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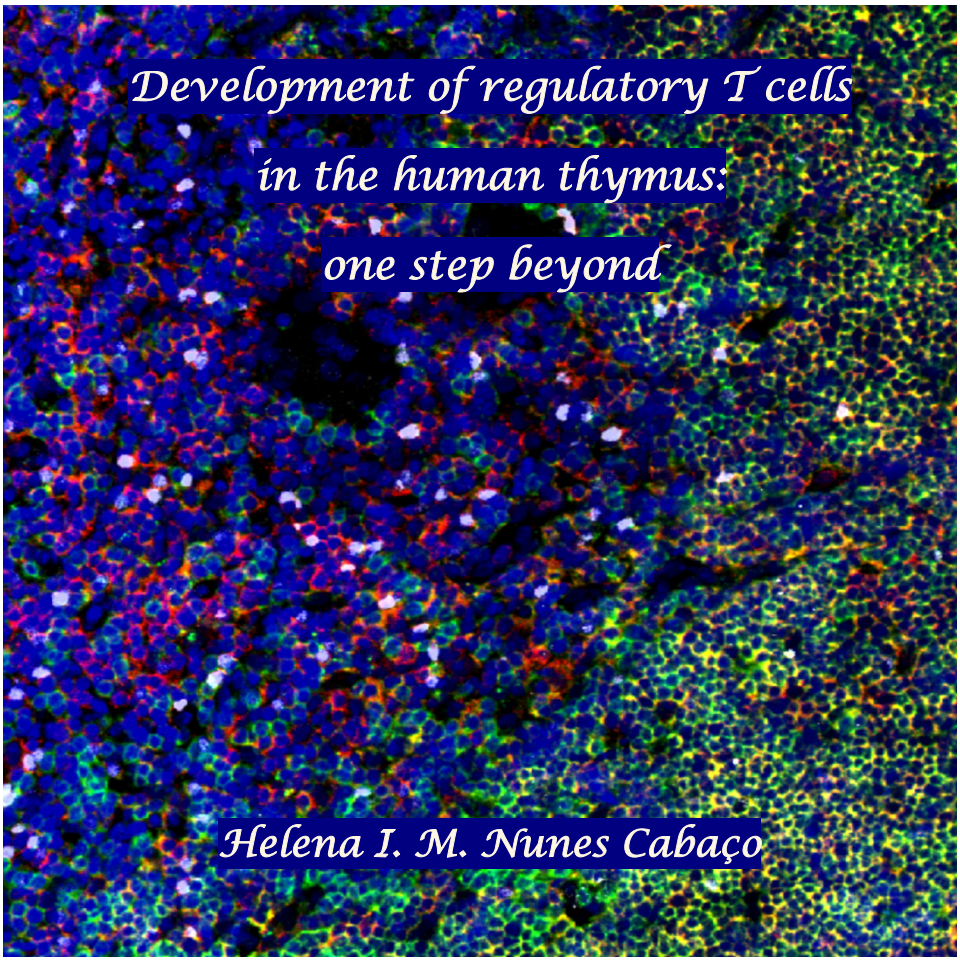

Doutoramento em Ciências Biomédicas

Especialidade de Ciências Biopatológicas

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Development of regulatory T cells in the human thymus: one step beyond

Desenvolvimento de células T reguladoras no timo humano

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Tese orientada pela Prof. Doutora Ana Espada de Sousa

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Cover and Back Images: ***Foxp3 expression in the human thymus*****.**

Foxp3-expressing cells are mostly observed in the medulla, which can be identified by the "dark wholes" (Hassall's corpuscles) and by the scarcity of thymocytes as compared to the cortical area, where cells are densely packed. Some Foxp3+ cells can also be found in this region, where the more immature CD4+CD8+ double positive thymocytes (yellow) prevail.

Frozen sections of a human pediatric thymus were stained with antibodies to CD8 (green), CD4 (red), Foxp3 (white in cover figure; blue in figure in the back) and, in the cover figure, DAPI (blue; nucleic acid counterstain). Image was obtained using a Zeiss LSM510 META Laser Scanning Confocal Microscope (at Instituto de Medicina Molecular).

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ABBREVIATIONS	
Abbreviation	**Description**
AIRE	Autoimmune regulator
APC	Antigen presenting cell
Bcl-2	B-cell lymphoma 2
CCR	Chemokine receptor
CD	Cluster of differentiation
cjTRECs	Coding-joint TRECs
CMJ	Corticomedullary junction
cTEC	Cortical thymic epithelial cell
CTLA-4	Cytotoxic T-lymphocyte antigen 4
DAPT	N-[N-(3,5-difluorophenyl)-L-alanyl]-sphenylglycine t-butyl ester
DC	Dendritic cell
DL1	Delta-like 1
DN	Double negative
DNA	Deoxyribonucleic acid
DP	Double positive
EDP	Early double positive
Foxp3	Forkhead box P3
FTOC	Fetal thymus organ culture
γc	Common γ chain
GALT	Gut-associated lymphoid tissues
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GARP	Glycoprotein A repetitions predominant
GATA3	GATA-binding protein
GFP	Green fluorescent protein
GITR	Glucocorticoid-induced tumor necrosis factor (TNF) receptor family-related gene/protein
GW	Gestational week
hmFTOC	Human/mouse hybrid FTOC
ICOS	Inducible T cell co-stimulator
IL	Interleukin
IPEX	Immune iysregulation, polyendocrinopathy, enteropathy, X-linked syndrome
ISP	Immature single positive

Abbreviation	Description
Jak	Janus kinase
LAP	Latency-associated peptide
mAb	Monoclonal antibody
MHC	Major histocompatibility comlex
mTEC	Medullary thymic epithelial cell
NK	Natural killer
nTreg	"Natural" Treg
PCR	Polymerase chain reaction
PD-1	Programmed cell death 1
PDC	Plasmacytoid dendritic cell
PECAM-1	Platelet endothelial cell adhesion molecule 1 (CD31)
pTα	Pre-TCRα
RAG	Recombinase activating gene
RNA	Ribonucleic acid
RTE	Recent thymic emigrant
RTOC	Reaggregate thymic organ culture
RT-PCR	Reverse transcription PCR
RUNX	Runt-related transcription factor
SCID	Severe combined immune deficiency
SEM	Standard error of mean
sjTRECs	Signal-joint TRECs
SOCS1	Suppressor of cytokine signaling 1
SP	Single positive
STAT5	Signal transducer and activator of transcription 5
TCR	T cell receptor
TEA	T-early α
TEC	Thymic epithelial cell
TGF-β	Transforming growth factor β
Th	T-helper
Th-POK	T-helper inducing POZ/Kruppel-like factor
TN	Triple negative
TNF	Tumor necrosis factor
TOC	Thymic organ culture

Abbreviation	Description
TOX	Thymus high mobility group (HMG) box protein
Tr1	Type 1 regulatory T cells
TRA	Tissue restricted antigens
TREC	TCR rearrangement excision circles
Treg	Regulatory T cell
TSLP	Thymic stromal lymphopoietin

RESUMO

O timo é um órgão linfóide primário essencial para o desenvolvimento das células T, sendo responsável pela formação de um repertório capaz de combater possíveis patogéneos estranhos ao organismo sem comprometer a tolerância ao próprio. Esta tolerência é maioritariamente garantida pelo timo, quer pela eliminação de linfócitos T potencialmente auto-reactivos, quer pela produção de uma população de células T, denominadas células T regulatoras (Treg), devotada ao controlo da resposta imune, particularmente no contexto de processos autoimunes e inflamatórios.

A diferenciação no timo de progenitores provenientes da medula óssea em células T CD4 ou CD8 maduras ocorre através de uma sequência de estadios de desenvolvimento que podem ser definidos com base na expressão das moléculas CD3, CD4 e CD8. Em humanos, células CD4- CD8- CD3- triplamente negativas (TN) adquirem inicialmente CD4 (estadio CD4 monopositivo imaturo, CD4ISP) e posteriormente CD8, tornando-se células CD4+ CD8+ duplamente positivas (DP). Na sequência de rearranjos genéticos do receptor de células T (TCR), este é expresso à superfície e é testada a sua capacidade de reconhecer o complexo de histocompatibilidade *major* (MHC), fundamental para a apresentação de antigénios. Células cujo TCR não reconheça MHC morrem por apoptose ("morte por negligência"). Apenas células apresentando TCRs que reconhecem MHC do próprio recebem sinais de sobrevivência ("selecção positiva") e de diferenciação em células T CD4 ou CD8 maturas. Estudos no ratinho mostram que a intensidade e duração da sinalização pelo TCR durante a diferenciação tímica são determinantes na decisão de uma célula se tornar uma célula T CD4 ou CD8. Uma sinalização excessivamente intensa pelo TCR induz "selecção negativa", resultando na apoptose de linfócitos potencialmente autoreactivos. A sinalização pelo TCR é também essencial para o desenvolvimento tímico de Tregs. Pensa-se que a sua selecção ocorre num intervalo muito restrito de afinidade das ligações TCR-ligando, entre a selecção positiva de células T convencionais e a selecção negativa de linfócitos T autoreactivos com TCR de alta afinidade. Neste sentido, tem sido atribuída às Tregs a característica peculiar de possuírem uma autoreactividade aumentada.

Actualmente, o factor de transcrição Foxp3 (forkhead box P3) é considerado o marcador mais adequado para a identificação de Tregs. O seu papel essencial no desenvolvimento e função de Tregs é suportado pela associação de mutações de Foxp3

com o síndrome fatal IPEX (*immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome*, síndrome auto-imune linfoproliferativo severo) e também pelo síndrome autoimune fatal observado em ratinhos *scurfy*, "naturalmente" deficientes em Foxp3. Outras moléculas cuja expressão tem também sido associada a Tregs incluem a cadeia α do receptor de interleucina 2 (IL-2Rα, CD25), CTLA-4 (cytotoxic T-lymphocyte antigen 4), o ectoapirase CD39 e baixos níveis da cadeia α do receptor de IL-7 (IL-7Rα, CD127).

Neste estudo investigámos o desenvolvimento de Tregs, identificadas pela expressão de Foxp3 e de outros marcadores associados, em timos de indivíduos em idade pediátrica. Em particular, perguntámos quando ocorre o comprometimento dos timócitos para a linhagem de Tregs durante o desenvolvimento, uma vez que esta questão é relevante para o repertório das Tregs. Perguntámos ainda como se compara o desenvolvimento das Tregs com o de linfócitos T convencionais.

Na primeira parte do trabalho analisámos o padrão de expressão de Foxp3 em timócitos humanos e murinos. Os nossos resultados revelam a expressão de Foxp3 em fases anteriores ao estadio DP, nomeadamente em timócitos no estadio CD4ISP em humanos e no estadio CD4- CD8- duplamente negativo 4 (DN4) em ratinhos. Mostram ainda que, em ratinhos, células Foxp3+ DN4 possuem rearranjos genéticos da cadeia α do TCR (TCRα). Recorrendo ao ratinho deficiente em TCRα como uma ferramenta genética mostramos que, na ausência de TCRα, a expressão de Foxp3 no estadio DN4 é abolida. Colectivamente, os nossos dados revelam a expressão de Foxp3 em fases pré-DP em timócitos humanos e murinos e confirmam que a expressão precoce de Foxp3 é dependente do TCR.

Na segunda parte do trabalho estudámos a indução de Foxp3 no estadio DP e a possível diferenciação de timócitos Foxp3+ DP no estadio mais maturo Foxp3+ SP. Usando um ensaio de reexpressão, que consiste na clivagem de moléculas de superfície usando pronase e na síntese e reexpressão em cultura de moléculas transcripcionalmente activas, mostramos que a expressão de Foxp3 na fase DP ocorre em "verdadeiras" células DP. Mostramos ainda que os timócitos Foxp3+ DP apresentam um fenótipo maturo e uma distribuição das famílias TCR Vβ que se assemelha à dos timócitos DP que não expressam Foxp3. Identificamos também uma população de timócitos Foxp3+ DP que apresenta um aumento do programa transcripcional associado com o fenótipo activado/supressor na periferia, expressando elevados níveis de marcadores associados com Tregs, tais como CD25, CTLA-4, CD39 e MHC classe II (HLA-DR). Esta população está também presente em timócitos Foxp3+ CD4SP, nos quais a intensidade de expressão

dos marcadores supramencionados parece decair antes da exportação das células do timo para a periferia. Os timócitos Foxp3+ CD8SP não apresentam a referida população, mas expressam a molécula CD103 (α_E), constituinte da integrina $\alpha_E\beta_7$. A expressão de CD103 está associada com a migração para a mucosa, o que pode explicar os níveis reduzidos de Tregs CD8 circulantes. Mostramos ainda que a molécula CD103 está já presente em células Foxp3+ no estadio DP, o que sugere uma possível relação precursor-progenia entre os timócitos Foxp3+ CD103+ DP e CD8SP. A possível diferenciação de timócitos Foxp3+ DP em Foxp3+ SP foi analisada com base num modelo estatístico de análise de regressão múltipla. Este modelo suporta um contributo significativo das células Foxp3+ DP para o *pool* Foxp3+ SP. Em resumo, os nossos dados indicam que Foxp3 é induzido na fase DP e que uma parte significativa dos timócitos Foxp3+ DP se diferencienciam em células Foxp3+ SP, que irão incorporar o *pool* de Tregs na periferia.

Em contraste com o que é observado na periferia, mostramos que uma fracção significativa dos timócitos Foxp3+ DP expressa níveis consideráveis do receptor de IL-7. A funcionalidade deste receptor à superfície dos timócitos Foxp3+ DP foi confirmada através do estado da fosforilação da molécula STAT5 (*signal transducer and activator of transcription 5*), associada à acção da IL-7, após estimulação de timócitos com esta citoquina. Mostramos ainda que IL-7 induz o aumento de intensidade de CD25 nos timócitos Foxp3+ DP em cultura. Estes resultados sugerem que a IL-7 poderá ter um papel significativo no desenvolvimento timico de Tregs em humanos. Esta observação pode ser de importância particularmente significativa em situações onde há alterações dos níveis de IL-7, tal como no contexto de situações clínicas de linfopénia como observado, por exemplo, na patologia do HIV.

Em conclusão, os nossos dados suportam que a expressão de Foxp3 em timócitos humanos requer sinalização pelo TCR e ocorre antes da decisão CD4 vs. CD8. Mostramos que os timócitos Foxp3+ DP expressam um fenótipo activado semelhante ao apresentado em Tregs activadas na periferia mas associado à expressão de receptor de IL-7 funcional, o que naquela fase possivelmente protege os timócitos de apoptose/selecção negativa. Identificámos ainda uma subpopulação das células Foxp3+ DP que expressa CD103 e que está provavelmente na origem das células Foxp3+ CD8SP, com migração preferencial para a mucosa. Finalmente, modelos de análise de regressão múltipla suportam que um comprometimento significativo para a linhagem Treg ocorre na fase DP, com possíveis implicações para o repertório e função da população de Treg.

Palavras-chave: Estudos humanos, Timo, Desenvolvimento de Células T, Células T reguladoras, Foxp3.

SUMMARY

The thymus is a primary lymphoid organ crucially involved in the development of T cells. While producing a self-tolerant T cell repertoire that warrants surveillance to foreign pathogenic threats, the thymus also produces a lineage of regulatory T cells (Tregs) that are deeply involved in the control of immune responses, particularly in the context of inflammatory and autoimmune processes.

Differentiation of blood-derived T cell progenitors to mature CD4 or CD8 T cells in the thymus is accomplished by sequential development through a series of stages that can be defined by the expression of CD3, CD4 and CD8. In humans, CD4-CD8-CD3- triple negative (TN) cells first acquire CD4 (CD4 immature single positive stage, CD4ISP stage) and then CD8 to become CD4+CD8+ (double positive stage, DP stage) cells. Upon T cell receptor (TCR) gene rearrangements, DP cells "test" their TCR for recognition of the antigen-presenting molecules named major histocompatibility complex (MHC). While cells that are unable to recognize self-MHC undergo "death by neglect", DP thymocytes bearing TCRαβ complexes that can engage self-MHC molecules are signaled to survive, a process designated as "positive selection", and to differentiate into functionally mature CD4 single positive (CD4+CD8-CD3high, CD4SP) or CD8 single positive (CD4-CD8+CD3high, CD8SP) T cells. Data from mouse models suggest that the strength and duration of TCR signaling is determinant in CD4 vs. CD8 lineage decision during thymic differentiation. Strong TCR signaling induces "negative selection", resulting in programmed cell death of potentially auto-reactive cells. TCR signaling has been shown to be essential for Treg differentiation in the thymus. It has been proposed that Treg selection may occur within a very narrow window of affinity of TCR-ligand interactions, between positive selection of conventional CD4 T cells and negative selection of high-affinity self-reactive T cells. In agreement, increased self- reactivity is considered a unique feature of Tregs.

At present, the forkhead winged-helix transcription factor Foxp3 is considered the best available marker to identify Tregs. The essential role of Foxp3 in Treg development and function is supported by the association of Foxp3 defects with the fatal condition IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome) and also by the lethal autoimmune syndrome observed in *scurfy* and Foxp3 knock-out mice. Other molecules whose expression has also been associated with Treg

function include the interleukin 2 receptor α chain (IL-2Rα, CD25), the cytotoxic T-lymphocyte antigen 4 (CTLA-4), the ectoapyrase CD39 and low levels of the IL-7 receptor α chain (IL-7Rα, CD127).

We have investigated the development of human Tregs, as identified by the expression of Foxp3, as well as of other Treg-associated markers, in human pediatric thymuses. Specifically, we asked when commitment to the Treg lineage occurred during T cell development, since this is particularly relevant for the generation of the Treg repertoire. In addition, we assessed the developmental pathway of Tregs in the thymus. In particular, we asked whether Treg development occurred similarly to what is observed in conventional, Foxp3- T cells.

In the first part of the work we dissected the early expression pattern of Foxp3 in human and murine thymocytes and showed that Foxp3 expression can be detected in pre-double positive (pre-DP) subsets, particularly in CD4ISP thymocytes in humans and in double negative stage 4 (DN4) cells in mice. We further showed that Foxp3-expressing murine thymocytes at the DN4 stage possess productive TCRα gene rearrangements. In addition, using the TCRα-deficient mouse model as a genetic tool we have found that Foxp3 expression was abolished in TCRα-deficient DN thymocytes. Collectively, our data have identified the pre-DP expression of Foxp3 in human and murine thymocytes and confirmed the TCR-dependency of the early Foxp3 expression in pre-DP thymocytes.

In the second part of the work we studied the induction of Foxp3 at the DP stage and the possible differentiation of Foxp3+ DP thymocytes into the SP stages. Using a re-expression assay, where surface molecules were cleaved using pronase and transcriptionally active molecules were allowed to be synthesized and re-expressed, we were able to show that Foxp3 expression at the DP stage occurs in *bona fide* DP thymocytes. We also showed that Foxp3+ DP thymocytes have a mature phenotype and a TCR Vβ family distribution that closely resembled that of Foxp3 negative DP thymocytes. We further found that a population within Foxp3-expressing DP thymocytes presented an up-regulation of the transcriptional program associated with an activated/suppressor phenotype in the periphery and expressed high levels of the Treg-associated markers CD25, CTLA-4, CD39 and MHC class II (HLA-DR). This population was also found within Foxp3+ CD4SP thymocytes, in which the expression of those markers appeared to decline prior to thymic egress. While Foxp3+ CD8SP

thymocytes were devoid of high expression of those markers, they homogeneously expressed CD103 (α_E), which is part of the $\alpha_E\beta_7$ integrin. CD103 expression is associated with migration to the mucosa, which might explain the very low levels of circulating CD8 Tregs. In addition, we found that Foxp3+ cells expressing CD103 were already present at the DP stage, indicating possible precursor-progeny relationship between Foxp3+CD103+ DP and CD8SP thymocytes. In order to further investigate a possible direct precursor-progeny association between Foxp3-expressing DP and SP cells we performed statistical analysis of our data using multiple regression analysis. This model supported the significant weight of Foxp3+ DP cells to the SP pool. Overall, our data thus indicate that considerable Foxp3 induction occurs at the DP stage and that a significant part of Foxp3+ DP thymocytes probably progresses to the SP stages, and will incorporate the Treg pool upon thymus egress.

In addition, and in clear contrast to what is observed in the periphery, we found that a large proportion of Foxp3+ DP thymocytes expressed considerable levels of IL-7 receptor (IL-7R). We assessed the functionality of IL-7R at the surface of Foxp3+ DP cells and showed that phosphorylation of STAT5 ("signal transducer and activator of transcription 5"), a downstream target of IL-7, was readily observed upon exposure to that cytokine. Furthermore, short-term culture with IL-7 induced the upregulation of CD25 on Foxp3+ DP thymocytes. Together, these results suggest that IL-7 may play a role in the development of human Tregs. Although requiring further investigation, this finding may be of particular significance in the context of situations where IL-7 levels are altered, such as lymphopenia, as observed in HIV pathology.

In conclusion, our data support that the expression of Foxp3 in human thymocytes requires TCR signaling and occurs prior to CD4 or CD8 lineage commitment. Foxp3+ DP thymocytes were shown to express an activated phenotype that is reminiscent of that found in peripheral activated Tregs but is associated with the expression of a functional IL-7 receptor, which may protect them from cell death/negative selection. A subpopulation of Foxp3+ DP expressing CD103 is likely to give rise to a population of Foxp3 CD8 T cells with preferential homing to the mucosa. Finally, multiple regression models support that a significant commitment to the Treg lineage occurs at the DP stage, with possible implications for Treg repertoire and function.

Keywords: Human studies, Thymus, T cell development, Regulatory T cell, Foxp3.

Chapter I

Introduction

CHAPTER I: INTRODUCTION

A. HUMAN T CELL DEVELOPMENT AND INSIGHTS FROM MOUSE MODELS

1. THE THYMUS: BRIEF HISTORICAL PERSPECTIVE

The word "thymus" is thought to come from the Latin derivation of the greek *θυμός* ("*thymos*"), meaning "warty excrescence", but also "soul" or "spirit", and the ancient Greeks believed it to be the seat of the soul. Later, it was also considered to be the organ of purification of the nervous system, a protective thoracic cushion and a center of regulation of fetal and neonatal respiration (1).

Originally described by Claudius Galen of Pergamum (130-200 AD) during his precise descriptions and studies of neurological functions and anatomy, the thymus was considered an "organ of mystery" for almost 2000 years. Galen observed that the thymus was proportionally largest in size during infancy and that it shrunk with aging (1). Studies on the evolution of thymic size during fetal and infant life were also performed by William Hewson in 1777. He further noted that the thymus was filled with 'particles' resembling those in lymph and blood, and believed that "the thymus exists during the early periods of life only when those particles seem to be the most wanted" (2).

By the mid 50's the lymphopoietic function of the thymus was established. However, immunologists still did not believe it to have an immunological function and considered the thymus an evolutionary redundant "lymphocyte graveyard" (2). In the early 1960's, Jacques Miller's work on the role of the thymus in mouse lymphocytic leukemia and the impact of neonatal thymectomy in tolerance (3-5) lead the way for the recognition of the thymus as a non-redundant specialized organ of the immune system with a unique function in the establishment and maintenance of the T cell pool.

2. PHYLOGENY, ONTOGENY AND STRUCTURE OF THE THYMUS

The thymic structure has not been identified in species more primitive than jawed vertebrates and is a common feature to all of them (6). Its precise embryological origin, the number of organs per animal and the final anatomical positions all vary markedly in different species. In spite of this, they share their origin from pharyngeal pouches, which arise as specialized pockets of the foregut endodermal tube and harbor primordia for organs and tissues later found in chest, neck, or head regions, including the thymus and the parathyroid gland (7).

The human thymus is located anatomically in the anterosuperior mediastinum, in front of the heart and behind the sternum. It achieves the maximum absolute size during the first year of life (8). The thymus undergoes a process named "involution", whereby it starts reducing its mass and changing its architecture with age, and extensively fills with adipose tissue after puberty (1, 8).

The thymus consists of two distinct lobes joined by a connective tissue isthmus. A thin connective tissue capsule surrounds each lobe and, in most species (although not in mouse), gives rise to septa that partially subdivide the thymus into interconnecting lobules of variable size and orientation. It is morphologically similar across species, being divided into cortical and medullary regions separated by a vascular corticomedullary zone (9, 10). Those areas can be distinguished histologically (Figure 1). While the cortex contains densely packed immature thymocytes within a sparse thymic epithelial framework, the medulla is less dense and contains prominent epithelial cells and Hassall's bodies (or Hassall's corpuscle). First described by the British physician Arthur Hill Hassall in 1849, Hassall's bodies are concentric structures composed of stratified keratinizing epithelium that are rare in rodents but rather frequently found in humans and other primates (9-11). They were suggested to represent the "graveyard" for dead thymocytes or the "privileged" area for the maturation of medullary thymocytes, and also to be active in cell signaling, transcription and metabolism mediated by cytokines or growth factor receptors (11-17).

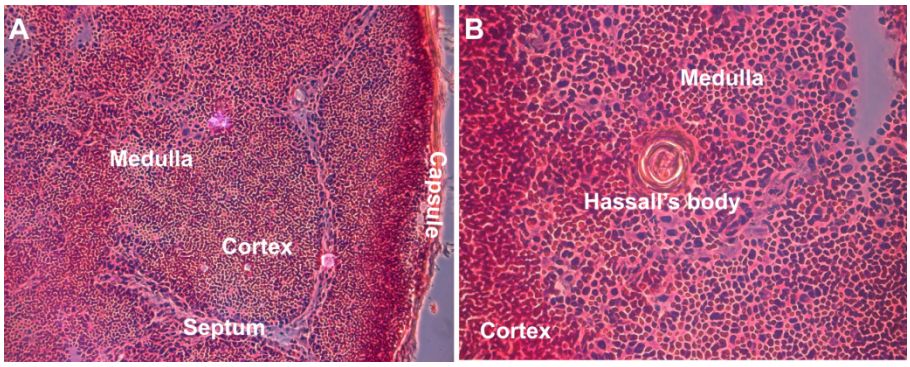


Figure 1: Structure of the human thymus. Hematoxylin/eosin staining of a section of a pediatric thymus (7 year old boy). The cortex is composed of densely packed thymocytes (A, B) while in the medulla thymocyte density is lower and contains Hassall's bodies (B). Magnification: 10x (A), 40x (B).

The epithelial thymic primordium begins its development at the end of the 4th gestational week (GW) and it is present by the 7th GW, with a distinct organization into a peripheral and central cell layer by the 8th GW (18). Seeding of the thymus is a very early event, with the first lymphoid progenitor cells arriving by the 8th GW via the peripheral blood (18-20). At this time progenitor cells that seed the thymus originate from the fetal liver (19, 20). Colonization of the thymic primordium by T cell progenitors is necessary for further development of the thymic microenvironment, including the compartmentalization into cortex and medulla and the development of Hassall's corpuscles (21, 22). Mature T cells are not detected in the thymus before 12 to 13 GW and progressive colonization of the periphery occurs between 13 and 14 GW (23-26). After the 16th GW fetal liver precursor cells colonize the bone marrow, which becomes the main source of thymic progenitors from the 22nd GW onwards throughout adult life (20).

The thymus has no afferent lymphatics, and thymic progenitors are thought to enter the thymus through the large venules at the corticomedullary junction (CMJ). T cells re-enter the circulation through the vascular lining of post-capillary venules at the CMJ, where efferent lymphatics drain into an adjacent pair of lymph nodes (10).

3. COMPOSITION OF THE THYMIC STROMA

The unique capacity of the thymus to efficiently promote T cell differentiation and repertoire selection is mediated by thymic epithelial cells (TECs), which are the major constituents of the thymic stroma. This epithelial nature of the thymus makes it a unique lymphoid organ (10). Cortical and medullary TECs are currently thought to be mainly derived from a bipotential cTEC-mTEC progenitor population present in the thymus anlage and possibly persisting beyond the embryonic period (27-29). Nonetheless, epithelial cells of the thymic microenvironment are very heterogeneous, and distinct cell types occur in the subcapsular zone, cortex, medulla and Hassall's corpuscles (18). They differ in their ultrasctructural characteristics, antigenic expression and their capacity to synthesize thymic hormones, which are important for intrathymic maturation and modulation of lymphocyte responsiveness (10).

The thymic stroma also comprises several non-epithelial cell types, such as endothelial cells, which contribute to the vasculature, and mesenchymal cells, that influence TECs and T cell progenitors (29). Bone-marrow derived cells are also present, including dendritic cells (DC), B cells and macrophages, involved in the shaping of the T cell repertoire and the elimination of apoptotic thymocytes (9, 29). Macrophages are

found throughout the thymus but occur at higher density in the cortex, while DCs are frequently present along the thymic CMJ and medulla (18) and B cells are mostly found in the medulla (30, 31).

4. OVERVIEW OF T CELL DEVELOPMENT

The highly ordered thymic architecture plays a crucial role in normal thymic function. Phenotypically distinct thymocyte populations are present in specific thymic areas (Figure 2). Progenitor cells lacking CD4 or CD8 expression (double negative cells, DN) enter the thymus at the CMJ and move through the cortex to the subcapsular zone, where they undergo early events. Large, mitotically active lymphoblasts can be found in the subcapsular zone (10, 29). The thymic cortex contains smaller CD4+CD8+ double positive (DP) thymocytes, while in the medulla single positive CD4+ CD8- (CD4SP) and CD4- CD8+ (CD8SP) T cells are screened for self reactivity before leaving the thymus through the CMJ (9, 29).

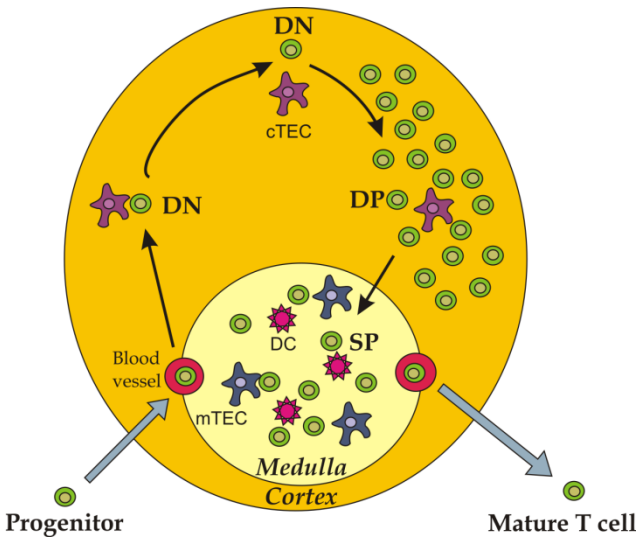


Figure 2: Thymic T cell development. T cell progenitors enter the thymus through the blood vessels at the CMJ. Development of CD4-CD8- double negative (DN) cells is accompanied by an outward movement towards the subcapsular zone. CD4+CD8+ double positive (DP) cells scan the cortex for positively selecting ligands. After positive selection and lineage commitment, single positive (SP) cells move to the medulla, where they scan medullary antigen presenting cells, mainly dendritic cells (DCs) and medullary thymic epithelial cells (mTECs), before they leave the thymus. cTEC: cortical TEC. Adapted from (32).

5. T CELL COMMITMENT AND EARLY T CELL DEVELOPMENT

Hematopoietic precursors in humans are present within a population of cells that express CD34 (33). CD34 is expressed on pluripotent stem cells, as well as on progenitor cells that are already committed to particular hematopoietic lineages, but not on their differentiated progeny (34-36). In the thymus, CD34+CD38lo cells include the most immature population, based on TCR rearrangement status (Figure 3) (37) and on their T, natural killer (NK) and DC precursor potential (36, 38). The presence of multipotential precursors in the human thymus indicates that T cell commitment takes place within the thymus itself (39). This is also supported by the fact that TCR gene rearrangements, which are the most definite marker for T cell commitment, were not found in fetal liver or cord blood CD34+ cells (40, 41). Neonatal cord blood CD34+ cells were also found not to express recombinase activating gene (RAG-1) or pre-TCRα mRNA (41). Thus, at least a proportion of the precursors that seed the human thymus are multipotential and commit to the T cell lineage in the thymus (20).

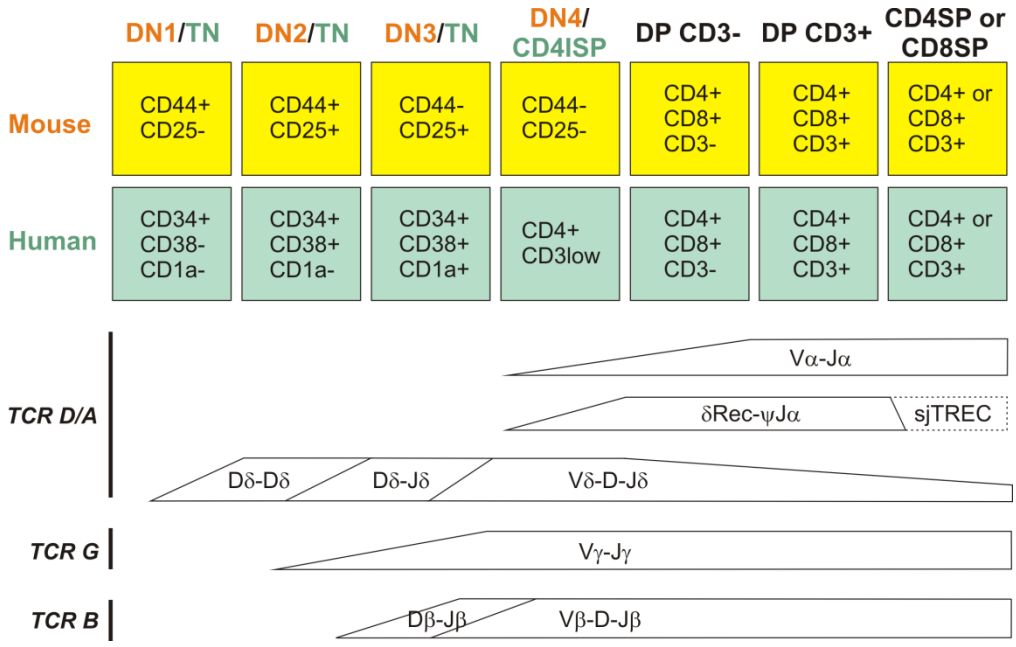


Figure 3: Stages of T cell development in human and mouse thymus and occurrence of TCR rearrangements. Parallel between the stages of T cell development in human and mouse and timing of TCR rearrangements. TN, triple negative (CD4-CD8-CD3-); CD4ISP, CD4 immature single positive (CD4+CD8-CD3low). Adapted from (37).

Cells expressing the highest levels of CD34 lack CD1a, a molecule that is widely expressed in cortical thymocytes (42, 43). Appearance of CD1a on CD34+ thymocytes marks an important checkpoint in early T cell development, since it is associated with the induction of T cell commitment, as CD34+CD1a+ thymocytes largely lose the ability to develop into non-T cells (36). CD34+CD1a+ cells, in contrast to CD34+CD1a- precursors, have strong T cell, but little NK-cell, and no DC or plasmacytoid DC (PDC) precursor activity (36). CD34 is downregulated in the subsequent population, which expresses CD4 but not CD8 or surface CD3, and is known as CD4 immature single positive (CD4ISP) (42, 44). CD4ISP cells are fully committed to the T cell lineage, since they lack NK or DC precursor activity but can develop into T cells, as demonstrated using a hybrid human-mouse fetal thymic organ culture (hmFTOC) system in which human thymocytes are cultured in murine fetal thymic lobes (45).

Human T cell development is critically dependent on the cytokine interleukin 7 (IL-7). The IL-7 receptor consists of two chains, an α chain (IL-7Rα) and a common γ chain (γc), shared with the receptors for IL-2, IL-4, IL-9, IL-15 and IL-21. Genetic defects in genes encoding for γc (46, 47), IL-7Rα (48, 49) or the Janus kinase Jak3, a component of the IL-7-induced signaling transduction pathway (50, 51), are associated with profound T cell deficiency and account for most severe combined immune deficiencies (SCID). The precise function of IL-7 on human T cell development is not completely understood. CD34+ thymic T cell progenitors proliferate in response to IL-7, and development from CD34+ precursors in a fetal thymic organ culture (FTOC) in the presence of inhibition of IL-7R signaling almost completely blocks the transition of CD34+CD1a+ cells into CD4ISP (52). Furthermore, IL-7 may be directly involved in the induction of TCRβ V-D-J rearrangements, although this is yet to be confirmed (53). In support of this, IL-7 addition enhances the levels of episomal DNA resulting from TCR gene rearrangements, known as TCR rearrangement excision circles (TREC; see section 7), in human thymic organ cultures (TOCs) (54).

Notch proteins constitute a family of highly conserved transmembrane receptors whose receptor-ligand interactions have also been shown to play a crucial role in T cell development (55-58). This is supported by studies where cord-blood and bone marrow CD34+ cells co-cultured with the murine bone marrow stromal cell line OP9 expressing the Notch ligand DL1 (delta-like 1) were shown to undergo full T cell development (58, 59). On the other hand, the γ-secretase inhibitor DAPT (N-[N-(3,5-difluorophenyl)-L-

alanyl]-Sphenylglycine t-butyl ester), which inhibits Notch signaling, strongly impaired T cell development in both the FTOC (60) and OP9-DL1 (61) systems. However, inhibition of TCRαβ development by DAPT was rescued by forced expression of intracellular Notch1 by retrovirus-mediated gene transfer (61). Furthermore, TCRβ V-DJ but not D-Jβ rearrangements were strongly inhibited by DAPT (60). These findings indicate that Notch is required for induction and maintenance of T cell specification.

6. EARLY TCR REARRANGEMENTS AND β-SELECTION OF DEVELOPING THYMOCYTES

The TCR expressed by the majority of T cells is a heterodimer composed of α and β chains. TCR diversity is generated by random rearrangement of multiple germline-encoded variable (42 Vα and 47 Vβ segments), diversity (2 Dβ segments), and joining (61 Jα and 13 Jβ segments) gene segments, non-germline-encoded N region insertions, and α and β chain pairing (62, 63). In humans, an estimated $>10^{18}$ different TCR can be produced and the diversity of TCR in a human at any given time has been estimated to be $> 2x10^7$ (63).

During the early stages of T cell development, the TCR loci undergo rearrangement in the sequence *TCR D>G>B>A* (37, 64, 65). The current model of TCR rearrangements in early T cell development, as described by Dik et al. (37), is depicted in Figure 3. However, there is some controversy with respect to the cell phenotype in which each rearrangement of particular loci occurs, probably due to the sensitivity of the methods used to analyze TCR rearrangements.

Studies in the mouse have shown that productive rearrangement of *TCRB* genes leads to the transport to the cell surface of a pre-TCR complex consisting of TCRβ protein associated with an invariant pre-TCRα (pTα) chain and the CD3 complex (66-69), and the presence and association of those molecules has also been shown to occur in human thymocytes (70, 71). Signaling through the pre-TCR induces survival, proliferation, differentiation and initiation of *TCRA* gene rearrangements (69, 71). This process, known as "β-selection", represents the first checkpoint of T cell development, as cells that fail to generate a TCRβ chain do not proceed along the αβ lineage differentiation pathway (69). In the mouse, this checkpoint mainly occurs at the DN3 stage and has been associated with the expression of CD27 (72, 73)

In humans, occurrence of TCRβ rearrangements and ensuing β-selection has generated some debate. While a PCR-based and GeneScalling analysis found some productive TCRβ V-DJ rearrangements within CD34+CD1a+ cells (37), a less sensitive southern blot analysis detected the first TCRβ V-DJ rearrangements in CD4ISP cells (65). Intracellular TCRβ protein can be detected in CD4ISP cells and at higher frequency at the subsequent CD4+CD8α+CD8β- stage (early double positive stage, EDP) (65, 74). However, intracellular TCRβ negative populations have been found in CD4+CD8α+β- DP cells (65, 75), indicating that not all populations downstream of CD34+CD1a+CD4- cells are post-β selection. A study by the Toribio group (75) has located the β-selection checkpoint later in development, at the transition from the CD4+CD8α+CD8β- to the CD4+CD8α+CD8β+ stage, since the later subset expresses surface TCRβ and pTα. However, in this study no surface pre-TCR was found in EDP cells, while a low level of CD3 was detected on a small proportion of EDP in the study of Blom et al. (65). Furthermore, pTα mRNA is mainly present at the CD4ISP stage (70, 71, 76) and is downregulated but still expressed in EDP and TCRαβ- DP cells (65, 70, 71), while it is absent at other stages of development (70, 71), supporting the possibility that β-selection occurs at early stages of human T cell development.

Recently, CD28 expression at the CD4ISP stage has been described as a marker of cells that have passed the β-selection checkpoint (76). CD4ISP CD28+ thymocytes express intracellular TCRβ and differentiate into CD3+ TCRαβ+ cells in the OP9 system, in the absence of Notch signaling (76). In this study pTα mRNA was shown to be highly expressed in CD4ISP CD28- cells and almost completely downregulated in CD4ISP CD28+ thymocytes. In contrast, expression of TCR Cα, which marks opening of the TCRα locus (73), was strongly and specifically induced in CD4ISP CD28+ thymocytes (76). CD4ISP TCRβ+ cells had been previously suggested to be in cycle, to express elevated levels of CD1a, CD4, CD28, CD45RO and CD71 and to differentiate in FTOC into TCRαβ+ T cells with faster kinetics than CD4ISP TCRβ- cells (39). These data further indicate that β-selection can occur within the CD4ISP population.

Overall, it is considered that β-selection in human thymocytes may not be directly associated with a particular stage. It may begin in a small subset of CD34+CD1a+CD4- cells, while a larger proportion occurs in cells that have up-regulated the expression of CD4, and subsequently CD8α or both CD8α and CD8β.

7. TCRα REARRANGEMENTS IN IMMATURE THYMOCYTES

Rearrangements at the *TCRA* locus are initiated after TCRβ-expressing cells have expanded, as a result of β-selection, and returned to slow cycle conditions (53, 71).

The human *TCRA* and *TCRD* gene segments are interspersed on chromosome 14q11, with the *TCRD* locus being located between the Vα and Jα gene segments (Figure 4) (77). Therefore, deletion of the *TCRD* gene plays an important role in the differentiation of TCRαβ cells. Excision of the δ locus during TCRα gene rearrangements results in the generation of DNA circles that persist episomally, producing two types of TRECs that are identical in ~70% of T cells (78). Two non-functional TCRδ-deleting gene segments, δRec and ψJα, have been identified that flank the major part of the TCRδ gene (79). The high frequency of δRec-ψJα rearrangements in human thymocytes suggests that a major fraction of the thymocytes use this rearrangement to delete their TCRδ genes (79). Rearrangement of δRec to ψJα produces signal-joint (sj) TRECs. Subsequent Vα-Jα rearrangement deletes the remaining Vδ gene segment and produces coding-joint (cj) TRECs. Since the α locus is not subject to allelic exclusion (80, 81), a maximum of two sjTRECs and two cjTRECs may be present per cell (53).

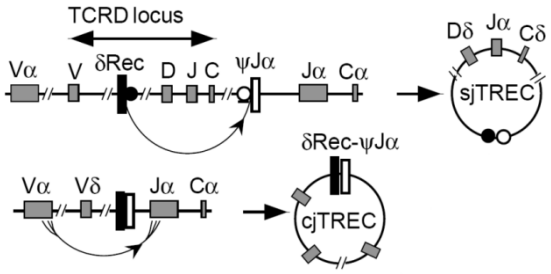


Figure 4: Generation of sjTRECs and cjTRECs. Simplified representation of the *TCRD* locus flanked by portions of the *TCRA* locus (Vα, Jα and Cα). From (82).

TCRA rearrangements were proposed to occur mainly in the small CD3- DP population (64, 71). In the mouse, productive *TCRA* rearrangements and transcripts have been shown to already be present at the DN stage (83), despite being generally associated with the wave of expression of RAG-1/RAG-2 at the DP stage (84). In agreement, Dik et al. recently showed, using the more sensitive real time quantitative PCR technique, that δRec-ψJα rearrangements in humans can already be detected at the CD4ISP stage (37). Furthermore, analysis of T-early α (TEA), whose transcription initiates TCRA recombination by opening the 5' site of the Jα cluster, showed that TEA-Cα transcripts are already detected at the CD34+CD38+CD1a+ stage (37).

8. TCRαß T CELL DEVELOPMENT: INSIGHTS FROM MOUSE MODELS

a. Positive and negative selection of developing thymocytes

Studies in the mouse have shown that thymocytes expressing surface TCRαβ will only survive if their TCR forms an MHC-restricted receptor that is signaled by low affinity self-peptides, a process called "positive selection". Thymocytes that fail to signal through their TCR undergo apoptosis, which is termed "death by neglect", while high-affinity contacts will also result in programmed cell death, and this is known as "negative selection" or "clonal deletion" (Figure 5) (53). Experimental affinity measurements thus support the idea that TCR affinity for positive selection ligands is lower than that for negative selection ligands (85-87). However, the "selection paradox" that recognition of self is essential for thymocyte survival but may also induce cell death remains an incompletely resolved issue (32).

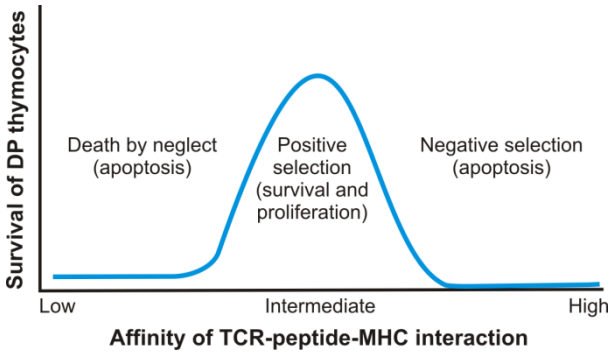


Figure 5: The affinity model of thymocyte selection. According to this model, the affinity of the TCR-peptide–MHC interaction is the key determinant of T cell selection. Only thymocytes with intermediate affinity receive a survival signal and undergo positive selection, CD4 or CD8 T cell lineage commitment and maturation to become part of the peripheral T cell pool. Low affinity for self-peptide–MHC complexes results in death by neglect, which is thought to account for 80–90% of the loss of thymocytes during thymic selection, while high-affinity binding induces cell death by apoptosis (negative selection or clonal deletion). DP: double positive. Adapted from (32).

Despite an inherent propensity of the TCRαβ receptor to bind to MHC, probably enforced by the expression of CD4 and CD8, the generation of MHC-restricted receptors from random α/β pairs is thought to be relatively infrequent (88-91). However, the α locus is structured such that multiple V/J recombination events can occur on the same allele, thus allowing several productive TCRα gene rearrangements to be tested per cell (92).

Efficient positive selection of developing thymocytes requires interactions with self-peptide-MHC complexes displayed by cTECs (93-96), and some studies have also assigned a role for cTECs in negative selection (29, 97). cTECs provide specialized

accessory interactions that MHC+ epithelial cells from other tissues do not (98). These accessory molecules are poorly defined, although CD83 has been shown to be involved in CD4 T cell development (29, 99). Despite studies demonstrating that intrathymic expression of MHC molecules by non-TECs, including thymocytes themselves, can support positive selection, the relative efficiency of this process and the nature of the TCR repertoire that is selected are unclear (100-104).

The physiological peptide-MHC repertoire expressed by cTECs *in vivo,* including the number and nature of the self-peptides involved in positive selection, and the way that these complexes might shape the polyclonal αβ T cell repertoire are largely unknown (32, 105). Positive selection of functional T cells apparently requires peptides with antagonist or partial agonist properties (106) that are structurally related to ligands that can fully activate mature T cells (32), thus emphasizing the importance of the quality (affinity) rather than the quantity (avidity) of the TCR–peptide–MHC interaction (32, 85). Furthermore, the generation of a fully diverse T cell repertoire *in vivo* has been shown to be positively correlated with the complexity of selecting ligands (105).

Evidence suggests that the TCR must remain engaged and sustain signaling for the duration of positive selection (107). In a study where Bousso et al. used two-photon microscopy and reaggregate thymic organ cultures (RTOCs), a system that allows the organotypic reaggregation of discrete thymocyte populations with stromal cells, to study thymocyte-stromal cell interactions these were shown to last on average 6 to 12h when MHC recognition occurred (108). Those interactions took place both as long-lived cellular associations displaying stable cell-cell contacts and as shorter, highly dynamic contacts (108).

Positive selection induces a signal that results in RAG gene repression, long-term survival, migration into the medulla and differentiation into mature T cells (105). The quality control of developing T cells through interactions with peptide-MHC complexes on medullary APCs is indispensible for central tolerance (32), as revealed by the severe manifestations of systemic autoimmunity in murine models where there is inhibition of positively thymocytes to enter the medulla or premature egress of thymocytes (109, 110), disorganization of the medullary architecture (111) or disrupted development of mTECs (112-116).

mTECs are unique in their "promiscuous" gene expression ability (117-119). A subset of mTEC selectively expresses the gene that encodes the autoimmune regulator AIRE, a transcriptional regulator of target genes that greatly controls the ectopic transcription of

numerous tissue restricted antigens (TRAs) in the thymus and is essential for T cell tolerance (119). AIRE is defective in the human autoimmune condition APECED (polyendocrinopathy-candidiasis-ectodermal dystrophy) (120, 121), and its deficiency in mice results in a reduction in the expression of TRAs in mTECs, defective tolerance induction and organ-specific autoimmunity (118, 122, 123). AIRE expression in mTECs enables intrathymic self-antigen presentation, thereby aiding in the establishment of thymocyte tolerance to peripheral antigens (29).

Promiscuous gene expression is thought to be a stochastic process, with only 1 to 5% of mTECs expressing a particular TRA (117, 124). However, it was recently found that mature mTECs are replaced every 2 to 3 weeks and are thus continuously altering the topology of antigen expression within the medulla (125, 126). Tolerance induction towards TRAs expressed in few dispersed mTECs may result from the numerous encounters with APCs in the 4 to 5 days that thymocytes spend in the medulla (127, 128) and/or from the transfer of mTEC-derived TRAs to neighbouring DCs (129, 130), which are known to have a central role as APCs in negative selection in the medulla and also in the cortex (100, 127, 131), thus increasing the probability that such antigens are encountered by developing thymocytes.

b. CD4 and CD8 lineage decision of developing TCRαß T cells

Positively selected DP thymocytes ultimately develop into CD4SP or CD8SP T cells, depending on whether they received signals through MCH class II or MHC class I, respectively (132). Classical models considered that CD4/CD8 lineage choice resulted in the transcriptional termination of one or the other coreceptor gene as a consequence of the same TCR signaling event that mediated positive selection, with the random or instructed nature of the termination of transcription underlying the "stochastic" (133-138) or "instructive" (139-142) models. Currently, CD4/CD8 lineage choice seems to be best explained by the kinetic signaling model (Figure 6), which proposes that lineage choice is determined by TCR-signal duration and that cytokines, such as IL-7, serve as "sensors" that detect that duration (143-145).

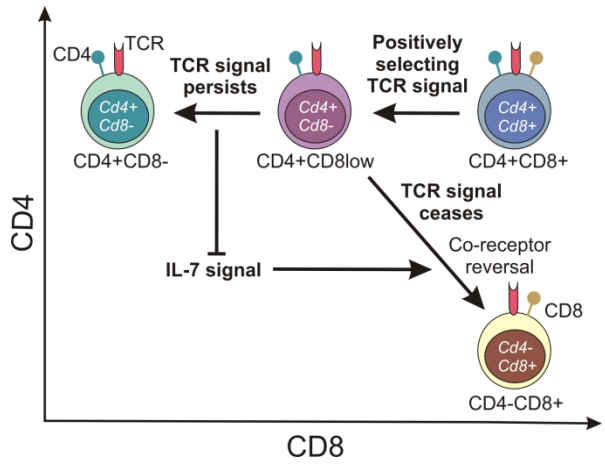


Figure 6: Kinetic signaling model of CD4/CD8 lineage choice in T cell development. According to this model, positively-selecting TCR signals induce DP thymocytes to downregulate *Cd8* transcription and thus become CD4+ CD8low. TCR-signal duration and cytokine signaling then determine whether a given thymocyte commits to the CD4 or the CD8 lineage. Adapted from (142).

In order to identify potentially useful TCRs, unselected DP thymocytes are unresponsive to other survival signals, such as IL-7, both through the downregulation of surface cytokine receptors and the expression of suppressor of cytokine signaling 1 (SOCS1), which is a potent intracellular suppressor of cytokine signal transduction (146, 147).

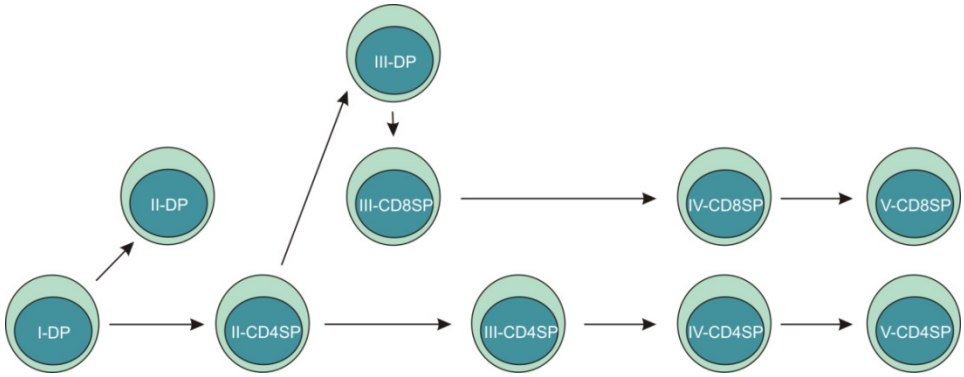
According to the kinetic model, TCR-signaled DP thymocytes first terminate their *Cd8* gene transcription and then assess the effect of the absence of CD8 on TCR signaling (142, 143). Whether TCR-mediated positive selection signals persist or cease in the absence of CD8 determines if the cell becomes a CD4 or CD8 T cell, respectively. This implies that positive selection and lineage choice are sequential and not simultaneous events, and that TCR-mediated positive selection signals terminate *Cd8* gene transcription and convert DP thymocytes into intermediate lineage-uncommitted *Cd4+Cd8-* cells, with lineage decision at this stage depending on whether TCR signaling persists or ceases (142). Since the progressive decrease in cell-surface CD8 eventually disrupts CD8-dependent signaling by MHC-class I-restricted TCRs, IL-7 or other γ chain cytokines are important for cell survival in the absence of TCR signaling (142, 148). Furthermore, IL-7 and other γ chain cytokines have been shown *in vitro* to promote "co-receptor reversal" by enhancing *Cd4* silencing and promoting reinitiation of *Cd8* gene transcription (143, 148).

Several transcription and nuclear factors have been identified that are involved in the regulation of *Cd4* and *Cd8* gene transcription. Th-POK (T-helper inducing POZ/Kruppel-like factor) seems to be both necessary and sufficient for CD4-lineage specification, probably acting as a master regulator of CD4 T cell differentiation (149,

150), while RUNX (runt-related transcription factor) proteins, in particular RUNX3, seem to promote CD8 T cell differentiation (151-153). Th-POK and RUNX3 negatively regulate each other's expression and thus reinforce lineage choices (154-158). TOX (thymus high mobility group (HMG) box protein) (159) and GATA3 (GATA-binding protein) (160) also appear to play a role in lineage commitment, particularly in CD4 lineage choice (142).

9. DEVELOPMENT OF HUMAN TCRαß T CELLS

The transitional stages that thymocytes undergo during their differentiation and maturation in the human thymus have mainly been studied using hmFTOCs and SCID-hu mice, a model that consists in transplanting small pieces of human fetal liver and thymus under the kidney capsule of SCID mice (Figure 7) (161-164).



CD3-TCR	Low	Low/+	Low/+	+	+	+	+
CD1a	+	+	+	+	+	-	-
CD69	-	+	+	+	+	+	Low
CD27	-	-	-	+	+	+	+
CD45RA	-	-	-	-	-	-	+
CD45RO	+	+	+	+	+	+	-
RAG transcripts	++	+	+	-	-	-	-
Development in hmFTOC	CD4SP and CD8SP	None	CD4SP and CD8SP	CD8SP	CD4SP	CD8SP or CD4SP respectively	CD8SP or CD4SP respectively

Figure 7: Stages of human T cell development. Delineation of human T cell development stages, as defined by the studies of the Vandekerckhove group in the 1990's using hmFTOCs, SCID-hu mice and pronase re-expression assays (161-164). Adapted from (53).

Expression of CD69 on human thymocytes is induced at the CD3low to CD3high transition and has been linked to positive selection (162). In support of this, CD69- CD3+ thymocytes were found to be corticosteroid-sensitive, whereas CD69+ CD3+ cells were resistant (162), and these populations express high and low levels of RAG-1 mRNA, respectively (163). Post-selection CD69+ thymocytes, which are initially CD27- CD1a+ CD45RA-, sequentially up-regulate CD27, down-regulate CD1a and eventually acquire CD45RA upon maturation (161-164). The identification of an early postselection CD69+CD27−CD4SP population that gives rise to both CD4SP and CD8SP cells when cultured in hmFTOC has been suggested to indicate that, similarly to what has been described in the mouse model, human DP thymocytes down-regulate CD8 expression soon after positive selection (162, 163). Cells committed to the CD4 lineage will then permanently stop the production of CD8, while CD8 lineage-committed cells will terminate the production of CD4 and start re-expressing CD8 (163).

Down-regulation of CD8β during positive selection appears to parallel CD69 up-regulation (163). The expression of CD8α and CD8β chains is differentially regulated on human thymocytes (Table 1) (74). On cells differentiating into CD4SP the expression of CD8β chains is more rapidly lost, whereas CD8αα chains may remain expressed well after the acquisition of CD27 (162).

TABLE 1: REGULATION OF CD8α AND CD8β EXPRESSION DURING HUMAN T CELL DEVELOPMENT.

Antigen	TN	CD4ISP	EDP	DP	CD4SP	CD8SP
CD4	-	+	+	+	+	-
CD8α	-	-	+	+	-	+
CD8β	-	-	-	+	-	+

TN: triple negative; ISP: immature single positive; DP: double positive; EDP: early DP; SP: single positive.

Interestingly, one study has suggested that CD69 expression might be maintained by the thymic microenvironment and only lost during or after emigration from the thymus (162). In the SCID-hu model, where virtually all human peripheral cells can be considered to be recent thymic emigrants (RTEs) due to their short half-life (<24h), RTEs were found to resemble that of cord blood T cells, being mostly CD3highCD69-CD27+CD1a-CD45RA+ (162). However, around 10 to 30% of the emigrant T cells have a CD45RA-/dullCD45RO+ phenotype, indicating that both CD45RA+CD45ROdull and CD45RA-/dullCD45RO+ thymocytes emigrate from the thymus (162). Accordingly, it has been shown that a minor fraction of cord blood T cells are CD45RA-/dull, and that

these cells are functionally immature, likely corresponding to recently emigrated thymocytes (165).

TRECs (see section 7) have been proposed by Douek et al. to represent a molecular marker of recent thymic emigrants in humans, as had been previously proposed in chickens (166), allowing the assessment of thymic output in the periphery (82). Studies in the mouse have shown that TRECs are stable (167) and are not duplicated during mitosis, and are thus diluted out with cell proliferation (168). sjTREC levels were found to be predominantly present in CD45RA+ naïve but not in CD45RO+ memory peripheral blood, reflecting the enrichment in RTEs and/or the lack of extensive cell division of the former population (82). Moreover, they were lower in thymectomized individuals as compared to age-matched controls, further supporting the thymic origin of sjTRECs (82). Although sjTRECs do decline with age, they are still found in 80 year old individuals, indicating that thymic function persists throughout life (82).

Other markers that have been described to be preferentially, but not exclusively, expressed on RTEs are the cell-surface molecules CD31 (platelet endothelial cell adhesion molecule 1, PECAM-1) (169) and CD103 (α_E component of the $\alpha_E\beta_7$ integrin) (170) on naïve CD4 and CD8 T cells, respectively, which identify populations enriched in TRECs.

B. DEVELOPMENT OF REGULATORY T CELLS

1. REGULATORY T CELLS: IDENTIFICATION AND ROLES IN HEALTH AND DISEASE

Although the existence of T-cell mediated immune suppression was highly controversial several decades ago and the suppressor-T-cell concept was abandoned in the 1980's due to its elusive nature, studies in 1995 by Sakaguchi et al. hinted at a rebirth of the idea of a population of cells specialized in immune suppression (171). Classical experiments showed that thymectomy of mice 3 days after birth prevented the export of Tregs from the thymus into the periphery and predisposed the mice to systemic autoimmune diseases, which could be ameliorated through the transfer of thymocytes or splenocytes from adult euthymic mice (11, 172-175). Sakaguchi and colleagues described a population of IL-2 receptor α chain (CD25)-expressing CD4+ T cells that were capable of suppressing immune responses in a variety of experimental models, including neonatal thymectomy (171). Several mechanisms of Treg-mediated suppression have been described, mediated by secreted soluble factors, including the production of anti-inflammatory cytokines, by direct cell-cell contact or by modulating the activation state and function of antigen presenting cells (176). Noteworthy, once activated by a particular antigen, Tregs can suppress responder T cells irrespective of whether they share antigen specificity with the Treg (177).

The presence in humans of CD4+CD25+ T cells that exhibited similar features to the homologous mouse CD4+CD25+ regulatory T cell subset has also been demonstrated (178-182). However, CD25 expression in human peripheral blood CD4 T cells does not define a distinct population, as it does in mouse. Furthermore, while up to 30% of human CD4 T cells express low levels of CD25, only the 2–4% expressing the highest levels of CD25 have been shown to be functionally suppressive and are considered Tregs (183). The majority of CD4+ CD25high cells express the memory marker CD45RO, CD62L (L-selectin) and the IL-2Rβ chain (CD122) (182, 183). Within this population, HLA-DR expressing cells have been shown to have the highest suppressive capacity (184). The *in vitro* characteristics of the human CD4+CD25high T cells were similar to those observed in the murine population. For instance, Tregs did not proliferate when stimulated through their TCR in the absence of exogenous IL-2 and were able to suppress the activation of other T cells in a cell-cell contact dependent manner (183). Furthermore, Tregs in both humans and mice could suppress both proliferation and

cytokine production of CD4+CD25− responder T cells in response to a number of different polyclonal stimuli, as measured by *in vitro* assays (176, 183, 185).

There is now solid evidence that a population of regulatory T cells (Tregs) is physiologically relevant and that disruption of its development or function is a primary cause of autoimmune and inflammatory diseases in humans and animals. Depletion of thymic-generated Tregs, also known as "naturally-occurring" (or "natural") Tregs, has been shown both to elicit autoimmunity and to augment immune responses to non-self-antigens (186). Treg depletion is associated with inflammatory bowel disease, which likely results from excessive immune responses to commensal bacteria in the intestine (187). In addition to their roles in maintenance of self-tolerance and the control of autoimmunity (188, 189), the prevention of escape responses to pathogens or allergens and the balance with microbial flora (190), Tregs also facilitate the escape of tumors from immunological monitoring (191). Tregs may act to impede immune surveillance against cancers in normal individuals and suppress potential responsiveness to autologous tumors in cancer patients (192). Furthermore, Tregs have been shown to suppress allergy, establish tolerance to organ grafts, prevent graft-versus-host disease after bone marrow transplantation and promote feto-maternal tolerance (186). Tregs thus represent a promising clinical approach for the treatment of autoimmune disease and inflammation and for cancer immunotherapy (193).

2. FOXP3 AS A MOLECULAR Treg MARKER

"Naturally-occurring" Tregs express the transcription factor Foxp3 (forkhead box P3), a member of the forkhead/winged-helix family of transcription factors. Members of the Fox family are both transcriptional activators and transcriptional repressors, many of which have been implicated in regulating immune system development and function (194). For example, mutations in the Foxn1 gene are responsible for the phenotype seen in nude mice, rats and humans (195, 196), characterized by abnormal development of the epidermis, lack of hair, and absence of a thymus.

The Foxp3 gene was first identified as the defective gene in *scurfy* mice as an X-linked recessive mutant that is lethal in hemizygous males within a month after birth, exhibiting hyperactivation of CD4+ T cells and overproduction of proinflammatory cytokines (197). The severe autoimmune diseases and allergy in *scurfy* resemble the diseases observed following depletion of CD25+CD4+ Tregs in rodents, and inoculation

of CD25+ CD4+ T cells from normal mice has been shown to prevent severe systemic inflammation in *scurfy* mice (198). It has also been shown that continued expression of Foxp3 is needed to maintain the lineage identity and function of peripheral mature Treg cells, since ablation of Foxp3 in mature Treg cells resulted in the loss of their suppressive function and of the transcriptional program established by Foxp3 during regulatory T cell lineage commitment in the thymus (199).

Mutations of the human gene *FOXP3*, located at Xq11.23–Xq13.3, have also been shown to be closely related to the genetic disease IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome) (200, 201), which represents the human counterpart of the *scurfy* phenotype. Immunopathology associated with the IPEX syndrome becomes apparent around the first month after birth and includes eczema or psoriasis-like lesions, watery diarrhea, type 1 diabetes mellitus, excessive cytokine production and chronic inflammation, leading to death (202). To date, there are over 20 known FOXP3 mutations resulting in IPEX syndrome with these manifestations. (202).

Importantly, ectopic retroviral transduction of the Foxp3 gene in naïve CD25− CD4+ T cells can convert them to CD25+ CD4+ Treg-like cells that are able to suppress proliferation of other T cells *in vitro* and inhibit the development of autoimmune disease and inflammatory bowel disease *in vivo* (198, 203). Foxp3 transduction in naïve T cells also up-regulates the expression of CD25 and other Treg-associated molecules, such as cytotoxic T cell associated antigen-4 (CTLA-4) and glucocorticoid-induced tumor necrosis factor (TNF) receptor family-related gene/protein (GITR), whereas it represses the production of IL-2, IFN-γ, and IL-4 (198). Similarly, Foxp3 gene transfer confers suppressor function to naïve human CD4+ T cells (204, 205). Interestingly, Foxp3 was less efficient in reprogramming the memory T-cell subset into regulatory cells (205).

Two main isoforms of Foxp3 are expressed in humans. While the complete isoform represents the ortholog to mouse Foxp3, a deletional isoform lacking the proline-rich exon 2 (amino acids 71–105) has been described that has not been reported in mouse CD4+CD25+ Tregs (206). Whether the same T cell expresses both isoforms simultaneously is currently not known. In addition, it is unclear if there is a functional difference between the two isoforms (206), although either isoform can confer regulatory function when strongly overexpressed (204, 207, 208).

In the mouse, Foxp3 has been shown to directly or indirectly control hundreds of genes and to directly bind ~10% of them (209, 210), including those encoding signal transduction molecules, transcription factors, cytokines (such as IL-2), cell-surface

molecules (such as IL-2Rα and CTLA-4), enzymes for cell metabolism and intergenic microRNAs (such as miR-155). It functions as an activator as well as a repressor of transcription depending on the target (189).

Foxp3 is currently the most reliable molecular marker for natural Tregs in both humans and mice (189). However, it has become evident in recent years that Foxp3+ Treg cells could also be generated *in vitro* and *in vivo* outside the thymus from conventional Foxp3- CD4 T cells under a variety of conditions, including TCR stimulation, the cytokines transforming growth factor β (TGF-β) and IL-2 or retinoic acid (Figure 8) (189, 211). The differentiation of these so called "inducible" or "adaptive" Tregs is apparently associated to particular environments, such as the gut-associated lymphoid tissues (GALT) and mesenteric lymph nodes, with their generation probably being induced by distinct gut-associated antigens such as food and commensal microbiota-derived antigens (185). Nevertheless, the different microenvironments of adaptive Treg cell development *in vivo* are still incompletely understood, as is the contribution of adaptive Tregs to the peripheral pool of Foxp3+ Tregs (189, 211). Furthermore, there are differences in Foxp3 expression upon activation of conventional T cells in human and mice (206). Although stable high expression of Foxp3 is restricted to Treg cells in both species, Foxp3 is induced at low levels upon stimulation of human T cells (207, 212-215). However, this Foxp3 expression is transient and does not result in the acquisition of a Treg cell phenotype and suppressor function by activated conventional human T cells (212, 215). The transient Foxp3 expression by activated T cells has been proposed to be a consequence of the methylation status at the *FOXP3* promoter region, with only the complete demethylation of the conserved *FOXP3* promoter region supporting stable long term Foxp3 expression and a committed Treg phenotype in humans (216-218). On the other hand, populations of Foxp3-negative adaptive Tregs have also been described, such as type 1 regulatory T cells (Tr1) and T helper-3 T cells (Th3) (Figure 8), that may play a role in the control of the development of autoimmunity and in promotion of transplantation tolerance (202).

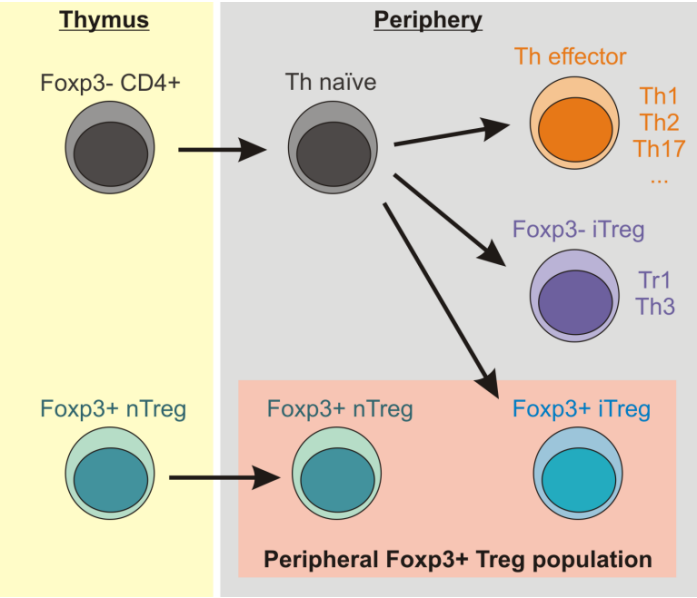


Figure 8: Thymic and peripheral generation of Foxp3+ Tregs. The peripheral Treg population comprises both "natural" Tregs (nTreg), which differentiate in the thymus and migrate to peripheral tissues, and "adaptive" or "inducible" Tregs (iTregs), which differentiate in secondary lymphoid organs and tissues. Adapted from (211).

Tregs are thus currently best identified in both mice and humans by the high levels of Foxp3, and/or CD25, in situations where there is no apparent immune activation *in vivo* (219). In addition, it has been shown that a further discriminating marker for this population can be the low levels of CD127 (220-222). Of note, up-regulation of CD127 has been recently reported in activated Tregs in mice (223). The expression of CD39 (ectonucleoside triphosphate diphosphohydrolase 1) has also been linked to the Treg phenotype and function in both humans and mice, which is in great part related to the generation of the immunosuppressive nucleoside adenosine in association with the 5'-ectonucleotidase CD73 (224, 225). Expression of the mucosal-homing associated molecule CD103, the α_E subunit of the $\alpha_E\beta_7$ integrin, has also been described as a marker of Tregs specialized for the crosstalk with epithelial environments, although its significance in mice is apparently more pronounced than in humans (226-229). A list of markers associated with Tregs is presented in Table 2.

Importantly, Foxp3+ natural Tregs retain their suppressive function after expansion *in vivo* and *in vitro* (230, 231), allowing envision of the use of clonally expanded antigen-specific natural Tregs in the therapeutic or clinical context, namely in transplantation tolerance and suppression of graft rejection.

TABLE 2: TREG CELL MARKERS.

Transcription factor	Activation and memory	Homing and origin	Suppressive and effector function	Apoptosis, survival or other
Foxp3	CD45RA	CCR4	CTLA4	CD27
	CD45RO	CCR6	ICOS	OX40
	CD25	CCR9	CD39–CD73	CD95
	HLA-DR	CD103	LAP	PD-1
	Lack of CD127	CD304	Granzyme B	GITR
	CD69	CD31	Galectin 1	Galectin 3
		Lack of CD49d	Galectin 10	GARP
			TRANCE	MS4A4B
			CD80 and CD86	IL-1R
			IL-10	CD6
			CD2	
			Lack of IL-2	

CCR, chemokine receptor; GARP, glycoprotein A repetitions predominant; ICOS, inducible T cell co-stimulator; LAP, latency-associated peptide; MS4A4B, membrane-spanning 4-domains, subfamily A, member 4B; PD-1, programmed cell death 1; TRANCE, TNF-related activation-induced cytokine. Adapted from (193).

3. THYMIC Treg DEVELOPMENT: INSIGHTS FROM MOUSE MODELS

Foxp3+ thymocytes have been observed in the thymus at a late CD4+CD8+ and constitute ~5% of mature CD4SP thymocytes and less than 1% of CD8SP or DN thymocytes (232). Very few Foxp3+ thymocytes develop in mice deficient in both MHC class I and II, indicating a crucial role for TCR signaling and self-peptide/MHC ligands in the development Foxp3+ thymocytes or their Foxp3- precursors (Figure 9) (232). Moreover, commitment to the Treg lineage must directly or indirectly reflect differential signals received through the TCR, since natural Tregs are thought to possess TCRs with higher affinity for thymic MHC/self-peptide ligands than those of other T cells (189). Engagement by agonist ligands favors the selection of Treg cells either by inducing differentiation along the lineage, as observed in transgenic systems (233, 234), or because Foxp3+ cells are inherently more resistant to clonal deletion (122, 235, 236). In addition, the intensity of the interaction between T cell accessory molecules and their ligands on thymic stromal cells contributes to the generation of natural Tregs (Figure 9). For example, deficiency of CD28, CD40 or CD80 and CD86 results in a substantial reduction of CD25+ CD4+ Tregs in the thymus and periphery (186).

Although the overwhelming majority of Foxp3+ cells are found within the medulla of the thymus, scattered cortical Foxp3+ thymocytes are also present in the cortex (232). The relative role of cortex vs. medulla in Treg induction and in the determination of the Treg fate is still matter of debate. In a study where thymic migration from the cortex to

the medulla was blocked by administering pertussis toxin to mice there was an accumulation of cortical CD4SP Foxp3+ thymocytes, indicating that the thymic cortex is sufficient to initiate Foxp3 expression (237). Furthermore, CCR7-deficient mice, which have impaired thymocyte trafficking from the cortex to the medulla, also present an increase in Foxp3+ Tregs in the cortex (109, 237). In another study where the role of cortical MHC class II was examined it was shown that mice expressing MHC class II only in the cortex were able to give rise to Tregs, although at 60% of normal numbers (238). These studies show that murine Tregs are capable of developing within the cortical region of the thymus. Nevertheless, other co-stimulatory signals appear to be required for Treg lineage commitment and/or survival (202).

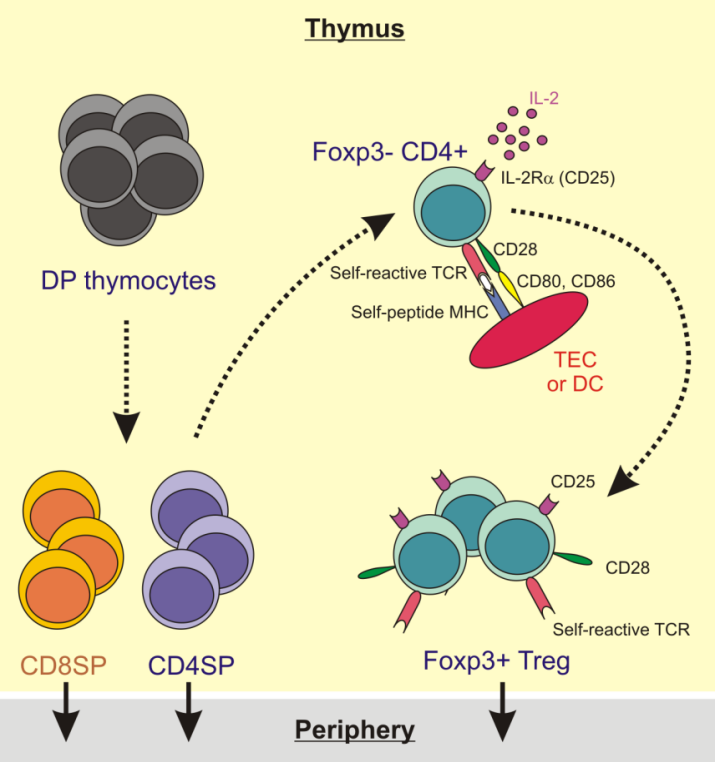


Figure 9: Differentiation of thymic Tregs. According to the current view of murine Treg development (adapted from (185)) most Foxp3+ thymic Treg cells differentiate from Foxp3-CD4SP thymocytes. Foxp3 induction/Treg differentiation requires increased T cell receptor (TCR) stimulation by self-peptide-MHC complexes presented by thymic epithelial cells (TECs) or dendritic cells (DCs), CD28 signaling by CD80/CD86 ligand expressed on antigen-presenting cells, and high-affinity IL-2 receptor (and/or other γc cytokine receptor) signaling.

The Hsieh and Farrar groups have proposed a two-step model of Treg cell differentiation. According to this model, recognition of high-affinity self-peptides by developing thymocytes and consequent increased TCR signal result in the up-regulation of CD25, increasing the responsiveness/survival of Treg precursor cells to consequent IL-2 signals within the medulla, which results in induction of Foxp3 and progression into the Treg lineage (Figure 9) (239, 240). This importance of IL-2 in eliciting Foxp3

expression is consistent with the profound Treg cell defects in mice lacking the IL-2 receptor or the IL-2 signal transducer STAT5 (signal transducer and activator of transcription 5) (241-243). STAT5, activated downstream of IL-2R and other common γ-chain cytokine receptors, does represent a likely candidate transcription factor for direct regulation of Foxp3 expression (239, 243).

A recent study has suggested that AIRE+ mTECs expressing TRAs may induce or facilitate the development of TRA-specific Tregs in normal thymus, and that scarcity of TRA-specific Tregs may contribute to the occurrence of autoimmune disease in AIRE deficiency (244). However, in another study AIRE deficiency was shown not to result in a reduced generation of Tregs but in a compromised negative selection of pathogenic self-reactive effector T cells in thymus, suggesting that AIRE may not be involved in the positive selection of Tregs (245). The possible role of AIRE in Treg selection is thus not completely understood.

4. THYMIC DEVELOPMENT OF HUMAN Tregs

As aforementioned, the development of Treg in the murine thymus and their export out of the thymus seem to be slightly delayed compared with that of conventional T cells, as shown by neonatal day 3 thymectomy experiments (11). In contrast, human CD25+ CD4SP Tregs are already present in the thymus as early as the 13th GW and in peripheral organs by the 14th GW (26). Importantly, the generation of CD25+CD4SP Tregs appears concomitant to the generation of other mature CD25- CD4SP or CD8SP cells (26). Furthermore, fetal thymic CD25+ CD4SP Tregs presented phenotypic and functional characteristics of Tregs present in adults, since they expressed intracellular CTLA-4, surface CD45RO and CD62L and *FOXP3* mRNA and already suppressed T cell proliferative responses (26, 246). These characteristics were also observed in CD25+ CD4SP human postnatal thymocytes (247-249). Moreover, postnatal CD25+ CD4SP thymocytes were found to be poor producers of IL-2, IL-4, IL-5, IL-13 and interferon γ after polyclonal activation, while a few of them produced IL-10 (249). The suppressive activity of those cells was contact-dependent and was completely blocked by a mixture of anti-CTLA-4 and anti-TGF-β1 monoclonal mAbs (249). Immunohistochemical analysis of CD25 expression in the human postnatal thymus localized this population to the medulla and fibrous septa (249, 250).

Studies in human fetal and postnatal thymuses have revealed the presence of CD25 on other populations besides the CD4SP population. The earliest stage where CD25 expression was reported was the DP stage, and CD25 expression in DP thymocytes was associated with high levels of CD3 (26, 246) and with the acquisition of the maturation marker CD27 (246) in fetal thymocytes. Furthermore, most CD25+ DP cells expressed the early activation marker CD69, suggestive of TCR signaling and of possible commitment to the Treg lineage during positive selection (246). This population constitutively expressed *FOXP3* mRNA and was able to suppress the proliferation of autologous CD25- CD4SP thymocytes, as shown in human postnatal thymuses (251). Although this suggests some degree of maturity of DP CD25+ thymocytes, it has been reported that this population expresses *RAG-2* mRNA (252). In addition, CD25 expression was also described in fetal (26, 246) and postnatal (253) CD8SP thymocytes, although at lower levels as compared to CD4SP thymocytes (246, 253). CD8SP CD25+ thymocytes expressed intracellular CTLA-4, *GITR* and *FOXP3* mRNA, were localized to the thymic medulla and septa and suppressed the proliferation of autologous CD25- T cells via a contact-dependent mechanism (253). Recent studies confirmed the expression of Foxp3 expression on postnatal DP, CD4SP and CD8SP thymocytes (252), and identified earlier, previously unacknowledged populations expressing that Treg marker (254).

Of interest, CD25+ DP cells in human fetuses reportedly up-regulated CD127 expression earlier that CD25- DP cells, to levels that were lower than those observed in CD25- SP thymocytes (246). The authors state that the majority of CD25+ DP cells do not express the thymic stromal lymphopoietin (TSLP) receptor complex, formed by CD127 in association with the TSLP receptor (TSLPR), indicating that IL-7 and not TSLPR should act directly on that population (246). In contrast to these findings, a recent study on human postnatal thymuses does not find significant expression of CD127 in Foxp3+ DP thymocytes (252).

Studies on the Treg developmental pathway in the human thymus have also attributed an important role to both plasmacytoid and myeloid DCs in thymic Treg differentiation (250, 255, 256). It was recently suggested that human Treg progenitors may selectively reside within mature DP thymocytes expressing high levels of CD69 and TCRαβ, since these cells develop into CD4SP Treg in response to activated autologous plasmacytoid and myeloid DCs (256). Induction of Foxp3 at the DP stage was suggested to involve signaling through CD28 in humans, as previously demonstrated in mice (250, 256, 257). CD25- CD4+ CD8- thymocytes have also been

shown to be able to differentiate into Foxp3+ CD25+ CD4 Tregs upon co-culture with mature DCs, and a role for the TSLP produced in the Hassall's bodies in DC maturation and Treg development has been proposed (250, 255).

Treg development in the human thymus thus remains largely undefined, and additional studies are essential and mandatory to provide valuable knowledge on the generation of this peculiar but fundamental lineage in immune homeostasis.

BIBLIOGRAPHY

1. Corrado Lavini, C.M., Uliano Morandi, Rudolf Schoenhuber. 2008. *Thymus Gland Pathology: Clinical, Diagnostic and Therapeutic Features*. Milan, Italy: Springer.

2. Miller, J.F. 2002. The discovery of thymus function and of thymus-derived lymphocytes. *Immunol Rev* 185:7-14.

3. Miller, J.F. 1960. Studies on mouse leukaemia. The fate of thymus homografts in immunologically tolerant mice. *Br J Cancer* 14:244-255.

4. Miller, J.F. 1963. Role of the Thymus in Immunity. *Br Med J* 2:459-464.

5. Cross, A.M., Leuchars, E., and Miller, J.F. 1964. Studies on the Recovery of the Immune Response in Irradiated Mice Thymectomized in Adult Life. *J Exp Med* 119:837-850.

6. Boehm, T., and Bleul, C.C. 2007. The evolutionary history of lymphoid organs. *Nat Immunol* 8:131-135.

7. Rodewald, H.R. 2008. Thymus organogenesis. *Annu Rev Immunol* 26:355-388.

8. Steinmann, G.G., Klaus, B., and Muller-Hermelink, H.K. 1985. The involution of the ageing human thymic epithelium is independent of puberty. A morphometric study. *Scand J Immunol* 22:563-575.

9. Takahama, Y. 2006. Journey through the thymus: stromal guides for T-cell development and selection. *Nat Rev Immunol* 6:127-135.

10. Pearse, G. 2006. Normal structure, function and histology of the thymus. *Toxicol Pathol* 34:504-514.

11. Liu, Y.J. 2006. A unified theory of central tolerance in the thymus. *Trends Immunol* 27:215-221.

12. Le, P.T., Lazorick, S., Whichard, L.P., Haynes, B.F., and Singer, K.H. 1991. Regulation of cytokine production in the human thymus: epidermal growth factor and transforming growth factor alpha regulate mRNA levels of interleukin 1 alpha (IL-1 alpha), IL-1 beta, and IL-6 in human thymic epithelial cells at a post-transcriptional level. *J Exp Med* 174:1147-1157.

13. He, W., Zhang, Y., Deng, Y., and Kabelitz, D. 1995. Induction of TCR-gamma delta expression on triple-negative (CD3-4-8-) human thymocytes. Comparative analysis of the effects of IL-4 and IL-7. *J Immunol* 154:3726-3731.

14. Romagnani, P., Annunziato, F., Manetti, R., Mavilia, C., Lasagni, L., Manuelli, C., Vannelli, G.B., Vanini, V., Maggi, E., Pupilli, C., et al. 1998. High CD30 ligand expression by epithelial cells and Hassal's corpuscles in the medulla of human thymus. *Blood* 91:3323-3332.

15. Annunziato, F., Romagnani, P., Cosmi, L., Beltrame, C., Steiner, B.H., Lazzeri, E., Raport, C.J., Galli, G., Manetti, R., Mavilia, C., et al. 2000. Macrophage-derived chemokine and EBI1-ligand chemokine attract human thymocytes in different stage of development and are produced by distinct subsets of medullary

epithelial cells: possible implications for negative selection. *J Immunol* 165:238-246.

16. Nishio, H., Matsui, K., Tsuji, H., Tamura, A., and Suzuki, K. 2001. Immunolocalization of the mitogen-activated protein kinase signaling pathway in Hassall's corpuscles of the human thymus. *Acta Histochem* 103:89-98.

17. Zaitseva, M., Kawamura, T., Loomis, R., Goldstein, H., Blauvelt, A., and Golding, H. 2002. Stromal-derived factor 1 expression in the human thymus. *J Immunol* 168:2609-2617.

18. Muller-Hermelink, H.K., Wilisch, A., Schultz, A., and Marx, A. 1997. Characterization of the human thymic microenvironment: lymphoepithelial interaction in normal thymus and thymoma. *Arch Histol Cytol* 60:9-28.

19. Gale, R.P. 1987. Development of the immune system in human fetal liver. *Thymus* 10:45-56.

20. Res, P., and Spits, H. 1999. Developmental stages in the human thymus. *Semin Immunol* 11:39-46.

21. Haynes, B.F. 1984. The human thymic microenvironment. *Adv Immunol* 36:87-142.

22. Haynes, B.F., and Heinly, C.S. 1995. Early human T cell development: analysis of the human thymus at the time of initial entry of hematopoietic stem cells into the fetal thymic microenvironment. *J Exp Med* 181:1445-1458.

23. Stites, D.P., and Pavia, C.S. 1979. Ontogeny of human T cells. *Pediatrics* 64:795-802.

24. Royo, C., Touraine, J.L., and de Bouteiller, O. 1987. Ontogeny of T lymphocyte differentiation in the human fetus: acquisition of phenotype and functions. *Thymus* 10:57-73.

25. Haynes, B.F., Denning, S.M., Singer, K.H., and Kurtzberg, J. 1989. Ontogeny of T-cell precursors: a model for the initial stages of human T-cell development. *Immunol Today* 10:87-91.

26. Darrasse-Jeze, G., Marodon, G., Salomon, B.L., Catala, M., and Klatzmann, D. 2005. Ontogeny of CD4+CD25+ regulatory/suppressor T cells in human fetuses. *Blood* 105:4715-4721.

27. Rossi, S.W., Jenkinson, W.E., Anderson, G., and Jenkinson, E.J. 2006. Clonal analysis reveals a common progenitor for thymic cortical and medullary epithelium. *Nature* 441:988-991.

28. Bleul, C.C., Corbeaux, T., Reuter, A., Fisch, P., Monting, J.S., and Boehm, T. 2006. Formation of a functional thymus initiated by a postnatal epithelial progenitor cell. *Nature* 441:992-996.

29. Anderson, G., Lane, P.J., and Jenkinson, E.J. 2007. Generating intrathymic microenvironments to establish T-cell tolerance. *Nat Rev Immunol* 7:954-963.

30. Isaacson, P.G., Norton, A.J., and Addis, B.J. 1987. The human thymus contains a novel population of B lymphocytes. *Lancet* 2:1488-1491.

31. Flores, K.G., Li, J., and Hale, L.P. 2001. B cells in epithelial and perivascular compartments of human adult thymus. *Hum Pathol* 32:926-934.

32. Klein, L., Hinterberger, M., Wirnsberger, G., and Kyewski, B. 2009. Antigen presentation in the thymus for positive selection and central tolerance induction. *Nat Rev Immunol* 9:833-844.

33. Payne, K.J., and Crooks, G.M. 2002. Human hematopoietic lineage commitment. *Immunol Rev* 187:48-64.

34. Terstappen, L.W., Huang, S., Safford, M., Lansdorp, P.M., and Loken, M.R. 1991. Sequential generations of hematopoietic colonies derived from single nonlineage-committed CD34+CD38- progenitor cells. *Blood* 77:1218-1227.

35. Baum, C.M., Weissman, I.L., Tsukamoto, A.S., Buckle, A.M., and Peault, B. 1992. Isolation of a candidate human hematopoietic stem-cell population. *Proc Natl Acad Sci U S A* 89:2804-2808.

36. Spits, H., Blom, B., Jaleco, A.C., Weijer, K., Verschuren, M.C., van Dongen, J.J., Heemskerk, M.H., and Res, P.C. 1998. Early stages in the development of human T, natural killer and thymic dendritic cells. *Immunol Rev* 165:75-86.

37. Dik, W.A., Pike-Overzet, K., Weerkamp, F., de Ridder, D., de Haas, E.F., Baert, M.R., van der Spek, P., Koster, E.E., Reinders, M.J., van Dongen, J.J., et al. 2005. New insights on human T cell development by quantitative T cell receptor gene rearrangement studies and gene expression profiling. *J Exp Med* 201:1715-1723.

38. Res, P., Martinez-Caceres, E., Cristina Jaleco, A., Staal, F., Noteboom, E., Weijer, K., and Spits, H. 1996. CD34+CD38dim cells in the human thymus can differentiate into T, natural killer, and dendritic cells but are distinct from pluripotent stem cells. *Blood* 87:5196-5206.

39. Blom, B., and Spits, H. 2006. Development of human lymphoid cells. *Annu Rev Immunol* 24:287-320.

40. Jaleco, A.C., Blom, B., Res, P., Weijer, K., Lanier, L.L., Phillips, J.H., and Spits, H. 1997. Fetal liver contains committed NK progenitors, but is not a site for development of CD34+ cells into T cells. *J Immunol* 159:694-702.

41. Blom, B., Res, P., Noteboom, E., Weijer, K., and Spits, H. 1997. Prethymic CD34+ progenitors capable of developing into T cells are not committed to the T cell lineage. *J Immunol* 158:3571-3577.

42. Galy, A., Verma, S., Barcena, A., and Spits, H. 1993. Precursors of CD3+CD4+CD8+ cells in the human thymus are defined by expression of CD34. Delineation of early events in human thymic development. *J Exp Med* 178:391-401.

43. Marquez, C., Trigueros, C., Fernandez, E., and Toribio, M.L. 1995. The development of T and non-T cell lineages from CD34+ human thymic precursors can be traced by the differential expression of CD44. *J Exp Med* 181:475-483.

44. Kraft, D.L., Weissman, I.L., and Waller, E.K. 1993. Differentiation of CD3-4-8- human fetal thymocytes in vivo: characterization of a CD3-4+8- intermediate. *J Exp Med* 178:265-277.

45. Blom, B., Heemskerk, M.H., Verschuren, M.C., van Dongen, J.J., Stegmann, A.P., Bakker, A.Q., Couwenberg, F., Res, P.C., and Spits, H. 1999. Disruption of alpha beta but not of gamma delta T cell development by overexpression of the helix-loop-helix protein Id3 in committed T cell progenitors. *EMBO J* 18:2793-2802.

46. Noguchi, M., Yi, H., Rosenblatt, H.M., Filipovich, A.H., Adelstein, S., Modi, W.S., McBride, O.W., and Leonard, W.J. 1993. Interleukin-2 receptor gamma chain mutation results in X-linked severe combined immunodeficiency in humans. *Cell* 73:147-157.

47. Russell, S.M., Johnston, J.A., Noguchi, M., Kawamura, M., Bacon, C.M., Friedmann, M., Berg, M., McVicar, D.W., Witthuhn, B.A., Silvennoinen, O., et al. 1994. Interaction of IL-2R beta and gamma c chains with Jak1 and Jak3: implications for XSCID and XCID. *Science* 266:1042-1045.

48. Puel, A., Ziegler, S.F., Buckley, R.H., and Leonard, W.J. 1998. Defective IL7R expression in T(-)B(+)NK(+) severe combined immunodeficiency. *Nat Genet* 20:394-397.

49. Giliani, S., Mori, L., de Saint Basile, G., Le Deist, F., Rodriguez-Perez, C., Forino, C., Mazzolari, E., Dupuis, S., Elhasid, R., Kessel, A., et al. 2005. Interleukin-7 receptor alpha (IL-7Ralpha) deficiency: cellular and molecular bases. Analysis of clinical, immunological, and molecular features in 16 novel patients. *Immunol Rev* 203:110-126.

50. Macchi, P., Villa, A., Giliani, S., Sacco, M.G., Frattini, A., Porta, F., Ugazio, A.G., Johnston, J.A., Candotti, F., O'Shea, J.J., et al. 1995. Mutations of Jak-3 gene in patients with autosomal severe combined immune deficiency (SCID). *Nature* 377:65-68.

51. Russell, S.M., Tayebi, N., Nakajima, H., Riedy, M.C., Roberts, J.L., Aman, M.J., Migone, T.S., Noguchi, M., Markert, M.L., Buckley, R.H., et al. 1995. Mutation of Jak3 in a patient with SCID: essential role of Jak3 in lymphoid development. *Science* 270:797-800.

52. Plum, J., De Smedt, M., Leclercq, G., Verhasselt, B., and Vandekerckhove, B. 1996. Interleukin-7 is a critical growth factor in early human T-cell development. *Blood* 88:4239-4245.

53. Spits, H. 2002. Development of alphabeta T cells in the human thymus. *Nat Rev Immunol* 2:760-772.

54. Okamoto, Y., Douek, D.C., McFarland, R.D., and Koup, R.A. 2002. Effects of exogenous interleukin-7 on human thymus function. *Blood* 99:2851-2858.

55. Radtke, F., Wilson, A., Stark, G., Bauer, M., van Meerwijk, J., MacDonald, H.R., and Aguet, M. 1999. Deficient T cell fate specification in mice with an induced inactivation of Notch1. *Immunity* 10:547-558.

56. De Smedt, M., Reynvoet, K., Kerre, T., Taghon, T., Verhasselt, B., Vandekerckhove, B., Leclercq, G., and Plum, J. 2002. Active form of Notch imposes T cell fate in human progenitor cells. *J Immunol* 169:3021-3029.

57. Schmitt, T.M., de Pooter, R.F., Gronski, M.A., Cho, S.K., Ohashi, P.S., and Zuniga-Pflucker, J.C. 2004. Induction of T cell development and establishment of T cell competence from embryonic stem cells differentiated in vitro. *Nat Immunol* 5:410-417.

58. La Motte-Mohs, R.N., Herer, E., and Zuniga-Pflucker, J.C. 2005. Induction of T-cell development from human cord blood hematopoietic stem cells by Delta-like 1 in vitro. *Blood* 105:1431-1439.

59. De Smedt, M., Hoebeke, I., and Plum, J. 2004. Human bone marrow CD34+ progenitor cells mature to T cells on OP9-DL1 stromal cell line without thymus microenvironment. *Blood Cells Mol Dis* 33:227-232.

60. De Smedt, M., Hoebeke, I., Reynvoet, K., Leclercq, G., and Plum, J. 2005. Different thresholds of Notch signaling bias human precursor cells toward B-, NK-, monocytic/dendritic-, or T-cell lineage in thymus microenvironment. *Blood* 106:3498-3506.

61. Dontje, W., Schotte, R., Cupedo, T., Nagasawa, M., Scheeren, F., Gimeno, R., Spits, H., and Blom, B. 2006. Delta-like1-induced Notch1 signaling regulates the human plasmacytoid dendritic cell versus T-cell lineage decision through control of GATA-3 and Spi-B. *Blood* 107:2446-2452.

62. Wilson, R.K., Lai, E., Concannon, P., Barth, R.K., and Hood, L.E. 1988. Structure, organization and polymorphism of murine and human T-cell receptor alpha and beta chain gene families. *Immunol Rev* 101:149-172.

63. Arstila, T.P., Casrouge, A., Baron, V., Even, J., Kanellopoulos, J., and Kourilsky, P. 1999. A direct estimate of the human alphabeta T cell receptor diversity. *Science* 286:958-961.

64. Verschuren, M.C., Blom, B., Bogers, A.J., Spits, H., and van Dongen, J.J. 1998. PJA-BP expression and TCR delta deletion during human T cell differentiation. *Int Immunol* 10:1873-1880.

65. Blom, B., Verschuren, M.C., Heemskerk, M.H., Bakker, A.Q., van Gastel-Mol, E.J., Wolvers-Tettero, I.L., van Dongen, J.J., and Spits, H. 1999. TCR gene rearrangements and expression of the pre-T cell receptor complex during human T-cell differentiation. *Blood* 93:3033-3043.

66. Groettrup, M., Ungewiss, K., Azogui, O., Palacios, R., Owen, M.J., Hayday, A.C., and von Boehmer, H. 1993. A novel disulfide-linked heterodimer on pre-T cells consists of the T cell receptor beta chain and a 33 kd glycoprotein. *Cell* 75:283-294.

67. Saint-Ruf, C., Ungewiss, K., Groettrup, M., Bruno, L., Fehling, H.J., and von Boehmer, H. 1994. Analysis and expression of a cloned pre-T cell receptor gene. *Science* 266:1208-1212.

68. Fehling, H.J., Krotkova, A., Saint-Ruf, C., and von Boehmer, H. 1995. Crucial role of the pre-T-cell receptor alpha gene in development of alpha beta but not gamma delta T cells. *Nature* 375:795-798.

69. von Boehmer, H., and Fehling, H.J. 1997. Structure and function of the pre-T cell receptor. *Annu Rev Immunol* 15:433-452.

70. Ramiro, A.R., Trigueros, C., Marquez, C., San Millan, J.L., and Toribio, M.L. 1996. Regulation of pre-T cell receptor (pT alpha-TCR beta) gene expression during human thymic development. *J Exp Med* 184:519-530.

71. Trigueros, C., Ramiro, A.R., Carrasco, Y.R., de Yebenes, V.G., Albar, J.P., and Toribio, M.L. 1998. Identification of a late stage of small noncycling pTalpha- pre-T cells as immediate precursors of T cell receptor alpha/beta+ thymocytes. *J Exp Med* 188:1401-1412.

72. Hoffman, E.S., Passoni, L., Crompton, T., Leu, T.M., Schatz, D.G., Koff, A., Owen, M.J., and Hayday, A.C. 1996. Productive T-cell receptor beta-chain gene rearrangement: coincident regulation of cell cycle and clonality during development in vivo. *Genes Dev* 10:948-962.

73. Taghon, T., Yui, M.A., Pant, R., Diamond, R.A., and Rothenberg, E.V. 2006. Developmental and molecular characterization of emerging beta- and gammadelta-selected pre-T cells in the adult mouse thymus. *Immunity* 24:53-64.

74. Hori, T., Cupp, J., Wrighton, N., Lee, F., and Spits, H. 1991. Identification of a novel human thymocyte subset with a phenotype of CD3- CD4+ CD8 alpha + beta-1. Possible progeny of the CD3- CD4- CD8- subset. *J Immunol* 146:4078-4084.

75. Carrasco, Y.R., Trigueros, C., Ramiro, A.R., de Yebenes, V.G., and Toribio, M.L. 1999. Beta-selection is associated with the onset of CD8beta chain expression on CD4(+)CD8alphaalpha(+) pre-T cells during human intrathymic development. *Blood* 94:3491-3498.

76. Taghon, T., Van de Walle, I., De Smet, G., De Smedt, M., Leclercq, G., Vandekerckhove, B., and Plum, J. 2009. Notch signaling is required for proliferation but not for differentiation at a well-defined beta-selection checkpoint during human T-cell development. *Blood* 113:3254-3263.

77. Satyanarayana, K., Hata, S., Devlin, P., Roncarolo, M.G., De Vries, J.E., Spits, H., Strominger, J.L., and Krangel, M.S. 1988. Genomic organization of the human T-cell antigen-receptor alpha/delta locus. *Proc Natl Acad Sci U S A* 85:8166-8170.

78. Verschuren, M.C., Wolvers-Tettero, I.L., Breit, T.M., Noordzij, J., van Wering, E.R., and van Dongen, J.J. 1997. Preferential rearrangements of the T cell receptor-delta-deleting elements in human T cells. *J Immunol* 158:1208-1216.

79. de Villartay, J.P., Hockett, R.D., Coran, D., Korsmeyer, S.J., and Cohen, D.I. 1988. Deletion of the human T-cell receptor delta-gene by a site-specific recombination. *Nature* 335:170-174.

80. Malissen, M., Trucy, J., Jouvin-Marche, E., Cazenave, P.A., Scollay, R., and Malissen, B. 1992. Regulation of TCR alpha and beta gene allelic exclusion during T-cell development. *Immunol Today* 13:315-322.

81. Gascoigne, N.R., and Alam, S.M. 1999. Allelic exclusion of the T cell receptor alpha-chain: developmental regulation of a post-translational event. *Semin Immunol* 11:337-347.

82. Douek, D.C., McFarland, R.D., Keiser, P.H., Gage, E.A., Massey, J.M., Haynes, B.F., Polis, M.A., Haase, A.T., Feinberg, M.B., Sullivan, J.L., et al. 1998. Changes in thymic function with age and during the treatment of HIV infection. *Nature* 396:690-695.

83. Mancini, S.J., Candeias, S.M., Di Santo, J.P., Ferrier, P., Marche, P.N., and Jouvin-Marche, E. 2001. TCRA gene rearrangement in immature thymocytes in absence of CD3, pre-TCR, and TCR signaling. *J Immunol* 167:4485-4493.

84. Wilson, A., Held, W., and MacDonald, H.R. 1994. Two waves of recombinase gene expression in developing thymocytes. *J Exp Med* 179:1355-1360.

85. Alam, S.M., Travers, P.J., Wung, J.L., Nasholds, W., Redpath, S., Jameson, S.C., and Gascoigne, N.R. 1996. T-cell-receptor affinity and thymocyte positive selection. *Nature* 381:616-620.

86. Liu, C.P., Crawford, F., Marrack, P., and Kappler, J. 1998. T cell positive selection by a high density, low affinity ligand. *Proc Natl Acad Sci U S A* 95:4522-4526.

87. Williams, C.B., Engle, D.L., Kersh, G.J., Michael White, J., and Allen, P.M. 1999. A kinetic threshold between negative and positive selection based on the longevity of the T cell receptor-ligand complex. *J Exp Med* 189:1531-1544.

88. Jerne, N.K. 1971. The somatic generation of immune recognition. *Eur J Immunol* 1:1-9.

89. Dai, S., Huseby, E.S., Rubtsova, K., Scott-Browne, J., Crawford, F., Macdonald, W.A., Marrack, P., and Kappler, J.W. 2008. Crossreactive T Cells spotlight the germline rules for alphabeta T cell-receptor interactions with MHC molecules. *Immunity* 28:324-334.

90. Zerrahn, J., Held, W., and Raulet, D.H. 1997. The MHC reactivity of the T cell repertoire prior to positive and negative selection. *Cell* 88:627-636.

91. Van Laethem, F., Sarafova, S.D., Park, J.H., Tai, X., Pobezinsky, L., Guinter, T.I., Adoro, S., Adams, A., Sharrow, S.O., Feigenbaum, L., et al. 2007. Deletion of CD4 and CD8 coreceptors permits generation of alphabetaT cells that recognize antigens independently of the MHC. *Immunity* 27:735-750.

92. Petrie, H.T., Livak, F., Schatz, D.G., Strasser, A., Crispe, I.N., and Shortman, K. 1993. Multiple rearrangements in T cell receptor alpha chain genes maximize the production of useful thymocytes. *J Exp Med* 178:615-622.

93. Bill, J., and Palmer, E. 1989. Positive selection of CD4+ T cells mediated by MHC class II-bearing stromal cell in the thymic cortex. *Nature* 341:649-651.

94. Benoist, C., and Mathis, D. 1989. Positive selection of the T cell repertoire: where and when does it occur? *Cell* 58:1027-1033.

95. Berg, L.J., Pullen, A.M., Fazekas de St Groth, B., Mathis, D., Benoist, C., and Davis, M.M. 1989. Antigen/MHC-specific T cells are preferentially exported from the thymus in the presence of their MHC ligand. *Cell* 58:1035-1046.

96. Anderson, G., Owen, J.J., Moore, N.C., and Jenkinson, E.J. 1994. Thymic epithelial cells provide unique signals for positive selection of CD4+CD8+ thymocytes in vitro. *J Exp Med* 179:2027-2031.

97. Goldman, K.P., Park, C.S., Kim, M., Matzinger, P., and Anderson, C.C. 2005. Thymic cortical epithelium induces self tolerance. *Eur J Immunol* 35:709-717.

98. Hare, K.J., Jenkinson, E.J., and Anderson, G. 2001. Specialisation of thymic epithelial cells for positive selection of CD4+8+ thymocytes. *Cell Mol Biol (Noisy-le-grand)* 47:119-127.

99. Fujimoto, Y., Tu, L., Miller, A.S., Bock, C., Fujimoto, M., Doyle, C., Steeber, D.A., and Tedder, T.F. 2002. CD83 expression influences CD4+ T cell development in the thymus. *Cell* 108:755-767.

100. Brocker, T., Riedinger, M., and Karjalainen, K. 1997. Targeted expression of major histocompatibility complex (MHC) class II molecules demonstrates that dendritic cells can induce negative but not positive selection of thymocytes in vivo. *J Exp Med* 185:541-550.

101. Choi, E.Y., Park, W.S., Jung, K.C., Chung, D.H., Bae, Y.M., Kim, T.J., Song, H.G., Kim, S.H., Ham, D.I., Hahn, J.H., et al. 1997. Thymocytes positively select thymocytes in human system. *Hum Immunol* 54:15-20.

102. Martinic, M.M., Rulicke, T., Althage, A., Odermatt, B., Hochli, M., Lamarre, A., Dumrese, T., Speiser, D.E., Kyburz, D., Hengartner, H., et al. 2003. Efficient T cell repertoire selection in tetraparental chimeric mice independent of thymic epithelial MHC. *Proc Natl Acad Sci U S A* 100:1861-1866.

103. Choi, E.Y., Jung, K.C., Park, H.J., Chung, D.H., Song, J.S., Yang, S.D., Simpson, E., and Park, S.H. 2005. Thymocyte-thymocyte interaction for efficient positive selection and maturation of CD4 T cells. *Immunity* 23:387-396.

104. Li, W., Kim, M.G., Gourley, T.S., McCarthy, B.P., Sant'Angelo, D.B., and Chang, C.H. 2005. An alternate pathway for CD4 T cell development: thymocyte-expressed MHC class II selects a distinct T cell population. *Immunity* 23:375-386.

105. Starr, T.K., Jameson, S.C., and Hogquist, K.A. 2003. Positive and negative selection of T cells. *Annu Rev Immunol* 21:139-176.

106. Hogquist, K.A., Jameson, S.C., Heath, W.R., Howard, J.L., Bevan, M.J., and Carbone, F.R. 1994. T cell receptor antagonist peptides induce positive selection. *Cell* 76:17-27.

107. Wilkinson, R.W., Anderson, G., Owen, J.J., and Jenkinson, E.J. 1995. Positive selection of thymocytes involves sustained interactions with the thymic microenvironment. *J Immunol* 155:5234-5240.

108. Bousso, P., Bhakta, N.R., Lewis, R.S., and Robey, E. 2002. Dynamics of thymocyte-stromal cell interactions visualized by two-photon microscopy. *Science* 296:1876-1880.

109. Kurobe, H., Liu, C., Ueno, T., Saito, F., Ohigashi, I., Seach, N., Arakaki, R., Hayashi, Y., Kitagawa, T., Lipp, M., et al. 2006. CCR7-dependent cortex-to-medulla migration of positively selected thymocytes is essential for establishing central tolerance. *Immunity* 24:165-177.

110. Srivatsan, S., and Peng, S.L. 2005. Cutting edge: Foxj1 protects against autoimmunity and inhibits thymocyte egress. *J Immunol* 175:7805-7809.

111. Boehm, T., Scheu, S., Pfeffer, K., and Bleul, C.C. 2003. Thymic medullary epithelial cell differentiation, thymocyte emigration, and the control of autoimmunity require lympho-epithelial cross talk via LTbetaR. *J Exp Med* 198:757-769.

112. Akiyama, T., Maeda, S., Yamane, S., Ogino, K., Kasai, M., Kajiura, F., Matsumoto, M., and Inoue, J. 2005. Dependence of self-tolerance on TRAF6-directed development of thymic stroma. *Science* 308:248-251.

113. Rossi, S.W., Kim, M.Y., Leibbrandt, A., Parnell, S.M., Jenkinson, W.E., Glanville, S.H., McConnell, F.M., Scott, H.S., Penninger, J.M., Jenkinson, E.J., et al. 2007. RANK signals from CD4(+)3(-) inducer cells regulate development of Aire-expressing epithelial cells in the thymic medulla. *J Exp Med* 204:1267-1272.

114. Akiyama, T., Shimo, Y., Yanai, H., Qin, J., Ohshima, D., Maruyama, Y., Asaumi, Y., Kitazawa, J., Takayanagi, H., Penninger, J.M., et al. 2008. The tumor necrosis factor family receptors RANK and CD40 cooperatively establish the thymic medullary microenvironment and self-tolerance. *Immunity* 29:423-437.

115. Hikosaka, Y., Nitta, T., Ohigashi, I., Yano, K., Ishimaru, N., Hayashi, Y., Matsumoto, M., Matsuo, K., Penninger, J.M., Takayanagi, H., et al. 2008. The cytokine RANKL produced by positively selected thymocytes fosters medullary thymic epithelial cells that express autoimmune regulator. *Immunity* 29:438-450.

116. Irla, M., Hugues, S., Gill, J., Nitta, T., Hikosaka, Y., Williams, I.R., Hubert, F.X., Scott, H.S., Takahama, Y., Hollander, G.A., et al. 2008. Autoantigen-specific interactions with CD4+ thymocytes control mature medullary thymic epithelial cell cellularity. *Immunity* 29:451-463.

117. Derbinski, J., Schulte, A., Kyewski, B., and Klein, L. 2001. Promiscuous gene expression in medullary thymic epithelial cells mirrors the peripheral self. *Nat Immunol* 2:1032-1039.

118. Anderson, M.S., Venanzi, E.S., Klein, L., Chen, Z., Berzins, S.P., Turley, S.J., von Boehmer, H., Bronson, R., Dierich, A., Benoist, C., et al. 2002. Projection of an immunological self shadow within the thymus by the aire protein. *Science* 298:1395-1401.

119. Kyewski, B., and Klein, L. 2006. A central role for central tolerance. *Annu Rev Immunol* 24:571-606.

120. Nagamine, K., Peterson, P., Scott, H.S., Kudoh, J., Minoshima, S., Heino, M., Krohn, K.J., Lalioti, M.D., Mullis, P.E., Antonarakis, S.E., et al. 1997. Positional cloning of the APECED gene. *Nat Genet* 17:393-398.

121. Bjorses, P., Aaltonen, J., Horelli-Kuitunen, N., Yaspo, M.L., and Peltonen, L. 1998. Gene defect behind APECED: a new clue to autoimmunity. *Hum Mol Genet* 7:1547-1553.

122. Liston, A., Lesage, S., Wilson, J., Peltonen, L., and Goodnow, C.C. 2003. Aire regulates negative selection of organ-specific T cells. *Nat Immunol* 4:350-354.

123. Derbinski, J., Gabler, J., Brors, B., Tierling, S., Jonnakuty, S., Hergenhahn, M., Peltonen, L., Walter, J., and Kyewski, B. 2005. Promiscuous gene expression in thymic epithelial cells is regulated at multiple levels. *J Exp Med* 202:33-45.

124. Derbinski, J., Pinto, S., Rosch, S., Hexel, K., and Kyewski, B. 2008. Promiscuous gene expression patterns in single medullary thymic epithelial cells argue for a stochastic mechanism. *Proc Natl Acad Sci U S A* 105:657-662.

125. Gabler, J., Arnold, J., and Kyewski, B. 2007. Promiscuous gene expression and the developmental dynamics of medullary thymic epithelial cells. *Eur J Immunol* 37:3363-3372.

126. Gray, D., Abramson, J., Benoist, C., and Mathis, D. 2007. Proliferative arrest and rapid turnover of thymic epithelial cells expressing Aire. *J Exp Med* 204:2521-2528.

127. McCaughtry, T.M., Wilken, M.S., and Hogquist, K.A. 2007. Thymic emigration revisited. *J Exp Med* 204:2513-2520.

128. Le Borgne, M., Ladi, E., Dzhagalov, I., Herzmark, P., Liao, Y.F., Chakraborty, A.K., and Robey, E.A. 2009. The impact of negative selection on thymocyte migration in the medulla. *Nat Immunol* 10:823-830.

129. Gallegos, A.M., and Bevan, M.J. 2004. Central tolerance to tissue-specific antigens mediated by direct and indirect antigen presentation. *J Exp Med* 200:1039-1049.

130. Koble, C., and Kyewski, B. 2009. The thymic medulla: a unique microenvironment for intercellular self-antigen transfer. *J Exp Med* 206:1505-1513.

131. Jenkinson, E.J., Anderson, G., and Owen, J.J. 1992. Studies on T cell maturation on defined thymic stromal cell populations in vitro. *J Exp Med* 176:845-853.

132. Teh, H.S., Kisielow, P., Scott, B., Kishi, H., Uematsu, Y., Bluthmann, H., and von Boehmer, H. 1988. Thymic major histocompatibility complex antigens and the alpha beta T-cell receptor determine the CD4/CD8 phenotype of T cells. *Nature* 335:229-233.

133. Chan, S.H., Cosgrove, D., Waltzinger, C., Benoist, C., and Mathis, D. 1993. Another view of the selective model of thymocyte selection. *Cell* 73:225-236.

134. Davis, C.B., Killeen, N., Crooks, M.E., Raulet, D., and Littman, D.R. 1993. Evidence for a stochastic mechanism in the differentiation of mature subsets of T lymphocytes. *Cell* 73:237-247.

135. Itano, A., Kioussis, D., and Robey, E. 1994. Stochastic component to development of class I major histocompatibility complex-specific T cells. *Proc Natl Acad Sci U S A* 91:220-224.

136. Robey, E., Itano, A., Fanslow, W.C., and Fowlkes, B.J. 1994. Constitutive CD8 expression allows inefficient maturation of CD4+ helper T cells in class II major histocompatibility complex mutant mice. *J Exp Med* 179:1997-2004.

137. Chan, S.H., Waltzinger, C., Baron, A., Benoist, C., and Mathis, D. 1994. Role of coreceptors in positive selection and lineage commitment. *EMBO J* 13:4482-4489.

138. Leung, R.K., Thomson, K., Gallimore, A., Jones, E., Van den Broek, M., Sierro, S., Alsheikhly, A.R., McMichael, A., and Rahemtulla, A. 2001. Deletion of the CD4 silencer element supports a stochastic mechanism of thymocyte lineage commitment. *Nat Immunol* 2:1167-1173.

139. Seong, R.H., Chamberlain, J.W., and Parnes, J.R. 1992. Signal for T-cell differentiation to a CD4 cell lineage is delivered by CD4 transmembrane region and/or cytoplasmic tail. *Nature* 356:718-720.

140. Itano, A., Salmon, P., Kioussis, D., Tolaini, M., Corbella, P., and Robey, E. 1996. The cytoplasmic domain of CD4 promotes the development of CD4 lineage T cells. *J Exp Med* 183:731-741.

141. Yasutomo, K., Doyle, C., Miele, L., Fuchs, C., and Germain, R.N. 2000. The duration of antigen receptor signalling determines CD4+ versus CD8+ T-cell lineage fate. *Nature* 404:506-510.

142. Singer, A., Adoro, S., and Park, J.H. 2008. Lineage fate and intense debate: myths, models and mechanisms of CD4- versus CD8-lineage choice. *Nat Rev Immunol* 8:788-801.

143. Brugnera, E., Bhandoola, A., Cibotti, R., Yu, Q., Guinter, T.I., Yamashita, Y., Sharrow, S.O., and Singer, A. 2000. Coreceptor reversal in the thymus: signaled CD4+8+ thymocytes initially terminate CD8 transcription even when differentiating into CD8+ T cells. *Immunity* 13:59-71.

144. Singer, A. 2002. New perspectives on a developmental dilemma: the kinetic signaling model and the importance of signal duration for the CD4/CD8 lineage decision. *Curr Opin Immunol* 14:207-215.

145. Singer, A., and Bosselut, R. 2004. CD4/CD8 coreceptors in thymocyte development, selection, and lineage commitment: analysis of the CD4/CD8 lineage decision. *Adv Immunol* 83:91-131.

146. Chong, M.M., Cornish, A.L., Darwiche, R., Stanley, E.G., Purton, J.F., Godfrey, D.I., Hilton, D.J., Starr, R., Alexander, W.S., and Kay, T.W. 2003. Suppressor of cytokine signaling-1 is a critical regulator of interleukin-7-dependent CD8+ T cell differentiation. *Immunity* 18:475-487.

147. Yu, Q., Park, J.H., Doan, L.L., Erman, B., Feigenbaum, L., and Singer, A. 2006. Cytokine signal transduction is suppressed in preselection double-positive thymocytes and restored by positive selection. *J Exp Med* 203:165-175.

148. Yu, Q., Erman, B., Bhandoola, A., Sharrow, S.O., and Singer, A. 2003. In vitro evidence that cytokine receptor signals are required for differentiation of double positive thymocytes into functionally mature CD8+ T cells. *J Exp Med* 197:475-487.

149. He, X., Dave, V.P., Zhang, Y., Hua, X., Nicolas, E., Xu, W., Roe, B.A., and Kappes, D.J. 2005. The zinc finger transcription factor Th-POK regulates CD4 versus CD8 T-cell lineage commitment. *Nature* 433:826-833.

150. Kappes, D.J., and He, X. 2006. Role of the transcription factor Th-POK in CD4:CD8 lineage commitment. *Immunol Rev* 209:237-252.

151. Taniuchi, I., Osato, M., Egawa, T., Sunshine, M.J., Bae, S.C., Komori, T., Ito, Y., and Littman, D.R. 2002. Differential requirements for Runx proteins in CD4 repression and epigenetic silencing during T lymphocyte development. *Cell* 111:621-633.

152. Woolf, E., Xiao, C., Fainaru, O., Lotem, J., Rosen, D., Negreanu, V., Bernstein, Y., Goldenberg, D., Brenner, O., Berke, G., et al. 2003. Runx3 and Runx1 are required for CD8 T cell development during thymopoiesis. *Proc Natl Acad Sci U S A* 100:7731-7736.

153. Sato, T., Ohno, S., Hayashi, T., Sato, C., Kohu, K., Satake, M., and Habu, S. 2005. Dual functions of Runx proteins for reactivating CD8 and silencing CD4 at the commitment process into CD8 thymocytes. *Immunity* 22:317-328.

154. He, X., Park, K., Wang, H., Zhang, Y., Hua, X., Li, Y., and Kappes, D.J. 2008. CD4-CD8 lineage commitment is regulated by a silencer element at the ThPOK transcription-factor locus. *Immunity* 28:346-358.

155. Setoguchi, R., Tachibana, M., Naoe, Y., Muroi, S., Akiyama, K., Tezuka, C., Okuda, T., and Taniuchi, I. 2008. Repression of the transcription factor Th-POK by Runx complexes in cytotoxic T cell development. *Science* 319:822-825.

156. Egawa, T., and Littman, D.R. 2008. ThPOK acts late in specification of the helper T cell lineage and suppresses Runx-mediated commitment to the cytotoxic T cell lineage. *Nat Immunol* 9:1131-1139.

157. Egawa, T., and Taniuchi, I. 2009. Antagonistic interplay between ThPOK and Runx in lineage choice of thymocytes. *Blood Cells Mol Dis* 43:27-29.

158. Wang, L., and Bosselut, R. 2009. CD4-CD8 lineage differentiation: Thpok-ing into the nucleus. *J Immunol* 183:2903-2910.

159. Aliahmad, P., and Kaye, J. 2008. Development of all CD4 T lineages requires nuclear factor TOX. *J Exp Med* 205:245-256.

160. Hernandez-Hoyos, G., Anderson, M.K., Wang, C., Rothenberg, E.V., and Alberola-Ila, J. 2003. GATA-3 expression is controlled by TCR signals and regulates CD4/CD8 differentiation. *Immunity* 19:83-94.

161. Vanhecke, D., Verhasselt, B., Debacker, V., Leclercq, G., Plum, J., and Vandekerckhove, B. 1995. Differentiation to T helper cells in the thymus. Gradual acquisition of T helper cell function by CD3+CD4+ cells. *J Immunol* 155:4711-4718.

162. Vanhecke, D., Leclercq, G., Plum, J., and Vandekerckhove, B. 1995. Characterization of distinct stages during the differentiation of human CD69+CD3+ thymocytes and identification of thymic emigrants. *J Immunol* 155:1862-1872.

163. Vanhecke, D., Verhasselt, B., De Smedt, M., Leclercq, G., Plum, J., and Vandekerckhove, B. 1997. Human thymocytes become lineage committed at an early postselection CD69+ stage, before the onset of functional maturation. *J Immunol* 159:5973-5983.

164. Vanhecke, D., Verhasselt, B., De Smedt, M., De Paepe, B., Leclercq, G., Plum, J., and Vandekerckhove, B. 1997. MHC class II molecules are required for initiation of positive selection but not during terminal differentiation of human CD4 single positive thymocytes. *J Immunol* 158:3730-3737.

165. Bofill, M., Akbar, A.N., Salmon, M., Robinson, M., Burford, G., and Janossy, G. 1994. Immature CD45RA(low)RO(low) T cells in the human cord blood. I. Antecedents of CD45RA+ unprimed T cells. *J Immunol* 152:5613-5623.

166. Kong, F., Chen, C.H., and Cooper, M.D. 1998. Thymic function can be accurately monitored by the level of recent T cell emigrants in the circulation. *Immunity* 8:97-104.

167. Livak, F., and Schatz, D.G. 1996. T-cell receptor alpha locus V(D)J recombination by-products are abundant in thymocytes and mature T cells. *Mol Cell Biol* 16:609-618.

168. Takeshita, S., Toda, M., and Yamagishi, H. 1989. Excision products of the T cell receptor gene support a progressive rearrangement model of the alpha/delta locus. *EMBO J* 8:3261-3270.

169. Kimmig, S., Przybylski, G.K., Schmidt, C.A., Laurisch, K., Mowes, B., Radbruch, A., and Thiel, A. 2002. Two subsets of naive T helper cells with distinct T cell receptor excision circle content in human adult peripheral blood. *J Exp Med* 195:789-794.

170. McFarland, R.D., Douek, D.C., Koup, R.A., and Picker, L.J. 2000. Identification of a human recent thymic emigrant phenotype. *Proc Natl Acad Sci U S A* 97:4215-4220.

171. Sakaguchi, S., Sakaguchi, N., Asano, M., Itoh, M., and Toda, M. 1995. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J Immunol* 155:1151-1164.

172. Nishizuka, Y., and Sakakura, T. 1969. Thymus and reproduction: sex-linked dysgenesia of the gonad after neonatal thymectomy in mice. *Science* 166:753-755.

173. Asano, M., Toda, M., Sakaguchi, N., and Sakaguchi, S. 1996. Autoimmune disease as a consequence of developmental abnormality of a T cell subpopulation. *J Exp Med* 184:387-396.

174. Bonomo, A., Kehn, P.J., Payer, E., Rizzo, L., Cheever, A.W., and Shevach, E.M. 1995. Pathogenesis of post-thymectomy autoimmunity. Role of syngeneic MLR-reactive T cells. *J Immunol* 154:6602-6611.

175. Sakaguchi, S., Takahashi, T., and Nishizuka, Y. 1982. Study on cellular events in postthymectomy autoimmune oophoritis in mice. I. Requirement of Lyt-1 effector cells for oocytes damage after adoptive transfer. *J Exp Med* 156:1565-1576.

176. Shevach, E.M. 2009. Mechanisms of foxp3+ T regulatory cell-mediated suppression. *Immunity* 30:636-645.

177. Takahashi, T., Kuniyasu, Y., Toda, M., Sakaguchi, N., Itoh, M., Iwata, M., Shimizu, J., and Sakaguchi, S. 1998. Immunologic self-tolerance maintained by CD25+CD4+ naturally anergic and suppressive T cells: induction of autoimmune disease by breaking their anergic/suppressive state. *Int Immunol* 10:1969-1980.

178. Taams, L.S., Smith, J., Rustin, M.H., Salmon, M., Poulter, L.W., and Akbar, A.N. 2001. Human anergic/suppressive CD4(+)CD25(+) T cells: a highly differentiated and apoptosis-prone population. *Eur J Immunol* 31:1122-1131.

179. Dieckmann, D., Plottner, H., Berchtold, S., Berger, T., and Schuler, G. 2001. Ex vivo isolation and characterization of CD4(+)CD25(+) T cells with regulatory properties from human blood. *J Exp Med* 193:1303-1310.

180. Jonuleit, H., Schmitt, E., Stassen, M., Tuettenberg, A., Knop, J., and Enk, A.H. 2001. Identification and functional characterization of human CD4(+)CD25(+) T cells with regulatory properties isolated from peripheral blood. *J Exp Med* 193:1285-1294.

181. Levings, M.K., Sangregorio, R., and Roncarolo, M.G. 2001. Human cd25(+)cd4(+) t regulatory cells suppress naive and memory T cell proliferation and can be expanded in vitro without loss of function. *J Exp Med* 193:1295-1302.

182. Baecher-Allan, C., Brown, J.A., Freeman, G.J., and Hafler, D.A. 2001. CD4+CD25high regulatory cells in human peripheral blood. *J Immunol* 167:1245-1253.

183. Baecher-Allan, C., Viglietta, V., and Hafler, D.A. 2004. Human CD4+CD25+ regulatory T cells. *Semin Immunol* 16:89-98.

184. Baecher-Allan, C., Wolf, E., and Hafler, D.A. 2006. MHC class II expression identifies functionally distinct human regulatory T cells. *J Immunol* 176:4622-4631.

185. Josefowicz, S.Z., and Rudensky, A. 2009. Control of regulatory T cell lineage commitment and maintenance. *Immunity* 30:616-625.

186. Sakaguchi, S., and Sakaguchi, N. 2005. Regulatory T cells in immunologic self-tolerance and autoimmune disease. *Int Rev Immunol* 24:211-226.

187. Singh, B., Read, S., Asseman, C., Malmstrom, V., Mottet, C., Stephens, L.A., Stepankova, R., Tlaskalova, H., and Powrie, F. 2001. Control of intestinal inflammation by regulatory T cells. *Immunol Rev* 182:190-200.

188. Sakaguchi, S., Ono, M., Setoguchi, R., Yagi, H., Hori, S., Fehervari, Z., Shimizu, J., Takahashi, T., and Nomura, T. 2006. Foxp3+ CD25+ CD4+ natural regulatory T cells in dominant self-tolerance and autoimmune disease. *Immunol Rev* 212:8-27.

189. Sakaguchi, S., Yamaguchi, T., Nomura, T., and Ono, M. 2008. Regulatory T cells and immune tolerance. *Cell* 133:775-787.

190. Belkaid, Y., and Tarbell, K. 2009. Regulatory T cells in the control of host-microorganism interactions (*). *Annu Rev Immunol* 27:551-589.

191. Yamaguchi, T., and Sakaguchi, S. 2006. Regulatory T cells in immune surveillance and treatment of cancer. *Semin Cancer Biol* 16:115-123.

192. Belkaid, Y., and Rouse, B.T. 2005. Natural regulatory T cells in infectious disease. *Nat Immunol* 6:353-360.

193. Sakaguchi, S., Miyara, M., Costantino, C.M., and Hafler, D.A. 2010. FOXP3+ regulatory T cells in the human immune system. *Nat Rev Immunol* 10:490-500.

194. Coffer, P.J., and Burgering, B.M. 2004. Forkhead-box transcription factors and their role in the immune system. *Nat Rev Immunol* 4:889-899.

195. Pignata, C., Gaetaniello, L., Masci, A.M., Frank, J., Christiano, A., Matrecano, E., and Racioppi, L. 2001. Human equivalent of the mouse Nude/SCID phenotype: long-term evaluation of immunologic reconstitution after bone marrow transplantation. *Blood* 97:880-885.

196. Nehls, M., Pfeifer, D., Schorpp, M., Hedrich, H., and Boehm, T. 1994. New member of the winged-helix protein family disrupted in mouse and rat nude mutations. *Nature* 372:103-107.

197. Brunkow, M.E., Jeffery, E.W., Hjerrild, K.A., Paeper, B., Clark, L.B., Yasayko, S.A., Wilkinson, J.E., Galas, D., Ziegler, S.F., and Ramsdell, F. 2001. Disruption of a new forkhead/winged-helix protein, scurfin, results in the fatal lymphoproliferative disorder of the scurfy mouse. *Nat Genet* 27:68-73.

198. Fontenot, J.D., Gavin, M.A., and Rudensky, A.Y. 2003. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. *Nat Immunol* 4:330-336.

199. Williams, L.M., and Rudensky, A.Y. 2007. Maintenance of the Foxp3-dependent developmental program in mature regulatory T cells requires continued expression of Foxp3. *Nat Immunol* 8:277-284.

200. Bennett, C.L., Christie, J., Ramsdell, F., Brunkow, M.E., Ferguson, P.J., Whitesell, L., Kelly, T.E., Saulsbury, F.T., Chance, P.F., and Ochs, H.D. 2001. The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3. *Nat Genet* 27:20-21.

201. Wildin, R.S., Ramsdell, F., Peake, J., Faravelli, F., Casanova, J.L., Buist, N., Levy-Lahad, E., Mazzella, M., Goulet, O., Perroni, L., et al. 2001. X-linked neonatal diabetes mellitus, enteropathy and endocrinopathy syndrome is the human equivalent of mouse scurfy. *Nat Genet* 27:18-20.

202. Bettini, M.L., and Vignali, D.A. 2010. Development of thymically derived natural regulatory T cells. *Ann N Y Acad Sci* 1183:1-12.

203. Hori, S., Nomura, T., and Sakaguchi, S. 2003. Control of regulatory T cell development by the transcription factor Foxp3. *Science* 299:1057-1061.

204. Yagi, H., Nomura, T., Nakamura, K., Yamazaki, S., Kitawaki, T., Hori, S., Maeda, M., Onodera, M., Uchiyama, T., Fujii, S., et al. 2004. Crucial role of FOXP3 in the development and function of human CD25+CD4+ regulatory T cells. *Int Immunol* 16:1643-1656.

205. Oswald-Richter, K., Grill, S.M., Shariat, N., Leelawong, M., Sundrud, M.S., Haas, D.W., and Unutmaz, D. 2004. HIV infection of naturally occurring and genetically reprogrammed human regulatory T-cells. *PLoS Biol* 2:E198.

206. Ziegler, S.F. 2006. FOXP3: of mice and men. *Annu Rev Immunol* 24:209-226.

207. Allan, S.E., Passerini, L., Bacchetta, R., Crellin, N., Dai, M., Orban, P.C., Ziegler, S.F., Roncarolo, M.G., and Levings, M.K. 2005. The role of 2 FOXP3 isoforms in the generation of human CD4+ Tregs. *J Clin Invest* 115:3276-3284.

208. Aarts-Riemens, T., Emmelot, M.E., Verdonck, L.F., and Mutis, T. 2008. Forced overexpression of either of the two common human Foxp3 isoforms can induce regulatory T cells from CD4(+)CD25(-) cells. *Eur J Immunol* 38:1381-1390.

209. Marson, A., Kretschmer, K., Frampton, G.M., Jacobsen, E.S., Polansky, J.K., MacIsaac, K.D., Levine, S.S., Fraenkel, E., von Boehmer, H., and Young, R.A. 2007. Foxp3 occupancy and regulation of key target genes during T-cell stimulation. *Nature* 445:931-935.

210. Zheng, Y., and Rudensky, A.Y. 2007. Foxp3 in control of the regulatory T cell lineage. *Nat Immunol* 8:457-462.

211. Curotto de Lafaille, M.A., and Lafaille, J.J. 2009. Natural and adaptive foxp3+ regulatory T cells: more of the same or a division of labor? *Immunity* 30:626-635.

212. Gavin, M.A., Torgerson, T.R., Houston, E., DeRoos, P., Ho, W.Y., Stray-Pedersen, A., Ocheltree, E.L., Greenberg, P.D., Ochs, H.D., and Rudensky, A.Y. 2006. Single-cell analysis of normal and FOXP3-mutant human T cells: FOXP3 expression without regulatory T cell development. *Proc Natl Acad Sci U S A* 103:6659-6664.

213. Morgan, M.E., van Bilsen, J.H., Bakker, A.M., Heemskerk, B., Schilham, M.W., Hartgers, F.C., Elferink, B.G., van der Zanden, L., de Vries, R.R., Huizinga, T.W., et al. 2005. Expression of FOXP3 mRNA is not confined to CD4+CD25+ T regulatory cells in humans. *Hum Immunol* 66:13-20.

214. Walker, M.R., Kasprowicz, D.J., Gersuk, V.H., Benard, A., Van Landeghen, M., Buckner, J.H., and Ziegler, S.F. 2003. Induction of FoxP3 and acquisition of T regulatory activity by stimulated human CD4+CD25- T cells. *J Clin Invest* 112:1437-1443.

215. Wang, J., Ioan-Facsinay, A., van der Voort, E.I., Huizinga, T.W., and Toes, R.E. 2007. Transient expression of FOXP3 in human activated nonregulatory CD4+ T cells. *Eur J Immunol* 37:129-138.

216. Baron, U., Floess, S., Wieczorek, G., Baumann, K., Grutzkau, A., Dong, J., Thiel, A., Boeld, T.J., Hoffmann, P., Edinger, M., et al. 2007. DNA demethylation in the human FOXP3 locus discriminates regulatory T cells from activated FOXP3(+) conventional T cells. *Eur J Immunol* 37:2378-2389.

217. Janson, P.C., Winerdal, M.E., Marits, P., Thorn, M., Ohlsson, R., and Winqvist, O. 2008. FOXP3 promoter demethylation reveals the committed Treg population in humans. *PLoS One* 3:e1612.

218. Miyara, M., Yoshioka, Y., Kitoh, A., Shima, T., Wing, K., Niwa, A., Parizot, C., Taflin, C., Heike, T., Valeyre, D., et al. 2009. Functional delineation and differentiation dynamics of human CD4+ T cells expressing the FoxP3 transcription factor. *Immunity* 30:899-911.

219. Akbar, A.N., Vukmanovic-Stejic, M., Taams, L.S., and Macallan, D.C. 2007. The dynamic co-evolution of memory and regulatory CD4+ T cells in the periphery. *Nat Rev Immunol* 7:231-237.

220. Seddiki, N., Santner-Nanan, B., Martinson, J., Zaunders, J., Sasson, S., Landay, A., Solomon, M., Selby, W., Alexander, S.I., Nanan, R., et al. 2006. Expression of interleukin (IL)-2 and IL-7 receptors discriminates between human regulatory and activated T cells. *J Exp Med* 203:1693-1700.

221. Liu, W., Putnam, A.L., Xu-Yu, Z., Szot, G.L., Lee, M.R., Zhu, S., Gottlieb, P.A., Kapranov, P., Gingeras, T.R., Fazekas de St Groth, B., et al. 2006. CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4+ T reg cells. *J Exp Med* 203:1701-1711.

222. Cozzo, C., Larkin, J., 3rd, and Caton, A.J. 2003. Cutting edge: self-peptides drive the peripheral expansion of CD4+CD25+ regulatory T cells. *J Immunol* 171:5678-5682.

223. Simonetta, F., Chiali, A., Cordier, C., Urrutia, A., Girault, I., Bloquet, S., Tanchot, C., and Bourgeois, C. 2010. Increased CD127 expression on activated FOXP3(+)CD4(+) regulatory T cells. *Eur J Immunol*.

224. Borsellino, G., Kleinewietfeld, M., Di Mitri, D., Sternjak, A., Diamantini, A., Giometto, R., Hopner, S., Centonze, D., Bernardi, G., Dell'Acqua, M.L., et al. 2007. Expression of ectonucleotidase CD39 by Foxp3+ Treg cells: hydrolysis of extracellular ATP and immune suppression. *Blood* 110:1225-1232.

225. Deaglio, S., Dwyer, K.M., Gao, W., Friedman, D., Usheva, A., Erat, A., Chen, J.F., Enjyoji, K., Linden, J., Oukka, M., et al. 2007. Adenosine generation catalyzed by CD39 and CD73 expressed on regulatory T cells mediates immune suppression. *J Exp Med* 204:1257-1265.

226. Annunziato, F., Cosmi, L., Liotta, F., Lazzeri, E., Romagnani, P., Angeli, R., Lasagni, L., Manetti, R., Marra, F., Gerard, C., et al. 2006. CXCR3 and alphaEbeta7 integrin identify a subset of CD8+ mature thymocytes that share phenotypic and functional properties with CD8+ gut intraepithelial lymphocytes. *Gut* 55:961-968.

227. Lehmann, J., Huehn, J., de la Rosa, M., Maszyna, F., Kretschmer, U., Krenn, V., Brunner, M., Scheffold, A., and Hamann, A. 2002. Expression of the integrin

alpha Ebeta 7 identifies unique subsets of CD25+ as well as CD25- regulatory T cells. *Proc Natl Acad Sci U S A* 99:13031-13036.

228. Allakhverdi, Z., Fitzpatrick, D., Boisvert, A., Baba, N., Bouguermouh, S., Sarfati, M., and Delespesse, G. 2006. Expression of CD103 identifies human regulatory T-cell subsets. *J Allergy Clin Immunol* 118:1342-1349.

229. Rotzschke, O., Borsellino, G., Battistini, L., Falk, K., and Kleinewietfeld, M. 2009. In vivo-activated CD103+ Foxp3+ Tregs: of men and mice. *Blood* 113:2119-2120; author reply 2120.

230. Klein, L., Khazaie, K., and von Boehmer, H. 2003. In vivo dynamics of antigen-specific regulatory T cells not predicted from behavior in vitro. *Proc Natl Acad Sci U S A* 100:8886-8891.

231. Fehervari, Z., and Sakaguchi, S. 2004. Control of Foxp3+ CD25+CD4+ regulatory cell activation and function by dendritic cells. *Int Immunol* 16:1769-1780.

232. Fontenot, J.D., Rasmussen, J.P., Williams, L.M., Dooley, J.L., Farr, A.G., and Rudensky, A.Y. 2005. Regulatory T cell lineage specification by the forkhead transcription factor foxp3. *Immunity* 22:329-341.

233. Jordan, M.S., Boesteanu, A., Reed, A.J., Petrone, A.L., Holenbeck, A.E., Lerman, M.A., Naji, A., and Caton, A.J. 2001. Thymic selection of CD4+CD25+ regulatory T cells induced by an agonist self-peptide. *Nat Immunol* 2:301-306.

234. Apostolou, I., Sarukhan, A., Klein, L., and von Boehmer, H. 2002. Origin of regulatory T cells with known specificity for antigen. *Nat Immunol* 3:756-763.

235. van Santen, H.M., Benoist, C., and Mathis, D. 2004. Number of T reg cells that differentiate does not increase upon encounter of agonist ligand on thymic epithelial cells. *J Exp Med* 200:1221-1230.

236. Bonasio, R., Scimone, M.L., Schaerli, P., Grabie, N., Lichtman, A.H., and von Andrian, U.H. 2006. Clonal deletion of thymocytes by circulating dendritic cells homing to the thymus. *Nat Immunol* 7:1092-1100.

237. Liston, A., Nutsch, K.M., Farr, A.G., Lund, J.M., Rasmussen, J.P., Koni, P.A., and Rudensky, A.Y. 2008. Differentiation of regulatory Foxp3+ T cells in the thymic cortex. *Proc Natl Acad Sci U S A* 105:11903-11908.

238. Bensinger, S.J., Bandeira, A., Jordan, M.S., Caton, A.J., and Laufer, T.M. 2001. Major histocompatibility complex class II-positive cortical epithelium mediates the selection of CD4(+)25(+) immunoregulatory T cells. *J Exp Med* 194:427-438.

239. Burchill, M.A., Yang, J., Vang, K.B., Moon, J.J., Chu, H.H., Lio, C.W., Vegoe, A.L., Hsieh, C.S., Jenkins, M.K., and Farrar, M.A. 2008. Linked T cell receptor and cytokine signaling govern the development of the regulatory T cell repertoire. *Immunity* 28:112-121.

240. Lio, C.W., and Hsieh, C.S. 2008. A two-step process for thymic regulatory T cell development. *Immunity* 28:100-111.

241. Burchill, M.A., Yang, J., Vogtenhuber, C., Blazar, B.R., and Farrar, M.A. 2007. IL-2 receptor beta-dependent STAT5 activation is required for the development of Foxp3+ regulatory T cells. *J Immunol* 178:280-290.

242. Yao, Z., Kanno, Y., Kerenyi, M., Stephens, G., Durant, L., Watford, W.T., Laurence, A., Robinson, G.W., Shevach, E.M., Moriggl, R., et al. 2007. Nonredundant roles for Stat5a/b in directly regulating Foxp3. *Blood* 109:4368-4375.

243. Feuerer, M., Hill, J.A., Mathis, D., and Benoist, C. 2009. Foxp3+ regulatory T cells: differentiation, specification, subphenotypes. *Nat Immunol* 10:689-695.

244. Aschenbrenner, K., D'Cruz, L.M., Vollmann, E.H., Hinterberger, M., Emmerich, J., Swee, L.K., Rolink, A., and Klein, L. 2007. Selection of Foxp3+ regulatory T cells specific for self antigen expressed and presented by Aire+ medullary thymic epithelial cells. *Nat Immunol* 8:351-358.

245. Anderson, M.S., Venanzi, E.S., Chen, Z., Berzins, S.P., Benoist, C., and Mathis, D. 2005. The cellular mechanism of Aire control of T cell tolerance. *Immunity* 23:227-239.

246. Cupedo, T., Nagasawa, M., Weijer, K., Blom, B., and Spits, H. 2005. Development and activation of regulatory T cells in the human fetus. *Eur J Immunol* 35:383-390.

247. Stephens, L.A., Mottet, C., Mason, D., and Powrie, F. 2001. Human CD4(+)CD25(+) thymocytes and peripheral T cells have immune suppressive activity in vitro. *Eur J Immunol* 31:1247-1254.

248. Wing, K., Larsson, P., Sandstrom, K., Lundin, S.B., Suri-Payer, E., and Rudin, A. 2005. CD4+ CD25+ FOXP3+ regulatory T cells from human thymus and cord blood suppress antigen-specific T cell responses. *Immunology* 115:516-525.

249. Annunziato, F., Cosmi, L., Liotta, F., Lazzeri, E., Manetti, R., Vanini, V., Romagnani, P., Maggi, E., and Romagnani, S. 2002. Phenotype, localization, and mechanism of suppression of CD4(+)CD25(+) human thymocytes. *J Exp Med* 196:379-387.

250. Watanabe, N., Wang, Y.H., Lee, H.K., Ito, T., Cao, W., and Liu, Y.J. 2005. Hassall's corpuscles instruct dendritic cells to induce CD4+CD25+ regulatory T cells in human thymus. *Nature* 436:1181-1185.

251. Liotta, F., Cosmi, L., Romagnani, P., Maggi, E., Romagnani, S., and Annunziato, F. 2005. Functional features of human CD25+ regulatory thymocytes. *Microbes Infect* 7:1017-1022.

252. Tuovinen, H., Pekkarinen, P.T., Rossi, L.H., Mattila, I., and Arstila, A.T. 2008. The FOXP3(+) subset of human CD4(+)CD8(+) thymocytes is immature and subject to intrathymic selection. *Immunol Cell Biol*.

253. Cosmi, L., Liotta, F., Lazzeri, E., Francalanci, M., Angeli, R., Mazzinghi, B., Santarlasci, V., Manetti, R., Vanini, V., Romagnani, P., et al. 2003. Human CD8+CD25+ thymocytes share phenotypic and functional features with CD4+CD25+ regulatory thymocytes. *Blood* 102:4107-4114.

254. Tuovinen, H., Kekalainen, E., Rossi, L.H., Puntila, J., and Arstila, T.P. 2008. Cutting edge: human CD4-CD8- thymocytes express FOXP3 in the absence of a TCR. *J Immunol* 180:3651-3654.

255. Hanabuchi, S., Ito, T., Park, W.R., Watanabe, N., Shaw, J.L., Roman, E., Arima, K., Wang, Y.H., Voo, K.S., Cao, W., et al. 2010. Thymic stromal lymphopoietin-activated plasmacytoid dendritic cells induce the generation of FOXP3+ regulatory T cells in human thymus. *J Immunol* 184:2999-3007.

256. Martin-Gayo, E., Sierra-Filardi, E., Corbi, A.L., and Toribio, M.L. 2010. Plasmacytoid dendritic cells resident in human thymus drive natural Treg cell development. *Blood* 115:5366-5375.

257. Tai, X., Cowan, M., Feigenbaum, L., and Singer, A. 2005. CD28 costimulation of developing thymocytes induces Foxp3 expression and regulatory T cell differentiation independently of interleukin 2. *Nat Immunol* 6:152-162.

Chapter II

Objectives and Workplan

CHAPTER II: OBJECTIVES AND WORKPLAN

Thymic-derived Tregs constitute a T cell lineage dedicated to immune regulation that is vital for the prevention of autoimmunity and the maintenance of immune homeostasis.

The overall objective of this work was to investigate the development of Tregs in the human thymus in order to clarify the developmental stage at which the Treg lineage diverges from other T cell fates and the developmental program that Treg precursors undergo to give rise to mature Foxp3+ cells.

At the time this work was initiated previous studies relied on CD25 expression as a marker of Tregs, and the few studies examining the expression of the then recently described Foxp3 transcription factor did so only at the RNA level, due to the lack of Foxp3-specific human antibodies. We took advantage of the collaboration with Hospital de Santa Cruz, which allowed us to study human thymuses from pediatric cardiac surgeries that would otherwise be discarded, and of the recent availability of human anti-Foxp3 antibodies to investigate the development of human Tregs.

Our first specific aim pertained to the earliest stage during human T cell development where the expression of Foxp3, thus far considered the best marker of Tregs, could be detected. With this purpose, we examined the expression of both Foxp3 RNA and protein throughout the developmental stages described for human T cell development based on CD3, CD4 and CD8 expression in 21 pediatric thymuses. Although the mechanisms of Treg generation have been the focus of intense investigation during the last decade in the mouse system, several issues are still matter of debate, namely pre-DP Foxp3 expression. Identification of early, pre-DP Foxp3 expression in the human thymus raised the question of whether this would also be observed in the mouse model, and, promoted the use of murine tools, such as the Foxp3-GFP and TCRα-deficient mice, to evaluate the TCR-dependency of this early Foxp3 expression during T cell development. The results of this study are presented in Chapter III.

Our second specific aim concerned the expression of Foxp3 at the DP stage and the relationship between Foxp3-expressing DP and SP thymocytes. Several approaches were used to address these questions. First, the existence of *bona fide* Foxp3+ DP

thymocytes was attested using a re-expression assay, consisting on the stripping of surface molecules and the re-expression in culture of transcriptionally active molecules. Then, we aimed to estimate the induction of Foxp3 at the DP stage and the relative contribution of Tregs generated at this stage for the mature Treg pool. Maturation and proliferation markers, as well as several Treg associated-markers, were used to characterize Foxp3+ DP thymocyte differentiation using up to 10-parameter flow cytometry, in addition to *in vitro* assays. Moreover, in order to assess a possible precursor-progeny relationship between Foxp3-expressind DP and SP thymocytes we developed a regression analysis model to fit the data. Results from this study are presented in Chapter IV.

In agreement with Decreto-Lei 388/70, art. 8º, parágrafo 2, the results presented here were published or are currently being prepared for publication in the following scientific journals:

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Immunology and Cell Biology (2010), 88 (5): 523-28.

Nunes-Cabaço H, Caramalho I, Sepúlveda N, Sousa AE

Regulatory T cell differentiation in the human thymus occurs mainly at the double positive stage.

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Chapter III

Foxp3 expression in pre-DP human and murine thymocytes

Immunology and Cell Biology 2010; 88 (5); 523-8

Foxp3 induction in human and murine thymus precedes the CD4+ CD8+ stage but requires early T cell receptor expression.

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CHAPTER III: FOXP3 EXPRESSION IN PRE-DP HUMAN AND MURINE THYMOCYTES

ABSTRACT

The thymus generates a T cell lineage dedicated to immune regulation, "naturally-occurring" regulatory T cells, best specified by the forkhead family transcription factor Foxp3. Here we have conducted a parallel study in humans and mice where we dissected the earliest stages of Foxp3 induction during thymocyte development. By analyzing a large collection of 21 human thymuses we show that Foxp3 can be consistently detected in CD4 immature single positive thymocytes that precede the CD4(+)CD8(+) (double positive, DP) stage. The reduced levels of CD3 expression found at this stage of human thymocyte development raise the question of TCR requirement for Foxp3 induction. We further show that, in mice, Foxp3 expression was also detected in pre-DP thymocytes of TCRα-sufficient but not in TCRα-deficient animals, genetically demonstrating the TCR-dependence of Foxp3 expression at pre-DP stages of T cell development.

INTRODUCTION

The existence of a thymic-derived lineage devoted to regulatory function ensures the maintenance of a peripheral regulatory T cell (Treg) pool throughout life [1, 2]. The forkhead family transcription factor, Foxp3, remains the best, albeit non-specific, Treg marker in both humans and mice [3, 4]. The biological importance of Foxp3 is attested by the highly aggressive multi-organ autoimmune disease that develops in its absence or mutation both in mice and humans, as demonstrated by the association of IPEX syndrome in humans (immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome) with *foxp3* mutations [5-7]. Foxp3 induction and regulation are therefore of crucial importance for the development of Treg-based therapeutic strategies for tolerance achievement and control of immunopathology. Over the last decade many reports have highlighted the important roles played by Treg in the control of immune responses, particularly in the context of inflammatory or autoimmune processes [8, 9], and Treg-based therapeutic strategies are currently being tested in the clinic [9].

The thymic cortex has been shown to fully support the induction of Foxp3 in the murine system [10-12]. In spite of previous data suggesting an absolute requirement for TCRαβ expression for Foxp3 gene induction in murine thymocytes [13], it was recently reported that human CD4- CD8- (double negative, DN) thymocytes can express Foxp3 the in the absence of surface TCR staining [14]. However, the possibility of Foxp3 induction independently of TCRαβ expression was subsequently questioned by a study showing that the large majority of the DN Foxp3+ cells were CD3/TCRαβlow [15].

DP thymocytes are traditionally regarded as the first population to complete TCRα rearrangements and thus express a mature TCRαβ complex. However, it has been clearly demonstrated that productive TCRα rearrangements and transcripts can be found in mouse DN thymocytes [16, 17]. Moreover, TCRβ selection and initiation of TCRα rearrangements in humans was also recently shown to occur earlier than previously thought, namely at the CD34+ CD38+ CD1a+ stage of thymocyte development [18].

Here we carefully dissected the expression pattern of Foxp3 in human and murine early thymocyte development and formally tested the requirement of TCRα expression, the rate-limiting factor for the assembly of the mature TCRαβ complex, for Foxp3 gene induction.

RESULTS

FOXP3 EXPRESSION IN EARLY HUMAN T CELL DEVELOPMENT

Data on Foxp3 expression in the human thymus are scarce [14, 19-21]. Human T cell development is known to proceed through a series of phenotypically distinct stages. T cell progenitors contained within the early CD3- CD4- CD8- triple negative population (TN) initially acquire CD4 (CD4 immature single positive cells, CD4ISP) and subsequently CD8 to become DP cells. A progressive increase in surface expression of CD3 occurs in parallel with surface TCRαβ in DP cells, followed by differentiation into CD4 or CD8 SP cells. We used 8-parameter flow cytometry to quantify Foxp3 protein expression in 21 thymuses of immunologically healthy children aged 5 days to 13 years, and found no significant differences according to age (r=-0.1799, n=17) or sex (p=0.3139, n=17), in agreement with previous reports [19, 22].

In order to assess the early stages of thymocyte development we applied stringent gating criteria using a panel of lineage markers. Foxp3 expression in TN thymocytes was not uniformly observed in all thymuses assessed (Fig. 1A and B and Supplemental Fig. 1A). Within CD4ISP cells we could observe consistent Foxp3 expression, although at low levels (Fig. 1A and B and Supplemental Fig. 1A). Moreover, within DP cells Foxp3 expression was found in cells with negative/low CD3 levels and increased with the upregulation of surface CD3 (Fig. 1A and B). The same profile was found when assessing the expression of Foxp3 in relation to TCRαβ within DP (Fig. 1A). Of note, similar results were observed with different commercially available anti-Foxp3 antibody clonotypes (Supplemental Fig. 1A and B).

To further elucidate the expression of Foxp3 in the early phases of T cell development we sorted the different subsets to purity and analyzed *foxp3* mRNA expression. The levels of transcriptional *foxp3* assessed by real-time PCR were significantly correlated with the levels of protein expression measured by flow cytometry in total thymocytes (r=0.7956, p=0.0059, n=10). The real-time PCR data confirmed the inconsistency of *foxp3* expression in TN cells, since amplification was only observed in 2 out of 4 thymuses and at low levels. *foxp3* transcripts were detected in CD4ISP and in DP CD3-/low cells, though at much lower levels than those found in DP CD3+/high cells and in SP thymocytes (Fig. 1C).

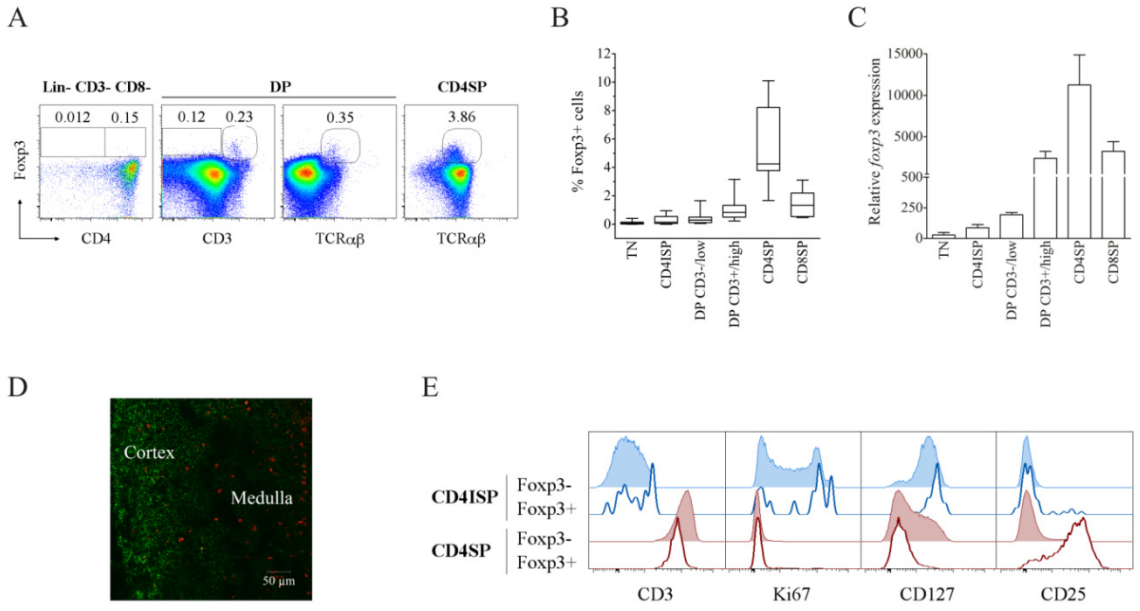

Fig. 1. Foxp3 expression in the early stages of human thymocyte development. (**A**) Representative dot-plots of Foxp3 protein expression (clone PCH101) in the human thymus as assessed by 6-color flow cytometry using a FACSCanto. Lineage- CD3- CD8- cells were analyzed according to absence or presence of CD4 expression in order to discriminate the TN and CD4ISP populations, respectively. Numbers represent the percentage of Foxp3+ cells as defined by the illustrated gates within the thymocyte populations mentioned in the top of the dot-plots. (**B**) Proportion of Foxp3+ cells within the different thymocyte subsets in 14 thymuses assessed with the same staining protocol. (**C**) Quantitative RT-PCR (qPCR) for *foxp3* in sorted thymocyte subsets from 4 thymuses (children aged 12 days, 2 months, 2 years and 7 years). A combination of lineage markers (CD14, CD16, CD20, CD56 and HLA-DR) with CD3, CD8 and CD4 were used to sort TN (Lin-CD3-CD8-CD4-) and CD4ISP (Lin-CD3-CD8-CD4+) thymocytes. *gapdh* was used as housekeeping gene for normalization of cDNA content. Bars represent mean±SEM. **(D)** Immunofluorescence confocal microscopy analysis of a thymic frozen section from a 46 day old child stained for Foxp3 (clone PCH101; red) and a cortical marker, CD1a (green), illustrating the expression of Foxp3 in the thymic cortex. **(E)** Representative analysis of the phenotype of CD4ISP Foxp3+ thymocytes. Comparison with CD4SP thymocytes was performed in order to confirm the immaturity of this population. Graphs show the expression of the cycling marker Ki67 and the surface levels of CD3, CD25 and CD127 within Foxp3+CD4ISP, Foxp3-CD4ISP, Foxp3+CD4SP and Foxp3-CD4SP gated cells.

Thymocyte development is known to be topographically organized within the thymus with the initial stages occurring in the cortex and mature cells being found in the medulla. Using immunostaining in thymic sections we confirmed the presence of Foxp3 in cortical CD1a+ thymocytes, although, as expected, the majority of Foxp3 positive cells were found in the medulla and corticomedullary junction (Fig. 1D). Moreover, to attest the immature phenotype of CD4ISP, we analyzed the expression of surface markers CD127, CD25 and CD3, and assessed their proliferative status by Ki67 staining. The results show that CD4ISP are proliferating cells that display a CD3lowCD25lowCD127+ phenotype characteristic of pre-DP thymocytes (Fig. 1E).

These data demonstrate that Foxp3 is expressed at the immature CD4ISP stage of human thymocyte development.

FOXP3 EXPRESSION IN EARLY MOUSE T CELL DEVELOPMENT

We next asked whether Foxp3 expression was also present in pre-DP stages of murine T cell development, since a careful dissection of Foxp3 expression in early thymocyte subsets has not been described. Importantly, Treg development is strongly influenced by cortical events that affect DN progenitors[23]. We subdivided DN cells into well-established developmental stages characterized by CD44, CD25 and c-Kit expression [24], and analyzed *foxp3* mRNA levels by quantitative RT-PCR. The first T cell-committed DN subset (CD44+ CD25+ c-Kit+), known as pro-T or stage 2 (DN2), as well as the subsequent pre-T DN3 stage, both lacked *foxp3* message; however, pre-DP DN4 cells expressed a low but clearly detectable level of *foxp3* transcripts, which was approximately 10-fold lower than in mature CD4SP thymocytes but 5-fold higher than in DP cells (Fig. 2A).

Analysis at protein level, revealed a population of Foxp3+ cells within the DN4 subset (<1% compared to ~4% for CD4SP thymocytes; Supplemental Fig. 2A and B) by intracellular staining. Moreover, taking advantage of a murine genetic tool, namely Foxp3 reporter mice, in which the GFP gene has been knocked-in into the *foxp3* locus [13], we showed that, while the DN3 subset was devoid of GFP+ cells, DN4 pre-DP thymocytes clearly contained a small population of GFP+ cells, with a frequency comparable to that observed in DP thymocytes (Fig. 2B and C and Supplemental Fig. 2C). These cells expressed high levels of HSA (CD24) (Fig. 2D), a well-established marker for thymocyte immaturity[25,26], thus excluding contamination of the DN compartment with HSA-negative mature T cells (Fig. 2D) that would have down-regulated CD4/ CD8.

These results collectively show that the induction of murine Foxp3 transcription and translation precedes the DP stage and occurs at the DN4 stage.

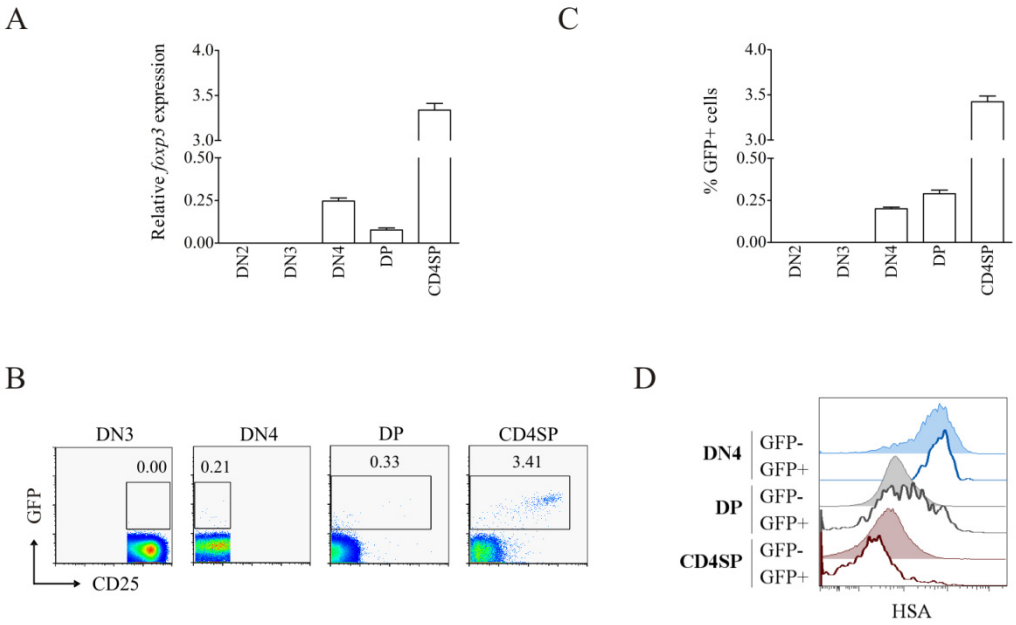

Fig. 2. Murine Foxp3 is expressed in pre-DP thymocytes. (**A**) Quantitative RT-PCR (qPCR) for *foxp3* in sorted thymocyte subsets from adult C57Bl/6 mice. DN2 are CD4- CD8- TCRγδ- NK1.1- B220- (Lin-) CD44+ CD25+, DN3 are Lin- CD44- CD25+, DN4 are Lin- CD44- CD25-, DP are CD4+ CD8+, and CD4SP are CD4+ CD8- thymocytes. *hprt* was used for normalization. (**B**) Foxp3 protein expression as revealed by GFP fluorescence in thymocyte subsets isolated from Foxp3-GFP knock-in reporter mice using the gating criteria described above. Each panel of this figure is representative of duplicate or triplicate experiments involving at least 4 animals each. (**C**) Proportion of Foxp3-GFP+ cells within the different thymocyte subsets in three separate experiments involving at least 4 animals each. (**D**) Representative characterization of GFP+ DN4 cells regarding the expression of the immaturity marker HSA. HSA levels were compared between GFP+ and GFP- cells within the DN4, DP and CD4SP subsets from pooled Foxp3-GFP mice (at least 2 animals).

REQUIREMENT FOR TCRα EXPRESSION IN FOXP3 INDUCTION

The timing of rearrangement of the various TCR genes in the human thymus has been recently reevaluated with TCRα rearrangements being shown to occur earlier than the CD4ISP stage [18]. These data are in agreement with the quantification of the TCR excision DNA circles resultant from the excision of TCRδ (sjTREC), that are known to be required for TCRα rearrangements [27]. Therefore, it is plausible that productive TCRα rearrangements have occurred in CD4ISP thymocytes.

Although the majority of murine DN thymocytes express negligible amounts of surface TCRαβ complexes, some display intermediate/low levels [24]. When we analyzed *foxp3* expression in both subpopulations, we were able to detect transcripts in TCRlow but not in TCRnegative DN thymocytes (Fig. 3A), demonstrating that early *foxp3* induction is

conserved in mice, but apparently correlates with the "premature" (pre-DP) expression of TCR complexes. Consistent with this, GFP+ DN4 cells expressed TCRβ on the cell surface and exhibited hallmarks of TCR signaling [28], such as CD5 upregulation (Fig. 3B). Of note, all GFP+ DN4 and DP thymocytes expressed high levels of CD5 (Fig. 3B), suggesting that early *foxp3* induction is associated with TCR signaling before the SP stage.

We further confirmed that productively rearranged and transcribed TCRα genes were expressed in our sorted C57Bl/6 "wild type" (WT) DN4 cells (Fig. 3C), as well as in their DN2/DN3 precursors, prompting us to test the direct involvement of TCRα, the rate-limiting component of TCRαβ assembly [29], in early *foxp3* induction. We employed another murine genetic tool, TCRα-deficient animals [30], and were able to show that DN4 cells isolated from TCRα-deficient thymuses are devoid of *foxp3* transcripts (Fig. 3D, 3E and 3F). We also observed a significant decrease to "background staining levels" of Foxp3 protein expression in TCRα-deficient DN4 and DP cells (Supplemental Fig. 2B). Thus, the mature TCR is absolutely required for Foxp3 expression in pre-DP thymocytes, just as it is at the DP stage (Fig. 3E). *foxp3* transcription in DN4 cells could be observed throughout WT mouse development, from newborn to adult, but was always absent in TCRα-deficient thymocytes (Fig. 3F). At that stage the pre-TCR was not able to substitute for the mature TCR (Fig. 3E). An involvement of TCR signaling was further suggested by the higher expression of CD5 in Foxp3+ DN4 cells as compared to their Foxp3- counterparts (Fig. 3B), as well as by the severe decrease in Foxp3 expression within DN4 cells in MHC class II-deficient mice, which also implies a requirement for self-peptide presentation (Supplemental Fig. 2B).

These data demonstrate that *foxp3* transcription in the murine thymus is first induced at the pre-DP stage through a TCR-dependent mechanism.

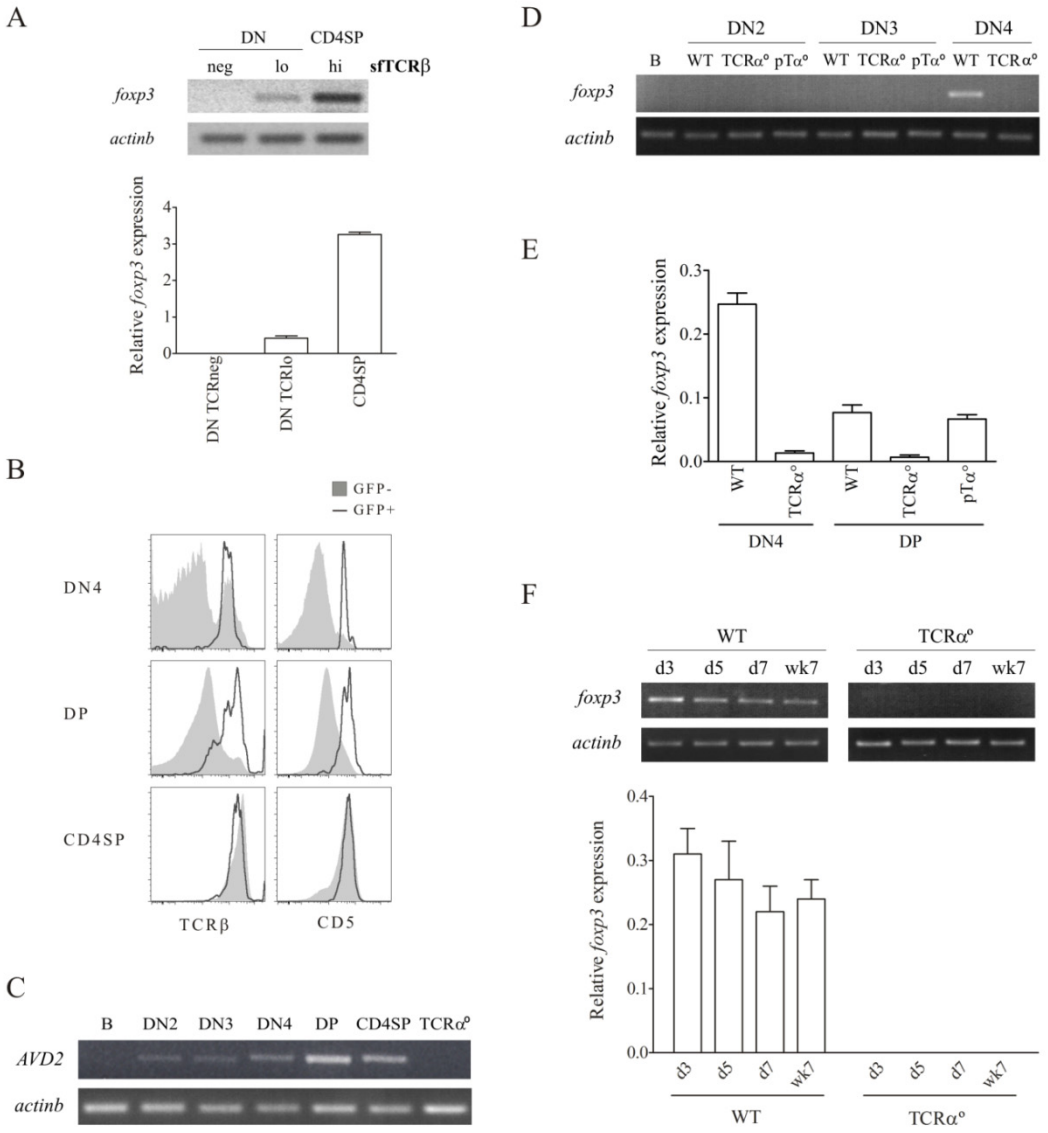

Fig. 3. TCRα expression is required for Foxp3 induction in pre-DP thymocytes. (**A**) Semi-quantitative RT-PCR and qPCR for *foxp3* in CD4- CD8- (DN) and CD4+ CD8- (CD4SP) murine (C57Bl/6) thymocytes FACSorted according to surface TCRβ (sfTCR) levels. *actinb* was used as housekeeping gene for normalization of cDNA content. (**B**) Representative analysis of surface TCRβ and CD5 expression levels within GFP+ DN4 cells from pooled Foxp3-GFP mice (at least 2 animals). GFP+ and GFP- cells were compared within the DN4, DP and CD4SP subsets. (**C**) Semi-quantitative RT-PCR for *tcra* (*AVD2*) transcripts in sorted thymocyte subsets from C57Bl/6 mice, except TCRα-deficient (TCRα^o) DP thymocytes in the last lane (negative control). Sorted B cells are shown in the first lane as an additional negative control. (**D**) Semi-quantitative RT-PCR for *foxp3* in DN subsets from adult C57Bl/6 (wild type, WT) mice, TCRα-deficient (TCRα^o) and pTα-deficient (pTα^o) mice. Sorted B cells are also shown in the first lane as a negative control. (**E**) qPCR for *foxp3* in DN4 and DP thymocytes from adult C57Bl/6 (wild type, WT) mice, TCRα-deficient (TCRα^o) and pTα-deficient (pTα^o) mice. (**F**) Semi-quantitative RT-PCR and qPCR for *foxp3* in DN4 thymocytes sorted from C57Bl/6 (wild type, WT) or TCRα-deficient (TCRα^o) mice at different ages: 3-5-7 days or 7 weeks (wk7, adult) postnatal. Duplicate or triplicate experiments involving at least 6 animals each were performed.

DISCUSSION

Immune-suppressive mechanisms are crucial for homeostasis of the T cell compartment and prevention of the immunopathology associated with inflammatory responses. The existence of a thymically-derived lineage devoted to regulatory function ensures the maintenance of a peripheral Treg pool throughout life. Understanding the mechanisms underlying Treg development, and in particular the key induction of Foxp3, is essential for the clinical manipulation of Treg.

Here we showed that Foxp3 is induced in pre-DP stages of both mouse and human T cell development. Foxp3 expression was clearly observed at the murine DN4 and the human CD4ISP stages, defined according to a series of maturation markers that exclude contamination with mature re-circulating cells.

GFP+ and GFP- DN4 thymocytes in Foxp3-GFP reporter mice were found to be at a similar stage of maturation, as indicated by the uniform high levels of surface HSA [25]. However, upregulation of CD5 expression, which has been closely associated with TCR signaling [28], was essentially restricted to GFP+ cells.

Mature TCRαβ complexes are first detectable by conventional antibody-staining techniques at the DP stage of both human and mouse T cell development, but productive rearrangement and mRNA expression of TCRβ and TCRα have clearly been shown to precede the DP stage [16-18]. This phenomenon has important repercussions for all TCR-dependent events, which could therefore be initiated at the DN stage in the thymic cortex. Using a combination of genetic tools, we formally demonstrated that TCR expression is required for murine Foxp3 induction at pre-DP stages. Of note, cumulative data from different groups have demonstrated that the thymic cortex epithelium can fully support the differentiation of mouse Tregs [10-12].

Our data emphasize the parallel between Foxp3 expression during human and murine thymocyte development despite the known differences in ontogeny [22, 31, 32], and resolve the conflicting observations that previously stemmed from independent mouse [13] and human [14, 15] studies.

We therefore propose that Foxp3 transcription is intrinsically downstream of TCRαβ signaling at all stages of T cell development, supporting previous observations that TCR signals are required for Foxp3 induction in DP/CD4SP thymocytes [13, 33], and extending them to pre-DP thymocyte stages of mice and humans. Our data thus suggest that Treg commitment at these early stages essentially follows the same rules described for CD4SP

thymocytes, namely TCR signaling upon self-peptide presentation by MHC. However, Treg commitment in pre-DP versus DP/CD4SP subsets is expected to occur in distinct anatomical locations within the thymus: cortex versus medulla, respectively. The repertoire of the self-peptide presented by the corresponding epithelial cells are known to be intrinsically different, which has been partly attributed to differential Aire expression[34]. It will therefore be important to determine whether early induction of Foxp3 is associated with distinct TCR specificities as compared to mature SP thymocytes, which could have critical implications for the design of novel Treg-based therapeutic strategies.

METHODS

HUMAN THYMIC SAMPLES

Thymic specimens were obtained from routine thymectomy performed during pediatric corrective cardiac surgery at the Hospital de Santa Cruz, Carnaxide, Portugal, after parent's written informed consent. Study was approved by the Ethical Board of the Faculty of Medicine of Lisbon. 21 thymuses were studied. Cells were recovered through tissue dispersion and separated on a Ficoll-Paque density gradient.

ANIMALS

TCRα-deficient mice and Foxp3-GFP reporter mice have been previously described [13, 30]. The latter were a kind gift from Prof. A. Rudensky. All mice, including C57Bl/6 mice, were maintained under barrier conditions and used as adults at 6-8 weeks of age.

FLOW CYTOMETRY

After surface staining cells were fixed, permeabilized and stained for Foxp3 using eBioscience (San Diego, CA, USA) Foxp3 staining sets (FJK-16s for mice and PCH101, 236A/E7 or 150D/E4 for human samples), according to the manufacturer's protocol, as well as for Ki67 (B56) or CTLA-4 (BNI3). The mAbs used for surface staining were anti-human CD3 (UCHT1), CD4 (RPA-T4), CD8α (SK1), CD14 (CLB-mon1/ 8G3), CD16 (3G8), CD20 (2H7), CD56 (MEM188), TCRβ (IP26), HLA-DR (L243); and anti-mouse CD4 (RM4-5), CD5 (53-7.3), CD8α (53-6.7), CD25 (PC61.5), CD44 (IM7), CD45 (30-F11), TCRβ (H57-597), HSA (M1/69), B220 (RA3-6B2) and NK1.1 (PK136). Antibodies were from eBioscience or BD Biosciences (San Jose, CA, USA). Cells were acquired on FACSCanto or FACSCalibur (BD Biosciences) and analyzed using FlowJo (TreeStar, Ashland, Oregon, USA). Doublet exclusion was confirmed using Side Scatter Width.

CELL SORTING

Cell sorting of human and murine thymocytes was performed with a FACSAria high-speed Cell Sorter (BD Biosciences). Murine thymocytes were purified according to previously described protocols [23]. The lineage markers CD14, CD16, CD20, CD56 and HLA-DR were combined with CD3, CD4 and CD8 staining to sort human TN and CD4ISP thymocytes. Purity was routinely >96%.

IMMUNOHISTOCHEMISTRY

6μm frozen sections of thymic tissue were fixed with acetone, blocked with a solution containing BSA and 5% normal goat serum and stained with affinity purified rat anti-human Foxp3 (PCH101) and mouse anti-human CD1a or PBS (controls) overnight at 4ºC. The following day the tissue sections were incubated with Alexa Fluor® 568 goat anti-rat and Alexa Fluor® 514 goat anti-mouse (Molecular Probes, Invitrogen, Carlsbad, CA,USA). Images were obtained using a Zeiss LSM510 META Laser Scanning Confocal Microscope.

RT-PCR

Quantitative (real-time) PCR for human *foxp3* was performed as described [35], as well as semi-quantitative and quantitative (real-time) PCR for murine *foxp3* [23], following the same RNA isolation and reverse transcription procedures. For amplification of murine *AVD2* transcripts, the PCR strategy described by Mancini et al. was used [17].

STATISTICAL ANALYSIS

Statistical analysis was performed using GraphPad Prism v5.01 (GraphPad Software Inc., San Diego, CA, USA). Results are presented as arithmetic mean ± SEM. Data were tested for Gaussian distribution and compared using *t* test and Pearson's correlation coefficient as appropriate. *p*-values <0.05 were considered to be significant.

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REFERENCES

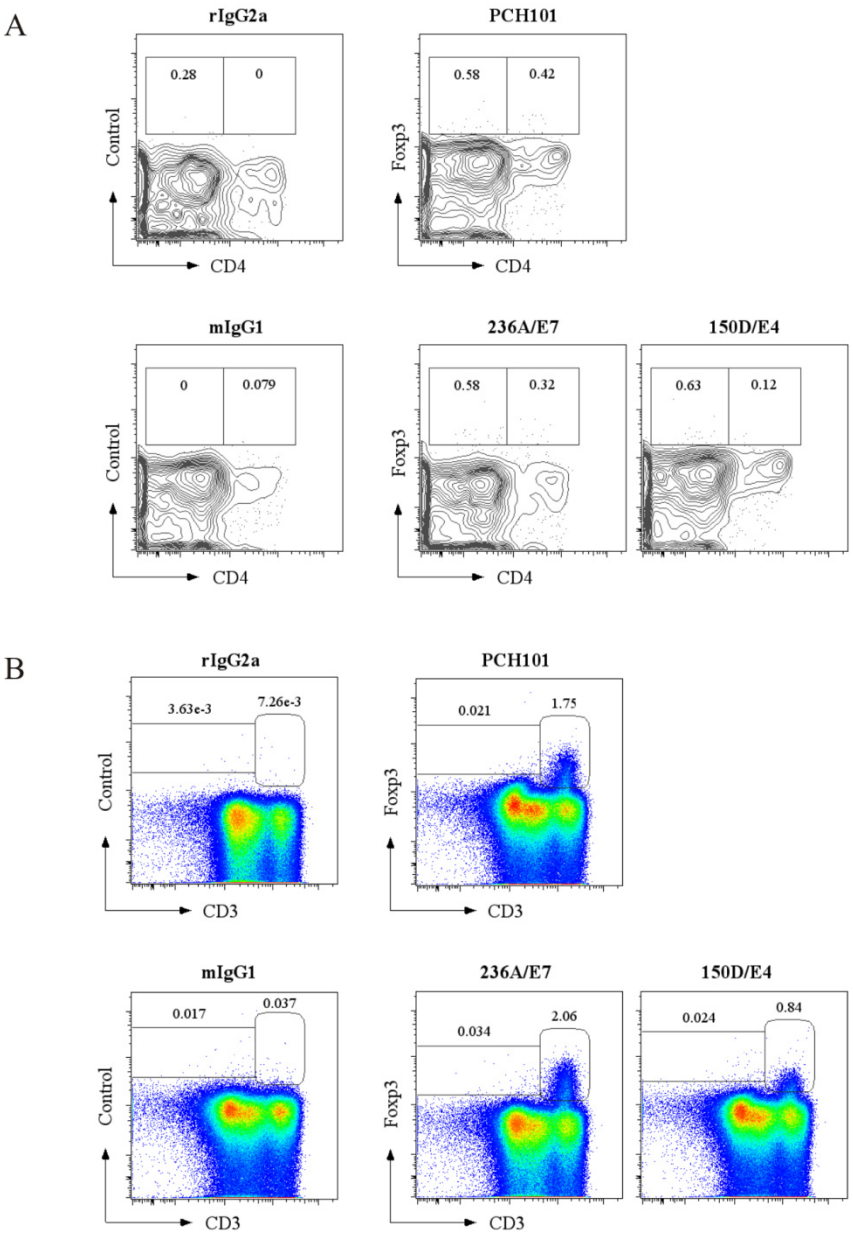
1. Hori S, Nomura T, and Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. Science. 2003; 299: 1057-1061.
2. Miyara M, Yoshioka Y, Kitoh A, Shima T, Wing K, Niwa A, et al. Functional delineation and differentiation dynamics of human CD4+ T cells expressing the FoxP3 transcription factor. Immunity. 2009; 30: 899-911.
3. Roncarolo MG, and Gregori S. Is FOXP3 a bona fide marker for human regulatory T cells? Eur J Immunol. 2008; 38: 925-927.
4. Ziegler SF. FOXP3: of mice and men. Annu Rev Immunol. 2006; 24: 209-226.
5. Bennett CL, Christie J, Ramsdell F, Brunkow ME, Ferguson PJ, Whitesell L, et al. The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3. Nat Genet. 2001; 27: 20-21.
6. Wildin RS, Ramsdell F, Peake J, Faravelli F, Casanova JL, Buist N, et al. X-linked neonatal diabetes mellitus, enteropathy and endocrinopathy syndrome is the human equivalent of mouse scurfy. Nat Genet. 2001; 27: 18-20.
7. Zheng Y, and Rudensky AY. Foxp3 in control of the regulatory T cell lineage. Nat Immunol. 2007; 8: 457-462.
8. Sakaguchi S, Yamaguchi T, Nomura T, and Ono M. Regulatory T cells and immune tolerance. Cell. 2008; 133: 775-787.
9. Roncarolo MG, and Battaglia M. Regulatory T-cell immunotherapy for tolerance to self antigens and alloantigens in humans. Nat Rev Immunol. 2007; 7: 585-598.
10. Ribot J, Romagnoli P, and van Meerwijk JP. Agonist ligands expressed by thymic epithelium enhance positive selection of regulatory T lymphocytes from precursors with a normally diverse TCR repertoire. J Immunol. 2006; 177: 1101-1107.
11. Ribot J, Enault G, Pilipenko S, Huchenq A, Calise M, Hudrisier D, et al. Shaping of the autoreactive regulatory T cell repertoire by thymic cortical positive selection. J Immunol. 2007; 179: 6741-6748.
12. Liston A, Nutsch KM, Farr AG, Lund JM, Rasmussen JP, Koni PA, et al. Differentiation of regulatory Foxp3+ T cells in the thymic cortex. Proc Natl Acad Sci U S A. 2008; 105: 11903-11908.

13. Fontenot JD, Rasmussen JP, Williams LM, Dooley JL, Farr AG, and Rudensky AY. Regulatory T cell lineage specification by the forkhead transcription factor foxp3. Immunity. 2005; 22: 329-341.
14. Tuovinen H, Kekalainen E, Rossi LH, Puntila J, and Arstila TP. Cutting edge: human CD4-CD8- thymocytes express FOXP3 in the absence of a TCR. J Immunol. 2008; 180: 3651-3654.
15. Battaglia A, Evoli A, Scambia G, and Fattorossi A. Comment on "Cutting Edge: Human CD4-CD8- Thymocytes Express FOXP3 in the Absence of a TCR". J Immunol. 2008; 181: 857-858.
16. Mertsching E, Wilson A, MacDonald HR, and Ceredig R. T cell receptor alpha gene rearrangement and transcription in adult thymic gamma delta cells. Eur J Immunol. 1997; 27: 389-396.
17. Mancini SJ, Candeias SM, Di Santo JP, Ferrier P, Marche PN, and Jouvin-Marche E. TCRA gene rearrangement in immature thymocytes in absence of CD3, pre-TCR, and TCR signaling. J Immunol. 2001; 167: 4485-4493.
18. Dik WA, Pike-Overzet K, Weerkamp F, de Ridder D, de Haas EF, Baert MR, et al. New insights on human T cell development by quantitative T cell receptor gene rearrangement studies and gene expression profiling. J Exp Med. 2005; 201: 1715-1723.
19. Tuovinen H, Laurinolli TT, Rossi LH, Pekkarinen PT, Mattila I, and Arstila TP. Thymic production of human FOXP3(+) regulatory T cells is stable but does not correlate with peripheral FOXP3 expression. Immunol Lett. 2008; 117: 146-153.
20. Seddiki N, Santner-Nanan B, Martinson J, Zaunders J, Sasson S, Landay A, et al. Expression of interleukin (IL)-2 and IL-7 receptors discriminates between human regulatory and activated T cells. J Exp Med. 2006; 203: 1693-1700.
21. Tuovinen H, Pekkarinen PT, Rossi LH, Mattila I, and Arstila AT. The FOXP3(+) subset of human CD4(+)CD8(+) thymocytes is immature and subject to intrathymic selection. Immunol Cell Biol. 2008.
22. Darrasse-Jeze G, Marodon G, Salomon BL, Catala M, and Klatzmann D. Ontogeny of CD4+CD25+ regulatory/suppressor T cells in human fetuses. Blood. 2005; 105: 4715-4721.
23. Pennington DJ, Silva-Santos B, Silberzahn T, Escorcio-Correia M, Woodward MJ, Roberts SJ, et al. Early events in the thymus affect the balance of effector and regulatory T cells. Nature. 2006; 444: 1073-1077.

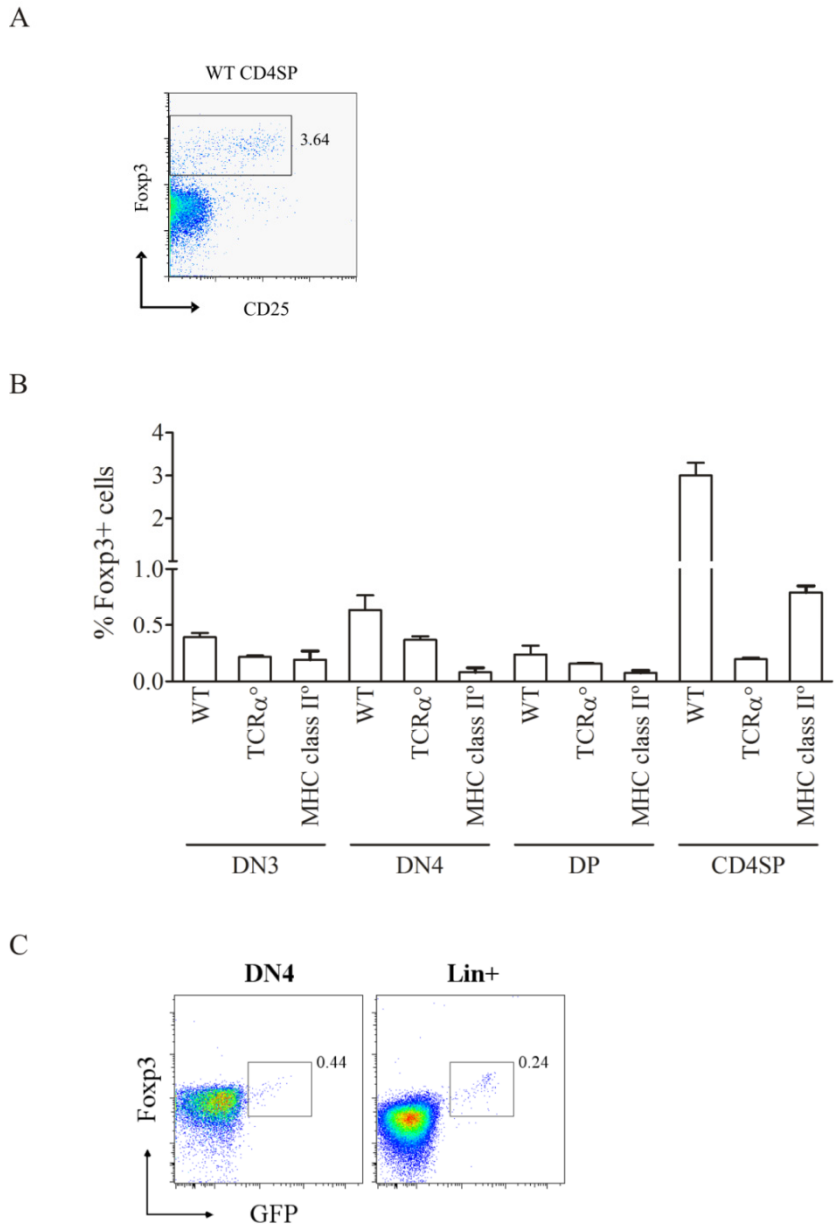
24. Godfrey DI, Kennedy J, Suda T, and Zlotnik A. A developmental pathway involving four phenotypically and functionally distinct subsets of CD3-CD4-CD8- triple-negative adult mouse thymocytes defined by CD44 and CD25 expression. J Immunol. 1993; 150: 4244-4252.
25. Hough MR, Takei F, Humphries RK, and Kay R. Defective development of thymocytes overexpressing the costimulatory molecule, heat-stable antigen. J Exp Med. 1994; 179: 177-184.
26. Ribot JC, deBarros A, Pang DJ, Neves JF, Peperzak V, Roberts SJ, et al. CD27 is a thymic determinant of the balance between interferon-gamma- and interleukin 17-producing gammadelta T cell subsets. Nat Immunol. 2009; 10: 427-436.
27. Dion ML, Poulin JF, Bordi R, Sylvestre M, Corsini R, Kettaf N, et al. HIV infection rapidly induces and maintains a substantial suppression of thymocyte proliferation. Immunity. 2004; 21: 757-768.
28. Azzam HS, Grinberg A, Lui K, Shen H, Shores EW, and Love PE. CD5 expression is developmentally regulated by T cell receptor (TCR) signals and TCR avidity. J Exp Med. 1998; 188: 2301-2311.
29. Borowski C, Li X, Aifantis I, Gounari F, and von Boehmer H. Pre-TCRalpha and TCRalpha are not interchangeable partners of TCRbeta during T lymphocyte development. J Exp Med. 2004; 199: 607-615.
30. Mombaerts P, Clarke AR, Rudnicki MA, Iacomini J, Itohara S, Lafaille JJ, et al. Mutations in T-cell antigen receptor genes alpha and beta block thymocyte development at different stages. Nature. 1992; 360: 225-231.
31. Michaelsson J, Mold JE, McCune JM, and Nixon DF. Regulation of T cell responses in the developing human fetus. J Immunol. 2006; 176: 5741-5748.
32. Cupedo T, Nagasawa M, Weijer K, Blom B, and Spits H. Development and activation of regulatory T cells in the human fetus. Eur J Immunol. 2005; 35: 383-390.
33. Jordan MS, Boesteanu A, Reed AJ, Petrone AL, Holenbeck AE, Lerman MA, et al. Thymic selection of CD4+CD25+ regulatory T cells induced by an agonist self-peptide. Nat Immunol. 2001; 2: 301-306.
34. Derbinski J, Gabler J, Brors B, Tierling S, Jonnakuty S, Hergenhahn M, et al. Promiscuous gene expression in thymic epithelial cells is regulated at multiple levels. J Exp Med. 2005; 202: 33-45.

35. Pereira-Santos MC, Baptista AP, Melo A, Alves RR, Soares RS, Pedro E, et al. Expansion of circulating Foxp3+CD25bright CD4+ T cells during specific venom immunotherapy. Clin Exp Allergy. 2008; 38: 291-297.

SUPPLEMENTARY FIGURES



Supplemental Fig. 1. Comparison of three different clones of anti-human Foxp3 mAb. Representative dot-plots of Foxp3 protein or adequate isotypic controls in the human thymus as assessed by 6-color flow cytometry using a FACSCanto. The clone PCH101 used in this study is compared with the clones 236A/E7 and 150D/E4. All the mAb are from eBioscience. **(A)** Analysis against CD4 expression within lineage (CD3, CD8, CD14, CD16, CD20, CD56) negative cells. 300 000 cells were acquired within a lineage negative gate. The percentages of TN (Lineage-CD4-; left gate) or CD4ISP (Lineage-CD4+; right gate) cells are presented. **(B)** Analysis against CD3 expression within total thymocytes.



Supplemental Fig. 2. Intracellular protein staining for Foxp3 in thymocyte subsets of C57Bl/6 (wild type, WT), TCRα-deficient (TCRα°), MHC class II-deficient (MHC class II°) or Foxp3-GFP mice. (A) Representative plot of intracellular Foxp3 versus surface CD25 expression in WT CD4SP thymocytes. (B) Quantification of Foxp3+ cells within each analyzed thymocyte subset. The background staining of the isotype control averaged 0.1- 0.2%. (C) Representative plot of intracellular Foxp3 versus GFP expression within DN4 or Lineage (CD4, CD8, NK1.1, TCRγδ) positive thymocytes of Foxp3-GFP reporter mice.

Chapter IV

Differentiation of human Foxp3+ DP thymocytes

Manuscript submitted.

Regulatory T cell differentiation in the human thymus occurs mainly at the double positive stage.

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CHAPTER IV: DIFFERENTIATION OF HUMAN FOXP3+ DP THYMOCYTES

ABSTRACT

Natural regulatory T cells (Treg), best identified by the expression of the transcription factor Foxp3 (*forkhead* box P3), constitute an independent thymus-derived T cell lineage that plays a crucial role in maintaining self-tolerance. The developmental program that Treg precursors undergo to give rise to mature Foxp3+ cells in humans is still ill-defined. We provide evidence of a Treg differentiation pathway initiated at the double-positive (DP) stage, prior to commitment to the CD4 or CD8 lineage, in pediatric thymuses. Foxp3+ DP thymocytes expressed positive selection and maturation markers, consistent with the requirement of TCR signaling for Foxp3 induction. Additionally, Foxp3+ DP cells displayed functional IL-7 receptor and regulatory markers, whose expression declined as the cells differentiated into the mature CD4 single positive (SP) stage. A subpopulation of Foxp3+ DP thymocytes expressed CD103, and likely gave rise to Foxp3+ CD8SP cells, that homogeneously expressed this mucosal homing molecule. Finally, multiple linear regression analysis supported the derivation of a significant proportion of Foxp3+ SP from Foxp3+ DP thymocytes. Overall, our data suggest that a significant commitment to the Treg lineage occurs at the DP stage, with possible implications for the diversity and autoreactivity of the human natural Treg repertoire.

INTRODUCTION

The thymus is essential for the establishment and renewal of the peripheral T cell compartment, and for the maintenance of a diverse T cell repertoire able to efficiently respond to novel pathogens but tolerant to self-antigens. Tolerance is at least partly achieved through the thymic production of a subset of T cells termed "natural" regulatory T cells (Treg). Treg modulate the activation, function and proliferation of other T cells, as well as of many other cell populations of the immune system (Shevach, 2009), playing important roles not only in the prevention of autoimmune diseases but also in other clinical settings such as tumor immunity and persistent infections (Belkaid and Tarbell, 2009; Nishikawa and Sakaguchi, 2010; Sakaguchi et al., 2008). Thymically-derived Treg are also considered fundamental for the regulation of T cell homeostasis. Although understanding Treg development in the human thymus is critical for their manipulation (Allan et al., 2008; Brusko et al., 2008; Miyara et al., 2009a), the developmental program that Treg precursors undergo to give rise to mature Foxp3+ cells is still unclear. Moreover, the developmental stage at which the Treg lineage diverges from other T cell fates in humans is not known.

T cell development proceeds through a series of stages that can be tracked by the expression of CD3, CD4 and CD8. In humans, CD4-CD8-CD3- triple negative cells first acquire CD4 (CD4 immature single positive, CD4ISP) and then CD8 to become CD4+CD8+ (double positive, DP) cells. DP cells bearing TCRαβ complexes able to engage self-MHC molecules are signaled to survive (positive selection) and to differentiate into functionally mature CD4 single positive (CD4+CD8-CD3high, CD4SP) or CD8 single positive (CD4-CD8+CD3high, CD8SP) T cells. Data from mouse models suggest that the strength and duration of TCR signaling drives the CD4 vs CD8 fate-decision during thymic differentiation, and the negative selection of cells with potentially auto-reactive TCRs (Singer et al., 2008; Starr et al., 2003). TCR signaling has also been shown to be essential for Treg differentiation in the thymus (Bettini and Vignali, 2010; Josefowicz and Rudensky, 2009). It has been proposed that this process may occur within a very narrow window of affinity of TCR-ligand interactions between positive selection of conventional CD4+ T cells and negative selection of high-affinity self-reactive T cells (Apostolou et al., 2002; Jordan et al., 2001). In fact, increased self-reactivity is considered a hallmark of Tregs (Hsieh et al., 2004).

Initial studies in human fetal and pediatric thymuses, relying on IL-2 receptor α chain (IL-2Rα, CD25) as a Treg marker, identified CD25 expression in CD4SP as well as in DP and CD8SP thymocytes (Annunziato et al., 2002; Cosmi et al., 2003; Cupedo et al., 2005; Darrasse-Jeze et al., 2005; Liotta et al., 2005; Stephens et al., 2001; Wing et al., 2002). Suppressive function was ascribed to CD4SP CD25+ thymocytes, but also to the DP and CD8SP CD25+ populations (Annunziato et al., 2002; Cosmi et al., 2003; Cupedo et al., 2005; Liotta et al., 2005; Stephens et al., 2001; Wing et al., 2005). Currently, the forkhead winged-helix transcription factor Foxp3 is considered the best available marker to identify Tregs. The essential role of Foxp3 in Treg development and function is supported by the association of Foxp3 defects with the fatal condition IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked) syndrome (Bennett et al., 2001; Wildin et al., 2001), and also by the lethal autoimmune syndrome observed in *scurfy* and Foxp3 KO mice (Brunkow et al., 2001; Fontenot et al., 2003). We and others have assessed Foxp3 expression in the human thymus and reported its expression before CD4 and CD8 lineage decision (Nunes-Cabaco et al., 2010; Tuovinen et al., 2008a). Treg induction at these early stages of T cell development is likely to impact on the diversity and autoreactivity of the Treg repertoire.

Here we investigated the contribution of Foxp3+ DP thymocytes to the human Treg pool. We provide evidence that a considerable fraction of Foxp3+SP cells derive from Foxp3+ DP thymocytes. We also show that Foxp3 induction in human DP thymocytes is associated with the expression of functional IL-7 receptor, as well as of markers of Treg suppressor function, which apparently decline as thymocytes mature. Conversely, the mucosal homing molecule CD103 is expressed by a subpopulation of Foxp3+ DP thymocytes and by the large majority of Foxp3+ CD8SP cells, but not by Foxp3+ CD4SP thymocytes, supporting that Foxp3+ CD8SP cells arise from Foxp3+ DP precursors and exit the thymus with a preferential mucosal homing phenotype.

RESULTS

1. HUMAN FOXP3+ DOUBLE POSITIVE THYMOCYTES COMPRISE A SIGNIFICANT PROPORTION OF CELLS UNCOMMITTED TO THE CD4 OR CD8 LINEAGE

Foxp3 has been shown to be expressed throughout human T cell development (Nunes-Cabaco et al., 2010; Tuovinen et al., 2008b), but the most immature population where consistent amounts of Foxp3 mRNA and protein are detected are DP thymocytes (Nunes-Cabaco et al., 2010). Accordingly, in all 35 thymuses analyzed here a population of Foxp3+ DP thymocytes, corresponding approximately to a third of the total Foxp3+ CD4SP thymocytes and twice the total of Foxp3+ CD8SP cells, was identified after careful exclusion of doublets (Fig. 1A and B). The latter technical detail is relevant, as overestimation of Foxp3+ DP cells in the murine thymus, due to doublet formation, has been described (Lee and Hsieh, 2009).

It is known that DP cells may already be committed to either the CD4SP or CD8SP lineage, and have thus shut down the synthesis of either CD4 or CD8, but still express both co-receptors at their surface (Suzuki et al., 1995; Vanhecke et al., 1997). This may be of particular relevance in Treg development, since Foxp3 induction has been linked to CD4 lineage commitment (Feuerer et al., 2009). Therefore, our first aim was to experimentally assess whether the Foxp3+ DP population we detected in the human thymus were "true" DP cells. For this purpose, we employed a re-expression assay, which consisted of stripping surface molecules with pronase, and analyzing their re-expression after short-term culture (Suzuki et al., 1995; Vanhecke et al., 1997). This technique allowed us to identify DP cells and CD4SP- or CD8SP-committed DP-like cells. Pronase treatment of sorted DP cells completely abolished surface CD4 and CD8 expression, and no re-expression of these molecules was observed when thymocytes were kept at 4ºC (Fig. 1C). In contrast, pronase-treated DP thymocytes cultured at 37ºC clearly re-expressed CD4 and/or CD8. Importantly, we found that Foxp3+ DP cells re-expressed both CD4 and CD8 (Fig. 1C and D), thus confirming the active synthesis of both co-receptors.

Overall, these results show that *bona fide*, CD4 and CD8 lineage-uncommitted, Foxp3+ DP cells are present in the human thymus.

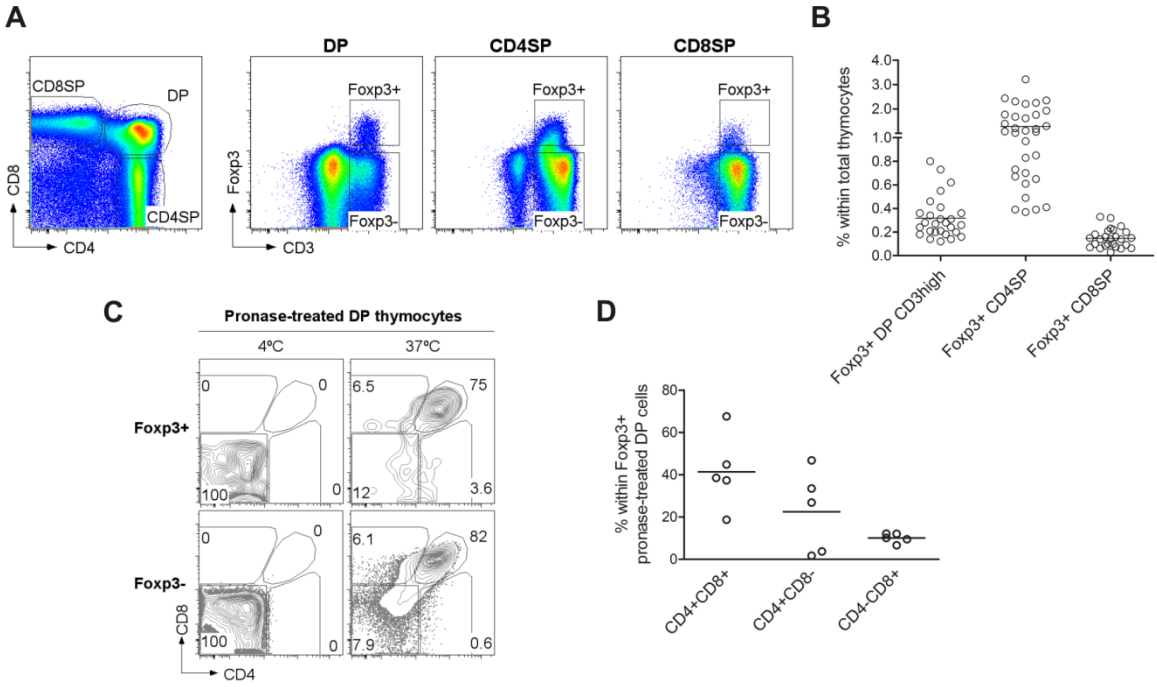

Figure 1: Foxp3 expression in human DP thymocytes. **(A)** Flow cytometry analysis of Foxp3 expression in a representative thymus (3 year old boy). The dot plot on the left shows total thymocytes after doublet exclusion and illustrates the definition of DP, CD4SP and CD8SP subsets. Foxp3 expression in CD3high thymocytes within DP, CD4SP and CD8SP subsets and the counterpart Foxp3- population are shown. **(B)** Frequency of Foxp3+ DP CD3high, Foxp3+ CD4SP and Foxp3+ CD8SP cells within total thymocytes. **(C)** Representative example of the re-expression assay of DP thymocytes. Sorted DP cells were treated with pronase and cultured overnight at 37ºC. Controls included pronase-treated DP cells left at 4ºC. Dot plots show re-expression of pronase-cleaved surface molecules and controls within Foxp3+ and Foxp3- cells after doublet and dead cell exclusion. **(D)** Frequency of thymocytes re-expressing CD4 (CD4+CD8-), CD8 (CD8+CD4-) or both (DP) co-receptors within Foxp3+ cells after overnight culture at 37ºC of pronase-treated purified DP cells. Each circle represents a thymus.

2. FOXP3+ CELLS AT THE DP CD3HIGH STAGE DISPLAY AN EARLY MATURE PHENOTYPE AND A DIVERSE TCR Vβ REPERTOIRE

Next we evaluated the phenotype of Foxp3+ DP cells. Foxp3 expression within DP cells has been mainly associated with high levels of CD3 (Nunes-Cabaco et al., 2010; Tuovinen et al., 2008b). Therefore, an accurate comparison of Foxp3+ and Foxp3- DP cells must take into account the late differentiation stage of the former. We thus compared Foxp3+ cells at the DP CD3high stage with Foxp3- DP cells expressing similarly high levels of CD3 (Fig. 1A). Higher levels of surface TCRαβ expression were found on Foxp3+ DP CD3high cells than on their Foxp3- counterparts (Fig. 2A). Of interest, Foxp3+ DP CD3high thymocytes showed levels of surface TCRαβ levels comparable to Foxp3+ SP stages, in clear contrast with Foxp3- DP CD3high cells, which presented much lower levels of surface TCRαβ as compared to the Foxp3- CD4SP population (Fig. 2A). Foxp3+

DP CD3high cells expressed significantly higher levels of the early activation marker CD69, usually associated with positive selection (Spits, 2002; Vanhecke et al., 1995), than Foxp3- DP CD3high cells (Fig. 2B). Moreover, we found that more than 90% of Foxp3+ DP CD3high cells already expressed the maturation marker CD27 (Vanhecke et al., 1995), in contrast to approximately 20% of Foxp3- cells at the same developmental stage (Fig. 2C). Together, these results suggest that Foxp3+ DP cells constitute a mature, positively selected population. Of note, Foxp3+ cells were found to homogeneously express CD27 at the SP stages, whereas only approximately 60% of Foxp3- CD4SP cells and 85% of Foxp3- CD8SP cells were CD27+ (Fig. 2C). Signaling through CD28 has been shown to be particularly important in Treg development, both in mice and humans (Hanabuchi et al., 2010; Martin-Gayo et al., 2010; Salomon et al., 2000; Tai et al., 2005). Accordingly, in all thymuses analyzed we found that CD28 expression was higher on Foxp3+ DP CD3high cells than on their Foxp3- counterpart (Fig. 2D).

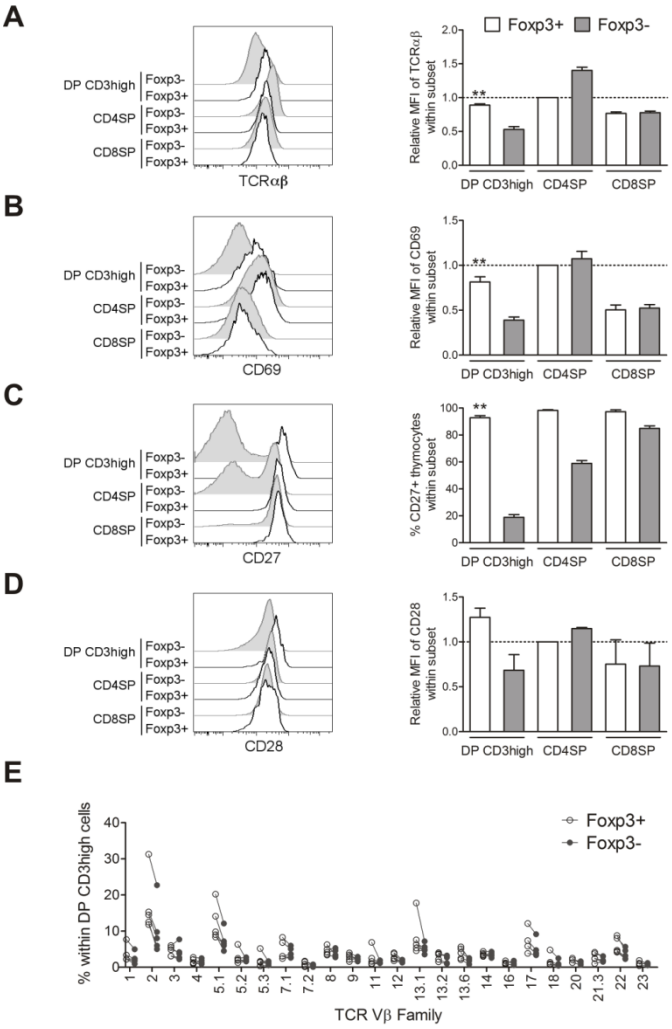

Figure 2: Foxp3+ cells present an early mature phenotype and a diverse repertoire at the DP CD3high stage. Histogram overlays and graphs show the expression, as analyzed by flow cytometry, of TCRαβ **(A)**, CD69 **(B)**, CD27 **(C)** and CD28 **(D)** in Foxp3+ (clear histograms and bars) or Foxp3- (filled histograms and bars) within CD3high thymocytes at the DP, CD4SP and CD8SP stages. Graphs in (**A**), (**B**) and (**D**) show the relative MFI (median fluorescence intensity), defined as the ratio between the MFI of a marker in a given population and the MFI of the same marker within Foxp3+ CD4SP cells, of TCRαβ (n=8), CD69 (n=11; Foxp3+ DP CD3high: n=10; Foxp3+ CD8SP: n=6) and CD28 (n=4), respectively. Graph (**C**) presents the frequency of CD27+ cells (n=12; Foxp3+ DP CD3high: n=10; Foxp3+ CD8SP: n=7). Bars represent mean±SEM. * refers to the comparison between Foxp3+ DP CD3high and Foxp3- DP CD3high populations. **, $P<0.01$. (**E**) TCR Vβ family distribution at the DP CD3high stage within Foxp3+ (open circles) or Foxp3- (grey circles) cells assessed by flow cytometry. Each line connects the frequency of a given family within the Foxp3+ DP CD3high and Foxp3- DP CD3high populations of the same thymus.

Overall, our data show that Foxp3+ DP CD3high cells present a more mature phenotype relative to Foxp3- cells, even when compared with thymocytes matched for their developmental stage in terms of CD3 expression levels. In order to further understand how Foxp3+ DP CD3high cells relate to Foxp3- cells at that stage we compared their TCR Vβ family distribution. Overall, we found very similar TCR Vβ family profiles between Foxp3+ and Foxp3- DP CD3high cells (Fig. 2E). Thus, Foxp3+ DP CD3high cells represent a pool of mature, post-selected thymocytes that display a diverse repertoire.

3. FOXP3+ DP CD3HIGH CELLS EXPRESS OTHER TREG-ASSOCIATED MARKERS AND INCLUDE A UNIQUE ACTIVATED SUBPOPULATION.

We next asked how the expression of Foxp3 related to the expression of other Treg-associated markers at the DP and SP stages.

CD25 and CTLA-4 were clearly expressed in DP CD3high thymocytes, and Foxp3 was associated with the highest levels of these markers (Fig. 3A-C). This was also observed in CD4SP cells, whilst CD8SP cells expressed lower levels of these molecules (Fig. 3A-C). Notably, within Foxp3+ cells the levels of CD25 and CTLA-4 were frequently higher in DP CD3high than in CD4SP cells (Fig. 3A and C).

CD39 (ectonucleoside triphosphate diphosphohydrolase 1) is an ATPDase that has also been associated with Treg activity (Borsellino et al., 2007). CD39high cells defined a population that comprised the brightest CD25 and CTLA-4 expressing cells and included a significant proportion Foxp3+ cells (Fig. 3D and Supplemental Fig. 1). Within the Foxp3+ population the expression of CD39 was also found to be higher in DP CD3high than in CD4SP cells (Fig. 3A-C). Of note, CD39high cells did not co-express CD45RA at the CD4SP stage (Supplemental Fig. 1), indicating that egress from the thymus with a CD45RA phenotype would require down-regulation of CD39 expression. CD8SP cells did not express high levels of CD39, in line with their lack of a CD25high population (Fig. 3A-C). Of note, CD73 (ecto-5'-ectonucleotidase), which dephosphorylates the CD39 product AMP to generate the immunosuppressive nucleoside adenosine (Borsellino et al., 2007; Resta et al., 1998), was not concomitantly expressed with CD39, being mainly expressed on CD8SP cells irrespective of concomitant Foxp3 expression (Supplemental Figure 1).

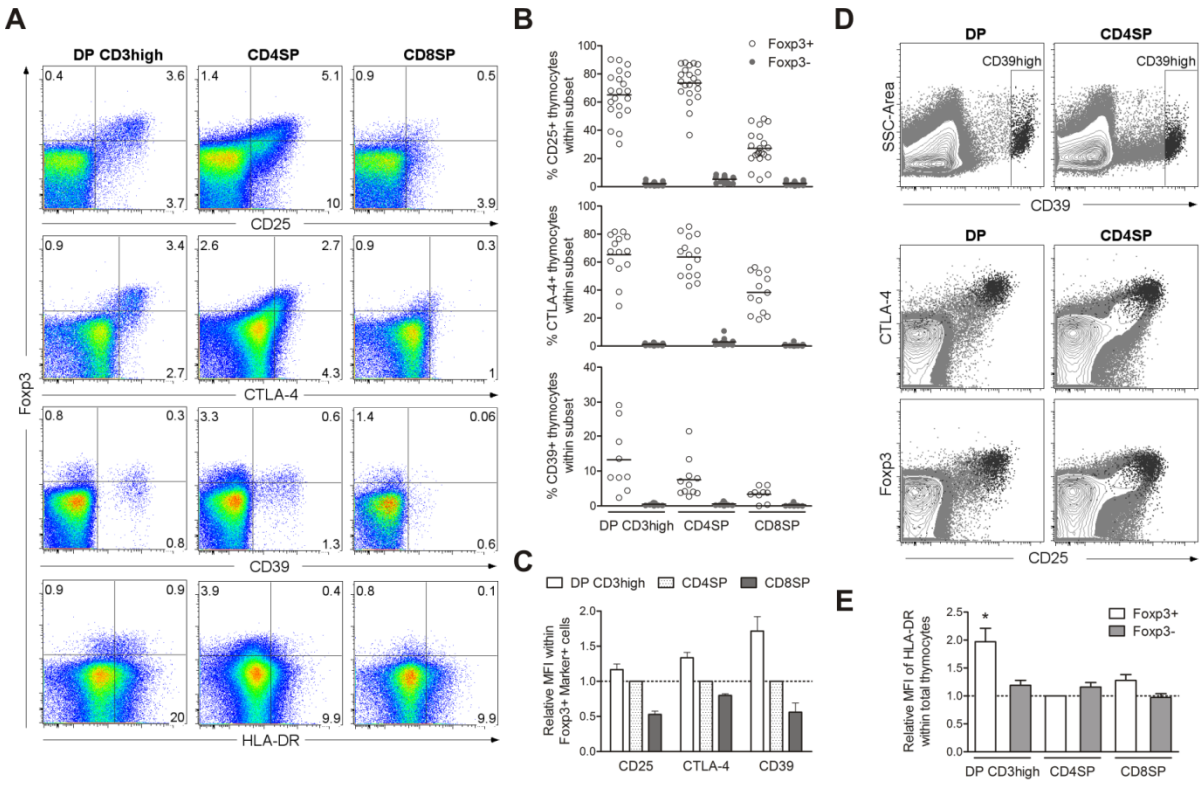

Figure 3: Foxp3+ DP CD3high cells in the human thymus co-express other Treg-associated markers and present an activated phenotype. **(A)** Representative flow cytometry analysis of Foxp3 and Treg-associated markers CD25, CTLA-4, CD39 and HLA-DR within CD3high DP, CD4SP and CD8SP cells. **(B)** Frequency of these Treg-associated markers within Foxp3+ (open circles) or Foxp3- (grey circles) cells at the CD3high DP, CD4SP and CD8SP stages. Each circle represents a thymus. **(C)** Relative MFI (ratio between the MFI of a marker in a given population and the MFI of the same marker within Foxp3+ CD4SP cells) of CD25, CTLA-4 and CD39 cells within Foxp3+ cells expressing the marker at each stage shown in (**B**). **(D)** Representative flow cytometry analysis of CD39 expression in relation to CTLA-4, CD25 and Foxp3 within DP, CD4SP and CD8SP thymocytes. CD39high cells, defined as shown in the upper dot plots, are highlighted in black over the contour plots representing the whole population (grey). **(E)** Relative MFI of HLA-DR within Foxp3+ (open bars) or Foxp3- (grey bars) cells at the CD3high DP, CD4SP and CD8SP stages, as related to the HLA-DR MFI within Foxp3+ CD4SP cells (n=8). Bars represent mean±SEM. * refers to the comparison between Foxp3+ DP CD3high and Foxp3- DP CD3high populations. * $P<0.05$.

HLA-DR expression has been reported to identify a mature, functionally distinct Treg subset that expresses higher levels of Foxp3 and mediates contact-dependent suppression (Baecher-Allan et al., 2006). Expression of HLA-DR was found to be clearly higher at the Foxp3+ DP CD3high stage (Fig. 3A and E), being particularly marked in the CD25high population, and also related to the expression of CD39 (Supplemental Fig. 1D and data not shown).

In summary, our results show that Foxp3+ DP CD3high cells already express other Treg-associated markers and include an activated population expressing HLA-DR and high levels of CD39, CD25 and CTLA-4. The decline of the expression levels of these markers

at the SP stage suggests that this activation is transitory and that Treg exit the thymus with a resting phenotype similar to that of peripheral naïve-like Treg (Miyara et al., 2009b).

4. FOXP3+ DP CD3HIGH CELLS EXPRESS FUNCTIONAL IL-7Rα RECEPTOR

Low levels of the IL-7 receptor alpha chain (IL-7Rα, CD127) are associated with a regulatory phenotype (Liu et al., 2006; Seddiki et al., 2006a). In agreement, we found lower expression of CD127 on Foxp3+ as compared to Foxp3- cells at the SP stages (Fig. 4A). In contrast, higher levels of CD127 were found on Foxp3+ than Foxp3- DP CD3high cells (Fig. 4A).

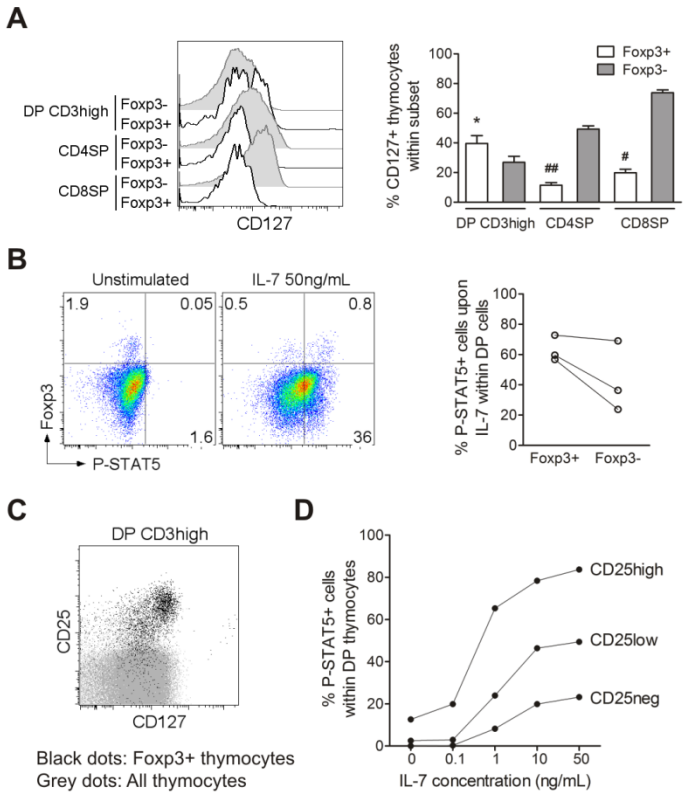

Figure 4: Foxp3+ DP cells in the human thymus express functional IL-7 receptor. **(A)** Histogram overlays and graph show the expression of CD127 (IL-7Rα), as analyzed by flow cytometry, in Foxp3+ (clear histograms and bars) and Foxp3- (filled histograms and bars) within CD3high thymocytes at the DP, CD4SP and CD8SP stages (n=8). Bars represent mean±SEM. * refers to the comparison between Foxp3+ DP CD3high and Foxp3- DP CD3high populations; *, $P<0.05$. # refers to the comparison between a Foxp3+ SP population and the corresponding Foxp3+ DP CD3high population; #, $P<0.05$; ##, $P<0.01$. **(B)** Representative dot plots showing phospho-STAT5 (P-STAT5) expression in relation to Foxp3 within DP thymocytes after exposure to IL-7 (50ng/mL) or PBS (control) and graph showing the frequency of P-STAT5+ cells within Foxp3+ or Foxp3- DP thymocytes upon IL-7 stimulation. Each line connects data from the same thymus. **(C)** Dot-plot illustrates the concomitant expression of CD127 and CD25 on DP CD3high thymocytes (grey dots) with Foxp3+ cells highlighted in black. **(D)** Frequency of P-STAT5+ cells within CD25neg, CD25low or CD25high DP cells from a representative experiment out of 3 upon exposure to different concentrations of IL-7 (0.1, 1 and 10ng/mL, n=2; 50ng/mL).

In order to assess whether CD127 expression on Foxp3+DP cells is functional, we exposed total thymocytes to IL-7 and measured the degree of STAT5 phosphorylation

(P-STAT5). The clear up-regulation of P-STAT5 observed confirmed that Foxp3+ DP thymocytes express IL-7Rα and are responsive to IL-7, at levels similar to or higher than their Foxp3- counterpart (Fig. 4B). Since CD127 expression in Foxp3+ DP cells was associated with high levels of CD25 (Fig. 4C) we also compared the expression of P-STAT5 in CD25negative, CD25low and CD25high DP cells in response to different concentrations of IL-7. STAT5 phosphorylation was strongest in DP cells expressing high levels of CD25, while CD25low and CD25negative cells were less susceptible to IL-7 stimulation (Fig. 4D and Supplemental Fig. 2).

Given the concomitant expression of IL-7Rα and IL-2Rα on Foxp3+ DP thymocytes we asked whether IL-7 or IL-2 were involved in the modulation of Foxp3 and/or CD25 expression on these cells, using short-term cultures of total thymocytes in the presence of IL-2, IL-7 or TCR stimulation (anti-CD3 plus anti-CD28 mAbs). To minimize the contribution of cell proliferation to the study, cells were assayed at 6h and 22h of culture. We found that IL-2 was able to increase both Foxp3 and CD25 expression levels within Foxp3+ DP thymocytes (Fig. 5A-C). Interestingly, although IL-7 did not directly impact on Foxp3 expression, it did increase the levels of CD25 within Foxp3+ DP cells in all experiments (Fig. 5A-C). These results confirm that Foxp3+ DP cells are susceptible to IL-7-mediated signals, which consequently lead to an up-regulation of CD25 levels on Foxp3+ DP cells and/or a preferential survival of CD25high Foxp3+ cells in culture. Of interest, Foxp3+ DP cells were mostly refractory to TCR stimulation, at the dose of anti-CD3 and anti-CD28 mAbs used (Fig. 5A-C).

Given the activated phenotype of Foxp3+ DP CD3high cells, we further assessed whether IL-2, IL-7 or TCR stimulation would impact on the thymocyte population that expressed the activation/suppression markers CD39 and HLA-DR. Surprisingly, the expression of both molecules was severely reduced upon culture, irrespective of the conditions (Supplemental Fig. 3A and B), suggesting that other cues, such as interaction with thymic stroma, are required for their induction/maintenance. Interestingly, both IL-2 and IL-7, but not TCR stimulation, were able to increase the frequency of Foxp3+ cells within CD4SP as well as CD8SP thymocytes after 6h culture (Supplemental Fig. 3C and D). The quick up-regulation of Foxp3 upon exposure to IL-2 or IL-7, but not to TCR stimulation at the dose used, suggest that both CD4SP and CD8SP thymocytes contain Treg-committed cells that have down regulated Foxp3 to levels below the threshold of the assay.

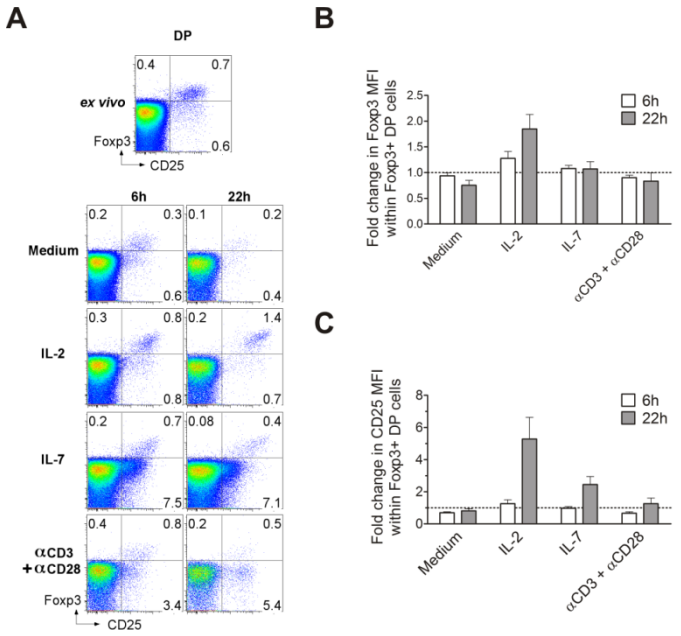

Figure 5: Up-regulation of Foxp3 and/or CD25 on Foxp3+ DP thymocytes upon short-term culture with IL-2 or IL-7. (**A**) Representative dot plots of Foxp3 and CD25 expression within DP thymocytes *ex vivo* (0h) and after 6h or 22h culture of total thymocytes in the presence or absence of IL-2 (10U/mL), IL-7 (10ng/mL) or anti-CD3 plus anti-CD28 (both at 1µg/mL). Fold change in the MFI of Foxp3 (**B**) and CD25 (**C**) within Foxp3+ DP cells after 6h culture (clear bars, n=3) and 22h culture (filled bars, n=4) in the conditions above described as compared to baseline expression (0h). Bars represent mean±SEM.

Overall, these data show that Foxp3+ DP human thymocytes express functional IL-7Rα, linking IL-7 to human Treg development.

5. CD103 IS ALREADY PRESENT IN FOXP3+ DP CD3HIGH CELLS AND IT IS EXPRESSED IN MOST FOXP3+ CD8SP CELLS

CD103 (integrin alpha E, αE) is expressed by a subset of peripheral Tregs (Allakhverdi et al., 2006; Banz et al., 2003; Huehn et al., 2004; Lehmann et al., 2002), and is thought to be important for T cell homing to the intestinal and other mucosal sites (Annunziato et al., 2006; Huehn et al., 2004). When we analyzed the expression of CD103 in the human thymus we found that it was already expressed at the DP CD3high stage on 12.9±2.4% of Foxp3+ cells but only on 0.87±0.25% of Foxp3- cells (Fig. 6A and B). Notably, CD103 expression at the DP CD3high stage was associated with low levels of CD25 (Fig. 6A). At the SP stages, CD103 was significantly more expressed on CD8SP than CD4SP cells within Foxp3+ subset (Fig. 6A and B). The large majority of Foxp3+ CD8SP cells expressed CD103, while only approximately 10% of Foxp3- CD8SP cells expressed it (Fig. 6A and B). The observation of high levels of expression of a mucosal homing molecule within Foxp3+ CD8SP is particularly relevant given that Foxp3+ CD8 T cells are rarely found in human peripheral blood (Chaput et al., 2009; Siegmund et al., 2009).

We further assessed whether the expression of CD103 in CD8SP Foxp3+ cells had an impact on the TCR Vβ distribution of the TCR repertoire by comparing Foxp3+ CD8SP

CD103+ cells with Foxp3- CD8SP CD103+ and CD103- cells. Overall, we did not find any striking differences in the TCR Vβ patterns of these populations (Supplemental Fig. 4).

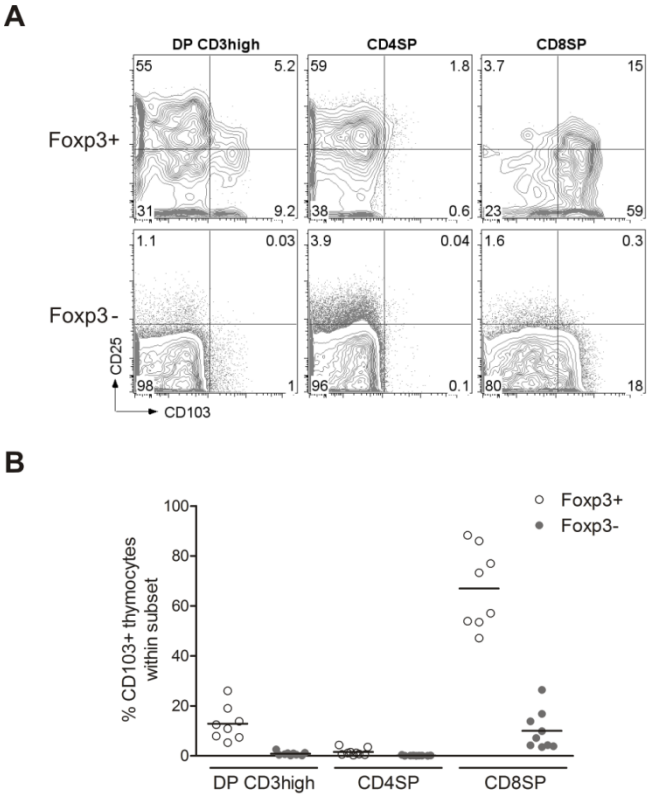

Figure 6: CD103 is expressed in Foxp3+ DP CD3high and in the majority of Foxp3+ CD8SP human thymocytes. (**A**) Representative contour plots showing the expression of CD103 in relation to CD25 within Foxp3+ (upper panels) and Foxp3- (lower panels) thymocytes at the CD3high DP, CD4SP and CD8SP stages. (**B**) Frequency of CD103+ cells within Foxp3+ (open circles) and Foxp3- (grey circles) thymocytes at the CD3high DP, CD4SP and CD8SP stages. Each circle represents a thymus.

Although the CD4/CD8 ratio was much higher in Foxp3+ as compared to Foxp3- SP subsets in the human thymus (9.9±0.9 vs. 3.1±0.3 for CD4SP/CD8SP ratio in Foxp3+ and Foxp3- cells, respectively; n=19), a distinct Foxp3+ CD8SP population was identified in all the thymuses evaluated (Fig.1A and B). Notably, 11.6±1.9% of the CD103+ CD8SP population expressed Foxp3.

Our data thus support the possibility that Foxp3+ CD8SP cells generated in the human thymus already express the mucosal homing molecule CD103 and that this commitment may in part occur at the DP stage.

6. MULTIPLE REGRESSION ANALYSIS SUPPORTS A PRECURSOR-PROGENY RELATIONSHIP BETWEEN FOXP3+ DP CD3HIGH AND FOXP3+ SP THYMOCYTES

In order to ascertain how Foxp3+ DP CD3high cells relate to mature Foxp3+ SP thymocytes, we first performed a canonical correlation analysis using Foxp3+ DP CD3high and Foxp3- DP CD3high as the first set of variables (DP variables) and Foxp3+ CD4SP, Foxp3- CD4SP, Foxp3+ CD8SP and Foxp3- CD8SP as the second set of variables (SP variables). Statistical analysis was performed using the number of live cells of the different populations, determined as described in Figure 1A. For the sake of simplicity, we focused the analysis on the first pair of canonical components, one referring to the DP variables and another to the SP variables. With respect to the former variables, the first canonical component was under strong influence of the Foxp3+ DP CD3high population. For the latter variables, the first canonical component was highly explained by Foxp3+ CD4SP, Foxp3+ CD8SP and Foxp3- CD8SP cells (Table I). Since this first pair of canonical components was intimately correlated to each other (correlation=0.95), the above result suggested that Foxp3+ DP CD3high cells was likely the most important thymocyte population contributing to variations in size of Foxp3+ CD4SP, Foxp3+ CD8SP and Foxp3- CD8SP cell subsets.

TABLE I: CANONICAL CORRELATION ANALYSIS.

	Variables	1st Canonical (corr=0.95)
Set 1	Foxp3+ DP CD3high	-3.01
	Foxp3- DP CD3high	0.25
Set 2	Foxp3+ CD4SP	-2.23
	Foxp3+ CD8SP	-0.08
	Foxp3- CD4SP	1.47
	Foxp3- CD8SP	-1.58

Using Log10 of the number of live thymocytes, analyzed as shown in Figure 1A.

To further explore the statistical dependence between DP and SP populations, we performed a multiple regression analysis considering all SP sub-populations as dependent variables and different DP and SP variables as putative explanatory variables. When fitted to the data (Fig. 7A), the models revealed a positive effect of Foxp3+ DP

CD3high cells on Foxp3+ CD4SP and Foxp3+ CD8SP but not on Foxp3- CD4SP and Foxp3- CD8SP cells, irrespective of the number of thymocytes (Table II). A similar qualitative result was obtained using the log10 of cell numbers (Supplemental Table I). According to the fitted models, 1,000 DP CD3high cells could explain approximately 510 and 3300 Foxp3+ CD8SP and Foxp3+ CD4SP cells, respectively.

TABLE II: MULTIPLE REGRESSION ANALYSIS.

Coefficient	Foxp3+ CD4SP (R^2 adjust= 0.89)		Foxp3+ CD8SP (R^2 adjust= 0.69)	
	Estimate	*P*-value	Estimate	*P*-value
Intercept	-1480	0.37	-93	0.79
Foxp3+ DP CD3high	3311	<0.001	509.9	0.01
Foxp3- CD4SP	58.31	<0.001	9.40	0.21
Foxp3- DP CD3high	-13.49	0.55	-0.92	0.86
Total thymocytes	-3.69	0.36	-0.40	0.70

Using the absolute number of live thymocytes of each subset (analyzed as shown in Figure 1A).

It is worth noting that Foxp3+ CD4SP cells also seem to be influenced by their Foxp3- counterpart (Supplemental Fig. 6). In this case, the model estimates that 58 Foxp3+ CD4SP cells could be explained by any 1,000 Foxp3- CD4SP cells. For Foxp3+ CD8SP cells, this number drops to approximately 10 cells per 1,000 Foxp3- CD8SP cells. This rather small estimate might offer a likely explanation for the lack of statistical significance of Foxp3- CD8SP in the respective model (Table II and Supplemental Table I).

Therefore, the multiple regression analysis supports the notion that: 1) at least part of the developmental pathway of Foxp3+ cells involves a sequential progression from the DP CD3high to the SP stages; and 2) the induction of Foxp3+ expression at the SP stage is likely a rare event. Notably, the distribution of TCR Vβ families of Foxp3+ cells at the DP CD3high, CD4SP and CD8SP stages exhibited comparable profiles among those populations (Supplemental Fig. 5).

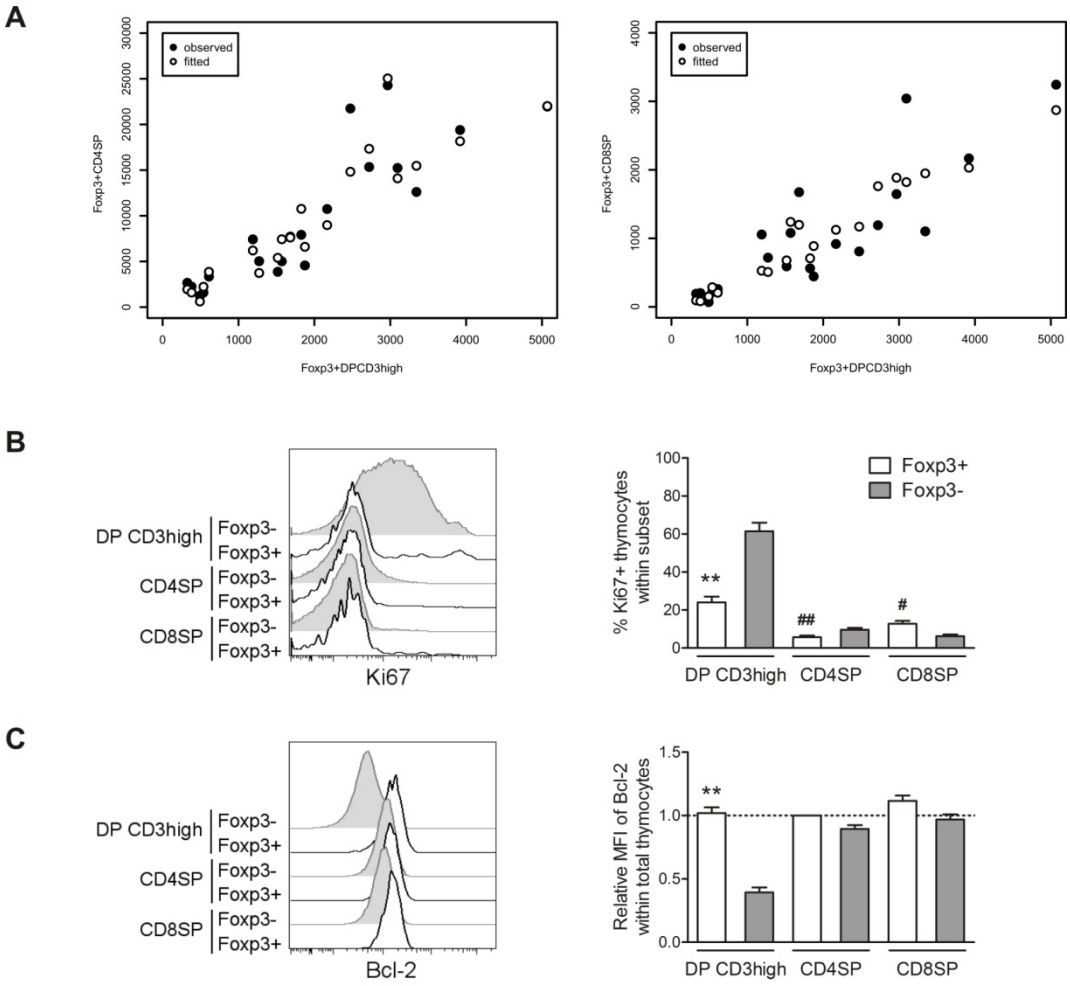

Figure 7: Associations between DP CD3high and SP stages of Foxp3+ and Foxp3- human thymocytes. (**A**) Relationship of Foxp3+ CD4SP (left panel) and Foxp3+ CD8SP (right panel) with Foxp3+ DP CD3high thymocytes. Multiple regression modeling was performed to assess which thymocyte populations have an effect on Foxp3+ SP populations (statistical results are depicted in Table 2) using absolute cell numbers estimated as described in Figure 1A. Observed (filled circles) and fitted (empty circles) values are shown. Each circle represents one thymus. Representative flow cytometry analysis of the expression of Ki67 (**B**) or Bcl-2 (**C**) within Foxp3+ (clear histograms) or Foxp3- (filled histograms) thymocytes at the CD3high DP, CD4SP and CD8SP stages. Graphs show: (**B**) the frequency of Ki67+ cells (n=11 except for Foxp3+ DP CD3high, n=9, and Foxp3+ CD8SP, n=6); and (**C**) the relative MFI of Bcl-2 (ratio between the MFI in a given population and the MFI within Foxp3+ CD4SP cells; n=10 except for Foxp3+ CD8SP: n=5). Bars represent mean±SEM. * refers to the comparison between Foxp3+ DP CD3high and Foxp3- DP CD3high populations; **, $P<0.01$. # refers to the comparison between a Foxp3+ SP population and the corresponding Foxp3+ DP CD3high population; #, $P<0.05$; ##, $P<0.01$.

The strong positive effect of Foxp3+ DP CD3high cells on Foxp3+ SP populations also supports a distinct contribution of cell death and/or proliferation during Foxp3+ T cell development as compared to Foxp3- cells. Indeed, we found the proportion of Ki67-expressing cells to be significantly lower in Foxp3+ as compared to Foxp3- cells at the DP CD3high and CD4SP stages (Fig. 7B). Furthermore, the Foxp3+ DP CD3high cell subset expressed significantly higher levels of Bcl-2 than Foxp3- cells, indicating that Foxp3+ cells may be more resistant to apoptosis (Fig. 7C).

Together these results suggest that a significant proportion of Foxp3+ cells at the CD4SP and CD8SP stage originate from Foxp3+ DP CD3high cells, likely through different mechanisms than those involved in the development of Foxp3- thymocytes.

DISCUSSION

We investigated here the Treg development in the human thymus and provided evidence for induction of Foxp3 prior to CD4 or CD8 lineage commitment. Foxp3 expression at the DP stage was observed in association with the activation of a transcriptional program similar to that observed in peripheral fully differentiated suppressive Treg, with the exception of the considerable levels of functional IL-7Rα expressed. Moreover, we found that the mucosal homing molecule CD103 was expressed by a subpopulation of Foxp3+ DP thymocytes as well as by the large majority of human Foxp3+ CD8SP cells. Finally, our statistical analysis of progenitor-progeny relationships in these pediatric thymuses suggests that a significant proportion of Foxp3+ SP cells might indeed derive from Foxp3+ DP cells, with likely implications for the autoreactivity and diversity of the natural Treg repertoire.

We demonstrated that Foxp3 expression in DP human thymocytes occurs prior to CD4 or CD8 lineage commitment using a re-expression assay, consistently showing active synthesis of both CD4 and CD8 and lack of commitment to either T cell lineage in Foxp3+ DP thymocytes. The dissociation between Foxp3 expression and CD4/CD8 lineage commitment is in agreement to what has been described in the mouse model (Cabarrocas et al., 2006; Fontenot et al., 2005; Ribot et al., 2007).

The majority of Foxp3+ DP thymocytes displayed a phenotype associated with positive selection and maturity in contrast with Foxp3- DP cells, thus supporting an upregulation of Foxp3 expression concomitant with, or immediately following, CD27 upregulation. An association between CD27 and CD25 acquisition had been previously described in human fetal thymuses (Cupedo et al., 2005). We extend here this observation to Foxp3 expression in pediatric thymuses. It was recently suggested that human Treg progenitors may selectively reside within mature DP thymocytes expressing high levels of CD69 and TCRαβ, since these cells develop into CD4SP Treg in response to activated autologous plasmacytoid and myeloid dendritic cells (DC) (Martin-Gayo et al., 2010). In spite of the mature phenotype of CD25+ DP thymocytes (Cupedo et al., 2005; Darrasse-Jeze et al., 2005), the expression of recombination-activating gene 2 (RAG-2) in CD25+ DP cells has been interpreted as possible sign of immaturity of these cells (Tuovinen et al., 2008b).

We also found that Foxp3+ DP thymocytes express IL-7Rα, which contrasted with the low levels found in Foxp3+ SP cells, a feature associated with the peripheral Treg phenotype (Liu et al., 2006; Seddiki et al., 2006a). This is in agreement with a previous

report that showed CD25 expression on DP cells of human thymic fetuses in conjunction with significant levels of IL-7Rα (Cupedo et al., 2005), though not in line with recent data regarding Foxp3 expression in DP cells in postnatal thymuses (Tuovinen et al., 2008b). Importantly, we further demonstrated that the IL-7Rα expressed in Foxp3+ DP thymocytes was functional, as indicated by the downstream phosphorylation of STAT5 in a significant fraction of cells upon IL-7 stimulation and the associated up-regulation of CD25 on these cells. The presence of functional IL-7Rα on Foxp3+ DP thymocytes suggests an involvement of IL-7 in human Treg development. This hypothesis is further supported by our observation of significantly higher levels of Bcl-2, an anti-apoptotic family member induced by IL-7, in the Foxp3+ than the Foxp3- DP subset. The high levels of Bcl-2 and the activated/suppressor phenotype reported in the Foxp3+ populations may contribute to some protection from apoptosis/negative selection as previously suggested for mouse thymic Treg (van Santen et al., 2004). This is in agreement with the clearly distinct precursor-progeny relationships that we documented in the Foxp3+ as compared to the Foxp3- populations.

The genetic program associated with effector-suppressive Tregs in the periphery that we found to be induced in Foxp3+ DP thymocytes included concomitant high level expression of CD25, CTLA-4, HLA-DR and CD39 and was likely dependent on thymic-stroma derived signals. These molecules were also expressed by a subpopulation of Foxp3+ CD4SP cells, though at lower levels, supporting the idea that the activated Foxp3+ DP cells preferentially differentiate into the CD4 lineage and progressively resemble resting cells before leaving the thymus. In agreement, activated Foxp3+ DP and CD4SP cells still expressed considerable levels of IL-7Rα, but no CD45RA, a marker associated with T cell egress from the thymus and with naïve T cells in the periphery (Seddiki et al., 2006b). Consequently, T cells exiting the thymus as CD45RA+ would display lower levels of CD25, CTLA-4 and Foxp3 and would likely be CD39 and IL-7Rα negative. Also in support of this view, the fraction of Foxp3+ CD45RA+ cells expressing CD39 in the periphery has been shown to be negligible (Booth et al., 2010).

Conversely, we showed that the expression of CD73, the other ectoenzyme that, in conjunction with CD39, generates the suppressive molecule adenosine, was mainly restricted to CD8SP cells, irrespective of Foxp3 expression. This is in agreement with the recent finding that CD73 is not expressed by circulating CD4+ Treg in humans (Booth et al., 2010). Furthermore, our results restrict the acquisition of CD73 to CD8SP cells in the

thymus, which, on egress, will most likely enrich the pool of CD73+ naïve CD8 T cells that has been described in humans (Dianzani et al., 1993).

We identified a considerable Foxp3+ CD8SP population in all the 35 thymuses evaluated, in agreement with previous reports (Cosmi et al., 2003; Tuovinen et al., 2008b). Although Foxp3+ CD8SP did not share the activated phenotype observed in Foxp3+ DP and CD4SP cells, previous studies have shown that CD8SP cells purified on the basis of CD25 expression have suppressive properties (Cosmi et al., 2003). Foxp3+ CD8 T cells are rarely found in the peripheral blood (Chaput et al., 2009; Siegmund et al., 2009). Interestingly, we found that the vast majority of Foxp3+ CD8SP cells in the human thymus express CD103, suggesting that they have the capacity to preferentially home to the mucosa. In fact, CD25+ Foxp3+ CD8+ T cells have been identified in normal colonic tissue (Chaput et al., 2009) and were found to be increased in colorectal cancer tissue (Chaput et al., 2009), suggesting a role for this population in mucosal tumors. A population of CD103+ CXCR3+ CD8SP cells was previously described in the human postnatal thymus, and featured an effector phenotype, given their expression of perforin and granzyme A and their ability to produce interferon γ and IL-2 (Annunziato et al., 2006). In agreement, we also found a population of CD103+ CD8SP cells that do not express Foxp3, and it would be of interest to evaluate the similarity of the TCR repertoire between these distinct CD8SP sub-populations. However, current technical limitations preclude this evaluation. The human thymus may thus concomitantly produce two populations of CD8SP cells expressing CD103, with regulatory and effector phenotypes, able to migrate to mucosal tissues, where the Foxp3+ subset may serve to counteract the activity of their Foxp3- counterparts. Furthermore, our data support a scenario whereby Foxp3+ DP cells that express CD103 may be diverted into the CD8 lineage. Despite the association of CD103 with peripheral regulatory CD4 T cells, particularly in mice (Allakhverdi et al., 2006; Banz et al., 2003; Huehn et al., 2004; Lehmann et al., 2002; Rotzschke et al., 2009), we found very little expression of this molecule within CD4SP cells, regardless of Foxp3 expression. This in agreement with what has been described in cord blood, where CD103+ CD4 T cells are virtually absent (Allakhverdi et al., 2006).

The multiple regression and canonical correlation analyses support a significant statistical dependency of Foxp3+ DP and SP populations. The numbers obtained are compatible with a direct precursor-progeny association with a significant commitment to the Treg lineage at the DP stage, taking in consideration the current estimations of the time-lag incurred maturation of cells in the thymic medulla (McCaughtry et al., 2007),

resulting in an accumulation of Foxp3+ SP cells prior to their exit. Recent murine studies have also demonstrated the possibility of Treg generation in the cortex, followed by rapid migration into the medulla, where they mature (Cabarrocas et al., 2006; Liston et al., 2008; Ribot et al., 2007; Schneider et al., 2007). These data do not exclude the possibility of Foxp3 induction at the SP stages (Hanabuchi et al., 2010; Martin-Gayo et al., 2010; Watanabe et al., 2005). Our findings are particularly relevant in view of the recent cumulative evidence that the thymus has a limited capacity to produce Treg (Bautista et al., 2009; Leung et al., 2009). The preferential induction of Foxp3 at the DP stage may represent a way of regulating the likely thymic niche that consequently limits Treg production in the thymus.

In conclusion, we show here that the expression of Foxp3 in human DP thymocytes occurs after positive selection, but prior to CD4 or CD8 lineage commitment. Foxp3+ DP thymocytes were shown to express an activated phenotype that is reminiscent of that found in peripheral activated Tregs but is associated with the expression of a functional IL-7 receptor, which may serve to protect them from cell death/negative selection. A subpopulation of Foxp3+ DP expressing CD103 is likely to give rise to a population of Foxp3 CD8 T cells with preferential homing to the mucosa. Finally, multiple regression models support a significant commitment to the Treg lineage at the DP stage, with possible implications for their repertoire and function.

MATERIAL AND METHODS

SAMPLES AND CELL PREPARATION

Thymic specimens were obtained from routine thymectomy performed during pediatric corrective cardiac surgery at the Hospital de Santa Cruz, Carnaxide, Portugal, after parent's written informed consent. Study was approved by the Ethical Board of the Faculty of Medicine of Lisbon. Pediatric thymuses were from patients raging from newborns to 13 year old children. Cells were recovered through tissue dispersion and separated on a Ficoll-Paque PLUS (GE Healthcare, Uppsala, Sweden) density gradient.

MONOCLONAL ANTIBODIES AND FLOW CYTOMETRY ANALYSIS

The following anti-human monoclonal antibodies were used: CD3 (UCHT1 or OKT3), CD4 (RPA-T4), CD8α (SK1 or RPA-T8), CD25 (2A3), CD27 (O323), CD28 (CD28.2), CD39 (eBioA1), CD69 (FN50), CD73 (AD2), CD103 (B-Ly7), CD127 (40131), HLA-DR (L243), Bcl-2 (124), CTLA-4 (BNI3), Foxp3 (PCH101), Ki67 (B56), TCRαβ (IP26). Antibodies were from BD Biosciences (San Jose, CA, USA), eBioscience (San Diego, CA, USA), DakoCytomation (Glostrup, Denmark), Miltenyi Biotec (Bergisch Gladbach, Germany) or R&D Systems (Minneapolis, MN, USA). Analysis was performed with 6- to 8-color combinations of the antibodies conjugated to FITC/ PE/ PerCP or PerCP-Cy5.5/ PE-Cy7/ APC or Alexa Fluor 647/ APC-Cy7, APC-Alexa Fluor 750 or APC-H7. Surface staining was performed for 20 minutes at room temperature. Cells were subsequently fixed, permeabilized and stained for Foxp3 as well as other intracellular antibodies using a Foxp3 staining kit from eBioscience, according to the manufacturer's protocol. Up to $1.5x10^6$ events were acquired on a FACSCanto or a FACSAria (BD Biosciences). Analysis was performed using FlowJo (TreeStar, Ashland, Oregon, USA). Doublets were stringently excluded based on area and width parameters of both forward and side scatter.

FLOW CYTOMETRY ANALYSIS OF THE TCR Vβ REPERTOIRE

TCR Vβ repertoire was determined using the IOTest Beta Mark kit (Beckman Coulter, Marseille, France). Cells were incubated for 10 minutes at room temperature with the reagent mixtures provided by the kit and subsequently stained for surface molecules and intracellular Foxp3 as described above.

CO-RECEPTOR RE-EXPRESSION ASSAY

DP thymocytes were sorted to a high degree of purity using a FACSAria (BD Biosciences). Sorted cells were treated with 0.5mg/mL pronase (Calbiochem, La Jolla, CA, USA) in PBS for 30 minutes at 37ºC and subsequently cultured at 37ºC or 4ºC in complete medium (RPMI1640 supplemented with FCS, L-glutamine and antibiotics, all from GIBCO-Invitrogen, Paisley, UK) overnight. As a control, cells that were not treated with pronase were cultured under the same conditions. Analysis of the reexpression of CD4 and CD8 was performed by sequentially staining for surface molecules (30 minutes on ice) followed by intracellular Foxp3, as described above. Dead cells were excluded from the analysis using LiveDead Fixable Viability Dye (Molecular Probes, Invitrogen, Carlsbad, CA, USA).

FLOW CYTOMETRY ANALYSIS OF PHOSPHO-STAT5

Total thymocytes were stained for surface molecules, washed and incubated with IL-7 (0.1, 1, 10 or 50ng/mL; R&D Systems) or PBS for 15 minutes at 37ºC. For analysis based on CD25 expression, cells were immediately fixed in formaldehyde 2% for 10 minutes at 37ºC and subsequently permeabilized with ice-cold 90% methanol for 30 minutes on ice. Thymocytes were then washed and incubated with anti phospho-STAT5 (Y694; BD Biosciences) for 1h at room temperature (Azevedo et al., 2009). For Foxp3 analysis an additional step of intracellular staining (see above) was performed after incubation with IL-7 and before fixation in 2% formaldehyde.

THYMOCYTE CULTURES

Total thymocytes were cultured at $3x10^6$ cells/mL in complete medium alone or supplemented with IL-2 (10U/mL), IL-7 (10ng/mL; R&D) or soluble anti-CD3 plus anti-CD28 mAbs (both 1µg/mL; eBioscience) for 6h or 22h. Cells were then stained for surface molecules and Foxp3 as described above. The following reagent was obtained through the AIDS Research and Reference Program, Division of AIDS, NIAID, NIH: Human rIL-2 from Dr. Maurice Gately, Hoffmann – La Roche Inc. (Lahm and Stein, 1985).

STATISTICAL ANALYSIS

Statistical analysis was performed using GraphPad Prism v5.01 (GraphPad Software Inc., San Diego, CA, USA) and R software (www.r-project.org). Results are presented as arithmetic mean±SEM. Two-sample data were compared using Wilcoxon matched pairs test. To tackle the statistical dependency between sizes of DP and SP populations, a canonical correlation analysis (CCA) was first performed using log10 of cell numbers. In general, CCA aims to relate two sets of variables by means of appropriate linear combinations. The method computes linear combinations for a set of variables that are "matched" with other linear combinations for the second set of variables. The different pairs of linear combinations are called "canonical components", sorted by the respective correlation. The interpretation of the respective linear coefficients provides a way of assessing which variables of one set might be important to explain the variability observed in the variables of the other set. For the sake of simplicity, the first canonical component, which provides the maximum correlation between the variables of both sets, was only analyzed.

To quantitatively assess the effect of different thymocyte populations on SP populations, multiple regression models were fitted to the data. To this end, the dependent variables of the models were the sizes of the four SP T cell populations (Foxp3+ CD4SP, Foxp3- CD4SP, Foxp3+ CD8SP and Foxp3- CD8SP), while the explanatory variables were the sizes of both DP populations (Foxp3+ DPCD3 high and Foxp3+ DP CD3high) and those of other relevant SP compartments. We also chose to include the total cell numbers as a putative explanatory factor, as recommended for the analysis of this type of data (Aitchinson, 1982). The estimation of the models was done using the maximum likelihood method. Statistical significance of each explanatory variables in the model was assessed by traditional Z-score tests using a 5% significance level (i.e., $P<0.05$ imply that the respective explanatory variable is significant in the fitted model). For a better interpretation of regression coefficients, the respective estimates were multiplied by 1,000, which indicate how many additional cells of a certain thymocyte population are expected to be generated when increasing 1,000 cells of the corresponding explanatory variable. An appropriate residual analysis was also performed in order to validate the models in statistical terms.

In all analyses, the level of significance was set at 5% (i.e., statistical significance was achieved when $P<0.05$).

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REFERENCES

Aitchinson, J. 1982. The Statistical Analysis of Compositional Data. Journal of Royal Statistical Society.

Allakhverdi, Z., D. Fitzpatrick, A. Boisvert, N. Baba, S. Bouguermouh, M. Sarfati, and G. Delespesse. 2006. Expression of CD103 identifies human regulatory T-cell subsets. *J Allergy Clin Immunol*. 118:1342-9.

Allan, S.E., R. Broady, S. Gregori, M.E. Himmel, N. Locke, M.G. Roncarolo, R. Bacchetta, and M.K. Levings. 2008. CD4+ T-regulatory cells: toward therapy for human diseases. *Immunol Rev*. 223:391-421.

Annunziato, F., L. Cosmi, F. Liotta, E. Lazzeri, R. Manetti, V. Vanini, P. Romagnani, E. Maggi, and S. Romagnani. 2002. Phenotype, localization, and mechanism of suppression of CD4(+)CD25(+) human thymocytes. *J Exp Med*. 196:379-87.

Annunziato, F., L. Cosmi, F. Liotta, E. Lazzeri, P. Romagnani, R. Angeli, L. Lasagni, R. Manetti, F. Marra, C. Gerard, I. Petrai, P. Dello Sbarba, F. Tonelli, E. Maggi, and S. Romagnani. 2006. CXCR3 and alphaEbeta7 integrin identify a subset of CD8+ mature thymocytes that share phenotypic and functional properties with CD8+ gut intraepithelial lymphocytes. *Gut*. 55:961-8.

Apostolou, I., A. Sarukhan, L. Klein, and H. von Boehmer. 2002. Origin of regulatory T cells with known specificity for antigen. *Nat Immunol*. 3:756-63.

Azevedo, R.I., M.V. Soares, J.T. Barata, R. Tendeiro, A. Serra-Caetano, R.M. Victorino, and A.E. Sousa. 2009. IL-7 sustains CD31 expression in human naive CD4+ T cells and preferentially expands the CD31+ subset in a PI3K-dependent manner. *Blood*. 113:2999-3007.

Baecher-Allan, C., E. Wolf, and D.A. Hafler. 2006. MHC class II expression identifies functionally distinct human regulatory T cells. *J Immunol*. 176:4622-31.

Banz, A., A. Peixoto, C. Pontoux, C. Cordier, B. Rocha, and M. Papiernik. 2003. A unique subpopulation of CD4+ regulatory T cells controls wasting disease, IL-10 secretion and T cell homeostasis. *Eur J Immunol*. 33:2419-28.

Bautista, J.L., C.W. Lio, S.K. Lathrop, K. Forbush, Y. Liang, J. Luo, A.Y. Rudensky, and C.S. Hsieh. 2009. Intraclonal competition limits the fate determination of regulatory T cells in the thymus. *Nat Immunol*. 10:610-7.

Belkaid, Y., and K. Tarbell. 2009. Regulatory T cells in the control of host-microorganism interactions (*). *Annu Rev Immunol*. 27:551-89.

Bennett, C.L., J. Christie, F. Ramsdell, M.E. Brunkow, P.J. Ferguson, L. Whitesell, T.E. Kelly, F.T. Saulsbury, P.F. Chance, and H.D. Ochs. 2001. The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3. *Nat Genet*. 27:20-1.

Bettini, M.L., and D.A. Vignali. 2010. Development of thymically derived natural regulatory T cells. *Ann N Y Acad Sci*. 1183:1-12.

Booth, N.J., A.J. McQuaid, T. Sobande, S. Kissane, E. Agius, S.E. Jackson, M. Salmon, F. Falciani, K. Yong, M.H. Rustin, A.N. Akbar, and M. Vukmanovic-Stejic. 2010. Different proliferative potential and migratory characteristics of human CD4+ regulatory T cells that express either CD45RA or CD45RO. *J Immunol*. 184:4317-26.

Borsellino, G., M. Kleinewietfeld, D. Di Mitri, A. Sternjak, A. Diamantini, R. Giometto, S. Hopner, D. Centonze, G. Bernardi, M.L. Dell'Acqua, P.M. Rossini, L. Battistini, O. Rotzschke, and K. Falk. 2007. Expression of ectonucleotidase CD39 by Foxp3+ Treg cells: hydrolysis of extracellular ATP and immune suppression. *Blood*. 110:1225-32.

Brunkow, M.E., E.W. Jeffery, K.A. Hjerrild, B. Paeper, L.B. Clark, S.A. Yasayko, J.E. Wilkinson, D. Galas, S.F. Ziegler, and F. Ramsdell. 2001. Disruption of a new forkhead/winged-helix protein, scurfin, results in the fatal lymphoproliferative disorder of the scurfy mouse. *Nat Genet*. 27:68-73.

Brusko, T.M., A.L. Putnam, and J.A. Bluestone. 2008. Human regulatory T cells: role in autoimmune disease and therapeutic opportunities. *Immunol Rev*. 223:371-90.

Cabarrocas, J., C. Cassan, F. Magnusson, E. Piaggio, L. Mars, J. Derbinski, B. Kyewski, D.A. Gross, B.L. Salomon, K. Khazaie, A. Saoudi, and R.S. Liblau. 2006. Foxp3+ CD25+ regulatory T cells specific for a neo-self-antigen develop at the double-positive thymic stage. *Proc Natl Acad Sci U S A*. 103:8453-8.

Chaput, N., S. Louafi, A. Bardier, F. Charlotte, J.C. Vaillant, F. Menegaux, M. Rosenzwajg, F. Lemoine, D. Klatzmann, and J. Taieb. 2009. Identification of CD8+CD25+Foxp3+ suppressive T cells in colorectal cancer tissue. *Gut*. 58:520-9.

Cosmi, L., F. Liotta, E. Lazzeri, M. Francalanci, R. Angeli, B. Mazzinghi, V. Santarlasci, R. Manetti, V. Vanini, P. Romagnani, E. Maggi, S. Romagnani, and F. Annunziato. 2003. Human CD8+CD25+ thymocytes share phenotypic and functional features with CD4+CD25+ regulatory thymocytes. *Blood*. 102:4107-14.

Cupedo, T., M. Nagasawa, K. Weijer, B. Blom, and H. Spits. 2005. Development and activation of regulatory T cells in the human fetus. *Eur J Immunol*. 35:383-90.

Darrasse-Jeze, G., G. Marodon, B.L. Salomon, M. Catala, and D. Klatzmann. 2005. Ontogeny of CD4+CD25+ regulatory/suppressor T cells in human fetuses. *Blood*. 105:4715-21.

Dianzani, U., V. Redoglia, M. Bragardo, C. Attisano, A. Bianchi, D. Di Franco, U. Ramenghi, H. Wolff, L.F. Thompson, A. Pileri, and et al. 1993. Co-stimulatory signal delivered by CD73 molecule to human CD45RAhiCD45ROlo (naive) CD8+ T lymphocytes. *J Immunol*. 151:3961-70.

Feuerer, M., J.A. Hill, D. Mathis, and C. Benoist. 2009. Foxp3+ regulatory T cells: differentiation, specification, subphenotypes. *Nat Immunol*. 10:689-95.

Fontenot, J.D., M.A. Gavin, and A.Y. Rudensky. 2003. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. *Nat Immunol*. 4:330-6.

Fontenot, J.D., J.P. Rasmussen, L.M. Williams, J.L. Dooley, A.G. Farr, and A.Y. Rudensky. 2005. Regulatory T cell lineage specification by the forkhead transcription factor foxp3. *Immunity*. 22:329-41.

Hanabuchi, S., T. Ito, W.R. Park, N. Watanabe, J.L. Shaw, E. Roman, K. Arima, Y.H. Wang, K.S. Voo, W. Cao, and Y.J. Liu. 2010. Thymic stromal lymphopoietin-activated plasmacytoid dendritic cells induce the generation of FOXP3+ regulatory T cells in human thymus. *J Immunol*. 184:2999-3007.

Hsieh, C.S., Y. Liang, A.J. Tyznik, S.G. Self, D. Liggitt, and A.Y. Rudensky. 2004. Recognition of the peripheral self by naturally arising CD25+ CD4+ T cell receptors. *Immunity*. 21:267-77.

Huehn, J., K. Siegmund, J.C. Lehmann, C. Siewert, U. Haubold, M. Feuerer, G.F. Debes, J. Lauber, O. Frey, G.K. Przybylski, U. Niesner, M. de la Rosa, C.A. Schmidt, R. Brauer, J. Buer, A. Scheffold, and A. Hamann. 2004. Developmental stage, phenotype, and migration distinguish naive- and effector/memory-like CD4+ regulatory T cells. *J Exp Med*. 199:303-13.

Jordan, M.S., A. Boesteanu, A.J. Reed, A.L. Petrone, A.E. Holenbeck, M.A. Lerman, A. Naji, and A.J. Caton. 2001. Thymic selection of CD4+CD25+ regulatory T cells induced by an agonist self-peptide. *Nat Immunol*. 2:301-6.

Josefowicz, S.Z., and A. Rudensky. 2009. Control of regulatory T cell lineage commitment and maintenance. *Immunity*. 30:616-25.

Lahm, H.W., and S. Stein. 1985. Characterization of recombinant human interleukin-2 with micromethods. *J Chromatogr*. 326:357-61.

Lee, H.M., and C.S. Hsieh. 2009. Rare development of Foxp3+ thymocytes in the CD4+CD8+ subset. *J Immunol*. 183:2261-6.

Lehmann, J., J. Huehn, M. de la Rosa, F. Maszyna, U. Kretschmer, V. Krenn, M. Brunner, A. Scheffold, and A. Hamann. 2002. Expression of the integrin alpha Ebeta 7

identifies unique subsets of CD25+ as well as CD25- regulatory T cells. *Proc Natl Acad Sci U S A*. 99:13031-6.

Leung, M.W., S. Shen, and J.J. Lafaille. 2009. TCR-dependent differentiation of thymic Foxp3+ cells is limited to small clonal sizes. *J Exp Med*. 206:2121-30.

Liotta, F., L. Cosmi, P. Romagnani, E. Maggi, S. Romagnani, and F. Annunziato. 2005. Functional features of human CD25+ regulatory thymocytes. *Microbes Infect*. 7:1017-22.

Liston, A., K.M. Nutsch, A.G. Farr, J.M. Lund, J.P. Rasmussen, P.A. Koni, and A.Y. Rudensky. 2008. Differentiation of regulatory Foxp3+ T cells in the thymic cortex. *Proc Natl Acad Sci U S A*. 105:11903-8.

Liu, W., A.L. Putnam, Z. Xu-Yu, G.L. Szot, M.R. Lee, S. Zhu, P.A. Gottlieb, P. Kapranov, T.R. Gingeras, B. Fazekas de St Groth, C. Clayberger, D.M. Soper, S.F. Ziegler, and J.A. Bluestone. 2006. CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4+ T reg cells. *J Exp Med*. 203:1701-11.

Martin-Gayo, E., E. Sierra-Filardi, A.L. Corbi, and M.L. Toribio. 2010. Plasmacytoid dendritic cells resident in human thymus drive natural Treg cell development. *Blood*. 115:5366-75.

McCaughtry, T.M., M.S. Wilken, and K.A. Hogquist. 2007. Thymic emigration revisited. *J Exp Med*. 204:2513-20.

Miyara, M., K. Wing, and S. Sakaguchi. 2009a. Therapeutic approaches to allergy and autoimmunity based on FoxP3+ regulatory T-cell activation and expansion. *J Allergy Clin Immunol*. 123:749-55; quiz 756-7.

Miyara, M., Y. Yoshioka, A. Kitoh, T. Shima, K. Wing, A. Niwa, C. Parizot, C. Taflin, T. Heike, D. Valeyre, A. Mathian, T. Nakahata, T. Yamaguchi, T. Nomura, M. Ono, Z. Amoura, G. Gorochov, and S. Sakaguchi. 2009b. Functional delineation and differentiation dynamics of human CD4+ T cells expressing the FoxP3 transcription factor. *Immunity*. 30:899-911.

Nishikawa, H., and S. Sakaguchi. 2010. Regulatory T cells in tumor immunity. *Int J Cancer*. 127:759-67.

Nunes-Cabaco, H., J.C. Ribot, I. Caramalho, A. Serra-Caetano, B. Silva-Santos, and A.E. Sousa. 2010. Foxp3 induction in human and murine thymus precedes the CD4+ CD8+ stage but requires early T-cell receptor expression. *Immunol Cell Biol*. 88:523-8.

Resta, R., Y. Yamashita, and L.F. Thompson. 1998. Ecto-enzyme and signaling functions of lymphocyte CD73. *Immunol Rev*. 161:95-109.

Ribot, J., G. Enault, S. Pilipenko, A. Huchenq, M. Calise, D. Hudrisier, P. Romagnoli, and J.P. van Meerwijk. 2007. Shaping of the autoreactive regulatory T cell repertoire by thymic cortical positive selection. *J Immunol*. 179:6741-8.

Rotzschke, O., G. Borsellino, L. Battistini, K. Falk, and M. Kleinewietfeld. 2009. In vivo-activated CD103+ Foxp3+ Tregs: of men and mice. *Blood*. 113:2119-20; author reply 2120.

Sakaguchi, S., T. Yamaguchi, T. Nomura, and M. Ono. 2008. Regulatory T cells and immune tolerance. *Cell*. 133:775-87.

Salomon, B., D.J. Lenschow, L. Rhee, N. Ashourian, B. Singh, A. Sharpe, and J.A. Bluestone. 2000. B7/CD28 costimulation is essential for the homeostasis of the CD4+CD25+ immunoregulatory T cells that control autoimmune diabetes. *Immunity*. 12:431-40.

Schneider, M.A., J.G. Meingassner, M. Lipp, H.D. Moore, and A. Rot. 2007. CCR7 is required for the in vivo function of CD4+ CD25+ regulatory T cells. *J Exp Med*. 204:735-45.

Seddiki, N., B. Santner-Nanan, J. Martinson, J. Zaunders, S. Sasson, A. Landay, M. Solomon, W. Selby, S.I. Alexander, R. Nanan, A. Kelleher, and B. Fazekas de St Groth. 2006a. Expression of interleukin (IL)-2 and IL-7 receptors discriminates between human regulatory and activated T cells. *J Exp Med*. 203:1693-700.

Seddiki, N., B. Santner-Nanan, S.G. Tangye, S.I. Alexander, M. Solomon, S. Lee, R. Nanan, and B. Fazekas de Saint Groth. 2006b. Persistence of naive CD45RA+ regulatory T cells in adult life. *Blood*. 107:2830-8.

Shevach, E.M. 2009. Mechanisms of foxp3+ T regulatory cell-mediated suppression. *Immunity*. 30:636-45.

Siegmund, K., B. Ruckert, N. Ouaked, S. Burgler, A. Speiser, C.A. Akdis, and C.B. Schmidt-Weber. 2009. Unique phenotype of human tonsillar and in vitro-induced FOXP3+CD8+ T cells. *J Immunol*. 182:2124-30.

Singer, A., S. Adoro, and J.H. Park. 2008. Lineage fate and intense debate: myths, models and mechanisms of CD4- versus CD8-lineage choice. *Nat Rev Immunol*. 8:788-801.

Spits, H. 2002. Development of alphabeta T cells in the human thymus. *Nat Rev Immunol*. 2:760-72.

Starr, T.K., S.C. Jameson, and K.A. Hogquist. 2003. Positive and negative selection of T cells. *Annu Rev Immunol*. 21:139-76.

Stephens, L.A., C. Mottet, D. Mason, and F. Powrie. 2001. Human CD4(+)CD25(+) thymocytes and peripheral T cells have immune suppressive activity in vitro. *Eur J Immunol*. 31:1247-54.

Suzuki, H., J.A. Punt, L.G. Granger, and A. Singer. 1995. Asymmetric signaling requirements for thymocyte commitment to the CD4+ versus CD8+ T cell lineages: a new perspective on thymic commitment and selection. *Immunity*. 2:413-25.

Tai, X., M. Cowan, L. Feigenbaum, and A. Singer. 2005. CD28 costimulation of developing thymocytes induces Foxp3 expression and regulatory T cell differentiation independently of interleukin 2. *Nat Immunol*. 6:152-62.

Tuovinen, H., E. Kekalainen, L.H. Rossi, J. Puntila, and T.P. Arstila. 2008a. Cutting edge: human CD4-CD8- thymocytes express FOXP3 in the absence of a TCR. *J Immunol*. 180:3651-4.

Tuovinen, H., P.T. Pekkarinen, L.H. Rossi, I. Mattila, and A.T. Arstila. 2008b. The FOXP3(+) subset of human CD4(+)CD8(+) thymocytes is immature and subject to intrathymic selection. *Immunol Cell Biol*.

van Santen, H.M., C. Benoist, and D. Mathis. 2004. Number of T reg cells that differentiate does not increase upon encounter of agonist ligand on thymic epithelial cells. *J Exp Med*. 200:1221-30.

Vanhecke, D., G. Leclercq, J. Plum, and B. Vandekerckhove. 1995. Characterization of distinct stages during the differentiation of human CD69+CD3+ thymocytes and identification of thymic emigrants. *J Immunol*. 155:1862-72.

Vanhecke, D., B. Verhasselt, M. De Smedt, G. Leclercq, J. Plum, and B. Vandekerckhove. 1997. Human thymocytes become lineage committed at an early postselection CD69+ stage, before the onset of functional maturation. *J Immunol*. 159:5973-83.

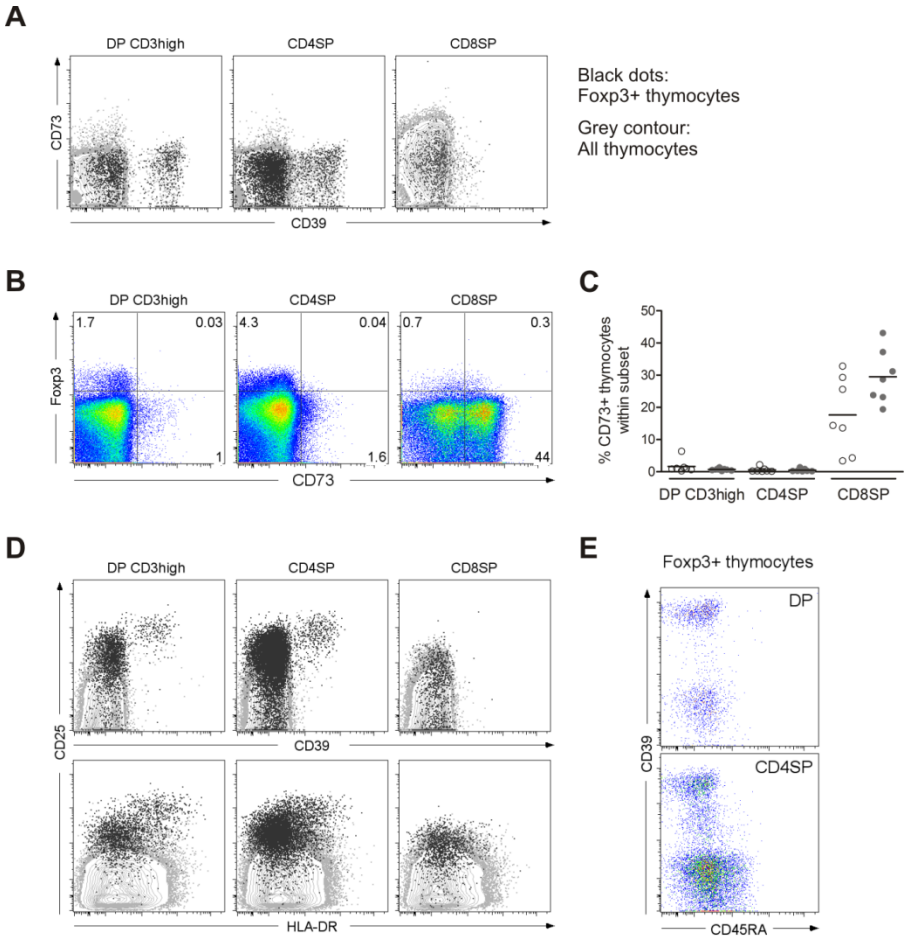
Watanabe, N., Y.H. Wang, H.K. Lee, T. Ito, W. Cao, and Y.J. Liu. 2005. Hassall's corpuscles instruct dendritic cells to induce CD4+CD25+ regulatory T cells in human thymus. *Nature*. 436:1181-5.

Wildin, R.S., F. Ramsdell, J. Peake, F. Faravelli, J.L. Casanova, N. Buist, E. Levy-Lahad, M. Mazzella, O. Goulet, L. Perroni, F.D. Bricarelli, G. Byrne, M. McEuen, S. Proll, M. Appleby, and M.E. Brunkow. 2001. X-linked neonatal diabetes mellitus, enteropathy and endocrinopathy syndrome is the human equivalent of mouse scurfy. *Nat Genet*. 27:18-20.

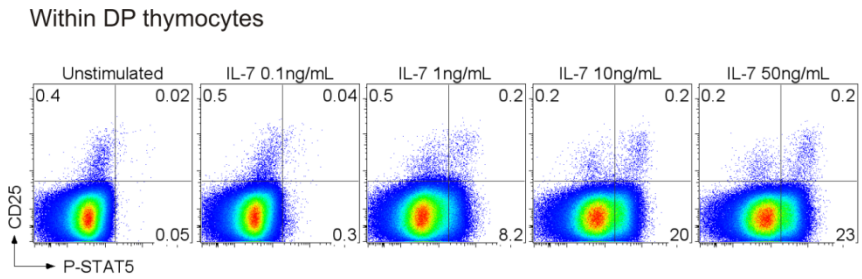
Wing, K., A. Ekmark, H. Karlsson, A. Rudin, and E. Suri-Payer. 2002. Characterization of human CD25+ CD4+ T cells in thymus, cord and adult blood. *Immunology*. 106:190-9.

Wing, K., P. Larsson, K. Sandstrom, S.B. Lundin, E. Suri-Payer, and A. Rudin. 2005. CD4+ CD25+ FOXP3+ regulatory T cells from human thymus and cord blood suppress antigen-specific T cell responses. *Immunology*. 115:516-25.

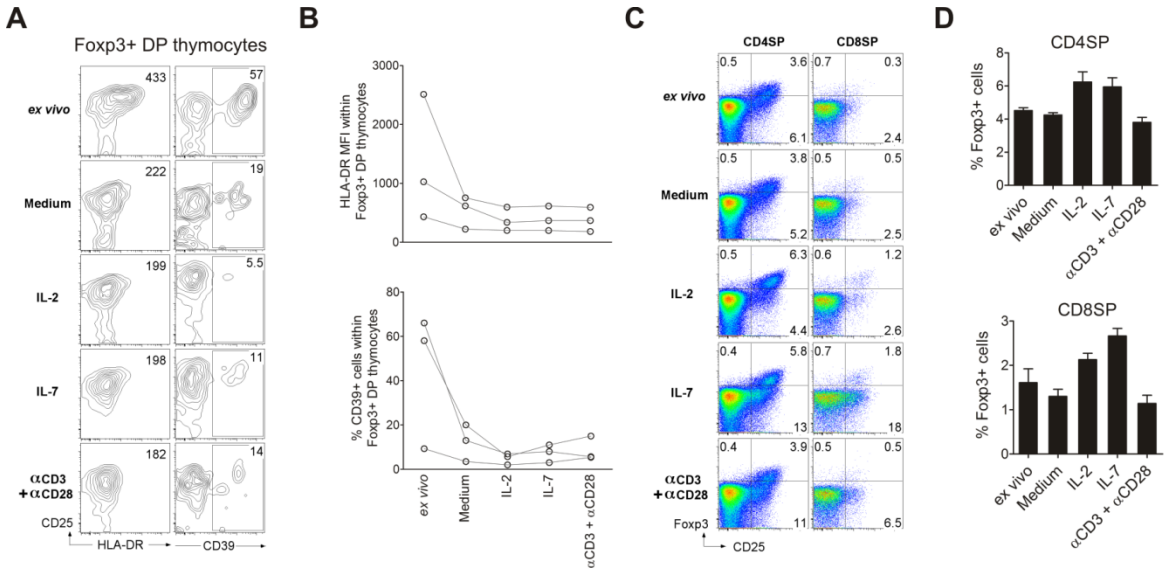
SUPPLEMENTARY FIGURES



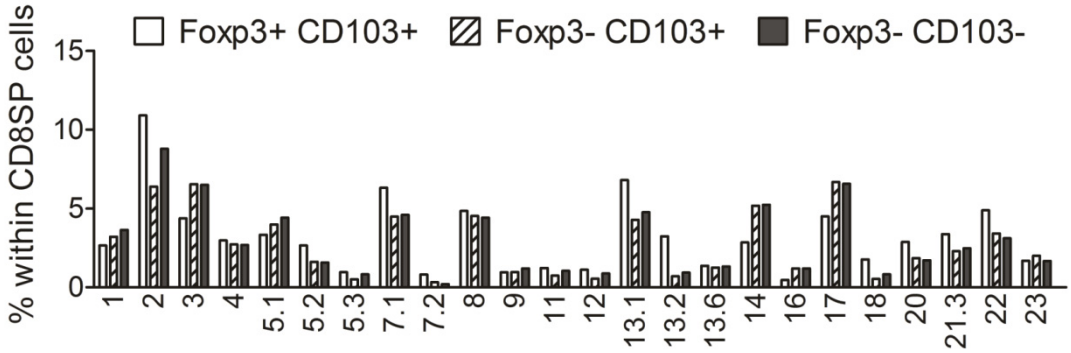
Supplemental Figure 1: Analysis of the co-expression of CD39, CD73, CD25 and HLA-DR within CD3high DP, CD4SP and CD8SP subsets in the human thymus. (A) Representative dot plots of the expression of CD39 and CD73 showing that CD39 and CD73 are not coexpressed by Foxp3+ or Foxp3- cells at the CD3high DP, CD4SP and CD8SP cells. (B) Expression of CD73 in relation to Foxp3 at these stages of human T cell development in a representative thymus. (C) Frequency of CD73 within Foxp3+ (open circles) or Foxp3- (grey circles) cells at the CD3high DP, CD4SP and CD8SP stages. Each circle represents a thymus. (D) Representative dot plots of the expression of CD25 versus CD39 or HLA-DR showing that CD25high cells express CD39 and HLA-DR. Foxp3+ cells are highlighted in black over the light grey contour plot representing the whole population. (E) Expression of CD39 in relation to CD45RA within Foxp3+ thymocytes at the DP (upper panel) or CD4SP (bottom panel) in a representative thymus, showing that CD45RA is not expressed in CD39high cells.



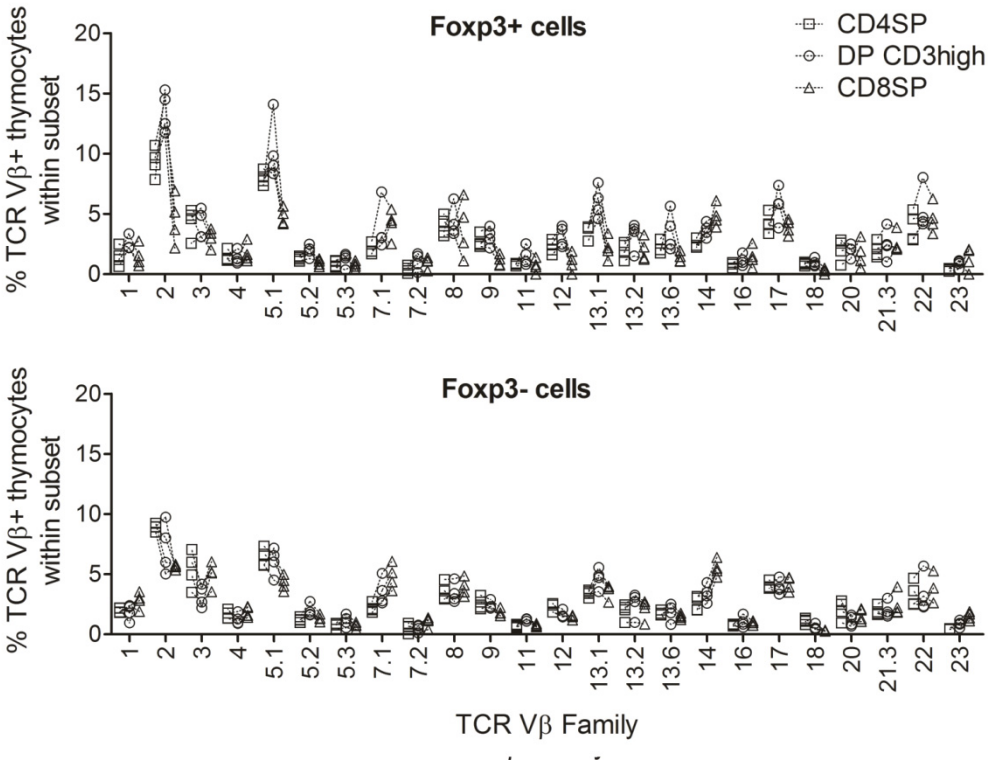
Supplemental Figure 2: Analysis of the expression of P-STAT5 and CD25+ within human DP thymocytes upon IL-7 stimulation. Representative flow cytometry analysis of the expression of P-STAT5 in relation to CD25 within DP, CD4SP and CD8SP thymocytes after exposure to IL-7 (0.1, 1, 10 or 50ng/mL) or PBS (control).



Supplemental Figure 3: Loss of the activated phenotype of Foxp3+ DP thymocytes in culture and up-regulation of Foxp3 on SP thymocytes upon short-term culture with IL-2 or IL-7. (A) Representative analysis within Foxp3+ DP thymocytes of the expression of CD25 and HLA-DR (left) or CD39 (right) ex-vivo (0h) and after 6h or 22h, respectively, of culture of total thymocytes in the presence or absence of IL-2 (10U/mL), IL-7 (10ng/mL) or anti-CD3 plus anti-CD28 (both at 1g/mL). The MFI of HLA-DR and the frequency of CD39+ cells are shown in these representative experiments and in 3 thymuses studied (B). Each line connects results from the same thymus. (C) Representative dot plots of Foxp3 and CD25 expression within CD4SP and CD8SP thymocytes after 6h culture in the conditions described in (A), with graphs showing the frequency of Foxp3+ cells within CD4SP and CD8SP thymocytes (D). Bars represent mean±SEM.

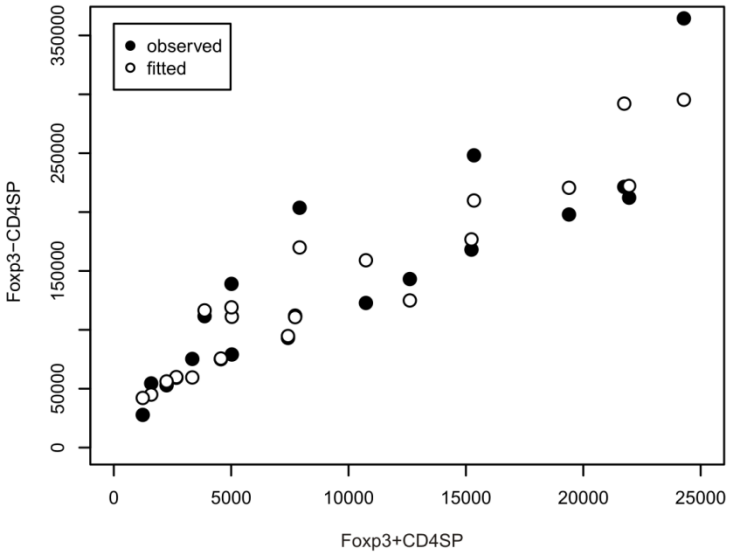


Supplemental Figure 4: Comparison of the TCR Vβ family distribution within CD8SP subsets defined according to Foxp3 and CD103 expression. TCR Vβ family distribution at the CD8SP stage within Foxp3+ CD103+ (open bars), Foxp3- CD103+ (dashed bars) and Foxp3- CD103- (grey bars) thymocytes assessed by flow cytometry. Results of a representative thymus out of 3 different experiments are shown.



Supplemental Figure 5: Relationship between the DP, CD4SP and CD8SP subsets in the human thymus according to Foxp3 expression. TCR Vβ family distribution at the CD4SP (square), DP CD3high (circle), CD8SP (triangle) stages within Foxp3+ (upper graph) or Foxp3- (lower graph) subsets assessed by flow cytometry. Each line connects data from the same thymus.

A



B

Coefficient	Foxp3- CD4SP			
	Using cell numbers within total thymocytes (R^2 adjust= 0.8337)		Using Log10 of cell numbers within total thymocytes (R^2 adjust= 0.8816)	
	Estimate	p-value	Estimate	p-value
Intercept	1.72e+04	0.44	0.96	0.31
Foxp3+ DP CD3high	-2.80e+04	0.06	-0.28	0.17
Foxp3+ CD4SP	1.10e+04	< 0.001	0.63	< 0.001
Foxp3- DP CD3high	2.32e+02	0.46	0.10	0.52
Total thymocytes	8.15e+01	0.13	0.36	0.18

Supplemental Figure 6: Multiple regression analysis relating the cell numbers of Foxp3+CD4SP to those of Foxp3-CD4SP thymocytes. (A) Observed and fitted values for the relation between Foxp3+ CD4SP and Foxp3- CD4 SP thymocytes. (B) Estimates for the coefficients of the models using cell numbers, or considering log10 of cell numbers. P-values <0.05 indicate that the respective explanatory variable is significant in the model.

SUPPLEMENTAL TABLE I: MULTIPLE REGRESSION ANALYSIS.

Coefficient	Foxp3+ CD4SP (R^2 adjust= 0.91)		Foxp3+ CD8SP (R^2 adjust= 0.78)	
	Estimate	**p-value**	**Estimate**	**p-value**
Intercept	- 0.96	0.41	- 2.04	0.34
Foxp3+ DP CD3high	0.69	0.001	0.89	0.01
Foxp3- CD4SP	0.96	< 0.001	0.48	0.19
Foxp3- DP CD3high	- 0.17	0.37	- 0.29	0.39
Total thymocytes	- 0.25	0.46	0.22	0.75

Using Log10 of the number of live thymocytes, analyzed as shown in Figure 1A.

Chapter V

Conclusions and Future Perspectives

CHAPTER V: CONCLUSIONS AND FUTURE PERSPECTIVES

The fundamental role of the thymus in the replenishment of the peripheral compartment with a diverse, yet self-tolerant repertoire that is able to respond to potential pathogens has long been recognized. At the same time that it produces T cells that can effectively fight foreign pathogenic threats, ideally without inducing damage to self, the thymus also generates a lineage of regulatory T cells that play an important role in the control of immune responses, particularly in the context of inflammatory or autoimmune processes. Human Treg development is poorly understood, with many aspects remaining elusive. Knowledge on this subject is essential in order to develop therapeutic strategies directly impacting on the Treg repertoire.

As a model, the mouse is undoubtedly a valuable tool to approach many features of Treg differentiation. However, and despite the extensive list of publications in the area, many central questions on Treg development in the mouse are still not fully understood. It is also essential to confirm that the knowledge generated in the mouse model also applies to humans. Despite the many similarities between the two systems, it is well known that significant differences do exist. A major difference concerns the timing of T cell generation during the early life of both species. While in humans the T cell repertoire is mostly formed at birth (1-4), in mouse it is not until the first days of life that the Treg population is produced (5-8). Furthermore, differences are also present in the developmental stages that thymocytes undergo in order to become a mature T cell, such as the absence of a CD4ISP stage in the mouse (9). In addition, both the genetic background and history of exposure to pathogens are greatly variable in humans, while in mouse studies those factors are highly controlled.

In the first part of this work, described in Chapter III, we studied the early stages of Treg development in both human and mouse thymus. In humans we consistently found the expression of both *FOXP3* mRNA and protein at the CD4ISP stage, thus before the DP stage. We also confirmed the presence of Foxp3+ cells in the cortex, supporting the expression of Foxp3 at early stages of T cell development. We further attested the immature phenotype of Foxp3+ CD4ISP thymocytes. We then turned to the mouse model in order to characterize the early stages of Treg development in that system, since this had not been specifically addressed to date. We were also able to find a pre-DP population expressing Foxp3 in the mouse, namely at the DN4 stage. The use of Foxp3-GFP mice allowed us to clearly identify this rare population. Foxp3 expression at pre-DP

stages raised the question of its TCR-dependency, since TCR signals have been shown to be required for Foxp3 expression at later stages of thymic development in the mouse. Using TCRα-deficient mice we were able to show that Foxp3 was no longer expressed in the DN4 population in the absence of TCRα, supporting TCR-dependency of Foxp3 expression at that stage. Although it is generally assumed that TCRα gene rearrangements are only started at the DP stage, it has been shown that they are already found in pre-DP stages both in humans and mouse models (10, 11), supporting the possibility that TCRαβ complexes may already be expressed at those stages. In agreement, we show that Foxp3+ DN4 thymocytes do express TCRβ at their surface. The possibility that pTα may have a role in Foxp3 induction was excluded by the fact that TCRα-deficient mice still express pTα and by the absence of an alteration on Foxp3 expression in pTα-deficient mice as compared to wild-type.

In humans it has been reported that Foxp3 is expressed in DN cells that lack surface TCRαβ (12). However, in this report the expression of CD3 was not examined, as later pointed out by an independent group (13). In agreement to what this group mentioned, we also found that many of the DN thymocytes expressing Foxp3 are CD3+. Although we cannot formally exclude the possibility that in some thymuses there is an earlier induction of Foxp3 expression at the TN stage, our data does not support the consistent presence of a Foxp3+ TN population in the human thymus. In sorted TN populations we could only detect Foxp3 expression in 2 out of 4 thymuses and always at low levels. Furthermore, and although flow cytometry is a powerful tool to characterize populations at the single cell level, it is generally not possible to distinguish negative from low expression of a molecule in a cell based on cytometric analysis alone. However, the current inability to sort live cells based on intracellular expression of molecules in humans precludes further analysis of Foxp3+ cells. Furthermore, the irreversible damage induced by the fixation and permeabilization agents to RNA (14) makes it impossible to analyze the pattern of transcription of sorted cells. However, it is feasible to perform genomic DNA-based PCR after intracellular staining and sorting (14), and it would thus be interesting to confirm the occurrence of *TCRA* gene rearrangements in the Foxp3+ CD4ISP population.

A question that follows from the identification of such an early population of Foxp3-expressing thymocytes regards its further development in the thymus. With the availability of Foxp3-GFP mice it would be expected that the Foxp3+ DN4 population could be easily sorted and cultured. However, the estimation of ~200 cells/mouse

created several technical difficulties. We tried a number of different strategies to optimize the differentiation of the few Foxp3+ DN4 sorted cells (up to 1000 cells from as many as 20 mice): (1) cultures with anti-CD3 plus APCs; (2) reconstitution of TOCs of RAG-deficient mice; (3) intrathymic injections; (4) co-cultures with OP9-DL1 stromal cells. However, we were not able to consistently and convincingly find a significant number of Foxp3-GFP+ cells after culture in order to attest their phenotype. It is possible that further optimization of these techniques for such a low number of cells or the use of embryonic FTOCs, at the expense of dozens of Foxp3-GFP mice for confirmation of reproducibility of the results, will allow the differentiation and study of that population. This would allow the investigation of another important question that arises from this study regarding the impact of such an early selection on the repertoire of Foxp3+ cells. Using the Foxp3-GFP mouse and an optimized differentiation system it would be possible to compare the repertoire of SP cells originated from the Foxp3+ DN4 population with that of wild type Foxp3+ SP thymocytes and determine the contribution of this early component to the overall Foxp3+ SP population.

The current inability to sort live cells based on an intracellularly expressed molecule precludes studies of differentiation of human Foxp3+ CD4ISP thymocytes. However, as previously mentioned, a DNA-based multiplex PCR has been developed that allows the clonotypic characterization of fixed and permeabilized sorted Foxp3+ cells (14). Using this method it would be interesting to compare the repertoire of Foxp3+ CD4ISP cells with that of more mature Foxp3+ thymocytes.

In the second part of this work, described in Chapter IV, we studied the DP and SP stages of human Treg development. We confirmed that a population of Foxp3-expressing cells at the DP stage is still CD4 or CD8 lineage uncommitted, as transcriptional shut down of either co-receptor has not taken place. This notwithstanding, Foxp3+ DP cells, which are mostly CD3high, present a mature phenotype when compared to their equivalent Foxp3- counterpart, suggesting that Foxp3 acquisition is associated with the expression of maturation markers and/or it is a late event during differentiation. Furthermore, we observed an up-regulation of Treg-associated markers at the Foxp3+ DP CD3high stage, indicating an activation of the suppressive transcriptional program observed in the periphery. However, in contrast to what is observed in the periphery (15, 16), we also found considerable expression of IL-7Rα in Foxp3+ DP CD3high cells, indicating a role for IL-7 in the development of Foxp3+ cells in the human thymus. In agreement, we show that IL-7Rα expressed at that stage is

functional and induces the up-regulation of CD25 expression in Foxp3+ DP cells. When we analyzed the SP populations expressing Foxp3 we found that CD4SP cells strikingly resembled the Foxp3+ DP CD3high population, while the CD8SP population was rather different. Most Foxp3+ CD8SP thymocytes actually expressed CD103, a mucosal homing-associated molecule that has also been linked to a regulatory phenotype (17, 18). Interestingly, we also found a population of Foxp3+ DP CD3high expressing CD103, raising the possibility that this might be the precursor population of Foxp3+ CD8SP CD103+ cells. Importantly, statistical analysis supported the hypothesis of a direct progeny-product relationship between Foxp3+ DP CD3high and Foxp3+ SP populations, and this was not observed for Foxp3- cells. Different impact of proliferation and selection on Foxp3+ vs Foxp3- populations was further supported by the differential expression of Ki67 and Bcl-2, respectively.

Our data thus indicate that a significant part of Foxp3+ cells selected at the DP CD3high stage directly progresses to the CD4SP or CD8SP stages, and this is supported by multiple regression analyses. A recent study by the Toribio group also suggested that human Treg progenitors may selectively reside within mature DP thymocytes expressing high levels of CD69 and TCRαβ, since these cells develop into CD4SP Treg in response to activated autologous plasmacytoid and myeloid DCs (19). As a large proportion of CD25+ DP CD3high cells are Foxp3+, it would be interesting to sort and differentiate this population in TOCs in order to understand the Treg-generating potential/pathway of these cells in comparison to the CD25- DP CD3high population. In addition, this system would allow the assessment of proliferation and selection in both populations, for example using CFSE and Annexin V, respectively. Furthermore, and since our results indicate a potential role for IL-7 in Treg differentiation, addition of IL-7 or other cytokines to the cultures would help devise the cytokine-mediated effects that might take place during Treg development. Ideally, all these experiments would be performed with sorted Foxp3+ DP CD3high thymocytes. In order for this to be possible, it would be necessary to devise and optimize a new strategy, such as the transduction of CD34+ cells with a retroviral vector (20-22) comprised of the Foxp3 promoter and enhanced GFP as a marker gene and to differentiate that population in RTOCs or co-culture with TECs. Sorted GFP+ or GFP- DP CD3high thymocytes could then be reintroduced into TOCs in order to study their development. A possible caveat of this procedure would be the difficulty to use autologous TECs in both steps. In order to

circumvent this limitation and assure similar MHC presentation at all stages of development, hybrid hmFTOCs could be employed, as has been described (20, 21).

Our results do not exclude the possibility that Foxp3 is induced at the SP stage, as has been shown in mouse, where the Foxp3- CD25high CD4SP population has been described as being highly enriched in Treg precursors (23, 24). In fact, we show that IL-2 and IL-7 are able to increase the frequency of Foxp3+ cells within both CD4SP and CD8SP thymocytes in as little as 6h in culture. Formal evidence to support this would require the sorting of SP thymocytes based on both Foxp3 and CD25 expression, which would only be possible with the use of Foxp3-GFP transduced thymocytes. However, it is possible to sort and culture CD4SP cells expressing different levels of CD25 in the presence of those cytokines while controlling for cell numbers, proliferation and death in order to determine which populations undergo cytokine-mediated increase of their Foxp3 frequency.

Comparison of the TCR Vβ distribution of DP or SP Foxp3+ and Foxp3- cells did not reveal differences among those populations. Previous studies based on CD25 expression also did not find differences in TCR Vβ distribution between CD4SP CD25+ and CD4SP CD25- thymocytes (25). We also found high similarity between the TCR Vβ distribution of Foxp3+ DP CD3high and SP stages. However, a recent study where the TCR Vβ repertoire of DP CD25+, CD4SP CD25+ and CD4SP CD25- thymocytes was analyzed based on the length of CDR3 sequences described higher similarity between CD4SP CD25+ and CD4SP CD25- cells than between DP CD25+ and CD4SP CD25+ thymocytes (26). Nevertheless, direct analysis of the repertoire of Foxp3+ thymocytes is still lacking. The above-mentioned DNA-based PCR would provide valuable information on this field, as would differentiated Foxp3-GFP-transduced CD34+ cells.

We described a subpopulation within Foxp3+ DP CD3high thymocytes, and also within Foxp3+ CD4SP thymocytes, that could be identified by the high expression of CD25 and CD39 and that also expressed HLA-DR. This could either mean that those populations have a direct progeny-precursor relationship or that similar signals are delivered at those stages of development that induce the up-regulation of similar molecules. Interestingly, both populations dramatically lost CD39 and HLA-DR expression in culture, even in the presence of IL-2, IL-7, IL-15 or anti-CD3 plus anti-CD28 antibodies. The induction/maintenance of this phenotype thus seems to be very much dependent on thymic-specific signals, probably delivered by the thymic stroma. Alternatively, we may not have provided the adequate cytokines or cytokine

combinations, or co-stimulatory signals might be necessary that were not present in the culture system. It would be interesting to determine what signals are able to preserve the phenotype of CD25+ CD39+ cells. For instance, the role of TECs could be studied by co-culturing total thymocytes with isolated TECs in the presence or absence of the above-mentioned cytokines. However, we cannot exclude the possibility that this population is apoptosis-prone, as observed in peripheral activated Tregs (27), and cannot be extensively cultured. It is thus important to determine whether the loss of the phenotype corresponds to death of the cells or down-regulation of CD39 and HLA-DR expression.

We also observed that cells emigrating from the thymus, as defined by the expression of CD45RA, would express lower levels of CD25 and CD39. Overall, our data suggest that the population of Foxp3+ DP CD3high cells expressing high levels of CD25 and CD39 sequentially becomes CD4SP and down-regulates CD25, CD39 and also Foxp3 before acquiring CD45RA and leaving the thymus. However, this hypothesis must be validated through the isolation and culture of CD25+ CD39+ DP thymocytes, possibly using co-cultures with TECs or in RTOCs. It would also be important to compare the development of those cells to that of CD25+ CD39- DP cells in order to determine whether there is an association between them. These studies will allow the investigation of the role of the up-regulation of those markers and the possible contribution of these cells to the peripheral compartment.

We have found that, while displaying a mature phenotype, at least part of Foxp3+ DP thymocytes are still uncommitted to the CD4 or CD8 lineage, since shut down of CD4 or CD8α transcription has not yet occurred. However, considering that most Foxp3+ thymocytes are indeed CD4SP and our results support direct differentiation from the DP stage, together with data in the mouse that links TCR signaling to Foxp3 induction, it would be expected that some degree of commitment to the CD4 lineage is present at the DP stage. A possible explanation for this discrepancy may lie in the expression of the other subunit of the CD8 heterodimer, CD8β, which is known to be regulated differently from CD8α in humans (28). We found that Foxp3+ cells actually express lower levels of CD8β than the equivalent Foxp3- cells (Figure 1A), suggesting that either CD8β down-regulation is faster in Foxp3+ DP cells or Foxp3 is acquired after CD8β has been down-regulated. The strong TCR signal endured by the developing Foxp3+ DP thymocytes, due to the envisaged higher affinity of their TCR, may then divert the cell towards the CD4SP lineage. In further support of a direct association between TCR signaling and Foxp3, we found that the expression of CD5, a molecule that

has been associated with TCR signaling in both human and mouse (29-31), is higher in Foxp3+ than Foxp3- thymocytes at the DP CD3high stage (Figure 1B).

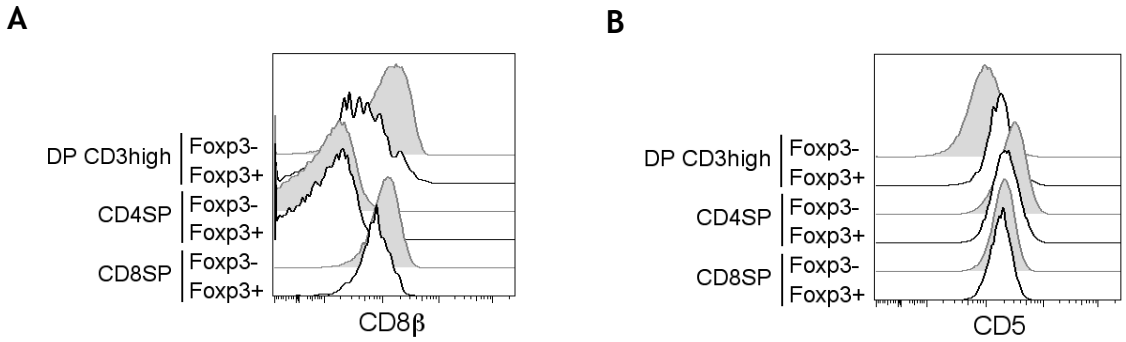


Figure 1: Expression of CD8ß and CD5 in human Foxp3+ and Foxp3- DP and SP thymocytes. **(A)** Lower CD8β expression in Foxp3+ DP CD3high than in Foxp3- DP CD3high thymocytes. **(B)** At the DP CD3high stage CD5 expression is higher in Foxp3+ than in Foxp3- thymocytes, indicating stronger TCR signaling in the former. Representative flow cytometric analysis.

Although a large proportion of Foxp3-expressing thymocytes are found at the CD4SP stage, we also found a small but consistent population of Foxp3+ CD8SP cells. The existence of CD8SP thymocytes with Treg features and functions in the human postnatal thymus had been described based on CD25 expression (32). However, identification of CD8SP Tregs based on CD25 expression underestimates their frequency, which can be revealed by the assessment of Foxp3 expression. Importantly, we found that most Foxp3+ CD8SP cells express the mucosal-associated molecule CD103, which is part of the E-cadherin ligand α_E(CD103)β_7 integrin. CD103 has also been considered a RTE marker for CD8 T cells in the periphery, based on the higher TREC content on naïve CD8 CD103+ T cells than on naïve CD8 CD103- T cells and the age-related decline in frequency of CD8 CD103+ T cells, which is enhanced by thymectomy (33). Remarkably, Foxp3+ CD8SP cells are rarely found in circulation (34), and thus expression of CD103 in CD8 Tregs may indeed direct them to mucosal sites. In agreement, Foxp3+ CD25+ CD8SP suppressive T cells have been found in normal colonic tissue and are enhanced in colorectal cancer tissue (34), suggesting a role for this population in tumor pathology. Although in this study the authors did not examine CD103 expression, this molecule has long been associated with intestinal lymphocytes (35) and it is expressed in virtually all CD8 T cells in the gut (36).

The presence of CD103 in Foxp3+ CD8SP thymocytes further suggested that CD103-expressing cells might be diverted to E-cadherin rich regions in the medulla, leading to the generation of a singular repertoire that might be shared with Foxp3- CD8SP CD103+ thymocytes. Although we did not find major differences in the TCR Vβ family distribution among those populations, a more careful analysis of their repertoire, maybe using the above-mentioned multiplex DNA-based PCR (14), might reveal finer differences. It would also be interesting to confirm histologically the localization of those populations in the human thymus.

CD8SP CD103+ cells thymocytes have been shown to proliferate in co-culture with TECs, and this proliferation was blocked by the addition of anti-CD103 antibodies (37). Additionally, the CD8SP CD103- thymocyte subset was found to have TREC levels 3 to 4 times higher than the CD103+ subset, which is consistent with the later subset having undergone additional divisions since TCR rearrangement (33). Importantly, TREC levels were considerably higher in those CD8SP populations than in peripheral blood T cells of the same subject, indicating thymic rather than peripheral origin of CD8SP CD103+ cells (33). In agreement with these studies, we also found a significantly higher frequency of Ki67+ cells within Foxp3+ CD8SP cells in comparison to Foxp3- CD8SP cells, probably due to the enrichment in CD103+ cells in the first population. TREC quantification in sorted Foxp3+ and Foxp3- CD8SP thymocytes expressing or lacking CD103 would provide additional information regarding the proliferation history of those populations.

We have also identified a population of Foxp3+ DP CD3high thymocytes that already expresses CD103. It is tempting to hypothesize that this constitutes the precursor population of Foxp3+ CD8SP thymocytes. It would be interesting to differentiate DP CD103+ and DP CD103- thymocytes, in co-culture with TECs or in TOCs, and to compare the generation of Foxp3+ CD8SP thymocytes and the expression of CD103 during differentiation. In addition, the proliferation kinetics of Foxp3+ CD8SP thymocytes could be studied through BrdU incorporation during cultures. Furthermore, such a system would allow the assessment of the role of IL-7 and other cytokines in CD8 Treg development.

Studies in the mouse have linked cytokine signaling to the CD8 lineage (38). According to the kinetic model, disruption of TCR signaling due to surface CD8 down-regulation of DP thymocytes renders them dependent on survival signals delivered by cytokines, which are required for differentiation of signaled DP thymocytes into mature CD8 T cells (38, 39). Given that TCR-unsignaled DP thymocytes are mainly refractory to

cytokine signaling and that IL-7 induces the expression of the CD8 lineage specification transcription factor Runx3 (38, 39), it would be expected that IL-7-susceptible DP thymocytes have been TCR-signaled and may potentially become CD8 rather than CD4 T cells. Although the kinetic model has not been confirmed in humans, where studies on that subject are missing, our results indicate that IL-7 may play a role in CD4 vs. CD8 lineage commitment of Foxp3+ DP CD3high cells. However, our data also suggest that the Foxp3+ DP CD3high CD127+ population may not uniformly yield CD8 T cells. For instance, around 40% of Foxp3+ DP CD3high thymocytes express CD127, which would predict a CD4/CD8 ratio of 1.5:1 if similar kinetics applied to both SP populations. Since the ratio we observed was approximately 10:1, major kinetic differences should exist, especially when we take into account the observation that Foxp3+ CD8SP cells may undergo higher proliferation than Foxp3+ CD4SP cells. It would thus be interesting to determine whether IL-7 is able to decrease the thymic CD4/CD8 ratio of Tregs using TOCs. Furthermore, since most thymocytes expressing CD127 are also CD25high and are sensitive to signals mediated by IL-2 and IL-7, as we have shown, the impact of IL-2 or IL-2 plus IL-7 on CD4/CD8 ratio in TOCs could also be studied. In addition, the CD4 vs. CD8 Treg potential of DP CD25low CD127- thymocytes could be compared to that of DP CD25high CD127+ upon differentiation in co-culture with TECs or in RTOCs.

According to our data, the kinetics of the DP CD3high to SP transition in Foxp3+ thymocytes is strikingly different from that of Foxp3- thymocytes. This is supported by the multiple regression analyses and by the differential expression of Ki67 and Bcl-2 in Foxp3+ and Foxp3- subsets. Additional support to that hypothesis could be obtained measuring BrdU incorporation and cell death in TOCs, or comparing the TREC content of sorted Foxp3+ and Foxp3- populations.

The thymus plays a primary role in the replenishment of the peripheral compartment with a diverse pool of T cells throughout life and is essential for its reconstitution in situations of immune depletion, such as bone marrow transplantation. It is also thought to be important for T cell maintenance in lymphopenic clinical settings, such as HIV infection. The thymus is also the source of a significant population of Tregs, which are important regulators of T cell homeostasis. We have studied Treg development in the human thymus and provided evidence for TCR-dependent induction of Foxp3 prior to CD4 or CD8 lineage commitment. Foxp3 expression at the DP stage was observed in association with the activation of a transcriptional program similar to that observed in peripheral fully differentiated activated/suppressive Treg,

with the exception of the considerable levels of functional IL-7Rα expressed, which suggest a role for IL-7 in human Treg development. Moreover, we found that the mucosal-homing molecule CD103 was expressed by a subpopulation of Foxp3+ DP thymocytes as well as by the large majority of human Foxp3+ CD8SP cells, raising the possibility of a direct relationship between those populations. Finally, our statistical analyses of progenitor-progeny relationships support that a significant proportion of Foxp3+ SP cells might indeed derive from Foxp3+ DP cells, with likely implications for the autoreactivity and diversity of the natural Treg repertoire. Overall, we provide evidence of an early thymic induction of a significant component of the human Treg pool. Further studies on human Treg development are essential in order to devise therapeutic strategies for the replenishment of the peripheral Treg compartment with a diverse repertoire enfolding the unique features of natural Treg.

BIBLIOGRAPHY

1. Gale, R.P. 1987. Development of the immune system in human fetal liver. *Thymus* 10:45-56.

2. Muller-Hermelink, H.K., Wilisch, A., Schultz, A., and Marx, A. 1997. Characterization of the human thymic microenvironment: lymphoepithelial interaction in normal thymus and thymoma. *Arch Histol Cytol* 60:9-28.

3. Res, P., and Spits, H. 1999. Developmental stages in the human thymus. *Semin Immunol* 11:39-46.

4. Darrasse-Jeze, G., Marodon, G., Salomon, B.L., Catala, M., and Klatzmann, D. 2005. Ontogeny of CD4+CD25+ regulatory/suppressor T cells in human fetuses. *Blood* 105:4715-4721.

5. Bonomo, A., Kehn, P.J., Payer, E., Rizzo, L., Cheever, A.W., and Shevach, E.M. 1995. Pathogenesis of post-thymectomy autoimmunity. Role of syngeneic MLR-reactive T cells. *J Immunol* 154:6602-6611.

6. Sakaguchi, S., Yamaguchi, T., Nomura, T., and Ono, M. 2008. Regulatory T cells and immune tolerance. *Cell* 133:775-787.

7. Josefowicz, S.Z., and Rudensky, A. 2009. Control of regulatory T cell lineage commitment and maintenance. *Immunity* 30:616-625.

8. Asano, M., Toda, M., Sakaguchi, N., and Sakaguchi, S. 1996. Autoimmune disease as a consequence of developmental abnormality of a T cell subpopulation. *J Exp Med* 184:387-396.

9. Spits, H., Blom, B., Jaleco, A.C., Weijer, K., Verschuren, M.C., van Dongen, J.J., Heemskerk, M.H., and Res, P.C. 1998. Early stages in the development of human T, natural killer and thymic dendritic cells. *Immunol Rev* 165:75-86.

10. Mancini, S.J., Candeias, S.M., Di Santo, J.P., Ferrier, P., Marche, P.N., and Jouvin-Marche, E. 2001. TCRA gene rearrangement in immature thymocytes in absence of CD3, pre-TCR, and TCR signaling. *J Immunol* 167:4485-4493.

11. Dik, W.A., Pike-Overzet, K., Weerkamp, F., de Ridder, D., de Haas, E.F., Baert, M.R., van der Spek, P., Koster, E.E., Reinders, M.J., van Dongen, J.J., et al. 2005. New insights on human T cell development by quantitative T cell receptor gene rearrangement studies and gene expression profiling. *J Exp Med* 201:1715-1723.

12. Tuovinen, H., Kekalainen, E., Rossi, L.H., Puntila, J., and Arstila, T.P. 2008. Cutting edge: human CD4-CD8- thymocytes express FOXP3 in the absence of a TCR. *J Immunol* 180:3651-3654.

13. Battaglia, A., Evoli, A., Scambia, G., and Fattorossi, A. 2008. Comment on "Cutting Edge: Human CD4-CD8- Thymocytes Express FOXP3 in the Absence of a TCR". *J Immunol* 181:857-858.

14. Scheinberg, P., Melenhorst, J.J., Hill, B.J., Keyvanfar, K., Barrett, A.J., Price, D.A., and Douek, D.C. 2007. The clonal composition of human CD4+CD25+Foxp3+ cells determined by a comprehensive DNA-based multiplex PCR for TCRB gene rearrangements. *J Immunol Methods* 321:107-120.

15. Seddiki, N., Santner-Nanan, B., Martinson, J., Zaunders, J., Sasson, S., Landay, A., Solomon, M., Selby, W., Alexander, S.I., Nanan, R., et al. 2006. Expression of interleukin (IL)-2 and IL-7 receptors discriminates between human regulatory and activated T cells. *J Exp Med* 203:1693-1700.

16. Liu, W., Putnam, A.L., Xu-Yu, Z., Szot, G.L., Lee, M.R., Zhu, S., Gottlieb, P.A., Kapranov, P., Gingeras, T.R., Fazekas de St Groth, B., et al. 2006. CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4+ T reg cells. *J Exp Med* 203:1701-1711.

17. Allakhverdi, Z., Fitzpatrick, D., Boisvert, A., Baba, N., Bouguermouh, S., Sarfati, M., and Delespesse, G. 2006. Expression of CD103 identifies human regulatory T-cell subsets. *J Allergy Clin Immunol* 118:1342-1349.

18. Koch, S.D., Uss, E., van Lier, R.A., and ten Berge, I.J. 2008. Alloantigen-induced regulatory CD8+CD103+ T cells. *Hum Immunol* 69:737-744.

19. Martin-Gayo, E., Sierra-Filardi, E., Corbi, A.L., and Toribio, M.L. 2010. Plasmacytoid dendritic cells resident in human thymus drive natural Treg cell development. *Blood* 115:5366-5375.

20. Heemskerk, M.H., Blom, B., Nolan, G., Stegmann, A.P., Bakker, A.Q., Weijer, K., Res, P.C., and Spits, H. 1997. Inhibition of T cell and promotion of natural killer cell development by the dominant negative helix loop helix factor Id3. *J Exp Med* 186:1597-1602.

21. Blom, B., Heemskerk, M.H., Verschuren, M.C., van Dongen, J.J., Stegmann, A.P., Bakker, A.Q., Couwenberg, F., Res, P.C., and Spits, H. 1999. Disruption of alpha beta but not of gamma delta T cell development by overexpression of the helix-loop-helix protein Id3 in committed T cell progenitors. *EMBO J* 18:2793-2802.

22. Owens, B.M., Hawley, R.G., and Spain, L.M. 2005. Retroviral transduction in fetal thymic organ culture. *Methods Mol Med* 105:311-322.

23. Lio, C.W., and Hsieh, C.S. 2008. A two-step process for thymic regulatory T cell development. *Immunity* 28:100-111.

24. Vang, K.B., Yang, J., Mahmud, S.A., Burchill, M.A., Vegoe, A.L., and Farrar, M.A. 2008. IL-2, -7, and -15, but not thymic stromal lymphopoeitin, redundantly govern CD4+Foxp3+ regulatory T cell development. *J Immunol* 181:3285-3290.

25. Kasow, K.A., Chen, X., Knowles, J., Wichlan, D., Handgretinger, R., and Riberdy, J.M. 2004. Human CD4+CD25+ regulatory T cells share equally complex and comparable repertoires with CD4+CD25- counterparts. *J Immunol* 172:6123-6128.

26. Tuovinen, H., Pekkarinen, P.T., Rossi, L.H., Mattila, I., and Arstila, A.T. 2008. The FOXP3(+) subset of human CD4(+)CD8(+) thymocytes is immature and subject to intrathymic selection. *Immunol Cell Biol*.

27. Miyara, M., Yoshioka, Y., Kitoh, A., Shima, T., Wing, K., Niwa, A., Parizot, C., Taflin, C., Heike, T., Valeyre, D., et al. 2009. Functional delineation and differentiation dynamics of human CD4+ T cells expressing the FoxP3 transcription factor. *Immunity* 30:899-911.

28. Hori, T., Cupp, J., Wrighton, N., Lee, F., and Spits, H. 1991. Identification of a novel human thymocyte subset with a phenotype of CD3- CD4+ CD8 alpha + beta-1. Possible progeny of the CD3- CD4- CD8- subset. *J Immunol* 146:4078-4084.

29. Azzam, H.S., Grinberg, A., Lui, K., Shen, H., Shores, E.W., and Love, P.E. 1998. CD5 expression is developmentally regulated by T cell receptor (TCR) signals and TCR avidity. *J Exp Med* 188:2301-2311.

30. Osman, N., Ley, S.C., and Crumpton, M.J. 1992. Evidence for an association between the T cell receptor/CD3 antigen complex and the CD5 antigen in human T lymphocytes. *Eur J Immunol* 22:2995-3000.

31. Gary-Gouy, H., Lang, V., Sarun, S., Boumsell, L., and Bismuth, G. 1997. In vivo association of CD5 with tyrosine-phosphorylated ZAP-70 and p21 phospho-zeta molecules in human CD3+ thymocytes. *J Immunol* 159:3739-3747.

32. Cosmi, L., Liotta, F., Lazzeri, E., Francalanci, M., Angeli, R., Mazzinghi, B., Santarlasci, V., Manetti, R., Vanini, V., Romagnani, P., et al. 2003. Human CD8+CD25+ thymocytes share phenotypic and functional features with CD4+CD25+ regulatory thymocytes. *Blood* 102:4107-4114.

33. McFarland, R.D., Douek, D.C., Koup, R.A., and Picker, L.J. 2000. Identification of a human recent thymic emigrant phenotype. *Proc Natl Acad Sci U S A* 97:4215-4220.

34. Chaput, N., Louafi, S., Bardier, A., Charlotte, F., Vaillant, J.C., Menegaux, F., Rosenzwajg, M., Lemoine, F., Klatzmann, D., and Taieb, J. 2009. Identification of CD8+CD25+Foxp3+ suppressive T cells in colorectal cancer tissue. *Gut* 58:520-529.

35. Kilshaw, P.J., and Murant, S.J. 1990. A new surface antigen on intraepithelial lymphocytes in the intestine. *Eur J Immunol* 20:2201-2207.

36. Annunziato, F., Cosmi, L., Liotta, F., Lazzeri, E., Romagnani, P., Angeli, R., Lasagni, L., Manetti, R., Marra, F., Gerard, C., et al. 2006. CXCR3 and alphaEbeta7 integrin identify a subset of CD8+ mature thymocytes that share phenotypic and functional properties with CD8+ gut intraepithelial lymphocytes. *Gut* 55:961-968.

37. Kutlesa, S., Wessels, J.T., Speiser, A., Steiert, I., Muller, C.A., and Klein, G. 2002. E-cadherin-mediated interactions of thymic epithelial cells with CD103+ thymocytes lead to enhanced thymocyte cell proliferation. *J Cell Sci* 115:4505-4515.

38. Singer, A., Adoro, S., and Park, J.H. 2008. Lineage fate and intense debate: myths, models and mechanisms of CD4- versus CD8-lineage choice. *Nat Rev Immunol* 8:788-801.

39. Park, J.H., Adoro, S., Guinter, T., Erman, B., Alag, A.S., Catalfamo, M., Kimura, M.Y., Cui, Y., Lucas, P.J., Gress, R.E., et al. 2010. Signaling by intrathymic cytokines, not T cell antigen receptors, specifies CD8 lineage choice and promotes the differentiation of cytotoxic-lineage T cells. *Nat Immunol* 11:257-264.