THE SAHLGRENSKA ACADEMY

Sex bias of regulatory T cells in thymus and blood

Degree Project in Medicine

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Abbreviations/Terms of importance

MHC   Major histocompatibility complex

NK cells   Natural killer cells

ILC   Innate lymphoid cell

TRECs   T cell receptor excision circles

AIRE   Autoimmune regulator

CD   Cluster of differentiation

FOXP3   Forkhead box P3

IL   Interleukin

TGF   Transforming growth factor

IPEX   Immunodysregulation polyendocrinopathy enteropathy X-linked

IBD   Inflammatory bowel disease

VSD   Ventricular septal defect

HLHS   Hypoplastic left heart syndrome

PBS   Phosphate-buffered saline

FCS   Fetal calf serum

EDTA   Ethylenediaminetetraacetic acid

DMSO   Dimethyl sulfoxide

FSC   Forward scatter

SSC   Side scatter

CHD   Congenital heart disease

Abstract

Background

At least three percent of the human population suffer from autoimmune diseases. There are differences between the sexes in the prevalence of auto immune diseases. Women generally have a higher prevalence. The reason to this sex bias in not known. One cell type of interest is the regulatory T cells that functions to regulate the immune response to prevent autoimmunity. The proportion between the sexes of regulatory T cells in thymus is not known.

Aim

To examine if there are sex differences in regulatory T in the thymus. And if so, explore in which stages of regulatory T cell development the sex bias is present.

Method

Antibodies attached to specific markers on the cells were used to analyze the samples in the flow cytometer for quantity and proportion of the different markers. The files were analyzed with the software FlowJo.

Results

No statistical significant difference of regulatory T cells in thymus between the sexes was found. This project shows the tendency of boys having higher proportion of regulatory T cells in peripheral blood and quite the opposite in the thymus, with a tendency towards higher proportion of regulatory T cells in females. This is not significant.

Conclusion

The results suggest that there may be a sex bias in regulatory T cells in thymus. Further studies are needed with more individuals included. It would especially be interesting to investigate children older than two months, the difference seen is perhaps related to the androgen peak boys get at 12 weeks of age. This is the first study to examine the proportion of regulatory T cells in the thymus with flow cytometry. Flow cytometry is a good method to further examine the underlaying causes to the sex bias seen in autoimmune diseases on cellular level.

Background

The immune system:

The immune system protects us against infections. The protection comprises of three lines of defense, with the outer lining of chemical and mechanical barriers on our skin and mucosa, for example hydrochloric acid in the stomach. If the pathogens pass the skin or the mucosa that line the outer surface of our body, the innate immune system is activated. The cells in the innate immune system recognize the pathogens and start an inflammatory response, and we feel pain and heat and see a redness and swelling at the site of inflammation. The third protection is the adaptive immune system that is more specific, but slower because it must be activated, and the cells must divide and mature before they can act (1).

All immune cells develop from pluripotent hematopoietic stem cells in the bone marrow. They form leukocytes (B cells and T cells) in the adaptive immune system and NK cells, ILC, dendritic cells, granulocytes, mast cells and macrophages in the innate immune system.

The immune system comprises of several organs in the body. The primary lymphoid tissues are the bone marrow, where the cells are produced, and the thymus where the T cells mature. The secondary lymphoid tissues with centers of immune cells, includes the spleen that filters the blood for pathogens, and the lymph nodes, filtering the lymph.

Thymus:

The thymus is found on top of the heart in the thoracic cavity, it is a bilobed organ organized in smaller lobules. It has its maximal size, relative to the body, in newborns (see figure 1) and

involutes during life, with gradually loss of function, shrinkage of lymphatic tissue and corresponding expansion of adipose tissue (2).
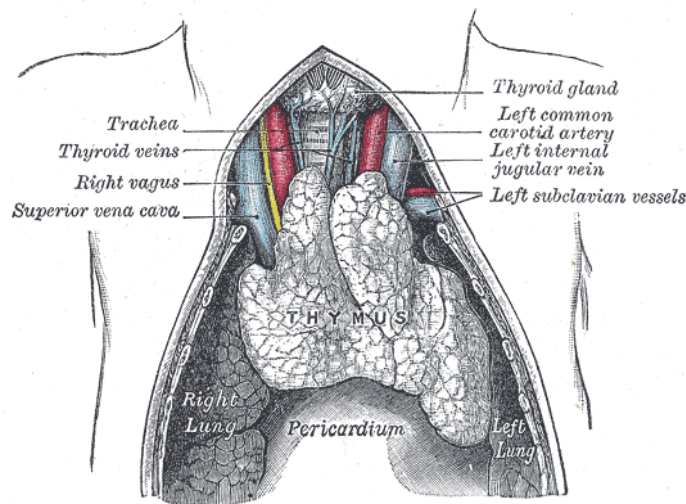

Figure 1. Thymus of a full-time fetus, located on top of the heart in the thoracic cavity. Anatomy of the Human Body, 20th ed. Gray, Henry. 1918.

Anatomically the lymphatic tissue consists of two regions, the cortex and the medulla, with surrounding connective tissue, vessels and adipose tissue. In addition to T cells some B cells exist as well, with largely unknown function, possibly acting as antigen presenting cells (3).

Thymus is an important organ in the immune system, since it is responsible for T cell development and maturation. The thymocytes (pre-T lymphocytes), produced by hematopoietic stem cells in the bone marrow, enter the thymic cortico-medullary junction via the blood stream. In the cortex signals from the thymic epithelial cells make the thymocytes express specific genes. Along the T cell development, the surface molecules change, it is combinations of these surface antigens that can be used for discrimination of T cells in different maturations stages.

First the thymocytes are double negative, they express neither CD4 nor CD8. Then they express both CD4 and CD8, i.e. double positive, and grow bigger to divide. They first go through the positive selection, where cells that do not recognize an MHC-peptide complex expressed by the epithelial cells with enough affinity undergo apoptosis. Cells that recognize an MHC-peptide complex are saved from apoptosis and become single positive and express either CD4 or CD8. In the medulla, epithelial cells present peripheral self-antigens to the thymocytes. The cells that recognize self-antigens with high affinity are eliminated. This is the negative selection (4).

Regulatory T cells:

Some of the cells going through the negative selection that have a high affinity for the MHC:self-peptide complex are not eliminated, but become regulatory T-cells in a process called agonist selection.

In recent years, it has been discovered that the regulatory T cell development is probably predetermined epigenetically. With specific sites where DNA demethylation occurs that general CD4+ single positive cells lack (5, 6).

During their maturation, the FOXP3 transcription factor is upregulated. These regulatory cells derived from the thymus are called natural regulatory T cells or nTregs.

The affinity model of T-cell selection

Number of thymocytes

positive selection

agonist selection of T$_{regs}$

no selection: death by neglect

negative selection: clonal deletion
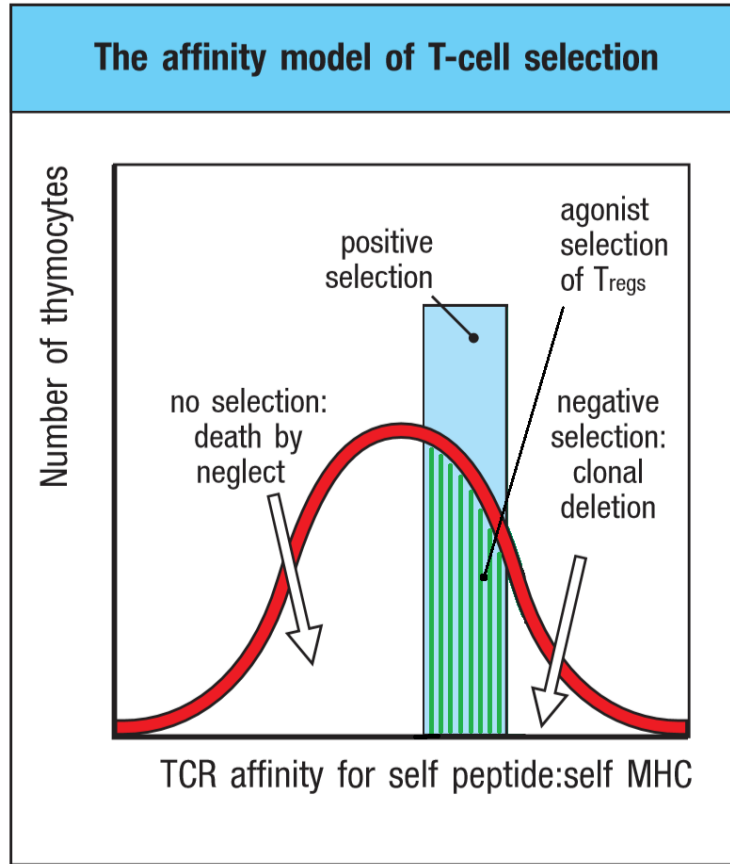
TCR affinity for self peptide:self MHC

Figure 2. T cells undergo positive and negative selection. In the positive selection, cells that do not recognize an MHC-peptide complex with enough affinity undergo apoptosis. In the negative selection, the thymocytes are presented to peripheral self-antigens. The cells that recognize self-antigens with high affinity are eliminated. Some cells are predetermined to become regulatory T cells, they have high affinity for the self-peptides in a process called agonist selection. Modified from Janeway's Immunobiology, 9th ed. Murphy and Weaver, p. 335.

Regulatory T cells can develop in the periphery as well, in secondary lymphoid tissues, from CD4+ T cells that have left the thymus (7). These peripherally derived T cells are called induced

regulatory T cells or iTregs. Both express FOXP3 and CD25 and have largely the same functions. 5-10 % of the CD4+ T cells in the peripheral blood are regulatory T cells in the healthy adult (8).

The regulatory T cells regulate the immune response to prevent autoimmunity and to modulate immune reactions against the bacteria in the intestines. The inhibitory actions through suppression of other T cells, B cells, natural killer cells and dendritic cells by cytokines (IL-35, IL-10, TGF-β), cytolysis (granzyme B, perforin), metabolic deprivation with IL-2 receptors (9) (in a feedback regulation loop with effector T cells, where the regulatory T cells bind all IL-2 leading to depletion of IL-2 for the T effectors cells) and suppression of dendritic cells (10, 11).

The transcription factor FOXP3 is important in the development of the regulatory T cells. In IPEX, a rare disorder caused by mutations in the *FOXP3* gene, the patients have several severe autoimmune diseases such as thyroiditis and diabetes mellitus type 1 (12).

Treatment with regulatory T cells is under investigation for both IBD and liver diseases (13, 14).

The regulatory T cells were discovered 1995 by Sakagushi et al. They isolated a group of CD4+ cells expressing CD25 in mice and showed that depletion of CD25+ cells caused various autoimmune diseases (15). Mason et al identified the suppressive CD25+ T cell subset in rats and in human (16, 17). In 2003 the transcription factor FOXP3 was identified as a more specific marker for regulatory T cells. Fontenot et al showed that mice missing the *Foxp3-gene* did not have any regulatory T cells (18).

Autoimmune diseases:

The immune system functions to protect the body from pathogens in the environment. When the immune system is impaired immune deficiency diseases appear. On the other hand, when the

immune system is attacking our own cells and tissue we get autoimmune diseases. There are more than 80 autoimmune diseases with a prevalence of at least 3% of the human population (19). The cause is not fully known but well studied. Popular theories of why autoimmunity develops include genetic predisposition since several autoimmune diseases are inherited and some diseases are mono genic disorders. Another theory is that environmental factors are of importance and data suggest that being less exposed to infectious agents is a risk for developing autoimmune diseases. On the other hand, some autoimmune diseases are triggered by an infectious disease (20). Less known theories include for example sleep disorders (21).

Sex bias:

Autoimmune diseases are more common in women than men. In some specific diseases, for example Sjögren syndrome, women have up to 8 times higher risk (22). The reason to this sex bias in not known. Women are more likely to develop disease after puberty; one theory is that sexual hormones play an important role in the women's preponderance. Several investigators have focused on the autoimmune regulator (AIRE) and showed that it is influenced by both estrogen and androgens, causing women to express lower levels of AIRE. It is unclear whether androgens could be protecting against disease, or estrogens could increase the risk (23, 24). The androgen levels differ between males and females throughout life, even during fetal period. In newborn boys the testosterone levels decrease from high levels at birth and escalate again for a period at 2 months of age (25).

It is known that boys have higher proportion of regulatory T-cells in peripheral blood (26, 27) and in cord blood (28) and the same for adults (29), but it is not studied if the proportion of regulatory T cells in thymus differ between boys and girls.

Very few scientific reports analyze their data by sex, and few report possible sex differences in the results section, even if they have both males and females in the study. In a systematic review from 2009 the field of immunology had the lowest amount of articles (out of 10 different fields) that specified which sex had been investigated both in animal and human articles (30), this in spite of the fact that there are differences between the sexes in immune response, in addition to the clear differences in sex hormones, there are differences on cellular level, where females have greater antibody response but males have higher frequency of NK cells, type 2 ILCs, CD8+ T cells (31) and as noted earlier more regulatory T cells (26-29).

Thymectomy:

Because of its substantial size in newborns, the thymus is routinely removed, totally or partially, when children undergo open cardiac surgery to correct congenital heart defects, for example ventricular septal defect, VSD, or hypoplastic left heart syndrome, HLHS. Approximately half of the heart surgeries in Sweden are performed at Queen Silvia Children´s Hospital in Gothenburg.

When the chest is opened, and the thymus is removed it is not recorded how much of the thymus is taken away, if it is the whole or just a part. During a few years the surgeons noted how much of thymus was taken away, but not during the recent years. This makes it difficult to know whether the children have any remaining thymus or not. In addition, it is not unusual with ectopic thymic tissue, often found connected to the lower parts of the thyroid gland (2). The ectopic thymic tissue can probably remain intact during the heart surgery. If it is possible the thymus, or parts thereof, is left during the surgery, because of the potentially negative impact of thymectomy in young ages. It is possible to measure if the individual has any remaining thymic function, by measuring small extrachromosomal circular DNA fragments, TRECs (T cell receptor excision circles), with PCR (32).

In previous studies from the research group it is showed that early thymectomy leads to T cell lymphopenia, with more frequently reported allergies and infections (33, 34). In addition, unpublished data from the same study indicate that there are sex differences between the thymectomized individuals, with more autoimmune diseases in the females (Olov Ekwall, personal communications).

Thymus transplantation is a rare, but promising, treatment for patients suffering from complete athymia, for example in complete diGeorge syndrome (22q.11 syndrome), where the children are born with severe immunodeficiency and are very susceptible to infections (35, 36).

Separation of lymphocytes:

CD3 is expressed by all T cells and is used to separate T cells from other lymphocytes. CD4 is expressed mainly by T helper cells and CD8 mainly by cytotoxic T cells. CD25 is the receptor for IL2 and it is expressed on the membrane of regulatory T-cells. However, CD25 is not solely expressed by regulatory T cells, but up to 40% of CD4+ cells (37). Therefore FoxP3, a nucleus marker for regulatory T cells, is used as well.

To separate between thymus derived T cells (nTregs) and peripherally induced regulatory T cells (iTregs) the nucleus marker Helios is used. Helios is expressed on nTregs.

CD45RA is expressed on naïve T cells that has not yet met their antigen. When they meet their antigen and become activated they switch to express CD45RO instead.

CD31 is a marker on recent thymic emigrants, i.e. T cells that just left the thymus (see table 1).

Table 1: Antigens used to identify regulatory T cells. The cells expressing the different antigens and the functions of the antigens.

CD antigen	Cellular expression	Functions
CD3	All T cells	Involved in the TCR cell-surface expression and signal transduction
CD4	Helper T cells	Co-receptor for MHC class II
CD8	Cytotoxic T cells	Co-receptor for MHC class I
FOXP3	Regulatory T cells	Transcription factor and master regulatory gene
CD25	Regulatory T cells	Receptor for IL-2
HELIOS	Regulatory T cells from thymus	Nuclear protein involved in T cell development
CD45RA	Naïve T cells (that not yet met their antigen)	Important for B- and T-cell receptor mediated activation
CD31	Recent thymic emigrants	Adhesion molecule

Aim

The aim of this pilot project is to investigate whether the regulatory T cells differ in number or proportion, in thymus and peripheral blood, between age matched boys and girls.

We wish to examine if the sex bias seen in peripheral blood and cord blood is present in thymus as well. And if so, explore in which stages of regulatory T cell development the sex bias is present. If it only is in the cells from the thymus (Helios), the peripherally produced cells, the naïve (CD45RA) cells or the recent thymic emigrants (CD31).

To answer these questions, we will use flow cytometry.

Method and material

Ethics:

The use of thymus and blood was approved by the regional ethics committee in Gothenburg (number 217-12) and written informed consent was obtained from the parents (see supplementary material). There is no risk for the patients to participate. With this type of cardiac surgery, it is standard to remove the thymus. In the research group as well as other groups past and ongoing studies examine whether the children are at risk by not having a thymus (33, 34, 38).

Sample collection:

The collected samples came from children, newborn up to 5 years, that underwent cardiac surgery 2016-2017, for example to correct ventricular septal defect, VSD. The children underwent surgery at Queen Silvia Children´s Hospital, where approximately half of the children in Sweden in need of cardiac surgery come. The thymus was removed (see figure 3) to have better access to the heart. The thymus was placed in phosphate-buffered saline (PBS) and put on ice. In addition, two milliliters of blood were obtained from eight of the children. The blood was

collected in an anticoagulant-treated sodium heparin tube kept in room temperature. All samples were frozen the same day.
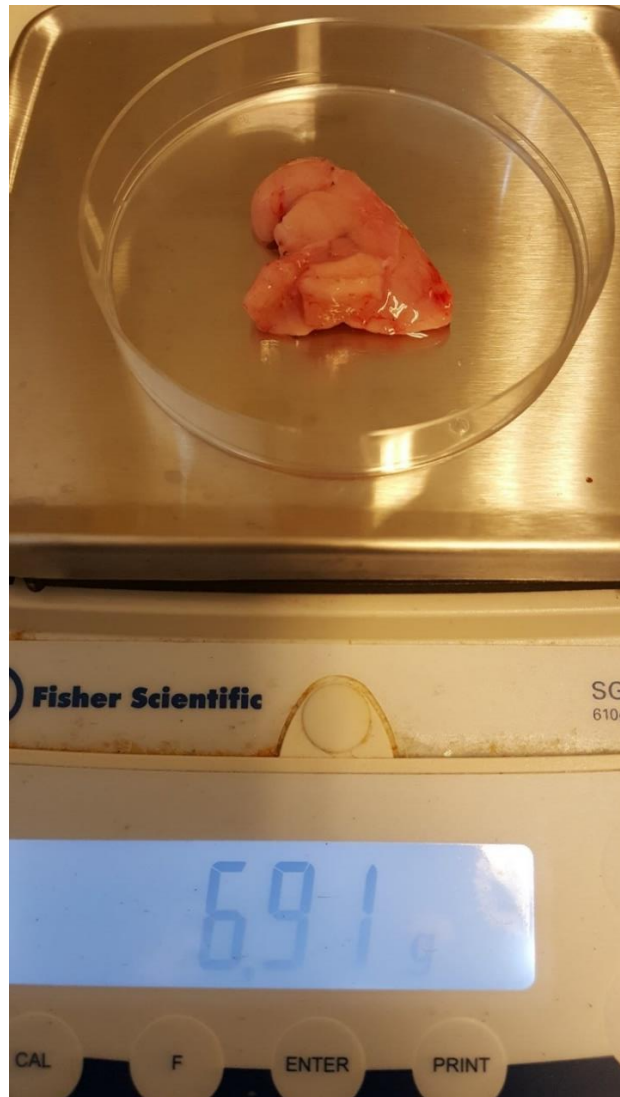

Figure 3. Weighing procedure of the thymic sample. Half thymus from a six-month-old

child. One gram of the thymic sample is cut to pieces to obtain the lymphocytes.

Freezing the cells:

To extract the thymic lymphocytes 1 gram of thymic tissue was injected with FACS buffer (1xPBS, 10% FCS, 10mM Hepes, 2mM EDTA), cut to pieces and pressed through a 40 µm cell strainer. The cells were washed by centrifugation twice (160g, 10 min, 20°C).

To isolate peripheral blood lymphocytes from the blood samples the density centrifugation medium Ficoll-Paque Plus was used. During centrifugation (400g, 30 min, 20°C, no brake) the lymphocytes form a separate layer floating on the Ficoll-Paque Plus, erythrocytes and granulocytes being heavier than the medium and sink to the bottom of the tube (see figure 4). The lymphocytes were gently drawn up with a Pasteur pipette, suspended in FACS buffer and centrifuged twice (160g, 10 min, 20°C).
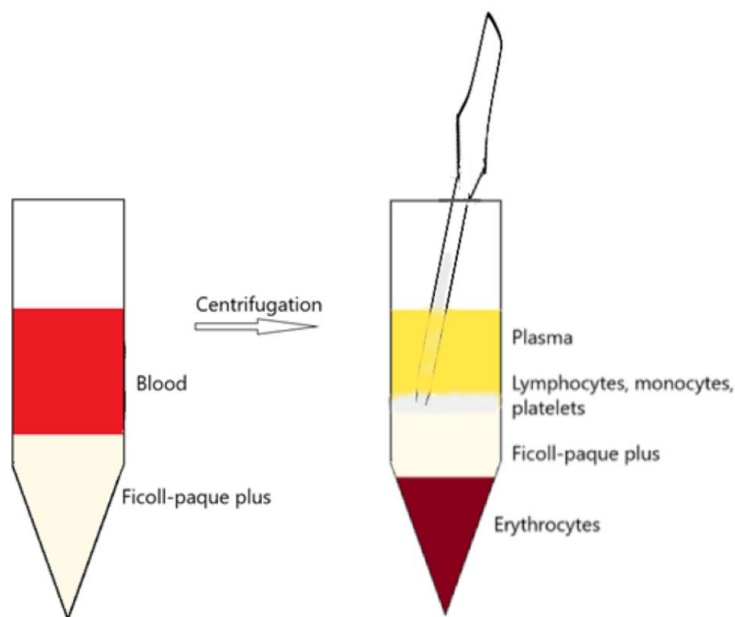

Figure 4. Isolation of lymphocytes with the density centrifugation medium Ficoll-Paque Plus. During centrifugation the lymphocytes form a separate layer floating on the Ficoll-Paque Plus. Erythrocytes and granulocytes are heavier than the medium and sink to the bottom of the tube. The lymphocytes were gently drawn up with a Pasteur pipette.

The blood and thymic lymphocytes were separately suspended in fetal calf serum (FSC) with 10% dimethyl sulfoxide (DMSO, a cryoprotectant). The cells were frozen in cryotubes approximately 1 degree per minute with the use of a "Mr Frosty" freezing container and stored at -140°C.

DMSO reduce water crystallization to protect against cell lysis in the freezing process.

Thawing the cells:

Peripheral blood mononuclear cells and thymic lymphocytes were thawed quickly (1 min in 37°C water bath), slowly diluted in FACS buffer and centrifuged twice (160g, 10 min, room temperature) to remove the DMSO and cell debris before resuspending the cells in FACS buffer.

Flow cytometry staining:

After thawing, the cells were blocked with mouse serum (1%) for 15 min (on ice), then centrifuged twice (300g, 3 min) to remove excess mouse serum.

The surface antigens (see table 2) were stained on ice for 20 minutes. The cells were washed two times (300g, 3 min) with FACS buffer.

The cells were fixed with fixation/permeabilization working solution (Invitrogen, eBioscience, San Diego, CA) for 1 hour in dark, room temperature, then centrifuged twice (500g, 3 min) with permeabilization buffer (Invitrogen, eBioscience, San Diego, CA).

The cells were blocked with hamster and mouse serum (2%) on ice for 15 min.

The intracellular antigens (see table 3) were stained on ice for 1 hour and centrifuged twice (500g, 3 min) with permeabilization buffer, before being resuspended in permeabilization buffer.

Isotypes are used to confirm the specificity of the intracellular antibodies and to correctly gate cells, subtracting any background fluorescence.

Flow cytometric acquisition was performed with FACSverse (BD, San Jose, CA) with the fluorochromes shown in tables 2 and 3. Automatic FACS-compensations were performed with compensation beads.

Before the real samples were analyzed we performed a test on infant thymus and adult peripheral blood monocyte cells to adjust the concentrations of the antibodies and laser settings (final concentrations in table 2 and 3).

Each thymus sample and peripheral blood sample were analyzed in flow cytometer for quantity and proportion of the different markers.

Table 2: The surface antigens of the lymphocytes and the fluorochrome-labeled antibodies that attach to the antigens of the cells. The fluorochromes are detected by the flow cytometer, to make it possible to quantity the different cell types in the sample.

Cell surface antigens	Fluorochrome-labeled antibodies
CD3	CD3 V450 1:60
CD4	CD4 PerCp-Cy5.5 1:120
CD8	CD8 APC-H7 1:60
CD25	CD25 PE 1:30
CD45RA	CD45RA PE-Cy7 1:60
CD31	CD31 BV510

Table 3: The intracellular antigens of the lymphocytes and the fluorochrome-labeled antibodies that attach to the antigens of the cells. The fluorochromes are detected by the flow cytometer, to make it possible to quantity the different cell types in the sample. Isotypes are used to confirm the specificity of the intracellular antibodies.

Intracellular antigens	Fluorochrome-labeled antibodies
FOXP3	FOXP3 AF647 BD 560045 1:25
HELIOS	HELIOS AF488 1:50
FOXP3 isotype control	AF 647 Mouse IgG1
HELIOS isotype control	AF 488 Hamster IgG

Flow cytometry theory:

The flow cytometer detects and counts cells passing a laser beam. It is used to study the properties of cells that can be marked by fluorochrome-labeled antibodies. In the flow cytometer the cells are ordered in a stream of single cells. Each cell passes a laser beam and scatters the laser light. Photo multiplier tubes sense the scattered light and the emitted fluorescence intensity from the fluorochrome-labeled antibodies. The amount of scattered light gives information about the size and granulation of the cell. The wave length of the emitted light from the fluorochromes gives information of proteins on the surface or, if intracellular staining, inside the cell. (4) Minimum 240 000 events were collected per sample.

Analysis:

To analyze the flow cytometer data the FlowJo software version 10.3.0 was used (FlowJo LLC). Each cell passing the laser beam in the flow cytometer generate a dot on the screen. By gating we can choose which cells to look at (see figure 5 and 6).
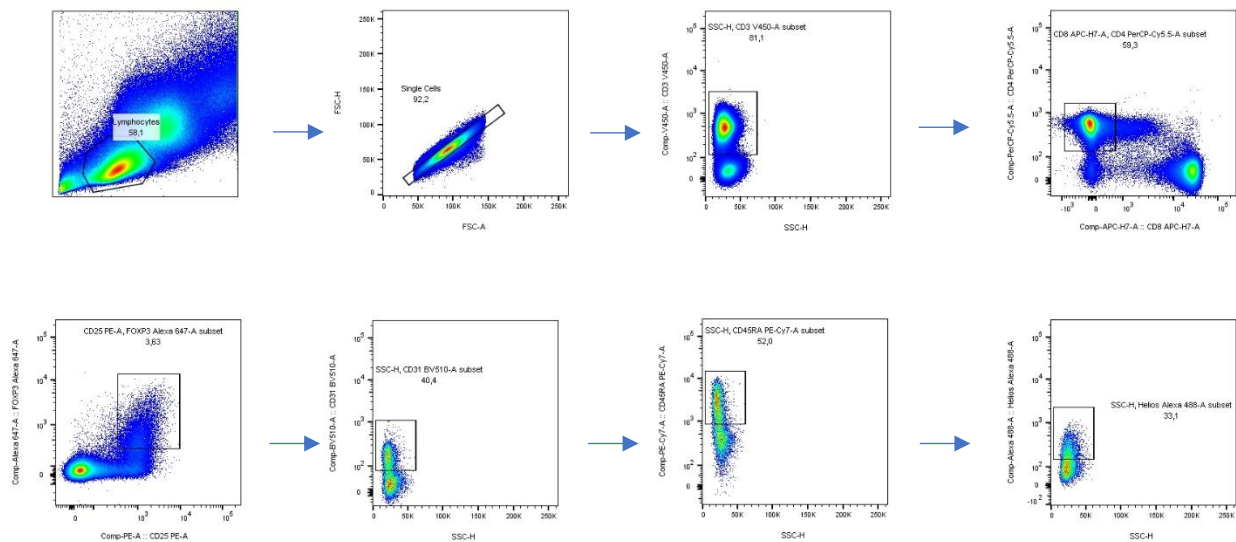

Figure 5. Flow cytometry gating strategy. The total amount of lymphocytes was first gated with forward scatter/side scatter (FS/SS) (A) and then gated for single cells (B). CD3+ cells (C) were gated on for CD4+CD8- (D). Among these we gated for FOXP3+ cells (E). These were further gated for the subsets of CD31+ (F), CD45RA+ (G), Helios+ (H).
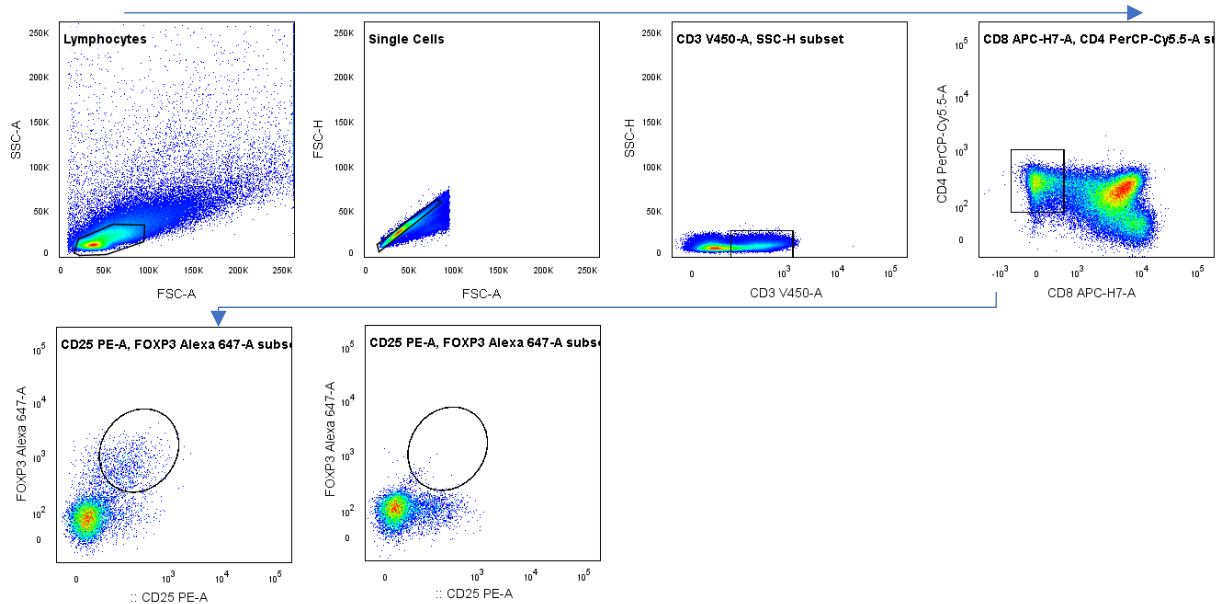
Figure 6. Flow cytometry gating strategy. Showing the use of isotype control to gate for FOXP3. The total amount of lymphocytes was first gated with forward scatter/side scatter (FS/SS) (A) and then gated for single cells (B). CD3+ cells (C) were gated on for CD4+CD8- (D). Among these we gated for FOXP3+ cells (E). By means of the isotype control we set the gate to include only the FOXP3+ cells (F).

The first gate in every experiment surround the lymphocytes by choosing the cells without granulation. Secondly, we gate the single cells, excluding the cells that did not go one by one through the flow cytometer. After that we look at the antigen expression, regulatory T cells are CD3+, CD4+, CD25+, and FOXP3+ (see table 4). By gating for these antigens, we find a population of regulatory T cells. Among these we look for cells that recently left the thymus (CD31), regulatory T cells originating from thymus (Helios) and cells not yet activated (CD45RA).

Table 4. Antigen expression, regulatory T cells are CD3+CD4+CD25+FOXP3+. Among the regulatory T cells some recently left the thymus (CD31+), regulatory T cells originating from thymus (Helios+) and cells not yet activated (CD45RA+).

CD antigen	Cellular expression
CD3	All T cells
CD4	Helper T cells
CD8	Cytotoxic T cells
FOXP3	Regulatory T cells
CD25	Regulatory T cells
HELIOS	Regulatory T cells from thymus
CD45RA	Naïve T cells (that not yet met their antigen)
CD31	Recent thymic emigrants

Fresh sample:

All our thymic samples and peripheral blood samples have been cryopreserved for various periods between 1 week and almost 2 years, and thawed before flow cytometry analyses.

To examine possible adverse effects on the tissues by the cryopreservation, we run one thymic sample and one peripheral blood sample in the flow cytometer directly on fresh tissue. According to literature the cryopreservation should not affect the cells (39).

The same protocol for obtaining lymphocytes and staining with antibodies were used, except that the thawing and the first steps of washing were excluded for obvious reasons. The remaining thymus and blood were frozen and used in the project.

Statistical analysis:

For the statistical analysis SPSS version 25 was used. $P<0.05$ was considered statistically significant. To evaluate the differences observed in proportion of FOXP3+ regulatory T cells, the Mann-Whitney-U test was used. Every individual was matched with an individual of the opposite sex with the same age.

Results

To explore whether there is a difference in the proportion of regulatory T cells in thymus from boys and girls under the age of 5 years, we analyzed lymphocytes by flow cytometry. The project was divided in two parts. One part where we analyzed regulatory T cells from six age matched boys and girls, examining both blood and thymus from the children, and the second part where we examined only the thymus from age matched boys and girls. All thymic samples were analyzed in two groups according to sex and divided into two age matched subgroups, with younger children, up to two months, and children older than two months.

The amount of regulatory T cells was measured in FlowJo, as percentage of CD4+ T helper cells.

Two of the samples (number 3 and number 4) were excluded because of technical problem with the flow cytometer.

Paired thymus and peripheral blood

The paired thymus plus PBMC comprise 3 boys and 3 girls, after exclusion of 1 pair.

Peripheral blood

The primary aim, to see if the regulatory T cells differ between males and females in the thymus and blood showed no significant difference. On the three paired samples there is a visible difference with the girls having lower percentage of FOXP3+ regulatory T cells in general (see figure 7).
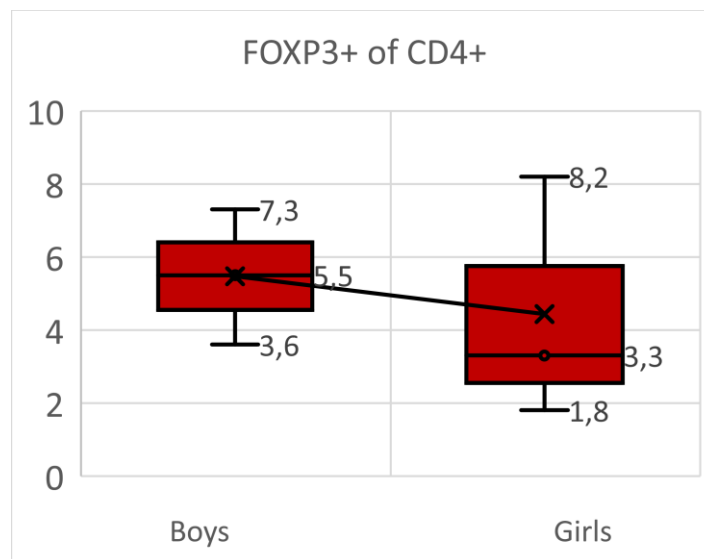

Figure 7. Percentage of FOXP3+ regulatory T cells in peripheral blood.

Age matched boys and girls. Percentage FOXP3+ of CD4+. P-value 0.51.

3+3 individuals, each number represent one individual.

Thymus

When examining the six thymic samples from the six individuals with paired peripheral blood, the girls are higher in regulatory T cells than two of the boys. The results are not significant with a p-value of 0.51 (see figure 8).

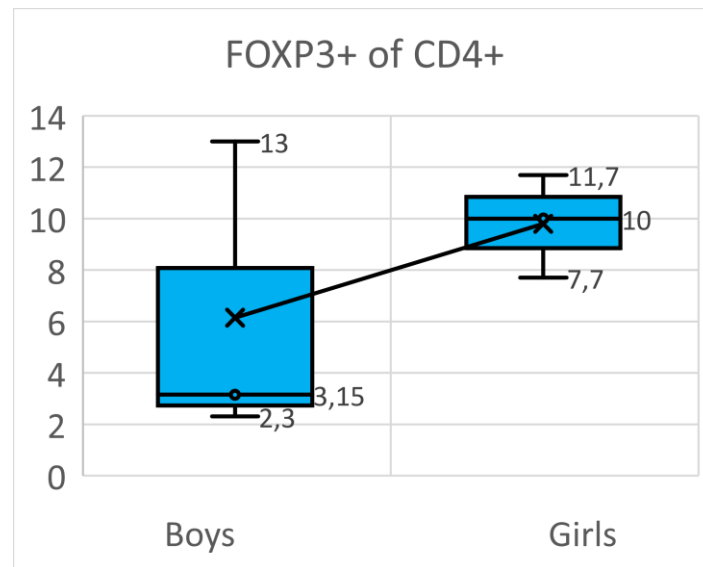*Figure 8. FOXP3+ regulatory T cells in thymus from 3+3 individuals, each*
number represent one individual. Thymus from individuals with paired
peripheral blood. Percentage FOXP3+ of CD4+. P-value 0.51.

Regulatory T cells in thymus

In the second part of the project we examined the proportion of regulatory T cells only in the thymus to see if there is a difference between infant males and females. In the total group with 18

thymuses from children 5 days to 1.5 years no significant difference was seen, although a

tendency is visible, that the girls lay marginally higher in their regulatory T cells (see figure 9).

This could however not be proven statistically.

Figure 9. FOXP3+ regulatory T cells in thymus from 9+9 individuals,

each number represent one individual. Differences between males and

females. Percentage FOXP3+ of CD4+. P-value 0.51.

Children ≤ 2 months

The youngest group comprise 5 boys and 5 girls, age matched in the age 0-2 months.

Almost no difference was seen between these young individuals (see figure 10).

Figure 10. FOXP3+ regulatory T cells in thymus from 5+5 individuals, each number

represent one individual. Sex differences between the youngest individuals

0-2 months. Percentage FOXP3+ of CD4+. P-value 0.53.

Children > 2 months

The second age group comprise 4 boys and 4 girls, in the age 5 months to 18 months. The amount of regulatory T cells was measured in FlowJo, as percentage of CD4+ T helper cells.

A tendency towards higher proportion of regulatory T cells in girls was seen (see figure 11).

However, this was not statistically significant, with the p-value 0.11.

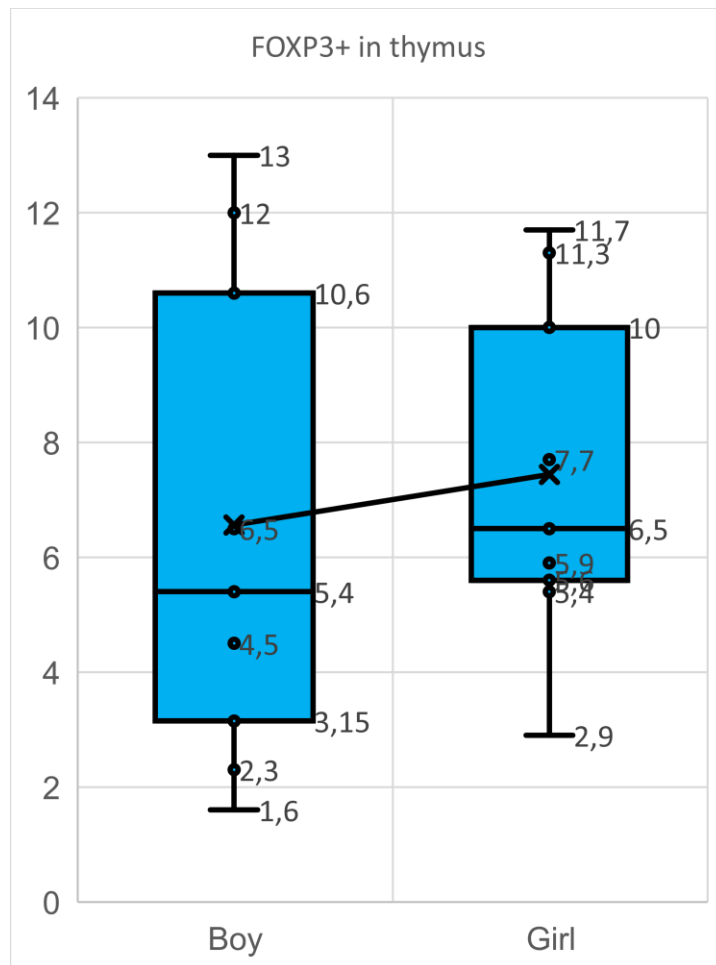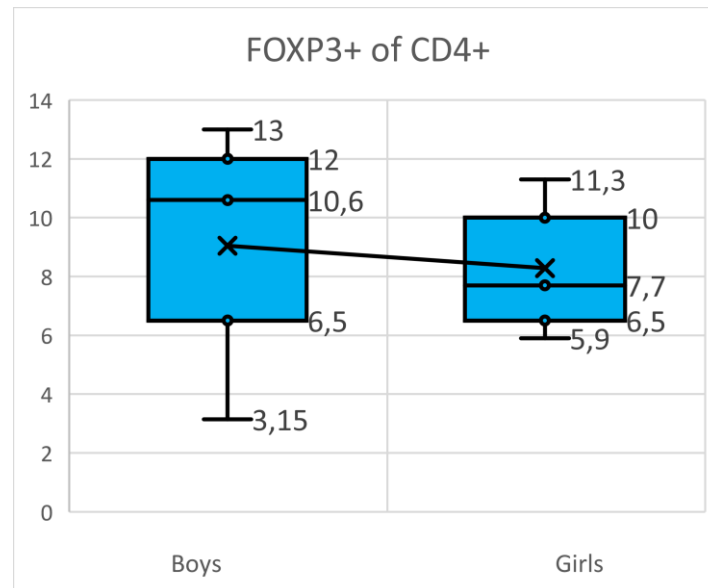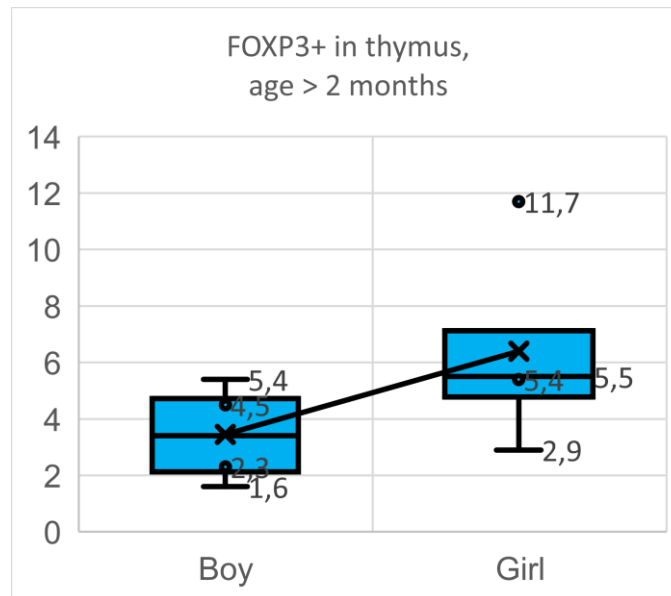*Figure 11. FOXP3+ regulatory T cells in thymus from 4+4 individuals, each number represent one individual. Sex differences between the older cases, 5-18 months. Percentage FOXP3+ of CD4+. P-value 0.11. 4+4 individuals.*

Helios, CD31, CD45RA

When looking into three additional markers: Helios (the marker for regulatory T cells from thymus), CD31 (marker for recent thymic emigrants) and CD45RA (marker for naïve T cells), the cells were gated for CD4+CD25+FOXP3+. When using the Helios isotype, the gate were set on the CD4+CD25+ isotype sample. When gating for CD4, CD25 and FOXP3/isotype, the subgroup Helios isotype is not visible anymore.

There are higher proportion of Helios+ regulatory T cells in female thymus compared to male, with p 0,05 (see table 5).

In CD45RA and CD31 no statistical significant difference is visible.

Table 5. Helios+, CD31+ and CD45RA+ cells as percentage of regulatory T cells in thymus. Differences between young males and females 0-18 months of age. Median value, ± interquartile range, (min-max). P-value calculated with Mann-Whitney-U test.

Parameter %	Males	Females	P-value
CD4+CD25+FOXP3+	5.4 ± 8.6 (1.6-13)	6.5 ± 5.2 (2.9-11.7)	0.5
Helios+ of regulatory T cells	92 ± 17 (49-97)	97 ± 7 (85-99)	0.05
CD31+ of regulatory T cells	12 ± 5.4 (7.5-14)	10.6 ± 2.7 (7.5-14)	0.2
CD45RA+ of regulatory T cells	21 ± 8 (15-39)	26 ± 13 (16-40)	0.1

median ± IQR (min-max)

Fresh vs frozen:

No major difference was seen between the samples after cryopreservation (see figure 12, for comparison between the samples). Both the thymic samples and the peripheral blood samples have largely the same values before and after the freezing and thawing.
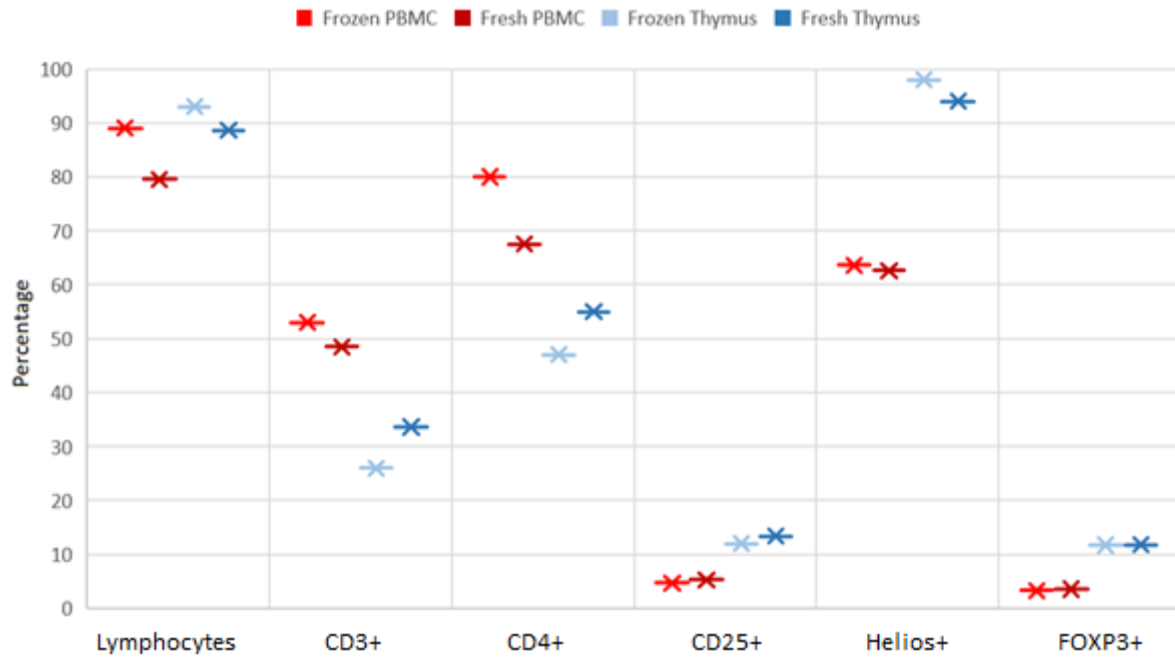
Figure 12. Comparison on fresh and frozen thymic samples and fresh and frozen peripheral blood samples. All dots are the same individual, thymus lymphocytes and peripheral blood lymphocytes before and after cryopreservation. Light red dots are the frozen PBMC, dark red dots are the fresh PBMC, light blue dots are the frozen thymic sample, dark blue are the fresh thymic sample. Percentage of the mother population in the gating; lymphocytes of total cells, CD3+ of single cells, CD4+ of CD3+, CD25+ of CD4+, Helios+ of regulatory T cells, FOXP3+ of CD4+.

Discussion

Discussion on the results

The regulatory T cells are of uttermost importance in the control of immune reactions and autoimmunity, therefore it is critical to fully understand their roles in autoimmunity and the sex bias seen in autoimmune diseases.

As far as we know this is the first study to examine the proportion of regulatory T cells in the thymus and compare to peripheral blood. We examined young children, the majority new born infants, the oldest 1,5 years old. Zhu et al (24) suggested that androgens play a major role in the sex bias seen in autoimmune diseases via the autoimmune regulator AIRE. The greatest difference is in the adults, in the children the androgen levels are more similar between the sexes, except for the first months of life, with the boys having considerable higher levels of androgens after birth (25). With this knowledge the included children of this study would be of optimal age, to be able to see sex differences in regulatory T cells, if they depend on the androgen levels.

The results from the three paired blood and thymic samples are very interesting and need to be examined further, in more individuals, for solid, reliable interpretation. Our results, although not significant, suggest that females have a higher proportion of regulatory T cells in the thymus in ages over 2 months, and lower proportion in the blood, compared to age matched boys, consentient with the present understanding in the field (26-28). Why the boys have higher proportion of regulatory T cells in the blood, but not in the thymus, where the T cells mature is interesting. Where would the difference start, if not in the thymus?

Recently in the research group, the proportion of FOXP3+ T cells in young male and female thymic samples were examined in a degree project by counting FOXP3+ cells in thymic tissue sections using confocal microscope (40). The results from that project showed a higher proportion of regulatory T cells in female thymus compared to male with statistical significant results. These findings correspond to the results from our study. The cells counted were however FOXP3+ regulatory T cells as a division of all countable cells and not only CD3+ cells.

Among the regulatory T cells, we saw higher amount of regulatory T cells stemming from thymus (Helios+) in thymus from girls compared to boys. Although the results on Helios sex bias showed the p-value 0,05 there are some doubts on whether Helios is a good marker to separate between natural and induced regulatory T cell (41), which may leave the results with some uncertainty. The results show a tendency of girls having higher values of naïve T cells (CD45RA+), and boys having higher amount of recent thymic emigrants (CD31+) in thymus. Although the results are uncertain because of the lacking significance, it is interesting to reflect on. If these results can be confirmed in future studies, it would be interesting to investigate further why females have less regulatory T cells in the blood and more in the thymus. Perhaps the regulatory T cells for some reason have trouble to exit the thymus, or perhaps there is a reason that the regulatory T cells originating from the thymus to stay in the thymus to a bigger extent in the girls, while in the boys the higher proportion of recent thymic emigrants can suggest that those cells to a higher extent are on their way out.

It is interesting that the field of immunology has the lowest amount of articles specifying which sex had been investigated (30), despite of the differences between the sexes in immune response (42). If all studies included both sexes and analyzed the data for sex differences, we would probably be able to see more differences.

Limitations

This study has some limitations. The main limitation was that the number of blood samples paired with thymic samples was rather small, and did not allow reliable interpretation of the association between regulatory T cells in thymus and blood. The collection of paired thymuses and blood samples will continue.

When analyzing the results from flow cytometry in FlowJo, a risk for uncertainty is the gating; It can be unprecise and arbitrary. To minimize the risk for that in these experiment gates were drawn in the same way in all samples, by one single investigator and the gating strategy is attached in the material section. Another risk in this study is that the person identifying the cell populations and analyzing the results was not blind to protocol outcome.

Every child is matched with a child of the opposite sex, and the same age. The matching is fairly precise, but we have no data on whether the children are premature, something that perhaps could be relevant in the youngest ages but should not matter to the older cases.

The FOXP3+ regulatory T cells are upregulated in environments with scarcity of oxygen (43). This could be a major source of error when examining thymus and blood from children operated for congenital heart defects, because some of the children have cyanotic heart defects (44). Potentially this could be the reason to sex bias seen when examining regulatory T cells in thymus and blood from children undergoing correction for congenital heart defects. In the present study we analysed the results separately for cyanotic and acyanotic heart defects, in thymus and blood and did not see any statistical significant bias (see supplementary material, table S1 and S2). In some children with congenital heart defects the abnormality is seen already in the foster, and in

those cases the child is provided supportive oxygen already at birth, to counteract any potential

hypoxia (45), perhaps leading to less effect of hypoxia on the regulatory T cells.

Future research

Unfortunately, the access of thymic samples paired with blood during the period for this student

project was unsatisfactory. The sampling of thymus and blood will continue, to get more reliable

data of the differences between infant males and females, and to be able to evaluate these results.

It would be interesting to see if these results are prevalent in older children as well. And

investigate if female regulatory T cells stay in the thymus to a higher extent.

This project emphasizes the need for further investigation on the sex bias of thymic regulatory T

cells, in human and in mice. Examining mice would give an easier access to adult thymus and in

addition lymph nodes. This is important to understand the differences in the immune system

between males and females, and to get more clues to why auto immune diseases are more

common in females than males.

Populärvetenskaplig sammanfattning på svenska

Bakgrund

Ungefär tre procent av befolkningen lider av autoimmuna sjukdomar, alltså sjukdomar där kroppens immunförsvar attackerar våra egna vävnader. Det är fler kvinnor än män som drabbas av dessa sjukdomar, men orsaken till det vet man inte riktigt. Det finns en celltyp i immunförsvaret som stänger av immunförsvaret när det attackerar våra egna vävnader, dessa celler kallas regulatoriska T celler och ska alltså motverka autoimmuna sjukdomar. Forskare har sett att det finns mer av den här celltypen i blod från män än blod från kvinnor, vilket skulle kunna vara en ledtråd till varför autoimmuna sjukdomar är vanligare hos kvinnor än män.

De regulatoriska T cellerna bildas i benmärgen och kommer sedan till brässen för att mogna. Brässen, belägen ovanpå hjärtat, är ett viktigt organ i immunförsvarets utveckling, framför allt under fosterlivet. I och med att de regulatoriska T cellerna mognar i brässen är det intressant att ta reda på om könsskillnaderna som man sett i blodet finns redan i brässen.

Syfte

Vi ville ta reda på om könnskillnaderna hos regulatoriska T celler som man sett i blod även finns i brässen, platsen för cellernas mognad.

Metod

En del av brässen tillvaratogs från tio flickor och tio pojkar, yngre än två år, som genomgick hjärtoperation av medfödda hjärtfel. Dessutom tillvaratogs två milliliter blod från sex av barnen. Vid hjärtoperation av så här små barn är brässen i vägen och tas rutinmässigt bort.

Immunförsvarscellerna vita blodkroppar separerades ut genom att brässen klipptes i små bitar och pressades genom en finmaskig sil. Med hjälp av centrifugering skiljdes de vita blodkropparna ut från den pressade brässen och blodet. Cellerna fryslagrades i -150°C.

När cellerna skulle analyseras tinades de och färgades med antikroppar som har små färgmolekyler fästa till sig. Sedan kördes cellerna i en speciell analysapparat, flödescytometern, som med hjälp av laser registrerade vilka färger som passerade. Resulaten analyserades i datorprogrammet FlowJo.

Results

Ingen statistiskt säkerställd skillnad av antalet regulatoriska T celler sågs mellan pojkar och flickor i det här experimentet. Slumpen kan alltså vara orsaken till de diskreta skillnader som sågs i form av att pojkar har aningen fler regulatoriska T celler i blod, medan flickor har aningen fler regulatoriska T celler i brässen. Den största skillnaden sågs hos de barn som var äldre än två månader, även om det inte heller här var någon statistiskt säkerställd skillnad.

Slutsats

Resultaten från det här projektet antyder att det kan vara en skillnad mellan pojkar och flickor i antalet regulatoriska T celler, de skillnader vi har fått fram (som dock kan bero på slumpen) är liktydiga med tidigare studier vilket stärker trovärdigheten till våra resultat.

Fler studier behövs med bräss och blod insamlade från fler barn. Det vore speciellt intressant att titta på barn äldre än två månader, efter som pojkarna i den här åldern har mer av könshormonet testosteron, vilket forskare tidigare sett påverkar autoimmunitet.

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Supplementary material

Table S1. Mean value of regulatory T cells in thymus, from children with cyanotic congenital heart disease (CHD), n=8, and children with acyanotic congenital heart disease, n=10. No difference is seen between the children with cyanotic CHD and acynotic CHD. P=0,33.

FOXP3 of CD4 in thymus

		N	Mean Rank	Sum of Ranks
FOXP3 of CD4	Cyanotic CHD	8	10.88	87.00
	Acyanotic CHD	10	8.40	84.00
	Total	18		

Table S2. Mean value of regulatory T cells in thymus, from children with cyanotic congenital heart disease (CHD), n=4, and children with acyanotic congenital heart disease, n=2. No difference is seen between the children with cyanotic CHD and acynotic CHD. P=0,64.

FOXP3 of CD4 in blood

		N	Mean Rank	Sum of Ranks
FOXP3 of CD4	Cyanotic CHD	4	3.25	13.00
	Acyanotic CHD	2	4.00	8.00
	Total	6		

Ethics – Patient information

Patientinformation för studien "Studier av central toleransutveckling i human tymus".

Härmed tillfrågas Ni som har ett barn som skall hjärtopereras om Ert barns deltagande i en forskningsstudien *"Studier av central toleransutveckling i human tymus"*.

Beskrivning av studien

Vid Drottning Silvias barn- och ungdomssjukhus pågår forskningsprojektet *"Studier av central toleransutveckling i human tymus"* som syftar till att studera bakomliggande mekanismer vid autoimmuna sjukdomar. Autoimmuna sjukdomar kännetecknas av att immunsystemet reagerar mot den egna kroppen och förstör organ i kroppen eller påverkar deras funktion. Sedan tidigare vet vi att funktionen hos tymus (brässen) är av betydelse för immunsystemets förmåga att skilja den egna kroppens strukturer från inkräktande bakterier, virus och andra främmande strukturer.

I studien undersöker vi tymusceller för att försöka förklara hur och varför immunsystemet felaktigt reagerar med den egna kroppen vid autoimmuna sjukdomar. I samband med hjärtoperationen tas rutinmässigt en bit av tymus bort för att komma åt hjärtat som sitter bakom tymus. Den bit av tymus som tas bort brukar normalt slängas bort. Vi önskar nu ta tillvara denna tymusbit från Ert barn och använda i den ovan beskrivna studien. Vi vill också ha möjlighet att ta ett extra blodprov om 2 ml via en befintlig venkanyl.

Risker

Medverkan i studien påverkar inte utförandet av operationen. Det medför inte någon ökad risk då vi tar till vara på en del av tymus som i annat fall skulle slängas bort. Om vi tar ett extra blodprov på ert barn sker det i en redan befintlig venkanyl, så det innebär inte något extra stick eller smärta för Ert barn.

Fördelar

Medverkan i studien medför vare sig fördelar eller nackdelar för ert barn.

Hantering av data och personuppgifter

Personuppgifter hanteras i enlighet med personuppgiftslagen (1998:204), och ansvarig för personuppgifterna är Sahlgrenska Universitetssjukhuset. Om ni har frågor om personuppgifterna kan ni vända er till sjukhusets personuppgiftsombud Susan Lindahl, tel. 031- 343 27 15.

Patientförsäkringen gäller för deltagare i studien. Resultaten kommer att behandlas så att inte obehöriga kan ta del av dem. Efter studiens genomförande kommer resultaten att publiceras i vetenskaplig tidskrift och resultaten kommer inte att kunna spåras till enskilda individer.

Biobank

Proverna kommer att förvaras i en biobank vid Sahlgrenska Universitetssjukhuset i enlighet med biobankslagen (SFS 2002:297). Proverna kommer att förvaras kodade vilket betyder att de inte direkt kan härledas till Ert barn. Proverna får endast användas på det sätt som Ni lämnat ditt samtycke till.

Ersättning

Ekonomisk ersättning utgår inte för deltagande i studien.

Deltagandet i studien är helt frivilligt och beslutet om deltagande påverkar inte på något sätt den planerade operationen. Ni har när som helst rätt att avbryta deltagandet och begära att proverna förstörs.

Göteborg 3 mars 2015

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Ethics - Consent

Studier av central toleransutveckling i human tymus

Vi har muntligen och skriftligen informerats om studien "Studier av central toleransutveckling i human tymus".

Vi har fått information om vilka prover och uppgifter som kommer att samlas in hur de kommer att användas i studien. Vi har informerats om att proverna kommer att sparas i en biobank i enlighet med biobankslagen och att vi när som helst och utan närmare förklaring kan begära att dessa prover förstörs. Vi har haft möjlighet att ställa frågor om studien.

Vi är medvetna om att deltagandet i studien är frivilligt.

Vi har förstått den information vi har fått, och härmed samtycker vi till att

_____ med

personnummer _____deltar i projektet

"Studier av central toleransutveckling i human tymus" samt till att prover lagras i en biobank.

_____ den _____

ort   datum

_____   _____

Vårdnadshavare 1   Vårdnadshavare 2

Ethics – Ethic approval

03/24/2015 Dnr 217-12

2015 -03- 2 7

THE SAHLGRENSKA ACADEMY

UNIVERSITY OF GOTHENBURG

Dnr _T 279-15_

Ad _217-12_

Betalt _2015.04.20_

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Ansökan om ändring av etikansökan för projektet "Studier av central toleransutveckling i human tymus", D-nr 217-12.

Ändring av etikansökan önskas enligt nedan för att utvidga rekryteringen av patienter till studien så att även barn som är äldre än sex månader gamla kan ingå. Då många barn äldre än sex månader genomgår hjärtkirugi skulle detta öka antalet möjliga deltagare. Utvidgning av åldern önskas så att barn upp till fem års ålder kan inkluderas. Vidare önskas att formuleringen "...i övrigt friska..." tas bort enligt nedan då barn med medfödda hjärtfel ofta har andra associerade missbildningar och att formuleringen i detta sammanhang kan förvirra.

Jag bedömer inte att de önskade ändringarna medför någon förändring gällande den etiska bedömningen som gjordes i samband med den ursprungliga etikprövningen.

Följande ändring önskas under punkt 2:4

Nuvarande formulering: _"Patienter som är aktuella för studien är i övrigt friska barn, yngre än sex månader, som opereras för medfödda hjärtfel."_

Önskad ny formulering: _"Patienter som är aktuella för studien är barn, yngre än fem år, som opereras för medfödda hjärtfel."_

Forskningsprogram där den förslagna ändringen är markerad bifogas. I övrigt innebär den föreslagna ändringen inte någon ändring av patientinformationen eller samtyckesblanketten.

Göteborg, 2015-03-27

Olov Ekwall

Granskad och godkänd som sekreterarärende
Göteborg 2015-03-31

Sven Wallerstedt, professor, vetenskapl. sekr
Med avd 2
Regionala etikprövningsnämnden i Göteborg

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1 (2)

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