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Short communication

Constitutive expression of genes encoding notch receptors and ligands in developing lymphocytes, nTreg cells and dendritic cells in the human thymus

Luciana Bento-de-Souza [a,c], Jefferson R. Victor [a], Luiz C. Bento-de-Souza [b], Magaly Arrais-Santos [b], Andréia C. Rangel-Santos [a], Érica Pereira-Costa [a], Elaine Raniero-Fernandes [d], Maria I. Seixas-Duarte [c], João B. Oliveira-Filho [d], Alberto J. Silva Duarte [a,b,*]

[a] Laboratory of Medical Investigation in Dermatology and Immunodeficiencies (LIM56), School of Medicine – USP, São Paulo, Brazil
[b] Heart Hospital – Hcor, São Paulo, Brazil
[c] Laboratory of Pathology of Transmissible Diseases, School of Medicine – USP, São Paulo, Brazil
[d] Institute of Integrative Medicine Professor Fernando Figueira – IMIP, Recife, PE, Brazil

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ABSTRACT

The thymus is the site of T cell maturation. Notch receptors (Notch1-4) and ligands (DLL1-3 and Jagged1-2) constitute one of several pathways involved in this process. Our data revealed differential constitutive expression of Notch genes and ligands in T lymphocytes and thymic dendritic cells (tDCs), suggesting their participation in human thymocyte maturation. nTreg analyses indicated that the Notch components function in parallel to promote maturation in the thymus.

1. Introduction

The thymus is a privileged and indispensable site for the generation and maturation of T cells in vivo, as this microenvironment induces and supports lineage commitment, differentiation, and survival of thymus-seeding cells. Indeed, the thymus is both where the T cell repertoire is generated and where T cells are shaped by positive and negative selection, giving rise to a broad functional MHC-restricted naïve TCR αβ repertoire [1].

T cell progenitors derived from hematopoietic stem cells migrate from the bone marrow and undergo development in the thymic environment to generate the T cell lineage. Notch signaling is one of several pathways involved in promoting T lineage commitment and maturation [2]. Notch receptors and their ligands are a family of trans membrane proteins involved in a variety of cell fate decisions that affect the developmental functions of many organs and systems, including hematopoiesis and the immune system. Mammalian genomes encode four Notch receptors (Notch 1–4, which are homologous to the single Notch receptor in *Drosophila*) and five ligands (Delta-like 1, 3, and 4 and Jagged 1 and 2, which are homologous to the Delta and Serrate ligands in *Drosophila*) [3]. The best established function for Notch signaling in the hematopoietic system is the essential role it plays in promoting T lineage commitment and maturation [4].

T lineage specification is mediated exclusively by Notch 1, and induced deletion of Notch 1 in hematopoietic progenitors results in a complete block of T cell development and the ectopic differentiation of immature B cells in the thymus [5]. Notch receptor-ligand interactions communicate signals between neighboring cells via highly conserved signaling mechanism activated through the interaction of the Notch receptor with its cognate ligand [6]. This pathway is initiated when Notch-ligand engagement induces two successive proteolytic cleavages, the second of which is mediated by a presenilin-containing complex exhibiting γ-secretase activity that releases the Notch intracellular domain (NICD). This activated form of Notch translocates to the nucleus where it binds to the CSL protein (also known as RBPJ, an important nuclear mediator of Notch signaling that is similar to the DNA-binding protein found in

* Corresponding author at: Laboratory of Medical Investigation in Dermatology and Immunodeficiencies (LIM56), School of Medicine – USP, São Paulo, Brazil.
E-mail address: adjsduar@usp.br (A.J. Silva Duarte).

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other species), thereby displacing co-repressors and recruiting co-activators [7].

Each Notch receptor interacts with a specific Notch ligand in a context-dependent manner. Numerous studies have identified transcripts for all Notch ligands in the thymus [8]. Members of the Delta-like family appear to be crucial for T lineage commitment and maturation. A report by Mohtashami and Zúgñiga-Pflücker [9] highlighted the importance of Delta-induced signals by demonstrating that the loss of DLL1 and DLL4 expression led to the inability of fresh, ex vivo thymic stromal monolayers to support T lineage specification following disruption of their three-dimensional organization. In contrast, the inactivation of the DLL1 ligand does not block T cell development, suggesting a redundant role for this and other DLL ligands, such as DLL4 [4]. These data suggest that Notch1 signaling in early thymocyte progenitors is restricted to DLL4 in the thymus [2].

T regulatory cells of the CD4+CD25+FOXP3+phenotype, also known as natural T regulatory cells (nTreg), are generated in the thymus as a specific T cell lineage and are critical for the maintenance of immune homeostasis and the suppression of naturally occurring self-reactive T cells [10]. The generation and maturation of this specific T cell lineage involve particular and complex processes within the thymus, and many signaling pathways participate in these processes. Ou-Yang and colleagues (2008) demonstrated that Notch signaling modulates the *Foxp3* promoter through a RBPJ- and HES-1-dependent mechanism (notch target genes with transcriptional repressor functions that negatively regulate gene transcription) [11]; however, further investigations will be required to determine the components of this pathway (in the context of Notch signaling) that are involved in the processes of nTreg commitment, maturation, and differentiation.

Within the thymic medullary region, groups of epithelial cells, termed Hassal's corpuscles, have been shown to play a critical role in the maturation of developing thymocytes [12] and the generation of nTreg [13]. Human Hassal's corpuscles express thymic stromal lymphopoietin (TLSP), which activates thymic dendritic cells (tDC) to express high levels of CD80 and CD86. Once activated, tDCs induce the proliferation and differentiation of CD4+CD8-CD25-thymic T cells into CD4+CD25+FOXP3+cells, suggesting that tDCs play a crucial role in dendritic cell-mediated secondary positive selection of nTreg [13].

In this study, we investigated the expression of the Notch receptor and ligand genes in immature thymocytes, nTreg and tDCs to evaluate the potential involvement of this pathway in the maturation of T lymphocyte and nTreg cells in the human thymus.

2. Materials and methods

2.1. Patient samples

Thymic tissues were obtained from 10 patients who underwent corrective cardiac surgery at the Hospital do Coração (HCor), SP, Brazil (mean 3.24 years). Each sample where analyzed in three independent experiments. The patients did not exhibit signs of immunodeficiency. The ethics committees at the Hospital do Coração and the School of Medicine at the University of São Paulo approved this study. Informed consent was obtained from the parents of all children.

2.2. Thymus tissue dissociation, cell isolation, and storage

Thymocytes and tDCs were released from the tissue samples using enzymatic dissociation. The thymus was cut into small fragments and added to 50-mL propylene conical centrifuge tubes. Next, an enzymatic solution (10 mL) containing RPMI pre-warmed to 37 °C, 0.5 mg/mL collagenase A, 0.02 mg/mL DNAse I (Roche Diagnostics, Mannheim, Germany), and 5% FBS was added, and incubation was performed for 10 min at 37 °C under continuous agitation. The digested fragments were homogenized gently and filtered through a plastic sieve with a 70-μM mesh screen (Cell Strainer, BD Falcon, CA, USA) to remove aggregates and stromal material. The resultant cell suspensions were washed twice with wash buffer 1 (50 mL RPMI [Gibco – Life Technologies, Grand Island, NY, USA]) pre-warmed to37 °C, 0.1 mg/mL collagenase A [Roche Diagnostics, Mannheim, Germany], and 0.02 mg/mL DNase I [Roche Diagnostics, Mannheim, Germany]), followed by centrifugation at 540 g for 5 min. Next, the pelleted cells were resuspended in a second wash buffer (50 mL cold PBS, 5 mM EDTA [Sigma Aldrich, Saint Louis, MO, USA], 0.02 mg/mL DNase I, and 5% FBS [Gibco – Life Technologies, USA]) and centrifuged at 540 g for 5 min. The pelleted cells were resuspended immediately in RPMI, and the low-density fraction was collected following centrifugation through Ficoll-Paque (GE Healthcare Bio Science, Uppsala, Sweden) at 540 g for 20 min. The cells were washed twice using RPMI and centrifuged at 1500 rpm for 10 min. The thymic cells were frozen and stored at −80 °C until use.

2.3. Flow cytometry and purification of thymic populations

Thawed cells were re suspended in pre-warmed RPMI, the number of cells per tube was adjusted, and the cells were subjected to fluorescence staining. To characterize cells populations in the human thymus, thymocytes were stained with human anti-CD3 PE-Cy7,-CD4 FITC or APC,-CD8 PE,-CD11cPE and -CD25 PE or FITC antibodies (BD Pharmingen, New Jersey, USA) and the cell populations were sorted into CD3-CD4-CD8-, CD4+CD8+, CD4-CD8+, CD4+CD8-, CD3+CD4+CD8-CD25^{high}, and CD11c+subsets using a FACSAria II flow cytometer (BD Pharmingen, New Jersey, USA). The purity of all sorted cell subsets was greater than 97%.

2.4. RNA purification and preparation of cDNA

After cell sorting, total RNA from the sorted populations was isolated using the RNeasy Plus mini and micro kits (QIAGEN, Hilden, Germany) and was retro transcribed using Sensiscript (QIAGEN, Hilden, Germany) in accordance with the manufacturer's instructions.

2.5. Real-time PCR

The *NOTCH1*, *NOTCH2*, *NOTCH3*, *DLL1*, *DLL3*,*DLL4*, *JAG1*, *JAG2*, and *FOXP3* genes were amplified using specific TaqMan® Gene Expression Assays and SYBR Green fluorescent dye (Applied Biosystems, California, USA) with an ABI 7600 SDS platform (Applied Biosystems, California, USA) in accordance with the manufacturer's instructions. The *NOTCH4* gene was not evaluated in any of the purified subsets of cells. The *GAPDH* housekeeping gene was used for normalization. HEK cells were used as a reference for gene expression because they express all evaluated genes (NIH, Bethesda, MD, USA). All the RT-PCR reactions were performed using controls for exogenous contamination and genomic DNA contamination. Gene expression was calculated as $2^{-\Delta Ct}$, which corresponds to the Ct value of the target gene in a population normalized using the Ct value of the housekeeping gene (GAPDH) in the same population and was expressed as the mean ± standard error. To perform relative gene analysis, we calculated $2^{-\Delta\Delta Ct}$ in accordance with the method of Livak et al. [14], and the data were expressed as the means ± standard errors. The following probes were used: NOTCH1 (Hs01062014), NOTCH2 (Hs01050702), NOTCH3 (Hs01128541), JAG1 (Hs01070032), JAG2 (Hs00171432),