 210]	-[85]	+[210]	
cortistatin		+[210]		+[210]		+[210]
IGF-1			+[125]		+[75, 125]	+[75]
T3/T4	+[85]		+[85]		+[93]	
Cortisol		+[96]	+[73]	-[96]	+[73]	
Estrogene	+[73, 79]					
Progesterone	+[73]					
Androgen	+[73, 79]					
hCG						+[74, 208]
Leptin					+[89, 211]	+[211]
Ghrelin					+[95, 212]	+[95, 212]
VIP		+[85]	+[85, 213]	+[85]	+[213]	+[213]
endorphins	+[85]	+[85]	+[85]	+[85]	+[79]	+[75, 209]
Insulin	+/-[85]	+[85]	-[85]			

TEC: thymic epithelial cells, PRL: prolactin, GH: growth hormone, ACTH: adrenocorticotropic hormone, LH: luteinizing hormone, FSH: follicle stimulating hormone, TSH: thyroid stimulating hormone, LHRH: luteinizing hormone releasing hormone, GHRH: growth hormone releasing hormone, CRH: corticotropin releasing hormone, TRH: thyrotropin releasing hormone, IGF-I: insulin-like growth factor-I, T3: triiodothyronine, T4: thyroxine, hCG: human chorionic gonadotropin, VIP: vasoactive intestinal peptide.

somatostatin and vasoactive intestinal peptide (VIP). These effects may be direct or indirect via enhanced secretion of thymulin by TEC.[73, 75, 85] In addition, PRL and leptin have been demonstrated to enhance thymocyte survival by increasing IL-7 levels *in vitro*.[73, 89] In line with these data, rodents with increased levels of GH, PRL, IGF-1 or T3 have increased thymocyte numbers.[85] Hormone levels may also influence thymocyte subpopulation composition. High GH levels are associated with an increase in the percentage of DP thymocytes and a decrease in percentages of DN thymocytes, while the thymus of GH deficient dwarf mice (DW/J mice) contains a reduced percentage of DP thymocytes.[79] High levels of glucocorticoids and estradiol also reduce the

percentages of DP due to increased apoptosis of DP thymocytes.[85] As a result of this a concomitant increase in the percentages of DN and SP thymocytes is observed.[85]

Evidence for neuro-endocrine regulation of TCR rearrangements is largely lacking so far. One study demonstrated that the percentage of thymocytes expressing Vβ6, Vβ8 or Vβ11 gene products was increased in mice treated with estradiol.[85] Moreover, prolactin was found to induce TCRγ expression and repress TCRβ expression in one study.[85]
MHC molecule expression and apoptosis largely determine positive and negative selection processes. Glucocorticoid hormones increase MHC-I expression on thymic DCs *in vitro*[85] and induce apoptosis in DP thymocytes, although low levels of glucocorticoids partially rescue thymocytes from apoptosis.[85] GH, PRL, leptin and VIP were found to inhibit the apoptotic effects of dexamethasone *in vitro*.[85, 89, 90]
These data indicate that the neuro-endocrine system contributes to the regulation of TCR rearrangements, MHC molecule expression and apoptosis during T-cell development and that multiple hormones act in concert. The specific effects of the neuro-endocrine hormones on TCR rearrangements and positive and negative selection however, need to be further defined.[85]

T-cell responses
Adaptive immune responses were found to be suppressed in pituitary-deficient rodents.[91, 92] Based on these studies it was suggested that apart from the cytokine milieu, also relevant hormone levels regulate adaptive immune responses. This is further supported by the notion that many hormonal changes occur during the acute phase of infection. PRL, GH, corticotrophin releasing hormone (CRH), insulin, endorphin and leptin serum levels increase, while luteinizing hormone (LH), follicle-stimulating hormone (FSH), sex hormones, progesterone and thyroid hormones levels decrease.[91, 92]
Many hormones including PRL, GH, leptin, substance P and β-endorphin enhance proliferation in activated T cells.[74-76, 89, 90, 93, 94] In contrast, somatostatin, ghrelin, VIP and human chorionic gonadotropin (hCG) reduce proliferation in activated T cells.[74, 76, 95] Moreover, the generation of cytotoxic T cells is enhanced by GH, while inhibited by hCG. [74-76]
Hormones may also alter the Th1/Th2 balance. Melatonin, leptin, dehydroepiandrosterone (DHEAS), adrenalin, VIP and adenosine are associated with Th1 skewing, while progesterone, corticosteroids, histamine, noradrenalin, ghrelin and 1,25 vitamin D induce Th2 skewing.[71, 72, 78, 90, 95, 96] Moreover, it is thought that during pregnancy hormones such as hCG, progesterone and estrogen are responsible for a skewing of the immune system towards Th2 responses, thereby suppressing rejection of the fetus.[97]
In conclusion, it is clear that T-cell development is regulated by a complex network of neuro-endocrine-immune interactions. So not only changes within the immune system, but also in the endocrine system, might largely affect T-cell development and functioning. In this thesis we will further focus on the effects of TSH on T-cell development.

Effects of TSH within the T-cell compartment

TSH and its receptor

TSH operates within the hypothalamus-pituitary-thyroid axis in which it regulates endocrine function of the thyroid gland.[98] TSH synthesis in the anterior pituitary is stimulated by thyrotropin-releasing hormone (TRH) released from the hypothalamus and is inhibited by thyroid hormones in a negative-feedback loop.[98] TSH induces iodine uptake, production and release of thyroid hormones, and promotion of thyroid cell growth in the thyroid gland. Moreover TSH plays an important role in ontogeny of the thyroid gland.[98]

TSH is a 28- to 30-kDa glycoprotein and belongs to the family of glycoprotein hormones that also include FSH, LH and hCG.[98] TSH consists of two non-covalently linked subunits, a common α-subunit, which is similar to the α-subunits of FSH, LH and hCG and a unique β-subunit which is responsible for the specific binding to and activation of the TSHR.[98]

TSH binds to the TSHR, a member of the seven-transmembrane domain, G-protein-coupled-receptor (GPCR) superfamily.[98] TSHR activated by TSH induces G-protein signaling pathways.[99] Activation of $G\alpha_s$ protein results in the activation of the adenylate cyclase (AC) signaling pathway with subsequent intracellular cyclic adenosine monophosphate (cAMP) release.[99] At higher concentrations TSH can also activate the $G\alpha_q$ protein which is associated with the activation of the inositol 1,4,5-triphosphate (IP3) and diaglycerol (DAG) pathways resulting in increased cytosolic Ca^{2+} levels.[99]

The TSHR is traditionally thought to be expressed exclusively within the pituitary and the follicular epithelial cells of the thyroid gland. However, recent studies identified TSHR expression in several other tissues and cells as well, including adipose tissue, retro-ocular fibroblasts, neuronal cells, astrocytes, muscle, hair follicles, bone and in the immune system.[100-105] Moreover, a TSHR mRNA variant consisting of exon 1 to 8 and lacking the transmembrane domain encoding exons has been found in thyroid tissue, lymphocytes, ocular muscle, fat and fibroblasts. Whether expression of this variant mRNA results in protein expression is unclear so far.[106, 107]

Expression of TSH and TSHR by T cells

Immune cells such as mouse splenocytes, the human MOLT4 T-lymphoma cell line and human intraepithelial T cells (IELs) have been found to produce TSH. Moreover, human peripheral blood mononuclear cells (PBMCs) stimulated with the T-cell mitogen staphylococcus enterotoxin-A (SEA) were found to secrete TSH as well[108-111] while TRH increased TSH production by mouse splenocytes, the MOLT4 T-lymphoma cell line and human IELs.[109-111]

TSHR expression has been described in monocytes/macrophages, NK cells, and DCs.[112-115] However, these data are inconclusive as another study could not detect TSHR expression in PBMCs.[103] THSR expression has not been demonstrated on most B and T cells, although a small population of naive T cells within the lymph node and IELs did express TSHR.[111, 113] In line with this, mouse T and B cells were unresponsive to TSH, while TSH did induce a cAMP response in mouse splenic DCs.[112, 113] TSHR expression has also been described in total rodent[116] and human[103, 117-119] thymic tissues. Moreover, TSH was able to induce a cAMP response in total rat thymic tissue, suggesting expression of functional TSHR in the thymus.[116] In other studies, however, the presence of TSHR

expression in the thymus could not be confirmed.[113, 120]
Within the thymus, TSHR immunoreactivity was demonstrated in thymic medullary cells including Hassall's corpuscles and epithelial cells.[118, 121] Data on TSHR expression on thymocytes, however, are contradictory.[117 118, 121]
Overall, it can be concluded that the TSHR is present in the thymus, although it is still unclear which cell types do express the receptor. Additional insights into the *in vivo* role for TSH in the regulation of T-cell biology might be obtained from animal models of hyperthyroidism and hypothyroidism or from human thyroid diseases.

Mouse models of hyperthyroidism and hypothyroidism
Several mouse models to investigate hypothyroidism and hyperthyroidism are known. In the most commonly used models, hypothyroidism is induced by thyroidectomy or thyrostatic drugs, while hyperthyroidism is induced by thyroid hormone administration.[122] In these mouse models, hypothyroidism results in involution of the thymus, the spleen and lymph nodes,[123, 124] while on the other hand, hyperthyroidism increases the size of the thymus and lymph nodes.[125, 126] This effect may very well be thyroid hormone dependent as in hyperthyroid mice increased TEC proliferation was found, although this could not be confirmed in murine TEC culture systems *in vitro*.[127, 128] In some studies also decreased peripheral blood T-cell numbers were found in rodents following induction of hypothyroidism, but others did not confirm this.[124, 129, 130] Thymulin plasma levels and thymulin containing cells in the thymus were found to be decreased in hypothyroid rodents.[131] Despite the clear opposite effects of hypothyroidism and hyperthyroidism on the cellular composition of the immune system, both hypothyroidism and hyperthyroidism are associated with normal or depressed humoral and cell-mediated immune responses.[124-126, 129]

Next to the hyperthyroidism mouse model induced by thyroid hormone production, several models have now been developed to mimic the autoimmune response of Graves' disease (GD). In these models, mice are injected with cells transfected with the human TSHR and MHC-II molecules or with plasmid or adenovirus containing the human TSHR, to stimulate the production of TSHR autoantibodies by the animals.[122] In these models especially the Th1/Th2 balance in GD has been investigated, however with conflicting results,[122] which may be related to the type of adjuvants used to induce the immune response, but possibly also by the induction of an immune response against plasmids and adenoviruses used.[122]

The disadvantage of the above described models is that in all models both TSH and thyroid hormone levels are altered. Therefore, both TSH and thyroid hormones might be responsible for the alterations. However, a new mouse model of hypothyroidism might be used to investigate selectively the effects of TSHR signaling on the T-cell compartment, the *hyt/hyt* mouse model. *Hyt/hyt* mice have a point mutation in the TSHR gene resulting in severe hypothyroidism due to diminished TSH binding capacity and TSHR functionality.[132, 133] In these mice, supplementation of thyroid hormones will result in normal thyroid hormone levels, but TSHR signaling will still be impaired. These mice when kept hypothyroid have decreased absolute numbers of bone marrow, thymus and spleen cells, similar to other hypothyroid mice.[134, 135]
So far, only one study investigated the immune system in thyroid-hormone-substituted *hyt/hyt*

mice, demonstrating a reduction in TSHR expressing IELs, and normal T-cell numbers in the spleen.[111]

Patients with hyperthyroidism and hypothyroidism

GD is the most common cause of hyperthyroidism in humans.[136] GD is an autoimmune disease caused by antibodies directed against the TSHR. These TSHR autoantibodies can have either an activating, a neutral or an inhibiting effect on the TSHR.[136] Activating TSHR autoantibodies stimulate the thyroid gland to produce an excess of thyroid hormones, which ultimately results in hyperthyroidism.[136] Hashimoto's thyroiditis (HT), an autoimmune disorder characterized by autoimmune destruction of the thyroid gland, is the most common cause of hypothyroidism in humans.[137] Autoreactive T cells play a dominant role in this destructive process.

Despite the autoimmune basis of GD and HT, the composition of the peripheral blood immune compartment is not well documented and for both diseases contradictory data exist. In both GD and HT, T-cell numbers have been reported as being increased, normal and even decreased.[138-141] Also percentages of naive and memory subpopulations have been found to be increased as well as decreased.[142, 143] In contrast, an increased CD4/CD8 ratio is a rather consistent finding in GD.[140, 141, 143, 144] Moreover, activation of the T-cell compartment based on increased percentages of HLA-DR$^+$, CD25$^+$ and CD69$^+$ cells has consistently been described in GD.[140, 145] A recent study suggested that thymic output might be increased in GD and HT, as they found increased levels of naive CD4$^+$CD45RA$^+$ T cells and CD4$^+$CD31$^+$ RTEs.[142] HT is considered a typical T-cell mediated, Th1-driven autoimmune disease, while GD is considered to be an autoantibody-mediated, Th2-driven autoimmune disease.[146, 147] Activated T cells and TSHR responsive T-cell clones isolated from GD thyroid glands were demonstrated to produce increased levels of IL-5. In contrast, activated T cells isolated from HT thyroid glands produced more IFN-γ.[148] Also serum IL-5 levels were increased in GD patients, while IL-12 and IFN-γ levels were increased in HT patients, although one study also described increased IL-5 serum levels in HT patients.[149, 150] despite the evidence of a Th2 response in GD TSHR antibodies are predominantly IgG1, a Th1 associated subclass.[151, 152] Moreover, upon PMA/ionomycine activation of peripheral blood T cells from GD patients, Th1 as well as Th2 dominated responses have been described.[144, 147, 153]

nTregs have been described to be normally present or increased in GD as well as HT,[154, 155] although, in one study the suppressive function was reduced.[154] No studies investigated immune function or responsiveness in GD and HT patients.

Thymic hyperplasia in GD

Similar to the animal models of hyperthyroidism, also hyperthyroidism in humans is associated with an increased size of lymphoid tissues, especially an increased size of the thymus has been described (table 2).[126] The association between GD and thymic hyperplasia was first described at the beginning of the 20th century.[156, 157] In one larger study, 33% of patients with GD were described to have microscopic changes (medullary lymphoid follicle formation) of the thymus. Total thymic size, however, was not determined in this study.[158] In a more recent study, thymic size and density (measured by CT-scan) were found increased in GD patients compared to age matched healthy controls.[117]

Table 2. Association between Graves' disease and thymic hyperplasia

Ref.	No. of patients	Thymic hyperplasia		Effects of treatment		Histology (biopsy or thymectomy)
		detection	*size*	*TSHR AB*	*Thymic size*	
[214]	1	MRI	5x8cm		↓	
[215]	1	CT			↓	True hyperplasia
[216]	1	CT	11.5x7.5 x3 cm			Follicular hyperplasia Germinal centers
[164]	1	CT	3.8x3cm		↓	
[217]	1	CT			↓	
[119]	1	CT	226cm^3	↓	↓	Normal tissue
[163]	2	CT	7x3cm		↓	
[117]	23	CT		↓	↓	
[218]	1	CT				Follicular hyperplasia
[219]	2					Thymic hyperplasia Lymphoid hyperplasia
[161]	1	CT		↓		Thymic hyperplasia
[220]	3	CT			↓	Benign hyperplasia Lymphoid follicles
[159]	1	X-ray				Thymic hyperplasia Lymphoid follicles
[158]	50		ND			Lymphoid follicles (18 out of 49 samples)

TSHR AB: thyroid stimulating hormone receptor antibodies, MRI: magnetic resonance imaging, CT: computed tomography.

Thymic hyperplasia in association with GD is characterized by hyperplasia of the lymphoid compartment of the thymus and the existence of structures that resemble active germinal centers.[158-160] The lymphoid hyperplasia mainly comprises T cells as <1% of the lymphoid cells were B cells.[161]
Thymic hyperplasia is only rarely described in association with other thyroid disorders, including HT and non-toxic nodular goiter.[158] On the other hand thymic hyperplasia is also described in association with other autoimmune disorders, such as myasthenia gravis.[162]

Thymic hyperplasia has long been thought to play an etiologic role in the pathophysiology of GD. However, because thymic hyperplasia generally resolves with anti-thyroid drug therapy while thymectomy does not reverse hyperthyroidism, it is now thought that thymic hyperplasia is a phenomenon secondary to GD.[117, 163, 164]
Similar to animal models, hyperthyroidism in humans is associated with increased thymulin plasma levels, while hypothyroidism is associated with decreased thymulin plasma levels which correlated with serum thyroid hormone levels.[165] The increased level of thymulin in GD patients

has been hypothesized to contribute to the development of thymic hyperplasia.[163, 165] Moreover, IgGs from patients with GD can bind to thymocytes.[161] In two out of six patients tested, IgGs possessed direct thymic mitogenic activity, suggesting that these antibodies might contribute to the development of thymic hyperplasia.[117, 161, 163]

From above described literature it can be concluded that hormones involved in the hypothalamus-pituitary-thyroid axis are able to affect T-cell biology *in vivo*. But so far, the specific contribution of TSH and thyroid hormones and the exact mechanisms are still largely unclear. Therefore, further detailed studies on the effects of TSH on the T-cell compartment are required.

ADIPOSE-IMMUNE INTERACTIONS WITHIN THE T-CELL COMPARTMENT

Similar to neuro-endocrine-immune interactions, also the metabolic and the immune system have been shown to be closely interacting systems, as they share common hormones, cytokines, transcription factors and bioactive lipids to regulate their own as well as each other's function.[166, 167] While adipose tissue traditionally has been viewed as inert tissue, it is now generally recognized that adipocytes produce large amounts of hormones and cytokines that affect not only metabolism but also immunity. Such factors are also referred to as adipokines.[166] Cytokines produced in adipose tissue include IL-1, IL-6, TNF-α and IFN-γ. Additionally, several hormones, some with pro-inflammatory effects, such as leptin, resistin and visfatin, and others with anti-inflammatory effects, such as adiponectin and acylated ghrelin are produced in adipose tissue.[166, 167] Next to immune-adipose interactions via circulating molecules, also direct anatomical interactions exist within the specialized immune and adipose microenvironments.[168] Hematopoietic cells, including macrophages and T cells, are present in the stromal-vascular fraction (SVF) of healthy adipose tissue.[169] Adipocytes are resident cells within the bone marrow and thymic stromal cell compartment, and also lymph nodes are surrounded by specialized adipose tissue.[170, 171] Although the exact function of adipose tissue within the lymphoid compartment is not known, it likely serves as a local energy reservoire.[171] Accumulation of adipose tissue in the bone marrow and the thymus occurs during ageing and is associated with decreased hematopoietic activity in these organs.[170]

The interplay between immunological and metabolic processes is thought to function in the maintenance of homeostasis during physiological conditions. For example, during antigen exposure, metabolic adaptations occur to support the high energy demands of an inflammatory response.[167, 172] This intimate relationship also implicates that disturbances in one system will affect the functionality of the other system as well. For example, malnutrition but also overnutrition has been associated with impaired immune responses, while chronic inflammatory diseases are also associated with the development of metabolic aberrations including insulin resistance.[167, 170, 173, 174] Furthermore, interactions between immune function and obesity have been described and will be described in more detail in the following sections.

Obesity

Obesity is a major cause of preventable death in the Western world,[175] and its prevalence is rapidly increasing.[176] The World Health Organization (WHO) has accepted the body mass index (BMI) as the appropriate method to define normal weight (BMI of 18.5-24.9 kg/m^2), overweight (BMI of 25-29.9 kg/m^2) and obesity (30 kg/m^2 or higher).[177] Based on these criteria, the WHO estimated that in 2008 1.5 billion adults, 20 years of age and older, were overweight worldwide.[178] In the Netherlands, in 2008, 46.9% of adults, with an age of >20 years, were overweight, while 11.1% were obese.[179] Obesity is closely associated with a variety of chronic conditions of different severity including diabetes mellitus type-II (DM-II), hypertension, high cholesterol, stroke, cardiovascular heart disease, certain cancers, and typical immunological disorders such as psoriasis and arthritis. DM-II, cardiovascular heart disease and certain types of cancer are responsible for the majority of obesity-related morbidity and mortality.[176]
Although obesity is primarily considered to be a metabolic disease, the picture emerges that obesity is also associated with immunological abnormalities.[167] These immunological abnormalities manifest itself by a chronic low-grade inflammatory state within the adipose tissue, which is most likely metabolically induced.[174, 180] The first evidence for chronic inflammation in obesity originates from studies in which increased TNF-α expression was found within adipose tissue from obese mice.[181] This state of chronic low-grade inflammation is nowadays considered an important early event in the development of obesity-related complications.[180, 182-184]

Low-grade chronic inflammation in obesity

The state of obesity-related chronic low-grade inflammation is thought to be initiated by the adipose tissue.[185] Specific processes in metabolic cells including, adipocyte cell death, reactive oxygen species (ROS) production, endoplasmatic reticulum (ER) stress and increased levels of lipid production are thought to be involved in the initiation and maintenance of this inflammatory state (figure 5A).[180]

Obesity is characterized by increased fat deposition in adipose cells, resulting in hypertrophy of adipocytes. Hypertrophy supports the initiation of inflammation in several ways. Hypertrophic adipocytes produce increased amounts of pro-inflammatory hormones and cytokines. Moreover, insufficient blood flow in the expanded adipose tissue causes local hypoxia. As a result of this, adipocytes die, which subsequently activates inflammatory responses in neighbouring cells and recruits macrophages into the tissue.[180, 184, 186] Local hypoxia also enhances ROS production in mitochondria.[167, 187, 188] Additionally, metabolic overload of the ER with lipids initiates ER-stress resulting in the so-called unfolded protein response (UPR). Both ROS and the UPR activate pro-inflammatory signaling pathways.[167, 187, 188]

Inflammatory signaling pathways are also triggered directly by lipids. Innate immune receptors such as Toll-like receptor 4 (TLR4) are expressed by adipocytes and macrophages and their expression is increased in obese subjects.[187] Free fatty acids (FFAs), especially saturated FFAs, bind TLR4 thereby initiating activation of pro-inflammatory signaling pathways *in vitro*.[189] Moreover, fatty acid binding proteins (FABP), proteins involved in intracellular transport of

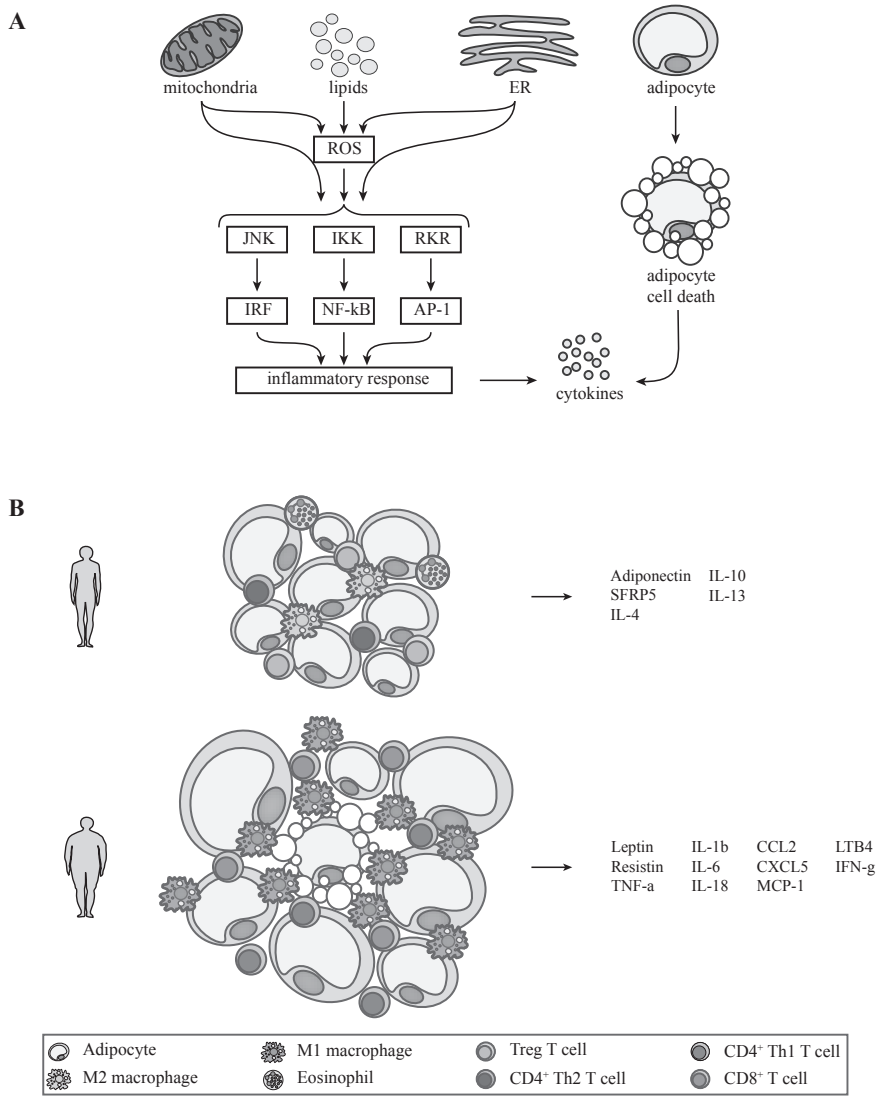

Figure 5. Schematic overview of the different pathways that are activated by increased fat deposition in adipose tissue resulting in a pro-inflammatory microenvironment. (A) Oxidative stress, increased levels of lipid production and ER stress result in ROS production, which will via several molecular pathways result in the activation of inflammatory responses. Moreover adipocyte cell death will stimulate cytokine production and immune cell recruitment. (B) Alterations in the adipose tissue immune compartment and adipokine production during the development of obesity. *See Chapter VII for full-color figure.*

FFAs, have been associated with inflammation.[187] The exact role of TLR signaling and FABPs in the development of obesity-related inflammation, however, is yet not fully understood.[172, 187]

All together, in obesity, a pro-inflammatory microenvironment is created in which increased secretion of cytokines and chemokines by adipocytes as well as adipocyte death result in recruitment of immune cells into the adipose tissue (figure 5B).[185]
Macrophages were the first immune cells recognized to accumulate in obese adipose tissues. It has been demonstrated that in obese mice as well as humans macrophages comprise up to 40% of the total adipose tissue cell count.[190, 191] Moreover, as much as 90% of adipose tissue macrophages in obese mice and humans localize selectively to sites of necrotic-like adipocyte death, where they form so-called 'crown-like' structures.[184] This macrophage accumulation co-occurs with a shift from an anti-inflammatory M2 to a pro-inflammatory M1 phenotype with concomitant production of pro-inflammatory cytokines such as TNF-α and IL-6 which largely contributes to the state of chronic low-grade inflammation.[169] More recently, it has been demonstrated that also T cells accumulate in adipose tissue early on during the development of obesity. [192-195]

Obesity and the T-cell compartment

Adipose tissue
In lean conditions, T cells can be found in the SVF of the adipose tissue where they play an important role in immune monitoring.[169, 196, 197] These T cells are predominantly nTregs and Th2 T cells, T-cell subpopulations with anti-inflammatory properties.[169, 196, 197]
Diet induced obesity (DIO) in both mice and humans was found to be associated with T-cell accumulation within adipose tissue.[192-195] This concerned mainly the accumulation of effector and memory $CD8^+$ T cells,[194-196] while the percentage of $CD4^+$ T cells and nTreg cells decreased.[196-198] $CD8^+$ effector T-cell infiltration was demonstrated to precede macrophage infiltration.[196, 199] Moreover, depletion of $CD8^+$ T cells prevented the accumulation of M1 macrophages. These data suggest that T cells are involved in the recruitment of macrophages into the obese adipose tissue. Depletion of $CD8^+$ T cells or the total T-cell pool improved insulin sensitivity in DIO mice.[196, 198] In contrast, DIO did not reduce macrophage accumulation or affect the development of insulin resistance in *RAG1*$^{-/-}$ or *RAG2*$^{-/-}$ mice, mice which lack mature T and B cells.[198, 199]

Peripheral T-cell compartment
So far, only a limited number of studies have investigated the composition of the peripheral blood immune system in obesity, with contradictory results. In most studies, a positive correlation was found between BMI and total white blood cell count[200-203] and T-cell numbers in peripheral blood[200-202, 204], but also negative correlations between BMI and T-cell numbers in peripheral blood have been found.[205] Additionally, decreased numbers of $CD4^+$ and $CD8^+$ T cells have been described, while other studies report increased $CD4^+$ and normal $CD8^+$ T-cell numbers.[200, 201, 205]
Finally, several studies have described a limited *TCRB* repertoire of T cells isolated from adipose tissue of obese mice. Based on these data it has been suggested that an antigen driven immune response might occur towards main antigens present within obese adipose tissue.[196, 206]

Thymus

A recent study in DIO mice demonstrated that obesity resulted in increased fat deposits within the thymus.[207] This was associated with decreased total thymocyte cell numbers and a reduced frequency of DP and SP thymocytes.[207] Moreover, a reduction in the percentages of peripheral blood naive T cells together with a decreased δREC-ψJα TREC content in $CD4^+$ T cells suggested that thymic output is impaired in these mice.[207]

Although all these data suggest that obesity might result in alterations in the T-cell compartment, which might be involved in inflammation and the development of obesity-related comorbidities, the exact involvement of alterations in the T-cell compartment is so far unknown. Therefore, further detailed studies on the T-cell compartment in obesity are required.

AIMS OF THE THESIS

From the above it can be concluded that a complex network of interactions exists between the neuro-endocrine, the metabolic and the immune system. The aim of the research described in this thesis is to increase our knowledge of neuro-endocrine-immune and adipose-immune interactions.

In humans, in a clinical setting, it is difficult to investigate thymic function directly because thymic tissue is difficult to obtain; a thymic biopsy is a risky procedure. Therefore, other ways to determine thymic function within peripheral blood have been investigated. In **chapter II** a novel TREC-based method was investigated to determine intra-thymic as well as peripheral T-cell proliferation. Using this method the contribution of intra-thymic and peripheral T-cell proliferation to T-cell homeostasis was investigated in young and aged humans.
Previous studies in our laboratory demonstrated that many hormone receptors are present within the thymus, suggesting a role for hormones in the normal regulation of T-cell development in the thymus. In **chapter III and IV** the interactions between the T-cell compartment and the hypothalamus-pituitary-thyroid axis are investigated. **Chapter III** describes the expression pattern and functionality of the TSHR in normal human thymic tissues. In addition, the significance of TSHR signaling during human and mouse thymocyte development was investigated using a mouse model lacking functional TSHR expression and two human thymocyte culture systems. In **chapter IV** a detailed analysis of the T- and B-cell compartment in GD patients is performed. Recently, it was demonstrated that obesity is associated with alterations within the T-cell compartment. In **chapter V** the effects of morbid obesity on the peripheral T-cell compartment are investigated with flow cytometric analyses. Additional TREC analysis and cytokine measurements were employed to obtain more insights into the mechanisms that are involved in the alterations found within the peripheral T-cell compartment. **Chapter VI** discusses the significance of the obtained data in the context of literature and provides directions for future investigations.

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Chapter II

Combined TCRG and TCRA TREC analysis reveals increased peripheral T-lymphocyte but constant intra-thymic proliferative history upon ageing

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Adapted from: Mol Immunol. 2013 Mar;53(3):302-12.

ABSTRACT

T-cell receptor (TCR) repertoire diversity, thymic output, clonal size and peripheral T-cell numbers largely depend on intra-thymic and post-thymic T-cell proliferation. However, quantitative insight into thymocyte and T-cell proliferation is still lacking. We developed a new *TCRG*-based TCR excision circle (TREC) assay, the Vγ-Jγ TREC assay, which we used together with an adjusted δREC-ψJα TREC assay to quantify the proliferative history of human thymocyte and T-cell subpopulations from children and adults. This revealed that thymocytes undergo ~6-8 intra-thymic cell divisions from the double negative (DN) 3 developmental stage onwards, which appeared independent of age. Thus thymocyte proliferation after the DN3 developmental stages is stable and therefore not contributing to the reduced thymic output upon ageing. Cord blood naive T cells had already undergone ~2-3 post-thymic cell divisions, which increased to ~6-7 cell divisions in naive T cells of middle-aged adults, indicating the importance of homeostatic naive T-cell proliferation from a young age onwards in the maintenance of peripheral T-cell numbers. In conclusion, our data provide quantitative insight into the proliferative history of thymocyte and T-cell subpopulations and alterations herein upon ageing. This novel TREC assay approach could prove valuable in immune status monitoring in a variety of conditions.

INTRODUCTION

The intricate process of T-cell development in the thymus is essential in the formation and maintenance of the peripheral T-cell compartment with a diverse T-cell receptor (TCR) repertoire.[1, 2] In the thymus, bone-marrow derived T-cell precursors develop through a highly complex process into mature naive T cells. An essential step during T-cell development is the rearrangement process of the variable domains of the different TCR loci, in which variable (V), diversity (D), and joining (J) genes are recombined to generate a unique TCR.[3] This process determines the diversity of the TCR repertoire and is thus critical in the establishment of a peripheral T-cell compartment that is well equipped to fight off foreign antigens.[4, 5]
Intra-thymic proliferation of developing T cells is an important determinant of thymic function as it largely determines thymic output.[6, 7] Moreover, preTCR controlled proliferation largely contributes to TCRαβ repertoire diversity.[8] After thymic emigration, post-thymic homeostatic peripheral T-cell proliferation results in further expansion of the naive T-cell compartment, without further expansion of the pre-existing repertoire.[4]

Thymic function is maximal early in life, during which T-cell homeostasis is mainly sustained by thymic output.[9] With ageing, however, the thymus involutes, eventually resulting in strongly diminished thymic output.[10, 11] To compensate for this, post-thymic homeostatic T-cell proliferation is enhanced to maintain peripheral naive T-cell numbers at desired levels during ageing.[1] Nevertheless, ageing will eventually result in a less diverse TCR repertoire, as repertoire formation largely depends on thymic output of *de novo* generated naive T cells.[12] Despite the importance of both intra-thymic and post-thymic T-cell proliferation in T-cell homeostasis, it remains so far unknown to what extent thymic output and homeostatic T-cell proliferation contribute to the maintenance of the peripheral T-cell compartment and how this is affected by ageing.

The possibility to quantify intra-thymic and post-thymic T-cell proliferation would enhance our knowledge about the normal physiology of T-cell development. Also, such measurement can provide information about the relative contribution of thymic output and peripheral homeostatic T-cell proliferation to the maintenance of the peripheral T-cell compartment. Moreover, quantitative determination of the intra-thymic proliferative history within the peripheral blood T-cell compartment might in principle provide a new marker of thymic function. In such, it will provide important additional information on the immune status of an individual, for which currently only limited diagnostic tools such as peptide-MHC tetramers and GeneScan analysis of TCR repertoire diversity are available.[13, 14]

V(D)J recombination of TCR genes results in the formation of a coding joint in the genomic DNA and a signal joint on the so-called T-cell receptor excision circle (TREC).[15, 16] TRECs are stable circular DNA structures that contain DNA sequences that are removed from the TCR loci during TCR gene rearrangements. In contrast to genomic DNA, TRECs do not duplicate during cell proliferation. Consequently, they will dilute out during consecutive cell divisions. The latter makes them useful markers to determine the proliferative history of T cells.[17]
The effect of proliferation on TREC dilution has been demonstrated clearly for the classical

δREC-ψJα TREC assay. In this assay the presence of the δREC-ψJα TREC, which is formed early during *TCRA* rearrangements, is determined in peripheral blood T cells as a marker for thymic output.[15-17] However, not only thymic output but also peripheral T-cell proliferation affects the TREC content.[17]

Additionally, the ratio between the classical δREC-ψJα TREC and multiple βTRECs, which are formed during *TCRB* rearrangements, has been used to measure intra-thymic proliferation.[7] As all TRECs are affected similarly by peripheral events such as proliferation and apoptosis, the δREC-ψJα TREC/βTRECs ratio only reflects intra-thymic proliferation.[7] This approach does however not allow quantitative evaluation of intra-thymic proliferation.

Based on the principles of the kappa-deleting recombination excision circle (KREC) assay that we have previously developed to quantitatively determine the proliferative history in B cells,[18] we designed a novel TREC-based assay, the Vγ-Jγ TREC assay. This assay is based on measurements of the early occurring, single-step *TCRG* gene rearrangement.[3] This rearrangement results in the formation of a Vγ-Jγ coding joint ($CJ_{V\gamma\text{-}J\gamma}$) which remains stably present in the genomic DNA, and a Vγ-Jγ signal joint ($SJ_{V\gamma\text{-}J\gamma}$) on the corresponding excision circle (Vγ-Jγ TREC). From the difference between $CJ_{V\gamma\text{-}J\gamma}$ and $SJ_{V\gamma\text{-}J\gamma}$ levels, the number of cell divisions undergone can be calculated.[18] The designed Vγ-Jγ TREC assay specifically measures the rearrangement of the *Vγ8* gene with the highly homologous *Jγ1.3* and *Jγ2.3* genes. Using this novel assay in combination with the δREC-ψJα TREC assay we investigated the contribution of intra-thymic and post-thymic peripheral T-cell proliferation upon human ageing.

MATERIALS AND METHODS

Patients

Thymic tissue was obtained from healthy children (aged one week to thirteen years) undergoing cardiac surgery and from adults undergoing thymectomy because of thymoma or myasthenia gravis. Umbilical cord blood was obtained from healthy newborns. Peripheral blood was taken from healthy adults. Informed consent was obtained and the experimental protocol was approved by the Medical Ethical Committee of the Erasmus MC, University Medical Center, Rotterdam, The Netherlands.

Material preparation

Thymocytes were isolated by dissection of the thymic lobes into smaller pieces and subsequent squeezing through a metal mesh. For all experiments, we used thymocytes obtained after Ficoll density separation. Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll density separation.

High-speed cell sorting of T-cell subpopulations

Thymocyte and T-cell subpopulations were sorted with a FACSAria cell sorter (BD Biosciences, San Jose, CA, USA) to a purity of >90% in all samples. Thymocyte subpopulations were isolated as described previously[3] using pooled thymocytes or thymocytes from a single donor. The following T-cell subpopulations were isolated from PBMCs: naive $CD4^+TCR\alpha\beta^+$ T cells ($CD3^+TCR\gamma\delta^-CD4^+CD45RO^-CD27^+$), memory $CD4^+TCR\alpha\beta^+$ T cells ($CD3^+TCR\gamma\delta^-CD4^+CD45RO^+$), naive $CD8^+TCR\alpha\beta^+$ T cells ($CD3^+TCR\gamma\delta^-CD8^+CD45RO^-CD27^+$) and memory $CD8^+TCR\alpha\beta^+$ T cells ($CD3^+TCR\gamma\delta^-CD8^+CD45RO^+$). Antibodies used for sorting experiments are summarized in supplementary table I.

TREC analyses

DNA extraction was done using the GeneElute Mammalian Genomic DNA miniprep kit (Sigma Aldrich Logistic GmbH, Schnelldorf, Germany).

Primers and probes specific for the δREC-ψJα signal joint ($SJ_{\delta REC-\psi J\alpha}$) were used as previously.[17, 19] Primers and probes recognizing the δREC-ψJα coding joint ($CJ_{\delta REC-\psi J\alpha}$), Vγ8-Jγ1.3/2.3 signal joints ($SJ_{V\gamma-J\gamma}$) and Vγ8-Jγ1.3/2.3 coding joints ($CJ_{V\gamma-J\gamma}$) were designed using OLIGO primer analysis software (Molecular Biology Insights, Cascade, CO, USA). Sequences are given in supplementary table II.

For detection of the $CJ_{V\gamma-J\gamma}$ and $SJ_{V\gamma-J\gamma}$ a 15-cycle pre-amplification PCR was performed. For this purpose a random sequence of 10 nucleotides was added to the 5' primer tail, which provides a common sequence ensuring comparable annealing efficiency for each primer.[20, 21] The twenty-five µl pre-amplification PCR mixes contained 50 ng of DNA of interest, 1x PCR Gold Buffer (Applied Biosystems, Foster City, CA, USA), 200 µM dNTPs (GE Healthcare, Diegem, Belgium), 1.5 mM $MgCl_2$ (Applied Biosystems), 2% BSA (Sigma Aldrich), 0.5 U AmpliTaq Gold (Applied Biosystems) and 1200 nM forward and reverse primer.

Two µl from the pre-amplification PCR product was used in subsequent real-time quantitative (RQ)-PCR reactions.

RQ-PCR reactions to quantitatively detect all signal and coding joints were performed using an ABI Prism 7900 machine (Applied Biosystems). Fifteen µl RQ-PCR mixes contained 50 ng DNA of interest or 2 µl PCR product from the pre-amplification step, 1x Taqman Universal MasterMix (Applied Biosystems), 2% BSA (Sigma Aldrich), 300-500 nM forward and reverse primer, and 167-200 nM probe.

All PCR experiments were performed in duplicate. To normalize for the amount of input DNA, an albumin PCR was included as an internal control as described previously.[22] For analyses SDS 2.3 software (Applied Biosystems) was used, the detection threshold was set at 0.12 in all experiments.

The mean number of cell divisions was calculated as the ΔCt:[18]

$$(Ct_{SJ_{V\gamma-J\gamma}} - Ct_{CJ_{V\gamma-J\gamma}})$$

The percentage of alleles expressing δREC-ψJα or Vγ8-Jγ1.3/2.3 rearrangements was calculated as levels relative to albumin:

$$(2^{Ct\ Alb - Ct\ CJ\ or\ SJ})$$

Validation of PCR efficiency

For control experiments, PCR products of $CJ_{\delta REC\text{-}\psi J\alpha}$, $SJ_{\delta REC\text{-}\psi J\alpha}$, $CJ_{V\gamma\text{-}J\gamma}$ and $SJ_{V\gamma\text{-}J\gamma}$ amplified from DNA isolated from adult human PBMCs were cloned into the pGEMT-easy vector (Promega, Leiden, The Netherlands). Primer efficiency was tested on serial dilutions of the constructs. Primers recognizing the pGEMT-easy vector backbone were added to correct for input.[18] Primer efficiency of the albumin and the $CJ_{\delta REC\text{-}\psi J\alpha}$ primer were compared on HSB2 T-cell line DNA containing an in-frame δREC-ψJα recombination on one allele.[15] Primer efficiency of the albumin and the $CJ_{V\gamma\text{-}J\gamma}$ primer were compared on Jurkat T-cell line DNA containing an in-frame Vγ8-Jγ1.3/2.3 recombination on one allele.[23]

Validation of sensitivity of TREC detection

To investigate accuracy of TREC dilution during proliferation, sorted naive T cells were incubated with carboxyfluorescein diacetate succinimidyl ester (CFSE) (5μg/ml; Molecular Probes Europe, Leiden, The Netherlands) for 10 minutes at 37°C.[24] The reaction was stopped by adding RPMI-1650 containing 10% FCS. Cells ($1x10^5$ cells/ml) were plated into a 96-wells plate in T-cell medium; IMDM without phenol red (Biowhittaker, Walkersville, MD) containing 10% FCS (Sigma-Aldrich), $1x10^{-5}$ M β-mercapto-ethanol (Merck, Darmstadt, Germany), 4 mM Ultra-glutamine (Lonza, Verviers, Belgium), penicillin (100 U/ml; Biowhittaker), streptomycin (100 μg/ml; Biowhittaker). After a 24-hour culture period (37°C and 5% CO_2), cells were either collected for CFSE measurement and DNA isolation or were cultured for another three days in the presence of anti-CD3 (0.3 μg/ml; Sanquin Reagents, Amsterdam, The Netherlands) and anti-CD28 (0.4 μg/ml; Sanquin Reagents) to stimulate proliferation. After three days, cells were harvested and sorted based on CFSE peaks. DNA isolated from the sorted cell populations was used for TREC analysis.

Validation of proliferation in thymocyte subpopulations

To investigate proliferation within different thymocyte subpopulations, Ki67 expression was examined by flow cytometry. Freshly isolated thymocytes (0-5 years of age) were stained for surface markers, fixed and permeabilized (lysing solution, BD Biosciences) and stained with a Ki67 antibody (DAKO, Carpinteria, CA, USA). Measurements were done with a FACS LSR-II (BD Biosciences) and analysed with FlowJo software (TreeStar, Ashland, OR, USA). Antibodies used for flow cytometric analyses are summarized in supplementary table I.

Statistical analysis

The exact Mann-Whitney U test was used for statistical comparisons between two groups. All statistical analyses were performed with SPSS software version 15.0. A p-value of less than 0.05 (two-tailed) was considered statistically significant. To describe subject characteristics the range is shown. Error bars are expressed as the standard error of the mean (s.e.m.).

RESULTS

Vγ-Jγ TREC analysis RQ-PCR design

In order to calculate the proliferative history of T cells we designed RQ-PCR assays recognizing the genomic Vγ8-Jγ1.3/2.3 coding joint ($CJ_{V\gamma\text{-}J\gamma}$) and the corresponding TREC containing the Vγ-Jγ signal joint ($SJ_{V\gamma\text{-}J\gamma}$; figure 1A). In addition we used RQ-PCR assays recognizing the genomic δREC-ψJα coding joint ($CJ_{\delta REC\text{-}\psi J\alpha}$) and the corresponding TREC containing the δREC-ψJα signal joint ($SJ_{\delta REC\text{-}\psi J\alpha}$; figure 1B). Upon ongoing Vα-Jα recombination, the $CJ_{\delta REC\text{-}\psi J\alpha}$ will also be deleted from the genomic DNA and become part of a novel TREC, and will thus dilute out as well (figure 1B).[25]

The efficiency of all RQ-PCR assays was determined using serial dilutions of constructs containing the CJ or SJ of interest. The $CJ_{\delta REC\text{-}\psi J\alpha}$ and $SJ_{\delta REC\text{-}\psi J\alpha}$ RQ-PCR assays were equally efficient, as were the $CJ_{V\gamma\text{-}J\gamma}$ and $SJ_{V\gamma\text{-}J\gamma}$ RQ-PCR assays (figure 2A). RQ-PCR reactions on serial dilutions of DNA from the HSB2 cell line (which contains an in-frame δREC-ψJα rearrangement on one allele)[15] and DNA from the Jurkat cell line (which contains an in-frame Vγ8-Jγ1.3/2.3 rearrangement on one allele)[23] revealed equal RQ-PCR efficiencies for albumin and $CJ_{\delta REC\text{-}\psi J\alpha}$ and for albumin and $CJ_{V\gamma\text{-}J\gamma}$ (figure 2B).

Sensitivity of low level $SJ_{V\gamma\text{-}J\gamma}$ detection

Because $SJ_{V\gamma\text{-}J\gamma}$ levels were low in PBMCs (Ct values ~ 38 to 42) PCR pre-amplification was performed. Duplicate experiments demonstrated highly reproducible results after the 15-cycle pre-amplification step (figure 2C). In thymocyte subpopulations, pre-amplification similarly reduced Ct values for albumin, $CJ_{V\gamma\text{-}J\gamma}$ and $SJ_{V\gamma\text{-}J\gamma}$. In PBMCs, the reduction in Ct values for albumin and the $CJ_{V\gamma\text{-}J\gamma}$ were comparable to those seen in the thymocyte subpopulations, while the Ct value reduction for the $SJ_{V\gamma\text{-}J\gamma}$ clearly diverged from that seen in thymocyte subpopulations (figure 2D). Only the samples with a $SJ_{V\gamma\text{-}J\gamma}$ Ct value < 29 after RQ-PCR with pre-amplification (closed circles) showed Ct value reductions comparable to those seen in thymocyte subpopulations. To determine whether this discrepancy in Ct-value reduction for the $SJ_{V\gamma\text{-}J\gamma}$ was related to limits of the assay, RQ-PCR with pre-amplification was performed on serial dilutions of a $SJ_{V\gamma\text{-}J\gamma}$ containing construct. In all dilutions that contained at least 7 vector particles ($Ct_{7\ vector\ particles}$ range 29.1 - 30.4) reproducible signals were measured (figure 3A), indicating that the Vγ-Jγ TREC analysis (RQ-PCR with pre-amplification) can reliably be used for the detection of 7 or more copies of DNA of interest.

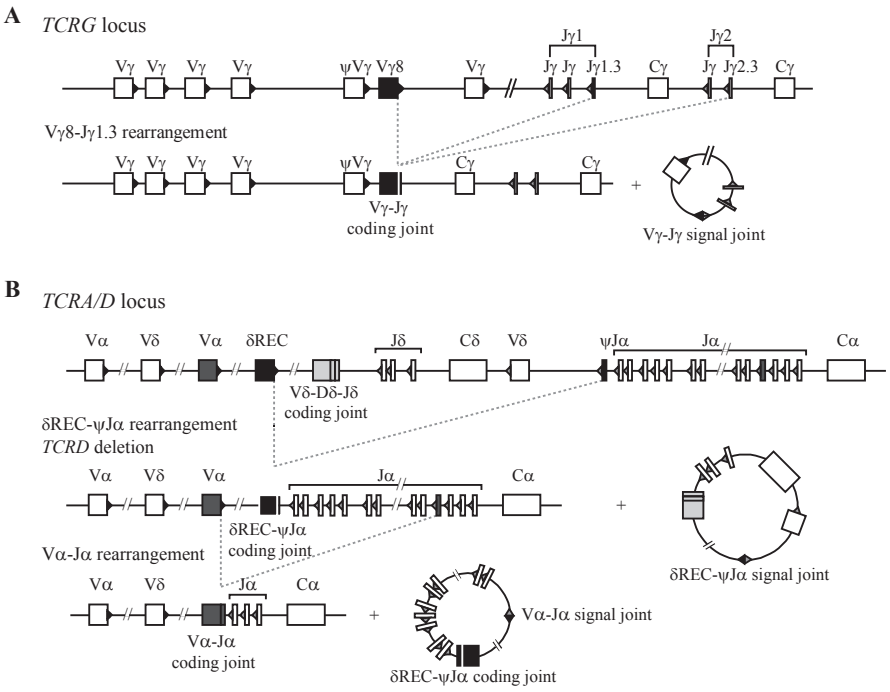

Figure 1. Detection of $CJ_{\delta REC\text{-}\psi J\alpha}$, $SJ_{\delta REC\text{-}\psi J\alpha}$, $CJ_{V\gamma\text{-}J\gamma}$, $SJ_{V\gamma\text{-}J\gamma}$. (A) During *TCRG* rearrangements Vγ8-Jγ1.3/2.3 rearrangements result in the formation of a $CJ_{V\gamma\text{-}J\gamma}$ present in the genomic DNA, and a $SJ_{V\gamma\text{-}J\gamma}$ on the corresponding TREC. (B) Just before *TCRA* rearrangements in preTCR$^+$ T cells, the *TCRD* locus is deleted, preferentially via the δREC-ψJα rearrangement. This rearrangement results in a $CJ_{\delta REC\text{-}\psi J\alpha}$ present in the genomic DNA, and a $SJ_{\delta REC\text{-}\psi J\alpha}$ on the corresponding TREC. Due to ongoing Vα-Jα recombination the $CJ_{\delta REC\text{-}\psi J\alpha}$ will also be excised and become a signal joint on the corresponding TREC.[25]

To determine whether Vγ-Jγ TREC analysis measurements can be used to determine proliferative history of T cells in peripheral blood, $CJ_{V\gamma\text{-}J\gamma}$, $SJ_{V\gamma\text{-}J\gamma}$ and $SJ_{\delta REC\text{-}\psi J\alpha}$ levels were quantified in naive T cells, sorted based on CFSE peaks, that had undergone 0, 1, 2, 3 or 4 cell divisions after 4 days of stimulation with anti-CD3 and anti-CD28 antibodies. As expected, the $SJ_{\delta REC\text{-}\psi J\alpha}$ signal diluted out upon consecutive cell divisions, and the $CJ_{V\gamma\text{-}J\gamma}$ signal remained stably present in the genomic DNA. The $SJ_{V\gamma\text{-}J\gamma}$ signal, however, did not dilute out upon consecutive cell divisions (figure 3B). This indicates that the $SJ_{V\gamma\text{-}J\gamma}$ level in peripheral blood T cells is too low to allow determination of the proliferative history by Vγ-Jγ TREC analysis.

Proliferative history of human thymocytes

We next determined $CJ_{\delta REC\text{-}\psi J\alpha}$, $SJ_{\delta REC\text{-}\psi J\alpha}$ $CJ_{V\gamma\text{-}J\gamma}$ and $SJ_{V\gamma\text{-}J\gamma}$ levels in sorted thymocyte subpopulations (figure 4A). The $CJ_{V\gamma\text{-}J\gamma}$ was readily detectable in DN3 thymocytes, and its presence increased in the ISP stage of development, indicating that Vγ8-Jγ1.3/2.3 rearrangements occur during these

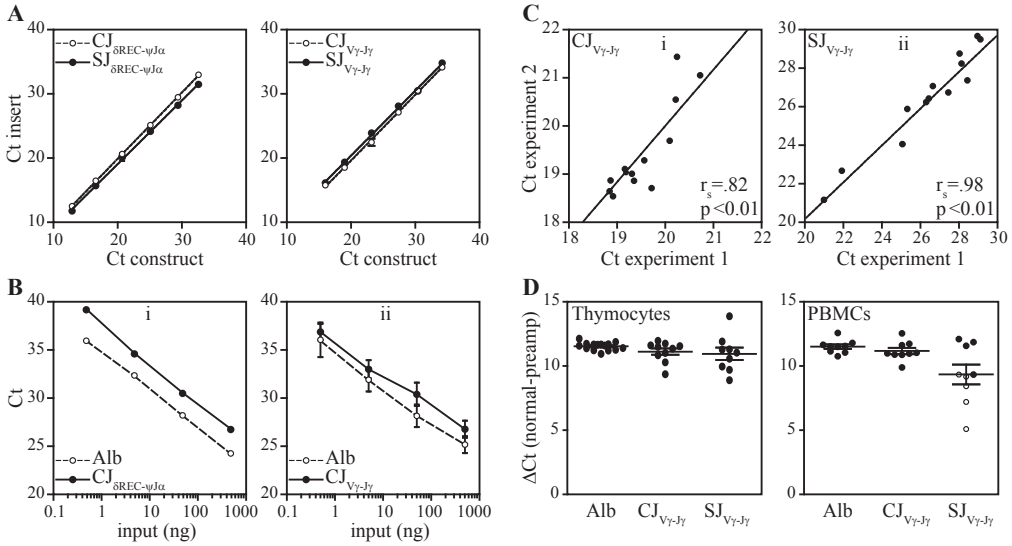

Figure 2. Sensitivity of $CJ_{\delta REC\text{-}\psi J\alpha}$, $SJ_{\delta REC\text{-}\psi J\alpha}$, $CJ_{V\gamma\text{-}J\gamma}$ and $SJ_{V\gamma\text{-}J\gamma}$ detection and quantification. (A) Efficiency of $CJ_{\delta REC\text{-}\psi J\alpha}$, $SJ_{\delta REC\text{-}\psi J\alpha}$, $CJ_{V\gamma\text{-}J\gamma}$ and $SJ_{V\gamma\text{-}J\gamma}$ RQ-PCR assays was tested using tenfold dilution series of constructs containing the $CJ_{\delta REC\text{-}\psi J\alpha}$, $SJ_{\delta REC\text{-}\psi J\alpha}$, $CJ_{V\gamma\text{-}J\gamma}$ or $SJ_{V\gamma\text{-}J\gamma}$ DNA sequence (Ct insert). Primers and probe recognizing the pGEMT-easy vector backbone were added to correct for input (Ct construct), n=3. (B) Comparison of albumin and $CJ_{\delta REC\text{-}\psi J\alpha}$ RQ-PCR assays using tenfold dilution series of the HSB2 cell line (i), comparison of albumin and $CJ_{V\gamma\text{-}J\gamma}$ RQ-PCR assays using tenfold dilution series of the Jurkat cell line (ii), n=3. (C) Duplicate Ct value measurements of $CJ_{\delta REC\text{-}\psi J\alpha}$ and $SJ_{\delta REC\text{-}\psi J\alpha}$ (i) and $CJ_{V\gamma\text{-}J\gamma}$ and $SJ_{V\gamma\text{-}J\gamma}$ (ii) in DNA from constructs, thymocyte subpopulations and T-cell subpopulations. $CJ_{V\gamma\text{-}J\gamma}$ and $SJ_{V\gamma\text{-}J\gamma}$ measurements included a pre-amplification step. (D) Measurement of Ct values for albumin, $CJ_{V\gamma\text{-}J\gamma}$ and $SJ_{V\gamma\text{-}J\gamma}$ with and without pre-amplification step in DNA isolated from TCR$\alpha\beta^+$ T cells of 9 healthy adult subjects. Alb: albumin.

stages. Thereafter, the $CJ_{V\gamma\text{-}J\gamma}$ remained relatively stable present in the genomic DNA (~5% of alleles). Also the $SJ_{V\gamma\text{-}J\gamma}$ became clearly detectable in the DN3 stage of development, and declined again after the ISP stage of development, indicating dilution based on cell proliferation. The $CJ_{\delta REC\text{-}\psi J\alpha}$ and $SJ_{\delta REC\text{-}\psi J\alpha}$ were first found at low levels in the DN3-ISP developmental stages, and their levels strongly increased in the $DP3^-$ stage of development from where on they remained stably present throughout thymic development. Based on the calculation:

$$(Ct_{SJ_{V\gamma-J\gamma}} - Ct_{CJ_{V\gamma-J\gamma}})$$

it was deduced that developing thymocyte subpopulations, from the DN3 stage onwards, had undergone ~6-8 cell divisions after initiation of *TCRG* rearrangements (figure 4B). Most of these cell divisions occurred during the DN3-ISP-$DP3^-$ developmental stages (figure 4B). In concordance with this, the percentage of $Ki67^+$ thymocytes declined during consecutive stages of T-cell development (figure 4C).

Ageing barely influences proliferative activity of developing thymocytes

To investigate the effects of ageing on thymocyte proliferation, thymocyte subpopulations isolated from thymic tissues of different age categories (children 1-2 weeks, 3-5 years and 9-13 years of age and adults 29-77 years of age) were subjected to Vγ-Jγ TREC analysis. Adult thymic tissues and young thymic tissues displayed only minor differences in thymocyte subpopulation composition, with adult tissues containing slightly higher percentages of DN thymocytes (figure 5A). Ki67 analysis on freshly isolated thymocytes from two adult thymi demonstrated that early thymocyte subpopulations exhibited a proliferative pattern comparable to those isolated from young thymic tissues, while the $DP3^+$ and $SP4^+$ subpopulations from adult thymic tissue displayed slightly higher Ki67 expression than did these subpopulations from young thymic tissues (figure 5B). Vγ-Jγ TREC analysis of the different thymic tissues (children 1-2 weeks (figure 5C), 3-5 years (figure 5D) and 9-13 years (figure 5E) of age and adults (figure 5G-J) 29-77 years of age) showed no clear alterations in proliferative activity of thymocytes among the different age groups examined (figure 5F). Thus the cell-intrinsic capacity of thymocytes to proliferate is not altered upon ageing.

Proliferative history in human peripheral blood T-cell subpopulations

Having established a novel assay to quantitatively determine intra-thymic proliferation, we next investigated the possibilities to quantitatively measure post-thymic proliferation. For this purpose, the $CJ_{V\gamma\text{-}J\gamma}$, $SJ_{V\gamma\text{-}J\gamma}$ and $SJ_{\delta REC\text{-}\psi J\alpha}$ levels were measured in naive and memory T cells from cord blood, young adults (21-26 years of age) and middle aged adults (59-62 years of age). This revealed that $CD4^+$ and $CD8^+$ naive T cells from cord blood had undergone ~10-11 cell divisions since the occurrence of Vγ-Jγ rearrangements (figure 6A). In naive and memory T cells from

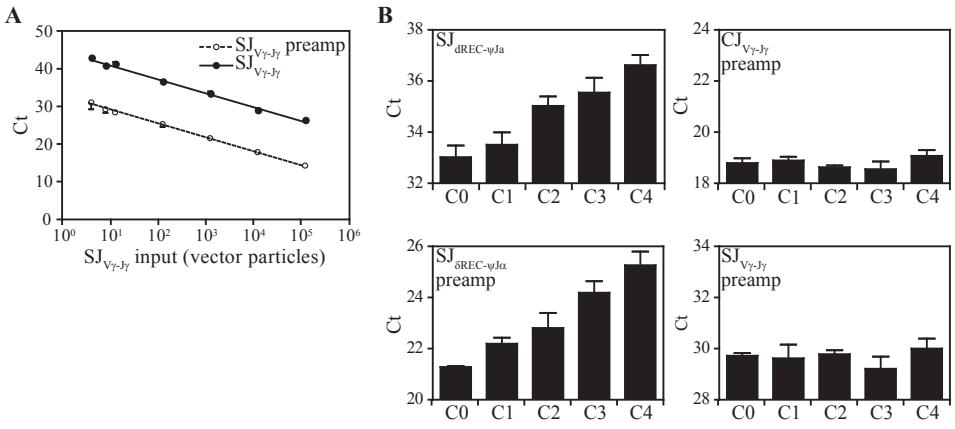

Figure 3. Detection limit of the $SJ_{V\gamma\text{-}J\gamma}$. (A) Tenfold dilution series of construct containing $SJ_{V\gamma\text{-}J\gamma}$ with and without preamplification of 15 cycles, n=3. (B) $SJ_{\delta REC\text{-}\psi J\alpha}$, $CJ_{V\gamma\text{-}J\gamma}$ and $SJ_{V\gamma\text{-}J\gamma}$ levels in naive T cells after one to four cell divisions, n=3. C0, C1, C2, C3, C4: cells that had undergone 0, 1, 2, 3 and 4 cell divisions, respectively.

young and middle-aged adults $SJ_{V\gamma\text{-}J\gamma}$ levels were below the detection limit demonstrating that at least 10-11 cell divisions had occurred. The exact amount of cell divisions, however, could not be determined reliably (figure 6A, data not shown).
Alternatively, we used an adjusted δREC-ψJα TREC analysis to investigate proliferation in naive and memory T-cell subpopulations from young and middle-aged adults. Based on the assumptions that in healthy individuals thymic proliferation and thus the TREC content in SP thymocytes is similar and changes in TREC content in the peripheral blood are mainly due to proliferation, the number of cell divisions in peripheral blood can be calculated as the $SJ_{\delta REC\text{-}\psi J\alpha}$ ΔCt of SP thymocytes and the peripheral blood T-cell subpopulation of interest, as given by:[26]

$$((Ct_{SJ_{\delta REC-\psi J\alpha}\ SP} - Ct_{alb\ SP}) - (Ct_{SJ_{\delta REC-\psi J\alpha}\ T-cell\ population} - Ct_{alb\ T-cell\ population}))$$

Based on $SJ_{\delta REC\text{-}\psi J\alpha}$ levels, a proliferative history of ~2-3 cell divisions in peripheral blood was calculated for naive T cells from cord blood, which is comparable to the number of cell divisions calculated on the basis of Vγ-Jγ measurements: $\text{Proliferative history}_{\text{naive T cells from cord blood}}$ – $\text{Proliferative history}_{\text{SP thymocytes}}$ = ~2-4 cell divisions.
The proliferative history of naive T cells increased from ~2-3 cell divisions in naive T cells

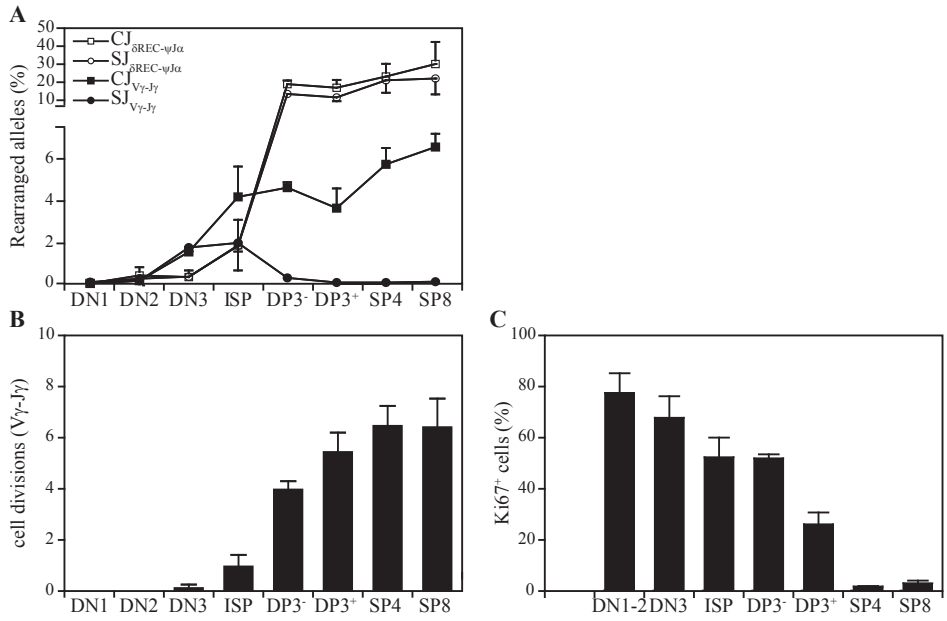

Figure 4. Proliferative history in consecutive thymocyte subpopulations. (A) Measurement of $CJ_{\delta REC\text{-}\psi J\alpha}$, $SJ_{\delta REC\text{-}\psi J\alpha}$, $CJ_{V\gamma\text{-}J\gamma}$ and $SJ_{V\gamma\text{-}J\gamma}$ levels in sorted thymocyte subpopulations (pooled samples, 6 days to 13 years of age), n=3. (B) Proliferative history in sorted thymocyte subpopulations (pooled samples, 6 days to 13 years of age), n=3. (C) Flow cytometric analysis of Ki67 expression in sorted thymocyte subpopulations (2 months to 5 years of age), n=3. DN: double negative, ISP: immature single positive, DP: double positive, SP: single positive.

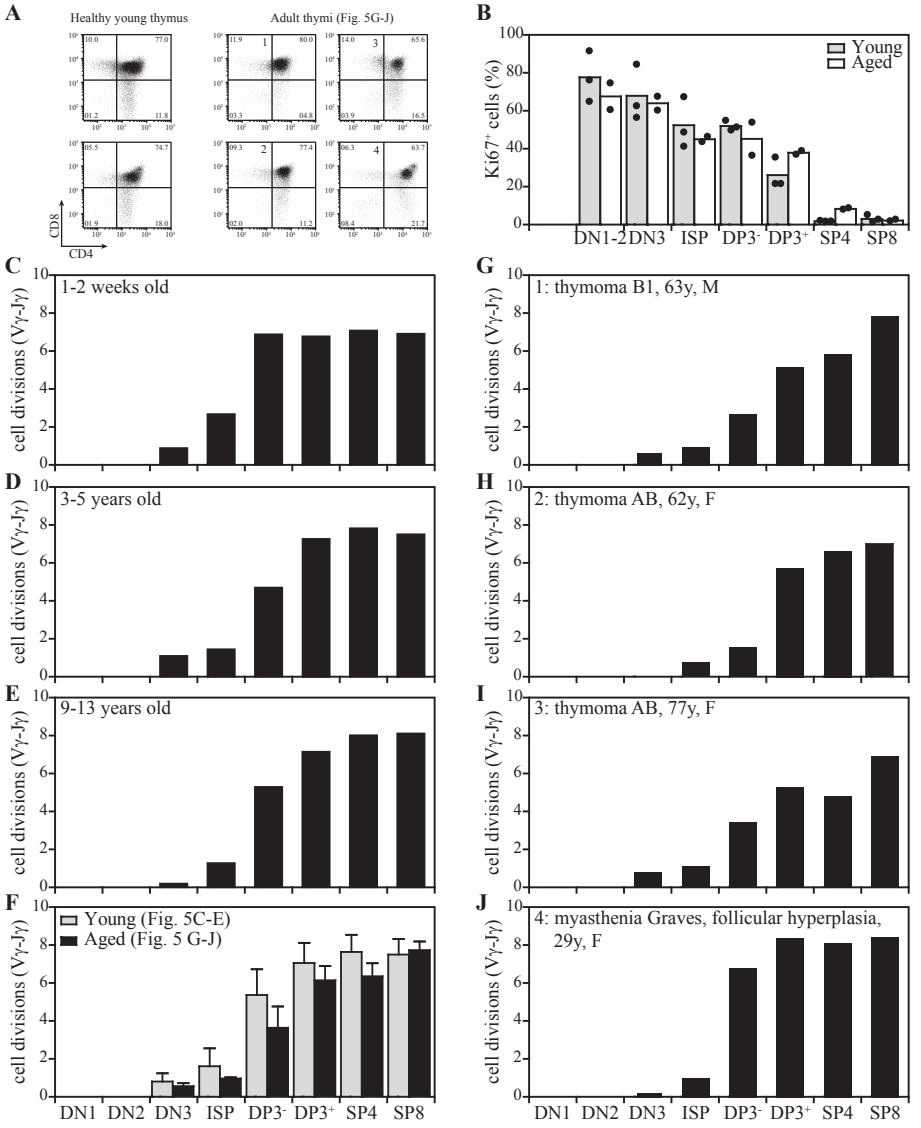

Figure 5. Intra-thymic proliferative history during ageing. (A) Flow cytometric analysis of thymocytes from healthy young thymic tissues (2 months and 5 years of age, n=2) and adult thymic tissues (29, 62, 63 and 77 years of age, n=4) based on CD4 and CD8 expression. (B) Ki67 expression in young (2 months to 5 years of age, n=3) and adult (62 and 63 years of age, n=2) thymocyte subpopulations. Bars represent means, dots represent single samples. Proliferative history based on Vγ-Jγ TREC analysis in sorted thymocyte subpopulations isolated from thymic tissue of (C) children 1 and 2 weeks of age, n=2; (D) children 3 and 5 years of age, n=2; (E) children 9 and 13 years of age, n=2; (F) young and adult thymic tissues; (G-J) adults 29, 62, 63 and 77 years of age, n=4. DN: double negative, ISP: immature single positive, DP: double positive, SP: single positive.

from cord blood to ~3-4 cell divisions in naive T cells from young adults (figure 6B). Moreover, a proliferative history of ~9-10 cell divisions was found in memory T cells from young adults indicating substantial proliferation after antigen encounter (figure 6B). In addition, the proliferative history of both naive and memory T cells was increased in middle-aged adults compared to young adults (figure 6C).

DISCUSSION

Proliferation is crucial for the formation and maintenance of the human T-cell compartment. Intra-thymic proliferation largely determines the size of the progenitor T-cell pool and TCR repertoire diversity.[4, 8] Clonal size and peripheral blood T-cell numbers are determined by intra-thymic and post-thymic proliferation.[4, 8] Moreover, antigenic responses depend on clonal expansion of antigen-specific T cells. Therefore, quantitative measurements of intra-thymic and post-thymic proliferation provide important information about the physiology of T-cell compartment formation and maintenance. In this study we developed a novel Vγ-Jγ TREC assay which we used to quantitatively determine intra-thymic proliferation in thymocyte subpopulations. Additionally, we used an adjusted δREC-ψJα analysis to calculate post-thymic proliferation in T-cell subpopulations. Together, these assays provide a tool to measure the proliferative history of human T cells. This is valuable to investigate the immune status in a variety of diseases, in vaccination studies and in ageing studies.

The novel Vγ-Jγ TREC assay, is based on measurement of $CJ_{V\gamma\text{-}J\gamma}$ and $SJ_{V\gamma\text{-}J\gamma}$ levels, which are simultaneously formed during the Vγ8-Jγ1.3/2.3 rearrangements. Vγ8-Jγ1.3/2.3 are two of the more frequently occurring rearrangements in TCRαβ$^+$ T cells (unpublished observations).[27] Moreover, *Jγ1.3* and *Jγ2.3* are the most downstream *Jγ* genes in the *Jγ1* and the *Jγ2* regions respectively, making further rearrangements unlikely to occur.
Vγ8-Jγ1.3/2.3 rearrangements occur early during T-cell development in the DN3 stage of development. Thus the Vγ-Jγ TREC assay allows quantitative determination of intra-thymic proliferative history of developing thymocytes from this early point onwards.
Moreover, the $CJ_{V\gamma\text{-}J\gamma}$ remains present in the genomic DNA of thymocytes that further mature into the αβ-lineage. Rearranged *TCRG* loci are indeed present in ~95% of mature human TCRαβ$^+$ thymocytes.[28] Moreover, we demonstrate that the specific Vγ8-Jγ1.3/2.3 rearrangements are present in ~5% of alleles (~10% of cells) in human thymocyte subpopulations and TCRαβ$^+$ T cells. Due to the stable presence of the $CJ_{V\gamma\text{-}J\gamma}$ within the genomic DNA the Vγ-Jγ TREC assay can be used for quantitative proliferation measurements in TCRαβ$^+$ T cells. Although our analysis measures Vγ8-Jγ1.3/2.3 rearrangements and TRECs only, there is no reason to doubt that the results obtained with this assay are representative for the total population.
It is important to realize that the Vγ-Jγ TREC assay is a population-based measurement, allowing quantification of the proliferation history of a population of cells, not of a single cell. Therefore, it is essential to select homogeneous populations for the calculations, as in heterogeneous populations alterations in composition may affect the results.

Vγ-Jγ TREC analysis of human thymocyte subpopulations revealed that ~6-8 cell divisions occur

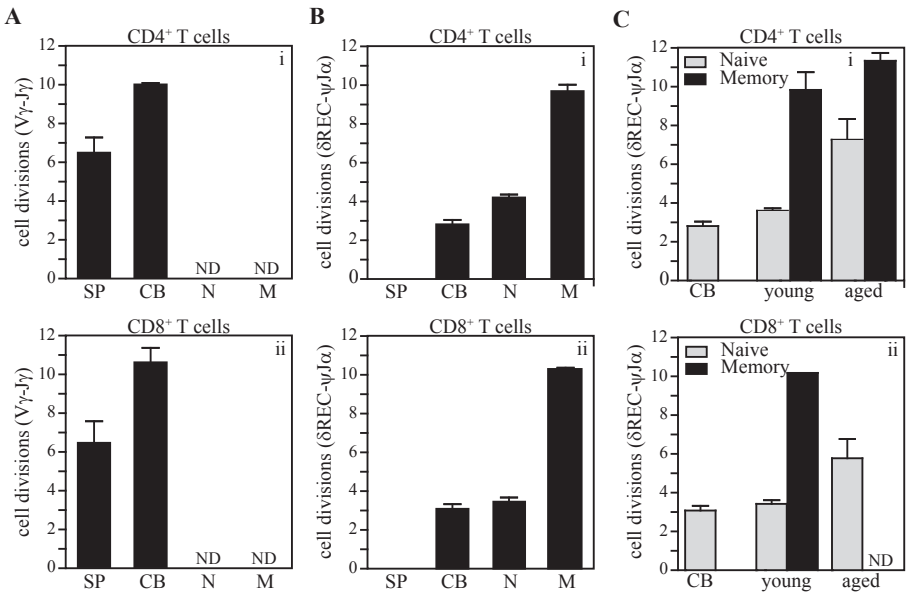

Figure 6. Proliferative history in peripheral T-cell subpopulations. (A) Proliferative history in SP thymocytes, cord blood naive TCRαβ$^+$ T cells and young adult naive and memory TCRαβ$^+$ T cells based on Vγ-Jγ TREC analysis; CD4$^+$ T cells (i), CD8$^+$ T cells (ii). (B) Proliferative history in SP thymocytes, cord blood naive TCRαβ$^+$ T cells and young adult naive and memory TCRαβ$^+$ T cells based on adjusted δREC-ψJα TREC analysis; CD4$^+$ T cells (i), CD8$^+$ T cells (ii). (C) Proliferative history in cord blood naive TCRαβ$^+$ T cells and young and middle-aged adult naive and memory TCRαβ$^+$ T cells based on adjusted δREC-ψJα TREC analysis; CD4$^+$ T cells (i), CD8$^+$ T cells (ii). Cord blood n=3, young adults (21-26 years of age) n=3, middle aged adults (59-62 years of age) n=3. SP: single positive thymocytes, CB: cord blood naive TCRαβ$^+$ T cells, N: naive TCRαβ$^+$ T cells, M: memory TCRαβ$^+$ T cells.

in the thymus after rearrangements of the *TCRG* locus. Ki67 analysis revealed high proliferative activity in the most immature thymocytes (DN1-3), while Vγ-Jγ rearrangements first occur in the DN3 stage of development.[3] Therefore Vγ-Jγ TREC analysis of intra-thymic proliferation underestimates the total number of intra-thymic cell divisions that thymocytes have undergone upon thymic exit as it does not include cell divisions undergone in the DN1-2 developmental stages. Based on thymic repopulation studies in mice it has been estimated that at least 10 cell divisions occur in these early stages of development.[29] There are, however, no single-step stable TCR rearrangements that occur earlier than in the DN3 stage of development to solve this issue, as the *TCRD* rearrangement consists of two recombination steps and most coding joints will be deleted during ongoing *TCRA* rearrangements.

Thus, The Vγ-Jγ TREC assay is the first assay that provides quantitative measurements of intra-thymic proliferation from the DN3 to the SP stages of development. Both Ki67 analysis and TREC analysis demonstrate that highly active proliferation occurs in the DN3, ISP and DP3$^-$ developmental stages, the stages in which thymocyte proliferation is largely driven by preTCR signaling.[30, 31]

PreTCR driven proliferation occurs between *TCRB* and *TCRA* rearrangements and enhances TCR diversity as daughter cells sharing the same TCRβ chain will each generate their own unique TCRα chain.[8] *TCRB* rearrangements are first detectable at the DN3 stages of development and in-frame *TCRB* rearrangements first appear in the ISP stage, while *TCRA* rearrangements start to appear in the $DP3^-$ stage of development.[3] Based on the proliferative history of ~4-5 cell cycles in $DP3^-$ thymocytes that occurred since the DN3 stage of development we can deduce that ~16-32 DP thymocytes with the same TCRβ chain are formed, each of which will generate its own unique TCRα chain. These intra-thymic data support those of a previous study that estimated that a TCRβ chain of a certain specificity must pair with at least 25 TCRα chains to explain the TCRαβ repertoire diversity that is found in the peripheral T-cell compartment.[32]

Due to involution of the thymus, characterized by atrophy of the epithelial layers, infiltration of adipocytes and a reduction in thymocyte cellularity,[10, 33] thymic output gradually declines during ageing.[10, 11] So far, however, the exact mechanisms that are involved in the reduced thymic output upon ageing are still under debate. We demonstrate that ageing does not alter intra-thymic proliferation from the DN3 to the SP stages of development. A drawback of the ageing studies is that we could not use healthy adult thymic tissues as removal is unethical. Therefore, we used thymic tissues from three patients with a thymoma, and one patient with a hyperplastic thymus due to myasthenia gravis. We found only small differences in thymocyte subpopulation composition compared to young thymi, and healthy adult thymic tissues from previous studies.[11, 34] Moreover, it was demonstrated that thymoma thymic stroma still has the ability to support T-cell development and T-cell development was largely unaffected in thymomas, especially in the type B1 and AB thymomas that were used in our study.[35] Therefore, we consider the thymocytes we used as representative for healthy adult thymocytes and regard it unlikely that the use of these tissues influenced our conclusions.

As the proliferative program after initiation of the first *TCRG* rearrangements was demonstrated to be stable and not changing upon ageing, we hypothesize that the decrease in thymic output with ageing is mainly determined by a reduction in thymocyte progenitors originating from the bone marrow and/or decreased proliferation in the early stages of T-cell development (DN1-2) but not by proliferation after initiation of *TCRG* rearrangements. This is supported by thymic repopulation studies in mice in which the number of DN thymocytes largely determines the number of DP thymocytes.[6, 36]

These data on intra-thymic proliferation in adults are in contrast to a previous study in which a reduction of the δREC-ψJα TREC/βTRECs ratio was observed with ageing, suggesting decreased intra-thymic proliferation between *TCRB* and *TCRA* rearrangements upon ageing.[7] Although we cannot fully explain the reasons for these contrary results, we think the discrepancies might be explained by the independent formation of the δREC-ψJα TREC and the βTRECs versus the simultaneous formation of the $CJ_{V\gamma\text{-}J\gamma}$ and the $SJ_{V\gamma\text{-}J\gamma}$. For example, alterations in cell death due to negative selection after βTREC formation will affect βTREC levels independent from δREC-ψJα levels which will directly affect measurement of intra-thymic proliferation based on the δREC-ψJα TREC/βTRECs ratio. Moreover, ageing might be associated with alterations in the differential usage/selection of specific rearrangements measured in the δREC-ψJα TREC/

βTRECs assay which will also affect the ratio. This is not the case for the Vγ-Jγ TREC assay as $CJ_{V\gamma\text{-}J\gamma}$ and $SJ_{V\gamma\text{-}J\gamma}$ are formed at the same stage in the same cell.
In addition, contamination by TCRγδ$^+$ T cells present in the samples might have affected the results since the TRECs were measured in PBMCs, as we previously demonstrated that *TCRB* rearrangements and thus also their corresponding excision circles can be found in mature TCRγδ$^+$ T cells, while this is not the case for the δREC-ψJα rearrangement as this would result in *TCRD* deletion. Measurement of βTRECs in TCRγδ$^+$ T cells might therefore also result in a reduction of the δREC-ψJα TREC/βTRECs ratio.
Overall, both technical as well as biological variations might differentially affect the levels of the δREC-ψJα TREC and the βTRECs, while $CJ_{V\gamma\text{-}J\gamma}$ and $SJ_{V\gamma\text{-}J\gamma}$ levels will be affected similarly, thereby enhancing the reliability of our novel Vγ-Jγ TREC assay.

Formation of a diverse TCR repertoire is essential in the formation of a functional T-cell compartment, but also a minimal clonal size for each naive T-cell specificity is required.[26] Clonal size of naive TCRαβ$^+$ T cells is determined by intra-thymic proliferation after *TCRA* rearrangements followed by homeostatic proliferation in the peripheral T-cell compartment. In cord blood naive T cells, Vγ-Jγ TREC analysis demonstrated an increase in proliferative history of ~3-4 cell divisions from the DP3$^+$ stage of development onwards. From this we deduce that the average clonal size of cord blood naive T cells approaches ~8-16 cells. These numbers are comparable to previous estimates of clonal size in human neonatal blood samples in which a clonal size of 10-20 cells was calculated based on adjusted δREC-ψJα TREC analysis,[26] and in mouse naive splenocytes in which TCR repertoire sequencing predicted a clonal size of 10 cells.[37]

The novel Vγ-Jγ TREC analysis enabled us to quantitatively measure the proliferative history in thymocyte subpopulations and cord blood T cells, but low $SJ_{V\gamma\text{-}J\gamma}$ expression hampered the possibility to determine the proliferative history in peripheral blood T-cell subpopulations. Therefore, we used an alternative approach, an adjusted δREC-ψJα TREC analysis, to examine the proliferative history of adult naive and memory T-cell subpopulations.[26]
As expected, adjusted δREC-ψJα TREC analysis demonstrated that homeostatic proliferation within the naive T-cell compartment increased with higher age, ranging from ~2-3 cell divisions in naive T cells from cord blood to ~6-7 cell divisions in naive T cells from middle-aged adults. This age-associated increase in homeostatic proliferation within the naive T-cell compartment can be expected to result in a ~8-fold increase in the number of naive T cells to compensate for reduced thymic output, demonstrating the importance of increased homeostatic proliferation to maintain peripheral T-cell numbers at desired levels with ageing.

In conclusion, using the novel Vγ-Jγ TREC assay and an adjusted δREC-ψJα TREC analysis, we quantified the proliferative history of thymocyte and T-cell subpopulations in children and adults (figure 7). In the thymus, ~6-8 cell divisions occur from the DN3 to the SP stages of development. This intra-thymic proliferation clearly enhances TCR repertoire diversity as ~4-5 of these cell divisions occur between TCRβ and TCRα generation. Intra-thymic proliferation from the DN3 to SP stages of development seems barely affected by ageing, suggesting that other factors such as a reduction in thymus-seeding progenitors and/or decreased proliferation during the DN1 and DN2 stages of development are involved in the age-related reduction in thymic output. In the peripheral

T-cell compartment, already ~2-3 cell divisions of homeostatic proliferation were found in cord blood naive T cells, demonstrating the importance of homeostatic proliferation in the naive T-cell compartment from an early age onwards. This number largely increased to ~6-7 cell divisions upon ageing, probably to compensate for the reduction in thymic output upon ageing. Finally, in memory T cells, at least ~9-10 cell divisions occurred after exit from the thymus. It is of great interest to use the Vγ-Jγ and adjusted δREC-ψJα TREC assays in a variety of disease entities in which T cells are involved, including those in which thymic output is affected such as HIV disease.

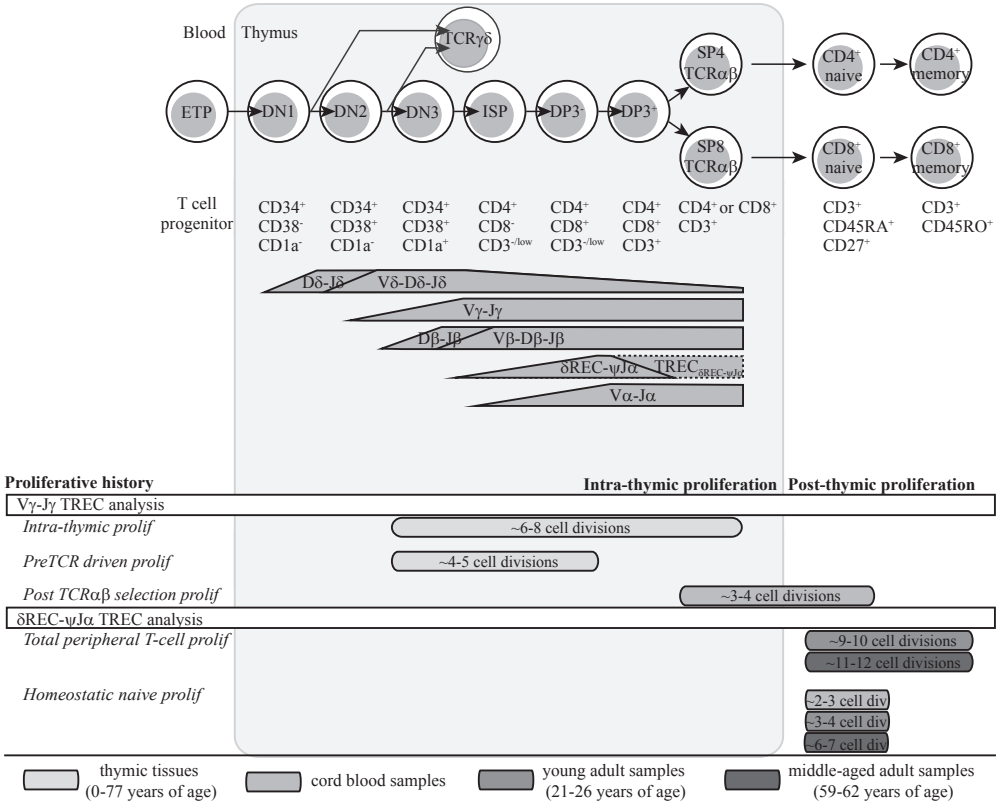

Figure 7. Schematic overview of proliferation during human T-cell development. Human T-cell developmental stages and V(D)J recombination scheme are adapted from Dik et al.[3] Intra-thymic and post-thymic proliferation was determined with Vγ-Jγ TREC and adjusted δREC-ψJα TREC analysis on thymocyte and peripheral T-cell subpopulations for different age categories. DN: double negative, ISP: immature single positive, DP: double positive, SP: single positive, cell div: cell divisions, prolif: proliferation.

ACKNOWLEDGEMENTS

The authors thank Joyce Vermeulen and Ashley van der Spek for their assistance with the TREC analyses, Edwin de Haas and Benjamin Bartol for their assistance with cell sorting, Sandra de Bruin–Versteeg for her assistance with the figures, Menno van Zelm and Mirjam van der Burg for participation in discussions, the surgeons and pathologists that helped us collect all the thymic tissues. This work was supported by internal grants from the departments of Internal Medicine and Immunology. FJTS is supported in part by Kika, ZonMW and AICR.

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SUPPLEMENTAL DATA

Supplementary table I. Antibody conjugates used for flow cytometry and sorting experiments

	Fluorochrome	Clone	Company
CD1a	PE	T6	Beckman Coulter (Brea, CA, USA)
CD3	FITC	SK7	BD Biosciences (San Jose, CA, USA)
	PerCP	SK7	BD Biosciences (San Jose, CA, USA)
	APC-cy7	SK7	BD Biosciences (San Jose, CA, USA)
CD4	PE	SK3	BD Biosciences (San Jose, CA, USA)
	PE-cy7	SK3	BD Biosciences (San Jose, CA, USA)
CD8	PerCP	SK1	BD Biosciences (San Jose, CA, USA)
	APC	SK1	BD Biosciences (San Jose, CA, USA)
CD27	APC	L128	BD Biosciences (San Jose, CA, USA)
CD34	FITC	8G12	BD Biosciences (San Jose, CA, USA)
	APC	8G12	BD Biosciences (San Jose, CA, USA)
CD38	APC	HB7	BD Biosciences (San Jose, CA, USA)
CD45	PerCP	2D1	BD Biosciences (San Jose, CA, USA)
CD45RO	FITC	UCHL1	DAKO (Carpinteria, CA, USA)
Ki67	FITC	KI-67	DAKO (Carpinteria, CA, USA)
TCRγδ	PE	11F2	BD Biosciences (San Jose, CA, USA)

Supplementary table II. Primers and probes used for TREC analysis

$CJ_{\delta REC-\psi Ja}$	Forward primer	5'-CAACATCACTCTGTGTCTAGCACGTA-3'
	Reverse primer	5'-GGCACATTAGAATCTCTCACTGA-3'
	Probe	5'-CCAGAGGTGCGGGCCCCA-3'
$SJ_{\psi J\alpha-\delta REC}$[1]	Forward primer	5'-CCATGCTGACACCTCTGGTT-3'
	Reverse primer	5'-TCGTGAGAACGGTGAATGAAG-3'
	Probe	5'-CACGGTGATGCATAGGCACCTGC-3'
$CJ_{V\gamma8-J\gamma1.3/2.3}$	Forward primer	5'-TCAGGAATCAGTCGAGAAAAGTATCA-3'
	Reverse primer	5'-GTGTTGTTCCACTGCCAAAGAG-3'
	Probe	5'-AAATTTAAGGCTCTTCCCTGTGCTTGCATAAGT-3'
$SJ_{J\gamma1.3/2.3-V\gamma8}$	Forward primer	5'-CCAGACCACCAGAATTTGAAGAA-3'
	Reverse primer	5'-CAGGAGTAGTCACAAGGCAGATTTT-3'
	Probe	5'-TCAGTCCATATCAAAAACTCTAATTCAGTTTCCCTGA-3'
Albumin[2]	Forward primer	5'-TGAACAGGCGACCATGCTT-3'
	Reverse primer	5'-CTCTCCTTCTCAGAAAGTGTGCATAT-3'
	Probe	5'-TGCTGAAACATTCACCTTCCATGCAGA-3'
pGEMTez[3]	Forward primer	5'-GTCACCTAAATAGCTTGGCGTAATC-3'
	Reverse primer	5'-CACGACAGGTTTCCCGACTG-3'
	Probe	5'-CCACACAACATACGAGCCGGAAGCATAA-3'

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Chapter III

Thyroid stimulating hormone acts as a T-cell developmental factor in mice and humans

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Submitted for publication.

ABSTRACT

Using gene expression profiling, we detected differential thyroid stimulating hormone receptor (TSHR) expression during human T-cell development in the thymus. This expression pattern indicated a potential role for the TSHR within the thymus, independent of its function in the thyroid gland. Here we demonstrate that TSHR expression is thymus-specific within the immune system. TSH is able to bind and activate the TSHR present on thymocytes, thereby activating calcium signaling and cAMP signaling pathways. Transplantation of bone marrow cells lacking a functional TSHR into *Rag2*$^{-/-}$ recipient mice results in a marked delay in thymic reconstitution. Moreover, addition of TSH to co-cultures of human thymocytes enhances T-cell development. Thus, TSH acts as a previously unrecognized growth factor for developing T cells with potential clinical use to enhance thymic output and thereby the functional T-cell repertoire in the periphery. The direct effects of TSH on thymocytes may also explain the thus far enigmatic thymic hyperplasia in Graves' disease.

INTRODUCTION

T-cell development in the thymus is a highly complex process in which bone-marrow derived precursor cells develop into mature T cells in the context of the specific microenvironment provided by thymic epithelium.[1-3] This process is critical in the formation of a functional peripheral T-cell compartment with a large T-cell-receptor (TCR) diversity to fight off foreign antigens. Key features during T-cell development are lineage commitment, TCR gene rearrangements, selection processes, and thymic emigration.[1-3] All these processes are strictly regulated by transcription factors, cytokines, growth factors and adhesion molecules.[1-5] Although many of these factors are produced by the developing thymocytes themselves and the microenvironment, also factors of extra-thymic origin such as hormones like corticosteroids and growth hormone are capable to regulate T-cell development.[6]

Quantitative defects in the peripheral T-cell pool are associated with several clinical conditions such as specific primary immunodeficiencies, HIV and other chronic viral infections, and after hematopoietic stem cell transplantation (HSCT).[7-9] Moreover, during physiological aging T-cell numbers and especially TCR diversity decline.[10] In many of these conditions, reconstitution of the T-cell pool is essential to reduce the risk of opportunistic infections, reactivation of latent viruses, malignancy, and autoimmunity.[11, 12]
T-cell pool reconstitution depends on active thymopoiesis as well as on peripheral expansion of T cells. Peripheral expansion increases total T-cell numbers without increasing TCR diversity. In contrast, thymopoiesis selectively complements the naive T-cell pool and thus increases TCR diversity and thereby T-cell function. Renewed thymopoiesis is therefore essential for functional T-cell recovery and repertoire development.[13]
Many strategies to enhance T-cell reconstitution have been investigated without success, largely because effects were mainly restricted to peripheral expansion of T cells.[14] Therefore, detailed understanding of intra-thymic T-cell development and the factors that regulate this process is necessary for the development of new treatment modalities that selectively stimulate thymopoiesis, resulting in the recovery of a functional and diverse T-cell pool.

We previously performed microarray analyses on consecutive human thymocyte subsets to identify factors involved in the regulation of T-cell development.[5] Upon reinvestigating these data, we noticed marked up-regulation of several hormone receptors coinciding with T-cell lineage commitment. Specifically, thyroid stimulating hormone receptor (TSHR) was abundantly expressed during the earlier stages of T-cell development (figure 1A), where lineage commitment, proliferation and TCR gene rearrangements take place.[4, 5, 15] This expression pattern suggests that thyroid stimulating hormone (TSH), the main natural ligand for the TSHR, might be involved in T-cell development.

Immune-neuro-endocrine interactions have been recognized for several decades. Many hormones and their receptors are expressed within the immune system, including the thymus.[6, 16] A functional role for growth hormone, steroid hormones, and reproductive hormones in the thymus is now clearly defined.[6] However, considerably less is known about the expression and function of hormones of the hypothalamus-pituitary-thyroid axis in the thymus. TSH is an important

hormone within the hypothalamus-pituitary-thyroid axis where it regulates the endocrine function of the thyroid gland through TSHR signaling. Although the TSHR is traditionally thought to be expressed exclusively within the pituitary and the thyroid gland, several studies have now identified novel functions for TSH and TSHR signaling in various organ systems, including the immune system. TSHR expression has been described in human[17, 18] and rat thymus,[19] as well as in several other hematopoietic cell types,[20, 21] however, without identification of specific subpopulations. Moreover, a clear association has been described between Graves' disease and thymic hyperplasia,[18] characterized mainly by an increase in developing thymocytes.[22] As thymic hyperplasia is mainly seen in Graves' disease and less in other forms of hyperthyroidism,[23] TSH or TSHR antibodies are more likely to be involved than thyroid hormone, suggesting a functional role for the TSHR present in the thymus. Functional studies investigating the role of TSH and its receptor within the immune system, however, have produced contradictory results.

We therefore set out to investigate thymic TSH and TSHR expression in greater detail and to investigate the possible roles for TSHR signaling during T-cell development. We here report that the TSHR is functionally expressed on specific thymocyte subsets. Moreover, we provide the first evidence that TSHR signaling might be a previously unrecognized growth and differentiation factor for thymocytes both in mice and man. We propose that the TSHR might be an interesting novel treatment target to enhance thymic output in T-cell immunodeficiencies. Finally, we discuss the possibility that our results might explain the previously enigmatic thymic hyperplasia described in Graves' disease patients that have specific TSHR autoantibodies.

MATERIALS AND METHODS

Mice

Balb-c *hyt/hyt (hyt$^{-/-}$)* and Balb-c *Rag2$^{-/-}$* mice, originally purchased from the Jackson Laboratory (Bar Harbor, ME, USA), were bred and maintained in the specified pathogen-free (SPF) breeding facilities of the animal facility, Erasmus Medical Center, Rotterdam, the Netherlands.
Breeding pairs of *hyt/hyt* mice consisted of *hyt$^{-/-}$* or *hyt$^{+/-}$* males and *hyt$^{+/-}$* females. To restore thyroid hormone levels, hypothyroid *hyt$^{-/-}$* mice received T3 [3,3',5-Triiodo-L-thyronine sodium salt (Sigma Aldrich Logistic GmbH, Schnelldorf, Germany), 0.2 μg/ml] supplementation in the drinking water directly from weaning.
This study was performed in accordance with the legal regulations in the Netherlands and with the approval of the local institutional Animal Ethical Committee (Permit Number: EUR1559).

Lentiviral vector production and transduction

Lentiviral vectors were produced using a four-plasmid system in 293T cells as described previously.[24] 293T cells were transfected transiently with the transfer plasmid pRRLsin-18.PPT. PGK.MCS.IRES.GFP.pre and helper plasmids, pRSV.Rev, pMDLg/p.RRE (expressing gag/pol) and pMD.G (large scale production by Plasmid Factory, Bielefeld, Germany) using Fugene6

(Roche Nederland, Woerden, The Netherlands). Supernatants were harvested 24, 36 and 48 h after transfection. Supernatants were filtered (0.22 μm), pooled, concentrated by ultracentrifugation for 16 h at 18,000xg at 4ºC and stored at -80ºC until use. Functional titers were determined by titration in HeLa cells. For transduction lineage negative bone-marrow cells from *hyt*$^{-/-}$ and *hyt*$^{+/-}$ mice were grown in StemSpan serum free medium (stemcell Technologies Inc, Vancouver, BC, Canada) supplemented with rmFLT3-L (50 ng/ml), rmTPO (10 ng/ml) and rmSCF (100 ng/ml) for one day. Cells were transduced with viral supernatant (MOI of 5) or mock by spin-occulation in the presence of 4 μg/ml protamine sulphate (Sigma-Aldrich) at 800xg, 32ºC for one hour. After transduction cells were grown for two more days in the presence of the described cytokines.

Transplantation experiments

For transplantation experiments, peripheral blood, spleen, thymus and bone marrow were collected from CO_2-euthanized *hyt*$^{-/-}$ and *hyt*$^{+/-}$ mice (8-12 weeks of age). To obtain single-cell suspensions, spleen and thymus were squeezed through a 70 μm filter. Bone marrow was flushed and passed through a 70 μm filter. Cells were counted and used for immunophenotyping, or frozen viably for transplantation experiments. Lineage negative cells were isolated from freshly isolated bone marrow cells using the autoMACS mouse lineage depletion kit (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany).

For non-competitive transplantation experiments, pooled total bone marrow cells (2 x 10E^6/mouse) from *hyt*$^{-/-}$ or *hyt*$^{+/-}$ mice or lineage negative bone marrow cells (1 x 10E^5/mouse) from *hyt*$^{+/-}$ or *hyt*$^{-/-}$ mice mixed with supportive *Rag2*$^{-/-}$ spleen cells (5 x 10E^5) were injected. For competitive transplantation experiments, GFP transduced *hyt*$^{+/-}$ (2.5 x 10E^4) and mock transduced *hyt*$^{-/-}$ cells (2.5 x 10E^4) or GFP transduced *hyt*$^{-/-}$ cells (2.5 x 10E^4) and mock transduced *hyt*$^{+/-}$ (2.5 x 10E^4) were injected in combination with supportive *Rag2*$^{-/-}$ spleen cells (5 x 10E^5). The GFP transduced cells were sorted with a purity >90%.

In a second set of experiments, lineage negative cells from *hyt*$^{+/-}$ or *hyt*$^{-/-}$ mice were transduced with a MSCV-GFP (green) or MSCV-D-Tomato (red) retrovirus, mixed and transplanted in a 1:1 ratio into *Rag2*$^{-/-}$ mice with spleen support.

Cells were injected into the tail vein of lethally irradiated (8-8.5 Gy) female, Balb-c *Rag2*$^{-/-}$ recipient mice (6-12 weeks of age). Recipient mice were killed four to ten weeks after transplantation and peripheral blood, spleen, thymus and bone marrow were collected for flow cytometric analyses.

Thymus samples

Thymi were obtained from children (0 to 6 years of age) undergoing cardiac surgery at Erasmus Medical Center, Rotterdam, the Netherlands. The children did not have any immunological abnormalities. Informed consent was given by the parents. Collection of the samples was approved by the local Medical Ethical Committee. Thymocytes and epithelial cells were isolated as described before.[5] Thymocytes were isolated by cutting thymic lobes into smaller pieces and squeezing them through a metal mesh. Thymocytes were washed and frozen viably for further analyses. For all experiments, we used thymocytes obtained after Ficoll density separation. Stromal cells were isolated from thymic tissue after removal of the majority of thymocytes. To

obtain a single stromal-cell suspension, tissue was digested during three sequential incubation steps (20 min, 37°C) with dispase II, collagenase and DNAse, followed by three sequential incubation steps (15 min, 37°C) with trypsine and DNAse. Remaining $CD45^+$ thymocytes were depleted by AutoMACS using CD45 beads (Miltenyi Biotec GmbH).

Cell lines

The cell line JP09, a Chinese Hamster Ovary (CHO) cell line stably transfected with the human TSHR, was provided by Prof. G. Vassart, Université Libre, Brussels, Belgium.[25] Wild type CHO cells were provided by Dr. L.J. Hofland, Erasmus Medical Center, Rotterdam, the Netherlands (Sigma-Aldrich). The cell line OP9DL1, a mouse BM stromal cell line expressing the Notch ligand Delta-like-1 was provided by Dr. B. Vandekerckhove, University Hospital, Ghent, Belgium.[26, 27]

Flow cytometry and cell sorting

Antibodies used for flow-cytometric analyses and cell sorting are summarized in supplementary table I.
Stained cells were measured using a FACSCalibur and/or a FACSCanto (BD Biosciences, San Jose, CA, USA). For sorting, a FACSAria (BD Biosciences) was used. Analyses were done with FlowJo software (TreeStar, Ashland, OR, USA).

Microarray analysis

To examine the effect of TSH on gene expression profiles in thymocytes, sorted ISPs were cultured with 1 nM recombinant human TSH (rhTSH; Thyrogen; Genzyme Europe BV, Naarden, The Netherlands) dissolved in PBS/0.1% BSA or vehicle. RNA was extracted using RNeasy MinElute columns (Qiagen, Hilden, Germany). Quality of RNA was assessed using the Agilent 2100 BioAnalyzer.
Biotin-labeled cRNA was prepared using the Affymetrix two-cycle target labeling kit and the MEGAscript T7 kit. Subsequently, 15 μg of biotin-labeled cRNA was fragmented and hybridized to a Human Genome U133 Plus 2.0 microarray. Thereafter, microarrays were washed and stained using the Affymetrix Fluidic Station F450 and scanned at 570 nm.

GeneScan analysis for in-frame TCRB gene rearrangements

To determine T-cell receptor (TCR) diversity we performed GeneScan analysis with DNA of sorted ISP thymocytes (defined as $CD4^+CD8^-CD3^-$), after being cultured for three to six hours in the presence of 1 nM rhTSH or vehicle, to determine in-frame Vβ-Jβ gene rearrangements with multiplex PCR as previously described.[28]

RNA isolation and RQ-PCR analysis

RNA was extracted using RNeasy columns (Qiagen) and RNA was reverse transcribed into cDNA as described.[5] For detection of gene expression levels of *TSHR*, *TSHB*, *GPHB5* (thyostimulin, β-subunit), *LHB* (luteinizing hormone) and *CGB* (human chorionic gonadotropin), primers and probes were obtained from Applied Biosystems, Foster City, CA, USA (Assay-on-demand^{tm}). Expression levels were normalized to *ABL* gene expression which is expressed at very stable levels in both healthy en leukemic lymphoid cells and thus an optimal choice as housekeeping gene.[29] Primers and probes for the detection of gene expression levels of *CXCL2*, *CXCL3*, *EGR3*, *MAFF*, *PTX3*, *ATP6*, *CREB1*, *CREB5*, *GNA11*, *GNAS*, *HLA-DRA*, *PDE3B* and *PDE4D* were designed using OLIGO primer analysis software (Molecular Biology Insights, Cascade, CO, USA). Sequences are provided upon request.

Immunohistochemistry

Immunohistochemistry was done as described previously.[30] Thymic and thyroid cryosections (6 μm) and cytospins of sorted thymocyte subsets were fixed in acetone/0.05% H_2O_2 and stained overnight with a TSHR specific antibody (MCA1571, 20 ug/ml, AbD Serotec, Dusseldorf, Germany) or an isotype control (BD Biosciences). Subsequently, sections were incubated with a goat-antimouse-horseradish peroxidase-labeled monoclonal antibody (Dako B.V., Glostrup, Denmark). For visualization of horseradish peroxidase activity, we used 3-amino-9-ethylcarbazole substrate (Sigma Co., St. Quentin Fallavier, France) dissolved in 50 mM sodium acetate/0.02% hydroxyperoxide. Sections were embedded in Kaiser's glycerol/gelatin (Boom B.V., Meppel, the Netherlands).

Labeling studies

Labeling studies were performed as described previously.[31] To demonstrate binding of TSH to the TSHR on thymocytes, 50 mg of thymocyte membrane was incubated for one hour at 4°C in medium containing 10 mM Tris-HCl and 1% BSA (pH 7.4) with I^{125} labeled bovine TSH (bTSH; 50 uCi/ug, BRAHMS, Berlin, Germany) with or without addition of an overload of unlabeled bTSH (250 mIU/ml, Sigma Aldrich). JP09 cell membranes served as a positive control. Radioactivity was counted in a γ-counter. Specific binding was calculated as the difference between binding in the absence and in the presence of unlabeled bTSH.

Calcium flux

Calcium-flux experiments were essentially performed as described previously.[32] Additionally, in order to allow distinction between thymocyte subsets, cells were stained for CD3, CD8 and CD4.

cAMP measurements

For cAMP measurements $5x10^5$ sorted DN, ISP, DP and SP thymocytes and a confluent layer of

CHO-WT or JP09 (CHO-TSHR) cells were stimulated at 37°C and 5% CO_2, in de presence of 1 mM IBMX (Sigma-Aldrich) with vehicle, 10 mM forskolin (Sigma-Aldrich) or 10mM bTSH (Sigma-Aldrich) for 30 minutes. Cyclic AMP production was measured in the supernatant using a RIA cAMP kit (Immunotech, Beckman Coulter, Marseille, France).

In vitro T-cell differentiation

To investigate the effect of TSH on human thymocyte development *in vitro,* immature $CD34^+$ human thymocytes were isolated from pooled total thymocytes using autoMACS beads (Miltenyi Biotec GmbH). Subsequently, these $CD34^+$ positive immature thymocytes were cultured in fetal thymic organ cultures (FTOC) and OP9DL1 co-cultures as described previously.[33, 34] Twice weekly rhIL-7 (1 ng/ml), rhSCF (5 ng/ml) and different concentrations of rhTSH (0.1-10 nM) were added.

Statistical analysis

The exact Mann-Whitney U test was used for statistical comparisons. Statistical analyses were performed with SPSS software version 15.0. A p-value of less than 0.05 (two tailed) was considered statistically significant. Error bars are expressed as s.e.m.

RESULTS

TSHR expression in the thymus

Upon extensive data mining of our previously published gene expression profiling studies of human thymi,[5] with the aim to identify novel regulators of T-cell lineage commitment, the expression pattern of the TSHR was striking (figure 1A). Differential TSHR mRNA expression was validated by RQ-PCR (figure 1B, left panel) showing an expression pattern similar to that obtained with microarray analyses, with the highest expression of TSHR mRNA in ISP and DP thymocytes (figure 1B, left panel). In addition TSHR mRNA was found to be expressed exclusively on thymocytes (figure 1B, left panel). No TSHR mRNA expression was observed in sorted peripheral blood mononuclear cells (figure 1B, left panel). TSHR expression in thymocytes was ~4 times lower than in thyroid tissue (figure 1B, right panel). In mice, the TSHR mRNA expression pattern in developing thymocytes was comparable to the human situation (data not shown).

To investigate TSHR protein expression, frozen human thymic tissues were analyzed by immunohistochemistry. In thymic tissues (figure 1C, left panel) as well as in thyroid control tissue (data not shown), a clear membrane staining for the TSHR was found. No immunoreactivity was observed when tissues were stained with isotype control (figure 1C, right panel). Cytospin stainings of sorted thymocyte subsets also demonstrated TSHR immunoreactivity in all thymocyte subsets, in a pattern similar to the mRNA expression data with the highest reactivity in ISP and

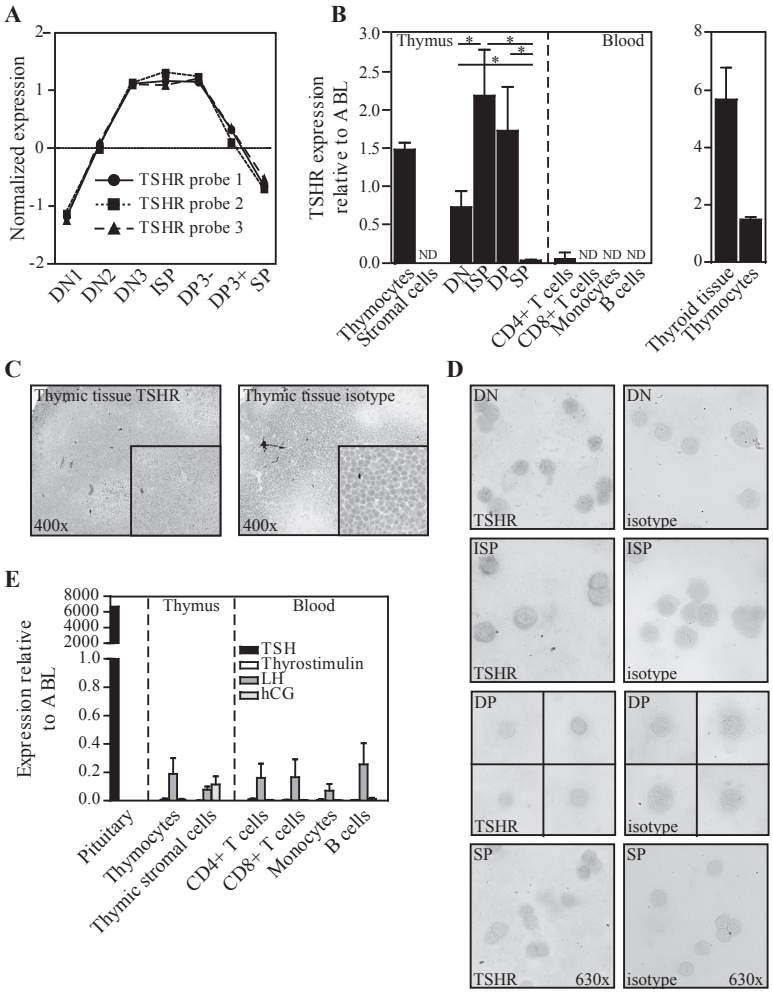


Figure 1. The TSHR is selectively expressed on thymocytes subsets, without expression on peripheral mononuclear blood cells. (A) Expression of the TSHR in human thymocyte subsets using microarray analysis. (B) TSHR mRNA expression relative to ABL mRNA in human thymocytes, CD45$^-$ thymic stromal cells, thymocyte subsets and PBMCs (left panel) and thyroid tissue (right panel); RQ-PCR, data are shown as mean ± SEM from three or more independent samples. (C) TSHR immunohistochemistry staining of thymic tissue using a TSHR specific antibody or isotype control; data are from a representative experiment. Shown at x400 original magnification. (D) TSHR immunohistochemistry staining of cytospins of sorted thymocyte subsets using a TSHR specific antibody or isotype control; data are from a representative experiment. Shown at x630 original magnification. (E) Expression of TSH, thyrostimulin, LH and hCG mRNA relative to ABL mRNA in human thymocytes, CD45$^-$ thymic stromal cells and PBMCs; RQ-PCR, data are shown as mean ± SEM from three independent samples. DN: double negative thymocytes (CD4$^-$CD8$^-$), ISP: immature single positive thymocytes (CD4$^+$CD3$^-$), DP: double positive thymocytes (CD4$^+$CD8$^+$), SP: single positive thymocytes (CD4$^+$CD8$^-$ or CD4$^-$CD8$^+$), LH: luteinizing hormone, hCG: human chorionic gonadotrofin, ND: not detected. * significance (2-tailed) $p<0.05$. *See Chapter VII for full-color figure.*

DP thymocytes (figure 1D, upper panel), while no immunoreactivity was observed in thymocytes stained with isotype control (figure 1D, lower panel). Thus, the TSHR is selectively expressed in developing T cells within the thymus, especially during ISP and DP stages of development.

Besides TSH, which is the natural ligand for the TSHR, several other hormones (e.g. luteinizing hormone (LH), human chorionic gonadotropin (hCG) and thyrostimulin) can also bind and activate the TSHR, albeit with much lower affinity than TSH.[35, 36] We therefore investigated mRNA expression of these hormones in thymocytes, thymic stromal cells and peripheral blood mononuclear cells. TSH expression was undetectable in thymocytes, thymic stromal cells and mature immune cells, while high TSH expression, as expected, was detected in pituitary tissue (figure 1E). Among the other TSHR binding hormones, only LH mRNA was occasionally found in all the cell types tested, albeit at very low levels (figure 1E).

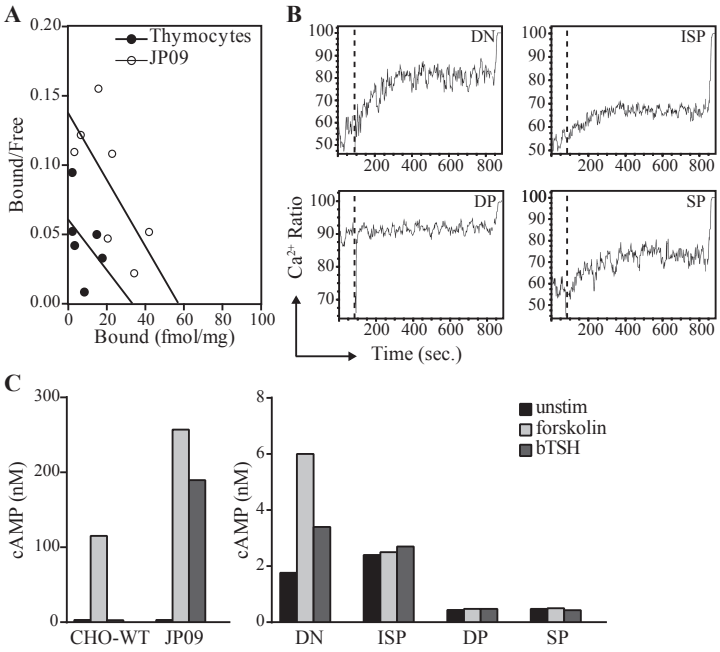


Figure 2. TSH binds and activates the TSHR expressed on human thymocytes. (A) Scatchard plot demonstrating specific binding of I^{125} labeled bTSH to TSHR on isolated thymocyte membranes and membranes of the JP09 cell line (Chinese Hamster Ovary (CHO) cell line stably transfected with the human TSHR); data are from a representative experiment. (B) Flow cytometric calcium flux analyses in thymocytes following stimulation with 10 nM rhTSH; data are from a representative experiment. (C) Measurement of cAMP production in CHO-WT cells, JP09 cells and thymocyte subpopulations following stimulation with 10 mU bTSH; data are from a representative experiment. DN: double negative thymocytes ($CD4^-CD8^-$), ISP: immature single positive thymocytes ($CD4^+CD3^-$), DP: double positive thymocytes ($CD4^+CD8^+$), SP: single positive thymocytes ($CD4^+CD8^-$ or $CD4^-CD8^+$).

TSH binds and activates the TSHR present on thymocytes

Scatchard analysis revealed that I^{125} labeled bTSH bound to human thymocytes with a dissociation constant (Kd 0.55 nM) similar to that obtained with TSHR positive control cells (JP09; Kd 0.42 nM), indicating that TSH binding on thymocytes is indeed via the TSHR. Maximal binding of bTSH by human thymocytes was 34 fmol/mg protein; maximal binding of bTSH by JP09 membranes was 57 fmol/mg protein (figure 2A).

Thyroidal TSHR activation results in the activation of several second messenger pathways, including the PIP_2/Ca^{2+} and the adenylate cyclase/cAMP transduction cascades.[37] We therefore investigated whether TSH activates these pathways in thymocytes. After stimulation with rhTSH, free intracellular calcium increased in ~15% of thymocytes in all subsets except for DP thymocytes that have very high (~90%) spontaneous calcium fluxes (figure 2B) virtually excluding detection of additional free calcium in this subpopulation. Moreover, an increase in cAMP production was clearly shown in DN thymocytes, after stimulation with bTSH (figure 2C). No responses were found in ISP, DP and SP thymocytes, but in these subpopulations also no response to forskolin could be demonstrated. The likely explanation for the unresponsiveness to these stimuli is that due to long and extensive processing of the cells (an 8-hour sort was needed to sort enough cells of all subpopulations to be able to measure cAMP production), cells were insufficiently viable to do the experiment needed. In contrast, the calcium flux experiments do not require cell sorting as the relevant subpopulations can be identified simply by electronic gating while measuring total thymocytes, thereby allowing direct measurement of the functionality of the TSHR. Therefore, our data demonstrate that in responsive thymocyte subpopulations TSH is able to induce a cAMP response.

In addition, we stimulated flow-sorted ISP thymocytes which have high TSHR expression for three or six hours with rhTSH and subsequently performed microarray analysis (for this experiment a shorter sorting time was needed and stimulation time could be extended to 3-6 hours). Gene-expression analysis of genes involved in G-protein-coupled receptor (GPCR) signaling demonstrated differential expression of many probe sets in rhTSH relative to vehicle-stimulated ISPs, suggesting that TSHR signaling is induced (supplementary figure IA). After three hours of culture, 4 of the 399 probe sets involved in GPCR signaling were more than 1.5-fold differentially expressed. This number increased to 14 after six hours of culture. Importantly, after six hours of culture with rhTSH, expression of the transcription factor *CREB5*, an important target gene of cAMP signaling, was up-regulated. Collectively, these data demonstrate that TSH binds and signals via the TSHR on thymocytes.

TSHR activation in ISP thymocytes results in changes in gene expression profile

To identify molecular pathways by which TSHR signaling might be involved in T-cell development, above described microarrays of flow-sorted ISP thymocytes were analyzed for differential expression of genes important for T-cell development. After three hours of culture, 32 probe sets were more than 2-fold differentially expressed in rhTSH relative to vehicle-stimulated ISPs. This number increased up to 180 probe sets after six hours of culture. A substantial number of these

probes represented genes that are known to play a role in different processes involved in T-cell development and TSHR signaling (supplementary figure IB), such as *HLA*, *TGF-βR*, *MAF* and *EGR*. Many of the differentially expressed genes function to drive T-cell development after the pre TCRβ-selection point (e.g., Egr, c-maf, various HLA Class I molecules). The microarray analyses were validated by performing RQ-PCR analyses for selected genes that were found to be differentially expressed, demonstrating a comparable pattern (figure 3A).

Moreover, we performed GeneScan analysis of *TCRB* rearrangements in DNA from flow-sorted ISP thymocytes stimulated for six hours with vehicle or rhTSH. In rhTSH stimulated ISP thymocytes the pattern of 3-nucleotide peaks was more readily visible than in vehicle stimulated thymocytes, demonstrating that more in-frame TCRB rearrangements were found after TSH stimulation. This indicates that more preTCR β-selection occurred in rhTSH stimulated ISP thymocytes. (figure 3B).[5] Overall, these data, together with the marked increase of TSHR expression on ISP and early DP thymocytes, indicate that one of the functions of TSHR signaling is to enhance post β-selection T-cell differentiation and proliferation.

Lack of functional TSHR expression alters normal thymic composition and development

To investigate whether TSHR signaling is involved in T-cell development *in vivo*, we used *hyt/hyt* mice as an *in vivo* model. *Hyt/hyt* mice have a point mutation in the *TSHR* gene resulting in severely diminished TSH binding capacity and TSHR functionality.[38] Consequently, these mice are hypothyroid. In order to study the effects of absence of TSHR signaling on the development of the immune system without additional effects of thyroid hormone abnormalities, the mice were supplemented with T3 from weaning.

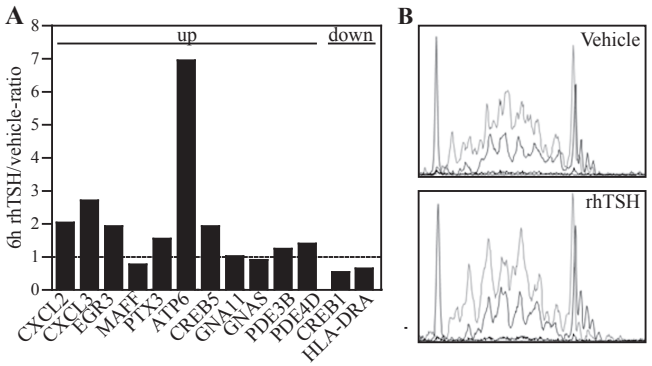


Figure 3. Induction of TSHR signaling in ISP thymocytes induces changes in expression of genes involved in T-cell development. (A) RQ-PCR analysis of several genes that were found to be differentially expressed in the microarray analysis in flow-sorted ISP thymocytes that were cultured for three to six hours in the presence of 1 nM rhTSH and vehicle, n=2. (B) GeneScan analysis of Vβ-Jβ rearrangements (Vβ+Jβ1.1 t/m Jβ1.6+Jβ2.2+Jβ2.6+Jβ2.7) in flow-sorted ISP thymocytes that were cultured for three to six hours in the presence of 1 nM rhTSH and vehicle; Primers for the Jβ1 cluster were HEX-labeled (green line), primers for the Jβ2 cluster were FAM-labeled (blue line). Note the higher peaks in rhTSH treated thymocytes at a 3 nucleotide distance. *See Chapter VII for full-color figure.*

Flow-cytometric analysis of thymocyte subsets demonstrated that *hyt*$^{-/-}$ mice had a relative block in T-cell development at the ISP or DP stages, resulting in decreased frequency of SP thymocytes and in some experiments DP thymocytes compared to heterozygous littermates (figure 4A). This did not result in decreased T-cell frequencies or numbers in blood and spleen (data not shown), most likely due to peripheral homeostatic expansion.

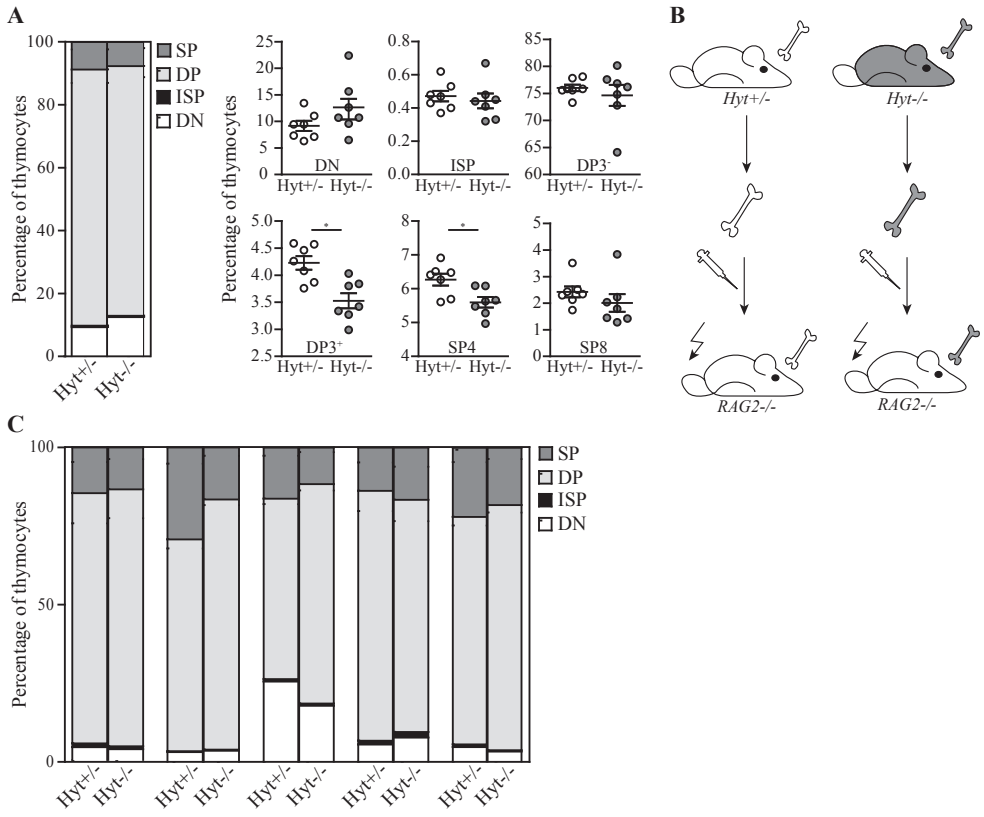


Figure 4. Lack of TSHR signaling alters normal thymic composition in mice. (A) Flow cytometric analysis of the thymus of eight week old *hyt*$^{+/-}$ and *hyt*$^{-/-}$ mice. Left panel: thymic compositition, right panel: percentages of thymocyte subsets, * significance (2-tailed) DP3^{+} p=0.007; SP4 p=0.02. Data are from a representative experiment (n=7 for heterozygous group, n=7 for homozygous group). (B) Bone marrow transplantation protocol. Bone marrow from *hyt*$^{+/-}$ and *hyt*$^{-/-}$ was injected into irradiated *RAG2*$^{-/-}$ recipient mice. (C) Flow cytometric analysis of the thymus after non-competitive bone marrow transplantation. *Rag2*$^{-/-}$ recipient mice received *hyt*$^{+/-}$ or *hyt*$^{-/-}$ bone marrow cells. Percentages of thymocyte subsets present in the thymus five to ten weeks after transplantation. (n≥4 for all groups). DN: double negative thymocytes (CD4^{-}CD8^{-}), ISP: immature single positive thymocytes (CD8^{+}CD3^{-}), DP3^{-}: double positive thymocytes (CD4^{+}CD8^{+}CD3^{-}), DP3^{+}: double positive thymocytes (CD4^{+}CD8^{+}CD3^{+}), SP: single positive thymocytes (CD4^{+}CD8^{-} or CD4^{-}CD8^{+}).

HSC lacking a functional TSHR have thymic repopulation disadvantage in competitive bone marrow transplantation

To specifically investigate the effects of absence of TSHR signaling on T-cell development and immune reconstitution, we performed bone-marrow transplantation experiments in which bone marrow from *hyt/hyt* mice was transplanted into lethally irradiated *Rag2*$^{-/-}$ mice.
Because *Rag2*$^{-/-}$ mice have a developmental block in T-cell development at the DN3 stage[39] only

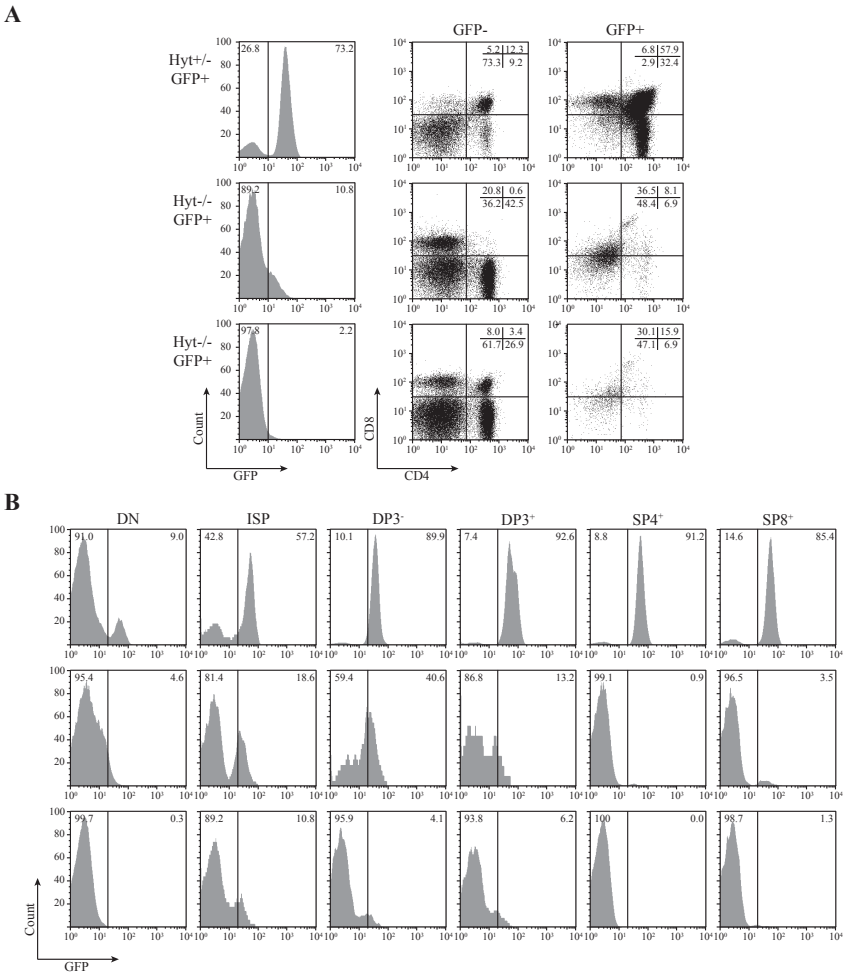
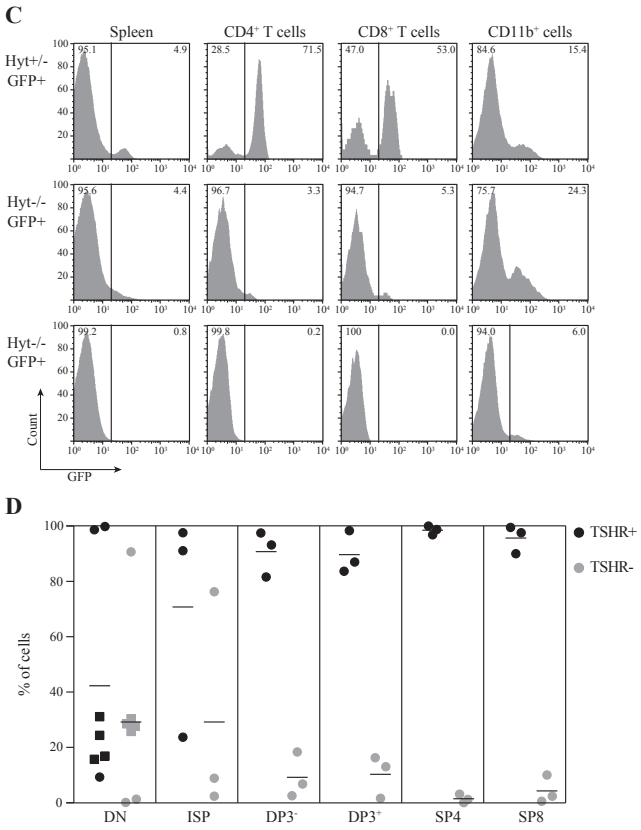


Figure 5. Lack of TSHR signalling results in a repopulation disadvantage in competitive bone marrow transplantation. Flow cytometric analysis of lymphoid organs after competitive bone marrow transplantation. *Rag2*$^{-/-}$ recipient mice received *hyt*$^{+/-}$ GFP transduced cells mixed with *hyt*$^{-/-}$ mock transduced cells or *hyt*$^{+/-}$ mock transduced cells mixed with *hyt*$^{-/-}$ GFP transduced cells in a 1:1 ratio. (A) Thymic chimerism per mouse, percentage of GFP^{+} thymocytes and the presence of thymocyte subsets within GFP^{-} and GFP^{+} populations defined by CD4 and CD8. (B) Percentages of GFP^{+} cells within thymocyte subsets per mouse.

stem cells derived from the donor are able to undergo full functional T-cell development. The recipient mice provide a TSHR competent environment for the hematopoietic cells derived from the TSHR deficient stem cells (figure 4B).
In non-competitive *hyt*$^{-/-}$ and *hyt*$^{+/-}$ total bone marrow transplantations large variations were present between individual mice, but there was a trend towards similar differences in thymic composition as found in thymi of *hyt*$^{-/-}$ and *hyt*$^{+/-}$ mice, measured at several time points after transplantation (figure 4C).



(C) Percentages of GFP^{+} cells in total splenocytes, CD4^{+} T lymphocytes, CD8^{+} T lymphocytes and CD11b^{+} myeloid cells. (D) Combined flow cytometric analyses of lymphoid organs after the different competitive bone marrow transplantations. Circles represent transplantations in which *Rag2*$^{-/-}$ recipient mice received *hyt*$^{+/-}$ GFP transduced cells mixed with *hyt*$^{-/-}$ mock transduced cells or *hyt*$^{+/-}$ mock transduced cells mixed with *hyt*$^{-/-}$ GFP transduced cells in a 1:1 ratio. Rectangles represent transplantation in which *Rag2*$^{-/-}$ recipient mice received *hyt*$^{+/-}$ GFP transduced cells mixed with *hyt*$^{-/-}$ Tomato red transduced cells or *hyt*$^{+/-}$ Tomato red transduced cells mixed with *hyt*$^{-/-}$ GFP transduced cells in a 1:1 ratio. Percentage of *hyt*$^{+/-}$ TSHR^{+} thymocytes and percentage of *hyt*$^{-/-}$ TSHR^{-} thymocytes per developmental stage are indicated, n=7. DN: double negative thymocytes (CD4^{-}CD8^{-}), ISP: immature single positive thymocytes (CD8^{+}CD3^{-}), DP3^{-}: double positive thymocytes (CD4^{+}CD8^{+}CD3^{-}), DP3^{+}: double positive thymocytes (CD4^{+}CD8^{+}CD3^{+}), SP: single positive thymocytes (CD4^{+}CD8^{-} or CD4^{-}CD8^{+}).

In order to reduce variation between mice we performed two different competitive transplantation experiments in which *hyt*$^{-/-}$ and *hyt*$^{+/-}$ lineage-negative bone marrow cells were transplanted in a 1:1 ratio. To be able to distinguish *hyt*$^{-/-}$ and *hyt*$^{+/-}$ cells in competitive transplantations, *hyt*$^{-/-}$ and *hyt*$^{+/-}$ cells were either GFP or mock lentivirally-transduced in the first experiment. In the second experiment we distinguished the *hyt*$^{+/-}$ *and hyt*$^{-/-}$ cells by transducing them with two different fluorescent labels, namely GFP (green) and Tomato (red). In both experiments we switched fluorescent markers in half of the mice, so that not all *hyt*$^{+/-}$ would be marked with one color and all *hyt*$^{-/-}$ transduced cells with the other, thereby avoiding possible selective effects based on the type of marker used rather than on TSHR deficiency.
In the first experiment four mice received *hyt*$^{+/-}$ GFP-transduced cells mixed with *hyt*$^{-/-}$ mock-transduced cells, while the other four mice received *hyt*$^{+/-}$ mock-transduced cells mixed with *hyt*$^{-/-}$ GFP-transduced cells. Seven weeks after transplantation we determined reconstitution and chimerism in thymocytes and T cells by flow-cytometric analysis. In three out of the eight mice normal reconstitution of the T-cell compartment was found as measured by the presence of $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells in the blood. The low number of mice with reconstitution is probably due to the low number of lineage-negative bone marrow cells that were injected for the purpose of discerning even subtle effects in T-cell reconstitution by TSHR-dysfunctional HSC. Of the mice with normal reconstitution, one mouse received *hyt*$^{+/-}$ GFP-tranduced cells, while the other two mice received *hyt*$^{-/-}$ GFP-transduced cells. Chimerism analysis in the thymus showed that thymocytes were largely GFP^+ in mice receiving *hyt*$^{+/-}$ GFP-transduced cells, while thymocytes were largely GFP^- in the other mice (figure 5A). Further subset analysis showed that both GFP^+ and GFP^- cells could be found in all mice in the DN and ISP stages of development, while in the DP stages clear selection for GFP^+ cells took place in mice receiving *hyt*$^{+/-}$ GFP-transduced cells, while selection of GFP^- cells took place in the other mice (figure 5B). In peripheral blood and spleen a similar pattern was found for $CD4^+$ and $CD8^+$ T cells. In contrast, within the $CD11b^+$ myeloid population both GFP^+ and GFP^- cells could be found (figure 5C), indicating that neither *hyt*$^{+/-}$ nor *hyt*$^{-/-}$ HSC had a selective (dis)advantage to develop into myeloid lineage cells.
In the second experiment, unfortunately, analysis 4 weeks after transplantation showed that our marking, especially for GFP but largely also for Tomato, was so efficient that it resulted in such high expression that fluorescent spill over occurred from the GFP (FITC) channel into all other fluorochromes until the far red tandem dyes. The only flurochrome used that remained detectable was CD4 as it was conjugated to PE-Cy7. Therefore, we could only reliably discern the DN population in red or green transduced cells (thereby excluding endogenous $Rag2^{-/-}$ thymocytes in DN). This however, still allowed us to draw some conclusions on T-cell development, albeit much more limited than we set out to do initially. We took a diminished percentage of DN thymocytes gated on either GFP or Tomato as indication for accelerated thymic development. In doing so, in all mice analyzed, the TSHR positive cells had fewer DN cells compared to the TSHR deficient cells, suggesting a positive effect on T-cell development by the TSHR (figure 5D).
These combined data indicate that progenitor cells with a functional TSHR have a selective repopulation advantage after the ISP stage of development in the thymus compared to progenitor cells without a functional TSHR (figure 5D).

rhTSH accelerates human T-cell development in vitro

Because we were ultimately interested in the effects of rhTSH on human T-cell development, we tested rhTSH in two culture systems supportive for human T-cell development: the OP9DL1 culture system and the fetal thymic organ culture system (FTOC). Both culture systems support T-cell development from DN up to at least the DP stages of development, and even functional SP thymocytes can be found in some of these cultures.[40, 41]

Flow-cytometric analysis showed that harvested cells were fully T-cell committed, with clear

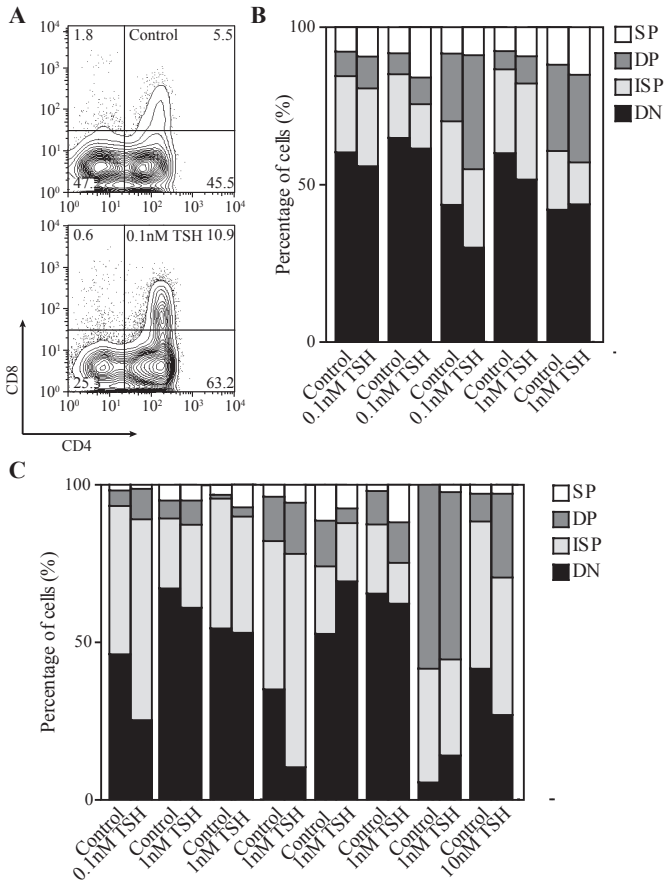


Figure 6. rhTSH enhances human T-cell development in vitro. Sorted human $CD34^+$ thymocytes were cultures in T-cell development supporting systems in the presence of 0.1-10 nM rhTSH or vehicle for two to three weeks. After harvesting, relative expression of the different thymocyte subsets was measured using flow cytometry. (A) Example of flow cytometric analysis of an FTOC experiment. (B) OP9DL1 culture system; n=5 experiments. (C) FTOC culture system; n=8 experiments. DN: double negative thymocytes ($CD4^-CD8^-$), ISP: immature single positive thymocytes ($CD4^+CD3^-$), DP: double positive thymocytes ($CD4^+CD8^+$), SP: single positive thymocytes ($CD4^+CD8^-$ or $CD4^-CD8^+$).

differentiation into ISP and DP thymocytes (figure 6A). When TSH was added, an increase in the more mature ISP and DP thymocytes relative to DN thymocytes was seen in 4 out of 5 co-cultures using the OP9DL1 system (figure 6B) and 6 out of 8 co-cultures using the FTOC system (figure 6C) indicating that rhTSH induced acceleration of human T-cell development *in vitro*.

DISCUSSION

The TSHR is well recognized for its essential role in thyroid homeostasis, and was long thought to be uniquely expressed within the hypothalamus-pituitary-thyroid axis. Recently, however, TSHR was shown to be also expressed in several non-thyroidal tissues.[42-44] Here, we provide a detailed study on TSHR expression pattern within the immune system and demonstrate for the first time a functional role for TSHR signaling in T-cell development.

In the present study, TSHR mRNA and protein expression, within the immune system was found to be restricted to developing T cells, with highest expression during the ISP and DP stages of development. These findings extend earlier observations of TSHR expression in total rodent[19] and human[17, 18] thymic tissue. We did not find TSHR expression in peripheral blood mononuclear cells, but published data on the presence of TSHR expression in these cells is contradictory. While some studies support our data,[17] others did detect TSHR expression on peripheral blood mononuclear cells, especially monocytes.[21] Also, a small population of naive T cells within the lymph node[20] and specific intestinal lymphoid cells (intraepithelial lymphocytes, IELs)[45] have been found to express TSHR. In general, given the high sensitivity of RQ-PCR compared to other techniques used, we conclude that TSHR expression within the immune system is restricted mainly to developing T cells within the thymus, although rare peripheral T-cell subpopulations might express TSHR as well. Compared to thyroid tissue, TSHR expression in thymocytes was only ~4 times lower. These expression levels support the notion that TSHR signaling in thymocytes might be functional.
We demonstrated that TSH specifically binds thymocytes and that TSH activated the PIP_2/Ca^{2+} and adenylate cyclase/cAMP signaling pathways which are downstream of the TSHR. Moreover, we have been able to demonstrate cAMP production and calcium fluxes in thymocyte subpopulations. TSH stimulation did not increase calcium flux in DP thymocytes, which could be related to apoptosis-induced calcium flux, a well known feature of DP thymocytes.[46] Calcium flux was already present in ~90% of DP thymocytes before stimulation, which will hamper the detection of rhTSH-induced calcium flux. The detection of these two types of second messengers after TSH stimulations demonstrates that the TSHR is functionally expressed in the thymus.
Functional expression of the TSHR on developing T cells suggests the presence of a previously unrecognized neuro-endocrine-immune pathway that involves pituitary-derived TSH and the thymus. Similar hormonal interactions with the thymus have been described for other hormones, such as growth hormone and cortisol.[6] We found no evidence of intra-thymic TSH production; therefore, we favor the explanation that pituitary-derived TSH is involved in this neuro-endocrine-immune pathway. A role for locally produced LH, the only other hormone with TSHR binding capacities that we infrequently detected in thymic tissue, is less obvious as it was expressed at very low levels and it has a substantially lower affinity for the TSHR compared to TSH.[35, 36]

A likely mechanism whereby TSHR signaling affects T-cell development is through combined effects on differentiation and apoptosis protection. In ISP thymocytes, rhTSH changed the expression patterns of genes involved in several processes related to T-cell development. Overall, expression levels of genes related to apoptosis induction and proliferation were reduced, while expression levels of genes that stimulate differentiation were increased.
Moreover, the abundant expression of the TSHR during the DN3, ISP and DP stages of development in which the processes of β-selection and *TCRA* rearrangements take place,[5] as well as the presence of more in-frame *TCRB* rearrangements in TSH-stimulated ISP thymocytes, demonstrate that TSHR signaling is involved in preTCR-signaling and β-selection and/or subsequent induction of *TCRA* rearrangements. Several changes in gene expression pattern found in the microarray analyses of TSH-stimulated ISP thymocytes support this notion. In particular, *EGR3* expression was clearly upregulated in rhTSH-stimulated ISP thymocytes. Recently, it was demonstrated that preTCR signaling induced *EGR1-3* expression in mouse thymocytes. Moreover, overexpression of *EGR3* enhanced progression trough the β-selection checkpoint in preTCR signaling deficient thymocytes.[47]
Finally, similar to its effect on thyroid cells, rhTSH increased cytosolic calcium levels and *CREB* expression in ISP thymocytes. Interestingly, cytosolic calcium is an important signaling molecule in preTCR signaling.[48] Moreover, preTCR signaling also enhances expression and phosphorylation of *CREB*, and several genes involved in β-selection (e.g. *CD3δ*, *TCRα*, and *TCRβ*) have binding sites for *CREB* in their enhancer or promoter regions.[49, 50] Additionally, inhibition of *CREB* activity has been shown to ameliorate the development of DP thymocytes in FTOC cultures.[51]

Based on our *in vitro* results we investigated the T-cell compartment in *hyt/hyt* mice *in vivo*. *Hyt*$^{-/-}$ mice had lower frequency of SP thymocytes and *hyt*$^{-/-}$ cells had a clear repopulation disadvantage in competitive transplantation experiments. Importantly, the repopulation disadvantage occurred in the ISP and DP stages in which we demonstrated up-regulation of the TSHR in normal mouse and human thymocytes. Moreover, during normal development β-selection and *TCRA* rearrangements occur in these stages of development. Consistent with our observations, others showed that also TSHR expressing IELs were reduced in thyroid-hormone-substituted *hyt*$^{-/-}$ mice.[45] In normal mice, TSH treatment did not affect these IELS, effects of TSH on developing thymocytes were not investigated in this study.[52]

Ultimately we demonstrate in two human *ex-vivo* systems that TSHR signaling enhanced normal T-cell development, even in the presence of IL-7 and SCF, two cytokines crucially important for normal T-cell development.[53] Factors that stimulate T-cell development are of special interest for the treatment of conditions associated with T-cell deficiencies. Such factors have been sought after, but many, including IL-2[54] and IL-7,[55] show strong effects on peripheral T-cell expansion but not on thymic output due to the expression of their receptors on peripheral mature T cells. Activation of the TSHR, most likely by rhTSH or a TSH-analogue, might be an interesting new therapeutic target in T-cell deficiencies as a selective effect on developing T lymphocytes is expected based on to the specific expression pattern of the TSHR in the T-cell compartment. Moreover, in contrast to the other factors, anticipated side effects of rhTSH are limited to the

thyroid gland, mainly being hyperthyroidism during rhTSH treatment. These side effects can be blocked relatively simple by treatment with anti-thyroid drugs, which have a long history of clinical use.

Our data demonstrate that TSHR signaling has selective stimulatory effects on human T-cell development in the thymus, which could be relevant in physiological and pathophysiological conditions. Interestingly, in the first hours after birth, a cold-induced increase in TSH levels up to 100mU/l (TSH surge) has been described.[56] As T-cell numbers are still largely increasing just after birth[57] it is tempting to hypothesize that this TSH surge could support the highly active process of T-cell development in the thymus just after birth. Moreover, an association between thymic hyperplasia, mainly due to an increase in lymphoid cells, and Graves' disease has been described,[18, 22] but the cause for this has remained elusive. Based on our results we postulate that Graves' disease associated stimulating TSHR autoantibodies activate thymocytes, resulting in enhanced T-cell development, increased thymocyte numbers and subsequent thymic hyperplasia. In support of this, we observed that purified immunoglobulins from sera of some patients with Graves' disease were capable of enhancing T-cell development in the OP9DL1 co-culture system to a comparable level of rhTSH (unpublished data).

Collectively, our data support the notion of a novel neuro-endocrine-immune interaction in which TSHR signaling in the thymus, most likely mediated by TSH, enhances thymic T-cell development, possibly by affecting preTCR signaling and β-selection. Due to the selective expression pattern within the immune system, the TSHR might be an interesting therapeutic target for primary and acquired T-cell immunodeficiencies to enhance thymic output.

ACKNOWLEDGEMENTS

The authors thank Edwin de Haas and Benjamin Bartol for their assistance with cell sorting, Tiago Luis for his assistance with transplantations, Peter Koetsveld for assistance with I^{125} TSH labeling studies, Hans van Toor and Yolanda de Rijke for measuring T3 levels in mouse plasma samples, Sandra de Bruin-Versteeg for her assistance with the figures and all other members of the Staal laboratory and Dik laboratory for their technical assistance. We thank Prof. G. Vassart, Dr. B. Vandekerckhove and Prof L.J. Hofland for providing the JP09, OP9DL1 and CHO-WT cell lines. This work was supported by internal grants from the departments of Internal Medicine and Immunology. FJTS is supported in part by Kika, ZonMW and AICR.

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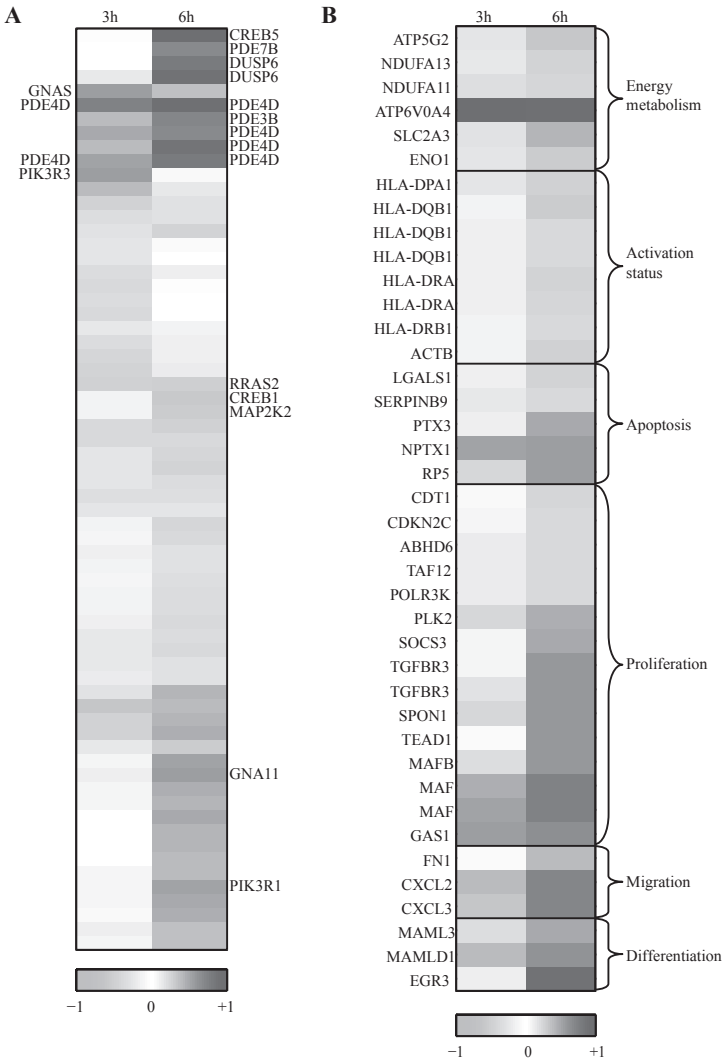
SUPPLEMENTAL DATA

Supplementary table I. Fluorochromes used for flow cytometry and sorting experiments

Human	Fluorochrome	Clone	Company
CD1a	PE	HI149	BD Biosciences (San Jose, CA, USA)
CD3	APC-cy7, FITC	SK7	BD Biosciences (San Jose, CA, USA)
CD4	PE-cy7	SK3	BD Biosciences (San Jose, CA, USA)
CD8	APC, APC-cy7	SK1	BD Biosciences (San Jose, CA, USA)
CD14	APC	M5E2	BD Biosciences (San Jose, CA, USA)
CD16	PE	B73.1	BD Biosciences (San Jose, CA, USA)
CD19	PerCP	SJ25C1	BD Biosciences (San Jose, CA, USA)
CD34	FITC	8G12	BD Biosciences (San Jose, CA, USA)
CD45	PerCP	2D1	BD Biosciences (San Jose, CA, USA)
CD56	PE	C5.9	DAKO (Carpinteria, CA, USA)
Mouse	**Fluorochrome**	**Clone**	**Company**
CD2	Bio	RM2-5	BD Biosciences (San Jose, CA, USA)
CD3	FITC, APC	145-2C11	BD Biosciences (San Jose, CA, USA)
CD4	PE-cy7	RM4-5	BD Biosciences (San Jose, CA, USA)
CD8	PerCP, APC	53-6.7	BD Biosciences (San Jose, CA, USA)
CD11b	Bio, PerCP	M1/70	BD Biosciences (San Jose, CA, USA)
CD19	PE	ID3	BD Biosciences (San Jose, CA, USA)
CD25	FITC	7D4	BD Biosciences (San Jose, CA, USA)
CD44	PE	IM7	BD Biosciences (San Jose, CA, USA)
TCRβ	FITC	H57-597	BD Biosciences (San Jose, CA, USA)
B220	PerCP	Ra3-6B2	BD Biosciences (San Jose, CA, USA)
NK1.1	Bio	PK136	BD Biosciences (San Jose, CA, USA)
Ter119	Bio	Ly-76	BD Biosciences (San Jose, CA, USA)
TCRγδ	PE	GL3	BD Biosciences (San Jose, CA, USA)
GR1	Bio	RB6-8C5	BD Biosciences (San Jose, CA, USA)
IgD	PE	11-26	eBioscience (San Diego, CA, USA)
IgM	PE-cy7	II/41	eBioscience (San Diego, CA, USA)
cμ	FITC		Jackson ImmunoResearch Laboratories (West Grove, PA, USA)
streptavidin	APC-Cy7		BD Biociences (San Jose, CA, USA)



Supplementary figure I. TSH stimulation of ISPs activates TSHR signaling pathways and induces changes in expression of genes involved in T-cell development. Microarray analysis of flow-sorted ISP thymocytes which were cultured for three to six hours in the presence of 1 nM rhTSH or vehicle. (A) Heatmap showing log2-transformed expression ratio (rhTSH/vehicle) of the 65 genes involved in G-protein-coupled-receptor signaling which were most differentially expressed after three and six hours of culture. Gene names demonstrate the genes that were more than 1.5-fold differentially expressed. Genes were selected using Ingenuity Software. (B) Heatmap of selected genes with a more than 2-fold different expression levels in rhTSH treated thymocytes compared to vehicle. Genes involved in different processes were categorized, but can be inhibiting or stimulating these processes. *See Chapter VII for full-color figure.*

Chapter IV

The peripheral blood compartment in patients with Graves' disease; activated T lymphocytes and increased transitional and pre-naive mature B lymphocytes

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Submitted for publication.

ABSTRACT

Introduction: Graves' disease (GD) is an autoimmune disease that involves aberrant B- and T-cell responses. Detailed knowledge about lymphocyte subpopulation composition will therefore enhance our understanding of the pathogenesis of GD and might support the development of new immunomodulatory treatment approaches.

Aim of the study: To gain detailed insight into the composition of the peripheral blood lymphocyte compartment in GD before and during anti-thyroid drug therapy.

Materials and methods: Major B- and T-cell subpopulations were investigated by flow cytometry in peripheral blood from newly diagnosed GD patients (n=5), GD patients treated with anti-thyroid drugs (n=4), patients with recurrent GD (n=7) and healthy controls (HC; n=10). Moreover, the effects of thyroid hormone on B-cell precursor distribution in bone marrow (BM) from TSHR signaling deficient *hyt/hyt* mice were examined.

Results: in GD patients numbers of activated T cells (HLA-DR$^+$ and CD25$^+$) were increased. The B-cell compartment in GD was characterized by significantly higher numbers of transitional ($CD38^{high}CD27^-$, $p<0.03$) and pre-naive mature ($CD38^{low}CD27^-IgD^+CD5^+$, $p<0.04$) B cells, while memory populations were slightly decreased. BM from hypothyroid *hyt*/*hyt* mice displayed reduced numbers of B-cell precursors which were restored to normal upon T3 supplementation.

Conclusion: GD is associated with increased numbers of activated T cells and transitional and pre-naive mature CD5$^+$ B cells within the peripheral blood. We provide evidence that elevated thyroid hormone levels present in GD might contribute to the rise in transitional and pre-naive mature CD5$^+$ B cells by enhancing B-cell development within the BM.

INTRODUCTION

Graves' disease (GD) is an autoimmune disease of the thyroid gland, characterized by hyperthyroidism which can be accompanied by extra-thyroidal symptoms such as ophthalmopathy and dermopathy.[1] GD is an autoantibody-mediated autoimmune disease in which hyperthyroidism is caused by thyroid stimulating hormone receptor (TSHR) specific autoantibodies (TRAb) which mimic the effects of pituitary TSH.[1] In addition, neutral and inhibiting TRAb and antibodies to other thyroid autoantigens such as thyroglobulin (Tg) and thyroid peroxidase (TPO) can also be found in the serum of GD patients. Thus, autoantibody producing B cells are important contributors to GD pathogenesis. Moreover, lymphoid infiltrates present in thyroid and retro-orbital tissue of GD patients consist of both secondary lymphoid follicles containing B cells and T cells.[2, 3] Consequently, GD is currently considered as a B-cell mediated T-cell dependent autoimmune disease.[4]

T-cell involvement in GD is evident by infiltration of activated memory T cells ($CD45RO^+HLA\text{-}DR^+$) into the thyroid gland.[5, 6] Also T cells in the peripheral blood exhibit signs of activation as reflected by increased expression of HLA-DR, CD25 and CD69.[7-12] However, contradictory data exist on the composition of the peripheral blood T-cell compartment. Naive and memory T-cell subpopulations have been described as being normal, increased, or decreased.[7, 8, 10, 13-15] Also, regulatory T cells (Tregs; $CD4^+CD25^+FoxP3^+$) have been described at normal or increased levels in GD.[16, 17] Due to the central pathogenic role of TRAb in GD, T-helper (Th) 2 cells are considered as important contributers to GD.[4] This is further supported by increased levels of typical Th2 associated cytokines such as IL-4, IL-5 and IL-13 in GD serum and the *in vitro* production of these cytokines by stimulated T cells from GD patients. But, increased levels of the Th1 associated cytokines IFN-γ and IL-12 have also frequently been reported, especially in serum from early GD patients with ophthalmopathy, suggesting involvement of Th1 responses in GD as well.[9, 18-20]

The B-cell mediated immune response in GD is characterized by autoantibody formation and infiltration of memory, germinal center and marginal zone B cells into the thyroid gland.[21, 22] In addition, increased peripheral blood B-cell numbers, in particular $CD5^+$ B cells, have been reported in GD.[23, 24] TRAb are mainly of the immunoglobulin (Ig)G1 subclass,[25] an IgG subclass formed in the presence of the Th1 cytokine IFN-γ, which underscores the importance of T-cell dependent B-cell responses in GD. The occurrence of IgM, IgA and IgE deposits in thyroid and extra-ocular muscle tissues suggests that B cells producing Ig subclasses other than IgG contribute to GD as well.[26, 27]

Despite the autoimmune pathogenesis of GD, current treatment modalities mainly focus on ablation of thyroid function by anti-thyroid drug therapy with thionamides, radioactive iodine therapy or thyroidectomy.[28] These therapies, however, do not largely affect the underlying pathogenic autoimmune response, although it has been suggested that thionamides have some immunomodulatory actions.[28, 29] Currently, B-cell-directed therapy with anti-CD20 (Rituximab) is investigated in GD ophthalmopathy. Early clinical studies report promising results on clinical improvement of ophthalmopathy, but the effects on hyperthyroidism are less pronounced.[28, 30]

In depth knowledge with regard to alterations in the composition of the peripheral blood lymphocyte compartment in GD will contribute to improved understanding of its pathogenesis and may lead to new immunomodulatory treatment strategies. However, detailed phenotypic studies on peripheral blood B-cell and T-cell subpopulations are lacking to date. In this study, we confirm activation of the T-cell compartment in GD being present in non-treated and treated GD patients. Anti-thyroid drug therapy does thus not markedly affect the activation status of T cells. In addition, we demonstrate for the first time increased numbers of transitional and pre-naive mature B cells in GD, while memory B-cell numbers are slightly decreased. Using a mouse model, we provide functional experimental data suggesting that increased thyroid hormone levels in GD may contribute to this increase in transitional and pre-naive mature B-cell numbers.

MATERIALS AND METHODS

Patients and controls

Seventeen patients with Graves' disease (GD) and ten healthy controls (HC) were included in this study. The GD patients were divided in three groups, a group of recently diagnosed patients prior to anti-thyroid drug therapy, a group that received anti-thyroid drug therapy for 2-4 months, and a group of patients with recurrent GD receiving anti-thyroid drug therapy for a second period of time. Characteristics of the subjects are summarized in table 1. GD was diagnosed based on typical clinical symptoms including diffuse enlargement of the thyroid and homogeneous increased uptake in a I^{123} thyroid scan combined with the presence of TRAb, suppressed TSH and increased free thyroxine (fT4) serum levels (figure 1A-C). One patient had clinically active GD ophthalmopathy. The patients had no coexistent other autoimmune diseases and had not used corticosteroids or antibiotics during the last three months before study inclusion. All subjects gave their written informed consent. The study was approved by the medical ethical committee of the Erasmus Medical Center, Rotterdam, The Netherlands and the Reinier de Graaf hospital, Delft, The Netherlands.

Study design

Blood was collected prior to the I^{123} thyroid scan. All patients receiving anti-thyroid drug therapy stopped using thyroid hormone supplementation three weeks before and thyrostatics one week before the blood collection and the pre-therapy I^{123} thyroid scan. Blood was collected in BD Vacutainer® SST™ II advance tubes for serum collection and in BD Vacutainer® lithium heparin tubes for collection of PBMCs (BD, Plymouth, UK) and further processed within 1 h after collection. Serum was isolated by centrifugation and frozen for further analyses. Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll density separation and viably frozen for further analyses.

Table 1. Characteristics of GD patients and HC

	HC	GD diagnosis	GD treatment	GD recurrent
Group size	10	5	4	7
Age (yr)	31.5 ± 6.1	47.2 ± 6.5	30.8 ± 8.5	32.9 ± 8.7
Gender				
female	8	4	4	4
male	2	1	0	3
Treatment				
thiamazole			3	6
propylthiouracil (PTU)			2	1
levothyroxine			1	5
β-adrenergic blocking drugs		1	1	1
Ophthalmopathy				1

Laboratory testing

TRAb were measured by radioimmunoassay with Dyno tests (BRAHMS, Berlin, Germany; normal range 0.0-0.9 IU/l). Serum free T4 (fT4) and TSH were measured by chemoluminescence assays (Vitros ECi Immunodiagnostic System, Ortho-Clinical Diagnostics, Amersham, UK; TSH normal range 0.4-4.3 mU/l; fT4 normal range 11-25 pmol/l).

Flow cytometry

Total leukocyte count was determined in freshly collected blood using a CoulterCounter (Beckman Coulter B.V., Woerden, The Netherlands) and leukocyte subpopulations were identified by flow cytometry based on CD45 expression and sideward scatter.

For immunophenotypic characterization of T- and B-cell subpopulations viably frozen PBMCs were used. T-cell subpopulations were defined as naive (T_N; $CD45RA^+CCR7^+CD27^+CD28^+$), central memory ($T_{CM}$; $CD45RO^+CCR7^+CD27^+CD28^+$), effector memory ($T_{EM}$; $CD45RO^+CCR7^-$) and terminally differentiated (T_{TD}; $CD45RA^+CCR7^-$). Regulatory T cells (Tregs; $CD4^+CD25^+FoxP3^+$), were identified by intracellular detection of the transcription factor FoxP3 using a FoxP3 staining kit (eBioscience, San Diego, CA, USA). In order to identify Th1, Th2 and Th17 lymphocytes, PBMCs were stimulated with phorbol-12-myristate-13-acetate (PMA; 50 ng/ml; Sigma-Aldrich, Saint Louis, MO, USA) and ionomycin (500 ng/ml; Invitrogen Ltd, Paisley, UK) for four hours in the presence of GolgiStop (BD Biosciences, San Jose, CA, USA). Thereafter, cells were first stained for extracellular markers, then fixed with 2% paraformaldehyde, permeabilized with 0.5% saponin, followed by intracellular staining for IFN-γ, IL-4 and IL-17A. Th1 T cells were defined as $CD4^+IFN\text{-}\gamma^+$, Th2 T cells as $CD4^+IL\text{-}4^+$ and Th17 T cells as $CD4^+IL\text{-}17A^+$.[31]

B-cell subpopulations were defined as transitional ($CD38^{high}CD27^-$), pre-naive mature

($CD38^{low}CD27^{-}IgD^{+}CD5^{+}$), naive mature ($CD38^{low}CD27^{-}IgD^{+}CD5^{-}$), natural effector ($CD38^{low}CD27^{+}IgD^{+}$), $IgG^{+}CD27^{+}$ memory ($CD38^{low}IgD^{-}IgM^{-}IgG^{+}CD27^{+}$), $IgA^{+}CD27^{+}$ memory ($CD38^{low}IgD^{-}IgM^{-}IgA^{+}CD27^{+}$), $IgG^{+}CD27^{-}$ memory ($CD38^{low}IgD^{-}IgM^{-}IgG^{+}CD27^{-}$), $IgA^{+}CD27^{-}$ memory ($CD38^{low}IgD^{-}IgM^{-}IgA^{+}CD27^{-}$), and $IgM^{+}CD27^{+}$ memory ($CD38^{low}IgD^{-}IgM^{+}CD27^{+}$) based on previous studies, with small adjustments.[32]
For flow-cytometric analyses, stained cells were measured using a FACS LSR-II (BD Biosciences) and data were analyzed with BD FACSDiva software version 6.1.2 (BD Biosciences).

Cytokine analysis

Plasma levels of IFN-γ, IL-4 and IL-17A were measured simultaneously using bead-based FlowCytomix simplex kits (Bender Medsystems GmbH, Vienna, Austria).

Mice

Balb/C *hyt*$^{-/-}$ mice, originally purchased from the Jackson Laboratory (Bar Harbor, ME, USA), were bred and maintained in the specified pathogen-free (SPF) breeding facilities of the animal facility, Erasmus Medical Center, Rotterdam, The Netherlands. To restore thyroid hormone levels, hypothyroid *hyt*$^{-/-}$ mice received 3,3',5-Triiodo-L-thyronine sodium salt (T3, 0.2 μg/ml; Sigma Aldrich Logistic GmbH, Schnelldorf, Germany) supplementation in the drinking water directly from weaning. This study was performed in accordance with the legal regulations in The Netherlands and with the approval of the local institutional Animal Ethical Committee.
Femurs were collected from CO_2-euthanized *hyt*$^{-/-}$, *hyt*$^{+/-}$ and WT mice (8-16 weeks of age). To obtain single bone marrow cell suspensions the femurs were flushed and suspensions were passed through a 70 μm filter and incubated with specific antibodies to identify sequential B-cell developmental stages. Mouse bone marrow subpopulations were defined as pro-B cells ($B220^{+}IgM^{-}CD2^{-}$), large pre-B cells ($B220^{+}IgM^{-}CD2^{+}c\mu^{+}$), small pre-B cells ($B220^{+}IgM^{-}CD2^{+}c\mu^{+}$), immature B cells ($B220^{+}IgM^{+}$) and mature B cells ($B220^{high}IgM^{+}$).

Statistical analysis

Subject characteristics are described as mean ± SD. The Kruskal Wallis test was used to identify statistical significant differences between the 4 groups, when a difference was found the exact Mann-Whitney U test was used to compare the different groups to each other. Statistics are displayed as median (range). All statistical analyses were performed with SPSS software version 15.0. A p-value < 0.05 (two-tailed) was considered statistically significant. Box-and-whisker plots display the 2.5 to 97.5 percentiles. Error bars are expressed as the standard error of the mean (s.e.m.).

RESULTS

Patient characteristics

Newly diagnosed GD patients were hyperthyroid (fT4 35.0 mU/l [33.7 – 90.0 mU/l], GD diagnosis vs HC p=0.001) with suppressed TSH levels (TSH 0.004 mU/l [0 – 0.004 mU/l], GD diagnosis vs HC p=0.001) and increased TRAb levels (7.8 IU/l [2.6 – 167.8 IU/l], GD diagnosis vs HC p=0.003; figure 1A-C). In treated GD patients fT4 levels had normalized (24.2 mU/l [14.6 – 36.5 mU/l], GD treatment vs HC p=0.15) while TSH levels were still suppressed (TSH 0 mU/l [0 – 1.09 mU/l], GD treatment vs HC p=0.007) and TRAb levels were still slightly elevated (9.0 IU/l [4.2 – 25.0 IU/l], GD treatment vs HC p=0.003; figure 1A-C). Also in patients with recurrent GD, TRAb were slightly elevated (3.0 IU/l [0.1 – 3.7 IU/l], GD recurrent vs HC p=0.02), while thyroid function was normal (fT4 14.6 mU/l [10.6 – 21.4 mU/l], GD recurrent vs HC p=0.37) under anti-thyroid drug therapy (figure 1A-C).
A general flow cytometric blood analysis revealed no significant differences in cell numbers of the main leukocyte populations between the different groups of GD patients and HC (figure 2A). Within the lymphocyte compartment, total numbers of NK cells, B cells, $CD4^+$ T cells and $CD8^+$ T cells were comparable between the different groups of patients and HC (figure 2B).

GD is characterized by an activated T-cell compartment

Newly diagnosed non-treated GD patients displayed elevated numbers of activated T cells compared to HC, as reflected by the significantly ($p<0.01$) higher numbers of HLA-DR expressing $CD4^+$ T cells and a trend towards increased numbers of CD25 expressing $CD4^+$ T cells (figure 3A-B). Numbers of HLA-DR expressing $CD4^+$ T cells normalized in GD patients treated with anti-thyroid drug therapy while the trend of elevated $CD4^+CD25^+$ T-cell numbers increased in this patient group (figure 3A-B). Similar trends in HLA-DR and CD25 expression were seen in $CD8^+$

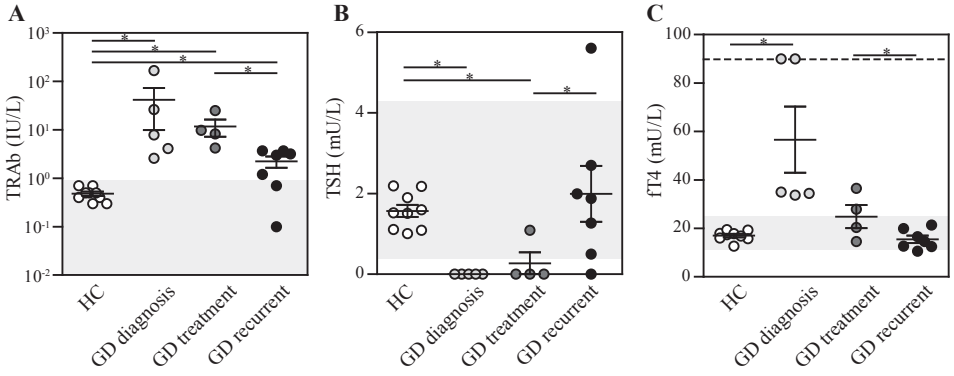

Figure 1. Serum levels of (A) TRAb, (B) TSH and (C) fT4 in HC (n = 10), GD diagnosis (n = 5), GD treatment (n = 4), GD recurrent (n = 7). Gray area: normal values, dashed line: upper limit, HC: healthy controls, GD: Graves' disease. * $p<0.05$.

T cells (figure 3A-B).
Absolute numbers of Tregs, Th1 (IFN-γ producing), Th2 (IL-4 producing) and Th17 (IL-17A producing) T cells were similar in all groups of GD patients and HC (figure 3C). In line with this, serum levels of IFN-γ, IL-4 and IL-17A did not differ between the study groups (data not shown). Absolute numbers of $CD4^+$ (figure 3D, left panel) and $CD8^+$ (figure 3D, right panel) naive, central memory, effector memory and terminally differentiated T-cell subpopulations were not significantly different in the different study groups. However, a trend towards increased numbers of naive T cells and $CD31^+$ recent thymic emigrants (RTE) was seen in GD patients receiving anti-thyroid drug therapy (figure 3D, data not shown).

GD is characterized by increased transitional B cells and decreased memory B cells

Transitional B-cell numbers were significantly (p=0.01) increased in newly diagnosed untreated GD patients when compared to HC. Also a trend towards increased numbers of pre-naive mature and naive mature B cells was found (figure 4A). In contrast, a trend towards decreased memory B-cell numbers was found. In GD patients receiving anti-thyroid drug therapy, transitional (p=0.03) and pre-naive mature (p=0.04) B-cell numbers were increased compared to HC, while the trend towards decreased memory B cells as seen in the newly diagnosed group was normalized (figure 4A). In patients treated for recurrent GD, numbers of all B-cell subpopulations were comparable to HC (figure 4A).
More detailed analysis of the major memory subpopulations showed a tendency towards decreased natural effector, $CD27^+IgM^+$, $CD27^+IgG^+$ and $CD27^+IgA^+$ memory B-cell numbers in newly diagnosed GD compared to HC, treated GD and recurrent GD, being only significant for the $CD27^+IgA^+$ memory B-cell population (GD diagnosis vc HC p=0.005, figure 4B). The examined $CD27^-$ memory B-cell populations did not differ between the study groups.
GD has previously been associated with increased percentages of $CD5^+$ B cells.[33] In line with this we found increased $CD5^+$ B-cell numbers in newly diagnosed and treated GD patients (GD diagnosis vs HC p=0.04; GD treatment vs HC p=0.02, figure 4C). In humans, CD5 is mainly

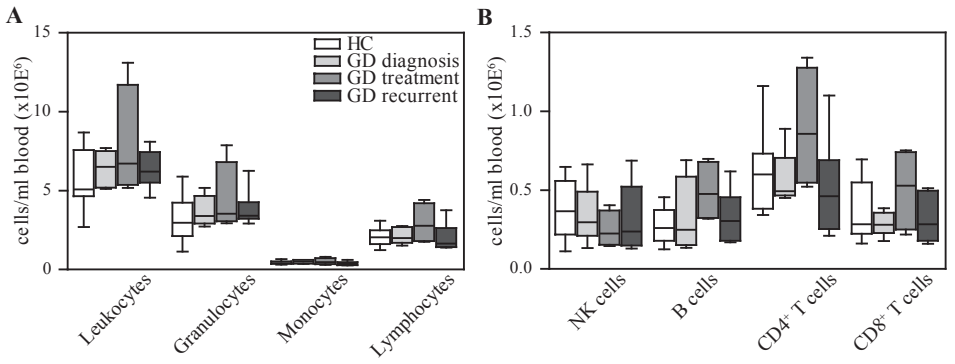

Figure 2. Absolute counts of (A) leukocyte subpopulations and (B) lymphocyte subpopulations in peripheral blood of HC (n = 10), GD diagnosis (n = 5), GD treatment (n = 4), GD recurrent (n = 7). HC: healthy controls, GD: Graves' disease, CD4: $CD4^+$ T cells, CD8: $CD8^+$ T cells. * $p<0.05$.

expressed on transitional and pre-naive mature B cells,[34] which is in line with the increased numbers of transitional and pre-naive mature B cells we observed in these GD groups.

Thyroid hormone stimulates B-cell development in *hyt/hyt* mice

Transitional B cells represent recent bone marrow (BM) emigrants.[35] The increased transitional B-cell numbers in peripheral blood of GD patients may therefore result from enhanced B-cell

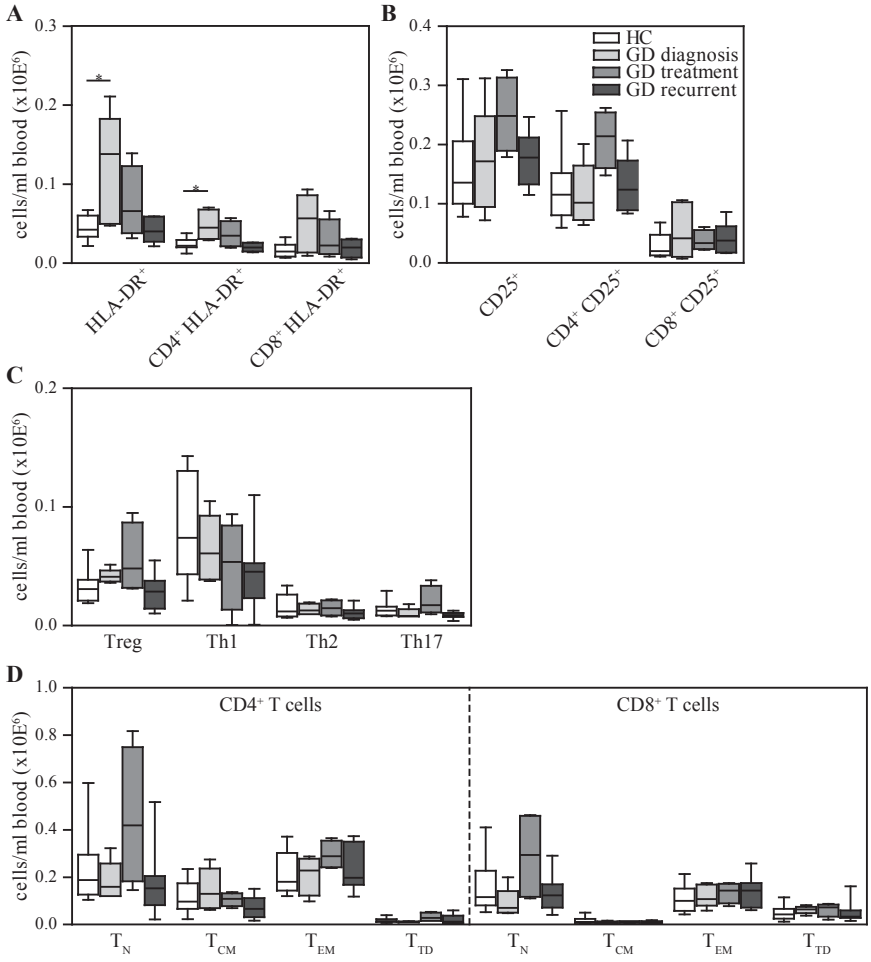

Figure 3. Absolute counts of (A) HLA-DR$^+$ T cells, (B) CD25$^+$ (FoxP3$^-$) T cells, (C) CD4$^+$CD25$^+$FoxP3$^+$ Treg cells, IFN-γ producing (Th1) cells, IL-4 producing (Th2) cells and IL-17A producing (Th17) cells and (D) naive and memory T-cell subpopulations in peripheral blood of HC (n = 10), GD diagnosis (n = 5), GD treatment (n = 4), GD recurrent (n = 7). HC: healthy controls, GD: Graves' disease, CD4: CD4$^+$ T cells, CD8: CD8$^+$ T cells, T_N: naive T cells, T_{CM}: central memory T cells, T_{EM}: effector memory T cells, T_{TD}: terminally differentiated T cells. * $p<0.05$.

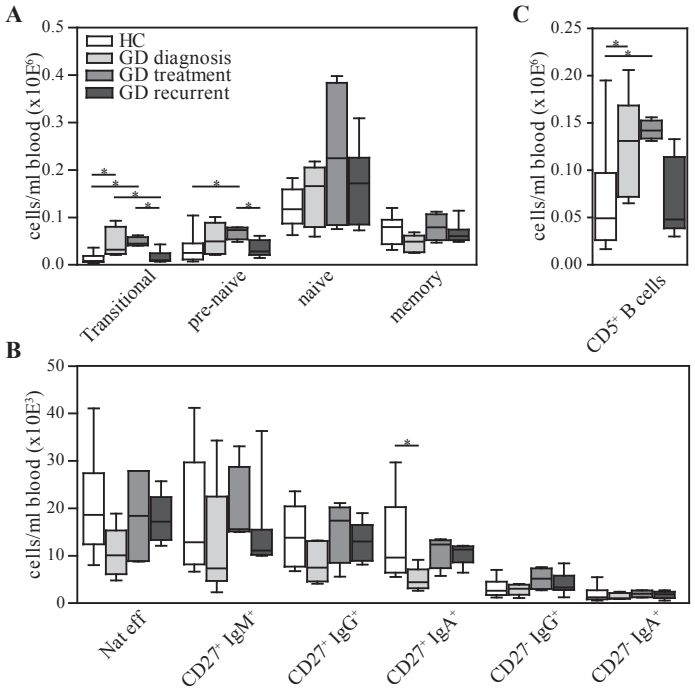

Figure 4. Absolute counts of (A) transitional, pre-naive mature, naive mature and memory B cells, (B) memory B-cell subpopulations and (C) $CD5^+$ B cells in peripheral blood of HC (n = 10), GD diagnosis (n = 5), GD treatment (n = 4), GD recurrent (n = 7). HC: healthy controls, GD: Graves' disease. * $p<0.05$.

development and/or BM output in these patients. As BM from GD patients is not commonly available, we used a mouse model to address this question. In order to examine the effect of disturbed thyroid hormone levels on B-cell development, we investigated B-cell development in the BM of a mouse strain (*hyt/hyt* mouse model) that harbor a point mutation in the TSHR receptor resulting in overt hypothyroidism. As expected, serum T3 levels were decreased in homozygous *hyt/hyt* mice ($hyt^{+/+}$) compared to wild type *Balb/C* (WT) mice and heterozygous *hyt/hyt* mice ($hyt^{+/-}$ mice), which could be restored by T3 supplementation via the drinking water (figure 5A). Hypothyroid $hyt^{+/+}$ mice had reduced total BM cell numbers compared to WT and $hyt^{+/-}$ mice (figure 5B). In addition, composition of the different B-cell developmental stages was altered in BM from $hyt^{+/+}$ mice characterized by increased percentages of pro B and immature B cells (figure 5C). Similar changes, although less pronounced, were found in $hyt^{+/-}$ mice (figure 5C). T3 supplementation resulted in normalization of BM cell numbers and size of the five main B-cell developmental stages in $hyt^{+/+}$ mice (figure 5B-C).

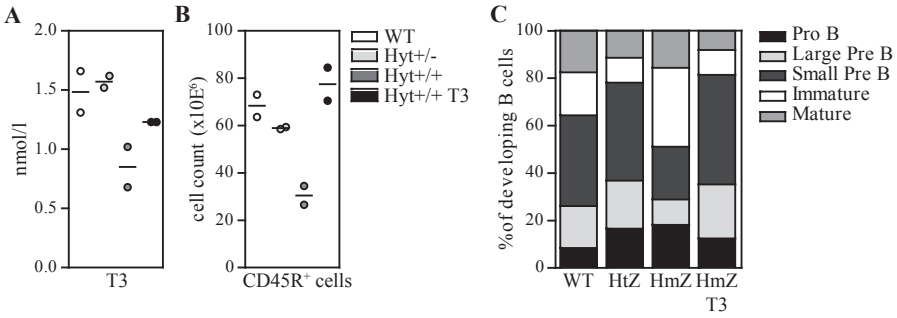

Figure 5. (A) Serum T3 levels in WT mice (n = 2), *hyt*$^{+/-}$ mice (n = 2), *hyt*$^{+/+}$ mice (n = 2) and T3 treated *hyt*$^{+/+}$ mice (n = 2). (B) Absolute BM cells counts in WT mice (n = 2), *hyt*$^{+/-}$ mice (n = 2), *hyt*$^{+/+}$ mice (n = 2) and T3 treated *hyt*$^{+/+}$ mice (n = 2). (C) B-cell progenitor composition in the BM of WT mice (n = 2), *hyt*$^{+/-}$ mice (n = 2), *hyt*$^{+/+}$ mice (n = 2) and T3 treated *hyt*$^{+/+}$ mice (n = 2). WT mice: wild type mice, *hyt*$^{+/-}$ mice: heterozygous *hyt/hyt* mice, *hyt*$^{+/+}$ mice: homozygous *hyt/hyt* mice, BM: bone marrow, T3: 3,3',5-Triiodo-L-thyronine sodium salt.

DISCUSSION

This study is the first to combine extensive immunophenotypic analysis of both the B- and T-cell compartment in peripheral blood from GD patients. We confirm the presence of increased activated T-cell and CD5$^+$ B-cell numbers in peripheral blood from GD patients. Importantly, we demonstrate for the first time that the increase of CD5$^+$ B cells in the blood of GD patients is largely attributable to increased numbers of transitional and pre-naive mature B cells. This increase may partly be due to increased thyroid hormone levels affecting B-cell development.

The peripheral blood T-cell compartment of the newly diagnosed untreated GD patients in our study showed increased numbers of activated CD4$^+$ T cells (HLA-DR$^+$ and CD25$^+$), similar to other studies.[5, 7, 11] HLA-DR$^+$ T-cell numbers were normalized to the level found in HC in GD patients receiving anti-thyroid drug therapy. In concordance with this, a reduction of HLA-DR expression in thyrocytes has been described upon initiation of anti-thyroid drug therapy.[36] In contrast, CD25$^+$ T-cell numbers did not normalize in GD patients receiving anti-thyroid drug therapy. Previous studies reported increased percentages of both HLA-DR$^+$ and CD25$^+$ T cells in GD patients receiving anti-thyroid drug therapy.[7, 11] Possibly, differences in duration of treatment or methodological differences (percentage of cells vs absolute cell numbers) account for the discrepancies between studies. Nevertheless, all available data so far point at the presence of an activated T-cell compartment in GD, and this present study shows that this is only marginally influenced by anti-thyroid drug therapy. These data suggest that T-cell directed therapy could be attempted in GD.

An increased percentage of naive T cells and RTEs has been described in euthyroid GD patients receiving anti-thyroid drug therapy compared to HC.[15] Moreover, it has been demonstrated that TSH enhances human T-cell development via functional TSHR expression on human thymocytes, suggesting that TRAb might also enhance T-cell development and thus thymic output of naive

T cells. (van der Weerd et al, submitted). In line with this, our study revealed a trend towards increased naive T-cell and RTE numbers in GD patients receiving anti-thyroid drug therapy compared to HC, albeit not statistically significant. Such a trend was however not observed in newly diagnosed GD patients. As the newly diagnosed GD patients were significantly older than the other groups (table 1) we cannot formally exclude an age-dependent effect on naive T-cell numbers in this group. However, only naive $CD8^+$ T-cell numbers correlated with age ($r_s = -0.6$; $p < 0.01$) while naive $CD4^+$ T-cell numbers ($r_s = -0.35$; $p = 0.08$) and RTE numbers ($r_s = -0.26$; $p = 0.19$) did not. Overall, these observations support the notion that circulating naive T-cell and RTE populations are increased in patients receiving anti-thyroid drug therapy. Moreover, in our study peripheral blood naive T-cell and RTE numbers did not correlate with TRAb or fT4 levels. We therefore suggest that the increased naive T-cell and RTE numbers merely reflect an effect of anti-thyroid drug therapy rather than being part of the pathogenesis of GD.

The peripheral blood B-cell compartment of patients with GD has previously been shown to contain an increase in $CD5^+$ B cells,[24] which is in line with our observation. CD5 expression on peripheral blood B cells is specifically confined to transitional and pre-naive mature B cells.[34] Transitional B cells represent recent BM emigrants that subsequently differentiate via the intermediate pre-naive mature B-cell stage into $CD5^-$ naive mature B cells.[34, 35] Here we demonstrated for the first time that the increase in $CD5^+$ B cells in GD is attributable to an increase in transitional and pre-naive mature B-cell numbers. In contrast to this, memory subpopulations showed a tendency to decreased cell numbers in GD.

Autoantibody-mediated autoimmune diseases such as systemic lupus erythematosus (SLE), rheumatoid arthritis (RA) and Sjögren's syndrome (SS) have also been associated with increased percentages of $CD5^+$ and transitional B cells, although absolute cell numbers were normal in these studies.[33, 34, 37-39] Several mechanisms have been suggested to explain this increase in transitional B cells in auto-immune diseases. Disturbed negative selection in the BM might account for increased BM emigration by immature B cells and subsequent peripheral blood entrance as transitional B cells.[34, 37, 40] In addition, chronic inflammation might enhance transitional B-cell numbers, as it has been demonstrated that especially IL-4 facilitates survival of transitional and pre-naive mature B cells.[34, 37] Moreover, factors such as B-cell activating factor (BAFF) and macrophage migration inhibitory factor (MIF), both crucial survival factors for naive B cells,[35, 41] have been found elevated in GD serum.[42, 43] In concordance with this, BAFF inhibition reduced total peripheral B-cell numbers in a hyperthyroid GD mouse model.[44] These factors may thus contribute to the expansion of transitional and pre-naive mature B cells in GD.
In addition, in this study we provide evidence that elevated thyroid hormone levels in GD may also contribute to the increase in transitional B-cell numbers. We demonstrate that hypothyroid *hyt*$^{+/+}$ mice have decreased numbers of developing B-cell progenitors in the BM, similar to other studies,[45] which could be restored by thyroid hormone supplementation. In line with this, reduced total BM cell numbers have been demonstrated in *T3Rα*$^{-/-}$ mice, and thyroid hormones have been found to stimulate pro-B-cell proliferation, thereby increasing total numbers of B-cell progenitors.[46, 47] Moreover, hypothyroidism in humans is also associated with decreased bone marrow output.[48] Based on this data we hypothesise that increased thyroid hormone levels in GD influence bone marrow activity and thereby contribute to the increased transitional and pre-naive

B-cell numbers as we found in the peripheral blood from GD patients. This hypothesis is further supported by the significant positive correlation ($r_s = 0.68$; $p < 0.01$) we observed between fT4 levels and the number of transitional B cells in our total study cohort (HC and GD patients).

Despite the important pathogenic role of autoantibodies in GD, we observed a slight reduction in memory B cells and plasma cells involved in Ig production in newly diagnosed GD patients compared to HC. It has, however, been shown that intra-thyroidal B cells mainly consist of marginal zone and memory B cells.[21, 22] This suggests that redistribution of memory cells into thyroid tissue may be responsible for the reduction in peripheral blood memory B-cell numbers in newly diagnosed GD.

In conclusion, combining detailed immunophenotypic analysis of B- and T-cell subpopulations in peripheral blood, we confirm the presence of an activated T-cell compartment in GD patients, which is not largely affected by anti-thyroid drug therapy. Moreover, we demonstrate for the first time an increase in transitional and pre-naive mature $CD5^+$ B-cell numbers in peripheral blood of GD patients. In addition, we provide evidence that increased thyroid hormone levels may contribute to the increase in transitional and pre-naive mature $CD5^+$ B-cell numbers in peripheral blood from GD patients by enhancing B-cell development in BM.

ACKNOWLEDGEMENTS

The authors thank Sandra de Bruin–Versteeg for her assistance with the figures, Menno van Zelm for assistance with B cell stainings and analyses, the endocrinologists and nuclear physicians who helped us with including GD patients, Hans van Toor and Yolanda de Rijke for measuring T3, T4 and TSH levels in mouse and human sera. This work was supported by the 'Nederlandse vereniging van Graves' patiënten (NVGP)' and internal grants from the departments of Internal Medicine and Immunology. FJTS is supported in part by Kika, ZonMW and AICR.

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Chapter V

Morbid obese human subjects have increased peripheral blood CD4$^+$ T lymphocytes with skewing towards a Treg and Th2 dominated phenotype

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Adapted from: Diabetes. 2012 Feb;61(2):401-8.

ABSTRACT

Obesity is associated with local T-cell abnormalities in adipose tissue. Systemic obesity-related abnormalities in the peripheral blood T-cell compartment are not well defined. In this study we investigated the peripheral blood T-cell compartment of morbid obese and lean subjects. We determined all major T-cell subpopulations via 6-color flow-cytometry, including $CD8^+$ and $CD4^+$ T cells, $CD4^+$ T-helper (Th) subpopulations and natural $CD4^+CD25^+FoxP3^+$ T-regulatory (nTreg) cells. Moreover, molecular analyses to assess thymic output, T-cell proliferation (TREC-analysis) and T-cell-receptor-β (*TCRB*) repertoire (GeneScan-analysis) were performed. In addition we determined plasma levels of pro-inflammatory cytokines and cytokines associated with Th subpopulations and T-cell proliferation.

Morbid obese subjects had a selective increase in peripheral blood naive, memory, nTreg and Th2 $CD4^+$ T cells, while $CD8^+$ T cells were normal. $CD4^+$ and $CD8^+$ T-cell proliferation was increased, while the *TCRB* repertoire was not significantly altered. Plasma levels of CCL5 and IL-7 were elevated. $CD4^+$ T-cell numbers correlated positively with fasting insulin levels.

The peripheral blood T-cell compartment of morbid obese subjects is characterized by increased homeostatic T-cell proliferation to which cytokines as IL-7 and CCL5 amongst others might contribute. This is associated with increased $CD4^+$ T cells with skewing towards a Treg and Th2 dominated phenotype, suggesting a more anti-inflammatory set-point.

INTRODUCTION

Obesity is a major cause of preventable death in the Western world,[1] and its prevalence is rapidly increasing.[2] Diabetes mellitus type II and cardiovascular disease are responsible for the majority of obesity-related morbidity and mortality.[2] Obesity is primarily considered to be a metabolic disease. However, in recent years it has become clear that obesity is also associated with immunological abnormalities.[3] These abnormalities probably result from intricate adipose-immune interactions,[4] and contribute to a great deal to obesity-related morbidity.[5]

Immunological abnormalities associated with obesity are often seen as a state of chronic low-grade inflammation. This state of chronic low-grade inflammation is nowadays considered to be crucial in the development of long-term complications of obesity such as diabetes[6, 7] and atherosclerosis.[8] The state of chronic low-grade inflammation has long been thought to be primarily due to an accumulation of pro-inflammatory macrophages within the adipose tissue and the production of pro-inflammatory cytokines by adipocytes and macrophages, such as TNF-α and IL-6.[9] Recently however, T-cell accumulation was demonstrated in both mouse and human obese adipose tissue,[10-12] which even preceded macrophage accumulation.[13, 14] Therefore, T cells are thought to be important participants in the initiation of adipose tissue inflammation.[9] This idea is further supported by the finding that T-cell depletion reduced adipose tissue macrophage accumulation and improved insulin sensitivity in high fat diet (HFD) fed mice.[13, 15] All together, several lines of evidence suggest a direct link between obesity and a deregulated T-cell accumulation within adipose tissue.[9]

Given the systemic nature of obesity it can be anticipated that the peripheral blood T-cell compartment is affected as well. So far however, only a limited number of studies have investigated the composition of the peripheral blood immune system in obesity. Positive correlations have been reported between body mass index (BMI) and total white blood cell count[16-19] and T-cell numbers in peripheral blood,[16-18, 20] but conflicting data have been published as well.[21] In the peripheral blood T-cell compartment increased $CD4^+$ and normal $CD8^+$ T-cell numbers have been found,[16, 17] while both subpopulations were found to be decreased in another study.[21] To date, however, studies on $CD4^+$ T-cell subpopulations, T-cell proliferation history and T-cell diversity are lacking.

In this study, we performed a detailed analysis of the peripheral blood T-cell compartment in morbid obese and lean subjects. For this purpose we determined the absolute counts and relative frequencies of all major T-cell subpopulations via 6-color flow cytometry including $CD8^+$ T cells, $CD4^+$ T cells and the $CD4^+$ T-cell subpopulations T-helper (Th)1, Th2, Th17 T cells and natural $CD4^+CD25^+FoxP3^+$ T regulatory (Treg) T cells. These numerical analyses were combined with molecular analyses to assess thymic output and T-cell proliferation (T-cell-receptor excision circle (TREC)-analysis) and T-cell-receptor β (TCRB) repertoire usage (GeneScan analysis). In addition, we determined plasma levels of pro-inflammatory cytokines (IL-6 and TNF-α), cytokines associated with Th1, Th2 or Th17 subpopulations (IFN-γ, IL-4, IL-17A), and cytokines involved in T-cell proliferation, survival and recruitment (CCL5, IL-2, IL-7).

MATERIALS AND METHODS

Patients and controls

Thirteen morbid obese (BMI > 40 kg/m^2) subjects and twenty-five lean (BMI < 25 kg/m^2) healthy controls were included in this study. Subjects with overt diabetes mellitus type II or liver enzyme test abnormalities were excluded. The presence of concomitant medical illness was excluded by medical history assessment in morbid obese and lean subjects. Characteristics of the subjects are summarized in Table 1. All subjects gave their written informed consent. The study was approved by the medical ethical committee of the Erasmus MC, Rotterdam, The Netherlands.

Table 1. Characteristics of morbid obese and lean healthy subjects

Flow cytometric and cytokine analysis		
	Lean subjects	Morbid obese subjects
Group size	11	8
BMI (kg/m^2)	23.2 ± 1.4	42.4 ± 6.7
Age (yr)	34 ± 9	45 ± 10
Female/male	9/2	8/0

TREC and GeneScan analysis		
	Lean subjects	Morbid obese subjects
Group size	14	13
BMI (kg/m^2)	23.9 ± 1.9	42.1 ± 5.9
Age (yr)	31 ± 7	48 ± 11
Female/male	12/2	13/0

Study design

Blood was obtained using vacuette sodium heparin-containing tubes (Greiner Bio-one, Alphen a/d Rijn, The Netherlands) and further processed within 1 h after collection. Plasma was isolated by centrifugation and frozen for further analyses. Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll density separation and viably frozen for further analyses.

Flow cytometry and cell sorting

Total leukocyte count was measured in freshly collected blood using a CoulterCounter (Beckman Coulter B.V., Woerden, The Netherlands) and leukocyte subpopulations were determined by flow cytometry based on CD45 expression and sideward scatter. For immunophenotyping of T-cell

subpopulations viably frozen PBMCs were used. Antibodies used for flow cytometric analyses and sorting experiments are summarized in supplementary table 1. T-cell subpopulations were defined as naive ($CD45RA^+CD27^+$), central memory ($CD45RO^+CD27^+$), effector memory ($CD45RO^+CD27^-$; although it is known that a small population of effector memory cells does still express CD27[22]), and terminally differentiated ($CD45RA^+CD27^-$). Natural regulatory T (nTreg) cells were defined as $CD4^+CD25^+FoxP3^+$. For intracellular cytokine detection, PBMCs were stimulated with phorbol-12-myristate-13-acetate (PMA; 50 ng/ml; Sigma-Aldrich, Saint Louis, MO, USA) and ionomycin (500 ng/ml; Invitrogen Ltd, Paisley, UK) for four hours in the presence of GolgiStop (BD Biosciences, San Jose, CA, USA). Thereafter, cells were first stained for extracellular markers, then fixed with 2% paraformaldehyde, permeabilized with 0.5% saponin, followed by intracellular staining for IFN-γ, IL-4 and IL-17A. Stained cells were measured using a FACS LSR-II (BD Biosciences) and data were analyzed with FlowJo software (TreeStar, Ashland, OR, USA). T-helper 1 (Th1) T cells were defined as $CD4^+IFN\text{-}\gamma^+$, Th2 T cells as $CD4^+IL\text{-}4^+$ and Th17 T cells as $CD4^+IL\text{-}17A^+$.
The following T-cell subpopulations were sorted with a purity of >90% in all samples using a FACSAria (BD Biosciences): $CD4^+$ naive ($CD3^+TCR\gamma\delta^-CD4^+CD45RO^-CD27^+$), $CD4^+$ memory ($CD3^+TCR\gamma\delta^-CD4^+CD45RO^+$), $CD8^+$ naive ($CD3^+TCR\gamma\delta^-CD8^+CD45RO^-CD27^+$), and $CD8^+$ memory ($CD3^+TCR\gamma\delta^-CD8^+CD45RO^+$) T cells. Total $TCR\alpha\beta^+$ T cells were isolated with a purity of >90% in all samples using AutoMACS with APC-labeled anti-TCRαβ antibodies and anti-APC beads (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany).

Signal joint T-cell-receptor excision circle analysis

Signal joint T-cell-receptor excision circle (sjTREC) analysis was used to evaluate thymic output and peripheral T-cell proliferation. The sjTREC is a stable circular DNA structure that is formed during T-cell-receptor alpha (*TCRA*) rearrangements in developing thymocytes (supplementary figure IA).[23] Because the sjTREC, in contrast to genomic DNA, is not duplicated during cell proliferation it will dilute out during consecutive cell divisions, making it a useful marker to determine proliferation history in $TCR\alpha\beta^+$ T cells (supplementary figure IB).[24]
To determine sjTREC dilution, DNA was extracted from different T-cell subpopulations using the GeneElute Mammalian Genomic DNA miniprep kit (Sigma-Aldrich). The sjTREC was detected by RQ-PCR using an ABI Prism 7900 machine (Applied Biosystems, Foster City, CA, USA; Table 2). To correct for total DNA-input, the amount of albumin gene was determined (Table

Table 2. Primers and probes used for TREC analysis

sjTREC[24]	Forward primer	5'-CCATGCTGACACCTGGTT-3'
	Reverse primer	5'-TCGTGAGAACGGTGAATGAAG-3'
	Probe	5'-CACGGTGATGCATAGGCACCTGC-3'
Albumin[25]	Forward primer	5'-TGAACAGGCGACCATGCTT-3'
	Reverse primer	5'-CTCTCCTTCTCAGAAAGTGTGCATAT-3'
	Probe	5'-TGCTGAAACATTCACCTTCCATGCAGA-3'

2).[25] Cycle threshold (Ct)-values for sjTREC were normalized to albumin Ct-values.[25] For copy number quantification serial dilutions of a pGEM-T Easy vector (Promega Benelux BV, Leiden, The Netherlands) in which the sjTREC sequence was cloned were used.[24]

GeneScan analysis for in-frame TCRB gene rearrangements

To determine T-cell-receptor beta (*TCRB*) diversity we performed GeneScan analysis for Vβ-Jβ gene rearrangements on DNA of sorted T-cell subpopulations with multiplex PCR as previously described.[26]

Cytokine analysis

Plasma levels of IL-2 (sensitivity >16.4 pg/ml), IL-4 (sensitivity >20.8 pg/ml), IL-6 (sensitivity >1.2 pg/ml), IL-10 (sensitivity >1.9 pg/ml), IL-12p70 (sensitivity >1.5 pg/ml), IL-17A (sensitivity >2.5 pg/ml), IFN-γ (sensitivity >1.6 pg/ml), CCL5 (sensitivity >25 pg/ml), and TNF-α (sensitivity >3.2 pg/ml) were measured simultaneously using bead-based FlowCytomix simplex kits (Bender Medsystems GmbH, Vienna, Austria). Plasma levels of IL-7 were measured by ELISA (Invitrogen Ltd).

Glucose and insulin measurements

Fasting blood glucose levels were measured with a Hitachi 917 Chemistry Analyzer (Roche Diagnostics). Fasting insulin levels were measured using a chemiluminescent immunometric assay (Immulite 2000, Siemens Medical Solutions Diagnostics, Los Angeles, CA, USA).

Statistical analysis

Subject characteristics are described as mean ± SD. The exact Mann-Whitney U test was used for statistical comparisons between morbid obese and lean subjects. Statistics are displayed as median (range). All statistical analyses were performed with SPSS software version 15.0. A p-value of less than 0.05 (two-tailed) was considered statistically significant. Box-and-whisker plots display the 2.5 to 97.5 percentiles. Error bars are expressed as the standard error of the mean (s.e.m.).

RESULTS

Peripheral blood $CD4^+$ T-cell numbers are increased in morbid obesity

An initial general examination of the blood samples did not reveal clear differences in the total leukocyte number or the numbers of distinct leukocyte subpopulations between morbid obese and lean subjects. However, a trend towards increased lymphocyte numbers was present in morbid

obese subjects (figure 1A). Detailed flow cytometric analyses on isolated PBMCs revealed that NK- and B-cell numbers did not differ between the morbid obese and lean subjects (figure 1B). T-cell numbers, however, were significantly ($p<0.01$) increased in morbid obese subjects. This was mainly due to a 2-fold increase in $CD4^+$ T cells ($p<0.01$), while $CD8^+$ T-cell numbers remained normal ($p=0.35$, figure 1C)**.** This resulted in an increased CD4/CD8 ratio (morbid obese 2.82 (1.62 - 6.17) vs lean 1.54 (1.29 - 5.23), $p=0.03$, data not shown).

Peripheral blood $CD4^+$ T-cell subpopulations that display an anti-inflammatory phenotype are increased in morbid obesity

Next, we performed extensive flow cytometric analyses to determine whether distinct T-cell subpopulations are affected in morbid obese subjects. Within the $CD8^+$ T-cell compartment, cell numbers within the different subpopulations were similar in morbid obese and lean subjects (figure 2A). Within the $CD4^+$ T-cell compartment, increased numbers of naive ($p=0.04$), central memory ($p<0.01$) and terminally differentiated ($p=0.03$) T cells were found in morbid obese subjects, while no differences were found in the effector memory subpopulation between morbid obese and lean subjects (figure 2A). Also absolute counts of natural $CD4^+CD25^+FoxP3^+$ Treg T cells were increased ($p<0.01$, figure 2B). Effector $CD4^+$ T-cell subpopulations were determined by measuring the intracellular cytokine profile after stimulation with PMA and ionomycin. The numbers of IFN-γ producing T cells (Th1) and IL-17A producing T cells (Th17) were similar in morbid obese and lean subjects, while the number of IL-4 producing T cells (Th2) was increased in morbid obese subjects ($p=0.03$, figure 2B).
Additional correlation analyses demonstrated also a significant correlation between BMI and the number of total T cells as well as the numbers of $CD4^+$ T cells, naive $CD4^+$ T cells, terminally differentiated $CD4^+$ T cells, central memory $CD4^+$ T cells, natural $CD4^+CD25^+FoxP3^+$ Treg T cells and Th2 T cells. For age, only a significant correlation was observed with the number of Th2 T cells (supplementary table II).

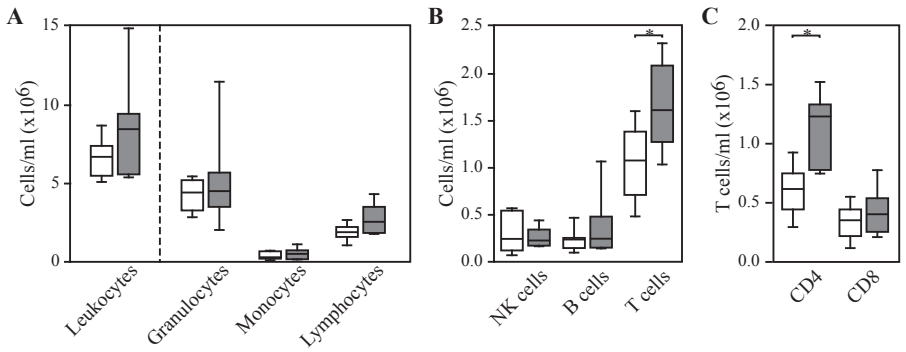

Figure 1. Absolute counts of (A) leukocyte subpopulations, (B) PBMC subpopulations and (C) $CD4^+$ and $CD8^+$ T cells in peripheral blood of morbid obese and lean subjects. CD4: $CD4^+$ T cells, CD8: $CD8^+$ T cells. White bars: lean, n=11 (BMI 23.2 ± 1.4 kg/m^2); gray bars: morbid obese, n=8 (BMI 42.4 ± 6.7 kg/m^2). * $p<0.05$.

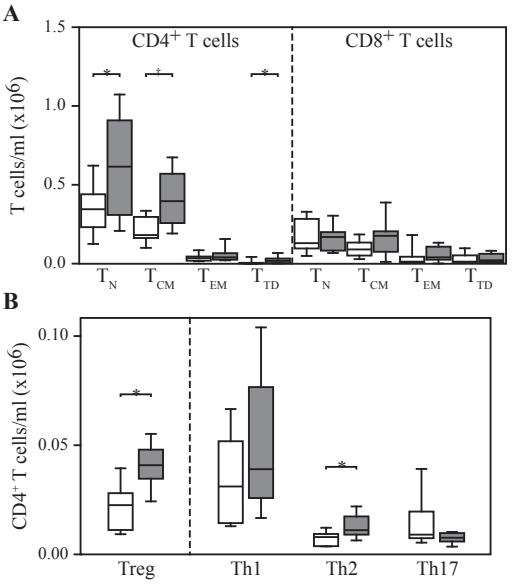

Figure 2. (A) Absolute counts of $CD4^+$ and $CD8^+$ T-cell subpopulations in peripheral blood of morbid obese and lean subjects. (B) Absolute counts of natural $CD4^+CD25^+FoxP3^+$ Treg T cells, IFN-γ producing (Th1) T cells, IL-4 producing (Th2) T cells and IL-17A producing (Th17) T cells in peripheral blood of morbid obese and lean subjects within the $CD4^+$ T-cell gate. T_N: naive T cells, T_{CM}: central memory T cells, T_{EM}: effector memory T cells, T_{TD}: terminally differentiated T cells. White bars: lean, n=11 (BMI 23.2 ± 1.4 kg/m^2); gray bars: morbid obese, n=8 (BMI 42.4 ± 6.7 kg/m^2). * $p<0.05$, † $p<0.01$.

Taken together, these data demonstrate that morbid obesity is associated with increased naive and memory $CD4^+$ T cells and with increased numbers of anti-inflammatory natural $CD4^+CD25^+FoxP3^+$ Treg T cells and Th2 T cells.

Proliferation of $CD4^+$ and $CD8^+$ T cells is increased in morbid obesity

Several mechanisms including increased thymic output, increased peripheral proliferation, decreased apoptosis or altered redistribution can account for the observed increased $CD4^+$ T-cell numbers found in morbid obese subjects. In order to distinguish between increased thymic output and increased peripheral proliferation or survival, the sjTREC content in peripheral blood T-cell subpopulations was determined (supplementary figure IC). A significantly lower sjTREC content, which together with increased cell numbers resembles increased proliferation (supplemetary figure IC), was found in total $TCR\alpha\beta^+$ T cells ($p<0.01$), $CD4^+$ naive ($p=0.03$), $CD4^+$ memory ($p=0.02$) and $CD8^+$ naive ($p=0.02$) T-cell subpopulations of morbid obese subjects (figure 3A). Moreover, a significant negative correlation ($p<0.01$) was found between $TCR\alpha\beta^+$ T-cell sjTREC content and BMI (figure 3B, left panel).
Previously, a negative correlation between sjTREC content and age has been reported,[25] but although the morbid obese group was significantly older than the lean control group (morbid

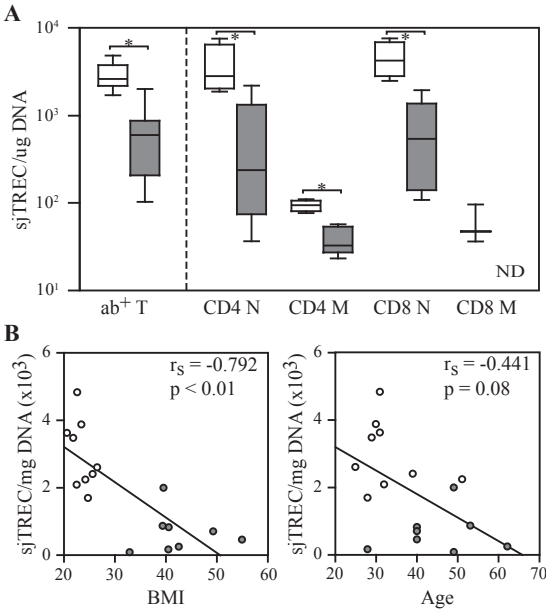

Figure 3. (A) SjTREC content in total TCRαβ$^+$ T cells and T-cell subpopulations of morbid obese and lean subjects. (B) Correlation between sjTREC content and BMI (left panel) and correlation between sjTREC content and age (right panel) in morbid obese and lean subjects. αβ$^+$ T: TCRαβ$^+$ T cells, CD4 N: CD4$^+$ naive cells, CD4 M: CD4$^+$ memory T cells, CD8 N: CD8$^+$ naive T cells, CD8 M: CD8$^+$ memory T cells, ND: not detectable. White bars or dots: lean, TCRαβ$^+$ T cells n=10 (BMI 24.0 ± 2.2 kg/m^2), T-cell subpopulations n=4 (BMI 23.6 ± 1.0 kg/m^2); gray bars or dots: morbid obese, TCRαβ$^+$ T cells n=8 (BMI 42.4 ± 6.7 kg/m^2), T-cell subpopulations n=5 (BMI 41.6 ± 5.1 kg/m^2). * p<0.05.

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obese 45 years of age (28 - 62) vs lean 31 years of age (25 - 51); p=0.02), the sjTREC content in TCRαβ$^+$ T cells did not correlate significantly with age (figure 3B, right panel), although we cannot exclude that this might be due to the relatively limited number of subjects studied. Moreover, in multiple regression analysis the BMI was the only variable significantly associated with the TREC content (R=0.8, R^2=0.58, p_{total}=0.001; p_{age}=0.28; p_{BMI}=0.002), demonstrating that the decreased sjTREC content in morbid obesity is mainly determined by obesity and not by age. Overall, the decreased sjTREC content together with the increased T-cell numbers in morbid obese subjects is indicative of increased proliferation within the T-cell compartment of these subjects.

Increased T-cell proliferation in morbid obesity is not driven by dominant antigens

Several studies have described a reduced diversity within the T-cell-receptor β (*TCRB*) repertoire of T cells isolated from adipose tissue of obese mice, suggesting a local antigen driven immune response towards the main antigens present within adipose tissue.[13, 27] We determined *TCRB* diversity in peripheral blood T-cell subpopulations. We observed a diverse *TCRB* repertoire in

$CD4^+$ and $CD8^+$ naive and memory T cells from both morbid obese and lean subjects (figure 4A-B). Minor alterations in the, normally Gaussian distributed, *TCRB* repertoire were found to a limited extent in the $CD4^+$ memory T cells and $CD8^+$ naive and memory T cells of morbid obese subjects, suggesting the existence of a slightly skewed *TCRB* repertoire in morbid obese subjects (figure 4A-B). In addition, we obtained fat tissue that was removed during surgery from five other morbid obese subjects. From these patients no peripheral blood T cells were available, as only adipose tissue and plasma samples were stored. Nevertheless, it gave us an opportunity to investigate the *TCRB* repertoire of adipose tissue T cells. Hence we performed GeneScan analysis on the T cells present in the adipose tissue. We observed a polyclonal *TCRB* repertoire (supplementary figure II) indicating that there was no strong skewing towards particular T-cell clones. Instead a rather broad TCR repertoire was present in the adipose tissue T cells.

T-cell growth factors in plasma are elevated in morbid obesity

Because obesity is characterized by abnormal production of pro-inflammatory cytokines,[28] we hypothesized that cytokines involved in T-cell proliferation, survival and recruitment might also

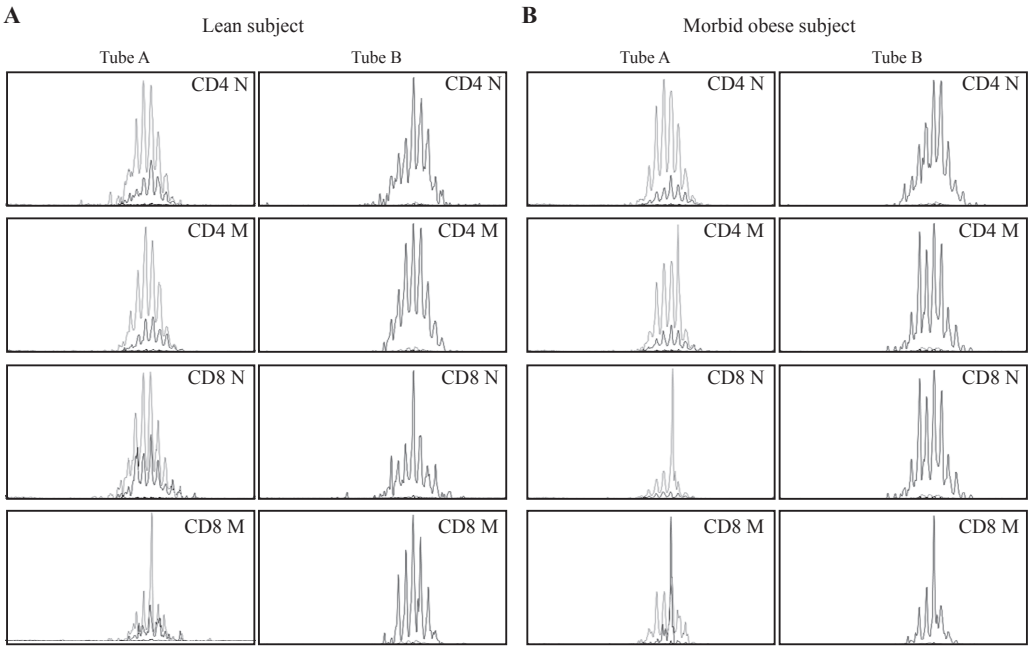

Figure 4. GeneScan analysis of Vβ-Jβ rearrangements in $CD4^+$ and $CD8^+$ naive and memory T-cell subpopulations in a representative (A) lean and (B) morbid obese subject. Tube A: Vβ+ Jβ1.1 to Jβ1.6+Jβ2.2+Jβ2.6+Jβ2.7; Tube B: Vβ+Jβ2.3+Jβ2.4+Jβ2.5; Primers for the Jβ1 cluster were HEX-labeled (green line), primers for the Jβ2 cluster were FAM-labeled (blue line). CD4 N: $CD4^+$ naive T cells, CD4 M: $CD4^+$ memory T cells, CD8 N: $CD8^+$ naive T cells, CD8 M: $CD8^+$ memory T cells. Lean n=4 (BMI 23.6 ± 1.0 kg/m^2); morbid obese n=5 (BMI 41.6 ± 5.1 kg/m^2). *See Chapter VII for full-color figure.*

be produced in excess in morbid obese subjects. Therefore, we determined a broad panel of cytokines in plasma from morbid obese and lean subjects.

Plasma levels of the pro-inflammatory cytokines IL-6 and TNF-α did not differ between morbid obese and lean subjects (figure 5A). Also, plasma levels of IFN-γ, IL-4 and IL-17A, cytokines

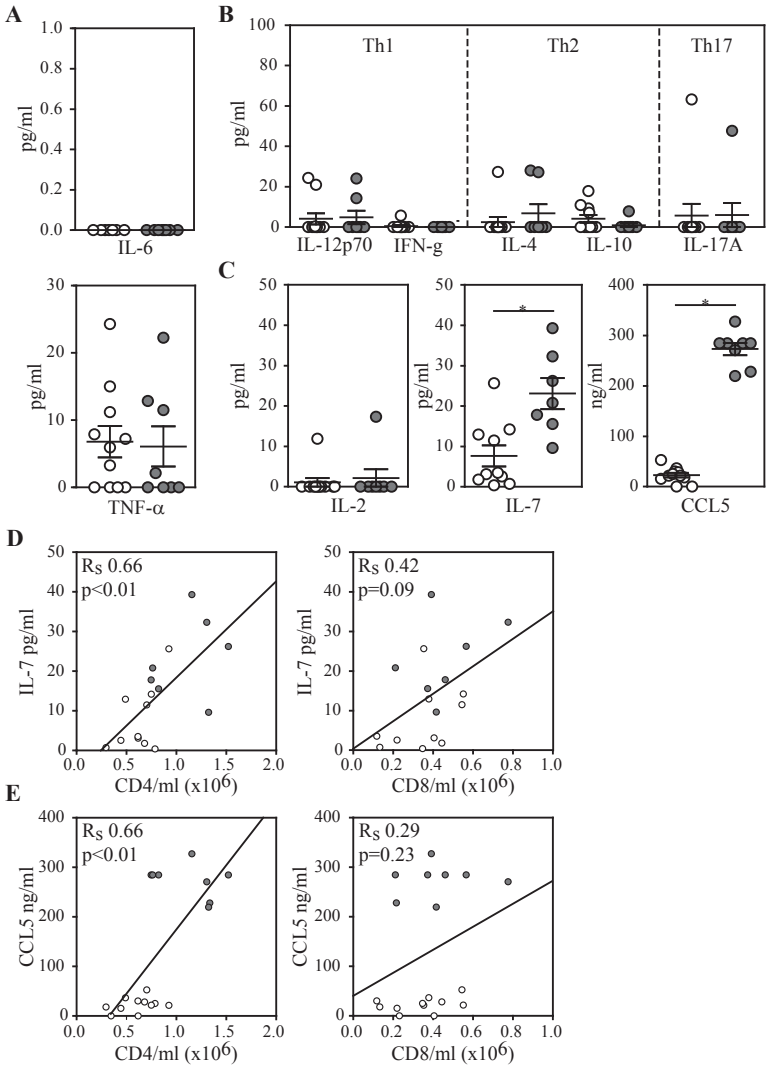

Figure 5. Plasma levels of (A) IL-6 and TNF-α, (B) Th1 cytokines IL-12p70 and IFN-γ, Th2 cytokines IL-4 and IL-10 and the Th17 cytokine IL-17A and (C) IL-2, IL-7 and CCL5 in morbid obese and lean subjects. (D) Correlation between IL-7 plasma levels and $CD4^+$ or $CD8^+$ T-cell counts in peripheral blood. (E) Correlation between CCL5 plasma levels and $CD4^+$ or $CD8^+$ T-cell numbers in peripheral blood. White dots: lean, n=11 (BMI 23.2 ± 1.4 kg/m^2); gray dots: morbid obese, n=8 (BMI 42.4 ± 6.7 kg/m^2). * p<0.05.

respectively associated with Th1, Th2 or Th17 subpopulations, were similar in morbid obese and lean subjects (figure 5B).
The cytokines CCL5, IL-2 and IL-7 enhance T-cell proliferation, survival and recruitment.[29-31] Plasma levels of CCL5 ($p<0.01$) and IL-7 ($p<0.01$) were significantly elevated in morbid obese subjects (figure 5C) and correlated positively with BMI (supplementary table III). IL-2 plasma levels were similar in morbid obese and lean subjects (figure 5C).
As expected, IL-7 and CCL5 plasma levels positively correlated with total $CD4^+$ T-cell numbers but not with total $CD8^+$ T-cell numbers (figure 5D-E). In the $CD4^+$ T-cell compartment, also a positive correlation was found between IL-7 plasma levels and the number of naive $CD4^+$ T cells, terminally differentiated $CD4^+$ T cells, central memory $CD4^+$ T cells and natural $CD4^+CD25^+FoxP3^+$ Treg T cells; CCL5 plasma levels correlated positively with the number of terminally differentiated $CD4^+$ T cells, central memory $CD4^+$ T cells and natural $CD4^+CD25^+FoxP3^+$ Treg T cells (supplementary table IV).

Increased $CD4^+$ T-cell numbers correlate with fasting insulin levels in morbid obesity

Although the morbid obese subjects did not have diabetes mellitus type II, we investigated the correlations between the increased $CD4^+$ T-cell numbers and metabolic measures. Fasting glucose and insulin levels were only determined in the morbid obese group.[32] A significant correlation was found between fasting insulin levels and $CD4^+$ T-cell numbers (figure 6A). Moreover, the glucose/

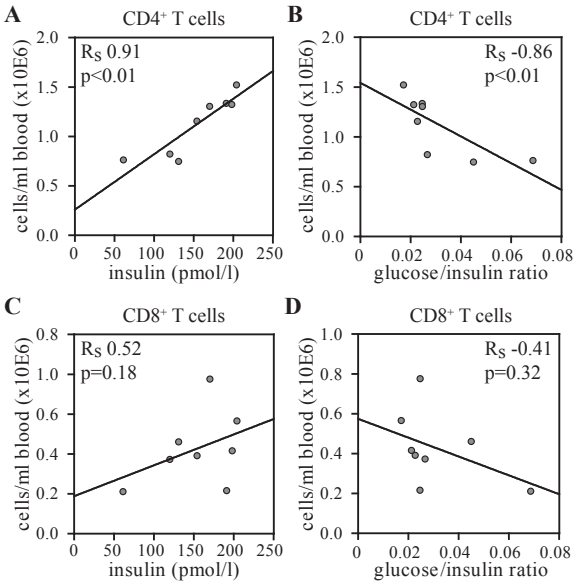

Figure 6. Correlation between $CD4^+$ T-cell count in peripheral blood and (A) fasting insulin levels and (B) insulin sensitivity (fasting glucose/fasting insulin ratio). Correlation between $CD8^+$ T-cell count in peripheral blood and (C) fasting insulin levels and (D) insulin sensitivity (fasting glucose/fasting insulin ratio). Gray dots: morbid obese, n=8 (BMI 42.4 ± 6.7 kg/m^2).

insulin ratio was calculated as a measure of insulin sensitivity. This ratio also correlated with $CD4^+$ T-cell numbers (figure 6B). Fasting insulin levels and insulin sensitivity did not correlate with $CD8^+$ T-cell numbers (figure 6C-D). No significant correlations were found between fasting blood glucose levels and T-cell subpopulations (data not shown).

DISCUSSION

This study is the first that comprehensively studied the peripheral blood T-cell compartment of morbid obese subjects. Our main finding was a selective increase in $CD4^+$ T-cell numbers within the peripheral blood T-cell compartment of morbid obese subjects. Peripheral blood $CD8^+$ T-cell numbers were normal in morbid obese subjects. This latter observation is in contrast with the increased numbers of local effector and memory $CD8^+$ T cells described in adipose tissue of obese subjects.[12, 13]

In mice, diet-induced obesity results in a reduced sjTREC content in splenic $CD4^+$ T cells.[33] This is accompanied by a reduction in naive T cells and a more restricted TCRB repertoire, suggesting that in this mouse model the decrease in sjTREC content is mainly due to reduced thymic output.[33] As ageing is also associated with a reduction in thymic output resulting in a reduced TREC content and a reduction in naive T-cell numbers, it was suggested that obesity is related with accelerated ageing of the T-cell compartment.[33, 34]
In our study, we found a decreased sjTREC content in peripheral blood T-cell subpopulations of morbid obese subjects. However, in contrast to observations in ageing studies,[35, 36] the decreased sjTREC content was accompanied by increased numbers of naive as well as memory $CD4^+$ T cells and only an insignificant skewing of the *TCRB* repertoire. Therefore, despite the limitation of the significant age difference between morbid obese and lean subjects in our study, we conclude from the increased naive T-cell numbers, that the decrease in sjTREC content in morbid obese subjects predominantly results from increased proliferation rather than accelerated ageing and decreased thymic output. This notion is further supported by the decreased telomere length observed in leukocytes of obese subjects.[37]

The increased proliferation within the peripheral blood T-cell compartment is more likely of homeostatic nature rather than being driven by dominant antigens, as the latter would result in increased memory and effector T-cell subpopulations with prominent skewing of the *TCRB* repertoire, while the naive T-cell compartment would remain unaffected. In our cohort we do not see such changes in the peripheral blood T-cell compartment. Moreover, in an additional analysis a rather polyclonal TCR repertoire was observed in adipose tissue T cells of morbid obese subjects. It therefore seems likely that there is no vast change in TCR repertoire in the adipose tissue T cells in our cohort. However, we formally cannot exclude the possibility of some skewing of the *TCRB* repertoire within adipose tissue T cells.
Several T-cell mitogenic factors, as adipokines, fatty acids or bacterial products, can be elevated in plasma of morbid obese subjects.[38-40] Also increased levels of IL-7 and CCL5, cytokines capable of stimulating homeostatic T-cell proliferation, survival and recruitment,[29-31] have been found in adipose tissue of obese mice and men.[41-43] We also found highly elevated plasma levels of IL-7

and CCL5 in morbid obese subjects in this study, which positively correlated with peripheral blood $CD4^+$ T-cell numbers. Based on these data, we hypothesize that IL-7 and CCL5 as well as the other T-cell mitogenic factors might contribute to the increased homeostatic $CD4^+$ T-cell proliferation in morbid obese subjects.

Despite the selective increase in $CD4^+$ T-cell numbers in peripheral blood, $CD8^+$ T cells also displayed decreased sjTREC content, indicating that $CD8^+$ T cells also undergo increased homeostatic proliferation due to the increased IL-7 and CCL5 cytokine levels in morbid obese subjects. However, peripheral blood $CD8^+$ T-cell numbers were not increased, suggesting a selective redistribution of $CD8^+$ T cells into adipose tissue, which is in line with the described preferential accumulation of $CD8^+$ T cells in obese adipose tissue.[12, 13] Also, CCL5 is a more potent chemoattractant for $CD4^+$ T cells than for $CD8^+$ T cells,[44] and in obesity systemic levels of CCL5 are ~100-fold higher than those locally produced within adipose tissue.[41] Therefore, the elevated CCL5 plasma levels that we observed may contribute to the selective retention of $CD4^+$ T cells in peripheral blood of morbid obese subjects.

With regard to the increased numbers of peripheral blood $CD4^+$ T cells, we observed that this was accompanied by a selective increase in natural $CD4^+CD25^+FoxP3^+$ Treg T cell numbers. In addition, stimulation of PBMCs with PMA/ionomycin specifically induced a Th2 phenotype within the $CD4^+$ T-cell compartment of morbid obese subjects. This indicates that the numerically elevated peripheral blood $CD4^+$ T-cell compartment of morbid obese subjects is skewed towards a Treg and Th2 dominated phenotype, suggesting a more anti-inflammatory set point. Despite this clear skewing, plasma levels of cytokines associated with the Th2 phenotype were mostly undetectable in plasma from both morbid obese and lean subjects, as was the case for cytokines associated with the Th1 and Th17 phenotypes.

Natural $CD4^+CD25^+FoxP3^+$ Treg T cells and Th2 T cells are capable of polarizing monocytes/macrophages towards an anti-inflammatory M2 phenotype, which is characterized by the production of anti-inflammatory mediators such as IL-1 receptor antagonist, IL-10 and TGF-β.[45, 46] We hypothesize that the preferential skewing of the $CD4^+$ T-cell compartment towards an Treg and Th2 dominated phenotype can be considered as a mechanism to counter regulate the pro-inflammatory activity that exists systemically and locally within the monocyte/macrophage compartment in obesity.[47-50] The absence of increased IL-6 and TNF-α plasma levels in our cohort of morbid obese subjects supports this notion.

We demonstrated this anti-inflammatory T-cell set point in a morbid obese cohort which was selected on the basis of being non-diabetic and thus relatively healthy and free of comorbidities (although we are not informed on the atherosclerotic state of our patients). To date, it is unknown whether changes in this set point away from the anti-inflammatory phenotype are associated with the development of obesity-related comorbidities.
Atherosclerosis, which frequently occurs during obesity, is characterized by accumulation of Th1 $CD4^+$ T cells within the plaques,[51] while $CD4^+$ T-cell depletion reduces the development of atherosclerosis in mice.[52] Also the development of diabetes mellitus type II is delayed in mice with diet induced obesity when T cells are depleted.[13, 15] In addition, we also demonstrated that

$CD4^+$ T-cell numbers positively correlated with fasting insulin levels.
Based on these literature data and our own here presented data it is thus tempting to speculate that changes away from the Treg and Th2 dominated phenotype towards a more pro-inflammatory Th1 or Th17 dominated set point may prove an important indicator, or even mediator, for the development of atherosclerosis or diabetes in morbid obese subjects. Longitudinal studies in morbid obese subjects will be important to further address these issues.

In conclusion, the peripheral blood T-cell compartment of morbid obese subjects is characterized by an increased homeostatic proliferation of both $CD4^+$ and $CD8^+$ T cells to which cytokines such as IL-7 and CCL5 probably contribute. This increased homeostatic proliferation is associated with an increase in peripheral blood $CD4^+$ T-cell numbers with skewing towards a Treg and Th2 dominated phenotype, suggesting an anti-inflammatory set point of the peripheral blood $CD4^+$ T-cell compartment.

ACKNOWLEDGEMENTS

The authors thank Sjanneke Heuvelmans, Erasmus MC, for collecting materials, Edwin de Haas and Benjamin Bartol, Erasmus MC, for assistance with cell sorting, Henk Wind, Jeroen te Marvelde and Dennis Tielemans, Erasmus MC, for their assistance with flow cytometry, Joyce Vermeulen and Ingrid Wolvers, Erasmus MC, for performing GeneScan analyses, Sandra de Bruin-Versteeg, Erasmus MC, for assistance with the figures, Jon Laman, Erasmus MC, for participation in discussions and all other members of the laboratories of Frank Staal, LUMC, en Wim Dik, Erasmus MC, for their technical assistance. This work was supported by internal grants from the departments of Internal Medicine and Immunology of the Erasmus Medical Center Rotterdam. Frank J.T. Staal is supported in part by Kika, ZonMW and AICR. P.M. van Hagen is the guarantor of this manuscript.

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SUPPLEMENTAL DATA

Supplementary table I. Fluorochrome-antibody combinations used for flow cytometry and sorting experiments

Human	Fluorochrome	Clone	Company
CD3	PerCP	SK7	BD Biosciences (San Jose, CA, USA)
CD4	PE-cy7	SK3	BD Biosciences (San Jose, CA, USA)
CD8	APC-cy7	SK1	BD Biosciences (San Jose, CA, USA)
CD14	FITC	My4	Beckman Coulter (Brea, CA, USA)
CD16	PE	B73.1	BD Biosciences (San Jose, CA, USA)
CD19	APC	SJ25C1	BD Biosciences (San Jose, CA, USA)
CD25	APC	2A3	BD Biosciences (San Jose, CA, USA)
CD27	APC	L128	BD Biosciences (San Jose, CA, USA)
CD31	FITC	WM59	BD Biosciences (San Jose, CA, USA)
CD45RA	PE	2H4	Beckman Coulter (Brea, CA, USA)
CD45RO	FITC	UCHL1	DAKO (Carpinteria, CA, USA)
CD56	PE	C5.9	DAKO (Carpinteria, CA, USA)
FoxP3	PE	PCH101	eBioscience (San Diego, CA, USA)
IL-4	PE	MP4-25D2	BD Biosciences (San Jose, CA, USA)
IL17A	PE	64DEC17	eBioscience (San Diego, CA, USA)
IFNγ	APC	B27	BD Biosciences (San Jose, CA, USA)
TCRαβ	APC	H57-597	BD Biosciences (San Jose, CA, USA)
TCRγδ	PE	11FF2	BD Biosciences (San Jose, CA, USA)

Supplementary table II. Correlations between age, BMI and different immunological cell populations

		Leuko cytes	Granulo cytes	Lympho cytes	Mono cytes	NK cells	B cells	T cells	CD4 T cells	CD8 T cells
Age	r_S	-0.208	- 0.260		- 0.191	- 0.118	0.096	0.160	0.202	- 0.012
	p	0.392	0.282	0.728	0.433	0.630	0.695	0.514	0.407	0.960
BMI	r_S	0.235	0.066	0.371	0.087	- 0.321	0.262	0.499*	0.716†	0.086
	p	0.332	0.789	0.118	0.722	0.180	0.279	0.030	0.001	0.726

		CD4n	CD4td	CD4cm	CD4em	CD8n	CD8td	CD8cm	CD8em
Age	r_S	0.113	0.011	0.348	- 0.090	- 0.354	0.117	0.188	0.144
	p	0.645	0.963	0.144	0.714	0.137	0.633	0.441	0.557
BMI	r_S	0.485*	0.470*	0.529*	0.232	- 0.118	0.370	0.367	0.298
	p	0.035	0.043	0.020	0.340	0.632	0.119	0.122	0.215

		Treg	Th1	Th2	Th17
Age	r_S	0.419	0.253	0.524*	- 0.025
	p	0.074	0.296	0.021	0.920
BMI	r_S	0.728†	0.225	0.520*	- 0.332
	p	0.000	0.355	0.022	0.165

r_S = spearman correlation coefficient, * $p < 0.05$ (2-tailed), † $p < 0.01$ (2-tailed).

Supplementary table III. Correlations between age and BMI and plasma levels of IL-7 and CCL5

		Age	BMI	IL-7	CCL5
Age	r_S	1.000	0.416	0.150	0.458*
	p		0.076	0.551	0.049
BMI	r_S		1.000	0.662†	0.837†
	p			0.003	0.000
IL-7	r_S			1.000	0.693†
	p				0.001
CCL5	r_S				1.000
	p				

r_S = spearman correlation coefficient * $p < 0.05$ (2-tailed), † $p < 0.01$ (2-tailed).

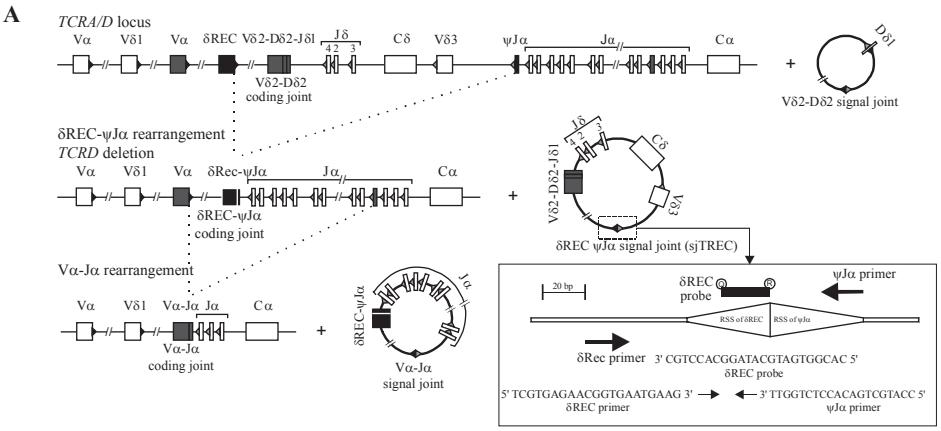
Supplementary table IV. Correlations between IL-7 and CCL5 plasma levels and different immunological cell populations

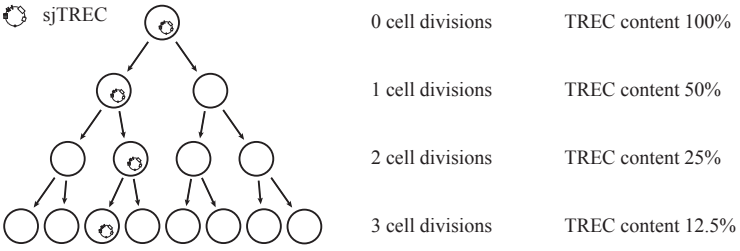
		Leuko cytes	Granulo cytes	Lympho cytes	Mono cytes	NK cells	B cells	T cells	CD4 T cells	CD8 T cells
IL-7	r_S	0.637†	0.459	0.447	0.344	- 0.042	0.375	0.657†	0.707†	0.265
	p	0.004	0.055	0.063	0.162	0.868	0.126	0.003	0.001	0.287
CCL5	r_S	0.348	0.218	0.286	0.172	- 0.368	0.105	0.585†	0.658†	0.288
	p	0.145	0.371	0.236	0.481	0.122	0.669	0.008	0.002	0.231

		CD4n	CD4td	CD4cm	CD4em	CD8n	CD8td	CD8cm	CD8em
IL-7	r_S	0.476*	0.593†	0.515*	0.368	0.106	0.418	0.302	0.354
	p	0.046	0.009	0.029	0.132	0.675	0.084	0.223	0.150
CCL5	r_S	0.400	0.561*	0.631†	0.432	- 0.107	0.376	0.454	0.515*
	p	0.090	0.012	0.004	0.065	0.664	0.112	0.051	0.024

		Treg	Th1	Th2	Th17
IL-7	r_S	0.593†	0.267	0.342	- 0.385
	p	0.009	0.284	0.165	0.115
CCL5	r_S	0.604†	0.339	0.426	- 0.299
	p	0.006	0.155	0.069	0.214

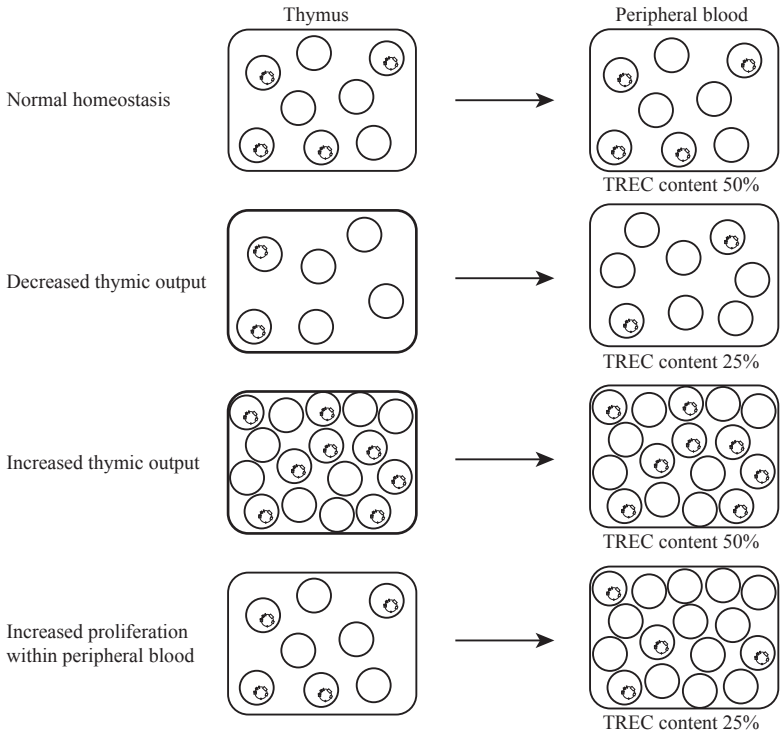
r_S = spearman correlation coefficient, * $p < 0.05$ (2-tailed), † $p < 0.01$ (2-tailed).



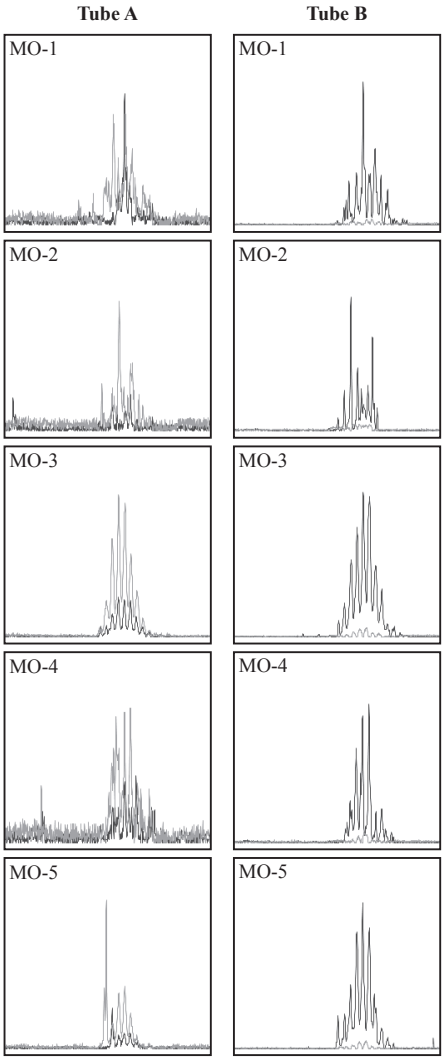


Supplementary figure I. (A) Schematic diagram of sequential rearrangements in the *TCRAD* locus. Before *TCRA* rearrangements occur within the TCRαβ lineage, the *TCRD* gene is deleted, preferably by the δREC-ψJα rearrangement. During this rearrangement the δREC-ψJα signal joint TREC (sjTREC) is formed. The deletion of the *TCRD* gene is immediately followed by *TCRA* (Vα-Jα) rearrangements. Position and sequence of the RQ-PCR primers and TaqMan probe for the detection and quantification of the δREC-ψJα signal joint TREC (sjTREC) are shown in the box. (B) Schematic diagram of the dilution of the sjTREC during consecutive cell divisions.

C



(C) Schematic diagram of the effects of changes in thymic output or proliferation on the TREC content in peripheral blood T cells.



Supplementary figure II. GeneScan analysis of Vβ-Jβ rearrangements in adipose tissue samples of five non-diabetic morbid obese subjects (BMI 44.9 ± 7.4 kg/m^2; Age 37.8 ± 3.4 years of age). DNA was extracted from frozen total adipose tissue samples using the GeneElute Mammalian Genomic DNA miniprep kit (Sigma-Aldrich). Tube A: Vβ+ Jβ1.1 to Jβ1.6+Jβ2.2+Jβ2.6+Jβ2.7; Tube B: Vβ+Jβ2.3+Jβ2.4+Jβ2.5. Primers for the Jβ1 cluster were HEX-labeled (green line), primers for the Jβ2 cluster were FAM-labeled (blue line). *See Chapter VII for full-color figure.*

Chapter VI

General discussion

Manuscript in preparation.

During the last century a large number of studies have demonstrated that complex interplay exists between the immune and the neuro-endocrine systems. This interplay, via shared cytokines, hormones and their respective receptors and nervous innervations, results in a highly organized integrated surveillance system capable of preserving homeostasis of the body to a large numbers of disturbances. Within this surveillance system the immune system recognizes external (viruses, bacteria, fungi, parasites) and internal (tissue damage) threats that might disturb homeostasis while the neuro-endocrine system recognizes internal threats (physical or emotional stress) that might disturb homeostasis. Ultimately, an integrated response of both systems will be initiated against both internal and external threats to preserve homeostasis of the body.[1-9]
The functional interplay between the immune and endocrine systems can be illustrated using an acute infection as example. Acute infections initiate tightly orchestrated immunological and endocrine responses with the aim to eradicate the pathogen. Recognition of a pathogen will primarily activate innate and adaptive immune responses to eliminate the pathogen. Additionally, cytokines such as IL-1β, IL-6 and TNF-α, produced by the activated immune system will stimulate the neuro-endocrine system resulting in adaptations in energy metabolism and behavior (sleep, appetite, malaise). Increased levels of hormones such as growth hormone (GH), oxytocin, leptin and adrenalin together with decreased levels of thyroid stimulating hormone (TSH) and gonadotrophin reduce energy expenditure and enhance catabolism, thereby providing energy to support the mounted immune response. Moreover, altered hormone levels within the neuro-endocrine system, of which GH, prolactin and glucocorticoids are well known examples, will directly affect the magnitude of the immune response to retain homeostasis. Higher GH and prolactin levels support the immune response, while increased glucocorticoid and vasopressin levels contribute to immune suppression in order to prevent extensive tissue damage.[2, 4, 6-8, 10-14]

As a result of this integration aberrations within the immune system will automatically affect the endocrine system, and vice versa. This is for example illustrated by the increased susceptibility to infections in patients with Cushing's disease.[15] Moreover, alterations in gonadal steroid hormones affect chronic inflammatory responses and contribute to an increased susceptibility of females for certain autoimmune diseases such as systemic lupus erythematosis (SLE), myasthenia gravis (MG) and rheumatoid arthritis (RA). Also, autoimmune disorders tend to get worse (SLE) or better (RA, Graves' disease; GD) during pregnancy.[3, 16]
In addition, abnormalities in the crosstalk between the immune and the neuro-endocrine system might cause diseases. Neuro-endocrine regulation of inflammation is thought to be aberrant in many chronic inflammatory diseases. For example, deficiencies or mutations of vasoactive intestinal peptide (VIP) or its receptor increase susceptibility to RA development and increase immune activation in SLE. Indeed VIP, but also cortistatin and ghrelin, can reduce disease severity in multiple experimental animal models of chronic inflammatory diseases including collagen-induced arthritis, inflammatory bowel disease, type I diabetes mellitus and multiple sclerosis.[1, 16, 17]

Despite the well-recognized neuro-endocrine-immune integration, more detailed understanding of the various interactions between the immune and the neuro-endocrine system is necessary. This will provide new insights in endocrine and immunological disorders and may lead to the development of new therapeutic strategies for both endocrine and immune mediated diseases.

This thesis provides new insights into two specific interactions that exist between T-cell development and the neuro-endocrine and metabolic systems. In **chapter II** a new technique to investigate intra-thymic proliferation, the Vγ-Jγ assay was developed and studied. In **chapters III and IV** of this thesis the effects of TSH on T-cell development in the thymus were investigated. In the **chapter V** of this thesis the focus was on aberrations in the peripheral T-cell compartment in obesity.

THYMIC FUNCTIONING

Detailed insight into thymic functioning is a prerequisite for understanding the contribution of neuro-endocrine-immune interactions to T-cell development. Therefore, the first part of this thesis, focused on the development of novel and reliable methods to measure thymic function.

Measurement of thymic functioning

Direct quantitative measurements of thymic functional output in humans are largely lacking, mostly due to the impossibility to obtain thymic tissue samples for analysis, in a clinical setting.[18] Therefore, several indirect assays to measure thymic function, have been developed.[19]
Indirect assays to estimate thymic function are performed on blood and most of them are based on distinguishing recent thymic emigrants (RTE) from mature naive T cells and other T-cell subpopulations. In mice, RTEs can be traced by intra-thymic labeling of thymocytes (e.g. with BrdU, CFSE or FITC) and subsequent label detection in the peripheral blood.[18, 19] In humans, it is recently proposed that RTEs can be recognized by several specific surface markers ($CD31^+$; $PTK7^+$; $CD8^+CD103^+$).[20-22]
Currently, the most commonly used RTE-based method to estimate thymic function in humans is T-cell-receptor excision circle (TREC) analysis.[18, 23] TRECs are stable circular DNA structures formed during TCR gene rearrangements,[24, 25] that do not replicate during cell division.[24, 26-28] In the classical δREC-ψJα TREC analysis, as initially developed by Van Dongen en coworkers, the presence of a specific TREC, the δREC-ψJα TREC formed early during *TCRA* rearrangements, is determined in peripheral blood T cells.[23, 27, 28] As the amount of δREC-ψJα TRECs will be higher in RTEs than in mature naive or memory T cells, higher TREC levels are used as an indicator for enhanced thymic output. RTE numbers are, however, not only determined by thymic output but also by peripheral T-cell proliferation.[23] Therefore, RTE quantification in peripheral blood more likely reflects a combination of thymic output and peripheral blood T-cell proliferation, rather than solely thymic output and thus thymic function.[23]
Several studies suggest that intra-thymic proliferation of developing thymocytes largely determines thymic output.[29, 30] Therefore, measurement of intra-thymic proliferation would be a good approach to determine thymic function. The ratio between the classical δREC-ψJα TREC and multiple βTRECs (formed during *TCRB* rearrangements) has been suggested to reflect intra-thymic proliferation.[30] The disadvantage of this approach is however that it does not allow quantitative evaluation of intra-thymic proliferation.
Quantitative measurements of T-cell proliferation in the thymus could provide valuable information

about proliferation during different stages of development and the impact of proliferation during certain stages of development on TCR repertoire. In contrast to the δREC-ψJα TREC/ βTRECs assay, the novel TREC-based method described in **chapter II** of this thesis allows true quantitative measurement of intra-thymic proliferation. This novel assay was designed according to the principles of the KREC assay[31] and is based on detection of the genomic Vγ-Jγ coding joint ($CJ_{Vγ\text{-}Jγ}$) and the TREC located Vγ-Jγ signal joint ($SJ_{Vγ\text{-}Jγ}$; therefore hereafter referred to as the Vγ-Jγ TREC assay) which are formed simultaneously early during T-cell development (DN3).[32] This approach allows determination of the number of cell divisions undergone based on the difference between $CJ_{Vγ\text{-}Jγ}$ and $SJ_{Vγ\text{-}Jγ}$ levels.[31] The relevance of the Vγ-Jγ TREC analysis to determine thymic output is, however, questionable.

It is well known that thymic function and output deteriorate with ageing.[33, 34] Interestingly, in **chapter II** thymocytes from aged thymic tissue (29-77 years of age) did not differ in number of cell divisions undergone compared to thymocytes from young thymic tissue (1-2 weeks of age). This data suggest that the reduced thymic output upon ageing does not result from diminished proliferation from the DN3 developmental stage onwards. Other mechanisms are therefore likely to contribute to the reduction in thymic output upon ageing; for example a diminished entry of progenitors into the thymus or increased apoptosis or reduced proliferation in the early DN1 or DN2 stages of development.[29, 35-37] It can be concluded that measurement of intra-thymic proliferation without taking into account processes such as thymic seeding (progenitor entrance) and apoptosis does not allow reliable measurement of thymic function. Therefore, at present the best method to determine thymic function in peripheral blood T cells is still the combination of δREC-ψJα TREC analysis and quantification of RTEs in naive T cells.

Intra-thymic and peripheral T-cell proliferation

Although Vγ-Jγ TREC analysis may not seem to give a reliable indication of all aspects of thymic function, it does provide a new tool enabling reliable quantitative investigation of human intra-thymic proliferation.
In **chapter II** it was demonstrated that in the human thymus maturation from the DN3 stages onwards encompasses ~6-8 cell divisions. Vγ-Jγ TREC analysis does not take into account the number of cell divisions that occurred in the DN1 and DN2 T-cell developmental stages. However it has been estimated that murine DN1 ($CD44^{+}CD25^{-}$) and DN2 ($CD44^{+}CD25^{+}$) developmental stages (the stages that resemble human DN1 and DN2 stages[32]) undergo at least 10 cell divisions.[38] Overall these data suggest that total intra-thymic proliferation of developing thymocytes in humans could consist of ~16-18 cell divisions (figure 1A-D).
In addition, Vγ-Jγ TREC analysis provides a tool to study the effects of intra-thymic proliferation on TCR diversity and clonal size. TCR diversity is increased by pre-TCR driven proliferation (DN3 - $DP3^{-}$ stages of development)[32] as cells that share the same TCRβ chain will each generate their own unique TCRα chain.[39] The study in **chapter II** demonstrates that ~4-5 cell divisions occur during pre-TCR driven proliferation (figure 1D), indicating that the TCRαβ repertoire will increase ~16-32 fold due to pre-TCR proliferation. These data are supported by a previous study that estimated that a TCRβ chain of a certain specificity must pair with at least 25 TCRα chains to explain the TCRαβ repertoire diversity that is found in the peripheral T-cell compartment.[40]

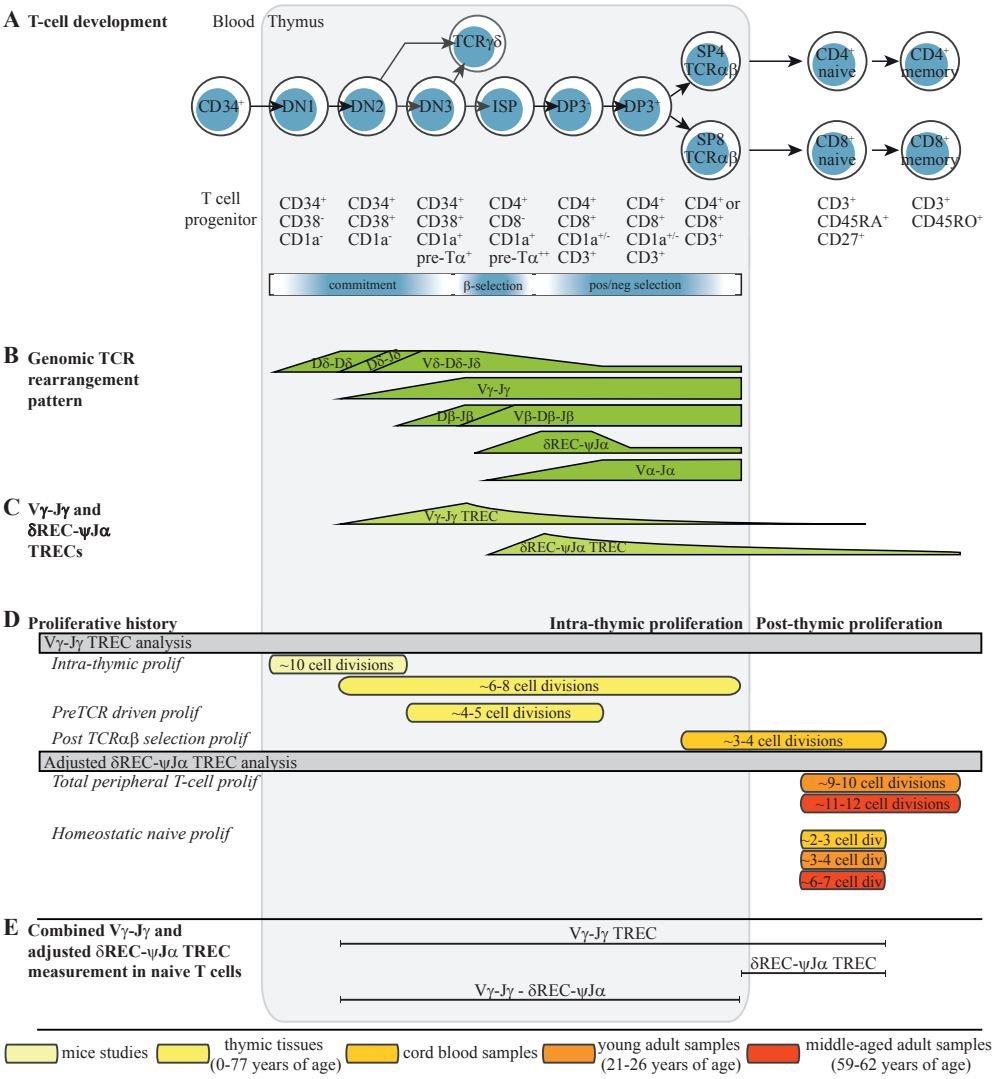


Figure 1. Proposed model of proliferation during human T-cell development. (A) Human T-cell developmental stages and (B) Genomic TCR rearrangement patterns, adapted from Dik et al.[32] (C) Intra-thymic and peripheral T-cell Vγ-Jγ and δREC-ψJα TREC dynamics. (D) Intra-thymic and post-thymic proliferation determined by Vγ-Jγ and adjusted δREC-ψJα TREC analysis on thymocyte and peripheral T-cell subpopulations for different age categories. (E) Schematic overview of measurement of intra-thymic and peripheral T-cell proliferation based on combined Vγ-Jγ and adjusted δREC-ψJα TREC analysis. DN: double negative thymocytes, ISP: immature single positive thymocytes, DP: double positive thymocytes, SP: single positive thymocytes, cell div: cell divisions, prolif: proliferation.

The total size of clonal naive T-cell populations is mainly determined by proliferation that occurs post TCRαβ selection (from $DP3^+$ stage of development onwards)[32] and by apoptosis. **Chapter II** demonstrated that ~3-4 cell divisions occur after TCRαβ selection (figure 1D), indicating that the average size of a population of clonal naive T cells in cord blood approaches a total of ~8-16 cells. These numbers are comparable to previous estimates of a clonal size of ~10-20 cells in human neonatal blood samples based on adjusted δREC-ψJα TREC analysis.[41] Also, in mouse, TCR repertoire sequencing of naive splenocytes predicted a clonal size of ~10 cells.[42]

Correct usage and interpretation of proliferation measurements using the Vγ-Jγ TREC analysis requires that one realizes that TREC assays are a population-based measurement, with results reflecting the average number of cell divisions for the total cell sample. Therefore, in heterogeneous populations (such as PBMCs or total T cells) alterations in composition may affect the results. Moreover, measurement of proliferation by Vγ-Jγ TREC analysis in peripheral blood samples is determined by both intra-thymic and peripheral blood T-cell proliferation. Recently, it has been demonstrated that in humans, in contrast to mice, T-cell maintenance largely depends on peripheral proliferation rather than on thymic output.[43] This is further illustrated by data from **chapter II** that demonstrate that naive T-cell populations in adults undergo ~6-7 cell divisions in peripheral blood (figure 1D). Therefore, especially in humans, Vγ-Jγ TREC analysis in naive T cells in blood will be largely affected by peripheral blood T-cell proliferation.
Intra-thymic and peripheral blood T-cell proliferation might however be distinguished from each other by combining Vγ-Jγ TREC analysis and adjusted δREC-ψJα TREC analysis. While Vγ-Jγ TREC analysis measures cell divisions from the DN3 stage of T-cell development onwards, the adjusted δREC-ψJα TREC analysis only measures cell divisions from the SP stages of T-cell development onwards (representing naive T-cell proliferation). Theoretically, the number of intra-thymic cell divisions can be calculated by subtracting the number of naive T-cell divisions measured with adjusted δREC-ψJα TREC analysis from the total number of intra-thymic and peripheral blood cell divisions measured by Vγ-Jγ TREC analysis (figure 1E).

However, as demonstrated in **chapter II**, due to the low expression of the $SJ_{V\gamma\text{-}J\gamma}$ it was impossible to use the Vγ-Jγ TREC analysis in peripheral blood T cells. As a consequence the current assay is not suitable for peripheral blood analysis. Therefore, a challenge for future research will be to increase sensitivity of the method to ensure usage in peripheral blood T cells, and thus making it a useful tool to measure intra-thymic and peripheral blood T-cell proliferation in a clinical setting (as will be discussed in more depth later in this chapter).
A likely possibility is to use simultaneous detection of multiple Vγ rearrangements to Jγ1.3/2.3 to increase the sensitivity, although inclusion of more proximal Vγ loci will increase the risk of ongoing rearrangements. The risk that this will hamper the assay, however, will be minimalized when only rearrangements to the Jγ1.3 and Jγ2.3 loci are measured, which are the most distal Jγ loci.

Clinical applications of the Vγ-Jγ TREC analysis

In a clinical setting, measurement of intra-thymic and peripheral blood T-cell proliferation by Vγ-Jγ and adjusted δREC-ψJα TREC analysis will be of importance in many conditions

with defects in T-cell development and/or the peripheral T-cell compartment, such as primary immunodeficiencies, viral infections (e.g. HIV or CMV), after hematopoietic stem cell transplantation, during corticosteroid treatment or even during physiological ageing.[44-46] In these conditions, analysis of intra-thymic and peripheral blood T-cell proliferation can generate new insights in pathogenesis.
Moreover, reconstitution of the T-cell compartment in the above mentioned conditions is essential to reduce the risk of opportunistic infections, reactivation of latent viruses, malignancy and the development of autoimmunity.[47-51] T-cell compartment reconstitution depends on both peripheral expansion and active thymopoiesis. Peripheral expansion leads to an increase in T-cell numbers without increasing TCR diversity, while thymopoiesis increases naive T-cell numbers, resulting in an increase in TCR diversity and thereby T-cell function. Renewed thymopoiesis is therefore essential for functional T-cell recovery.[52, 53] Measurement of intra-thymic and peripheral blood proliferation by combined Vγ-Jγ and δREC-ψJα TREC analysis may thus largely add in monitoring T-cell reconstitution, possibly providing information about the relative contribution of intra-thymic and peripheral homeostatic T-cell proliferation to reconstitution.

Concluding remarks

Overall, although it is questionable whether intra-thymic T-cell proliferation determined with the current version of the Vγ-Jγ TREC analysis can be used as a measure of thymic function, measurement of intra-thymic proliferation will clearly add much to our knowledge about human T-cell development in the thymus. Future challenges include increasing sensitivity of the Vγ-Jγ TREC analysis to a level that when combined with the adjusted δREC-ψJα TREC analysis in peripheral blood it can be used as a tool to investigate both intra-thymic and peripheral blood T-cell proliferation in a clinical setting.

EFFECTS OF TSHR SIGNALING ON T-CELL DEVELOPMENT

The TSHR was traditionally thought to be expressed exclusively within the pituitary and the follicular epithelial cells of the thyroid gland as the natural receptor for TSH, an important hormone within the hypothalamus-pituitary-thyroid axis regulating endocrine function of the thyroid gland.[54] However, several studies have now identified novel functions for TSH and TSHR signaling in various organ systems, including the immune system.[55-60] TSHR expression has been described in thymic tissue from rats,[61] and humans,[58, 62] however without identification of specific thymocyte subpopulations. **Chapter III** describes that the TSHR is present on developing thymocytes and that its activation contributes to T-cell development, mainly during the ISP and DP developmental stages (figure 2).

TSHR expression and signaling in T-cell development

TSH, the natural ligand for the TSHR is able to bind TSHR expressed by immune cells.[61, 63-65] **Chapter III** demonstrates the ability of TSH to bind thymocytes and to activate second messenger

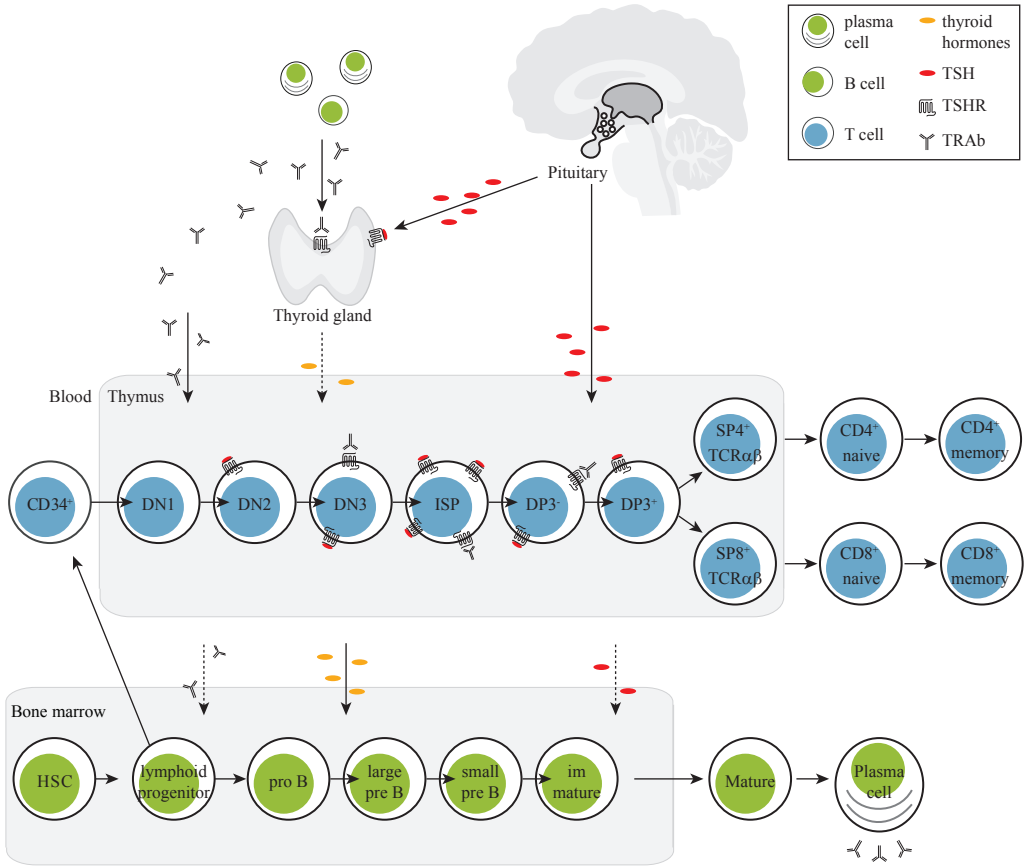


Figure 2. Proposed model of neuro-endocrine-immune interactions between the HPT-axis hormones TSH and thyroid hormones and T- and B-cell development. Pituitary derived TSH and TSHR antibodies formed during GD (TRAb) enhance T-cell development via the TSHR present on especially ISP and DP thymocytes. Thyroid hormones enhance both T-cell and B-cell development. DN: double negative thymocytes, ISP: immature single positive thymocytes, DP: double positive thymocytes, SP: single positive thymocytes, HSC: hematopoietic stem cell.

pathways, including the PIP_2/Ca^{2+} and the adenylate cyclase/cAMP transduction cascades, that are involved in TSHR signaling. Because the natural TSHR ligands, TSH and thyrostimulin, were not detected in thymic tissue (thymocytes and epithelial cells), pituitary-derived TSH most likely activates the TSHR expressed by developing thymocytes (figure 2). This is further supported by the observation that thymocytes were activated by TSH concentrations ranging from 0.1-1nM TSH (~15-150 mU/L), which are concentrations quite similar to healthy human serum concentrations.

TSHR signaling in the thyroid gland is necessary for normal thyrocyte functioning; TSH promotes proliferation and protects from apoptosis, and stimulates iodine uptake, protein iodination, thyroid hormone synthesis, and thyroid hormone secretion.[54, 66, 67] Evidence for local networks of HPT-axis hormones (TRH, TSH and T4) was found in the gut and the skin.[59, 65, 68] Despite the presence of TSHR on thymocytes, within the thymus no evidence was found for a local HPT-axis hormone network in **chapter III** of this thesis. TSH was not found to be produced locally and micro-array analyses of ISP thymocytes stimulated with TSH did not demonstrate increased expression of classical TSH target genes such as *NIS*, *TPO* or *Tg* (unpublished data) in thymocytes. TSH did however reduce expression of apoptosis-related genes in thymocytes and enhanced expression of genes related to proliferation (**chapter III**). Moreover, expression levels of genes that stimulate differentiation were increased. This suggests that TSHR signaling in thymocytes promotes thymocyte proliferation and differentiation and protects from apoptosis, which is in part comparable to its effects in thyrocytes.[54, 66, 67] TSHR is abundantly expressed during the ISP and DP stages of thymocyte development in which the processes of β-selection and *TCRA* rearrangements take place.[32] Therefore, also based on data from **chapter III** demonstrating more in-frame *TCRB* rearrangements in TSH-stimulated ISP thymocytes, it can be hypothesized that TSHR signaling is involved in preTCR-signaling and β-selection and/or subsequent induction of *TCRA* rearrangements.

Selective investigation of the effects of TSH on the immune system can be performed using *hyt/hyt* mice[69, 70] or TSHR KO mice.[71] When supplemented with thyroid hormone these mouse models enable to study the effects of lack of TSHR signaling under euthyroid conditions. Ultimately, by transplanting bone marrow cells incapable of TSHR signaling into a mouse strain with a normal HPT-axis the effects of lack of TSHR signaling within immune cells can be selectively investigated. The studies in **chapter III** show slightly delayed T-cell development in thyroid hormone supplemented *hyt/hyt* mice, while competitive transplantation experiments demonstrated that T cells expressing a functional TSHR had a slight developmental advantage over T cells that lack functional TSHR expression. In addition, activation of TSHR signaling enhanced T-cell development in two different types of *ex-vivo* human T-cell developmental systems, even in the presence of IL-7 and SCF, two cytokines crucially important for normal T-cell development.[72]

Additional insights into the *in vivo* role for TSHR signaling in the regulation of T-cell development in humans might be obtained from patients with thyroid diseases.
GD is a autoantibody-mediated autoimmune disease in which hyperthyroidism is caused by TSHR specific autoantibodies (TRAb) which mimic the effects of pituitary TSH.[73] Due to the presence of high levels of TSHR stimulating TRAb, this disease is an interesting model to investigate the effects of TSHR signaling on the T-cell compartment.
Both thymic and peripheral blood T-cell aberrations have indeed been described in GD. Within the peripheral blood, GD is mainly characterized by increased numbers of activated T cells as part of the autoimmune response,[74, 75] as confirmed by studies conducted in **chapter IV** of this thesis. Moreover, the thymus as well as other secondary lymphoid tissues can be increased in size in GD.[62, 76] This thymic hyperplasia is mainly characterized by increased amounts of developing thymocytes.[77]

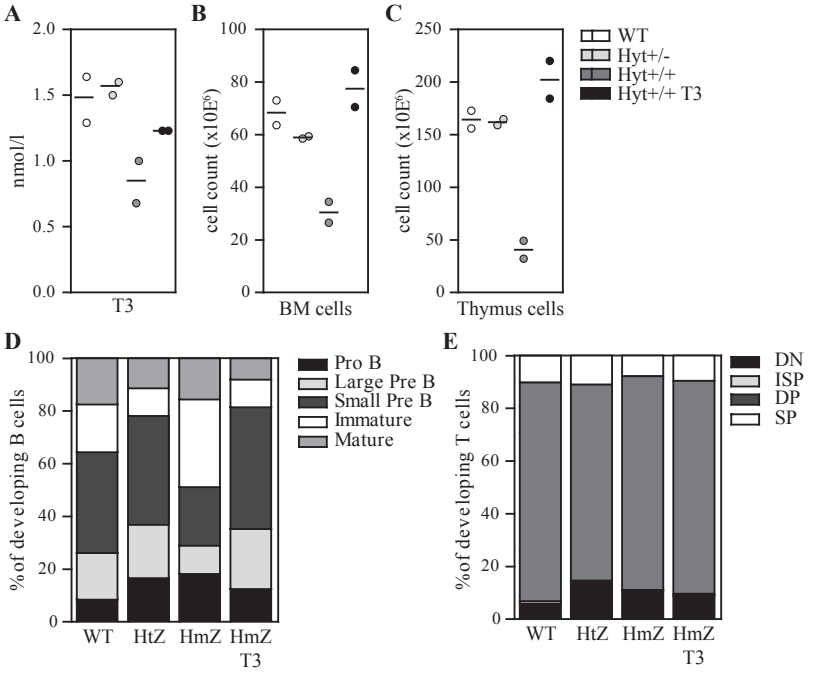


Figure 3. Thyroid hormones stimulate B- and T-cell development. (A) Serum T3 levels, (B) absolute BM cells counts, (C) absolute thymocyte cells counts, (D) B-cell progenitor composition in the BM and (E) T-cell progenitor (thymocyte) composition in the thymus in WT mice, *hyt*$^{+/-}$ mice, *hyt*$^{+/+}$ mice and T3 treated *hyt*$^{+/+}$ mice. WT mice: wild type mice, *hyt*$^{+/-}$ mice: heterozygous *hyt/hyt* mice, *hyt*$^{+/+}$ mice: homozygous *hyt/hyt* mice, BM: bone marrow, T3: 3,3',5-Triiodo-L-thyronine sodium salt.

Thymic hyperplasia

It has been suggested that a thyroid hormone induced rise of the thymic hormone thymulin might contribute to thymic hyperplasia.[78, 79] But increased thyroid hormone levels may also be directly involved in inducing thymic hyperplasia, similar to the effects of thyroid hormones on B-cell development as described in **chapter IV**. This is supported by the observation that exogenous thyroid hormone administration in mice increases thymocyte numbers in the thymus.[80] Also, hypothyroid *hyt*$^{+/+}$ mice (figure 3) and *T3Rα*$^{-/-}$ mice[81] have reduced thymocyte numbers which could be restored with thyroid hormone supplementation. Moreover, thyroidectomy reduces thymic weight.[62]

Interestingly, thymic hyperplasia is mainly seen in GD and less in other forms of hyperthyroidism,[82] suggesting that factors other than thyroid hormone contribute to thymic hyperplasia in GD. Based on the findings in **chapter III** it can be postulated that GD associated TRAb enhance T-cell development by activating TSHR signaling in developing thymocytes. In support of this, recent publications demonstrated that IgGs from GD patients are able to bind to thymocytes,[62, 77, 78] Moreover, in preliminary experiments, we observed that purified immunoglobulins from sera of some (but not all) GD patients were capable of enhancing T-cell development in the OP9DL1 co-

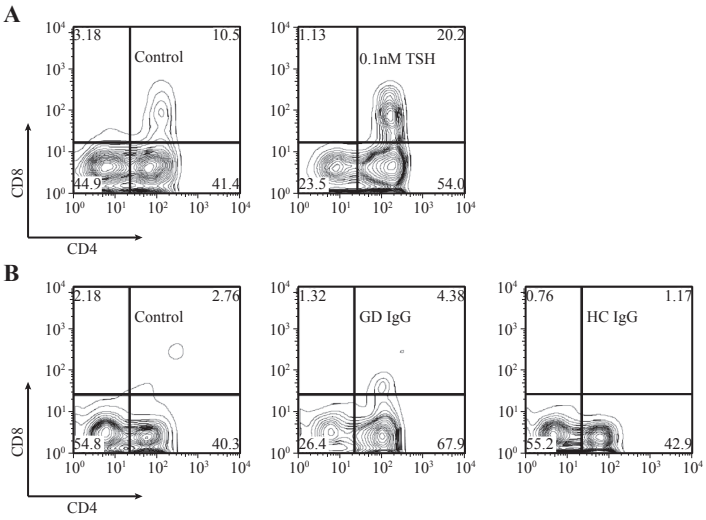


Figure 4. rhTSH and TRAb enhance human T-cell development in vitro. Sorted human $CD34^+$ thymocytes were cultures in T-cell development supporting systems in the presence of 0.1-10 nM rhTSH, 10% IgG from GD patients or vehicle for two to three weeks. Flow cytometric analysis of an OP9Dl1 experiment with (A) rhTSH and with (B) GD IgG. DN: double negative thymocytes ($CD4^-CD8^-$), ISP: immature single positive thymocytes ($CD4^+CD3^-$), DP: double positive thymocytes ($CD4^+CD8^+$), SP: single positive thymocytes ($CD4^+CD8^-$ or $CD4^-CD8^+$).

culture system, as effective as rhTSH (unpublished data; figure 4).

The presence of lymphoid thymic hyperplasia in GD suggests that thymic output might be increased, resulting in increased numbers of peripheral blood RTEs and naive T cells. Recently, an increase in naive $CD4^+CD45RA^+$ T cells and $CD4^+CD31^+$ RTEs has been described in euthyroid GD patients receiving anti-thyroid drug treatment.[83] Although a similar trend towards increased naive and $CD31^+$ T cells in GD patients receiving anti-thyroid drug therapy was observed in **chapter IV**, this trend did not exist in newly diagnosed GD patients. Moreover, no correlations existed between TRAb levels and naive $CD4^+CD45RA^+$ T cells or $CD4^+CD31^+$ RTEs. However, none of the patients in the cohort examined in **chapter IV** had signs of thymic hyperplasia as determined by I^{123} scan of the thymus (10 minutes and 24 hours after intake of I^{123}, as it was described that I^{123} is taken up by the hyperplastic thymus;[84-87]unpublished data). Therefore, it remains uncertain whether thymic hyperplasia in GD is associated with increased thymic output.

Although GD is a good model to investigate the effects of the *in vivo* role for TSHR signaling in the regulation of T-cell development, some drawbacks must be taken into account. Firstly, in GD, thyroid hormone and TRAb levels are increased. As both thyroid hormones and TRAb have been suggested to affect T-cell development, interpretation of the data is difficult. Moreover, GD is an autoantibody mediated autoimmune disorder in which T-cell compartment aberrations are part of the pathophysiology of the disease. Therefore, to selectively study the effects of TSH or TRAb on human T-cell development *in vivo* a different study design is needed. For such a study hypothyroid patients with no remnant thyroid function who are euthyroid due to treatment with

thyroid hormone would be most suitable. In this experimental set-up, the effects of controlled doses of TSH on T-cell development may be investigated under euthyroid conditions.

Clinical applications

All together, the data presented in **chapters III and IV** support a role for TSHR signaling in T-cell development, although under normal physiological conditions the contribution is difficult to estimate next to the effects of generally known regulators of T-cell development such as IL-7. Possibly, under normal physiological conditions TSHR signaling is more involved in fine tuning the regular developmental process than being a true driver.
It will be interesting to investigate the effects of TSH on T-cell development during, for example infections, as infections rapidly deplete DP thymocytes, which are highly susceptible to stress-induced apoptosis. Within the first hours following infection a rise in TSH levels can be seen.[88] Moreover, viral infection of the gut increases local TSH production that enhances the intestinal immune response[89] and PBMCs produce TSH upon stimulation with the T-cell mitogen staphylococcus enterotoxin-A (SEA).[65, 90-92] It is however unknown whether inflammation/ infection influences TSHR expression and signaling in thymocytes in the human thymus. Based on above data it could be argued that under pathological conditions such as infection and tissue damage increased systemic TSH levels and locally produced TSH might also enhance thymic T-cell development in order to enhance immunological responses.

In addition, factors that stimulate T-cell development are of special interest for the treatment of conditions associated with T-cell deficiencies such as primary immunodeficiencies, viral infections (e.g. HIV or CMV), after hematopoietic stem cell transplantation, during corticosteroid treatment or even during physiological ageing.[44-46] Despite treatment, longstanding T-cell deficiency is an important problem, and even with re-establishment of normal T-cell numbers, immune function may remain impaired due to restricted TCR diversity.[52] This T-cell deficiency leads to high morbidity and mortality rates due to a high incidence of opportunistic infections like CMV and EBV, reactivation of latent viruses,[47, 48] and the association with malignancies[50, 51] and autoimmunity.[49]
Currently, several strategies to enhance T-cell reconstitution are being developed. Treatment with IL-7 was one of the first recognized potential strategies to enhance T-cell reconstitution. Although IL-7 treatment resulted in T-cell pool expansion, this was almost completely due to enhanced peripheral T-cell expansion rather than enhanced thymopoiesis, both in mice and humans.[93, 94] Alternative strategies such as administration of Fms-like tyrosine kinase-3 (Flt-3) ligand and keratinocyte growth factor (KGF) exhibited more profound effects on thymopoiesis in mice.[95-97]
Recently, several hormonal therapies were investigated as well. GH was found to enhance both thymic function and peripheral T-cell proliferation as demonstrated by the presence of thymic hyperplasia and increased T-cell numbers. But GH did not significantly increase $TREC^+$ naive T cells.[98] Chemical castration with luteinizing hormone releasing hormone (LHRH) agonists in patients undergoing hematopoietic stem cell transplantation (HSCT) did result in increased $TREC^+$ naive T cells and increased TCR diversity.[99]
Based on the studies in **chapter III and IV**, it can be hypothesised that TSH (rhTSH or a TSH-analogue) could also be a new therapeutic modality to enhance T-cell reconstitution. A

theoretical advantage of TSH is the selective expression of the TSHR on developing thymocytes, suggesting selective support of thymopoiesis while large effects on peripheral T cells are not expected. Moreover, anticipated side effects of TSH are limited to the thyroid gland, mainly being hyperthyroidism. Such effects of TSH on the thyroid gland can be blocked relatively easily by treatment with anti-thyroid drugs, which have a long history of clinical use. Such drugs may therefore overcome the disadvantages associated with the use of TSH-analogues to improve thymic function.

Concluding remarks

Above described data support the presence of a complex neuro-endocrine-immune network between hormones of the HPT-axis and B- and T-cell development. Pituitary derived TSH and TRAb seem to enhance T-cell development via the TSHR present mainly on ISP and DP thymocytes. Moreover, thyroid hormones might also increase thymocyte numbers. Within the bone marrow, thyroid hormones largely affect B-cell development, while effects of TSH and TRAb are less well known (figure 2). The presence of this network seems to be important especially in hypothyroid and hyperthyroid states as well as during infections. Further studies could investigate the efficiency of TSH to enhance T-cell development in T-cell deficient states.

THE PERIPHERAL BLOOD T-CELL COMPARTMENT IN OBESITY

Obesity is generally considered as a metabolic/endocrine disease, but immunological aberrations have been found as well, contributing to the development of obesity as well as its co-morbidities.[100, 101] Immune cells such as macrophages, eosinophils, some T cells and B cells are normally present in adipose tissue where they are mainly in an anti-inflammatory state and are thought to be involved in immune surveillance and regeneration.[102-105] Obesity is characterized by infiltration of additional immune cells, including macrophages, T cells, B cells and mast cells into adipose tissue. Both resident and the newly infiltrated immune cells within the obese adipose tissue exhibit a more activated and pro-inflammatory state. This is reflected by skewing of macrophages from an anti-inflammatory M2 phenotype towards the more pro-inflammatory M1 phenotype.[104] Also T-cell skewing towards pro-inflammatory Th1 and Th17 phenotypes occurs in obese adipose tissue.[104] In line with this, the production of especially pro-inflammatory cytokines (e.g. IL-1β, IL-6, TNF-α) is largely increased in adipose tissue (figure 5A-B).[106, 107]

During the last decade, research on the pro-inflammatory state of adipose tissue mainly focused on the balance between M1 and M2 macrophages,[102] but nowadays T cells are considered important participants in the initiation of adipose tissue inflammation.[104] T-cell accumulation in obese adipose tissue is rapid and even precedes macrophage accumulation.[108, 109] Moreover, T-cell depletion reduces adipose tissue macrophage accumulation and improves insulin sensitivity in high fat diet (HFD) fed mice.[108, 110] Interestingly, elevated T-cell numbers, mainly $CD4^+$ T cells, have also been observed in peripheral blood during the development of obesity, which correlated positively with BMI.[111-114] Although decreased numbers of both $CD4^+$ and $CD8^+$ T cells have been

described as well.[115] The data in **chapter V** show that peripheral blood from morbid obese subjects contains increased numbers of naive and memory $CD4^+$ T cells, while $CD8^+$ T-cell numbers are within the normal range.

Increased peripheral blood T-cell proliferation in obesity

The decreased δREC-ψJα TREC content in both naive en memory $CD4^+$ T-cell subpopulations of morbid obese subjects (**chapter V)**, suggests that increased proliferation rather than reduced apoptosis or increased thymic output accounts for the increase in $CD4^+$ T cells numbers in peripheral blood. In line with this notion is the observation of shortened telomere length in leukocytes of obese subjects.[116]
The polyclonal *TCRB* repertoire in both peripheral blood T cells and adipose tissue T cells from morbid obese subjects (**chapter V**) suggests that the increased peripheral blood T-cell proliferation in morbid obese subjects is of homeostatic nature instead of being driven by dominant antigens. This latter type of proliferation would have been associated with prominent skewing of the *TCRB* repertoire and an increase in memory and effector T cells, but without affecting naive T-cell numbers. Other studies did however find a limited *TCRB* repertoire within adipose tissue T cells, and therefore do suggest an immune response towards dominant antigens within obese adipose tissue.[108, 110, 117] However, the finding that the T-cell mitogens IL-7 and CCL5 are increased in peripheral blood of morbid obese subjects which correlated positively with peripheral blood $CD4^+$ T-cell numbers (**chapter V**) again supports homeostatic non-antigen dependent expansion of the T-cell pool. Moreover, several other T-cell mitogenic factors, such as adipokines, fatty acids or bacterial products, have been found to be elevated in plasma of morbid obese subjects, and can enhance homeostatic proliferation as well.[118-120]
$CD8^+$ T cells also displayed decreased δREC-ψJα TREC content (**chapter V)** indicating increased homeostatic proliferation of these cells in morbid obese subjects as well. Peripheral blood $CD8^+$ T-cell numbers were, however, not increased (**chapter V**) and it has been described that especially $CD8^+$ T cells accumulate in obese adipose tissue.[108, 121] This data suggest selective redistribution of $CD8^+$ T cells during obesity into other body compartments most likely being adipose tissue (figure 5B). What factors would specifically recruit $CD8^+$ T cells into adipose tissue in unclear so far.

Th1/Th2 skewing in peripheral blood in obesity

The adipose tissue T-cell compartment in obesity consists mainly of $CD8^+$ T effector and memory cells and Th1 cells[108, 121-124] with decreased percentages of Th2 cells and Treg cells.[105, 108, 110] One study found increased percentages of Treg and Th2 cells in adipose tissue from non-diabetic obese subjects.[125] Despite the fact that adipose tissue studies suggest T-cell involvement in obesity, the peripheral blood T-cell compartment is so far hardly studied in obesity. The studies in **chapter V** show that the increase in $CD4^+$ T cells is accompanied by a skewing towards a Treg and Th2 dominated phenotype. This suggests that the peripheral blood T-cell compartment in morbid obese patients is skewed towards an anti-inflammatory set point. Importantly, the study cohort in **chapter V** contained non-diabetic obese subjects that were also free of other co-

morbidities, thus being relatively healthy. This is important as other studies found that obesity associated with diabetes mellitus type II (DM-II) or impaired glucose tolerance is characterized by a predominance of Th1 cells in peripheral blood.[126-128] As described above, similar alterations were found within adipose tissue with Th1/Th17 skewing in most studies[108, 121-124] but Th2/Treg skewing in a study with only non-diabetic obese subjects.[125] Therefore, it is tempting to speculate that T-cell skewing away from the anti-inflammatory Treg and Th2 dominated phenotype towards a more pro-inflammatory state dominated by Th1 or Th17 cells may reflect or even mediate the development of co-morbidities such as DM-II or atherosclerosis in morbid obese subjects (figure 5A-C). This hypothesis is further supported by studies demonstrating that atherosclerosis, which frequently occurs during morbid obesity, is characterized by accumulation of Th1 cells within the plaques.[129] Also, depletion of $CD4^+$ T cells has been shown to delay the development of atherosclerosis and DM-II in experimental mouse models.[108, 110, 130] Furthermore, in **chapter V**, $CD4^+$ T-cell numbers in the non-diabetic cohort correlated positively with insulin sensitivity (measured as glucose/insulin ratio), while the increased Th1/Th2 ratio found in one study with a largely diabetic cohort[126] correlated positively with insulin resistance (measured by homeostasis model assessment index; HOMA-IR).

Interestingly, both leptin and insulin have profound effects on T-cell skewing. While insulin supports anti-inflammatory Th2 cell skewing,[131] leptin enhances differentiation of pro-inflammatory Th1 cells.[132] Obesity is associated with increased plasma levels of both insulin and leptin and the development of insulin and leptin resistance in many tissues.[101, 133] Therefore, altered insulin or leptin levels or altered sensitivity of T cells to insulin or leptin may be involved in obesity associated Th-cell differentiation. Whether T cells can become resistant to insulin and/or leptin has hardly been studied so far, but a recent study demonstrated that systemic insulin resistance is associated with inability of insulin to induce Th2 cell development.[126] This does suggest that T cells in obese subjects can indeed become insulin resistant. Therefore, it can be hypothesized that changes in insulin and/or leptin plasma levels or resistance might affect Th-cell differentiation. It could be argued that leptin resistance in obese non-diabetic subjects would allow Th2 skewing in response to insulin, while the subsequent development of insulin resistance and ultimately DM-II would again counteract this skewing, ultimately resulting in a Th1/Th17 skewed proinflammatory T-cell compartment in obese diabetic subjects.

Clinical applications

Collectively, the data described above suggest that Th-cell differentiation analysis in peripheral blood might be a clinically useful test to identify patients at risk for developing obesity related co-morbidity. Longitudinal studies in morbid obese subjects will, therefore, be important to further investigate this hypothesis. Such studies should correlate changes in Th-cell composition in peripheral blood with the development of obesity-related co-morbidities as well as with plasma levels of insulin and leptin. Moreover, simultaneous collection of peripheral blood T cells and adipose tissue T cells will be important to investigate whether changes found in peripheral blood directly relate to changes within adipose tissue, for example whether decreased $CD8^+$ T-cell numbers in peripheral blood correlate with increased $CD8^+$ T-cell numbers in adipose tissue and whether Th-cell differentiation in peripheral blood and adipose tissue correlate.

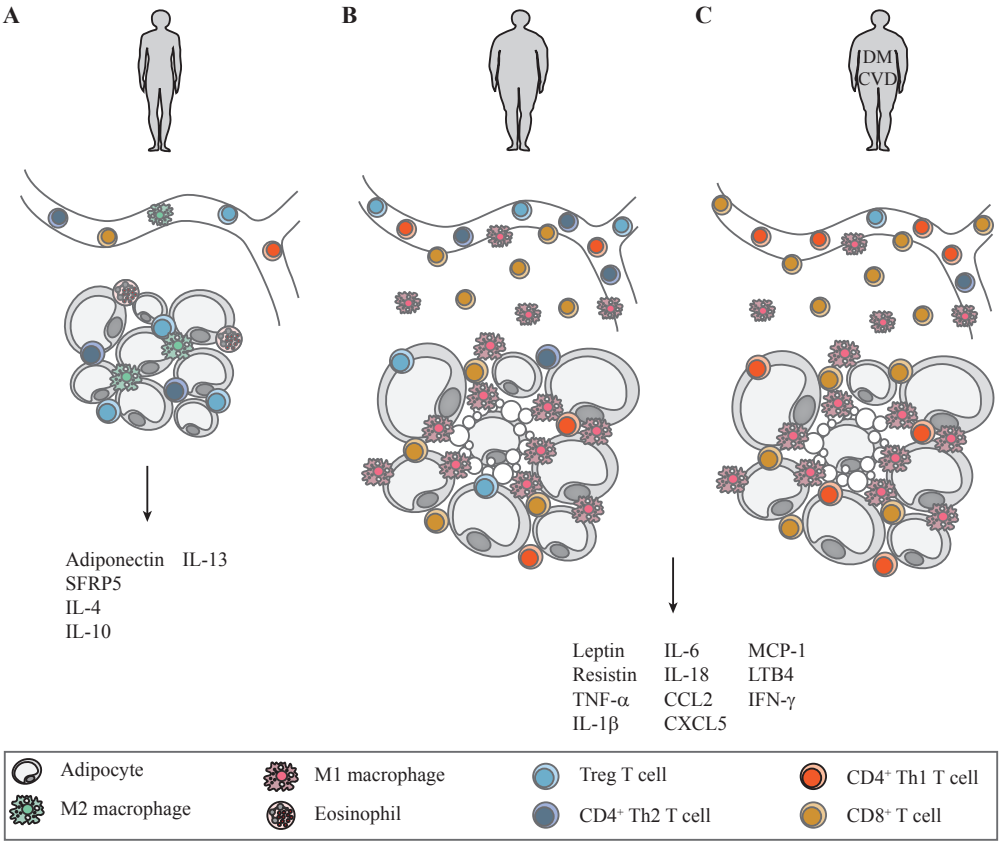


Figure 5. Proposed model of alterations in the peripheral blood and adipose tissue T-cell department during obesity and development of co-morbidities. The development of obesity is associated with increased Th2 and Treg cells in peripheral blood, $CD8^+$ T cells in adipose tissue and increased production of pro-inflammatory cytokines. The development of co-morbidities is associated with an additional skewing towards a more pro-inflammatory Th1/Th17 set point in peripheral blood. DM: diabetes mellitus type II, CVD: cardiovascular disease.

In addition, knowledge of immunological aberrations in obesity might allow the development of new treatment modalities for obesity and its co-morbidities. Although obesity is traditionally thought to be a metabolic disorder, the state of chronic low-grade inflammation present in obesity is now recognized as crucial in the development of co-morbidities such as diabetes[100, 101] and atherosclerosis.[134] Moreover, obesity is associated with the development of several autoimmune disorders (e.g. RA, psoriasis, asthma, autoimmune thyroid disease and SLE),[135-138] increased susceptibility to a broad spectrum of infectious diseases[135, 139, 140] and an increased frequency of certain forms of cancer.[141] Also here, the state of chronic low-grade inflammation is thought to be crucial.[142] Therefore, next to weight reduction strategies, still the golden standard for treatment of obesity and prevention of co-morbidities,[143] immune-modulatory therapies could be useful as

novel treatment modalities to delay or even prevent obesity associated co-morbidities.
Some immune-modulatory treatments have been investigated in clinical trials, mostly in diabetic patients. TNF-α blockers, peroxisome proliferator-activated receptor agonists (PPAR) α and γ and non-steroidal anti-inflammatory drugs (NSAIDs) all slightly enhanced insulin sensitivity in diabetic subjects, but overall effects were small. Moreover, IL-1β receptor antagonists reduced CRP and IL-6 levels and NSAIDs increased IL-4 and IL-10 levels, suggesting modulatory effects on inflammation.[144]
Based on data in **chapter V** and above described literature, targeting of T cells might be considered as a novel treatment modality. It has already been demonstrated in diet induced obesity (DIO) mice that lack of $CD8^+$ T cells (after anti-CD8 treatment or in $CD8^+$ T-cell deficient mice) decreased production of IL-6 and TNF-α and reduced M1 macrophage infiltration in adipose tissue.[108] Moreover, Anti-CD3 treatment in DIO mice reduced insulin resistance.[110] On the other hand macrophage accumulation still occurred in adipose tissue from DIO *RAG2*$^{-/-}$ mice.[109] Therefore, further studies are necessary to investigate the role for immune-modulatory therapies in diabetic patients, but importantly also in obese subjects.

Concluding remarks

Obesity is associated with clear immunological aberrations.[100, 101] The obese adipose tissue T-cell compartment consists mainly of $CD8^+$ T cells and Th1 $CD4^+$ T cells.[108, 121-124] Within peripheral blood, the T-cell compartment has been described as rather anti-inflammatory with Th2/Treg skewing in metabolically relatively healthy obese subjects (**chapter V**) and more pro-inflammatory with Th1/Th17 T-cell skewing in diabetic obese subjects.[126-128] Additional longitudinal studies are needed to investigate whether changes in T-cell set point in peripheral blood of obese subjects are indeed indicative or even responsible for the development of co-morbidities such as atherosclerosis or DM-II in morbid obese subjects. Moreover, future studies could investigate the role of immunomodulatory therapies for the prevention of obesity associated co-morbidities.

OVERALL CONCLUDING REMARKS

In conclusion, the studies in this thesis illustrate that important immune-neuro-endocrine and immune-metabolic interactions exist. **Chapter III and IV** of this thesis demonstrate the presence of interactions between T-cell development in the thymus and hormones from the HPT-axis in which TSH stimulates T-cell development *in vitro*. **Chapter V** demonstrates the presence of T-cell aberrations during obesity which could be markers or even part of pathogenesis of obesity and associated co-morbidities.
These examples support that detailed knowledge of these interactions might not only enhance our knowledge of the pathogenesis of immune and endocrine disorders, but might also provide a whole new range of therapeutic modalities including hormonal therapies in immune disorders such as hormonal stimulation of T-cell development in T-cell deficiencies and immunomodulatory therapies in endocrine disorders such as anti-inflammatory treatment in obesity to reduce the risk of development of co-morbidities.

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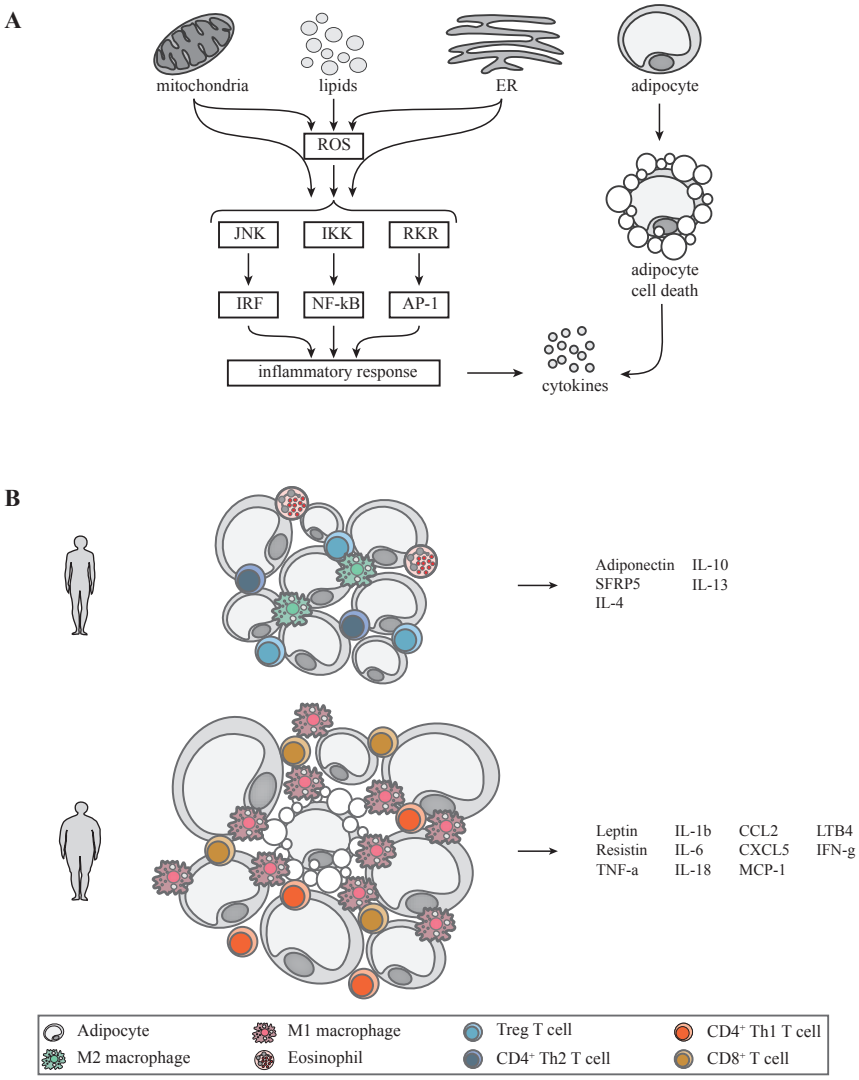
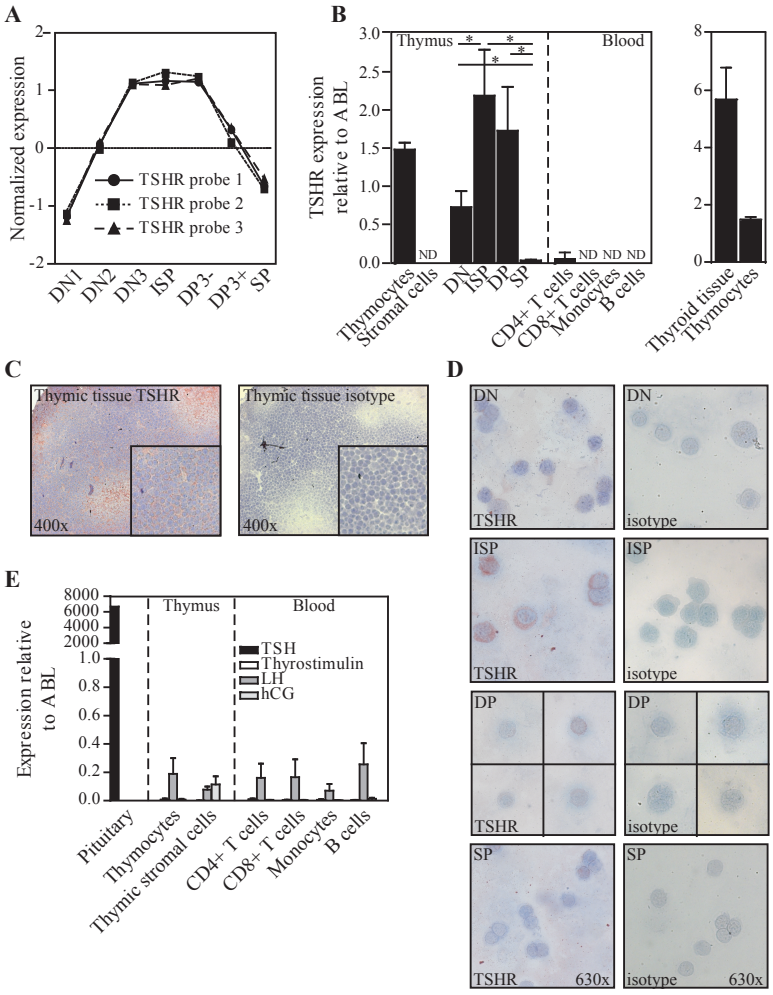
Chapter VII

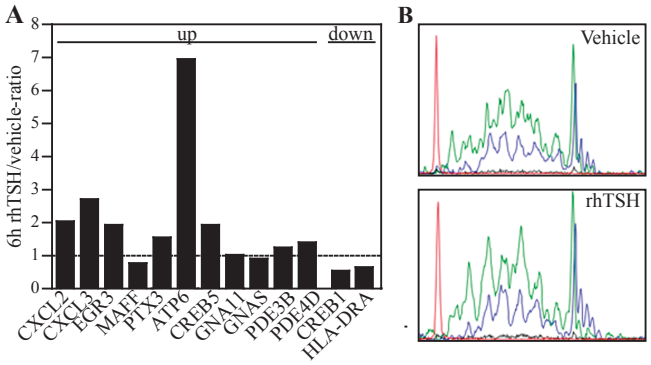
Full-color figures



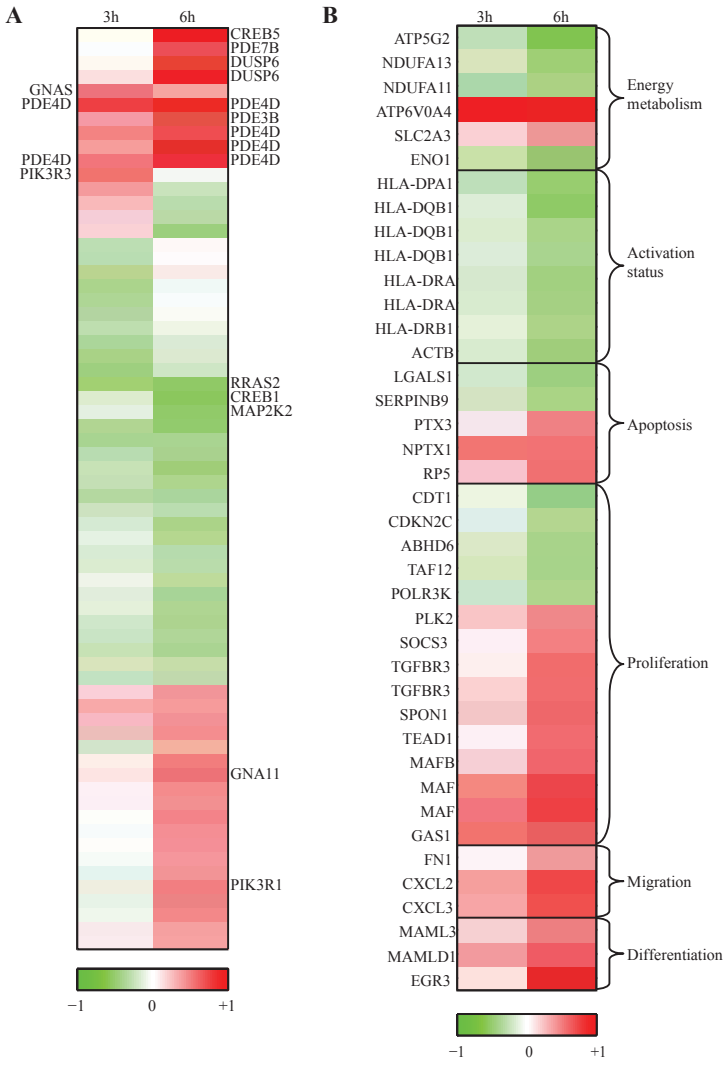
Chapter I - Figure 5. Schematic overview of the different pathways that are activated by increased fat deposition in adipose tissue resulting in a pro-inflammatory microenvironment. (A) Oxidative stress, increased levels of lipid production and ER stress result in ROS production, which will via several molecular pathways result in the activation of inflammatory responses. Moreover adipocyte cell death will stimulate cytokine production and immune cell recruitment. (B) Alterations in the adipose tissue immune compartment and adipokine production during the development of obesity.



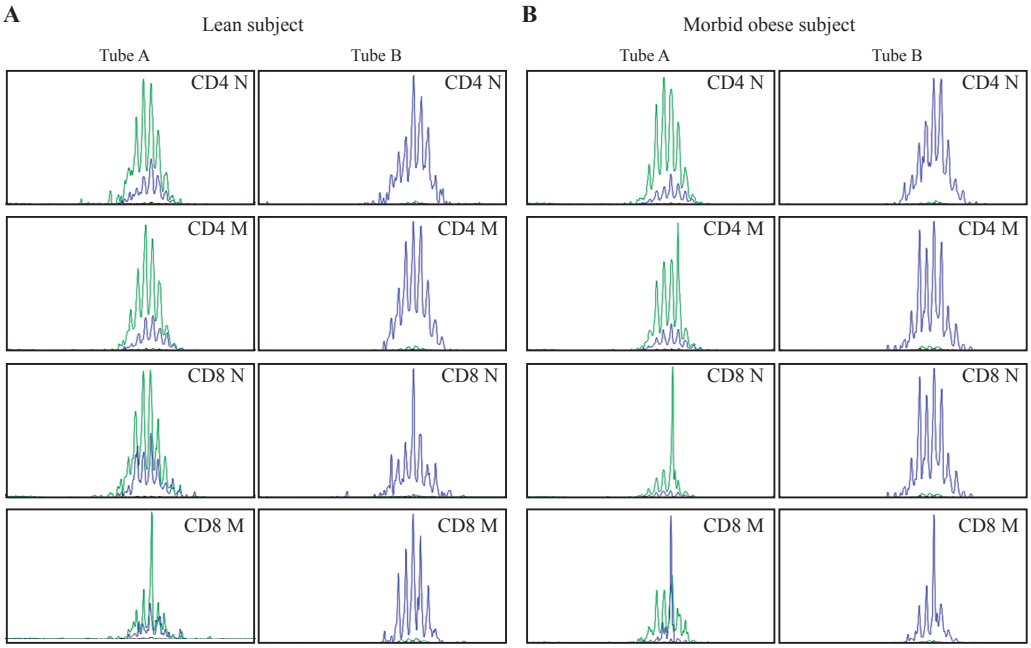
Chapter III - Figure 1. The TSHR is selectively expressed on thymocytes subsets, without expression on peripheral mononuclear blood cells. (A) Expression of the TSHR in human thymocyte subsets using microarray analysis. (B) TSHR mRNA expression relative to ABL mRNA in human thymocytes, CD45$^-$ thymic stromal cells, thymocyte subsets and PBMCs (left panel) and thyroid tissue (right panel); RQ-PCR, data are shown as mean ± SEM from three or more independent samples. (C) TSHR immunohistochemistry staining of thymic tissue using a TSHR specific antibody or isotype control; data are from a representative experiment. Shown at x400 original magnification. (D) TSHR immunohistochemistry staining of cytospins of sorted thymocyte subsets using a TSHR specific antibody or isotype control; data are from a representative experiment. Shown at x630 original magnification. (E) Expression of TSH, thyrostimulin, LH and hCG mRNA relative to ABL mRNA in human thymocytes, CD45$^-$ thymic stromal cells and PBMCs; RQ-PCR, data are shown as mean ± SEM from three independent samples. DN: double negative thymocytes (CD4$^-$CD8$^-$), ISP: immature single positive thymocytes (CD4$^+$CD3$^-$), DP: double positive thymocytes (CD4$^+$CD8$^+$), SP: single positive thymocytes (CD4$^+$CD8$^-$ or CD4$^-$CD8$^+$), LH: luteinizing hormone, hCG: human chorionic gonadotrofin, ND: not detected. * significance (2-tailed) $p<0.05$.



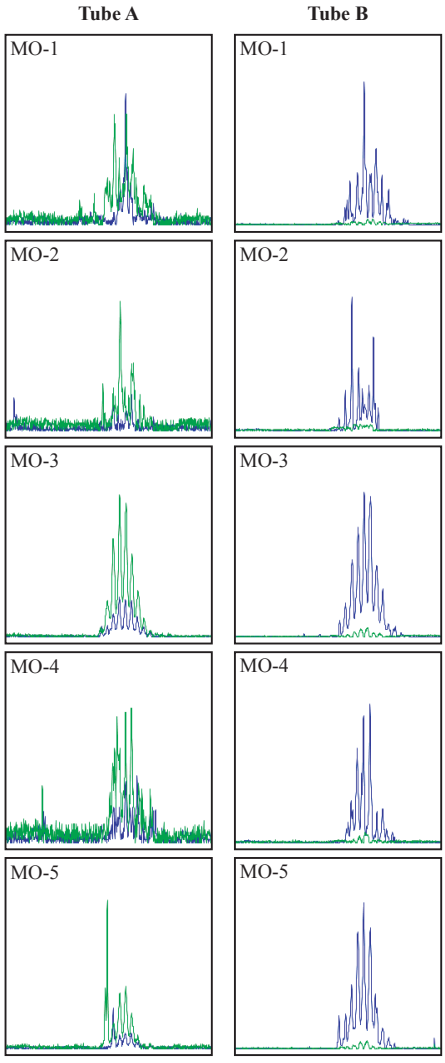
Chapter III - Figure 3. Induction of TSHR signaling in ISP thymocytes induces changes in expression of genes involved in T-cell development. (A) RQ-PCR analysis of several genes that were found to be differentially expressed in the microarray analysis in flow-sorted ISP thymocytes that were cultured for three to six hours in the presence of 1 nM rhTSH and vehicle, n=2. (B) GeneScan analysis of Vβ-Jβ rearrangements (Vβ+Jβ1.1 t/m Jβ1.6+Jβ2.2+Jβ2.6+Jβ2.7) in flow-sorted ISP thymocytes that were cultured for three to six hours in the presence of 1 nM rhTSH and vehicle; Primers for the Jβ1 cluster were HEX-labeled (green line), primers for the Jβ2 cluster were FAM-labeled (blue line). Note the higher peaks in rhTSH treated thymocytes at a 3 nucleotide distance.



Chapter III - Supplementary figure I. TSH stimulation of ISPs activates TSHR signaling pathways and induces changes in expression of genes involved in T-cell development. Microarray analysis of flow-sorted ISP thymocytes which were cultured for three to six hours in the presence of 1 nM rhTSH or vehicle. (A) Heatmap showing log2-transformed expression ratio (rhTSH/vehicle) of the 65 genes involved in G-protein-coupled-receptor signaling which were most differentially expressed after three and six hours of culture. Gene names demonstrate the genes that were more than 1.5-fold differentially expressed. Genes were selected using Ingenuity Software. (B) Heatmap of selected genes with a more than 2-fold different expression levels in rhTSH treated thymocytes compared to vehicle. Genes involved in different processes were categorized, but can be inhibiting or stimulating these processes.



Chapter V - Figure 4. GeneScan analysis of Vβ-Jβ rearrangements in $CD4^+$ and $CD8^+$ naive and memory T-cell subpopulations in a representative (A) lean and (B) morbid obese subject. Tube A: Vβ+ Jβ1.1 to Jβ1.6+Jβ2.2+Jβ2.6+Jβ2.7; Tube B: Vβ+Jβ2.3+Jβ2.4+Jβ2.5; Primers for the Jβ1 cluster were HEX-labeled (green line), primers for the Jβ2 cluster were FAM-labeled (blue line). CD4 N: $CD4^+$ naive T cells, CD4 M: $CD4^+$ memory T cells, CD8 N: $CD8^+$ naive T cells, CD8 M: $CD8^+$ memory T cells. Lean n=4 (BMI 23.6 ± 1.0 kg/m^2); morbid obese n=5 (BMI 41.6 ± 5.1 kg/m^2).



Chapter V - Supplementary figure II. GeneScan analysis of Vβ-Jβ rearrangements in adipose tissue samples of five non-diabetic morbid obese subjects (BMI 44.9 ± 7.4 kg/m^2; Age 37.8 ± 3.4 years of age). DNA was extracted from frozen total adipose tissue samples using the GeneElute Mammalian Genomic DNA miniprep kit (Sigma-Aldrich). Tube A: Vβ+ Jβ1.1 to Jβ1.6+Jβ2.2+Jβ2.6+Jβ2.7; Tube B: Vβ+Jβ2.3+Jβ2.4+Jβ2.5. Primers for the Jβ1 cluster were HEX-labeled (green line), primers for the Jβ2 cluster were FAM-labeled (blue line).

Chapter VIII

Summary
Samenvatting

SUMMARY

All cells of the immune system develop from hematopoietic stem cells. Most immune cells undergo their major differentiation steps in the bone marrow, except for T cells. T cells develop from bone-marrow derived precursors in the thymus, a specialized organ that provides a unique microenvironment that supports the complex processes required for T-cell development including lineage restriction and specification, lineage commitment, (pre)TCR formation, positive and negative selection and thymic emigration.

T-cell proliferation is crucial for formation of the T-cell compartment in the thymus as well as maintenance of the human T-cell compartment in peripheral blood. T-cell proliferation largely determines TCR repertoire diversity, clonal size, peripheral blood T-cell numbers and antigenic response size. Quantitative measurements of intra-thymic and post-thymic proliferation, however, are currently not available.
In **chapter II** of this thesis, a new technique to investigate intra-thymic and peripheral T-cell proliferation, the Vγ-Jγ TREC assay, was developed and studied. Vγ-Jγ TREC analysis combined with adjusted δREC-ψJα analysis demonstrated that human thymocytes, independent of age, undergo ~6-8 intra-thymic cell divisions from the double negative (DN) 3 developmental stage onwards. Additional calculations illustrated the importance of T-cell proliferation for TCR repertoire diversity and clonal size. Moreover, the importance of homeostatic T-cell proliferation in peripheral blood in the maintenance of the peripheral T-cell compartment was emphasized by the observation that naive T cells in cord blood already displayed post-thymic homeostatic cell divisions. In addition, the number of homeostatic cell divisions in naive T cells increased substantially with ageing.
In a clinical setting measurement of intra-thymic and peripheral blood T-cell proliferation could provide valuable information about T-cell compartment physiology and about pathophysiology in conditions associated with T-cell deficiencies. Moreover, these measurements could be used in monitoring T-cell reconstitution.

All processes important for T-cell development and maintenance such as differentiation, survival and proliferation, are strictly regulated by a variety of extracellular signals, such as growth factors, cytokines, chemokines and adhesion molecules as well as specific transcription factors. Although many of these stimuli are produced by the developing cells themselves and the microenvironment, factors of extra-thymic origin have been demonstrated to affect T-cell development as well. There is growing evidence that the neuro-endocrine and metabolic systems significantly affect immune system development and function.

The neuro-endocrine and immune systems are tightly interconnected via shared cytokines, hormones, receptors and nervous innervations. The resulting highly organized surveillance system is thought to support the induction of coordinated responses to internal and external danger to maintain homeostasis of the body. Well recognized examples of neuro-endocrine-immune interactions include the depletion of DP thymocytes upon corticosteroid treatment and the increased susceptibility of women to certain autoimmune diseases such as systemic lupus erythematosis, myasthenia gravis and rheumatoid arthritis, but many other regulatory pathways

likely exist. In this thesis, interactions between hormones of the hypothalamus-pituitary-thyroid axis and B- and T-cell development were examined.
Microarray analyses of thymocyte subpopulations demonstrated the expression of several hormone receptors on thymocytes, suggesting a role for their ligands in T-cell development. One of these receptors was the TSHR, the receptor for TSH, a hormone mainly involved in the hypothalamus-pituitary-thyroid axis. In **chapter III** the expression pattern and function of the TSHR in the thymus was studied. It was demonstrated that the TSHR is selectively expressed on developing T cells in the thymus, mainly ISP and DP thymocytes. In addition, TSH was able to bind and activate the TSHR present on human thymocytes. Finally, both animal experiments and observations in *in vitro* human systems of T-cell development supported the notion that TSH enhanced T-cell development.
In addition, in **chapter IV**, the *in vivo* effects of TSHR signaling on the human T-cell compartment were investigated in patients with Graves' disease (GD). GD is an interesting model to investigate the effects of TSHR signaling on the T-cell compartment as high levels of TSHR stimulating TRAb are present. Moreover, GD has been associated with thymic hyperplasia due to increased thymocyte numbers. However, no increase in thymic output or naive T-cell numbers was found in the cohort of GD patients described in **chapter IV**. Also, thymic hyperplasia could not be demonstrated in these patients.
The peripheral blood T-cell compartment was, however, characterized by increased activated T-cell numbers, probably as part of the autoimmune response present in patients with GD. In addition, within the peripheral blood B-cell compartment of GD patients, an increase in transitional and pre-naive mature $CD5^+$ B-cell numbers was found. The data from **chapter IV** suggest that this increase in transitional and pre-naive mature B cells might (at least partly) be due to increased thyroid hormone levels that enhance B-cell development in BM.
Collectively, the data in **chapters III and IV** support the existence of a complex neuro-endocrine-immune network between hormones of the HPT-axis and developing B- and T-cells. The presence of this network becomes important especially when disturbances in one of the systems occur, thereby affecting the other system such as in patients with thyroid disorders or during infections.

Similar to neuro-endocrine-immune interactions, also the metabolic and the immune system closely interact via shared hormones, cytokines, and bioactive lipids. These interactions are prominently involved in obesity in which immunological aberrations are now widely recognized to contribute to the development of obesity itself as well as its co-morbidities. In this thesis detailed studies on the T-cell compartment in morbid obesity were performed.
Obesity is associated with the accumulation of pro-inflammatory T cells in adipose tissue, which has been suggested to be associated with the development of co-morbidities such as atherosclerosis and diabetes mellitus type II (DM-II). Whether alterations also occur in the peripheral blood T-cell compartment of obese subjects is so far largely unknown. **Chapter V** demonstrates that morbid obesity is associated with increased numbers of T cells in the peripheral blood, mainly due to increased homeostatic proliferation. In this morbid obese cohort without co-morbidities a skewing towards a Treg and Th2 dominated phenotype was found. This suggests an anti-inflammatory set point of the peripheral blood T-cell compartment. Based on the studies in **chapter V** and studies by others demonstrating a more pro-inflammatory set point of peripheral blood T cells in obese subjects with co-morbidities, it can be hypothesized that T-cell skewing away from the

anti-inflammatory Treg and Th2 dominated phenotype towards a more pro-inflammatory state dominated by Th1 or Th17 cells may reflect or even mediate the development of co-morbidities such as DM-II or atherosclerosis in morbid obese subjects.

In conclusion, the studies described in this thesis provided new insights into the interactions between the immune and neuro-endocrine and metabolic systems. Detailed investigation of these interactions will significantly enhance our knowledge about pathogenesis of immune and endocrine disorders and thereby possibly provide a whole new range of therapeutic modalities.

SAMENVATTING

Alle cellen van ons immuunsysteem ontwikkelen zich vanuit hematopoietische stamcellen. De meeste immuuncellen ontwikkelen zich geheel in het beenmerg, met uitzondering van T-cellen. T-cellen ontwikkelen zich in de thymus vanuit voorlopercellen afkomstig uit het beenmerg. De thymus is een gespecialiseerd orgaan met een unieke structuur dat de gecompliceerde processen die noodzakelijk zijn voor verdere T-cel ontwikkeling ondersteunt. Belangrijke processen die plaatsvinden in de thymus zijn cellijn restrictie, formatie van een (pre)TCR, positieve en negatieve selectie en emigratie uit de thymus.

T-cel proliferatie is van cruciaal belang tijdens T-cel ontwikkeling in de thymus, maar ook voor het onderhoud van het perifere T-cel compartiment. T-cel proliferatie is verder van grote invloed op de diversiteit van het TCR repertoire, de grootte van klonale naïeve T-cel populaties, het aantal T-cellen in het bloed en de grootte van de antigeenrespons. Kwantitatieve metingen van intra-thymale en post-thymale proliferatie zijn echter momenteel niet beschikbaar.
In **hoofdstuk II** van dit proefschrift werd een nieuwe techniek om intra-thymale en perifere T-cel proliferatie te bestuderen, de Vγ-Jγ TREC assay, ontwikkeld en getest. Met gecombineerde Vγ-Jγ TREC en aangepaste δREC-ψJα TREC analyse werd aangetoond dat humane thymocyten, ongeacht de leeftijd, ongeveer 6-8 intra-thymale celdelingen ondergaan vanaf het DN3 ontwikkelingsstadium. Aanvullende berekeningen illustreerden het belang van T-cel proliferatie voor de diversiteit van het TCR repertoire en de grootte van klonale naïeve T-cel populaties. Daarnaast werd het belang van homeostatische T-cel proliferatie in het bloed voor onderhoud van het perifere T-cel compartiment benadrukt doordat er al homeostatische celdelingen werden gevonden in naïeve T-cellen in navelstrengbloed. Het aantal homeostatische celdelingen nam sterk toe met de leeftijd.
Analyse van intra-thymale en perifere T-cel proliferatie in de kliniek zou belangrijke informatie kunnen geven over de fysiologie van het T-cel compartiment en over de pathofysiologie van ziektes die geassocieerd zijn met T-cel deficiënties. Ook zouden deze analyses gebruikt kunnen worden voor monitoring van T-cel reconstitutie.

Processen die van belang zijn voor de ontwikkeling en het onderhoud van het T-cel compartiment zoals differentiatie, overleving en proliferatie worden strak gereguleerd door vele extracellulaire signalen zoals groeifactoren, cytokines, chemokines en adhesiemoleculen, en daarnaast transcriptiefactoren. De meeste van deze stimuli worden lokaal geproduceerd in het gespecialiseerde thymusweefsel. Er zijn echter ook factoren van extra-thymale origine die in staat zijn T-cel ontwikkeling te reguleren. Er komt steeds meer bewijs voor een mogelijke rol van het neuro-endocriene/metabole systeem in de ontwikkeling en de functie van het immuunsysteem.

Het neuro-endocriene systeem en het immuunsysteem zijn sterk geïntegreerd door de aanwezigheid van gedeelde cytokines, hormonen, receptoren en innervatie. Deze integratie resulteert in een goed georganiseerd surveillance systeem dat gecoördineerde acties kan initiëren in reactie op interne en externe gevaren om homeostase in het lichaam te behouden of te herstellen. Bekende neuro-endocriene-immuun interacties zijn apoptose van DP thymocyten tijdens behandeling met corticosteroïden en het verhoogde risico op bepaalde auto-immuunziekten zoals systemische

lupus erythematose, myastenia gravis en reumatoïde artritis in vrouwen ten opzichte van mannen. Wellicht bestaan er nog vele andere regulatoire interacties. In dit proefschrift werden interacties tussen hormonen van de hypothalamus-hypofyse-schildklier as en B- en T-cel ontwikkeling onderzocht.

Micro array analyses toonden aan dat verschillende hormoonreceptoren tot expressie komen op thymocyten, wat suggereert dat de liganden voor deze receptoren een rol in de T-cel ontwikkeling kunnen spelen. Eén van deze receptoren was de TSHR, de receptor voor TSH, een hormoon dat voornamelijk van belang is in de hypothalamus-hypofyse-schildklier as. In **hoofdstuk III** werden het expressiepatroon en de functie van de TSHR in de thymus verder onderzocht. De TSHR komt selectief tot expressie op zich ontwikkelende T-cellen in de thymus, voornamelijk tijdens de ISP en DP stadia van ontwikkeling. Tevens werd aangetoond dat TSH zich aan de TSHR op thymocyten kan binden en deze kan activeren. Ten slotte ondersteunden dierenexperimenten en observaties in *in vitro* humane systemen van T-cel ontwikkeling ook een stimulerende rol voor TSH tijdens de T-cel ontwikkeling.

In aanvulling op deze *in vitro* studies werden in **hoofdstuk IV** de *in vivo* effecten van TSHR signalering op de humane T-cel ontwikkeling onderzocht in patiënten met de ziekte van Graves (GD).

GD is een interessant model om de effecten van TSHR signalering op het T-cel compartiment te onderzoeken vanwege de aanwezigheid van TSHR stimulerende TRAb. Tevens is GD geassocieerd met thymus hyperplasie, gekenmerkt door een toename van het aantal ontwikkelende T-cellen. Er werd echter geen verhoogde thymic output of een toename van het aantal naïve T-cellen gevonden in het cohort van GD patiënten beschreven in **hoofdstuk IV**. Ook werd in geen van de GD patiënten thymus hyperplasie aangetoond.

Het perifere bloed T-cel compartiment in patiënten met GD werd echter wel gekarakteriseerd door een toename van geactiveerde T-cellen, meest waarschijnlijk als onderdeel van de autoimmuunrespons die aanwezig is in GD patiënten. Daarnaast werd in het perifere bloed B-cel compartiment een toename in transitionele en pre-naïeve mature $CD5^+$ B-cellen gevonden. Aanvullende studies in **hoofdstuk IV** toonden aan dat deze toename van transitionele en pre-naïeve mature B cellen mogelijk (tenminste deels) wordt veroorzaakt door de verhoogde aanmaak van schildklierhormonen die de B-cel ontwikkeling in het beenmerg stimuleren.

Concluderend ondersteunen de data beschreven in **hoofdstuk III en IV** de aanwezigheid van een neuro-endocrien-immuun netwerk van hormonen van de hypothalamus-hypofyse-schildklier as en ontwikkelende B- en T-cellen. De aanwezigheid van dit netwerk is in het bijzonder van belang is situaties waarin veranderingen optreden in één van deze systemen, waardoor ook het andere systeem wordt aangedaan zoals bij patiënten met schildklierziekten of infecties.

Communicatie zoals beschreven tussen het neuro-endocriene en het immuunsysteem vindt ook plaats tussen het metabole en het immuunsysteem via gedeelde hormonen, cytokines en bioactieve lipiden. Deze interactie in van groot belang in obesitas, waarbij immunologische afwijkingen een belangrijke bijdrage leveren aan de ontwikkeling van co-morbiditeiten. In dit proefschrift werd het T-cel compartiment in morbide obesitas in detail bestudeerd.

Obesitas is geassocieerd met de accumulatie van pro-inflammatoire T-cellen in vetweefsel, wat mogelijk gerelateerd is aan de ontwikkeling van atherosclerose en diabetes mellitus type II. Het is momenteel onduidelijk of obesitas ook is geassocieerd met afwijkingen in het perifere bloed

T-cel compartiment. In **hoofdstuk V** werd aangetoond dat obesitas wordt gekenmerkt door een toename van het aantal T-cellen in het perifere bloed, wat voornamelijk wordt veroorzaakt door een toename van homeostatische T-cel proliferatie. Ook werd er in dit cohort van morbide obese mensen zonder co-morbiditeiten een verschuiving naar een Treg en Th2 gedomineerd fenotype waargenomen. De data in **hoofdstuk V** suggereren de aanwezigheid van een anti-inflammatoir setpoint van het perifere bloed T-cel compartiment in mensen met morbide obesitas zonder co-morbiditeiten. Daarentegen tonen studies van anderen een meer pro-inflammatoir setpoint in mensen met obesitas en co-morbiditeiten. Deze gecombineerde studies suggereren dat verschuiving van een anti-inflammatoir Treg en Th2 gedomineerd fenotype naar een meer pro-inflammatoir Th1 en Th17 gedomineerd fenotype geassocieerd zou kunnen zijn met, of zelfs zou kunnen bijdragen aan, de ontwikkeling van co-morbiditeiten zoals diabetes mellitus type II of atherosclerose in mensen met obesitas.

Concluderend geven de studies beschreven in dit proefschrift nieuwe inzichten in de sterke integratie van het immuunsysteem, het neuro-endocriene systeem en het metabole systeem. Uitgebreid onderzoek naar deze interacties zal in belangrijke mate bijdragen aan onze kennis van immunologisch en endocriene ziektes en zou daardoor kunnen bijdragen aan de ontwikkeling van nieuwe aangrijpingspunten voor behandeling van deze ziektes.

Chapter IX

Abbreviations
Dankwoord
Curriculum Vitae
PhD portfolio
Publications

ABBREVIATIONS

Ab	antibody
AC	adenylate cyclase
ACTH	adrenocorticotropin
AITD	autoimmune thyroid disease
APC	allophycocyanin/antigen presenting cell
Ag	antigen
BM	bone marrow
BMI	body mass index
BrdU	bromodeoxyuridine
BREC	B-cell receptor recombination excision circle
BSA	bovine serum albumin
cAMP	3',5'-cyclic adenosine monophosphate
CB	cord blood
CCL	C-C chemokine ligand
CCR	C-C chemokine receptor
CD	cluster of differentiation
CFSE	carboxyfluorescein diacetate succinimidyl ester
Ci	Curie
CJ	coding joint
CMJ	cortico-medullary junction
cpm	counts per minute
CRH	corticotropin releasing hormone
Ct	cycle threshold
CT	computed tomography
CTL	cytotoxic T cell
CXCL	CXC chemokine ligand
CXCR	CXC chemokine receptor
DAG	diaglycerol
DC	dendritic cell
DIO	diet induced obesity
DM	diabetes mellitus
DMEM	Dulbecco's Modified Eagle's Medium
DN	double negative
DNA	deoxyribonucleic acid
DP	double positive
ECM	extracellular matrix
EDTA	ethylenediamine tetra-acetate
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmatic reticulum
ETP	early thymic progenitor
FABP	fatty acid binding proteins
FACS	fluorescence activated cell sorter

Fas-L	Fas ligand
FCS	fetal calf serum
FFA	free fatty acid
FITC	fluorescein isothiocyanate
FoxP3	forkhead box P3
FSH	follicle-stimulating hormone
FTOC	fetal thymic organ culture
GC	glucocorticoids
GD	Graves' disease
GH	growth hormone
GHRH	growth hormone releasing hormone
GPCR	G-protein-coupled-receptor
GR	glucocorticoid receptor
hCG	human chorionic gonadotropin
HFD	high fat diet
HLA	human leukocyte antigen
HPA-axis	hypothalamic-pituitary-adrenal axis
HPT-axis	hypothalamic-pituitary-thyroid axis
HRP	horseradisch peroxidase
HSC	hematopoietic stem cell
HT	Hasimoto's thyroiditis
IBMX	isobutylmethylxanthine
IEL	intraepithelial T cell
IFN-γ	interferon-gamma
IMDM	Iscove's Modified Eagle's Medium
Ig	immunoglobulin
IGF-1	insulin-like growth factor 1
IL	interleukin
IP3	inositol 1,4,5-triphosphate
ISP	immature single positive
KREC	κ-deleting recombination excision circle
LH	luteinizing hormone
LH-RH	luteinizing hormone releasing hormone
MACS	magnetic cell sorting system
MALT	mucosa-associated lymphoid tissue
MHC	major histocompatibility complex
mRNA	messenger ribonucleic acid
NK-cell	natural killer cell
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PE	phycoerythrin
PerCP	peridinin chlorophyl protein
PHA	phytohaemagglutinin
PMA	phorbol-12-myristate-13-acetate

PKA	protein kinase A
PRL	prolactin
PTU	propylthiouracil
RA	rheumatoid arthritis
RAG	recombination activating gene
RANTES	regulated on activation normal T cell expressed and secreted
ROR-γt	retinoic acid-related orphan receptor-γt
ROS	reactive oxygen species
RPMI-1640	Roswell Park Memorial Institute-1640 medium
RQ-PCR	real-time quantitative polymerase chain reaction
RSS	recombination signal sequence
RTE	recent thymic emigrant
SEA	staphylococcus enterotoxin-A
S1P	sphingosine 1-phosphate
$S1P_1R$	sphingosine 1-phosphate type 1 receptor
SAV	streptavidin
SCF	stem cell factor
SJ	signal joint
SLE	systemic lupus erythematosis
SP	single positive
SPF	specified pathogen-free
SS	somatostatin
SVF	stromal-vascular fraction
T3	triiodothyronine
(f)T4	(free) thyroxine
T_{CM}	central memory T cell
TCR	T cell receptor
TEC	thymic epithelial cell
T_{EM}	effector memory T cell
Tfh	T follicular helper cell
Tg	thyroglobulin
TGF-β	transforming growth factor-β
Th	T helper cell
TH	thyroid hormone
TLR	Toll-like receptor
T_N	naive T cell
TNF-α	tumor necrosis factor-alpha
TRAb	TSHR specific autoantibodies
TREC	T-cell receptor recombination excision circle
Treg	regulatory T cell
TPO	thyroid peroxidase
TRH	thyrotropin-releasing hormone
TSH	thyroid-stimulating hormone (thyrotropin)
TSHR	thyroid-stimulating hormone receptor

T_{TD}	terminally differentiated T cell
UPR	unfolded protein response
VIP	vasoactive intestinal peptide
WHO	World Health Organization

DANKWOORD

Ruim vijf jaar later is mijn proefschrift dan bijna klaar! Maar uiteraard kan dat niet zonder iedereen die mij hierbij heeft geholpen enorm te bedanken, want zonder de lieve hulp en enorme inzet van vele mensen was dit boekje zeker niet tot stand gekomen! Ik kan helaas niet iedereen persoonlijk noemen, maar ook degenen die niet bij naam worden genoemd ongelofelijk bedankt voor jullie hulp en fijne samenwerking.

Prof. Van Hagen, beste Martin. Ruim 5 jaar geleden werd ik aan jou voorgesteld als de nieuwe promovendus op een van jouw projecten. Ondanks dat we elkaar toen nog nauwelijks kenden heb je me sindsdien onvoorwaardelijk gesteund. Dank je voor alle wijze raad, nieuwe ideeën en gezelligheid op het werk en tijdens congressen. Ik hoop dat je me de komende jaren met even goede raad wil bijstaan tijdens mijn opleiding interne geneeskunde.

Prof. Staal, beste Frank. Je kreeg met mij een arts in het lab zonder enige "labskills", wat in het begin een enorme uitdaging moet zijn geweest. Maar je hebt me alle kansen en hulp geboden bij het opbouwen van laboratorium vaardigheden en het goed opzetten van nieuwe experimenten. Ik heb veel geleerd van jou onuitputtelijke immunologische kennis. Tijdens de moeilijke momenten had ik veel aan jouw enthousiasme en positieve instelling. Ik ben je enorm dankbaar dat je me ook na de verhuizing naar Leiden en de nieuwe verantwoordelijkheden als professor nog zo intensief begeleid hebt.

Dr. Dik, beste Wim. Dank je dat je mij na de verhuizing van Frank naar Leiden zo vanzelfsprekend in je groep hebt ontvangen. Jou enthousiaste en resultaatgerichte begeleiding waren een enorme houvast de afgelopen jaren en mijn artikelen zijn door jouw kritische blik een stuk scherper geworden.

Prof. Benner, Prof. Van Dongen en Prof. Hooijkaas. Dank jullie voor de gastvrijheid die jullie me hebben geboden om mijn onderzoek op de afdeling immunologie uit te voeren, zowel voor als na de verhuizing van Frank.
Beste Jacques, ik heb het altijd erg gewaardeerd dat ik, ook na de switch naar de Medische immunologie deel heb mogen nemen aan de researchmeeting van de Moleculaire immunologie op dinsdagmorgen. Jouw scherpe inzichten en de kritisch stimulerende discussies met de mensen van de Moleculaire immunologie hebben mijn studies zeker verbeterd.

Prof. Van der Lelij. Dank je voor de mogelijkheid die je me hebt geboden om dit promotieonderzoek op te starten. Ook tijdens het traject heb ik veel gehad aan de goede inhoudelijke discussies die we hebben gehad, in het bijzonder over de voor het onderzoek benodigde METC protocollen. Tevens wil ik, Theo Visser en Leo Hofland bedanken voor de fijne, openhartige en intensieve samenwerking met de afdeling endocrinologie. Maarten van Aken en Rosalie Kiewiet, dankzij jullie enthousiaste begeleiding tijdens mijn afstudeeronderzoek is mijn interesse in onderzoek aangewakkerd en ben ik aan dit promotietraject begonnen.

Leendert en Jeroen. Ik zou hier niet kunnen staan zonder jullie aan mijn zij, fijn dat jullie mijn

paranimfen willen zijn. Ik heb een supertijd met jullie gehad! Ik zal jullie gezelligheid, goede discussies over statistiek en alle andere uitdagingen (zowel werk gerelateerd als daarbuiten), en de liters koffie met bergen snoep enorm missen.

Sjanneke, Benjamin en Conny. Ik wil jullie bedanken voor alles wat jullie mij op het lab hebben geleerd, zonder al jullie tips en tricks was er geen experiment geslaagd. Daarnaast hebben jullie me ook veel werk uit handen genomen. Sjanneke ik ben je erg dankbaar voor je hulp met de vele co-kweken en Benjamin en Conny voor de vele TREC analyses en de enorme FACS experimenten. Conny, heerlijk dat jij me af en toe ook wat vrouwelijke ondersteuning gaf als de mannen op het lab weer eens helemaal los gingen.

Al mijn labgenoten. Karin, Peter, Tiago, Gita, Edwin, Ozden, Floor, Miranda, Sjanneke, Machteld, Kim, Anna, Rianne, Tom, Mark, Conny, Gemma, Jan Willem, Marten, Nicole, Jeroen, Leendert, Dagmar, Marja en Marion. Jullie hebben me voor het eerst wegwijs gemaakt op een laboratorium, dank jullie voor alle gezelligheid op het lab en uiteraard de hulp bij de muizenproeven.

Al mijn mede-OIOs, Karin, Kim, Ozden, Nicole, Magda, Marten, Jan Willem, Leendert, Jeroen, Hanna, Ferry, Jan-Piet, Fleur en vele anderen, dank voor jullie steun, gezelligheid, en leuke AIO-weekenden. Heel veel plezier en succes met jullie carriëres!!

Paul van Daele, Jan van Laar, Virgil, Kiki, Jasper en Lieke, ondanks dat ik niet vaak in de kliniek te vinden was, was ik er wel altijd welkom, dank jullie voor de gezellige congressen bijvoorbeeld in Antwerpen.

Vele andere mensen hebben me telkens weer praktische hulp geboden op het lab. Ik wil Sandra bedanken voor haar hulp aan mijn figuren; Yvette en Kim voor de ondersteuning in het EDC; Ferry, Jan-Piet en Patrick voor hulp en antistoffen bij de FACS experimenten; Patricia voor de hulp bij het ontwerpen van primers en probes; Ashley, Joyce en Ingrid voor hulp bij TREC en GeneScan experimenten; Tom en Sigrid Swagemakers voor de micro-array experimenten; Edwin en Benjamin voor de lange, lange cell-sorts; Jeroen, Henk, Dennis en Romana voor alle antilichamen en FACS adviezen; Peter Koetsveld voor hulp bij TSH bindingsstudies en cAMP experimenten; Hans van Toor en Yolanda de Rijke voor de T3 metingen.

Ook heb ik ongelovelijk veel hulp gehad bij het verzamelen van al het patiëntenmateriaal dat is gebruikt in de studies die worden beschreven in dit proefschrift. Robin Peeters, Wouter de Herder, Richard Feelders en alle andere endocrinologen, Mark ten Broek, Dik Kwekkeboom, Ad Bogers, Alexander Maat, Francien van Nederveen en de andere pathologen, Dave Schweitzer en de afdeling neonatologie in het SFG, dank jullie voor jullie langdurige, enthousiaste hulp bij het verzamelen van patiënten en patiëntenmateriaal voor de verschillende studies.

Ik wil al mijn nieuwe collega's uit het Reinier de Graaf Gasthuis en het Havenziekenhuis bedanken voor de support tijdens mijn eerste stapjes weer terug in de medische wereld na een aantal jaren promotieonderzoek. Ook waardeer ik het zeer dat jullie mij de vrijheid en ruimte hebben gegeven om dit promotieonderzoek tot een goed einde te brengen.

Familie en vrienden, dank jullie voor al jullie lieve ondersteuning de afgelopen jaren, ook als ik eens wat minder tijd had, maar vooral ook voor alle gezelligheid en afleiding die jullie met hebben geboden buiten het werk.

Lieve Daan, we hebben het gehaald, allebei een boekje op de plank! Zowel werkgerelateerd, maar zeker ook daar buiten hebben we een paar bizarre, maar heerlijke, onvergetelijke jaren gehad! Dank je dat je er altijd voor me was, ondanks dat je eigen boekje ook nog af moest en je daarna een tijdje in Duitsland zat. Ik kijk nu al uit naar al nieuwe uitdagingen die voor ons liggen de komende jaren!!

Bedankt!!

Kim

CURRICULUM VITAE

Kim van der Weerd was Born in Almelo on the 28th of March in 1983. In 2001 she graduated *cum laude* from secondary school at the OSG West-Friesland in Hoorn. Thereafter, she attended medical school at the Rijksuniversiteit Groningen (RuG) from 2001 to 2007. During her medical studies she did a research internship entitled 'Een studie naar de invloed van ghrelin op het hongergevoel bij patienten met morbide obesitas' at the department of Endocrinology, Erasmus MC, University Medical Center, Rotterdam, The Netherlands (supervisors Dr. M.O. van Aken, Drs. R.M. Kiewiet, Prof. dr. B.H.R. Wolffenbuttel). In 2007 she obtained the degree in medicine, after which she started her PhD project entitled 'Endocrine regulation of T-cell development and peripheral T-cell maturation' at the department of Immunology, Erasmus MC, University medical Center, Rotterdam, The Netherlands (supervisors Prof. dr. P.M. van Hagen, Prof. dr. F.J.T. Staal, Dr. W.A. Dik). In January 2012 she started her residency of internal medicine at the Havenziekenhuis in Rotterdam.

PHD PORTFOLIO

Name PhD student:	Kim van der Weerd
Erasmus MC Department:	Immunology
Research School:	Molecular Medicine (MolMed)
PhD period:	September 2007 – June 2013
Promotores:	Prof. Dr. P.M. van Hagen, Prof. Dr. F.J.T. Staal
Copromotor:	Dr. W.A. Dik

PhD training	Year	Workload (ECTS)
General courses		
- BROK ('Basiscursus Regelgeving Klinisch Onderzoek')	2008	1
- Laboratory Animal Science	2008	2
- Biomedical English Writing and Communication	2010	4
- Molecular Immunology	2008	3
- Basic Data Analysis on Gene Expression Arrays I	2008	1.2
- Molecular Diagnostics IV	2009	1
- Basic and Translational Endocrinology	2011	2
Seminars and workshops		
- Seminars immunology	2007-2011	2
- Journal clubs	2007-2011	2
(Inter)national conferences		
- NVVI	2007	0.6
- NVVH	2008	0.6
- The XIIIth anuual symposium of the Dutch thyroid club	2008	0.2
- Wetenschapsdagen (poster presentation, 3rd prize poster)	2008	0.2
- MolMed day (poster presentation)	2008	0.2
- IMID (poster presentation)	2008	0.6
- Wetenschapsdagen (oral presentation)	2009	1
- NVVH (oral presentation)	2009	1
- MolMed day (oral presentation)	2009	1
- ETA (oral presentation)	2009	1
- IMID (poster presentation)	2009	0.6
- NVVI (poster presentation)	2009	0.6
- Wetenschapsdagen (poster presentation)	2010	0.2
- MolMed day (poster presentation)	2010	0.2
- IMID (poster presentation)	2010	0.6
- NVVI (poster presentation)	2010	0.6
- Wetenschapsdagen (poster presentation)	2011	0.2
- MolMed day (poster presentation, poster prize)	2011	0.2
- ECE (poster presentation)	2011	0.6
- 1st International Conference on ImmunoMetabolism	2011	0.6
- (poster presentation)		

Teaching	Year	Workload (ECTS)
Lectures and supervision of practicals		
- 2nd year Master Immunity and Infection (lecture)	2011	0.2
- 2nd year medical student VO (supervision)	2008-2011	6
- Proefstuderen (supervision)	2009-2011	2

PUBLICATIONS

K. van der Weerd, P.M. van Hagen, B. Schrijver, D. Kwekkeboom, W.W. de Herder, M.R.J. ten Broek, P.T.E. Postema, J.J.M. van Dongen, F.J.T. Staal, W.A. Dik. The peripheral blood compartment in patients with Graves' disease; activated T lymphocytes and increased transitional and pre-naive mature B lymphocytes. *Submitted for publication.*

K. van der Weerd, P.M. van Hagen, B. Schrijver, S.J.W.M. Heuvelmans, L.J. Hofland, S.M.A. Swagemakers, A.J.J.C. Bogers, W.A. Dik, T.J. Visser, J.J.M. van Dongen, A.J. van der Lelij, F.J.T. Staal. Thyroid stimulating hormone acts as a T-cell developmental factor in mice and humans. *Submitted for publication.*

K. van der Weerd, W.A. Dik, B. Schrijver, A.J.J.C. Bogers, A.P.W.M. Maat, F.H. van Nederveen, P.M. van Hagen, J.J.M. van Dongen, A.W. Langerak, F.J.T. Staal. Combined TCRG and TCRA TREC analysis reveals increased peripheral T-lymphocyte but constant intra-thymic proliferative history upon ageing. *Mol Immunol. 2013 Mar;53(3):302-12. doi: 10.1016/j.molimm.2012.08.019. Epub 2012 Sep 19.*

K. van der Weerd, W.A. Dik, B. Schrijver, D.H. Schweitzer, A.W. Langerak, H.A. Drexhage, R.M. Kiewiet, M.O. van Aken, A. van Huisstede, J.J.M. van Dongen, A.J. van der Lelij, F.J.T. Staal, P.M. van Hagen. Morbid obese human subjects have increased peripheral blood $CD4^+$ T lymphocytes with skewing towards a Treg and Th2 dominated phenotype. *Diabetes. 2012 Feb;61(2):401-8. Epub 2012 Jan 6.*

R.M. Kiewiet, M.J. Hazell, M.O. van Aken, **K. van der Weerd**, J.A. Visser, A.P. Themmen, A.J. van der Lelij. Acute effects of acylated and unacylated ghrelin on total and high molecular weight adiponectin inmorbidly obese subjects. *J Endocrinol Invest. 2011 Jun;34(6):434-8. Epub 2010 Oct 15.*

R.M. Kiewiet, M.O. van Aken, **K. van der Weerd**, P. Uitterlinden, A.P.N. Themmen, L.J. Hofland, Y.B. de Rijke, P.J.D. Delhanty, E. Ghigo, T. Abribat, A.J. van der Lely. Effects of acute administration of acylated and unacylated ghrelin on glucose and insulin concentrations in morbidly obese subjects without overt diabetes. *Eur J Endocrinol 2009; 161(4):567-73. Epub 2009 Jul 23.*