Thymic Studies
Investigations into the effects of childhood thymectomy, and characterization of thymic B cells and Hassall's corpuscles

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Curiosity killed the cat
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ABSTRACT

This thesis focuses on the human thymus, a primary lymphoid organ responsible for the maturation of T cells. Progenitors arrive from the bone marrow and start to randomly assemble their T cell receptor (TCR) followed by a thorough selection process in which the TCR is tested for functionality and autoreactivity. The selection process is carried out with the help of different types of antigen presenting cells to ensure that only functional mature T cells that do not react towards the body’s own structures are released into the periphery. In the selection process, also T regulatory cells that can maintain tolerance by acting immunosuppressive are generated from subset of the autoreactive T cells. Only around 3% of the progenitors that enter the thymus leave as mature T cells two-three weeks later and the net output is approximated to 1.7 x10^7 cells/day. The thymus is most active during childhood. Starting at puberty the thymus gradually involutes, but even though only a fraction of its original capacity eventually remains it is functional throughout life.

In paper I we investigated the effect of early thymectomy on the diversity of the TCR in the peripheral T cell pool. We followed up on thymectomized children 18 years after thymectomy by analyzing peripheral blood samples. In these children, more than 90% of the thymus had been removed during heart surgery before the age of six months. T and B cells were sorted out from peripheral blood, DNA encoding TCR was sequenced, and the results were compared with age and gender matched controls. Thymectomized children showed reduced diversity of the T cell receptor repertoire in the periphery compared with controls, which may lead to reduced infection control and
blunted regulatory functions of the T cell pool. The B cell receptor diversity was unaffected.

Paper II focuses on thymic B cells, a small population that while consisting of less than 1% of the total cell count in the thymus, covers a relatively large area of the medulla. We discovered that a significant fraction of these B cells underwent immunoglobulin class switching, a process that usually takes place in germinal centers after the body encounters an infection, which should be a rare event in a newborn infant. The thymic B cells displayed a mature phenotype and expressed high levels of co-receptors for T cell communication along with the transcription factor AIRE, which would imply a role as an antigen presenting cell (APC) that may aid in the T cell selection process.

Paper III aims to characterize a prominent structure in the human thymic medulla, the Hassall’s corpuscles. Since the medullary epithelial cells (mTEC) in and surrounding the structure are difficult to digest into a single cell suspension, they were cut out using laser microdissection for further studies. Analyses of the retrieved sections using RNA sequencing and proteomics showed an increasing similarity with skin epidermis the more differentiated and closer to the Hassall core the cells were located. The center, devoid of nuclei, also contained bacterial defense proteins, further emphasizing similarity to the skin. The mTEC differentiation is thought to be influenced by the expression of the AIRE gene. Comparisons between Down syndrome thymus (three copies of AIRE) and control thymus showed larger corpuscles in the former, perhaps due to a higher turn-over and differentiation of mTECs than in control tissue. In mouse models in which the Aire gene is knocked out, the corpuscle like structures in the thymus were fewer and smaller, and the skin was thinner.

**Keywords:** thymus, thymectomy, TCR, B cells, APC, Hassall’s corpuscles, AIRE

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Avhandlings titel är Thymusstudier, undersökningar av effekten av thymektomi i barndomen, och karakterisering av B-celler och Hassallska korpuskler i thymus. Den beskriver funktioner hos human thymus, vad som händer med immunsystemet om thymus tas bort och beskriver olika cellers funktion och utveckling i thymus.


Det andra arbetet karakteriserar de B-celler som återfinns i thymus i relativt lågt antal. De utvecklas ur samma stamceller i benmärgen som T-celler men stannar i benmärgen under den första mognadsfasen och är inte beroende av thymus för sin fortsatta utveckling. Vi upptäckte att en betydande del av B-celler i thymus hos nyfödda barn hade en mogen fenotyp som annars inte förekommer innan kroppen genomgått upprepade infektioner, något som spädbarn normalt sett inte haft. Dessa celler hade även högre nivåer av receptorer som används för att kommunicera med T-celler, vilket gör att vi tror att deras funktion i thymus är att hjälpa epitelceller att utbilda T-celler.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

* These authors contributed equally to this work.

* These authors contributed equally to this work.
‡ These authors contributed equally to this work.

PUBLICATIONS NOT INCLUDED IN THE THESIS


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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AIRE/Aire</td>
<td>Human/mouse Autoimmune Regulator gene</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
</tr>
<tr>
<td>APS1</td>
<td>Autoimmune polyendocrine syndrome type 1</td>
</tr>
<tr>
<td>BCR</td>
<td>B cell receptor</td>
</tr>
<tr>
<td>CDR3</td>
<td>Complementary determining region 3</td>
</tr>
<tr>
<td>cTEC</td>
<td>Cortical thymic epithelial cell</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td>DN</td>
<td>Double negative thymocyte</td>
</tr>
<tr>
<td>DP</td>
<td>Double positive thymocyte</td>
</tr>
<tr>
<td>HC</td>
<td>Hassall’s corpuscle</td>
</tr>
<tr>
<td>IGH</td>
<td>Immunoglobulin heavy chain</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MMR</td>
<td>Measles, mumps and rubella vaccine</td>
</tr>
<tr>
<td>mTEC</td>
<td>Medullary thymic epithelial cell</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cell</td>
</tr>
<tr>
<td>RTE</td>
<td>Recent thymic emigrant</td>
</tr>
<tr>
<td>SLE</td>
<td>Systemic lupus erythematosus</td>
</tr>
<tr>
<td>sjTREC</td>
<td>Signal joint T cell receptor rearrangement excision circle</td>
</tr>
<tr>
<td>SP</td>
<td>Single positive thymocyte</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>TBE</td>
<td>Tick-borne encephalitis</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TRA</td>
<td>Tissue restricted antigen</td>
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<tr>
<td>TREC</td>
<td>T cell receptor excision circle</td>
</tr>
<tr>
<td>Tx</td>
<td>Thymectomy</td>
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1 INTRODUCTION

The body needs to balance the need of having a well-functioning immune response to pathogens against not reacting with self-structures causing autoimmunity. Part of this balance is exacted in the thymus, a primary lymphoid organ situated on top of the heart in the thoracic cavity (Figure 1). Here the developing T cells form a functioning adaptive immune system that does not react to self. The works included in this thesis are focused mainly on the human thymus.

Figure 1. Thymus in a child, located on top of the heart in the thoracic cavity. Anatomy of the Human Body, 20th ed. Gray, Henry. 1918.

1.1 THYMUS IN THE PAST

The earliest mention of the thymus gland in medical literature is from the first century AD by Rufus of Ephesus in Greece who described the thymus anatomically as a gland located over the heart. (Rufus Med. De corporis humani appellationibus 168.1–169.1)\(^2\). An interesting theory about the origin of the word thymus has been put forward by Konstantinos Laois. Thymus might originate from Indo-European with the meaning of “vapor” or “fume”. Since the involution of the organ was difficult to investigate at that time the disappearance of the organ could have been linked to vapor, or going up in smoke\(^2\). Thymus has also been attributed to a Greek word for heart or soul. The interpretation being that the proportionally big thymus seated above the heart in young animals must be the base of the soul\(^3,4\).
In the beginning of the 20th century a large thymus was seen as a condition of sickness in young children. The organ was thought to put pressure on the lungs and impede breathing, treatment with irradiation was sometimes recommended. This belief might have risen due to the many autopsies performed on children diseased from serious illnesses such as diphtheria. The shrunken thymus seen in these children might have become the norm.

In “The Anatomy of the Thymus Gland” from 1832 a detailed description of the human thymus is recorded. The author, Sir Astley Cooper, dissected and uncovered that the two thymic lobes are divided into smaller lobes that can be unraveled in a serpentine manner, comparing the organ to a necklace of beads (Figure 2).

Veins, arteries and mucous membranes needed to be removed for the thymus to unravel in this fashion. The different lobules were connected allowing communication between them with a spiral cavity in the center of the gland. He demonstrated the connection between the lobes by injecting mercury into one lobe and followed the diffusion into the adjacent lobe. He also described the thick fluid coming out from the organ as filled with particles, and described

Figure 2. 1. The serpentine form of the lobes. 2. The lobes partially unraveled. From “The Anatomy of the Thymus Gland” by Sir Astley Cooper. 1832.
it as the same particles found in blood. These particles, or blood lymphocytes, and the function of the thymus was not generally accepted until 1960s and were long considered without a role in immunity until Jacques Miller showed dramatic effects on the immune system in mice thymectomized at birth.

Before the role of the thymus was revealed, it became famous in Swedish media. In 1952 a Swedish newspaper published a story about veterinarian Elias Sandberg and how he had discovered a new medicine for cancer. He defended his thesis about the calf thymus a decade earlier and believed that the key to immunological resistance lay in the thymus. He had started to treat people suffering from terminal cancer with injections of THX, a calf thymus extract, which became national news. This was the start of a prolonged conflict between Sandberg, medical doctors and the state, which lasted until his death in 1989. Until 2009 there was still a registered alternative medicine, Enzythym, based on Elias Sandberg’s theories.

1.2 THYMUS TODAY

Huge progress has been made in the field of immunology and thymus research since its function was first described by Miller.

The lobules of the human thymus consist of two distinct areas; medulla and cortex. The cortex consists mainly of immature thymocytes, heavily branched cortical epithelial cells (cTECs) and macrophages with the main function to clear apoptotic thymocytes. The medulla is much sparser and mainly consists

![Figure 3. Human thymus section stained with Hoechst to show the nuclei. Cortex (C) is the dense area and the medulla (M) is the sparse area. Scale bar 200µm.](image-url)
of single positive thymocytes, medullary epithelial cells (mTEC), macrophages, dendritic cells (DC), and B cells.

Other cell types have also been reported to inhabit the thymus, such as neutrophils, eosinophils, and mast cells. One of the most unexpected cells found in the thymic medulla was the myoid cell, containing myofibrils, and from these cells a cell line was established that expressed a functional acetyl choline receptor. The latest cell type to be uncovered in the human thymus was the tuft cell, usually seen in the gastrointestinal tract.

1.2.1 THYMOCYTE DEVELOPMENT

The T cell progenitors from the bone marrow enter the thymus in the corticomedullary junction. The capillaries extending into the cortex are impermeable, but venules in the corticomedullary junction are fenestrated, allowing progenitors to enter the thymus. The so-called blood thymus barrier prevents antigens from reaching the developing thymocytes in the cortex, but is incomplete in the medulla, allowing antigens through from the blood.

When the thymocytes enter the cortex, they are double negative (DN), expressing neither of the T cell markers CD4 or CD8. At the third double negative stage the thymocytes begin to re-arrange their T cell receptor (TCR), starting with the β-chain, and if successful they receive signaling through their pre-TCR. The pre-TCR consist of the rearranged β-chain and a pre-alpha chain. The thymocyte then rearranges the α-chain until it results in a productive αβ-TCR. The theoretical TCR diversity has been calculated up to $10^{20}$ possible clones. At this stage the thymocytes have a short lifespan and are destined to apoptosis, and if they are not rescued by a survival signal from binding to MHC molecules on cTECs they die by neglect. cTECs have constitutive autophagy degrading their intracellular proteins to be presented on both MHC class I and II to the developing thymocytes. There is also growing evidence for a negative selection process in the cortex, which seems to be dependent on presentation of self-antigens by dendritic cells.

The surviving thymocytes migrate into the medulla as single positive, either for CD8 or CD4 depending on if the survival signal came from binding MHC class I or II. In the medulla, self-antigens are presented to the thymocytes by mTECs or DCs which results in one of three main outcomes depending on the affinity for the antigens presented; negative selection (by activation induced apoptosis), diversion into the regulatory T cell lineage or egress from the thymus as an effector T cell. The mTECs express a vast number of tissue restricted antigens (TRAs) under the influence of AIRE, and a high constitutive
autophagy activity for the generation of numerous self-peptides. When these are presented to the thymocytes, autoreactive clones will effectively be removed or be directed into the regulatory T cell lineage\textsuperscript{24-26}. The TRAs produced by the mTECs have also been shown to be transferred to DCs to enlist them in the negative selection process\textsuperscript{27}. This transfer has been suggested to be partly mediated via exosomes carrying MHC-peptide complexes emanating from the mTECs\textsuperscript{28}. Eventually, approximately 3\% of the thymocytes exit the thymus as mature T cells\textsuperscript{29}.

The importance of the generation of a regulatory T cell population expressing FoxP3 for preventing autoimmunity is illustrated by the disease immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) caused by mutations in the \textit{FOXP3} gene. It is a rare, severe, autoimmune disease with bowel and skin inflammation, autoimmune diabetes and other autoimmune manifestations presenting already in the neonatal period\textsuperscript{30}. It was recently shown that regulatory T cells can arise from two different development programs, where one path develops through agonist selection similar to negative selection with high affinity to self and the other path shows more similarities with positive selection and display a broader repertoire\textsuperscript{31}.

B cells have also been suggested to be of importance for the development of regulatory T cells, having MHC class II, and costimulatory molecules such as CD80, CD86 and CD40. A mouse strain lacking B cells shows no difference in CD4\textsuperscript{+} and CD8\textsuperscript{+} thymocytes but has lower numbers of regulatory T cells in the thymus\textsuperscript{32}.

1.2.2 THYMIC INVOLUTION

The thymus grows in size until puberty when the involution starts, this process continues throughout life and if extrapolated it has been estimated that the thymus would be completely absent at 120 years of age\textsuperscript{33}.

Signs of involution, such as widening of trabeculae and of the perivascular space, has been attributed as early as after the first year of life\textsuperscript{34}. The impact of puberty on thymic involution has been debated\textsuperscript{35}, and peak cellularity has been proposed to occur as early as at 6 months of age\textsuperscript{36}. In an effort to better quantify involution and thymus senescence a labeling technique with a modified form of Sudan black (binding lipofuscin) was developed by Barbouti and co-workers. They demonstrated that infant and young thymi showed no cellular senescence but during adolescence senescence seems to be activated\textsuperscript{37}. Involution does not seem to be due to intrinsic aging of the lymphohematopoietic stem cells and early T cell progenitors, but rather
changes in the thymic environment\textsuperscript{38}. For example, FoxN1, which is of vital importance for mTEC development and function, is shown to gradually decrease with age in mTECs\textsuperscript{39}. 
2  PAPER I: THYMECTOMY

2.1 THYMECTOMY

Thymectomy (Tx) for a non-medical reason is performed on children undergoing cardiac surgery to correct congenital heart defects. The thymus blocks the surgeon’s access to the heart and is removed routinely. This type of surgeries started to become more common after 1970 when surgical techniques, as the cardiopulmonary bypass, allowed more lifesaving interventions. Heart defects affect approximately 1% of all children of which 1/4 to 1/3 undergo open surgery including thymectomy. Roughly 200 Txs are performed each year in Sweden (Figure 4). Individuals that have undergone Tx are increasing in number and age, which makes it important to study the immunological and clinical consequences of thymectomy thoroughly.

Figure 4. Thymectomies performed in Sweden over time. Adapted from Gudmundsdottir et al.¹
2.1.1 THYMIC OUTPUT

2.1.2 TRACING THYMIC OUTPUT

When T cell progenitors enter the cortex, they start to rearrange the T cell receptor (TCR), beginning with the β-chain during the DN3 stage. After successful rearrangement of the β-chain the thymocyte undergoes proliferation and progresses into the DN4 stage, and the TCR α-chain rearranges. TCR α-chain can make multiple rearrangements, until the recombination is halted by positive selection, or the cell dies. Thymic nurse cells are believed to help in the multiple rearrangements of the α-chain.

In the rearrangement process of the TCR genes, TCR rearrangement excision circles (TREC) are generated. The most commonly measured variant is the signal joint TREC (sjTREC), circular DNA strands created during recombination of the α-chain. The rings are stable and not duplicated in mitosis, and are therefore diluted when the cells expand in the periphery to reconstitute the T cell pool. Recent thymic emigrants (RTE) have a higher level of TRECs than memory T cells, due to that less cell divisions have occurred in RTEs. A drop of 1-1.5 log10 is expected during a lifetime. TRECs are still detectable in elderly people, while no TREC can be measured in patients with complete Di George syndrome, that lack a thymus.

TRECs represent a useful way to quantify thymic output, however, it can be misleading since naïve T cells are long-lived and TRECs can remain in non-dividing cells the whole lifetime. Thus, a TREC containing naïve T cell is not necessarily recently produced by the thymus. Adult thymectomy, when the individual has an established repertoire, does not lead to a rapid decline in TREC levels.

2.1.3 THYMIC OUTPUT WITH AGE AND THYMECTOMY

Thymic involution is a process were the active lymphoid tissue is replaced by fat and connective tissue. This process takes place slowly over a long period of time, with an increase at puberty and periods of fast involution with following rebound, such as after pregnancy and corticosteroid treatment. The pregnancy studies were mainly performed in mice although the same pattern should be expected in humans. A newly released study in humans showed no difference in TREC levels in naïve T cells during pregnancy compared to non-pregnant controls, arguing that the thymic output is maintained in humans. However, due to the longevity of naïve cells and the limited time of a pregnancy, it is difficult to draw any firm conclusions. An
argument against a long-lasting impact of sex hormones on the thymus is that the observed castration-induced involution in mice is short lived\textsuperscript{50}.

Involution normally starts 10-15 years later than a childhood Tx and proceed at a slow pace, with TRECs still detectable up in high ages since adult thymus contains areas of active tissue\textsuperscript{51}. Even though the decrease in TREC levels between 25 and 60 years of age has been shown to be more than 95\%, the TCR diversity at 60-65 years did not differ too much from young adults, with a clone diversity comprising 20 million different \(\beta\)-chains. After 70 years of age the repertoire diversity decreased drastically to a clone diversity of 200,000\textsuperscript{52}. An aging immune system, with involution of the thymus, correlates with an increase of infections and autoimmune diseases, and is referred to as immunosenescence\textsuperscript{53}.

Thymectomy at a young age would be expected to affect the peripheral T cell pool in a similar but accelerated way as seen in the process of aging. Disruption of the T cell compartment after thymectomy was shown already in 1970\textsuperscript{s}\textsuperscript{54}. Although some studies have shown no apparent effects on the immune system\textsuperscript{55-57}, the majority of studies performed have found that early thymectomy leads to an impairment of the T cell compartment. Lower T cells numbers, lower TRECs and fewer RTEs have also been reported\textsuperscript{58-60} together with alterations in the CD4 and CD8 ratio\textsuperscript{58, 61}. Reduction of naïve T cells in thymectomized individuals together with an increase in Ki67 indicate that an expansion of T cells in the periphery compensate for absent thymic output\textsuperscript{62}. A recent study shows lower CD4 and CD8 naïve cell counts, but a preserved regulatory T cell compartment, in Tx individuals\textsuperscript{63}. Earlier the same group suggested that homeostatic proliferation of peripheral regulatory T cells explained their increased numbers\textsuperscript{64}. These observations regarding increased numbers of regulatory T cells after thymectomy is confirmed in a separate study in which an increase of T regulatory cells and their cytokine production was detected during the first years after thymectomy\textsuperscript{65}. Peripheral proliferation of T regulatory cells could potentially play an important role in limiting the amount of autoimmune diseases after Tx.

Thymectomized individuals have also been demonstrated to have increased frequencies of autoantibodies, for example autoantibodies associated with autoimmune liver disease and SLE\textsuperscript{63, 66}. 
2.2 THYMECTOMY FOLLOW UP

2.2.1 THYMECTOMY FOLLOW UP STUDY

Paper I is part of a study that was started in 1993 by Solveig Oskarsdottir and Anders Fasth. Children under the age of 6 months that got more than 90% of their thymus removed during the cardiac surgery at the Queen Silvia Children’s Hospital in Gothenburg were included in the study. Blood samples were taken preoperatively, at 18 months and 18 years of age and compared to matched controls.

Childhood thymectomy resulted in immunological changes resembling premature aging. The thymectomy resulted in lower absolute numbers of naïve CD4\(^+\) cells, CD31\(^+\) cells and T regulatory cells, although the proportions were mainly unaffected. TREC levels among the thymectomized patients were low to non-detectable. The telomeres were shorter among CD8\(^-\) cells, indicating peripheral expansion. Signs of repertoire oligoclonality were discovered using flow cytometry analysis for TCR variable \(\beta\)-chain\(^67\), which prompted us to follow up with immunorepertoire sequencing (Paper I), to enable a more detailed repertoire analysis.

DNA from sorted CD4\(^+\), CD8\(^+\) and CD19\(^+\) cells was sequenced and analyzed for T cell receptor \(\beta\) chain (TCR\(\beta\)) and immunoglobulin heavy chain (IGH) usage. This allowed a more detailed investigation than possible with flow cytometry. It did not only give information about the genes used but also deletions, insertions and CDR3 length and composition.

The method used to quantify the clonality is based on the occurrence of coincidences\(^68\). The sample was divided into six reactions that were amplified and sequenced individually. If the same clone appeared in more than one reaction it was termed a coincidence. Based on the coincidences it is possible to calculate a clonality score. Our main result from Paper I was the significantly increased clonality among CD4\(^+\) and CD8\(^+\) T cells in the thymectomized patients. As an internal control we could, as expected, not detect any difference in the clonality of CD19\(^+\) B cells between thymectomized individuals and controls. The clonality score among T cells were negatively correlated with the number of CD4\(^+\) and CD8\(^+\) T cells in peripheral blood, further strengthening the results.

2.2.2 THYMECTOMY LONG TERM EFFECTS

Responses to vaccines obtained previous to Tx, e.g. MMR seem relatively unaltered, with similar MMR-specific IgG concentration as controls.
Responses to vaccinations after thymectomy, e.g. tick-borne encephalitis, was delayed, with a normal response first after the third vaccination\textsuperscript{69}. Age at Tx correlated with TBE-specific IgG antibody levels, with higher levels the later the Tx was performed, which is supported by the observation that thymectomized children show significantly lower total counts and percentages of naïve T cells, which correlated with the time passed since Tx, compared to controls\textsuperscript{70}. Hepatitis B vaccination in individuals with no thymic activity revealed undetectable or low levels of Hepatitis B-specific IgG\textsuperscript{71}.

Ageing mice have an impaired immune response against influenza virus. Their aged immune system suffers from a restricted diversity of CD8\textsuperscript{+} T cells, resulting in holes in the repertoire, which hampers the immune response. The same effect was seen in thymectomized mice, consistent with the decreased repertoire, where absolute number of CD8\textsuperscript{+} T cells was unchanged, but a reduced response in influenza specific CD8\textsuperscript{+} T cells was observed. These results strengthen the arguments for links between decreased diversity, age and less responsiveness to infections\textsuperscript{72}. In a larger register study, Gudmundsdottir et al reported an increased risk for autoimmune diseases such as hypothyroidism and type 1 diabetes and infections in thymectomized patients compared to surgery controls. The study included 5664 thymectomized individuals, but due to the relatively low average age of the patients (mean 14 years) the follow up time was still short. The amount of thymus tissue removed during surgery was not reported, but far from all subjects had undergone total thymectomy, which might lead to an underestimation of the differences between the compared groups\textsuperscript{1}.

Two studies that studied atopy in thymectomized patients reported different findings. In the first study, heart surgery was associated with increased frequencies of atopic disorders, possibly due to an altered T cell repertoire. They showed that thymectomy significantly increased the development or worsening of atopic symptoms, mainly asthma. The patients had undergone heart transplantation and were treated with immunosuppression, which may have affected the results\textsuperscript{73}. A second Danish study of risk for atopic dermatitis among thymectomized infants showed that the risk for atopic dermatitis was reduced in the surgery group compared to controls\textsuperscript{74}. This was also shown by Gudmundsdottir et al in the register study mentioned above\textsuperscript{1} and may be explained by the decreased T cell efflux following thymectomy.

### 2.2.3 Peripheral Expansion

Studies of the effects of human thymectomy generally show relatively mild clinical outcome. This supports the notion that homeostatic proliferation of
naive T cells in the periphery is effective and can compensate for a decreased thymic output. This regulation is particularly active in lymphopenic hosts, such as elderly individuals and thymectomized patients.\textsuperscript{75}

Patients thymectomized during their first 30 days of life that were followed up showed lower TREC levels and higher levels of IL-7 in serum. The levels of IL-7 correlated negatively with absolute CD4\textsuperscript{+} T cell counts two years post-thymectomy.\textsuperscript{61} Another article also reported significantly elevated levels of IL-7 the first years after thymectomy.\textsuperscript{76} Further findings supported the idea that peripheral expansion counteract the decrease in thymic output to maintain T cell homeostasis. The altered equilibrium has also been illustrated by higher levels of Ki67 in naïve T cells after thymectomy, which did not normalize until ten years post-thymectomy.\textsuperscript{62}

Most centenarians have undetectable TREC\textsuperscript{s} and lower levels of CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells than both young controls and middle-aged individuals. An important factor for the thymic T cell production and the maintenance and survival of the peripheral T cell pool is IL-7, and interestingly plasma levels of IL-7 were higher in women, which have been speculated to be a factor involved in the higher number of female centenarians.\textsuperscript{77} Furthermore, IL-7 given to aged macaques increased the thymic output measured by TREC\textsuperscript{s} and resulted in an increase of central memory cells.\textsuperscript{78} Thus, the higher IL-7 among female centenarians is possibly resulting in a better conservation of the lymphocyte pool.

In mice the maintenance of the peripheral naïve T cell pool is sustained by thymic output throughout their lifetime, and almost all naïve T cells originate from thymic output in mice, even at old age. The T cells have a short life span of approximately 7 weeks for CD4\textsuperscript{+} and 11 weeks for CD8\textsuperscript{+}.\textsuperscript{29} In contrast, the human T cell pool is more dependent on peripheral T cell division,\textsuperscript{79} which makes comparisons between human and mouse less relevant and can probably account for the relatively mild clinical manifestations of childhood thymectomy observed in the clinical follow-ups so far.

A diverse repertoire can have an impact on health later in life. In a study on glioblastoma multiforme, where advanced age is a predictor for poor clinical outcome, a favorable prognosis correlated better with CD8\textsuperscript{+} RTE levels measures, as measured by TREC\textsuperscript{s}, than with age.\textsuperscript{80} The age dependent decreased thymic output of CD8\textsuperscript{+} T cells could possibly influence the age-related cancer mortality. An immune model was used to show the association with cancer and thymic involution rather than with age, although it normally accompanies each other. An interesting speculation was that the reduced
cancer risk observed in certain shark species could be due to the thymus not involuting.

Thymic output is thought to be vital during T cell repertoire establishment, but not essential for repertoire maintenance during adulthood, at least for a limited time. The relative diversity seen in thymectomized individuals and the proportions between naïve and memory T cells are often reported to be sustained during a long time. Due to that the peripheral expansion is so efficient in humans, it may take a long time before the full effects of thymectomy are shown as clinical manifestations. With an emerging group of thymectomized patients, and a population growing older, treating diseases of aging by targeting the thymus, the thymic output or the peripheral expansion represents interesting therapeutic possibilities.
3  PAPER II: THYMIC B CELLS

3.1  THYMIC B CELLS

B cells constitute about 1 % of the total cell number in both human and murine thymus. They were first discovered in the human thymus in 1987 by immunohistochemistry, which revealed the presence of these cells almost exclusively in the medulla.

3.1.1  MOUSE THYMUS

The B cells in the mouse thymus have been reported to emanate from progenitor cells within the thymus, with the recruitment from the periphery playing only a minor part. The progenitors are located in the cortex area while more mature B cells reside in the medulla. However, other studies have reported that peripheral immigration contribute substantially to the establishment and maintenance of the thymic B cell population.

Thymic B cells are characterized by the expression of Aire, CD80, CD86 and high levels of MHC class II and CD40. These specific features of the thymic B cells are acquired in the thymic environment, which was shown by Yamano et al. They injected IgM⁺IgD⁺MHCII⁺CD80⁻Aire⁻ B cells and later finding them in the thymus with higher levels of MHCII and positive for CD80 and Aire.

Interestingly, even though the percentage of B cells in the thymus increases with age, the absolute number of B cells goes down. The expression of Aire and self-antigens appear to diminish with age, and if aged B cells are injected intra-thymically in young mice, this expression is not restored. These results suggest that the inability to express Aire and self-antigens due to aging is an intrinsic feature of the B cells.

The Ig switching of the thymic B cells in mice is thought to take place intrathymically, and is dependent on the B-T cell interaction where the CD40-CD40L interaction plays an important role. This interaction is also crucial for the maintenance and proliferation of the thymic B cells. The repertoire of the thymic B cells is distinct, with a high degree of autoreactivity, making the B cells capable of acting as effective APCs for self-antigens during T cell selection, which suggests an important role in shaping the CD4⁺ T cell repertoire. Similarly to dendritic cells, the thymic B cells are reported to be able to aid in the negative but not the positive selection.
Thymic B cells have also been proposed to play a role both in the induction of T regulatory cells\textsuperscript{91, 92} and in the deletion of autoreactive thymocytes in an experimental murine system using myelin oligodendrocyte (MOG) reactive thymocytes and B cells expressing MOG on MHC-class II\textsuperscript{93, 94}.

A specific thymic B cell population in the mouse, expressing sialidase, was discovered in 2004\textsuperscript{95}. It has been proposed that these B cells, together with mTECs, remove sialic acid on thymocytes to aid interaction with APCs in the negative selection process. SP thymocytes have higher levels of sialic acid covering D-galactose residues. This can be shown by staining with peanut agglutinin (PNA), which binds the galactose residues in the DP thymocytes in the cortex whereas staining is impaired in the SP thymocytes with higher level of sialic acid. It has been proposed that in order to allow tight interactions between maturing thymocytes and APCs this sialic acid needs to be removed\textsuperscript{96-98}.

We have seen a similar staining pattern as it has been described in mouse thymus when staining with PNA in human thymus tissue. (Figure 5)

Figure 5. PNA (green) and nuclear stain Hoechst (gray) staining of the same area, showing PNA staining in the immature thymocytes in the cortex. Scale bar 200 µm.

A study in non-obese diabetic (NOD) mice showed an increased activity of the thymic B cells in the prediabetic phase. The thymic tissue showed an accumulation of thymic B cells in the cortico-medullary junction and formation of germinal centers. Autoantibodies binding cytokeratin 5\(^+\) epithelial cells were found in the NOD mice together with a higher level of apoptosis among these cells. The antibodies, presumably produced by the accumulated B cells, could be inducing apoptosis in mTECs, including insulin expressing mTECs. This was thought to impair the thymic negative selection of insulin reactive T cells driving the development of diabetes in the NOD mice\textsuperscript{99}. 
3.1.2 HUMAN

Less work has been done concerning human thymic B cells. Human thymic B cells are located in the medulla or in the perivascular spaces, similarly to the distribution in mice. The B cells in the perivascular area are thought to be plasma cells, secreting antibodies towards viral proteins. These cells are maintained throughout aging and are assumed to protect the thymus from infections.

The B cells located in the medulla are suggested to take part in the negative selection of thymocytes. Thymic B cells show a prominent reactivity towards peptide autoantigens, and by cloning and expressing antibodies from thymic B cells they appear to be more autoreactive than B cells in the bone marrow. It has also been shown that thymic B cells have a strong bias towards V(H)4, a gene segment family frequently encountered in autoimmunity.

According to a recent study, about half of the thymic B cells in humans are naïve B cells. As shown in this thesis and by others, the thymic B cells express AIRE and high levels of CD86, MHC class II and CD40. Moreover, human thymic B cells express tissue restricted antigens (TRAs) that are different from those expressed by mTECs. Together with their location in the medulla and their activated phenotype, the expression of AIRE and TRAs in human thymic B cells supports their possible involvement in negative selection and generation of regulatory T cells.

As in mice, the percentage of B cells in the thymus has been reported to rise in older children, but the levels of AIRE declines with age. Autoimmunity has been linked to abnormal B cell numbers in the thymus and germinal center formation has for example been observed in SLE and myasthenia gravis. In addition, CCL21 is overexpressed in MG thymus, attracting both T cells and naïve B cells from the periphery. Furthermore, an increase in T follicular helper cells has been reported in thymic tissue of MG patients. Similarly to T follicular helper cells in mice they might drive the B cell development by expressing IL21.

3.2 CD21\(^{-}\)/LOW B CELLS

Over the last decade a population of mature B cells with low expression of the complement receptor 2, (CD21\(^{-}\)/low), has been described in tonsils and peripheral blood of healthy individuals. This B cell population has also been found to expand with age and is more abundant in patients with chronic infections and autoimmune diseases.
In peripheral blood from healthy controls the CD21\(^{-}\)/low B cells are mainly memory cells, and account for approximately 5% of all B cells\(^{108}\). The population is absent in cord blood, suggesting that they are antigen-experienced cells. In tonsils they have been defined by their expression of the Fc-receptor-like protein 4 (FcRL4) and lack of CD27, a key marker for memory B cells. They are mainly isotype-switched and defined as tissue-based memory B cells\(^{109,111}\).

CD21\(^{-}\)/low B cells are expanded in conditions with chronic infection such as human immunodeficiency virus (HIV)\(^{112}\), hepatitis C virus (HCV)\(^{113}\) and malaria\(^{114}\), but also in immunological disorders and autoimmune conditions such as common variable immunodeficiency (CVID)\(^{115}\), rheumatoid arthritis (RA)\(^{116}\) and systemic lupus erythematosus (SLE)\(^{117}\). The persistent immune activation observed in these disorders makes the CD21\(^{-}\)/low B cells hyporesponsive to stimulation via the B-cell antigen receptor (BCR)\(^{110}\). The role of CD21\(^{-}\)/low B cells in health and disease is not fully understood.

### 3.3 CD21\(^{-}\)/LOW B CELLS IN THE THYMUS

In Paper II we show that half of the B cells residing in the human thymus early in life display a unique phenotype characterized by the lack of or low surface expression of CD21, (CD21\(^{-}\)/low).

The lack of, or low, surface expression of CD21 is typical for immature B cells, such as early transitional B cells in peripheral blood of infants\(^{118}\), although these cells also express CD10, separating them from mature cells. However, when comparing the thymic CD21\(^{-}\)/low B cells with cells from the same infants’ peripheral blood, we found that the vast majority of the B cells in the thymus were mature cells, being CD10\(^{-}\)CD34\(^{-}\).

Despite the thymic CD21\(^{-}\)/low cells being negative for the memory B-cell marker CD27, almost half of them were Ig class switched cells. This was unexpected considering that switched B cells were almost absent in the peripheral blood from the same infants. Ig class switching occurs for example after active immunization or an infection, and switched B cells are not generally found in peripheral blood from healthy neonates. The origin of the switched B cells in the thymus of newborns could potentially be maternal due to cell microchimerism, however we found that the thymic B cells originated from the child, disproving the hypothesis of a maternal origin. As in mice, class switching could be facilitated by cognate interaction with the thymocytes\(^{83}\).
Thymic Studies

where the interaction between CD40 on the B cells with CD40L on the T cells seems crucial.

The thymic CD21⁻/low B cells were large in size and expressed high levels of the typical activation markers CD69 and CD95. Their high levels of CD86, a costimulatory molecule highly expressed on professional APCs, together with the high levels of HLA-DR and CD40 and their localization in the medulla, suggest a role of the thymic B cells, and in particular the CD21⁻/low, in T-cell selection. This is supported by studies in mice where switched B cells play an important role in driving T-cell tolerance.⁸³

Thymic CD21⁻/low B cells expressed significant levels of AIRE, which was higher than in the CD21⁺ cells. In mice, thymic B cells express TRAs induced by AIRE, and are efficient APCs.⁸⁶, ⁸². Also, human thymic B cells express TRAs, which differ from the TRAs expressed by mTECs, suggesting a non-redundant contribution of thymic B cells to central T-cell tolerance.⁸⁴. Our results propose that the major contributor to this selection is the CD21⁻/low B cells, since they have an activated phenotype and express high levels of AIRE.

As mentioned above, CD21⁻/low B cells are found in both peripheral blood and tonsils from healthy individuals, and are expanded under conditions of chronic immune stimulation. In most of these conditions, the CD21⁻/low B cells express high levels of activation markers, with subsets that co-express T-bet, CD11c, FcRL4 and/or CXCR3. A CD21⁻/low population termed age associated B cells (ABCs), that express T-bet and/or CD11c, has also been described in both wild type and autoimmune prone mouse strains.⁸¹, ¹¹⁰, ¹¹¹, ¹¹². CD11c expression, which also is a hallmark of dendritic cells, potentiates the ability of ABCs to present antigen to T cells.¹²³ A subset of the CD21⁻/low B cells in the thymus expresses CD11c supporting their role as APC. The inhibitory receptor FcRL4 has been found to dampen BCR-signaling which would be consistent with most thymic CD21⁺ cells being FCRL4⁺ and respond to BCR agonists whereas some CD21⁻/low cells were FCRL4⁺ and showed a bi-modal response.

These findings, together with the findings that about half of thymic CD21⁻/low B cells were apoptosis prone and half were Ki67⁺ proliferating cells provide evidence that thymic B cells are heterogeneous and in a highly dynamic state. Thymic B cells communicate actively with the thymocytes, shown in our coculture experiments were CD21⁻/low B cells were able to induce CD25 upregulation in T cells more effectively than in the CD21⁺ counterparts.
4 PAPER III: HASSALL’S CORPUSCLES

4.1 THYMIC EPITHELIAL CELLS

The epithelial cells in the thymus are presumed to originate from a common progenitor. The common progenitor passes through a stage with expression of both cTEC and mTEC markers before differentiating into their respective lineages. However, there is also evidence for lineage committed progenitors, where the mTECs have been shown to originate from one progenitor clone, forming islets.

The mTECs differentiate from mTEC\textsuperscript{low} immature cells (Aire\textsuperscript{−} MHC II\textsuperscript{lo} CD80\textsuperscript{−}) to mTEC\textsuperscript{high} (Aire\textsuperscript{+} MHC II\textsuperscript{hi} CD80\textsuperscript{+}) and subsequently return to a state of mTEC\textsuperscript{low} (Aire\textsuperscript{−} MHC II\textsuperscript{lo} CD80\textsuperscript{−}). Both differentiation into the mTEC lineage and maturation from mTEC\textsuperscript{low} to mTEC\textsuperscript{high} requires activation of the NF-κB signaling pathway by members of the TNF-family, e.g. RANKL, which is produced by single positive thymocytes. Deficiency in RANKL leads to impaired medulla formation due to its importance for mTEC development. In adult mouse the halftime of Aire\textsuperscript{+} mTEC\textsuperscript{high} is about 2 weeks. A normal mTEC life cycle, including intact kinetics, seem to be needed for the organization of the medulla to be successful.

The transition to post-Aire mTEC\textsuperscript{low} is less studied than the conversion to mTEC\textsuperscript{high}. In the post-Aire state the cells start to express late-stage keratins and later form the Hassall’s corpuscles, in a process which may be supported by the expression of keratinocyte growth factor by single positive CD4 and CD8 thymocytes.

An important transcription factor in thymus ontogeny and development is FOXN1. A lack of function mutation in FOXN1 in humans is related to loss of hair, athymia and deficiencies in the T cell compartment. The mutation was first described in mice and gives rise to a nude phenotype, lacking hair and a functional T cell system. Foxn1 is not completely non-redundant for all epidermal differentiation in the skin as nude mice do not have an altered skin histopathology, apart from absence of hair.

4.1.1 AIRE

Aire is an mTEC defining gene that allows promiscuous gene expression, a process that aims to mirror the complete repertoire of peripheral self-antigens within the thymic medulla. mTECs can express more than 18 000 genes, approximately 85% of the coding genome. In the absence of Aire,
approximately 15,000 genes were still expressed in the epithelial cells suggesting that Aire is responsible for inducing 3-4,000 genes. Only a fraction of all TRAs are expressed in one cell at a given time point. The mTECs expressing a specific gene tend to localize in clusters in the medulla, about 1-3% of all mTECs express a particular TRA.

Aire interacts with unmethylated histone-3, found on inactive chromatin. It induces histone modifications at a low frequency resulting in the low number of specific TRAs expressed by each mTEC. A recently described transcription factor, Fezf2, has been reported to control a set of Aire independent TRAs in mTECs, even though the function of Fezf2 is not completely understood.

A deficiency of Aire in mice has been shown to cause failure in the thymic tolerance induction among single positive thymocytes. The thymus shows a defective removal of autoreactive thymocytes specific for Aire dependent antigens, and also an impaired generation of regulatory T cells, leading to multiple autoimmune manifestations. This indicates that the peripheral tolerance mechanisms are not enough to prevent autoimmunity, without negative selection taking place in the thymus.

Lack of functional AIRE in humans causes autoimmune polyendocrine syndrome type 1 (APS1) or autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED), a syndrome characterized by multiple organ-specific autoimmune diseases such as hypoparathyroidism, primary adrenal insufficiency (Addison’s disease) and mucocutaneous candidiasis. Although candidiasis in itself is an infection and not an autoimmune disease, the chronic mucocutaneous candidiasis in APS1 patients is thought to emerge as a result of neutralizing autoantibodies against IL-17 and IL-22, which hampers the defense against fungal infections. Other manifestations such as vitiligo, enamel hypoplasia, pernicious anemia, autoimmune hepatitis and type 1 diabetes are also common.

Even though AIRE is needed for a well-functioning negative selection, an overexpression of AIRE does not seem to improve the exposure of TRAs and result in a better central tolerance induction. Individuals with Down syndrome, or trisomy 21, have three copies of the AIRE gene, and it has been reported that higher levels of AIRE and also of insulin, a common AIRE dependent TRA, is expressed in their thymus. However, this overexpression of AIRE does not seem to be of benefit since both autoimmunity and infections are over-represented in Down syndrome. In Down syndrome, signs of accelerated mTEC maturation kinetics is seen, which may be a result of the high AIRE
levels. A signature of premature involution including a small thymus size with a high ratio of medullary versus cortical areas together with larger cystic involutions in the medulla is also seen\(^{148}\). Low levels of TREC is also confirming an impaired thymic function\(^{151}\).

The effects of increased AIRE expression have also been investigated in mice by creating a model in which extra copy of the human AIRE gene was added. This led to an altered maturation of the mTECs and a failure in depletion of autoreactive thymocytes\(^{152}\).

### 4.1.2 Diseases of the Thymic Epithelium

The most common acquired disease of the thymic epithelium is thymoma. It can originate from either cTEC or mTEC. There are associations between thymomas and autoimmune diseases, the most common being myasthenia gravis. The maturation of mTEC in the thymomas can be disturbed, with an impaired expression of AIRE and TRA which in turn can result in a dysfunction of the negative selection\(^{153}\).

Atrophy of the thymus is a common denominator in infectious diseases, mainly because of the depletion of thymocytes. This is mainly due to an increased apoptosis of DP thymocytes in the cortex and can be seen for example in AIDS and rabies\(^{154}\). It has been theorized that this can be due to a rise in circulating glucocorticoid levels during infections\(^{155}\). Thymocyte depletion in rabies can be prevented by adrenalectomy, showing that the thymic involution is depending on glucocorticoids\(^{156}\). The measles virus can attack the thymic epithelium itself, and can cause an arrest in cell growth and induce terminal differentiation\(^{157}\).

### 4.1.3 Late Stage Differentiation

The role of \textit{Aire} in the organization of the thymic medulla is not completely clear. After a transient activated stage with expression of \textit{Aire}, the mTECs continue into a post-\textit{Aire} stage. The loss of \textit{Aire} is accompanied by a decrease of MHC class II and CD80 expression\(^{158}\).

\textit{Aire} affects the differentiation of the thymic epithelium in mouse and the absence of \textit{Aire} causes morphological changes with reduced numbers of terminally differentiated mTECs expressing involucrin\(^{159}\). Deficiency of \textit{Aire} also results in altered transcriptional profile, disruption of the thymic cortico-medullary organization and a reduction of the medullary compartment. This indicates a role for \textit{Aire} in the differentiation and composition of thymic epithelium\(^{160}\).
By lineage tracing experiments it was shown that the Hassall’s corpuscles constitute the final developmental stage for the Aire\textsuperscript{+} mTECs, and that all involucrin positive cells have at one point also been positive for Aire\textsuperscript{158,161}.

### 4.2 HASSALL’S CORPUSCLES

The origin of the Hassall’s corpuscles has been the subject of considerable controversy. They were long considered to be degenerated endothelial cells from small vessels in the thymus. Later it was proposed that they originate from remnants of omnipotent embryonal epithelial cells. They have also been said to contain reticuloendothelial cells and striated myoid cells in the core. Furthermore, the proposed function has varied over time, from a site to destroy 'forbidden clones’ that are dangerous to the organism and recycling of materials, to storage of antibodies and antigens\textsuperscript{162-165}.

An interesting study was made where it was tested if Hassall’s corpuscles reacted to antigens. Tetanus toxin was injected to guinea pigs and the morphology of the thymus was investigated, showing proportionally more epithelial cells and larger Hassall’s\textsuperscript{166}. This was probably due to stress and corticosteroid induction of apoptosis within the thymocyte population and not an active response from the epithelial cells.

The cell layers immediately surrounding the corpuscles stain positive for various late-stage keratins such as cytokeratin 10 and involucrin, also found in the upper layers of the skin. Although the Hassall’s corpuscles originate from epithelial cells and their keratinization resembling skin was noted already in 1979\textsuperscript{167}, a reason for the many theories surrounding the structures could be the difficulty to study them microscopically due to the tendency of unspecific binding of histological stains and antibodies. The slow progress of deciphering the function of the Hassall’s corpuscles could also be due to the limited size and appearance of the corpuscles in mice.

It is likely that the Hassall’s corpuscles represent the last stage of the mTEC development, where the epithelial cells lose their nuclei and form the core of the Hassall’s corpuscles. The function, if any, of the corpuscles is still unknown and the ideas have varied over the years. The two most recent theories are the induction of regulatory T cells by Hassall’s production of thymic stromal lymphopoietin (TSLP)\textsuperscript{168} and a role as recruiters of IL-23 producing neutrophils by CXCR5 to induce plasmacytoid DCs to produce TNF\textalpha\textsuperscript{9}. 

An interesting sex difference has been reported, with a higher average number of Hassall’s corpuscles in male than female fetuses. This could be explained by the differences in sex hormones that female and male fetuses are exposed to during pregnancy. A sex difference in AIRE expression has also been noted with a higher AIRE expression in males, which in turn could drive the mTEC differentiation and result in a higher turnover and more Hassall’s corpuscles in males.

4.2.1 HASSALL’S CORPUSCLES AND SKIN

In the manuscript included as Paper III, we aimed at characterizing the Hassall’s corpuscles in the human thymus more extensively than had been done previously. It has been proven difficult to degrade the keratinized cell compartment of the Hassall’s corpuscles and create a single cell suspension to be able to sort the different cell populations by FACS. To overcome this problem and to acquire cells from the rim of the corpuscles as well as material from within the Hassall’s corpuscles we chose to use a laser microdissection technique.

The RNA sequencing revealed more upregulated genes (3285) in the cell layers surrounding the HC than downregulated genes (656), compared to Hassall’s corpuscle free thymic medulla. Gene ontology analysis revealed an enrichment of skin differentiation processes and a downregulation of T and B cell markers in the cells surrounding the corpuscles. This could be due to the enrichment of mTECs around the Hassall’s corpuscles, resulting in a relatively lower frequency of lymphocytes. An analysis of the chromosomal enrichment gives the highest hit on Chromosome 1q21, where the epidermal differentiation complex is located. This consists of several genes with important functions in the terminal differentiation of the epidermis, such as S100As, involucrin, loricrin and SPRRs.

The results from the proteomic analysis of the cells surrounding the corpuscles and the material within the Hassall’s corpuscle core show a downregulation of chromatin and RNA processing systems in the latter, as the mTECs lose their nuclei and end up in the amorphous core of the corpuscle. An enrichment of late-stage keratins and antibacterial proteins is seen in the core, mimicking the keratinization process observed in the skin. The observed similarities regarding RNA and protein content between human thymus and skin was not entirely surprising, since similarities have been described in mice where immunohistochemistry has shown similar staining patterns in thymus and skin.
As in mouse, the human mTEC\textsuperscript{low} stage is followed by mTEC\textsuperscript{high}, in which the cells express AIRE and TRAs to be presented to the developing thymocytes in the negative selection process. After the loss of AIRE, the cells transition into mTEC\textsuperscript{low}, expressing late-stage keratins and eventually forms the Hassall’s corpuscles.

As reported earlier, we demonstrated a higher level of AIRE expression in Down syndrome thymus, probably caused by the extra copy of $AIRE^{148, 149}$. The higher level of AIRE could be driving an increased turnover of the epithelial cells, resulting in the enlarged Hassall’s corpuscles typical for Down syndrome thymus. Unfortunately, we have not managed to acquire skin samples from Down syndrome patients to investigate if the differentiation of the epidermis is comparably affected. However, it is interesting in this context that Down syndrome patients have been reported to have increased frequencies of keratinization defects of the skin, resulting in diseases such as keratosis pilaris and xerosis\textsuperscript{174}.

In Aire\textsuperscript{−/−} mice we can report a decrease of involucrin positive areas, the mouse equivalent of Hassall’s corpuscles, in the thymic medulla. Interestingly, the skin of Aire\textsuperscript{−/−} mice showed a reduced thickness of the involucrin positive cell layer, suggesting an involvement of Aire in the development of both thymic epithelium and skin in mouse.

In theory, the Hassall’s size could correspond to the thickness of the skin of the animal species in question. The human skin is thicker than for example the mouse skin, which corresponds to the bigger Hassall’s corpuscles seen in humans. (see Figure 6). However, this theory remains unproven, as articles describing the thymus histology from animals with thick skin, such as Nile crocodiles and rhinoceros, do not mention the particular size of the Hassall’s corpuscles\textsuperscript{175, 176}.
One potential function of the Hassall’s corpuscles could simply be to provide skin specific antigens for T cell education. Interestingly, it has been shown that Hassall’s corpuscles for example express the pemphigus vulgaris-related autoantigens Desmoglein-1 and -3\textsuperscript{158}.

As the end stage mTECs around the Hassall’s cannot desquamate after being keratinized, in the same way as the keratinized cells of the skin do, they create these large formations of keratin, which probably are challenging for the thymus to break down. A question, yet to be addressed, is how the corpuscles are degraded in the thymus, since there has been no report of them accumulating with age to the extent expected if not being possible to degrade.

*Figure 6. Thymus staining with Involucrin (green, upper row) and skin (lower row) from human (left) and mouse (right). Nuclear stain (gray) is Hoechst. Scale bar 50µm.*
5 METHODS AND LIMITATIONS

For a detailed description of the methods used in the thesis I refer to the original papers.

5.1.1 TISSUE HANDLING

The thymic tissue was placed in cold PBS immediately after surgery and until further processing. Blood from the patient was collected in a heparin tube and kept at room temperature.

For analysis of lymphocytes, the tissue was cut finely and gently pressed over a 40µm cell strainer. The cells were passed through a cell strainer and the blood sample was centrifuged on a density gradient with Ficoll-Paque (GE Healthcare Life Sciences, Little Chalfont, Buckinghamshire, United Kingdom) to separate the lymphocytes and “clean” the sample.

For analysis of the epithelial cell compartment, mechanical and enzymatic digestion was needed. The tissue was cut into small pieces and incubated on rotation with RPMI media with Liberase TM (Roche) and DNase (Worthington) with intermittent mechanical degradation using a gentleMACS tissue dissasociator (Miltenyi).

Potential methodological problems could be that some lymphocytes might be left in the tissue and give a skewed cell population distribution, specifically cells close to the “sticky” keratinized part of the medulla could be more difficult to extract than cells in the cortex. The mechanical dissociation of the epithelial cells causes cell death, evident by the presence DNA and the need of DNase in the enzyme mixture. The enzymatical treatment might affect and strip away sensitive cell surface markers.

5.1.2 FLOW CYTOMETRY AND FACS

For Paper I peripheral blood mononuclear cells were sorted into CD4⁺, CD8⁺ T cells and CD19⁺ B cells with an i-Cyt Synergi cell sorter (Sony Biotechnology Inc, San Jose, California).

For Paper II CD19⁺ B cells were sorted into CD21⁺ and CD21⁻/low and Ig switched and unswitched populations for FISH analysis and functional co-culture experiments. Due to the small populations we did not manage to sort large samples, which was evident in the FISH analysis where the switched cell count did not reach 50. The flow cytometer used in the B cell paper was a
FACS lyric (BD Biosciences, San Diego, California) and the sorter a SH800Z (Sony Biotechnology Inc, San Jose, California).

All sorting experiments were performed with ≥95% purity.

The image stream images in paper II were acquired with ImageStream X Mark II imaging flow cytometer (Amnis, Seattle, Wash). Thymic epithelial cells were run in parallel to have a positive control when analyzing AIRE.

5.1.3 IMMUNE REPERTOIRE SEQUENCING

The benefit of the DNA sequencing-based analysis of receptor repertoire, used in paper I, over Flow cytometry analysis of the Vβ families is that it allows a detailed analysis of the repertoire and a resolution where it is possible to distinguish clones and not only chain usage. The samples came from DNA originally sorted for telomere length analysis. The sample populations were CD4+ and CD8+ T cells and CD19+ B cells.

Multiplex PCR was performed according to BIOMED-2 guidelines with specific primers to amplify Vβ or IGH chains. A possible problem with this approach is the primer efficacy, if it differs it could give a skewing in the amplification step. However, this would affect the controls and the thymectomy samples to the same extent, and therefore it should not affect the comparison between the two.

The amplified reads were sequenced on an MiSeq sequencer (Illumina, San Diego, California). The clonality was analyzed by a method described by Boyd were six parallel wells were amplified and sequenced and the occurrence of the same clone (determined by amino acid usage) in the separate wells is named coincidence. The coincidences are presumed to originate from the same T cell, meaning the fewer coincidences the more diverse repertoire.

We analyzed CD19+ B cells for somatic hypermutations (data not shown) to assess whether the lack for clonality among T cells affected the B cell compartment in Ig switching and affinity maturation in germinal centers. We could find no difference between the groups, although it would have increased the chances of finding differences if the sorted populations had been more specific and for example distinguished between naïve and memory B cells.
5.1.4 IMMUNOHISTOCHEMISTRY

Almost all sections used in both paper II & III were OCT embedded frozen samples. Although more time-consuming at the first tissue processing step the antigens are preserved without modification, which is an advantage compared to formaldehyde fixation that cause denaturation of proteins. Formaldehyde fixation gives a better morphology but risks that certain antigens are not recognized by the antibodies. The downstream steps of formaldehyde fixed samples require more time and the use of harmful chemicals, making frozen samples the preferred alternative.

All stains included in this thesis were well defined and the risk of detecting false positives as true staining were minimal. All images were acquired with an LSM700 confocal microscope (Carl Zeiss AG, Oberkochen, Germany).

5.1.5 LASER MICRODISSECTION

To analyze the Hassall’s corpuscles in paper III there was a dilemma of how to acquire samples to analyze. Creating single cell suspensions of late stage epithelial cells would require tough degradation, without knowing if all cells are properly released in the suspension and represented in the analyses. This problem has been approached by comparing TEC numbers acquired by flow cytometry and microscopy, showing a clear bias were not all cells are represented in suspension\textsuperscript{172}. To be able to acquire all cells from the area surrounding the Hassall’s corpuscles, and also the very center of the corpuscle, we chose to use laser microdissection. The corpuscles are distinct and identifiable without specific staining, making the protocol straightforward. Unfortunately, all the cells surrounding the structure are collected simultaneously. The epithelial cells of interest are diluted by other cell types present in the vicinity. One way of approaching this problem could be to subtract e.g. thymocyte markers from the sequencing data.

5.1.6 QPCR

qPCR was performed in Paper III with TaqMan assay. Duplex PCR was used to save cDNA from samples. In duplex two different primers are used in the same well, with different fluorescent molecules attached to the probes. The two duplexed assays were diluted previously to limit the primer amount of the more expressed gene to prevent it from consuming all reagent. Samples were run separately and in same well simultaneously to compare the CT curves. The samples were analyzed with a Viia 7 real time PCR system. (Thermo Fisher, Waltham, Massachusetts)
6 PATIENT SAMPLES

Thymic samples were collected at the Queen Silvias Children’s Hospital, Sahlgrenska University hospital, Gothenburg. Due to the location of the thymus in the upper chest cavity it blocks the surgeon’s access to the heart and needs to be removed during corrective cardiac surgery.

The health of children undergoing surgery can be variable and may affect our studies, this needs to be kept in mind when analyzing human samples. Up to 10% of children with congenital heart disease have anomalies that fit into a syndrome. This is not always fully investigated at time of surgery and therefore not always registered for the samples in our biobank.

Some heart diagnoses are more subjected to cyanosis than others. The most common cyanotic congenital heart diseases are tetralogy of Fallot and transposition of the great arteries (TGA). It is not known to what extent cyanosis might affect the thymus, newborns suffering from this affliction are also discovered early and treated. A study performed on human thymic tissue could not find any effects on thymic subset distributions between cyanotic and well saturated children. However, it was not specified what subsets were investigated. A later study found a difference in the numbers of FoxP3 expressing cells in the thymic medulla in children with a cyanotic congenital heart disease who had greater numbers than non-cyanotic controls. Cyanosis can induce FoxP3 through hypoxia inducible factor-1α and cause an unintentional bias. One of the most common cyanotic heart defects, TGA, afflicts males more often than females. This could introduce a gender bias if studying FoxP3.

The field of immunology had the fewest articles that specified sex out of ten research fields according to a review. This is surprising since there are large immunological differences reported between the sexes, for example many of the autoimmune diseases are more common in women. It has also been shown that males have higher levels of AIRE and that women express less AIRE after puberty. It was also shown that male castration decreases thymic AIRE expression. Cultures of human TEC showed a downregulation of AIRE upon addition of estrogen. The major differences arise in puberty when sex hormones are expressed in higher levels, but also more discrete changes of hormones, as in minipuberty, seems to affect the infant thymus during the first years of life. The hypothalamic-pituitary-gonadal axis is active in mid gestation but silenced towards full term. At birth the axis reactivates which
leads to a testosterone rise in males that peaks at 1-3 months of age known as “minipuberty”. In females it leads to an increase in oestradiol levels \(^{170, 189}\).

There are both limitations and benefits in working with human samples rather than mouse models. Some of the limitations includes less possibilities for \textit{in vivo} manipulations since all experiments on human thymic tissue need to be performed \textit{in vitro}. The sample access is more unreliable and difficult to plan. Despite all this it is a great opportunity to be able to study human tissue as animal models and cell lines are usually not as representative.
CONCLUDING REMARKS

In paper I we investigated the effects of thymectomy in early childhood on T and B cell receptor repertoires in peripheral blood. The T cell compartment showed a higher clonality, indicating an effect of the absent thymic output. The B cell clonality was not affected. The impact on the T cell compartment 18 years after thymectomy suggests an impending problem, which might also affect the B cell population with time.

Paper II reveals a new characteristic about the thymic B cells, half of them expressed low levels of CD21, associating them to an already known population of B cells usually found in diseases characterized by chronic immune stimulation. A surprising number of the thymic B cells were switched, higher among the CD21<sup>-low</sup> population than among the CD21<sup>+</sup>. They were also more effective in thymocyte crosstalk, probably due to their high levels of CD40 and MHC class II.

Paper III aims to characterize the last developmental stage of the thymic epithelial cells. The Hassall’s corpuscles have since their discovery been an enigmatic structure. We showed with the help of laser microdissection that the cell layers surrounding the Hassall’s have an enrichment of skin markers, and that the core of the structure itself contains late stage keratins and bacterial defensive proteins also found on the skin.
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