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"Characterization Of T-Cell Ontogeny And Other Cell Populations In Pediatric Human Thymus"

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ABSTRACT

Immune related diseases, including autoimmune disorders, allergies, graft rejection, tumour growth are still one of the main concerns of current medicine. The complexity of the immune system demands a continuous review of the knowledge employing available technologies. T lymphocytes play a central role in the coordination, regulation and execution of immune responses.

Maturation of T lymphocytes from the thymus towards the periphery elicit several questions regarding the relations between different immune cell populations, which are highly influenced by the processes taking place at the thymus. Such processes, related to differences into immature and mature cell populations of thymocytes, showing different patterns of expression of CD4 and CD8, along with other factors, could reveal useful information still unknown with respect to the spatial distribution and interactions of such cells. In addition, regulatory T cells (Treg) is a subtype of T cell specialised in the regulation of immune responses, and its ontogeny in the thymus also represent an interesting unexplored concept. Here we show extensive and detailed analysis based on characterization and identification of the distribution of different cell populations of thymocytes at the thymus, derived from flow cytometry and 2D images acquired by confocal microscopy.

The distribution of the four major thymic cell populations found is identified, ensuring results reported by previous publications, along with spatial identification of thymocytes, especially focusing on the interactions related to regulatory T cells. Our results demonstrate further reassurance of the presence of different patterns of FOXP3 expression, the marker that characterises Tregs, as well as the inability of CD45RA and CD45RO markers for providing reliable information at the thymus.

Therefore, analysis on the results obtained from a comparison between flow cytometry and confocal microscopy have high reliability and consistency with previous results reported, reinforcing the importance of identification, selection and maturation processes related to Treg cell population for further research focused on autoimmunity and maintenance of self-tolerance

Key words: Thymocytes, FOXP3, regulatory T cells (Tregs)

TABLE OF CONTENTS

1. INTRODUCTION 1

1.1 Fundamentals of thymus and immunity 1

1.1.1 Immune and lymphatic systems 1

1.1.2 Thymus anatomy 8

1.1.3 Ontogeny and maturation of T cells 10

1.1.4 Positive and negative selection. Immunogenicity and self-tolerance 15

1.2 Background on previous immunological research projects 17

1.2.1 Thymocytes classified into different cell populations 17

1.2.2 Regulatory T cell population 17

1.3 Project description 19

2. MATERIALS AND METHODS 20

2.1 Source of pediatric human thymus and preparation for analysis 20

2.1.1 Obtaining of pediatric human thymus for analysis 20

2.1.2 Human pediatric tissue processing 20

2.2 IHC methodology. Fixation, permeabilization and blocking steps 21

2.2.1 Immunohistochemistry/Immunofluorescence 21

2.2.2 Foundations on fixation, permeabilization and blocking steps 22

2.3 Flow cytometry characterization 22

2.3.1 Foundations on flow cytometry 22

2.3.2 Dissociation of human pediatric tissue for flow cytometry 24

2.3.3 Characterization of thymocytes from human pediatric tissue 25

2.3.4 Characterization of Treg thymocytes from human pediatric tissue 26

2.3.5 Analysis of sample cell suspension by flow cytometry 28

2.4 Characterization and identification of cell population distribution using confocal microscopy 29

2.4.1 Confocal microscopy structure and acquisition method 29

2.4.2 Preparation of samples for acquisition aided by confocal microscopy 31

2.4.3 Acquisition, processing and analysis of images obtained from confocal microscopy 32

2.4.4 Cell counting and CD4-CD8 colocalization analysis from confocal microscopy images using ImageJ 33

3. RESULTS ... 36

3.1 Flow cytometry results ... 36

3.1.1 Cell viability and identification of main populations ... 36

3.1.2 CD3 and CD45 characterization of cell populations ... 37

3.1.3 Characterization of active cell populations by HLA-DR expression ... 39

3.1.4 Identification of Treg cell population derived from expression of CD25 and CD127 ... 40

3.1.5 Identification and classification of Treg cell population derived from FOXP3 expression ... 41

3.1.6 Charactherization of Treg FOXP3+ cell population by expression of HLA-DR, CTLA-4 and CD39 ... 43

3.2 Images of paediatric human thymus obtained by confocal microscopy ... 44

3.2.1 Analysis of control images. Elimination of background and image processing. ... 44

3.2.2 THY670horiz series analysis ... 45

3.2.3 THY670vert series analysis ... 50

3.2.4 CD4-CD8 colocalization analysis ... 55

4. DISCUSSION ... 56

4.1 Discussion over the results obtained from previous analysis ... 56

4.1.1 Flow cytometry ... 56

4.1.2 Confocal microscopy ... 59

4.1.3 Limitations encountered during results obtaining and its analysis ... 61

4.2 Future research ... 62

4.2.1 Cell differentiation and fate derived from two subpopulations of FOXP3 thymocytes ... 62

4.2.2 Analysis of cell population distribution in 3D in vitro model ... 62

5. REGULATORY FRAMEWORK AND BUDGET ... 64

5.1 Regulatory framework and legal aspects related to the project ... 64

5.2 Project budget ... 64

6. BIBLIOGRAPHY ... 67

1. INTRODUCTION

1.1 Fundamentals of thymus and immunity

Over the years, several immunological studies have been performed with the aim of understanding the complete functioning of our immune system. However, the information obtained through investigation is not detailed enough and it is quite limited for proper development of further and advanced treatments for immune related diseases.

In order to establish the background of our research, it is important to gather and examine the previously obtained data and general knowledge, detecting those weak points still unknown and unresolved.

1.1.1 Immune and lymphatic systems

The immune and lymphatic systems are in charge of protecting the human organism from pathogens and external threats which could severely damage our internal and autoregulated balance.

In a detailed way, the immune response produced by the activation of our immune system is "a reaction to microbes as well as to molecules that are recognized as foreign, regardless of the physiologic or pathologic consequence of such a reaction"[1]. The immune system is divided in two essential components, called innate (non-specific) and adaptive (specific) immunity. Both innate and adaptive immunity regulate and control the immune response, by means of different pathways of recognition and eradication of potential hazards, although cooperative in turn.

Innate immunity is characterized by the presence of defences prior to infection, which are considered as a barrier for the pathogens. Its action guarantees the proper preparation of the specific defences by the adaptive system. When infection occurs, the adaptive system is prepared to thoroughly recognize pathogens, denominated antigens, and develop the proper response by exposure mechanisms; these later on, contribute to improving the adaptive immune response.

The immune response is properly accomplished by cooperative interaction between innate and adaptive immunities, which involve stimulation of the adaptive immune system beneath the innate immune response and, in turn, enhancing of the innate defense responses by adaptive immune action. Furthermore, there are several mechanisms for ensuring to a certain degree that unleashing of the immune

response is only produced when non-self-molecules are recognized, avoiding autoimmunity in most of the cases.

	Innate	Adaptive
Characteristics		
Specificity	For molecules shared by groups of related microbes and molecules produced by damaged host cells	For microbial and nonmicrobial antigens
Diversity	Limited; recognition molecules encoded by inherited (germline) genes	Very large; receptor genes are formed by somatic recombination of gene segments in lymphocytes
Memory	None or limited	Yes
Nonreactivity to self	Yes	Yes
Components		
Cellular and chemical barriers	Skin, mucosal epithelia; antimicrobial molecules	Lymphocytes in epithelia; antibodies secreted at epithelial surfaces
Blood proteins	Complement, various lectins and agglutinins	Antibodies
Cells	Phagocytes (macrophages, neutrophils), dendritic cells, natural killer cells, mast cells, innate lymphoid cells	Lymphocytes

Table 1-1 Characteristics of innate and adaptive immunity [1]

Besides, the immune response is greatly effective as it is systemic, as it will be later discussed, and it relies on positive feedback which produces activation of large groups of lymphocytes from exposure to a small number of them; or other mechanisms which close regulate the immune response development to avoid overaction of lymphocytes, which could be detrimental for the organism.

There are three features [1] which clearly differentiate the adaptive immune response from the innate one:

- Specificity and diversity: Lymphocytes have high specificity for detecting specifics parts of a wide variety of antigens, called “epitopes”. Besides, there are different clones of lymphocytes with the ability to recognized foreign antigens, independent from previous responses against said antigens.
  When an antigen is recognized, proliferation of the lymphocyte with the specific antigen receptors is produced, therefore there is a great variety of lymphocytes which can detect extremely large number of antigens. This process for generating specificity and diversity in lymphocytes is called “clonal selection”. As a result, the immune system is composed of large pools of clones of lymphocytes with the ability for react against a large number of antigens, specifically.
- Memory: Once the immune system has reacted against a specific antigen, some immune cells gain memory against this encounter, thus producing a larger and rapid response against the re-exposure of the antigen. These long-lived cells are classified as memory lymphocytes.

- Self-tolerance: Self-tolerance is the ability of the immune system to not react against an individual's own antigens. Therefore, lymphocytes are reactive against any foreign antigen whereas no immune response is to be produced against self-antigens. Lymphocytes which do not possess tolerance against one's own antigens are eliminated or suppressed by several mechanisms, such as selection during maturation or suppression mediated by regulatory immune cells.

For the sake of introducing relevant information regarding our research, information regarding adaptive immune system, dependent of B lymphocytes is avoided, thus offering useful background for understanding T lymphocytes mediated immune response, related to maturation of T cells from thymocytes.

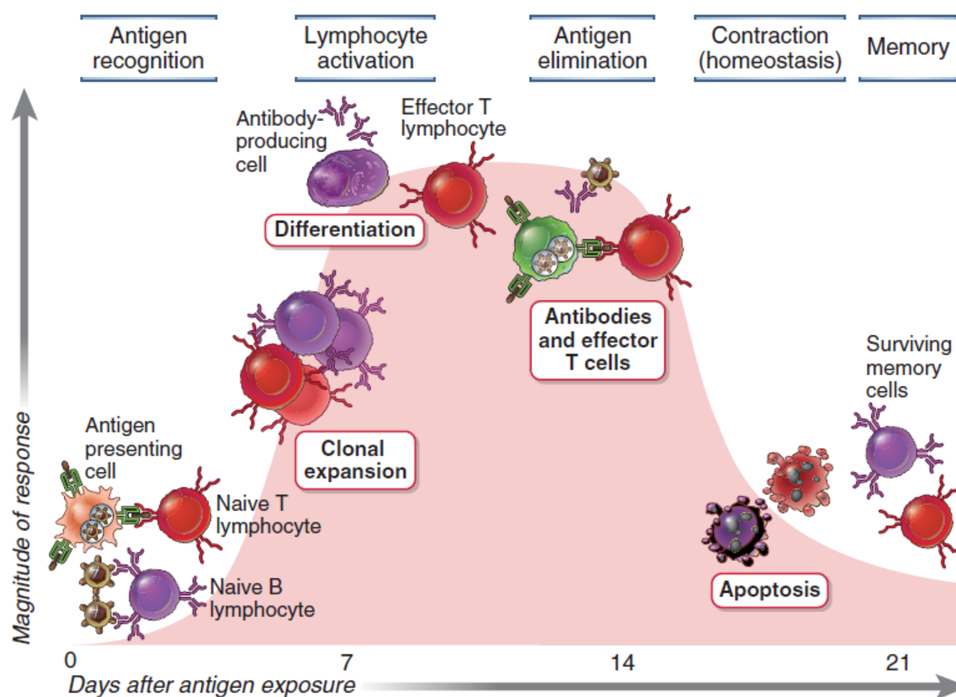


Fig. 1-1 Schematic of steps involving development of adaptive immune response, from identification of antigen, clonal selection and expansion, finishing with dead of effector cells and surviving of memory cells [1]

The immune response derived from action of T lymphocytes is called cell-mediated immunity, as it relies on interaction between immune cells and antigens presented in other cell populations. Recognition of antigens performed by T lymphocytes is based on presentation of such particles to immune cells from phagocytes, called antigen presenting cells (APCs). APCs engulf foreign antigens for presentation upon recognition of T lymphocytes for proper elimination of the harm.

In a detailed way, T lymphocytes recognize foreign antigens presented by binding of major histocompatibility complex (MHC) molecules to antigens at host cells (APCs)

In the majority of the cases, immune responses are regulated by communication between participating cells through secreted proteins called cytokines. Those proteins have several functions including promotion of grown, differentiation, effector immune cells activation or repression of specific functions or cells. Besides, after considering the importance of cytokines, receptors for such proteins are extensively used for determining the characteristic of immune cells, such as its cell linage. CD25 is part of the class II MHC complex with the ability of detecting Il-2, essential cytokine for development of Treg cell population.

T lymphocytes are formed by several cell populations, from which helper T cells (CD4) and cytotoxic T cells (CD8) are highly defined in immunology. Interactions are greatly found between these cell populations, as helper T cells, which secreted specific cytokines which support action of cytotoxic T cells, which in turn secreted specific molecules for elimination of antigens and the cells infected. In addition, there is a relevant group of T lymphocytes, which are called regulatory T cells (Treg) which repress immunity responses when necessary, maintaining tolerance and homeostasis.

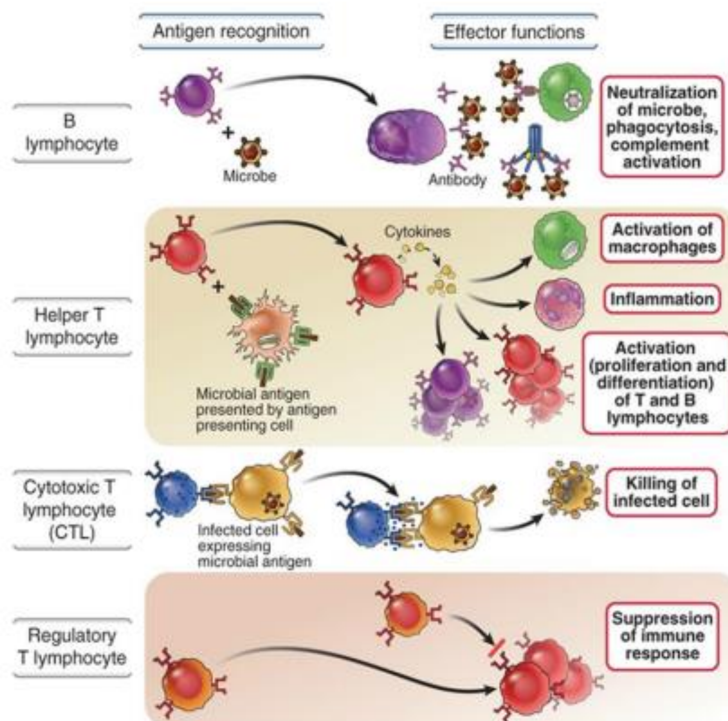


Fig. 1-2 Description of different functions of lymphocytes in a immune response, composed of antigen recognition and effector functioning phases [1]

As previously mentioned, the immune system is formed by innate and adaptive immunity. Although it is not touched upon in this project, a brief explanation of cells involved in the innate immunity is encouraged for a better understanding of the relations between both innate and adaptive immune systems.

Both systems are derived from hematopoietic stem cells (HSCs) which, as multipotent cells, differentiate into two different cell lines, called myeloid and lymphoid cell lineages. Phagocytes such as macrophages, monocytes or neutrophils, as well as other cells such as mast cells, dendritic cells, basophils or eosinophils are derived from myeloid cell lineage while lymphocytes and natural killer cells (which are classified as innate immune cells) are derived from lymphoid cell lineage.

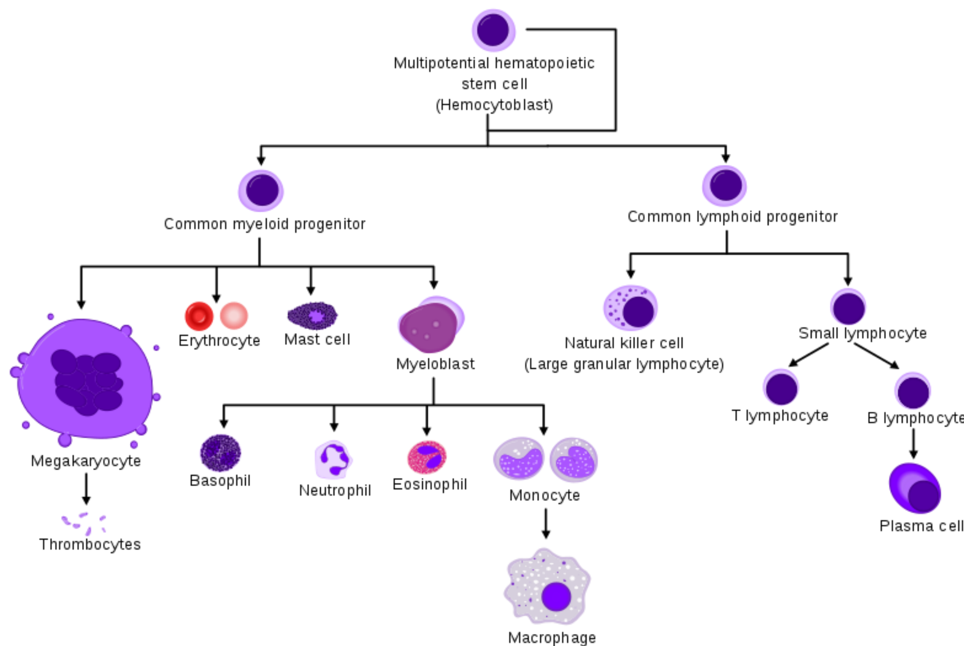


Fig. 1-3 Myeloid and lymphoid cell lineages [2]

Phagocytes are in charge of engulfing and destroying pathogens, usually microbes, along with removing damaged tissue. Both neutrophils and macrophages are classified as phagocytes, although they differ in rate of action and origin, as some macrophages are differentiated from monocytes at the tissue where the immune response is been produced. Macrophages are in a close relation with T lymphocytes, derived from their ability to secrete specific cytokines relevant for the adaptive immune response, as well as to serve as antigen presenting cells (APCs).

Mast cells, basophils and eosinophils greatly react against infections, microbes and allergic responses. In many cases, they release specific molecules which promote inflammatory process for parasite or infection protection of the organism. Mast cells and basophils are in charge of promoting inflammatory responses and

creating a good environment for the immune response, thus, they are responsible for allergic responses caused by exposure to secreted histamine. Eosinophils have similar functions with respect to previous cells, although its activation could lead to damage to host tissue.

Dendritic cells are considered the operators of immune response as they are in charge of detecting pathogens or microbes, leading to generation of immune response. They establish important communication between cells from both innate and adaptive immunities and are also classified as APCs, serving as an important component in cell mediated immunity performed by T lymphocytes. Dendritic cells are subdivided into conventional DCs (cDCs) and plasmacytoid DCs (pDCs), being the later specialized in antiviral responses involving T cells.

On the other hand, lymphocytes are derived from bone marrow and the thymus, which are classified as primary lymphoid organs, divided as B lymphocytes and T lymphocytes, respectively. In a more detailed way, T lymphocytes precursors are also originated at the bone marrow, although they migrate towards the thymus to undergo development and maturation of thymocytes into T lymphocytes.

				Percentage of Total Lymphocytes*		
Class	**Functions**	**Antigen Receptor and Specificity**	**Selected Phenotype Markers**	**Blood**	**Lymph Node**	**Spleen**
αβ T Lymphocytes						
$CD4^+$ helper T lymphocytes	B cell activation (humoral immunity) Macrophage activation (cell-mediated immunity) Stimulation of inflammation	αβ heterodimers Diverse specificities for peptide–class II MHC complexes	$CD3^+$, $CD4^+$, $CD8^-$	35–60†	50–60	50–60
$CD8^+$ cytotoxic T lymphocytes	Killing of cells infected with intracellular microbes, tumor cells	αβ heterodimers Diverse specificities for peptide–class I MHC complexes	$CD3^+$, $CD4^-$, $CD8^+$	15–40	15–20	10–15
Regulatory T cells	Suppress function of other T cells (regulation of immune responses, maintenance of self-tolerance)	αβ heterodimers Specific for self and some foreign antigens (peptide-class II MHC complexes)	$CD3^+$, $CD4^+$, $CD25^+$, $FoxP3^+$ (most common, but other phenotypes as well)	Rare	10	10
Natural killer T (NKT) cells	Suppress or activate innate and adaptive immune responses	αβ heterodimers Limited specificity for glycolipid-CD1 complexes	CD56, CD16 (Fc receptor for IgG), CD3	5-30	Rare	10
γδ T lymphocytes	Helper and cytotoxic functions (innate immunity)	γδ heterodimers Limited specificities for peptide and nonpeptide antigens	$CD3^+$, CD4 and CD8 variable	Rare	Rare	Rare

Table 1-2 Classification of T lymphocytes, describing its principal properties [1]

As explained before, helper CD4+ T lymphocytes and cytotoxic CD8+ T lymphocytes are the two major T cell populations, both of them expressing αβ T cell receptors (TCRs), the main component of cell mediated immunity derived from its affinity to foreign antigens. After lymphocytes have undergone the majority of the process concerning its maturation, they migrate towards secondary lymphoid organs. There, they are exposed to antingens, leading to clonal selection and expansion, finally developing into effector and memory lymphocytes. The transition from primary to secondary lymphoid organs is closely related to phenotipic changes in lymphocytes, transitioning from naïve to effector and memory lymphocytes.

Specifically, naïve T lymphocytes are defined as mature T cells which do not have encountered any antigen so far. Once they are stimulated by the expousure of a foreign antigen, they abadond their quiescent state into an active one, with the aim of eliminating the antigen. These cells are called effector T lymphocytes. After exposure to an antigen ceases, thus ending the immune response, effectors T lymphocytes perform apoptosis, leaving only memory T lymphocytes, prepared for any repeated encounter with the antigen exposed. Naïve, effector and memory T lymphocytes can be identified depeding on their expression pattern of CD45RA and CD45RO.

	Naive	Activated or Effector	Memory
T Lymphocytes			
Migration	Preferentially to secondary lymphoid organs	Preferentially to inflamed tissues	Preferentially to inflamed tissues, mucosal tissues
Frequency of cells responsive to particular antigen	Very low	High	Low
Effector functions	None	Cytokine secretion; cytotoxic activity	None
Cell cycling	No	Yes	±
Surface protein expression			
IL-2R (CD25)	Low	High	Low
L-selectin (CD62L)	High	Low	Variable
IL-7R (CD127)	Moderately high	Low	High
Adhesion molecules: integrins, CD44	Low	High	High
Chemokine receptor: CCR7	High	Low	Variable
Major CD45 isoform (humans only)	CD45RA	CD45RO	CD45RO; variable
Morphology	Small; scant cytoplasm	Large; more cytoplasm	Small

Table 1-3 Classification of T lymphocytes into naïve, effector and memory subpopulations[1]

Relations between these subpopulations of T lymphocytes are significant for understanding the homeostasis of adaptive immunity depending on T cells.

When the human body ages, the population of memory T lymphocytes increases as a consequence of the several encounters with a huge variety of foreign antigens, although, as we will see, population involving naïve T cells decreases as a result of encounters with different antigens, establishing a balance with memory T lymphocytes, ensuring homeostasis and tolerance.

It is not stange to postulate that obtaining of useful information is dependent of understanding the processes from which T cells differentiate from premature cells into mature T cells contributing to the human immunity. As it is commented previously, thymcytes precursor cells are formed at the bone marrow upon migrating to the thymus, the principal primary lymphoid organ. At the thymus, specific processes undergone by thymocytes are responsible for the complete maturation of T cells, obtaining phenotipic and functional modifications, such as expresssion of antigen receptors, based on positive and negative selection. Once thymic maturation of T cells is complete, T cells migrate towards secondary lymphoid organs, such as lymph nodes or the spleen, where they will be exposed to foreign antigens, thus completing the development into T lymphocytes.

Therefore, analysis on the structure of the thymus could provide useful inforamation regarding the maturation processes undergone, which are highly related to thymic cortex and medulla separation.

1.1.2 Thymus anatomy

The thymus is a specialized organ, which belongs to the lymphoid and immune systems. It is one of the most important organs in charge of the maintenance and development of a healthy immune system. Specifically, the thymus is the lymphoid organ where T cells maturation takes place. It is located anterior to the heart and posterior to the sternum, in the anterior superior mediastinum.

One of the unique characteristics of this organ is that it is the largest and most organ until puberty. Once puberty is reached, the thymus becomes atrophied, being modularly replaced by fat tissue. Therefore, thymus absence is fatal for the development of immune system, promoting autoimmunity and allergic diseases.

The thymus is composed of two lobes, connected by a structure similar to an isthmus, and formed by two different areas, the cortex and the medulla, both of them having different roles in the development of T cells. Both lobes composing the organ are subdivided into different lobules by a fibrous connective tissue called fibrous septa. Along the thymus, there is a clear separation between cortex and medulla, spatially distributed in the lobules, being the latter internal with respect to the thymic cortex. Whereas the cortex is populated by T lymphocytes, the thymic medulla harbours a great collection of immune cells, including lymphocytes, DCs and

macrophages. Interestingly, the thymus also contains a cell population which is not classified as lymphoid, corresponding to both cortical and medullary thymic epithelial cells.

Cortical thymic epithelial cells (cTECs) are reported to aid in the early processes regarding maturation of T cells, whereas medullary thymic epithelial cells (mTECs) have significant influence in the maturation of T cells, especially in the development of Treg cell population, based on presentation of self-antigens for eliminating those lymphocytes with no tolerance [1], [3], [4].

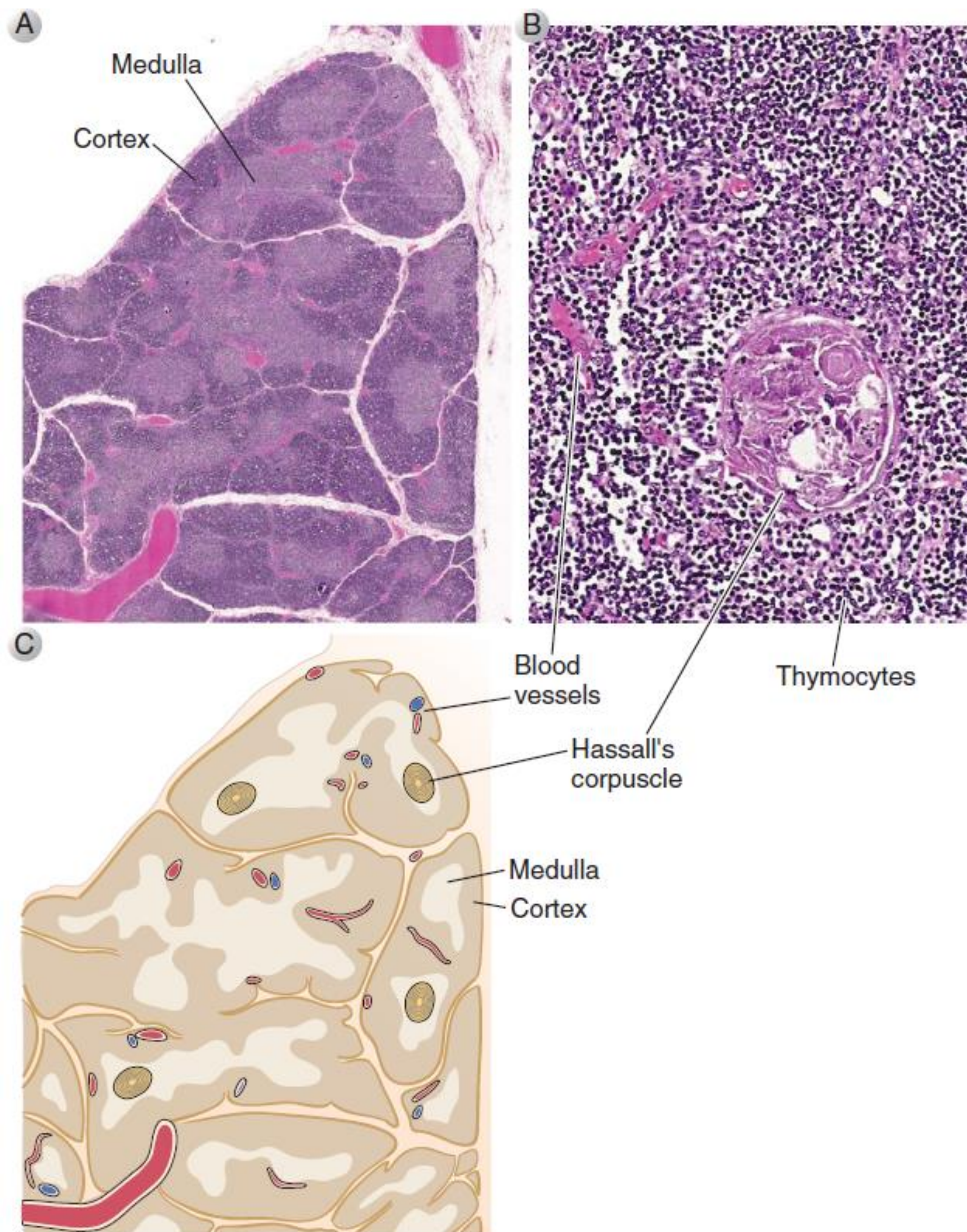


Fig. 1-4 Image obtained through light micrography, showing separation between thymic cortex and medulla (top-left). In addition, a detailed visualization of the thymic medulla is shown (top-rigth), involving different thymic structures. Finally, a schematic of the structure of the thymus is presented (bottom) [1]

In addition, there are specific anatomical structures called Hassall's corpuscles composed of thymic epithelial cells in a tubular fashion, derived from degeneration of mTECs. Although it is still under study, the main function of the Hassall's corpuscles is the secretion of thymic stromal lymphopoietin (TSLP), which is highly important for the maturation of T cells [5], [6].

Furthermore, there is a complex vasculature system surrounding the organ, with two different orientations for the blood vessels, radially distributed with respect to the medulla and tangentially distributed to it. Besides, the thymus possesses efferent lymphatic systems to lymph nodes located nearly, at the mediastinum.

Therefore, the anatomy and its implications are important when the thymus is to be extracted from a living being. It can be easily confused with fatty tissue, and the weight and shape can be different from specimen to specimen, even from related ones. Analysis of samples obtained from infants is extremely useful for the understanding of the processes related to maturation of T cells and the importance and influence of the structure of the thymus, especially when focusing on Treg cell population, which is quite minimal for proper analysis or development of treatments at the periphery.

1.1.3 Ontogeny and maturation of T cells

Commitment of T cell lineages for maturation of T lymphocytes starts by activation of Notch1 and GATA3 transcription factors, which promote further gene expression involved in the development of T cells, especially the formation of T cell receptor (TCRs). Further development of thymocytes, which would be covered later on, is performed by the secretion of Il-7 by cortical thymic epithelial cells (cTECs) upon maturation at the thymic medulla.

One of the most relevant processes in the maturation of T cells involved the random rearrangement of genes which encode for one of the two chains present at T cell antigen receptors, by means of V(D)J recombination. The rearrangement leads to expression of TCR β chain as a pre-antigen receptor, which is considered one of the selection processes for maturation of T cells. Later on, expression of the remaining chain for the TCR is produced following V(D)J recombination, leading to the expression of mature TCR. Once the TCR is completely formed, thymocytes undergo positive and negative selection, so they develop normal immune response against foreign antigens. According to its definition, V(D)J recombination is defined as "the rearrangement in individual lymphocytes of different variable (V) region gene segments with diversity (D) and joining (J) gene segments" [1].

In addition, gene sequence rearrangement related to TCRs and its expression greatly influences in the development of T lymphocytes. Therefore, such modifications affect the cell differentiation of thymocytes, leading to classification of thymocytes into different thymic cell population. As stated previously, immature thymocytes are located at the cortex whereas increase in maturation of T cells can be observed towards the medulla. In short, specific expression of CD4 or CD8 as single positive cells is produced when migrating from thymic cortex to medulla.

Most cortical thymocytes do not express neither TCR, although they possess the genes for its expression, nor CD4, CD8 and CD3. These thymocytes are classified as Double Negative thymocytes (DN). These thymocytes undergo an extensive selection program, based on V(D)J recombination of TCR genes for the maturation and expression of such receptors, as explained previously, resulting in expression of pre-antigen β chain TCR. Once this stage is reached, TCR α gene recombination is started, which will produce the differentiation of DN thymocytes into Double Positive thymocytes (DP). DP thymocytes express mature TCR, in association with CD3, along with both CD4 and CD8. As a consequence, thymocytes which fail in the maturation of αβ TCR perform apoptosis. Further maturation of thymocytes from DP population is determined by expressing CD4 or CD8, with differences in their function as T cells.

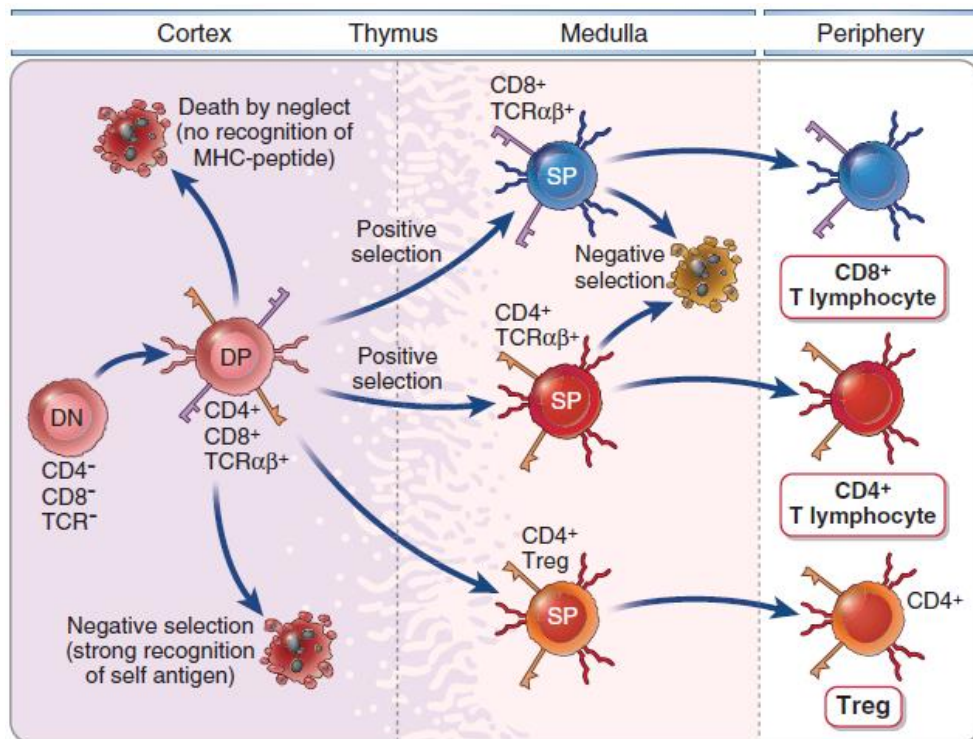


Fig. 1-5 Visual representation of the maturation process undergone by thymocytes, from immature DN thymocytes located at the cortex to mature T cells found at the periphery, derived from single positive thymocytes expressing CD4 and CD8 [1]

Following positive selection for recognition of self-antigens, DP thymocytes differentiate into thymocytes which recognize class I MHC, CD8+CD4- Single Positive thymocytes (CD8 SP) and those which recognize class II MHC, CD8-CD4+ Single Positive thymocytes (CD4 SP).

CD8 SP thymocytes which successfully overcome selection processes acquire specific features for developing its cytolytic functions, eliminating infected or tumour cells. The cytolytic mechanism is based on secretion of perforins and granzymes from granules present at CD8 T lymphocytes, upon recognition of an antigen from a target cell. Antigen presentation usually is performed by dendritic cells and the proliferation of activated CD8 T lymphocytes and its action are aided by CD4 T lymphocytes. The support from helper T lymphocytes provides stimulation of cytotoxic differentiation into memory cells and enhanced antigen presentation from APCs. Lastly, cytotoxic T lymphocytes secrete IFN-γ, which induce activation of macrophages and inflammatory processes.

CD4 SP thymocytes undergoing positive selection develop as mature T cells upon activation of its mechanisms for supporting other immune cells by secretion of cytokines, being this action subjected to recognition of antigens from APCs. These cytokines are directed to stimulation and activation of specific immune cells, such as macrophages, eosinophils, neutrophils, monocytes or cytotoxic T lymphocytes, enhancing the immune response by creating a suitable environment for the development of the response against the pathogen.

CD4 T lymphocytes are subdivided into three effector helper T cells, with specific interactions with different immune cells. Each subset of effector helper T cells is characterized by the secretion of specific cytokines directed to different immune cells.

- Th1 CD4 T lymphocytes secrete IFN-γ directed to macrophages, producing its stimulation and activation.
- Th2 CD4 T lymphocytes secrete different interleukins which activate eosinophils and mast cells, as well as enhance activation of macrophages. These cells can also stimulate B lymphocytes in the production of antibodies.
- Th17 CD4 T lymphocytes secrete different interleukins for recruitment and activation of neutrophils.

Each subset of effector helper T cells (Th1, Th2, Th17) has differences in their pattern of receptor expression, thus they have instructions to migrate towards specific parts of the infection. As for activation of CD8 T lymphocytes, antigen presentation is mainly performed by dendritic cells.

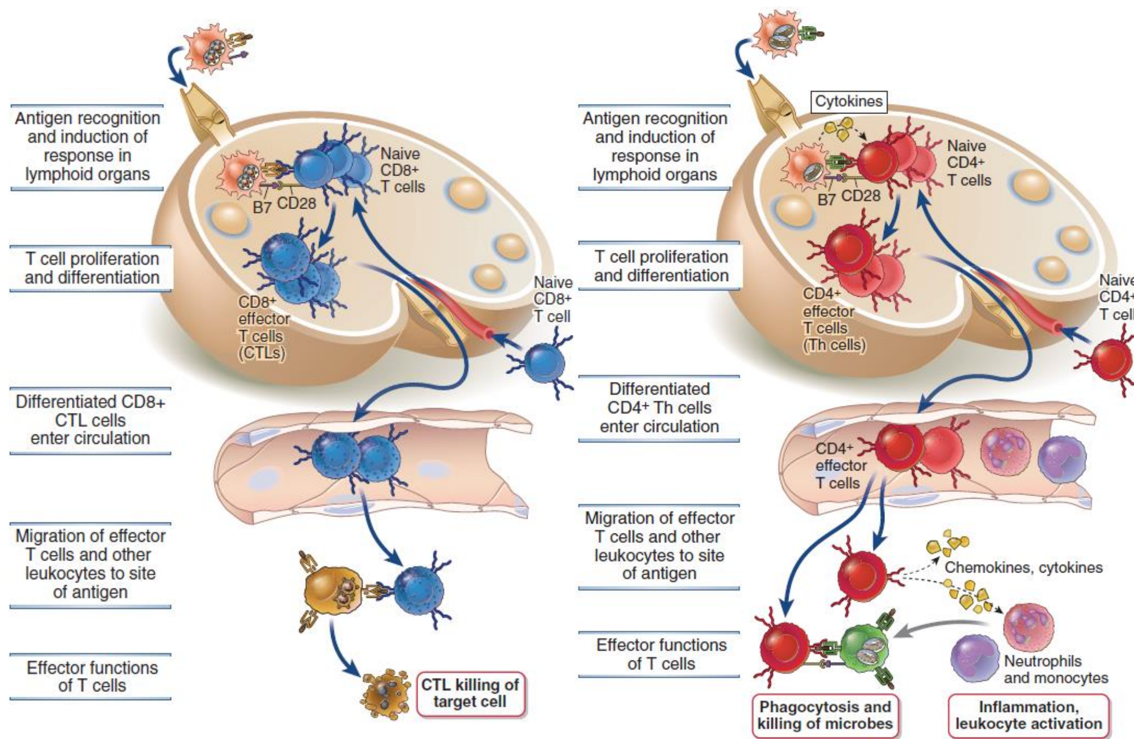


Fig. 1-6 Schematics describing the mechanism of activation of both CD8 cytotoxic T lymphocytes (left) and CD helper T lymphocytes, from their naïve stage at secondary lymphoid organs to their effector stage upon production of an immune response [1]

Besides, some CD4+ thymocytes located at the thymic medulla have large reactions against self-antigens during selection processes for maturation of T cells, even considering the deletion of thymocytes derived from negative selection. These cells differentiate into a different cell population called regulatory T cells (Treg). Regulatory T cells are in charge of maintaining peripheral T cell tolerance, deleting inhibiting excessive immune response of other immune cells against the organism and promoting the deletion of immune cells which have lost self-tolerance.

Treg cells expresses high amounts of CD25 and FOXP3, which greatly characterize this cell population, being the latter the major transcription factor for development and maturation of Treg cells and as well as CTLA-4, factor required for their suppression functions.

In addition, they express low levels of CD127 (Il-7 receptor) and have great dependence on Il-2 for maintenance of FOXP3 expression and their regulatory capabilities derived from their inability to produce Il-2. Their high expression of Il-2 receptor causes depletion of Il-2, thus controlling the proliferation rate of other immune cells

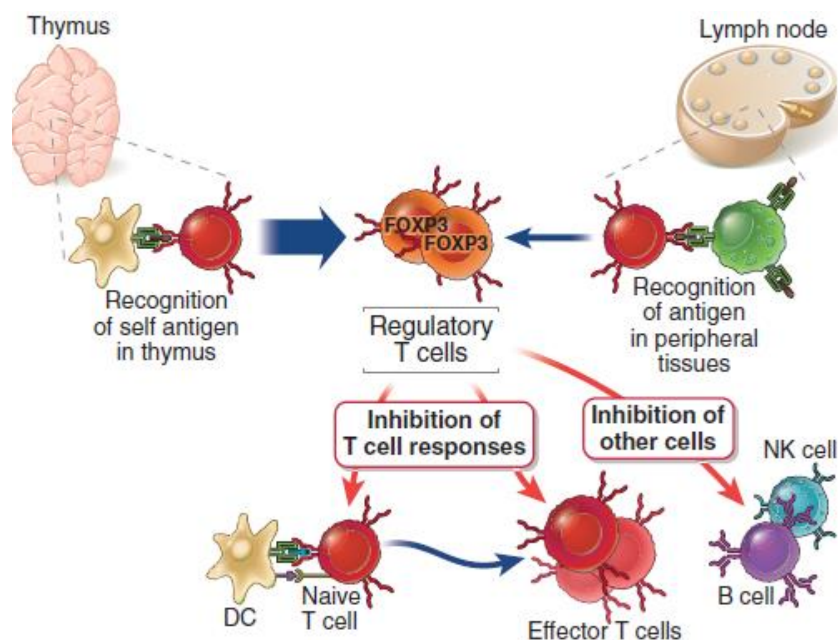


Fig. 1-7 Graphical representation of suppressive activity of regulatory T cells [1]

Regulatory T cells are derived both from the thymus and the periphery, by similar means based on self-antigen recognition. Furthermore, recent studies report different pathways for the development of regulatory T cells, involving action of dendritic cells, mTECs or cytokines such as Il-2, Il-15 and TFG-β [1], [6], [5]. Recently, studies show that the ratio between effector and regulatory T cells is key part of the determination of the allergies of the organism, so modification of the adaptive immune response could be implemented in the field of allergology and autoimmunity.

Finally, it is important to mention that mature T lymphocytes circulate along blood and lymphatic vessels, which also cause a recirculation of regulatory T lymphocytes towards the thymus. Lymphocyte recirculation of naïve T lymphocyte is observed from blood to secondary lymphoid organs, depending on the recruitment of such cell when an immune response is produced.

However, there is almost no information regarding the functions performed by regulatory T lymphocytes which recirculate towards the thymus, although it is believed that Treg cells support maturation of regulatory T cells and other immune cells at the primary lymphoid organ [6].

1.1.4 Positive and negative selection. Immunogenicity and self-tolerance

As it is commented previously, several selection processes are performed during the development of mature T cells, ensuring proper response against foreign and self-antigens.

Positive selection promotes survival of thymocytes that have low reactivity against class I and II MHC from the organism, as they are the mediators in the presentation of antigen from target cells in an immune response. In addition, positive selection induces separation of DP thymocytes into two different cell lineages, depending on their reactivity towards class I MHC (CD8 SP) or class II MHC (CD4 SP).

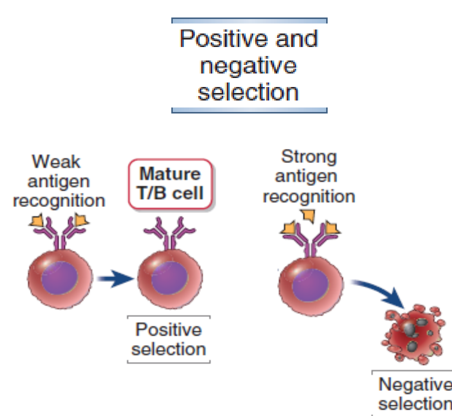


Fig. 1-8 Positive and negative selection [1]

On the other hand, negative selection ensures the elimination of thymocytes that overreact when exposed to self-antigen, producing harmful responses against the organism. Therefore, negative selection is the main mechanism for maintenance of central self-tolerance at the thymus. Moreover, no reactivity agains self-antigen presentation produce apoptosis of defective immature thymocytes.

Positive and negative selection processes are possible as eptithelial cells at the thymus posses a wide variety of antigens from different tissues along the human body. Therefore, thymocytes are exposed to several antigens distributed along different tissues, checking their reactivity agains self-antigens. The presentation of said antigens is regulated by autoimmune regulator protein (AIRE) which induces its exposure by means of mTECs.

As it is established previously, major histocompatibility complex (MHC) proteins present in APCs are in charge of properly binding to antigen fragments to present hem to T lymphocytes in cell-mediated immuntity. T cell receptor (TCR) is specialized in recognition of such MHC-antigen complexes. There are several cell populations classified as APCs, such as dendritic cells, macrophages or plasma cells (derived from B lymphocytes).

MHC proteins in the human organism are also called human leukocyte antigens (HLA). It is important to mention that class I MHC are expressed in cells possesing a nucleus (thus, erytrocytes do not express thys type of MHC) whereas only specific cell populations classified as APCs express class II MHC. The distribution of class I and II MHC depeding on the type of cell is induced by the mechanism by such proteins present fragment antigens to specific T lymphocytes, as commented previously.

CD8 T lymphocytes are directed to eliminate infected or tumor cells, thus it is reasonable that any nucleated cell could be classified as one of the targets for elimination, mediated by class MHC I. On the contrary, phagocytes and other immune cells are meant to engulf pathogens for the presentation of such particles to lymphocytes, therefore its antigen presentation mechanism is mediated by class II MHC, only present at APCs and detectable by CD4 T lymphocytes.

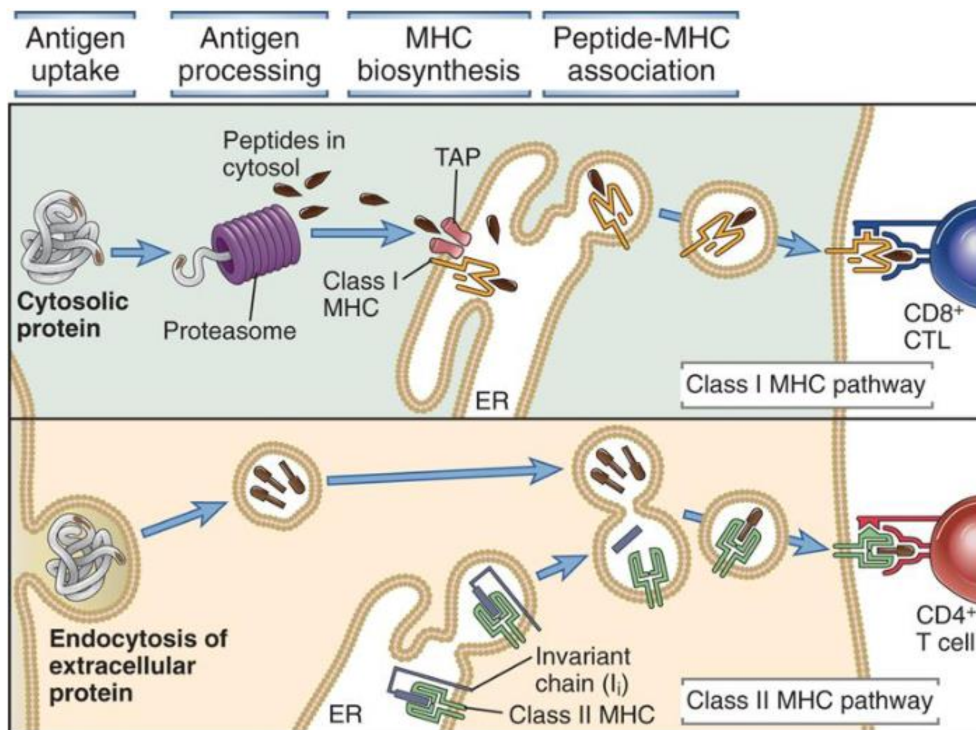


Fig. 1-9 Mechanism of antigen processing for both class I MHC (top) and class II MHC (bottom) [1]

Class I MHC processing of the antigen is based on digestion of antigen present at the cytosol, derived from infection or tumor development, by the proteosome. Peptides generated one the antigen is processed by the proteosome migrate to the endoplasmic reticulum where binding with class MHC protein is produced.

On the other hand, class II MHC processinf of the antigen is derived from endocytosis of the microbe, which is degradated in the lysosomes at APCs, upon binding with class II MHC proteins

1.2 Background on previous immunological research projects

Although extensive description of several concepts have been provided already, which ensure better understanding of the processes related to the subject under study in this project, there are some advanced definitions and concepts which should be addressed in the identification of key points for the present research project.

1.2.1 Thymocytes classified into different cell populations

As explained previously; naïve, effector and memory T lymphocytes can be separated into different populations by the analysis of the pattern expression of CD45RA and CD45RO. Several studies show the differences in proliferation when comparing naïve and memory T lymphocytes, characterized by CD45RA and CD45RO, respectively, in addition to relation between activation and proliferation of such cells with phenotypic changes from CD45RA to CD45RO expression [7].

Although FOXP3 and CD25 expression is closely linked to regulatory T cells, DP thymocytes, along with other thymic populations such as CD8 SP thymocytes have shown clear expression of FOXP3, even exhibiting suppressive capabilities [regulatory]. There is also proof of relation of cycling mature CD4 T lymphocytes and CD4 SP thymocytes, by means of exposure of Il-2 for proper development of immature T cells [6].

1.2.2 Regulatory T cell population

Detailed analysis have been performed to focus on identification and charactehrizaztion of different processes related to proliferation, maturation and activatin of regulatory T cell population.

The expression of FOXP3 is extremelly relevant for the proper functioning of Treg cell population, as its deletion produces increase in Il-2 production and differentiation into CD4 T lymphocytes subsets, such as Th1 CD4 T lymphocytes, losing their supressive function [5].

As commented before, there is a negative feedback circuit which regulates responses between non-regulatory and regulatory T cells based on Il-2 secretion. Furthermore, exposure of cells expressing low amounts of CD25 to FOXP3 produces phenotypic changes.

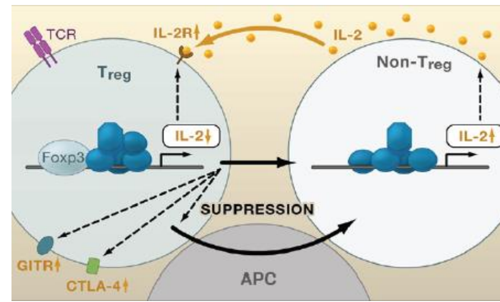


Fig. 1-10 Il-2 based negative feedback circuit for regulation of cell proliferation [5]

It results in development of immune cells possesing similar Treg cell functions, establishing the relevance of FOXP3 for the development of regulatory T cells, derived from the increase in production and expression of CD25 [8].

Also, observation related to CD4 SP thymocytes expressing FOXP3, which are prompted to differentiate into regulatory T cell exhibit traits of advanced developmental stage, with the required stimulation of TCR upon commitment to Treg cell lineage [5].

FOXP3, which, as a curiosity, was first detected from its defective absence in immune dysregulation poluendocrinopathy (IPEX), closely related to mouse strain scurfy, has extensive influence in the expression of suppresor regulators CTLA-4 and CD39, being the latter responsible for the transformation of ATP or ADP into AMP by selective cleavage. Along with CD73, AMP is cleaved to adenosine which is responsible for the suppresive functions of regulatory T cells, inhinbiting T cell proliferation [9], [10]. Besides, CTLA-4 is related to CD28, both of them mediating in the regulation of suppresive behabiour of regulatory T cells. [11].

Finally, involution of the thymus related to aging of the human body causes a decrease in naïve T cells population towards an increase in memory T cells, as well as it is highly related to the low presence of naïve CD45RA+ regulatory T cells [12]. However, results suggest that such population is essential for the maintaining of the regulatory T cell population, along with the self-tolerance. This observation is supported by the presence of naïve regulatory T cells which require alternative routes of development from its thymic origin [12]. Detailed analyses show clear differences in proliferation leading to maintenance of regulator T cell population based on CD45RA+ naïve Treg cells.

1.3 Project description

Detailed analysis of thymic cell populations, focused on the regulatory T cell population is of such importance for a better understanding of the different mechanisms related to immunology, including organ transplantation, allergic reactions and autoimmunology. In short, immunological research conducted previously is significant for the proper interpretation of results related to such concepts, which are a great foundation for further research and analysis.

Our aim for this research project is to properly define and characterize the different populations located at the thymus, analysing their spatial distribution and ensuring the reliability of the results with the previous scientific publications.

Furthermore, detailed analysis and discussion is conducted using novel human pediatric thymic tissue, for the characterization of regulatory T cells and their relation with other immune cells, which would be of great help in further investigations with the goal of unravelling the unknown concepts related to such fields of science.

2. MATERIALS AND METHODS

2.1 Source of pediatric human thymus and preparation for analysis

2.1.1 Obtaining of pediatric human thymus for analysis

Samples for the experiments at the *Laboratorio de Inmuno-Regulación* are provided by mutual agreement with the *Servicio de Cardiología Pediátrica and Servicio de Cirugía Cardiaca Infantil* at the *Hospital General Gregorio Marañón*. Pediatric patients which suffer from severe cardiac conditions are subjected in several cases to surgery in order to treat their condition. As it is explained previously, the size of the thymus of babies or infants difficult the cardiac surgery, thus requiring the extraction of the thymus, which is offered to us and other research groups as sample tissue to be used.

Sample tissues are obtained from a variety of patients, with differences in age, gender and diseases, which could be positive for the analysis of the viability of such researches for different fields of science, looking for differences between samples which could serve as a foundation for further analysis and as an acquisition of knowledge related to immunity. The samples used in the experiment correspond to a 12-month female (THY670)

Planification of the experiments to be performed is tightly organized depending on the schedule of cardiac surgery for the patients. Upon arrival, samples are properly storaged and maintained in TexMACS GMP cell culture medium (Miltenyi Biotec) with 1% of antibiotics to avoid contamination, for 1-2 days, until research is to be performed. Regulation related to extraction of the human sample tissue, transference to the designated research groups and further manipulation are highly regulated by GMP and other medical, ethical, research and professional protocols.

2.1.2 Human pediatric tissue processing

Research focused on characterization and analysis of cell population distribution is performed following two different analysis pathways: flow cytometry analysis and analysis of images acquired via confocal microscopy. Different protocols for proper tissue processing are performed to ensure the reliability and efficiency of the methods used in the research.

Such protocols required different routes of action for sample tissue in order to successfully perform the experiment and guarantee the quality of the results obtained.

2.2 IHC methodology. Fixation, permeabilization and blocking steps

2.2.1 Immunohistochemistry/Immunofluorescence

Immunohistochemistry is defined as the series of methods or processes from which specific distribution of cells with certain features can be obtained with high sensitivity, based on principles of affinity between antigens and antibodies. Antigens are defined as specific molecules, usually proteins. They can be found at the surface of cells or intracellularly, which have high affinity with specific antibodies directed to such antigens, minimizing interaction with other particles which are not considered the desired target, thus obtaining high sensitivity. As a reminder, antibodies are composed of two different areas, called fragment antigen-binding (Fab) and fragment crystallizable region (Fc). Epitopes from antigens are recognized by the Fab domain, which contains an area of large sensitivity and specificity for antigens, called paratope. Once the antibody has recognized an epitope from an antigen, the Fc domain is in charge of establishing the signalling with other cells, as it contains a conserved domain based on glycosylation.

Immunohistochemistry is the preferred method for identification of cells in a sample due to its high reliability and sensitivity. There are two different methods of immunohistochemistry, based on the interactions and the number of molecules participating in the reaction involving the identification of the antigen by a specific antibody. Specifically, immunofluorescence is derived from immunohistochemistry although molecules emitting a fluorescent signal are used.

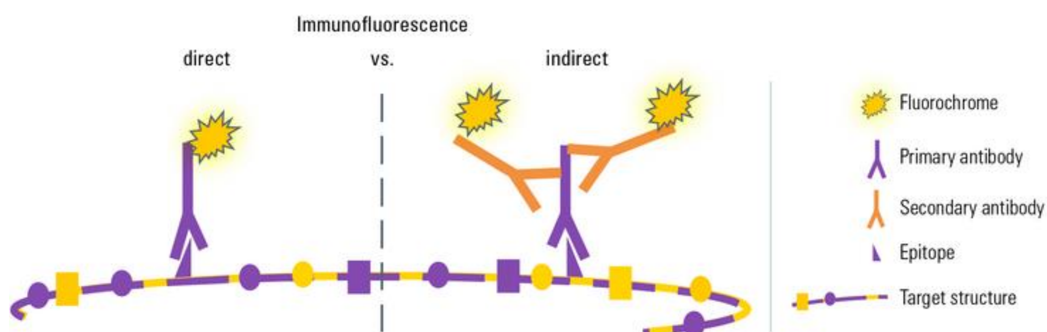


Fig. 2-1 Scheme of direct and indirect immunofluorescence [13]

A primary antibody is described as the one which is targeted to a specific antigen whereas a secondary antibody is directed to primary antibodies bounded to an antigen. The latter is conjugated with a fluorophore or other type of molecule which is able to emit a readable signal when interaction with the primary antibody is produced. For our purpose, direct immunofluorescence was the elected method as it was highly adaptive for our resources, equipment and necessities in addition to the simplification of the process, avoiding other steps such as blocking.

2.2.2 Foundations on fixation, permeabilization and blocking steps

Fixation is the process of protecting the tissue by avoiding the degradation of its structures, specifically the antigens as well as increasing the sample tissue robustness. It is essential for the preparation of the tissue for further IHC treatment, increasing the possibility of obtaining significant results.

In some cases, additional steps are required in the process of immunohistochemistry/immunofluorescence, derived from the antibodies or other features to be taken into consideration. Permeabilization is a process from which a sample tissue or a cell culture is chemically treated in such a manner that the cell membrane is permeable to the chosen antibodies, as the antigen of interest is located intracellularly. As a drawback, the cell culture or sample tissue subjected to permeabilization have modified characteristics derived from the chemicals used in the process, which could affect the performance of such cells.

Additionally, in some cases a blocking step is added to the IHC/IF protocol. Blocking is referred to the addition of substances which are directed to the blockage of unspecific signal and background signal derived from the interaction of the antibodies with the sample, antigens such as the ones presented at APCs or Fc receptors [14]. Generally, any protein which is not directed to the targeted antigen can be used although proteins obtained from the same species as the secondary antibodies used are recommended.

2.3 Flow cytometry characterization

One of the two methods selected for the analysis is flow cytometry. It offers high reliability and control over the sample, which is essential for the proper characterization of cell populations and its comparison with analysis performed using confocal microscopy.

2.3.1 Foundations on flow cytometry

Flow cytometry is a method for characterization and identification of different cell populations found in a heterogeneous cell suspension, based on cell size, volume and specific expression of factors, detected by the analysis of the signals emitted when exposing the single cells to a laser radiation. Cell suspension is prepared with a specific panel of antibodies detecting several proteins of interest, which will be analysed by flow cytometry, receiving specific outputs which can be used for separation of cell into populations [15], [16].

Inside the flow cytometer, cell suspension is surrounded by streams of fluid (sheath fluid) which redirect the cells into a narrow chamber where they flow one

by one, being exposed to a laser beam. Modifications to the machine could be introduced, charging the cells in such a manner that when subjected to EM fields, they are divided into different streams, flowing into independent chambers (cell sorter).

Laser exposition produces both scattering in several angles and absorption of the light when striking cells [15]. Typically, a fluorescent signal is emitted as a result of the excitation of fluorophores present in antibodies attached to cells, which is filtered through several dichroic mirrors, which separate the light into specific wavelengths. Scattering and the separation into wavelengths of the signal emitted is used for the analysis, characterization and identification of cell populations.

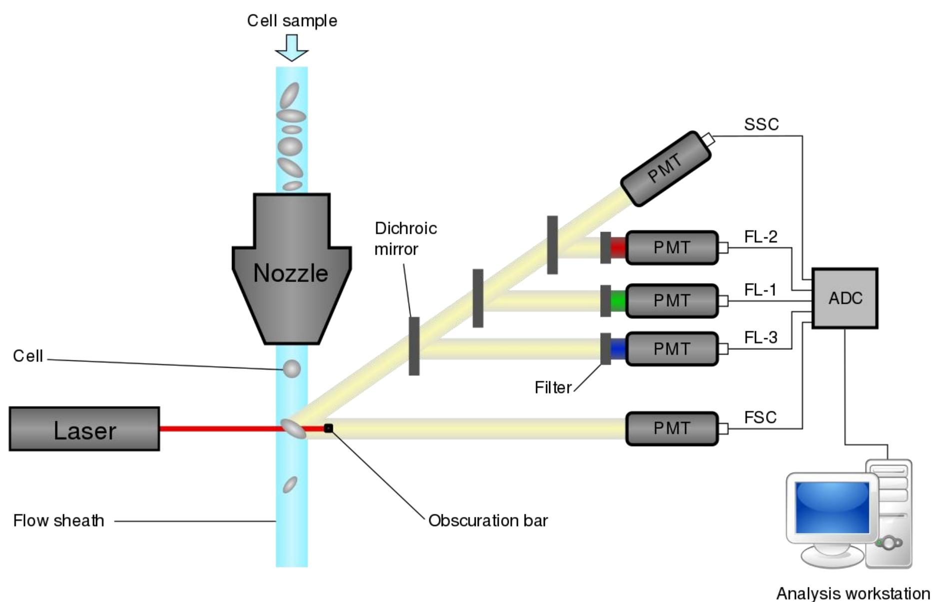


Fig. 2-2 Graphical representation of a flow cytometer [17]

Light scattering is decomposed in Forward Scatter (FS) and Side Scatter (SS) which are detected by a photodetector located in the same line as the laser beam and the sides, respectively. FS signal is related to cell size whereas SS signal is related to the complexity of the cell (presence of granules).

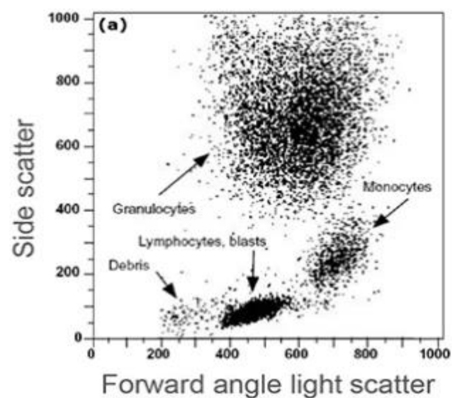


Fig. 2-3 2D dotplot representation of cell populations depending on their size and complexity [15]

Therefore, its relation is sufficient for the separation of several cells into different populations depending on their size and complexity of structure [15], [16]. As an example, cell suspension from a blood sample

can be analysed by flow cytometry. As it is depicted in the figure, cell populations are organized depending on their FS and SS signal, dividing the cell suspension into granulocytes, monocytes, lymphocytes and debris. As a result, cells characterized as low-medium size and low complexity are lymphocytes. Fluorescent signal emitted by cells is directed towards several dichroic mirrors, which separate the signal into different ranges of wavelengths, discarding the undesired ones upon reaching the specific photodetector with an additional filter. Each wavelength of interest is captured by a photodetector, with up to 10 different colours to be analysed.

Finally, results from the flow cytometry can be analysed using bioinformatics and properly represented in dotplots, comparing two biological parameters, selecting and characterizing the different cells population depending on the relation between them. Subdivisions between cell populations can be performed by the application of gates, based on fluorescent signal. Flow cytometry could analyse up to 30000 cells per second, with external and internal identification, with the addition of permeabilization methods, of essential factors for characterization and identification of cell populations.

2.3.2 Dissociation of human pediatric tissue for flow cytometry

Upon preparation of thymocytes substrate for flow cytometry, thymic tissue is subjected to mechanical lysis which is the preferred method for obtaining a high number of thymocytes for further analysis, although other methods such as enzymatic lysis are also viable.

The thymus is weighted, multiplying the result by two if the organ is composed of only one lobule, as the protocol is optimized for two lobules, and a small piece is cut off from the organ using sterile medical equipment, avoiding excessive presence of blood, at the laminar flow cabinet for biological samples. Sample tissue is turned into smaller pieces for easier dissociation and introduced in a tissue dissociator.

After dissociation of the sample tissue, the substrate is filtered in a 50 ml Falcon tube, obtaining a volume around 25-30 ml of cell suspension. The cell suspension is centrifuged at room temperature, 1500 rpm for 10 minutes. The cell suspension is resuspended, and the cell medium is renewed, before another filtration is performed, so no tissue aggregations are formed. After the supernatant is discarded using a pipette controller and the pellet formed is detached from the bottom, 45-50 ml of cell medium are added and the tubes containing the cell suspension are placed in a tube rotator, so aggregations are not formed.

During the rotation process, some 1:50 dilutions are made with PBS 1x as solvent. A 1.5 ml Eppendorf is filled with 200 µl of dilution, thus obtaining a considerable volume of cells to be counted (4 µl of our sample). Finally, cell counting is performed in a Neubauer's chamber [18], and the concentration is calculated following the equation:

$$\frac{Cells}{mL} = \frac{nº\ cells \cdot f_{dil}}{nº\ squares \cdot V_{square}(\mu l)} \times 1000$$

$$\frac{Cells}{mL} = \frac{(89 + 86 + 88 + 74) \cdot 50}{4 \cdot 0.1} \times 1000$$

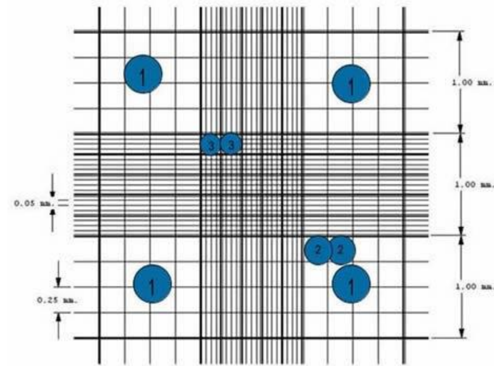


Fig. 2-4 Schematic of a Neubauer's chamber [18]

$$Nº\ cells = 4.2125 \cdot 10^7 \frac{cells}{ml} \cdot 45\ ml = 1.895625 \cdot 10^9 cells$$

The information is annotated in the tubes prior to incubating the antibodies and preparing the cell suspension for the analysis by flow cytometry.

2.3.3 Characterization of thymocytes from human pediatric tissue

Once the concentration of cells is known, a sample of volume containing around 10^6 thymocytes is washed with 2 ml of staining buffer (PBS 1x + 2% FBS) and it is introduced in the centrifuge, at 1500 rpm, 4ºC for 5 minutes. After the centrifugation, the supernatant is discarded, except from the last drop (~100 µl) and the sample is placed in the vortex.

IHC incubation includes the addition of a specific panel of antibodies which will mark the factors of interest in the cells. The following volumes are added to the final volume of 100 µl of cell suspension.

THY Linfos Thymocytes Panel		
CD45	FITC (BC)	10 µl
CD127	PE (BC)	8 µl
CD45RA	ECD (BC)	3 µl
CD45RO	PC 5.5 (BC)	5 µl
CD25	PC 7 (BD)	3 µl
HLA-DR	APC (IS)	1 µl
CD8	APC/Cy5 (BC)	1 µl
CD4	APC/Cy7 (BC)	1 µl
CD3	PO (Mty)	3 µl

BC: Beckman Coulter
BD: BD Bioscience
IS: Immunostep
Mty: Miltenyi Biotec

Table 2-1 THY Linfos Thymocytes Panel

After the antibodies are added, the solution is placed in the vortex and incubated at 4ºC, without light for 30 minutes. Lastly, a viability labelling is performed with Pacific Blue, so it is possible to identify the proportion of viable cells. For the viability labelling, the cells are washed with 2 ml of PBS 1x and introduced in the centrifuge at 1500 rpm, 4ºC for 5 minutes. Then, the supernatant is discarded and placed in the vortex, followed by the addition of 1 ml PBS 1x and the labelling of the cells with 1 µl of viability dye (Pacific Blue, eBioscience).

The solution is placed in the vortex and incubated for 30 minutes at 4ºC, without light. The cells are washed with 2 ml of staining buffer and placed in the centrifuge at 1500 rpm, 4ºC for 5 minutes. The solution is placed in the vortex after the supernatant is discarded and resuspended with 500 µl of staining buffer.

2.3.4 Characterization of Treg thymocytes from human pediatric tissue

For the characterization and identification of Treg cell population it is necessary to implement a different panel of antibodies as FOXP3 and CTLA4 are two essential markers which are present intracellularly, which require the addition of permeabilization and blocking processes. In addition, other different antibodies are added as the process will allow for a better understanding of the cells classified as Treg thymocytes.

There are two steps in the IHC labelling process as there are antigens of interest at the surface of cells and intracellularly. For the surface labelling, the following volume of antibodies is added to the sample containing a volume of 100 µl:

THY FOXP3 Thymocytes Panel		
CD39	FITC (Mty)	5 µl
CD45RA	ECD (BC)	5 µl
CD25	PC 7 (eBio)	2 µl
HLA-DR	PC 5.5 (BC)	5 µl
CD8	APC/Cy5 (BC)	1 µl
CD4	APC/Cy7 (BC)	1 µl
CD3	PO (Mty)	3 µl

BC: Beckman Coulter
eBio: eBioscience
Mty: Miltenyi Biotec

Table 2-2 THY FOXP3 Thymocytes Panel

Once the antibodies are added, the solution is placed in the vortex and antibody incubation is performed for 30 minutes, at 4ºC without light.

As previously performed with the first cell suspension, a cell viability labelling is important for further analysis. The cell suspension is washed with 2 ml of PBS 1X and introduced in the centrifuge at 1500 rpm, 4ºC for 5 minutes. Then, the supernatant is discarded, and the sample is placed in the vortex. A volume of 1 ml of PBS 1x and 1 µl of viability dye are added to the solution, which is placed in the vortex and incubated for 30 minutes at 4ºC, without light.

Prior to intracellular staining, permeabilization and blocking steps are performed so the sample is prepared for the correct intracellular IHC. The cells are centrifugated at 1500 rpm, 4ºC for 5 minutes, followed by the discarding of the supernatant. Then, the solution is placed in the vortex and the cells are resuspended in 1 ml of Fixation/Permeabilization Solution. The cell suspension is placed in the vortex and incubated for 20 minutes at 4ºC.

Later on, the cells are washed with 2 ml of Permeabilization/Wash Buffer and the cell suspension is introduced in the centrifuge for 5 minutes, at 1500 rpm, 4ºC. Finally, the supernatant is discarded, leaving the last drop (~100 µl) and the sample is placed in the vortex.

The last step of the preparation of cell suspension starts by the addition of the following panel of antibodies, which are directed to intracellular antigens of high interest:

THY Intracellular Panel		
FOXP3	PE (eBio)	5 µl
CTLA4	APC (BD)	2 µl

eBio: eBioscience
BD: BD Bioscience

Table 2-3 THY Intracellular Panel

Once the antibodies are added, the cell suspension is placed in the vortex and incubated at 4ºC, without light, for 30 minutes. Then, the cells are washed with 2 ml Permeabilization/Wash Buffer and introduced in the centrifuge at 1500 rpm, 4ºC for 5 minutes. Lastly, the supernatant is discarded, and the cell suspension is placed in the vortex a last time before resuspended in 500 µl of staining buffer. The cell suspension is prepared for the analysis by flow cytometry along with the one prepared previously.

2.3.5 Analysis of sample cell suspension by flow cytometry

Once the preparation of sample cell suspension is complete, it is introduced in the flow cytometer for the analysis. The flow cytometer (Gallios Flow Cytometer, 10 colours, 3 lasers, Beckman Coulter) is a complex and delicate device which requires high control over the process and good maintenance to obtain good results.

The sample cell suspensions are introduced in the correspondent tubes, followed by the tubes containing the solutions for the cleanse protocol. Both thymocyte and FOXP3 panels are strictly optimized following a specifically designed protocol for the characterization and identification of the cell population of interest.

Cleaning Protocol
Protocol Bleach
Protocol Water
Protocol Water
Protocol Bleach
Sample Analysis Protocol
THY Linfos Thymocytes Panel
THY FOXP3 Thymocytes Panel
Cleaning Protocol
Protocol Bleach
Protocol Water
Protocol Water
Protocol Bleach

Fig. 2-5 Gallios flow cytometer (Beckman Coulter) [19] (left) and the workflow for the flow cytometry (right)

Finally, results are analysed using Kaluza Analysis (Beckman Coulter), which is a program designed for the analysis of multi-colour data, such as the one obtained from the flow cytometry process, possessing algorithms for colour compensation, certain adjustments of the results and displaying of the data obtained [20]. The analysis from the software is regulated by an optimized protocol, as well as the flow cytometry panel analysis, with specific gates of interest. The protocol for Kaluza Analysis is meant to properly characterize the different cell populations encountered in the thymus, as well as maintaining certain standards which ensure the proper comparison of results and high reliability of such.

2.4 Characterization and identification of cell population distribution using confocal microscopy

As it has been explained before, flow cytometry is meant for the characterization and identification of cell population with high reliability. Unfortunately, it is not possible to properly determine the distribution of such populations as the mechanical lysis destroys the structure of the organ, so the heterogeneous cell suspension is formed from cells from different parts of the thymus. Confocal microscopy could offer a powerful solution to this problem as slides of the organ can be analysed using specific biomarkers and it would be possible to determine the distribution of cell populations in a 2D fashion, very similar of what it is found in vivo.

2.4.1 Confocal microscopy structure and acquisition method

Confocal microscopy is an optical method for acquisition of images derived from principles of fluorescent emission. It takes advantage of the presence of a spatial pinhole at a specific location, conjugate point of the point of emission in the sample, thus only the point under exposure is detected, eliminating light emission for the surroundings.

Fluorescent microscopy uses emissions of laser beams for the excitation of the fluorophores present in the sample. Although the target could be a specific position at the sample, the laser beam is directed evenly to the sample, thus emission of fluorescence is produced from different parts of the sample, decreasing the optical resolution of the image by the addition of undesired and unfocused signals to the final image. On the other hand, confocal microscopy uses a special configuration which allows for the detection of specific signals emitted by a desired point in the sample, through aiming of the laser beam to several pinholes which block out-of-focus signal of the sample.

The laser beam and the photodetector are positioned following an epi-fluorescent configuration, in which the light emitted is filtered to a certain wavelength and reflected in a dichroic mirror, so it strikes the sample. The fluorescent signal emitted by the sample passes through the dichroic mirror and a filter upon being detected by the photodetector. The confocal configuration includes two spatial pinholes at the laser and the photodetector as well as the lens, so the laser beam is properly focused at the target point in the sample.

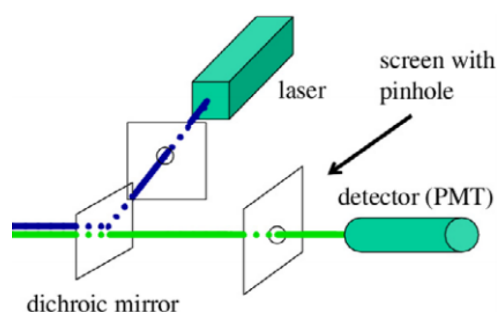


Fig. 2-6 Epi-fluorescent configuration [21]

In the confocal microscopy, the laser beam is directed through a pinhole upon being redirected by the dichroic mirror and focused on a specific point in the sample with the aid of a lens. The emitted signal is directed to another pinhole, which blocks any signasl except the one being emitted by the target point.

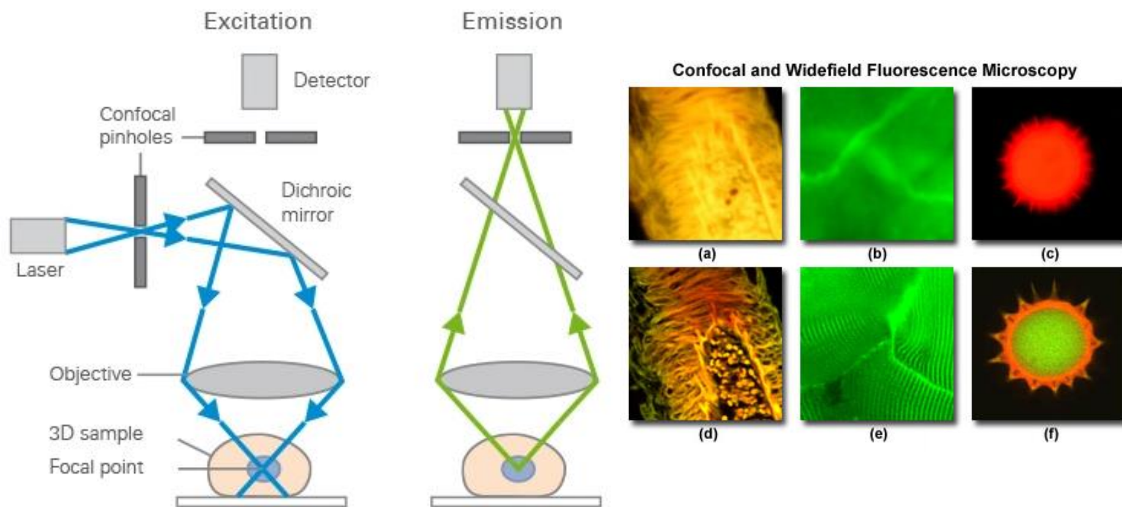


Fig. 2-7 Structure of a confocal microscope [22] (left) and representation of visual changes in image resolution when comparing widefield and confocal microscopy [23] (right)

Both pinholes are positioned in conjugation with the target point, creating two conjugated planes, so the laser emitted by the target creates an image at the pinhole when it passes through the lens. The target point can be selected by monitoring the orientation of the mirror in such a way that the sample can be analysed point by point, creating several planes at different heights which could be processed into a 3D image with external software.

Analysis of the points at a certain height (depth of view) is called optical tissue sectioning, as it simulates the image obtained if the tissue was physically sectioned and observed using a microscopy. Optical sectioning produces clear images from different depths of view at the confocal microscope, without manipulation of the sample, resulting in a z-stack for detailed analysis.

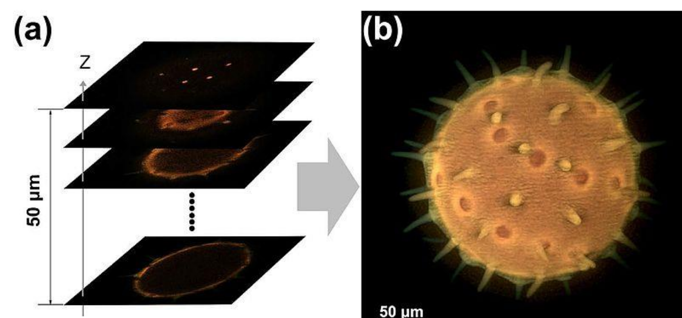


Fig. 2-8 Example of pollen grain optical sectioning [24]

2.4.2 Preparation of samples for acquisition aided by confocal microscopy

For the confocal microscopy acquisition, the stored sample organ is sectioned, processed and subjected to an IHC protocol, producing sample tissue with fixated structure, properties and marking of a specific proteins for further study and comparison with flow cytometry results.

Upon sectioning, the stored thymus is placed in an inset with medium and it is frozen in dry ice for 10 minutes at -80ºC. Once the tissue is ready, slices of 10 µm are sectioned from the organ using a cryostat and placed for drying at room temperature for 1 hour. Later on, the slices are treated with acetone by immersion for 15 min, which would fixate and permeabilize the sample tissue. The processes used in the preparation of the sample tissue are optimal for properly unaltered maintenance of antigens in the sample. After the fixation and permeabilization process, the sample tissue is subjected to drying for 1 hour and stored at -80ºC until the IHC staining process begins.

For the IHC staining process, the sample tissue is dehydrated with PBS 1x for 2 minutes and subjected to blocking process with human IgG as a blocking antibody for 5 minutes at room temperature in a humidity chamber. Blocking process will prevent undesired binding of our antibodies, as antigens presented by APCs are bounded to IgG (Fc blocking). A negative control is also processed and subjected to blocking, which will ensure the reliability of the results. Several antibodies are used in the protocol although FOXP3-PE has lower performance when compared with others in IHC staining. Therefore, a secondary antibody with a fluorophore with similar wavelength emission will be added to re-dye the antigens marked with our primary antibody FOXP3-PE.

Sample tissue is incubated with primary antibodies for 1 hour at room temperature in a humidity chamber. The volume of antibodies added to each slice should be between 1-5 µg/µl so proper dilutions with PBS 1x are prepared beforehand. The primary antibodies used in the IHC staining process are FOXP3-PE rat (eBioscience), CD4-APC Mouse (Beckman Coulter) and CD8-FITC Mouse (Beckman Coulter). Following the incubation with primary antibodies, sample tissue is washed by immersion in PBS 1x for 5 minutes, with a slight shake to ensure the cleaning of antibodies before the secondary antibody incubation.

The secondary antibody is added to the sample tissue and negative control, for incubation for 1 hour at room temperature in a humidity chamber. The negative control using secondary antibodies will ensure that there are no false positives if there is no signal detecting in its acquisition using confocal microscopy. The secondary antibody used in the IHC staining process is Anti-rat Cy3 Donkey (Jackson Immunoresearch).

Finally, sample tissue and negative control are washed by immersion in PBS 1x for 5 minutes before the addition of DAPI (Sigma-Aldrich) for cell staining of nucleic acids in a dilution of 1:10000 using PBS 1x as a solvent. The sample tissue and negative control are dried out for 5 minutes before mounting using Dako Fluorescence Mounting Medium. Both sample tissue and control are prepared for acquisition of images using confocal microscopy.

2.4.3 Acquisition, processing and analysis of images obtained from confocal microscopy

Image acquisition is performed using a confocal fluorescent microscope Leica TCS SPE (Leica Microsystems), which is manipulated with the supervision of a qualified technician. Images are stored in LIF format for processing and analysis using ImageJ [25], which is a digital image processing software.

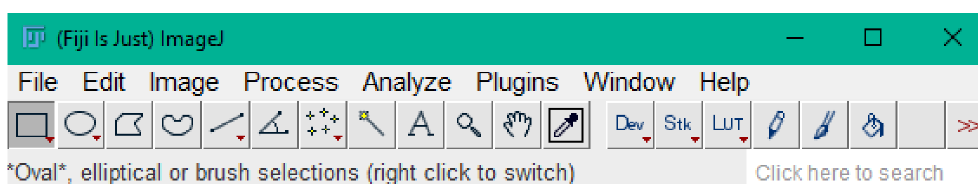


Fig. 2-9 ImageJ interface menu

When images are visualized using ImageJ, it can be seen that, although the sample is treated to avoid unspecific signals, there are background signals which should be minimized using image processing. Therefore, negative control images can be used for determining the minimum value from which modifications to brightness and contrast minimize the unspecific signal, using the marked bar at the figure (Image – Adjust – Brightness/Contrast). Once the values are determined, brightness and contrast of sample images are modified and smoothhing of the image noise is applied for resolution improvement. (Process – Smooth).

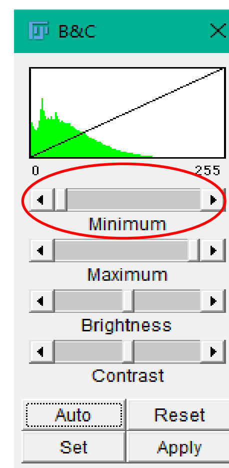


Fig. 2-10 Brightness and Contrast tool interface

For the analysis of images, a region of interest can be selected using the left click of the mouse on the predefined shapes. In addition, arragement of colour channels can be done for better interpretation of the results.

Two modification are used in the analysis:

- From Image – Color – Arrange Channels, those channels can be ordered as needed, as well as colour changed in the LUT of images for further detailed analysis.
- From Image – Color – Channels Tool, different representation of the colour channels are available, from composite to individual colour channels.

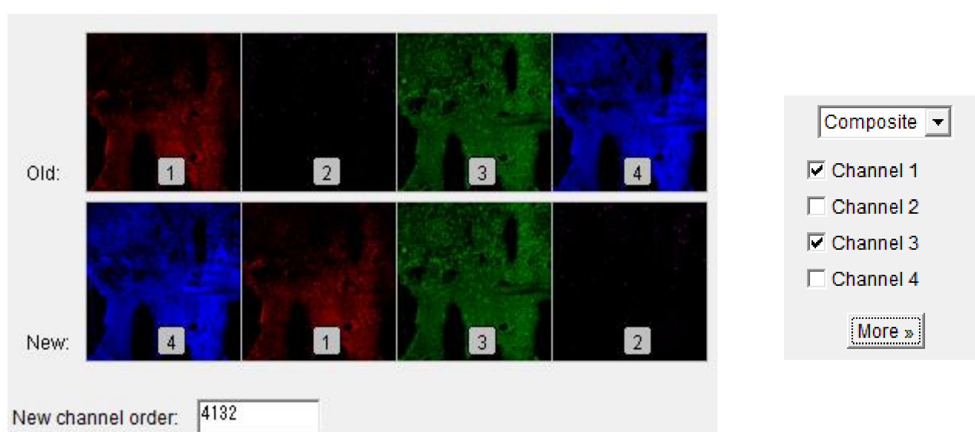


Fig. 2-11 Arrange Channels tool interface (left) and Channels Tool interface (right). Channel 1 in far-red was changed to red and Channel 2 was change to magenta from red, in addition to modification in the visualization of channels in composite, for better interpretation of results

Lastly, specific parts of the image can be zoomed in as well as saving a specific regions of interest of the images as needed (Image – Zoom and Image – Overlay – Add Selection, respectively).

2.4.4 Cell counting and CD4-CD8 colocalization analysis from confocal microscopy images using ImageJ

For the analysis of the cell population and its distribution, ImageJ offers a plugin which allows for the manual counting of the cells expressing specific patterns of factors of interest. Cell counting [26] is performed by "Cell Counter" plugin (Plugins – Analyse – Cell Counter).

It is possible to configure the different counters, establishing the features as needed, counting the cells by left clicking on each of those from the same population. Moreover, results are obtained in a format compatible with Microsoft Office Excel and the markers for each cell counter group can be saved in a file for further analysis.

Lastly, colocalization analysis is performed between CD4 and CD8 colour channels for detailed identification of DP thymocytes. Colocalization is defined as the presence of signal from two different colour channels at the same location, so the point under study has expression of both factors considered.

Colocalization analysis of the results is based on identification and study of pixel intensity and the correlation between both channels, therefore it is dependent on Manders Coefficients and the Costes approach [27], [28]. Manders Coefficients are proportional to the fluorescent signal detected in each colour channel, ranged from 0 to 1, expressing the amount of intensity of one of the channels in the other and vice versa.

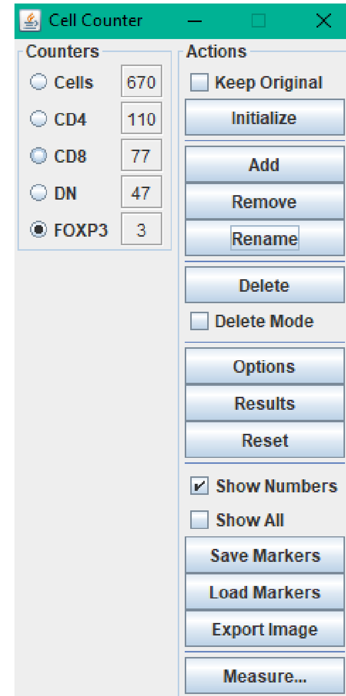


Fig. 2-12 Cell Counter tool interface

In addition, Costes approach evaluates the results obtained from Manders Coefficient using statistics to determine the significance of the results, when compared to mere coincidence. Simple evaluation of the intensity correlation is not considered to be a proper method for colocalization analysis as the human eye tends to modify the visual signals detected, providing deceiving information to the brain.

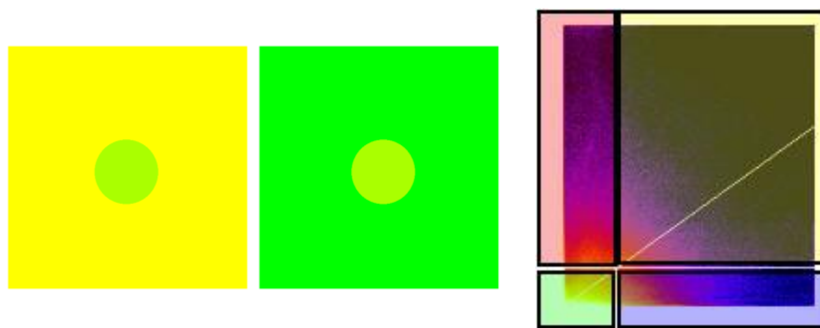


Fig. 2-13 Optical illusion (left). To the human eye, it seems that both circles are of different colours, although this change comes from the colours of the surroundings, as they have the same pixel value [26]. Scatterplot (right), from which it can be seen the linear correlation between channels (diagonal) and the thresholds for each of the channels separating the quadrants [26].

Therefore, analysis of a scatterplot, which represents the pixel values from each colour channels against one other, is the preferred method for the colocalization analysis of the results. For the analysis of the colocalization, Coloc 2 from ImageJ is used as it is optimized for the proper identfication of signifcant results, based on Mandes Coefficients and Costes approacch. Images are processed as previously stated, minimizing as possible the background noise, since the plugin has high sensitivity to background. A threshold is determined for each colour channel upon identification of the intensity correlation, thus providing significant results based on Costes approach. Those pixel bellow threshold are not considered in the analysis.

Looking at the scatterplot, points located in the red quadrant have values from the second channel above the threshold whereas values from the first channel are not above the threhold associated. Analogally, points located at the blue quadrant have values from the first channel above the threshold whereas values from the second channel are not above the threshold associated. The green quadrant represents the values below both threshold whereas the yellow quadrant is composed of those values above both thresholds.

From the results, the percentage of image volume colocalizing is provided as well as the number of colocalized voxels. These results are used for the analysis of the colocalization of CD4 and CD8 in the images obtained by confocal microscopy in addition to Manders Coefficients and the scatterplot.

3. RESULTS

3.1 Flow cytometry results

Flow cytometry was performed following two preestablished panels for cell separation into relevant populations:

- THY Linfos Thymocytes: Analysis focused on differentiation of cell population and other features of lymphocytes
- THY FOXP3 Thymocytes: Specific panel for the analysis of FOXP3 expression in thymocytes

3.1.1 Cell viability and identification of main populations

It is to be noticed that cell populations from the thymus are different when compared with other populations such as blood. It is ensured that almost no granulocytes, macrophages and monocytes are present in the substrate, as it is demonstrated from the analysis comparing FSC and SSC parameters. The majority of the cells identified were classified as lymphocytes (low-medium size and low complexity) although there is a group of cells corresponding to debris (low size and complexity). From the previous analysis, it is obtained a 99.46 % cell viability from the thymocytes using Pacific Blue dye expression.

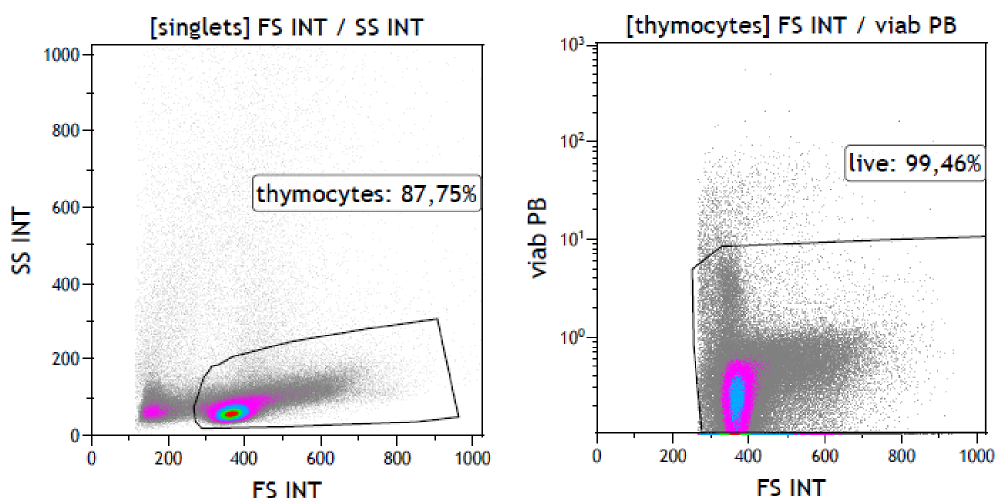


Fig. 3-1 Dotplot representation of the percentage of thymocytes present in the tissue (left). Dotplot representation of the viable thymocytes present in the tissue (right)

The pattern of expression obtained from CD4 and CD8 analysis provides distinction from four significant cell populations found in the thymus: Double

Positives (DP), Double Negatives (DN), CD4+ Single Positives (CD4 SP) and CD8+ Single Positives (CD8 SP).

Cells expressing both CD4 and CD8 (DP) account for the majority of cell encountered in the human thymus, followed by cells only expressing CD4 (CD4 SP) and those only expressing CD8 (CD8 SP), with a small difference in the number between this two groups. Lastly, low percentage of cells without expression of either CD4, CD8 or both (DN) is found.

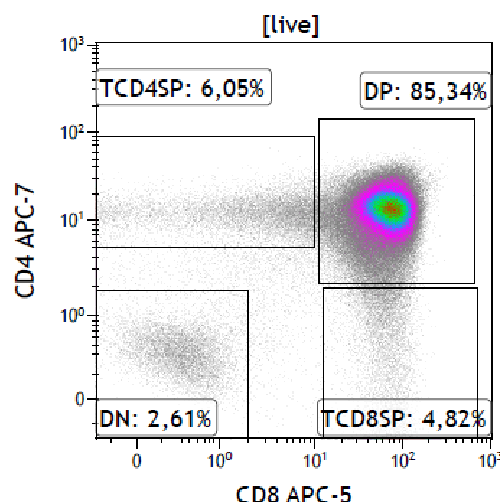


Fig. 3-2 Dotplot representation of the four major cell populations found in the thymus

3.1.2 CD3 and CD45 characterization of cell populations

CD3 expression is subjected to a progressive increase in its presence when comparing cells located inside of the thymus and lymphocytes located at the periphery (blood), CD3 serving as a marker for maturation of lymphocytes. The analysis is performed under the same gate of values, marked in the figures.

DP and DN thymocytes are expected to express low values of CD3 as they are in an early state of their development and maturation, as opposed to CD8 and CD4 SP thymocytes, which are at more advanced states when compared to precursors DP and DN thymocytes, continuing too express greater amounts of CD3 until achieving those values characteristic of the peripheral lymphocytes.

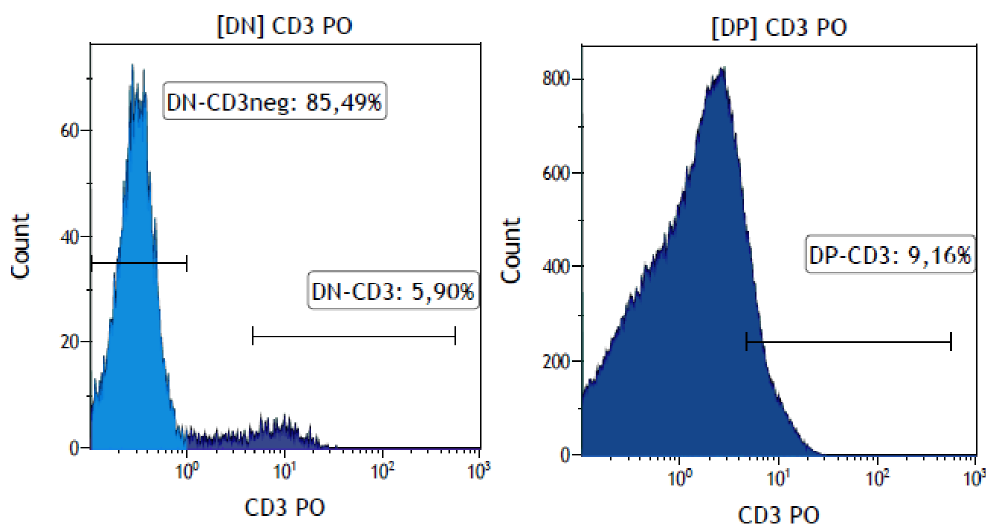


Fig. 3-3 Cell counting of DN thymocytes (left) and DP thymocytes (right) depending on their expression of CD3.

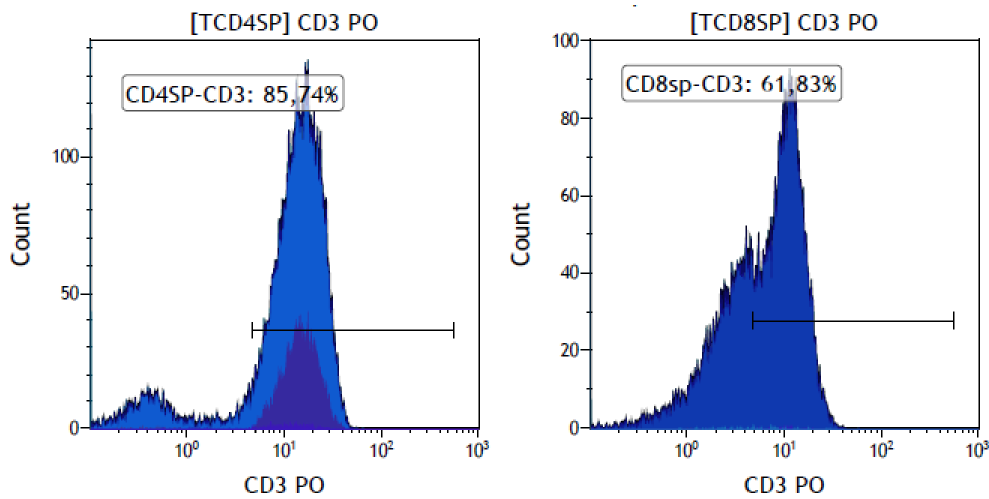


Fig. 3-4 Cell counting of CD4 SP thymocytes (left) and CD8 SP thymocytes (right) depending on their expression of CD3.

DN thymocytes have the lowest expression of CD3, with a slight increase when compared to DP thymocytes, whereas CD4 SP thymocytes have the greatest CD3 expression, being CD3 expression of CD8 SP thymocytes slightly below. In addition, thymocyte characterization is ascertain by their expression of CD45, which is complete by the separation in terms of their capacity of retaining information from previous encounters with allergens or pathogens from expression of one of the main two isoforms of CD45, CD45RA and CD45RO.

Surprisingly, only DN thymocytes have a greater expression of CD45RA than CD45RO, which is consistent with their naïve state, at early stages of development.

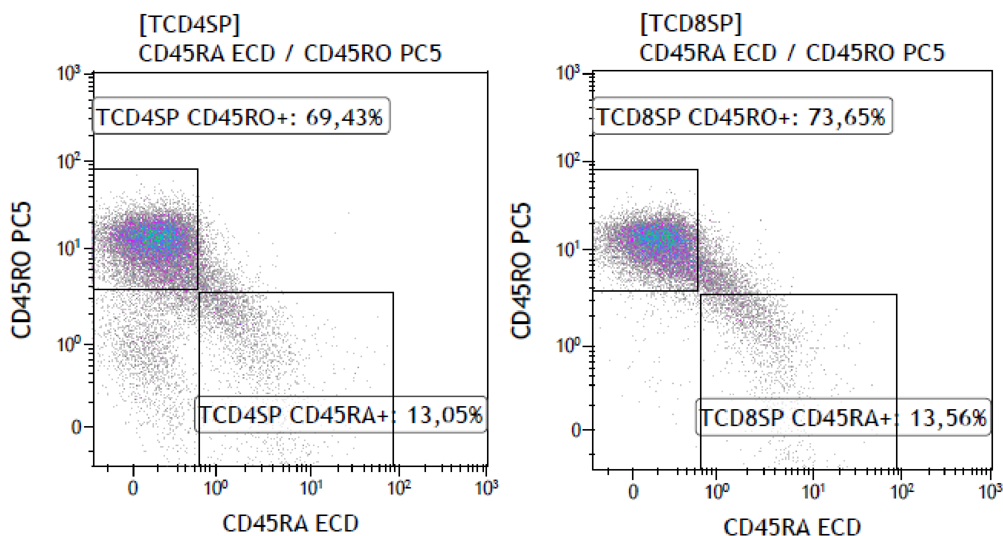


Fig. 3-5 Dotplot representation of CD4 SP thymocytes (left) and CD8 SP thymocytes (right) classified as naïve or memory thymocytes, depending on expression of CD45RA and CD45RO

The DP, CD4 SP and CD8 SP thymocytes significant expression of CD45RO, with low values of CD45RA expression suggest that these thymocytes have memory over previous encounters, although they do not have exited the thymus previously, which could be indicative of the ineffective analysis of CD45RO and CD45RA ECD antibodies for thymocytes prior to their release into the periphery, which is opposed to the high reliability of the characterization for peripheral lymphocytes.

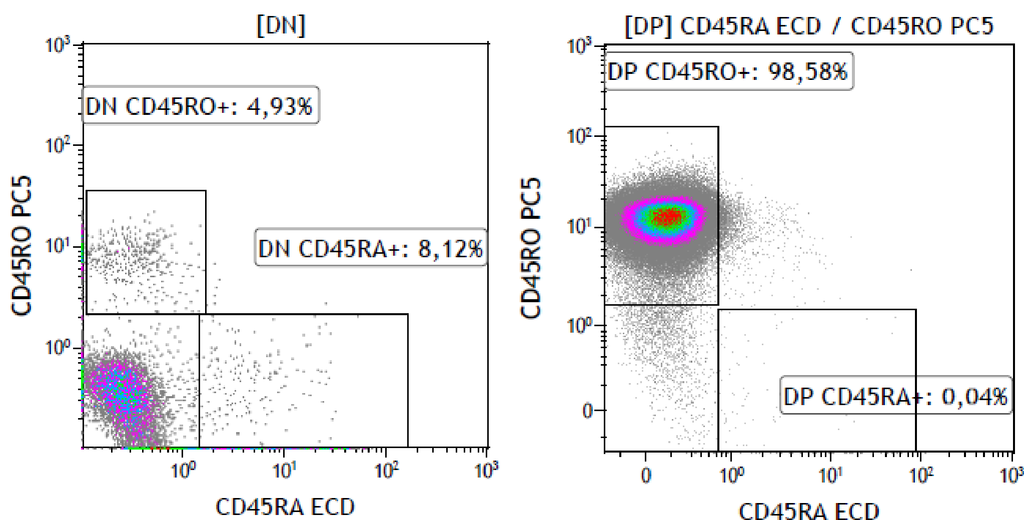


Fig. 3-6 Dotplot representation of DN thymocytes (left) and DP thymocytes (right) classified as naïve or memory thymocytes, depending on expression of CD45RA and CD45RO

Results from DN and DP thymocytes are quite interesting for analysis as DN thymocytes do not express neither CD45RA nor CD45RO in high amounts, which is completely opposite of what it is obtained from DP thymocytes, characterized by expression of CD45RO over the majority of its cell population.

3.1.3 Characterization of active cell populations by HLA-DR expression

Activation of immune cells at the periphery is tipically identified by analysis of HLA-DR expression, highly expressed in activated cells as a consequence of their maturation and development of MHC complex structure. All cell populations under study have low-medium values of HLA-DR expression as well as low values of CD45RA, thus showing no activation features of such cell populations, consistent with their absence of naïve characterization.

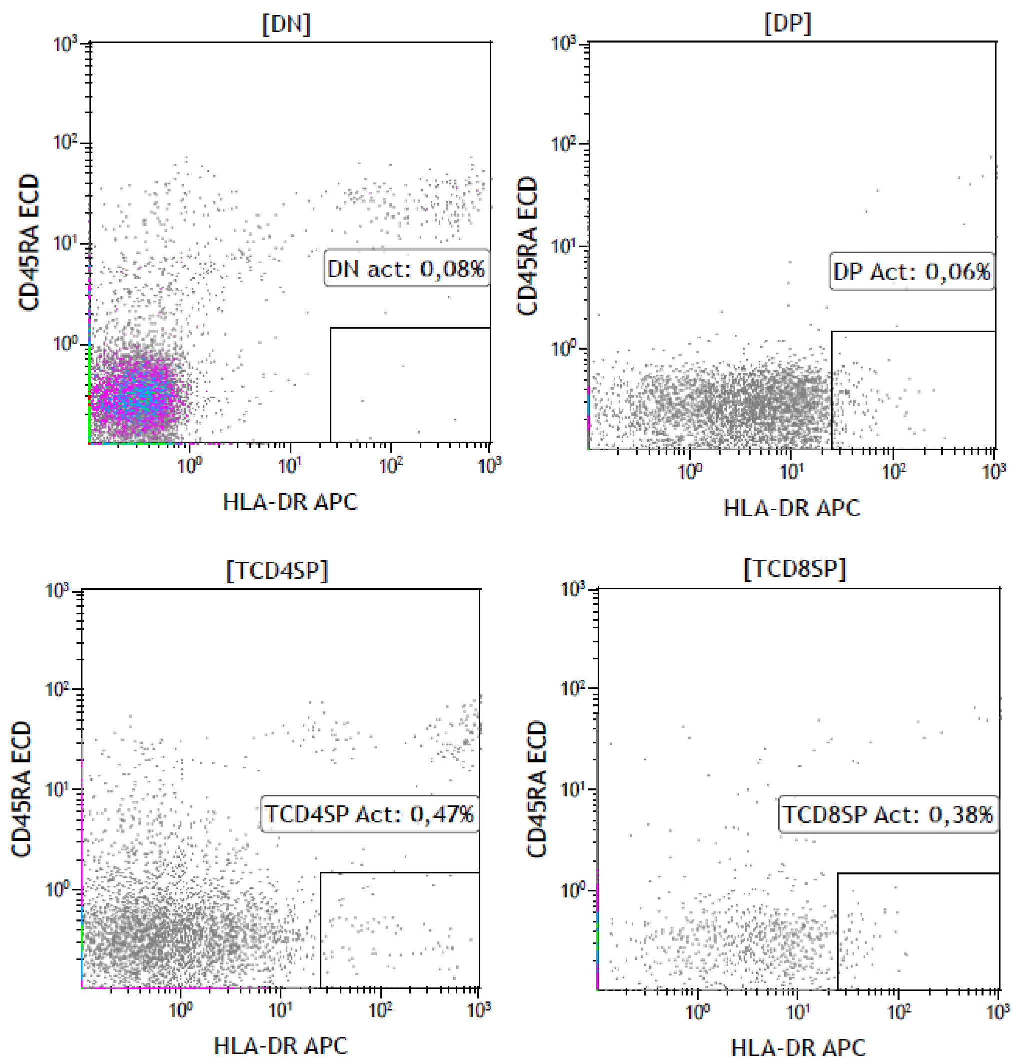


Fig. 3-7 Dotplot representation of DN thymocytes (top-left), DP thymocytes (top-right), CD4 SP thymocytes (bottom-left) and CD8 SP thymocytes (bottom-right) organized depending on their expression of activation factor HLA-DR

When comparing the results, DN thymocytes almost do not have activation traits in its cell population, with low values of HLA-DR, whereas DP, CD4 SP and CD8 SP thymocytes express slightly greater amounts of HLA-DR, notably showing more activation traits in CD4 SP and CD8 SP thymocytes, consistent with what was expected in terms of developmental state of cell populations.

3.1.4 Identification of Treg cell population derived from expression of CD25 and CD127

Treg cell population is characterized by expression of FOXP3, although they also can be identified by analysing the patterning of expression of CD25 and CD127.

Treg cells are characterized by high expression of CD25 whereas expressing low amounts of CD127. Comparing the results obtained with other experiment conducted previously, the total amount of Treg cell population and those who are specifically from CD4 SP cell population is consistent, with values up to 7% in some thymus analysed.

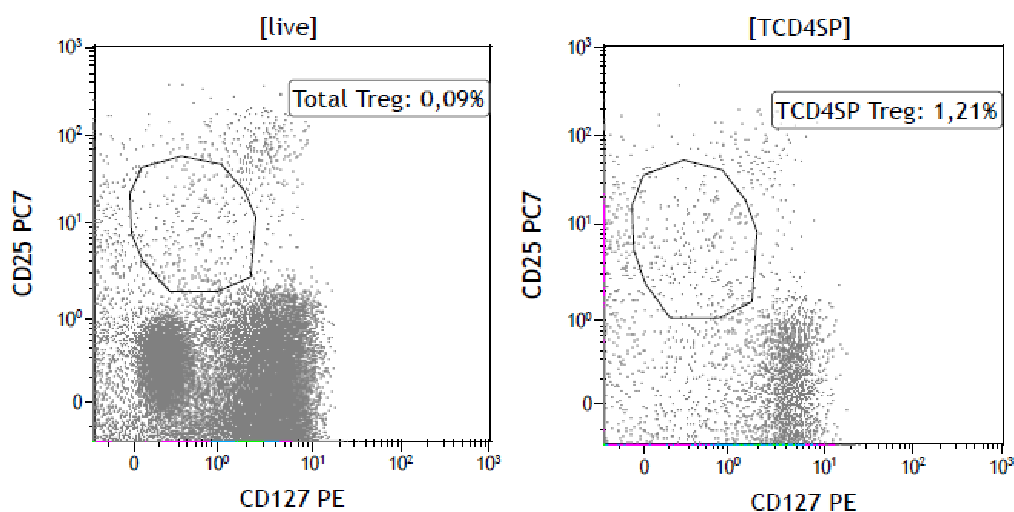


Fig. 3-8 Dotplot representation of total thymocytes (left) and CD4 SP thymocytes (right), with respect to the relation between expression of CD25 and CD127, as a classification to these cells as Treg cell population

3.1.5 Identification and classification of Treg cell population derived from FOXP3 expression

As it is stated before, expression of FOXP3 intracellularly is analysed for the identification of Treg cell population, which in turn can be classified in the four major groups of thymocytes. Treg cell population can be identified by the analysis of live thymocytes and their respective significant groups patterning of high FOXP3 expression, as well as their CD25 expression, as it was clarified in the previous analysis.

The amount of Treg cells found in the four major groups of thymocytes as well as in total is consistent with previous results obtained.

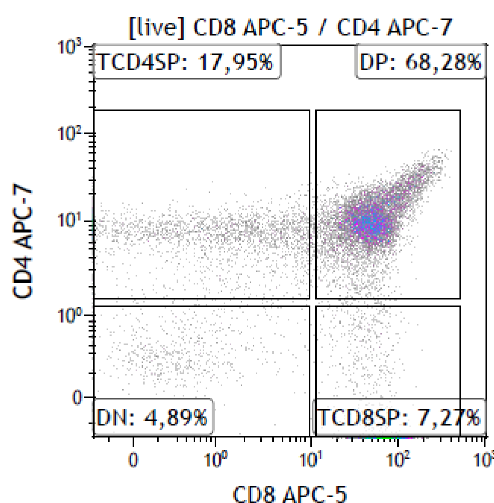


Fig. 3-9 Dotplot representation of the four major cell populations found in Treg cell population

After identification of Treg cell population in DN, DP, CD4 SP and CD8 SP cell populations another analysis is performed by identification of patterning of CD4 and CD8 expression from thymocytes expressing FOXP3.

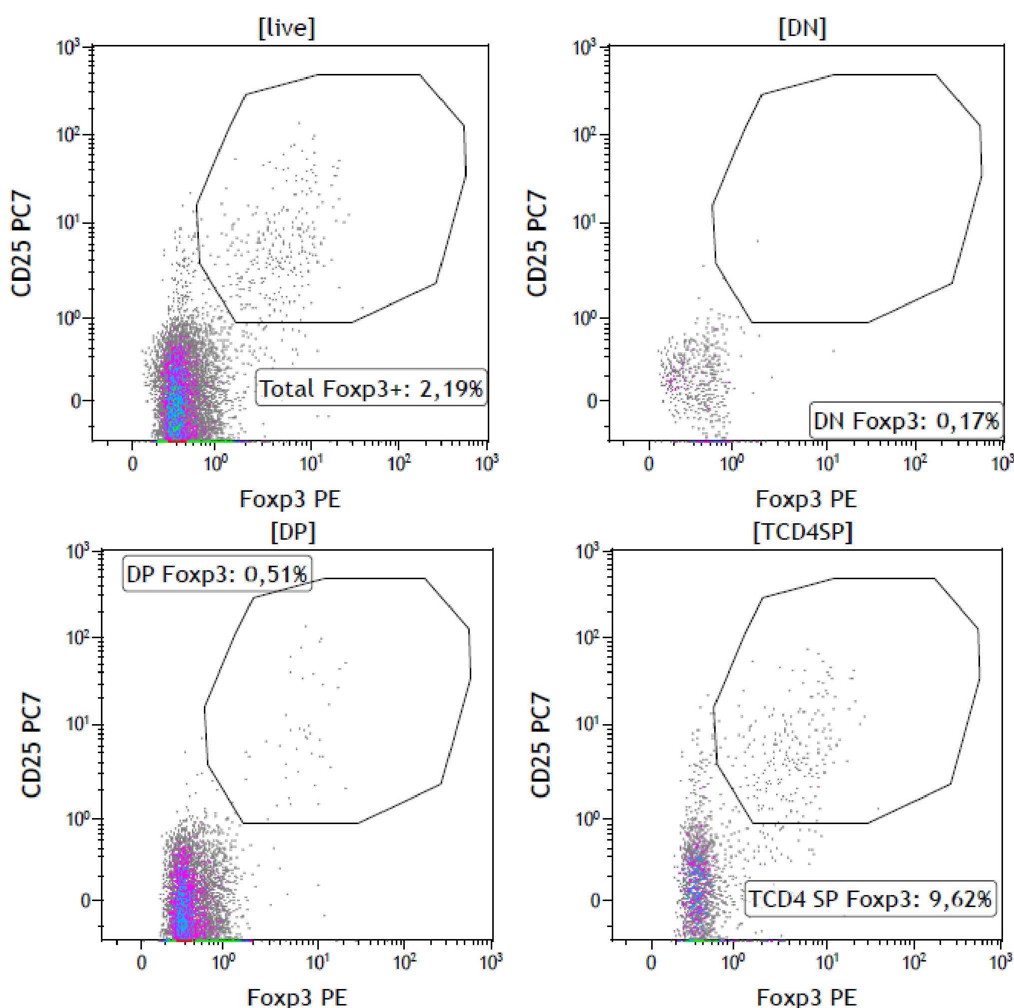


Fig. 3-10 Dotplot representation of live thymocytes (top-left), DN thymocytes (top-right), DP thymocytes (bottom-left) and CD4 SP thymocytes (bottom-right) classified as Treg cell population when expressing high amounts of FOXP3

Considering both analysis, CD4 SP thymocytes have a greater expression of FOXP3 than the rest of thymocytes, thus showing clear relation between CD4SP and Treg cell populations. DP thymocytes express FOXP3 in a lower amount given their precursor state, whereas CD8 SP and DN thymocytes have low expression of FOXP3.

Ensuring the results, Treg cell population greatly express CD4, with smaller percentage of such population expressing CD4 and CD8. Lastly, almost no Treg cell populations express either CD8 or do not express any cluster of differentiation.

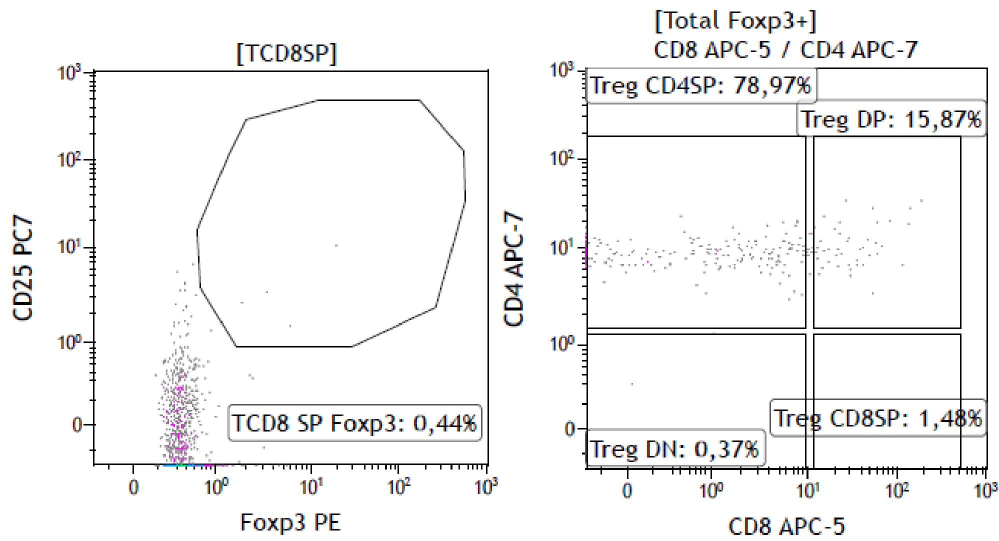


Fig. 3-11 Dotplot representation of CD8 SP thymocytes, classified as Treg cell population when expressing high amounts of FOXP3 (left) and dotplot representation of Treg cell population expressing FOXP3 organized in the four major groups of thymocytes (right)

3.1.6 Charactherization of Treg FOXP3+ cell population by expression of HLA-DR, CTLA-4 and CD39

As for other cell populations, expression of HLA-DR in Treg cells is observed in such cells showing activation traits, with absence of CD45RA, which determines the progressive activation and mature behaviour of those T cells. In turn, there is a percentage of Treg cells which are in a naïve state, as they are expressing CD45RA. However, results do not fit with respect to other studies performed by analysis of Treg cell population at the periphery, expressing high amount of CD45RA.

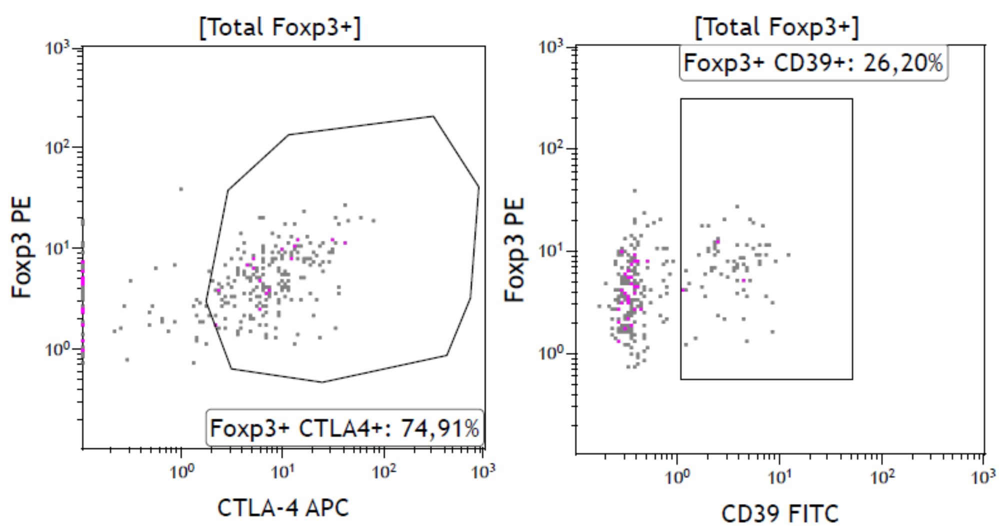


Fig. 3-12 Dotplot representation of Treg cell population FOXP3+ expressing other factors of interest, as CTLA-4 (left) and CD39 (right)

FOXP3+ Treg have specific markers for both its proliferation pathway as well as its activation against abnormal immune responses, as it is stated by the presence of subsets expressing CTLA4 and CD39.

The results are consistent with the data from other studies, as the Treg cell population is strongly in charge of suppressing CD8 T cells and maintaining of tolerance and homeostasis.

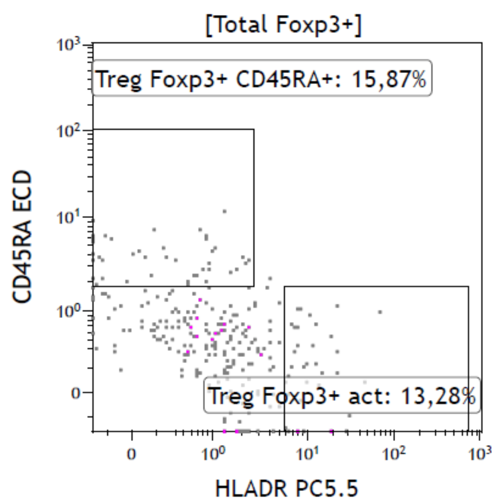


Fig. 3-13 Dotplot representation of Treg cell population FOXP3+ expressing behaviour factor CD45RA and activation factor HLA-DR

3.2 Images of paediatric human thymus obtained by confocal microscopy

Flow cytometry allows for accurately identifying and characterizing the different cell populations from our substrate of thymocytes, although there is no possible information regarding the structure and distribution as the analysis is performed in a sample obtained by mechanical lysis of thymic tissue.

Confocal microscopy and IHC fluorescent techniques could solve this problem, as the tissue is fixated and processed in such a manner that cells are more or less maintained in the original structure, which can be analysed following the pattern of fluorescent expression of specific markers for thymocytes.

As it is stated previously, images considered in the analysis are extracted from the same sample, although orientation is changed in order to study the thymocyte distribution.

3.2.1 Analysis of control images. Elimination of background and image processing.

Although the IHC protocol is optimized, there are unspecific interactions between antibodies and undesired targets, producing background noise which must be eliminated. Image processing of negative control images is performed, resulting in identification of minimum value to be settled in the analysis of subsequent images.

For the Thy670horiz control, minimum values for far-red, red and green channels are 15, 40 and 20, respectively, whereas minimum values for Thy670vert control are 25, 30 and 15. Lastly, images are smoothed using ImageJ process module

for better results. For a better understanding of the following results, a schematic representation of both sections of the sample tissue can be represent along with an image acquired using confocal microscopy.

As it has been explained previously, both sections are obtained from the same portion although the orientation is modified, thus obtaining different sliced from the thymic cortex and medulla.

DAPI visualization is quite useful for determining the differences in the structure between cortex and medulla, obtained from the slices to the sample tissue.

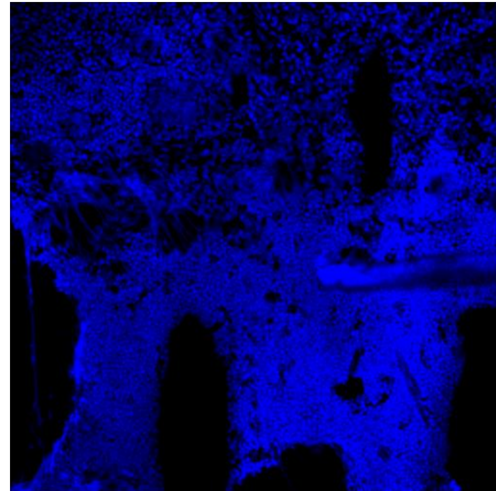


Fig. 3-19 DAPI visualization of medulla (top) and cortex (bottom) from vertical slice

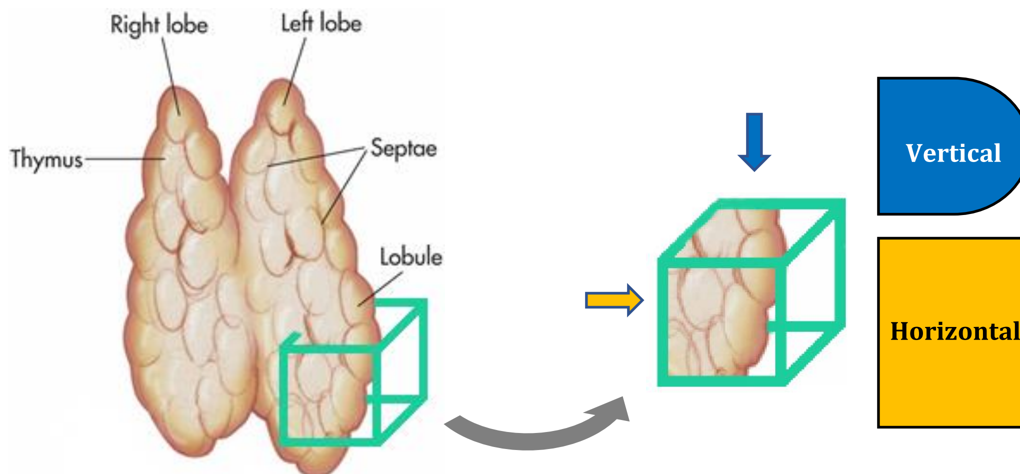


Fig. 3-20 Schematic representation of a human thymus (left) with the portion of the organ from which both slices were made (right). Vertical slices are associated with blue whereas horizontal ones are associated with orange.

3.2.2 THY670horiz series analysis

Four series were analysed following confocal microscopy acquisition. Images show clear and defined CD4 and CD8 patterns, at a surface level of thymocytes as well as high expression of FOXP3 intracellularly.

There is no clear separation between zones inside the thymus observed at Series001, although there are some areas with predominance of fluorescent signal emitted by CD4 or CD8.

Series002 is obtained by zooming at the left side of Series001. Analysis of FOXP3+ cells revealed that the majority of these are classified as DP thymocytes, which is consistent with the results obtained from flow cytometry.

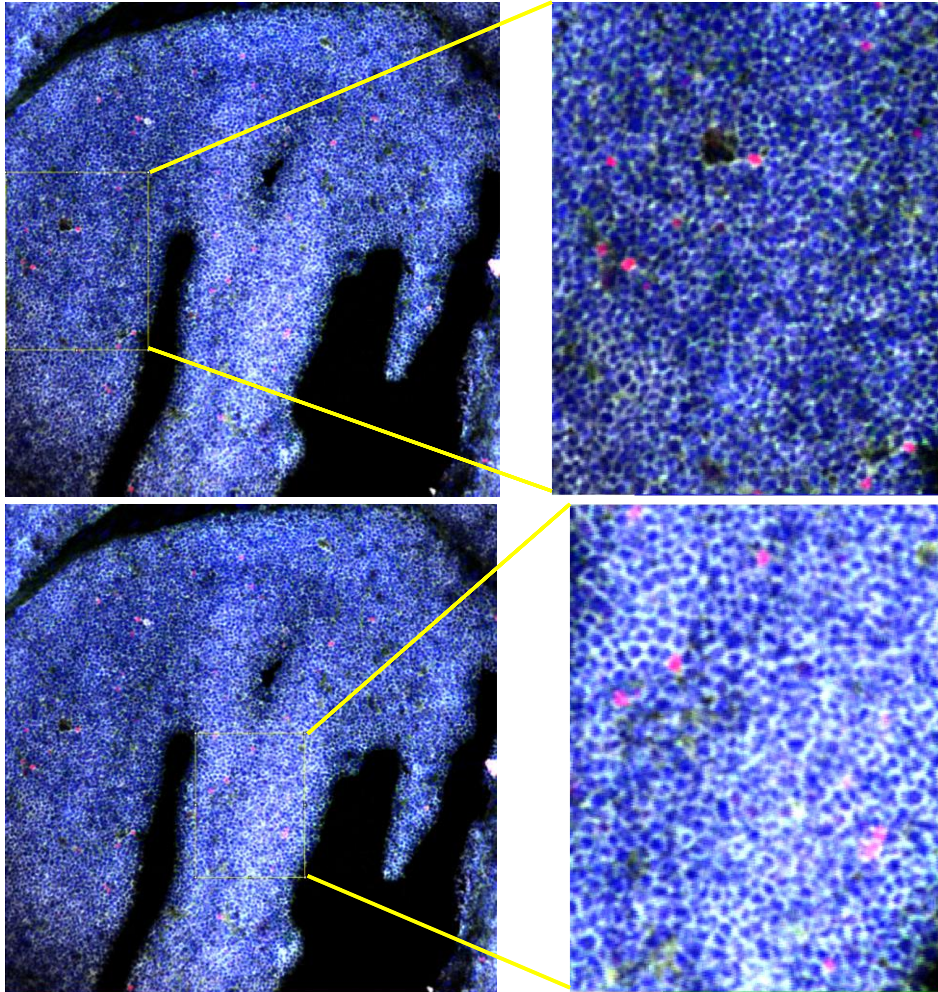


Fig. 3-21 Composite 10x image of Series001. Two different regions are zoomed for better visualization, being those corresponding to higher expression of FOXP3 (top) and high expression of overall factors (right)

There is a slightly bigger concentration of FOXP3 when comparing left and right side of the image. It is noticeable that the majority of the thymocytes observed are classified as DP thymocytes, with huge differences when compared with a low amount of other cell populations. LUTs values are modified so colocalization between CD4 (red) and CD8 (green) can be observed with a yellowish hue so identification of cell population distribution is easier.

As it is said previously, Series002 offers better view of thymocytes expressing FOXP3, from which two different groups could be extracted, depending on the intensity of the intracellular fluorescent signal.

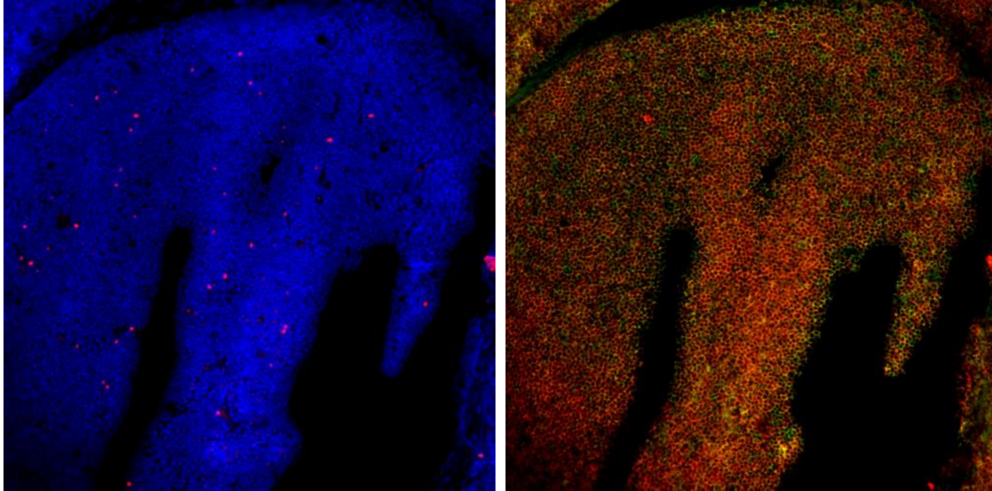


Fig. 3-22 DAPI+FOXP3 visualization of Series001 (left). Note the distribution of FOXP3+ cells, with a higher concentration of cells toward the left side of the sample. CD4+CD8 visualization of Series001 (right), with LUT modification of the CD8 colour channel from grey to red for better interpretation of results

Results from the cell counting with respect to FOXP3+ thymocytes, with differences between high and low expression of the transcription factor can be seen in the figure. A total number of 19 thymocytes are classified as FOXP3+, differencing between 11 with high expression and 9 with low expression of FOXP3.

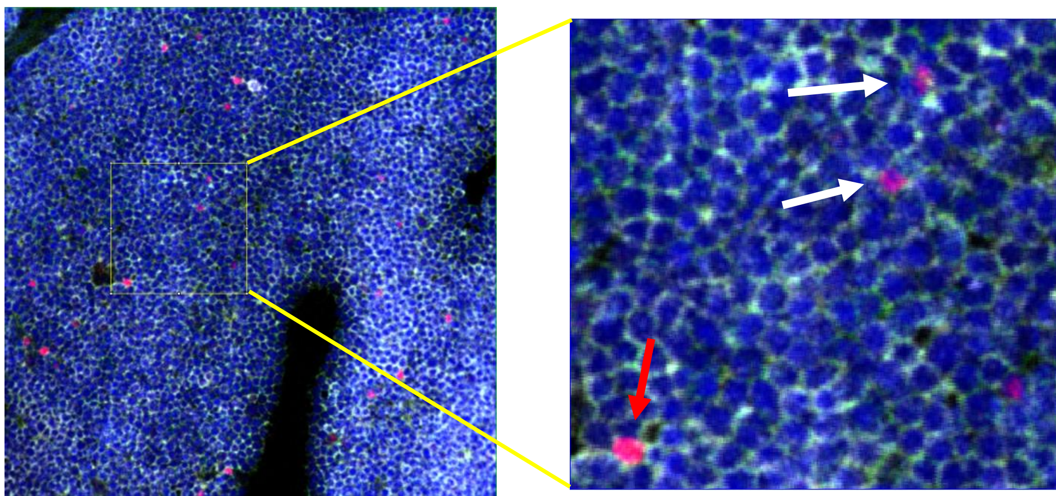


Fig. 3-23 Composite 20x image of Series002 (left). Detail of a section from which differences in the pattern of expression of FOXP3 can be observed (right), with high expression cell marked with a red arrow and low expression cells marked with a white one.

Zooming in on the selection in Series002 allows for a better observation of high expression of FOXP3 pattern, marked with a red arrow, opposite to low expression FOXP3 pattern, marked with white arrows.

For further analysis, a selected area from Series003 was subjected to cell counting for the determination of the major cell populations, as it shows better distribution of cells, with some areas characterized by presence of CD4 or CD8.

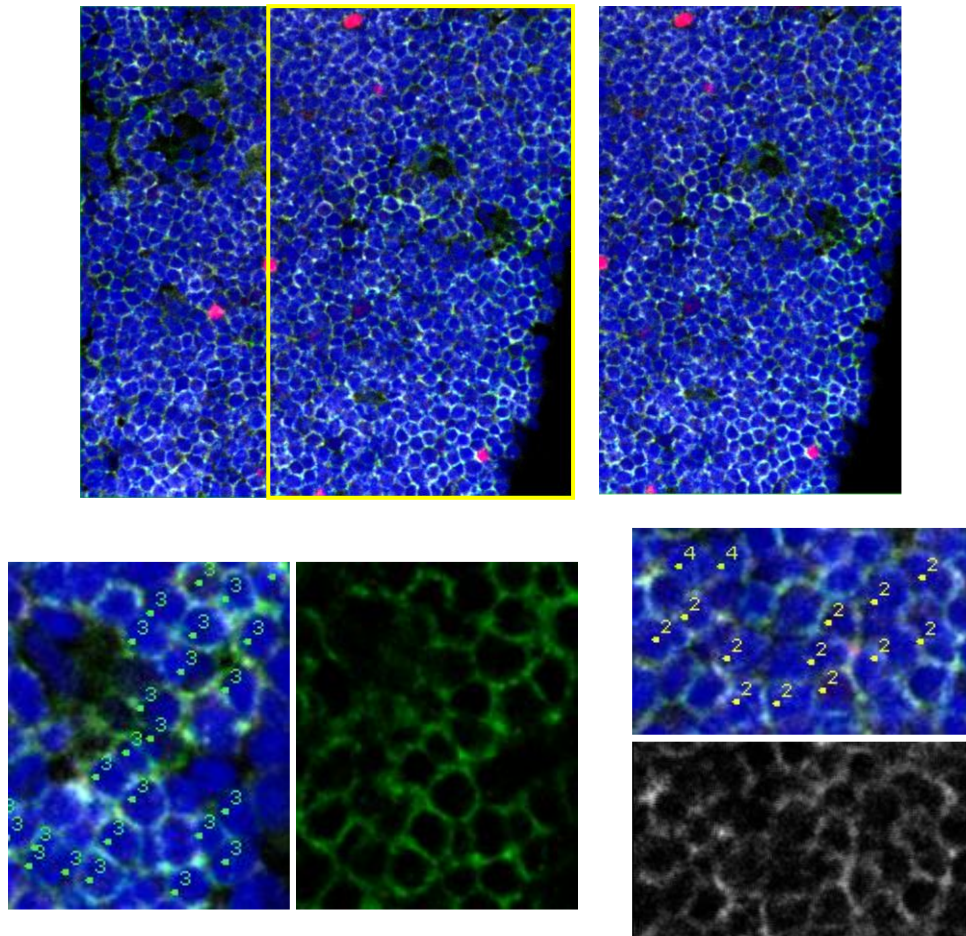


Fig. 3-24 Composite 10x image of Series003, with the marked region considered in the analysis (top). Detail of the CD8 SP thymocyte population in green (bottom-left) and CD4 SP thymocyte population in grey (bottom-right)

As it can be observed, CD4+ thymocytes are distributed evenly, contrary of what it is obtained from observation of CD8+ thymocytes, with a not so even distribution, without presence in some areas in the upper part of the image.

Cell counting was performed by classifying the cells expressing DAPI into different cell population, depending on their pattern expression of CD4 and CD8, similar to what is performed in flow cytometry analysis.

Cell population distribution observed in the image is similar to what was obtained from flow cytometry analysis, with similar amounts of thymocytes from CD4 SP and CD8 SP cell populations, with slight differences, low number from DN thymocytes and the majority of the cells group as DP thymocytes, obtained by simple deduction from the total number of cells and the cell population countered.

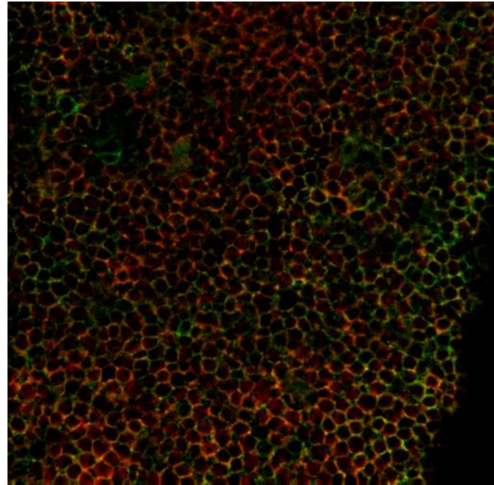


Fig. 3-25 CD4+CD8 visualization of Series003, with LUT modification of the CD8 colour channel from grey to red for better interpretation of results

Several thymocytes from different cell populations expressing FOXP3 are present in the image, being those marked with an orange arrow from CD4 SP thymocytes whereas the ones marked with a white arrow are from DP thymocytes.

It is to be noticed the presence of thymocytes with different patterns of FOXP3 expression, as it can be seen from DP thymocytes from the figure.

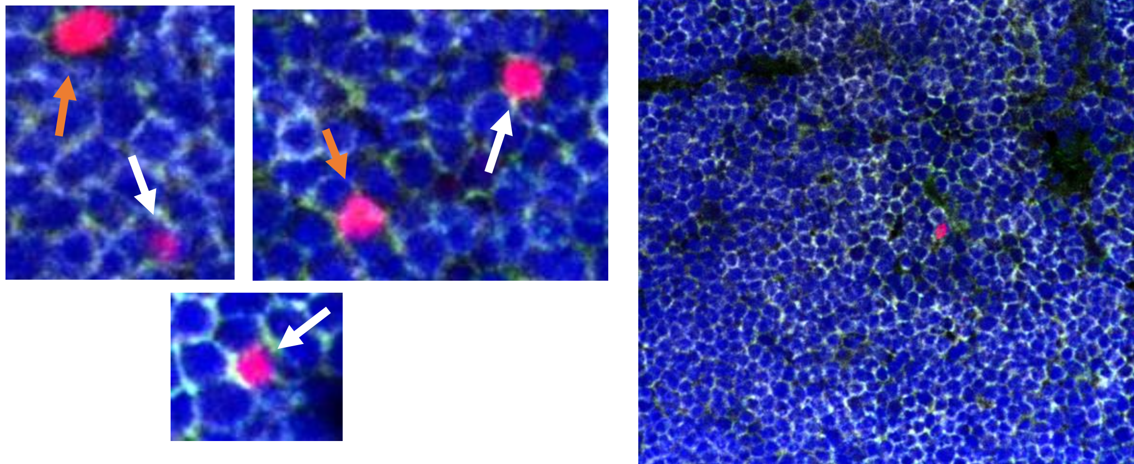


Fig. 3-26 Detail of FOXP3+ thymocytes classified as CD4 SP thymocytes (orange) and DP thymocytes (white), with some difference between FOXP3 pattern of expression (left). Composite 10x image of Series004 (right)

Lastly, Series004 show a similar distribution of cells when compared with previous results, although CD8 fluorescent signal is weaker than the one emitted by CD4+ thymocytes, with a clear difference at the upper-left part of the image.

Therefore, predominance of DP and CD4 SP thymocytes is ascertain from the observations up to some limit.

THY670horiz Series003	
Cells	670
CD4 SP	110
CD8 SP	77
DN	47
FOXP3	3

Fig. 3-27 Cell counting results from Series 003

3.2.3 THY670vert series analysis

For this samples, eight series are taken into consideration, although even ones are zoomed from areas present in odd images. Overall, images offer more results to be analysed, in which specific features can be taken under extensive study. As for the previous series, there are clear and defined patterns for CD4 and CD8 expression, as well as FOXP3, at the surface and inside the cell, respectively.

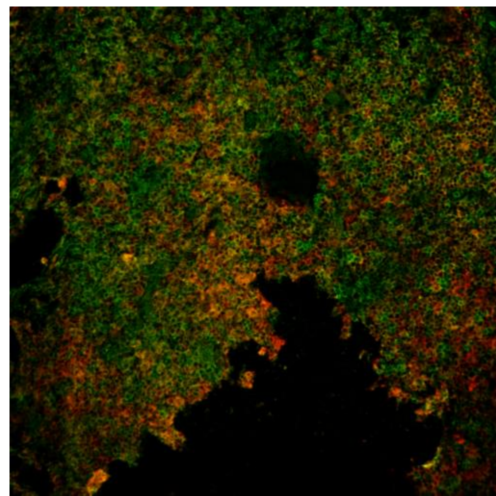
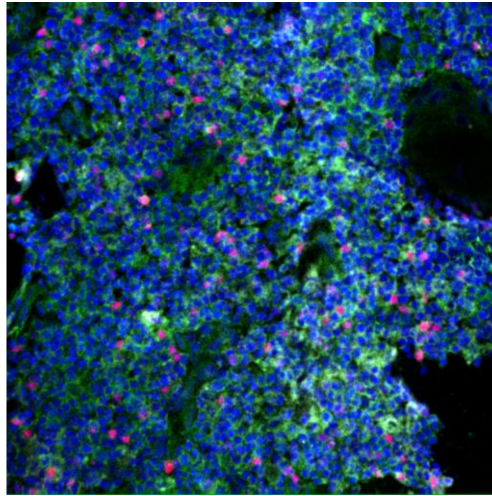


Fig. 3-28 CD4+CD8 visualization of Series001, with LUT modification of the CD8 colour channel from grey to red for better interpretation of results

Series001 and Series002 show normal distribution of cell population, with high percentage of thymocytes expressing FOXP3, related with CD4 SP and DP cell populations. FOXP3+ thymocytes are located slightly to the left part of the sample, with some differences between CD4 and CD8 pattern expression.

Results from cell counting are not quite consistent with previous analyses by flow cytometry and confocal microscopy, as there is a greater amount of CD8 SP than CD4 SP thymocytes, although percentages could be attributed to be similar of what it is to be expected, with the majority of cells expressing both CD4 and CD8, followed by single positives thymocytes. FOXP3+ thymocytes are classified as DP thymocytes at the majority, although this is extracted from a specific area of the sample tissue.

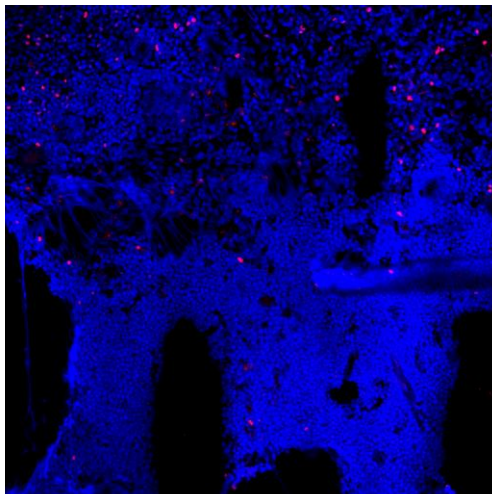
In addition, differences in the patterning of FOXP3 expression can also be seen in the image, locating high and low FOXP3+ expression. Lastly, DN thymocytes are found in resonance with our expectations.



THY670vert Series002	
Cells	1541
CD4 SP	43
CD8 SP	105
DN	15
FOXP3	105
FOXP3_H	61
FOXP3_L	44

Fig. 3-29 Composite 20x image of Series002 (left) and the results obtained from cell counting process of the same image (right)

Surprisingly, Series003 and Series004 seem to represent clear distinction between two areas with difference distribution of cell population, characterize by a vertical gradient of FOXP3+ thymocytes. Furthermore, CD4 SP thymocytes show bigger emission of fluorescent signal at the bottom part of the sample, with almost no signal found at the top, as well as a diffuse pattern of CD8 expression in this area.



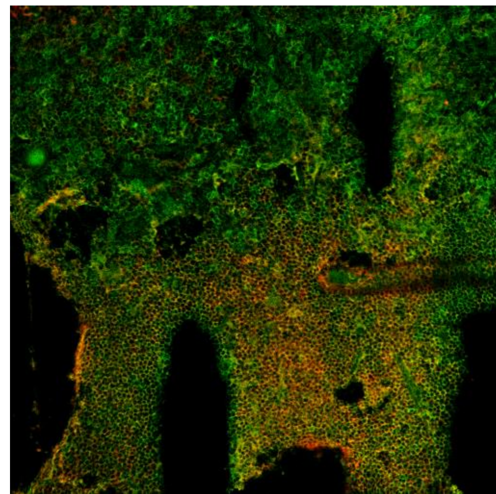
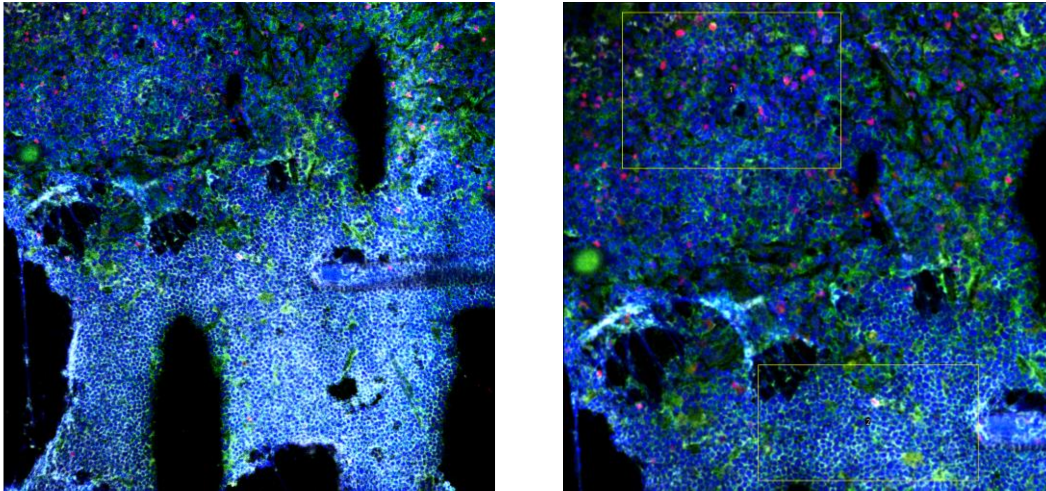


Fig. 3-30 DAPI+FOXP3 visualization of Series003 (left). Note the distribution of FOXP3+ cells, indicating differentiation between cortex and medulla. CD4+CD8 visualization of Series003 (right), with LUT modification of the CD8 colour channel from grey to red for better interpretation of results.

The amount of thymocytes classified into different cell populations is calculated over to regions of interest from Series004, representing the two areas of distribution. Results are consistent with respect to the previous series from THY670vert, although clear differences between singles positives are observed.

It is to be noticed that DP thymocytes are distributed evenly, being the ones found in the greatest amount, although it seems to be some distribution of DP thymocytes focus at the bottom of the sample, with CD4 SP thymocytes located at the same area that FOXP3+ cells.



THY670vert Series004 ROI1	
Cells	442
CD4 SP	11
CD8 SP	180
DN	8
FOXP3	29
FOXP3_H	16
FOXP3_L	13

THY670vert Series004 ROI2	
Cells	479
CD4 SP	3
CD8 SP	32
DN	0
FOXP3	1
FOXP3_H	1
FOXP3_L	0

Fig. 3-31 Composite 10x image of Series003 (top-left). Composite visualization of both two regions of interest considered, corresponding to medulla (ROI1) and cortex (ROI2) of Series004 (top-right). Cell counting results from ROI1 and ROI2 located at Series004 (bottom)

However, upon observation, FOXP3+ cells seem to be classified as CD8 SP thymocytes, which opposes our initial guest and the results previously obtained, which suggest the possibility of low expression of CD4 which was not considered in the classification, thus classifiying those cells as DP thymocytes, which is consistent.

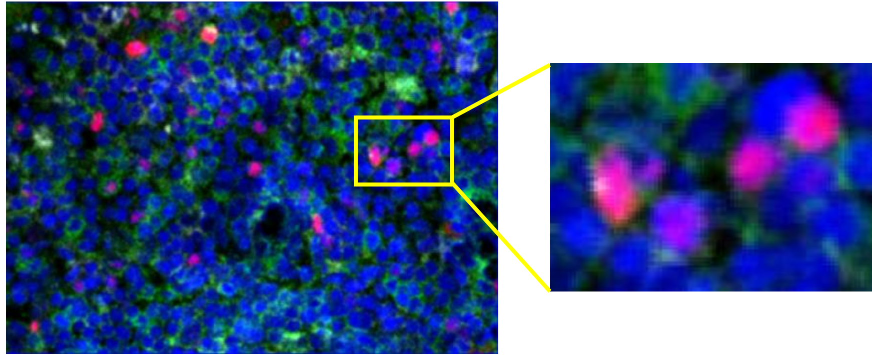


Fig. 3-32 Detail of ROI1 from Series004. Some of the FOXP3+ thymocytes have greater values of CD8 than CD4

Series005 clearly show the expression partern of FOXP3, which is charactherized for being intracellular, arranged in a dotted-like fashion, with distiction between high and low expression of the transcription factor.

From observations, thymocytes in which FOXP3 expression is present are classified as DP thymocytes. Moreover, the distribution and number of cells from each cell population follows the same tendecy that previous resuls, even considerating the disintegration of the sample tissue. However, fluorescent signal from CD4 expression is weaker than the one emitted by CD8, which difficults the charachterization of cells.

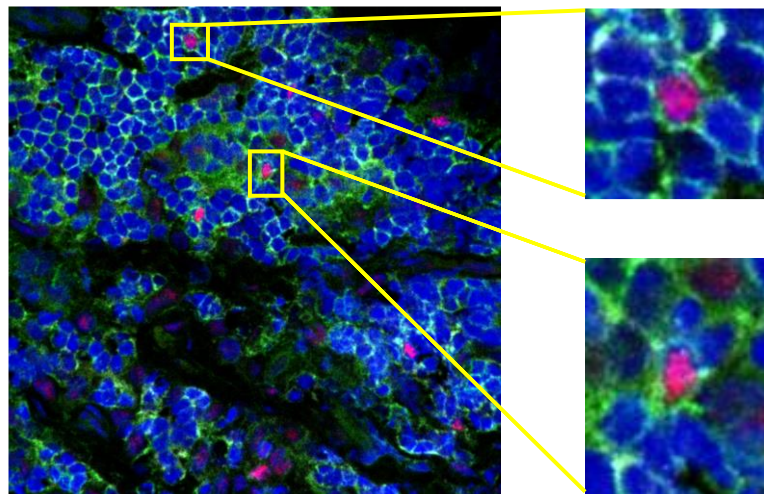


Fig. 3-33 Detail of FOXP3+ thymocytes from Series005. It is possible to observe the dotted pattern of expression of FOXP3 intracellularly, with high and low FOXP3 expression

Lastly, Series007 and Series008 have low resolution of DAPI expression with respect to other samples, which highly difficults the charactherization of the cell population and its distribution. All considered, the distribution of CD4 and CD8

expression is even along the image, with particularly clear expression of FOXP3. CD4 expression is stronger at the bottom part of the image with similar distribution when compared to other images of the sample tissue.

THY670vert Series005	
Cells	338
CD4 SP	1
CD8 SP	53
DN	7
FOXP3	7
FOXP3_H	4
FOXP3_L	3

THY670vert Series008	
Cells	141
CD4 SP	1
CD8 SP	37
DN	6
FOXP3	3

Fig. 3-34 Cell counting results from Series005 (left) and Series008 (right)

From Series007, distribution of thymocytes expressing FOXP3 can be calculated. Therefore, the total number of FOXP3+ thymocytes is 10, subdivided in high expression patterns which account for 6 thymocytes and the rest classified as low expression of FOXP3.

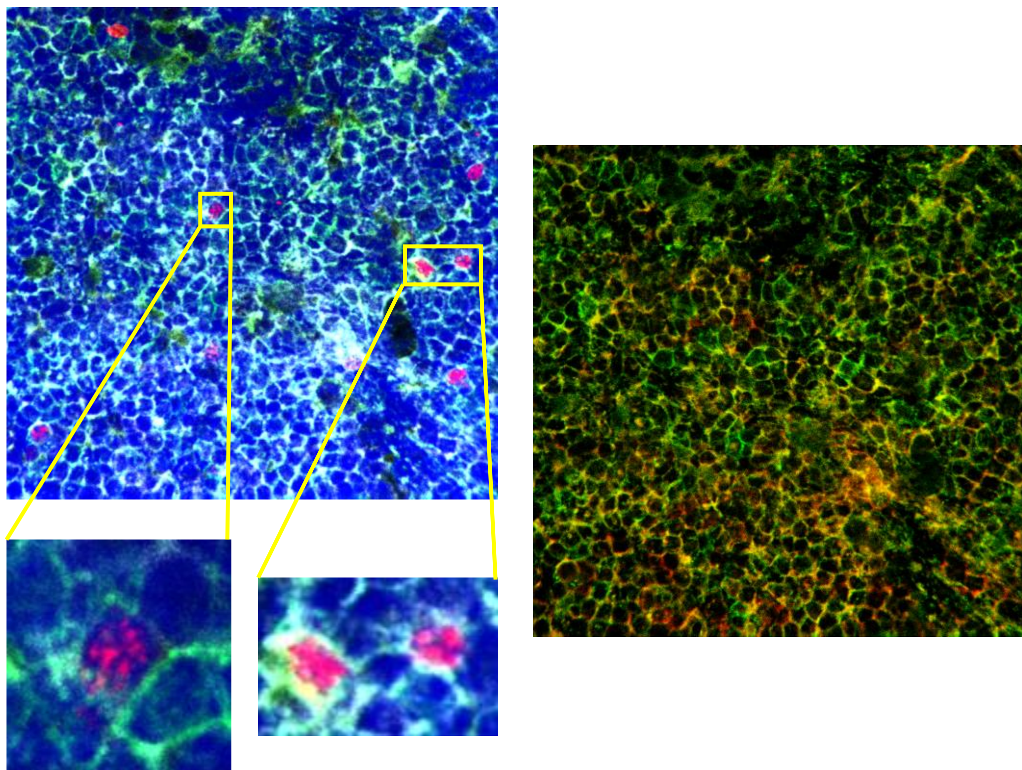


Fig. 3-35 Detail of FOXP3+ thymocytes from Series007. Note the low resolution of DAPI as well as the specific pattern of FOXP3 expression (left). CD4+CD8 visualization of Series007 (right), with LUT modification of the CD8 colour channel from grey to red for better interpretation of results.

3.2.4 CD4-CD8 colocalization analysis

Further analysis can be performed in order to ensure the similarity of the results from confocal microscopy and flow cytometry. Through analysis of the amount of specific signal for a channel found in another one we can calculate the colocalization between CD4 and CD8 pattern expression.

	Images	Rtotal	m	b	Ch1 thresh	Ch2 thresh	Rcoloc	R<threshold	M1	M2
Horiz_Series001	CD4 - CD8	0,625	2,296	8	6	22	0,4974	-0,007	**0,9962**	**0,8977**
Horiz_Series003	CD4 - CD8	0,561	1,804	0,5	11	20	0,4049	0	**0,9679**	**0,8473**
Vert_Series001	CD4 - CD8	0,547	1,38	-14,8	17	9	0,4325	0,009	**0,9233**	**0,9893**
Vert_Series003	CD4 - CD8	0,598	1,025	-23,4	30	7	0,4605	0,017	**0,8025**	**0,9993**
Vert_Series005	CD4 - CD8	0,637	0,352	-5,7	23	2	0,5147	-0,015	**0,6028**	**0,9988**
Vert_Series007	CD4 - CD8	0,726	1,043	-19,5	30	12	0,5978	0,029	**0,9047**	**0,9983**

	tM1	tM2	Ncoloc	%Volume	%Ch1 Vol	%Ch2 Vol	%Ch1 Int	%Ch2 Int	%Ch1 Int > thresh	%Ch2 Int >thresh
Horiz_Series001	0,9663	0,8528	553582	52,79%	90,93%	76,11%	95,38%	82,88%	96,09%	84,69%
Horiz_Series003	0,8136	0,6863	312786	29,83%	73,52%	60,78%	76,28%	63,85%	81,79%	70,97%
Vert_Series001	0,8563	0,9137	524523	50,02%	81,14%	85,09%	83,16%	89,80%	86,75%	90,89%
Vert_Series003	0,7412	0,9462	444575	42,40%	70,11%	86,79%	70,61%	93,44%	76,51%	94,18%
Vert_Series005	0,5847	0,9572	254364	24,26%	49,74%	85,76%	55,39%	94,85%	62,17%	95,04%
Vert_Series007	0,8291	0,9294	523610	49,94%	78,93%	85,00%	79,30%	91,36%	86,31%	92,90%

Fig. 3-36 CD4-CD8 colocalization results. CD4 expression is represented in Channel 1 whereas CD8 expression is represented in Channel 2.

M1 and M2 are the parameters defining the colocalization between channels of the image. Images which are taken into consideration are the ones offering the best view of the sample tissue, so reliability is improved.

4. DISCUSSION

4.1 Discussion over the results obtained from previous analysis

In this chapter, results over cell population's characterization and distribution obtained through flow cytometry and confocal microscopy will be compared with other analysis and experiments performed, in a detailed way; for determining the implications of the results, the possible solutions to some questions which are not answered yet and the different paths available for future research.

4.1.1 Flow cytometry

Overall, results obtained using flow cytometry are highly consistent with previous analyses of other sample tissues as well as essential states surrounding the ontogeny of T cells in a human pediatric thymus. The percentages of cell population obtained through flow cytometry classify thymocytes under four different cell populations, from DP thymocytes to DN thymocytes, along with CD4 SP and CD8 SP thymocytes, being consistent to previous knowledge with respect to cell populations, as they highly depend on positive and negative selection and T cell development [1].

Furthermore, an increase in the percentage of thymocytes expressing CD3 when comparing double positive to single positives is a consequence of the maturation process of T cells, as CD3 is an essential signalling molecule located in T cell receptor complex (TCR complex) [1].

As it is pointed out in the results, the expression of CD45RA and CD45RO do not match the paradigm established for these populations in peripheral blood, as markers of naïve and memory states, respectively. However, pattern of expression of these isoforms are studied over T cells from the periphery whereas thymocytes are the target from our experiment, which suggest that expression of CD45RA and CD45RO in thymocytes will have a different function or significance in the thymus environment. As it is commented previously, there is a relation between CD45RA and CD45RO with other proteins present in thymocytes, such as CD25 or HLA-DR, both expressed in thymocytes showing traits of activation, could lead to modification in the isoforms, resulting in expression of CD45RO in CD4 SP, CD8 SP and DP thymocytes, highly related to expression of activation factors in such cell populations. Moreover, the majority of Treg cells expressing FOXP3+ found at the periphery expressed high amounts of CD45RO, which could be explained with the results obtained from flow cytometry [12].

Lastly, maturation of T cells involving activation of thymocytes could be related to the increase in proliferation between CD45RO compared to CD45RA, thus showing a connection between activation traits, proliferation and CD45RA transition to CD45RO expression [7].

Similar to CD3 expression in thymocytes, HLA-DR is highly related to activation traits in thymic cells as it is quite relevant as a cell receptor from class II MHC, participating in processes involving cell-mediated immune responses, being this type of responses exclusive from T lymphocytes, derived from maturation of thymocytes. Therefore, it is not to be unexpected that CD4 SP and CD8 SP thymocyte cell populations have greater amounts of HLA-DR, showing the activation of thymocytes for further development into lymphocytes capable of participate in immune responses.

Characterization of Treg cell populations from specific patterns of expression of both CD127 and CD25 is highly reported in other studies, characterized by low expression of CD127, inversely proportional to FOXP3 expression [29], which is used as a common marker for Treg cells. Moreover, high expression of activation factor CD25 is to be expected in Treg cells as thymocytes located in an advanced state of maturation, are highly dependent of expression of FOXP3. [30]

As stated before, FOXP3+ thymocytes are characterized by high expression of CD25, thus serving its relation for the identification of Treg cell population. Furthermore, percentages of FOXP3+ thymocytes are consistent with previous studies and experiments, with high expression of FOXP3 in CD4 SP cell population, precursor of Treg periphery cell population [31], [32], although in this particular case, the sample thymus had lower amount of Treg cells when compared to other samples, which could be attributed to physical conditions inherent to the organ, such as the patient's condition or age, which are important factors affecting the maturation of T cells [33]. Therefore, it is established the relation between CD25, CD4 and FOXP3 patterns of expression which characterize Treg cell population and its suppressor activity [32]. Later on, images obtained using confocal microscopy could be of great help to understand the maturation process of T cells, its relation with FOXP3 and development of thymocytes into Treg cell population.

Surprisingly, flow cytometry analysis of expression patterns of both CD25 and FOXP3 elucidate slight differences between two independent populations of thymocytes expressing FOXP3, classified as Low and High FOXP3, as previously reported [34]. Differences between patter of FOXP3 expression are clearly observed in analysis from images obtained using confocal microscopy, which could be proof of the existence of subpopulations between FOXP+ thymocytes with different features and cell fates in the Treg cell and other cell lineages in the thymus [34].

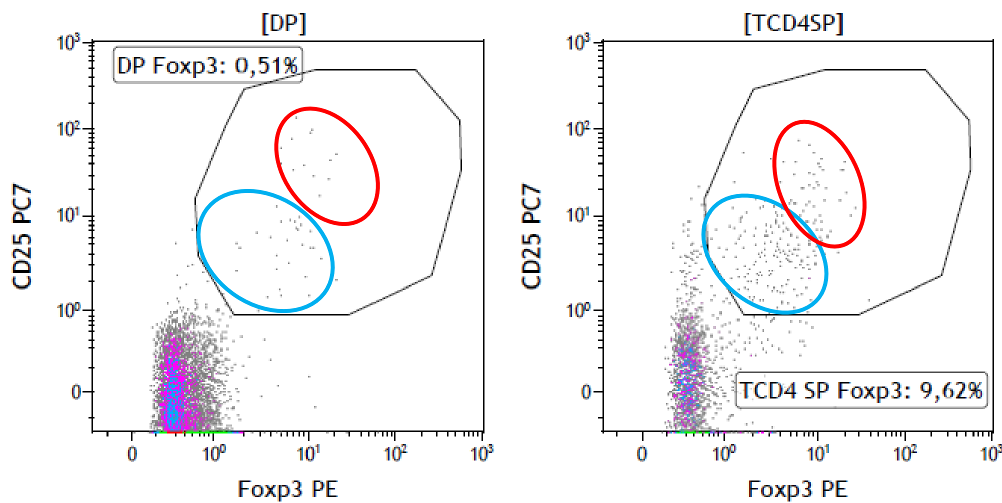


Fig. 4-1 Dotplot representation of DP thymocytes (left) and CD4 SP thymocytes (right) classified as subpopulations of FOXP3+ thymocytes. Low pattern of FOXP3 expression is marked with a blue oval whereas high pattern is marked with a red one.

Lastly, FOXP3 is also related to other factors such as CDTLA-4 and CD39, which participate in the establishing of suppressor activity of Treg cell population. It is not unusual to find high percentages of FOXP3+ thymocytes expressing these factors in high amounts, as reported previously, characterizing Treg cells with the function of regulating the self-tolerance of immune system [33], [35], [9], [10], [11]. In addition, CD39 serve as replacement for other more common markers, such as CD25 or FOXP3, as its presence in Treg cell population is essential for its proper suppressor functioning [10], even considering the presence of FOXP3 in other subtypes of cells in the thymus not being included as Treg cell population, such as activated T cells.

However, it is not likely to be of such importance when analysing thymic populations, as in many cases, the majority of FOXP3 expression is found in CD4 SP thymocytes which are highly related to Treg cell lineage, as oppose to difficulties found when separating Treg cell population from others in the periphery by means of FOXP3 expression, as Treg cell lineage is not exclusive to thymic origin [10], [12]. In addition, CD39 expression in FOXP3+ thymocytes is lower than results obtained from analysis of peripheral T cells which could represent an early stage of development of Treg cell population, increasing the presence of such factor with maturation of thymocytes. CTLA-4 and CD39 are highly related to suppression capabilities, being its absence extremely detrimental to the immune system, with fatal autoimmunity and inflammation due to uncontroled immune responses [5].

4.1.2 Confocal microscopy

As well as for previous results obtained from flow cytometry, images acquired with confocal microscopy are consistent with other scientific publications in terms of thymic population classification, and other important aspects related to FOXP3 expression and Treg cell population.

Analysis on the expression of CD4 and CD8 show predominance of thymocytes expressing both factors (DP thymocytes) as well as significant amounts of CD4 SP and CD8 SP thymocytes, as opposed to low presence of DN thymocytes [6], [5]. Distribution of thymic subpopulations is observed in several images, following reported patterns over distribution of thymocytes in medulla and cortex [3], [4]. As commented previously, differences in intensity are observed in FOXP3+ thymocytes, which could lead to separation of such cells into low FOXP3+ thymocytes and high FOXP3+, consistent with flow cytometry results and other publications [34]. Differences in expression of FOXP3 could be caused by different stage in cell differentiation of thymocytes, thus influencing the expression of FOXP3. Furthermore, low FOXP3+ thymocytes tend to differentiate in a slower rate than thymocytes with a high expression of FOXP3, which also advocates for a relation with the activation of thymocytes, considering the FOXP3 as a factor of expression in advanced stages of cell differentiation [34].

Analysis performed to Series003 image obtained from vertical orientation sample slices show clear differences between thymic cortex and medulla in terms of structure, as well as a distribution of FOXP3+ thymocytes towards the top side of the image, where the medulla could be located [6], [3], [4]

The Treg cell population is derived from CD4 SP FOXP+3 thymocytes as precursors, from maturation of T cells, as the main source of Treg cell pool at the human body, in addition to other sources, such as conversion of non-regulatory T cells into Treg cells by means of stimulation of FOXP3 expression mechanisms [12]. High reactivity towards self-antigen is a result from positive selection, which is performed at the thymic medulla, thus it is not strange to locate FOXP3+ thymocytes at this location [5].

Treg cell population is highly dependent on positive selection of CD4 SP thymocytes derived from precursors DP thymocytes, mediated by TCR stimulation. However, several methods for Treg cell maturation have been reported recently, as well as recirculation of mature Treg cells found at the periphery, although this process is still greatly unknown. FOXP3+ thymocytes located at the medulla, classified as CD4 SP thymocytes, have expressed commitment towards maturation into Treg cell when stimulated by pDC, mDC and mTECs. Even cTECs have specific features which could influence Treg differentiation at the cortex if necessary [3]. As

commented, mDCs activated by cytokine TSLP (Thymic stromal lymphopoietin), which is secreted by stromal cells found in Hassal's corpuscles have shown influence in Treg cell maturation process from medullary CD4+FOXP3+ thymocytes. Moreover, pDCs also show similar behaviour derived from its response to TSLP [5], [6].

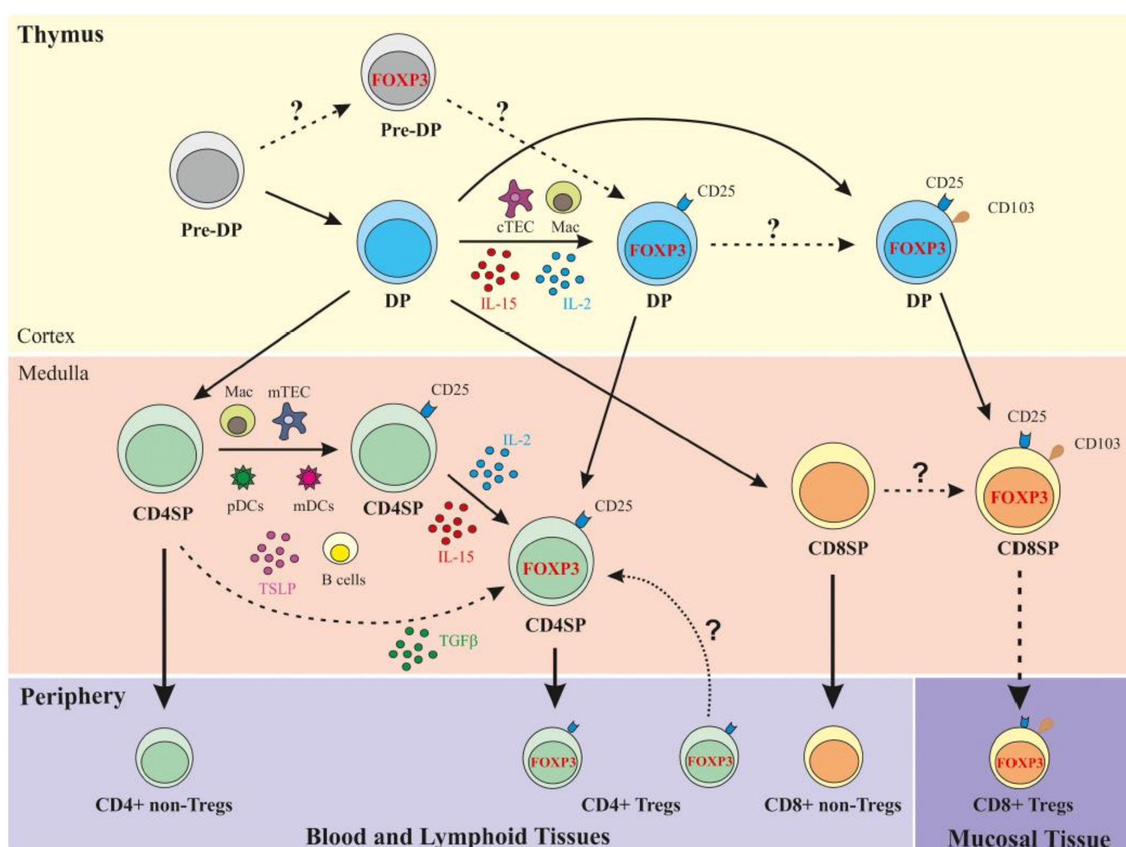


Fig. 4-2 Schematic representation of thymic cortex and medulla, establishing different mechanism for maturation of T cells, from DP thymocytes to SP thymocytes. Besides, different mechanisms for FOXP3 expression and development of Treg cell population are depicted [6]

Interestingly, mTECs in the presence of IL-2, which is secreted by non-regulatory T cells, also produce differentiation of thymocytes into Treg cell population. Lastly, at the periphery, T cells expressing CD45RA (naïve) can also express FOXP3 and differentiation into Treg cells in presence of TFG-β, which influence in the inhibition of Il-6 by retinoic acid generated by DCs. Il-6 inhibition decrease differentiation of naïve T cells to Th17 helper T cells. [5]

Therefore, there are several mechanisms from which CD4 SP thymocytes, in most cases expressing FOXP3, could be committed to differentiation into mature Treg cell, which could be found at the periphery. Thus, results from confocal

microscopy show consistency with respect to information related to distribution of cell population in the human thymus.

4.1.3 Limitations encountered during results obtaining and its analysis

Although flow cytometry and confocal microscopy results are highly reliable and have consistency with respect to other scientific publications, there are some factors which need to be addressed for improvement in successive researches. During the IHC process, an antibody against CD4 conjugated with APC was selected, which renders good results, although it has been reported that APC is highly sensitive to light fading. Therefore, properties related to its photostability could be the reason behind some results obtained from cell counting and qualitative analysis on images obtained from confocal microscopy, where unusual low amounts of CD4 SP thymocytes were found. In addition, confocal microscopy configuration is established for acquisition of images using up to 4 different colour channels, which restrict the number of combinations and possibilities for antibody analysis and screening, which affect the comparison with other results, such as the ones obtained from flow cytometry.

The comparison with other sources ensure the relevance of CD4 SP thymocytes presence in thymus, which is consistent with the results obtained using flow cytometry and the ones obtained from analysis of images acquired by confocal microscopy, taking into consideration the low intensity of CD4 expression, which could lead to falsely identification of cell populations.

Unfortunately, an absence of the extracellular matrix in the thymus leads to difficulties in determining the structure of the thymus in a qualitative way. Disintegration of sample tissue is derived from this feature, which could difficult the preparation of samples for analysis whereas cell counting is highly affected by the particular structure of thymus and the distribution of thymocytes spatially, as cells clusters are commonly formed, defaulting the identification of single cells and the factors being expressed.

As a consequence, algorithms for cell counting are not recommended for analysis of thymic samples, as the structure of the organ difficult the proper analysis and classification of thymocytes. Therefore, manual cell counting is to be performed, which is highly subjective and could lead to big changes when compared with other researches.

Finally, CD4-CD8 colocalization could represent a more automatized and precise tool for the classification of DP thymocytes, although it is important to remark the high sensitivity of M1 and M2 parameters to background noise, which in many cases is greatly difficult to be minimized properly.

4.2 Future research

As a conclusion, analysis of results obtained from flow cytometry and confocal microscopy have high consistency with other reported scientific publications, but also we obtained new findings that could be relevant for a better understating of the Treg ontogeny. Comparison between flow cytometry and confocal microscopy have been greatly useful for identification and characterization of the distribution of thymocytes, unravelling new pathways for analysis with the addition of supportive information for better understanding the distribution of thymocytes and the Treg cell population, in terms of both distribution and maturation.

4.2.1 Cell differentiation and fate derived from two subpopulations of FOXP3 thymocytes

Our results employing flow cytometry and confocal microscopy images have shown clear evidence of the possibility of existence of two different subpopulations of FOXP3+ thymocytes, depending on the intensity of expression [34]. Reported information has established different mechanism for development of Treg cell, and also differences in the expression of markers established as a paradigm for these cells in peripheral blood. Such mechanisms are based in stimulation of CD4 SP thymocytes from different cell populations, such as mTECs or DCs.

Therefore, there are enough pieces of information to conclude that further investigation regarding the cell differentiation processes related to low and high expressing FOXP3 thymocytes could be of great help for better understanding the Treg cell lineage and its cell differentiation program.

Research should be focused on clearly identifying and classifying such subpopulations of FOXP3+ thymocytes, performing extensive analysis on the specific expression of factors in those cells, which could provide significant information regarding the different pathways or mechanisms involved in the maturation of Treg cell from these subpopulations. Identification of cytokine responses could also support further research of the relations from which Treg cell differentiation program is based on, even providing useful information for development of effective and advanced treatments against autoimmunity or absence of proper development of immune system due to thymectomy.

4.2.2 Analysis of cell population distribution in 3D *in vitro* model

Through our analysis, relevant connections have been made between flow cytometry analysis of thymic cell populations and 2D distribution and characterization of those populations. As a result, it is obvious that further research

centered on classification and identification of relations between thymocytes influencing maturation of T cells should be provided by development of *in vitro* 3D models. *In vitro* 3D has significant advantages when compared to a bidimensional analysis of sample tissue, thus obtaining more genuine information about the distribution of thymic cell populations and the microenvironment which comes into play for proper maturation of T cells at the human thymus.

Considering the absence of an extracellular matrix, which is a significant feature of thymic tissue, it is not surprising to think about free scaffold techniques for cell culture of *in vitro* 3D models, based on aggregations between thymic cells, and their signalling, resulting in self-formation of organoids or spheroids, denominated as "thymospheres" which could offer valuable information closer to in vivo development of T cells. Stem cells properties, in specific TEC population at the thymus, have been previously reported, with thymosphere formation characterized by the absence of FOXN1, with relevant relations with respect to mTECs and cTECs [36]. Further analysis and determination of effective protocols for development of a 3D *in vitro* model could possibly serve as a new advanced pathway when compared to 2D sample tissue analysis.

Lastly, the analysis of full organ samples have been proposed, based on obtaining of images using SPIM microscopy. Closer investigation was performed for determining the possibilities of analysing 3D portion samples of organs using tissue clearing protocols, IF staining and acquisition of images using SPIM microscopy.

Optimization of tissue clearing protocols have greatly contributed to the possibility of further investment in 3D acquisition of images, which could offer real identification of specific markers for thymocytes and the 3D spatial distribution of different thymic cell populations. However, low resolution and small magnifications used for acquisition of images using SPIM microscopy are the two main drawbacks for the analysis of 3D sample organ, thus showing another pathway which could benefit from extensive and detailed research for obtaining of results offering better results.

5. REGULATORY FRAMEWORK AND BUDGET

5.1 Regulatory framework and legal aspects related to the project

The regulatory framework is controlled by the *Comité de Ética de la Investigación con Medicamentos (CEIm)*, which is in charge of regulating the development of observational studies or scientific research and ensuring the adequacy with the law and the ethics related to the subjects under study.

Researches conducted under the supervision of the CEIm are subjected to the Nuremberg Code (1947), the Declaration of Helsinki (1964) and the Belmont Report (1978). Furthermore, this commission is under the *Comunidad de Madrid*'s jurisdiction, subjected to the following laws [37]:

- *Decreto 39/1994, de 28 de abril, de Consejo de Gobierno, por la que se regulan las competencias de la Comunidad de Madrid en materia de Ensayos Clínicos con medicamentos*
- *Real Decreto 1090/2015, de 4 de diciembre, por el que se regulan los Ensayos Clínicos con medicamentos*
- *Ley 14/2007, de 3 de julio, de investigación biomédica*

Therefore, the conducting of the experiment detailed in this document was authorized by the CEIm, according to the law, by means of approbation of an application presented by the *Laboratorio de Immuno-Regulación*, following the corresponding requirements. In addition, as the experiment involved the participation of minors, parental consent was required and accepted, in all terms for the purpose of the experiment, and notified to the *Ministerio Fiscal de Menores*.

5.2 Project budget

There are several aspects which come into play for the estimation of the project budget, although they can be summarized depending on if they are derived from human resources, laboratory material and machinery and softwares used.

As a bachelor's thesis, human resources are correlated to the number of hours of work dedicated by both the student and the director/co-director. Laboratory equipment is subdivided into specific devices and softwares and services which are offered based on economic fees for the researches. Lastly, laboratory material and solutions are considered [38].

However, the project budget depicted in this document is an estimation of the genuine economic investment required for the proper development of the experiment, therefore, some values could differ from different sources, producing fluctuations in the estimated budget.

Human Resources			
Personnel	**Cost/hour**	**Hours**	**Total cost**
Director/Co-director	30,00 €	300	9.000,00 €
Student	25,00 €	450	11.250,00 €
		TOTAL	20.250,00 €

Laboratory Equipment and Softwares				
Equipment	**Cost**	**Depriciation/year**	**Hours of use**	**Total estimated cost**
Leica Application Suite X Software	- €	- €	0	- €
Tissue dissociator	3.000,00 €	990,00 €	0,25	0,12 €
Bechtop centrifuge	10.000,00 €	3.300,00 €	1,5	2,38 €
Kaluza Analysis Software	2.000,00 €	- €	0	2.000,00 €
ImageJ Software	- €	- €	0	- €
Microsof Office	70,00 €	- €	0	70,00 €
Personal computer	1.800,00 €	594,00 €	200	57,12 €
Cryostat	16.000,00 €	2.400,00 €	1	1,15 €
			TOTAL	2.130,77 €

Equipment and Services based on fees			
Equipment	**Fee (per hour)**	**Hours of use**	**Total cost**
Leica TCS SPE confocal microscope	10,00 €	4	40,00 €
Gallios flow cytometer	6,00 €	1,5	9,00 €
Cell culture biochamber	0,50 €	6,5	3,25 €
Pipette controller	- €	0	- €
Tube rotator	- €	0	- €
Magnetic Stirrer	- €	0	- €
Hemocytometer	- €	0	- €
Manual counter	- €	0	- €
Cell culture incubator	0,06 €	3,5	0,22 €
		TOTAL	52,47 €

Fig. 5-1 Breakdown of human resources, laboratory equipment and software and equipment and services based on fees estimation of budget [38], [39]

Laboratory material and Solutions	
Material/Solution	**Total cost**
TexMACS GMP cell culture medium (1 L) + 1% Ab	180,00 €
PBS 1x solution (100 ml)	15,00 €
FBS solution (500 ml)	400,00 €
IHC primary antibodies	5.600,00 €
Anti-rat Cy3 Donkey (Jackson Immunoresearch)	250,00 €
Pacific Blue cell dye (eBioscience)	132,00 €
Foxp3 / Transcription Factor Staining Buffer Set	156,00 €
Rat serum (eBioscience, 100 µl)	30,00 €
Cleaning Agent (Flow cytometry)	50,00 €
Acetone and dry ice	70,00 €
IgG blocking buffer (50 ml)	80,00 €
DAPI (Sigma Aldrich)	40,00 €
Other materials	50,00 €
TOTAL	7.053,00 €

Fig. 5-2 Breakdown of laboratory material and solutions estimation of budget [38], [39]

The estimated budget for the development of the experiments conducted and explained in this document amounts to 29.486,24 €

Finally, as it is represented, services offered by different units at the laboratory possess specific fees for the usage of each of the laboratory devices available. The information can be revised at the IISGM website [38].

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