Universidade de Lisboa
Faculdade de Medicina de Lisboa

Living without thymus:
lessons from adults thymectomized in early infancy

Susana Clara Barão Lopes da Silva dos Anjos

Orientadores: Professora Doutora Ana Cristina Gomes Espada de Sousa
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Tese especialmente elaborada para obtenção do grau de Doutor em Medicina, especialidade de Imunologia Clínica

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O trabalho aqui apresentado foi co-financiado pela Fundação para a Ciência e Tecnologia e pelo Programa Operacional Ciência e Inovação (2010/IC/83068/2007).

2016

A impressão desta tese foi aprovada pelo Conselho Científico da Faculdade de Medicina de Lisboa, em reunião de 19 de Julho de 2016.

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A presente dissertação foi realizada na Unidade de Imunodeficiência Humana e Reconstituição Imune do Instituto de Medicina Molecular, Faculdade de Medicina da Universidade de Lisboa.

Para o Pedro

Para o Rui

Para os meus Pais

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ACKNOWLEDGEMENTS

Finishing a PhD is not something you accomplish on your own!

Behind the work presented in this Thesis is the support of many people that, in different ways, have helped me throughout this long journey. They have certainly made the difference during these years and I do feel really grateful for that. I would like to express my sincerest gratitude to

Professora Ana Espada de Sousa, my mentor, for her guidance, for constant stimulus and support;
Professor Manuel Barbosa, for trusting me, for believing that this project will ultimately benefit the all group and the patients;
Sara P. Silva, for looking after our patients, for being so many times the other part of me, at the hospital;
Adriana that taught me everything, from the basics in the Lab;

Colleagues from the Lab (some of them real Friends), that helped in many different ways, from discussions to bench and laughter: Professora Conceição, Alcinda, Ana Luísa, Maria Soares, Iris, Rita Azevedo, Ana Rita, Paula, Helena, Russell, Rita T, Catarina Mota, Susana Fernandes, , Francisca;
Professor Rui Victorino, for his constant support and enthusiasm;
Colleagues from Serviço de Imunoalergologia for their constant support, particularly to Dra Elisa Pedro, Dra Amélia Spínola, Anabela Lopes and Manuel Branco-Ferreira;
Ana Graça for an enormous help in the beginning of this work, helping to find thymectomized patients in old, dusty, hand-written files;
Serviço de Cirurgia Cardíaca from Hospital de Santa Cruz, particularly Dr José Neves for welcoming and support, and nurses;
Dário Ligeiro and Rémi Cheynier for their interested and stimulating cooperation in this work;
Thesis Committee (Professora Maria Gomes da Silva, João Taborda Barata and Bruno Silva-Santos), for discussions, enthusiasm and support;

Thymectomized patients and healthy controls included in the studies;
Patients, that kept encouraging me;

And always

D. Berta, that made my life so much easier;
My really great Friends, for their constant support and for staying;
Sisters and wonderful Family for understanding and supporting;
Dearest Mom and Dad, that have been always there, for their infinite support, for a life of dedication and the best examples;
Rui, for always being with me, supporting, believing and understanding
Pedro, …for always making my eyes and heart smile, just by writing his name!

(I am so lucky!!)

ABBREVIATIONS

ART:	Antiretroviral treatment
APC:	Antigen-presenting cells
CCR:	Chemokine receptor
CD4ISP:	CD4 immature single positive cells
CD:	Cluster of differentiation
CD4SP:	CD4 single positive cells
CD8SP:	CD8 single positive cells
CM:	Central-memory cells
cTEC	Cortical thymic epithelial cell
cjTREC:	Coding joint (cj) TREC
DC:	Dendritic cells
DN:	Double negative cells
DP:	Double positive cells
DUSP6:	Dual-specificity protein phosphatase 6
EDP:	Early double-positive cells
EM:	Effector-memory cells
ERK:	Extracellular signal regulated kinase
FACS:	Fluorescence-activated cell sorting
Foxp3/FOXP3:	Forkhead box P3
γc:	Common-gamma chain
HIV	Human Immunodeficiency Virus
HLA:	Human leucocyte antigen
HSCT:	Hematopoietic stem cell transplantation
Ig:	Immunoglobulin
IL:	Interleukin
IL-2R:	Interleukin-2 receptor
IL-2Rα:	Interleukin-2 receptor alpha chain
IL-2Rβ:	Interleukin-2 receptor beta chain
IL-7R:	Interleukin-7 receptor
ITIM:	Immune-receptor tyrosine-based inhibitory motifs
JAK:	Receptor-associated Janus kinases
LFA-1:	Lymphocyte function-associated antigen 1
MHC:	Major histocompatibility complex

miRNA:	MicroRNA
mTEC	Medullary thymic epithelial cell
mTOR:	Mammalian target of rapamycin
NGS:	Next generation sequencing
NK:	Natural killer cells
PD-1:	Programmed cell death protein 1
PECAM-1:	Platelet endothelial cell adhesion molecule-1
PI3K	Phosphoinositide 3-kinase
RTE:	Recent thymic emigrants
SCF:	Stem cell factor
sjTREC:	Signal joint (sj) TREC
SLO:	Secondary lymphoid organs
SP:	Single positive
STAT:	Signal transducer and activator of transcription
TCR:	T-cell receptor
TEC	Thymic epithelial cell
TN:	Triple negative cells
TREC:	T-cell receptor excision circle
Tonv:	Conventional T-cell
Treg:	Regulatory T-cell

SUMMARY

The maintenance of the naïve CD4 T-cell pool is critical for immune competence and is ensured by continuous thymic output. Moreover, the naïve population of FoxP3-expressing regulatory T-cells (naïve-Tregs) is essential to control immune responses, given their enrichment in thymus-committed autoreactive suppressor cells.

We investigated the ability of peripheral homeostatic mechanisms to counteract thymus impairment. For this purpose, adults thymectomized early in infancy, during corrective cardiac surgery, were grouped based on presence/absence of thymopoiesis and compared with age-matched controls. We focused on the role of IL-7, which is known to induce naïve cell survival, and to distinctively target the peripheral naïve subset expressing CD31 to induce low-level proliferation.

We found that the homeostasis of the $CD31^-$ naïve compartment was independent of the thymus and that its size is tightly preserved by peripheral mechanisms, including extended cell survival. Conversely, a significant contraction of the $CD31^+$ subset was observed in the absence of thymic activity, in association with impairment of *in vitro* PI3K-dependent responses of purified naïve CD4 T-cells to IL-7, namely proliferation and up-regulation of CD31 expression.

Notably, naïve-Tregs were remarkably well preserved irrespective of thymectomy. Additionally, we provided *ex vivo* evidence of ongoing IL-7 responses and, our *in vitro* data supported that naïve-Tregs are able to up-regulate Bcl-2 and to proliferate in a PI3K-dependent manner in response to IL-7, whilst preserving their naïve phenotype and suppressive capacity. Complete thymectomized adults featured no clinical autoimmunity or increased prevalence of allergy/IgE-sensitizations, although higher levels of IgG against a cluster of antigens linked to autoimmune diseases were found using a large array.

Our data show for the first time that IL-7 contributes to naïve-Treg homeostasis and to thymus-independent survival of functional naïve-Tregs. Moreover, our results

revealed the requirement of effective thymopoiesis for IL-7-mediated immune reconstitution of conventional naïve CD4 T-cells.

Key words

Naïve CD4 T-cells; Regulatory T-cells; IL-7; Thymus; Thymectomy.

SUMÁRIO

Homeostasia das células T CD4 *naïve* em adultos timectomizados na infância.

A manutenção do compartimento de células T CD4 *naïve* é importante para garantir a imunocompetência. Uma subpopulação destas células expressa FoxP3 (*naïve*-Tregs) e tem um papel fundamental na regulação de respostas imunes e supressão de autoreactividade.

Neste trabalho questionámos a capacidade de mecanismos homeostáticos periféricos compensarem a diminuição da actividade tímica, e consequente perda de produção de células T *naïve*. Adultos timectomizados na infância durante cirurgia cardíaca correctiva foram agrupados de acordo com presença/ausência de timopoiese e comparados com controlos emparelhados por idade. Investigámos particularmente o papel da IL-7 no aumento da sobrevivência celular e indução de proliferação de células *naïve* que expressam CD31.

Documentámos manutenção da subpopulação $CD31^-$ independentemente do timo, sendo a dimensão deste compartimento controlada por mecanismos periféricos, incluindo aumento da sobrevivência celular.

Em contraste, observou-se contracção significativa da subpopulação $CD31^+$ nos timectomizados sem evidência de timopoiese, associada a perda das respostas à IL-7 em cultura de células T CD4 *naïve* purificadas, afectando a sua proliferação e aumento da expressão de CD31, efeitos dependentes da via PI3K.

No entanto, mesmo na ausência de timopoiese, as células *naïve*-Treg estavam preservadas, com evidência *ex vivo* de resposta à IL-7 e aumento, em cultura na presença de IL-7, de marcadores de sobrevivência celular e da proliferação dependente da via PI3K, mantendo fenótipo *naïve* e supressor. É de notar que adultos submetidos a timectomia completa não evidenciaram aumento da prevalência de autoimunidade ou alergia/sensibilizações IgE-mediadas, embora se documentasse maior autoreactividade IgG contra um grupo de antigénios ligados a doenças autoimunes.

Em conclusão, demonstrámos pela primeira vez que a IL-7 contribui para a manutenção das células *naïve*-Treg independentemente do timo. Revelámos ainda que a reconstitutição do compartimento CD4 *naïve* mediada pela IL-7 depende da manutenção de timopoiese, com implicações para o uso clínico desta via na promoção da reconstituição imunológica.

Palavras Chave:
Células T CD4 *naïve*; Células T reguladoras; IL-7; Timo; Timectomia.

RESUMO EXTENSO

O timo é um orgão linfóide primário, essencial para o desenvolvimento das células T. As células T CD4 são exportadas para a periferia com fenótipo *naïve*, mantendo-se este processo activo até pelo menos à sexta década de vida, conforme demonstrado pela quantificação nos linfócitos circulantes do ADN episssomal resultante dos rearranjos de genes do receptor das células T (TRECs).

A homeostasia do compartimento T CD4 *naïve* resulta de um equilíbrio dinâmico entre o *output* tímico e mecanismos na periferia que visam contrariar o declínio progressivo da actividade tímica associado à idade e à morte ou diferenciação das células *naïve* em células de memória/effectoras. O objectivo é manter um número relativamente estável de células T CD4 *naïve* e preservar a diversidade do seu reportório ao longo da vida. Esta enorme diversidade resulta de um complexo processo de rearranjo genético intra-tímico do receptor das células T (TCR), permitindo manter capacidade de resposta a novos antigénios e tolerância ao próprio, ao longo da vida.

O principal objectivo deste trabalho foi estudar o impacto da perda de actividade tímica na homeostasia das células *naïve* e a eventual adaptação dos mecanismos homeostáticos da periferia. Indivíduos submetidos a timectomia na infância têm sido usados como modelo para responder a esta questão. No entanto, os resultados descritos são muito heterogéneos relativamente ao impacto da timectomia no compartimento *naïve* no adulto, o que se deve em parte à não avaliação da actividade tímica residual, que pode ser devida a tecido tímico ectópico e/ou regeneração tímica. Neste trabalho, avaliámos um coorte de adultos submetidos a timectomia na primeira infância, em contexto de cirurgia cardíaca correctiva, usando uma metodologia inovadora para estimar de forma mais precisa o contributo do *output* tímico para a manutenção do compartimento *naïve*. Os doentes foram estratificados de acordo com a ausência (∅Thy: n=8) ou presença (Thy: n=14) de timopoiese, com base nos respectivos relatórios cirúrgicos e no *output* tímico, estimado pela quantificação de sjTREC/ml. Foi feito um estudo transversal destes indivíduos, com mediana de 21 anos post-timectomia, em comparação com controlos (n=20), com distribuição semelhante por idade e sexo.

Observámos que nos indivíduos timectomizados com persistência de actividade tímica (Thy), o compartimento CD4 *naïve* estava mantido (frequência de células CD4 *naïve* no total de CD4 e número absoluto), e apresentava diversidade de TCR semelhante aos controlos. A análise do *ratio* sj/β TREC, que permite estimar a proliferação intra-tímica, revelou nestes indivíduos valores semelhantes aos controlos, sugerindo uma regeneração tímica post-timectomia. Estes dados reforçam a vantagem de priveligiar uma atitute conservadora, sempre que possível, em cirurgias correctivas cardíacas, evitando a timectomia total, em particular após o primeiro ano de vida.

Em contraste, os indivíduos timectomizados sem evidência de timopoiese (∅Thy) apresentavam uma contração marcada do compartimento CD4 *naïve*. Nestes indivíduos, a análise da expressão de um conjunto de genes relacionados com a regulação da quiescência/proliferação nas células T CD4 *naïve*, revelou um aumento da expressão de DUSP-6, que codifica uma fosfatase que aumenta o limiar de activação do TCR. No entanto, a resposta *in vitro* das células T CD4 *naïve* purificadas à estimulação por via do TCR e a sua consequente diferenciação em células de memória não revelou alterações, sendo assim pouco plausível que esta seja uma via preferencial de preservação do compartimento CD4 *naïve* na ausência de timopoiese.

O compartimento CD4 *naïve* contém dois *subsets* definidos pela expressão/ausência de CD31, tendo sido demonstrado pelo nosso grupo que a IL-7 induz proliferação preferencial das células $CD31^+$, onde se incluem as células recentemente emigradas do timo (RTE).

Em ambos os coortes de indivíduos submetidos a timectomia, o *subset* $CD31^-$ estava mantido, independentemente do seu grau de timopoiese. Esta manutenção associava-se ao aumento da proliferação das células CD4 *naïve* $CD31^-$ e da sua sobrevivência, estimada pela expressão de Bcl-2. Estes resultados sugerem que a homeostasia do *subset* $CD31^-$, é controlada de forma rigorosa por mecanismos da periferia, provavelmente, como previamente sugerido, por interações de baixa afinidade com péptidos do próprio apresentados no contexto de HLA. Os nossos dados são ainda

suportados por estudos anteriores que mostraram a manutenção do *subset* $CD31^-$ durante o envelhecimento, independentemente do declínio do *output* tímico.

No que respeita ao *subset* $CD31^+$, observámos a sua diminuição apenas nos timectomizados sem timopoiese activa. Esta diminuição verificou-se apesar das respostas homeostáticas, documentadas *ex vivo*, com aumento da proliferação e da expressão de Bcl-2. Subsequentemente questionámos se estas respostas eram mediadas por IL-7. Para este efeito, estudámos as respostas *in vitro* de células CD4 *naïve* purificadas de indivíduos timectomizados e saudáveis em cultura com IL-7 e IL-2 (controlo).

A IL-7 induz proliferação selectiva das células $CD31^+$ e mantém a expressão deste marcador, pela via de sinalização PI3K. Em paralelo, a sinalização pela IL-7 também promove o aumento da sobrevivência celular, induzindo o aumento da expressão de Bcl-2 por mecanismos independentes do PI3K. Em cultura com IL-7, o aumento da expressão de Bcl-2, estava preservado nos compartimentos $CD31^+$/$CD31^-$ de indivíduos timectomizados e saudáveis. No entanto, os indivíduos sem timopoiese não apresentaram aumentos significativos da proliferação das células CD4 *naïve* ou da intensidade de expressão de CD31. Com base nestes achados, concluimos que as respostas das células CD4 *naïve* à IL-7, mediadas pela sinalização pela via PI3K, estão dependentes da manutenção de actividade tímica.

No timo tem origem uma linhagem de células T com função reguladora (Treg) e caracterizadas pela expressão de FoxP3, que são exportadas com fenótipo *naïve* (*naïve*-Tregs). Este *subset* mantém um reportório diversificado no sangue periférico e é considerado fundamental para a prevenção de autoimunidade, alergia e contenção das respostas imunes. O conhecimento sobre a homeostasia das *naïve*-Treg é escasso. Assim, questionámos o impacto do declínio da timopoiese na manutenção do *subset naïve*-Treg. Observámos que os indivíduos sem timopoiese, mantinham as células *naïve*-Treg, apesar da contracção do *subset naïve* sem expressão de FoxP3 (*naïve*-Tconv). As células *naïve*-Treg apresentavam uma frequência de células a proliferar *ex vivo* superior à das *naïve*-Tconv, quer em timectomizados quer em saudáveis, contrariando a quiescência atribuída na literatura a esta população e levando-nos a

investigar os mecanismos envolvidos. Dada a elevada expressão do receptor para a IL-2 (CD25) e baixa expressão do receptor para a IL-7 (CD127) nas *naïve*-Treg não se antecipava um papel importante para a IL-7 na sua homeostasia. No entanto, reunimos evidência *ex vivo* do papel da IL-7 nomeadamente: 1) aumento de pSTAT5 *ex vivo*, indicador de sinalização pela IL-7, nas *naïve*-Treg em comparação com as *naïve*-Tconv e populações de memória; 2) recuperação da expressão de CD127 nas *naïve*-Treg após cultura de células CD4 *naïve* em privação de IL-7. Adicionalmente, verificámos que as células *naïve*-Treg proliferavam em cultura com IL-7, mantendo o fenótipo *naïve* e função reguladora. Em comparação com culturas com IL-2, a IL-7 induzia níveis mais elevados de expressão de Bcl-2 e frequências superiores de células a proliferar. O aumento na proliferação em resposta à IL-7 foi inclusivamente superior nas *naïve*-Treg, quando comparadas com as *naïve* -Tconv, sendo igualmente abolido pela inibição específica da via PI3K/mTor.

Os dados reunidos suportam assim, pela primeira vez, o papel da IL-7 na manutenção das células *naïve*-Treg em indivíduos saudáveis.

Os estudos anteriores não têm sido conclusivos no que respeita ao eventual aumento de frequência de manifestações clínicas de alergia e/ou autoimunidade após timectomia na infância. Tendo acesso a um grupo de indivíduos com idade homogénia e rigorosamente estratificados por grau de timopoiese, questionámos se a preservação do compartimento *naïve*-Treg poderia contribuir para evitar o aumento da incidência de doenças autoimunes e/ou alérgicas. A avaliação clínica de ambos os coortes de timectomizados (∅Thy e Thy) não revelou aumento da prevalência de doença alérgica ou autoimune. Quantificámos ainda, em indivíduos sem timopoiese (n=7), a expressão de anticorpos específicos para um painel alargado de 128 autoantigénios. Apesar de não encontrarmos diferenças na autoreactividade IgG, IgM, IgA, ou IgE que permitissem a diferenciação entre coortes; identificámos um conjunto de antigénios para os quais os indivíduos timectomizados tinham autoreactividade IgG significativamente aumentada, alguns destes antigénios com correlações clínicas descritas em doenças autoimunes. Em paralelo, a avaliação de IgEs específicas para 112 componentes de aeroalergénios e alergénios alimentares comuns não apresentou tendência para aumento de sensibilização IgE-mediada nos indíviduos sem timopoiese.

Em resumo, os nossos resultados sugerem que, 21 anos post-timectomia, apesar da contração do compartimento CD4 *naïve*, a preservação de mecanismos de tolerância na periferia e, em particular, a manutenção do compartimento *naïve*-Treg, poderá limitar a evolução para manifestações clínicas de autoimunidade e alergia. Por outro lado, a longo prazo, a diminuição da proliferação induzida pela IL-7 em células *naïve*-Tconv nos indivíduos sem timopoiese poderá contribuir para a redução da diversidade do reportório e eventual aumento do risco infeccioso, bem como da emergência de clones autoreactivos.

Um número crescente de crianças, têm sido submetidas a cirurgia cardíaca correctiva nos últimos 30 a 40 anos, traduzindo-se numa diminuição significativa da morbilidade associada a malformações cardíacas e no aumento da esperança de vida. O seguimento futuro destes indivíduos será particularmente informativo relativamente ao risco de autoimunidade/alergia e capacidade de resposta a novos antigénios/infecções.

Os resultados deste trabalho têm implicações para a homeostasia do compartimento CD4 *naïve* em saudáveis, em particular ao longo do processo de envelhecimento. Revelámos que a manutenção da proliferação homeostática das células T CD4 *naïve* em resposta à IL-7 depende da manutenção da actividade tímica e que esta citocina desempenha um papel importante na preservação do compartimento *naïve*-Treg. Estes achados têm potenciais implicações para o desenho e avaliação de terapêuticas dirigidas para o eixo IL-7/IL-7R, particularmente em contextos de autoimunidade, linfopénia e na reconstituição imunológica após transplante de células estaminais ou depleção linfocitária por doença oncológica ou autoimune. Terapêuticas dirigidas à preservação/regeneração tímica deverão ser ponderadas neste contexto.

Palavras Chave:
Células T CD4 *naïve*; Células T reguladoras; IL-7; Timo; Timectomia.

1. INTRODUCTION

1.1 Homeostasis of naïve CD4 T-cells

The immune system has evolved to protect the host from pathogenic microbes, that are themselves constantly evolving, and to help in the elimination of toxic or allergenic agents that contact with body surfaces. Therefore, the ability to discriminate self from non-self is obviously critical to ensure efficacy and safety of immune responses.

An efficient immune system relies on both innate and adaptive responses to overcome foreign assaults, with T and B-cells being the main players of the later [1]. T-cells are defined by the expression of T-cell receptor (TCR) on their surface, a transmembrane heterodimeric protein that binds processed antigens displayed by antigen presenting cells (APCs). TCRs are generated upon somatic rearrangement of few hundred germline gene elements in the thymus, allowing the formation of millions of different antigen receptors, each one with a potentially unique specificity for a different antigen [2, 3].

During their maturation in the thymus, T-cells differentiate into discrete subpopulations, each with defined repertoires of effector functions [4-6]. The major subsets of T-cells are defined by their selective surface expression of CD4 or CD8. In the blood and secondary lymphoid organs, 60-70% of T-cells are $CD4^{+}CD8^{-}$ that constitute the cornerstone of adaptive immune response, and are generally designated "helper cells" [4, 6, 7].

Newly generated T-cells continuously replenish the naïve CD4 T-cell compartment [8]. This thus represents the long-life reservoir of antigen inexperienced $CD4^{+}$ T-cells, that differentiate into functionally distinct subsets of memory-effector cells upon exposure to antigens in the periphery [9-11]. Memory-effector cells acquire specialized function/phenotype, depending on the nature of the cytokines present at the site of activation [6, 7, 12, 13].

1.1.1. Thymus and T-cell development

The thymus is the central organ for T-cell development, which occurs via a multistep pathway tightly controlled by the complex thymic environment [5]. Originally described by Claudius Galen of Pergamum (130-200 AD), the lymphopoietic function of the thymus was only recognized in the XX century [14], although it was still considered an evolutionary redundant "lymphocyte graveyard". In the early 1960's, Jacques Miller's work on the role of the thymus in mouse lymphocytic leukaemia and the impact of neonatal thymectomy in tolerance [15-17] led 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.

The thymus is found in all vertebrates [18, 19]. Its development in humans closely follows that observed in the murine model and in most land mammalian animals [19]. In the mouse, the thymic rudiment is first evident on day 11 of gestation, having evolved from the endoderm of the third pharyngeal pouch [20]. The human thymus is thought to develop from either the third pharyngeal pouch or from both the third and fourth pharyngeal pouches [21]. The thymic primordium is colonized by T-cell progenitors during the 8th week of gestation, and mature T-cells are observed by the 12th to 13th week [21-23], already featuring a diverse T-cell repertoire [22]. Remarkably, as a primary site for T-cell lymphopoiesis, the thymus is active, not only during the foetal stages of development, but also throughout post-natal life with variable degrees across age (as reviewed in 1.1.3.1).

The thymus architecture is essentially conserved across species. It consists of distinct anatomical compartments which include the subcapsular area, the cortex, the cortico-medullary junction and an inner medulla [24, 25]. Thymic stroma is very important, as it provides the specialized microenvironment that actively supports T-cell development, guiding thymocytes across the different anatomical compartments [25]. Thymic epithelial cells (TECs) constitute the major component of the stromal population, and can be divided into cortical (cTEC) and medullary (mTEC) epithelial cells, with different functional, morphological, and antigen presentation capacities [25, 26]. Thymic stroma also comprises other cell types, including populations of

haematopoietic origin, namely dendritic cells (DC), macrophages and B-cells, and other populations, like fibroblasts and endothelial cells [27, 28].

A combination of factors controls thymocytes proliferation, survival and differentiation, including stromal cell-derived signals (namely interleukin-7 (IL-7), stem cell factor (SCF)) secreted by TEC, Wnt molecules, Hedgehog (an essential positive regulator of T-cell progenitor differentiation), and Notch1, amongst others [27, 29-31].

Bone-marrow derived lymphoid progenitor cells, expressing CD34, migrate to the thymus to undergo T-cell development [5], as illustrated in the diagram of Figure 1. These precursor cells enter the human thymus at the cortico-medullary junction [5, 27] and migrate towards the cortical region where proliferation and differentiation are initiated, via interactions with the thymic stroma [5].

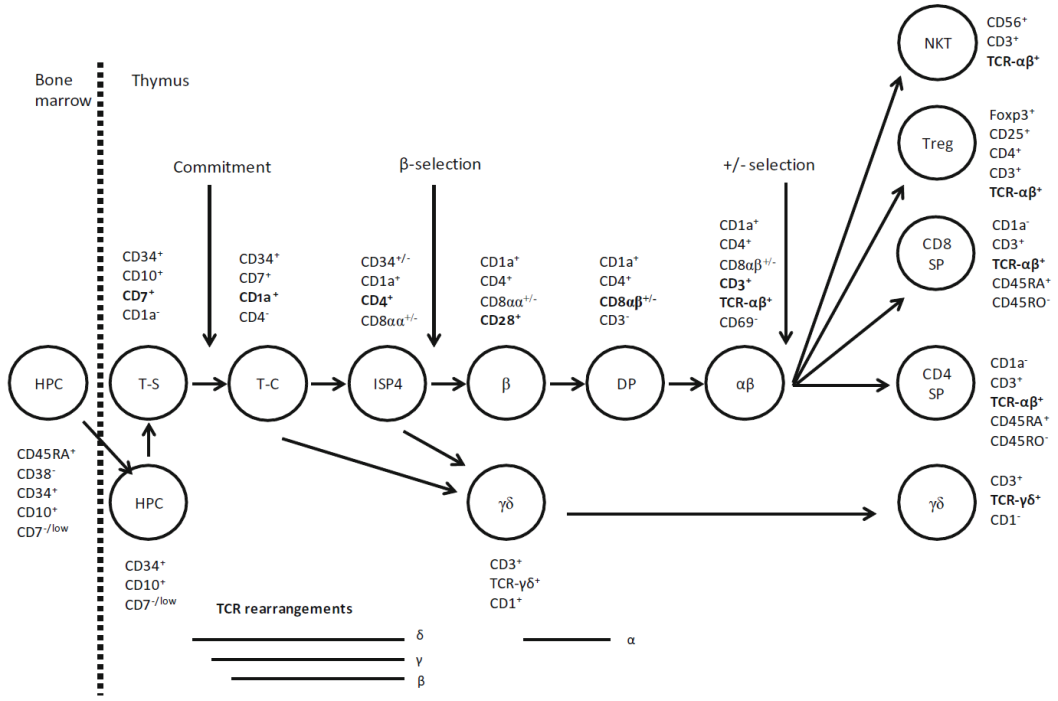

Figure 1 ***Schematic overview of the different developmental stages that characterize human T-cell development.*** HSC: hematopoietic stem cell, T-S: T-lineage specified progenitor, T-C: T-cell committed progenitor, ISP4: CD4 immature single positive, β: rearranged T-cell receptor-β chain, DP: double positive, αβ T-cell receptor: αβ positive cell, NKT: natural killer T-cell, Treg: regulatory T-cell, CD8 SP: CD8 single positive, CD4 SP: CD4 single positive, γδT-cell: receptor-γδ positive cell. *Adapted from [32].*

The expression of the CD1a molecule in progenitor cells is strongly associated with the commitment to the T-cell lineage, at which stage cells begin to undergo rearrangement of their TCR β, γ and δ loci [33]. Undifferentiated $CD34^+CD1a^+$ thymocytes do not express either CD4 or CD8 and thus are named double negative (DN). In the cortex $CD34^+CD1a^+$ thymocytes lose CD34 expression and start to express CD4, thus becoming immature CD4 single positive (CD4ISP) cells, that can either become αβ T-cells or γδ T-cells. The CD4ISP stage is followed by an early double-positive (EDP) stage, in which cells express CD4 and the α chain of CD8 [34], followed by double-positive (DP) cells, that co-express CD4 and the α and β chains of CD8. During the DP stage the levels of CD3 progressively increase and the cells acquire a functional TCR allowing positive and negative selection. Following this DP stage, cells differentiate into $CD4^+$ or $CD8^+$ SP T-cells, mature and exit the thymus [5, 24, 35, 36]

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 [37, 38]. D-to-J recombination occurs first in the β chain of the TCR [5], followed by Vβ-to-DβJβ rearrangements leading to the formation of the TCR Cβ chain. β-selection is the process by which precursor T-cells, with a productive rearrangement of the TCRβ locus, are then selected to undergo further differentiation, in the form of TCRα rearrangements [32]. Thymocytes harbouring a rearranged TCRβ locus generate a TCRβ molecule that pairs covalently with the invariant pre-TCRα (pTα), and non-covalently with CD3 signal-transducing molecules, resulting in formation of a pre-TCR complex at the cell surface [32]. DP thymocytes proliferate extensively and rearrange their TCRα [5, 34]. The subsequent assembly of the β and α chains results in formation of the αβ-TCR that is expressed on the majority of T-cells.

Developing T-cells undergo a filtering process in the thymus that establishes a particular T-cell repertoire for each individual. The TCR diversity that can be generated within the thymocytes pool has been estimated to be as large as 10^{18} α/β-chain combinations [3, 38]. Life or death decisions leading to their selection are mainly

dictated by the strength of TCR signalling, upon interaction with peptide/major histocompatibility complexes (MHC) complexes, as schematically illustrated in Figure 2, ultimately resulting in the generation of CD4 or CD8 SP cells, based on MHC-II or MHC-I restriction, respectively [2, 3, 5, 39]. These interactions are currently thought to important for the peripheral homeostasis of the naïve compartment, by providing tonic signals that promote cell survival and low-level homeostatic proliferation [3].

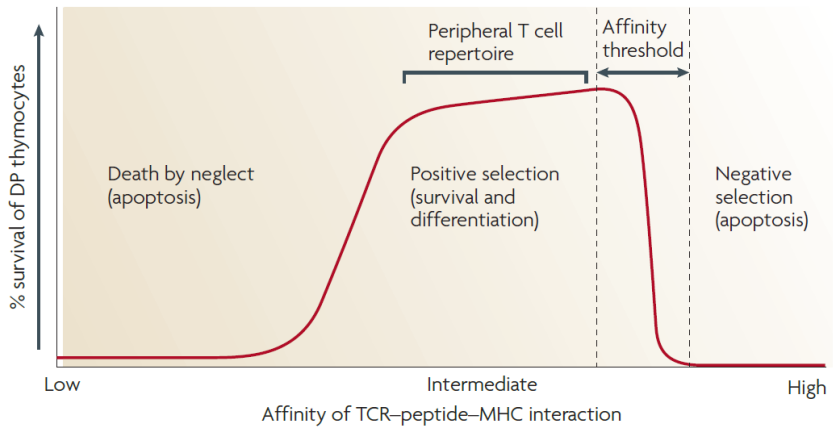

Figure 2. ***The affinity model of thymocyte selection.***
According to this model, the affinity of the TCR-peptide/MHC interaction is the key determinant of T-cell selection. Thymocytes with intermediate affinity for self-peptide/MHC complexes receive a survival signal, undergo positive selection, CD4 or CD8 T-cell lineage commitment and maturation to become part of the T-cell pool. *Adapted from [40]*

Immature $CD4^+CD8^+$ DP αβ T-cells are selected by intermediate/high-affinity interaction of the TCRαβ heterodimer with self-peptides complexed with MHC antigens expressed on cTECs, a stringent process termed positive selection [3, 39]. Positively selected DP thymocytes differentiate into either $CD4^+$ or $CD8^+$ SP T-cells depending on their MHC-II or MHC-I restriction, respectively. Most DP cells (around 98%) cannot recognise any antigen in the context of either MHC-I or MHC-II molecules and these cells die rapidly *in situ* from "neglect" (lack of a TCR signal) [39].

Cells that survive positive selection then move to the thymic medulla, where thymocytes expressing high-affinity for self-peptide/MHC complexes expressed on thymic DCs are deleted and die through apoptosis, a process known as negative selection [5]. Hassall's bodies, structures observed in the thymic medulla, are likely the

"graveyards" for dead thymocytes [27]. Finally, CD4SP and CD8SP thymocytes with a stringently selected TCR repertoire and expressing the naïve-associated marker CD45RA exit the thymus and migrate to the periphery, incorporating the naïve T-cell pool [5, 27].

A distinct lineage of suppressive $CD4^+$ T-cells, defined by FoxP3 expression is also committed in the thymus. These thymus-derived Tregs are thought to be enriched in self-reactive TCRs and to be critical for the maintenance of self-tolerance [41]. Cumulative evidence supports the existence of different pathways of Treg commitment in the human thymus that may occur at different stages of thymocytes differentiation, as illustrated in Figure 3 [42]. In parallel with the proposed role of TCR signalling strength in human Treg commitment, it has become increasingly clear that common gamma chain (γc) cytokines, particularly IL-2 and IL-15, are important mediators of lineage stabilization [42].

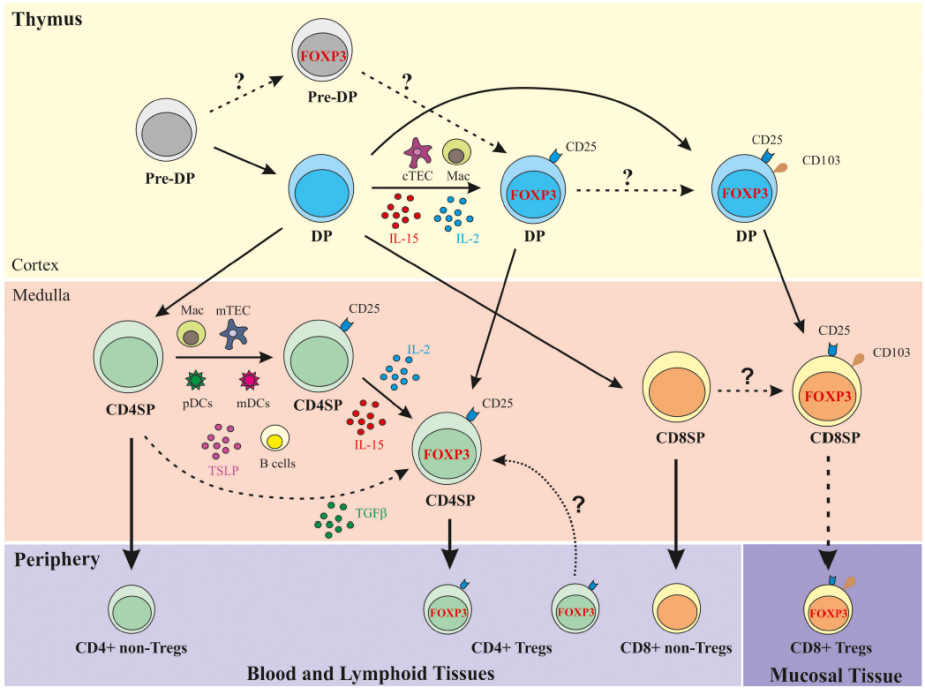

Figure 3. ***Schematic representation of human Treg development in the thymus.***
DP: double-positive ($CD4^+CD8^+$), CD4SP: CD4 single-positive ($CD4^+CD8^-$), CD8SP: CD8 single-positive ($CD8^+CD4^-$), cTEC: cortical thymic epithelial cell, mTEC: medullary TEC, Mac: macrophage, FOXP3: Forkhead box P3, TSLP: thymic stromal lymphopoietin. *Adapted from [42]*

A variety of T-cell receptor excision circles (TRECs) are formed from the excised DNA generated by the rearrangements of the TCRα, β and δ loci described above [43-48]. As

the TCRδ gene segments are embedded within the TCRα locus, the V-to-Jα rearrangements lead to deletion of the δ locus from the chromosome [5, 49]. The end-to-end ligation of the recombination signal sequences flanking the δ-rec locus and the ψ-Jα locus generates a single TREC containing a signal joint (sj) sequence (sjTREC), as illustrated in Figure 4. Coding-joint (cj)TRECs are then produced during the TCRα rearrangement of V to J gene segments.

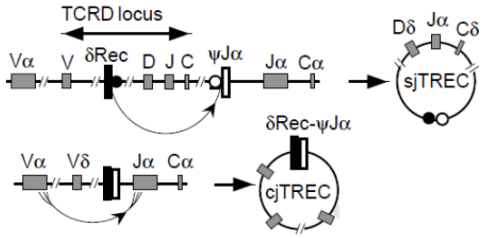

Figure 4 Generation of signal-joint (sj) and coding-joint cjTRECs. *Adapted from [43].*

TREC levels have been used to assess thymic function, through their quantification in peripheral blood using real time PCR technology [50]. This PCR-based assay performed in circulating lymphocytes is also currently used in the neonatal screening of major T-cell defects [51]. The quantification of sjTREC as a measure of thymic output was first utilized by Douek *et al* [43]. As expected, given the gradual loss of thymic function during aging, they showed a decreasing number of sjTRECs with age in healthy individuals [43].

However, it is important to note that the measurement of sjTREC concentration as an estimate of thymic function requires careful consideration. TRECs are not duplicated during mitosis, and are therefore diluted out with each cellular division [45, 52]. Because sjTRECs are not replicated during cell division, their levels can also be influenced by other events occurring in the periphery, such as cell proliferation and differentiation, redistribution, or alterations in cell survival [53]. All of these can lead to a dilution of TREC-bearing cells in the periphery. Of note, it has been reported that quantification of TRECs/μl is less influenced by proliferation in the periphery than quantification of TRECs in a given subpopulation [50, 54].

The level of thymic output has been shown to be primarily determined by the intra-thymic proliferation of precursor T-cells [55]. A new assay was developed to estimate the relative changes in intra-thymic proliferation occurring between the TN and early DP stages [56], as represented in Figure 5. This assay quantified sjTRECs, generated at the DP stage, and DβJβTREC created during the DβJβ rearrangement and organization of the TCRβ locus at the TN stage. The proliferation occurring between these stages can thus be estimated by the ratio of sjTREC to βTREC (sj/βTREC ratio). This marker is not influenced by peripheral T-cell homeostasis, given that both types of TREC are equally affected by the rates of proliferation and death of peripheral T-cells [50].

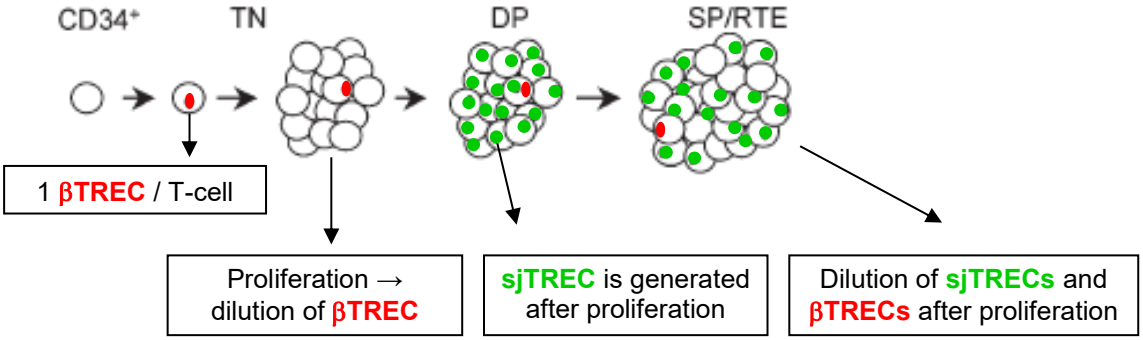

Figure 5: ***Schematic representation of intra-thymic proliferation occurring between late TN and early DP thymocyte differentiation stages and its effects on βTREC and sjTREC levels.***
TN: Triple Negative stage; DP: Double Positive stage; SP/RTE: Single Positive/ Recent Thymic Emigrant stage. *Adapted from [56]*.

1.1.2. Naïve CD4 T-cells as key players in the immune system

Naïve T-cells have been defined as mature T-cells that have not yet encountered their cognate antigen in the periphery. Prior to contact with antigen, CD4 naïve T-cells continuously recirculate between blood and secondary lymphoid tissues (SLO), mainly the spleen and lymph nodes [57, 58]. High endothelial venules (HEV) serve as the entry point into lymph nodes and Peyer's patches, and endothelial cells in these vessels express a unique set of ligands that are recognized by homing receptors expressed by naïve T-cells [59]. The expression of the chemokine receptor CCR7 and L-selectin (CD62L), that engage chemokine CCL21 and vascular addressins in endothelial cells respectively, enables this continuous migration, which is a key mechanism in providing antigenic surveillance [11, 57, 59].

Within lymphoid tissues, antigens are presented to T-cells in the form of peptide fragments bound to MHC molecules expressed upon specialized APC, in particular DC. These cells are strategically positioned within a dense network in the T-cell zones. Once naïve cells encounter their cognate antigen/MHC complex, they undergo proliferation and differentiation towards a memory phenotype, with inherent migratory and functional properties (reviewed in [60]). Clonal selection, upon activation with foreign antigen, therefore leads to predictable loss of TCR diversity in the memory compartment [61]

Isoforms of the transmembrane phosphatase CD45 were initially considered the crucial markers of naïve and memory T-cells, with naïve cells expressing the CD45RA isoform and memory cells expressing CD45RO [62-64]. Presently, distinction between naïve and memory subsets no longer relies solely on CD45 isoforms, as both antigen-primed $CD8^+$ [65, 66] and $CD4^+$ [67-69] T-cells have since been reported to be able to re-express CD45RA in humans, in settings of terminal effector differentiation. Moreover, it has also been suggested that some thymocytes may egress the thymus before switching from CD45RO to CD45RA and only acquire the typical $CD45RA^+$ naïve phenotype in the periphery [70]. Other markers associated with lymphocyte differentiation, such as co-stimulatory molecules (CD27, CD28) [65, 71] or chemokine

receptors (CCR7, CD62L) [71, 72] are currently used to distinguish naïve and memory T-cell subsets within the CD4 and CD8 T-cell pools.

Naïve CD4 T-cells are usually seen as a homogeneous population regarding stage of maturation and cell differentiation, although their phenotypic and functional variety is increasingly recognized [73-77]. Age is an obvious determinant of naïve T-cell biology, impacting both on thymic activity and on SLO microenvironment where peripheral homeostatic mechanisms operate [78, 79]. The functional properties of naïve CD4 T-cells change along infancy, with a clear trend to differentiation into a Th2 profile of cytokine production in early life [80, 81]. Additionally, thymic generated functional populations, that are hardly found beyond the first decade of life have been described with a yet unclear role in immunity, as illustrated by the IL-8 producing subset [74, 76].

1.1.3. Mechanisms involved in the maintenance of the naïve CD4 T-cell compartment throughout life

The size of circulating naïve T-cell pool remains relatively constant throughout adult life, despite continuous environmental antigenic stimulation and the age-associated reduction in thymic output [43, 44].

It has been suggested that the naïve T-cell pool in mice is almost entirely maintained by thymic output [82]. In contrast, the maintenance of human naïve T-cell compartment is sustained by dynamic equilibrium between new T-cell generation in the thymus and mechanisms operating in the periphery, namely peripheral T-cell proliferation, extended survival and death or differentiation into the memory-effector compartment [8, 82], as illustrated in Figure 6. In agreement with studies using manipulated murine models, the cell-intrinsic properties in terms of turnover, survival and threshold for TCR activation are also modulated by a prolonged time-span in circulation [11, 83-86].

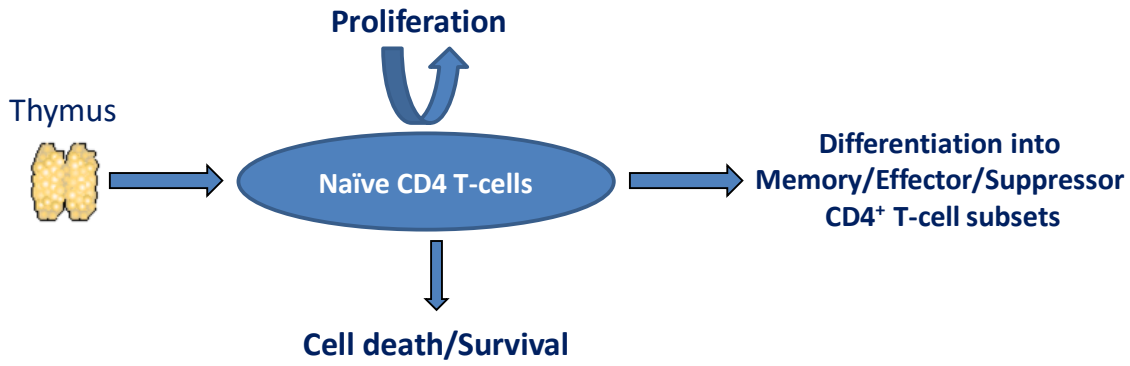

Figure 6. ***Homeostasis of the naïve CD4 T-cell compartment.***

We will briefly review the contribution of these different mechanisms to the homeostasis of the naïve CD4 T-cell compartment.

1.1.3.1. Thymic output

The thymus plays a central role in the generation and maintenance of naïve T-cells early in life [87]. Both thymic epithelial cell development defects, namely DiGeorge syndrome and FOXN1 deficiency, as well as defects of hematopoietic progenitors, have severe clinical impact, which illustrates the non-redundant thymic contribution for the establishment of the T-cell compartment [88, 89].

Despite this, the role of the thymus in T-cell homeostasis after the first years of life was neglected for many years, because of the acknowledged age-dependent thymic involution [78, 90, 91]. However, there is now clear data supporting life-long thymic activity [43, 92]. Particularly, the thymus is known to be active until at least the sixth decade of life [43]. Moreover, data from patients with different clinical conditions associated to T-cell depletion, such as human immunodeficiency virus (HIV) infection [44, 93], and chemotherapy/bone-marrow transplantation [94-99] have confirmed that the thymus is still capable of producing new T-cells in these settings, which might be relevant to the reconstitution of immune function.

The rate of thymic involution has been differently estimated [100], and this heterogeneity of results is possibly influenced by the methods used, namely histology *versus* measurement of thymic output by TREC quantification [50]. This information may be complemented with imaging studies, although these are difficult to perform in adults and usually lack functional correlation [101]. Some authors consider that after a peak of thymic output, thymus involution starts at the age of 1 year and thymic output decreases at a rate of approximately 3% per year until young adulthood, and a rate of 1% per year thereafter [100]. The speed of involution changes during mid-life and leads to a strongly reduced involution afterwards [100, 102]. Other authors consider that thymic output only begins to decrease in early adulthood and continues to further decline with aging [103].

The assessment of the relative contribution of thymic output *versus* peripheral expansion to naïve T-cell homeostasis requires markers able to distinguish the so called Recent Thymic Emigrants (RTEs) from naïve T-cells that have undergone post-

thymic proliferation. High expression of CD31 has been suggested to identify RTEs, but its use alone is not sufficient [43, 104, 105]. Work from our Laboratory has shown that CD31 expression may be maintained upon homeostatic proliferation in the periphery [106] and therefore although, the $CD31^+$ naïve CD4 T-cell subset include the RTEs, there is a large population of $CD31^+$ naïve cells that are not RTEs [61, 104, 106]. Protein tyrosine kinase 7 (PTK7) was also described as a novel marker of human CD4 RTEs [75]. The authors showed that a fraction of the $CD31^+$ naïve CD4 subset expresses PTK7 and that the frequency of $PTK7^+$ cells within the naïve subset decreases progressively with age [75], however not all RTEs are likely to express this marker [75, 107].

1.1.3.2. Cell-Proliferation – 2 main conductors: IL-7 and low-affinity self-peptide/MHC interactions

Although thymic involution progressively limits the replenishment of the peripheral naïve T-cell pool by RTEs, the size of the naïve pool only slowly declines in aging humans [43, 104, 108, 109]. TREC numbers among CD4 T-cells decrease 50-100 times during aging [43, 44], while absolute numbers of naïve CD4 T-cells, as characterized by the expression of CD45RA, decrease only by a factor of 2 or 3 with age [108, 109]. Additionally, several *in silico* studies suggest that thymic output *per se* is insufficient to guarantee the size of the peripheral naïve T-cell compartment without a major contribution of cell proliferation in the periphery [82, 83, 87, 110-113]. Overall, these data support that peripheral post-thymic T-cell proliferation must contribute to the maintenance of the naïve T-cell pool, implying that naïve T-cells are able to proliferate in the periphery whilst retaining their phenotypic and functional properties [105, 114].

The naïve CD4 T-cell compartment has been shown to comprise two subsets with distinct proliferative histories in the periphery, that can be distinguished by the expression of the platelet endothelial cell adhesion molecule-1 (PECAM-1 or CD31) [73]. Of note, both $CD31^+$ and $CD31^-$ naïve CD4 T-cells express surface markers of naivety, such as CD45RA, CD27 and CD62L, and may secrete IL-2, in the absence of significant effector cytokine production after polyclonal stimulation [73]. In addition,

both $CD31^+/CD31^-$ naïve CD4 T-cells are able to differentiate into memory-effector cells upon encounter with antigen [73].

The absolute numbers, as well as the frequency of $CD31^+$ naïve CD4 T-cells in human peripheral blood decrease with aging, in parallel with the decline in their TREC content [61, 73, 104, 115]. Of note, whereas in cord blood 90-95% of $CD45RA^+$ CD4 T-cells express CD31, the majority of the cells lack CD31 expression in the elderly [61]. In accordance with their phenotypic proximity to thymocytes, which also express CD31 [116], the sjTREC content of $CD31^+$ naïve CD4 T-cells is only slightly reduced compared with thymocytes, supporting that this subset contains RTEs.

In contrast, the absolute numbers of $CD31^-$ naïve CD4 T-cells remain relatively constant throughout adult life despite thymic involution [61, 73, 104, 115]. The proportion of $CD31^-$ cells within the naïve $CD4^+$ T-cell population increases with age, allowing the maintenance of naïve T-cell numbers in the elderly through peripheral expansion [61, 73, 108, 109, 115, 117]. The markedly reduced sjTREC content of $CD31^-$ naïve CD4 T-cells strongly implies peripheral proliferation in the generation/maintenance of this subset [61].

The homeostatic proliferation of naïve T-cells is dependent on TCR interaction with self-peptide/MHC complexes plus IL-7 signalling [61, 105, 118-124]. The peripheral expansion/survival in $CD31^+$ compartment is thought to be mainly mediated by IL-7, whereas the $CD31^-$ subset is thought to mainly proliferate in response to TCR stimulation by low-affinity self-peptide/MHC [73, 105], as illustrated in Figure 7.

IL-7, a γ_C cytokine, is mainly produced by non-lymphoid cells within stromal cells in the bone-marrow and SLO, and epithelial cells in the thymus and gut [10, 125-128]. IL-7 has been described as a non-redundant cytokine in the development of T-cells in mice and humans [129-132], as well as being essential for the survival and proliferation of naïve and memory T-cells in the periphery [114, 133-136].

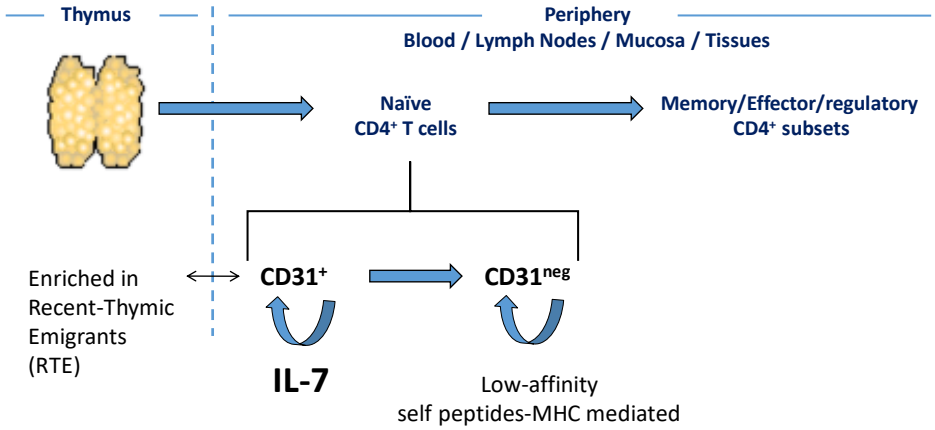

Figure 7. ***Post-thymic proliferation of human naïve CD4 T-cells.***

IL-7 signalling appears to be tightly regulated by the expression of the α-chain of its receptor (IL-7Rα, CD127). The expression of IL-7Rα on lymphocytes varies according to the stage of development or activation [137]. IL-7 itself [138, 139], other γ_C cytokines [138, 140] and TCR stimulation [118, 139, 141] down-modulate IL-7Rα expression, which is up-regulated in the absence of its cognate cytokine [138, 139]. IL-7 induces several pro-survival pathways, particularly through the modulation of the expression of Bcl-2 family members [142-144], in addition to promoting cell proliferation, growth and metabolic activity [141, 145-149].

IL-7 signal transduction involves different pathways, as illustrated in Figure 8. Ligation of IL-7 to IL-7Rα induces the hetero-dimerization of IL-7Rα with the γ_C [150], and consequent activation of the receptor-associated Janus kinases (JAK) -1 and -3 [151]. JAK1 and JAK3, respectively associated with the γ_C chain and IL-7Rα, phosphorylate each other and then IL-7Rα, creating docking sites for the signal transducers and activators of transcription (STAT) factors, such as STAT1, -3 and -5 [152-154]. STAT5, the most relevant STAT in IL-7-induced signalling, comprises two isoforms: STAT5a and STAT5b [155], that are phosphorylated by JAK1/3. The STAT5 signalling pathway promotes cell survival through the modulation of Bcl-2 family members, up-regulating the expression of anti-apoptotic proteins Bcl-2 and Bcl-xL and down-regulating the pro-apoptotic proteins Bax and Bad [156-158]. STAT5 signalling also leads to the

inhibition of protein kinase C θ (PKCθ), and subsequently to the down-modulation of the cyclin-dependent kinase inhibitor $p27^{kip1}$, inducing cell cycle entry [159].

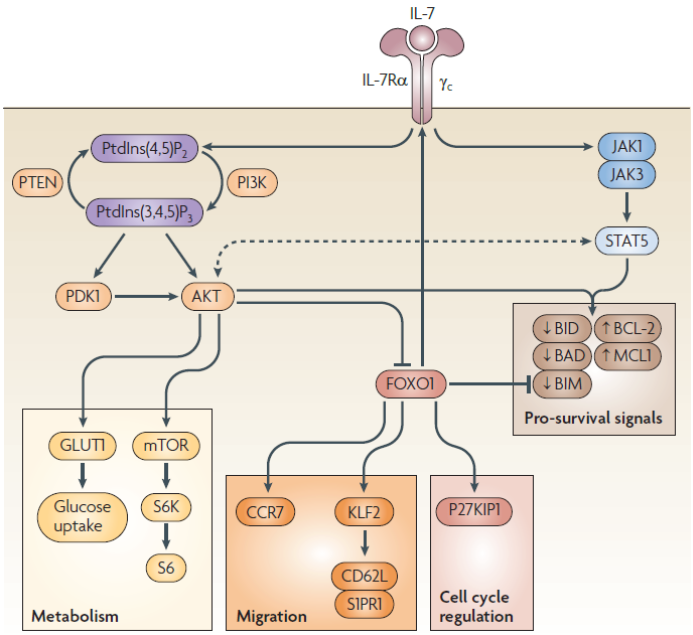

Figure 8. ***IL-7 signal transduction pathways.*** The main IL-7R pathways — through JAK1/JAK3 and then STAT5 and STAT5b or through PI3K and AKT — and some of their key downstream targets involved in naïve T-cell homeostasis are depicted.
Adapted from Takada et al 2009 [9]

The phosphoinositide 3-kinase (PI3K) pathway is central in IL-7 signal transduction, playing a key role in the regulation of cell survival, growth, metabolism and proliferation [160]. The major substrate of PI3K is Akt, a serine/threonine kinase [161]. Activation of PI3K by growth factors or cytokines induces the recruitment of Akt to the plasma membrane, where it becomes fully activated [162]. The substrates of Akt include several molecules that impact on cell survival and proliferation, such as pro- and anti-apoptotic Bcl-2 family members, caspases and forkhead transcription factors [163]. Akt inactivates the fork head transcription factor FOXO3a through phosphorylation, which leads to the down-regulation of the pro-apoptotic protein Bim [164]. The PI3K pathway is also required for the IL-7-induced increase of GLUT1 expression, a key glucose transporter in T-cells, thus promoting glucose uptake and metabolic activity [146, 149].

TREC content within $CD31^+$ naïve $CD4^+$ T-cells was reported to decrease slightly with age [104] and following IL-7 administration in humans [165]. Furthermore, the absolute numbers of $CD31^+$ naïve CD4 T-cells in the elderly are higher than those estimated by the assessment of thymic output through the quantification of TREC levels [43]. Hence the $CD31^+$ naïve CD4 T-cell subset appears to undergo post-thymic

proliferation, which is likely induced by a TCR-independent mechanism, driven by homeostatic cues, such as γ_C cytokines [105]. Results from our Laboratory showed, for the first time, that IL-7 plays a role in the maintenance of CD31$^+$ naïve CD4 T-cells during adult life [106], preferentially driving the proliferation of the CD31$^+$ naïve CD4 T-cells, while sustaining their expression of CD31 [106].

Other authors have shown a preferential ability of RTEs to proliferate in response to IL-7 [75]. RTEs have been shown to proliferate in response to IL-7 in an antigen-independent manner, more efficiently than naïve T-cells from adult peripheral blood [85, 166, 167], which is in agreement with the higher proliferation of naïve CD4 T-cells expressing PTK7 in response to IL-7 [75]. The ability of IL-7 to promote the homeostatic proliferation of RTEs has been proposed to allow the maintenance of the peripheral naïve CD4 T-cell pool whilst preserving a diverse TCR repertoire [166].

CD31 is a trans-membrane glycoprotein from the immunoglobulin super-family which is expressed by a variety of cell types, including endothelial cells, platelets, monocytes, neutrophils and T-cells [168-170]. CD31 has been shown to be required for the trans-endothelial migration of neutrophils and monocytes [171], hence it might play a role on the migration of CD31$^+$ naïve T-cells into SLO [73], a proposed site for homeostatic proliferation of naïve T-cells [172]. Furthermore, CD31 engagement has been shown to inhibit TCR-mediated signal transduction via immune-receptor tyrosine-based inhibitory motifs (ITIMs), present in its cytoplasmic domain [169], raising the hypothesis that CD31 might hamper peripheral proliferation of CD31$^+$ naïve CD4 T-cells upon TCR triggering with self-peptide/MHC complexes [105].

As mentioned above, the term naïve CD4 T-cells derives from the assumption that these are antigen-inexperienced T-cells when they egress from the thymus until they are primed by foreign antigen and differentiate into memory/effector CD4 T-cells. During recent years, a substantial number of studies have highlighted the importance of continuous low-affinity contact with MHC-II peptides for naïve CD4 T-cells, promoting their survival and low-level homeostatic proliferation [121, 122, 173-179].

According to experimental studies, the peptides presented by MHC-II in a non-immunogenic fashion could be derived from self-antigens, related to those displayed in the thymus. In this scenario [174, 175, 178, 179], naïve CD4 T-cell proliferation could be regarded as a peripheral positive selection of $CD4^+$ T-cells, in which their repertoire is shaped/restricted to those cells selected to undergo peripheral post-thymic proliferation, as illustrated in Figure 9. Of note, a role for exogenous antigens cannot be ruled out in these processes.

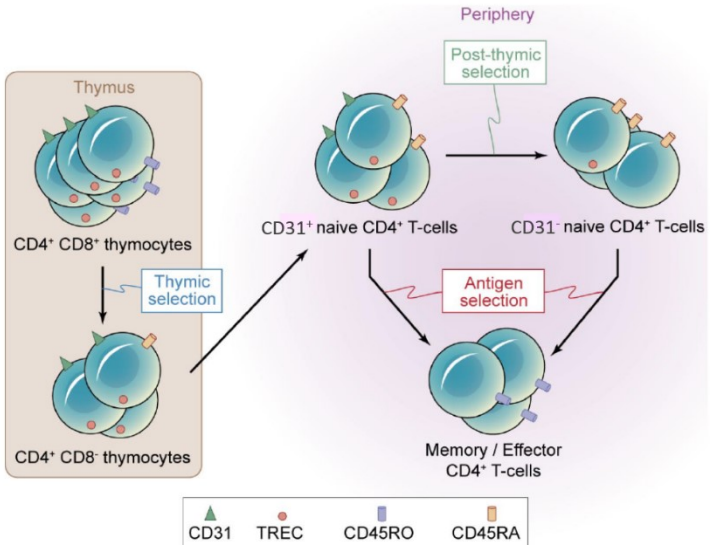

Figure 9. ***Post-thymic proliferation of human naïve CD4 T-cells.*** RTEs emigrate from the thymus and are incorporated into the pool of $CD31^+$ naïve CD4 T-cells, with high TREC content. Some $CD31^+$ naïve CD4 T-cells undergo peripheral postthymic selection; $CD31^-$ naïve CD4 T-cells are induced and maintain the size of the naïve CD4 T-cell pool. On cognate interaction with foreign antigens, both subsets can differentiate directly into memory/effector CD4 T-cells.
Adapted from[105]

The expression of CD31 in both cord blood and adult naïve CD4 T-cells has been shown to be down-regulated upon activation with anti-CD3+IL-2 [180]. However, this overt TCR activation also leads to the differentiation into a $CD45RO^+CD62L^-$ memory phenotype [180]. Thus, it has been proposed that the $CD31^-$ naïve CD4 T-cell subset could result from TCR triggering by low-affinity antigens, thus inducing the loss of CD31 without affecting the overall naïve phenotype [61, 73].

Moreover, $CD31^-$ naïve CD4 T-cells have been shown to express higher levels of the anti-apoptotic BFL1/A1 than their $CD31^+$ counterparts [61]. This marker is specifically induced by TCR, but not cytokines like IL-4, IL-7 and IL-15 stimulation, pointing to an involvement of TCR signalling in the generation and/or maintenance of the $CD31^-$ naïve CD4 subset. These data have raised the possibility that the non-immunogenic signals triggered by self-peptide/MHC complexes, might play a role in the generation and/or maintenance of the $CD31^-$ naïve CD4 T-cell subset [121, 122, 173, 175-178]. As

a result, the proliferation of $CD31^-$ naïve CD4 T-cell subset is thought to cause a contraction of the naïve TCR repertoire [61, 181, 182].

1.1.3.3. Cell survival / death

The maintenance of the naïve T-cell pool is also thought to depend upon survival signals, such as those provided through TCR engagement of self-peptide/MHC complexes and by IL-7 [85, 114, 119, 183, 184]. Naïve T-cells moving along HEV's encounter IL-7, self-peptide/MHC complexes and CCR7 ligands, all of which cooperate to produce homeostatic survival signals [185].

As mentioned above, the binding of IL-7 to its receptor induces several signalling cascades (Figure 8), such as the JAK–STAT and the PI3K pathways, that promote lymphocyte survival [10, 186] through the up-regulation of the anti-apoptotic molecule Bcl-2 [187].

The relevance of cell survival pathways is further supported by the progressive loss of naïve CD4 T-cells in association with defective Bcl-2 expression in patients with MST1 deficiency [188]. An increase in the peripheral survival of naïve CD4 T-cells in the elderly has been predicted by *in silico* models, in agreement with experimental data in mice [189].

1.1.3.4. Cell differentiation into the memory compartment

The dynamics of the naïve T-cell compartment is also constrained by pressure to memory-effector differentiation [8]. It has been suggested that naïve CD4 T-cells may adjust their threshold for TCR activation with the length of time in circulation since thymus export [11, 83, 190] and with the aging of the individual [190-192]. T-cell activation is triggered by a series of steps (Figure 10): an initial interaction between a TCR and its cognate peptide/MHC complex presented by a DC (signal 1), followed by co-stimulatory signals delivered by the DC, such as those provided through CD28 (signal 2).

When they encounter their antigen in SLOs, activated CD4 T-cells proliferate rapidly and differentiate into memory-effector T helper subsets namely Th1, Th2, Th17, Th9, T follicular helper (TFH) cells, depending on the pathogen encountered and initiating stimulation signal. Each cell type is distinct in terms of the type of immune response provided and cytokine produced [12, 13]. Some conventional naïve CD4 T-cells may also up-regulate FoxP3 and differentiate into induced-regulatory T-cells (Tregs) in the periphery [77].

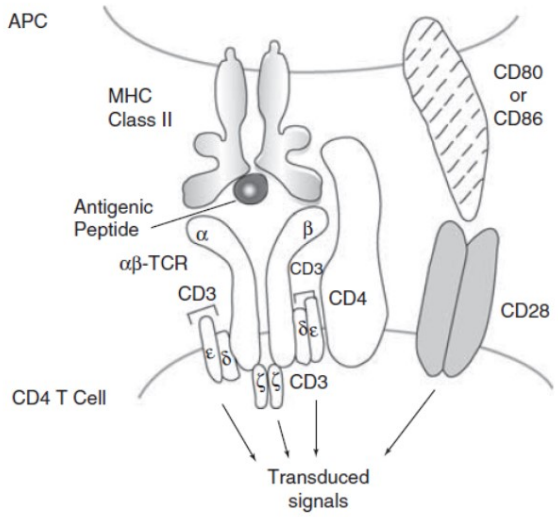

Figure 10. ***CD4 T-cell recognition of antigen and activation.***
Adapted from Lewis 2011 et al [107]

Upon antigen clearance, the expansion of the T-cell population is followed by a contraction phase during which most cells perish through apoptosis, although a proportion of the expanded population is preserved to ensure long-term protection against subsequent antigenic challenge. Around 5-10% of both CD4 and CD8 T-cells persist as memory T-cells which can be broadly subdivided into: central-memory (CM) CD4 or CD8 T-cells that express CCR7 and CD45RO and are mainly present in secondary lymphoid tissues; and effector-memory (EM) CD4 or CD8 T-cells that also express CD45RO but not CCR7 [6, 12].

Memory T-cells constitute a highly heterogeneous population, differing not only in cell surface phenotype, but also in functional ability and history of antigen encounter [4, 6, 193]. Memory T-cells provide more rapid and effective immunity against previously encountered antigens, as they can be activated by lower doses of antigen, and accumulate as well as perform effector functions quicker than their naïve counterparts upon antigen re-exposure [194-196]. Furthermore, the distinct migratory capacity of memory T-cells allows them to enter non-lymphoid tissues, potentially detecting and responding to infection earlier on [4, 6, 197].

1.1.4. The uniqueness of FoxP3$^+$ regulatory T-cells within the naïve compartment

The human naïve CD4 T-cell compartment comprises a population of thymus-generated naïve-like cells expressing FoxP3 (naïve-Tregs) that continuously replenish the pool of fully-suppressive activated Tregs expressing memory markers [42, 77, 198-202].

Tregs maintain tolerance to self and to the environment, and are central players in the control of immune responses [41, 77]. Expression of the transcription factor FoxP3, considered a master regulator of Treg development and function, is essential for their role in the maintenance of dominant tolerance [41].

Both naïve and activated Tregs possess a fully functional *FOXP3* gene, hardly secrete cytokines and potently suppress proliferation [77]. Naïve-Tregs have been considered resting/quiescent population in steady-state conditions in non-lymphopenic individuals, in opposition to the activated memory-Tregs [77]. In addition to thymus-derived Tregs, the peripheral Treg compartment includes a peripheral population converted from conventional CD25$^-$FoxP3$^-$ CD4 T-cell precursors, particularly in the gut mucosa and inflammatory tissue sites, likely via a transforming growth factor beta (TGF-β)-dependent mechanism (peripheral or induced Tregs).

Despite extensive research on Treg development using murine models in the past 20 years, many questions remain unanswered regarding the mechanisms involved in the commitment to the Treg lineage in the thymus [42, 201, 203]. The mechanisms governing naïve-Treg homeostasis, in parallel with age-associated thymic involution, also remain largely unknown [202]. The investigation of naïve-Treg homeostasis has been hampered by the difficulty in clearly identifying this subset in murine models [204].

A distinct homeostasis of the naïve-Treg and conventional naïve (naïve-Tconv) CD4 T-cell compartments have been suggested by a longitudinal study that showed naïve-

Treg preservation up to 1 year post-thymectomy [205], although long-term data are lacking.

In the thymus, increased affinity-TCR engagement is required for initiation of the Treg cell differentiation program and induction of Foxp3 expression [42, 206]. As a consequence, Tregs exported to the periphery exhibit a TCR repertoire skewed towards self-recognition [207, 208]. Naïve-Tregs are thus likely to display high-affinity for self-peptides, and to rapidly differentiate into memory-Tregs upon TCR-stimulation [42, 77, 198-200, 202]. Thus, self-peptide/MHC stimulation is likely to result in loss of their naïve phenotype. Of note, the maintenance of murine naïve-like FoxP3$^+$ CD4 T-cells was recently demonstrated to be dependent of TCR-driven signals [209].

On the other hand, in terms of the contribution of cytokine-driven proliferation, naïve-Tregs express low levels of receptors for the main homeostatic cytokines. CD25, the α-chain of the IL-2 receptor, is expressed at only intermediate/dim levels [77], questioning whether naïve-Tregs, like their memory counterparts, depend on IL-2 [202]. Moreover, Tregs *per se* typically express low levels of IL-7Rα, and there are controversial reports on the IL-7 impact on human and murine Tregs [210-214].

Overall, the mechanisms involved in the homeostasis of human naïve-Tregs remain largely unclear.

1.2 Clinical models to study naïve CD4 T-cell homeostasis

1.2.1. Immune reconstitution settings

Lymphopenic settings, such as the drastic reductions of peripheral T-cell numbers observed following chemotherapy [215], bone-marrow transplantation [94, 97, 216, 217], HIV infection [44, 218, 219], or increasingly used lymphocyte-depleting therapies in autoimmunity [220] provide important clinical scenarios to investigate the mechanisms of homeostatic responses, e.g. cytokine-induced proliferation, to restore the size of the T-cell pool.

As previously detailed, IL-7 is a key player in T-cell homeostasis, and is essential for T-cell development in humans [114, 129-136]. This has provided a rationale for the consideration of IL-7 as a therapeutic agent in a variety of settings, particularly in the improvement of immune reconstitution in lymphopenia and following hematopoietic stem cell transplantation (HSCT) or lymphocyte depletion in the context of autoimmune disease or malignancy [124, 221, 222].

The first clinical trial using recombinant human (rh)IL-7 in humans was performed with lymphopenic patients with non-lymphoid cancer that was refractory to standard therapy [165]. Phase I/II clinical trials performed in lymphopenic cancer patients have reported that rhIL-7 administration induces T-cell survival and cycling *in vivo*, increasing CD4 and CD8 T-cell numbers [165, 223, 224], with preferential expansion of the naïve subsets [165, 223, 224]. Of note, the absolute numbers of $CD31^+$ naïve CD4 T-cells, were increased following rhIL-7 administration, leading to the generation of a diverse TCR repertoire even in older individuals [165, 224]. These effects of rhIL-7 on naïve T-cell numbers and repertoire diversity seem to be due to increased proliferation of RTEs rather than augmented thymic output, since they are age-independent and no thymic enlargement was observed [165]. These observations, however, do not preclude a potential effect of rhIL-7 therapy in thymic output.

The safety and efficacy of IL-7 administration to HIV-1-infected subjects have been evaluated in a phase I/IIa trial, in which rhIL-7 was administrated to HIV-1 infected patients presenting persistently low CD4 T-cell counts, despite virological suppression, under combination antiretroviral therapy (ART-Discordants) [225]. IL-7 therapy was associated with a significant increase in circulating naïve and memory CD4 and CD8 T-cells, while maintaining their functional profile and cytokine production in response to HIV antigen [225]. In addition, in a randomized placebo-controlled dose escalation trial of 3 weekly doses of rhIL-7 in ART-treated HIV-infected patients, with low CD4 T-cell counts and undetectable plasma HIV levels, CD4 increases of predominantly naïve and central-memory T-cells were brisk and lasted up to 1 year [226]. Of note, IL-7 seemed to increase thymic output and tended to improve the TCR repertoire in patients with low TCR diversity [226]. Thus, these initial human trials suggested that IL-7 therapy may improve T-cell recovery and function in the context of ART-treated HIV-1 infection. However, recently it has been debated the putative inefficacy of IL-7 in purging the HIV reservoirs during ART, as it was shown to enhance residual levels of viral production and induce proliferation of latently infected cells. These data question the possible role of IL-7 as a suitable therapeutic strategy for HIV eradication [227]

Systemic administration of rhL-7 to bone-marrow transplant recipients has also been shown to increase thymopoiesis and improve immune function following bone-marrow transplantation [228-231]. The increase in naïve CD4 T-cell compartment size and TCR diversity was reported in a phase I trial of rhIL-7 in recipients of T-cell depleted allo-HSCTs, which was considered very promising, particularly in younger patients [232, 233]. IL-7Rα expression was shown to be down-regulated during continued IL-7 administration, which might constitute a negative feed-back loop that hinders uncontrolled T-cell expansion thus preventing the development of lymphoproliferative disorders in response to IL-7 [165, 221].

1.2.2. Thymectomy as a privileged model to study naïve CD4 homeostasis

The importance of thymus in immunity was first recognized in murine models. It was observed that neonatal thymectomy resulted in severe immunodeficiency mainly affecting cell-mediated immune responses [234]. Importantly, in rodents, as thymic maturity at birth differs across species, the impact of thymectomy is also heterogeneous. In some strains, neonatal thymectomy causes severe immunodeficiency [234, 235], whereas thymectomy at 2-4 days after birth leads to autoimmune disease, associated with a decrease in regulatory T-cell number [236, 237].

Children born with congenital cardiac defects frequently require corrective cardiac surgery in infancy. In order to increase exposure of the surgical field and reduce the risk of bleeding, surgeons frequently need to perform partial or complete thymectomy during those procedures. The value of these thymectomized individuals as a model to study the homeostasis of T-cells has been long recognized [238].

Several groups have analysed the impact of thymectomy on T-cell subpopulations in thymectomized individuals [76, 238-250]**.** A decrease in CD4 and CD8 T-cells was reported as compared to healthy subjects [238, 239, 242, 245], mainly affecting the naïve T-cell pool [238-240, 242, 245-247].

Of note, few studies have addressed specifically the long-term impact of thymectomy in the homeostasis of the naïve CD4 T-cell compartment and their results are highly heterogeneous [76, 243-245, 247]. Recently, there were reports on the restoration of T-cell compartment, including naïve CD4 T-cell counts, beyond 5 years post-cardiac surgery upon complete thymectomy performed in the first 4 months of life [76, 243]. This was found in more than 75% of the thymectomized individuals and was accompanied by a restoration of TREC levels and of the size of the $CD31^+$ naïve CD4 T-cell compartment [76, 243].

The recovery of the naïve CD4 T-cell compartment was attributed to thymic tissue regeneration, which is thought to be more effective when thymectomy is performed in younger ages [76, 243]. In these studies, the longitudinal data are limited to few patients, namely 3 individuals, in whom naïve CD4 T-cell counts and TREC levels do not support the restoration of the naïve compartment at an individual level [243].

Most studies in the literature do not provide information on the extent of the thymectomy performed [239, 245, 248, 249], or exclude the presence of residual thymus [239, 245, 249]. Given the fact that younger individuals are more likely to undergo incomplete thymectomy [245, 250], the interpretation of the heterogeneous results on the impact of thymectomy in the naïve CD4 T-cell compartment, would benefit from a strict (sequential) quantification of the remaining thymic activity. Of note, the existence of ectopic thymus is very frequent in humans [251].

The investigation of putative compensatory mechanisms on thymectomized individuals has revealed an increase in the proliferation of naïve CD4 T-cells in the patients with contracted naïve CD4 T-cell subset [242]. This was associated with shorter telomeres in the total CD4 T-cell compartment 18 years post-thymectomy, although this decrease was not significant, as compared to age-matched controls [247]. Moreover, higher turnover of naïve CD4 T-cells was found to be inversely correlated with the size of the $CD31^+$ pool [245]. Gent et al, reported a transient increase in the frequency of Ki-67^+ cells within the naïve CD4 T-cells, that was no longer detectable 5 years post-thymectomy and restoration of the naïve CD4 T-cell compartment size [243].

Importantly, no differences were reported between thymectomized and controls in the levels of STAT5 phosphorylation within CD8 and CD4 T-cells upon stimulation with rhIL-2 or rhIL-7, indicating that responsiveness to homeostatic cytokines such as IL-2 or IL-7 is not impaired following thymectomy [245].

Despite their young age, a subgroup of adults submitted to thymectomy in infancy presented particularly marked alterations in the T-cell compartment, similar to those usually found in individuals older than 75 years [245]. They featured marked loss of

naïve CD4 and CD8 T-cells, with accumulation of oligoclonal memory cells and high plasma levels of the pro-inflammatory cytokines IL-1β, IL-8, and eotaxin [245]. Notably, the TCR spectratyping profile of sorted CD4 and CD8 T-cells from these individuals revealed modest impact on the CD4 repertoire, and marked reduction of diversity of CD8 T-cells [245, 247].

It has also been reported that CMV infection may have an important role in the heterogeneity of alterations observed upon thymectomy [245]. Thymectomy or CMV seropositivity alone appeared to have *per s*e an effect on the contraction of the naïve T-cell compartment, although this was less marked than both combined [245].

The profound imbalance between naïve and memory T-cell frequencies, loss of T-cell repertoire diversity and evidence of inflammation, are typically associated with immune-senescence and are reminiscent of the immune risk phenotype, defined by gerontologists as a cluster of immune parameters that are predictive of early all-cause mortality in the elderly [252, 253].

The long-term impact of thymectomy remain debatable. Concerns about the consequences of early thymectomy for immune function in later life have been rising, as corrective cardiac surgery in infancy has become routinely practised for the last 3 to 4 decades [247]. The vast majority of the short- or long-term studies did not show an increase in the infectious risk [239, 248, 254, 255]. Of note, one research group reported partial immunodeficiency in humans after thymectomy [238], and another study has shown increased frequency of infections, without increased severity [242]. In a recently reported 18 years' follow-up study upon thymectomy below 6 months of age, the authors present clinical data on 11 patients showing increased frequency of recurrent respiratory infections and severe infections requiring admission, although the thymectomized individuals are not compared with a prospective/longitudinal cohort of controls, which may alter data interpretation [247].

The risk of allergy/autoimmunity is also a concern in these patients, given the central role of thymic-derived regulatory T-cells in the prevention/control of both. The impact of thymectomy on the pool of regulatory T-cells has been scarcely investigated,

although data available point to the maintenance of the proportion of circulating regulatory T-cells, although with decreased cell counts [240, 245, 247, 254]. In the studies with clinical information available, no increase in clinical manifestations of autoimmunity [239, 240, 242, 247, 248] or increased levels of autoantibodies [239, 240, 242, 249] have been found.

Importantly, most individuals included in these studies were children and adults below the 4th decade of life. Immunological and clinical check-ups of thymectomized patients may therefore be advised to monitor the development of an immune risk phenotype and potentially related clinical manifestations. Premature immune-senescence after thymectomy may be of clinical relevance as the patients get older, possibly increasing the incidence of diseases of the elderly, such as infectious complications, reduced response to vaccines, atherosclerosis, neurodegenerative disorders and malignancies. Thymectomy is, thus, expected to have an impact on immunity later in life, which could be particularly critical in case the patients developed a lymphopenic condition that required reconstitution of the immune function [256].

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2. AIM AND WORK PLAN

Aim and Work Plan

CD4 T-cells play a central role in the complex puzzle of the human immune system, being at the intersection of innate and adaptive immune responses. The naïve CD4 T-cell compartment can be considered the lifelong reservoir of cells with a diverse repertoire, maintaining ability to mount specific responses to new antigens and providing continuous replenishment of the memory-effector compartments. Moreover, the diversity of naïve regulatory ($FoxP3^+$) CD4 T-cells is thought to be crucial to ensure the preservation of tolerance to self.

Naïve CD4 T-cells are generated in the thymus and significantly expand in the periphery to establish the naïve CD4 T-cell compartment. They are maintained throughout life via a combination of thymic output and peripheral expansion/survival, as well as death/differentiation into the memory-effector compartment. The peripheral expansion/survival is thought to be mediated by both homeostatic cytokines, particularly IL-7, and low affinity self-peptide/MHC interactions with TCR, which are likely to have distinct impacts on cell diversity/function.

The main objective of this thesis is to investigate the interplay of these different mechanisms operating in the homeostasis of the naïve CD4 T-cell compartment.

For this purpose, a cohort of adults thymectomized in early infancy was organized. Patients were strictly stratified according to degree of residual thymic activity, based on surgery report on the degree of thymectomy performed and estimative of thymic output at the time of our evaluation. Two groups were generated, based on evidence of complete absence of thymic activity or not, and compared with age-matched healthy subjects.

The following specific questions were addressed:

1) How is the naïve CD4 T-cell compartment maintained in the absence of thymic output?

We asked whether partial/total thymectomy would be associated with distinct long-term impacts on size, quality or TCR diversity of the naïve CD4 T-cell compartment. We hypothesized that in the absence of thymic output, thymectomized individuals would adjust their threshold for TCR activation of naïve CD4 T-cells and subsequent differentiation into memory phenotype. Moreover, we investigated the possibility that thymectomy could also be associated with an altered response of the naïve CD4 T-cell compartment to IL-7, in terms of proliferation and/or survival. These results are included in chapter 3.1

2) How is the subset of naïve regulatory (Foxp3+) T-cells maintained?

Research on human regulatory T-cell homeostasis has been particularly scarce, despite its lifelong relevance in the maintenance of tolerance and therapeutic potential. Our first aim was to compare the ability of individuals submitted to complete thymectomy to preserve naïve regulatory *versus* naïve conventional CD4 T-cell subsets, in order to evaluate the requirement of thymic output to the long-term maintenance of naïve regulatory T-cells. We next investigated the impact of homeostatic cytokines, IL-7 and IL-2, on naïve-Treg compartment. These results are included in chapter 3.2

3) Is the complete thymectomy performed early in infancy associated with an increase in clinical and subclinical markers of autoimmunity and allergy?

We hypothesized that in the absence of thymic output, the contraction of naïve T-cell repertoire and the pressure to differentiate into memory phenotype would favour the emergence of autoreactive clones, leading to increased/dysregulated production of autoantibodies. An extensive evaluation of the reactivity to self and/or to innocuous allergens in patients submitted to complete thymectomy aimed to reveal a possible tendency to autoimmunity and/or allergy, and evaluate the central role of thymus in the establishment and lifelong maintenance of tolerance. These results are included in chapter 3.3.

In agreement with the *Decreto-Lei 388/70, art. 8º, parágrafo 2*, the results presented here were published or are currently submitted for publication:

Susana L. Silva, Adriana S. Albuquerque, Paula Matoso, Rémi Cheynier, Bénédicte Charmeteau de-Muylder, Dário Ligeiro, Miguel Abecasis, Rui Anjos, João T. Barata, Rui M. M. Victorino, and Ana E. Sousa

IL-7-induced proliferation of human naïve CD4 T-cells requires maintenance of thymic activity

Manuscript under submission.

Susana L. Silva[*], Adriana S. Albuquerque[*], Ana Serra-Caetano, Russell B. Foxall, Ana R. Pires, Paula Matoso, Susana M. Fernandes, João Ferreira, Rémi Cheynier, Rui M. M. Victorino, Iris Caramalho, João T. Barata, and Ana E. Sousa

[*]SLS and ASA contributed equally to this study

Human naïve regulatory T-cells feature high steady-state turnover and are maintained by IL-7

Oncotarget 2016;15:12163-75 (see facsimile in Annexes)

Susana L. Silva, Adriana Albuquerque, Andreia J. Amaral, Quan-Zhen Li, Catarina Mota, Rémi Cheynier, Rui M. M. Victorino, M. Conceição Pereira-Santos, and Ana E. Sousa

Autoimmunity and allergy in adults submitted to complete thymectomy early in infancy

Manuscript under submission.

3. RESULTS

3.1 IL-7-induced proliferation of human naïve CD4 T-cells requires maintenance of thymic activity

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Keywords

Naïve CD4 T-cells, T-cell Homeostasis, IL-7, Thymus, Thymectomy

Manuscript under submission.

3.1.1. Abstract

Naïve CD4 T-cell maintenance is critical for immune competence. We investigated here the fine-tuning of homeostatic mechanisms of the naïve compartment to counteract the loss of *de novo* CD4 T-cell generation. Adults thymectomized in early childhood during corrective cardiac surgery were grouped based on presence or absence of thymopoiesis and compared with age-matched controls. We found that the preservation of the $CD31^-$ subset was independent of the thymus and that its size is tightly controlled by peripheral mechanisms, including prolonged cell survival as attested by Bcl-2 levels. Conversely, a significant contraction of the $CD31^+$ naïve subset was observed in the absence of thymic activity. This was associated with impaired PI3K-dependent responses of purified naïve CD4 T-cells to IL-7, namely *in vitro* proliferation and up-regulation of CD31 expression, which likely potentiated the decline in recent thymic emigrants. Additionally, we found no apparent constraint in the differentiation of naïve cells into the memory compartment in individuals completely lacking thymic activity despite upregulation of *DUSP6*, a phosphatase associated with increased TCR threshold. Of note, thymectomized individuals featuring some degree of thymopoiesis were able to preserve the size and diversity of the naïve CD4 compartment, further arguing against complete thymectomy in infancy. Overall, our data suggest that robust peripheral mechanisms ensure the homeostasis of $CD31^-$ naïve CD4 pool and point to the requirement of continuous thymic activity to the maintenance of IL-7–driven homeostatic proliferation of $CD31^+$ naïve CD4 T-cells, which is essential to secure T-cell diversity throughout life.

3.1.2. Introduction

Long-term preservation of the naïve CD4 T-cell pool is vital to ensure immunity to foreign antigens and to maintain peripheral tolerance (1, 2). Naïve CD4 T-cells are preserved throughout life due to a dynamic balance between thymic generation, and peripheral proliferation, survival, death, or differentiation into memory/effector cells (1, 3).

The thymus is known to be functional up to the sixth decade of life, even though an age-dependent decline in thymic activity occurs (4). Thymic output can be estimated through the quantification of TCR rearrangement circles (TRECs), which are excision by-products generated during T-cell development in the thymus (4). These episomal DNA fragments are progressively diluted with cell division in the periphery (5), being thus enriched in recent thymic emigrant cells (RTEs). Aging is associated with a progressive reduction in TREC levels (4). This decline is much more striking than the one observed in naïve CD4 T-cell counts, indicating that the loss of cell replenishment due to thymic involution is complemented by peripheral dilution due to cell proliferation (4, 6-8). In fact, in contrast to mice, the establishment and maintenance of the human naïve CD4 T-cell compartment is currently thought to significantly rely on post-thymic T-cell proliferation (3, 9, 10). Several *in silico* studies suggest that thymic output *per se* is insufficient to guarantee the size of the peripheral naïve T-cell compartment without a major contribution of cell proliferation in the periphery (3, 9, 10). This homeostatic proliferation is driven by self-peptide/MHC interaction and/or cytokines, namely IL-7 (9, 11, 12).

IL-7 is essential for thymopoiesis and plays a key role in peripheral naïve T-cell survival through the induction of Bcl-2 (11, 13). In addition, IL-7 induces low-level naïve T-cell proliferation (11, 13), which is particularly important in lymphopenic clinical settings (11, 13). In steady-state conditions, this homeostatic proliferation within the naïve CD4 T-cell compartment is mainly restricted to the subset expressing CD31 (Platelet endothelial cell adhesion molecule, PECAM-1) (14), a population that includes the RTEs and is thought to have a broadly diverse TCR repertoire (12). We have also shown that IL-7 increases the levels of expression of CD31 in this subset (14). The biological

significance of CD31 expression is still debatable, though it has been suggested that it may limit TCR-mediated naïve CD4 T-cell responses through inhibitory signalling ascribed to its cytoplasmic immune-receptor tyrosine-base inhibitory motifs (ITIMS) (15). In agreement, the homeostatic proliferation of $CD31^-$ naïve CD4 T-cells is thought to be mainly mediated by low-affinity self-peptide/MHC interactions (16). Of note, CD31 expression is lost after TCR stimulation of naïve CD4 T-cells (2, 17-19).

There are few studies on human naïve CD4 T-cell homeostasis, and the interplay between peripheral mechanisms and the age-associated decline in thymic output remains unclear (3, 9, 10). Adults thymectomized early in infancy due to corrective cardiac surgery provide a unique setting to address this issue (20-27). Using this clinical model, we show here that thymic activity is required to ensure IL-7-mediated peripheral homeostatic proliferation, whereas the homeostasis of the $CD31^-$ compartment is preserved in the absence of thymic activity.

3.1.3. Material and Methods

Study design

Blood was collected from 22 adult patients submitted to thymectomy during corrective cardiac surgery in early childhood, and 20 age-matched healthy controls. All subjects gave written informed consent for blood sampling and processing. The study was approved by the Ethical Boards of Faculdade de Medicina da Universidade de Lisboa, Centro Hospitalar Lisboa Norte, and Hospital de Santa Cruz, Portugal.

Cell isolation and cell culture

PBMC (Peripheral blood mononuclear cells) were isolated from freshly-collected heparinized blood via Ficoll-Paque PLUS (GE Healthcare, Uppsala, Sweden). Naïve CD4 T-cells were subsequently purified by negative selection (purity >96%, StemCell Technologies, Grenoble, France). Purified naïve CD4 T-cells were cultured at $1x10^6$ cells/ml with either IL-7 (10ng/ml; R&D Systems, Minneapolis, MN) or IL-2 (20IU/ml; NIH/AIDS Research and Reference Program, Division of AIDS, NIAID, Hoffman-La Roche), for up to 13 days (d), with media replacement at d3 and d7, as we have previously described (14, 28).

Flow-cytometry

Ex vivo phenotypic analysis was performed in freshly-collected whole blood using an eight-colour staining protocol and a panel of monoclonal antibodies previously described (28). Purified naïve CD4 T-cells were surface stained *ex vivo* and upon culture, followed by intracellular staining using eBioscience FoxP3 kit (eBioscience, San Diego, CA), as described (28). At least 150,000 events were acquired for each sample on a BD LSRFortessa (BD Biosciences, San Jose, CA). Data were analysed using FlowJo software (TreeStar, Ashland, OR) after doublet exclusion. Results are presented as proportion of a cell population or as mean fluorescence intensity (MFI) of a given marker within the specified population.

TCR threshold

Purified untouched naïve CD4 T-cells were cultured at $1x10^6$ cells/ml (25000 cells/well) and stimulated with increasing concentrations of beads coated with anti-

CD3 and anti-CD28 monoclonal antibodies (Dynabeads, Thermo Fischer Scientific). In agreement with a previously reported protocol (29), antibody against CD40L (clone 89-76, BD Bioscience) was added to the culture media to improve the staining. After 6h, cells were collected and surface stained, as described (28), for the marker of cell differentiation (CD45RO) and the additional activation marker (CD69).

TREC quantification

Signal joint (sj) and DβJβ TREC analyses were conducted as described (5, 30). Briefly, multiplex PCR amplification for sjTREC, DJβ1TRECs (Dβ1-Jβ1.1 to 1.6) or DJβ2TRECs (Dβ2-Jβ2.1 to 2.7), together with the CD3γ chain was performed in triplicate on lysed PBMC. TREC and CD3γ quantifications were then performed using a LightCycler™ in independent experiments, with the same first-round serial dilution standard curve. This highly sensitive nested quantitative PCR assay allowed detection of one copy in 10^5 cells for any excision circle. The sj/βTREC ratio, (sjTREC/10^5cells/(DJβ1TRECs/10^5cells+DJβ2TRECs/10^5cells)), was calculated as described (30).

mRNA quantification

Total RNA was extracted from purified naïve CD4 T-cells using Quick-RNA MicroPrep (Zymo Research Corporation, Irvine, USA). cDNA was synthesized from 50ng of RNA (SuperScript III Reverse Transcriptase, Thermo Fischer Scientific), and used to quantify the expression levels of *KLF2, FOXP1, P21, BIM, DUSP4,* and *DUSP6* in duplicates, using TaqMan Gene Expression Assays on a ViiA7 Sequence Detection system (both from Thermo Fischer Scientific). Results are expressed as ΔCT normalized to the medium CT levels of *GAPDH* and *HPRT*.

TCR Spectratyping analysis

Total RNA was extracted from 10^5 to 10^6 cells with RNeasy kit (Qiagen, MD, USA), and first strand cDNA synthesized from 1-2μg of RNA (SuperScript III) using an equivolume mixture of random hexamers and oligo (dT). Amplification of the TCRVβ CDR3 was performed using primers specific for each TRBV family, and a common TRCB reverse primer (31); followed by a run-off reaction that extends each different PCR product with a second TRCB FAM labeled primer; and a third step, in which each different

fluorescent TRBV-TRBC PCR fragment was separated using a capillary electrophoresis based DNA automated sequencer. Data were collected and analyzed with GeneMapper v4.0 (Thermo Fischer Scientific) for size and fluorescence intensity determination.

Statistical analysis

Statistical analysis was performed with Graph Prism Version 5.01 (GraphPad Software, San Diego, CA). The following tests were used for analyzing epidemiological data and results from *ex vivo* studies as appropriate: Wilcoxon-Signed Rank/paired T-test for pairwise comparisons; unpaired T-test/Mann-Whitney for unpaired comparisons; for Gaussian and non-Gaussian distribution respectively. Cultures were analyzed using one-way ANOVA (Kruskal-Wallis Test). Results were expressed as median (interquartile range or range when n<4). *P*-values <0.05 were considered significant.

3.1.4. Results

Evidence of thymus activity in adults thymectomized during the first year of life

We studied a cohort of 22 adults submitted to thymectomy in early childhood during corrective cardiac surgery, and 20 age-matched healthy controls (Table 1 and Supplementary Table 1). Of note, the thymic function is relatively stable in healthy individuals during the age-period spanned (4). The thymectomized patients were stratified into two groups according to evidence of residual thymic activity (Table 1 and Supplementary Table 1). No thymic activity (∅Thy) strictly refers to cases with surgical reports of complete thymus removal and levels of sjTRECs clearly below the lower level found in controls (P<0.0001, Figure 1A), as we previously reported (28).

Table 1. ***Clinical-epidemiological characteristics of cohorts***

	healthy	**Thy[a]**	**ØThy[a]**
Number (male/female)	20 (8/12)	14 (6/8)	7 (5/3)
Age, years	22 [18-29]	25 [18-30]	23 [20-27]
Age at thymectomy, months	NA	8 [1-60]	21 [12-72]
Total lymphocytes/μl	2408 [1430-3502]	2219 [1230-3400]	2005 [934-2618]*
% T-cells (CD3⁺)	72.7 [50.2-79.0]	70.5 [57.3-82.7]	66.6 [42.7-71.2]*;#
% CD4 T-cells	39.9 [31.2-60.0]	41.0 [33.8-55.3]	42.7 [20.20-46.4]
Serum IL-7[b], pg/ml	15.0 [6.5-23.3]	12.8 [5.3-16.2]*	14.8 [8.3-19.8]

NA indicates not applicable. Results are shown as median and range in brackets; *, ** *P*-value <0.05 and 0.01, respectively, in comparison with healthy; #, ##, *P*-value <0.05 and 0.01, respectively, in comparison with Thy. [a] Thymectomy was performed during reconstructive cardiac surgery to facilitate surgical access to the heart and great vessels; patients with syndromatic cardiopathy were excluded (e.g. Trisomy 21, Velocardiofacial or DiGeorge Syndrome); individuals were not treated with drugs known to influence the immune system; ØThy - No thymic activity based on surgical reports of complete thymus removal and levels of sjTRECs clearly below the lower level found in controls; Thy - some degree of thymic activity attested by sjTREC levels within the range of age-matched controls. [b] Serum IL-7 levels were quantified using Human IL-7 Quantikine HS ELISA kit (R&D Systems).

Individuals with some degree of thymic activity (Thy) featured sjTREC levels within the range of age-matched controls, though significantly lower (P=0.0061, Figure 1A).

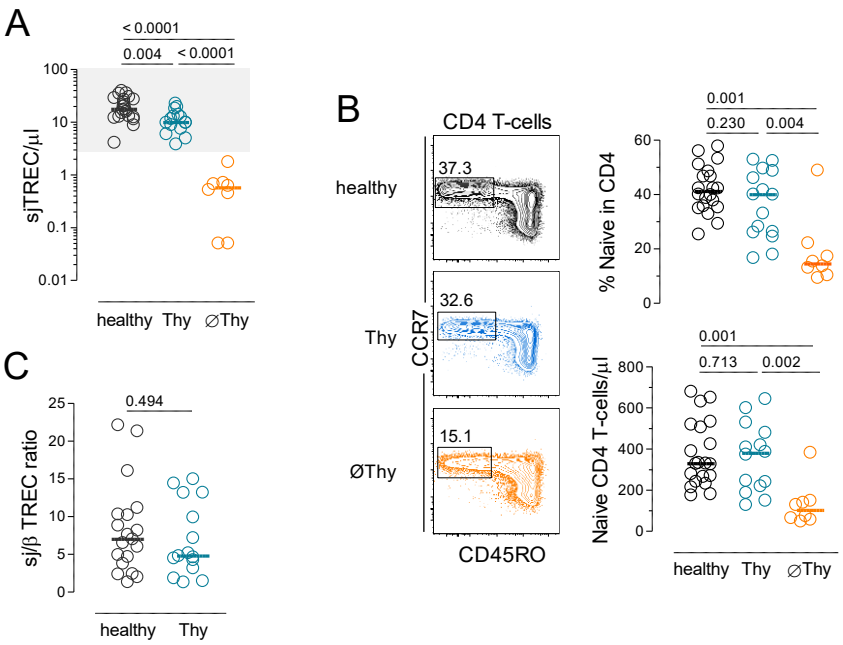

Figure 1. ***Naïve CD4 T-cell compartment in adults thymectomized early in life.***
(A) Quantification of sjTREC levels in PBMCs from thymectomized patients without (∅Thy), or with evidence of thymic activity (Thy), and in age-matched healthy individuals. **(B)** Contour plots illustrating CD45RO and CCR7 analysis within circulating CD4 T-cells of representative individuals from the 3 cohorts; graphs show naïve CD4 T-cell frequency (top) and counts (bottom). **(C)** sj/β TREC ratio quantified in PBMCs from Thy and healthy individuals. Each dot represents one individual, bars represent median, and *P*-values are shown.

Interestingly, these two groups showed almost no overlap between age at thymectomy, which was performed during the first year of life in all Thy cases except two, and later on in all ∅Thy patients (Supplementary Table 1). A previous study also found an association between preservation of thymic activity and younger age at thymectomy, which was attributed to loss of thymus regenerative capacity in children older than eighteen months-old (24, 32). It is also noteworthy that complete thymic tissue removal is more likely to occur after the first year of life due to the surgical procedures required for the type of cardiac defects (Supplementary Table 1), as well as due to age-related anatomic specificities (21) Of note, individuals with syndromatic cardiac defects were not included (Supplementary Table 1).

In agreement with complete lack of thymic activity, the ∅Thy group featured decreases in naïve CD4 T-cell frequency and absolute numbers, that were statistically significant not only in comparison with healthy individuals (*P*=0.0012 and *P*=0.0006 respectively, Figure 1B), but also with Thy (*P*=0.0041 and *P*=0.0019 respectively, Figure 1B). These cells featured a truly naïve phenotype based on an extensive panel of naïve markers and lack of expression of molecules associated with a memory phenotype, as we have previously reported (28).

On the other hand, Thy patients showed no reduction in lymphocyte counts (Table 1), and maintained the naïve CD4 T-cell compartment (Figure 1B). In order to estimate their effective thymic output, we quantified the sj/βTREC ratio, which reflects the number of proliferation cycles undergone by precursor T-cells during their intra-thymic differentiation, and directly correlates with thymic activity (30). We observed similar levels of sj/βTREC ratio in Thy and age-matched healthy individuals (Figure 1C). Together with close to normal sjTREC contents, this observation supports a major contribution of thymic recovery to the maintenance of the size of naïve CD4 T-cell compartment upon partial thymectomy.

We further assessed the impact of the degree of thymectomy on the structural diversity of naïve CD4 T-cells by spectratyping analysis of their TCR repertoire. The distribution of the CDR3 lengths within each different Vβ family is considered to reflect the overall sequence diversity (33). A diverse polyclonal TCR repertoire is associated with a Gaussian distribution of CDR3 lengths, whereas skewed TCR repertoires feature a reduced number of peaks. A relatively preserved TCR diversity was observed in Thy patients as compared to age-matched controls *(P*=0.2620), supporting that their degree of thymic activity was sufficient to ensure the preservation of the quality of the naïve CD4 T-cell compartment (Figure 2A and 2B). Patients with no thymic activity exhibited higher numbers of non-polyclonal Gaussian families than both Thy (*P*<0.0001) and healthy individuals *(P*=0.0001), as shown in Figure 2.

Of note, these differences between the two thymectomized cohorts could not be attributed to a distinct prevalence of CMV infection, since a similar proportion of individuals with IgG seropositivity against CMV was observed in ∅Thy and Thy cohorts

(Supplementary Table 1). Moreover, the contraction of the naïve CD4 T-cell compartment in ∅Thy individuals was not restricted to those CMV+, as previously reported (21).

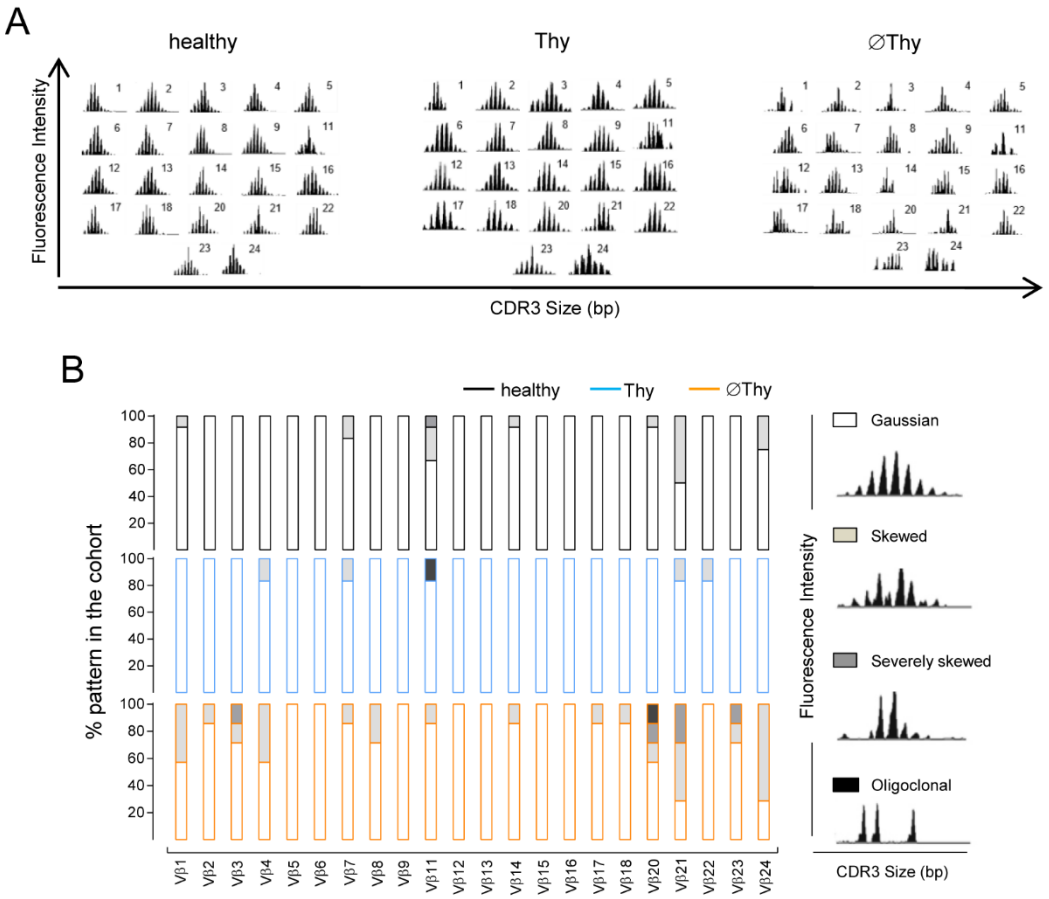

Figure 2. ***Spectratyping analysis of naïve CD4 T-cell diversity in adults thymectomized early in life.*** CD3 length distribution within each of the 22 Vβ families of purified naïve CD4 T-cells analyzed by spectratyping: **(A)** Illustrative examples from thymectomized patients without (right, ∅Thy1 in Supplemental Table 1), and with evidence of thymic activity (middle, Thy6 in Supplemental Table 1), as well as age-matched healthy (left) individuals; **(B)** Proportion of the illustrated patterns within each Vβ family in the three cohorts (∅Thy, bottom, n=7; Thy, middle, n=6; healthy, top, n=12).

Overall, peripheral homeostatic mechanisms were unable to prevent the contraction of the naïve CD4 T-cell compartment upon complete thymus removal in infancy, whereas the maintenance of some degree of thymic activity allowed preservation of naïve CD4 T-cells with a diverse TCR repertoire into adulthood.

Lack of thymic activity does not associate with increased threshold for TCR-activation of naïve CD4 T-cells

The maintenance of naïve T-cells is also determined by the rate of their differentiation into memory-effector cells. Of note, both central- and effector-memory CD4 T-cell counts in individuals lacking thymic activity were found to be similar to those in age-matched healthy controls (Figure 3A). Therefore, we assessed the expression of a panel of genes known to be involved in the regulation of cell quiescence and/or of the threshold for TCR-mediated cell activation in purified naïve CD4 T-cells from thymectomized and healthy individuals (Figure 3B). No alterations were found in the expression levels of the following genes: krüppel-like factor 2 (*KLF-2*) (34); the transcription factor *FOXP1* (35); *CDKN1A* (encoding the cyclin-dependent kinase inhibitor $p21^{cip1/waf}$) (36); the pro-apoptotic Bcl-2 family member *BIM* (37); and the dual-specificity protein phosphatase *DUSP4* (38). However, individuals with no thymic activity featured significantly higher *DUSP6* transcript levels than controls, an increase not observed in those with some preservation of thymopoiesis (Figure 3B).

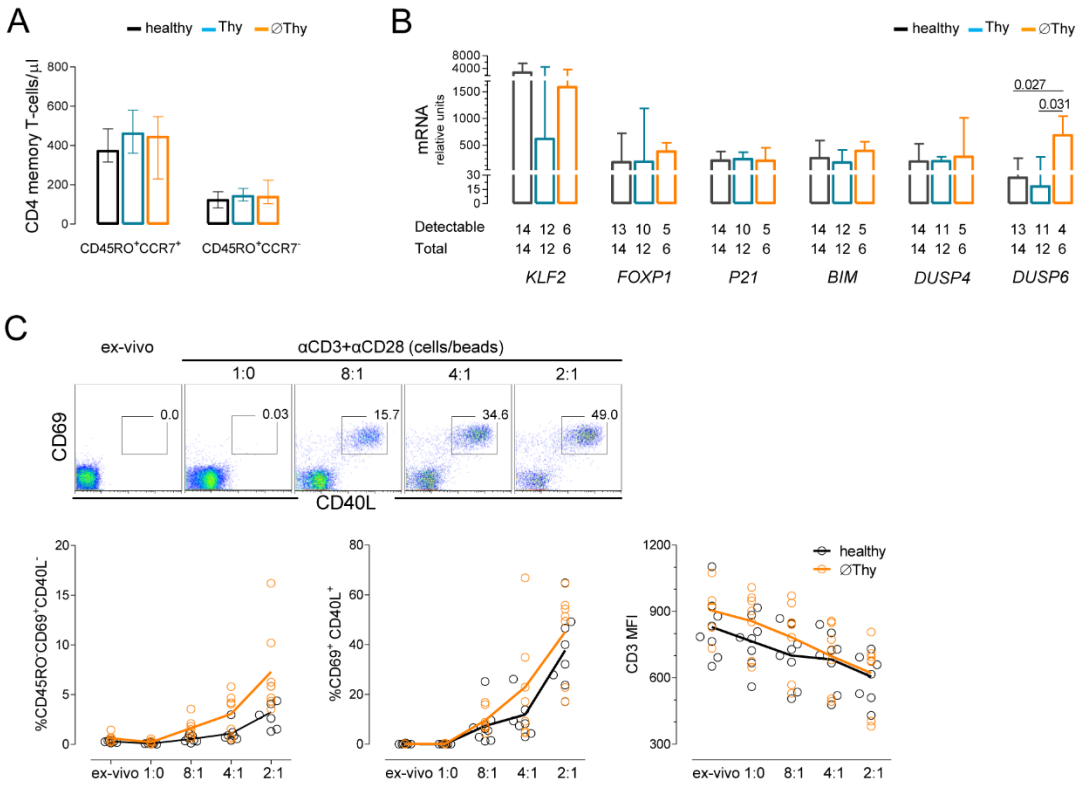

Figure 3. ***Maintenance of naïve CD4 T-cell quiescence upon thymectomy.*** **(A)** Absolute numbers of circulating central-memory ($CD45RO^+CCR7^+$), and effector-memory ($CD45RO^+CCR7^-$) cells in thymectomized patients without (∅Thy), and with evidence of thymic activity (Thy), as well as age-

matched healthy individuals. **(B)** mRNA expression levels of genes involved in cell cycle or maintenance of naïve phenotype quantified in purified naïve CD4 T-cells from the three cohorts; ΔCT normalized to the medium CT levels of *GAPDH* and *HPRT* are shown; numbers below the graph indicate the total number of samples tested and those with levels above the detection threshold of the respective gene. **(C)** Purified naïve CD4 T-cells from ∅Thy and healthy individuals were stimulated (6h) with increasing concentrations of beads coated with anti-CD3 and anti-CD28 monoclonal antibodies with dot-plots illustrating the up-regulation of the activation markers CD40L and CD69 in one ∅Thy subject, and graphs showing frequencies of $CD69^{+}CD40L^{-}$ cells (left), $CD69^{+}CD40L^{+}$ cells (middle), and CD3 MFI (right); each dot represents one individual, lines connect means, and the two cohorts were compared with 2-way ANOVA. Bars represent median and interquartile range. $P<0.05$ are shown.

DUSP6 is highly specific for ERKs, leading to reduction of ERK activity that is critical for efficient TCR-signalling (38, 39). Therefore, high *DUSP6* levels might be associated with an increase in the threshold for TCR-induced activation in ∅Thy. To test this possibility, we performed a dose-response TCR stimulation of purified naïve CD4 T-cells, and quantified the up-regulation of the activation markers CD40L and CD69, in parallel with the down-regulation of CD3 expression and induction of the memory marker CD45RO. Contrarily to our expectation, individuals completely lacking thymic activity responded to TCR stimulation as efficiently as healthy subjects (Figure 3C).

In conclusion, we found no apparent constraint in the differentiation of naïve CD4 T-cells into the memory compartment in individuals completely lacking thymic activity.

Preservation of the $CD31^{-}$ compartment of CD4 naïve T-cells in the absence of thymic activity

Next, we investigated the contribution of peripheral cell-survival and cell-cycling to the homeostasis of the $CD31^{+}$ and $CD31^{-}$ naïve CD4 T-cell subsets according to the degree of thymic activity.

The $CD31^{+}$ compartment was preserved in Thy patients (Figure 4A and 4B). Conversely, it was significantly contracted in the ∅Thy cohort, both in frequency (Figure 4A) and absolute counts (Figure 4B), as expected in the absence of thymic activity (3, 20). Importantly, despite the marked naïve CD4 T-cell lymphopenia, ∅Thy featured preserved $CD31^{-}$ naïve CD4 T-cell counts (Figure 4B). This finding adds to

previous data on aged individuals reporting preservation of the CD31⁻ compartment in parallel with the progressive decline of CD31⁺ cell counts (40), and argues in favor of the robustness of the homeostasis of CD31⁻ naïve CD4 T-cells in individuals lacking thymic activity.

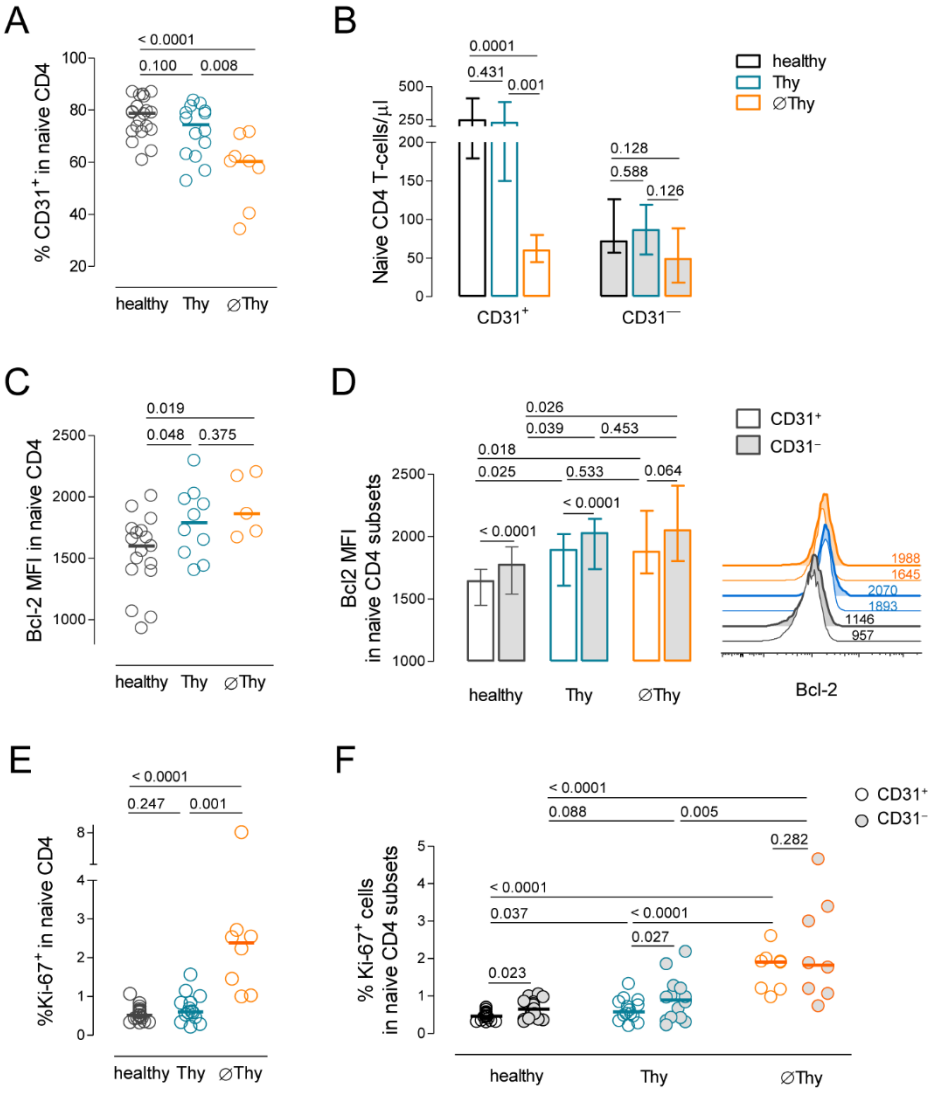

Figure 4. ***Homeostasis of the CD31⁺ and CD31⁻ subsets of naïve CD4 T-cells upon thymectomy.*** **(A)** Frequency of CD31⁺ cells within naïve CD4 T-cells in thymectomized patients without (∅Thy), and with evidence of thymic activity (Thy), as well as age-matched healthy individuals. **(B)** Absolute numbers of CD31⁺ and CD31⁻ naïve CD4 T-cells in the three cohorts. **(C-D)** Bcl-2 MFI within naïve CD4 T-cells **(C)**, and within the CD31⁺ and CD31⁻ subsets **(D)** with the overlay histogram illustrating Bcl-2 expression within CD31⁺ and CD31⁻ naïve CD4 T-cells in ∅Thy, Thy and healthy individuals (numbers indicate Bcl-2 MFI). **(E-F)** Frequency of cycling cells (Ki-67⁺) within naïve CD4 T-cells **(E)** and within the CD31⁺ and CD31⁻ subsets **(F)** in the three cohorts. In scatter graphs each dot represents one individual, and bars represent median; bar graphs show median and interquartile range; *P*-values are shown.

The expression levels of the survival marker Bcl-2 were up-regulated in both thymectomized cohorts as compared to healthy controls (∅Thy: *P*=0.0187; Thy: *P*=0.0487; Figure 4C), suggesting increased naïve CD4 T-cell survival irrespectively of the presence of thymic activity that persisted for more than 20 years post-thymectomy (Table 1). This increase was observed in both $CD31^+$ and $CD31^-$ naïve T-cells (Figure 4D). Of note, we found that the $CD31^-$ compartment featured significantly higher Bcl-2 MFI than $CD31^+$ cells both in healthy and Thy individuals, a difference that was attenuated in ∅Thy (Figure 4D).

Regarding proliferation, a significant increase in the frequency of cycling cells within total naïve CD4 T-cells was found only in ∅Thy (*P*<0.0001 to healthy, *P*=0.0007 to Thy; Figure 4E). The proportion of Ki-67^+ cells was significantly higher in the $CD31^-$ than in the $CD31^+$ compartment in both healthy individuals and in Thy patients (Figure 4F). Nevertheless, its relative contribution to the pool of proliferating naïve CD4 T-cells is minor (<1%), given the large overrepresentation of $CD31^+$ cells in these individuals (Figure 4A). On the other hand, ∅Thy individuals featured an increase in the relative representation of the $CD31^-$ subset, associated with the loss of $CD31^+$ cells (Figure 4A), in parallel with a significant increase in the frequency of cycling cells irrespective of CD31 expression ($CD31^-$ subset: *P*<0.0001 to healthy; *P*=0.0048 to Thy; $CD31^+$ subset: *P*<0.0001 to healthy; *P*<0.0001 to Thy; Figure 4F).

Thus, we showed that the $CD31^-$ naïve CD4 T-cell compartment was maintained in the absence of thymic output, in association with both expanded cell survival and increased proliferation.

Naïve CD4 T-cells feature reduced proliferative response to IL-7 in vitro in the absence of thymic activity in vivo

Cytokine-driven homeostatic mechanisms are crucial for naïve CD4 T-cell maintenance, and IL-7 is considered the key cytokine in these processes (13). Therefore, we hypothesized that naïve CD4 T-cells adjust their intrinsic ability to respond to IL-7 in order to counteract the decline in thymic output. Thus, we

investigated the impact of IL-7 on purified naïve CD4 T-cells, using a 13d culture system previously optimized in our laboratory (14, 28). We found comparable *ex vivo* levels of IL-7Rα-chain (CD127) expression within naïve CD4 T-cells in healthy and thymectomized individuals, irrespective of the degree of thymopoiesis (Figure 5A), and others have shown that the proximal signalling through the IL-7 receptor is preserved in adults thymectomized in infancy, as assessed by STAT5 phosphorylation upon short-term stimulation with IL-7 (20).

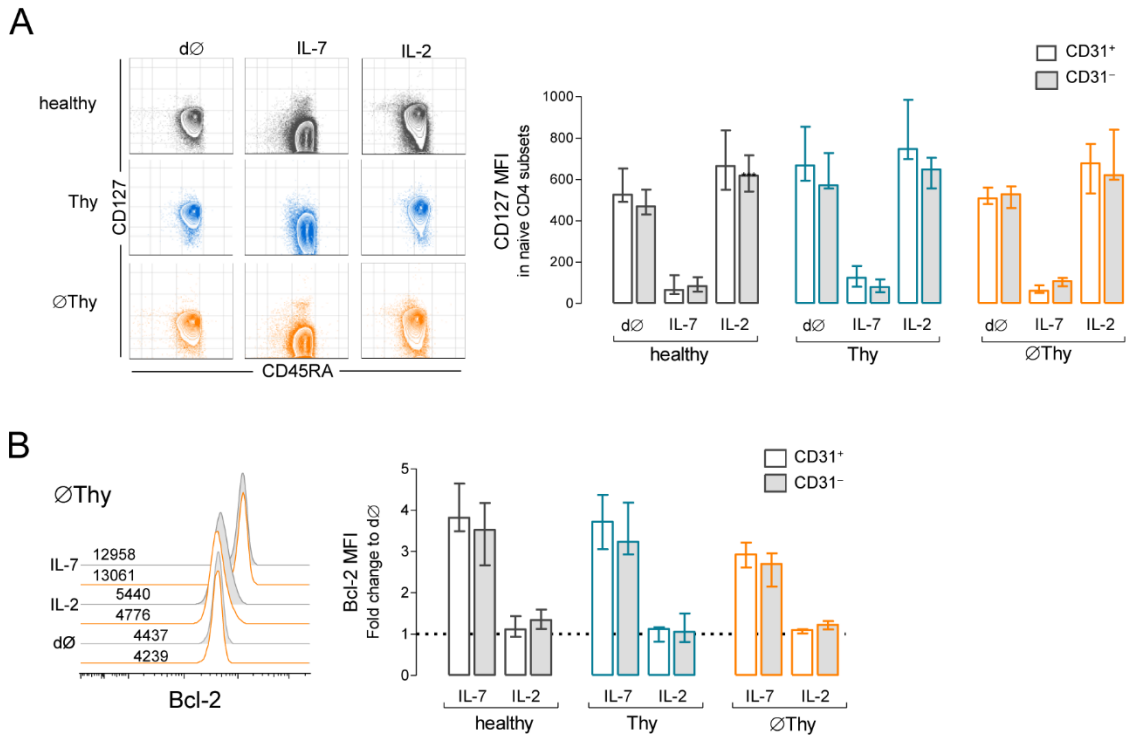

Figure 5. ***Impact of thymectomy on naïve CD4 T-cell ability to respond to IL-7.*** Purified naïve CD4 T-cells from thymectomized individuals without (∅Thy) or with evidence of thymic activity (Thy), and age-matched healthy controls were cultured for 13d with IL-7 or IL-2. **(A)** Illustrative contour-plots from one individual of each cohort showing maintenance of naïve phenotype ($CD45RA^+$) and down-regulation of IL-7Rα upon culture with IL-7, but not with IL-2; graph shows CD127 MFI within the $CD31^+$ and $CD31^-$ compartments *ex vivo* (d∅) and upon culture with IL-2 or IL-7 in the three cohorts. **(B)** Overlay histogram illustrating Bcl-2 expression within $CD31^+$ and $CD31^-$ naïve CD4 T-cells in one ∅Thy patient *ex vivo* (d∅) and upon culture; graph shows the MFI fold change to d∅ of Bcl-2 expression level in $CD31^+$ and $CD31^-$ naïve CD4 T-cells upon culture with IL-7 or IL-2 in the three cohorts. Bars represent median and interquartile range. The down-regulation of CD127 and the up-regulation of Bcl-2 in response to IL-7 in all the cell populations evaluated were statistically significant as compared to both d∅ and IL-2 ($P<0.05$), and no significant differences were found between d∅ and IL-2 ($P>0.05$).

As illustrated in Figure 5A, the cells preserved their naïve phenotype upon culture with either IL-7 or IL-2, including those from ∅Thy individuals. The expected IL-7-mediated

down-regulation of CD127 expression (14, 41) was comparable in all individuals, and, therefore, independent of the degree of thymic activity (Figure 5A). Of note, no changes occurred in the control culture condition with IL-2 (Figure 5A). Additionally, the up-regulation of CD25 and CD95 by IL-7 (14, 42) was also similar in all cohorts, both in the $CD31^+$ and $CD31^-$ compartments (data not shown).

An important physiological role of IL-7 relies on Bcl-2 induction (43). We found a clear up-regulation of Bcl-2 expression, in both $CD31^+$ and $CD31^-$ subsets in cultures with IL-7 in all thymectomized individuals, which was not observed with IL-2 (Figure 5B).

We then investigated the proliferative response of naïve CD4 T-cells using the cell-cycling marker Ki-67, which we have shown to be the best approach to reveal low-level IL-7-driven proliferation (14). Unexpectedly, we found that in contrast to healthy and Thy cohorts, ∅Thy featured no significant increase in the frequency of cycling cells in response to IL-7, as compared to both *ex vivo* and cultures with IL-2 (Figure 6A and 6B). Naïve CD4 T-cell recovery per well was significantly higher upon culture with IL-7 than IL-2 in healthy (fold change 1.07[0.86-1.54] vs 0.79[0.51-1.63]; P=0.0137) and Thy (fold change 1.15[0.16-1.55] vs 0.65[0.06-1.32]; P=0.0259), but not in ∅Thy (fold change 0.87[0.65-1.77] vs 0.54[0.34-1.66]; P=0.2234). The cell recovery upon culture with IL-7 was significantly lower in ∅Thy as compared to healthy individuals (P=0.0236).

We have previously shown that the IL-7-induced proliferation was restricted to the $CD31^+$ subset in healthy subjects (14), which was confirmed here (Figure 6B). Notably, a similar profile was observed in Thy individuals, in striking contrast with patients lacking thymic activity (Figure 6B).

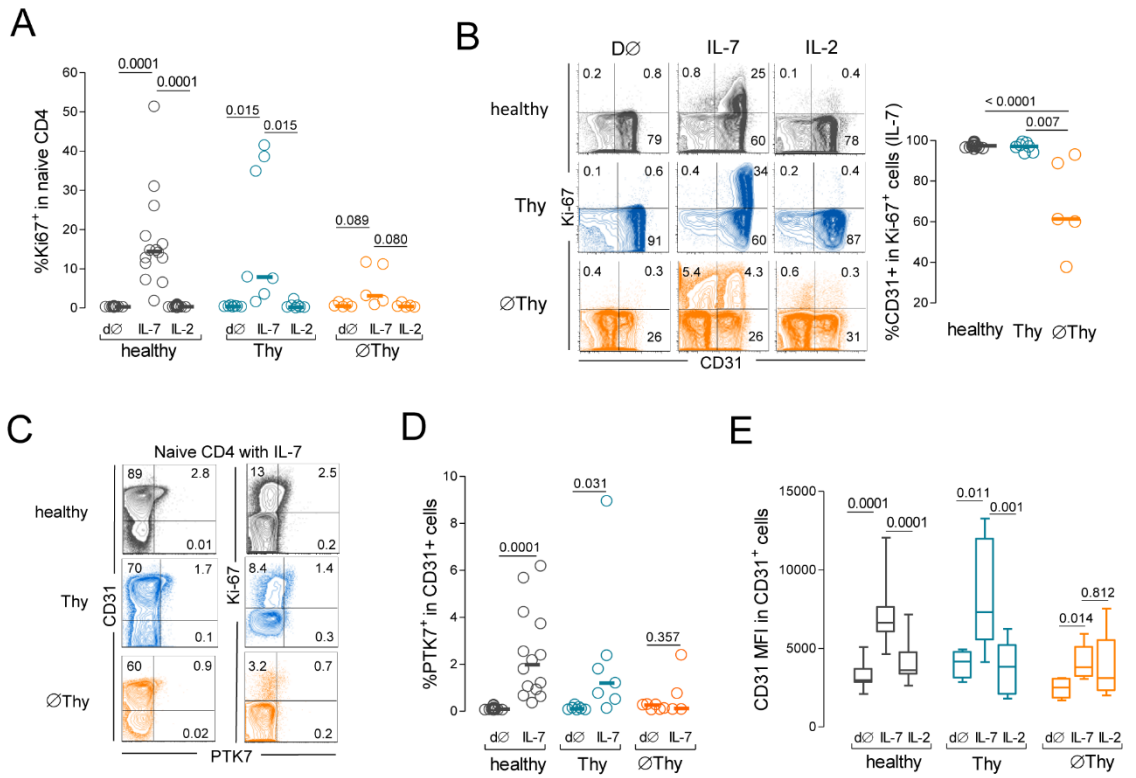

Figure 6. ***Impact of thymectomy on naïve CD4 T-cell ability to proliferate in vitro in response to IL-7.*** Purified naïve CD4 T-cells from thymectomized patients without (∅Thy) or with evidence of thymic activity (Thy), and from age-matched healthy controls were cultured for 13d with IL-7 or IL-2. **(A)** Frequency of cycling cells (Ki-67⁺) within total naïve CD4 T-cells ex vivo (d∅) and upon culture with IL-7 or IL-2. **(B)** Contour-plots illustrate the analysis of Ki-67 *versus* CD31 in representative ∅Thy, Thy and healthy individuals, and graph shows the frequency of CD31⁺ cells within Ki-67⁺ cells. **(C)** Illustrative contour-plots of the analysis of PTK7 *versus* CD31 or Ki-67 within naïve CD4 T-cells cultured with IL-7. **(D)** Frequency of PTK7 *ex vivo* (d∅) and upon culture with IL-7. **(E)** Levels of expression of CD31 (MFI) within the CD31⁺ compartment *ex vivo* (d∅) and upon culture with IL-7 or IL-2. Each dot represents one individual, and bars represent median; *P*-values comparing conditions within cohorts (A, C, D) and between cohorts (B) are shown.

Naïve CD4 T-cells expressing protein tyrosine kinase 7 (PTK7) have been shown to display higher ability to proliferate in response to IL-7 (44). In spite of the very low (<1%) frequencies of PTK7⁺ cells within naïve CD31⁺ CD4 T-cells *ex vivo*, we demonstrated, in cultures with IL-7 from healthy individuals, that PTK7⁺ cells expressed the highest levels of CD31 and were all cycling (Figure 6C), leading to an increase in their overall frequency at the end of the culture (Figure 6D). The same pattern of response was observed in individuals with evidence of some degree of thymic activity (Figure 6C and 6D). In contrast, ∅Thy cases featured cycling cells in both CD31⁺ and CD31⁻ naïve CD4 T-cell compartments (Figure 6B), and no expansion of PTK7⁺ cells (Figure 6C and 6D).

We have also previously shown that IL-7 up-regulates the levels of expression of CD31 within the CD31$^+$ naïve CD4 T-cell subset in a PI3K-dependent manner (14, 28). Of note, the ∅Thy cohort reached significantly lower levels of up-regulation of CD31 MFI within the CD31$^+$ subset in response to IL-7, as compared to healthy (P=0.0035), despite featuring comparable *ex vivo* levels (P=0.1052) (Figure 6E). Moreover, the up-regulation of CD31 MFI was also significantly lower in ∅Thy than in Thy (P=0.0177) individuals, although the latter featured significantly higher *ex vivo* levels of CD31 MFI within the CD31$^+$ subset (P=0.0177) (Figure 6E). These findings suggest that naïve CD4 T-cells from ∅Thy patients lost the ability to respond to IL-7 through the PI3K pathway, in agreement with their impaired proliferation. Conversely, they preserved the ability to up-regulate Bcl-2 in response to IL-7, which we have shown previously that is not PI3K-dependent (14, 28).

Altogether, these data showed that the ability of naïve CD4 T-cells to proliferate and upregulate CD31 in response to IL-7 was impaired in individuals completely lacking thymic activity

3.1.5. Discussion

We investigated here mechanisms of peripheral naïve CD4 T-cell homeostasis in adults with different degrees of thymus impairment since early infancy. We found that the size of the $CD31^-$ compartment was similar in healthy and thymectomized subjects, supporting the existence of thymus-independent homeostasis, possibly driven by self-peptide/MHC. On the other hand, proliferation mediated by IL-7, the main homeostatic cytokine, was severely impaired in the absence of thymopoiesis.

Thymectomy performed during corrective cardiac surgery in infancy is widely recognized as a powerful model to investigate the thymus contribution to naïve T-cell maintenance beyond the establishment of the T-cell compartment. Nevertheless, a wide heterogeneity of findings has been reported (22-26, 32, 45-48). Our study focused on adults thymectomized during infancy/early childhood within a relatively narrow age range, which were grouped according to absence (∅Thy) or presence (Thy) of thymopoiesis based on circulating sjTRECs/µl (5). Our molecular strategy to stringently rule out the existence of thymic output in thymectomized patients overcomes the limitations of other approaches based solely on surgical reports (21, 23, 32, 45-47) and/or thoracic imaging (21, 32, 46, 49), which may have neglected thymic regeneration or ectopic thymus (50).

Of note, after the exclusion of the thymectomized patients lacking thymic activity, we found that both size and diversity of the naïve CD4 T-cell compartment were preserved to a median of 21y post-thymectomy. This likely occurred through both peripheral mechanisms and thymus regeneration, as supported by our finding of sj/βTREC ratios in Thy patients within the range of healthy age-matched controls. These data strengthen the recommendation to avoid complete thymectomy during cardiac surgery (45, 51), which is particularly relevant after the first year of life given the observed association between younger age at thymectomy and thymic recovery (24, 32).

The thymus provides a unique environment to generate a diverse TCR-repertoire (52). This process that involves genomic recombination and gene editing at the individual

cell level (53), imposes major challenges to the quantification of TCR diversity, particularly when sample availability is limited (53-58), leading us to opt for a standard approach using spectratyping. To our knowledge, there is only one study assessing the diversity of purified naïve CD4 T-cells from 3 thymectomized children/adolescents that reported conservation of the spectratyping profiles (25). We showed here that the diversity of the TCR repertoire within the naïve CD4 T-cell compartment was preserved in thymectomized individuals with some degree of remaining thymic activity, and significantly contracted in patients completely lacking thymopoiesis. This profile of premature immune senescence (21, 22, 47, 59) is likely to have clinical implications not yet evaluated, since successful corrective cardiac surgery in young children only became a routine practice 3 decades ago, precluding extended follow-up studies (46, 60).

Of note, patients lacking thymic activity featured no major change of the transcript levels of genes involved in cell quiescence and survival of naïve CD4 T-cells, except for the significant increase in *DUSP6*. This phosphatase enhances the TCR activation threshold by decreasing ERK phosphorylation (39). However, no significant change was observed in the activation of purified naïve CD4 T-cells, suggesting that this pathway does not limit their purging into the memory-effector pool in completely thymectomized patients. Accordingly, they featured an increase in cycling cells within the $CD31^-$ subset that is thought to mainly proliferate in response to TCR stimulation by low-affinity self-peptide/MHC (2, 18).

We showed here that the maintenance of the $CD31^-$ subset is independent of thymic output, and that robust peripheral mechanisms ensure the homeostasis of this population. This is in agreement with the $CD31^-$ preservation that others have reported during age-associated thymic involution (12, 40). Our study revealed that, even in healthy young adults, the levels of the pro-survival molecule Bcl-2 were significantly higher in $CD31^-$ than in $CD31^+$ naïve CD4 T-cells, emphasizing the contribution of anti-apoptotic pathways for the homeostasis of the $CD31^-$ subset (61).

IL-7 is known to play a crucial role in naïve CD4 T-cell homeostasis, not only by enhancing thymopoiesis (62, 63), but also through the peripheral induction of survival

and proliferation (14, 28, 57, 64). Our data support the notion that in the absence of thymopoiesis there is a significant impairment in the peripheral responses to IL-7 that are PI3K-dependent, namely proliferation and CD31 up-regulation, whereas Bcl-2 induction, which does not rely on this pathway, is preserved.

Functional heterogeneity within naïve CD4 T-cells may result from the maturation process that RTEs undergo in the periphery, which may vary throughout life (65-67). It is expectable that cells with privileged response to IL-7 are more abundant in the first years of life, when accelerated growth and constant exposure to new antigens demand for peripheral expansion to ensure continuous replenishment of the naïve compartment. IL-7-induced proliferation is known to be higher in mature single positive thymocytes than peripheral T-cells (28, 67), and in cord blood than adult naïve CD4 T-cells (14). Moreover, it is plausible that in elderly an impaired ability of circulating naïve CD4 T-cells to proliferate in response to IL-7 contributes to their decline, in parallel with thymic involution (40).

Our data suggest a scenario where proliferative responses to IL-7 would be favored in a narrow window of time upon thymic egress, which has important implications to the therapeutic use of IL-7 in clinical settings known to be associated with thymic injury, namely HIV/AIDS (68, 69) and chemotherapy (70, 71). Therefore, the requirement for ongoing thymopoiesis questions the suggested benefit of IL-7 therapy in the recovery of lymphopenia in thymectomized individuals (21).

In conclusion, our investigation of the interplay of thymic output and peripheral mechanisms to the maintenance of the naïve CD4 T-cell compartment uncovered the need for continued thymic activity to the IL-7-driven peripheral proliferation of naïve CD4 T-cells. These findings are of particular relevance for lymphopenic clinical settings and aging, demanding the appraisal of thymus targeting strategies in order to maximize the peripheral effect of IL-7.

Acknowledgments

We thank the sample donors for their collaboration, as well as Rita Tendeiro, Helena Nunes-Cabaço, Maria Conceição Pereira-Santos, Ana Serra-Caetano, Russell B. Foxall, Ana R. Pires and Ana Isabel Pinheiro for technical assistance and discussion of the results.

Funding

This work was supported by Fundação para a Ciência e Tecnologia (FCT; POCI2010/IC/83068/2007 to RMMV; PTDC/SAU-MIC/109786/2009 to AES), and Gulbenkian Foundation (P132532/2013 to AES). SLS, ASA, and PM received FCT scholarships.

Author contributions

SLS, ASA, JTB, RMMV, and AES designed the study; SLS, ASA, PM, DL and RC performed research; SLS. MA, and RA collected clinical data; AES supervised the study; SLS and AES wrote the paper.

Competing interests

The authors declare that they have no competing interests.

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3.1.7. Supplemental Data

Supplemental Table 1. ***Epidemiological, clinical and immunological characteristics of thymectomized individuals***

Patient	Age (years) / gender	Cardiac defect	Age at thymectomy (months)	Surgical report of thymus removal	sjTREC/µl	Naïve CD4+ T-cells		CMV[a] (IgG)
						% within total CD4	cells/µl	
Thy 1	21/M	Tricuspid atresia	10	partial	12.83	48.7	407.5	+
Thy 2	24/M	Coarctation of the aorta	3	partial	7.53	49.5	478.9	–
Thy 3	26/F	Anomalous pulmonary venous return	11	partial	8.85	17.9	149.5	+
Thy 4	28/F	Transposition Great Arteries	11	partial	22.64	41.6	529.2	–
Thy 5	20/F	Transposition Great Arteries	1	partial	9.90	24.5	239.8	+
Thy 6	19/F	Ventricular septal defect	8	partial	11.52	28.2	187.0	+
Thy 7	18/M	Ventricular and atrial septal defects	8	partial	12.52	26.3	418.4	+
Thy 8	26/F	Double outlet right ventricle	60	complete	4.97	26.0	218.6	+
Thy 9	25/M	Transposition Great Arteries+pulmonary stenosis	15	complete	9.81	46.0	373.1	–
Thy 10	28/F	Transposition Great Arteries	7	partial	13.76	37.0	599.1	+
Thy 11	29/F	Transposition Great Arteries+ventricular sept defect	3	partial	3.80	16.6	127.1	–
Thy 12	30/M	Transposition Great Arteries	3	partial	9.68	33.0	245.7	+
Thy 13	27/F	Transposition Great Arteries	8	partial	19.45	39.9	385.9	–
Thy 14	17/M	Anomalous pulmonary venous return	7	partial	18.18	52.8	642.6	+
∅Thy 1	20/F	Tetralogy of Fallot	18	complete	0.62	13.6	63.7	+
∅Thy 2	22/F	Tetralogy of Fallot	23	complete	0.52	13.4	138.5	+
∅Thy 3	24/M	Tetralogy of Fallot	72	complete	0.05	48.8	381.9	–
∅Thy 4	22/F	Tetralogy of Fallot	48	complete	1.76	15.3	128.6	–
∅Thy 5	22/M	Pulmonary atresia	12	complete	0.71	22.1	56.2	+
∅Thy 6	26/M	Tetralogy of Fallot	48	complete	0.45	17.2	148.1	+
∅Thy 7	27/M	Tetralogy of Fallot	12	complete	0.67	10.3	47.0	–
∅Thy 8	26/M	Tetralogy of Fallot	20	complete	0.05	9.28	74.2	+

[a] CMV specific IgM was negative in all patients.

3.2 Human naïve regulatory T-cells feature high steady-state turnover and are maintained by IL-7

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Keywords

Human Regulatory T-cells, Naïve Regulatory T-cells, Regulatory T-cell Homeostasis, Thymectomy, IL-7.

Published

Silva, S.L., et al., Human naïve regulatory T-cells feature high steady-state turnover and are maintained by IL-7. *Oncotarget* 2016;15:12163-75

3.2.1. Abstract

Naïve FoxP3-expressing regulatory T-cells (Tregs) are essential to control immune responses via continuous replenishment of the activated-Treg pool with thymus-committed suppressor cells. The mechanisms underlying naïve-Treg maintenance throughout life in face of the age-associated thymic involution remain unclear. We found that in adults thymectomized early in infancy the naïve-Treg pool is remarkably well preserved, in contrast to conventional naïve CD4 T-cells. Naïve-Tregs featured high levels of cycling and pro-survival markers, even in healthy individuals, and contrasted with other circulating naïve/memory CD4 T-cell subsets in terms of their strong γc-cytokine-dependent signalling, particularly in response to IL-7. Accordingly, *ex vivo* stimulation of naïve-Tregs with IL-7 induced robust cytokine-dependent signalling, Bcl-2 expression, and phosphatidylinositol 3-kinase (PI3K)-dependent proliferation, whilst preserving naïve phenotype and suppressive capacity. Altogether, our data strongly implicate IL-7 in the thymus-independent long-term survival of functional naïve-Tregs, and highlight the potential of targeting the IL-7 pathway to modulate Tregs in different clinical settings.

3.2.2. Introduction

FoxP3-expressing regulatory T-cells (Treg) maintain tolerance to self and to the environment, and are central players in the control of immune responses in general [1]. Notwithstanding their relevance in limiting immune-mediated pathology, and their therapeutic potential, research focused on human Treg homeostasis has been scarce [2]. Human Tregs comprise a compartment of thymus-generated naïve-like cells (naïve-Tregs) that continuously replenish the pool of fully-suppressive activated Tregs expressing memory markers [1-7]. The mechanisms governing naïve-Treg homeostasis, in parallel with age-associated thymic involution, remain largely unknown. Moreover, the investigation of naïve-Treg homeostasis has been hampered by the difficulty in clearly identifying this subset in murine models [8].

The establishment and maintenance of the human naïve T-cell compartment is known to rely on both thymic output and peripheral expansion, with their relative contributions still the subject of intense debate. Increasing evidence points to a major contribution of peripheral proliferation in order to explain the relatively stable size of the naïve compartment in adulthood despite thymic involution [9, 10].

Individuals submitted to total thymectomy in early infancy due to corrective cardiac surgery provide an ideal clinical setting to address the peripheral contribution to long-term maintenance of naïve T-cell subsets. Contraction of the entire naïve compartment upon thymectomy has been consistently reported, confirming the central role of the thymus [11-14]. Notwithstanding this, distinct homeostasis of the naïve-Treg and conventional naïve (naïve-Tconv) CD4 T-cell compartments have been suggested by a longitudinal study that showed naïve-Treg preservation up to 1 year post-thymectomy [15], although long-term data are lacking.

Peripheral homeostasis of naïve-Tconvs relies upon a slow rate of cell-turnover resulting from both cytokine-driven proliferation, and TCR-stimulation by low-affinity self-peptides [16]. Additionally, naïve-Tconv homeostasis is known to rely on pro-survival factors, such as Bcl-2, which are up-regulated by homeostatic cytokines, particularly IL-7 [16, 17].

Naïve-Tregs are characterized by high-affinity for self-peptides, and by rapidly differentiating into memory-Tregs upon TCR-stimulation [1-6]. Thus, MHC-self peptide stimulation is likely to result in loss of their naïve phenotype. On the other hand, in terms of the contribution of cytokine-driven proliferation, naïve-Tregs express low levels of receptors for the main homeostatic cytokines. CD25, the α-chain of the IL-2 receptor, is expressed at only intermediate/dim levels [1], questioning whether naïve-Tregs, like their memory counterparts, depend on IL-2 [2]. Moreover, Tregs *per se* typically express low levels of the α-chain of the IL-7 receptor (IL-7Rα), and there are controversial reports on the IL-7 impact on human and murine Tregs [18-22].

We investigated here the impact of IL-7 and IL-2 on peripheral naïve-Tregs from blood and secondary lymphoid organs (SLO), as well as on mature FoxP3$^+$ thymocytes, and provide evidence for a role of IL-7 in human naïve-Treg homeostasis. We show for the first time that naïve-Tregs feature much higher levels of the pro-survival molecule Bcl-2 and significantly higher turnover than naïve-Tconvs in healthy individuals. These parameters further increased in the absence of thymic replenishment, ensuring the long-term maintenance of the naïve-Treg compartment in total thymectomized individuals.

3.2.3. Materials and Methods

Study design

Blood was collected from healthy and age-matched adults thymectomized in infancy (Table 1). Complete thymectomy was confirmed based on the corrective cardiac surgery report and severely reduced levels of sjTREC. Patients with syndromatic cardiopathy were excluded. Circulating naïve-Tregs were further compared to their precursors, CD4SP thymocytes, isolated from thymic tissue obtained from children during routine corrective cardiac surgery.

Tonsil tissue and blood collected at the same time from children submitted to tonsillectomy were used to study in parallel naïve CD4 T-cells in blood and SLO. All subjects/legal guardians gave written informed consent for blood and/or tissue sampling. Study was approved by Ethical Boards of Faculty of Medicine of University of Lisbon, and from Hospital de Santa Cruz for thymic tissue collection, Portugal.

Cell isolation

PBMCs were isolated from freshly-collected heparinized blood via Ficoll-Paque PLUS (GE Healthcare, Uppsala, Sweden). Total or naïve CD4 T-cells were subsequently purified by negative selection (purity >96%, StemCell Technologies, Grenoble, France). Naïve-Tregs ($CD45RO^{-}CD25^{high}CD127^{-}$), naïve-Tconvs ($CD45RO^{-}CD25^{-}CD127^{high}$), and memory-Tregs ($CD45RO^{+}CD25^{high}CD127^{-}$) were sorted from purified CD4 T-cells using a FACSAria (purity >95%; BD Biosciences, San Jose, CA). Tonsillar mononuclear cells and thymocytes were recovered using Ficoll-Paque PLUS after mechanical dispersion of tonsil and thymic tissue. CD4SP thymocytes were subsequently purified as $CD3^{high}CD8^{-}CD4^{+}$ cells using a FACSAria (purity >97%).

Cell culture

Purified naïve CD4 T-cells, naïve-Tregs or naïve-Tconvs, were cultured at $1x10^{6}$ cells/ml in complete medium as described [17], with either IL-7 (10ng/ml; R&D Systems, Minneapolis, MN) or IL-2 (20IU/ml; NIH/AIDS Research and Reference Program, Division of AIDS, NIAID, Hoffman-La Roche), for up to 13 days, with media replacement at day 3 and day 7. Naïve CD4 T-cell recovery per well was significantly higher upon culture with IL-7 than IL-2 (200,615[144,000-262,167] *versus*

95,958[60,063-178,125]; n=16; *P*=0.0006). In some experiments, anti-IL-2 blocking monoclonal antibody (clone 5334, 10µg/ml, R&D Systems) or isotype control IgG1 (10µg/ml, eBioscience, San Diego, CA) was added to cultures with IL-7. Cultures were also performed for 7 days in presence of PI3K inhibitor LY294002 (10µM, Merck Biosciences, Nottingham, UK); mTOR inhibitor rapamycin (100nM, Sigma-Aldrich); or drug vehicle DMSO (Sigma-Aldrich). For serum deprivation experiments, purified CD4 T-cells were cultured for 24 hours in medium without AB serum, or with 40% autologous serum alone or supplemented with IL-7, IL-2, anti-IL-2 blocking mAb, or isotype control.

Flow-cytometry

Ex vivo phenotypic analysis was performed in freshly-collected whole blood. Eight-color staining was performed using mAbs listed in Supplementary Table 1. Intracellular staining was done using eBioscience FoxP3 kit. At least 150,000 events were acquired for each sample on a BD LSRFortessa (BD Biosciences) and data analyzed using FlowJo software (TreeStar, Ashland, OR). Dead cells were excluded according to FSC/SSC characteristics and/or LifeDead staining. After lymphogate definition, doublets were excluded, and cells analyzed within the mentioned gates.

STAT5 phosphorylation

pSTAT5 was quantified in whole blood immediately after collection. After surface staining, red blood cells were lysed, and cells fixed and permeabilized using eBioscience FoxP3 protocol, followed by BD Cytofix and BD Phosflow (BD Biosciences), and 1h incubation at 4°C with anti–pSTAT5 mAb and other intracellular markers (Supplementary Table 1). pSTAT5 was also quantified upon stimulation of surface stained purified CD4 T-cells, with increasing concentrations of recombinant IL-7 (0.1/1/10/50ng/ml) or IL-2 (2/20/100/200IU/ml) for 15 minutes at 37°C, as described [44].

Thymidine analogue 5-ethynyl-2'-deoxy-uridine (EdU) incorporation

EdU (5µM) was added to cell cultures for 12 hours. Immediately after collection, cells were surface and intracellularly stained, resuspension in ice-cold 1% formaldehyde (15 minutes), kept on ice on 70% ethanol (10 minutes), and then washed several times

in PBS with 0.05% Triton-X100 before detection of EdU-substituted DNA using the Click-iT® EdU HCS Assay (Thermo Fischer Scientific) according to manufacturer´s instructions.

Cytokine quantification

Cytokine production at the single-cell level was assessed after 4 hours stimulation with PMA+Ionomycin and Brefeldin A, as described [44]. Serum IL-7 levels were quantified using Human IL-7 Quantikine HS ELISA kit (R&D Systems) [42].

In vitro suppression assay

Purified naïve-Tregs were incubated with IL-7, IL-2, or medium alone for 4 hours followed by co-culture (ratio 1:1) with autologous naïve-Tconvs labeled with carboxyfluorescein succinimidyl ester (CFSE), as described [17], stimulated with plate bound anti-CD3 (0.5μg/ml, clone OKT3, eBiosciences) in the presence of irradiated autologous PBMCs (4000rad). CFSE intensity decline was assessed at d4 by flow-cytometry; % of suppression of naïve-Tconv proliferation = [(% proliferating naïve-Tconvs plated alone - % proliferating naïve-Tconvs co-cultured with Treg)/ % proliferating naïve-Tconvs plated alone]x100.

sjTREC quantification

A highly sensitive nested quantitative PCR assay (detection-limit 1copy/10^5 cells) was used as described [24]. Triplicate multiplex PCR amplification of sjTREC, together with the CD3γ chain was performed on lysed PBMCs; quantifications were then performed using a LightCycler™ in independent experiments, with the same first-round serial dilution standard curve.

FOXP3 mRNA quantification

Total RNA (Zymo Research kits) was used to generate cDNA (Superscript III Reverse Transcriptase, Thermo Fischer Scientific). mRNA levels of *FOXP3* (primers/probe described in [45]), and *GAPDH* (Taqman Gene Expression Assay) were quantified in duplicates (ViiA 7 Real-Time PCR System, all from Thermo Fischer Scientific) using standard curves generated by serial dilutions of cDNA from pooled PBMCs for *GAPDH*

and a plasmid with *FOXP3* sequence. Relative copy numbers of *FOXP3* were calculated upon normalization to *GAPDH*.

Immunofluorescence staining

Human tonsils were placed in OCT (VWR, Radnor, PA) and snap-frozen in liquid nitrogen. 3µm sections were stained overnight at 4°C using antibodies listed in Supplementary Table 1, and DAPI as a nuclear counter stain. Image processing was performed using FIJI software.

Statistical analysis

Statistical analysis was performed with Graph Prism Version 5.01 (GraphPad Software, San Diego, CA). The following tests were used: Friedman for variance; Wilcoxon-Signed Rank for pairwise comparisons; Mann-Whitney for unpaired comparisons; Spearman's coefficient for correlations. Results were expressed as median [interquartile range or range (n<4)]. *P*-values <0.05 were considered significant.

3.2.4. Results

Preservation of the naïve-Treg compartment following thymus removal

Adults submitted to total thymectomy early in life provide a unique setting to investigate human naïve compartment homeostasis. However, published studies have been hampered by the lack of clear information regarding possible residual thymic activity that can result from either ectopic thymus or post-thymectomy regeneration [11, 12, 14, 23]. We applied here strict criteria to exclude residual thymic activity based on detailed surgical reports and single-joint TCR excision circles (sjTREC) levels clearly below the lowest level observed in healthy adults (Table 1).

Table 1. ***Characterization of the cohorts***

	Healthy	Thymectomized
Number	22	7
Age, years	21 [18-30]	24 [22-27]
Age at thymectomy, months	NA	23 [12-72]
sjTRECs/µl	16.6 [4.1-39.3]	0.5 [0.1-1.8][a]
Lymphocytes/µl	2413 [1430-3502]	2010 [933-2618]
% CD4 T-cells	40 [29-52]	43 [23-47]
% Naïve[b] in CD4	40 [29-58]	15 [9-49][a]
% FoxP3$^+$ in CD4	2.9 [1.2-5.4]	4.1 [3.0-5.6]
FoxP3$^+$ cells/µL	21 [9-51]	28 [11-39]
Serum IL-7, pg/ml	15 [7-23]	14 [8-20]

Data are presented as median with limits in square brackets.
NA - not applicable. [a] $P<0.0001$. [b] Naïve cells defined as CCR7$^+$CD45RO$^-$.

sjTRECs are by-products of TCR rearrangements during T-cell development that are progressively lost as cells divide in the periphery, and thus used to identify recent thymic

emigrant cells [24]. Adults, with a median of 21y (18-24.5y) after total thymectomy, were compared with age-matched healthy individuals (Table 1).

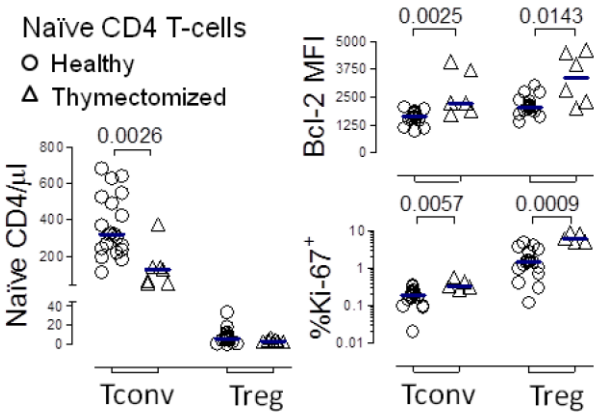

Figure 1. ***Preservation of the naïve-Treg compartment following thymus removal***. Circulating naïve-Treg and naïve-Tconv counts; as well as % of cycling-cells, and Bcl-2 MFI within these subsets in healthy and thymectomized adults; each symbol represents one individual; bars represent median; significant *P*-values of comparisons within each subset are shown.

We observed a significant decrease in circulating naïve-Tconvs, both in frequency within CD4 T-cells (*P*=0.0042; Supplementary Figure 1A) and absolute numbers (*P*=0.0026; Figure 1), in agreement with previous data from other thymectomized cohorts [11-14]. Conversely, the naïve-Treg pool size was preserved, as compared to healthy subjects (Figure 1 and Supplementary Figure 1A).

The healthy cohort spanned an age period associated with relatively stable thymic function and naïve-Treg numbers [6], and the size of the circulating naïve-Treg pool was within the range previously described [4-6]. We sorted naïve-Tregs, and confirmed that their *FOXP3* mRNA expression levels were comparable to those found in memory-Tregs (4526-21104 *versus* 1540-14363 relative copy numbers, respectively, n=3 healthy adults), and much higher than those in naïve-Tconvs (34-115 relative copy numbers), confirming them as *bona fide* Tregs [1].

Circulating naïve-Tregs were confirmed to have a truly naïve phenotype both in healthy and thymectomized subjects, based on the expression of a panel of naïve markers and reduced CD95 expression, as well as expressing Treg function-associated markers (CTLA-4, HLA-DR, CD39) at lower levels than memory-Tregs (Figure 1 and Supplementary Figure 1B). Although Helios has been proposed as a marker of thymus-

derived Tregs, we showed that in both cohorts a significant proportion of circulating naïve-Tregs lacked Helios expression (Figure 1 and Supplementary Figure 1B), as already observed in human mature FoxP3$^+$ CD4 single-positive (CD4SP) thymocytes (Supplementary Figure 1C), questioning its usefulness as a marker of thymic-derived Tregs [25]. Regarding the CD31$^+$ subset, a population known to be enriched in recent thymic emigrants [16], no significant contraction was observed within naïve-Tregs of thymectomized, as compared to healthy individuals (P=0.1708, Supplementary Figure 1B), in contrast to the significant reduction observed within naïve-Tconvs (P=0.0122, Supplementary Figure 1B). This finding suggests that distinct homeostatic mechanisms are operating in the two naïve compartments upon thymectomy. Interestingly, we have previously shown that IL-7 up-regulates CD31 expression [17], further validating the importance of investigating the impact of IL-7 on naïve-Tregs, as detailed below.

In order to investigate the mechanisms underlying the preservation of the naïve-Treg compartment, we quantified the expression levels of markers of cell survival and cell cycling in thymectomized, as compared to healthy individuals. Bcl-2 expression was significantly higher, supporting a contribution of increased naïve-Treg survival to their maintenance upon thymectomy (P=0.0143; Figure 1). Additionally, significantly higher frequencies of cycling-cells, as assessed by Ki-67, were documented (P=0.0009; Figure 1). The degree of Bcl-2 increase in thymectomized was comparable in the two naïve compartments, whereas the increase in Ki-67$^+$ cell frequency was much more striking within naïve-Tregs (4-fold) than within naïve-Tconvs (1.7-fold), supporting an important contribution of increased cell-turnover to the better preservation of the naïve-Treg compartment.

Altogether, our data indicate that naïve-Tregs are preserved in adults submitted to total thymectomy in early infancy, mainly through increased homeostatic proliferation.

High steady-state turnover of the naïve-Treg compartment

The finding of a preserved naïve-Treg compartment in thymectomized adults prompted us to further investigate the mechanisms underlying naïve-Treg homeostasis under steady-state conditions in healthy young adults with stable thymic output.

We found that naïve-Tregs displayed significantly higher levels of Bcl-2 than both naïve-Tconvs and memory-Tregs (Figure 2A), suggesting that enhanced survival plays a major role in their maintenance. Therefore, in this respect, naïve-Tregs are distinct from memory-Tregs, which have been shown to have a pro-apoptotic profile [1, 4].

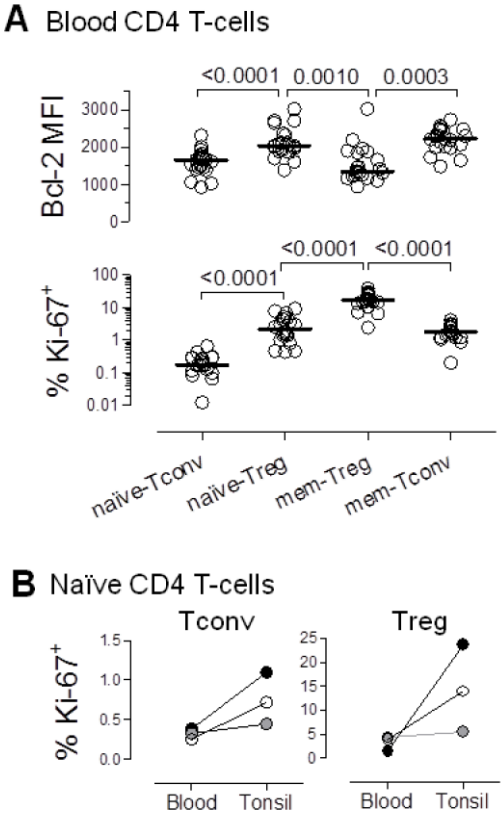

Figure 2. ***High steady-state turnover of the naïve-Treg compartment.*** **(A)** Bcl-2 MFI and % of cycling-cells, within circulating CD4 T-cell subsets; each dot represents one individual; bars represent median; significant *P*-values are shown. **(B)** % of cycling-cells within naïve-Tregs and naïve-Tconvs from matched blood and tonsil samples (each symbol represents one child: 2y/open, 6y/grey, and 7y/black).

Additionally, naïve-Tregs featured a much higher *in vivo* turnover than naïve-Tconvs (Figure 2A), with significantly elevated proportion of cycling-cells (2.23%[1.12-4.75]

versus 0.17%[0.10-0.29], P<0.0001). Thus, although naïve-Tregs are frequently labeled as "quiescent" as opposed to memory-Tregs, we show here that they featured levels of cycling comparable to those found in memory-Tconvs (Figure 2A).

Homeostatic naïve-Tconv proliferation occurs mainly in SLO [16]. We performed a parallel study of blood and tonsils, used here as an example of SLO due to ease of access to clinical samples. Tonsil naïve-Tregs featured a much higher proportion of cycling-cells than blood naïve-Tregs (Figure 2B). Notably, the fold-increase in cycling-cells in tonsils as compared to blood was much more striking in the naïve-Treg than in the naïve-Tconv compartment (4-fold *versus* 2-fold, respectively). The presence of naïve-Tregs expressing Ki-67 in tonsil tissue was further confirmed by immunofluorescence (Supplementary Figure 2A). Additionally, we showed that naïve-Tregs from freshly-collected tonsils consistently entered S-phase at higher rates than naïve-Tconvs, as estimated by the *ex vivo* incorporation of 5-ethynyl-2'-deoxy-uridine (Edu), a thymidine analogue (Supplementary Figure 2B; 12 hours incubation: naïve-Treg: 1.23-1.70%; naïve-Tconv: 0.37-0.42%; n=3). Of note, in tonsils, naïve-Tregs featured significantly higher proportion of Ki-67$^+$ cells than naïve-Tconvs (Figure 2B), as well as Bcl-2 MFI (mean fluorescence intensity; range 2777-3161 *versus* 1873-1992, n=3, respectively). These results suggest that SLO may provide appropriate niches for naïve-Treg homeostatic proliferation.

Overall, our data show that, in healthy young adults, naïve-Tregs featured markers of enhanced survival and increased turnover in comparison to naïve-Tconvs, supporting an important contribution of peripheral proliferation to the maintenance of the naïve-Treg compartment.

Ex vivo evidence for ongoing naïve-Treg response to IL-7

Next, we investigated the putative contribution of γc-cytokines to the increased turnover and survival of naïve-Tregs. *Ex vivo* levels of pSTAT5, a downstream marker of both IL-7 and IL-2-mediated signalling, were significantly higher in naïve-Treg than in naïve-Tconv and memory CD4 subsets, analyzed in freshly-collected whole blood from healthy subjects (Figure 3A), irrespectively of the levels of expression of IL-7Rα and IL-2Rα (Supplementary Figure 1B).

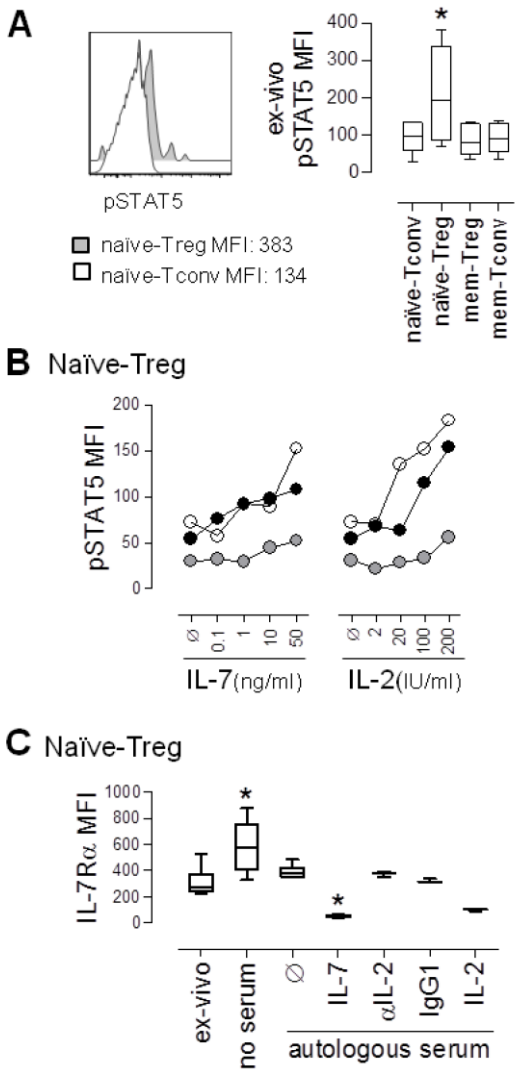

Figure 3. ***Ex vivo evidence for ongoing naïve-Treg response to IL-7.*** **(A)** Representative histogram of *ex vivo* pSTAT5 levels in circulating naïve-Tregs and naïve-Tconvs, with graph showing pSTAT5 MFI within these and counterpart memory CD4 T-cell subsets; comparison of pSTAT5 levels only revealed significant differences between naïve-Tregs and all the other subsets (* $P<0.05$; n=6). **(B)** Analysis of pSTAT5 MFI within gated naïve-Tregs upon *in vitro* stimulation with increasing concentrations of IL-7 or IL-2; each symbol represents one individual. **(C)** IL-7Rα MFI within naïve-Tregs analyzed *ex vivo* and after 24 hours-culture without serum or supplemented with 40% autologous serum alone or with IL-7 (n=6); as well as with IL-2, anti-IL-2 blocking monoclonal antibody, or isotype-control (n=3); * $P<0.05$ as compared to *ex vivo* levels.

Additionally, we stimulated purified CD4 T-cells with increasing concentrations of IL-7 or IL-2, and clearly showed that naïve-Tregs responded to both cytokines, in a dose-dependent manner (Figure 3B and Supplementary Figure 3A). Our data extend previous human and murine studies on the ability of Tregs to phosphorylate STAT5 in response to IL-7 *in vitro* [20, 26-28], through the comparison of naïve and memory Tregs, as well as their FoxP3 negative counterparts. Since IL-7 signalling induces the

down-regulation of its receptor [17], we reasoned that the level of recovery of IL-7Rα expression upon IL-7 deprivation would reflect the extent of ongoing IL-7 signalling *in vivo*. IL-7Rα expression levels were quantified in purified CD4 T-cells from healthy subjects following 24 hours in different culture conditions (Figure 3C and Supplementary Figure 3B). Naïve-Tregs featured a significant recovery of IL-7Rα expression in the absence of IL-7 (culture in serum free medium), as compared to *ex vivo* levels, in agreement with a significant ongoing IL-7 response. Notably, IL-7Rα expression was relatively preserved in the presence of autologous serum (IL-7 levels range: 15.8-23.5pg/ml). Moreover, the possible contribution of IL-2, either derived from serum or produced by cells in culture, was excluded by documenting a lack of impact of IL-2 blockade. Addition of exogenous IL-7 (10ng/ml) or IL-2 (20IU/ml) led to the expected down-regulation of IL-7Rα [29].

In conclusion, we showed that naïve-Tregs featured high *ex vivo* pSTAT5 levels, and recovered IL-7Rα expression after IL-7 deprivation, supporting their ongoing response to IL-7 *in vivo*.

Preserved naïve phenotype, Treg-markers and suppressive capacity upon naïve-Treg response to IL-7.

We subsequently investigated the impact of IL-7 on naïve-Treg phenotype and suppressive capacity. For this purpose, total naïve CD4 T-cells were purified from freshly-collected blood of healthy donors, and cultured for up to 13 days in the presence of IL-7 or IL-2, using conditions previously optimized in our laboratory [17]. Analysis was performed within naïve-Tregs and naïve-Tconvs defined according to their FoxP3 expression, as illustrated in Figure 4A.

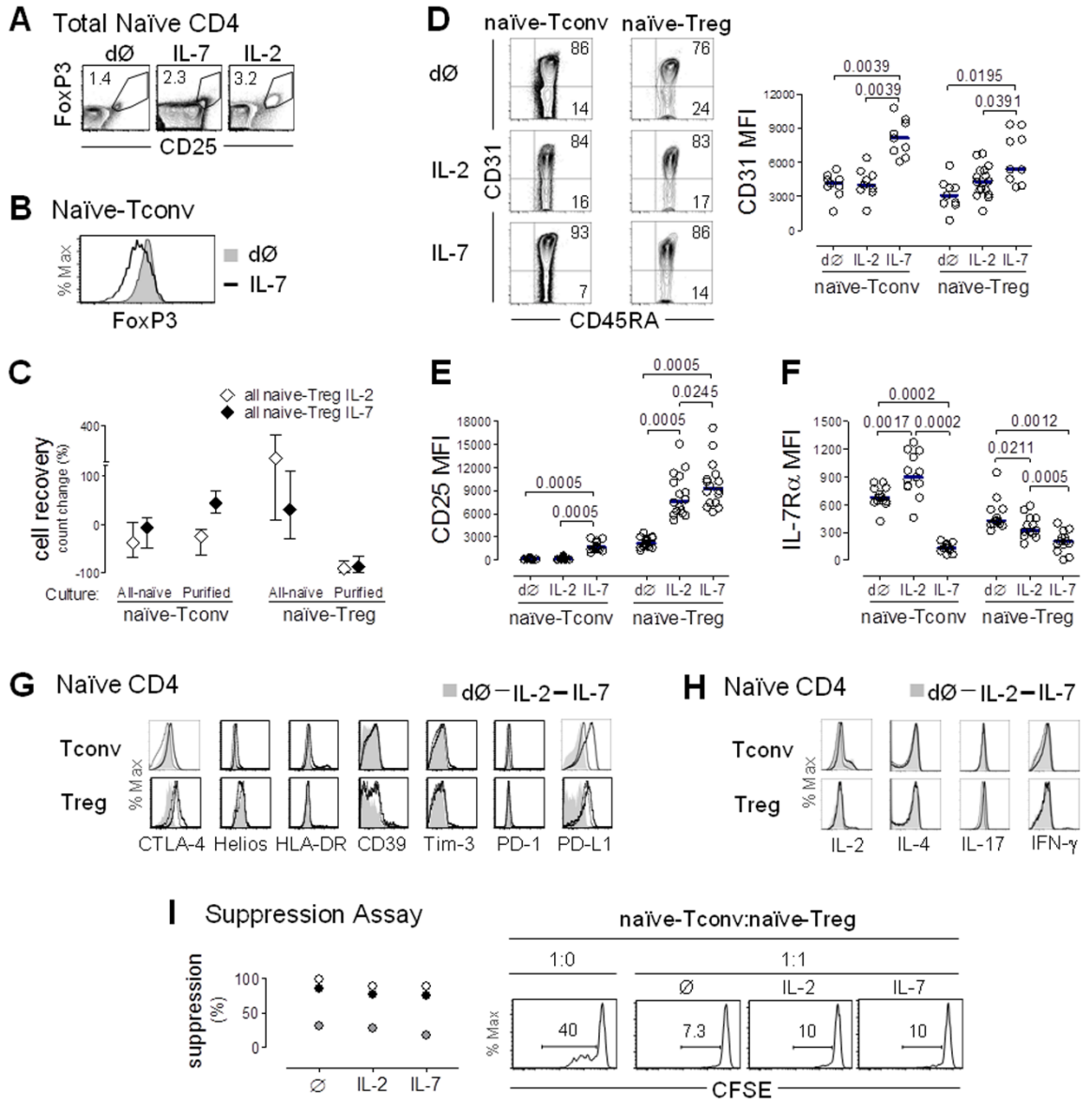

Figure 4. ***Evidence of naïve-Treg response to IL-7 while preserving their naïve and suppressive phenotype*****. (A)** Representative contour-plots of FoxP3 and CD25 expression within total naïve CD4 T-cells *ex vivo* (dØ) and upon 13 day-culture with IL-7 or IL-2. **(B)** Illustrative histogram demonstrating absence of FoxP3 induction in purified naïve-Tconvs following 13 day-culture with IL-7. **(C)** Cell recovery upon 13 day-culture with IL-7 or IL-2 of total naïve CD4 T-cells (n=11), purified naïve-Tregs (n=3) or purified naïve-Tconvs (n=3); graph shows median and interquartile range or range (purified populations). **(D-F)** Analysis of naïve-Tregs and naïve-Tconvs *ex vivo* and post-13 day-culture of naïve CD4 T-cells with IL-7 or IL-2 showing representative contour-plots of CD31/CD45RA expression **(D),** and graphs of MFI of CD31 **(D)**, CD25 **(E)**, and IL-7Rα **(F)**; each dot represents one individual; bars represent median; comparisons done within each subset; significant *P*-values are shown. **(G)** Illustrative histograms of CTLA-4, Helios, HLA-DR, CD39, Tim-3, PD-1 and PD-L1 expression within naïve-Tregs and naïve-Tconvs *ex vivo* and post-13 day-culture with IL-7 or IL-2 (one/3-14). **(H)** IL-2, IL-4, IL-17 and IFN-γ production after short-term PMA/Ionomycin stimulation *ex vivo* and post-13 day-culture with IL-7 or IL-2 (one/3). **(I)** Illustrative histograms of CFSE expression within naïve-Tconvs cultured alone or with naïve-Tregs pre-incubated with medium alone, IL-7 or IL-2 (numbers represent % of cells that divided at least once); graph shows % of suppression of naïve-Tconv proliferation in 3 individuals.

We confirmed that FoxP3 was not induced in naïve-Tconvs in the presence of IL-7, using sorted naïve-Tconvs cultured alone with IL-7, and showing no increase in FoxP3 expression either at the protein level, by flow-cytometry (Figure 4B), or at the transcriptional level by real-time PCR (*ex vivo* range 34-115 *versus* 14-62 FOXP3 relative copy numbers after 13 day-culture, n=3). FoxP3 induction was also not observed in cultures of purified naïve-Tconvs with IL-2 (data not shown). This is in agreement with the requirement for TCR stimulation for *de novo* induction of FoxP3 in conventional CD4 T-cells [1].

Importantly, despite the clear impact of both IL-7 and IL-2 on the recovery of naïve-Tregs in cultures of total naïve CD4 T-cells, neither cytokine was able to maintain sort-purified naïve-Tregs when they were cultured alone (Figure 4C). This finding revealed a requirement for naïve-Tregs to interact with naïve-Tconvs to ensure their survival, which was not overcome by the addition of IL-2 (Figure 4C). These data precluded purified naïve-Treg culture in isolation.

Of note, the overall proportion of naïve-Tregs within total naïve CD4 T-cells significantly increased upon 13 day-culture with IL-7 (from 0.99%[0.60-1.43] to 2.15%[1.29-3.21], P=0.0048, n=16). This increase was more evident upon culture with IL-2 (7.94%[3.65-11.75], P=0.0005), likely due to the reduced impact of IL-2 on naïve-Tconvs (Figure 4C).

As illustrated in Figure 4D, FoxP3$^+$ cells maintained their naïve phenotype following 13 day-culture with either IL-7 or IL-2. Our previous work showed that IL-7 increases CD31 expression in naïve CD31$^+$ CD4 T-cells [17]. Here, we showed that naïve-Tregs cultured in the presence of IL-7 up-regulated CD31 MFI to levels comparable to naïve-Tconvs (fold-increase: 2.17[1.45-3.69] *versus* 2.17[1.73-2.46], P=0.4333, respectively; Figure 4D). Moreover, naïve-Tregs up-regulated CD25 (fold-increase: 3.85[3.29-6.01], Figure 4E), and down-regulated IL-7Rα (fold-reduction: 0.43[0.23-0.67], Figure 4F) upon IL-7 stimulation, although less markedly than naïve-Tconvs (11.54[7.80-14.96], P<0.0001 for CD25; and 0.21[0.15-0.27], P=0.0058 for IL-7Rα expression). These findings support an ability of naïve-Tregs to respond to IL-7.

The impact of IL-2 on naïve-Tregs followed a similar pattern, albeit significantly less striking than that observed for IL-7 (Figure 4D-F). Conversely, in naïve-Tconvs both CD25 and CD31 expression levels were unaltered in response to IL-2, whereas IL-7Rα expression increased (Figure 4D-F), possibly due to a dominant effect of IL-7 deprivation [17, 29].

Concerning Treg function-associated markers, naïve-Treg FoxP3 MFI (*ex vivo*: 778[650-1009], n=16) significantly increased upon culture with IL-7 (1166[863-1522], *P*=0.0214), and even more so with IL-2 (1229[902-1916], *P*=0.0280 as compared to *ex vivo*, *P*=0.0362 as compared to IL-7). Furthermore, we observed a sustained or moderate increase in CTLA-4, CD39 and HLA-DR expression upon culture with either IL-7 or IL-2 (Figure 4G), suggesting maintenance of regulatory function. Additionally, IL-7 induced a major up-regulation of PD-L1, but not PD-1, on both naïve subsets (Figure 4G).

Importantly, naïve-Tregs remained unable to produce IL-2, IL-4, IL-17 or IFN-γ after 13 day-culture in the presence of IL-7 or IL-2, as evaluated upon further short-term stimulation with PMA plus Ionomycin (Figure 4H).

Finally, we asked whether exposure to IL-7 impairs the suppressive capacity of naïve-Tregs. For this purpose, we sorted naïve-Tregs and compared their suppressive capacity upon 4h pre-incubation with culture medium alone or supplemented with IL-7 or IL-2. We observed that upon treatment with either cytokine, naïve-Tregs maintained their ability to suppress autologous naïve-Tconv proliferation in response to TCR stimulation (Figure 4I).

Altogether, these results support an ability of naïve-Tregs to respond to IL-7 whilst maintaining both their naïve and suppressive phenotype.

IL-7 induced naïve-Treg survival and proliferation

We then asked whether IL-7 induces the expression of markers of survival (Bcl-2) and cell cycling (Ki-67) in naïve-Tregs. We observed a clear induction of Bcl-2 expression

in naïve-Tregs cultured in the presence of IL-7 (Figure 5A-B), although less marked than in naïve-Tconvs (fold-increase: 3.02[2.33-3.72] *versus* 3.93[3.29-5.05], respectively, $P<0.0001$). Bcl-2 up-regulation in naïve-Tregs was significantly higher upon IL-7 stimulation than with IL-2 (Figure 5A-B), using optimal concentrations of both cytokines [17].

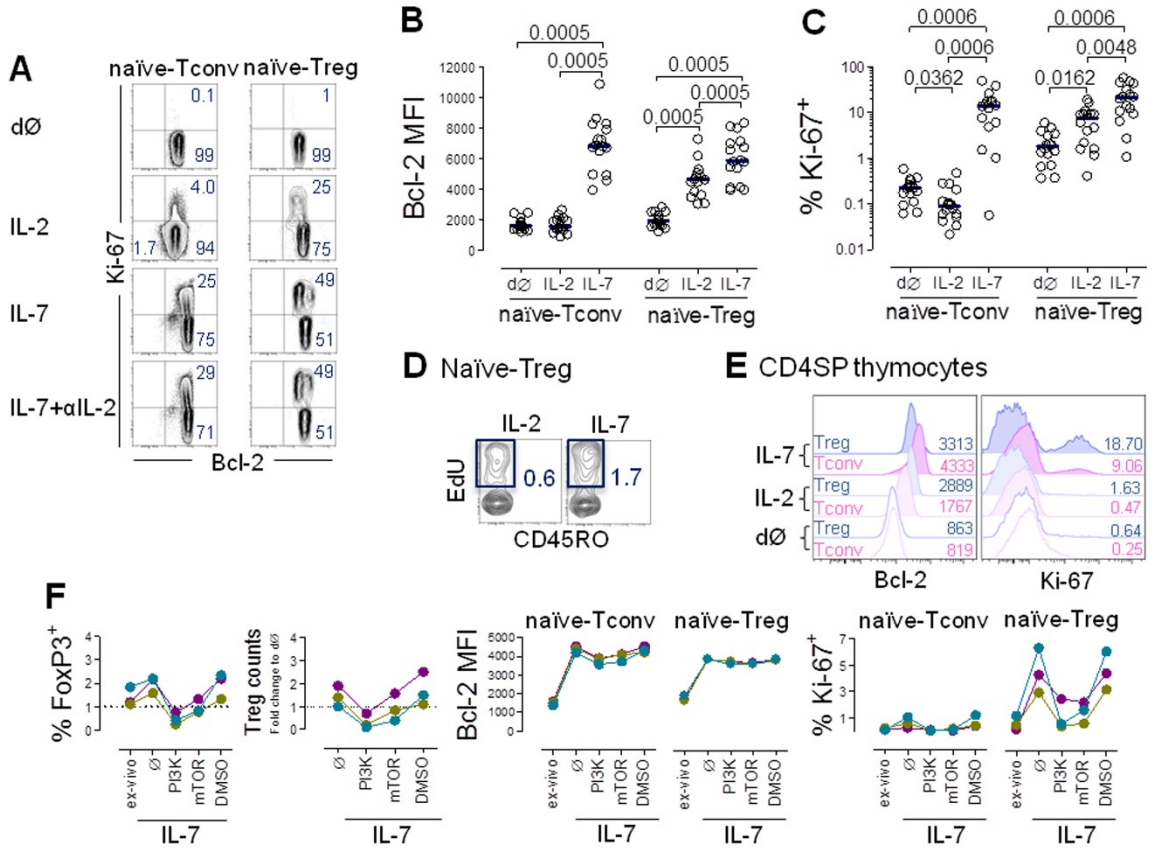

Figure 5. ***IL-7 induced naïve-Treg survival and proliferation.*** **(A-D)** Analysis of naïve-Tregs and naïve-Tconvs *ex vivo* (dØ) and post 13 day-culture of purified naïve CD4 T-cells with IL-7 or IL-2 or IL-7 plus anti-IL-2 blocking monoclonal antibody: **(A)** Representative contour-plots of Bcl-2 and Ki-67 expression; graphs show Bcl-2 MFI **(B)** and % of cycling-cells **(C)**, bars represent median; significant *P*-values of comparisons within each subset are shown; **(D)** Contour-plots illustrating the impact of IL-7 or IL-2 on EdU incorporation by naïve-Tregs (final 12 hours-culture, n=2). **(E)** Illustrative histograms of Bcl-2 and Ki-67 expression within gated FoxP3$^+$ or FoxP3$^-$ cells *ex vivo* and post 7 day-culture of purified CD4SP thymocytes with IL-7 or IL-2 (one/3). **(F)** Impact of the PI3K-inhibitor (LY294002), mTOR-inhibitor (rapamycin), or vehicle control (DMSO), on naïve CD4 T-cells upon 7 day-culture with IL-7; graphs show % of naïve-Treg and change in cell recovery, Bcl-2 MFI and % of cycling-cells within naïve-Tregs and naïve-Tconvs, as compared to *ex vivo* levels; each color represents one individual.

Additionally, IL-7 induced cycling of naïve-Tregs (Figure 5A and 5C). The proportion of cells expressing Ki-67 (21.06%[9.62-40.40]) was higher than that observed within

naïve-Tconvs (13.92%[5.11-17.63], *P*=0.0186), and within naïve-Tregs cultured with IL-2 (7.31%[1.74-9.35], *P*=0.0019). These findings are even more striking considering the much higher IL-7Rα expression levels on naïve-Tconvs (Supplementary Figure 1B). We further showed that IL-7 was superior to IL-2 in promoting naïve-Treg entry into cell-cycle, via assessment of the frequency of EdU-incorporating cells in the last 12 hours of the culture (Figure 5D).

Proliferative studies using CFSE-labeled cells were not performed, as we have previously demonstrated that this approach does not reproducibly reveal cytokine-mediated proliferation of naïve CD4 T-cells [17].

Importantly, as shown in Figure 5A, IL-7 induction of Bcl-2 and cell cycling was unaltered in the presence of an anti-IL-2 blocking monoclonal antibody, thus supporting a major role for IL-7 in the maintenance of naïve-Treg homeostasis independently of IL-2.

Human FoxP3$^+$CD4SP thymocytes were already able to proliferate in culture, and to up-regulate Bcl-2 in response to IL-7 (Figure 5E), in agreement with our previous data showing an increase of pSTAT5 in FoxP3$^+$ thymocytes upon stimulation with IL-7 [30].

Finally, we investigated whether the signalling pathways involved in IL-7 response differed between naïve-Tregs and naïve-Tconvs. IL-7 signalling uses both JAK3/STAT5 and PI3K/Akt pathways to promote T-cell survival and proliferation [17]. We have previously demonstrated that IL-7-mediated naïve CD4 T-cell proliferation, but not Bcl-2 up-regulation, critically depends upon PI3K activity [17]. On the other hand, Treg metabolism is known to rely on mammalian target of rapamycin (mTOR) [2]. To investigate the relative importance of these pathways, we added the cell permeable PI3K-specific inhibitor (LY294002) or mTOR inhibitor (rapamycin) to naïve CD4 T-cells cultured with IL-7 or IL-2. A 7 day-culture assay was selected to avoid possible deleterious impact of long-term exposure to the inhibitors, and was found to be sufficient to reveal Bcl-2 and Ki-67 induction albeit at lower levels than after 13 days culture (Figure 5F). Inhibition of the PI3K pathway led to a major decrease in naïve-Treg recovery and proliferation upon stimulation with IL-7. Conversely, Bcl-2

expression was not significantly altered. Similar trends were observed upon mTOR inhibition, supporting its participation in the signalling cascade leading to proliferation. Moreover, the addition of these inhibitors to IL-2 stimulated cultures generated comparable profiles in naïve-Tregs, indicating that these pathways are utilized by both cytokines (data not shown).

Overall, our *in vitro* data reveal that both IL-7 and IL-2 are able to promote proliferation of naïve-Tregs via activation of PI3K/mTOR signalling, and that IL-7 promotes the survival and proliferation of FoxP3$^+$ mature thymocytes and circulating naïve-Tregs.

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3.2.5. Discussion

Naïve-Tregs are thymus-committed regulatory T-cells with a high degree of self-reactivity that continuously replenish the activated memory-Treg pool. Our finding of a preserved naïve-Treg compartment in adults submitted to total thymectomy in infancy support the relevance of peripheral mechanisms to ensure naïve-Treg homeostasis. We further showed that in steady-state conditions human naïve-Treg homeostasis occurs through a combination of high turnover rate and extended survival mediated by IL-7 and/or IL-2, based on the following evidence: 1) higher levels of the pro-survival molecule Bcl-2 and proportion of cycling-cells than naïve-Tconvs in both blood and tonsils; 2) the highest *ex vivo* levels of pSTAT5 as compared to other naïve/memory CD4 T-cell subsets, which further increases, in a dose-dependent manner, following IL-7 or IL-2 stimulation; 3) recovery of IL-7Rα expression upon IL-7 deprivation, consistent with an ongoing *in vivo* response to IL-7; 4) response to IL-7 and IL-2 in culture indicated by up-regulation of Bcl-2, and induction of PI3K/mTOR-mediated proliferation; and 5) maintenance of naïve and suppressive phenotype upon exposure to IL-7. Additionally, we showed that naïve-Treg precursors, namely FoxP3^{+}CD4SP thymocytes, already proliferate and up-regulate Bcl-2 in response to IL-7 stimulation *in vitro*.

Thus, our results challenge the assumption that naïve-Tregs are a quiescent population in steady-state conditions in non-lymphopenic individuals [1]. Human naïve-Tregs have been shown to feature a functional and molecular profile partially overlapping with both memory-Tregs and naïve-Tconvs [1, 4, 6]. Regarding their homeostasis, as for the memory compartment we found a much higher cell-turnover in naïve-Tregs than in naïve-Tconvs, suggesting an intrinsic propensity of Tregs to enter cell cycle. On the other hand, in agreement with their naïve character, naïve-Treg turnover was lower and Bcl-2 levels higher than in their memory counterparts. Additionally, similar pathways were apparently operating in response to IL-7 in naïve-Tregs and naïve-Tconvs.

In fact, we revealed here, for the first time, a role for IL-7 in naïve-Treg survival and homeostatic proliferation. These findings are particularly relevant for understanding

the impact of *IL-7/IL-7Rα* polymorphisms on autoimmunity [31], and the mechanisms underlying Treg expansion in lymphopenic settings, with implications for the therapeutic use of IL-7.

Our data do not exclude the contribution of self-peptide/MHC stimulation to the naïve-Treg proliferation, which may be particularly important given their enrichment in self-antigen reactivities [6]. However, TCR stimulation of naïve-Tregs has been associated with rapid acquisition of a memory phenotype, with the potential risk of contraction of the naïve compartment [1, 4]. Of note, the maintenance of murine naïve-like FoxP3$^+$ CD4 T-cells was recently demonstrated to be independent of TCR-driven signals [32].

Importantly, we showed that naïve-Treg proliferation in response to IL-7 or IL-2 was associated with preservation of the naïve-like phenotype, in parallel with the maintenance or even increase of regulatory markers. We further showed that prior exposure of naïve-Tregs to IL-7 did not impair their suppressive capacity.

IL-7, like IL-2, is known to induce PD-1 and PD-L1 on Tconvs [33] and Tregs [20]. Our observations extended these reports, by showing that IL-7 up-regulated PD-L1, but not PD-1, on both naïve-Tregs and naïve-Tconvs. These results suggest that IL-7 contributes not only to T-cell homeostasis, but also to the containment of immunopathology, by up-regulating the inhibitory molecule PD-L1 on naïve CD4 T-cells, irrespective of their FoxP3 status. On the other hand, this may have implications for tumor immunity, given the clinically relevant suppression of tumor specific responses mediated by the PD-1/PD-L1 pathway [34].

SLO are an important source of IL-7 [10, 17]. Our results from tonsil tissue, using both flow-cytometry and immunofluorescence, confirmed a high local naïve-Treg turnover, suggesting that SLO contain appropriate niches for naïve-Treg homeostasis. Nevertheless, more detailed studies are required to decipher the topography of naïve-Tregs in SLO, as well as the contribution of their possible interactions with IL-7-producing stromal reticular cells, dendritic cells or IL-2-producing memory T-cells.

Our data argue for the sufficiency of peripheral proliferation and survival to achieve long-term maintenance of the naïve-Treg compartment even in the absence of thymic output. These results in adults expand upon a recent report describing preservation of naïve-Treg counts during short-term follow-up of children submitted to total thymectomy in early infancy [15].

Although a previous study showed a reduction of naïve-Treg frequency within total Tregs [13], this was not related to an actual contraction of naïve-Tregs upon thymectomy, but rather to the relatively high frequency of total Tregs, as also observed in our study (Table I; proportion of naïve-Tregs within total Treg compartment: 11.60%[9.08-18.00] in thymectomized *versus* 14.85%[12.05-23.95] in healthy subjects, *P*=0.3776).

The increase in naïve-Treg cycling in thymectomized adults was more likely mediated by IL-7 than by self-peptide/MHC interaction, given the conserved frequency of $CD31^+$ cells, which are typically lost following TCR stimulation [16], and maintained by IL-7 [17]. We further showed that the $CD31^+$ subset of naïve-Tregs was preferentially expanded by IL-7 *in vitro*, in agreement with our previous data generated with total naïve CD4 T-cells [17]. Adults thymectomized in infancy feature an unexpectedly low prevalence of autoimmune diseases and allergy [12] , which may be related to the maintenance of the broadly-reactive naïve-Treg pool.

To our knowledge, there are no published data specifically addressing the changes in the naïve-Treg compartment in individuals receiving IL-7 therapy. Nevertheless, an increase in total Treg numbers was documented in clinical trials, although the expansion of the Tconv compartment was much higher, leading to maintenance [35, 36] or a relative decrease [37-39] in Treg proportions. In agreement with our *in vitro* data, naïve-Treg expansion was reported upon IL-2 therapy [28, 40, 41].
In severe lymphopenic settings associated with elevated serum IL-7 levels, such as HIV infection [42], there is an apparent preservation of circulating naïve-Tregs [43], raising the possibility that these cells are responding to IL-7. We did not find a correlation between the size of the naïve-Treg compartment and IL-7 serum levels in either our thymectomized individuals or the study population as a whole, which may be due to

the narrow range of IL-7 levels (Table 1). Our data are also relevant in other lymphopenic settings, particularly in the context of immune reconstitution following hematopoietic stem cell transplantation, where IL-7-driven proliferation of naïve-Tregs may help to control graft *versus* host disease. Our results support a model in which the increased availability of IL-7 in lymphopenic settings may contribute to the expansion of the naïve-Treg compartment, thereby helping prevent immunopathology in the context of a constrained TCR repertoire.

In conclusion, naïve-Tregs feature high turnover in healthy adults, which further increases to compensate the loss of thymic replenishment upon total thymectomy. Moreover, our data reveal a role for IL-7 in naïve-Treg maintenance, both through up-regulation of the survival molecule Bcl-2 and induction of PI3K/mTOR-mediated proliferation. Clinical use and evaluation of therapies targeting the IL-7 pathway should take into account the contribution of IL-7 to naïve-Treg homeostasis.

Acknowledgments

We thank the sample donors for their collaboration; Cardiothoracic Department, Hospital de Santa Cruz, for thymus collection; ENT Department, Hospital de Santa Maria, for tonsil collection; R Tendeiro, R Azevedo, H Nunes-Cabaço, MC Pereira-Santos, and AI Pinheiro for technical assistance; B Silva-Santos for fruitful discussions.

Disclosure of potential conflicts of interest

The authors declare no conflict of interest.

Grant support

This work was supported by Fundação para a Ciência e Tecnologia (FCT; POCI2010/IC/83068/2007 to RMMV; PTDC/SAU-MIC/109786/2009 to AES), and Gulbenkian Foundation (96526/2009 to JF; P132532/2013 to AES). SLS, ASA, RBF, ARP, PM and SMF received FCT scholarships.

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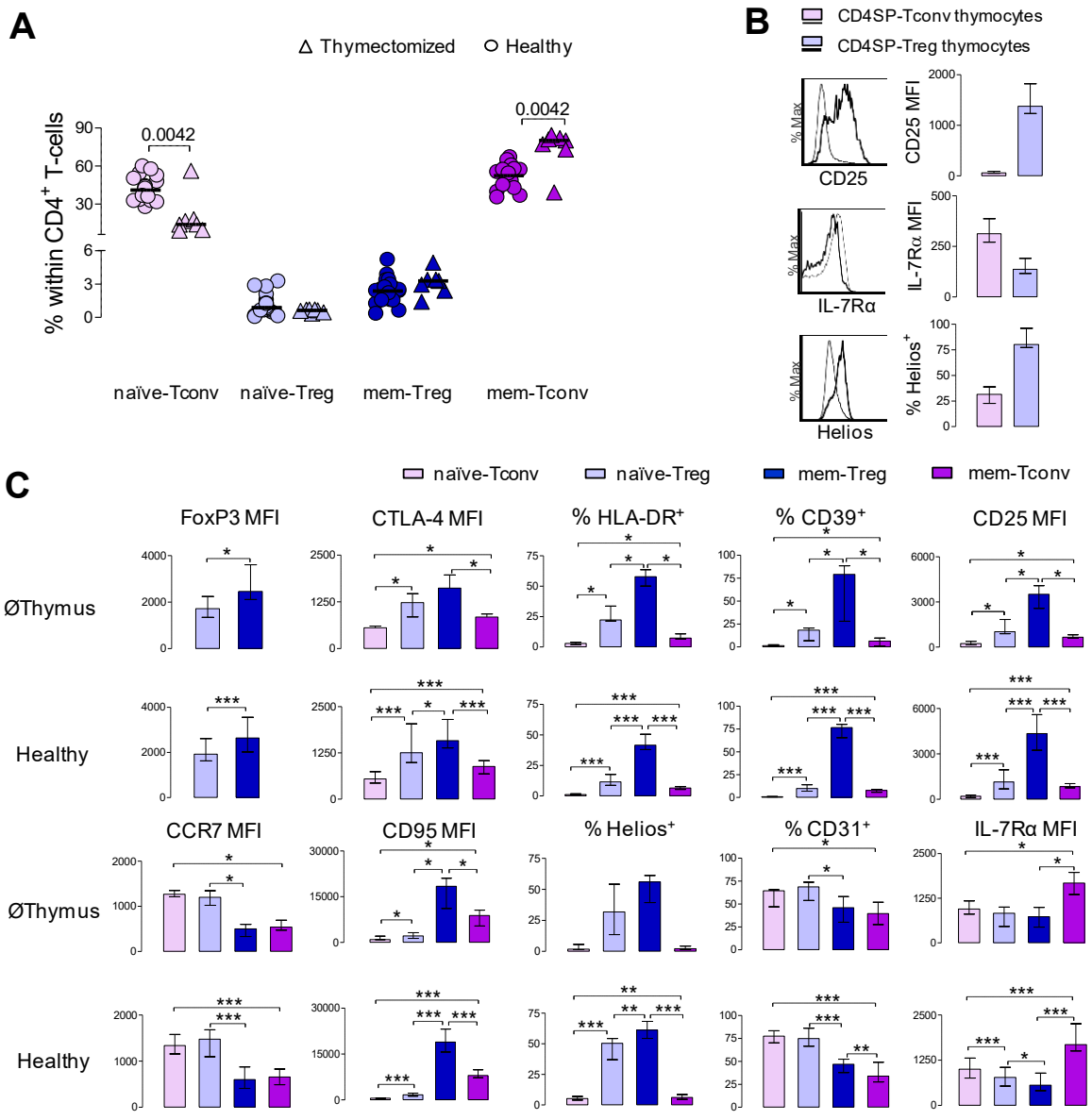
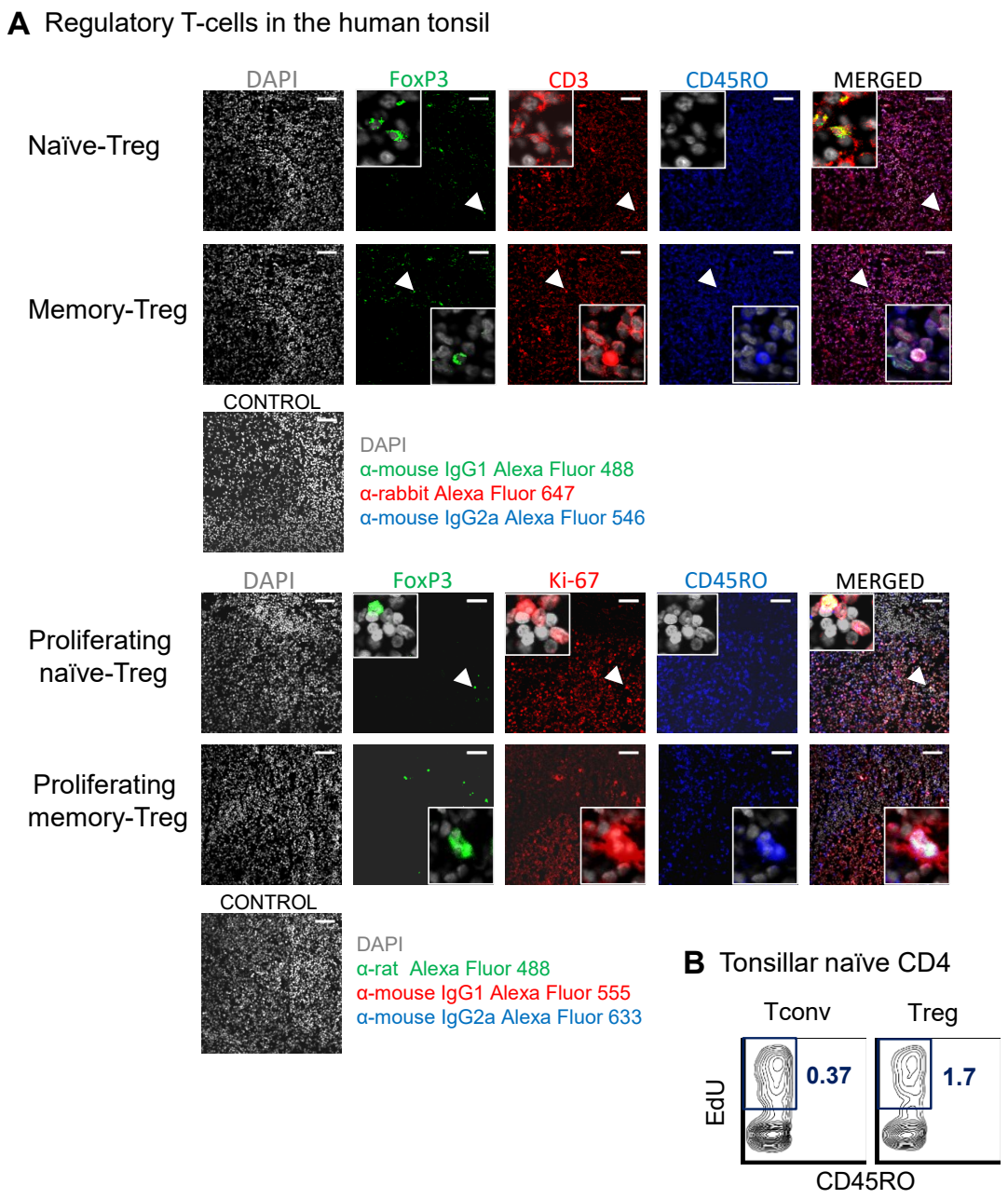
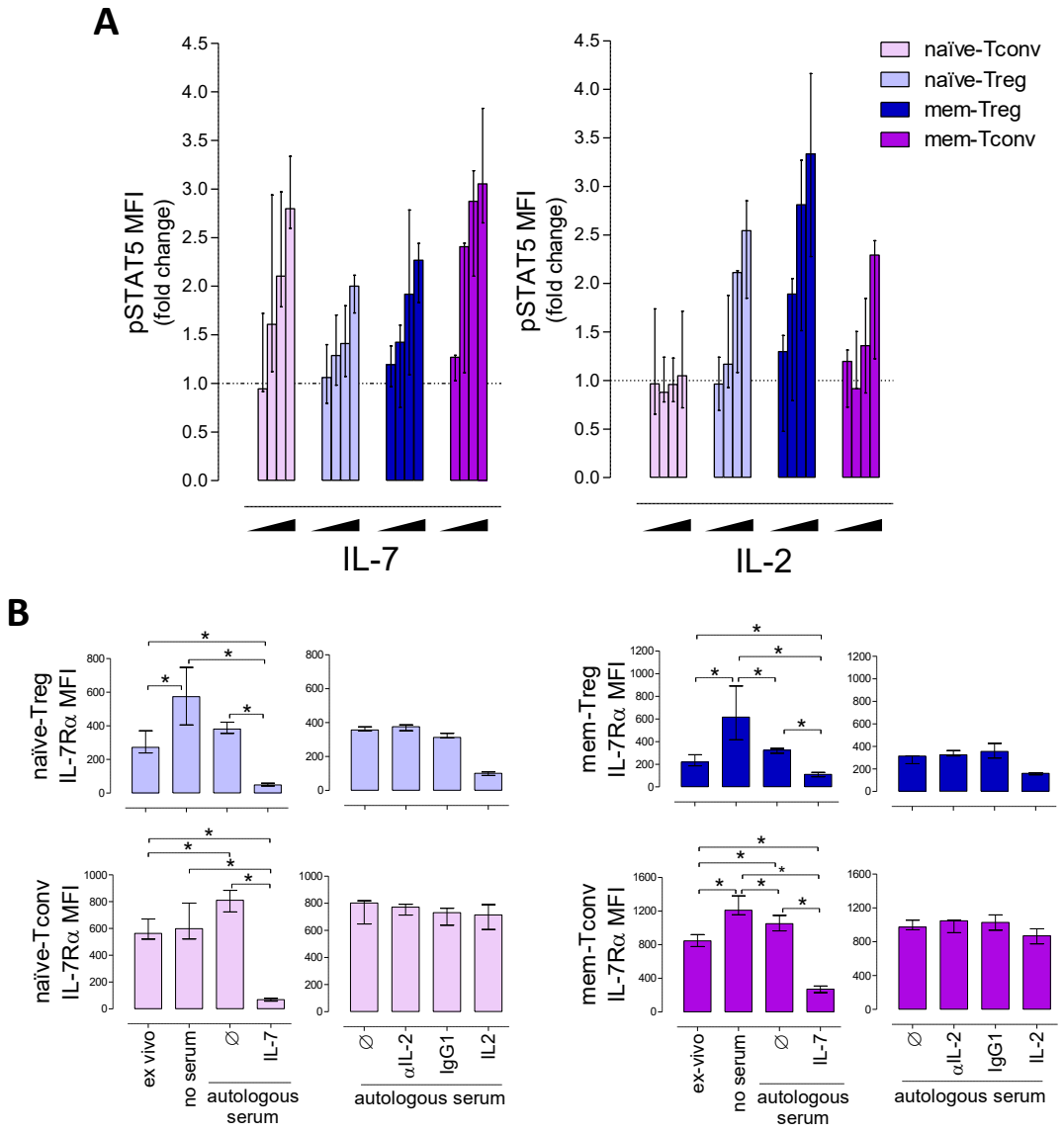
3.2.7. Supplementary Data



Supplementary Figure 1. ***Imbalances of CD4 T-cell subsets in adults submitted to total thymectomy in infancy.*** **(A)** Frequency of naïve-Tconv, naïve-Treg, mem-Treg, and mem-Tconv within circulating CD4+ T-cells in thymectomized and age-matched healthy subjects; each dot represents one individual; comparisons performed for each subset between the two cohorts; significant *P*-values are shown. **(B)** Subset phenotype (numbers of thymectomized and healthy, respectively, shown in brackets): FoxP3 (7/22), CTLA-4 (6/21), HLA-DR (7/22), CD39 (7/22), CD25 (6/21), CCR7 (7/21), CD95 (7/21), Helios (5/17), CD31 (7/16), and IL-7Rα (6/16); subsets within naïve and memory, as well as within Treg and Tconv compartments were compared; bars represent median and range; significant *P*-values are shown: * $P<0.05$, ** $P<0.005$, *** $P<0.001$. **(C)** CD25, IL-7Rα and Helios, within FoxP3+ (CD4SP-Treg) and FoxP3− (CD4SP-Tconv) CD4SP thymocytes from three children (1, 3, and 9m-old); bars represent median and range; % of mature CD4SP thymocytes ($CD45RA^{bright}$) lacking Helios expression varied between 4.5% and 19.6%.



Supplementary Figure 2. ***Naïve-Treg proliferation in the human tonsil.*** **(A)** Illustrative immunofluorescence analysis of regulatory T-cells in human tonsil showing naïve ($CD3^+CD45RO^-$) and memory ($CD3^+CD45RO^+$) $FoxP3^+$ Treg in the top; and proliferating ($Ki\text{-}67^+$) naïve-Treg ($CD45RO^-FoxP3^+$) and memory-Treg ($CD45RO^+FoxP3^+$) in the bottom; DAPI was used to counter-stain nuclei. Illustrative images of single stainings, merged image, as well as the control staining in the absence of the primary antibodies are shown (resolution: 1024x1024; insert magnification: 200x, scale bar: 2.409 pixels/µm; length shown: 60µm); images were acquired using a Zeiss LSM710 microscope (Zeiss, Oberkochen, Germany) with a dry objective Plan-Apochromat with a numerical aperture of 0.8. **(B)** Contour-plots illustrating proportion of tonsillar naïve-Tregs and naïve-Tconvs incorporating EdU upon 12h-culture of freshly-isolated CD4 T-cells.



Supplementary Figure 3. ***Ex vivo evidence of naïve-Treg response to IL-7*****. (A)** IL-7 and IL-2 dose-dependent induction of pSTAT5 following 15min *in vitro* stimulation of purified CD4 T-cells; graphs show pSTAT5 MFI fold change in relation to non-stimulated cells in the gated CD4 T-cell subsets in three individuals; bars represent median with range. **(B)** IL-7Rα modulation in CD4 T-cell subsets according to exposure to IL-7 or IL-2; purified CD4 T-cells were cultured for 24h in the absence of IL-7/IL-2 (no serum), or with 40% autologous serum either alone or further supplemented with: IL-7 (10ng/ml), IL-2 (20IU/mL), anti-IL-2 blocking mAb (10μg/mL), or isotype-control (IgG1, 10μg/mL); graphs show the IL-7Rα MFI within gated CD4 T-cell subsets *ex vivo* and post-culture, in six (left-hand graphs) and three (right-hand graphs) individuals; bars represent median with interquartile range (left-hand graphs; significant *P*-values are shown: **P*<0.05) or median with range (right-hand graphs).

Supplementary Table 1. ***Antibodies used in Flow-Cytometry and Immunofluorescence***

Antibodies for Flow Cytometry			
Antibody	Fluorochrome	Clone	Source
CD3	PerCP-Cy5.5/eFluor450	OKT3	eBioscience [a]
CD3	APC	UCHT1	eBioscience
CD3	V500	UCHT1	BD Bioscience [b]
CD4	PE/PerCP-Cy5.5/PE-Cy7/APC	RPA-T4	eBioscience
CD4	V500	RPA-T4	BD Bioscience
CD11c	FITC	3.9	eBioscience
CD14	FITC	61D3	eBioscience
CD19	FITC	HIB19	eBioscience
CD123	FITC	6H6	eBioscience
CD45RA	FITC/PerCP-Cy5.5/APC	HI100	eBioscience
CD45RA	eFluor450	2D1	eBioscience
CD45RA	PE-Cy7	L48	BD Bioscience
CD45RO	FITC/PerCP eFluor710/APC-H7	UCHL1	eBioscience
CCR7	FITC	150503	R&D Systems [c]
CCR7	PE	3D12	R&D Systems
CD25	PE-Cy7	2A3	BD Bioscience
CD27	PE/PE-Cy7	O323	eBioscience
CD31	PE/APC	WM-59	eBioscience
CD39	FITC/APC	eBioA1	eBioscience
CD95	PE-Cy7	DX2	eBioscience
CD127	Alexa Fluor660/APC eFluor780	eBioRDR5	eBioscience
HLA-DR	FITC	L243	BD Bioscience
HLA-DR	V500	G46.6	BD Bioscience
PD-1	FITC	MIH-4	eBioscience
PD-L1	APC	BIH1	eBioscience
FoxP3	PE/PerCP-Cy5.5/eFluor450	PCH101	eBioscience
CTLA-4	PE	BNI3	BD Bioscience
Helios	Alexa Fluor488	22F6	Biolegend [d]
Ki-67	FITC/PE/PerCP-Cy5.5/Alexa Fluor647	B56	BD Bioscience
Bcl-2	FITC	124	DAKO [e]
IL-2	Brilliant violet421	MQ1-17H12	Biolegend
IL-4	APC	8D4-8	eBioscience
IL-17A	PerCP-Cy5.5	eBio64DEC17	eBioscience
IFN-γ	PE-Cy7	4S.B3	eBioscience
pSTAT5	PerCP-Cy5.5	47/pY694	BD Bioscience
LIVE/DEAD®	APC-Cy7	-	Thermo Fischer Scientific [f]
Primary Antibodies for Immunofluorescence			
Antigen	Species/isotype	Clone	Source
FoxP3	Mouse/IgG1	236/E7	eBioscience
FoxP3	Rat/IgG2a	PCH101	eBioscience
Ki-67	Mouse/IgG1	B56	BD Bioscience
CD45RO	Mouse/IgG2a	UCHL1	eBioscience
CD3	Rabbit/NA	Polyclonal	Abcam [g]
Secondary Antibodies for Immunofluorescence			
Specificity	Species	Fluorochrome	Source
Mouse IgG1	Goat	Alexa Fluor 488	Thermo Fischer Scientific
Mouse IgG2a	Goat	Alexa Fluor 546	Thermo Fischer Scientific
Rabbit	Goat	Alexa Fluor 647	Thermo Fischer Scientific
Rat	Goat	Alexa Fluor 488	Thermo Fischer Scientific
Mouse IgG1	Goat	Alexa Fluor 555	Thermo Fischer Scientific
Mouse IgG2a	Goat	Alexa Fluor 633	Thermo Fischer Scientific

[a] eBioscience, San Diego, CA; [b] BD Bioscience, San Jose, CA; [c] R&D Systems, Minneapolis, MN; [d] Biolegend, San Diego, CA; [e] DAKO, Glostrup, Denmark; [f] Thermo Fischer Scientific, Walthan, MA; [g] Abcam, Cambridge, UK. NA- Not Applicable

3.3 Autoimmunity and allergy in adults submitted to complete thymectomy early in infancy

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Key-words

Thymus, thymectomy, allergy, autoimmunity, autoreactivity, tolerance, sensitization, regulatory T-cells, autoantibody microarray, specific IgE microarray

Manuscript under submission

3.3.1. Manuscript

To the Editor

The thymus is essential to the establishment of the "peripheral" T-cell compartment before birth and during the accelerated somatic growth of childhood, and contributes to its continuous replenishment until at least the sixth decade of life[1]. Thymus removal early in infancy during corrective cardiac surgery is, therefore, associated with marked contraction of the naïve T-cell subset, as well as with the presence of markers of premature immune senescence as a result of homeostatic naïve T-cell proliferation[1]. This is thought to occur mainly in response to self and environmental antigens, raising the question whether early thymectomy leads to an increased risk of autoimmunity and/or allergic disease. The few studies available are not conclusive, in part due to cohort heterogeneity regarding length of follow-up post-thymectomy and degree of residual thymic activity[1-3]. Notably, thymic recovery has been reported in some individuals[4].

Autoimmunity and allergy are controlled by a subset of regulatory CD4 T-cells (Tregs), defined by FoxP3 expression. Tregs generated in the thymus are particularly implicated in the maintenance of self-tolerance. They egress from the thymus with a naïve phenotype (naïve-Tregs), and continuously replenish the fully-suppressor memory-Treg compartment throughout life. We recently reported that naïve-Tregs are preserved in adults more than 18y (median 21y) after complete thymectomy early in infancy, in contrast with the marked contraction of conventional naïve CD4 T-cells[5]. Importantly, in contrast to others[2, 3], we specifically excluded individuals with evidence of remaining thymic activity, based on sjTREC quantification[5]. Naïve-Treg preservation was also found in a cohort of recently thymectomized children[6].

We hypothesized that the maintenance of naïve-Tregs limits the development of autoimmunity and/or allergy likely associated with the skewed conventional T-cell repertoire upon thymectomy. We applied an extensive structured questionnaire to assess clinical manifestations of autoimmunity and allergy, in parallel with the evaluation of serum reactivity to large panels of autoantigens and allergen components, to our cohort of complete thymectomized adults[5] and age-matched

individuals (Table 1, see supplementary methods in this article's Online Repository at www.jacionline.org).

In spite of the very low naïve CD4 and CD8 T-cell counts (Table 1), we found no autoimmune manifestations. Therefore, it is possible that Treg preservation, with evidence of suppressive phenotype based on FoxP3, CTLA-4 and CD39 expression levels (Table 1), may contribute to maintain self-tolerance.

In order to reveal a possible subclinical increase in IgG, IgM, IgA and/or IgE autoreactivity, we took advantage of autoantigen array-based technology[7], an emergent approach with high sensitivity for early and large-scale autoantibody identification (see supplementary methods in this article's Online Repository at www.jacionline.org). We compared the complete thymectomized adults with 7 representative controls (Table 1). By fitting a linear model to the normalized data, we did not find significant differences in serum IgG, IgM, IgA and IgE reactivity between cohorts ($P>0.05$); moreover, clustering analysis showed a clear inability to correctly assign thymectomized and controls (Figure 1, and see Table E1 and in this article's Online Repository at www.jacionline.org).

Regarding IgG reactivity, we identified 18 autoantibodies with increased expression within thymectomized ($P<0.05$, before multiple testing correction), and for which we obtained better clustering of samples according to their cohort of origin (Fig 1). Their targets included nuclear antigens (Sp100, chromatin, histone H1, ribophosphoprotein P1, SmD2, SmD3, TIF1gamma, Sm), cytoplasmic/membrane proteins (mitochondrial antigen, tissue transglutaminase, liver cytosolic 1 antigen, sphingomyelin), cell matrix proteins (fibronectin), glomeruli-specific proteins (glomerular basement membrane) and circulating proteins (complement C3a, complement C4, complement C8, factor D). Interestingly, these are common autoantigens identified in systemic lupus erythematosus (SLE) patients[7], some of them with high specificity, such as SmD proteins.

Table 1. ***Clinical, epidemiologic and immunologic characterization***

		Age/Gender	Autoimmunity	Allergy	Atopy Phadiatop®	ImmunoCAP ISAC®	CD8 T-cells/µl	% naïve within CD8[a]	CD4 T-cells/µl	% naïve within CD4[a]	% FoxP3$^+$ within CD4	% CD39$^+$ within mem Treg[b]	CTLA4 MFI within mem Treg[b]
Thymectomized													
Individual Code (Fig.1)	T1 (+)	22/F	No	No	-	-	367	7.3	799	13.0	3.1	21.7	879
	T2 (-)	24/M	No	Rhinitis	+	+[c]	239	25.8	654	48.8	5.3	79.2	1327
	T3 (-)	22/F	No	No	-	-	567	13.8	1153	15.3	5.6	76.4	1005
	T4 (+)	22/M	No	No	-	-	186	23.6	406	22.1	4.7	28.0	1229
	T5 (+)	26/M	No	No	-	-	366	4.2	1000	17.2	5.0	88.4	970
	T6 (+)	27/M	No	No	-	-	386	9.8	407	9.3	3.1	79.1	1346
	T7 (+)	26/M	No	Rhinitis	+	+[c]	352	4.8	785	10.3	5.1	90.6	1189
Cohort (n=7)		24 [22-27] 2F/5M	0	2	2	2	366 * [186–567]	9.8 *** [4.2-25.8]	785 [406–1153]	15.3 ** [9.3-48.8]	4.9% * [3.1-5.6]	77.8% [21.7-90.6]	1189 [879-13461
Controls													
Included in arrays (n=7)		23 [21-25] 3F/4M	0	2[d]	1[d]	2[d]	501 [307–863]	44.1 [31.5-56.1]	942 [588–1192]	40.0 [34.9-46.6]	2.9% [1.6-5.4]	75.1% [34.4-80.6]	1247 [808-1831]
Total (n=20)		21 [18-29] 12F/8M	0	7[e]	n.a.	n.a.	583 [307–921]	48.3 [22.9-70.6]	967 [566–1315]	42.2 [29.2-57.7]	2.9% [1.2-5.4]	76.5% [34.4-84.7]	1197 [808-1950]

n.a. Not applicable; F - female; M - male; Results are shown as median and range in brackets; *, **,*** p value <0.05; 0.01; 0.001 respectively, in comparison with controls (n=20). [a] Naïve cells were defined as CCR7$^+$RO$^-$. [b] mem Treg (memory Treg) were defined as CD4$^+$RO$^+$FoxP3$^+$. [c] ISAC® detectable specific IgE (KU/l) – T2: rDer f2: 1.4; rDer p2: 2.2; rLep d2: 12; rOle e1: 2.2; T7: rBlo t5: 2.1; nDer f1: 14; rDer f2: 22; nDer p1: 19; rDer p2 :34. [d] Allergic manifestations, Phadiatop®, ISAC® detectable specific IgE (KU/l) in the controls included in the arrays- C3: rhinitis, Phadiatop® +, rOlee1: 6.8 ; nPhp4: 3; rAlta1:5.3nDerf1 4.3; rDerf2 7.1; nDerp1:11; rDerp2 11; C6: peach allergy, Phadiatop® -, rPru p3: 22; nJug r3: 4. [e] Allergic manifestations in controls: rhinitis (n=4), rhinitis and asthma (n=1), atopic dermatitis and rhinitis (n=1); peach allergy (n=1)

Previous studies in SLE patients revealed the association of particular IgG autoantibody clusters with overall disease activity and lupus nephritis, one of which was partially reproduced in our thymectomized cohort (Figure 1, right; glomerular basement membrane/chromatin antibodies). Additionally, a cluster combining IgG autoantibodies to liver cytosolic antigen 1/mitochondrial antigen/tissue transglutaminase, antigens clearly associated with autoimmune liver disease[8], was also found in our thymectomized adults (Fig 1).

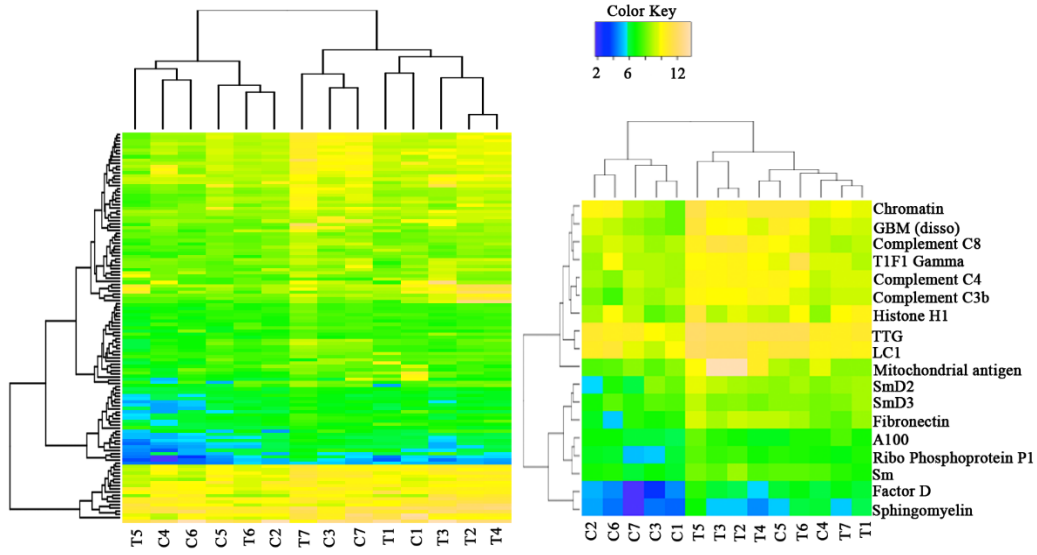

Figure 1. ***IgG autoantibody profiles*** Heatmap (left) of serum IgG autoantibody reactivities in adults thymectomized early in infancy (T) and controls (C) clustered by autoantigen (listed in Table E1 in this article's Online Repository at www.jacionline.org) and subject group. Reactivity intensity was normalized and log2-transformed, and 121 autoantibodies meeting minimal net fluorescence intensity requirements are presented. Heatmap (right) of the 18 autoantibodies with higher expression in thymectomized as compared to control individuals (*P*-value<0.05). Key color bar corresponds to quantified reactivity.

Recent data recall attention for a role of IgE reactivity against self-peptides in autoimmunity[9]. Using the same panel of antigens, we were able to identify IgE reactivity to a relatively small number of antigens in both cohorts (a median of 34 per individual in thymectomized *versus* 31 in controls in a total of 125 included in the array, *P*>0.05). There were 4 IgE autoantibodies, not previously reported[9], for which we observed different levels of reactivity between thymectomized and control individuals (*P*<0.05, before multiple testing correction), although without a coherent trend between the two cohorts, in contrast to IgG (see Fig E2 in this article's Online

Repository at www.jacionline.org). These findings reinforce the need for further investigation on IgE autoreactivity[9].

Regarding allergy, we found no significant differences in the prevalence of clinical manifestations (Table 1). Allergic rhinitis was the most frequent disease in both cohorts, as expected in young adulthood. In order to expose a putative increase in the frequency of IgE sensitizations (sIgE) upon complete thymectomy, we used a microarray with a large panel of allergen components (Table 1 and see supplementary methods in this article's Online Repository at www.jacionline.org). There were no differences in serum total IgE between thymectomized and controls (data not shown), and atopy was documented only in individuals with rhinitis (Table 1). The microarray revealed positive sIgE in patients with allergic disease, in agreement with their clinical manifestations (Table 1). Our findings suggest that thymectomy in early age has no major impact on the degree of sensitization.

Cohorts of adults submitted to thymectomy in infancy are expanding due to generalization of the access to early corrective/palliative cardiac surgery. Nevertheless, the eldest individuals are still in the fourth decade of life. Our finding of increased subclinical IgG autoreactivity against antigens with relevant clinical associations, stress the importance of extended patient follow-up to ultimately reveal whether peripheral tolerance will be able to limit overt progression towards autoimmune disease.

Overall, our study, that was strictly designed to include only adults without evidence of thymic activity upon thymus removal in infancy, revealed no increase in clinical/subclinical manifestations of allergy or in autoimmune diseases, in parallel with the preservation of the Treg compartment and contraction of the conventional naïve T-cell pool.

Author contributions

SLS, ASA, RMMV, and AES designed the study; SLS, ASA and MCPS performed research; SLS collected clinical data; SLS, AJA, QZL and CM analysed the data, AES supervised the study; SLS and AES wrote the paper.

Acknowledgments

We thank the sample donors for their collaboration; and Cardiothoracic Department, Hospital de Santa Cruz, for providing us access to the thymectomized cohort. We thank Ana R. Pires for technical assistance and Thermo-Fischer scientific for kindly providing the ImmunoCAP ISAC® Assay kits.

Funding

This work was supported by Fundação para a Ciência e Tecnologia (FCT; POCI2010/IC/83068/2007 to RMMV; PTDC/SAU-MIC/109786/2009 to AES), and Gulbenkian Foundation (P132532/2013 to AES). SLS, ASA and AJA received FCT scholarships. AJA was supported by an EMBO long-term fellowship (ALT-33-2010) and a Marie Curie European Integration Grant (PERG-GA-2009-256595).

3.3.2. References

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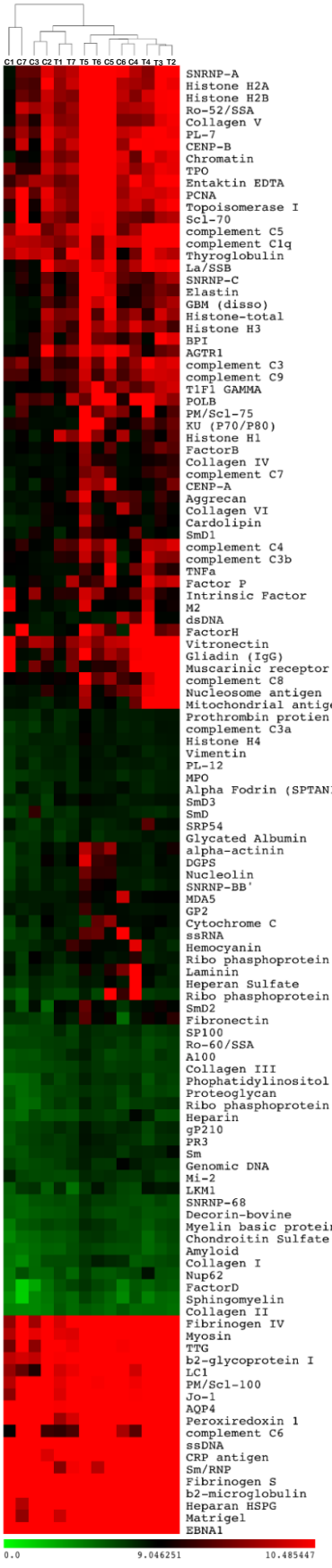
3.3.3. Supplemental Data

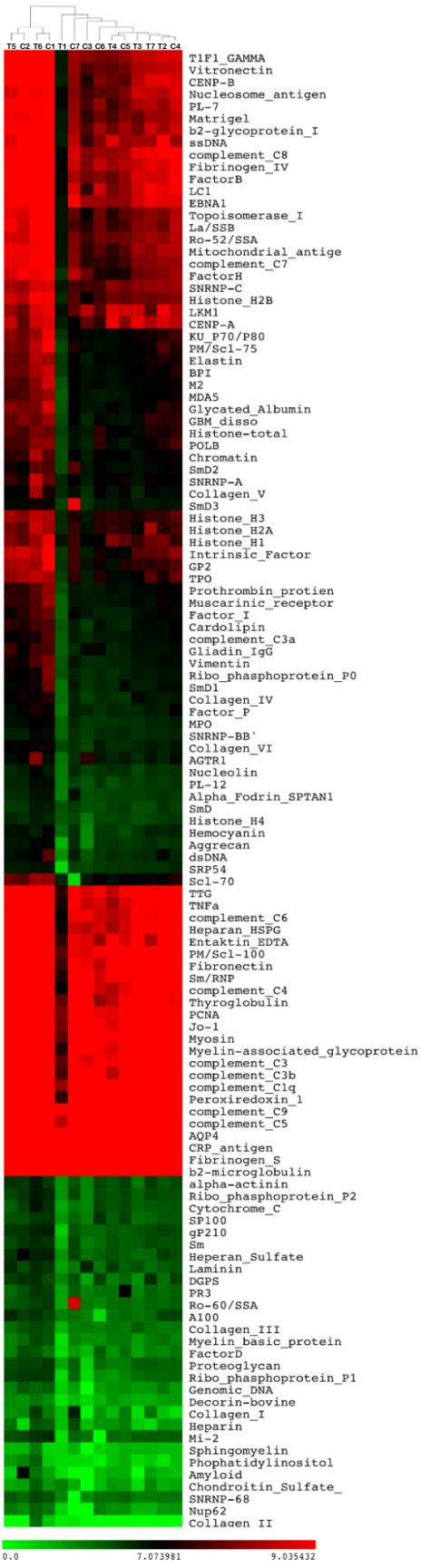
Supplemental Table E1. ***Sequence of antigens whose IgG reactivities are represented in heatmap for IgG autoreactivity (Fig 1).***

Sequence of antigens interested in heatmap for IgG autoreactivity (top-down order)					
1	SNRNP-A	42	TNFa	82	A100
2	Histone H2A	43	Factor P	83	Collagen III
3	Histone H2B	44	Intrinsic Factor	84	Phophatidylinositol
4	Ro-52/SSA	45	M2	85	Proteoglycan
5	Collagen V	46	dsDNA	86	Ribo phasphoprotein P1
6	PL-7	47	FactorH	87	Heparin
7	CENP-B	48	Vitronectin	88	gP210
8	Chromatin	49	Gliadin (IgG)	89	PR3
9	TPO	50	Muscarinic receptor	90	Sm
10	Entaktin EDTA	51	complement C8	91	Genomic DNA
11	PCNA	52	Nucleosome antigen	92	Mi-2
12	Topoisomerase I	53	Mitochondrial antige	93	LKM1
13	Scl-70	54	Prothrombin protien	94	SNRNP-68
14	complement C5	55	complement C3a	95	Decorin-bovine
15	complement C1q	56	Histone H4	96	Myelin basic protein
16	Thyroglobulin	57	Vimentin	97	Chondroitin Sulfate
17	La/SSB	58	PL-12	98	Amyloid
18	SNRNP-C	59	MPO	99	Collagen I
19	Elastin	60	Alpha Fodrin (SPTAN1)	100	Nup62
20	GBM (disso)	61	SmD3	101	FactorD
21	Histone-total	62	SmD	102	Sphingomyelin
22	Histone H3	63	SRP54	103	Collagen II
23	BPI	64	Glycated Albumin	104	Fibrinogen IV
24	AGTR1	65	alpha-actinin	105	Myosin
25	complement C3	66	DGPS	106	TTG
26	complement C9	67	Nucleolin	107	β2-glycoprotein I
27	T1F1 GAMMA	68	SNRNP-BB'	108	LC1
28	POLB	69	MDA5	109	PM/Scl-100
29	PM/Scl-75	70	GP2	110	Jo-1
30	KU (P70/P80)	71	Cytochrome C	111	AQP4
31	Histone H1	72	ssRNA	112	Peroxiredoxin 1
32	FactorB	73	Hemocyanin	113	complement C6
33	Collagen IV	74	Ribo phasphoprotein P2	114	ssDNA
34	complement C7	75	Laminin	115	CRP antigen
35	CENP-A	76	Heperan Sulfate	116	Sm/RNP
36	Aggrecan	77	Ribo phasphoprotein P0	117	Fibrinogen S
37	Collagen VI	78	SmD2	118	β2-microglobulin
38	Cardolipin	79	Fibronectin	119	Heparan HSPG
39	SmD1	80	SP100	120	Matrigel
40	complement C4	81	Ro-60/SSA	121	EBNA1
41	complement C3b				

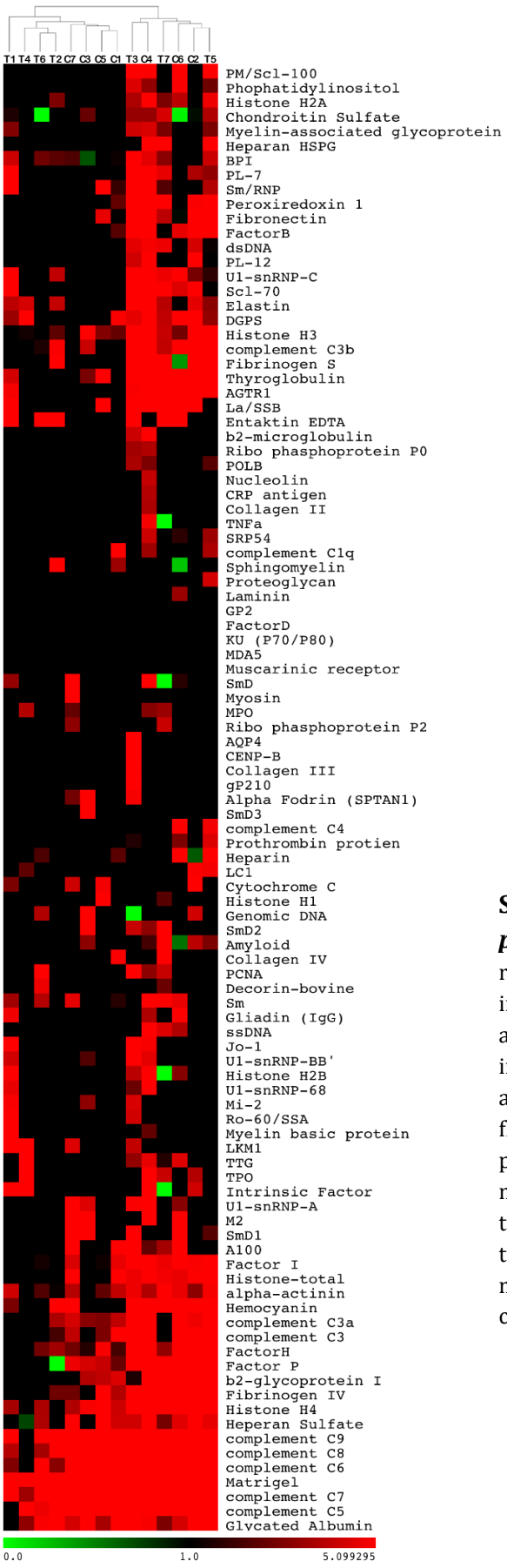
IgA

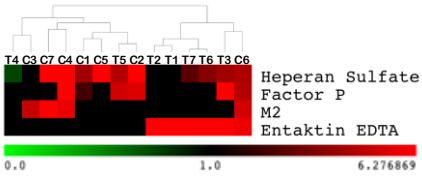

C1 C7 C3 C2 T1 T7 T5 T6 C5 C6 C4 T4 T3 T2
SNRNP-A
Histone H2A
Histone H2B
Ro-52/SSA
Collagen V
PL-7
CENP-B
Chromatin
TPO
Entaktin EDTA
PCNA
Topoisomerase I
Scl-70
complement C5
complement C1q
Thyroglobulin
La/SSB
SNRNP-C
Elastin
GBM (disso)
Histone-total
Histone H3
BPI
AGTR1
complement C3
complement C9
T1F1 GAMMA
POLB
PM/Scl-75
KU (P70/P80)
Histone H1
FactorB
Collagen IV
complement C7
CENP-A
Aggrecan
Collagen VI
Cardolipin
SmD1
complement C4
complement C3b
TNFa
Factor P
Intrinsic Factor
M2
dsDNA
FactorH
Vitronectin
Gliadin (IgG)
Muscarinic receptor
complement C8
Nucleosome antigen
Mitochondrial antige
Prothrombin protien
complement C3a
Histone H4
Vimentin
PL-12
MPO
Alpha Fodrin (SPTAN1)
SmD3
SmD
SRP54
Glycated Albumin
alpha-actinin
DGPS
Nucleolin
SNRNP-BB'
MDA5
GP2
Cytochrome C
ssRNA
Hemocyanin
Ribo phasphoprotein P2
Laminin
Heperan Sulfate
Ribo phasphoprotein P0
SmD2
Fibronectin
SP100
Ro-60/SSA
A100
Collagen III
Phophatidylinositol
Proteoglycan
Ribo phasphoprotein P1
Heparin
gP210
PR3
Sm
Genomic DNA
Mi-2
LKM1
SNRNP-68
Decorin-bovine
Myelin basic protein
Chondroitin Sulfate
Amyloid
Collagen I
Nup62
FactorD
Sphingomyelin
Collagen II
Fibrinogen IV
Myosin
TTG
b2-glycoprotein I
LC1
PM/Scl-100
Jo-1
AQP4
Peroxiredoxin 1
complement C6
ssDNA
CRP antigen
Sm/RNP
Fibrinogen S
b2-microglobulin
Heparan HSPG
Matrigel
EBNA1
0.0
9.046251
10.485447

IgM


T5 C2 T6 C1 T1 C7 C3 C6 T4 C5 T3 T7 T2 C4
T1F1_GAMMA
Vitronectin
CENP-B
Nucleosome_antigen
PL-7
Matrigel
b2-glycoprotein_I
ssDNA
complement_C8
Fibrinogen_IV
FactorB
LC1
EBNA1
Topoisomerase_I
La/SSB
Ro-52/SSA
Mitochondrial_antige
complement_C7
FactorH
SNRNP-C
Histone_H2B
LKM1
CENP-A
KU_P70/P80
PM/Scl-75
Elastin
BPI
M2
MDA5
Glycated_Albumin
GBM_disso
Histone-total
POLB
Chromatin
SmD2
SNRNP-A
Collagen_V
SmD3
Histone_H3
Histone_H2A
Histone_H1
Intrinsic_Factor
GP2
TPO
Prothrombin_protien
Muscarinic_receptor
Factor_I
Cardolipin
complement_C3a
Gliadin_IgG
Vimentin
Ribo_phasphoprotein_P0
SmD1
Collagen_IV
Factor_P
MPO
SNRNP-BB'
Collagen_VI
AGTR1
Nucleolin
PL-12
Alpha_Fodrin_SPTAN1
SmD
Histone_H4
Hemocyanin
Aggrecan
dsDNA
SRP54
Scl-70
TTG
TNFa
complement_C6
Heparan_HSPG
Entaktin_EDTA
PM/Scl-100
Fibronectin
Sm/RNP
complement_C4
Thyroglobulin
PCNA
Jo-1
Myosin
Myelin-associated_glycoprotein
complement_C3
complement_C3b
complement_C1q
Peroxiredoxin_1
complement_C9
complement_C5
AQP4
CRP_antigen
Fibrinogen_S
b2-microglobulin
alpha-actinin
Ribo_phasphoprotein_P2
Cytochrome_C
SP100
gP210
Sm
Heperan_Sulfate
Laminin
DGPS
PR3
Ro-60/SSA
A100
Collagen_III
Myelin_basic_protein
FactorD
Proteoglycan
Ribo_phasphoprotein_P1
Genomic_DNA
Decorin-bovine
Collagen_I
Heparin
Mi-2
Sphingomyelin
Phophatidylinositol
Amyloid
Chondroitin_Sulfate_
SNRNP-68
Nup62
Collagen II
0.0
7.073981
9.035432

Supplemental Figure E1 – *IgA and IgM autoantibody profiles.* Heatmaps of serum IgA (left) and IgM (right) autoantibody reactivities in adults thymectomized early in infancy (T) and controls (C), clustered by autoantigen and subject group. Reactivity intensities were normalized and log2-transformed; 121 IgA and 122 IgM autoantibodies meeting minimal net fluorescence intensity requirements are presented. Key color bars correspond to quantified reactivity.





Supplemental Figure E2. ***IgE autoantibody profiles.*** Heatmaps of serum IgE autoantibody reactivities in adults thymectomized early in infancy (T) and controls (C) clustered by autoantigen and subject group. Reactivity intensity was normalized and log2-transformed, and 100 IgE autoantibodies meeting minimal net fluorescence intensity requirements are presented in the heat map on the left. The heat map on the right shows IgE autoantibody profiles to 4 IgE autoantibodies distinctly expressed in thymectomized individuals (P<0.05, before multiple testing correction). Key color bars correspond to quantified reactivity.

Online Repository : Supplemental Methods

Study Design

A cohort of 7 adults submitted to thymectomy early in infancy, that we previously reported[1], was included in this study. Patients were thymectomized during corrective cardiac surgery for no syndromatic cardiopathy and complete thymectomy was confirmed based on the surgical report and severely reduced levels of sjTREC/µl. Thymectomized individuals were compared with age-matched controls (Table 1). Both cohorts were screened for clinical manifestations of autoimmunity and allergy. Blood was collected for *ex vivo* immune-phenotypic analysis and serum samples were stored at -80ºC. All subjects gave written informed consent for blood sampling. The study was approved by the Ethical Boards of Faculdade de Medicina da Universidade de Lisboa, Centro Hospitalar Lisboa Norte, and Hospital de Santa Cruz, Portugal.

Clinical evaluation of allergy and autoimmunity

An extensive structured questionnaire was applied to all individuals by the same investigator. Thymectomized and controls were inquired regarding previous diagnosis and clinical signs/symptoms of autoimmune or allergic diseases, namely rhinitis, asthma, eczema and food allergy. Modules of ISAAC Questionnaire validated for Portuguese speaking adults were included in this evaluation[2, 3].

Flow-cytometry

Ex vivo phenotypic analysis was performed in freshly-collected whole blood. Eight-color staining was performed using the following anti-human Abs, clones in brackets: CD3-eFluor450 (OKT3), CD8-eFluor 780 (RPA-T8), CD38-PE (HB7), CD45RO- APC-H7/APC (UCHL1), CD39-APC (eBioA1), FOXP3-eFluor450 (PCH101), HLA-DR PerCP eFluor 710 (L243), all from eBioscience, San Diego, CA; CD4-V500 (RPA-T4), CTLA-4-PE (BNI3) from BD Biosciences, San Jose, CA; CCR7-FITC (150503) R&D Systems, Minneapolis, MN. Intracellular staining was done using eBioscience FoxP3 kit. At least 150,000 events were acquired for each sample on a BD LSRFortessa (BD Biosciences) and data analysed using FlowJo software (TreeStar, Ashland, OR). Total cell numbers were calculated by multiplying the percentage of each population within total

lymphocytes by the peripheral blood lymphocyte count obtained at the clinical laboratory on the day of sampling.

sjTREC quantification

A highly sensitive nested quantitative PCR assay (detection-limit 1 copy/10^5 cells) was used as described[1, 4]. Triplicate multiplex PCR amplification of sjTREC, together with the CD3γ chain was performed on lysed PBMCs; quantifications were then performed using a LightCycler™ in independent experiments, with the same first-round serial dilution standard curve.

ImmunoCAP Phadiatop®

ImmunoCAP Phadiatop® (TermoFischer scientific, Waltham, MA) was used to assess the presence of IgE antibodies in the serum to a balanced mixture of relevant environmental allergens. Results were expressed as positive or negative, indicating the patient is atopic/non-atopic, respectively. ImmunoCAP Phadiatop® was performed according to manufacturer´s instructions.

Microarray for IgE sensitization

ImmunoCAP ISAC® (TermoFischer scientific), a miniaturized immunoassay platform was used to assess serum specific IgE (sIgE) to 112 allergen components from 51 aeroallergen or food sources. The purified, natural or recombinant allergen components are spotted in triplets and covalently immobilized on a polymer-coated slide. IgE antibodies in the sample bound to the immobilized allergen components are detected by a second fluorescence-labelled anti-IgE antibody. Fluorescence is measured with a laser scanner and results are evaluated using Phadia Microarray Image Analysis (MIA) software, on a semiquantitative basis. IgE values are presented in arbitrary units called ISAC standardized units (from 0.3 to 100 ISU). Values of >0.3 ISU were considered positive. All individuals were evaluated in the same day. ImmunoCap ISAC® was performed according to manufacturer´s instructions.

Autoantigen microarrays

Autoantigen microarrays were used to detect IgG/IgM or IgA/IgE autoantibodies in serum to a panels of 125 autoantigens as previously described[5]. A complete list of

the probes used and their sources is available from the authors on request. The same chip was used to test all individuals for each isotype. The samples were treated with DNAse I, diluted 1:50, and incubated with autoantigen arrays. The autoantibodies were detected in parallel arrays, with Cy3-labelled anti-human IgG and Cy5-labelled anti-human IgM (Jackson ImmunoResearch, West Grove, PA, USA), or Cy3-labeled anti-human IgA (Jackson ImmunoResearch) and TRITC-conjugated anti-human IgE (ThermoFisher Scientific) for imaging. Fluorescence was quantified using GenePix 4400A scanner with appropriate laser wavelengths and generated Tiff images. Genepix Pro 7.0 software was used to analyse the image and create the Gene Pix Results (GPR) file. The net fluorescence intensities were normalized using purified human IgG/IgM/IgA/gE spotted onto each array. Data obtained from duplicate spots were averaged. Signal-to-noise ratio (SNR) is used as a quantitative measure of the ability to resolve true signal from background noise. A higher SNR indicates higher signal over background noise. SNR equal or bigger than 3 are considered true signal from background noise.Normalized profiles of antigen-microarrays were log2 transformed. Using ggplots package[6] we have generated non-supervised hierarquical clustering heatmaps of the Euclidean distance matrix estimated from serum autoantibody reactivities.

Statistical analysis

Statistical analysis for epidemiological, clinical and flow-cytometry data, was performed with Graph Prism Version 5.01. Unpaired T-test or Mann-Whitney were used as appropriate; *P*-values <0.05 were considered significant.

To identify autoantigens with significantly different reactivity between controls and thymectomized patients, significance analysis of antigen-microarrays was performed using R statistical environment[7] and Limma package[8].

Supplemental References

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4. CONCLUSION AND FUTURE PERSPECTIVES

Conclusion and Future Perspectives

The thymus is the central organ for T-cell production that continuously replenishes the periphery with newly generated naïve CD4 T-cells [1]. The naïve compartment represents the lifelong reservoir of T-cells with a diverse repertoire ensuring ability to mount specific responses to new antigens [2-4]. Moreover, it includes a distinct lineage of cells with suppressor functions that expresses FoxP3 (naïve-Tregs) and are enriched in self-reactivities, thus considered essential to prevent autoimmunity [5, 6]

In spite of the continuous environment antigenic stimulation, and the age-associated reduction in thymic output, the size of the human naïve T-cell pool features only a slight decline throughout adulthood [7-10]. This is due to a dynamic equilibrium between thymic output and homeostatic proliferation of peripheral naïve CD4 T-cells, which replace those cells lost by death or conversion into memory-effector cells [3, 4].

The relative contribution of thymic output and homeostatic proliferation to the composition of the naïve CD4 T-cell pool throughout aging remains debatable [1]. It is known that human thymus is active until at least the sixth decade of life [7]. However, in contrast to mice, the establishment and maintenance of the human naïve CD4 T-cell compartment is currently thought to significantly rely on extensive post-thymic T-cell proliferation [1, 11-16]. Several *in silico* studies suggest that thymic output *per se* is insufficient to guarantee the size of the peripheral naïve T-cell compartment without a major contribution of cell proliferation in the periphery [1, 11-16].

The main objective of this work was to investigate the interplay between the different mechanisms operating in the homeostasis of the naïve CD4 T-cell compartment. Specifically, we investigated the impact of thymus impairment on peripheral homeostatic mechanisms and how naïve CD4 T-cells adjust to different degrees of thymic impairment. We used as a model adults thymectomized early in infancy, stratified according to the actual degree of thymopoiesis.

Our major findings are:

a) Adults thymectomized in infancy that featured some degree of actual thymopoiesis were able to preserve the size of the naïve CD4 T-cell compartment with a conserved spectratyping profile, in association with sj/β TREC ratio within the range of age-matched controls, supporting recovery of intra-thymic proliferation and of thymic output;
b) In contrast, those completely lacking thymopoiesis featured a contraction in the size and diversity of naïve CD4 T-cell compartment;
c) Although we documented significantly higher transcriptional levels of DUSP6, a gene involved in the fine-tuning of TCR activation threshold, in patients lacking thymopoiesis, this was not associated with impairment in the ability of purified naïve CD4 T-cells to respond to TCR stimulation and to differentiate into memory cells;
d) The $CD31^-$ naïve compartment was maintained independently of the thymus, and its size was tightly preserved by peripheral mechanisms;
e) The maintenance of IL-7-driven homeostatic proliferation of naïve CD4 T-cells required continuous thymic output;
f) Naïve-Treg cells were remarkably well preserved, irrespective of thymectomy;
g) Naïve-Treg cells were able to up-regulate Bcl-2 and to proliferate in a PI3K-dependent manner in response to IL-7, whilst preserving their naïve phenotype and suppressive capacity;
h) Patients lacking thymopoiesis featured no clinical autoimmunity or increased prevalence of allergy/IgE-sensitizations, although higher levels of IgG against a cluster of antigens linked to autoimmune diseases were found using a large array of autoantigens.

These data add significantly to the current literature and have important clinical implications, which are discussed below, in parallel with the future perspectives to pursue this work, organized in five major topics.

1. *Children thymectomized early in infancy provide a unique model to address the contribution of thymus and peripheral mechanisms to naïve CD4 T-cell homeostasis.*

Thymectomy is widely recognized as a powerful model to investigate the contribution of the thymus to the naïve T-cell maintenance beyond the establishment of the T-cell compartment.

Our study features important differences from previous follow-up studies upon thymectomy. We only included adults thymectomized within a relatively narrow age range in infancy, and patients were evaluated upon a median of 21y post-thymectomy, in contrast with studies that include children/adolescents with variable length of time post-thymectomy [17-22]. Moreover, thymectomized individuals were grouped according to absence (ØThy) or presence (Thy) of thymopoiesis, based on circulating sjTRECs/μl [23]. Our molecular strategy to stringently rule out the existence of thymic output in thymectomized patients overcomes the limitations of other approaches based solely on surgical reports [21, 22, 24-27] and/or thoracic imaging [17, 25-28], which may have neglected thymic regeneration or ectopic thymus, which is known to be frequent in humans [29].

There is a wide heterogeneity of findings reported by human studies addressing the impact of thymectomy in the naïve T-cell compartment [17, 18, 20-22, 24, 25, 27, 30, 31]. Our stratification of patients allowed us to reveal that the contraction of the naïve CD4 T-cell size and repertoire diversity was observed in the group of patients lacking thymopoiesis, in agreement with a premature immune-senescence profile, previously reported in some patients [21, 26, 31, 32]. In contrast, thymectomized patients that featured some degree of thymopoiesis were able to restore the naïve CD4 T-cell compartment, in size and diversity, showing a conserved spectratyping profile. Importantly, these thymectomized individuals also featured similar levels of sj/β TREC ratios as compared to age-matched controls, supporting the contribution of thymic recovery to the maintenance of the naïve CD4 T-cell compartment. Thus, in a long-term perspective, our data support the advantages of avoiding complete thymectomy during cardiac surgery [24, 33], particularly after the first year of life, given the observed association between younger age at thymectomy and thymic recovery [20, 27].

We have used spectratyping in our study to assess the diversity of the TCR repertoire in naïve CD4 T-cells. In the future, the possibility of using NGS-based techniques, that are currently being improved to use in this context [34, 35], may provide a deeper characterization of the impact of thymectomy on the repertoire of the naïve T-cell compartment. An important question, will be to understand if holes in the repertoire, found with spectratyping, correspond to deletions or rather to clonal expansions in the periphery [35, 36].

Extending the follow-up of our cohort will also be instrumental, as complete/partial thymectomy was performed during corrective cardiac surgery, that has only become routinely performed worldwide in infancy for the last 3 to 4 decades.

The extent of thymic regeneration post-thymectomy, and possible dependency on the age at thymus removal, also remains under debate [20, 27]. It would be important to have long-term longitudinal studies, starting from thymectomy, with sequential quantification of thymic activity in correlation with immunological profile. The use of imaging techniques directed to the thymus size and activity in that study would also be informative.

2. *The lack of thymic activity has distinct impacts on the CD31$^+$ and CD31$^-$ subsets*

Our data reveal distinct impacts of the lack of thymus activity on the 2 compartments of naïve CD4 T-cells defined according to CD31 expression. This is in agreement with previous work from our Laboratory and others, showing their distinct proliferative histories, and data supporting that different homeostatic mechanisms are operating in these compartments throughout life [2, 37-39].

In the literature, we found no previous data on the homeostasis of the naïve CD31$^-$ compartment upon thymectomy. Interestingly, we observed that this compartment was maintained, irrespective of the degree of thymus impairment. Our finding is in agreement with the CD31$^-$ preservation reported during age-associated thymic involution [2, 8] and suggests that peripheral mechanisms are determining the

homeostasis of this population. We also observed that even in healthy young adults, the levels of expression of the pro-survival molecule Bcl-2 were significantly higher in $CD31^-$ than in $CD31^+$ naïve CD4 T-cells [40], emphasizing the contribution of the survival pathways for the homeostasis of the $CD31^-$ subset.

In contrast, contraction of the naïve $CD31^+$ compartment has been previously reported in patients submitted to thymectomy [26, 27, 41]. We reveal that this contraction was only significant in individuals lacking thymopoiesis, and occurred despite homeostatic responses observed in both thymectomized cohorts, including increase in cell-survival, estimated by the expression of the pro-survival molecule Bcl-2, that we showed for the first time.

It has been hypothesized that CD31 might play a role on the trans-endothelial migration of $CD31^+$ naïve T-cells into SLO [38], a proposed site for homeostatic proliferation of naïve T-cells [42]. Furthermore, CD31 engagement has been shown to inhibit TCR-mediated signal transduction [43], raising the hypothesis that CD31 might hamper TCR/antigen-driven peripheral proliferation of $CD31^+$ naïve $CD4^+$ T cells [39], thus favour cytokine-driven homeostatic proliferation.

The peripheral expansion/survival in $CD31^+$ compartment is thought to be mainly mediated by IL-7, whereas the $CD31^-$ subset is thought to mainly proliferate in response to TCR stimulation by low-affinity self-peptide/MHC [2, 38, 39]. Here, we also reported this pattern in healthy subjects and in thymectomized patients featuring some degree of thymopoiesis. Conversely, in thymectomized adults lacking thymic activity the functional differences between the two naïve subsets were not apparent, in association with a significant impairment of IL-7 driven proliferation *in vitro*. This effacement of functional heterogeneity is in agreement with a recent report [27]. In future studies, it would be interesting to address the role of CD31 expression by naïve CD4 T-cells and to investigate the pathways that concur with CD31 to modulate naïve CD4 T-cell responses in the periphery.

3. *IL-7-mediated homeostatic proliferation is impaired in the absence of thymic activity*

We and others have previously shown the preferential role of IL-7 on the homeostasis of the CD31$^+$ naïve compartment, not only by enhancing thymopoiesis [44, 45] but also through the induction of survival and proliferation in the periphery [37, 46]. In face of the contraction of CD31$^+$ compartment upon thymectomy, we thus questioned whether thymectomy could be associated with altered homeostatic responses of naïve CD4 T-cells to IL-7, in addition to the obvious reduction in thymic output.

We showed that IL-7-driven proliferation and up-regulation of CD31 within CD31$^+$ naïve CD4 T-cells, which we have previously found to be dependent on signalling via PI3K pathway, were significantly impaired in the absence of thymic output [37, 47]. In contrast, Bcl-2 up-regulation, thought to be PI3K-independent and mediated by STAT5 phosphorylation, was preserved in these individuals.

Although based on a limited number of patients, our data support that the absence of thymopoiesis significantly impairs PI3K-dependent responses to IL-7. Future studies will be important to further dissect the mechanisms underlying this impairment. Another interesting question is to identify the possible subpopulation within naïve CD31$^+$ cells that is missing or that has lost the/its ability to proliferate in a PI3K-dependent manner in the absence of thymic activity, which will require new phenotypic markers.

These results have implications to different clinical settings in which IL-7/IL-7R axis based therapies have been under evaluation, namely in autoimmunity, lymphopenia and in immune reconstitution following hematopoietic stem cell transplantation (HSCT) or lymphoid depletion in the context of autoimmune disease or malignancy [48-53]. Administration of recombinant human (rh)IL-7 was shown to induce preferential expansion of naïve CD4 T-cells with a diverse TCR repertoire in lymphopenia associated with HIV infection [54, 55] or cancer therapy [48, 49]. Our data stress the requirements for continuous thymic output in these settings, in order to optimize response in the naïve CD4 compartment.

Although IL-7 has been reported to have *per se* an enhancing effect on thymopoiesis, additional therapies targeting the thymus might be considered in order to optimize IL-7-mediated immune reconstitution. IL-22 [56], human growth hormone [57, 58], and sex steroid inhibition [59] have been pointed as potential strategies for enhancing thymic function, and are currently under evaluation in clinical trials. Many other strategies are in preclinical stage of development, including therapies targeting thymocytes (IL-12, IL-21, keratinocyte growth factor) and TECs (IGF-1)(reviewed in [59]).

Therapies targeting thymic preservation/regeneration could also be considered in the elderly, in order to support an adequate homeostatic post-thymic proliferation and to delay the development of an immune-senescence profile. With the increase in life expectancy there is a growing number of individuals reaching advanced age with a high-risk immunological profile that has been linked to an increased frequency of infections, insufficient response to vaccines, autoimmunity and neoplasia, due to a decline in immune surveillance [60].

4. *The uniqueness of the naïve regulatory CD4 T-cell compartment*

Research on human regulatory T-cell homeostasis has been particularly scarce, despite its lifelong relevance in the maintenance of tolerance and therapeutic potential [6] Overall, our data provide evidence for an essential role of IL-7 in the maintenance of naïve-Tregs. Additionally, we challenge the assumption that naïve-Tregs are a quiescent population in steady-state conditions in non-lymphopenic individuals [5], given our finding of much higher cell-turnover in naïve-Tregs than naïve-Tconvs. Interestingly, we showed increased frequency of cycling naïve-Tregs in the tonsils than in blood, using samples from children undergoing routine tonsillectomy. This increase is more marked than that observed comparing frequencies of cycling-cells in naïve-Tconvs in tonsils and blood; and prompts us to pursue studies to find the *niches* where this homeostatic proliferation is occurring.

We also show that in healthy adults, IL-7 induced Bcl-2 expression and PI3K-dependent proliferation of naïve-Tregs, whilst preserving their naïve phenotype and suppressive capacity. Notably their proliferation in response to IL-7 was higher than that observed in response to to IL-2, thus revealing an unexpected role for IL-7 in the maintenance of naïve-Tregs.

Importantly, our data reveal distinct homeostasis of these subpopulations upon thymectomy. We found that: 1) naïve-Tregs were remarkably well preserved in individuals lacking thymopoiesis, in contrast to naïve-Tconvs, and featured an *ex vivo* phenotype suggestive of extended survival and proliferation; 2) in culture of purified naïve CD4 T-cells with IL-7, naïve-Tregs from individuals lacking thymopoiesis maintained better ability to proliferate to IL-7 than naïve-Tconvs. Future studies should address whethe, in the absence of thymic activity, naïve-Tregs proliferation in response to IL-7 is also PI3K-dependent. Moreover, it would be important to understand when possibly during thymic commitment these differences in signalling/metabolism between naïve-Tregs and naïve-Tconvs were established. Functional studies comparing healthy and thymectomized individuals would be informative in this regard.

To our knowledge, there are no published data specifically addressing the changes in the naïve-Treg compartment in individuals receiving IL-7 therapy. Nevertheless, an increase in total Treg numbers was documented in clinical trials, although the expansion of the Tconv compartment was much higher, leading to maintenance [61, 62] or a relative decrease [48, 49, 63] in Treg proportions. Our data call for reappraisal of the results of previous studies.

5. *Long-term clinical consequences of ceasing thymic activity early in infancy*

Clinical consequences of thymectomy performed in early infancy in humans have been a matter of debate. The few studies available are not conclusive[17, 19, 26, 30, 31], in part due to cohort heterogeneity regarding length of follow-up post-thymectomy and degree of residual thymic activity [64], as discussed above. In this study, the same

investigator/clinician performed the clinical evaluation of all individuals, using extensive structured questionnaires. Of note, none of our thymectomized patients reported severe infections or increased frequency of minor infections.

In face of a better preservation of naïve-Tregs in patients lacking thymopoiesis, despite naïve-Tconv pool contraction, we focused on the risk for autoimmunity/allergy.

We found no significant increase in the frequency of autoimmune manifestations or allergy/IgE-sensitizations. It is important to stress that the number of individuals per group in our study is obviously limited to assess differences in the prevalence of allergy or autoimmunity between thymectomized individuals and controls. Our innovative evaluation of subclinical autoreactivity/IgE-sensitizations thus adds interesting input to this debate. It has been shown that autoantibodies frequently precede clinical manifestations of autoimmunity for many years [65]. Our finding of higher IgG autoreactivity to autoantigens associated with autoimmune diseases in patients lacking thymopoiesis reinforces the need to extend their follow-up. This will ultimately reveal whether peripheral tolerance will be able to limit overt progression towards autoimmune disease.

Final Conclusion

Overall, our study comparing adults thymectomized early in infancy and age-matched controls, provides lessons regarding naïve CD4 T-cell compartment homeostasis in healthy, with particular implications for aging. Our data show, for the first time that IL-7 contributes to naïve-Treg homeostasis and to a thymus-independent survival of functional naïve-Tregs and point to the requirement of ongoing thymopoiesis to the maintenance of IL-7-driven homeostatic proliferation of conventional naïve CD4 T-cells. These findings have important implications to lymphopenic clinical settings and aging, supporting the future appraisal of strategies targeting the thymus, in order to maximize the homeostatic effect of IL-7.

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5. ANNEXES

www.impactjournals.com/oncotarget/ Oncotarget, Vol. 7, No. 11

Human naïve regulatory T-cells feature high steady-state turnover and are maintained by IL-7

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Keywords: human regulatory T-cells, naïve regulatory T-cells, regulatory T-cell homeostasis, thymectomy, IL-7, Immunology and Microbiology Section, Immune response, Immunity

Received: *December 17, 2015* ***Accepted:*** *January 26, 2016* ***Published:*** *February 19, 2016*

ABSTRACT

Naïve FoxP3-expressing regulatory T-cells (Tregs) are essential to control immune responses via continuous replenishment of the activated-Treg pool with thymus-committed suppressor cells. The mechanisms underlying naïve-Treg maintenance throughout life in face of the age-associated thymic involution remain unclear. We found that in adults thymectomized early in infancy the naïve-Treg pool is remarkably well preserved, in contrast to conventional naïve CD4 T-cells. Naïve-Tregs featured high levels of cycling and pro-survival markers, even in healthy individuals, and contrasted with other circulating naïve/memory CD4 T-cell subsets in terms of their strong γc-cytokine-dependent signaling, particularly in response to IL-7. Accordingly, *ex-vivo* stimulation of naïve-Tregs with IL-7 induced robust cytokine-dependent signaling, Bcl-2 expression, and phosphatidylinositol 3-kinase (PI3K)-dependent proliferation, whilst preserving naïve phenotype and suppressive capacity. Altogether, our data strongly implicate IL-7 in the thymus-independent long-term survival of functional naïve-Tregs, and highlight the potential of targeting the IL-7 pathway to modulate Tregs in different clinical settings.

INTRODUCTION

FoxP3-expressing regulatory T-cells (Treg) maintain tolerance to self and to the environment, and are central players in the control of immune responses in general [1]. Notwithstanding their relevance in limiting immune-mediated pathology, and their therapeutic potential, research focused on human Treg homeostasis has been scarce [2]. Human Tregs comprise a compartment of thymus-generated naïve-like cells (naïve-Tregs) that continuously replenish the pool of fully-suppressive activated Tregs expressing memory markers [1-7]. The mechanisms governing naïve-Treg homeostasis, in parallel with age-associated thymic involution, remain largely unknown. Moreover, the investigation of naïve-Treg homeostasis has been hampered by the difficulty in clearly identifying this subset in murine models [8].

The establishment and maintenance of the human naïve T-cell compartment is known to rely on both thymic output and peripheral expansion, with their relative contributions still the subject of intense debate. Increasing evidence points to a major contribution of peripheral proliferation in order to explain the relatively stable size of the naïve compartment in adulthood despite thymic involution [9, 10].

Individuals submitted to total thymectomy in early infancy due to corrective cardiac surgery provide an ideal clinical setting to address the peripheral contribution to long-term maintenance of naïve T-cell subsets. Contraction of the entire naïve compartment upon thymectomy has

been consistently reported, confirming the central role of the thymus [11-14]. Notwithstanding this, distinct homeostasis of the naïve-Treg and conventional naïve (naïve-Tconv) CD4 T-cell compartments have been suggested by a longitudinal study that showed naïve-Treg preservation up to 1 year post-thymectomy [15], although long-term data are lacking.

Peripheral homeostasis of naïve-Tconvs relies upon a slow rate of cell-turnover resulting from both cytokine-driven proliferation, and TCR-stimulation by low-affinity self-peptides [16]. Additionally, naïve-Tconv homeostasis is known to rely on pro-survival factors, such as Bcl-2, which are up-regulated by homeostatic cytokines, particularly IL-7 [16, 17].

Naïve-Tregs are characterized by high-affinity for self-peptides, and by rapidly differentiating into memory-Tregs upon T-cell receptor (TCR)-stimulation [1-6]. Thus, major histocompatibility (MHC)-self peptide stimulation is likely to result in loss of their naïve phenotype. On the other hand, in terms of the contribution of cytokine-driven proliferation, naïve-Tregs express low levels of receptors for the main homeostatic cytokines. CD25, the α-chain of the IL-2 receptor, is expressed at only intermediate/dim levels [1], questioning whether naïve-Tregs, like their memory counterparts, depend on IL-2 [2]. Moreover, Tregs *per se* typically express low levels of the α-chain of the IL-7 receptor (IL-7Rα), and there are controversial reports on the IL-7 impact on human and murine Tregs [18-22].

We investigated here the impact of IL-7 and IL-2 on peripheral naïve-Tregs from blood and secondary lymphoid organs (SLO), as well as on mature FoxP3$^+$ thymocytes, and provide evidence for a role of IL-7 in human naïve-Treg homeostasis. We show for the first time that naïve-Tregs feature much higher levels of the pro-survival molecule Bcl-2 and significantly higher turnover than naïve-Tconvs in healthy individuals. These parameters further increased in the absence of thymic replenishment, ensuring the long-term maintenance of the naïve-Treg compartment in total thymectomized individuals.

RESULTS

Preservation of the naïve-Treg compartment following thymus removal

Adults submitted to total thymectomy early in life provide a unique setting to investigate human naïve compartment homeostasis. However, published studies have been hampered by the lack of clear information regarding possible residual thymic activity that can result from either ectopic thymus or post-thymectomy regeneration [11, 12, 14, 23]. We applied here strict criteria to exclude residual thymic activity based on detailed surgical reports and single-joint TCR excision circles (sjTREC) levels clearly below the lowest level observed in healthy adults (Table 1). sjTRECs are by-products of TCR rearrangements during T-cell development that are progressively lost as cells divide in the periphery, and thus used to identify recent thymic emigrant cells [24]. Adults, with a median of 21 years (18-24.5) after total thymectomy, were compared with age-matched healthy individuals (Table 1).

We observed a significant decrease in circulating naïve-Tconvs, both in frequency within CD4 T-cells (P = 0.0042; Supplementary Figure 1A) and absolute numbers (P = 0.0026; Figure 1), in agreement with previous data from other thymectomized cohorts [11-14]. Conversely, the naïve-Treg pool size was preserved, as compared to healthy subjects (Figure 1 and Supplementary Figure 1A).

The healthy cohort spanned an age period associated with relatively stable thymic function and naïve-Treg numbers [6], and the size of the circulating naïve-Treg pool was within the range previously described [4-6]. We sorted naïve-Tregs, and confirmed that their *FOXP3* mRNA expression levels were comparable to those found in memory-Tregs (4526-21104 *versus* 1540-14363 relative copy numbers, respectively, n = 3 healthy adults), and much higher than those in naïve-Tconvs (34-115 relative copy numbers), confirming them as *bona fide* Tregs [1].

Circulating naïve-Tregs were confirmed to have a truly naïve phenotype both in healthy and thymectomized subjects, based on the expression of a panel of naïve markers and reduced CD95 expression, as well as to express Treg function-associated markers (CTLA-4, HLA-DR, CD39) at lower levels than memory-Tregs (Figure 1 and Supplementary Figure 1B). Although Helios has been proposed as a marker of thymus-derived Tregs, we showed that in both cohorts a significant proportion of circulating naïve-Tregs lacked Helios expression (Figure 1 and Supplementary Figure 1B), as already observed in human mature FoxP3$^+$ CD4 single-positive (CD4SP) thymocytes (Supplementary Figure 1C), questioning its usefulness as a marker of thymic-derived Tregs [25]. Regarding the CD31$^+$ subset, a population known to be enriched in recent thymic emigrants [16], no significant contraction was observed within naïve-Tregs of thymectomized, as compared to healthy individuals (P = 0.1708, Supplementary Figure 1B), in contrast to the significant reduction observed within naïve-Tconvs (P = 0.0122, Supplementary Figure 1B). This finding suggests that distinct homeostatic mechanisms are operating in the two naïve compartments upon thymectomy. Interestingly, we have previously shown that IL-7 up-regulates CD31 expression [17], further validating the importance of investigating the impact of IL-7 on naïve-Tregs, as detailed below.

In order to investigate the mechanisms underlying the preservation of the naïve-Treg compartment, we quantified the expression levels of markers of cell survival and cell cycling in thymectomized, as compared to healthy individuals. Bcl-2 expression was significantly higher, supporting a contribution of increased naïve-Treg survival to their maintenance upon thymectomy (P = 0.0143; Figure 1). Additionally, significantly higher frequencies of cycling-cells, as assessed by Ki-67, were documented (P = 0.0009; Figure 1). The degree of Bcl-2 increase in thymectomized was comparable in the two naïve compartments, whereas the increase in Ki-67$^+$ cell frequency was much more striking within naïve-Tregs (4-fold) than within naïve-Tconvs (1.7-fold), supporting an important contribution of increased cell-turnover to the better preservation of the naïve-Treg compartment.

Altogether, our data indicate that naïve-Tregs are preserved in adults submitted to total thymectomy in early infancy, mainly through increased homeostatic proliferation.

High steady-state turnover of the naïve-Treg compartment

The finding of a preserved naïve-Treg compartment in thymectomized adults prompted us to further investigate the mechanisms underlying naïve-Treg homeostasis under steady-state conditions in healthy young adults with stable thymic output.

We found that naïve-Tregs displayed significantly higher levels of Bcl-2 than both naïve-Tconvs and memory-Tregs (Figure 2A), suggesting that enhanced survival plays a major role in their maintenance. Therefore, in this respect, naïve-Tregs are distinct from memory-Tregs, which have been shown to have a pro-apoptotic profile [1, 4].

Additionally, naïve-Tregs featured a much higher *in-vivo* turnover than naïve-Tconvs (Figure 2A), with significantly elevated proportion of cycling-cells (2.23%[1.12-4.75] *versus* 0.17%[0.10-0.29], $P < 0.0001$). Thus, although naïve-Tregs are frequently labeled as "quiescent" as opposed to memory-Tregs, we show here that they featured levels of cycling comparable to those found in memory-Tconvs (Figure 2A).

Homeostatic naïve-Tconv proliferation occurs mainly in SLO [16]. We performed a parallel study of blood and tonsils, used here as an example of SLO due to ease of access to clinical samples. Tonsil naïve-Tregs featured a much higher proportion of cycling-cells than blood naïve-Tregs (Figure 2B). Notably, the fold-increase in cycling-cells in tonsils as compared to blood was much more striking in the naïve-Treg than in the naïve-Tconv compartment (4-fold *versus* 2-fold, respectively). The presence of naïve-Tregs expressing Ki-67 in tonsil tissue was further confirmed by immunofluorescence (Supplementary Figure 2A). Additionally, we showed that naïve-Tregs from freshly-collected tonsils consistently entered S-phase at higher rates than naïve-Tconvs, as estimated by the *ex-vivo* incorporation of 5-ethynyl-2'-deoxy-uridine (EdU), a thymidine analogue (Supplementary Figure 2B; 12 hours incubation: naïve-Treg: 1.23-1.70%; naïve-Tconv: 0.37-0.42%; n = 3). Of note, in tonsils, naïve-Tregs featured significantly higher proportion of Ki-67$^+$ cells than naïve-Tconvs (Figure 2B), as well as Bcl-2 MFI (mean fluorescence intensity; range 2777-3161 *versus* 1873-1992, n = 3, respectively). These results suggest that SLO may provide appropriate niches for naïve-Treg homeostatic proliferation.

Overall, our data show that, in healthy young adults, naïve-Tregs featured markers of enhanced survival and increased turnover in comparison to naïve-Tconvs, supporting an important contribution of peripheral proliferation to the maintenance of the naïve-Treg compartment.

Ex-vivo evidence for ongoing naïve-Treg response to IL-7

Next, we investigated the putative contribution of γc-cytokines to the increased turnover and survival of naïve-Tregs. *Ex-vivo* levels of pSTAT5, a downstream marker of both IL-7 and IL-2-mediated signaling, were significantly higher in naïve-Treg than in naïve-Tconv and memory CD4 subsets, analyzed in freshly-collected whole blood from healthy subjects (Figure 3A), irrespectively of the levels of expression of IL-7Rα and IL-2Rα (Supplementary Figure 1B). Additionally, we stimulated purified CD4 T-cells with increasing concentrations of IL-7 or IL-2, and clearly showed that naïve-Tregs responded to both cytokines, in a dose-dependent manner (Figure 3B

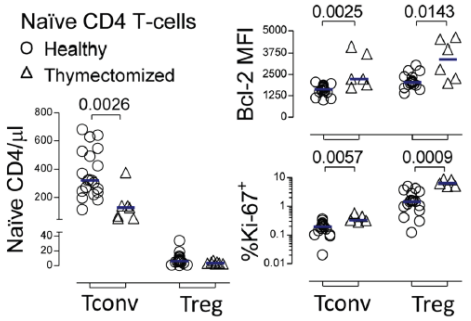

Figure 1: Preservation of the naïve-Treg compartment following thymus removal. Circulating naïve-Treg and naïve-Tconv counts; as well as % of cycling-cells, and Bcl-2 MFI within these subsets in healthy and thymectomized adults; each symbol represents one individual; bars represent median; significant P-values of comparisons within each subset are shown.

Table 1: Characterization of the cohorts

	Healthy	Thymectomized
Number	22	7
Age, years	21 [18-30]	24 [22-27]
Thymectomy, month of age	NA	23 [12-72]
sjTRECs/µl	16.6 [4.1-39.3]	0.5 [0.1-1.8]a
Lymphocytes/µl	2413 [1430-3502]	2010 [933-2618]
% CD4 T-cells	40 [29-52]	43 [23-47]
% Naïve[b] in CD4	40 [29-58]	15 [9-49][a]
% FoxP3$^+$ in CD4	2.9 [1.2-5.4]	4.1 [3.0-5.6]
FoxP3$^+$ cells/µl	21 [9-51]	28 [11-39]
Serum IL-7, pg/ml	15 [7-23]	14 [8-20]

Data are presented as median with limits in square brackets. NA - not applicable. [a]P<0.0001. [b]Naïve cells defined as CCR7+CD45RO−

and Supplementary Figure 3A). Our data extend previous human and murine studies on the ability of Tregs to phosphorylate STAT5 in response to IL-7 *in-vitro* [20, 26-28], through the comparison of naïve and memory Tregs, as well as their FoxP3 negative counterparts.

Since IL-7 signaling induces the down-regulation of its receptor [17], we reasoned that the level of recovery of IL-7Rα expression upon IL-7 deprivation would reflect the extent of ongoing IL-7 signaling *in-vivo*. IL-7Rα expression levels were quantified in purified CD4 T-cells from healthy subjects following 24 hours in different culture conditions (Figure 3C and Supplementary Figure 3B). Naïve-Tregs featured a significant recovery of IL-7Rα expression in the absence of IL-7 (culture in serum free medium), as compared to *ex-vivo* levels, in agreement with a significant ongoing IL-7 response. Notably, IL-7Rα expression was relatively preserved in the presence of autologous serum (IL-7 levels range: 15.8-23.5pg/ml). Moreover, the possible contribution of IL-2, either derived from serum or produced by cells in culture, was excluded by documenting a lack of impact of IL-2 blockade. Addition of exogenous IL-7 (10ng/ml) or IL-2 (20IU/ml) led to the expected down-regulation of IL-7Rα [29].

In conclusion, we showed that naïve-Tregs featured high *ex-vivo* pSTAT5 levels, and recovered IL-7Rα expression after IL-7 deprivation, supporting their ongoing response to IL-7 *in-vivo*.

Preserved naïve phenotype, Treg-markers and suppressive capacity upon naïve-Treg response to IL-7

We subsequently investigated the impact of IL-7 on naïve-Treg phenotype and suppressive capacity. For this purpose, total naïve CD4 T-cells were purified from freshly-collected blood of healthy donors, and cultured for up to 13 days in the presence of IL-7 or IL-2, using conditions previously optimized in our laboratory [17]. Analysis was performed within naïve-Tregs and naïve-Tconvs defined according to their FoxP3 expression, as illustrated in Figure 4A.

We confirmed that FoxP3 was not induced in naïve-Tconvs in the presence of IL-7, using sorted naïve-Tconvs cultured alone with IL-7, and showing no increase in FoxP3 expression either at the protein level, by flow-cytometry (Figure 4B), or at the transcriptional level by real-time PCR (*ex-vivo* range 34-115 *versus* 14-62 FOXP3 relative copy numbers after 13 day-culture, n = 3). FoxP3 induction was also not observed in cultures of purified naïve-Tconvs with IL-2 (data not shown). This is in agreement with the requirement for TCR stimulation for *de novo* induction of FoxP3 in conventional CD4 T-cells [1].

Importantly, despite the clear impact of both IL-7

and IL-2 on the recovery of naïve-Tregs in cultures of total naïve CD4 T-cells, neither cytokine was able to maintain sort-purified naïve-Tregs when they were cultured alone (Figure 4C). This finding revealed a requirement for naïve-Tregs to interact with naïve-Tconvs to ensure their survival, which was not overcome by the addition of IL-2 (Figure 4C). These data precluded purified naïve-Treg culture in isolation.

Of note, the overall proportion of naïve-Tregs within total naïve CD4 T-cells significantly increased upon 13 day-culture with IL-7 (from 0.99%[0.60-1.43] to 2.15%[1.29-3.21], $P = 0.0048$, $n = 16$). This increase was more evident upon culture with IL-2 (7.94%[3.65-11.75], $P = 0.0005$), likely due to the reduced impact of IL-2 on naïve-Tconvs (Figure 4C).

As illustrated in Figure 4D, FoxP3$^+$ cells maintained their naïve phenotype following 13 day-culture with either IL-7 or IL-2. Our previous work showed that IL-7 increases CD31 expression in naïve CD31$^+$ CD4 T-cells [17]. Here, we showed that naïve-Tregs cultured in the presence of IL-7 up-regulated CD31 MFI to levels comparable to naïve-Tconvs (fold-increase: 2.17[1.45-3.69] *versus* 2.17[1.73-2.46], $P = 0.4333$, respectively; Figure 4D). Moreover, naïve-Tregs up-regulated CD25 (fold-increase: 3.85[3.29-6.01], Figure 4E), and down-

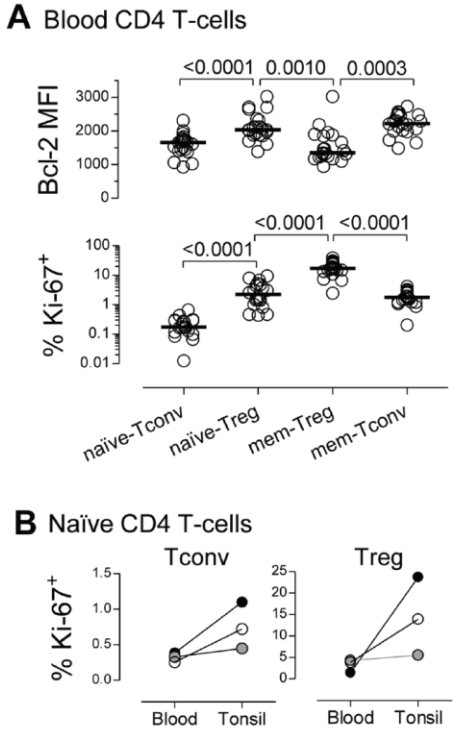

Figure 2: High steady-state turnover of the naïve-Treg compartment. A. Bcl-2 MFI and % of cycling-cells, within circulating CD4 T-cell subsets; each dot represents one individual; bars represent median; significant *P*-values are shown. **B.** % of cycling-cells within naïve-Tregs and naïve-Tconvs from matched blood and tonsil samples (each symbol represents one child: 2years/open, 6years/grey, and 7years/black).

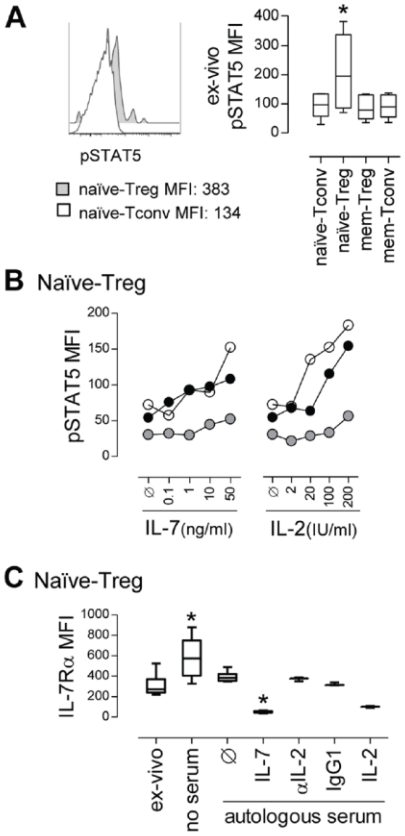

Figure 3: *Ex-vivo* evidence for ongoing naïve-Treg response to IL-7. A. Representative histogram of *ex-vivo* pSTAT5 levels in circulating naïve-Tregs and naïve-Tconvs, with graph showing pSTAT5 MFI within these and counterpart memory CD4 T-cell subsets; comparison of pSTAT5 levels only revealed significant differences between naïve-Tregs and all the other subsets (* $P < 0.05$; $n = 6$). **B.** Analysis of pSTAT5 MFI within gated naïve-Tregs upon *in-vitro* stimulation with increasing concentrations of IL-7 or IL-2; each symbol represents one individual. **C.** IL-7Rα MFI within naïve-Tregs analyzed *ex-vivo* and after 24 hour-culture without serum or supplemented with 40% autologous serum alone or with IL-7 ($n = 6$); as well as with IL-2, anti-IL-2 blocking monoclonal antibody, or isotype-control ($n = 3$); * $P < 0.05$ as compared to *ex-vivo* levels.

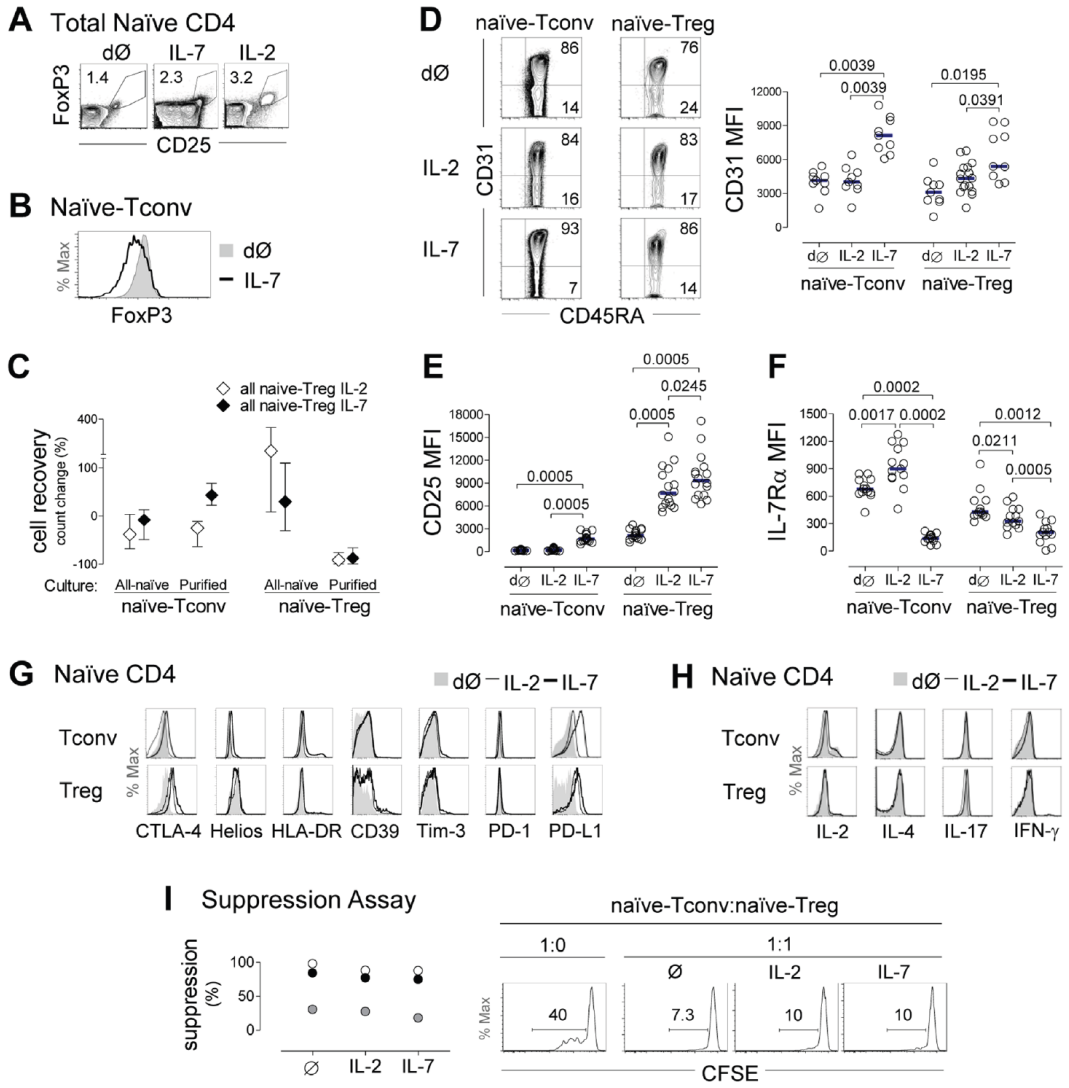

Figure 4: Evidence of naïve-Treg response to IL-7 while preserving their naïve and suppressive phenotype. A. Representative contour-plots of FoxP3 and CD25 expression within total naïve CD4 T-cells *ex-vivo* (dØ) and upon 13 day-culture with IL-7 or IL-2. **B.** Illustrative histogram demonstrating absence of FoxP3 induction in purified naïve-Tconvs following 13 day-culture with IL-7. **C.** Cell recovery upon 13 day-culture with IL-7 or IL-2 of total naïve CD4 T-cells ($n = 11$), purified naïve-Tregs ($n = 3$) or purified naïve-Tconvs ($n = 3$); graph shows median and interquartile range or range (purified populations). **E.-F.** Analysis of naïve-Tregs and naïve-Tconvs *ex-vivo* and post-13 day-culture of naïve CD4 T-cells with IL-7 or IL-2 showing representative contour-plots of CD31/CD45RA expression (D), and graphs of MFI of CD31 (D), CD25 (E), and IL-7Rα (F); each dot represents one individual; bars represent median; comparisons done within each subset; significant *P*-values are shown. **G.** Illustrative histograms of CTLA-4, Helios, HLA-DR, CD39, Tim-3, PD-1 and PD-L1 expression within naïve-Tregs and naïve-Tconvs *ex-vivo* and post-13 day-culture with IL-7 or IL-2 (one/3-14). **H.** IL-2, IL-4, IL-17 and IFN-γ production after short-term PMA/Ionomycin stimulation *ex-vivo* and post-13 day-culture with IL-7 or IL-2 (one/3). **I.** Illustrative histograms of CFSE expression within naïve-Tconvs cultured alone or with naïve-Tregs pre-incubated with medium alone, IL-7 or IL-2 (numbers represent % of cells that divided at least once); graph shows % of suppression of naïve-Tconv proliferation in 3 individuals.

regulated IL-7Rα (fold-reduction: 0.43[0.23-0.67], Figure 4F) upon IL-7 stimulation, although less markedly than naïve-Tconvs (11.54[7.80-14.96], $P < 0.0001$ for CD25; and 0.21[0.15-0.27], $P = 0.0058$ for IL-7Rα expression). These findings support an ability of naïve-Tregs to respond to IL-7.

The impact of IL-2 on naïve-Tregs followed a similar pattern, albeit significantly less striking than that observed for IL-7 (Figure 4D-F). Conversely, in naïve-Tconvs both CD25 and CD31 expression levels were unaltered in response to IL-2, whereas IL-7Rα expression increased (Figure 4D-4F), possibly due to a dominant effect of IL-7 deprivation [17, 29].

Concerning Treg function-associated markers, naïve-Treg FoxP3 MFI (*ex-vivo*: 778[650-1009], $n = 16$) significantly increased upon culture with IL-7 (1166[863-1522], $P = 0.0214$), and even more so with IL-2 (1229[902-1916], $P = 0.0280$ as compared to *ex-vivo*, $P = 0.0362$ as compared to IL-7). Furthermore, we observed a sustained or moderate increase in CTLA-4, CD39 and HLA-DR expression upon culture with either IL-7 or IL-2 (Figure 4G), suggesting maintenance of regulatory function. Additionally, IL-7 induced a major up-regulation of PD-L1, but not PD-1, on both naïve subsets (Figure 4G).

Importantly, naïve-Tregs remained unable to produce IL-2, IL-4, IL-17 or IFN-γ after 13 day-culture in the presence of IL-7 or IL-2, as evaluated upon further short-term stimulation with PMA plus Ionomycin (Figure 4H).

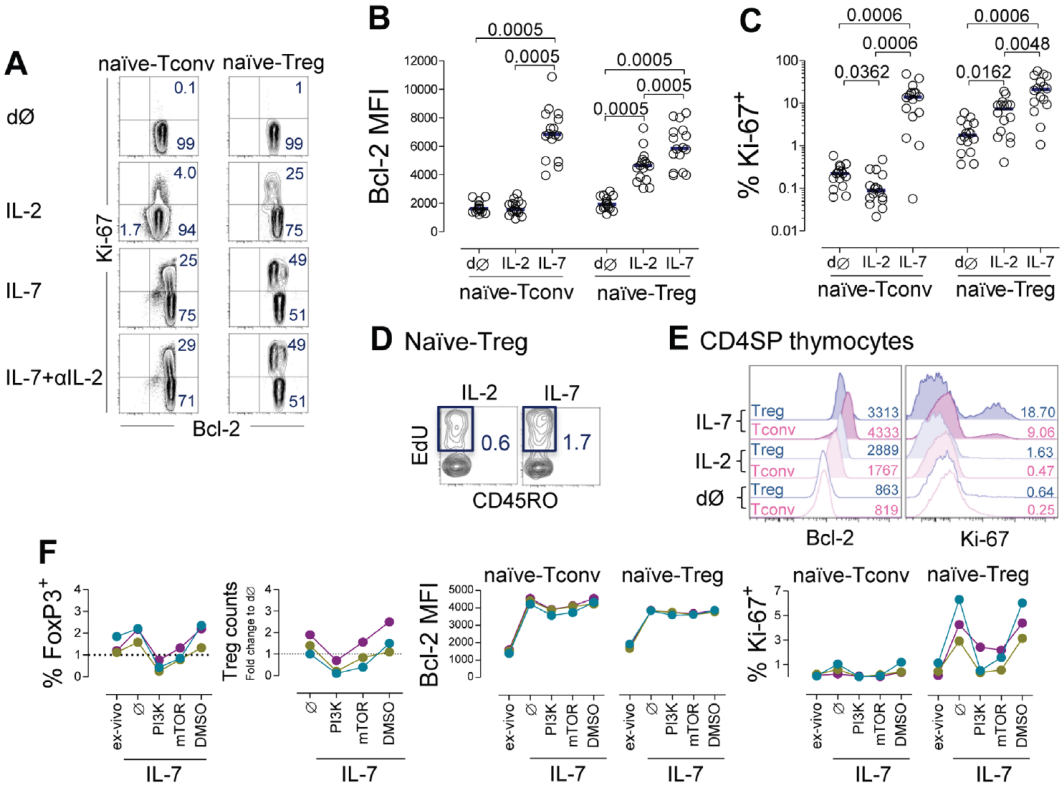

Figure 5: IL-7 induced naïve-Treg survival and proliferation. A.-D. Analysis of naïve-Tregs and naïve-Tconvs *ex-vivo* (dØ) and post 13 day-culture of purified naïve CD4 T-cells with IL-7 or IL-2 or IL-7 plus anti-IL-2 blocking monoclonal antibody: (A) Representative contour-plots of Bcl-2 and Ki-67 expression; graphs show Bcl-2 MFI (B) and % of cycling-cells (C), bars represent median; significant *P*-values of comparisons within each subset are shown; (D) Contour-plots illustrating the impact of IL-7 or IL-2 on EdU incorporation by naïve-Tregs (final 12 hours-culture, $n = 2$). **E.** Illustrative histograms of Bcl-2 and Ki-67 expression within gated FoxP3$^+$ or FoxP3$^-$ cells *ex-vivo* and post 7 day-culture of purified CD4SP thymocytes with IL-7 or IL-2 (one/3). **F.** Impact of the PI3K-inhibitor (LY294002), mTOR-inhibitor (rapamycin), or vehicle control (DMSO), on naïve CD4 T-cells upon 7 day-culture with IL-7; graphs show % of naïve-Treg and change in cell recovery, Bcl-2 MFI and % of cycling-cells within naïve-Tregs and naïve-Tconvs, as compared to *ex-vivo* levels; each color represents one individual.

Finally, we asked whether exposure to IL-7 impairs the suppressive capacity of naïve-Tregs. For this purpose we sorted naïve-Tregs and compared their suppressive capacity upon 4 hours pre-incubation with culture medium alone or supplemented with IL-7 or IL-2. We observed that upon treatment with either cytokine, naïve-Tregs maintained their ability to suppress autologous naïve-Tconv proliferation in response to TCR stimulation (Figure 4I).

Altogether, these results support an ability of naïve-Tregs to respond to IL-7 whilst maintaining both their naïve and suppressive phenotype.

IL-7 induced naïve-Treg survival and proliferation

We then asked whether IL-7 induces the expression of markers of survival (Bcl-2) and cell cycling (Ki-67) in naïve-Tregs. We observed a clear induction of Bcl-2 expression in naïve-Tregs cultured in the presence of IL-7 (Figure 5A-B), although less marked than in naïve-Tconvs (fold-increase: 3.02[2.33-3.72] *versus* 3.93[3.29-5.05], respectively, $P < 0.0001$). Bcl-2 up-regulation in naïve-Tregs was significantly higher upon IL-7 stimulation than with IL-2 (Figure 5A-5B), using optimal concentrations of both cytokines [17].

Additionally, IL-7 induced cycling of naïve-Tregs (Figure 5A and 5C). The proportion of cells expressing Ki-67 (21.06%[9.62-40.40]) was higher than that observed within naïve-Tconvs (13.92%[5.11-17.63], $P = 0.0186$), and within naïve-Tregs cultured with IL-2 (7.31%[1.74-9.35], $P = 0.0019$). These findings are even more striking considering the much higher IL-7Rα expression levels on naïve-Tconvs (Supplementary Figure 1B). We further showed that IL-7 was superior to IL-2 in promoting naïve-Treg entry into cell-cycle, *via* assessment of the frequency of EdU-incorporating cells in the last 12 hours of the culture (Figure 5D). Proliferative studies using carboxyfluorescein succinimidyl ester (CFSE)-labeled cells were not performed, as we have previously demonstrated that this approach does not reproducibly reveal cytokine-mediated proliferation of naïve CD4 T-cells [17].

Importantly, as shown in Figure 5A, IL-7 induction of Bcl-2 and cell cycling was unaltered in the presence of an anti-IL-2 blocking monoclonal antibody, thus supporting a major role for IL-7 in the maintenance of naïve-Treg homeostasis independently of IL-2.

Human FoxP3$^+$CD4SP thymocytes were already able to proliferate in culture, and to up-regulate Bcl-2 in response to IL-7 (Figure 5E), in agreement with our previous data showing an increase of pSTAT5 in FoxP3$^+$ thymocytes upon stimulation with IL-7 [30].

Finally, we investigated whether the signaling pathways involved in IL-7 response differed between naïve-Tregs and naïve-Tconvs. IL-7 signaling uses both JAK3/STAT5 and PI3K/Akt pathways to promote T-cell survival and proliferation [17]. We have previously demonstrated that IL-7-mediated naïve CD4 T-cell proliferation, but not Bcl-2 up-regulation, critically depends upon PI3K activity [17]. On the other hand, Treg metabolism is known to rely on mammalian target of rapamycin (mTOR) [2]. To investigate the relative importance of these pathways, we added the cell permeable PI3K-specific inhibitor (LY294002) or mTOR inhibitor (rapamycin) to naïve CD4 T-cells cultured with IL-7 or IL-2. A 7 day-culture assay was selected to avoid possible deleterious impact of long-term exposure to the inhibitors, and was found to be sufficient to reveal Bcl-2 and Ki-67 induction albeit at lower levels than after 13 day-culture (Figure 5F). Inhibition of the PI3K pathway led to a major decrease in naïve-Treg recovery and proliferation upon stimulation with IL-7. Conversely, Bcl-2 expression was not significantly altered. Similar trends were observed upon mTOR inhibition, supporting its participation in the signaling cascade leading to proliferation. Moreover, the addition of these inhibitors to IL-2 stimulated cultures generated comparable profiles in naïve-Tregs, indicating that these pathways are utilized by both cytokines (data not shown).

Overall, our *in-vitro* data reveal that both IL-7 and IL-2 are able to promote proliferation of naïve-Tregs *via* activation of PI3K/mTOR signaling, and that IL-7 promotes the survival and proliferation of FoxP3$^+$ mature thymocytes and circulating naïve-Tregs.

DISCUSSION

Naïve-Tregs are thymus-committed regulatory T cells with a high degree of self-reactivity that continuously replenish the activated memory-Treg pool. Our finding of a preserved naïve-Treg compartment in adults submitted to total thymectomy in infancy support the relevance of peripheral mechanisms to ensure naïve-Treg homeostasis. We further showed that in steady-state conditions human naïve-Treg homeostasis occurs through a combination of high turnover rate and extended survival mediated by IL-7 and/or IL-2, based on the following evidence: 1) higher levels of the pro-survival molecule Bcl-2 and proportion of cycling-cells than naïve-Tconvs in both blood and tonsils; 2) the highest *ex-vivo* levels of pSTAT5 as compared to other naïve/memory CD4 T-cell subsets, which further increases, in a dose-dependent manner, following IL-7 or IL-2 stimulation; 3) recovery of IL-7Rα expression upon IL-7 deprivation, consistent with an ongoing *in-vivo* response to IL-7; 4) response to IL-7 and IL-2 in culture indicated by up-regulation of Bcl-2, and induction of PI3K/mTOR-mediated proliferation; and 5) maintenance of naïve and suppressive phenotype upon exposure to IL-7. Additionally, we showed that naïve-Treg precursors, namely FoxP3$^+$CD4SP thymocytes, already proliferate and up-regulate Bcl-2 in response to IL-7 stimulation *in-vitro*.

Thus, our results challenge the assumption that

naïve-Tregs are a quiescent population in steady-state conditions in non-lymphopenic individuals [1]. Human naïve-Tregs have been shown to feature a functional and molecular profile partially overlapping with both memory-Tregs and naïve-Tconvs [1, 4, 6]. Regarding their homeostasis, as for the memory compartment we found a much higher cell-turnover in naïve-Tregs than in naïve-Tconvs, suggesting an intrinsic propensity of Tregs to enter cell cycle. On the other hand, in agreement with their naïve character, naïve-Treg turnover was lower and Bcl-2 levels higher than in their memory counterparts. Additionally, similar pathways were apparently operating in response to IL-7 in naïve-Tregs and naïve-Tconvs.

In fact, we revealed here, for the first time, a role for IL-7 in naïve-Treg survival and homeostatic proliferation. These findings are particularly relevant for understanding the impact of *IL-7/IL-7Rα* polymorphisms on autoimmunity [31], and the mechanisms underlying Treg expansion in lymphopenic settings, with implications for the therapeutic use of IL-7.

Our data do not exclude the contribution of self-peptide/MHC stimulation to the naïve-Treg proliferation, which may be particularly important given their enrichment in self-antigen reactivities [6]. However, TCR stimulation of naïve-Tregs has been associated with rapid acquisition of a memory phenotype, with the potential risk of contraction of the naïve compartment [1, 4]. Of note, the maintenance of murine naïve-like FoxP3$^+$ CD4 T-cells was recently demonstrated to be independent of TCR-driven signals [32].

Importantly, we showed that naïve-Treg proliferation in response to IL-7 or IL-2 was associated with preservation of the naïve-like phenotype, in parallel with the maintenance or even increase of regulatory markers. We further showed that prior exposure of naïve-Tregs to IL-7 did not impair their suppressive capacity.

IL-7, like IL-2, is known to induce PD-1 and PD-L1 on Tconvs [33] and Tregs [20]. Our observations extended these reports, by showing that IL-7 up-regulated PD-L1, but not PD-1, on both naïve-Tregs and naïve-Tconvs. These results suggest that IL-7 contributes not only to T-cell homeostasis, but also to the containment of immunopathology, by up-regulating the inhibitory molecule PD-L1 on naïve CD4 T-cells, irrespective of their FoxP3 status. On the other hand, this may have implications for tumor immunity, given the clinically relevant suppression of tumor specific responses mediated by the PD-1/PD-L1 pathway [34].

SLO are an important source of IL-7 [10, 17]. Our results from tonsil tissue, using both flow-cytometry and immunofluorescence, confirmed a high local naïve-Treg turnover, suggesting that SLO contain appropriate niches for naïve-Treg homeostasis. Nevertheless, more detailed studies are required to decipher the topography of naïve-Tregs in SLO, as well as the contribution of their possible interactions with IL-7-producing stromal reticular cells, dendritic cells or IL-2-producing memory T-cells.

Our data argue for the sufficiency of peripheral proliferation and survival to achieve long-term maintenance of the naïve-Treg compartment even in the absence of thymic output. These results in adults expand upon a recent report describing preservation of naïve-Treg counts during short-term follow-up of children submitted to total thymectomy in early infancy [15]. Although a previous study showed a reduction of naïve-Treg frequency within total Tregs [13], this was not related to an actual contraction of naïve-Tregs upon thymectomy, but rather to the relatively high frequency of total Tregs, as also observed in our study (Table 1; proportion of naïve-Tregs within total Treg compartment: 11.60%[9.08-18.00] in thymectomized *versus* 14.85%[12.05-23.95] in healthy subjects, $P = 0.3776$).

The increase in naïve-Treg cycling in thymectomized adults was more likely mediated by IL-7 than by self-peptide/MHC interaction, given the conserved frequency of CD31$^+$ cells, which are typically lost following TCR stimulation [16], and maintained by IL-7 [17]. We further showed that the CD31$^+$ subset of naïve-Tregs was preferentially expanded by IL-7 *in-vitro*, in agreement with our previous data generated with total naïve CD4 T-cells [17]. Adults thymectomized in infancy feature an unexpectedly low prevalence of autoimmune diseases and allergy [12], which may be related to the maintenance of the broadly-reactive naïve-Treg pool.

To our knowledge, there are no published data specifically addressing the changes in the naïve-Treg compartment in individuals receiving IL-7 therapy. Nevertheless, an increase in total Treg numbers was documented in clinical trials, although the expansion of the Tconv compartment was much higher, leading to maintenance [35, 36] or a relative decrease [37-39] in Treg proportions. In agreement with our *in-vitro* data, naïve-Treg expansion was reported upon IL-2 therapy [28, 40, 41].

In severe lymphopenic settings associated with elevated serum IL-7 levels, such as HIV infection [42], there is an apparent preservation of circulating naïve-Tregs [43], raising the possibility that these cells are responding to IL-7. We did not find a correlation between the size of the naïve-Treg compartment and IL-7 serum levels in either our thymectomized individuals or the study population as a whole, which may be due to the narrow range of IL-7 levels (Table 1). Our data are also relevant in other lymphopenic settings, particularly in the context of immune reconstitution following hematopoietic stem cell transplantation, where IL-7-driven proliferation of naïve-Tregs may help to control graft *versus* host disease. Our results support a model in which the increased availability of IL-7 in lymphopenic settings may contribute to the expansion of the naïve-Treg compartment, thereby helping prevent immunopathology in the context of a constrained TCR repertoire.

In conclusion, naïve-Tregs feature high turnover in healthy adults, which further increases to compensate the loss of thymic replenishment upon total thymectomy. Moreover, our data reveal a role for IL-7 in naïve-Treg maintenance, both through up-regulation of the survival molecule Bcl-2 and induction of PI3K/mTOR-mediated proliferation. Clinical use and evaluation of therapies targeting the IL-7 pathway should take into account the contribution of IL-7 to naïve-Treg homeostasis.

MATERIALS AND METHODS

Study design

Blood was collected from healthy and age-matched adults thymectomized in infancy (Table 1). Complete thymectomy was confirmed based on the corrective cardiac surgery report and severely reduced levels of sjTREC. Patients with syndromatic cardiopathy were excluded. Circulating naïve-Tregs were further compared to their precursors, CD4SP thymocytes, isolated from thymic tissue obtained from children during routine corrective cardiac surgery. Tonsil tissue and blood collected at the same time from children submitted to tonsillectomy were used to study in parallel naïve CD4 T-cells in blood and SLO. All subjects/legal guardians gave written informed consent for blood and/or tissue sampling. Study was approved by Ethical Boards of Faculty of Medicine of University of Lisbon, and from Hospital de Santa Cruz for thymic tissue collection, Portugal.

Cell isolation

PBMCs were isolated from freshly-collected heparinized blood *via* Ficoll-Paque PLUS (GE Healthcare, Uppsala, Sweden). Total or naïve CD4 T-cells were subsequently purified by negative selection (purity >96%, StemCell Technologies, Grenoble, France). Naïve-Tregs ($CD45RO^{-}CD25^{high}CD127^{-}$), naïve-Tconvs ($CD45RO^{-}CD25^{-}CD127^{high}$), and memory-Tregs ($CD45RO^{+}CD25^{high}CD127^{-}$) were sorted from purified CD4 T-cells using a FACSAria (purity >95%; BD Biosciences, San Jose, CA). Tonsillar mononuclear cells and thymocytes were recovered using Ficoll-Paque PLUS after mechanical dispersion of tonsil and thymic tissue. CD4SP thymocytes were subsequently purified as $CD3^{high}CD8^{-}CD4^{+}$ cells using a FACSAria (purity >97%).

Cell culture

Purified naïve CD4 T-cells, naïve-Tregs or naïve-Tconvs, were cultured at $1x10^{6}$ cells/ml in complete medium as described [17], with either IL-7 (10ng/ml; R&D Systems, Minneapolis, MN) or IL-2 (20IU/ml; NIH/AIDS Research and Reference Program, Division of AIDS, NIAID, Hoffman-La Roche), for up to 13 days, with media replacement at day 3 and day 7. Naïve CD4 T-cell recovery per well was significantly higher upon culture with IL-7 than IL-2 (200,615[144,000-262,167] *versus* 95,958[60,063-178,125]; $n = 16$; $P = 0.0006$). In some experiments, anti-IL-2 blocking monoclonal antibody (clone 5334, 10µg/ml, R&D Systems) or isotype control IgG1 (10µg/ml, eBioscience, San Diego, CA) was added to cultures with IL-7. Cultures were also performed for 7 days in presence of PI3K inhibitor LY294002 (10µM, Merck Biosciences, Nottingham, UK); mTOR inhibitor rapamycin (100nM, Sigma-Aldrich); or drug vehicle DMSO (Sigma-Aldrich). For serum deprivation experiments, purified CD4 T-cells were cultured for 24 hours in medium without AB serum, or with 40% autologous serum alone or supplemented with IL-7, IL-2, anti-IL-2 blocking monoclonal antibody, or isotype control.

Flow-cytometry

Ex-vivo phenotypic analysis was performed in freshly collected whole blood. Eight-color staining was performed using monoclonal antibodies listed in Supplementary Table 1. Intracellular staining was done using eBioscience FoxP3 kit. At least 150,000 events were acquired for each sample on a BD LSRFortessa (BD Biosciences) and data analyzed using FlowJo software (TreeStar, Ashland, OR). Dead cells were excluded according to FSC/SSC characteristics and/or LifeDead staining. After lymphogate definition, doublets were excluded, and cells analyzed within the mentioned gates.

STAT5 phosphorylation

pSTAT5 was quantified in whole blood immediately after collection. After surface staining, red blood cells were lysed, and cells fixed and permeabilized using eBioscience FoxP3 protocol, followed by BD Cytofix and BD Phosflow (BD Biosciences), and 1 hour incubation at 4°C with anti-pSTAT5 monoclonal antibody and other intracellular markers (Supplementary Table 1). pSTAT5 was also quantified upon stimulation of surface stained purified CD4 T-cells, with increasing concentrations of recombinant IL-7 (0.1/1/10/50ng/ml) or IL-2 (2/20/100/200IU/ml) for 15 minutes at 37°C, as described [44].

Thymidine analogue 5-ethynyl-2'-deoxy-uridine (EdU) incorporation

EdU (5μM) was added to cell cultures for 12 hours. Immediately after collection, cells were surface and intracellularly stained, resuspended in ice-cold 1% formaldehyde (15 minutes), kept on ice on 70% ethanol (10 minutes), and then washed several times in PBS with 0.05% Triton-X100 before detection of EdU-substituted DNA using the Click-iT® EdU HCS Assay (Thermo Fischer Scientific) according to manufacturer´s instructions.

Cytokine quantification

Cytokine production at the single-cell level was assessed after 4 hours stimulation with PMA+Ionomycin and Brefeldin A, as described [44]. Serum IL-7 levels were quantified using Human IL-7 Quantikine HS ELISA kit (R&D Systems) [42].

In vitro suppression assay

Purified naïve-Tregs were incubated with IL-7, IL-2, or medium alone for 4 hours followed by co-culture (ratio 1:1) with autologous naïve-Tconvs labeled with CFSE, as described [17], stimulated with plate bound anti-CD3 (0.5μg/ml, clone OKT3, eBiosciences) in the presence of irradiated autologous PBMCs (4000rad). CFSE intensity decline was assessed at day 4 by flow-cytometry; % of suppression of naïve-Tconv proliferation = [(% proliferating naïve-Tconvs plated alone - % proliferating naïve-Tconvs co-cultured with Treg)/ % proliferating naïve-Tconvs plated alone]x100.

sjTREC quantification

A highly sensitive nested quantitative PCR assay (detection-limit 1copy/10^5 cells) was used as described [24]. Triplicate multiplex PCR amplification of sjTREC, together with the CD3γ chain was performed on lysed PBMCs; quantifications were then performed using a LightCycler™ in independent experiments, with the same first-round serial dilution standard curve.

FOXP3 mRNA quantification

Total RNA (Zymo Research kits) was used to generate cDNA (Superscript III Reverse Transcriptase, Thermo Fischer Scientific). mRNA levels of *FOXP3* (primers/probe described in [45]), and *GAPDH* (Taqman Gene Expression Assay) were quantified in duplicates (ViiA 7 Real-Time PCR System, all from Thermo Fischer Scientific) using standard curves generated by serial dilutions of cDNA from pooled PBMCs for *GAPDH* and a plasmid with *FOXP3* sequence. Relative copy numbers of *FOXP3* were calculated upon normalization to *GAPDH*.

Immunofluorescence staining

Human tonsils were placed in OCT (VWR, Radnor, PA) and snap-frozen in liquid nitrogen. 3μm sections were stained overnight at 4°C using antibodies listed in Supplementary Table 1, and DAPI as a nuclear counter stain. Image processing was performed using FIJI software.

Statistical analysis

Statistical analysis was performed with Graph Prism Version 5.01 (GraphPad Software, San Diego, CA). The following tests were used: Friedman for variance; Wilcoxon-Signed Rank for pairwise comparisons; Mann-Whitney for unpaired comparisons; Spearman's coefficient for correlations. Results were expressed as median [interquartile range or range ($n < 4$)]. P-values < 0.05 were considered significant.

ACKNOWLEDGMENTS

We thank the sample donors for their collaboration; Cardiothoracic Department, Hospital de Santa Cruz, for thymus collection; ENT Department, Hospital de Santa Maria, for tonsil collection; R Tendeiro, R Azevedo, H Nunes-Cabaço, MC Pereira-Santos, and AI Pinheiro for technical assistance; B Silva-Santos for fruitful discussions.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

GRANT SUPPORT

This work was supported by Fundação para a Ciência e Tecnologia (FCT; POCI2010/IC/83068/2007 to RMMV; PTDC/SAU-MIC/109786/2009 to AES), and Gulbenkian Foundation (96526/2009 to JF; P132532/2013 to AES). SLS, ASA, RBF, ARP, PM and SMF received FCT scholarships.

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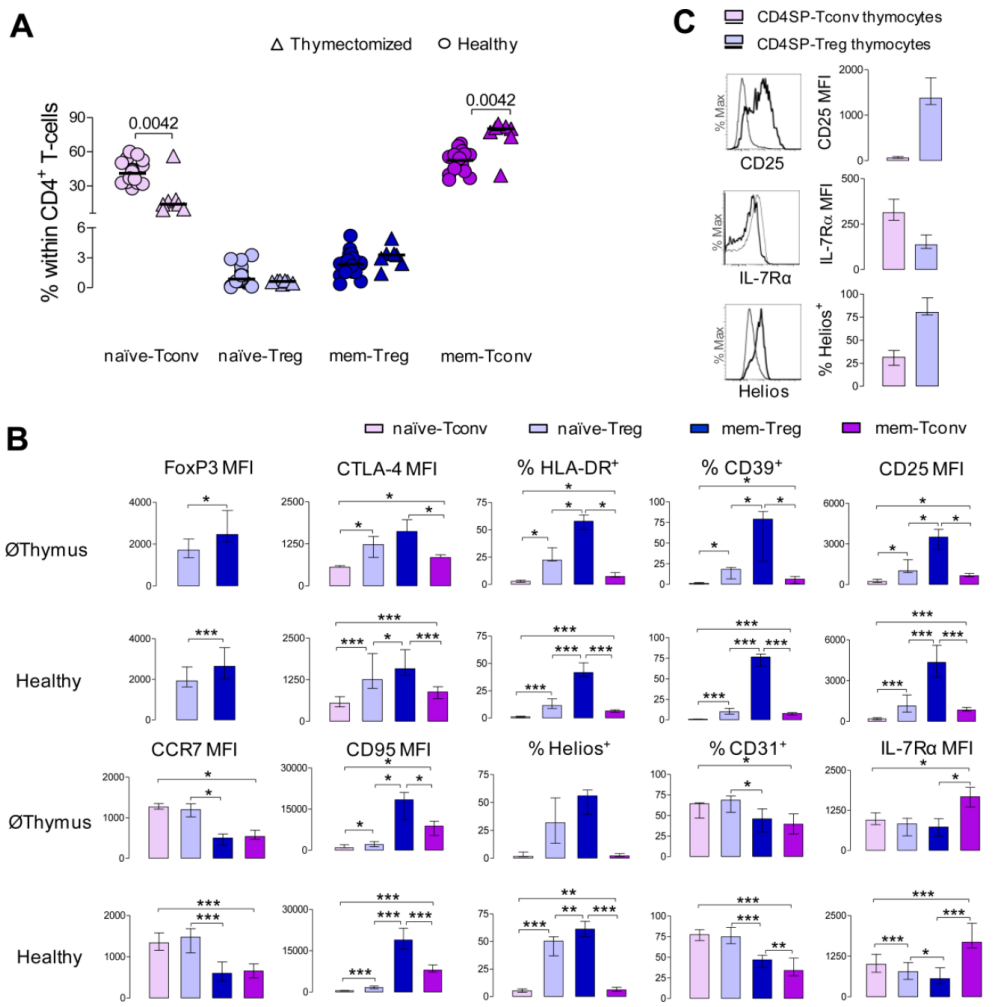
Human naïve regulatory T-cells feature high steady-state turnover and are maintained by IL-7

Supplementary Material

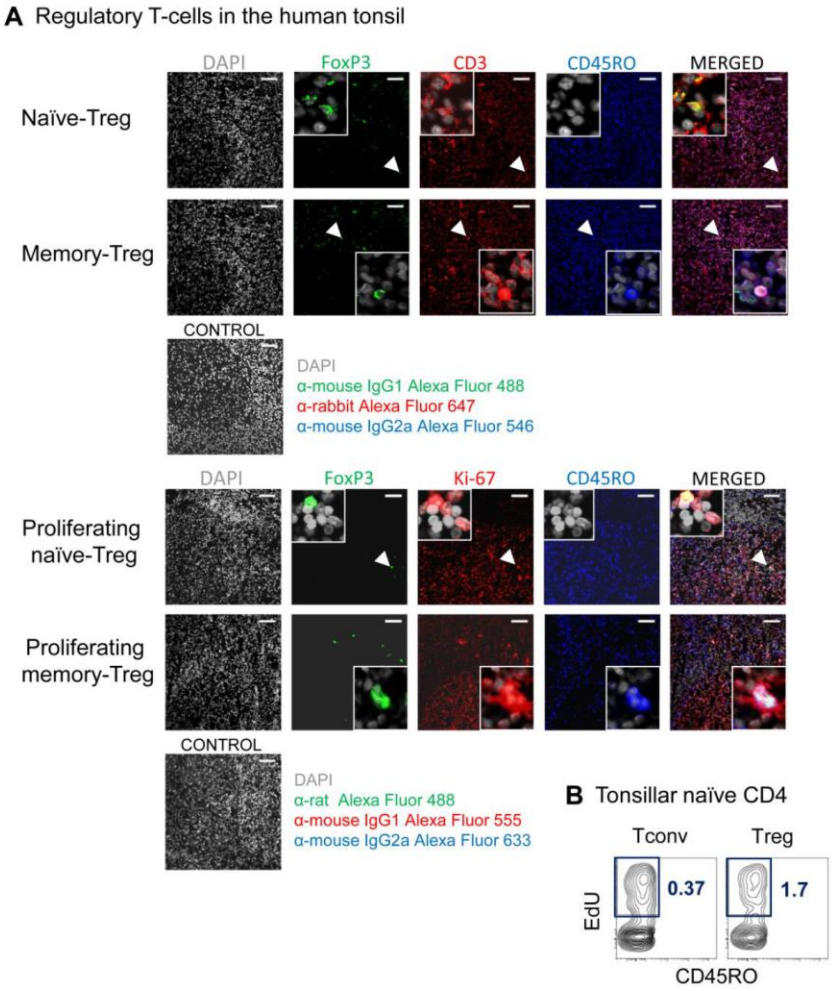
Supplementary Table 1. Antibodies used in Flow-Cytometry and Immunofluorescence

Antibodies for Flow Cytometry			
Antibody	**Fluorochrome**	**Clone**	**Source**
CD3	PerCP-Cy5.5/eFluor450	OKT3	eBioscience [a]
CD3	APC	UCHT1	eBioscience
CD3	V500	UCHT1	BD Bioscience [b]
CD4	PE/PerCP-Cy5.5/PE-Cy7/APC	RPA-T4	eBioscience
CD4	V500	RPA-T4	BD Bioscience
CD11c	FITC	3.9	eBioscience
CD14	FITC	61D3	eBioscience
CD19	FITC	HIB19	eBioscience
CD123	FITC	6H6	eBioscience
CD45RA	FITC/PerCP-Cy5.5/APC	HI100	eBioscience
CD45RA	eFluor450	2D1	eBioscience
CD45RA	PE-Cy7	L48	BD Bioscience
CD45RO	FITC/PerCP eFluor710/APC-H7	UCHL1	eBioscience
CCR7	FITC	150503	R&D Systems [c]
CCR7	PE	3D12	R&D Systems
CD25	PE-Cy7	2A3	BD Bioscience
CD27	PE/PE-Cy7	O323	eBioscience
CD31	PE/APC	WM-59	eBioscience
CD39	FITC/APC	eBioA1	eBioscience
CD95	PE-Cy7	DX2	eBioscience
CD127	Alexa Fluor660/APC eFluor780	eBioRDR5	eBioscience
HLA-DR	FITC	L243	BD Bioscience
HLA-DR	V500	G46.6	BD Bioscience
PD-1	FITC	MIH-4	eBioscience
PD-L1	APC	BIH1	eBioscience
FoxP3	PE/PerCP-Cy5.5/eFluor450	PCH101	eBioscience
CTLA-4	PE	BNI3	BD Bioscience
Helios	Alexa Fluor488	22F6	Biolegend [d]
Ki-67	FITC/PE/PerCP-Cy5.5/Alexa Fluor647	B56	BD Bioscience
Bcl-2	FITC	124	DAKO [e]
IL-2	Brilliant violet421	MQ1-17H12	Biolegend
IL-4	APC	8D4-8	eBioscience
IL-17A	PerCP-Cy5.5	eBio64DEC17	eBioscience
IFN-γ	PE-Cy7	4S.B3	eBioscience
pSTAT5	PerCP-Cy5.5	47/pY694	BD Bioscience
LIVE/DEAD®	APC-Cy7	-	Thermo Fischer Scientific [f]
Primary Antibodies for Immunofluorescence			
Antigen	**Species/isotype**	**Clone**	**Source**
FoxP3	Mouse/IgG1	236/E7	eBioscience
FoxP3	Rat/IgG2a	PCH101	eBioscience
Ki-67	Mouse/IgG1	B56	BD Bioscience
CD45RO	Mouse/IgG2a	UCHL1	eBioscience
CD3	Rabbit/NA	Polyclonal	Abcam [g]
Secondary Antibodies for Immunofluorescence			
Specificity	**Species**	**Fluorochrome**	**Source**
Mouse IgG1	Goat	Alexa Fluor 488	Thermo Fischer Scientific
Mouse IgG2a	Goat	Alexa Fluor 546	Thermo Fischer Scientific
Rabbit	Goat	Alexa Fluor 647	Thermo Fischer Scientific
Rat	Goat	Alexa Fluor 488	Thermo Fischer Scientific
Mouse IgG1	Goat	Alexa Fluor 555	Thermo Fischer Scientific
Mouse IgG2a	Goat	Alexa Fluor 633	Thermo Fischer Scientific

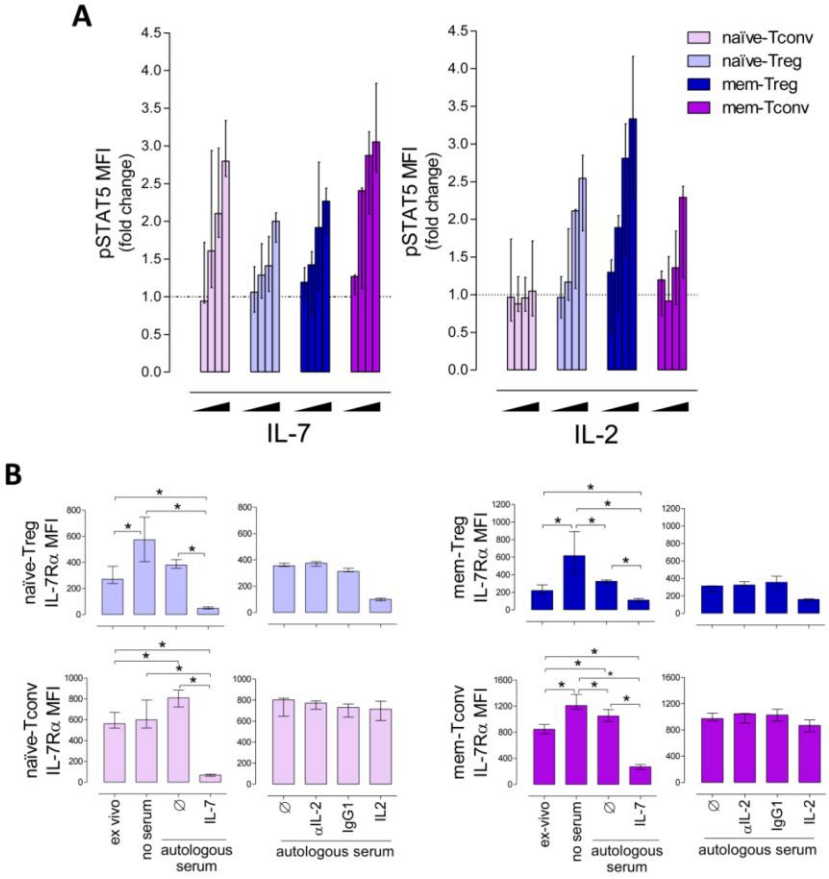
[a] eBioscience, San Diego, CA; [b] BD Bioscience, San Jose, CA; [c] R&D Systems, Minneapolis, MN; [d] Biolegend, San Diego, CA; [e] DAKO, Glostrup, Denmark; [f] Thermo Fischer Scientific, Walthan, MA; [g] Abcam, Cambridge, UK. NA- Not Applicable



Supplementary Figure 1. ***Imbalances of CD4 T-cell subsets in adults submitted to total thymectomy in infancy.*** **(A)** Frequency of naïve-Tconv, naïve-Treg, mem-Treg, and mem-Tconv within circulating $CD4^+$ T-cells in thymectomized and age-matched healthy subjects; each dot represents one individual; comparisons performed for each subset between the two cohorts; significant *P*-values are shown. **(B)** Phenotype of the subsets **(**numbers of thymectomized and healthy, respectively, shown in brackets): FoxP3 (7/22), CTLA-4 (6/21), HLA-DR (7/22), CD39 (7/22), CD25 (6/21), CCR7 (7/21), CD95 (7/21), Helios (5/17), CD31 (7/16), and IL-7Rα (6/16); subsets within naïve and memory, as well as within Treg and Tconv compartments were compared; bars represent median and range; significant *P*-values are shown: * $P<0.05$, ** $P<0.005$, *** $P<0.001$. **(C)** CD25, IL-7Rα and Helios, within $FoxP3^+$ (CD4SP-Treg) and $FoxP3^-$ (CD4SP-Tconv) CD4SP thymocytes from three children (1, 3, and 9 months-old); bars represent median and range; % of mature CD4SP thymocytes ($CD45RA^{bright}$) lacking Helios expression varied between 4.5% and 19.6%.



Supplementary Figure 2. ***Naïve-Treg proliferation in the human tonsil.*** **(A)** Illustrative immunofluorescence analysis of regulatory T-cells in human tonsil showing naïve ($CD3^+CD45RO^-$) and memory ($CD3^+CD45RO^+$) $FoxP3^+$ Treg in the top; and proliferating ($Ki\text{-}67^+$) naïve-Treg ($CD45RO^-FoxP3^+$) and memory-Treg ($CD45RO^+FoxP3^+$) in the bottom; DAPI was used to counter-stain nuclei. Illustrative images of single stainings, merged image, as well as the control staining in the absence of the primary antibodies are shown (resolution: 1024x1024; insert magnification: 200x, scale bar: 2.409 pixels/µm; length shown: 60µm); images were acquired using a Zeiss LSM710 microscope (Zeiss, Oberkochen, Germany) with a dry objective Plan-Apochromat with a numerical aperture of 0.8. **(B)** Contour-plots illustrating proportion of tonsillar naïve-Tregs and naïve-Tconvs incorporating EdU upon 12 hours-culture of freshly-isolated CD4 T-cells.



Supplementary Figure 3. ***Ex-vivo evidence of naïve-Treg response to IL-7.*** **(A)** IL-7 and IL-2 dose-dependent induction of pSTAT5 following 15 minutes *in-vitro* stimulation of purified CD4 T-cells; graphs show pSTAT5 MFI fold change in relation to non-stimulated cells in the gated CD4 T-cell subsets in three individuals; bars represent median with range. **(B)** IL-7Rα modulation in CD4 T-cell subsets according to exposure to IL-7 or IL-2; purified CD4 T-cells were cultured for 24 hours in the absence of IL-7/IL-2 (no serum), or with 40% autologous serum either alone or further supplemented with: IL-7 (10ng/ml), IL-2 (20IU/mL), anti-IL-2 blocking mAb (10µg/mL), or isotype-control (IgG1, 10µg/mL); graphs show the IL-7Rα MFI within gated CD4 T-cell subsets *ex-vivo* and post-culture, in six (left-hand graphs) and three (right-hand graphs) individuals; bars represent median with interquartile range (left-hand graphs; significant *P*-values are shown: **P*<0.05) or median with range (right-hand graphs).