Regulatory T cell Development in the Human Thymus
A Comprehensive Approach Combining Genome-wide Analysis and Single-cell Protein Expression by Computational Flow Cytometry

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1 RESEARCH PROBLEM

The immune responses need to be tightly controlled to avoid harmful effects. T cells are key players to orchestrate these immune processes. There is one T cell subset named regulatory T cells (Treg) devoted to suppress immune responses, which is defined by the expression of the transcription factor forkhead box P3 (FOXP3). Tregs can develop either in the thymus, the organ where T cells are produced, or be generated during immune responses.

Thymic Tregs are considered particularly important to ensure self-tolerance and prevent autoimmunity. There are very few data regarding the factors that determine the Treg lineage commitment in the human thymus, as well as those that contribute to their maintenance after leaving the thymus as naïve Tregs. Naïve Tregs are known to continuously replenish the memory fully suppressor Treg pool, but the mechanisms involved in their maintenance throughout life are largely unknown. Our main objective is to investigate these processes by using next-generation sequencing (NGS) and computational flow-cytometry approaches.

The currently available NGS data from human thymocytes are very limited. Additionally, flow-cytometry analysis has been mainly done based on a sequential gating strategy, which only focus on cell populations identified by pre-defined cellular markers. An unbiased approach will be more effective for exploring unknown developmental stages. Importantly, flow-cytometry generates multi-parameter protein expression profiles at the single-cell level. Applying computational analysis to these single-cell high dimensional data will provide relevant new relevant insights.

This study is expected to significantly improve our understanding of human Treg development and homeostasis, with implications for tolerance induction and autoimmune diseases.

2 OUTLINE OF OBJECTIVES

The main objective of this project is to investigate factors controlling regulatory T cell development in the human thymus and the homeostasis of naïve Tregs utilizing unbiased computational flow cytometry analysis and NGS approaches.

We aim to generate data that will help answering the following questions:

1. At which stage of T cell development can Treg commitment occur in the human thymu?.
2. Which pathways play a critical role in Treg lineage specification?
3. Which are the best epigenetic markers associated with human Treg lineage stability?
4. Which mechanisms are involved in Treg maturation both in the thymus and after thymus egress as circulating naïve Tregs?
5. Which factors contribute to the maintenance of circulating naïve Tregs, and what is the relative contribution of homeostatic cytokines and of self-peptides-MHC signaling?

The availability of whole-transcriptome data from human thymocytes is currently limited.

We plan to characterize the transcriptome of purified thymocyte populations along human T cell development using NGS.

As a strategy to select the best markers to define the populations to sort for the NGS studies, we will use unbiased approaches for multicolor flow-cytometry analysis that allow the analysis of the continuous maturation process.

In order to unravel the biological factors of cell fate stability we will also perform DNA methylation studies. Given the putative role of non-coding RNAs, samples will be collected to allow small RNA-seq studies in the populations that we will identify to be of particular relevance to investigate Treg commitment.

The ultimate goal is to generate an integrated model of human Treg development upon validation

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of the identified key molecules/pathways. These studies will be combined with the evaluation of the peripheral naïve T cell compartments.

3 STATE OF THE ART

Multipotent progenitors migrate from the bone marrow to the thymus, where they generate T cells. After commitment to the T cell lineage, triple negative cells (CD3-CD4-CD8-) develop into immature CD4 single positive (SP) cells, and subsequently acquire CD8 becoming double positive cells (DP) (Spits, 2002). During the DP stage there is a progressive increase in CD3/T cell receptor (TCR) expression. TCR recognition of self-peptides-MHC is important for surviving (positive selection). On the other hand, the cells with high TCR affinity for self-antigen are eliminated (negative selection), although the clearance of auto-reactive cells is not complete (Starr, Jameson and Hogquist, 2003). Finally they differentiate into CD4SP or CD8SP and they mature in the thymic medulla before thymus egress and incorporation in the naïve T cell pool.

Treg population is a subset defined by the expression of the transcription factor FOXP3 that is considered to be a main player in self-tolerance (Hori, Nomura and Sakaguchi, 2003). Tregs suppress many immune cells (Pandiyan et al, 2007; Iikuni et al, 2009; Gotot et al, 2012; Ralainirina et al, 2007;Liu et al, 2009). There is currently intense investigation to explore their function in innovative clinical therapies for autoimmune diseases (Miyara, Ito and Sakaguchi, 2014; Katzmann and Abbas, 2015; Spence et al, 2015), as well as in transplantation (Tang and Bluestone, 2013) and oncology (Wang, 2006).

Tregs are known to develop in the thymus or to be generated during immune responses in the periphery (Ito et al, 2008). There are no surface markers to distinguish these two populations (Povoleri et al, 2013). However, their TCR repertoire appears to be distinct (Relland et al, 2012). Thymic Tregs are enriched in self-reactive TCRs (Wong et al, 2007), but can also significantly recognize broad microflora-derived antigens (Cebula et al, 2013). Moreover, thymic Treg feature epigenetic markers that are associated with a higher phenotypical stability and function than peripheral derived Treg (Ohkura et al, 2012). Therefore, thymic Tregs are considered to be particularly dominant to ensure self-tolerance and to prevent autoimmunity.

Regarding the thymic Treg development, previous work has shown that both TCR stimulation and cytokines, namely IL-2 and IL-15, are required in the process (Caramalho et al, 2015a). IL-2 is also known to regulate circulating Treg homeostasis (Yu et al, 2009; Attridge et al, 2012). Recently, our lab has showed that IL-7 also plays a determinant role in naïve Treg maintenance (Silva et al, 2016).

However, it remains to be determined at which stage cell differentiation fate toward Treg lineage occurs, and which factors are implicated in these processes in the human thymus (Caramalho et al, 2015b). Also, many questions remain unclear regarding the mechanisms of their maintenance after leaving the thymus and being incorporated in the naïve Treg pool (Silva et al, 2016).

FOXP3 is considered the best available marker to define this population, and, therefore, the clarification of the mechanisms that regulate FOXP3 expression, including epigenetic control, is an important research area (Kitagawa, Ohkura and Sakaguchi, 2015). However, Tregs can develop in mice without functional FOXP3 (Lin et al, 2007), and other data support that the commitment to the Treg lineage is independent of FOXP3 expression (Wang, 2006). Our lab and others revealed that FOXP3 is already expressed in early stages of T cell development (Nunes-Cabaço et al, 2011), much before the SP stage and migration to the thymic medulla (Caramalho et al, 2015b). These results emphasize the lack of knowledge regarding the importance of FOXP3 in the Treg lineage specification in the human thymus.

We hypothesize that other factors have a critical role in triggering thymic Treg differentiation. Furthermore, most of the available data are from mouse thymus, and human studies are of utmost importance given the known significant differences between T cell development and homeostasis in the two species (Caramalho et al, 2015b).

This study is expected to significantly improve our understanding of human Treg development and to identify new targets for the development of immune-based therapies, useful not only for autoimmune diseases, but also for other clinical settings such as cancer, allergy and persistent infections.

Regarding methodological aspects, single cell analysis is of utmost importance to investigate heterogeneous cell populations. Flow cytometry is a potent technology to generate high dimensional data of protein expression at single-cell level. Nowadays, computational flow cytometry analysis tools have been developed using many algorithms similar to those used by other big data analysis for visualization, classification and clustering (Saeys,

Gassen and Lambrecht, 2016). These techniques allow a more clear separation of cell subpopulations without the conventional use of biased sequential gating approaches.

Although NGS is a high potential technology for comprehensive analysis such as genome wide study, there are very limited data regarding human thymocytes and Tregs. This method enables to predict genes or genome regions that have potential to affect the function in genome-wide level (Wang, Gerstein and Snyder, 2009; Nagalakshmi, Waern and Snyder, 2010).

Those unbiased approaches will be of particular relevance to investigate the process of Treg commitment.

4 METHODOLOGY

The overall aim is to investigate human T cell development, with a particular focus on Tregs.

Our laboratory has been generating a significant amount of multicolor flow-cytometry data using human thymic samples (Caramalho et al, 2015b; Nunes-Cabaço et al, 2011; Mota et al, 2014). We will take advantage of new software for analysis of these data (Infinicyt software, Cytognos, Salamanca, Spain), which will allow a better definition of maturation curves of human thymocytes. This will facilitate the identification of the best markers to be used to define the thymocyte populations of interest in our study. Moreover, we will use other computational approach with R program for investigation of the thymocyte cell populations from a broad viewpoint.

Additionally, we plan to do an *in silico* analysis of the public next generation sequencing (NGS) and microarray datasets of thymocytes, although most transcriptome analysis data were generated from mouse samples, to make full use of the available data to plan our NGS studies.

We will do whole transcriptome analysis of human thymocyte populations to identify different expressed genes and splicing variants by high depth sequence data. Lineage cell commitment will be further investigated using epigenetic studies (Tarakhovsky, 2010; Cedar and Bergman, 2011).

Ultimately, we will validate the identified factors and pathways using in-vitro assays, which have been extensively used in our laboratory (Caramalho et al, 2015a; Silva et al, 2016; Nunes-Cabaço et al, 2011; Mota et al, 2014). The comparison between conventional and putative Treg populations will help clarify the critical pathways for the specification and maintenance of the Treg lineage.

It has been suggested that naïve T cells complete their maturation process after thymus egress (Boursalian et al, 2004). Therefore, we will also compare mature single-positive thymocytes with naïve T cells from peripheral blood of healthy adults. These comparisons are expected to provide a maturation profile from the T cell progenitors to fully-mature naïve T cells.

Overall, according to our best knowledge, we will generate the first comprehensive NGS data regarding T cell development in the human thymus as well as of thymic Treg lineage specification.

4.1 Human Samples and Ethical Aspects

Thymic specimens are obtained from thymectomy during pediatric corrective cardiac surgery (newborns to 4-year old) at Santa Cruz Hospital, after parents' informed consent. Thymic tissue is collected by clinical indication, and would be otherwise discarded. Children with diseases potentially involving the immune system such as DiGeorge and Down syndromes are excluded.

The cord blood is obtained through a protocol with the Obstetrician Department of the Centro Hospitalar Lisboa Norte (CHLN), and the peripheral blood from volunteer healthy donors after written informed consent.

All samples were anonymized before use.

The study was approved by the Ethical Broads of the Faculty of Medicine of Lisbon, of CHLN, and of Santa Cruz Hospitals.

4.2 Flow Cytometry Analysis

Thymocyte and circulating T cell suspensions are prepared and stained as previously described (Silva et al, 2016; Nunes-Cabaço et al, 2011), using a broad panel of markers to investigate the possible maturation curves by multi-color flow-cytometry.

The analysis will be performed using the software Infinicyt (Cytognos, Salamanca, Spain), SPADE, tSNE, flowSOM and other algorithms. We expect to identify the appropriate cell populations to be used in the NGS studies.

4.3 Reanalyze the Microarray and NGS Data of Public Databases Related to T cell Development and Tregs

We will reanalyze the whole transcriptome data of microarray and NGS, including mice, in the public databases (Shumway, Cochrane and Sugawara, 2009).

From public database such as GEO, ImmGen, and ENCODE, we will collect the datasets of RNA-seq data by NGS of murine thymocyte, microarray data of human thymocyte and cord blood, and reanalyze them to gather all the information available. Also we will use histone modification data of human thymocyte from Roadmap epigenome project (Roadmap Epigenomics Consortium et al 2015), and integrate the gene expression data and epigenetics data.

4.4 Cell Sorting

The identified cell populations, including both putative Tregs and conventional T cells, will be sort-purified by flow cytometry using FACSAria (BD Bioscience).

4.5 Generation of NGS Data

We will extract RNA and DNA from the sorted cells, and use next generation sequencer for RNA-seq and small RNA-seq, as well as for the epigenetic studies to be defined according to the preliminary data obtained.

We plan to have at least three replicates of each condition to strengthen the statistical analysis.

4.6 Bioinformatics Analysis

For the whole transcriptome analysis, the output data from NGS will be processed using bioinformatics tools.

The RNA sequencing reads will be check for their qualities and filtered by quality check tools such as FastQC. After that, they will be aligned to the human genome to calculate the transcript expression values by bioinformatics tools, such as TopHat and Cufflinks, or discover the alternative splicing transcripts by MISO. On the other hand, small RNA-seq data will be aligned to the known small RNAs from database using miRtools, or we will predict novel small RNAs and their binding target genes using Mirdeep2.

Regarding the epigenetic studies, we will decide accordingly to the preliminary data obtained if it is worth to perform a whole-based approach using BS-seq or a more strategy focused on target regions of interest. BS-seq is an approach to determine the methylation site in genome wide level by bisulfite treatment of DNA. In the BS-seq, the data is aligned to the human genome by mapping tools and methylated genome regions are detected by tools such as Bismark and BSMAP. Additionally, we will consider other genome-wide approaches to investigate DNA methylation, as well as possibility of including histone modification studies using Chip-seq for revealing epigenetic regulation.

Data will be processed after establishing pipeline optimized parameters of each bioinformatics tool. We expect that integration of the results of transcriptome and methylation or histone modification data will identify several candidate genes or genome regions.

4.7 Integrated in Silico Analysis

We will combine the results of whole transcriptome and methylation/histone modification analysis, as well as small RNA data, and screen for the more influential factors from the possible candidates using the relevant algorithms (Conesa et al, 2016). We will compare results and predict the function of the differential expressed transcripts or genome regions and their impact on the biological mechanisms. With these kinds of NGS data, we will combine the results and identify the influential factors for Treg development and homeostasis.

4.8 Validation Experiments

Finally, we will select the more relevant factors likely to be involved in T cell/ Treg development and naïve T cell/Treg homeostasis, and will validate their expression by PCR and their function using the appropriate in-vitro assays.

We will take full profit of the methodologies to investigate Treg development and function previously optimized in our laboratory (Caramalho et al, 2015a; Silva et al, 2016; Nunes-Cabaço et al, 2011; Mota et al, 2014). In addition to the investigation of the impact of TCR signaling and cytokines, we will manipulate other possible pathways that will be inferred from our data.

4.9 Human Treg Development Model

The combination of the flow cytometry and NGS data will allow us to generate an integrated proposal for Treg development in the human thymus that we will test using mathematical modeling and system immunology.

5 EXPECTED OUTCOME

We expect to identify the Treg subpopulations including developmental stages by using computational flowcytometry data analysis. Additionally the combination of the flow cytometry and NGS data will allow us to generate an integrated proposal for Treg development in the human thymus that we will test using mathematical modeling and system immunology. These will improve our knowledge of the commitment and maintenance of Treg lineage.

6 STAGE OF THE RESEARCH

To investigate Treg differentiation process and identify the cell populations for the NGS studies, we used Infinicyt, flowSOM and SPADE to analyze human thymus flow cytometry data.

Firstly, we explored the Infinicyt software to define cell maturation stages. In APS (Automatic Population Separator), which is based on Principal component analysis, the result build by the levels of expression of CD3, CD4 and CD8 showed the two maturation paths toward to CD4SP and CD8SP cells (Figure 1).

Then we chose the one toward CD4SP, and validated the maturation process using other well-known developmental markers. This result suggests that this APS build based on CD3, CD4 and CD8 enables the drawing of the maturation path.

Next, we gated in total FOXP3+ thymocytes and FOXP3- thymocytes, and generated APS for creating the respective maturation curves with these populations.

The comparison of FOXP3+ and FOXP3- thymocytes revealed differences along the developmental stages regarding the expression level of several markers (Figure 2), providing us with a new tool to investigate these processes.

On the other hands FlowSOM and SPADE allowed us to build spanning trees and the possibility

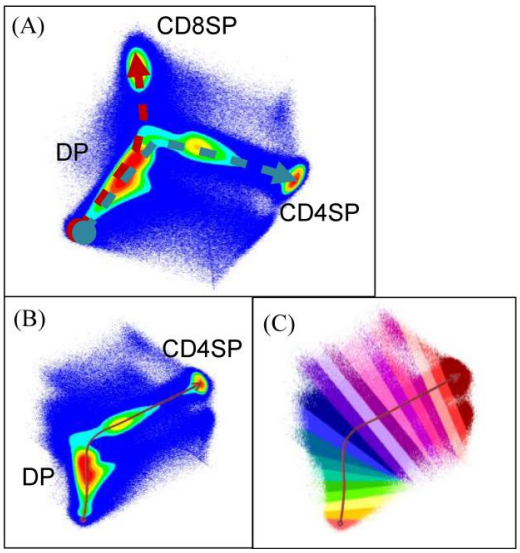

Figure 1: Maturation path toward to CD4SP and CD8SP cells (A) and illustrative automatic distribution of the 20 stages of the CD4SP maturation path after excluding CD8SP from the analysis (B, C).

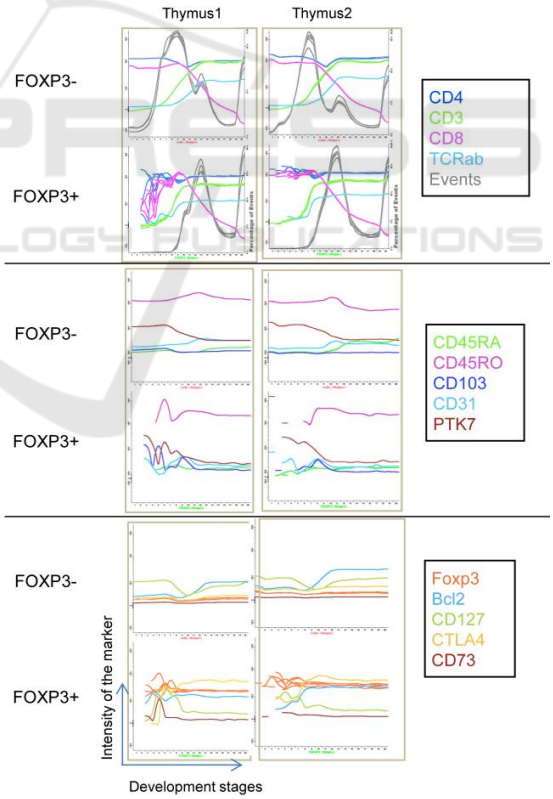

Figure 2: Expression of different markers along the stages of the maturation curve analysis of FOXP3+ cells and FOXP3- cells. We draw the maturation path toward to CD4SP in infinicyt both FOXP3- and FOXP3+ cells, and the marker expression levels in each development stage are calculated.

to analyze the branching of differentiation between Treg and conventional T cell.

We were able to characterize each node according to the levels of expression of the cellular markers used (Figure 3).

This result suggests that not only cellular markers but also Forward Scatter (FSC) and Side Scatter (SSC) levels influence to the cell population's characterization. Therefore the physical cellular size and complexity would be also important to find the subpopulations in thymocytes.

Our strategy is to identify the cell populations of Treg by spanning trees such as FlowSOM, and analyze continuous maturation process by maturation curves such as Infinicyt. We are currently optimizing the best combinations of markers to use in new flow cytometry tubes to fully explore these tools.

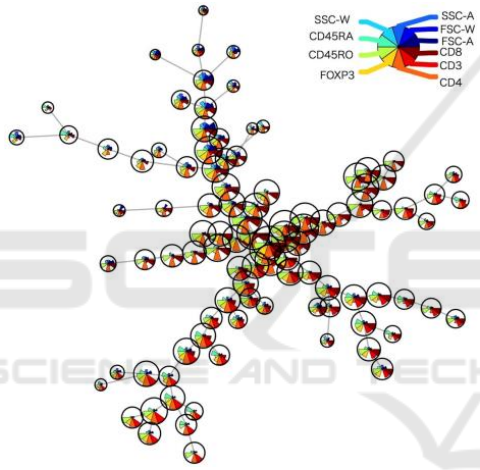

Figure 3: Representative example of a spanning tree generated using FlowSOM to analyze flow cytometry data from total thymocytes isolated from human thymus. The size of the circles corresponds to the number of events. Each color identifies one marker and the height corresponds to its level of expression.

Regarding RNA-seq analysis, we are using the available human thymocyte data to build the analysis pipeline and optimize it to identify different transcriptome expression profiles, including splicing variants between thymocyte cell populations of interest.

Additionally, we picked up microarray data from public database related to T cell development and Treg for reanalysis. The comparison of the gene expression profiles between thymocyte developmental stages showed different expressed genes related to ncRNA regulation, TCR signaling and cytokine signaling pathways.

Also we used Treg cord blood and peripheral blood data for comparing mature and immature Treg gene expression profiles. In this case, the identified different expressed genes were related to mitochondrion, hemopoiesis, regulation of cell death, zinc-finger.

We plan to further explore the role of these pathways in the T cell and Treg development processes.

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APPENDIX

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